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The Stinging Catfish, Heteropneustes fossilis, Exposed to Environmental Ammonia

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Abstract: **The stinging catfish, *Heteropneustes fossilis*, can tolerate high concentrations of environmental ammonia. Previously, it was regarded as ureogenic, having a functional ornithine-urea cycle (OUC) that could be up-regulated during ammonia-loading. However, contradictory results indicated that increased urea synthesis and switching to ureotelism could not explain its high ammonia tolerance. Hence, we re-examined the effects of exposure to 30 mmol l^{-1} NH_4Cl on its ammonia and urea excretion rates, and its tissue ammonia and urea concentrations. Our results confirmed that *H. fossilis* did not increase urea excretion or accumulation during 6 days of ammonia exposure, and lacked detectable carbamoyl phosphate synthetase I or III activity in its liver. However, we discovered that it could actively excrete ammonia during exposure to 8 mmol l^{-1} NH_4Cl. As active ammonia excretion is known to involve Na^+/K^+-ATPase (Nka) indirectly in several ammonia-tolerant fishes, we also cloned various *nkaα-subunit* isoforms from the gills of *H. fossilis*, and determined the effects of ammonia exposure on their branchial transcripts levels and protein abundances. Results obtained revealed the presence of five *nkaα-subunit* isoforms, with *nkaα1b* having the highest transcript level. Exposure to 30 mmol l^{-1} NH_4Cl led to significant increases in the transcript levels of *nkaα1b* (on day 6) and *nkaα1c1* (on day 1 and 3) as compared with the control. In addition, the protein abundances of Nkaα1c1, Nkaα1c2, and total NKAα increased significantly on day 6. Therefore, the high environmental ammonia tolerance of *H. fossilis* is attributable partly to its ability to actively excrete ammonia with the aid of Nka.**

Index Terms **- air-breathing fishes, carbamoyl phosphate synthetase, nitrogen metabolism, ornithine-urea cycle, Singhi catfish, ureogenesis**

INTRODUCTION

In fishes, proteins ingested would be first catabolized to amino acids and then further broken down through deamination or transamination with the eventual production of ammonia. Although the production of ammonia can occur in many tissues (Walton and Cowey, 1977), the liver is the main organ where it is produced (Pequin and Serfaty, 1963). Most teleost fishes are ammonotelic, keeping body ammonia levels low by excreting excess ammonia mainly as NH3 (>50% of the total nitrogenous wastes) across the gills (Wright et al., 1993). In aqueous solution, ammonia exists as molecular NH3 and cationic NH4+, based on the reaction NH3 H3O+ NH4+ H2O with a pK of 9.0–9.5. Hence, acidic environmental conditions augment ammonia excretion, as NH3 diffusing across the branchial epithelium is converted into NH4+ and trapped in the external environment. By contrast, an increase in the pH of the environment would impede the diffusion of NH3 into the external medium leading to the accumulation of endogenous ammonia in the body. When fishes emerge from water, there could be a reduction in the excretion of ammonia due to a lack of water to flush the gills. Furthermore, ammonia concentrations in the ambient water could increase due to the decomposition of organic matters or the addition of fertilizers, and impede ammonia excretion in fishes.

As aquatic hypoxia is a frequent event in tropical waters, air- breathing is a common occurrence (Graham, 1997). With the development of air-breathing abilities, some tropical fishes can leave water and make short excursions on land, while some even burrow into semi-solid mud during drought. When confronted with terrestrial conditions or low levels of environmental (exogenous) ammonia, fishes may have difficulties in excreting ammonia that is endogenously produced. However, when confronted with high environmental ammonia concentrations that result in a reversed PNH3 gradient, fishes would have to detoxify not only endogenous ammonia but also exogenous ammonia that could have penetrated into the body (ammonia- loading). Under all these adverse environmental conditions, ammonia accumulation in the fish's body would occur, but ammonia is toxic (see Ip et al., 2001a; Chew and Ip, 2014 for reviews) and fishes are generally very susceptible to elevated tissue ammonia levels. Therefore, many tropical fish species have evolved mechanisms to avoid ammonia accumulation and to ameliorate ammonia toxicity during terrestrial or ammonia exposure (see Ip et al., 2001a, 2004a,b; Chew et al., 2006; Ip and Chew, 2010, 2018; Chew and Ip, 2014, 2017 for reviews).

