UREOTELISM IS INDUCIBLE IN THE NEOTROPICAL FRESHWATER *Hoplias malabaricus* (TELEOSTEI, ERYTHRINIDAE)

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ABSTRACT

Increased environmental pH decreases ammonia transport through the gills, impairing nitrogenous waste. The consequent toxicity is usually drastic to most fishes. A few species are able to synthesize urea as a way to detoxify plasma ammonia. We studied three teleosts of the family Erythrinidae living in distinct environments, and assumed the biochemical behaviors would be different in spite of their being closely related species. Adult fish collected in the wild were submitted to alkaline water and the urea excretion rate was determined. The specific activity of urea cycle enzymes was determined in liver samples of fish from neutral waters. The studied species *Hoplias lacerdae*, *Hoplerithrynus unitaeniatus*, and *Hoplias malabaricus* are ureogenic. Urea synthesis is not a metabolic way to detoxify ammonia in *H. lacerdae* and *Hoplerithrynus unitaeniatus* exposed to an alkaline environment. The plasma ammonia profile of both species showed two distinct biochemical responses. Urea excretion of *H. malabaricus* was high in alkaline water, and the transition to ureotelism is proposed. The nitrogen excretion rate of *H. malabaricus* was among the highest values reported and the high urea excretion leads us to include this species as ureotelic in alkaline water.

Key words: ureotelism, ammonia, toxicity, urea cycle, *Hoplias malabaricus*.

RESUMO

Ureotelismo é indutível em *Hoplias malabaricus* (Teleostei, Erythrinidae)

O aumento do pH ambiental diminui o transporte de amônia pelas brânquias, prejudicando a saída de nitrogênio. A conseqüente toxidade é normalmente drástica para muitos peixes. Poucas espécies são capazes de sintetizar uréia como forma de detoxificar a amônia plasmática. Estudamos três telósteos da família Erythrinidae que vivem em ambientes distintos e apresentam respostas bioquímicas diferentes apesar de serem espécies extremamente próximas. Peixes adultos foram coletados no ambiente, sendo submetidos a águas alcalinas, e o grau de excreção de uréia foi determinado. As atividades específicas das enzimas do ciclo da uréia foram determinadas em amostras de fígado de peixe de águas cujo pH é neutro. As espécies estudadas, *Hoplias lacerdae*, *Hoplerithrynus unitaeniatus* e *Hoplias malabaricus*, são ureogênicas. A síntese de uréia não é a rota metabólica utilizada para detoxificar a amônia em *H. lacerdae* e *H. unitaeniatus* expostas a ambiente alcalino. O perfil da amônia plasmática em ambas as espécies apresentaram duas respostas bioquímica distintas. A excreção de uréia em *H. malabaricus* foi superior em águas alcalinas, e transição para o ureotelismo é proposta. O grau de excreção nitrogenada em *H. malabaricus* foi superior aos valores citados, e o aumento da excreção de uréia sugere que essa espécie é ureotélica em águas alcalinas.

Palavras-chave: ureotelismo, amônia, toxidade, ciclo da uréia, *Hoplias malabaricus*.

INTRODUCTION

Ammonia is the main nitrogenous excretion product of freshwater teleost fishes. Its waste basically occurs through the gills, and the ammonium ion NH_4^+ prevails over the NH_3, the practically impermeable form. Decrease of H^+ impairs nitrogen waste by increasing the NH_3/NH_4^+ ratio. Consequently, the increase of water pH raises plasma ammonia, leading to drastic physiological effects. To prevent such toxicity, a mechanism for clearing the wasted nitrogen must be triggered. Among the ways to detoxify ammonia, urea synthesis and excretion are independent of environmental pH. This biochemical pathway has been reported in a few teleosts living in alkaline lakes (Randall *et al.*, 1989). However, independently of the presence of urea cycle (UC) enzymes and the regular production of urea, the fish are usually ammoniotelic (Wood, 1993; Chadwick & Wright, 1999). Functional UC enzymes characterize the ureogenic species (Randall *et al.*, 1989; Saha & Ratha, 1987, 1989; Mommsen & Walsh *et al.*, 1989, 1991, 1992). However, only the nitrogen excretion pattern in ureogenic species makes it possible to establish ureotelism in fish, and to infer something about the strategy which detoxifies plasma ammonia.

