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Ammonia handling in freshwater teleosts: a comparative study

Hoe gaan zoetwater vissen om met ammoniak: een vergelijkende studie

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ABBREVIATIONS

96h LC_{50}: Lethal concentration for 50% of the population within 96 hours

ALT: Alanine aminotransaminase

AST: Aspartate aminotransaminase

COI: Cytochrome oxidase subunit 1

CR: Cortisol receptor

ES: Exhaustive swimming

ES: Exhaustive-swimming

ESAF: Exhaustive swum ammonia exposed fed

ESAS: Exhaustive swum ammonia exposed starved

FAAs: Free amino acids

GDH: Glutamate dehydrogenase

GH**:** Growth hormone

GH: Growth hormone

GHR: Growth hormone receptor

GSase: Glutamine synthetase

H^+-ATPase: Proton pump

HEA: High environmental ammonia

HSP70: Heat shock protein70

IGF-I: Insulin-like growth factor I

ILCM: Interlamellar cell mass

J_{amm}: Net ammonia flux rate

J^{K}_{net}: Net potassium flux rate

J^{Na}_{in}: Sodium influx rate

J^{Na}_{net}: Sodium net flux rate

J^{Na}_{out}: Sodium efflux rate

J_{urea}: Net urea flux rate

MCs: Mucous cells

MO_2: Oxygen consumption rate

MRCs: Mitochondria-rich cells

NHE: Na^+/H^+ exchanger

NKA: Na^+/K^+-ATPase

PRL: Prolactin

PRLR: Prolactin receptor

PVCs: Pavement cells

Rh: Rhesus

RIA: Radioimmunoassay

RS: Routine swimming

RS: Routine-swimming

RSAF: Routine swum ammonia exposed fed

RSAS: Routine swum ammonia exposed starved

SEM: Scanning electron microscope

SL: Somatolactin

T3: 3,5,3′-triiodo-L-thyronine

T4: L-thyroxin

T_{amm}: Total ammonia concentration

TEP: Transepithelial potential

TH: thyroid hormone

THR: Thyroid hormone receptor

U_{crit}: Critical swimming speed

UT: Urea transporter

α-KG: α-ketoglutarate

List of publication

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Chapter 1

INTRODUCTION

1.1. AMMONIA: OCCURRENCE AND PROPERTIES

Ammonia is a persistent pollutant of aquatic habitats, produced and excreted by the aquatic animals as an end product of protein catabolism. It also enters the environment as a result of municipal, agricultural, industrial and natural processes. Natural sources of ammonia include the decomposition or breakdown of organic waste matter, gas exchange with the atmosphere, forest fires, animal waste, the discharge of ammonia by biota, and nitrogen fixation processes. Ammonia spills due to production, processing, storage, and disposal of the chemicals are the major causes of ammonia pollution in Flanders waters and in most part of the world. In aqueous solutions, ammonia exists as unionized ammonia (NH_3) and ionized ammonium (NH_4^+). The NH_3/ NH_4^+ ratio varies with pH and NH_4^+ greatly predominating at normal water pHs. The pK_a of the reaction is about 9.5 in freshwater at 15 °C. Beside water pH which has a marked effect on both the NH_3/ NH_4^+ equilibrium and on ammonia toxicity; other environmental parameters such as temperature, ionic strength, pressure, dissolved oxygen, salinity and carbon dioxide also affect this equilibrium. Increasing the pH value by 1 unit at 10 ºC produces 10-fold increase in NH_3 concentration while increasing the temperature by 10º C approximately doubles the NH_3 concentration (Eddy, 2005). The toxicity of ammonia depends principally upon the presence of unionized form (NH_3), which can readily diffuse across the gill membranes due to its lipid solubility and lack of charge whereas the ionized form (NH_4^+) cannot readily pass through the hydrophobic micropores in the gill membrane. Since the body or the physiological pH is around 7.0-7.8; therefore, most ammonia entering the body as NH_3 will be rapidly converted to NH_4^+. Thus, more than 95% of total ammonia in the body exists as NH_4^+ and it is this chemical species which is responsible for toxic effects (Smart, 1976; Hillaby and Randall, 1979).

1.2. AMMONIA TOXICITY

Under high environmental ammonia (HEA) the outward flux (excretion) of ammonia through the gills of fish are reduced, and a reversed inward ammonia flux occurs, resulting in net uptake of ammonia from the environment. Consequently, blood and tissue ammonia levels increase and fish experience both chronic and acute toxic affects (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002). Ammonia toxicity to fishes has been a subject of extensive laboratory studies and reviews (Randall and Wright, 1987; Wood, 1993; Saha and Ratha, 1998; 2007a). Acute ammonia toxicity in fish leads to reduction in oxygen carrying capacity of haemoglobin (Sousa and Meade, 1977), increased oxygen consumption, respiratory rate and heart beat (Smart, 1978; Chen and Nan, 1993) and disturbance of acid-base, iono-regulatory and hormonal balance (Soderberg and Meade, 1992; Wilkie, 1997). Chronic ammonia toxicity in general results in the reduction of growth rate (Dosdat et al., 2003; Foss et al., 2004; Lemarie et al., 2004; Pinto et al., 2007), alters energy metabolism (Arillo et al., 1981b), causes gill hyperplasia, thickening of the gill epithelium, fusion of

lamellae, haemorrhaging (Burrows, 1964; Reichenbach-Klinke, 1967; Smart, 1976; Thurston et al., 1978; Ip et al., 2001c), alters hormone regulation (Dosdat et al., 2003; Knoph and Olsen, 1994), and at a very high levels even induces hyperexcitability, coma, convulsions and death (Ip et al., 2001a). Moreover, elevated endogenous ammonia level are reported to reduce swimming capacity in salmonids (Shingles et al., 2001; Wicks et al., 2002), probably increased NH_4^+ levels alter the metabolic status within the fish, which may lead to premature muscle fatigue. Moreover, depolarization of muscle membrane potential due to the substitution of K^+ with NH_4^+ has also been hypothesized for impairment of muscle contraction (Beaumont et al., 1995a,b, 2000a; Cooper and Plum, 1987; Raabe and Lin, 1985). Ammonia also interferes with energy metabolism by stimulating glycolysis via activation of phosphofructokinase in the muscle and through impairment of the tricarboxylic acid cycle (TCA) in the mitochondria (Arillo et al., 1981b). The suppression of TCA cycle is through depletion of α-ketoglutarate, which is converted to glutamate to remove ammonia and also due to inhibition of some key enzymes, including isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and pyruvate dehydrogenase, as in mammals (Cooper and Plum, 1987; Ip et al., 2001c). The level of pyruvate and lactate levels in the plasma have also been reported to increase as a consequence of a high ammonia load (Fromm and Gillette, 1968). Plasma lactate concentration has long been considered a useful indicator of aerobic limitations and anaerobic capabilities in exercise studies, a significant reduction in swimming capacity was reported in goldfish (*Carassius auratus*) (Sinha et al., 2012a), rainbow trout (*Oncorhynchus mykiss*) and sockeye salmon (*Oncorhynchus nerka*) when plasma lactate concentration augmented more than the threshold limits (Farrell et al., 1998; Jain and Farrell, 2003; Stevens and Black, 1966). Moreover, an increase of acid metabolites due to accumulation of pyruvate and lactate lowers the blood pH which would shift the oxygen saturation of haemoglobin and cause death by suffocation (Campbell, 1991).

Ammonia also exerts its toxic effects in the brain by disrupting cerebral blood flow (Andersson et al., 1981) and by interfering with amino acid transport (Mans et al., 1983) as well as excitatory amino acid neurotransmitter metabolism (Hindfelt et al., 1977). Exposure to HEA threatens the level cerebral glutamate which is the principle excitatory neurotransmitter in brain. Inhibition of glutamate uptake (Oppong et al., 1995) or increased glutamate release from neurons (Rose, 2002) and/or astrocytes as a consequence of elevated ammonia can cause an increase in extra-cellular glutamate (Michalak et al., 1996) that will initiate a series of deleterious effects. Furthermore, the uncoupling of oxidative phosphorylation by NH_4^+ is another adverse effect of ammonia to inhibit ATP production (Smart, 1978). Moreover, environmental challenges such as ammonia pollution may induce expression of various proteins and genes associated with short term and/or long term stress responses at the cellular level to the behavioural response (Lee et al., 2006b). These consequences have profound effect on overall performance of fish (Lee et al., 2006a,b; Sinha et al., 2010, 2012c).

Furthermore, teleosts are known to use their gills as the dominant site of gas exchange, osmoregulation, acid-base regulation, as well as for the excretion of nitrogenous wastes making the fish gill an excellent model to understand ammonia excretion. Since ammonia exerts its toxic effects by interfering with gill physiological processes, it is important to have a better understanding of fish gill structure and how ammonia excretion is handled by the fish gills.

1.3. FISH GILL STRUCTURE

Fish species studied in present work belong to the freshwater fishes which are hyper-osmotic to the surrounding environment and face osmotic gain of water and diffusional loss of NaCl across the gill epithelium. Consequently, these freshwater fish need to have highly efficient iono-osmoregulatory mechanisms to ensure body fluid homeostasis and subsequent normal function of all physiological processes (Hwang and Lee, 2007). To compensate for this water absorption and ion loss, large volumes of dilute urine are excreted via the kidney while active uptake of NaCl occurs at the gill (Evans, 2008). Although freshwater fish do have kidneys and these are equipped to minimize renal salt loss by tubular reabsorption, it is actually the gill that performs most of the ion regulatory functions (Evans et al., 2005). To fulfil all of these (and other) functions, gills must be highly specialized. Indeed, their structure is modelled in such a way that their highly complex vasculature and their high surface area are optimized to improve gas and ion exchange.

In general, individual branchial arches (4 on each side) in the teleosts consist of multiple filaments, which are further subdivided into thousands of lamellae, the sites of gas exchange (Fig.1.1). The flow of water is counter-current to the flow of blood that perfuses the lamellae, maximizing gas, ionic, and osmotic gradients. This facilitates gas exchange, but also enhances net ionic and osmotic movements that the fish must counter to maintain osmotic homeostasis (Evans et al., 1999). Fish gills are covered by a thin epithelium which is composed of various cell types: pavement cells (PVC), chloride cells (or mitochondria-rich cells 'MRC'), accessory cells and mucus cells. This thin gill epithelium separates the blood from external water. Among wide array of cells in freshwater fish gill, PVC are the predominant ones (>90%) and MRC the second in line (<10%) (Evans et al., 2005).

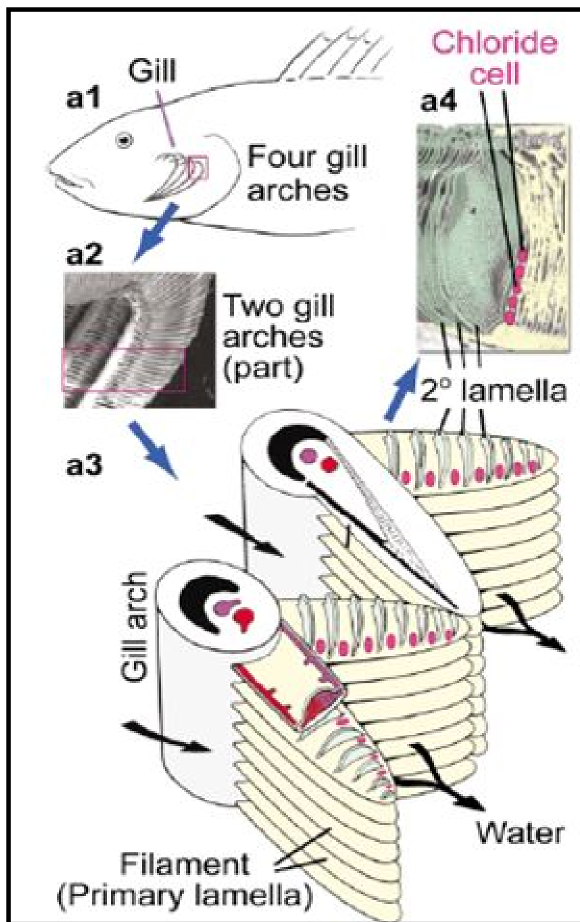

Figure.1.1. Structure of the gill. The gills contain 4 gill arches (a1-a2) which consist out of primary lamella, the filaments (a2-a3). These filaments are subdivided into secondary lamella (a3-a4). The gill epithelium is mostly made of pavement cells and mitochondria-rich cells (Fig. adapted from Hirose et al., 2003)

MRCs are believed to be responsible for ion uptake and are mostly located in the afferent edge of filaments and the inter-lamellar region. In freshwater fish, they often occur singly in the epithelium. MRCs have high densities of mitochondria in their cytoplasm and are round or ovoid shaped cells. Additionally, Na^+/K^+-ATPase (explained in detailed subsequently) is present in large quantities in these cells (Hirose et al., 2003).Previously, it was believed that PVCs did not contribute substantially to the ion transport (Perry, 1997); although recent evidence points to a subdivision of PVCs which are involved in ion uptake and acid-base transport (Evans et al., 2005). These particular PVCs appear to have a higher number of mitochondria present as well as V-type H^+-ATPase (Galvez et al., 2002; Goss et al., 2001). All PVCs are linked with intercellular tight junctions to adjacent cells, relatively impermeable for ions (Evans et al., 2005). Furthermore, they are thought to be important in gas exchange since they are thin cells with an extensive apical surface area and microvilli (Perry, 1997). In brief, freshwater fishes have evolved a relatively ion impermeable gill epithelium to minimize diffusive ion losses. Structurally, this low ion permeance is conferred by relatively deep paracellular tight junctions between adjacent gill epithelial cells. In contrast, many marine teleosts gills are characterized by relatively shallow tight junctions between the basolateral interfaces of adjacent

seawater type chloride cells or accessory cells, therefore offering the gills more permeable to ion loss in these marine fish.

1.4. MECHANISMS OF AMMONIA TRANSPORT ACROSS THE BRANCHIAL EPITHELIUM

Mechanisms of ammonia excretion have been reviewed in the last 5–10 years (e.g., Wood, 1993, 2001; Wilkie, 1997, 2002; Evans, 2005) suggesting transport either by simple diffusion and/or by the use of transport proteins pathways as discussed below in fig.1.2.

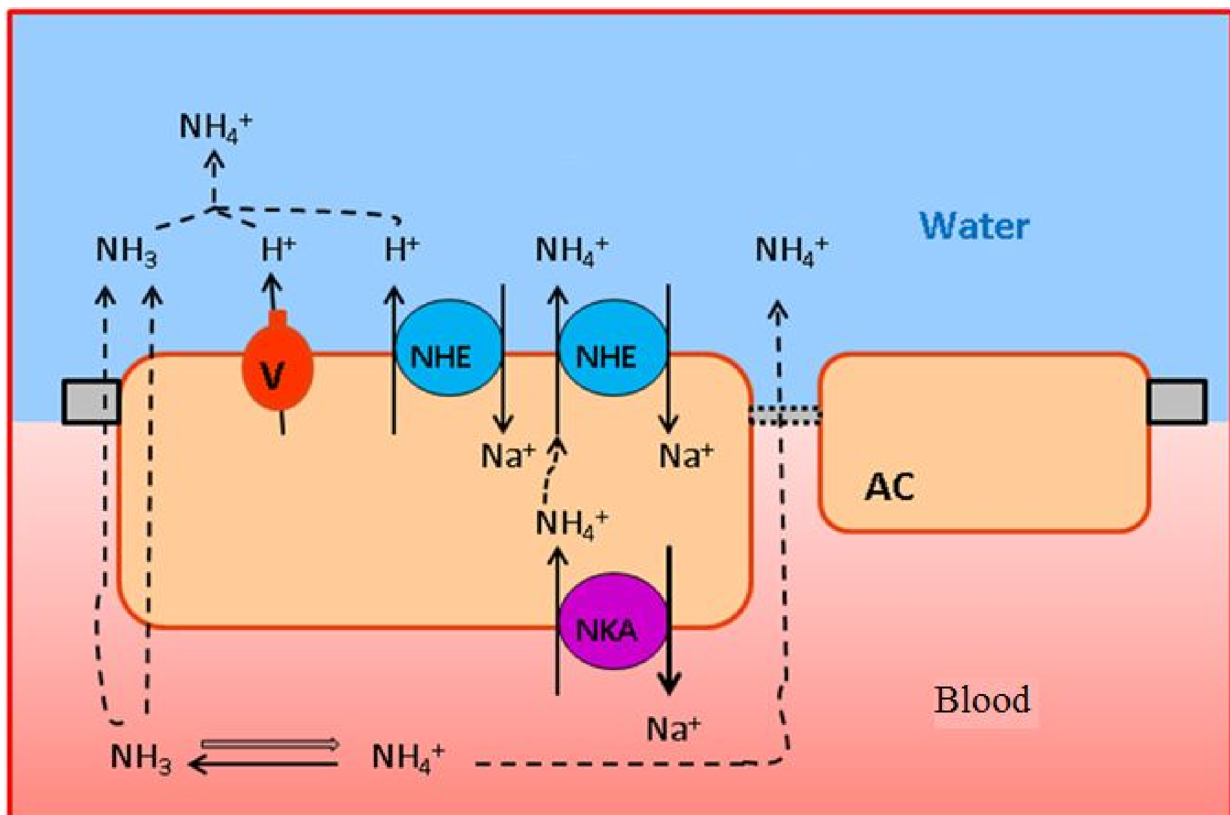

Figure. 1.2. Proposed pathways of ammonia excretion in branchial epithelium involving simple diffusion and transporters/exchangers (slightly modified from Evans et al., 2005)(NHE: Na^+/H^+ or Na^+/NH_4^+ exchanger; V-pump: H^+-ATPase pump; NKA: Na^+/K^+-ATPase pump, see text below for further explanation).

1.4.1. Diffusion pathways

NH_3 Diffusion: rely on blood-to-water diffusion gradients

The bulk of evidence generated over the last 25 years points to the simple diffusion of ammonia (NH_3) down the favourable blood-to-water gradient across the membrane in freshwater teleosts (Wilkie, 2002). At physiological pH the majority of ammonia exists as ammonium (NH_4^+) and due to its positive charge it cannot penetrate the lipid phase of cell membranes (Knepper et al., 1989). In addition, the gills of fresh water fish are relatively tight to cations, therefore, it is unlikely that appreciable passive NH_4^+ diffusion takes place under typical fresh water conditions. Though NH_3 is about 10-1000 times more permeable in gill epithelia than NH_4^+ (Wood, 1993), but NH_3 lipid solubility is only moderate in comparison to other neutral molecules such as carbon dioxide (Knepper et al., 1989). Since lipid solubility of NH_3 is not especially high, it therefore seems unlikely that simple diffusion can be responsible for the entirety of ammonia excretion.

In addition, beside lipid bilayers, aqueous pores (aquaporins) are the other possibility by which NH_3 moves through branchial epithelium (Wood, 1993). Since the solubility of NH_3 in water is many folds higher than that of CO_2 and O_2 (Cameron and Heisler, 1983; Boutilier et al., 1984), it should readily move down favourable P_{NH3} gradients via aqueous pores. There are evidences of NH_3 and NH_4^+ movement through aquaporin 1 (AQP1) expressed in oocytes of the African clawed frog (*Xenopus laevis*; Nakhoul et al., 2001) but the possible expression of aquaporins in fish gill epithelia is still debated.

Diffusion of NH_3: relies on proton secretion to "trap" ammonia as NH_4^+

This pathway is based on the production of protons (H^+) by the hydration of CO_2 to HCO_3^- and H^+. Measurements of inspired and expired gill water pH suggest that H^+ arising from CO_2 hydration can reduce the expired gill water pH's may be down to 1.5 units lower than bulk inspired gill water values (Playle and Wood, 1989). At low pH in the boundary water layer of the branchial epithelia, a H^+ traps NH_3 as NH_4^+ (Fig.1.2), preventing the excreted NH_4^+ from dissociating into NH_3 and H^+, thus avoiding the back diffusion of NH_3 into the body down the inwardly directed ΔP_{NH3}.

In addition to the hydration of CO_2, acidification of the gill water is also facilitated by other mechanisms such as the V-type H^+-ATPase pump and Na^+/H^+ exchanger present in the apical branchial epithelium (see below).

NH_4^+ diffusion

Significant NH_4^+ diffusion likely occurs across the marine fish gill, but it is unlikely in fresh water. Freshwater teleosts are characterized by the deep tight junctions between adjacent cells in the gill epithelium, minimizing the NH_4^+ diffusion through paracellular route. In contrast, marine fishes have

shallow tight junctions between chloride cells and adjacent accessory cells. This arrangement not only facilitates Na^+ excretion by marine teleosts (Marshall, 1995; Karnaky, 1998) but it may also promote passive NH_4^+ diffusion.

1.4.2. Mediated by transport systems: involvement of pumps and exchanger

Ammonia excretion in freshwater fish is linked to Na^+ uptake, a scheme first proposed by Krogh (1939), demonstrating the presence of electroneutral Na^+/ NH_4^+ exchange in fresh water fish gills. In this model, Na^+ uptake across the apical side of the gill is coupled to NH_4^+extrusion which replace H^+ on an electroneutral Na^+/H^+ antiport. However, the concentration of Na^+ in freshwater is not sufficient enough to drive $Na^+/H^+(NH_4^+)$ exchanger (Potts, 1994; Wilkie, 1997). Therefore, the involvement of Na^+/ NH_4^+ exchange is not very likely in freshwater environments, although it could be important in seawater where external Na^+ concentrations are sufficient to drive such an exchange. Therefore, Na^+ uptake in fresh water fish was hypothesized to be carried out through apical channels, down favourable electrochemical gradients generated via proton pump (V-type H^+-ATPase) mediated H^+ extrusion (Perry and Fryer 1997; Marshall, 2002). It emphasized the potential role of an electroneutral Na^+/H^+ exchanger (NHE) and/or a primary H^+ pump/Na^+ channel system for Na^+ uptake, which consequently facilitate ammonia excretion (Avella and Bornacin, 1989; Potts, 1994; Lin and Randall, 1995; Sullivan et al., 1995, 1996).There was some discrepancy among freshwater fish whether the occurrence of this mechanism of NHE exists or if it is mistaken for ammonia trapping, in which the same protein channels, NHEs and H^+-ATPase, pumps out protons and these protons traps NH_3 as NH_4^+. This controversy was resolved through the most recent model of ammonia excretion proposed by Wright and Wood (2009). This model suggests the occurrence of an apical "Na^+/NH_4^+ exchange complex" consisting of several membrane transporters working together as a metabolon and provides an acid trapping mechanism for apical ammonia excretion (Fig.1.3).

The force for Na^+ uptake is also generated across the basolateral membrane by Na^+/K^+-ATPase (or Na^+ pump). It is composed of two catalytic α subunits and two accompanying β subunits. It energizes salt transport across both absorptive and secretory epithelia by pumping out, for every molecule of ATP hydrolyzed, three Na^+ ions in exchange for two K^+ ions against their electrochemical gradients. The β subunit is thought to participate in anchoring the complex in the membrane.

Although the branchial enzyme Na^+/K^+-ATPase is primarily associated with sodium transport, it also plays an important role in ammonia excretion since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at the basolateral transport sites of branchial epithelia (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). As a result NH_4^+ is transported into the intracellular compartment and from there moves across the apical membrane in exchange of sodium through Na^+/H^+ (NH_4^+) exchanger (Evans et al., 2005; Wilkie, 2002). Therefore, Na^+/K^+-ATPase provides a

driving force for the exchange of Na^+ from the water for ammonia from the fish (Avella and Bornancin, 1989; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997). Besides all these studies on ammonia excretion mechanisms, the debate on how the NH_3 gas passed across the gill membranes continued till the discovery of the potential role of Rhesus (Rh) glycoprotein in ammonia excretion in fish.

1.4.3. Involvement of Rh glycoproteins in ammonia excretion

Previously, it was proposed that NH_3 diffusion occurs across the phospholipid bilayer of the branchial epithelium, but pioneer work in recent years has indicated that Rhesus (Rh) glycoproteins which have long been linked to antibody production in humans, also serve as ammonia transporters in the respiratory surfaces of fish. Piscine Rh proteins show a high degree of homology with the members of Mep (methylammonium permeases) and Amt (ammonia transporters protein) that mediate ammonia transport in bacteria (Huang and Peng, 2005), yeast (Marini et al., 1994), plant (Ninnemann et al.,1994) and nematods (Huang and Peng, 2005), suggesting a long evolutionary history. Vertebrates typically express four paralogous groups of Rh genes: Rh30, Rhag, Rhbg, Rhcg (Huang and Peng, 2005). Rhag, Rhbg and Rhcg facilitate ammonia transport whereas Rh30 (in humans RhD/RhCE) are nonglycosylated, nontransporting, and are associated with the erythrocyte Rhag complex. Compared with mammals, fish have many more copies of Rh genes (Huang and Peng, 2005).

Weihrauch et al. (2004) were the first to identify Rh gene expression in the gills of water-breathing animals in the marine crab *Carcinus maenas*. The cDNA of a Rh-like protein from *C. maenas* gills was found to share sequence similarity with human RhCG and RhBG, as well as with zebrafish sequences. In fish, Nawata et al. (2007) confirmed the expression of seven Rh genes in the gills of the rainbow trout. The expressions of mRNAs of these Rh proteins were up-regulated in the gills in response to ammonia exposure, suggesting a potential role of Rh protein in ammonia excretion. Similarly, in the gills of pufferfish (*Takifugu rubripes*) Rh protein homologs were identified, favouring their functional role in ammonia excretion (Nakada et al. 2007a). Since then, there has been a series of studies shedding light on the role of Rh proteins as vital ammonia conduits among freshwater fish. Across all fish species studied to date, Rhcg and Rhbg mRNA and/or protein have been detected in the gills and other tissues, and Rhag in red blood cells and erythroid tissues (Hung et al., 2007; Nakada et al., 2007a,b; Nawata et al., 2007; Tsui et al., 2009).