The stinging (or Singhi) catfish, Heteropneustes fossilis, belonging to Family Heteropneustidae (air sac catfishes), can be found in Pakistan, India, Bangladesh, Myanmar, and Thailand (Burgess, 1989; Munshi and Hughes, 1992). Its main habitats include ponds, ditches, swamps, marshes, and muddy rivers (Munshi and Choudhary, 1994). It can survive long periods of emersion and tolerate moderately high concentrations of environmental ammonia (Saha and Ratha, 1990, 1994; Saha et al., 2001). It had been reported that H. fossilis was ureogenic and contained a functional ornithine-urea cycle (OUC) that could be up-regulated during ammonia-loading (Saha and Ratha, 1987, 1989, 1994, 1998; Saha et al., 1997). However, as pointed out by Graham (1997), those reports also comprised intrinsically contradictory evidence, indicating that ureogenesis and shifting from ammonotelism (excreting > 50% of the total waste-N as ammonia) to ureotelism (excreting > 50% of the total waste- N as urea) could not be the "major" contributor to its high ammonia tolerance.

Saha and Ratha (1990) reported that H. fossilis absorbed ammonia from the external environment when exposed to 25,50, or 75 mmol l−1 NH4Cl for 28 days. There was an increase in urea excretion by 1.5- to 2-fold between day 10 and day 12, which remained high throughout. Thus, they concluded that "prolonged hyper-ammonia stress induced the shift from ammonotelism to ureotelism in H. fossilis." However, their results did not corroborate such a conclusion. This is because the rate of ammonia N excretion reported in the control fish fasted for 14 days was 7.82 µmol 48 h−1 g−1 (Table 1 in Saha and Ratha, 1990) and a fish exposed to 75 mmol l−1 NH4Cl for 14 days absorbed 56.37 µmol ammonia N 48 h−1 g−1. If one assumes that ammonia N excretion was totally impeded in a medium containing 75 mmol l−1 NH4Cl, the total ammonia N accrued would be 7.82 56.37 = 64 µmol 48 h−1 g−1. Yet, the increase in the rate of urea excretion after exposure to 75 mmol l−1 NH4Cl was reported to be only 2.86 (urea excretion rate in 75 mmol l−1 NH4Cl) 0.96 (urea excretion rate in water) = 1.90 µmol (or 3.80 µmol urea N) 48 h−1 g−1 (Saha and Ratha, 1990). Hence, the increase in urea excretion represented 5.93%, which is only a very small fraction, of the total accumulated ammonia N (64 µmol ammonia N 48 h−1 g−1). Alternatively, it can be interpreted that H. fossilis could have actively excreted a certain amount of ammonia during environmental ammonia exposure; however, this possibility had not been examined. Another enigma was that the rate of ammonia absorption from the external environment increased with increasing concentrations of external NH4Cl (Table 1 in Saha and Ratha, 1990), but the rate of urea excretion was relatively constant during exposure to different concentrations of external NH4Cl (Saha and Ratha, 1990). If indeed increased urea synthesis and shifting from ammonotelism to ureotelism were the "major" strategies adopted by H. fossilis to survive ammonia exposure, an increase in urea excretion rate should have occurred with an increase in the concentration of environmental ammonia. These discrepancies led Graham (1997) to state that "paradoxically, some of the species reported to have the OUC enzymes are not ureotelic; urea, in fact, amounts to a quite a small percentage of the total nitrogen excreted by Heteropneustes (even in 75 mmol l−1 NH4Cl)."

