Biochemical and environmental perspectives on nitrogen metabolism in fishes

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Abstract. Catabolism of nitrogen-containing substances makes a major contribution to the oxidative metabolism in teleostean fishes. In this review, we focus on aspects of the formation, transport, detoxification and excretion of the two most important nitrogenous products of fishes: ammonia (NH_3 plus NH_4^+) and urea. While NH_4^+ makes up the bulk of nitrogenous waste, it is in equilibrium with the highly toxic NH_3. Ammonia is generated in the liver and excreted through branchial, surface and renal routes. Innocuous urea is derived through hepatic uricolysis or argininolysis and voided through kidney, gill, skin or faeces.Under conditions hampering the release of ammonia, such as exposure to exogenous ammonia, water limitation, or alkaline conditions, some teleosts detoxify ammonia through synthesis of urea by the ornithine-urea cycle in liver. Ammonia and possibly alanine are the prevalent vehicles of internal nitrogen transport. Glutamine is immaterial to interorgan nitrogen transport in fishes, but plays a transient role in the detoxification of ammonia by brain glutamine synthetase.
Key words. Alanine; ammonia; argininolysis; detoxification; excretion; glutamine; ornithine-urea cycle; teleost fish; toxicity; transport; urea; uricolysis.

Catabolism of proteins and amino acid makes a major contribution to the total energy production of fishes. In fact, it has been estimated that oxidation of nitrogenous substances may account for 41% and 85% of total energy production. Such estimates are based on values published for the ammonia quotient, i.e. the moles of ammonia produced divided by the moles of oxygen consumed by fishes undergoing 'standard metabolism'[77]. These estimates would be even higher if total nitrogenous waste products rather than just ammonia had been used in the calculations. Metabolism of nitrogenous substances in fishes leads to the production of a number of nitrogen-containing excretory products. The relative contribution of the different compounds varies considerably between species and depends on the evolutionary position of a particular group of animals, the specific physiological roles of nitrogenous substances such as urea or glutamine, as well as the particular environmental conditions.

The most prominent nitrogenous excretory products in teleostean fishes are ammonia and urea, with ammonia (sum of NH_3 and NH_4^+) generally making up the bulk. The contribution of urea is variable, but usually falls into the 20–40% range (as % of total nitrogen excreted). In some selected cases, urea may constitute the exclusive nitrogenous excretory product, while in other situations, its share in nitrogen excretion may be negligible. Substances such as creatine, creatinine, purines or uric acid and its derivatives rarely play more than an ancillary role. In this review, we will focus our attention on different aspects of ammonia and urea production and excretion. In each case, different parameters have to be considered. Production of ammonia, for instance, requires little metabolic expenditure, but potentially yields the highly toxic un-ionized NH_3. As well, because of the dualism between un-ionized and ionized forms of ammonia (NH_4^+, the ammonium ion), ambient water conditions (e.g. salinity, pH) are likely to impact ammonia excretion. Urea, on the other hand, is non-toxic at concentrations normally encountered in teleostean fishes. It may be produced catabolically without energy input, or it may be synthesized from simple precursors at considerable metabolic cost. Further, ammonia, but not urea, occurs as an important environmental contaminant. Our discussion will highlight the teleostean fishes, although at times, for reasons of illustrating disparate concepts, we also draw on elasmobranch or mammalian examples.

Excretion of ammonia

In teleostean fishes, a direct relationship exists between protein intake and ammonia excretion. This relationship is probably best illustrated in the sockeye salmon (*Oncorhynchus nerka*) (fig. 1). In this species, which normally releases ammonia at a constant rate, each feeding event is soon followed by transient additional release of ammonia into the surrounding water[6]. It seems reasonable to conclude that the nitrogen excreted in the absorptive state is derived without much diversion from alpha-amino groups of amino acids taken up in surplus of amounts required for metabolic maintenance or growth. Of the major organs potentially liberating alpha-amino groups from amino acids (liver, intestine, kidney and gill) only the liver contains the complete complement of metabolic machinery to deal with an entire gamut of amino acids as well as additional nitrogenous compounds. Also, with reference to organ extraction of nitrogenous compounds, only liver and to a limited degree the kidney are able to liberate substantial amounts of ammonia from added nitrogenous substrates[81]. In support of this notion, generated from studies on isolated systems and enzymes, it was found that kidney and espe-

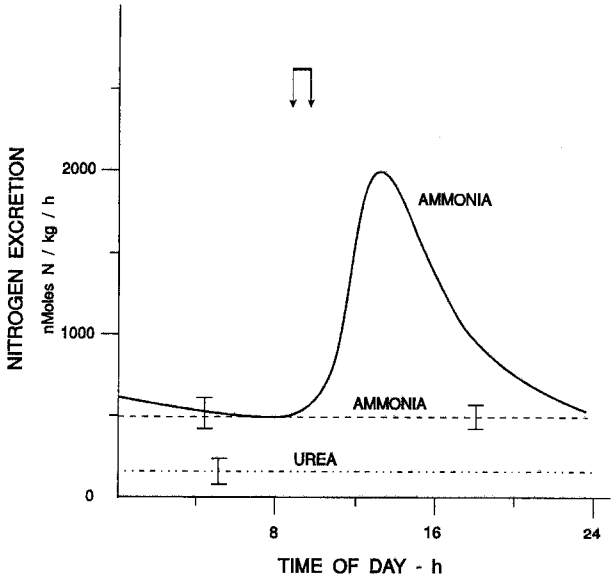

Figure 1. Ammonia and urea excretion in sockeye salmon (*Oncorhynchus nerka*). Ammonia (NH_3 plus NH_4^+) excretion was monitored for 24 h for starved fish (---) and for fish which received food (—). The arrows delineate the feeding period. Urea excretion was constant and identical in starved and recently fed animals (– · · –). Error bars indicate 2 SEM. Adapted from Brett and Zala[6].

cially liver were the major contributors of ammonia to the blood draining the two organs[81]. Considering the larger mass of the liver with its exceptionally high rate of perfusion[40], this organ appears to carry the main weight of transforming nitrogenous substrates destined for excretion into ammonia. Gill tissue is largely devoid of such ammonia generating ability, since venous-arterial differences for potentially ammoniogenic amino acids across the fish gill are insignificant[81]. Gill merely serves as the prevalent excretory site[52,83].