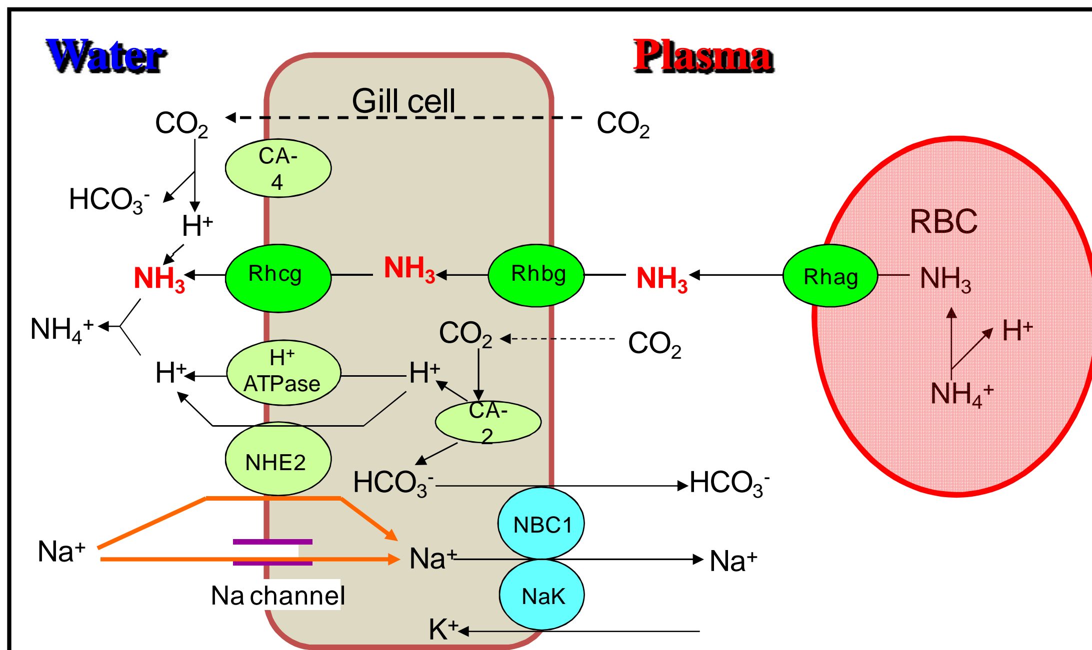

Figure.1.3. An updated model of ammonia excretion by freshwater fish, highlighting the potential role of Rh glycoprotein and "Na^+/NH_4^+ exchange complex" in brachial epithelium (Wright and Wood, 2009) (NHE: Na^+/H^+ exchanger; NKA: Na^+/K^+-ATPase; NBC: Na^+/HCO_3^- co-transporter; CA: carbonic anhydrase; RBC: red blood cells)

Based on the new Rh-related data available to date for freshwater fish (Nawata et al., 2007; Nawata and Wood, 2008; Nawata and Wood, 2009; Nakada et al., 2007a; Shih et al., 2008; Tsui et al.,2009; Braun et al., 2009b) Wright and Wood (2009) proposed the most advanced and widely accepted model for ammonia excretion in freshwater fish (Fig.1.3). This model suggests that Rhag proteins appear to be mainly restricted to erythrocytes whereas several isoforms of Rhbg and Rhcg proteins are expressed in many tissues. The general pattern suggests that in branchial epithelium Rhbg proteins occur on basolateral membranes whereas Rhcg proteins are expressed on apical membrane. According to this model ammonia is brought from red cells to plasma and is facilitated by Rhag present in erythrocytes. Then by diffusion NH_3 moves across the basolateral gill membrane via Rhbg, and across the apical gill membrane via Rhcg, down the partial pressure gradient of ammonia. Once NH_3 enters the water on the apical side, it combines with H^+ which is pumped from the cell by H^+-ATPase and/or by one or more Na^+/H^+ (NHE) exchange proteins, to form NH_4^+. It must be noted that different isoforms of Rhbg, Rhcg and NHE can be present based on inter-species variations. Many studies have confirmed that the mRNA expression of these Rh protein is up-regulated during exposure to high environmental ammonia or internal ammonia infusion, reinforcing their potential role in ammonia excretion (Nawata and Wood, 2009; Salama et al., 1999; Zimmer et al., 2010; Braun et al., 2009b; Hung et al., 2007; Nawata et al., 2007; Nawata et al., 2010b; Wood and Nawata, 2011).

This model favours that these Rh protein in fish function as ammonia channels, binding NH_4^+ but facilitating the diffusion of NH_3 (Nawata et al., 2010a). Since the actual species of ammonia moving through the fish Rh channels seems to be NH_3, the H^+ removed from NH_4^+ must be shuttled by another mechanism. To address this issue, Wright and Wood (2009) suggest that H^+ removed from the NH_4^+ at the intracellular binding site of the Rhcg proteins may be transferred to the external water by either or both of the V-type H^+-ATPase and/or the NHE. Both mechanisms would provide a coupling to Na^+ uptake. Direct exchange of Na^+ with H^+ is facilitated by NHE, and the V-type H^+-ATPase provide the necessary electromotive force to power the uptake of Na^+ from the water through a Na^+- channel (Wright and Wood, 2009, 2012). Therefore, Rhcg in particular appears to be coupled to H^+ excretion and Na^+ uptake mechanisms and as such this scheme suggests an apical "Na^+/ NH_4^+ exchange complex" consisting of several membrane transporters (Rhcg, V-type H^+-ATPase, Na^+/H^+ exchanger, Na^+ channel) working together as a metabolon provides an acid trapping mechanism for apical excretion.

1.5. HORMONAL CONTROL OF IONO-OSMOREGULATION AND ASSOCIATED AMMONIA TRANSPORT

High ammonia level can disrupt the delicately balanced ion-osmoregulation in fish (Knoph and Thorud, 1996; Wilson and Taylor, 1992). Several hormones such as cortisol, prolactin, growth hormone and thyroid hormones are involved in regulation of these regulatory processes. Therefore, a better knowledge of these hormones will offer us to understand adaptive responses in fish against ammonia toxicity.

Cortisol is the major corticosteroid produced by the activation of the hypothalamic pituitary-adrenal axis in stress situations and functions as both a glucocorticoid and mineralocorticoid. Cortisol acts to mobilize and synthesize metabolites and thus directly as well as indirectly influences many physiological processes during stress in fish (Babitha and Peter, 2010; Vijayan et al., 1997; Wendelaar Bonga, 1997). This hormone is reported to stimulate proliferation and differentiation of the chloride cells and the Na^+/K^+-ATPase expression in gill cells (Dang et al., 2000; Flik and Perry, 1989; Sloman et al., 2001). Furthermore, cortisol, together with increasing plasma NaCl concentrations, initiates salt secretion. McCormick (2001) demonstrated that high growth hormone concentration and low prolactin concentrations promote salt secretion while low growth hormone concentration and high prolactin concentration will result in ion uptake. Part of the interaction between growth hormone and cortisol is thought to be the ability of growth hormones to up regulate the number of gill cortisol receptors (Shrimpton and McCormick, 1999).

Prolactin (PRL) is produced in the pituitary gland, and is characterized by its function in water and electrolyte balance (Sangiao-Alvarellos et al., 2006). It has been well documented that in low ion-concentration environment, the expressions and the levels of PRL tend to increase to augment ion uptake and prevent ion loss and water uptake (Manzon, 2002; Sangiao-Alvarellos et al., 2006), making PRL the most important fresh water adapting hormone in euryhaline teleosts (Uchida et al., 2004). Nevertheless, differences of the importance of PRL in fresh water adaptation and in the action of PRL exist between and within species (Bern, 1983; Manzon, 2002). Moreover, it is equally crucial for metabolism, growth, development, reproduction, behaviour and immune-regulation (Power, 2005). The antagonism of PRL towards cortisol induction of salt secretory processes is an important aspect of the numerous functions of prolactin (McCormick, 2001; Tse et al., 2008). In general, PRL initiates its actions through binding to a specific cell surface PRL receptor.

Growth hormone (GH) is a member of the same protein family as PRL. GH is involved in the regulation of growth by the stimulation of insulin growth factor in liver and other tissues (Wood et al., 2005), osmoregulation, metabolism, reproduction and immunity (Lee et al., 2001; Yada, 2007). The role of GH in osmo-regulation can be different among species (Sakamoto and McCormick,

2006). In salmonids for instance, GH facilitates seawater acclimation through chloride cell proliferation and hence enhanced branchial Na^{+}/K^{+}-ATPase activity (Sakamoto et al., 1993). Depending on the species, GH can enhance the capacity to hyper- or hypo-osmoregulate in non-salmonid species (McCormick, 2001). Among teleosts, Insulin-like growth factor (IGF) is partly responsible for the osmo-regulatory actions of GH (McCormick et al., 1991; McCormick, 1996; McCormick, 2001), the functional role of IGF as feedback signal for GH production is well conserved.

Thyroid hormones (THs) play critical roles in differentiation, growth, development, aerobic energy metabolism and reproduction (Swapna and Senthilkumaran, 2007). The primary TH, tetraiodo-L-thyronine (thyroxine, T4) is produced in a single layer of specialized epithelial cells, the thyroid follicles (Swapna and Senthilkumaran, 2007; Yamano, 2005). The necessary iodine is predominantly absorbed through the gills from the environment. The pituitary produces a thyroid stimulating hormone (TSH) which regulates the production of the TH via TSH receptors. First, iodide that has been concentrated in the thyroid follicles is incorporated into the glycoprotein thyroglobulin. This molecule is subsequently hydrolyzed through endocytosis and proteolytic digestion in phagolysosomes to release thyroid hormones, mostly T4 (Iseki et al., 2000; Swapna and Senthilkumaran, 2007). T4 is then metabolized to triiodo-L-thyronine (T3) via deiodinases, mostly in peripheral tissues. The binding affinity of T3 to their hormone receptors is much greater than for T4, therefore T3 is considered as the most biologically active form of TH. Evidence indicates that most thyroid hormone effects are mediated via binding to the nuclear thyroid hormone receptors (THRs), of which two forms THRα and THRβ exist. THRβ shows higher expression in the muscle and gill compared to THRα (Filby and Tyler, 2007; Power et al., 2001; Swapna and Senthilkumaran, 2007). Moreover, circulating THs also regulate expression levels IGF-I and growth hormone receptor (GHR) which are involved in the control of growth (Higgs et al., 1982).

1.6. DEFENCE AGAINST AMMONIA TOXICITY

The main reason in adopting strategies for ameliorating ammonia toxicity in aquatic animals including fishes is to prevent accumulation of ammonia to a toxic level in various tissues. During high ammonia threat, the ammonia excretion is impeded causing retention of endogenous ammonia and uptake of exogenous ammonia, thereby leading to an accumulation of toxic ammonia internally. In piscine groups, a variety of mechanistic adaptations have been evolved to deal with the increase of body ammonia loading as a result of retention and/or reduction in ammonia excretion. These defense strategies in fish in response to elevated ammonia can be broadly classified into two major categories as discussed below and also illustrated in fig.1.4.

1.6.1. At the branchial and/or epithelial surfaces

Active excretion of ammonium ions

Active transport of NH_4^+ against the inwardly directed electrochemical gradient of ammonia was observed in the mudskipper (*Periophthalmodon schlosseri*) (Randall *et al*., 1999). African sharp-tooth catfish (*Clarias gariepinus*) are able to maintain the steady-state level of plasma ammonia by excreting NH_4^+ against a concentration gradient of ammonia (Ip et al. 2004a). Likewise, rainbow trout could excrete part of an ammonia load against high ambient levels in both freshwater and seawater (Wilson and Taylor, 1992), suggesting that NH_4^+ may be actively exchanged for H^+ in freshwater and Na^+ in seawater. Substitution of NH_4^+ for K^+ in Na^+/K^+-ATPase has been proposed as a method of branchial ammonia excretion in fish (Claiborne et al., 1982; Evans and Cameron, 1986). Indeed, the gill of *P. schlosseri* has very high activity of branchial Na^+/K^+-ATPase, approximately 10-fold higher than that of other fishes (Peng et al., 1998; Randall et al., 1999). There are reports of effective NH_4^+ substitution for K^+ on the Na^+/K^+-ATPase in the gills of mudskipper (Randall et al., 1999) which moves ammonium ions into the cell and are moved across the apical membrane in exchange for sodium via $Na^+//H^+(NH_4^+)$ exchanger present in the apical membrane. This mechanism would result in the accumulation of Na^+ in the blood and facilitate the excretion of ammonia out of the body across the gill epithelium.

Low NH_3 permeability of the respiratory surfaces

The branchial epithelial surface of aquatic teleosts plays a major role in gaseous exchange, therefore the permeability to NH_3 is considerably high. Air- breathing fish usually possess degenerated gills (Graham, 1997) so they depend largely on accessory organs for respiration.

Also, they usually hold air in their buccal cavities during immersion, which means their gills would not be exposed to ammonia even in high environment ammonia. Such an adaptation helps to reduce the influx of exogenous NH_3 through the gills during ammonia loading. However, many tropical air-

breathing fishes substitute branchial respiration by having highly vascularised skins. Hence it would essential for these fish to reduce the permeability of their skins to NH_3 despite the cell membranes being permeable to gaseous molecules like O_2 and CO_2, even though the permeability of NH_3 is less than those of O_2 and CO_2 (Marcaggi and Coles, 2001). NH_3 permeates the membrane by solvation and diffusion in the lipid bilayer and the limit is determined by the lipid properties of membrane (Ip et al., 2004a). The cholesterol and phospholipid fatty-acid contents of artificial membranes have been shown to affect the permeability of artificial membranes to ammonia, which decreases with decreasing membrane fluidity (Lande et al., 1994). However, there is no report of membrane fluidity manipulation in fish during ammonia stress, except possibly in the mudskipper (*P. schlosseri*). The skin of the mudskipper has a very high cholesterol content (4.5 µmol/g), which lowers the fluidity of biomembranes. The cholesterol content in the skin of (*P. schlosseri*) increased significantly to 5.5 µmol/g after 6 days of high environmental ammonia exposure (Ip et al., 2003a), suggesting a role for cholesterol as a defence mechanism against environmental ammonia toxicity. In the face of high environmental ammonia, a reduction in NH_3 permeability of the skin of the mudskipper would help to reduce the influx of NH_3. This is an important adaptation, which complements active NH_4^+ excretion through its gills because it would prevent back diffusion of ammonia through the cutaneous surfaces after build up of high ammonia concentrations in the external medium (Randall et al., 1999; Ip et al., 2003a,b).

NH_3 volatilization

In teleosts, ammonia volatilization was first reported in the temperate intertidal blenny (*Blennius pholis*) (Davenport and Sayer, 1986). The other tropical air-breathing fish that are capable of volatizing ammonia during aerial exposure are the leaping blenny (*Alticus kirki*) (Rozemeijer and Plaut, 1993), the oriental weatherloach (*Misgurnus anguillicaudatus*) (Chew et al., 2001) and the mangrove killifish (*Rivulus marmoratus*) (Frick and Wright, 2002). However, only the oriental weatherloach has been reported to be capable of volatizing NH_3 during HEA (Tsui et al., 2002). This species can accumulate ammonia in its body to an extent much higher than other fish (Chew et al., 2001) results in the alkalinity of the blood pH. The progressive increase in the amount of internal ammonia and alkalinization are the essential prerequisites for volatilization to occur. These together would facilitate the ΔP_{NH3} gradient that favours efflux of ammonia from the non-branchial epithelial surfaces such as gut as this loach uses its gut for gaseous exchange. Tsui et al. (2002) reported that the pH of the anterior part of its gut significantly elevated during ammonia exposure, suggesting that the anterior part of the gut may be the site of ammonia volatilization in this fish.

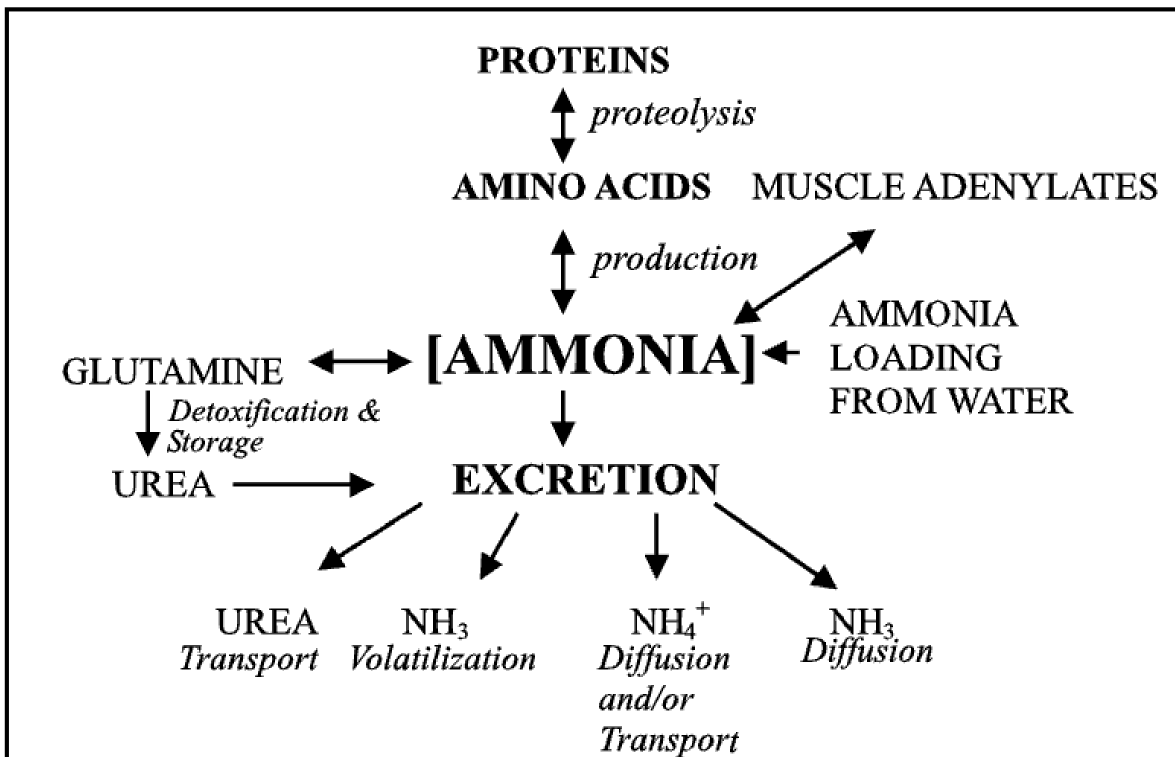

Fig.1.4. Summary of strategies utilized by fish to ameliorate ammonia toxicity (adapted from Ip et al., 2001b,c).

1.6.2. At the cellular and subcellular levels

Reduction in ammonia production

Many teleosts utilise a strategy of decreasing the rate of endogenous ammonia production from amino acid catabolism and thus are able to slow down the build up of ammonia internally. The steady-state concentrations of free amino acids (FAAs) in tissues are determined by the rates of their degradation and production. This defensive mechanism supports the suppression of ammonia production rate in fish by diminishing the rates of FAA catabolism (Ip et al., 2001b; Lim et al., 2001; Tsui et al., 2004). As such it changes the FAA metabolic equilibrium towards their synthesis, leading to FAA accumulation under HEA as previously shown in numerous teleosts (Anderson et al., 2002; Chew et al., 2001; Dabrowska and Wlasow, 1986; Ip et al., 2001c; Iwata, 1988; Iwata et al., 1981; Peng et al., 1998; Tsui et al., 2002; Wilson et al., 1998).

Glutamine synthesis

Glutamine formation is an important ammonia (exogenous and endogenous) detoxification strategy reported especially in the brain of many freshwater teleosts during exposure to HEA (Hernández et al., 1999; Ip et al., 2001b; Mommsen and Walsh, 1992; Saha et al., 2002; Wee et al., 2007; Wicks and Randall, 2002). Glutamine, a non-essential amino acid, is formed from glutamate and NH_4^+, the reaction is catalyzed by the enzyme glutamine synthetase (GSase). Glutamate is produced from α-ketoglutarate (α -KG) and NH_4^+ by glutamate dehydrogenase (GDH) or from α -KG and other amino

acids catalyzed by various transaminases. Therefore, starting with α -KG, formation of 1 mol of glutamine will detoxify 2 mol of NH_4^+. Upon exposure to ammonia, cerebral glutamine levels have been reported to increase many fold in Lake Magadi tilapia, *Oreochromis grahami* (Mommsen and Walsh, 1992), goldfish, *Carassius auratus* (Levi et al., 1974, Sinha et al., 2013), mudskippers, *Periophthalmus schlosseri* and *Boleophthalmus boddaerti* (Ip et al., 2001b, 2005; Peng et al., 1998), common carp, *Cyprinus carpio* (Dabrowska and Wlasow, 1986; Sinha et al., 2013), Nile tilapia, *Oreochromis niloticus* (Hegazi et al., 2010) and rainbow trout, *Oncorhynchus mykiss* (Sanderson et al., 2010; Wicks and Randall, 2002; Sinha et al., 2013).

Also, glutamine is stored within the body and can be used for other anabolic processes (e.g., syntheses of purine, pyrimidine, mucopolysaccharides) when environmental conditions become more favourable. Another advantage of glutamine synthesis is that it is energetically more efficient than other defensive strategies such as ureogenesis (see next section). During glutamine formation, every mole of ammonia detoxification consumes 2 mol of ATP while a total of 5 mol of ATP are hydrolyzed for each mole of urea synthesized (Ip et al., 2004c).

Partial amino acid catabolism

Certain amino acids such as arginine, glutamine, histidine, and proline can be converted to glutamate which can undergoe deamination catalysed by GDH, producing NH_4^+ and α-KG (Campbell, 1991). Glutamate can also undergo transamination with pyruvate, catalysed by alanine aminotransferase (ALT), producing α-KG without the release of ammonia (Ip et al., 2001c, Chew et al., 2003). This would facilitate the oxidation of the carbon chain of some amino acids without polluting the internal environment with ammonia. For those fish (e.g. mudskippers) which have difficulty in excreting endogenous ammonia, partial amino acid metabolism coupled with reduction in the rate of amino acid catabolism would be the most cost-effective strategy in reducing the rate of ammonia build-up in the body. It allows amino acids to be used as energy source during adverse conditions without releasing ammonia in the environment. However, it cannot be strictly considered as a strategy for detoxification of ammonia, because ammonia is not released and then converted back to alanine.

Urea synthesis

Some teleosts have the capacity to detoxify ammonia to the less toxic urea. The possible pathways for urea synthesis in teleosts are arginolysis, uricolysis and the ornithine urea cycle (OUC) (Mommsen and Walsh, 1989; 1992; Anderson and Walsh, 1995; Wright, 1995a; Walsh, 1997; Saha and Ratha, 2007). Though the majority of teleost fish are ammoniotelic, urea also constitutes about 10–30% of the total nitrogenous wastes in most of them (Saha and Ratha, 1998). To date, only a few teleosts (e.g.

Lake Magadi tilapia *Oreochromis grahami*, African lungfish *Protopterus dolloi*, Gulf toadfish *Opsanus beta*) are known to synthesis urea as a major strategy in detoxifying ammonia.

Ureogenesis in fish is energetically very expensive (as mentioned above), 5 mol of ATP are hydrolyzed for each mole of urea synthesized (Ip et al. 2001a). Therefore, detoxifying the exogenous (and endogenous) ammonia to urea and excreting it would not only result in a high expenditure of energy but also a loss of carbon. Presumably, because of these limitations, ureogenesis is not commonly adopted as a major strategy by teleosts to deal with ammonia toxicity during HEA.

1.7. ADDITIONAL STRESSORS

In addition to elevated levels of endogenous or exogenous ammonia, fish in natural and culture systems may face various other stressful situations such as feed limitation and exhaustive swimming. These additional stressors individually and/or in combination with HEA can have pronounced impacts on the physiological and ecological fitness of fish and their capacity to handle ammonia.

1.7.1. Feed limitation

Food restriction is a natural phenomenon in wild populations of fish and also occurs regularly for cultured fish. Aquaculture species are often subjected to periods of restricted feeding and fasting as management tools for water quality and disease (Robinson and Li, 1999), and to avoid build-up of waste products which may allow fish to maintain internal ammonia concentrations below toxic levels (Wicks and Randall, 2002b). Although reduction in feeding rates may be convenient and increase survival, it will undoubtedly hamper growth rate (Person-Le Ruyet et al., 1997; Sinha et al., 2012 a). It has already been demonstrated that nutritional status can have pronounced impacts on the performance and physiological condition of fish (Bucking and Wood, 2006a,b; Gaylord et al., 2001, 2005; Peterson and Small, 2004; Small et al., 2002; Small, 2005; Small and Peterson, 2005; Sinha et al., 2012 b). For example, the concentration of anaerobic fuels that are important for burst swimming, ionic status important for nerve conduction, and oxygen carrying tissues such as red blood cells all have the potential to be impacted by the nutritional status. When fish are able to feed adequately, they can accumulate energy stores, maintain the activity rates of important enzymes (Deng et al. 2004), conserve swimming ability and grow (Mendez and Wieser 1993). However, it is important to highlight that feeding not only induces an increased energy demand as part of the specific dynamic action (SDA), but it also leads to an increased endogenous production of ammonia (Randall and Tsui, 2002; Wicks and Randall, 2002b). In this scenario, it can be expected that feed deprivation may not be a problem but high endogenous ammonia production as a consequence of feeding can create undesirable effect. Interestingly, it was seen in many teleosts that fed fish are able to reduce internal ammonia load more efficiently than the fasted ones and is also true during HEA exposure (Wicks and Randall, 2002a,b; Zimmer et al.,2010; Sinha et al., 2012a,b,c). This indicates that fed fish are less sensitive to external ammonia than unfed fish and that fed fish are better equipped to tolerate HEA compared to starved fish. This protective effect is due to stimulation of glutamine synthetase activity during feeding. It facilitates rapid conversion of ammonia to glutamine which allows the animals to maintain lower plasma and tissue ammonia concentrations (Wicks and Randall, 2002b). Moreover, there is now growing evidence that feeding can up-regulate the transcript level of Rh protein (Zimmer et al., 2010) resulting in higher ammonia excretion rate in fed fish.