At present, the roles of ureogenesis and ureotelism in defending ammonia toxicity in H. fossilis exposed to environmental ammonia remain controversial. Therefore, this study was undertaken to re-examine the effects of exposure to 30 mmol l−1 NH4Cl on the rates of ammonia and urea excretion, and the tissue ammonia and urea concentrations, in H. fossilis. As results obtained contradicted information in the literature (Saha and Ratha, 1987, 1989, 1990), we decided to re-examine the presence of carbamoyl phosphate synthetase (CPS) III activity in its liver using an established radiometric method (Anderson et al., 1970; Anderson, 1980; Anderson and Walsh, 1995; Chew et al., 2003b, 2004; Tam et al., 2003; Ip et al., 2012a). This is because all the reports that indicated the possible presence of CPS III in the liver of H. fossilis were based on a coupled-enzyme colorimetric assay (Saha and Ratha, 1987; Saha et al., 1997). Subsequently, we obtained results that denoted a lack of detectable CPS III activity in the liver of H. fossilis, demanding an alternative explanation for its high ammonia tolerance. Thus, we made an effort to examine whether H. fossilis could actively excrete ammonia during ammonia exposure (8 mmol l−1 NH4Cl). This is because active ammonia excretion has been reported previously for the highly ammonia-tolerant giant mudskipper, Periophthalmodon schlosseri (Peng et al., 1998; Randall et al., 1999; Ip et al., 2005; Chew et al., 2007), climbing perch, Anabas testudineus (Tay et al., 2006), and African sharptooth catfish, Clarias gariepinus (Ip et al., 2004d). In the case of A. testudineus (Ip et al., 2012b,c; Loong et al., 2012) and P. schlosseri (Chew et al., 2014, 2015), Na+/K+-ATPase (Nka) is known to take part in a multi-component mechanism involved in active ammonia excretion. Therefore, we also cloned and sequenced isoforms of nkaα-subunits from the gills of H. fossilis, and determined the effects of ammonia exposure on their branchial transcript levels and protein abundances, which could serve as an indicator of the ability of H. fossilis to actively excrete ammonia.

MATERIALS AND METHODS

Catfish and Rearing Condition

Heteropneustes fossilis (approximately 20–60 g) were imported from India through a local fish importer (Qian Hu Fish Farm, Singapore). Its identity was confirmed by the very small dorsal fin that lacked a leading spine and was located in the anterior third of body, as well as the two tubular air sacs that extended from the gill cavity to the caudal peduncle (Burgess, 1989). The fish (without sex differentiation) were acclimated in plastic tanks containing dechlorinated fresh water at a temperature of 25–26◦C and under a photoperiod of 12 h illumination and 12 h darkness using artificial light. The fish were fed frozen bloodworms and the water in the tanks was changed daily for a week. Feeding of the fish was halted 2 days before experimentation. Approval of protocol (ARFSBS/NIE-A-0311) for fish maintenance and experimentation was granted by the Nanyang Technological University Institutional Animal Care and Use Committee.

Effects of Exposure to 30 mmol l−1 NH4Cl on Urea Excretion Rate Fish (20–30 g) were exposed individually in a tank containing 20 vol (volume to the mass of the fish) of either fresh water (five individuals in the control group) or fresh water containing 30 mmol l−1 NH4Cl (five individuals in the experimental group; pH 7.0) for 6 days. The water in the tanks was replaced daily. On day 1, 3, or 6 (after 24 h of water replacement), 2 ml of water was sampled, acidified with 0.2 ml of H2SO4, kept at 4◦C, and used for the analysis of urea within a week. The colorimetric method by Jow et al. (1999) was used for the determination of urea. The excretion rate was expressed as µmol urea−N g−1 day−1. A water sample (1 ml) was taken immediately as the 0 h sample. After 24, 48, and 72 h, 1 ml of water was again sampled. The water sampled was acidified as mentioned above. The water was kept at 4◦C and used for both ammonia and urea analyses within a week. A parallel set of containers without fish was set up and water was sampled for ammonia analysis to ensure that the ammonia concentration was not altered significantly by bacterial action. The colorimetric method by Anderson and Little (1986) was used for ammonia analysis. The rate of ammonia excretion was expressed as µmol ammonia−N g−1 day−1.