However, having made the above generalizations, a minor caveat should be included. There is some indication that the liver may not always be the exclusive site of ammonia production in fishes. Hepatectomized European eels (*Anguilla anguilla*), for instance, reveal unaltered rates of ammonia excretion over a period of ten days. When injected with alanine, however, the rate of ammonia excretion is drastically increased in control fish, while no such increase is noted for hepatectomized animals[32]. Perhaps the exploitation of complementary experimental approaches will make it possible to differentiate between potential processing sites for nitrogenous substances under varying conditions. At this point, it seems reasonable to conclude that one site, the liver, is responsible for the transitory increases in ammonia due to deamination of dietary nitrogenous compounds. Extrahepatic sources other than the gill are likely involved in the ammonia release at other times and their importance might be enhanced during hepatectomy or natural shifts in liver metabolic emphasis.

As numerous workers have shown, the ratio of NH_3 to NH_4^+ excretion across the gill epithelium is subject to pH, pK′, temperature, ionic strength of the fluids involved, and the relative permeabilities of the gill, and possibly other tissues, for NH_3 and NH_4^+. After having taken all these parameters into consideration, Cameron estimated that the ratio of NH_3 to NH_4^+ may reach 32 : 1 or be as low as 1 : 9[7]; a typical value is 10 : 1. Consequently, ammonia excretion (as NH_3) depends largely on the blood to water p_{NH_3} gradient. According to the model of Wright and co-workers[88], one way by which this gradient is kept directed strongly outward is that CO_2 excretion acidifies the layer of water at the gill-water boundary, allowing for the effective removal of ammonia by protonation to ammonium. Their model is based on data showing that ammonia excretion is critically impaired at low environmental proton concentrations (high pH) in a number of teleostean fishes. Under these conditions, which shift the NH_3 : NH_4^+ equilibrium towards ammonia, the diffusion of endogenous ammonia as NH_3 from fish plasma across the gill to the surrounding water is hampered. In addition, the generally high buffering capacity of naturally alkaline lakes renders the lamellar exchange of NH_4^+ for Na^+ less effective since it partially depends on the fishes' ability to acidify the gill boundary layer. This effect reaches its zenith in the Lake Magadi tilapia (*Oreochromis alcalicus grahami*) where pH 10 and high environmental carbonate/bicarbonate combined render ammonia excretion useless and urea synthesis is necessary[56,87].

Although, generally, the renal excretion of ammonia is secondary to branchial ammonia release, it is possible that under specific environmental conditions, renal excretion of ammonia prevails. Such conditions may exist in *Chalcalburnus tarichi*, a cyprinid living in a low-soda, alkaline lake in Turkey, which on average excretes about 63% of its nitrogenous waste as ammonia[19] with urea-nitrogen making up the remainder. Although the environmental conditions are likely to impair, if not prevent, branchial release of ammonia (see above), it is possible that instead of detoxifying ammonia into urea prior to excretion – de novo synthesis of urea is absent in the tissues of *C. tarichi*[19] – these fish release ammonia directly via the kidneys or utilize an as yet unknown mechanism to release ammonia across the skin.

In spite of the fact that actual rates of ammonia release through renal pathways may be small compared with total release of ammonia across the gill or, as in the case of marine fishes, across the skin, renal production and release of ammonia may play an important role in acid base regulation. Teleosts can and do use renal ammonia production, mainly from amino acid amides, to remove and excrete protons[33].

Production of ammonia

Ammonia is generated in the liver through a few complementary pathways. The most important route is the oxidative transdeamination pathway, consisting of a variety

of specific transaminases (eq. 1) followed by glutamate dehydrogenase (eq. 2). Overall, these reactions lead to the oxidative production of ammonium ion from the alpha-amino function of most amino acids.This list includes serine which, in fish liver, is transaminated into hydroxypyruvate[84], although the more common serine dehydratase reaction generating pyruvate and ammonia may occur in minute quantities[76].

$$\text{Amino acid}_1 + \text{2-oxoglutarate} \leftrightarrow \text{2-oxo-acid}_1 + \text{glutamate} \quad \text{(eq. 1)}$$

$$\text{Glutamate} + \text{NAD(P)}^+ \leftrightarrow \text{2-oxoglutarate} + \text{NAD(P)H} + \text{H}^+ + \text{NH}_4^+ \quad \text{(eq. 2)}$$

In fish liver, as in vertebrate liver in general, glutamate dehydrogenase is localized in the mitochondrial matrix, while some of the transaminases occur in cytosolic as well as mitochondrial isoforms. The ammonium ion is liberated in the mitochondrial matrix and has to cross mitochondrial as well as hepatocyte membranes on its way into the bloodstream. Depending on the ionic species actually crossing the membranes, a proton may be carried out of the mitochondrial compartment or NH_3 may undergo protonation during exit.

Because glutamine occupies a pivotal role in mammalian nitrogen metabolism – which it does not in teleosts – it is not surprising, that fish researchers have given particular attention to this amino acid. In the absence of ornithine-urea cycle activity (see below), glutamine may serve as a precursor of ammonia in three, somewhat independent, reactions. Glutamine may be hydrolyzed to glutamate and ammonia (eq. 3) in a reaction catalyzed by a phosphate-dependent glutaminase, at times called 'glutaminase I'. The enzyme has been found to be active in fish liver, gill, brain and kidney[13, 79, 83].

$$\text{Glutamine} + \text{H}_2\text{O} \rightarrow \text{glutamate} + \text{NH}_3 \quad \text{(eq. 3)}$$

In an alternate reaction, also present in fish liver[4], consisting of gamma-glutamyl-transpeptidase, glutamate is transferred onto a non-specific acceptor with the concurrent release of ammonia:

$$\text{Glutamine} + \text{gamma-glutamyl-acceptor} \leftrightarrow \text{gamma-glutamylpeptide} + \text{NH}_3 \quad \text{(eq. 4).}$$

An additional pathway, catalyzed by glutamine-2-oxo-acid transaminase and ω-amidase, leads to the production of an amino acid, containing the original alpha-amino group of glutamine, and 2-oxo-glutarate as well as NH_3 derived from the glutamine amide. This sequence of reactions is sometimes called 'glutaminase II' and to date has been shown to be present in white skeletal muscle of cod (together with glutaminase I)[65].