1.7.2. Exhaustive swimming or exercise

Like feeding, exercise is another important physiological activity of fish. Fish swim constantly, either in the form of low-intensity routine swimming, or high-intensity exhaustive swimming as a means of coping with water currents and predatory, hunting and migratory challenges.

Swimming has been shown to induce endogenous ammonia production and increases internal ammonia levels when compared with the resting situation (Knoph and Thorud, 1996). Ammonia accumulation in the plasma has been implicated in reducing swimming capacity in salmonids (Shingles et al., 2001; Wicks et al., 2002). Beaumont et al. (2000b) suggested that increased NH_4^+ levels alter the metabolic status within the fish, arising from the effects on a number of metabolic pathways which may lead to premature muscle fatigue. Besides, depolarization of muscle membrane potential due to the substitution of K^+ with NH_4^+ has also been hypothesized for impairment of muscle contraction (Beaumont et al., 1995a,b, 2000a; Cooper and Plum, 1987; Raabe and Lin, 1985). Furthermore, when fish are forced to swim at higher speed, the ammonia induced toxic responses in fish are amplified (Wicks et al., 2002). Therefore, exhaustive swimming in fish may act as an ecological stressor.

1.8. RESEARCH GOAL

We divided the objective of the present PhD work into three broad categories as described below

I. To investigate comparative ammonia toxicity and response

Despite of numerous studies conducted on different fish species concerning acute and chronic ammonia toxicity, compensatory mechanisms that respond to HEA are not yet fully understood. Therefore, there is a need to explore various adaptive responses at physiological, biochemical and at gene level in fish when confronted with ammonia polluted water. These will also provide an insight into the underlying mechanisms determining ammonia sensitivity among different fish species. Therefore, we did a comparative study to elucidate the compensatory responses in three commercially important freshwater fish (rainbow trout, common carp and goldfish) which have different tolerance limits to ammonia toxicity

Gill is the main organ for ammonia handling in freshwater fish and contains a number of transporters and associated mechanisms that are involved in maintenance of ammonia homeostasis (Avella and Bornancin, 1989; Evans, 1980; Evans et al., 1999; Wilkie, 1997).Therefore, it is of utmost importance to examine how ammonia is handled at the branchial epithelium by these experimental fish during HEA.

Therefore, first goal of our research was to do a comparative study to evaluate various mechanisms associated with ammonia handling such as the interaction of ammonia loading with the movement of Na^+ across the gill epithelia, iono/osmotic permeability in the gills in response to HEA and various physiological approach such ammonia and urea excretion rate and potential role of hormones.

Additionally, we wanted to evaluate the response of various pumps, transporter, exchanger and more importantly the role of Rh glycoprotein in the experimental animals as the potential means to deal with high ammonia load.

II. To examine detoxification and defensive response against ammonia at cellular and sub-cellular level

We already discussed (in above literature) that piscine group have evolved a number of different strategies to defend against ammonia toxicity at the cellular and the subcellular level and these countervailing responses vary greatly between species. However, there is limited information with regard to freshwater teleosts which have different tolerance limits to ammonia toxicity.

Therefore, goal of this part of research work is to compare the strategic approaches implemented by three freshwater fish (rainbow trout, goldfish and common carp) to offset ammonia toxicity.

III. To study possible interaction of ammonia with additional stressors-starvation and exhaustive swimming

Under natural conditions and/or in culture systems, the simultaneous occurrence of these three stressors (HEA, feed limitation and exercise) is expected; therefore it is advisable to study the interactive effect of these ecologically important metrics on the ecological fitness of fish. Therefore, our goal was to investigate the response of fish against the combined effects of these stressors at metabolic and physiological level, iono-osmoregulatory and hormonal level, and molecular (gene expression) level.

Chapter 2

INTRODUCTION TO EXPERIMENTAL WORKS AND RESEARCH OUTLINE

2.1. TEST SPECIES

In our experiments we used three commercial freshwater teleosts: rainbow trout (*Oncorhynchus mykiss,* Walbaum 1792), common carp (*Cyprinus carpio*, Linnaeus, 1758) and goldfish (*Carassius auratus,* Linnaeus, 1758). These fish species differs in their sensitivities to ammonia. Rainbow trout is ammonia-sensitive, common carp is moderately resistant while goldfish is very resistant to ammonia. The reported ammonia 96h LC_{50} values (expressed as total ammonia) for goldfish, common carp and trout are approximately 9 mM (pH 8.0), 2.6 mM (pH 7.5-7.8) and 1.7 mM (pH 8.0) respectively (Dowden and Bennett, 1965; Hasan and MacIntosh, 1986; Thurston et al., 1981).

Rainbow trout (Order: Salmoniformes; Family: Salmonidae) is a cold-water fish species, native to the tributaries of the Pacific Ocean and North America. They live primarily in freshwater and need well oxygenated, clean fast flowing streams or rivers to thrive in. They are carnivorous and feed on aquatic insects, molluscs, crustaceans, fish eggs, and other small fishes. Rainbow trout acquires a high commercial value as it is one of the top five sport fishes in North America, and it supports a big farming industry throughout the world. Moreover, it is an excellent food fish, which is marketed fresh and frozen.

Common carp (Order: Cypriniformes; Family: Cyprinidae) is native to Europe and thrives in a wide variety of conditions. They are usually found in still or slowly flowing waters at low altitudes, particularly in areas with abundant aquatic vegetation. The species can survive low oxygen concentrations (0.3-0.5 mg/L) as well as supersaturation. They are omnivorous, mainly fed on water insects, larvae of insects, worms, molluscs, and zooplankton. It is the most desired species for aquaculture purpose and has high market demand throughout the world.

Goldfish (Order: Cypriniformes; Family: Cyprinidae) is native of Eastern Asia, including China and adjacent regions. They are a close relative to the carp, typical habitat includes the quiet streams and pools, especially those with submerged aquatic vegetation. The goldfish is tolerant of high levels of turbidity, temperature fluctuations, and low levels of dissolved oxygen. It can thrive very well in ammonia polluted water. Like common carp, goldfish are also omnivorous and preferably fed on crustaceans, insect larvae, fish eggs and fry, benthic vegetation, and detritus. In general, goldfish is one of the most commonly used fish in aquaria and ornamental pools.

The optimal temperature for goldfish and common carp is around 20-22°C while for rainbow trout it is around 18°C.

2.2. RESEARCH TISSUES

Gills

The gills are the main entry routes for toxic agents in teleost fish and make this organ important target for the chemicals such as ammonia. Gills are also important organ for the regulation of water and ions in fish, and this is the main reason why many waterborne toxic agents are well known for disturbing iono-osmoregulatory homeostasis in fish. Moreover, gills are the organ at the interface with the contaminated environment and are thus the main tissue through which the toxicants enter in the organism. Therefore, it is well understood that this organ possesses a wide array of defensive mechanism. In this PhD work, ammonia was tested as a potent aquatic toxicant. Gills are the main organ in teleosts for ammonia excretion and serve many other purposes such as aquatic respiration, ammonia excretion, acid-base balance, modification of circulating metabolites and immune defense. Gill and all its functions are prime targets during ammonia exposure (see Introduction), which makes this organ an ideal tissue to study the effects of ammonia and the associated compensatory responses.

Brain

Ammonia is neurotoxic and the brain is one of the prone target organs for ammonia toxicity. It inhibits the formation of neurotransmitters (gamma-aminobutyric acid 'GABA' and glutamate) in brain, disrupts cerebral blood flow, causing astrocyte swelling and it alters the Krebs or TriCarboxylic Acid cycle (TCA) cycle. GABA is a major inhibitory neurotransmitter, whereas glutamate is a major excitatory neurotransmitter. Both neurotransmitters work together to control many processes. To protect against ammonia toxicity, fish brain must have a cascade of defensive mechanisms, therefore, this was the main tissues analyzed within the framework of this PhD to study defensive strategies against ammonia toxicity.

Plasma

Plasma serves as a transport system delivering various biological components to and from the cells. It is responsible for transporting gases, ions, nutrients, hormones, waste products and carrier molecules. Moreover, plasma proteins serve as carriers for other molecules. Many types of small molecules bind to specific plasma proteins and are transported from the organs that absorb these proteins to other tissues for utilization. These plasma proteins regulate the osmotic pressure and also help to keep the blood slightly basic at a stable pH. They do this by functioning as weak bases themselves to bind excess H^+ ions. By doing so, they remove excess H^+ from the blood which keeps it slightly basic.

Liver

Liver plays a key role in many physiological processes such as detoxification, digestion, and storage of glycogen and lipids. Liver comprises several cell types, such as biliary epithelial cells, macrophages, fat-storing cells and endothelial cells. A number of fish species, such as carp and goldfish, have characteristic high contents of glycogen in their hepatocytes and it is an important organ for assessing energy stores. Under certain toxic and stressful circumstances, the biochemical and physiological functions of the liver can be altered.

Muscle

There are two kinds of fish muscle, white muscle and red muscle. In cyprinids and salmonids there is a small strip of red aerobic muscle just under the skin on both sides of the body, running beneath the lateral line. In general, red muscle is trimmed out for biochemical analysis, and thus the results represent the major muscle type is white muscle. Muscle is another important tissue to measure energy stores, and ammonia and lactate content.

2.3. THESIS OUTLINE

To accomplish the goals mentioned in chapter 1, this thesis can be divided three major parts each containing several chapters.

Chapter 1: General Introduction

Chapter 2: Introduction to experimental works

Part I- Comparative ammonia toxicity and compensatory responses

Chapter 3- Gives an overview of the physiological and biochemical responses to acute high environmental ammonia (1mM and 1.4 or 5mM), particularly the linkages between branchial ammonia fluxes and unidirectional Na^+ fluxes, as well as urea excretion, cortisol, and indicators of gill permeability in three freshwater teleosts (rainbow trout, common carp and goldfish) differing in their sensitivities to ammonia. The results of study explain the differential tolerance of the three species during acute ammonia exposure.

Chapter 4- is the extension of chapter 3. It describes the physiological and molecular compensatory response in rainbow trout, common carp and goldfish during chronic ammonia exposure. Moreover, this chapter aims at getting a deeper insight in the potential role of Rhesus (Rh) glycoprotein and the involvement of 'Na^+/NH_4^+ exchange metabolon' in ammonia excretion.

Part II- Comparative detoxification and defense against ammonia toxicity

Chapter 5- describes different strategies implemented by trout, carp and goldfish to defend and detoxify ammonia mediated toxicity in the brain tissue. It mainly focuses on the regulation of amino acid synthesis and associated metabolic pathways to cope with ammonia toxicity.

Part III- Interactive studies with additional stressors- starvation and exhaustive swimming

Chapter 6- deals with the interactive study of high ammonia, nutritional status (fed *vs* starved) and exercise (routine swimming *vs* exhaustive swimming) on the metabolic and physiological performance in goldfish (*Carassius auratus* L.).

Chapter 7- is the continuation of chapter 6. In this chapter the interactive study was performed with the aim to observe the responses at hormonal and ion-regulatory processes.

Chapter 8- deals with the compensatory responses in a moderately ammonia tolerant fish species (the common carp, *Cyprinus carpio*) under ammonia exposure along with the additional effects of feed restriction and exercise.

Chapter 9- provides comprehensive information on the molecular mechanisms by which common carp (*Cyprinus carpio*) deals with an assortment of natural stressors (e.g. high ammonia, starvation and exercise). This work describes the modulation of mRNA transcript of potential biomarker genes controlling growth, ion-regulation and stress responses during above mentioned stressful conditions. The outcome of this study could permit to develop a "molecular biomarker system" to identify the underlying physiological processes.

Chapter 10- provides a common conclusion drawn from all the works described above and the possibility for further research.

Chapter 11: illustrates the morphological modifications in the gills of trout, common carp and goldfish when subjected to high level of ammonia. Macro-and ultrastructural components of gill filament and lamellae were examined by scanning electron microscope. These results provide an insight into the adaptive 'remodeling' of fish gill under ammonia threat. Light microscopy and transmission electron microscopy examinations will be carried out shortly which will offer a complete dynamics of gill transformation or remodeling.

The structure of the thesis is also provided in fig. 2.1 (refer below)

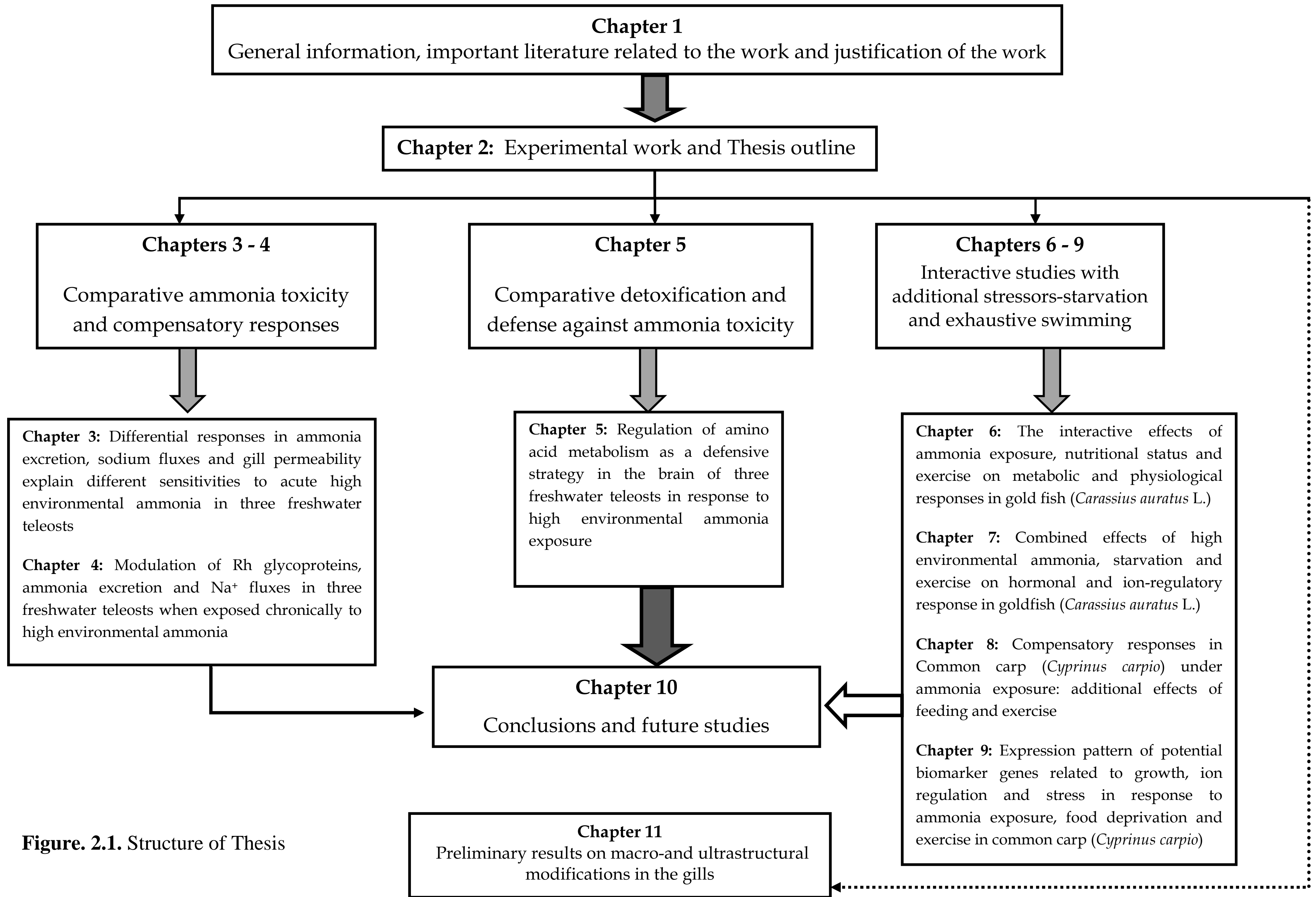

Figure. 2.1. Structure of Thesis

Part I

COMPARATIVE AMMONIA TOXICITY AND COMPENSATORY RESPONSES

Chapter 3

Differential responses in ammonia excretion, sodium fluxes and gill permeability explain different sensitivities to acute high environmental ammonia in three freshwater teleosts

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Abstract

We examined the acute physiological responses to high environmental ammonia (HEA), particularly the linkages between branchial ammonia fluxes and unidirectional Na^+ fluxes, as well as urea excretion, cortisol, and indicators of gill permeability in three freshwater teleosts differing in their sensitivities to ammonia; the highly sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less sensitive cyprinid *Cyprinus carpio* (common carp) and the highly resistant cyprinid *Carassius auratus* (goldfish). Fish were acutely exposed to two sub-lethal ammonia concentrations (as NH_4HCO_3) at pH 7.9: 1mM for a period of 12 h, identical for all species, and 5mM for the cyprinids and 1.4mM for the trout for 3 h. Elevation of plasma cortisol at both levels of HEA was apparent in all species. At 1mM, ammonia excretion (J_{amm}) was inhibited to a greater extent in trout than cyprinids and concurrently a significantly higher plasma ammonia level was evident in trout. However J_{amm} was reversed in all species at 5 or 1.4 mM. Goldfish showed a significant increase in urea excretion rate (J_{urea}) during HEA exposure. In carp and trout, neither level of HEA elevated J_{urea} but urea production was increased as evidenced by a considerable elevation of plasma urea. At 1mM HEA, Na^+ imbalance became progressively more severe in trout and carp due to a stimulation of unidirectional Na^+ efflux (J^{Na}_{out}) without a concomitant increase in unidirectional Na^+ influx (J^{Na}_{in}). Additionally, a transient reduction of J^{Na}_{in} was evident in trout. Goldfish showed an opposite trend for J^{Na}_{out} with reduced efflux rates and a positive Na^+ balance during the first few hours of HEA. However, after 12 h of exposure, both J^{Na}_{in} and J^{Na}_{out} were also increased in both carp and goldfish, whereas only J^{Na}_{out} was increased in trout, leading to a net Na^+ loss. Na^+ homeostasis was entirely disrupted in all three species when subjected to the 5 or 1.4mM ammonia for 3 h: J^{Na}_{in} was significantly inhibited while considerable activation of J^{Na}_{out} was observed. Diffusive water efflux rates and net K^+ loss rates across the gills were enhanced during HEA only in trout, indicating an increment in gill transcellular permeability. Transepithelial potential was increased in all the species during ammonia exposure, but to the least extent in goldfish. Overall, for several different physiological systems, trout were most disturbed, and goldfish were least disturbed by HEA, helping to explain the differential ammonia tolerance of the three species.

***Keywords*:** High environmental ammonia (HEA), Sodium flux, Gill permeability, Ammonia excretion, Urea excretion, Transepithelial Potential, Rainbow trout, Common carp, Goldfish.

3.1. INTRODUCTION

Ammonia is a major pollutant in aquatic environments, arising from sources such as sewage effluents, industrial wastes, agricultural run-off and decomposition of biological wastes (Randall and Tsui, 2002). Moreover, in confined waters or in aquaculture systems, a possible accumulation of metabolic waste products of fish, including ammonia, is a major concern. In aqueous solutions, ammonia exists as unionized ammonia (NH_3) and ionized ammonium (NH_4^+), with the latter greatly predominating at normal water pHs (Randall and Tsui, 2002; Wajsbrot et al., 1993). Most biological membranes are permeable to ammonia but relatively impermeable to ammonium ions. Consequently, the toxicity of ammonia is attributed to its unionized form (NH_3) which can readily diffuse across the gill membranes. Moreover, fish can excrete ammonia as NH_3 across the gill into the water providing there is an outwardly directed gradient, and this process is facilitated by Rhesus (Rh) glycoproteins (Nakada et al., 2007a; Nawata et al., 2007). However, under high environmental ammonia (HEA), the outward flux of ammonia through the gills is reduced, and a reversed inward ammonia flux occurs. As a result blood and tissue ammonia levels increase and fish experience both acute and chronic toxic effects (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002). Notable pathologies include decreased growth rates (Dosdat et al., 2003; Foss et al., 2004; Lemarie et al., 2004; Pinto et al., 2007; Sinha et al., 2012a), alterations in energy metabolism (Arillo et al.,1981a,b), disruption of ionic balance (Soderberg and Meade, 1992; Wilkie, 1997), increased vulnerability to disease, and histopathological changes in gill epithelia (Wilkie, 1997). Numerous studies on different fish species concerning acute and chronic ammonia toxicity already exist (Benli et al., 2008; Dosdat et al., 2003; Knoph and Olsen, 1994; Knoph and Thorud, 1996; Lemarie et al., 2004; Person-Le Ruyet et al., 1997, 1998, 2003; Tomasso, 1994; Weinstein and Kimmel, 1998; Wicks and Randall, 2002), but the various compensatory mechanisms that respond to HEA are not yet fully understood.

We postulated that such physiological responses may vary among fish species which have different tolerance limits to ammonia toxicity, the understanding of which might help to identify underlying mechanisms involved in ammonia sensitivity. Therefore, the focus of the present comparative study was to elucidate the physiological compensatory responses of three commercially important freshwater fish when exposed acutely to high environmental ammonia (HEA 1mM-5mM, at pH 7.9): a sensitive salmonid, the rainbow trout *Oncorhynchus mykiss*, a less sensitive cyprinid, the common carp, *Cyprinus carpio*, and the very resistant cyprinid, goldfish, *Carassius auratus*. The reported ammonia 96h LC_{50} value (expressed as total ammonia) for goldfish, common carp and trout are approximately 9 mM (pH 8.0), 2.6 mM (pH 7.5-7.8) and 1.7 mM (pH 8.0) respectively (Dowden and Bennett, 1965; Hasan and MacIntosh, 1986; Thurston et al., 1981).

We examined net ammonia (J_{amm}) and urea (J_{urea}) flux rates as well as plasma ammonia and urea concentrations to assess whether nitrogenous waste production and/or excretion processes are altered during acute HEA exposure. We also studied cortisol levels which play a crucial role in the stress response and in osmo-regulatory processes (McCormick, 2001; Wendelaar Bonga, 1997) and appears to augment the ammonia transport capacity of the gills (Ortega et al., 2005; Tsui et al., 2009; Wood and Nawata, 2011).

Unidirectional (J^{Na}_{in}, J^{Na}_{out}) and net (J^{Na}_{net}) sodium flux rates using ^{22}Na was also examined. A number of studies have shown that the perturbation of branchial ionic exchanges as a consequence of HEA may result in sodium imbalance in aquatic animals (Avella and Bornancin, 1989; Maetz and Garcia Romeu, 1964; Maetz, 1972, 1973; Shaw, 1960; Wilson and Taylor, 1992; Wright, 1975; Zimmer et al., 2010). Specifically, a reduction in the rate of Na^+ uptake has been reported in juvenile rainbow trout (Twitchen and Eddy, 1994; Zimmer et al., 2010), channel catfish (*Ictalurus punctatus*) (Tomasso et al., 1980) and goldfish (Maetz and Garcia Romeu, 1964) when subjected to HEA. However, the exact mechanisms underlying these effects are not well understood, and earlier suggestions of "Na^+/NH_4^+ exchange" pathways (e.g. Maetz and Garcia Romeu, 1964; Salama et al., 1999; Wilson et al., 1994; Wright and Wood, 1985) are now being re-evaluated as "Na^+/NH_4^+ exchange complexes or metabolons" in light of new findings on the involvement of Rh glycoproteins, Na^+/H^+ exchangers (NHE), and H^+-ATPase in facilitating both ammonia excretion and Na^+ uptake across the gills (Nawata et al., 2007; Weihrauch et al., 2009; Wright and Wood, 2009).

Finally, we also examined several indices of gill permeability during HEA exposures. In addition to the unidirectional Na^+ efflux rates (J^{Na}_{out}) measured during the Na^+ balance experiments, we also assessed net K^+ flux rates (J^{K}_{net}), diffusive water exchange rates (using ^3H_2O), and transepithelial potential (TEP). J^{K}_{net} (Lauren and McDonald, 1985; Wood et al., 2009) and diffusive water exchange rates (Isaia, 1984; McDonald et al., 1991; Wood et al., 2009) have been interpreted as indices of gill transcellular permeability. In freshwater fish, TEP is thought to mainly reflect a ''Na^+ diffusion potential'' originating from the relative passive permeabilities of the gills to Na^+ versus Cl^- ions. (Eddy 1975; House and Maetz 1974; McWilliams and Potts 1978; Potts, 1984; Potts et al., 1991; Potts and Hedges 1991; Wood and Grosell, 2008, 2009). Recent reports indicated that TEP (inside relative to external water as zero) shifted in a positive direction in rainbow trout during acute HEA exposure (Tsui et al., 2009; Wood and Nawata, 2011).

In brief, the purpose of this study was 3-fold. The first goal was to investigate how these three freshwater fish (rainbow trout, common carp and goldfish) regulate their ammonia excretion and/or metabolic conversion of ammonia in response to acute HEA exposure (1mM for 12 h for all species, and 5mM for 3 h for carp and goldfish or 1.4mM for 3 h for the more sensitive trout, which succumbed at the higher level in preliminary tests). The second was to determine the interaction of

ammonia loading with the movement of Na^+ across the gill epithelia by measuring J^{Na}_{in}, J^{Na}_{out} and J^{Na}_{net}. The third was to observe the effect of HEA on iono/osmotic permeability in the gills through investigation of TEP, diffusive water flux and J^{K}_{net}. Overall, the results indicate that these fish species show differential compensatory responses towards HEA in several different physiological systems. Goldfish deal with ammonia challenge better than carp while trout are weakest in virtually all responses, helping explain the differential ammonia tolerance of the three species.

