UREOGENESIS AND ITS REGULATION IN A FRESHWATER AIR-BREATHING TELEOST, *Heteropneustes fossilis*

ABSTRACT

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Nature of the excretory product in animals has been strictly dependent upon the availability of water. Freshwater teleosts are ammoniotelic excreting primarily ammonia mostly by diffusion through gills. Ammonia being highly toxic has to be excreted out immediately after its production from the catabolism of proteins and amino acids **in vivo.** It cannot be stored in the body due to its toxicity and needs large amount of water for its excretion. This has been possible in the aquatic animals such as fishes making them ammoniotelic. Fishes excrete ammonia by diffusion through gills to the abundantly available water in their natural habitat. Presence of small amount of urea in the excretory products and also in the tissues has been reported in some freshwater teleosts. However, the source of this urea and its physiological significance are not yet clear. In terrestrial animals such as amphibians and mammals ammonia diffusion was not possible. The accumulated ammonia was converted to urea through ornithine-urea (o-u) cycle. Urea is less toxic and being highly soluble needs lesser amount of water for excretion. It can also be retained in the body upto a considerable concentration. There have been reports of amphibians being ammoniotelic in water and ureotelic on land. This shift to ureotelism has been attributed to water restriction. Some fishes such as lungfish and mudskipper fish are capable of spending sometime outside water and some of them also undergo aestivation. Ammonia accumulates due to lack of its excretion during these conditions. The presence of functional o-u cycle and the conversion of greater part of accumulated ammonia to urea through o-u cycle as physiological adaptation during water restriction have been reported in these fish.

Urea is also known to play a significant role for osmoregulation in addition to detoxification of ammonia in some groups of animals. In all the marine amphibians, elasmobranchs and also in many primitive fishes urea has been reported to be synthesised actively via o-u cycle and retained inside the body mainly for maintaining osmotic balance.

Urea can be formed through uricolytic pathway or by the catabolism of dietary arginine besides o-u cycle. In all ureotelic animals urea is formed mostly through o-u cycle. Presence of a functional o-u cycle in freshwater teleosts was questioned by various workers. Traces of urea found in the excretory products and also in some tissues of freshwater teleosts were suggested to come either from uricolytic pathway or by the catabolism of dietary arginine or both. Ammonia excretion being easy and osmotic problem reversed during the evolution of freshwater teleosts, the presence of the energy linked complicated o-u cycle was not expected in freshwater fishes. Some attempts have been made to find out a functional o-u cycle in freshwater teleosts since 1960. Brown and Cohen (1960) could not detect some of the enzymes of o-u cycle in several freshwater teleosts studied and suggested that the genes responsible for these enzymes might have got deleted in freshwater teleosts in the process of evolution. This has been known as the 'deletion' hypothesis. Wilson (1973) also supported this view as he could not detect some of the enzymes of o-u cycle in the channel catfish, **Ictalurus punctatus**. However, Huggins **et al** (1969) could detect the full complement of o-u cycle in some freshwater teleosts with of course very low enzyme activity. They suggested that the expression of the genes responsible for the synthesis of o-u cycle enzymes in freshwater teleosts might have altered due to an adaptational change instead of being deleted. Most of the freshwater teleosts studied so far by various groups did not show any promise for adaptation to environmental changes at the level of ureogenesis particularly through o-u cycle.

However, there are some freshwater teleosts which are primarily aquatic but breath predominantly air through their secondary respiratory organs. Sometimes, they are exposed to air and tolerate short periods of dehydration. Some of them are capable of living successfully in sewage fed water bodies and in paddy fields with high ammonia concentration. Such freshwater teleosts might have some special adaptive physiological regulatory mechanism(s) in their nitrogen metabolism and excretion like amphibians and lungfishes to manage ammonia toxicity during the periods of water deprivation or higher ambient ammonia in aquatic medium. There has not been any systematic study on this aspect which can also throw some light on the evolutionary modifications in the nitrogen metabolism and excretion in freshwater fishes.

Several species of freshwater air-breathing teleosts are available in this sub-continent including **Heteropneustes fossilis, Clarias batrachus, Anabas testudineus, Channa punctatus** and **Amphipnous cuchia** which were used in the present study. They are primarily aquatic but breath predominantly air. They inhabit usually in stagnant and slow flowing shallow water bodies. During drought conditions, they live inside mud and tolerate temporary dehydration when kept outside water. **A. cuchia** has an eel or snake like body and has almost completely lost the power of aquatic respiration with rudimentary gills.It spends most of its time on the bank of ponds and rivers and also can burrow inside mud during drought conditions.

Therefore, the present study has been aimed to find out the following in the above mentioned five species of freshwater air-breathing teleosts.