The glutaminase responsible for eq. 3 is an enzyme of the inner mitochondrial membrane. The other three reactions are catalyzed by cytosolic enzymes. In addition, fish liver displays the enzymatic complement to deal in a selective fashion with other specific amino acids. Histidine, for instance, is degraded to urocanate and ammonia by histidase[62], while asparaginase removes the amide nitrogen (as ammonia)[79], in a reaction analogous to that catalyzed by glutaminase I (cf. eq. 3).

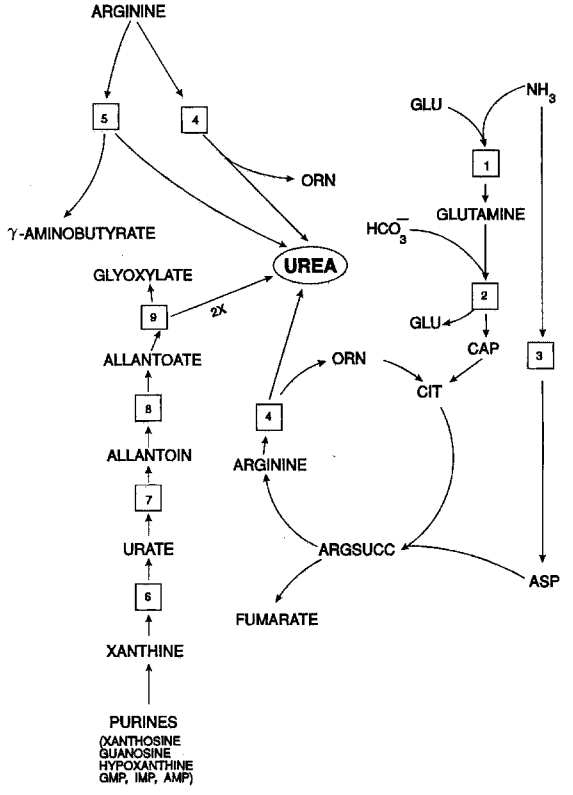

Figure 2. Metabolic routes potentially leading to the production of urea in fish tissues. Three different pathways are presented: Arginine breakdown, purine degradation and synthesis by the ornithine-urea cycle. Key enzymes involved are: 1. Glutamine synthetase; 2. Carbamoylphosphate synthetase (CPS III); 3. Glutamate dehydrogenase followed aspartate aminotransferse (cf. eq. 1 and 2); 4. Arginase; 5. The γ-guanidino urea hydrolase pathway; 6. Xanthine oxidase; 7. Urate oxidase; 8. Allantoinase; 9. Allantoicase. ATP-consuming reactions in the ornithine-urea cycle are: synthesis of glutamine from glutamate and ammonia (1 ATP), synthesis of carbamoylphosphate from bicarbonate and glutamine (2 ATP), and synthesis of argininosuccinate from citrulline and aspartate (2 ATP). Abbreviations: Argsucc-argininosuccinate, Asp-aspartate, CAP-carbamoylphosphate, Cit – citrulline, Glu – glutamate, Orn – ornithine.

Fish liver also comprises the metabolic machinery to liberate ammonia from additional nitrogenous substrates. A combined carp liver/intestine preparation, for example, rapidly liberates ammonia when adenosine, guanosine, GMP or ATP are added to the perfusate (cf. fig. 2). These compounds, together with amide groups of glutamine and asparagine, are much better precursors for ammonia than any of the common amino acids. The inclusion of adenine or AMP into the perfusate leads only to minor increases in ammonia production[81]. Considering the abundance of adenosine in fish blood, its potential role as a tissue hormone and the presence of highly specific adenosine receptors on fish tissues[64], the ability of intestine/liver to deaminate adenosine efficiently is intriguing and certainly deserves more in-depth study.

Although the fish liver is known to display AMP deaminase activity, an enzyme generating ammonia from adenylate (eq. 5) and forming an integral part of the purine nucleotide cycle[38], this activity is dwarfed by the activity of this enzyme in fish white muscle. As the above perfusion studies already indicate, from an ammoniogenic point of view, AMP deaminase activity in fish liver is trivial.

$$AMP + H_2O \rightarrow IMP + NH_3 \qquad \text{(eq. 5)}$$

Therefore the purine nucleotide cycle fails to contribute to hepatic ammonia production[9,78].

Once generated by the liver, the bulk of the ammonia is cleared from the circulatory system by the gill[69]. In this context, however, the word 'bulk' has to be taken with a grain of salt, since in some teleosts, especially marine species, ammonia excretion across the skin or through the urine may play more than just an ancillary role[50,63]. In five species of marine teleosts from different families and representing different life-styles, from intertidal to benthic, the latter two processes may account for more than half of the ammonia-nitrogen excreted. Working on an air-breathing, ammonia-resistant catfish (*Heteropneustes fossilis*) and pointing out potential differences in branchial *versus* renal nitrogen excretion, Saha and coworkers[60] found that the ratio of ammonia-nitrogen over urea-nitrogen excreted by the gill is 10, while it is only 0.12 for urine. As expected for a freshwater species and, substantiating the early results of Homer Smith[69], gill accounted for over 99% of the ammonia nitrogen excreted[60].