3.2. MATERIALS AND METHODS

Experimental system and animals

Rainbow trout, *Oncorhynchus mykiss*, were obtained from a fish farm - Bijnens, Zonhoven, Belgium; goldfish, *Carassius auratus*, were obtained from Aqua Hobby, Heist op den Berg, Belgium; common carp, *Cyprinus carpio,* were obtained from the fish hatchery at Wageningen University, The Netherlands. Fish were kept at the University of Antwerp in aquaria (200 L) for at least a month before the exposure started. A total of 75 goldfish and 75 common carp were distributed species wise into three 200 L tanks (n = 25 per tank) while 80 trout were placed in eight 200 L tanks (n=10 per tank). Each of these tanks was equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17±1°C and photoperiod was 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Water parameters were: pH 7.4 ± 0.2, dissolved oxygen 6.9-7.4 mg/L, total NH_3 0.006-0.009 mM, nitrite 0.0015- 0.0021mM, nitrate 0.015-0.042 mM, Ca^{2+} 0.8–1.0 mM, Mg^{2+} 0.19-0.21 mM, Na^+ 1.2-1.4 mM, K^+ 0.09-0.10 mM, Cl^- 0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 226 mg $CaCO_3$/L. Average mass (mean ± standard deviation) of rainbow trout was 210± 56 g, of common carp 18±4 g, and of goldfish 14± 4 g. Fish were acclimated for 2 weeks prior to the experiment and were fed ad libitum once a day with either commercial pellets (‘Hikari Staple’, Kyorin Food Ind. Ltd., Japan) for common carp and goldfish, or ‘Trouvit’ (Trouw Nutrition, Fontaine-les-Vervins, France) for rainbow trout. Feeding was suspended 2 days before experimentation.

Experimental protocol

Fish were placed in individual experimental containers the evening before an experiment and left overnight to settle with continuous aeration. These containers were placed in a climate chamber having the same temperature and photoperiod as for the fish holding. The containers for trout were 3 L (water volume set to 2.5 L) sealable Nalgene kitchen cutlery containers mounted on their sides; the horizontally flattened shape fitted the morphology of the fish. Similar Nalgene kitchen cutlery containers of 0.5 L (water volume set to 0.3 L) were employed for common carp and goldfish. The experimental compartments were shielded with black plastic to minimize visual disturbance and fitted

with individual air-stones. The experimental protocols consisted of exposing the fish (n= 8 per experiment) to HEA while simultaneously measuring unidirectional Na^+ fluxes with ^{22}Na (manufactured by Perkin Elmer Boston, MA, USA) and net ammonia and urea fluxes.

The exposures were run in two series. In the first series goldfish, carp and trout were exposed to 1mM HEA for a period of 3 h and 12 h. This concentration is sublethal for all 3 species (11%, 38% and 59% of the 96h LC_{50} respectively). Control groups (no HEA) were setup in parallel to 3 h and at 12 h.

In a second series, goldfish and common carp were subjected to a higher dose of 5mM HEA for a period of 0 h (control) and 3 h. Rainbow trout were exposed for the same periods to 1.4mM. These concentrations were close to 96h LC_{50} values for all species. Each exposure compartment was spiked with the required amount of a NH_4HCO_3 stock solution (Sigma, Germany). Water pH was monitored at 30-min intervals throughout the experiments and was maintained at 7.8-8.0 using dilute HCl and/or KOH.

At the start of each flux period, an aliquot of ^{22}Na (typically 2 µCi/L) was added to each container and allowed to equilibrate for 30 min. Water samples (4 x 5 mL for ^{22}Na, total Na^+ and K^+ measurements) were taken at the start of the experiment (0 h) and at subsequent 1-h intervals up to 3 h after the onset of ammonia exposure. For the ammonia and urea flux measurements an initial (0 h) and final water sample were also taken. When the experiment was terminated the animals were rapidly killed with a lethal dose of neutralized MS222 (ethyl 3-aminobenzoate methane-sulfonic acid, 1 g/L, Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, a blood sample was collected from the caudal blood vessel using a heparinized syringe. Blood was immediately centrifuged (for 1 min at 16,000 rpm at 4°C), and aliquots of plasma were frozen in liquid nitrogen and stored at -80°C for later analysis.

Transepithelial potential measurement

A parallel experiment was conducted with each fish species (n=8) to monitor changes in transepithelial potential (TEP). The intra-peritoneal catheter technique, which had been pioneered by Potts and Eddy (1973) and validated against blood catheter measurements by Wood and Grosell (2008), was employed. Fish were lightly anaesthetized by neutralized MS222. A saline-filled PE50 catheter (Clay-Adams; Becton–Dickinson, Sparks, MD, USA) was inserted 1-2 cm through the peritoneal wall into the coelom via a puncture site made with a 19-gauge needle, just lateral and anterior to the rectum. A 2 cm PE160 sleeve, heat-flared at both ends, was glued to the PE50 with cyanoacrylate resin and anchored to the body wall with several silk sutures to prevent the catheter from changing depth. After overnight recovery, TEP was measured under control conditions and after exposure to 1mM HEA for 3 h and 12 h, with all recordings being made on the same fish.

TEP was measured by means of 3mol/L KCl-agar bridges connected via Ag/AgCl electrodes (WPI, Sarasota, FL, USA) to a high impedance electrometer (Radiometer pHM 82 meter, Copenhagen, Denmark). The reference bridge was placed in the water in the fish chamber, and the measurement bridge was connected to the saline-filled intraperitoneal catheter. TEP measurements were expressed relative to the apical (water) side as 0 mV after correction for junction potential.

Diffusive water flux measurement

In a separate series of experiment, the diffusive exchange rate of water was measured by monitoring the efflux of tritiated water (^3H_2O; manufactured by Perkin Elmer, Boston, MA, USA). The protocol was modelled after that described by Wood et al. (2009). After overnight acclimatization in the experimental compartments (as explained above), individuals of each species (n=8) were injected intra-peritoneally with ^3H_2O (10μCi ^3H_2O in 200 μL Cortland saline for trout, 2μCi ^3H_2O in 40 μL Cortland saline for the smaller carp and goldfish). After 1-h equilibration, water samples were withdrawn at 0.5-h intervals for a period of 3 h. Separate groups of fish (n=8) were examined under control conditions (no HEA), after 3 h (i.e. injection at 0 h, sampling at 1-4 h), and after 12 h (i.e. injection at 10 h, sampling at 11-14 h) of 1mM HEA.

For all treatment groups, the compartments were then left closed with aeration for approximately 24 h after the original injection. A final water sample was taken to ascertain the exact dose of ^3H_2O which had been administered to each fish, because by this time the radioisotope had completely equilibrated between the fish and the water.

3.3. ANALYTICAL TECHNIQUES AND CALCULATIONS

Plasma cortisol levels were determined by radioimmunoassay (RIA) using a kit from MP Biomedicals (New York, U.S.A) as described by Balm et al., (1994). Water total ammonia was determined colorimetrically by using the salicylate-hypochlorite method (Verdouw et al., 1978) and urea concentrations by the diacetyl monoxime assay (Rahmatullah and Boyde, 1980). Ammonia levels in plasma were determined according to Wright et al. (1995a) using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

^{22}Na activities in water samples were measured by a gamma counter (Wallac wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Science, Turku, Finland). Na^+ and K^+ concentrations in water were measured using flame atomic absorption spectrophotometry (AAnalyst 800, Perkin Elmer). Measurements of ^3H_2O in water samples were done via liquid scintillation counting (LS6500, Beckman Coulter, Fullerton, CA) on 5 mL water samples added to 5 mL of Packard Ultima Gold AB fluor (Perkin Elmer, Wellesley, MA). Tests demonstrated that quenching was constant, so no correction was necessary.

Net flux rates (in µmol/kg/h) of Na^+ (J^{Na}_{net}) and K^+ were calculated from changes in concentration (in µmol/L), factored by the known fish mass (in kg), volume (in L), and time (in h). Net flux rates of ammonia (J_{amm}) and urea (J_{urea}) were calculated as for net flux rates of Na^+ (J^{Na}_{net}). Na^+ influx rates (J^{Na}_{in}, by convention positive) were calculated from the mean external specific activity, and the disappearance of counts from the external water.
Calculation of influx (J^{Na}_{in}) was done by the formulae

$$J_{in}^{Na} = ([CPM_i] - [CPM_f])\,(V) / (SA_{ext})\,(t)\,(M)$$

where, CPM_i is the initial ^{22}Na radioactivity in the water (cpm/mL) at the start of the flux period; CPM_f is the final ^{22}Na radioactivity in the water (cpm/mL) at the end of the flux period; V is the volume of water (in mL); SA_{ext} is the mean external specific activity (^{22}Na per total Na^+) in the water (cpm/nmol), calculated from measurements of water ^{22}Na radioactivity and total water $[Na^+]_{ext}$ at the start and end of the flux period; t is the time of flux period (h); M is the mass of the fish (g).

Na^+ unidirectional efflux rates (J^{Na}_{out}, by convention negative) were calculated by difference, as outlined in detail by Wood (1992). The equation is

$$J^{Na}_{out} = J^{Na}_{net} - J^{Na}_{in}$$

The rate constant of ^3H_2O efflux was calculated from the rate of decline in total ^3H_2O in the fish, which was approximately exponential with time (Evans, 1967):

$$k = (\ln CPM_1 - \ln CPM_2) \times 100 / (t_1 - t_2)$$

where, k is the rate constant of the efflux (% per h); CPM_1 is the total ^3H_2O radioactivity (cpm in the fish at time t_1 (h); CPM_2 is the total ^3H_2O radioactivity (cpm in the fish at time t_2 (h).

3. 4. STATISTICAL ANALYSIS

All data have been presented as mean values ± standard error (S.E). Some of the data (wherever applicable) were natural logarithm transformed to equalize the variances and to approximate a normal distribution prior to statistical analysis. For comparisons between different experimental groups a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. Student's two-tailed t- test was used for single comparisons. A probability level of 0.05 was used for rejection of the null hypothesis.
Within species, no significant differences were found between any of the control values at different sampling times. Therefore, pooled controls for each experimental group are shown for clarity of the figures.

3.5. RESULTS

3.5.1. Ammonia and urea flux

Ammonia excretion rate (J_{amm}) invariably declined in carp and trout during the first 3 h of 1mM ammonia exposure (Fig.3.1A). This response was more pronounced ($P < 0.05$) in trout which displayed a reversal of ammonia flux from a control value of -372µmol/kg/h to + 59 µmol/kg/h (i.e., net ammonia uptake). A significant inhibition was also observed in common carp during the first 3 h of exposure, while exposed goldfish were able to maintain an excretion rate near to control values. However, by 12 h of HEA, all three species were able to re-establish ammonia excretion to a value not significantly different from the control. The inhibition of ammonia excretion became much more intense after exposure to 5 or 1.4mM ammonia, with all three species experiencing a reversal of J_{amm} (Fig. 3.1B).

Urea excretion rate (J_{urea}) also exhibited some changes in the face of HEA. At 1mM a gradual increment with exposure time was seen in goldfish and the same trend was seen in carp but not in trout (Fig.3.2A). The response was most prominent in goldfish; J_{urea} at 12 h HEA exposure was several fold larger ($P < 0.01$) than the control. Under the high level of HEA (5 or 1.4mM), J_{urea} was elevated considerably ($P < 0.01$) only in goldfish, with a 2.2-fold increase compared to control (Fig.3.2B).

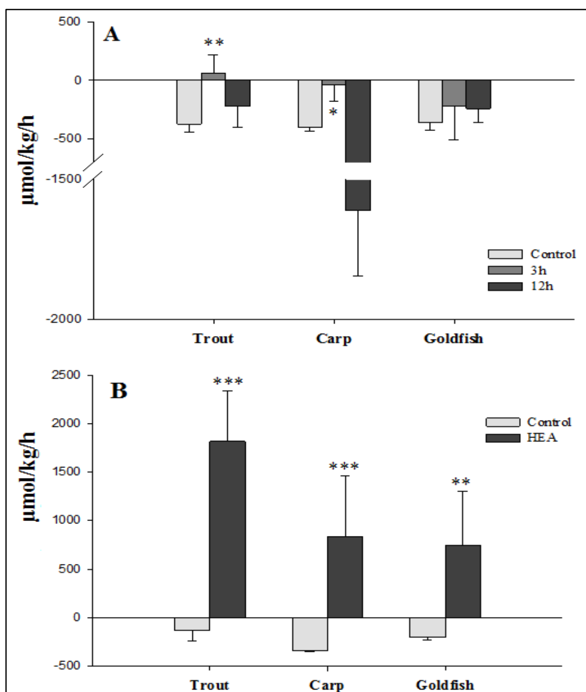

Figure. 3. 1. Net excretion rate of ammonia (J_{amm}) in rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

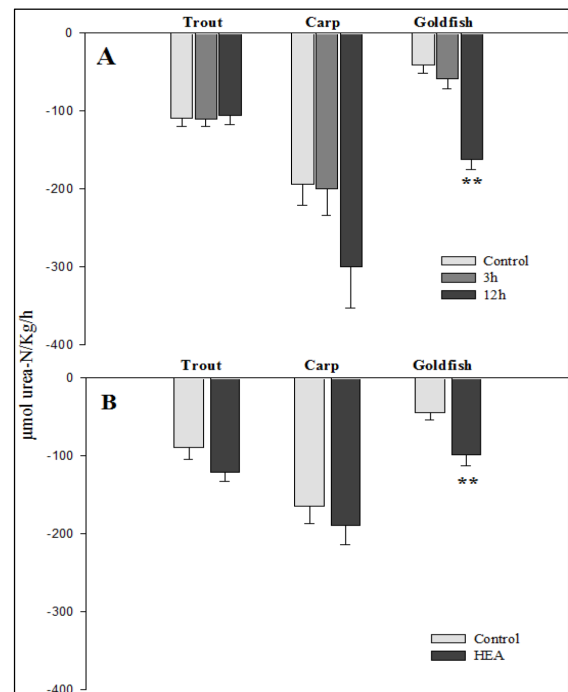

Figure. 3. 2. Net excretion rate of urea (J_{urea}) in rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (**$P < 0.01$).

3.5.2. Plasma metabolites

Plasma ammonia

Plasma total ammonia (T_{amm}) level was significantly elevated by 30% in trout after 3 h exposure at 1mM ammonia (Fig.3.3A). This increase was followed by a subsequent recovery at 12 h to a value not significantly different from the control group. A trend towards plasma ammonia accumulation was also observed in carp and goldfish but the levels were not statistically higher than their respective controls.

When each of these fish species were exposed to 5mM (or 1.4mM), they showed much higher (many fold increments, $P < 0.01$ or 0.001) accumulations of T_{amm} in comparison to the control groups (Fig.3.3B). The respective increments in trout, carp and goldfish were 270, 225% and 320% of control levels.

Plasma urea

In trout, plasma urea-N concentration was elevated considerably compared to control by 50% ($P < 0.05$) and 85 % ($P < 0.001$) respectively after 3 h and 12 h of 1mM HEA exposure (Fig.3.4A). Also common carp started to accumulate considerable ($P < 0.001$) amounts of urea-N when exposed to HEA and followed the same pattern as trout, with increases of 42% and 59% at 3 h and 12 h respectively. However, in goldfish no obvious differences ($P > 0.05$) were seen in any of the sampling periods. This illustrates a divergent pattern of urea-N accumulation between the two cyprinids upon exposure to 1mM HEA. Very similar patterns in plasma urea were seen at 5mM (or 1.4mM) HEA, confirming these inter-specific differences (Fig.3.4B).

Plasma cortisol

Exposure to 1mM ammonia elevated plasma cortisol level ($P < 0.05$) in carp and goldfish from 3 h onwards (Fig.3.5A). The increments in carp after 3 h and 12 h were 55% ($P < 0.05$) and 35% ($P < 0.05$) higher than the control. Likewise, in goldfish the respective augmentations were 30% ($P < 0.05$) and 40% ($P < 0.01$). In contrast, trout displayed a gentle ($P > 0.05$) rise at 3 h which became significant (22%; $P < 0.05$) after 12 h HEA.

A more severe effect was seen in all these species when exposed for 3 h at the higher level of HEA (5 or 1.4mM) (Fig.3.5B). In this case, the levels of cortisol in exposed trout, carp and goldfish were elevated by about 60% ($P < 0.01$), 140% ($P < 0.001$) and 95% ($P < 0.01$) above control values respectively. These increases occurred despite the fact that the level of cortisol in all the control groups (trout in particular) was high; most likely due to confinement stress.

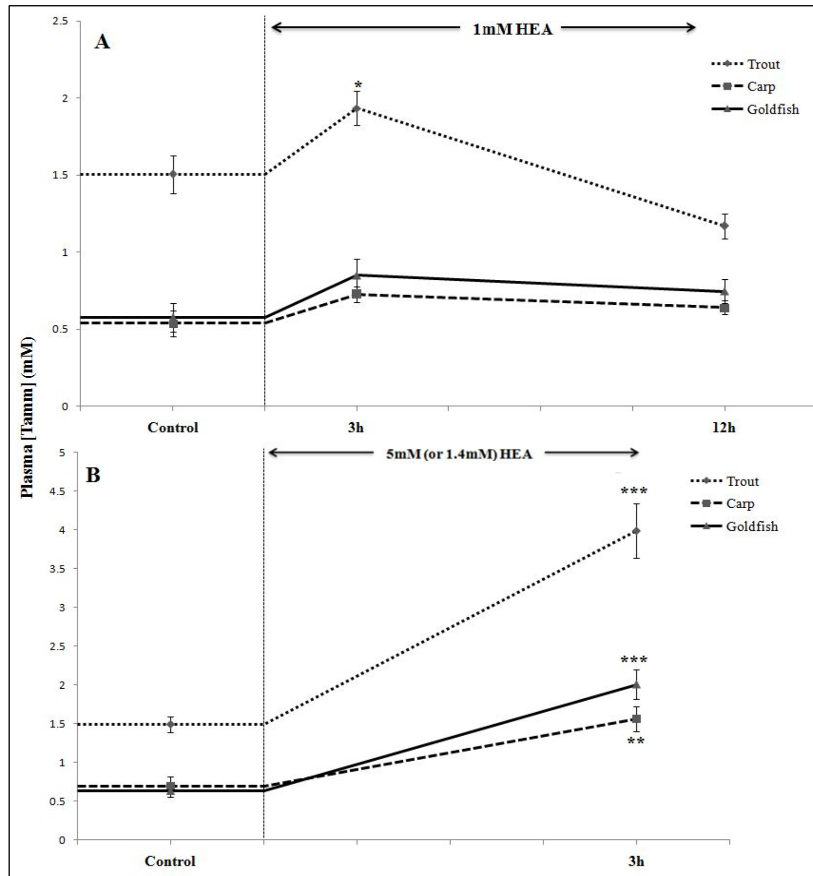

Figure. 3.3. Plasma ammonia accumulation in rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

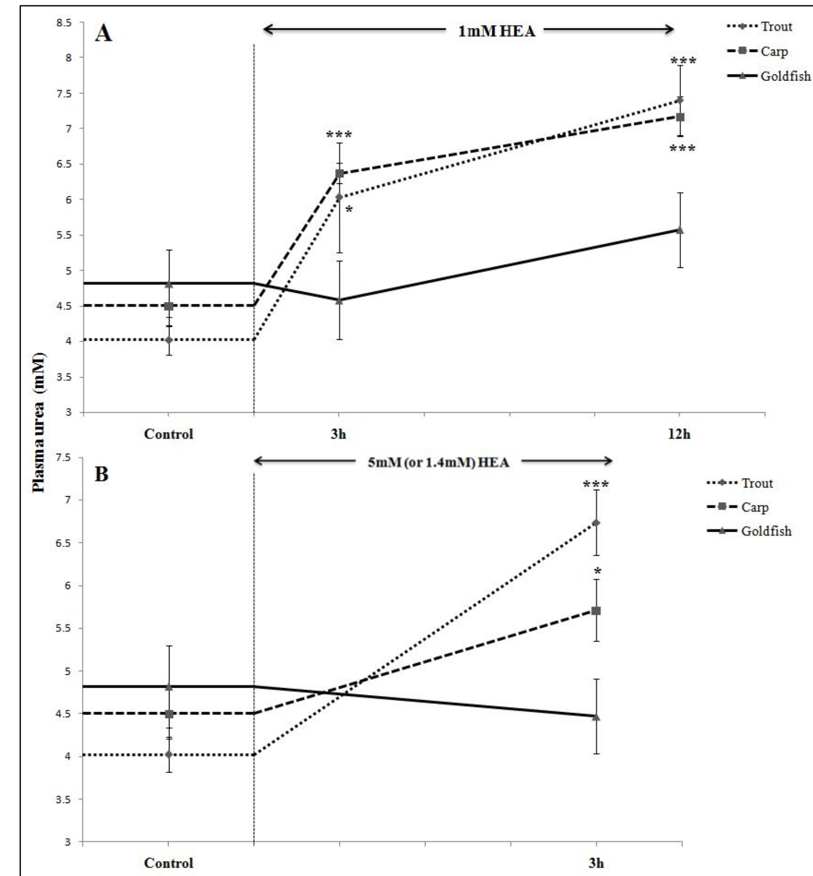

Figure. 3.4. Urea accumulation in plasma of rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$; ***$P < 0.001$).

3.5.3. Na^+ flux response to HEA

Unidirectional sodium influx rates (J^{Na}_{in}) did not change significantly relative to controls in common carp and goldfish during the first 3 h of exposure to 1mM HEA (Fig.3.6). However, a significant stimulation in Na^+ influx was observed in both species after 12 h of exposure. Under the same experimental condition, a temporary reduction ($P < 0.001$) in J^{Na}_{in} was seen in trout during the first hour only, with recovery thereafter.

During the first 3 h, the response of unidirectional Na^+ efflux (J^{Na}_{out}) in goldfish displayed an opposite trend to carp and trout. In goldfish, J^{Na}_{out} was inhibited ($P < 0.05$) in the first and third hours of exposure to 1mM HEA, and thus net Na^+ balance was maintained positive. On the contrary, in carp and trout the diffusive loss was stimulated, such that net Na^+ balance became more negative. In carp, the increment in J^{Na}_{out} ($P < 0.001$ or 0.01) was seen during the first and the third hour of exposure while in trout similar increases ($P < 0.001$) were observed only during the second and third hour of exposure. Strikingly, at 12 h all three species showed significant elevations of J^{Na}_{out}, resulting in highly negative values of J^{Na}_{net} (Fig.3.6).

At the higher exposure level (5 or 1.4mM), J^{Na}_{in} was significantly inhibited in all species (Fig.3.7). The effect was more prominent in goldfish where a reduction ($P < 0.05$) was seen from hour one onwards whereas in carp a significant reduction was evident only after the second hour of exposure. The reductions in goldfish as compared to control were about 75%, 50% and 45% at 1, 2 and 3 h respectively. While in carp the average reduction was 38% compared to its control value. J^{Na}_{in} in trout at 1.4mM followed the same trend as when subjected to 1mM. J^{Na}_{out} values in carp and trout were increased significantly from the first hour onwards respectively by 590% and 280%. However, such an increment was delayed in goldfish and became significant only after 2 h of exposure. Thus all three species were in markedly negative net Na^+ balance at the higher level of HEA exposure (Fig.3. 7).

3.5.4. Indices of gill permeability

Diffusive exchange of water during HEA

Rate constants (k) of diffusive water exchange, measured with ^3H_2O, were very similar (0.426-0.454% h^{-1}) in control group of all three species (Fig.3.8). The rate increased significantly in trout after 3 h when exposed to HEA (1mM) reaching 0.676 % h^{-1}. This activation at 3 h was followed thereafter by a partial recovery at 12 h. No significant changes occurred in carp and goldfish at either 3 h or 12 h of exposure to 1mM HEA.

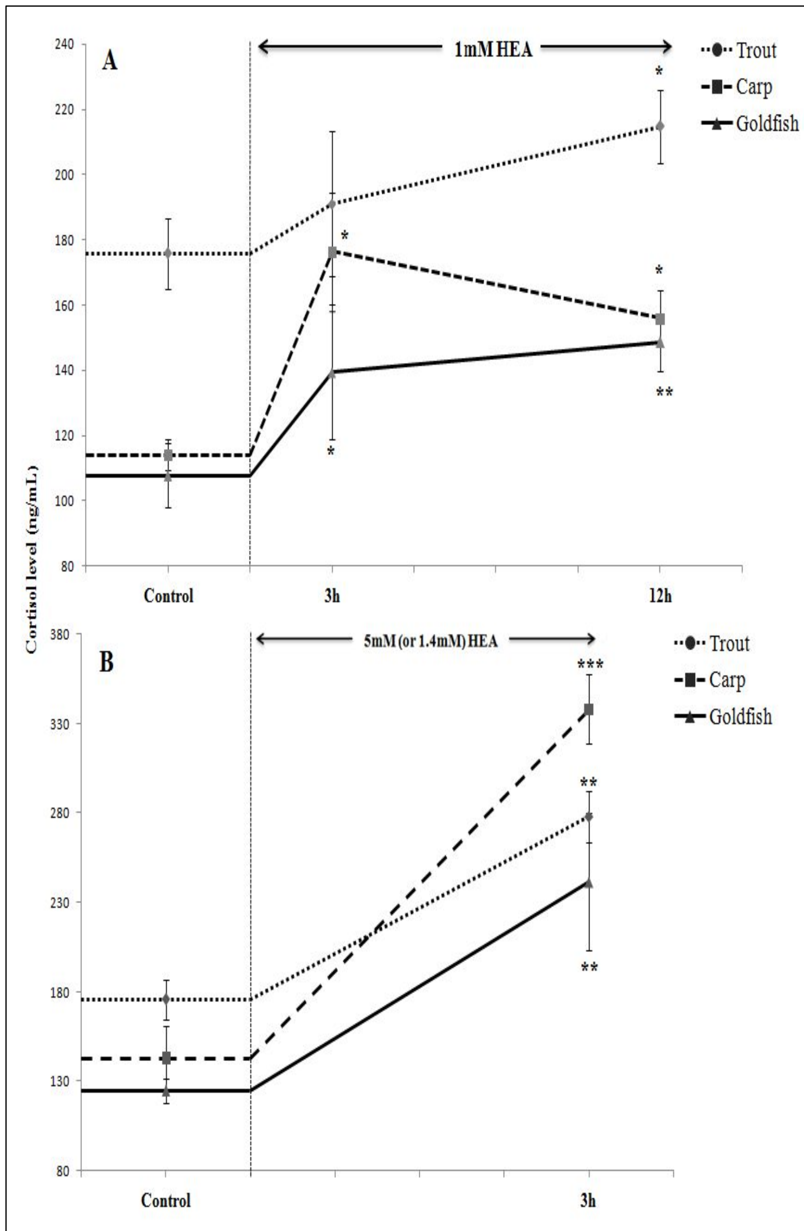

Figure. 3. 5. Plasma cortisol level in rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01;***P < 0.001).