Intensive photosynthetic activity usually raises environmental pH in numerous freshwater eutrophic systems in South America (Val & Almeida-Val, 1995). Organisms living in such environmental conditions should present metabolic strategies to overcome consequent disturbances. The freshwater teleosts *Hoplias malabaricus* (trahira), *Hoplias lacerdae* (trairão), and *Hoplerithrynus unitaeniatus* (aimara) belong to the family Erythrinidae, live in distinct habitats, and behave differently (Godoy, 1975). Trahira is usually found in lagoons and ponds, resisting very hard conditions such as hypoxia, dryness, and pH oscillations (Godoy, 1975; Wood & Lenfant, 1984). In contrast, trairão live in rivers of lotic waters and under more stable conditions. Aimara is commonly found in small rivers and lagoons. It usually slithers overland, using its fins to move to more convenient waters (Kramer *et al.*, 1978).

Do close related species living in distinct aquatic environments present different biochemical responses to cope with the same stressor? Could there be expected among them similar kinds of biochemical strategies in the face of environmental changes? Regardless of those three Erythrinids being phylogenetically related, we expected to find specific biochemical behaviors distinguishing them. The frequency of exposition to environmental pH oscillations probably differs among the there. Therefore, the UC enzyme pattern was studied to verify the capacity of those species to synthesize urea. Changes in the plasma nitrogen profile, and the urea and ammonia excretion rate were compared when the species were exposed to alkaline and acid water. Ureotelism, a rare biochemical feature in fish, is reported as the strategy of H. malabaricus for coping with environmental alkaline water.

MATERIAL AND METHODS

Adult H unitaeniatus and H. malabaricus, ranging from 20 ± 5 g (means ± SD) to 90 ± 10 g respectively, were collected from shallow ponds on the shore of the Mogi-Guaçu River (21°58'S-47°26'W), Pirassununga, SP, Brazil. Adult specimens *H. lacerdae*, weighing 450 ± 80 g, were obtained from Rio Grande Basin at Furnas Hydroelectric Power Plant, MG. The fish were brought to the aquaculture facilities of the National Center of Research on Tropical Fish (CEPTA-IBAMA) in Pirassununga, SP. Before the experiments, the fish were starved for 14 days in 500 L tanks provided with well-aerated water (pO_2 7.5 mg/L), pH 6.8 ± 0.2, and temperature 25 ± 2°C. The experimental tests were performed in January February.

Experimental design

Nitrogen excretion experiments were done in 5 L glass aquaria. The water was previously autoclaved, aerated (pO_2 7. 5 ppm), and kept at 25 ± 2°C. The water pH was adjusted to 5.0, 7.0, and 10.0 with $NaPO_4^{2-}/Na_2PO_4^-$; the fish were weighted, rinsed with clean water, and transferred to the aquaria. The ratio 10 g of fish per liter of water was kept for every test, and the specimens individually assayed. Samples of water were collected every 15 minutes to determine ammonia, urea, and uric acid. After 6 hours, blood samples were drawn from the caudal vein and the fish were sacrificed. The liver of fish from neutral waters was excised for enzyme analysis.

Blood and tissue treatments

The blood samples were immediately centrifuged at 12,000 x g for 3 min. The plasma was

separated, and 0.6 N perchloric acid (PCA) was added 3 : 1 (v/v) to remove the proteins. The protein pellets were discarded after centrifugation at 6,000 x g for 3 minutes, and ammonia, urea, and uric acid were determined in the supernatant. Liver samples were homogenized in a buffer solution with a motor-driven Teflon pestle under an ice-bath. The homogenization buffer contained: 0.01 M Tris, 0.02 M Na phosphate, 0.01 M glycine, 0.5 mM ethylendiamintetracetic acid (EDTA), and pH 7.0, in glycerol v/v. The homogenates were centrifuged at 3,000 x g at 4°C for 15 min and the pellets were discarded. The supernatants were centrifuged at 8,000 x g at 4°C for 20 min and used as an enzyme source.

Enzyme assays

Glutamine synthetase (GS) activity was measured by γ-glutamyl hydroxamate formation modified from Vorhaben *et al.* (1973). The incubation mixture, containing 50 mM HEPES, pH 7.0; 60 mM glutamine; 15 mM hydroxylamine; 0.4 mM ADP; 20 mM $NaAsO_4$; and 3 mM $MnCl_2$, was brought to a final volume of 1.5 ml after the addition of a suitable amount of enzyme. After 60 min of incubation at 25°C, the reaction was stopped by adding 300 µL of ferric-chloride-reagent. The reaction product γ-glutamyl monohydroxamate was estimated at 540 nm in the supernatant of the reaction mixture after centrifugation at 7,000 x g for 1 min at 5°C.