1. Pattern of excretion and tissue levels of ammonia and urea.

2. Probable sources of urea by studying o-u cycle and uricolytic pathway enzymes.
3. The regulation in the excretion of ammonia and urea, and ureogenesis under different environmental conditions such as higher ambient ammonia, dehydration and higher-osmolar ambient medium using one of the five species (**H. fossilis**) as a model. The results have been presented in five different chapters in the thesis as follows.

CHAPTER I: (Normal excretion pattern and tissue level of ammonia and urea) This chapter contains the studies on diurnal pattern of excretion and level of ammonia and urea in different tissues such as liver, kidney, muscle, brain, gill, skin and blood plasma of the five species of freshwater air-breathing teleosts in the aquatic medium. This chapter also includes the studies on diurnal pattern of renal and extra-renal excretion of ammonia and urea in **H. fossilis** while in water. The survival period outside water for the five species was also reported.

CHAPTER II: (Normal ureogenesis) This chapter includes the findings on the activity of five ornithine-urea cycle enzymes both in liver and kidney of above mentioned five species of freshwater air-breathing teleosts while in water. It also includes the results of the assay of the three uricolytic pathway enzymes in different tissues of **H. fossilis** in aquatic medium.

CHAPTER III: (Hyper-ammonia stress) This chapter presents the results on the tolerance limit for ambient ammonia and changes in excretion pattern and concentration of ammonia and urea in different tissues. Alterations in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with higher concentration of NH_4Cl in the medium for 28 days also have been included.

CHAPTER IV: (Dehydration stress) This chapter deals with the changes in excretion pattern and concentration of ammonia and urea in different tissues, and the alterations in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** kept ouside water for 48 hrs.

CHAPTER V: (Hyper-osmotic stress) This chapter presents the results on the osmotolerance limit and changes in excretion and concentration of ammonia and urea in different tissues. It also presents the changes in the activity of o-u cycle enzymes in the liver and kidney of **H. fossilis** treated with 250 mOsm mannitol for 28 days.

Physiological level of ammonia was found maximum in various tissues of **C. punctatus** and minimum in **A. cuchia**. However, urea level was found maximum in **A. cuchia** and minimum in **C. punctatus**. This has been correlated with their capacity to survive outside water. **A. cuchia** was found to survive maximum (90-100 hrs) while **C. punctatus** survived for minimum time (8-12 hrs) outside water. Both **H. fossilis** and **C. batrachus** survived for 60-70 hrs and **A. testudineus** for 24-36 hrs. In all the air-breathing fishes studied the urea level was found higher and ammonia level lower than other purely freshwater teleosts reported earlier.

Ammonia was found to be the major excretory product in the five species like other freshwater teleosts in aquatic medium. However, the rate of excretion of urea was found higher than reported earlier in other freshwater teleosts. Both ammonia and urea were excreted mostly through extra-renal sources in **H. fossilis.** Higher tissue level and higher rate of excretion of urea in four out of five species of freshwater air-breathing teleosts studied were suggestive of their capability for converting ammonia to urea **in vivo** through an active o-u cycle.

The above suggestion found support in the presence of functional o-u cycle in at least four (except **C. punctatus**) out of the five species studied. The activity of all the enzymes was found very high compared to purely freshwater teleosts, aquatic lungfish (**Neoceratodus forsteri**) and freshwater sting rays. The enzyme activities were similar or nearer to those of aestivating lungfish (**Protopterus**) and aquatic amphibia (**Xenopus laevis**) where urea synthesis through o-u cycle have been confirmed. The uricolytic pathway enzymes were also found to be present in **H. fossilis.** The activity of the enzymes of this pathway were found, in general lower than other freshwater teleosts. Synthesis of urea calculated from the relative activity of the enzymes seemed to be more through o-u cycle than uricolytic pathway in **H. fossilis.** The results obtained indicate that 'deletion' hypothesis is not applicable at least to freshwater air-breathing teleosts. The ability to synthesise urea by two distinct pathways by any freshwater teleost is a unique finding resembling more with their marine ancestors and aestivating dipnoan lungfish, **Protopterus.** Some of the freshwater teleosts such as the air-breathing species studied have still retained the functional o-u cycle besides uricolytic pathway for active ureogenesis. It was also found that the o-u cycle could be regulated by various environmental factors.