Catabolic urea production and urea excretion

In contrast to the production of ammonia, which usually can be traced back to individual enzymes or small assemblies of enzymes (cf. eq. 1–5), the metabolic routes leading to the availability of urea in plasma and excretory products of teleostean fishes are more indirect. Pathways potentially leading to the production of urea include the degradation of purines, such as adenine, inosine, guanine and xanthine into uric acid, followed by uricolysis, routine turnover of arginine, and synthesis through the ornithine-urea cycle (fig. 2). Although purines and arginine are normal components of any fish diet, the data presented in figure 1 contradict the notion of immediate urea production through the above routes. Rather, the constant, feeding- and also oxygen uptake-independent rate of urea excretion in the sockeye salmon[6] is likely a reflection of maintenance metabolism (fig. 1): the turnover of endogenous arginine and nucleic acids. It is not overly surprising that the guanidino group of arginine is not immediately committed to excretion as urea, since arginine constitutes an essential amino acid for most teleostean fishes, including the sockeye salmon[73].

In most teleostean fishes, the enzymes of the uricolytic pathway are ubiquitous and their activities in liver are substantial[17,28], especially compared with the minute – if they are present at all – activities of urea cycle enzymes[15,29,86]. Therefore, it is always assumed that urea released by largely ammoniotelic species is, indeed, derived through uricolysis, with key enzymes localized in hepatic peroxisomes[28]. Accordingly, the entire pathway from de novo synthesis of purines through uricolytic degradation could be demonstrated in carp[81]: after injection with ^{14}C-formate, which should be incorporated into positions 2 and 8 of the purine ring, carp plasma, liver and faeces contained labelled urea (cf. fig. 2). No incorporation of label was found for fish which had received labelled bicarbonate instead, substantiating the absence of a functional urea cycle in this species. This rather involved route of urea formation from purine degradation or even preceded by de novo synthesis of the entire purine ring, may explain the lack of feeding-dependent variability in urea excretion in the sockeye salmon[6]. The recent description of an active carbamoyl phosphate synthetase II (CPS II) in largemouth bass (*Micropterus salmoides*)[10] further substantiates the notion that the teleostean liver has a significant capacity to synthesize (and degrade) purines. The CPS II required for purine synthesis is a cytosolic enzyme, utilizing glutamine as the nitrogen donor and showing no dependence on *N*-acetylglutamate as a cofactor. However, not all purines synthesized or landing on the scrap-heap of metabolism from routine turnover, are voided from the fish's body as urea. An alternative, and at some life stages possibly quantitatively important, sink for purines is their deposition in fish skin. During smoltification in Atlantic salmon (*Salmo salar*), for instance, substantial amounts of guanine and hypoxanthine are deposited in skin and scale layers[31], resulting in increased silvering of the animals. Two alternative routes potentially lead to the catabolic production and excretion of urea. Both routes involve the guanidino function of arginine as the source of urea. The first is hydrolysis into urea and ornithine, catalyzed by the ubiquitous arginase[11,18,47,61,66], while the alternative route steps through 2-oxo-delta-guanidinovalerate and gamma-guanidinobutyrate en route to yielding urea and gamma-aminobutyrate (cf. fig. 2). The key enzyme in this pathway, the gamma-guanidino urea hydrolase (EC 3.5.3.7), is, in fact, more active in fish liver and kidney than arginase[80]. Unfortunately, the actual relative contribution of these two routes to arginine degradation remains to be elucidated.

Sites of urea excretion

The site of urea release varies among species and depends on such factors as environmental salinity, branchial urea permeability and urine production rates. Potentially, it can be voided through gills, skin, urine and faeces, or any combination of these four sites. Although largely branchial excretion was identified for freshwater species, a more recent study of five marine teleosts – in which

urea accounted for less than 20% of the nitrogenous waste[63] – showed that release across the skin or via the urine was two – to three-fold higher than branchial excretion. The potential separation between sites for ammonia and urea excretion is exceptionally well illustrated in an air-breathing catfish (*H. fossilis*), which releases more than 99% of its waste ammonia across the gill, while renal and urinary release accounts for more than 25% of total urea discharge[60]. As stated in the onset, urea normally makes up less than 40% of the nitrogenous waste and may build up in fish plasma and tissues to much higher concentrations (1–10 mM) than the toxic ammonia. This partial accumulation may indicate that voiding the system is not quite as straightforward as expected, and ultimately, it is possible that the molecule functions as a minor osmolyte in teleostean fishes. In contrast to the continuous release of ammonia, the excretion of urea may be intermittent[26,82]. Ureogenic toadfish, for instance, temporarily store urea in the bladder before pulsatile release into the surrounding water[82].

Urea is quite evenly distributed throughout the fish's body, since it readily crosses internal membranes. There are some restrictions to the free movement of urea, however, mainly pertaining to gill and membranes associated with the kidney. Even though elasmobranch fishes utilize urea as the important intracellular osmolyte, the elasmobranch kidney excretes urea. Nonetheless, a certain portion of urea is subsequently reabsorbed by the kidney tubules.

It is possible that the mere measurements of urea excretion in fishes will conceal the true rates of internal urea production. In marine elasmobranchs, which possess internal levels of urea in excess of 200 mM, a certain degree of urea destruction may occur internally. In sharks, this process is mediated by microbial processes in several tissues as well as in the lumen of the gastrointestinal tract[34]. In the teleost toadfish, which may achieve plasma urea concentrations in the 10 mM range and bladder concentrations approaching 20 mM, similar urea destruction occurs only in the intestine. The overall contribution of this microbial process to urea recycling and urea turnover in toadfish seems to be minor, since wholesale removal of gut bacteria by antibiotic treatment fails to increase the rate of urea excreton[82]. Nevertheless, it should be kept in mind that in some mammalian systems, similar processes in the gut may lead to the recycling of some 20% of the internal urea[27,57]. When dealing with aquatic organisms, it is also conceivable that microbial activity of the ambient water may lead to the hydrolysis of excreted urea resulting in artificially low rates of urea excretion.