Effects of Exposure to 30 mmol l−1 NH4Cl on Ammonia and Urea Concentrations in Tissues Fish (25–40 g) were exposed individually in a tank containing 30 mmol l−1 NH4Cl (pH 7.0) for 1 day (five individuals), 3 days (five individuals), or 6 days (15 individuals) with daily changes of NH4Cl solution. Fish kept individually in fresh water (15 individuals) served as controls. At the respective time interval, fish were sacrificed by immersing in 0.1% phenoxyethanol and then applying a blow to the head. Gills, muscle, and liver were dissected, frozen in liquid nitrogen, and kept in a 80◦C freezer. A separate group of fish (30–60 g) used for blood collection was exposed to either fresh water (10 individuals) or fresh water containing 30 mmol l−1 NH4Cl for 6 days (10 individuals). The fish was anesthetized and heparinized capillary tubes were used to collect the blood from the caudal peduncle. The plasma obtained after centrifugation of the blood at 4000 g for 10 min at 4◦C were deproteinized in two volumes of ice-cold 6% trichloroacetic acid. The clear supernatant obtained after centrifugation of the deproteinized samples at 10,000 g for 15 min at 4◦C was used for analyses of ammonia and urea concentrations.

The muscle and liver tissues collected were first ground to a powder in liquid nitrogen, before weighing and homogenizing in five volumes (w/v) of ice-cold 6% trichloroacetic acid using an Ultra-Turrax disperser (Ika-Werke, Staufen, Germany) set to 24,000 r/min for 20 s thrice. The clear supernatant obtained after centrifugation of the homogenate as mentioned above was used for analyses of ammonia and urea concentrations.

The method by Bergmeyer and Beutler (1985) was used for the determination of ammonia concentrations in the tissue samples while the urea concentrations were analyzed as mentioned above.

Determination of CPS Activities in the Liver

The extraction procedure was carried out on liver samples using the method of Ip et al. (2012a). Approximately 300 mg of liver was homogenized in five volumes (w/v) of ice-cold buffer containing 50 mmol l−1 Hepes (pH 7.6), 50 mmol l−1 KCl, 0.5 mmol l−1 EDTA, 0.5 mmol l−1 phenylmethanesulfonyl fluoride (PMSF), Effects of Exposure to 8 mmol l NH4Cl on Ammonia and Urea Excretion Rate Fish (20–25 g) were immersed in a small plastic container containing six volumes (volume to the mass of the fish) of fresh water (five individuals) or fresh water with 8 mmol l−1 NH4Cl (five individuals) (pH 7.0). Temperature was kept at 25–26◦C. and 1 mmol l−1 dithiothreitol as mentioned above. The homogenate was sonicated three times at 110 W, 20 kHz using a Misonix sonicater (Farmingdale, NY, United States) for 20 s each with two 10 s off-intervals. The sonicated homogenate was then centrifuged as mentioned above, and the supernatant collected was desalted using the Econo-Pac 10DG desalting column (Bio-Rad Laboratories, Hercules, CA, United States) equilibrated already with extraction buffer without EDTA and PMSF. The eluent collected was used for CPS assay. The protein concentrations of all samples (before and after elution) were determined using the Bio-Rad Protein assay dye (Bradford, 1976) in order to obtain the dilution factor to be used in the subsequent calculation for CPS activities. CPS activity was determined using the radiometric method described by Anderson and Walsh (1995). Briefly, the reaction mixture consisted of 0.05 mol l−1 Hepes (pH 7.5), 0.05 mol l−1 KCl, 0.024 mol l−1 MgCl2, 0.2 mmol l−1 EDTA, 0.4 mmol l−1 dithiothreitol, 0.02 mol l−1 ATP, and 5 mmol l−1 [14C]bicarbonate. The substrates tested were 10 mmol l−1 glutamine (for CPS III assay) or 100 mmol l−1 NH4Cl (for CPS I assay) or glutamine NH4Cl (for CPS III CPS I assay). The inclusion of 0.5 mmol l−1 N-acetylglutamate in the assay medium was used to activate CPS I or III activity, while 1 mmol l−1 UTP was added to inhibit the activity of CPS II. A Wallac 1414 liquid scintillation counter (Wallac Oy, Turku, Finland) was used for radioactivity measurement. CPS activity was expressed as µmol [14C]urea formed min−1 g−1.