Transepithelial potential (TEP)

Under control conditions TEP was negative in carp and goldfish but slightly positive in trout (Fig. 3.9). Upon exposure to HEA (1mM), TEP rose substantially in all three fish, an effect that was significant (P < 0.05 or 0.01) during all the exposure periods. After 3 h of exposure, common carp appeared to have the highest induction compared to goldfish and trout, TEP (in carp) rose from the control value of -7.8 mV to +11.4 mV. In goldfish and trout the increments at 3 h were from -3.4 mV to +2.4 mV and from +1.5 mV to +9.8 mV respectively. In all three species, the elevations in TEP remained significant (P < 0.05 or 0.01) at 12 h.

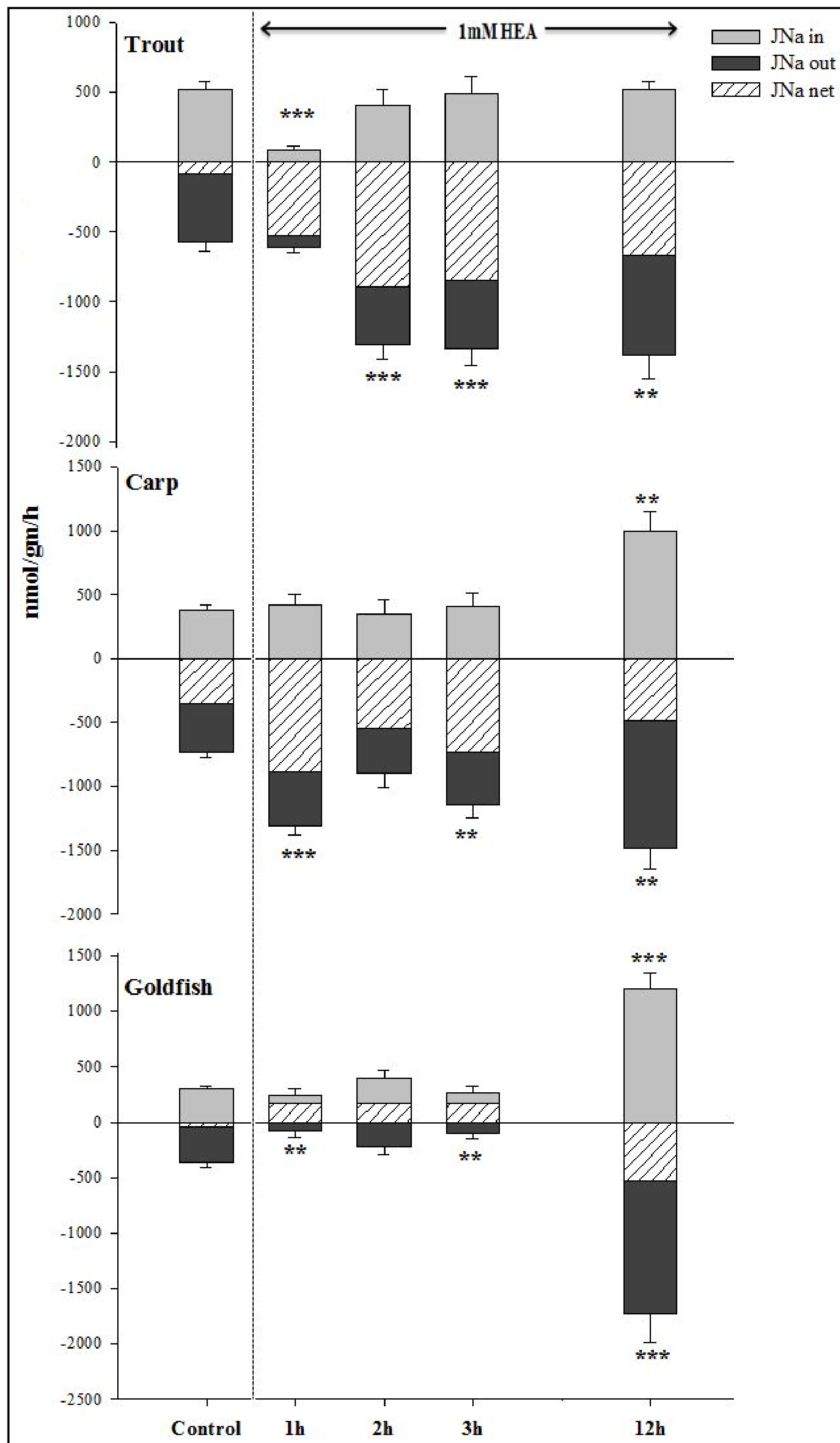

Figure. 3. 6. Na^+ unidirectional influx (J^{Na}_{in}, upward bars), Na^+ efflux(J^{Na}_{out}, downward bars) and Na^+ net flux (J^{Na}_{net}, hatched bars) rates in rainbow trout, common carp and goldfish during 1mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (**$P < 0.01$;***$P < 0.001$).

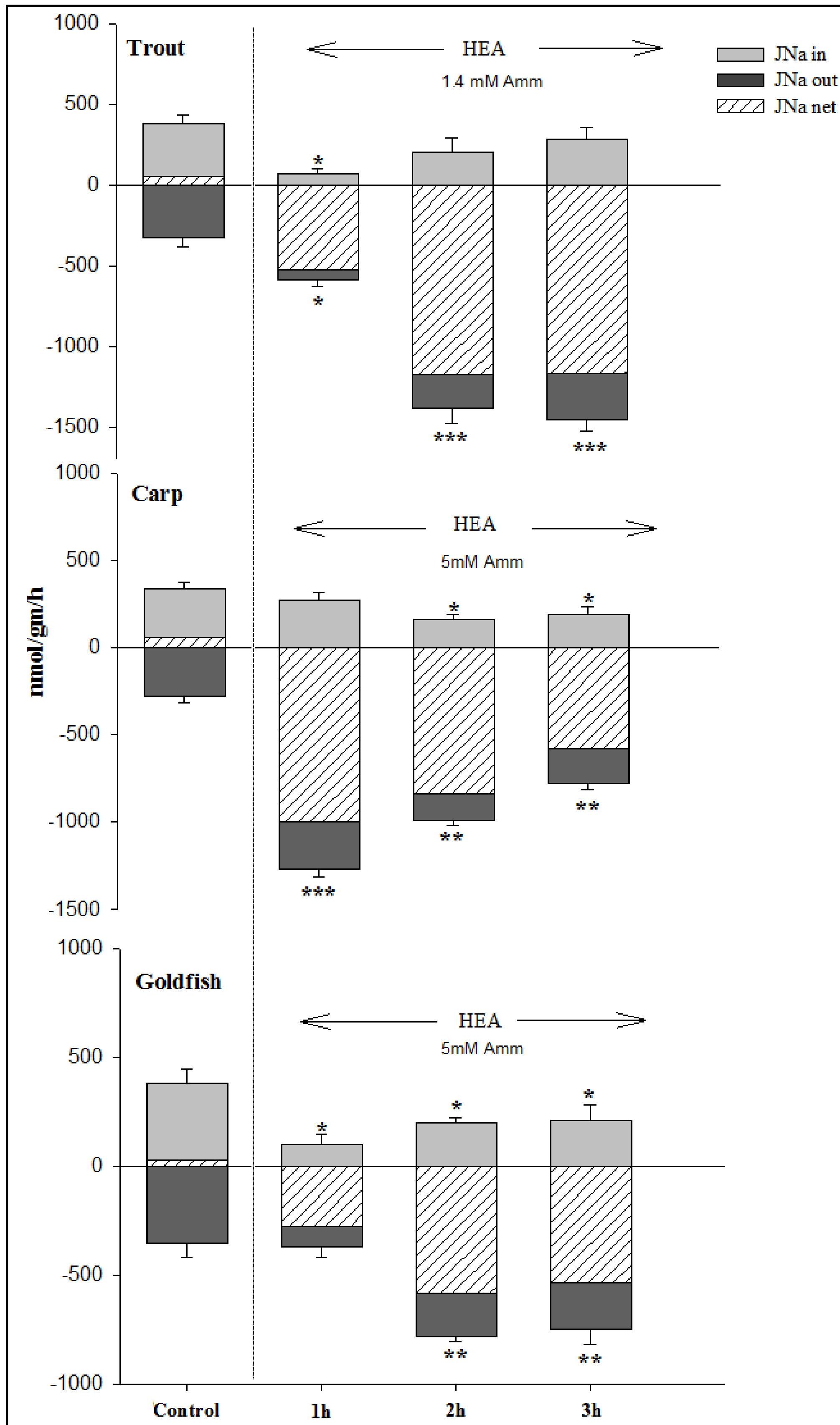

Figure. 3.7. Na^+ unidirectional influx (J^{Na}_{in}, upward bars), Na^+ efflux(J^{Na}_{out}, downward bars) and Na^+ net flux (J^{Na}_{net}, hatched bars) rates in rainbow trout, common carp and goldfish during 5mM or 1.4mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

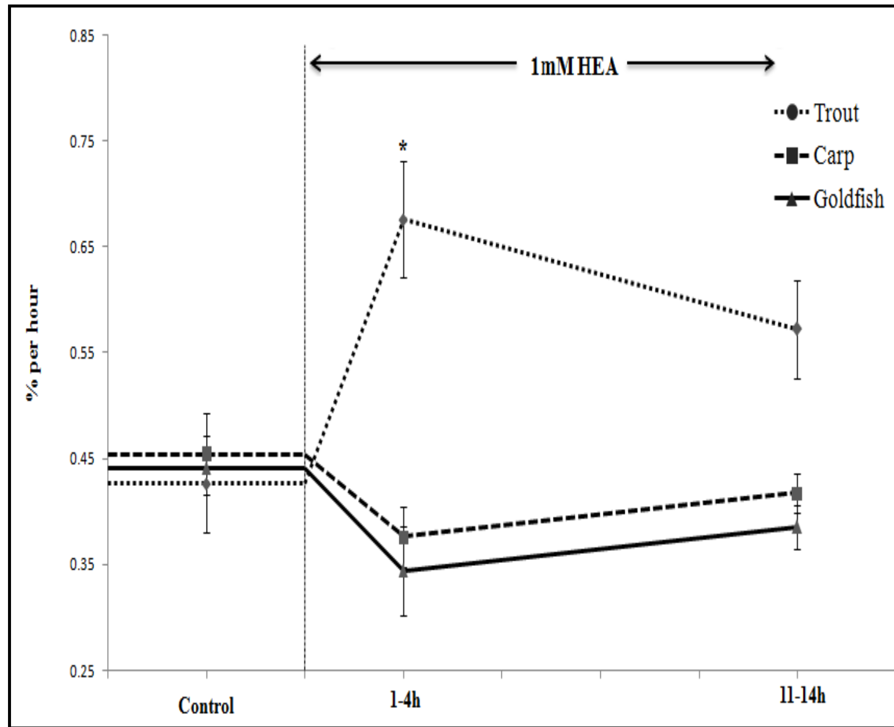

Figure. 3.8. Diffusive water efflux rates measured with ^3H_2O in rainbow trout, common carp and goldfish during 1mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$).

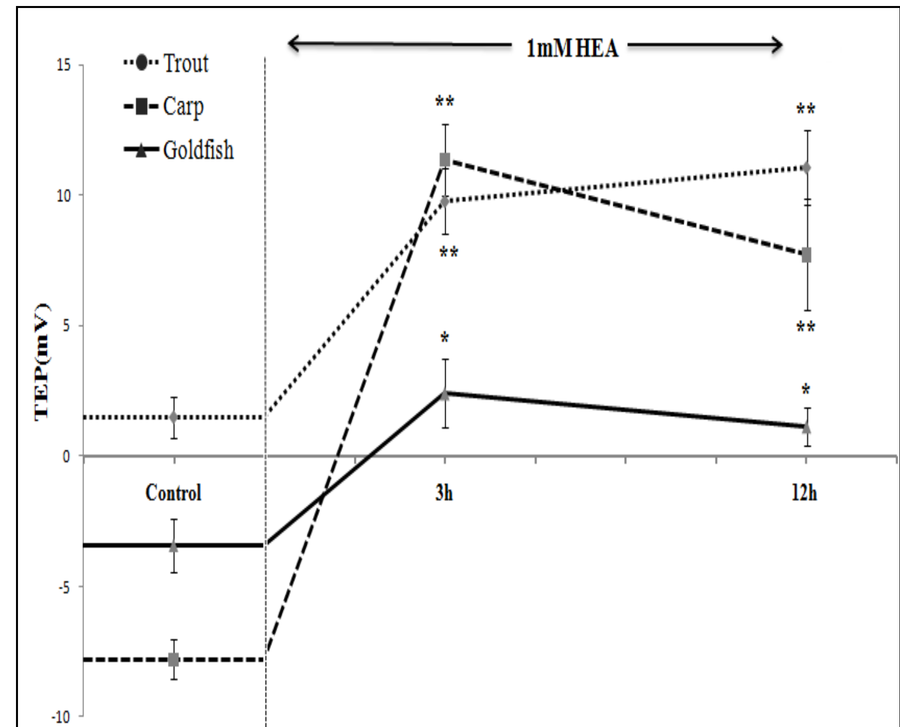

Figure. 3.9. Changes in transepithelial potential (TEP) in rainbow trout, common carp and goldfish during 1mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

3.5.5. Net K^+ flux rates via gills during acute HEA exposure

In all three species, net K^+ flux rates (J^{K}_{net}) were negative (i.e. net losses) under control conditions. During exposure to 1mM HEA, net loss rates increased significantly after 3 h and 12 h exposure (by 364% and 215%; $P < 0.01, 0.05$) in trout (Fig.3.10A). On the contrary, in carp and goldfish the rates of K^+ loss after 3 h of exposure decreased by 35% ($P > 0.05$) and 20% ($P > 0.05$) respectively compared to their controls. After 12 h of exposure, the K^+ flux was reversed in both cyprinids, resulting in a net uptake ($P < 0.05$ or 0.01).

When these fish species were exposed to the higher level of ammonia (5mM or 1.4mM), an increment in K^+ loss rate was observed all the three species (Fig.3.10B). In exposed trout, the loss rate was significantly increased by 55%. Likewise, in carp and goldfish the loss rate was augmented by 30% and 12% respectively but these increases were statistically insignificant compared to their control values.

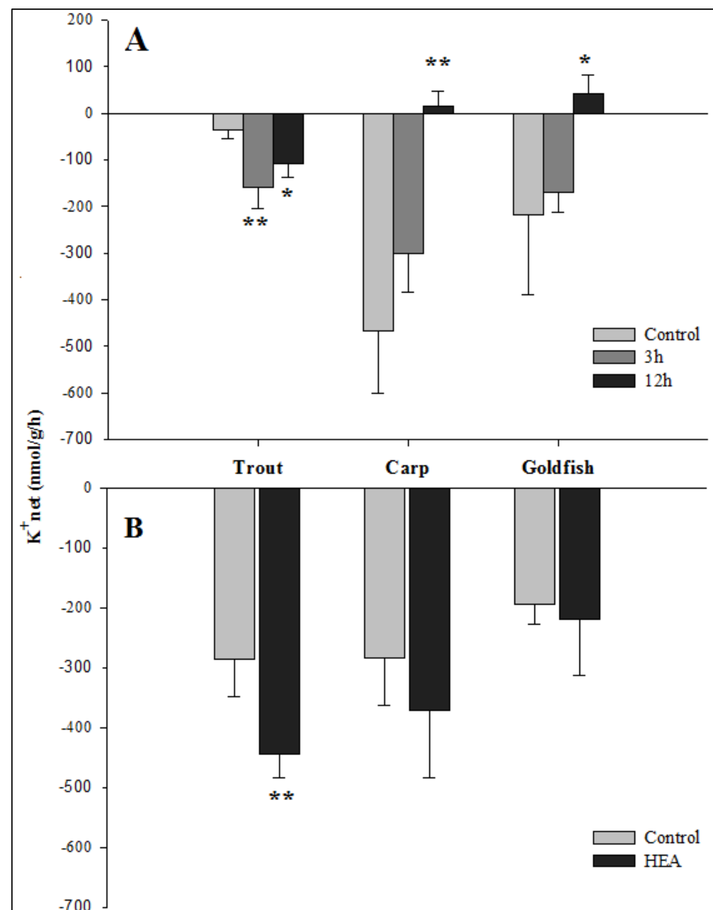

Figure. 3.10. Net flux rates of K^+ (nmol/g/h) in rainbow trout, common carp and goldfish during **(A)** 1mM ammonia exposure **(B)** 5mM (for cyprinids) or 1.4mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$)

3.6. DISCUSSION

3.6.1. Effects on ammonia metabolism

In trout and carp, ammonia excretion rate (J_{amm}) was depressed during the first 3 hours of 1mM HEA. J_{amm} was inhibited to a greater extent particularly in trout where an initial reversal of J_{amm} was observed at 3 h. Such inhibition has also been demonstrated in trout by Wilson et al. (1994), Nawata et al. (2007) and Zimmer et al. (2010) in response to HEA. A dramatic increase in plasma T_{amm} occurred in trout in conjunction with the reversal of J_{amm}. In carp J_{amm} was also reduced in response to HEA although the fall was not as large as in trout indicating that this cyprinid can compensate the ammonia load more efficiently than trout. Amazingly, the other, more resistant cyprinid, the goldfish managed to keep excreting ammonia, even against a concentration gradient (note that plasma T_{amm} levels remained < 1 mM). Indeed, all three species were able to restore ammonia excretion by 12 h exposure to 1 mM HEA. Recent findings (Nakada et al., 2007a; Nawata et al., 2007; Weihrauch et al,, 2009; Wright and Wood, 2009) that Rh glycoproteins present in the gill cell membranes are implicated as a putative mechanism of active ammonia transport in linkage with Na^{+} uptake, as discussed subsequently, may help explain these responses. A trend of decreasing plasma T_{amm} ensued in both cyprinids and trout at 12 h of HEA, as all three fish were able to re-establish ammonia excretion at this time, in concert with either recovery (in trout) or elevation (in carp and goldfish) of J^{Na}_{in}.

Notably, after exposure to a higher ammonia level (5 or 1.4mM), a prominent inhibition of J_{amm} was seen in all species; excretion rate was reversed to negative values, in concert with marked inhibitions of J^{Na}_{in}. These responses were accompanied by considerable increments in plasma T_{amm}, indicating that the ability to cope with ammonia transport might have severely been disrupted in all three species. However, again, increases in plasma T_{amm} were a lot lower in both carp and goldfish, than in trout, staying well below HEA levels.

3.6.2. Effects on cortisol

Cortisol, produced as an end product of the hypothalamic–pituitary–interrenal axis, plays a crucial role in the stress response and in osmoregulatory processes as well as in energy metabolism (McCormick, 2001; Wendelaar Bonga, 1997). The present study shows that the level of plasma cortisol in all three species increased during HEA, a commonly observed response in cyprinids and salmonids (Ortega et al., 2005; Sinha et al., 2102b; Tsui et al., 2009; Wood and Nawata, 2011). Moreover, cortisol has been shown to increase the ion-transporting capacity of the gills by the proliferation of chloride cells (Goss et al., 1992). It is also very likely that elevated cortisol level may stimulate ammonia and urea flux in fish. Ortega et al. (2005) reported a linear relationship between plasma cortisol levels and ammonia levels in rainbow trout exposed to HEA. In vitro studies on

cultured trout gill epithelia by Tsui et al. (2009) indicated that cortisol can play a role in activating the "Na^+/NH_4^+ exchange metabolon" involving the Rh glycoproteins, thereby augmenting ammonia transport capacity. In addition, elevated plasma cortisol was shown to increase urea-N excretion rates in the trout (McDonald and Wood, 2004b). However, no clear-cut relationship between elevated cortisol levels and ammonia and/or urea flux was perceptible in any of the fish species investigated in present work.

3.6.3. Response of sodium fluxes

From our results, it is clear that Na^+ imbalance was induced to a differential extent by HEA in the three species. At 1mM HEA, the interspecies difference between cyprinids and trout became apparent. In trout, HEA tended to depress J^{Na}_{in} transiently contrasting with the response of cyprinids, which showed no inhibition and increased J^{Na}_{in} after 12 h of exposure. Such inhibition of Na^+ uptake in trout may be a consequence of reduced proton excretion (through the electrogenic proton pump, H^+-ATPase which is thought to drive Na^+ uptake from water) resulting from potential intracellular alkalisation by NH_3 (Avella and Bornancin, 1989), coupled with the direct inhibition of Na^+ uptake by high external NH_4^+ competing for the Na^+ site on the Na^+/H^+ exchanger (NHE) (Twitchen and Eddy, 1994). Similar to our results, the inhibition of J^{Na}_{in} by HEA has been reported previously in rainbow trout (Twitchen and Eddy, 1994; Wilson et al., 1994; Zimmer et al., 2010). From the second hour of exposure J^{Na}_{in} recovered to normal values. This recovery of J^{Na}_{in} in trout along with a significant increment among the cyprinids (at 12 h) may be due to the activation of the branchial apical "Na^+/NH_4^+ exchange metabolon" which involves several membrane transporters and Rh glycoproteins (Rhcg in particular) working together to provide an acid trapping mechanism for apical ammonia excretion (Cameron and Heisler, 1983; McDonald and Prior, 1988; Wilson and Taylor, 1992; Wright and Wood, 1985, 2009, 2012). Since NH_4^+ ions are moved across the apical membrane in exchange for sodium through the Na^+/H^+ (or NH_4^+) exchanger (NHE), another possible explanation may be related to the increased enzyme activity of Na^+/K^+-ATPase present on the basolateral membrane of branchial cells (Evans et al., 2005; Wilkie, 2002). This basolaterally situated enzyme is believed to provide the major source of energy driving Na^+ influx, although not necessarily the only source (Avella and Bornancin, 1989; Lin and Randall, 1995; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997). An increased activity of Na^+/K^+-ATPase was reported in silver perch (*Bidyanus bidyanus*), golden perch (*Macquaria ambigua*) and African catfish (*Clarias gariepinus*) when exposed to ammonia polluted water (Alam and Frankel, 2006; Schram et al., 2010). This may increase Na^+ uptake and thereby enhance ammonia excretion (via the metabolon) as observed in our study. Therefore, investigation of these pumps and exchangers may be crucial in future experiments.

Furthermore, all three species suffered a significant inhibition of J^{Na}_{in} when subjected to more severe exposure (5 or 1.4mM). At this higher exposure level, NH_4^+ may directly compete with the Na^+ uptake mechanism, before these species can activate their metabolon involving the Rh glycoproteins in order to get rid of excess internal ammonia.

Unidirectional flux measurements indicate that Na^+ efflux (J^{Na}_{out}) was almost doubled in trout when exposed to 1mM and 1.4mM ammonia. Our result is in accordance with the findings of Twitchen and Eddy (1994) who reported that HEA increased the diffusive efflux of Na^+ across the gills, and lowered plasma [Na^+] in freshwater trout. Concomitantly, the increased Na^+ loss rate was also evident in carp (at both exposure levels) but the effect was less severe compared to trout. The observed increment in J^{Na}_{out} during HEA is likely due to the increased diffusive leakage of Na^+ - i.e. enhanced Na^+ permeability (transcellular and/or paracellular) of the gills (Gonzalez and McDonald, 1992). In this context, measurements of diffusive water flux across the gills were instructive. Diffusive water flux at fish gills is generally considered to occur by the transcellular route (Isaia, 1984; McDonald et al., 1991) and its rate constants (k) measured with ^3H_2O were higher in exposed trout in comparison to both cyprinids. The k value in the former increased respectively by 58% and 34% after 3 h and 12 h exposures to 1mM HEA compared to control value. Therefore increased transcellular leakage might be one of the possible explanations for intensified Na^+ loss rate in trout.

Beside diffusive water flux, K^+ loss rate (J^K_{net}) is another indicator of transcellular permeability because K^+ concentrations inside cells are many times greater than those in blood plasma. Therefore it has been proposed that K^+ loss rates at the gills of freshwater teleosts mainly reflect transcellular leakage (Lauren and McDonald, 1985). During HEA, J^K_{net} was markedly activated in trout signifying an augmentation in transcellular permeability, in accord with the observed increased rate of Na^+ loss in this species.

In addition, the increased Na^+ diffusion may also be driven by the observed positive shift in the transepithelial potential (TEP). This occurred in all three species, but to a much lesser extent in the goldfish. These findings therefore extend the original observations of increased TEP during HEA exposure on rainbow trout (Tsui et al., 2009; Wood and Nawata, 2011) to two more species. The net effect would be to retard NH_4^+ uptake but exacerbate Na^+ loss because the extracellular fluid of the fish became more positive relative to the external water. Originally TEP changes were proposed as a paracellular event (Gonzalez and McDonald, 1992), though recent evidence suggest that transcellular permeability changes may also be involved (Wood et al., 2009)

Interestingly, goldfish responded with a reduction in J^{Na}_{out} in the initial 3 h period when exposed to 1mM ammonia which is in contrast to trout and carp. It could be that goldfish are able to regulate gill permeability more efficiently during HEA and that gill remodelling might effectively shut-down membrane channels in gill epithelia cells. Indeed, a comparable phenomenon has been observed

during acute exposure to severe hypoxia in the Amazonian oscar, *Astronotus ocellatus* (Wood et al., 2009). This would be a manifestation of the 'channel arrest' hypothesis in the gills, originally proposed for brain and liver tissue to explain survival of the turtles and Crucian carp under extreme hypoxia conditions (Boutilier, 2001; Boutilier and St-Pierre, 2000; Hochachka, 1986; Hochachka and Lutz, 2001). However, at the higher ammonia level (5mM), it appears that even goldfish could not regulate gill permeability effectively, resulting in higher J^{Na}_{out} as seen for carp and trout as well. Furthermore, an analogous pattern was noticed after 12 h exposure among all these fish species; J^{Na}_{out} was elevated remarkably compared to respective controls. Interestingly, J^{Na}_{in} was also stimulated more or less in the same time frame among all species. It indicates some sort of recovery response towards normal Na^+ balance by enhancing Na^+ influx (Salama et al., 1999) as part of the ammonia excretion mechanism, and that an increase in J^{Na}_{out} might directly be coupled to an increase in J^{Na}_{in} through mechanisms such as carrier- mediated exchange diffusion transport system or through a leaky pump (Goss and Wood, 1990; Potts and McWilliams, 1989; Twitchen, 1990). Exchange diffusion has been observed during normoxia in many freshwater teleosts, including trout (Shaw, 1959; Wood and Randall, 1973) but until now, it has not been studied under ammonia exposure.