Ammonia toxicity

Ammonia may be introduced into the environment through a number of different routes, which include runoff from industrial or agricultural processes, sewage effluent, and production by organisms, as for instance in the case of high density fish farming. In aqueous solution, ammonia exists in an equilibrium between its unionized form (NH_3) and the ammonium ion (NH_4^+). The relative concentrations of NH_3 and NH_4^+ in aqueous solutions is not only determined by the pK_a of ammonia (9.5), and thus strongly dependent on the hydrogen ion concentration, but also influenced by temperature, pressure and the concentrations of other ions[21]. Toxicity to aquatic life is largely due to NH_3, while NH_4^+ is only a minor contributor to the toxic events reported for ammonia. However, to further complicate the relationship between different parameters, the toxicity of NH_3 to fishes may increase as ambient pH is decreased.

Exposure of teleostean fishes, even to non-lethal levels of NH_3, has numerous effects, ranging from pathological changes[22,67], through general stress effects[71,74], compromised food intake and growth[5], modified amino acid metabolism[2,37], altered oxygen delivery[70], and enzyme induction (Mommsen & Walsh, unpubl.) to the impairment of ion-exchange across the gill[16]. In addition, chronic ammonia exposure leads to indirect metabolic effects by increasing the levels of cortisol[71], a generalized stress hormone in fishes[53], in the circulatory system. Chronically increased cortisol levels, in turn, have been shown to significantly increase the number beta-adrenoceptors in rainbow trout hepatocytes[58], likely rendering the fish more sensitive to short-term stress. As well, the fish are increasingly susceptible to diseases due to the immunosuppressive effects of the corticosteroid[53].

Although teleostean fishes are generally considered to be more tolerant to ammonia than other vertebrates, a rather large range exists for actual ammonia tolerances. Rainbow trout, for instance, quickly develop convulsions and die if exposed to 36 μM NH_3[2]. Gulf toadfish, in contrast, survive exposure to 20 μM NH_3 indefinitely[82]. Another member of the toadfish family (Batrachoididae), the plainfin midshipman (*Porichthys notatus*), can survive 150 μM NH_3 for at least 10 days (Mommsen & Walsh, unpubl.), while a species of tilapia (*O. alcalicus grahami*) living in an alkaline soda lake can withstand half-millimolar levels of NH_3 without any apparent ill effects[87].

Ammonia detoxification

Whereas the toxic effects of exogenous ammonia to fish have been known for a long time, the exact mechanisms of action and the particular physiological parameters affected are still under debate. Some authors implicated NH_3-induced acidosis or NH_3-dependent hemolysis. The current consensus view appears to be that neural effects are at the root of NH_3 toxicity. Not unexpectedly, the fish brain is the tissue undergoing the largest and most uniform metabolic change. Several mechanisms are invoked to protect the brain from ammonia's toxic effects

and thus to remove this toxicant from the brain. In rainbow trout exposed to increasing levels of unionized NH_3, the most pronounced change is an increase in cerebral levels of glutamine at the expense the glutamate pool [2, 37] concomitant with an overall increase in the glutamate/glutamine pool. Most likely, ammonia entering the brain cells is promptly removed into glutamine by the activity of glutamine synthetase, a cytosolic enzyme with a binding constant for ammonia in the micromolar range (cf. eq. 6). The absolute dependence on ATP as a cofactor is reflected in the fact that increases in tissue glutamine are accompanied by a decrease in cerebral ATP levels.

$$\text{Glutamate} + \text{ATP} + NH_3 \leftrightarrow \text{glutamine} + \text{ADP} + \text{phosphate} \qquad \text{(eq. 6)}$$

In fact, brain tissue contains the highest concentration of glutamine synthetase. Other tissues do not enjoy such protection, and incur increases in intracellular ammonia, while only minor increases in tissue glutamine are observed [2, 30], observations that are congruent with the relatively lower titers of glutamine synthetase in non-cerebral tissues of non-ureogenic fish species [12, 13, 47, 68, 85]. At least in the rainbow trout, however, the rate of ammonia removal from the brain by glutamine synthetase is not rapid enough to prevent an increase in brain ammonia entirely under conditions of ammonia exposure [2].

Interestingly, in addition to the expected high activity of glutamine synthetase, the brain of an extremely ammonia tolerant mudskipper (*Periophthalmus cantonensis*), also contains 10-fold higher levels of glutamate dehydrogenase than five intolerant congeners [30]. It appears therefore that at least in this mudskipper, brain glutamate dehydrogenase functions in the direction of glutamate formation (cf. eq. 2) to complement the ammonia detoxification capacity of the brain supplied by glutamine synthetase. A similar mechanism, although not based on the observed activity of glutamate dehydrogenase, must be postulated for the rainbow trout brain, where the size of the glutamate/glutamine pool increases during ammonia exposure. This unusual situation of a potential two-step amination/amidation of 2-oxoglutarate into glutamine is intriguing from different angles. First, the activity of glutamate dehydrogenase (and, in fact, its direction) is known to be controlled, inter alia, by the redox state of the mitochondrion, while glutamine synthetase activity is controlled in vivo by the availability of cytosolic ATP. Not only do the two enzymes remove NH_3/NH_4^+ from different cellular compartments, but to be able to work in tandem to detoxify ammonia, the enzymes also have to rely on a constant supply of 2-oxoglutarate. The latter will have to be bled off the Krebs cycle, rendering an analysis of anaplerotic reactions in brain tissue an interesting topic. Further, the enzymes are dependent on an uninterrupted transport of glutamate from the mitochondrion – the location of glutamate formation by glutamate dehydrogenase – to glutamine synthetase. Brain glutamine synthetase is exclusively located in the cytosolic compartment. It is the concept of an efficient glutamate transporter in the mitochondrial membrane coupled with control of glutamate dehydrogenase which would make an excellent model system to study the interaction between glutamate dehydrogenase and glutamine synthetase.

In the short term, the fish brain accumulates glutamine during an ammonia insult, and it is conceivable that the tissue will increasingly export glutamine into the systemic circulation during and following the insult. The tissue most likely to deal with glutamine is liver, be it for generation and excretion of ammonia when the insult has subsided or for formation and excretion of urea – should the capability exist (see below). However, considering the limited size and perfusion of the fish brain, this tissue is prone to contribute an insignificant fraction to the total ammonia/urea output of the fish. Besides, the overall ability of the brain to detoxify ammonia is small [2] and thus temporally finite, and may be limited by the relative capacity to deliver 2-oxoglutarate and to remove glutamine. As long as both processes are unknown entities, we prefer to refrain from speculating on the role of the fish brain as an ammonia sink for the whole animal.