Extraction of Total RNA From Gills and cDNA Synthesis

The extraction and purification of total RNA from gill samples and the synthesis of cDNA from the purified RNA were performed following the method described in Chew et al. (2014). Briefly, Tri ReagentTM (Sigma–Aldrich Co., St. Louis, MO, United States) was used for the extraction while RNeasy Plus Mini Kit (Qiagen GmbH, Hilden, Germany) was used for the purification. The quantification of the RNA obtained was performed with a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, United States) and the integrity of the RNA was verified using gel electrophoresis. The purified RNA was converted to cDNA with oligo (dT)18 primers using the RevertAidTM First Strand cDNA synthesis kit (Thermo Fisher Scientific Inc., Waltham, MA, United States).

The removal of seasonal limitations of spawning for year-round breeding is a prerequisite for the full domestication of a fish species destined for intensive cultivation (Purdom 1993). Photoperiod and tem- perature are the most important environmental cues for triggering the gonadal maturation process in fish (Lam 1982). Manipulation of daylength has been used successfully for controlling spawning in sal- monids and flatfish (Scott, Baynes, Skarphedinson & Bye 1984). However, this technique is expensive, not always reliable and application is not possible in all locations. Exposure to a constant water temper- ature of 25°C with a normal photoperiod can extend the spawning period of mature African catfish, Clarias gariepinus (Burchell), to 10 or 11 months of the year (Richter, Viveen, Eding, Sukkel, Rothuis, Van Hoof, Van Den Berg & Van Oordt 1987b), but this approach is only successful to a small degree and is also not always reliable. Therefore, alternative methods of hormone manipulation are being developed to overcome the physiological constraints to gonadal recrudescence.

Testosterone, a male sex steroid, is known to produce all-male populations at low concentrations, and generally sterile populations or occasionally paradoxical feminization (feminization after andro- gen treatment) at high concentrations with pro- longed duration at early stages of gonadal development in teleosts (Hunter & Donaldson 1983). It also exerts a positive feedback on the gonadotro- phin-releasing hormone (GnRH) producing system in the hypothalamus, and thereby, triggers preco- cious maturation in juvenile rainbow trout, Oncorhynchus mykiss (Walbaum) (Crim & Evans 1982). Testosterone, together with GnRH analogues, was able to induce precocious ovarian maturation in milkfish, Chanos chanos (Forsskal), (Marte, Crim & Sherwood 1988).

Air-breathing fish are a major product of capture fisheries in India. These animals thrive well at higher stock densities in swamps, woody marshes and other derelict water bodies with poor oxygen content. How- ever, culture of these fish is at a nascent stage in India. Among catfish, the Indian catfish, Heteropneustes fos- silis (Bloch), commonly known as Singi, is highly regarded for its food value; it has a high protein con- tent (22.8%), is low in fat (0.6%) and has a very high iron content (226 mg 100 g–1 tissue) (Anonymous 1982). Indian catfish attains gonadal maturity at the end of its first year, when females measure about 12 cm in length (Dutta Munshi & Hughes 1992). Seeds are only available during the spawning season of this fish (between July and August). As a result, despite its beneficial traits, Indian catfish is not popular among fish farmers because of the non- availability of adequate seeds. Therefore, controlling the spawning season of Indian catfish under captivity for out-of-season egg production will have a major impact on its intensive culture.

Materials and methods

Experimental fish

The fish used in this study were one-day-old hatch- lings of Indian catfish, produced in a laboratory from gametes collected from sexually mature individuals during August 1995. The experiment was conducted in the present authors' laboratory from August 1995 to April 1996 under controlled temperature (26 ± 1°C) and photoperiod (8 h light:16 h dark). After absorption of the yolk sac, juvenile catfish were fed on Tubifex tubifex (Mu¨ ller) provided ad libitum throughout the experimental period. One- day-old hatchlings were randomly divided into four groups of 100 fish each. The groups were kept in four similar plastic pools of 10 L capacity and supplied with well- aerated water.