The carbamoyl phosphate synthetase (CPS) activity was determined in a reaction system containing 50 mM HEPES pH 8.0, 20 mM ATP, 10 mM glutamine (or 10 mM NH_4Cl), 10 mM ornithine, 5 mM acetylglutamate, 5 mM UTP, 10 mM $NaHCO_3$, 24 mM $MgSO_4$, and 5 UI OCT (Streptococus faecalis-Sigma). The reactions were incubated at 25°C for 60 min. and stopped by addition of 70% TCA (trichloroacetic acid). The reaction mixtures were centrifuged at 7,000 x g for 2 min. Citrulline as the reaction product was colorimetrically determined at 464 nm, as proposed by Boyde & Rahmatullah (1980).

Activity of ornithine carbamoyl transferase (OCT) was determined in a reaction system containing 50 mM HEPES pH 8.5, 10 mM ornithine, and 10 mM carbamoyl phosphate. The reactions were incubated at 25°C for 30 min. and halted by adding 70% TCA, after which they were centrifuged at 7,000 x g for 2 min. Citrulline, the reaction product, was colorimetrically determined at 464 nm, as proposed by Rahmatullah & Boyde (1980).

The enzyme system argininosuccinate synthetase, argininosuccinate lyase (AS-AL), was determined in a reaction mixture containing 50 mM HEPES pH 7.5, 16 mM ATP; 30 mM citrulline, 90 mM aspartic acid, and 5 mM $MgCl_2$. The reactions were incubated at 25°C for 60 min, stopped by the addition of 70% TCA and centrifuged at 7,000 x g for 2 min. Arginine was colorimetrically determined at 460 nm, as proposed by Campanini *et al.* (1970).

Arginase (ARG) activity was determined by measuring the formation of urea from arginine. The assay mixture contained 50 mM HEPES pH 10.0, 278 mM arginine, 10 mM $MnCl_2$, and an appropriate amount of enzyme so as to reach a final volume of 1.5 ml. The reactions were halted with 70% TCA and centrifuged at 7,000 x g for 1 min at 5°C. Urea was measured in the supernatant at 525 nm, following Boyde & Rahmatullah (1980).

Metabolite determination

Metabolites were colorimetrically determined. Urea was determined, following Rahmatullah & Boyde (1980) at 525 nm; ammonia following as Gentzkow & Masen (1942) at 420 nm; and uric acid, according to Henry *et al.* (1957) at 650 nm. All chemicals were of analytical grade, purchased from Sigma Chemical Co., or Merck. MS 222 was from Sandoz.

Statistics

Tests for significance were performed using Mann Whitney; significance level was set at $p < 0.05$. The Pearson correlation coefficient was used for some parameters and the critical values for (r) were set at 95%.

RESULTS

The enzymes of urea synthesis were detected in the liver of trahira, trairão, and aimara. Glutamine synthetase was active in the species, the lowest value being registered for trairão (Table 1). Carbamoyl phosphate synthetase assayed with glutamine as a substrate (CPS_{III}) was similar for the species. However, this catalytic activity with ammonia as a nitrogenous substrate (CPS_I) was very low. Orni-

thine carbamoyl transferase activity of trahira was the highest and the system argininosuccinate synthase and arginosuccninate lyase was quite similar among the three species. The maximum activity of arginase was observed in trairão.

Ammonia was the main nitrogenous excretion product in acid and neutral water, and urea was constant for the studied species (Table 2). Uric acid was not detected either as an excretion product or as a plasma component. In neutral to alkaline water, ammonia excretion was reduced. However, this response was more evident in aimara and trahira. In alkaline and neutral water, the chemical ratio ammonia/urea was very high in both species, particularly in trahira. When water pH rose, the plasma ammonia increased in aimara, however in trairão it was drastically reduced.

DISCUSSION

Ureogenesis has been considered less important for most teleosts. It was assumed for many years that genes of UC enzymes had been lost from the teleost genome. However, the presence of UC enzymes in early life stages is being reported in some species, and proposed as a fact for most.

TABLE 1
The urea cycle enzymes in erythrinids.