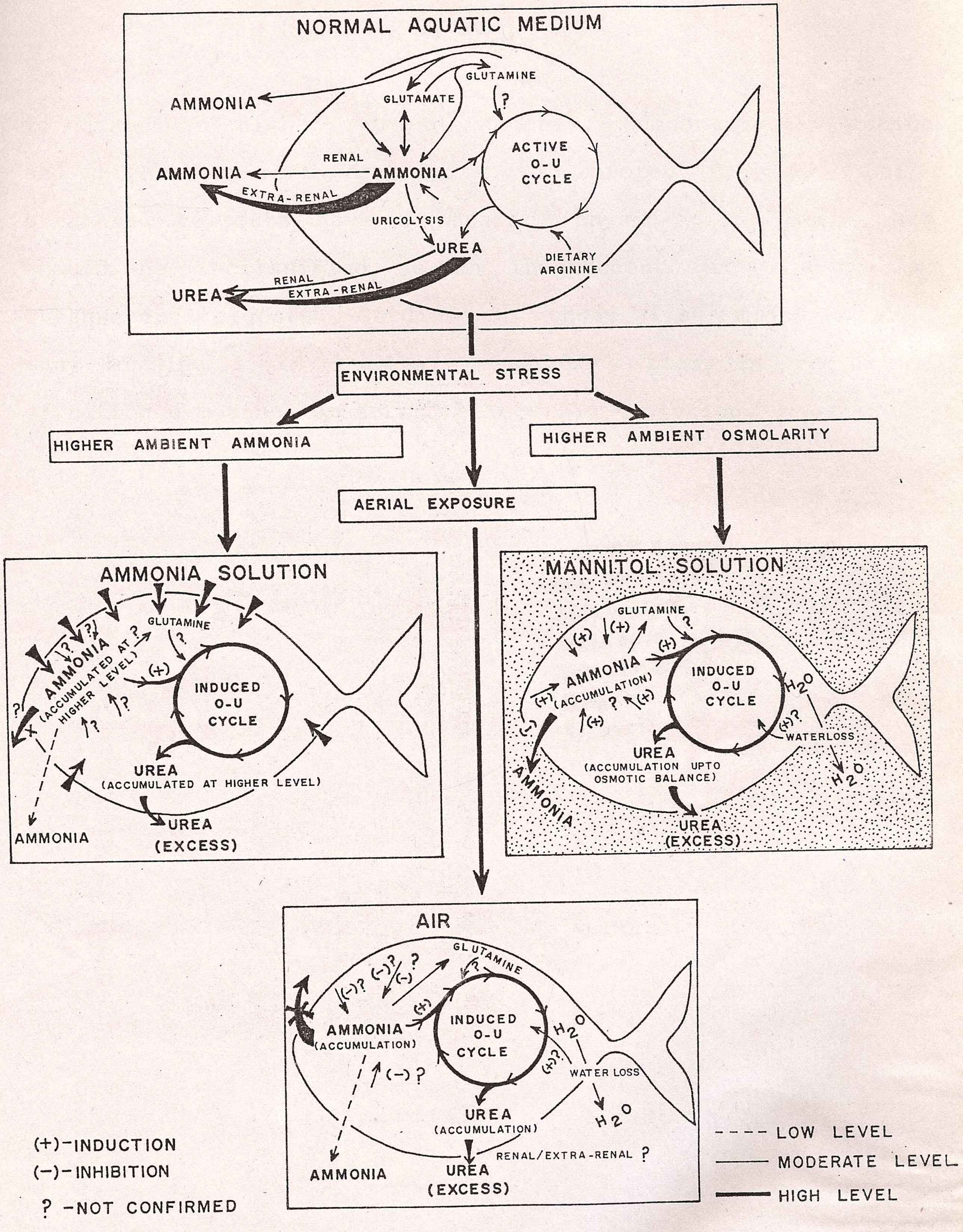
H. fossilis treated with different concentrations of NH_4Cl, tolerated upto 75 mM NH_4Cl for at least 28 days without any apparent deleterious effect. This high tolerance to ambient ammonia was several times higher than those reported for other freshwater teleosts and even aquatic amphibia, **Xenopus laevis. H. fossilis** was treated with 25, 50 and 75mM NH_4Cl solution for 28 days and the pattern of excretion, levels of ammonia and urea in different tissues and in blood plasma, and the alterations in the activity of o-u cycle enzymes in the liver and kidney were studied at different time intervals. Absorption of ammonia dominated over excretion during the exposure of the fish to all the concentrations of NH_4Cl. In 25 mM, ammonia excretion became normal during later periods of the experiment. Urea excretion was suppressed immediately after treatment in different concentrations of NH_4Cl. After 6-8 days, urea excretion rate increased by 2-3 fold and continued at that higher level till the end of the experiment. Absorption of ammonia from the medium was accompanied by the increase in the tissue ammonia level which reached its maximum on the 7th day of treatment. There was no further increase during the later periods of treatment. Accumulation of urea was also noticed in all tissues of treated fish probably for osmotic balance. All the enzymes of o-u cycle except arginase (ARG) were induced both in the liver and kidney of **H. fossilis** treated with 50 mM NH_4Cl. There were correlations between the accumulation of ammonia and urea with alterations in the rate of their excretion and the induction of o-u cycle enzymes. Increase in ammonia level in different tissues might be the primary factor for induction of o-u cycle enzymes for mainly detoxification of accumulated ammonia and retention of urea for osmoregulation. The urea excretion also enhanced after the induction of o-u cycle enzymes.