Overall, as a result of influx inhibition and efflux stimulation during HEA, trout appeared to have the highest net Na^+ loss compared to carp and goldfish, indicating a clear and sustained disruption of Na^+ homeostasis in trout. Such effects on Na^+ uptake and loss rates were much smaller in goldfish indicating that Na^+ balance was least disturbed in goldfish while trout suffer the most.

3.6.4. Detoxification of ammonia to urea

Ammonia is either excreted directly if feasible, or converted to some less toxic compound such as urea. Although the majority of teleost fishes are ammoniotelic, urea also constitutes about 10–30% of the total nitrogenous wastes in most of them (Saha and Ratha, 1998). Data presented in our study indicate that goldfish are able to cope quite well with HEA by significantly increasing the rate of urea excretion (J_{urea}). Goldfish exposed to 1mM HEA for 12 h showed a nearly identical activation in J_{urea} as when placed in 5mM HEA for 3 h. Thus the time course for the response was shortened when subjected to a very high ambient ammonia concentration. Similarly, Olson and Fromm (1971) also reported an increase in urea excretion rates in goldfish subjected to HEA. When ambient ammonia was increased J_{urea} did not rise significantly in carp (although an increasing trend was evident) or rainbow trout, but there were significant increments in plasma urea concentrations in trout and carp, though not in goldfish. We speculate that in trout and carp, the urea excretion mechanism was either very limited, or inhibited. It may reflect the inability of trout (and to some extent in carp) to prevent the build-up of blood ammonia during HEA as also evident from Fig 3.3.These consequences eventually may provide some clues as to why trout are so susceptible to HEA. However, the source of

urea production in teleosts and the involvement of the ornithine-urea cycle are still doubtful. Furthermore, it would be interesting to know the involvement of urea transporter (UT) in facilitating the diffusion of urea across basolateral membrane of gill cell. Though some studies have been conducted in rainbow trout (McDonald and Wood, 2004a; Wood and Nawata, 2011), the function of UT in carp and goldfish is yet to be evaluated.

3.7. CONCLUSION

Exposure to 1mM ammonia induced differential physiological responses among the three freshwater teleosts. Firstly, in trout, J_{amm} during HEA exposure was significantly inhibited (actually reversed), resulting in considerable accumulation of ammonia in plasma, whereas J_{amm} was partially (in carp) or fully maintained (in goldfish). Secondly, goldfish revealed a better capacity to detoxify ammonia since they were able to excrete greater quantities of urea than trout and carp, thereby preventing the build-up of blood ammonia. This may be a good indicator of the stronger ammonia tolerance of the goldfish relative to carp and trout. Thirdly, Na^+ balance was severely affected in trout and to a lesser extent in carp. Na^+ uptake was inhibited (temporarily) in trout while Na^+ efflux rate was activated in both species resulting in net loss of Na^+ through gills. In contrast, goldfish were able to maintain Na^+ homeostasis during ammonia stress as they repressed their Na^+ loss rate, and they exhibited the smallest changes in transepithelial potential. Lastly, regulation of gill permeability was disturbed in trout since diffusive water efflux and net K^+ loss rate (indicators of transcellular leakage) were increased during ammonia exposure. In summary, goldfish were able to implement these physiological and biochemical responses more effectively in response to HEA without compromising net Na^+ balance; whereas trout exhibited the weakest compensatory responses. However, many of these countervailing responses were also disturbed when goldfish were confronted with a very high ammonia level of 5mM indicating that exposure at this level would probably be detrimental even for this very resistant species, despite its capacity to cope with HEA at lower exposure levels.

Acknowledgments

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Chapter 4

Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia

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SUMMARY

We investigated relationships among branchial unidirectional Na^+ fluxes, ammonia excretion, urea excretion, plasma ammonia, plasma cortisol, and gill transporter expression and function in three freshwater fish differing in their sensitivity to high environmental ammonia (HEA). The highly ammonia-sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less ammonia-sensitive cyprinid *Cyprinus carpio* (common carp) and the highly ammonia-resistant cyprinid *Carassius auratus* (goldfish) were exposed chronically (12 h to 168 h) to 1 mM ammonia (as NH_4HCO_3; pH 7.9). During HEA, carp and goldfish elevated ammonia excretion (J_{amm}) and Na^+ influx rates (J^{Na}_{in}) while trout experienced higher plasma ammonia (T_{amm}) and were only able to restore control rates of J_{amm} and J^{Na}_{in}. All three species exhibited increases in Na^+ efflux rate (J^{Na}_{out}). At the molecular level, there was evidence for activation of a "Na^+/NH_4^+ exchange metabolon" likely in response to elevated plasma cortisol and T_{amm}, though surprisingly, some compensatory responses preceded molecular responses in all three species. Rhbg, Rhcg (Rhcg-a and Rhcg-b), H^+-ATPase (V-type, B-subunit) and Na^+/K^+-ATPase (NKA) mRNA expressions were up-regulated in goldfish, Rhcg-a and NKA in carp, and Rhcg2, NHE-2 (Na^+/H^+ exchanger) and H^+-ATPase in trout. Branchial H^+-ATPase activity was elevated in goldfish and trout, and NKA activity in goldfish and carp, but NKA did not appear to function preferentially as a Na^+/NH_4^+-ATPase in any species. Goldfish alone increased urea excretion rate during HEA, in concert with elevated urea transporter mRNA expression in gills. Overall, goldfish showed more effective compensatory responses towards HEA than carp, while trout were least effective.

Keywords: High environmental ammonia (HEA), Sodium flux, Rhesus (Rh) glycoproteins, Ammonia excretion, Urea excretion, Na^+/NH_4^+ exchange metabolon, Rainbow trout, Common carp, Goldfish.

4.1. INTRODUCTION

The majority (more than 80%) of metabolic ammonia is excreted *via* the gills in freshwater teleost fish. Gills contain a number of transporters and associated mechanisms that are involved in the maintenance of ammonia homeostasis (Avella and Bornancin, 1989; Evans, 1980; Evans et al., 1999; Evans et al., 2005; Wilkie, 1997; Wilkie, 2002). Ammonia excretion takes place either as NH_3, diffusion and/or as NH_4^+ transport linked in some manner to Na^+ uptake (Wilkie, 1997; Wilkie, 2002; Wood, 1993). While the exact mechanisms are not yet fully revealed, the process often manifests as a coupling of ammonia excretion to Na^+ uptake, the scheme first proposed by Krogh (1939). Current hypotheses include a direct coupling through Na^+/NH_4^+ exchangers, coupling with H^+ excretion by an apical Na^+/H^+ exchanger (NHE), and coupling with H^+ excretion via an apical H^+- pump which energizes Na^+ uptake through a putative Na^+ channel. The latter two mechanisms would acidify the gill boundary layer, thereby enhancing the "diffusion-trapping" of NH_3 as NH_4^+ and maintaining the partial pressure gradient for diffusive NH_3 efflux, thereby manifesting as an apparent Na^+/NH_4^+ exchange (Clarke and Potts, 1998; Wilson et al., 1994).

Recently, the key involvement of Rhesus (Rh) glycoproteins in this diffusive NH_3 efflux has been recognized (Nakada et al., 2007a; Nawata et al. 2007). These appear to function as ammonia channels, binding NH_4^+ but facilitating the diffusion of NH_3 (Nawata et al., 2010a). Rhag occurs in red blood cells, while Rhcg and Rhbg occur in the apical and basolateral membranes respectively of the branchial epithelium. On the apical membrane, the deprotonation of NH_4^+ at the cytoplasmic side of the Rhcg channel may provide a source of H^+ ions to drive Na^+ uptake under circumstances such as low external pH (Hirata et al., 2003; Kumai and Perry, 2011; Lin et al., 2012; Shih et al., 2012) which otherwise would seem thermodynamically challenging (Parks et al., 2008). In two studies, increased water Na^+ concentration resulted in elevated ammonia excretion (Shih et al., 2012; Wood et al., 2007). Furthermore, ammonia loading by infusion (Nawata and Wood, 2009; Salama et al., 1999), feeding (Zimmer et al., 2010), or high environmental ammonia (HEA) exposure (Braun et al., 2009b; Hung et al., 2007; Nawata et al., 2007; Nawata et al., 2010b; Wood and Nawata, 2011) resulted in both increased Na^+ uptake and increased mRNA expression of the gill Rh proteins, especially Rhcg, in a number of teleost species. In some freshwater teleosts the ammonia excretion is initially inhibited by exposure to HEA but with later recovery (Liew et al., 2013; Nawata et al., 2007; Payan et al., 1978; Wilkie et al., 2011; Wilson et al., 1994; Zimmer et al., 2010). Moreover, cortisol increases greatly during HEA exposure (Ortega et al., 2005; Tsui et al., 2009) and may play a role. The combined stimulus of HEA and elevated cortisol augmented both Na^+ uptake and ammonia transport capacity in cultured freshwater rainbow trout gill epithelia (Tsui et al., 2009). Therefore, there is evidence for several potential linkages between ammonia efflux (*via* Rh proteins) and Na^+ influx (*via* NHE and/or H^+-ATPase). As the coupling appears to be indirect and loose, involving several

interacting transporters, Wright and Wood (2009) have described it as a 'Na^+/NH_4^+ exchange complex or metabolon'.

In addition, Na^+/K^+-ATPase (NKA), present in the basolateral membrane of branchial cells, also plays a major role in the transport of Na^+ ions across gill membranes (Evans et al., 2005; Kultz and Somero, 1995; Wilkie, 1997). Since NH_3 or NH_4^+ are moved across the apical membrane in loose exchange for apical Na^+ uptake, NKA would contribute to the driving force to uptake Na^+ from the water in exchange for ammonia efflux out of the fish (Avella and Bornancin, 1989; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997). Furthermore, the possible direct involvement of NKA in ammonia excretion via basolateral transport of NH_4^+ has also been implicated since similarities in the hydration radius of K^+ and NH_4^+ may allow substitution at the K^+ transport site (Alam and Frankel, 2006; Randall et al., 1999). While this does not seem to occur in freshwater or seawater trout (Salama et al., 1999; Wood and Nawata, 2011), there are reports of effective NH_4^+ substitution for K^+ on the NKA in the gills of several other euryhaline and marine fish (Balm et al., 1988; Mallery, 1983; Nawata et al., 2010b; Randall et al., 1999).

While chronic toxicity during HEA exposure is well documented (Dosdat et al., 2003; Knoph and Thorud, 1996; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002), most studies on the Na^+/NH_4^+ exchange metabolon, particularly those focusing on the involvement of Rh proteins, have been short term. Recently, we have compared the acute physiological responses of three teleosts differing in their toxicological sensitivity to HEA (rainbow trout > common carp > goldfish; Liew et al., 2013). Although the Na^+/NH_4^+ exchange metabolon was not examined at a molecular level, trout clearly exhibited the largest disturbances in ammonia excretion, Na^+ balance, and gill permeability, while goldfish displayed the least disruptions. The present study extends this comparative investigation to chronic HEA exposure, with the additional incorporation of molecular endpoints and gill ATPase activity measurements so as to assess the relative contributions of the Na^+/NH_4^+ exchange metabolon.

Specifically, we investigated the combined physiological and molecular compensatory responses and the linkages between branchial ammonia fluxes and unidirectional Na^+ fluxes in three commercially important freshwater fish differing in their sensitivities to ammonia: a sensitive salmonid, the rainbow trout *Oncorhynchus mykiss*, the less sensitive cyprinid, the common carp, *Cyprinus carpio*, and the very resistant cyprinid goldfish, *Carassius auratus* when exposed chronically (up to 168 h) to HEA (1mM at pH 7.9) .

To achieve our goals we measured net ammonia (J_{amm}) and urea (J_{urea}) flux rates as well as plasma ammonia and urea concentrations, plasma cortisol levels, unidirectional (J^{Na}_{in}, J^{Na}_{out}) and net (J^{Na}_{net}) sodium flux rates and mRNA expression of Rh glycoproteins (Rhbg and Rhcg), H^+-ATPase (V-type, B subunit), NHE-2, NKA (alpha subunit) and urea transporter (UT) during HEA challenge.

The sequence information for Rh glycoproteins, H^+-ATPase, NKA, and UT was not available for the carp and goldfish, so we cloned partial sequences of these cDNAs. H^+-ATPase and NKA enzyme activities were measured and the ability of NH_4^+ to activate gill NKA was evaluated *in vitro* for all the three species. Moreover, we also assessed net K^+ flux rates (J^K_{net}) which are interpreted as indices of gill transcellular permeability (Lauren and McDonald, 1985; Wood et al., 2009).

Overall, we hypothesized that differential physiological and molecular compensatory responses to HEA would be seen among these fish species. Our results indicate that the components of "Na^+/NH_4^+ exchange complex" were more effective in goldfish in dealing with the ammonia challenge than those in carp and trout. This helps explain the high resistance of goldfish towards HEA.

4.2. MATERIALS AND METHODS

4.2.1. Experimental system and animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were obtained from a fish farm - Bijnens, Zonhoven, Belgium; goldfish (*Carassius auratus*, Linnaeus, 1758) were obtained from Aqua Hobby, Heist op den Berg, Belgium; common carp (*Cyprinus carpio*, Linnaeus, 1758) were obtained from the fish hatchery at the Wageningen University, The Netherlands. Fish were kept at the University of Antwerp in aquaria (200 l) for at least a month before the exposure started. A total of 80 goldfish and 80 carp were each distributed species wise into four 200 l tanks (N = 20 per tank) while 80 trout were placed in eight 200 l tanks (N=10 per tank because of the larger size of the trout). Each of these tanks was equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17±1°C and photoperiod was 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Water parameters were: pH 7.4 ± 0.2, dissolved oxygen 6.9-7.4 mg l^{-1}, total NH_3 0.006-0.009 mM, nitrite 0.0015- 0.0021 mM, nitrate 0.015-0.042 mM, Ca^{2+} 0.8–1.0 mM, Mg^{2+} 0.19-0.21 mM, Na^+ 1.2-1.4 mM, K^+ 0.09-0.10 mM, Cl^- 0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 226 mg $CaCO_3$ l^{-1}.

Average mass (mean ± standard deviation) of rainbow trout was 132 ± 22 g, of carp 16 ± 4 g, and of goldfish 15 ± 5 g. Fish were acclimated to the above mentioned constant temperature and photoperiod for 2 weeks prior to the experiment and were fed ad libitum once a day with either commercial pellets ('Hikari Staple', Kyorin Food Ind. Ltd., Japan) for carp and goldfish, or 'Trouvit' (Trouw Nutrition, Fontaine-les-Vervins, France) for rainbow trout. Feeding was suspended 2 days before experimentation. During the actual exposure, charcoal and lava stones were removed from the filter to prevent ammonia absorption. All animal experiments were approved by the local ethics committee (University of Antwerp), and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

4.2.2. Exposure and sampling intervals

Goldfish, carp and trout were exposed to 1 mM HEA for a period of 12 h, 84 h and 168 h for all measurements. An additional 40 h exposure was conducted for ammonia and urea flux determinations only. Each exposure tank was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 1.09 ± 0.08 mM of ammonia was maintained throughout the experiment. Control groups (no HEA) were set up in parallel to these exposure groups. Ammonia concentrations were measured (using the salicylate-hypochlorite method, Verdouw et al., 1978) each 6 h after the onset of treatment and the concentration of ammonia in the tanks was maintained by adding a calculated amount of the NH_4HCO_3 solution. Moreover, to avoid the microbial removal of ammonia and the accumulation of other waste products, 60–80% of the water was discarded every 2 days and replaced with fresh water containing the respective amount of ammonia. Water pH was maintained at 7.8-8.0 throughout the experimental period using dilute HCl and/or KOH.

4.2.3. Experimental protocol and sodium flux experiment

All the fish were placed in individual experimental containers with continuous aeration 12 h prior to sampling (the evening before measurement) to settle; water composition was identical to that of the exposure tanks. This series of experiment was done in another climate chamber maintaining the same temperature and photoperiod to which fish were acclimatized.

The containers for trout were 3 l (water volume set to 2.5 l) sealable Nalgene kitchen cutlery containers mounted on their sides; the horizontally flattened shape fitted the morphology of the fish. Similar Nalgene kitchen cutlery containers of 0.5 l (water volume set to 0.3 l) were employed for carp and goldfish. Black plastic shielding minimized visual disturbance.

For each experiment *N*=8 fish (4 from each of two tanks for cyprinids, 2 from each of four tanks for trout) were sampled and transferred individually into their respective experimental containers. The experimental protocols consisted of exposing the fish (*N*=8) to HEA while simultaneously measuring unidirectional Na^+ fluxes with ^{22}Na (manufactured by Perkin Elmer Boston, MA, USA) and net ammonia and urea fluxes. Control groups were conducted in parallel to exposure groups. Exposure to HEA started within the container for those groups of fish which were sampled at 12 h.

At the start of each flux period, an aliquot of ^{22}Na (typically 2 μCi l^{-1}) was added to each container and allowed to equilibrate for 30 min. Water samples (4 x 5 ml for ^{22}Na, total Na^+ and K^+ measurements) were taken at the start of the experiment and at subsequent 1-h intervals up to 4 h after the onset of ammonia exposure. For the ammonia and urea flux measurements, initial and final water samples were also taken. Following the last (i.e. 4 h) water sampling, the animals were

terminally anaesthetized with a lethal dose of neutralized (with 2 parts NaOH) MS222 (ethyl 3-aminobenzoate methane-sulfonic acid, 1 g l^{-1}, Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, approximately 0.6-0.7 ml blood samples (from each fish species) were withdrawn by caudal puncture into heparinized (2500 units ml^{-1} lithium heparin, Sigma, Munich, Germany) 1-ml syringes with 23-gauge needles. Blood was immediately centrifuged (for 1 min at 16,000 rpm at 4°C), and aliquots of plasma were frozen in liquid nitrogen and stored at -80°C for later analysis. Fish were dissected; gills were removed, washed with saline and blotted. One portion was added to five volumes of RNA*later* (Qiagen, GmbH, Hilden, Germany) and stored at 4°C for later molecular analysis, while the remaining gills were flash-frozen in liquid nitrogen and stored at -80°C for enzymatic assays.

4.2.4. Enzymatic analyses

NKA activity and H^+-ATPase activity were measured in crude gill homogenates of control fish and those exposed to HEA using methods from McCormick (1993) and Lin and Randall (1993) respectively, as modified by Nawata et al. (2007). Protein concentrations were measured with Bradford Reagent and BSA standards (Sigma-Aldrich). In order to assess whether NH_4^+ could activate NKA activity, the assay was performed under optimised conditions replacing the native concentration of KCl (10 mM) with same molar concentration of NH_4Cl in the reaction medium. Activities were then measured in the absence and presence of ouabain.

4.2.5. Molecular analyses

RNA extraction and cloning of gill transporters

Total RNA was isolated from gill samples using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. The extracted RNA samples were DNase treated to avoid genomic DNA contamination. The quantity of the RNA was evaluated by using Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and purity by measuring the OD_{260}/OD_{280} nm absorption ratio > 1.95.

For cloning, first strand cDNA was synthesized with SuperscriptII (Invitrogen, Burlington, ON, Canada). Partial sequences for carp and goldfish Rhbg, Rhcg, H^+-ATPase (vacuolar, B subunit), NKA (alpha subunit), and urea transporter (UT) were amplified using taq DNA polymerase (Invitrogen) and the primers listed in Table 4.1. Basic Local Alignment Search Tool (BLAST) analyses were done on the National Center for Biotechnology Information (NCBI) database.

Table 4.1

Cloning primers

Primer	5′→ 3′
Rhbg (carp/goldfish)	F: catcctcatcatcctctttggc R: cgtctacctgtggatgttctg
Rhcga (carp/goldfish)	F: tgggctcttctcatgcaaggctgg R: ctgcggtatcatctctacactgggat
Rhcgb (carp/goldfish)	F: gcacactgttcctgtggatg R: tctggggagatcctgctg
H^+-ATPase (carp)	F: cccgtggacagaagatccccatc R: ggatacatcacagagggacaggtg
H^+-ATPase (goldfish)	F: ctaccccgaggagatgatccag R: cagctgcacaacagacagatct
NKA (carp/goldfish)	F: aaggtggacaactcctccctgactg R:gcgtatggccaagaagaactgcctggt
UT (carp/goldfish)	F:gatggtgggcactcaatggttttgttgg R: caggccactacaatcaccacttccc

Real-time PCR

For quantitative real-time PCR (qPCR), a starting amount of 1 μg RNA was transcribed into first strand cDNA using the Revert Aid H minus First strand cDNA synthesis kit (Fermentas, Cambridgeshire, UK). mRNA expression in the gills of fish exposed to HEA for 12 h, 84 h and 168 h was compared to that in control fish by qPCR using the specific primers listed in Table 4.2. The primer sequences for rainbow trout were adopted from Wood and Nawata (2011).

qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). Reactions (20 μl) containing 4 μl of 5 x diluted cDNA, 4 pmol each of forward and reverse primers, 0.8 μl ROX dye (1:10 dilution), and 10 μl Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen), were performed at 50°C (2 min) and 95°C (2 min), followed by 40 cycles of 95°C (15 s) and 60°C (30 s). Melt curve analyses of the target genes and reference genes were performed which resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e. with water sample) for each set of genes was also performed to ensure no contamination of reagents, no primer–dimmers formation etc. In present study, the extracted RNA samples were subjected to DNase-treatment, and also melt curve analyses and gel analyses yielded single product, therefore, conducting runs with 'no RT' controls was not considered.

Comparison of several reference genes (beta-actin, elongation factor-1α, glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA) favoured beta-actin (β-actin) for cyprinids (goldfish and carp) and elongation factor-1α (EF-1α) for trout as the most stable genes across the samples (20

random samples were tested) and were used as endogenous standards to calculate relative mRNA expression by the standard curve method. Standard curves were generated by serial dilution of a random mixture of control samples.

Table 4.2

qPCR primer list and accession numbers, and efficiencies of the PCR reactions

Gene	Accession no.	Sequence of Primer (5´→ 3´)	Efficiency (%)
Trout			
Na^+/K^+-ATPase (α1a-subunit)	AY319391	F: ttgacctggatgaccacaag R: ggatctccttagcccgaac	87.2%
H^+-ATPase (V-type, B-subunit)	AF100042	F: tcagccttggttgtgagatg R: caacattggtgggaaacagg	103.7%
Rhbg	EF051113	F: cgacaacgacttttactaccgc R: gacgaagccctgcatgagag	75.3%
Rhcg1	DQ431244	F: catcctcagcctcatacatgc R: tgaatgacagacggagccaatc	80.1%
Rhcg2	AY619986	F: cctcttcggagtcttcatc R: ctatgtcgctggtgatgttg	88.7%
NHE-2	EF446605	F: tatggccattgtgacctgtg R: caggcctctccacactaagg	92.8%
UT	EF688013	F: gtataggccaggtgtatggg R: gatcgcctcaaatggagctg	106.0%
EF-1α	AF498320	F: ggaaagtcaaccaccacag R: gataccacgctccctctcag	88.6%
Common carp			
Na^+/K^+-ATPase	JX570881	F: aggtggacaactcctccctg R: atacgacccatgacagtacg	153%
H^+-ATPase	JX570880	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	102.5%
Rhbg	JX570877	F: tcccagtttccaggatgttc R: tggaaaaagccctgcataag	93.7%
Rhcg-a	JX570878	F: atcctgaacatcctccatgc R: aacttggccagaacatccac	113.2%

Rhcg-b	JX570879	F: cacaaagccacacacagtcc R: tctttttctcgccgttcttg	93.8%
UT	JX570882	F: agtgcatcttggttggtctc R: aggacttgtgggaagtggtg	129.8%
β-actin	M24113.1	F: cgtgatggactctggtgatg R: tcacggacaatttccctctc	110.9%
Goldfish			
Na^+/K^+-ATPase	JX570887	F: gtcatgggtcgtattgcatc R: gttacagtggcagggagacc	89.1%
H^+-ATPase	JX570886	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	79.1%
Rhbg	JX570883	F: atgatgaaacggatgccaag R: tcctggaaactgggataacg	95.1%
Rhcg-a	JX570884	F: gctggttccattctctggac R: atcttcggcatggaggacag	120.2%
Rhcg-b	JX570885	F: attgtgggcttcttctgtgg R: ggcacacgtttctcaaaagc	90.1%
UT	JX570888	F: tgttaaagggcagggtgaag R: cggatataacggcatcttgg	111.4%
β-actin	AB039726	F: ggcctccctgtctatcttcc R: ttgagaggtttgggttggtc	95.0%

The accession number refers to the registered sequence from GenBank. F: forward, R: reverse.

4.3. ANALYTICAL TECHNIQUES AND CALCULATION

It has been explained in details in previous chapter (refer: Chapter 3; section 3.3)

4.4. STATISTICAL ANALYSIS

All data have been presented as mean values ± standard error (s.e.m.), *N*= number of fish. Some of the data (wherever applicable) were natural logarithm transformed to stabilize the variance and to approximate a normal distribution prior to statistical analysis. For comparisons between different experimental groups a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. Student's two-tailed t- test was used for single comparisons. A probability level of 0.05 was used for rejection of the null hypothesis. No significant differences were

found between any of the control values at different sampling times. Therefore, pooled controls for each experimental group are shown for clarity of the figures.