	H. malabaricus	***H. lacerdae***	***H. unitaeniatus***
GS	1.4 ± 0.1	1.0 ± 0.1	1.6 ± 0.1
CPS – Gn	(46.3 ± 2.0) x 10^{-3}	(43.0 ± 8.0) x 10^{-3}	(40.4 ± 3.0) x 10^{-3}
CPS – NH_4	(0.1 ± 0.01) x 10^{-3}	(0.10 ± 0.01) x 10^{-3}	(0.2 ± 0.01) x 10^{-3}
OCT	0.90 ± 0.03	0.34 ± 0.05	0.69 ± 0.02
AS-AL	0.13 ± 0.01	0.17 ± 0.02	0.10 ± 0.01
ARG	10.45 ± 3.7	73.40 ± 6.2	16.34 ± 4.9

Specific activity of the urea cycle enzymes: (GS) glutamine synthetase, (CPS – Gn) carbamoyl phosphate synthetase glutamine dependent, (CPS – NH_4) carbamoyl phosphate synthetase-ammonia dependent, (OCT) ornithine carbamoyl transferase, (AS-AL) arginine succinate synthetase-arginine succinate lyase, and (ARG) arginase of *H. malabaricus*, *H. lacerdae*, and *H. unitaeniatus*, starved for 10 days at environmental pH 7.0. Enzyme activities are expressed in μmol x min^{-1} x g^{-1} of wet tissue.

TABLE 2
Excreted and plasma nitrogen in erythrinids.

	H. malabaricus			***H. lacerdae***			***H. unitaeniatus***		
pH	5.0	7.0	9.0	5.0	7.0	9.0	5.0	7.0	9.0
Plasma nitrogen									
Ammonia	79 ± 0.5	74 ± 0.3	90 ± 0.6*	329 ± 12	290 ± 20	40 ± 0.3*	105 ± 9	1.4 ± 0.01	290 ± 15*
Urea	83 ± 0.6	81 ± 0.5	140 ± 8*	31 ± 0.2	31 ± 0.1	21 ± 0.1*	52 ± 0.3	59 ± 0.3	63 ± 0.2
U/T.N.(%)	51.2	52.2	60.8*	8.6	9.7	34.4*	33.1	32.2	17.8*
Excreted nitrogen									
Ammonia	476 ± 21	450 ± 19	6 ± 0.04*	66 ± 0.5	40 ± 0.3	36 ± 0.3	210 ± 11	193 ± 11	9 ± 0.05*
Urea	7 ± 0.05	8 ± 0.06	17 ± 0.9*	2 ± 0.01	2 ± 0.01	2 ± 0.01	4 ± 0.02	5 ± 0.02	5 ± 0.01
U/T.N.(%)	1.5	1.7	73.9*	2.9	4.7	5.3	1.9	2.5	35.7*

Nitrogen concentration in plasma (mmol – $N{\cdot}10^{-2} \cdot L^{-1}$) and excreted nitrogen (mmol – $N{\cdot}10^{-2} \cdot Kg^{-1}$ x h^{-1}) in *H. malabaricus*, *H. lacerdae*, and *H. unitaeniatus* exposed to distinct environmental pHs. (U/T.N.) means Urea/Total nitrogen. (*) Statisticaly different for every species at $p < 0.05$ (N = 6) as compared to different environmental pHs.

Hitherto, it was not very clear whether ammonia excretion prevails over the urea. In spite of a few UC enzymes with different specific activities reported in many species (Nener, 1988; Mommsen & Walsh, 1989, 1991, 1992), the full set is uncommon. It was reported that in difficult environmental conditions such as alkaline waters and seasonal drought, urea cycle (UC) enzymes remain active in a few adult teleost species (Randall *et al.*, 1989).

Environmental H^+ oscillates in many water systems and an alkaline medium can often be observed. Such conditions should promote the urea synthesis in oder to prevent ammonia toxicity. This response must enhance the enzyme activity of the main UC enzymes. Glutamine synthetase, the glutamine source for urea synthesis, is a limiting factor (Mommsen & Walsh, 1989, 1991, 1992; Campbell & Anderson, 1991; Walsh, 1997) and it is much more active in elasmobranchs than teleosts (Anderson, 1980; Webb & Brown, 1980; Anderson & Casey, 1984; Grifth, 1991). The specific activity of GS in a study of erythrinids is close to that found in *Heteropneustis fossilis* (Saha & Ratha, 1997), a species with a functional UC. The second step in synthesizing urea consumes glutamine by carbamoyl phosphate synthetase. In the studied species this enzyme was very active, with N-acetyl glutamate as a co-factor in the reaction. The CPS of ureogenic species, such as *Clarias batrachus*, *Amphipnous cuchia*, and *Anabas testudineus* is slightly lower than that observed in the here-studied erythrinids (Huggins *et al.*, 1969; Cao *et al.*, 1991; Wright, 1993; Saha & Ratha, 1989, 1997).