Urea synthesis

An entirely different approach to longer-term ammonia detoxification or to the production of nitrogenous wastes is observed in some specially adapted teleostean fishes. This strategy is found in species that may routinely encounter conditions of erratic water availability, when dilution of toxic ammonia into the surrounding water is clearly finite, or when the external conditions negatively impact ammonia excretion. At present, this growing list includes representative species of clingfishes, toadfishes, catfishes, gouramis, mudskippers, snakeheads, frogfishes (?), a tilapia and several air-breathers. The African lungfishes also belong into this rather heterogeneous group. All these animals may be able to switch to preferential production of urea during periods of water limitation or they may be permanently ureogenic. However, instead of using urea liberated from the catabolism of purines or endogenous arginine, these fishes synthesize urea de novo [1, 8, 47]. They do so by use of the ornithine-urea cycle. Because of the substantial metabolic cost involved – about 5 high-energy phosphates are expended for two nitrogens fixed (cf. fig. 2) – it is usually assumed that aquatic fishes resort to this metabolic expenditure only under extreme physiological conditions. Thus, these species avoid the long-term buildup of toxic NH_3, while, as usual, the brain is protected from short-term impacts by the detoxification mechanisms outlined above.

Just as in mammals, the ornithine-urea cycle of fish liver is a compartmentalized pathway, but interesting differences exist in enzyme characteristics and compartmentation between the teleostean fishes, the coelacanth and the elasmobranch fishes ('fishes') on one side and the lung-

fishes, amphibians and mammals on the other. The first, committing, step of the cycle is the intramitochondrial synthesis of carbamoyl phosphate by carbamoyl phosphate synthetase (CPS). 'Fishes' possess a glutamine- and *N*-acetylglutamate-dependent CPS [8,47,48], the so-called CPS III [1] depicted in figure 2, while the CPS I of lungfishes and other vertebrates is inactive in the absence of ammonia and *N*-acetylglutamate. The last enzyme of the cycle, arginase is mitochondrial in 'fish' liver, but cytosolic in other vertebrates. Although arginase is common in fish liver, its activity tends to be scaled up in air-breathing, potentially ureogenic, species [66]. These differences in enzyme localization have repercussions on transport phenomena associated with the cycle. In fishes, citrulline (out), arginine (in) and urea (out) will have to cross the mitochondrial membrane in the course of one spin of the cycle, while in the other vertebrates, including the lungfish, urea is liberated in the cytosol, and ornithine (in) and citrulline (out) have to be shunted across the mitochondrial membrane. The biochemical differences have been exploited to show that in the vertebrate line, the ornithine-urea cycle is a monophyletic trait. In the course of vertebrate evolution, the cycle has undergone only a few, biochemically minor, changes which occurred most likely before the evolution of the lungfishes [47,48].The inferred positioning of the lungfishes, rather than the coelacanth, in proximity to the amphibians has since been supported by studies using molecular biology techniques [41].

An additional function of the ornithine-urea cycle, and possibly its ancient function, is the de novo synthesis of arginine from ornithine. A radiotracer study concluded that rainbow trout under different diet regimes can synthesize arginine from ornithine and citrulline [15]. However, actual rates of arginine production were minute, thus substantiating a previous study analyzing urea synthesis from bicarbonate in this species [20]. In addition, CPS activities were scaled up in fish fed a diet devoid of protein, supporting a role of the cycle in production of arginine, although the concurrent increase in kidney arginase is somewhat counterintuitive in this context [15]. Intriguingly , replacing 50% of dietary arginine with glutamate led to significantly reduced growth rates, while replacement with ornithine or citrulline depressed growth rates only slightly or not at all. In theory, glutamate carbon can be shunted into ornithine, but the pathway has not been analyzed for fishes and, as the above results indicate, is unlikely to be of physiological importance. This hypothesis is backed by the fact that ornithine is considered an essential amino acid for most teleostean fishes. It would be interesting to analyze whether ureogenic species also require dietary ornithine (or arginine for that matter), although overall the function of ornithine in the cycle is merely catalytic while other pathways, such as polyamine synthesis or transamination into delta [1]-pyrroline-5-carboxylate during oxidation, will result in actual decreases in the cellular ornithine pool. However, it should be kept in mind that substitution of arginine with other amino acids not only decreases the availability of an alleged essential amino acid [73], but also of one of the most potent insulinotropic substances in fishes [59]. Therefore, secondary growth effects due to altered insulin titers in treated fish cannot be dismissed. In addition, as recently shown for non-piscine vertebrate systems, arginine may also serve as the immediate precursor of nitric oxide, a potentially important intracellular messenger [23].

Ureogenesis versus ammoniogenesis

Although the regulatory mechanisms of the switch to ureogenesis from ammoniogenesis need to be clarified, many fishes follow a similar pattern: during their aquatic phase, ammonia is the major nitrogenous excretory substance, while under conditions of reduced access to water, ammonia is detoxified through synthesis of innocuous urea and, subsequently, urea makes up the bulk of the excretory nitrogen. Air exposure of the Chilean clingfish (*Sicyases sanguineus*) amply illustrates this strategy (table [25]).

Similarly, exposure of Gulf toadfish (*Opsanus beta*) to increasing concentrations of ambient ammonium chloride leads to increased urea synthesis and excretion (fig. 3). When analyzing air exposure in *O. beta*, possibly

Ammonia and urea excretion in Chilean clingfish (*Sicyases sanguineus*) in water and following air exposure. Values are given in mMoles N excreted per kg of live weight in 1 h ± SEM at 20° C for six observations. Recalculated from Gordon et al.[25].