Table 1
Percentages of paradoxical feminization and precociously matured females (PMF) after testosterone treatment of catfish hatchlings

Treatment	Fish sexed (n)	Females (%)	PMF (%)
Control	42	64	0
100 µ g	46	61	0
200 µ g	40	67	0
300 µ g	37	100	70

Hormonal treatments

Testosterone propionate (Sigma Chemical Co., St Louis, MO, USA) was first dissolved at various concentrations in absolute ethanol. One millilitre of these solutions were then added to 10 L of water to obtain the following steroid concentrations: 100, 200 and 300 □g L–1. At each concentration, three groups were subjected to immersion treatment for one month. The water was changed daily followed by hormone application. The remaining group of fish served as control.

Data collection and analyses

Fish, total length µ 5 cm, were sexed during April 1996 by gross morphological examination of the gonads. Females were identified by the presence of ovaries and males were identified by presence of testes. Fish were also sexed through the histological examination of gonads.

For determination of the gonado-somatic index (GSI) and histological examination of ovaries, 10 fish were sacrificed randomly from each experi- mental and control group. Gonad weights were compared with eviscerated body weights to deter- mine GSIs. Ovaries from each fish were fixed in Bouin's fluid, dehydrated in graded ethanol solu- tions, embedded in paraffin and sectioned at 7 □m using a microtome. The sections were stained with haematoxylin and eosin, mounted with DPX, and viewed under a light microscope.

Analysis for alteration in sex ratio was performed with the chi-square test. The GSI of precociously matured females (PMFs) was compared with non- PMFs from the treated and control groups with Student's t-test.

Results

Histological examination of gonads and sexing showed that the proportion of females in the control group and the two groups with lower doses of steroids ranged between 61–67% (a ratio of about two females: one male; Table 1). Therefore, no significant difference in sex ratio was observed between these control and the treatment groups. The highest dose (300 □g L–1) induced complete feminization in 70% of females showing a signific- antly high ($P < 0.001$) GSI (38 ± 4) in comparison to females in the remaining treated and control groups (GSI = 12 ± 3) during April 1996 (Fig. 1). Histological studies of ovaries from PMFs showed fully matured ova with thickened walls in compar- ison to those from non-PMFs, which showed few developing oocytes at yolk vesicle stage. Eggs pro- duced by PMFs were found to be viable with excellent hatchability after induced breeding by human chor- ionic gonadotrophin (hCG; Sigma Chemical Co.).

Discussion and Conclusion

The present study demonstrates paradoxical femin- ization concomitantly with precocious ovarian mat- uration in a fish for the first time. In addition, this technique has a high potential for developing a technology to advance the spawning season of Indian catfish. The present study also supports previous findings that paradoxical feminization occurs only at high concentration of testosterone used for prolonged duration (Hunter & Donaldson 1983). The physiological mechanisms involved in this phenomenon seems to be aromatization of testosterone to oestradiol-17□ in neural tissues of hatchlings, leading to paradoxical feminization (Piferrer & Donaldson 1991). This dramatic change in sex ratio at the highest dose is probably caused by production of higher amount of oestradiol-17□ from high dose of testosterone through aromatase activity, and such a concentration of oestradiol-17□ acts as a loading dose for induced feminization and precocious maturation in this catfish. Testosterone may have direct action on GnRH-secreting neurones because it can stimulate in vivo gonadotrophin secretion by increasing sGnRH contents in the brain and pituitary gland of rainbow trout (Breton & Sambroni 1996). Steroid activation of the hypothal- amo-hypophyseal-gonadal axis during the juvenile stages of Indian catfish might have initiated positive feedback regulation of sex steroids for rapid oocyte maturation, 3 months ahead of normal spawning period. However, further experiments with non- aromatizable androgen, aromatase activity and activity of GnRH secreting neurones are clearly necessary to confirm this hypothesis.

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