Ornithine carbamoyl transferase (OCT) is the second most active UC enzyme (Nener, 1988; De Gregório *et al.*, 1993). This enzyme activity is very high in the studied erythrinids, but compared with the water-breathing freshwater fishes just reported, the OCT of trahira is higher. Conversion of citrulline into arginine is performed by argininosuccinate synthetase and argininosuccinate lyase. There are few data concerning both enzymes in teleost fish (Saha & Ratha, 1987, 1994; Campbell & Anderson, 1991). These are very unstable and their catalytic activity is very low. The values observed for the studied erythrinids are closer to the ureogenic air-breathing teleosts *H. fossilis*, *C. batrachus*, *A. cuchia*, and *A. testudineus* (Saha & Ratha, 1989, 1997). This enzyme activity for water-breathing freshwater teleosts is very low compared to that for trahira, trairão, and aimara. Arginine conversion to ornithine is catalyzed by arginase. This enzyme is widely distributed and its activity in fishes is supposed to change with the ingested protein level (Berge *et al.*, 1997). The feeding habits of the studied species are distinct in many aspects, but all of them are insectivores/piscivores (Paiva, 1974; Caramashi, 1979), and the differences observed among these erythrinids are unlikely to be related to an excretory character. Therefore, the set of UC enzymes and the limiting urea-synthesis-enzyme glutamine synthetase are fully expressed in the ureogenic teleost fish trairão, aimara, and trahira.

Usually, ammoniotelism predominates among the teleosts, and the transition to ureotelism is not clear (Wright, 1995; Walsh, 1997; Julsrud *et al.*, 1998; McKenzie *et al.*, 1999). Facultative ureotelism is seldom observed among fishes and it is considered only when at least 50% of nitrogenous excretion is urea (Walsh *et al.*, 1990). Inhibition of ammonia excretion is the most common environmental inducer of ureotelism (Campbell & Anderson, 1991; Wood, 1993; Walsh, 1997). Alkaline environments frequently impair ammonia excretion by inhibiting NH_3 conversion to NH_4^+. This avoids diffusion through the gills (Timothy & Iwama, 1992; Wilkie *et al.*, 1993) and increases the plasma NH3 (McGeer & Eddy, 1998). The erythrinid responses to alkaline water were species specific.

The ammonia excretion rate of trairão in alkaline water fell by half. However, compared to aimara and trahira, the decrease in ammonia excretion was smooth. The plasma ammonia of trairão was drastically reduced in alkaline exposure but it is unlikely that nitrogen catabolism decreased equally. Glutamine synthesis is a bypass to reduce plasma ammonia (Mommsen & Walsh, 1989; Mommsen & Walsh, 1992; McKenzie *et al.*, 1999). Therefore, other biochemical pathways should be involved in hypoammonemia. Metabolic preferences can be shifted to lipid or carbohydrate catabolism to sustain energetic demands when trairão is exposed to alkaline waters. The metabolic behavior of aimara was distinct. Exposure to alkaline water reduced ammonia excretion with a

consequent increase of ammonemia but plasma urea and urea excretion were unchanged. A similar response is reported for *Opsannus beta*, a ureogenic teleost that shifts to ureotelism and reduces ammonia excretion. This fact is assumed to be more relevant than the increase of urea waste (Wood *et al.*, 1995). The excretion ratio (urea/total nitrogen) increased sharply in aimara, suggesting a transition to ureotelism. Trahira presents a particular strategy to cope with alkaline water. In this species, the nitrogen excretion rate is among the highest values reported recently in the literature. Compared with those of trairão and aimara, this rate decreased sharply when the fish was removed from acid to alkaline waters. However, the excretion ratio (urea/total nitrogen) was very high and the transition to ureotelism was evident. Our data allow us to conclude that, in spite of these three teleost species being close phylogenetically, they present different biochemical strategies to cope with the same kind of environmental stressor. Moreover, an biochemical characteristic uncommon among fishes is reported: The tropical teleost H. malabaricus exhibits ureotelism in alkaline water.

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