	Urea	Ammonia (% of total)
In water	0.76 ± 0.10	0.27 ± 0.02 (26)
After 20 h air exposure	2.62 ± 0.28	0.21 ± 0.02 (7)
After 36 h air exposure	8.58 ± 1.04	0.16 ± 0.02 (2)

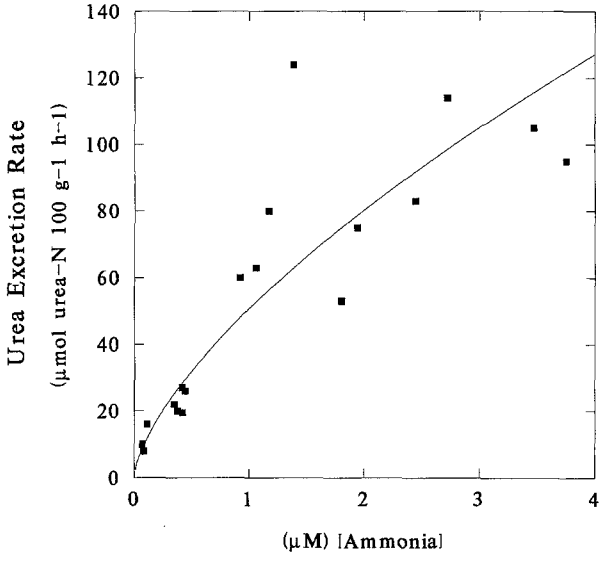

Figure 3. Effects of external NH_3 on urea excretion in the Gulf toadfish (*Opsanus beta*). Urea excretion was measured over 24 h. Water was changed every 6 h to keep control levels of ammonia at a minimum. Adapted from Walsh et al.[82].

a common metabolic challenge for toadfish which spawn and live around rocks in the intertidal areas, we noticed that fish could be assigned to two different groups depending on the prevailing metabolic strategy. Specimens which prior to air-exposure released more than 90% of their nitrogenous waste as urea, did not change the relative composition of nitrogenous waste after 8 h of air exposure. Specimens which in their aquatic phase preferentially released ammonia (90%), drastically increased the proportion of excretory urea (51%) after air exposure [82]. Interestingly, at least in the toadfish, few regulatory sites of the urea synthetic pathway could be identified. Of all urea cycle and ancillary enzymes analyzed only hepatic glutamine synthetase appeared to be inducible by administration of hormones [44]. Toadfish do not appear to alter their metabolic rate during air-exposure. Nevertheless, it can be postulated that some species may detoxify invading ammonia in the brain but otherwise may last through such adverse conditions by down-regulation or ceasing of metabolic activity.

When exposed to levels of ammonia in the 0.15 mM range, goldfish increase the rate of urea excretion by four- to fivefold, while no such increase is noted in rainbow trout [51]. Taking into account that activities of urea cycle enzymes are low or non-existent in the liver of these two species and that goldfish urea excretion is independent of previous ammonia exposure, argininolysis and purine degradation – or synthesis followed by breakdown – are the most likely sources of excretory urea.

Unfortunately, there is a paucity of data on the mechanisms by which ammonia or urea production can be regulated in fishes. Some results indicate that glucagon, which increases the rate of gluconeogenesis from amino acids in fishes [72], leads to increased release of ammonia into the surrounding medium [14], a phenomenon that is also observed in isolated toadfish hepatocytes [44]. In addition, overall utilization of amino acids by liver is increased in vitellogenic fish [35]. Possible repercussions of this strategy on nitrogen excretion need to be elucidated.

One special condition concerning the movement and excretion of ammonia is the case of developing embryos, where diffusion of metabolic end-products may be limited [75]. Preliminary data indicate that developing embryos detoxify ammonia into urea and display relatively high rates of bicarbonate incorporation into urea, an ability that is decreased or lost in larvae and adults [20].

Vehicles for nitrogen transport

In spite of the fact that glutamine can serve as a prime substrate for a number of fish tissues, including liver and gill, resulting in ammonia production from the amide as well as alpha-amino groups, ammonia is usually considered as the most important carrier molecule for nitrogen. This applies to nitrogen shuttling between tissues and, as shown above, vectorial transport from liver to gill. One weakness of this conjecture is that it is partly based on the rejection of glutamine as universal nitrogen vehicle, rather than being focussed on ammonia. At any rate, the accepted irrelevance of glutamine in this function clearly sets the fishes apart from other systems, especially the mammals where glutamine plays such a central, multifacetted role. During starvation, when teleostean as well as elasmobranch fishes break down muscle protein [36, 45], alanine and ammonia concentrations in plasma rise, while glutamine concentrations remain unaltered. Additionally, in contrast to mammalian systems, where muscle is characterized by its ability to export glutamine, inter alia expressed through a high ratio of glutamine synthetase to glutaminase, glutamine synthetase activities in fish muscle are small to insignificant and dwarfed by glutaminase activities [13]. Further, hepatic glutamine synthetase is prominent in ureogenic fishes only where it functions as a feeder enzyme to (glutamine-dependent) CPS III [47, 85], clearly identifying liver and not (as in mammals) extrahepatic tissues as sources of glutamine (cf. fig. 2). Also, the enzyme is distributed homogeneously over liver cells of varying density [49] and subject to hormonal control [43]. Both details are indicative of glutamine's central role to urea synthesis and imply that nitrogen delivery to the liver is as ammonia. In mammalian liver glutamine synthetase is exclusively localized in perivenous hepatocytes and thus serves to prevent efflux of ammonia from the tissue. Although fish brain contains substantial amounts of glutamine synthetase (see above), and thus may release glutamine into the circulation, its contribution to glutamine turnover is likely minute. More direct evidence against glutamine as nitrogen carrier is supplied by an analysis of blood amino acids simultaneously drawn from dorsal aorta and hepatic portal vein in rainbow trout. In fasted fish, glutamine incurs no changes in concentration [3]. These data indicate that glutamine was not supplied by muscle or by intestine in the post-absorptive state, while portal blood is clearly enriched in alanine which accounted for almost 50% of the amino acid increase observed between the two sampling sites. Even 3 h following feeding (cf. fig. 1), glutamine accounted for less than 2% of the amino acid increase observed [3], ruling out glutamine as a nitrogen or carbon carrier from gut to liver. It can be concluded that the contribution of glutamine to nitrogen transport is trifling, although direct analysis of glutamine turnover vis-à-vis turnover of other amino acids (alanine, glutamate, etc) and ammonia, is still outstanding.