4.5. RESULTS

4.5.1. Flux measurements

Sodium uptake and exchange

Trout exposed to 1mM ammonia (HEA) exhibited an initial 3.5 fold stimulation of J^{Na}_{out} ($P < 0.01$) at 12 h, but this had returned to control values by 84 h (Fig.4.1). J^{Na}_{in} remained statistically similar in exposed trout in comparison to the control group. In carp, both J^{Na}_{in} and J^{Na}_{out} were elevated considerably ($P < 0.01$ or 0.001) after 12 h exposure by 2.6 fold and 2.4 fold respectively, restored after 84 h and then increased again ($P < 0.05$) at the end of exposure period. In goldfish, 12 h HEA exposure induced increases ($P < 0.001$) in both J^{Na}_{in} and J^{Na}_{out} , by 5 to 6-fold compared to the control level. These elevations were followed thereafter by a decline (at 84 h and 168 h) but remained significantly higher than the control. As carp and goldfish exhibited persistent and synchronized stimulation in both influx and efflux under ammonia exposure, J^{Na}_{net} was affected to a lesser extent (values fluctuated within the range of control values) than in trout though J^{Na}_{net} values remained negative throughout in these fasted fish. In contrast, Na^+ balance was severely altered in trout at 12 h HEA, as J^{Na}_{net} decreased to -533 µmol $kg^{-1}h^{-1}$ from a control value of -1.06 µmol $kg^{-1}h^{-1}$ ($P < 0.05$), but beyond 12 h this species also was able to retain J^{Na}_{net} at the control level as seen for cyprinids.

Net K^+ flux rates

In all three species, net K^+ flux rates (J^{K}_{net}) were negative (i.e. net losses) under control conditions (Table 4.3). After 12 h of exposure, the K^+ flux was reversed in both cyprinids, resulting in a net uptake ($P < 0.05$ or 0.01). However, the net loss rates increased significantly in both trout (by 228%, $P < 0.01$) and carp (by 148%, $P < 0.01$) after 84 h HEA. J^{K}_{net} returned to control rates in goldfish at 84 h while in the other two at 168 h.

Table 4.3

Net flux rates of K^+ (μmol kg^{-1} h^{-1}) and plasma cortisol level (ng ml^{-1}) in rainbow trout, common carp and goldfish during exposure to 1 mM ammonia

	Control	12 h	84 h	168 h
K^+ (μmol kg^{-1} h^{-1})				
Rainbow trout	-63.4 ± 43.5	-108.6 ± 28.3	-208.1± 46.9**	-139.8± 15.0
Common carp	-178.6 ± 35.6	13.8 ± 34.2**	-443.3 ± 76.8**	-156.1± 23.5
Goldfish	-215.3 ± 26.0	42.2 ± 69.5*	-127.8 ± 34.4	-125.9 ± 57.0
Cortisol level (ng ml^{-1})				
Rainbow trout	170.8 ± 19.5	214.65 ± 19.5*	368.6 ± 32.3***	349.6 ± 30.2**
Common carp	114.1 ± 12.7	156.01 ± 14.1*	258.0 ± 39.2*	182.4 ± 44.5*
Goldfish	107.7 ± 16.0	148.5 ± 15.4*	233.7 ± 30.5*	174.3 ± 32.6*

Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (N=7-8) and its respective pooled control (N=22-24) (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

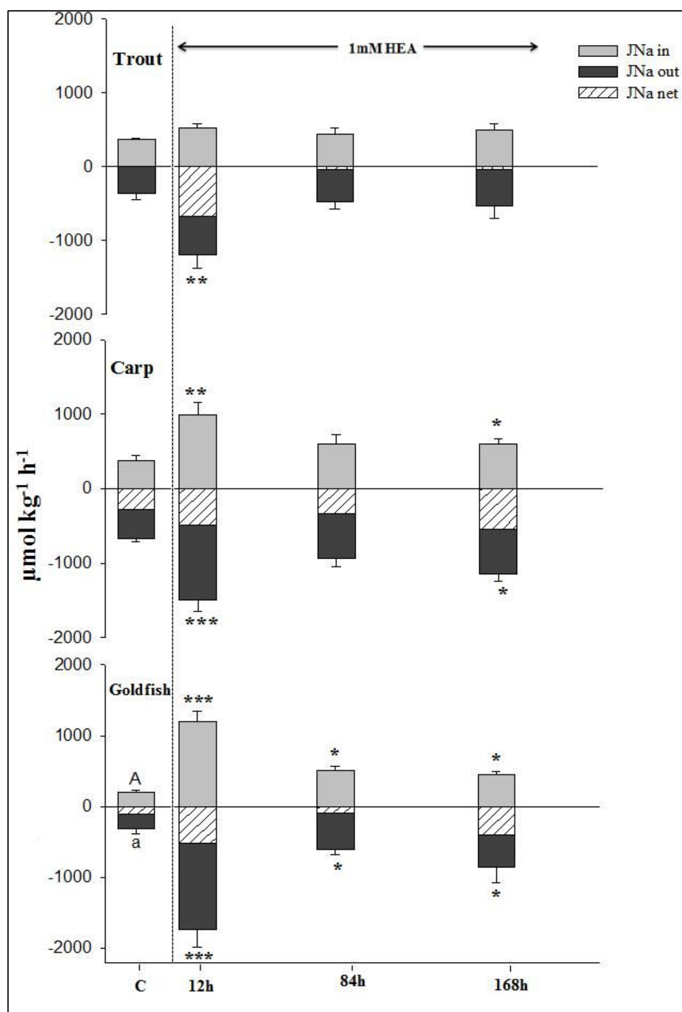

Figure. 4.1. Na^+ unidirectional influx (J^{Na}_{in}, upward bars), Na^+ efflux (J^{Na}_{out}, downward bars) and Na^+ net flux (J^{Na}_{net}, hatched bars) rates in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (N=8) and its respective pooled control (N=24) (*$P < 0.05$; **$P < 0.01$;***$P < 0.001$).

4.5.2. Ammonia and urea excretion rate

In contrast to cyprinids, trout exposed to HEA displayed no significant changes in ammonia excretion rates (J_{amm}) during any of the sampling periods, which remained at approximately -300 µmol kg^{-1}h^{-1} throughout despite the unfavourable gradient (Fig. 4.2). J_{amm} in carp was -402 µmol kg^{-1}h^{-1} under control conditions and increased significantly after 12 h HEA. The significant elevation persisted till the end of exposure period except at 40 h where a slight decline was observed. The relative J_{amm} increments at 12 h, 84 h and 168 h were 150% ($P < 0.01$), 70% ($P < 0.05$) and 186% ($P < 0.01$) higher than the control. In contrast, in goldfish, the control J_{amm} was -250 µmol kg^{-1}h^{-1} and there was no change at 12 h of HEA exposure. However, after 40 h, J_{amm} increased 2-fold ($P < 0.05$) followed by a return to control rates at 84 h HEA. At 168 h, there was a 3.9 fold increase ($P < 0.01$) over the control, similar to the pattern in carp.

Under control conditions, urea-N excretion rates were approximately 25%, 30%, and 13% of ammonia-N excretion rates in trout, carp, and goldfish respectively. The effect of HEA on urea excretion rate (J_{urea}) was notable only in goldfish (Fig.4.3). A significant rise was observed from 12 h onwards throughout the entire exposure course. The relative increments after 12 h, 40 h, 84 h and 168 h compared to control were 419% ($P < 0.001$), 130% ($P < 0.05$), 150% ($P < 0.05$), and 96% ($P < 0.05$) respectively. Notably, at 12 h of HEA, the urea-N excretion rate became almost equal to the ammonia-N excretion rate in the goldfish. In contrast, no significant effect ($P > 0.05$) of HEA on J_{urea} was seen either in carp or in trout.

4.5.3. Plasma metabolites

Ammonia and urea accumulation

Plasma total ammonia (T_{amm}) was significantly elevated in both cyprinids and trout from 84 h onwards and remained higher until the end of the HEA exposure period (168 h) (Fig.4.4). In the cyprinids at 84 h and 168 h, plasma T_{amm} levels were only slightly higher than the mean exposure level (1.09 mmol l^{-1}) while in trout T_{amm} was about two- fold higher than the ammonia level in the water.

Under control conditions, plasma urea-N levels were considerably higher than plasma T_{amm} in all species. During HEA exposure, plasma urea-N concentration in trout was elevated significantly compared to control by 68% ($P < 0.001$) and 58% ($P < 0.05$) respectively after 12 h and 168 h (Fig.4.5). Likewise, carp started to accumulate considerable amounts of urea-N when exposed to HEA and followed the same pattern as trout, with increases of 56% ($P < 0.01$) and 38% ($P < 0.05$) after 12 h and 168 h respectively. Curiously, urea-N concentrations at 84 h were similar to control values in both species. In contrast, no obvious differences ($P > 0.05$) were seen in goldfish during

any of the sampling periods, illustrating a divergent pattern of urea-N accumulation between the two cyprinids upon HEA exposure.

Cortisol

Cortisol levels in plasma were considerably ($P < 0.05$) elevated in all the species from 12 h HEA onwards (Table 4.3). The rise in trout after 12 h, 84 h and 168 h were 30% ($P < 0.05$) 115% ($P < 0.001$) and 104% ($P < 0.01$). Among cyprinids, the respective augmentations were 38% ($P < 0.05$), 116% ($P < 0.05$) and 62% ($P < 0.05$) in goldfish; while in carp the increments were 37% ($P < 0.05$), 126% ($P < 0.05$) and 60% ($P < 0.05$). These increases occurred despite the fact that the level of cortisol in all the control groups was relatively high, most likely due to confinement stress.

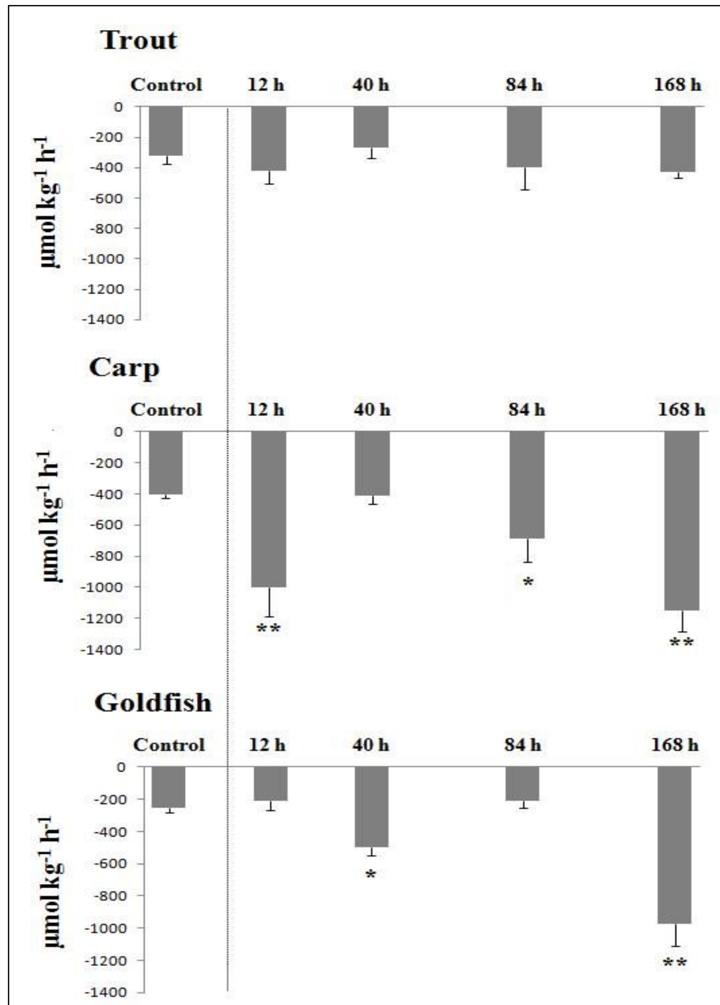

Figure. 4.2. Net excretion rate of ammonia (J_{amm}) in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (N=8) and its respective pooled control (N=32) (*$P < 0.05$;**$P < 0.01$).

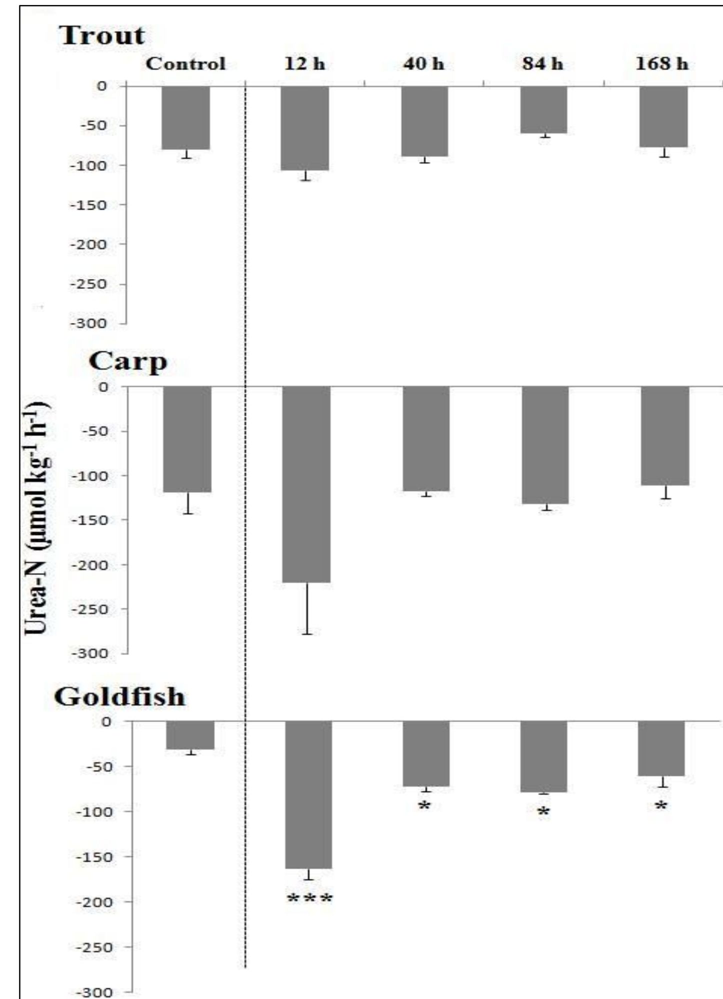

Figure. 4.3. Net excretion rate of urea (J_{urea}) in rainbow trout, common carp a goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (N= and its respective pooled control (N=32) (*$P < 0.05$;***$P < 0.001$).

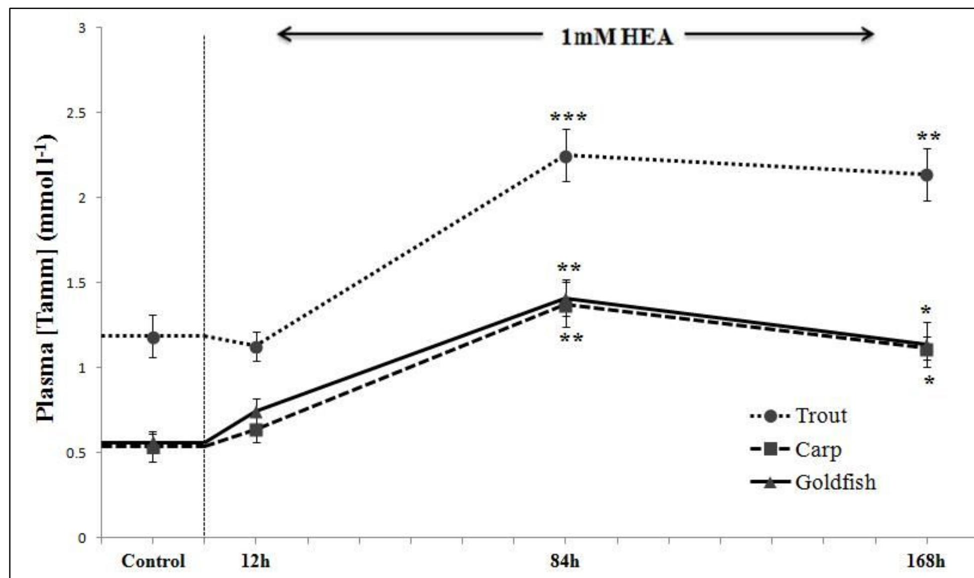

Figure. 4.4. Plasma total ammonia concentration (T_{amm}) in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

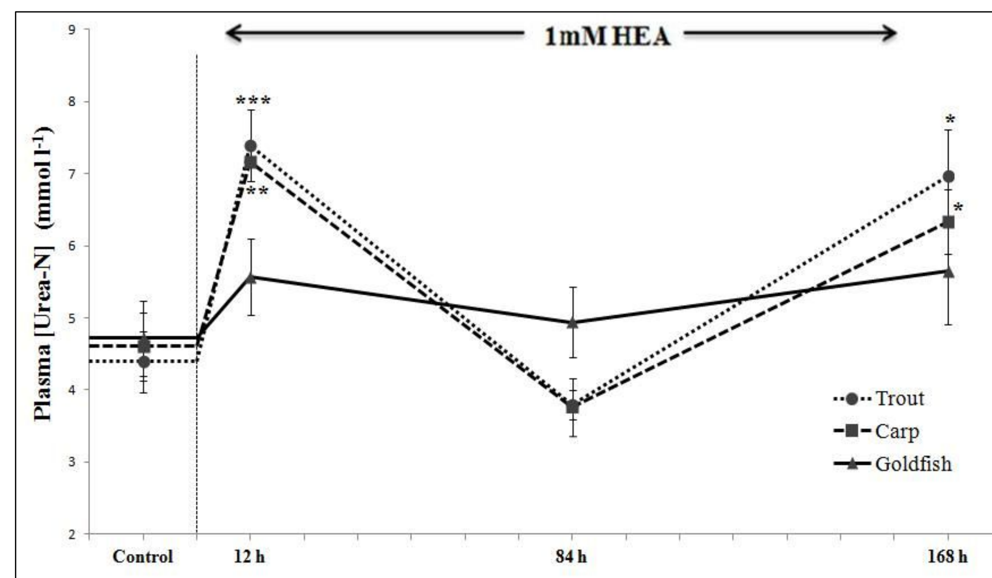

Figure. 4.5. Plasma urea-N concentration of rainbow trout, common carp and goldfish during during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

4.5.4. Gill mRNA expression

BLAST results show that the carp and goldfish Rhbg partial sequences are most similar to *Danio rerio* Rhbg (AAH49405) since *D. rerio* has the smallest Expect (E) values (2e-121 for carp and 3e-123 for goldfish) than any other species. The carp and goldfish Rhbg sequences are respectively 88% and 90% identical to the *D. rerio* Rhbg amino acid sequence and they are both 83% identical (E-value of 1e-116) to *O. mykiss* Rhbg (ACF70599). Rhcg-a is most similar to *D. rerio* Rhcga (NP_001083046) (89% amino acid identity and E-value of 3e-127 for both carp and goldfish). Also, Rhcg-a is more similar to *O. mykiss* Rhcg1 (NP_001118049) than to *O. mykiss* Rhcg2 (NP_001117995) (86% and 88% identity and E-values of 1e-121 and 2e-123 for carp and goldfish respectively *vs*. 85% identity and an E-value of 1e-120 for both carp and goldfish). Rhcg-b is most similar to *D. rerio* Rhcg-like2 (Q8JI14) with an E-value of 4e-110 and 86% identity to carp and E-value of 3e-108 and 85% identity to goldfish. Rhcg-b is more similar to *O. mykiss* Rhcg2 than *O. mykiss* Rhcg1 (E-value of 3e-100 with 78% identity to carp and an E-value of 1e-100 with 79% identity to goldfish *vs*. an E-value of 2e-96 with 80% identity to carp and E-value of 4e-94 with 79% identity to goldfish).

Rh glycoproteins and transporters

In goldfish gills, the mRNA expression level of Rhbg increased significantly at 12 h and 84 h HEA by 1.9 fold ($P < 0.05$) and 1.6 fold ($P < 0.05$) respectively (Fig.4.6). These increases were followed by a partial recovery at 168 h. In carp and trout, small increments in Rhbg transcript level were not significantly different from their respective control levels.

Rhcg-a expression level was significantly up-regulated in both cyprinids after HEA exposure (Fig.4.7A). In goldfish, a 5.1 fold ($P < 0.001$) and 2.4 fold ($P < 0.01$) increase in transcript level was noticed following 84 h and 168 h exposure. Likewise, 84 h HEA exposed carp exhibited a 2.1 fold ($P < 0.01$) elevation relative to the control which declined after 168 h exposure to control levels.

Rhcg-b mRNA level in goldfish gills increased significantly ($P < 0.01$) at 84 h with a 2.6 fold higher expression than in the control (Fig.4.7B). At the end of exposure period, a slight decline was noted but the mRNA level remained significantly higher (2 fold, $P < 0.05$) than in the control. In contrast, Rhcg-b expression in carp remained unchanged during HEA exposure.

In trout gills, Rhcg1 mRNA expression remained unchanged across the HEA exposure time period (Fig.4.8A) while Rhcg2 expression increased by 2.2 fold ($P < 0.01$) at 84 h and remained elevated by 2 fold at 168 h ($P < 0.05$) (Fig.4.8B). NHE-2 expression displayed a gradual rise with exposure time which became significant after 84 h exposure (Fig.4.8C). The augmentations over the control level after 84 h and 168 h were 40% ($P < 0.05$) and 50% ($P < 0.05$) respectively.

NKA and H^+-ATPase

Significant effects of HEA exposure were seen on NKA mRNA expression levels in gills of cyprinids only (Fig.4.9A). In goldfish, NKA expression increased significantly by 3.8 fold ($P < 0.001$) after 84 h of HEA exposure. Subsequently, the levels dropped at the end of exposure period, but the transcript activity remained significantly higher (1.8 fold elevation; $P < 0.05$) than control. Almost the same pattern was observed for carp, the mRNA level increasing significantly by 1.6 fold ($P < 0.05$) at 84 h HEA with recovery thereafter.

Pronounced effects ($P < 0.01$) of HEA were evident on the transcript levels of H^+-ATPase in gills of trout and goldfish while no significant effects ($P > 0.05$) were noted for carp (Fig.4.9B). In trout, 84 h and 168 h HEA resulted in a 1.5 ($P < 0.01$) and 1.7 fold ($P < 0.01$) elevations over the control. Likewise, H^+-ATPase expression level in goldfish increased gradually and became significant (2.2 fold, $P < 0.01$) at 84 h but returned to control values ($P > 0.05$) after 168 h of HEA exposure.

Urea transporter (UT)

Exposure of goldfish to HEA tended to increase UT mRNA expression in gills with a significant effect from12 h onwards (Fig.4.10). The relative elevations in expression level at 12 h, 84 h and 168 h were 12-fold ($P < 0.001$), 11-fold ($P < 0.001$) and 6-fold ($P < 0.01$) respectively. On the contrary, HEA did not appear to exert any notable effect ($P > 0.05$) on UT mRNA expression level in carp or trout.

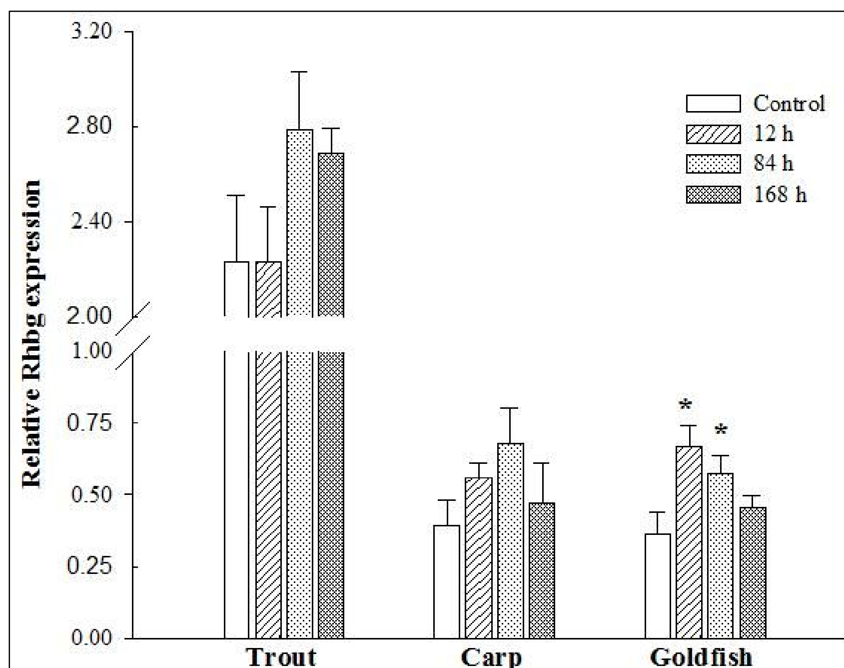

Figure. 4.6. Expression of Rhbg mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (*$P < 0.05$).

4.5.5. NKA and H^+-ATPase enzyme activity

In general, enzyme activity responses (Figs.4.11A,B) paralleled mRNA expression data (Figs. 4.9A,B) quite well for these two enzymes. Branchial NKA activity in carp and goldfish increased considerably at 84 h HEA exposure (Fig. 4.11A). Carp displayed an increase of 129% ($P < 0.001$) at 84 h exposure and a corresponding enhancement of 275% ($P < 0.001$) was observed in goldfish. In both cyprinids, the activity of NKA tended to decline again after 84 h exposure but remained significantly higher (at 168 h: 78% and 114% rise in carp and goldfish respectively) than their controls. In contrast to cyprinids, exposure to HEA did not induce noteworthy alterations in trout.

The ability of NH_4^+ to activate gill NKA activities in these three species was examined (Fig.4.11A). In the course of these trials, we found that NKA activities were always lower when NH_4^+ was substituted for K^+, regardless of the treatment, time, or species, although carp appeared to take NH_4^+ slightly better than goldfish and trout. Thus while NH_4^+ can support some Na^+/ NH_4^+-ATPase activity, it is not as effective as K^+ (i.e. Na^+/K^+-ATPase activity) on an equimolar (10 mM) basis in any of the three species. In carp and goldfish, the increases in Na^+/K^+-ATPase activities occurring at 84 h and 168 h were tracked by smaller increases in Na^+/NH_4^+ -ATPase activities (Fig.4.11A).