H. fossilis was subjected to dehydration stress by keeping outside water in glass jar for 48 hrs. There was an immediate suppression of both ammonia and urea excretion rate under aerial exposure. Ammonia excretion reduced by 75% at the initial stage which continued further to 85% at later period of emersion. However, the urea excretion rate which was reduced by 40% at the initial stage returned almost to normal level during the later period of the experiment. Decrease in ammonia and urea excretion was accompanied by linear increase in their levels in almost all tissues. All the enzymes of o-u cycle except ARG were also induced significantly both in liver and kidney of **H. fossilis** during dehydration. Accumulation of ammonia to the toxic level in different tissues was perhaps the main cause again for induction of o-u cycle enzymes reesulting in a shift towards ureotelism under dehydration stress in **H. fossilis**. This physiological adaptation might have helped the animal to avoid accumulation of toxic ammonia **in vivo**.

Treatment with different concentrations of mannitol indicated that **H. fossilis** could tolerate easily upto 300 mOsm of mannitol solution without any visible deleterious effect. However, the long term experiments were conducted in **H. fossilis** treated with 250 mOsm mannitol for 28 days. Immediately after treatment with 250 mOsm mannitol there was a suppression in the rate of excretion of both ammonia and urea by **H. fossilis**. The excretion of ammonia by treated fish remained at lower level than the control all through the experiment. However, the urea excretion rate returned to normal after 8-10 days and then increased significantly during the later period of the treatment. There was no increase in ammonia level in any tissue except in the kidney estimated on 7, 14, 21 and 28 days of treatment. However, accumulation of urea was noticed in all the tissues studied. Maximum accumulation was observed by 14th day and it was maintained at that level till the end of the experiment. More excretion and accumulation of urea were accompanied by the induction of all the enzymes of o-u cycle except ARG both in the liver and kidney of **H. fossilis.** It might be possible that increase in tissue ammonia level might have occurred due to the suppression of its excretion immediately (within a day or two) after exposure of **H. fossilis** to hyper-osmotic medium. That enhanced ammonia level possibly led to the induction of o-u cycle resulting in the decrease in ammonia level (by 7th day) and increase in urea level in various tissues for ammonia detoxification and osmoregulation. Thus, induction of o-u cycle for urea synthesis and its accumulation under hyper-osmolar stress were found to play a significant role in maintaining osmotic balance in **H. fossilis.**

Above findings have made it clear that the o-u cycle is not only functional in freshwater air-breathing teleosts but also can be physiologically regulated to provide better adaptation to the freshwater air-breathing teleosts under various environmental conditions such as higher ambient ammonia, dehydration and hyper-osmotic stress. Increase of ammonia level in different tissues under the above mentioned three environmental conditions was suggested to be the major factor for induction of o-u cycle enzymes in freshwater air-breathing teleosts. Water loss from the tissues might have also played some part in the process. A model has been proposed to explain the regulatory mechanism of o-u cycle in freshwater air-breathing teleosts under the three environmental conditions (see page 13).

Freshwater air-breathing teleosts have shown unique physiological adaptive mechanisms for ammonia metabolism which has given them the capacity to tolerate higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Thus, freshwater air-breathing teleosts show all the three urea producing characters such as ureogenic - having the full complement of o-u cycle enzymes, ureotelic - having the capacity to synthesise through o-u cycle and to excrete sufficient urea and ureosmotic - having the capacity to synthesise and accumulate urea for osmoregulation. It is further suggested that the freshwater air-breathing teleosts might be either relatively primitive to the present day freshwater teleosts and the genes for o-u cycle enzymes might have been repressed at a later stage of evolution. Alternately it could be possible that these genes might have been derepressed as a secondary modification in these fish for their ability



A diagramatic model for regulation of ureogenesis under environmental stress in freshwater air-breathing teleosts.

to adapt to higher ambient ammonia, temporary dehydration and higher ambient osmolarity. Therefore, freshwater air-breathing teleosts have a separate physiological status and should be reclassified as an independent group among the freshwater teleosts. Studies on these fish might connect many missing links in the evolutionary story of regulation of nitrogen metabolism during teleostean evolution.