What, then, are the internal sources, other than hepatic processes, for ammonia in fishes? First, under conditions of intermittent burst-type exercise, muscle has the potential to deliver ammonia into the circulation, due to the substantial activity of AMP deaminase (eq. 7) [55]. Since the enzyme, which is under tight control in fish muscle [54], produces NH_3, it can serve to reduce acidification during periods of high anaerobic energy production by muscle [46]. The NH_4^+ formed is largely released into the bloodstream [42, 46], although it is not clear how much of

the ammonia derived from muscle is actually excreted, since a nitrogen donor (aspartate) is required to replenish the adenylate pool of white muscle during recovery from exercise. Second, in vitro, glutamine is the best oxidative substrate for bowfin (*Amia calva*) and char (*Salvelinus namaycush*) red muscle mitochondria[13]. In both species, red muscle contains high amounts of glutaminase, and only minor levels of glutamine synthetase. The resulting high ratio of glutaminase over glutamine synthetase (200 in bowfin, 50 in char) is reminiscent of that in mammalian tumor cells which are known to oxidize glutamine at high rates[39]. In contrast, mammalian muscle synthesizes glutamine and subsequently releases it into the blood, a situation that is manifested in similar titers of glutaminase and glutamine synthetase, and the fact that isolated muscle mitochondria clearly prefer glutamate to glutamine as oxidative substrate[13]. However, considering the limited availability of glutamine within fish tissues and the insignificant difference in glutamine levels between hepatic portal vein and dorsal aorta, it seems unlikely that glutamine serves as an important in vivo source of muscular ammonia.

It should be pointed out that the above dualism between glutamine versus ammonia may be somewhat artificial. In addition to ammonia, amino acids may make a sizeable contribution to nitrogen transport in fishes. Two amino acids come to mind: alanine and asparagine. Although asparagine should be considered, because it is an excellent oxidative and gluconeogenic substrate for liver[13,24], its blood dynamics and extrahepatic concentrations tend to disqualify it. Alanine must be mentioned for four compelling reasons: first, because alanine can be, and apparently is, synthesized intramuscularly from almost all amino acids[36,45]; second, subsequent to its production in muscle, alanine is shunted into the bloodstream[36,45]; third, alanine makes the single most important contribution to the amino acid increase observed in one pass between dorsal aorta and hepatic portal vein[3]; and fourth, because it is a prime substrate for numerous hepatic processes[35,72].

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Research Articles

Structural characteristics and distribution of satellite cells along crayfish muscle fibers

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Abstract. The distribution of satellite cells (sc) in long-sarcomere muscle fibers from the carpopod extensor muscle of the crayfish (*Astacus fluviatilis*) has been studied electron-microscopically. The sc are spindle-shaped and are oriented parallel to the long axis of a fiber. The mean lengths of sc nuclei (17.00 μm) and that of myonuclei (18.35 μm) differ non-significantly. In older animals, the mean ratio of the number of sc nuclei to the total number of nuclei (sc nuclei + myonuclei) is 0.0716, 0.0848, and 0.034 for the tendon, central and shell segments, respectively. The corresponding values for younger animals are 0.158, 0.166, and 0.081. The mean numbers of sc nuclei per mm of a fiber are 94, 117, and 47 (older animals), and 164, 117, and 94 (younger animals) for the tendon, central and shell segments, respectively. The high incidence of sc per unit fiber length in crayfish may be related to the fact that crayfish muscle fibers have a much larger diameter than vertebrate muscle cells.
Key words. Crayfish muscle; satellite cells.

One of the most important problems in the study of muscle regeneration concerns the origin of myogenic cells. The results obtained from vertebrate skeletal muscle[1–4] have shown that myogenic cells originate from activated satellite cells. Satellite cells are mononuclear cells located between the external lamina and the plasma membrane of muscle fibers[5]. Their number, size and form vary in the course of muscle development[6, 7], and they are assumed to represent the stage of dormant or resting myoblasts. It is known that after local damage to muscular tissue in vertebrates mitotic activity of satellite cells is induced, with migration of the dividing cells from undamaged regions to the site of injury[8]. The mechanism of activation of satellite cells is as yet unknown.

In addition to satellite cells, a supplementary source of myogenic cells has been suggested in a study on regeneration of crustacean (crayfish) muscle fibers following mechanical injury[9]. Hemocytes, after penetration into damaged areas, undergo gradual transformation manifested in cell apposition, degranulation and the appearance of contractile filaments. The transformation of hemocytes has been assumed to be induced by activated satellite cells present in degenerated material.

To test this hypothesis, and to study systematically the process of myogenic transformation of satellite cells, basic data concerning structural characteristics and distribution of satellite cells along crayfish muscle fibers are necessary. The findings of the present study have already been reported in a preliminary form[10].

Materials and methods

For quantitative estimation of satellite cells, long-sarcomere muscle fibers from the carpopod extensor muscle of the crayfish (*Astacus fluviatilis*) were used. Two groups of animals of different ages were investigated: a) 12-cm-long crayfish (approx. 5 years old) and b) 6-cm-long crayfish (approx. 2 years old).

Following amputation of the first cheliped and opening of the shell, the muscle was exposed for 2 × 3 min to the fixative solution, 2% glutaraldehyde and 0.05% OsO_4 in 0.15 mol/l sodium cacodylate (pH 7.4)[11], and then fixed for 60 min in 2% glutaraldehyde in cacodylate buffer. The muscle fibers were then teased apart in a buffer solution to obtain single fibers. After post-fixation in 1% OsO_4 for 30 min and staining with 2% aqueous uranyl acetate overnight, the fibers were dehydrated in an