Though no stimulation of NKA activity was noted for HEA exposed trout, significant ($P < 0.05$ or 0.01) 1.4- and 1.6-fold increases in branchial H^+-ATPase activities were observed for this species after 84 h and 168 h of HEA exposure (Fig.4.11B). Also, in goldfish a significant elevation (3 fold, $P < 0.01$) was seen at 84 h, with a partial restoration at 168 h HEA. In contrast to trout and goldfish, increases in branchial H^+-ATPase activity in carp following HEA exposure were not significant.

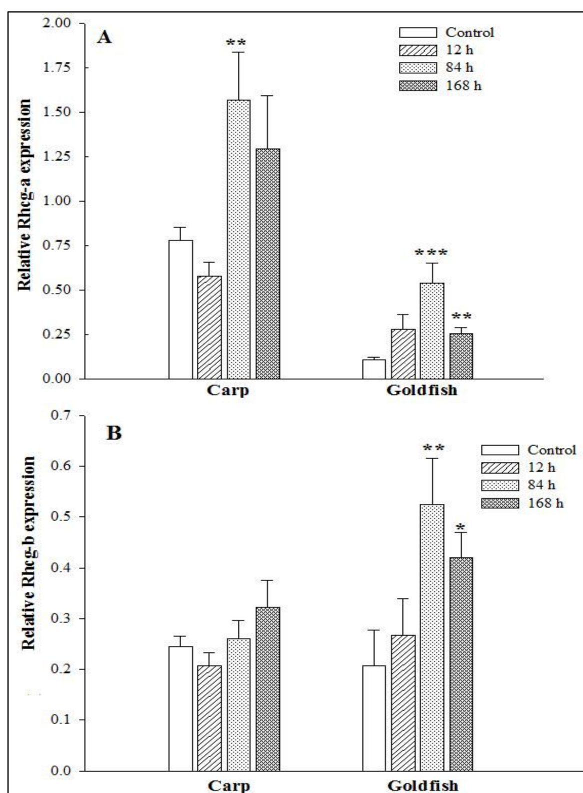

Figure. 4.7. Expression of **(A)** Rhcg-a and **(B)** Rhcg-b mRNA in the gills of common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (N=8) and its respective pooled control (N=24) (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

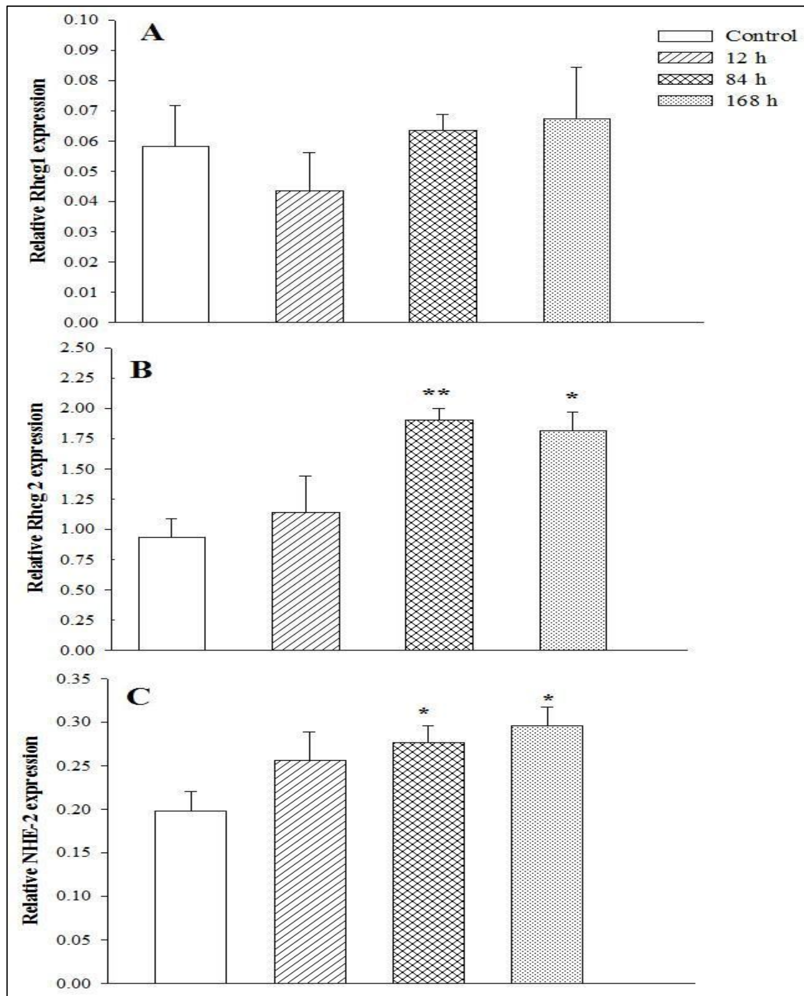

Figure. 4.8. Expression of **(A)** Rhcg1 **(B)** Rhcg2 and **(C)** NHE-2 mRNA in the gills of rainbow trout during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (*$P < 0.05$;**$P < 0.01$).

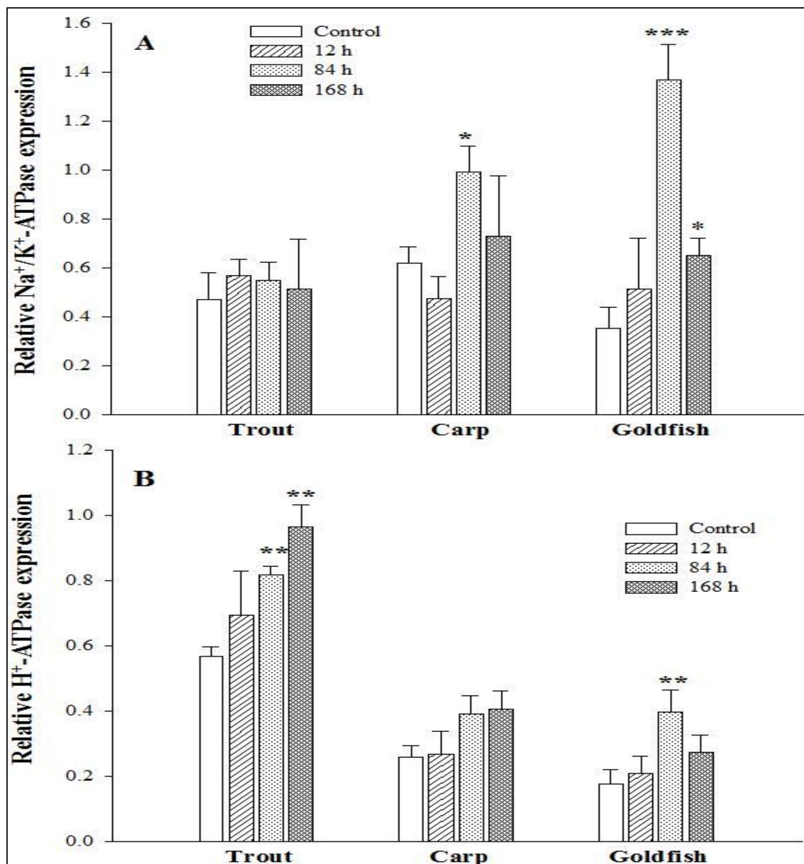

Figure. 4.9. Expression of **(A)** Na^+/K^+-ATPase and **(B)** H^+-ATPase mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

4.6. DISCUSSION

4.6.1. Ammonia flux and Rh glycoproteins

In our earlier study on acute exposure to the same level of HEA, we found that ammonia excretion (J_{amm}) was strongly inhibited or reversed in carp and trout during the first few hours (3 h) of exposure, but remained statistically unchanged in goldfish, and that all three species had restored control rates of J_{amm} by 12 h (Liew et al., 2013). The current results confirm the restoration of J_{amm} by 12 h in all three species, and indeed show a significant increase in the carp at this time (Fig.4.2). This sequence of initial J_{amm} inhibition followed by re-establishment has already been reported for goldfish and trout (Nawata et al., 2007; Wilkie et al., 2011; Wilson et al., 1994; Zimmer et al., 2010). Furthermore both cyprinids exhibited enhanced J_{amm} at several time points, signifying their ability to excrete ammonia under unfavourable circumstances. Assuming that similar pH's are maintained inside (i.e. in the blood) as outside these cyprinids (7.8-8.0), there would have been virtually no P_{NH3} gradient from the blood to the bulk water as T_{amm} concentrations became virtually identical in the two media by 84-168 h (Fig.4.4). In contrast, trout exposed to HEA were not able to increase the excretion rate above control levels and had plasma T_{amm} elevated more than 2-fold over water levels by this time. Goldfish and carp, therefore, appear to regulate ammonia homeostasis more efficiently than the trout.

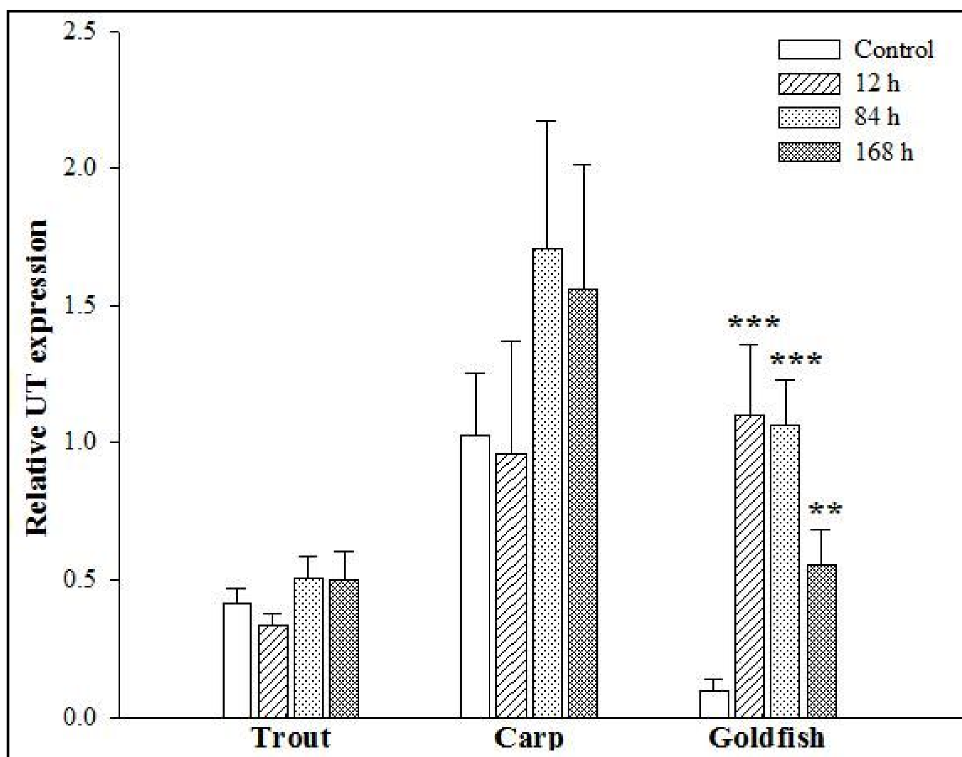

Figure. 4.10. Expression of urea transporter (UT) mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (**$P < 0.01$;***$P < 0.001$).

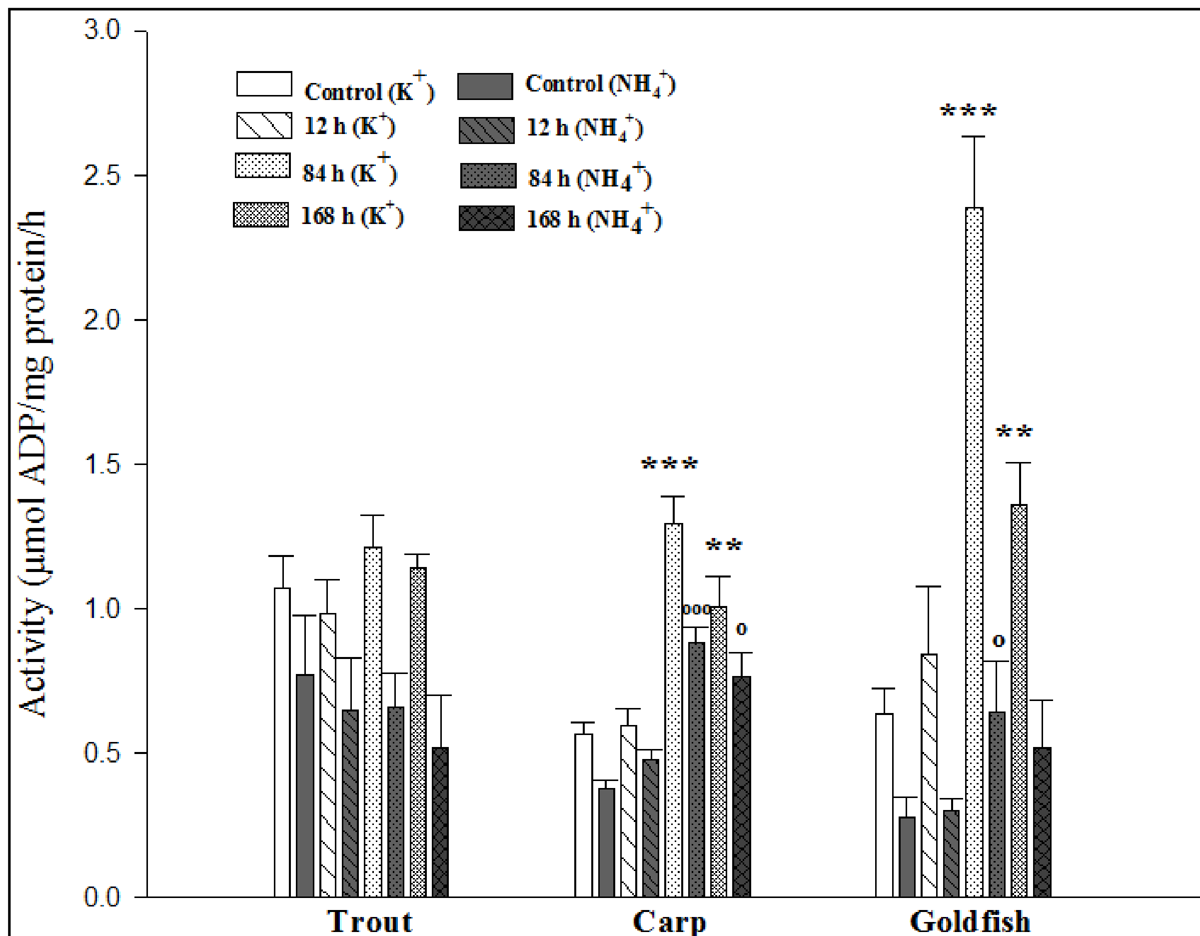

Figure. 4.11. Enzyme activities of **(A)** Na^+/K^+-ATPase (with K^+ present and with K^+ replaced by NH_4^+ in the reaction media) and **(B)** H^+-ATPase in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean ± s.e.m. Asterisk (*) indicates a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) when K^+ is present (i.e. Na^+/K^+-ATPase) and for H^+-ATPase ; circle (o) represent the significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) when K^+ is replaced with NH_4^+ (i.e. Na^+/NH_4^+-ATPase). ($^*P$ < 0.05;$^{**}P < 0.01$;$^{***}P < 0.001$ and $^{o}P < 0.05$; $^{ooo}P < 0.001$).

At least some of this increased ammonia transport may relate to the Rh glycoproteins present in the gill cell membranes which are implicated as a putative mechanism of ammonia transport and linked in some manner with Na^+ uptake (see Introduction). Indeed, Rh mRNA expression levels do increase in response to high external or internal ammonia in most reports on fish (Braun et al., 2009a; Hung et al., 2007; Nawata and Wood, 2009; Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011), but not in all (Nakada et al., 2007b).

In the present study we compared Rh gene expression in the gills of three species, using previously determined trout sequences (Nawata and Wood, 2008; Nawata et al., 2007) and newly identified partial sequences of three Rh cDNAs (Rhbg, Rhcg-a, and Rhcg-b) in the two cyprinids, goldfish and

common carp. Rhbg expression was up-regulated only in goldfish upon HEA (12 h-84 h) exposure (Fig.4.6). These increases in goldfish were accompanied by restored levels or significant increases in J_{amm} at these times (Fig.4.2), signifying that Rhbg may be involved in the maintenance or regulation of ammonia transport in goldfish. Likewise, in zebrafish larvae Rhbg was found to be highly expressed, broadly distributed and play an appreciably role in ammonia excretion (Braun et al., 2009a). In a previous study on rainbow trout, Nawata et al. (2007) also reported a lack of significant increment in mRNA expression of Rhbg in whole gill during 48 h exposure to 1.5 mM HEA. However, their study showed that a significant Rhbg induction occurred in pavement cells but not in mitochondrial rich cells. Since both cell types are involved in routine ammonia transport via Rh glycoproteins, the expression kinetics in the gill cell fractions need to be validated in future studies.

Rhcg present on the apical membrane of the branchial epithelium facilitates ammonia efflux out of the gills and appears to be coupled to H^+ excretion (through H^+-ATPase and/or NHE-2) and Na^+ uptake (Wright and Wood, 2009). In the present study we identified two Rhcg homologs (Rhcg-a and Rhcg-b) for both carp and goldfish. An increase of Rhcg-a expression was observed in both carp and goldfish during HEA, and of Rhcg-b expression only in goldfish (Fig.4.7). As J_{amm} was restored or increased at these same times, these results suggest that both Rhcgs may be involved in ammonia excretion in goldfish, and Rhcg-a appears to be involved in carp. In trout, which restored but did not elevate J_{amm} during chronic HEA exposure (Fig.4.2) Rhcg2 expression was up-regulated at 84-168 h, in accord with observations of Nawata et al. (2007) and Wood and Nawata (2011) over a shorter time frame in rainbow trout. However, Rhcg1 expression did not alter in trout during HEA exposure, corroborating previous studies on zebrafish, freshwater and seawater trout, and weatherloach (Braun et al., 2009b; Moreira-Silva et al., 2010; Nakada et al., 2007b; Nawata et al., 2007; Wood and Nawata, 2011). Rhcg1 does not seem to be connected to increased ammonia transport under HEA in trout, as even a progressive downregulation of Rhcg1 during HEA exposure has been reported in this species (Wood and Nawata, 2011).

4.6.2. Na^+ fluxes and the role of the "Na^+/NH_4^+ exchange complex"

The restoration of J^{Na}_{in} in trout (after an initial inhibition, Liew et al., 2013) or increases in cyprinids (Fig.4.1) could be due to the activation of the branchial apical "Na^+/NH_4^+ exchange metabolon" which involves several membrane transporters (H^+-ATPase, Na^+/H^+ exchanger, Na^+ channel) and Rh glycoproteins (Rhcg in particular) working together to provide an acid trapping mechanism for apical ammonia excretion (see Introduction).

NHE-2 mRNA was quantified only in the gills of trout (Fig.4.8C), where it is the dominant Na^+/H^+ exchange protein, although NHE-3 also occurs there (Ivanis et al., 2008). In trout, an increase in NHE-2 expression was seen during chronic HEA exposure (Fig. 4.8C), along with increases in H^+-

ATPase expression (Fig.4.9B), H^+-ATPase activity (Fig.4.11B), and Rhcg2 expression (Fig.4.8B), accompanied by restoration of J_{amm} to the control level (Fig.4.2) after initial reversal of J_{amm} (Liew et al., 2013). The activation of these transporters in trout may also help to explain the recovery of J^{Na}_{in} (Fig.4.1) after an initial inhibition in the first few hours of exposure (Liew et al., 2013). Increased NHE-2 and H^+-ATPase expression and/or activity, restored or increased J_{amm}, and increased J^{Na}_{in} have been seen in several other studies on ammonia-loaded trout (Nawata and Wood, 2009; Nawata et al., 2007; Tsui et al., 2009; Wood and Nawata, 2011; Zimmer et al., 2010).

Similar to trout, goldfish also seem to rely on an acid-trapping model for ammonia excretion as we observed augmentations in H^+-ATPase expression (Fig.4.9B) and activity (Fig.4.11B), accompanied by parallel up-regulation of Rhcg-a and Rhcg-b (Fig.4.7), elevated J_{amm} (Fig. 4.2), and elevated (J^{Na}_{in}) (Fig.4.1). In contrast, in carp neither the activity nor the expression level of H^+-ATPase was affected by HEA (Figs. 4.11B, 4.9B), but carp may utilise other components of the "Na^+/NH_4^+ exchange metabolon" as suggested by a significant up-regulation in Rhcg-a expression at 84 h (Fig. 4.7A).

In HEA exposed trout, Na^+ influx was maintained at the control level (Fig. 4.1), while H^+-ATPase activity (and expression) increased at 84 h and 168 h (Figs.4.11B, 4.9B). If these changes signify an increased H^+ efflux, this scenario envisages that Na^+ uptake may become partially uncoupled from H^+ flux and ammonia excretion under these circumstances. It is also tempting to speculate that during HEA exposure, NH_4^+ may act like a low dose of amiloride, limiting Na^+ uptake, while at the same time H^+ efflux and boundary layer acidification for trapping of NH_3 efflux can still occur (Nelson et al., 1997). Moreover, upregulation of NHE-2 would serve to maintain some degree of Na^+ uptake (Zimmer et al., 2010). Nevertheless, the limitations of this study need to be mentioned, as H^+ efflux was not measured.

Furthermore, it was surprising that branchial J^{Na}_{in} (Fig. 4.1) and J_{amm} (Fig. 4.2) in trout were restored by 12 h of HEA onwards, long before any significant increment (at 84 h -168 h) in H^+-ATPase activity (Fig. 4.11B) or genomic up-regulation of H^+-ATPase (Fig. 4.9B), Rhcg2 (Fig.4.8B), and NHE-2 (Fig.4.8C). A similar discrepancy was noted for cyprinids where J^{Na}_{in} (Fig. 4.1) and J_{amm} (Fig. 4.2) increased by 12 h and 12 h -40 h respectively while the responses of Rhcg ammonia transporters became significant only at 84 h-168 h HEA (Figs.4.7A,B). Notably, this delayed up-regulation of these transporters in all three species occurred in conjunction with the elevated plasma T_{amm} which was apparent at 84 h and 168 h (Fig. 4.4). These results reinforce previous proposals that elevated internal ammonia levels may be involved in the signalling mechanism for upregulation of the "Na^+/NH_4^+ exchange complex" (Nawata and Wood, 2009; Tsui et al., 2009). Other factors such as post-translational modifications (PTMs) of the Rh proteins and other associated transporters during HEA may be responsible for the temporary disconnect between the changes recorded in mRNA

expression and those in functional activity. In this regard, Nawata et al. (2010b) reported that the molecular weight of Rh proteins increased by ~8 kDa in pufferfish (*Takifugu rubripes*) when exposed to 1 mM NH_4HCO_3 for 48 h. Perhaps under normal conditions, the majority of these transporters are in a dormant state, requiring stimuli such as high ammonia (and perhaps cortisol, as argued subsequently) to instigate the changes necessary for full functionality.

Na^+ efflux rate (J^{Na}_{out}) increased when trout were exposed to HEA for 12 h and similar increases were also evident in carp and goldfish (Fig. 4.1). These are likely due to the increased diffusive leakage of Na^+ during HEA - i.e. enhanced Na^+ permeability (transcellular and/or paracellular) of the gills (Gonzalez and McDonald, 1992). K^+ loss rate (J^{K}_{net}), an indicator of transcellular leakage, was measured in the present study (Table 4.3) but did not show a consistent pattern in any of the fish species analogous with their J^{Na}_{out}. Therefore, investigation of other indices of gill permeability such as diffusive water flux and transepithelial potential (cf. Liew et al., 2013) may be crucial in future experiments.

In our previous experiment (Liew et al., 2013) we reported that during pre-12 h HEA exposure, the diffusive Na^+ loss (J^{Na}_{out}) was stimulated and exceeded influx rate (J^{Na}_{in}) in trout and in carp, resulting in net Na^+ loss in these two species. However, goldfish displayed an opposite trend and were able to maintain a positive net Na^+ balance, illustrating a divergent pattern between the two cyprinids during the early exposure period (< 12 h). In the present experiment, an interesting pattern was noticed among the cyprinids; J^{Na}_{in} was stimulated precisely at the same time when there was an increase in J^{Na}_{out} (Fig.4.1). Also, from 84 h onwards, trout were able to maintain net Na^+ flux closer to zero. These consequences may indicate some sort of recovery response in these experimental animals towards normal Na^+ balance as part of the ammonia excretion mechanism, and/or that an increase in J^{Na}_{out} among cyprinids might be coupled directly to an increase in J^{Na}_{in} through mechanisms such as a carrier- mediated exchange diffusion transport system or through a leaky pump (Goss and Wood, 1990; Potts and McWilliams, 1989; Twitchen, 1990). Exchange diffusion (Shaw, 1959) has been observed during normoxia in many freshwater animals, including trout (Wood and Randall, 1973) but until now, it has not been studied under HEA exposure.

4.6.3. NKA response

Basolaterally situated NKA is believed to provide the major source of energy driving Na^+ influx (Avella and Bornancin, 1989; Lin and Randall, 1995; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997). In the current study, mRNA expression levels (Fig. 4.9A) and activity (Fig. 4.11A) of NKA clearly responded to HEA in carp and goldfish gills, but not in trout. Increased activities of NKA have also been reported in silver perch, golden perch, climbing catfish, and

pufferfish when exposed to HEA (Alam and Frankel, 2006; Nawata et al, 2010b; Schram et al., 2010), but not in previous studies on rainbow trout (Nawata et al., 2007; Wood and Nawata, 2011). These enhanced responses in HEA exposed carp and goldfish might provide another explanation for increased J^{Na}_{in} in these species (Fig. 4.1).

Potentially, the NKA enzyme may serve another role. In addition to Rhbg channels, which were only upregulated at the mRNA level in goldfish (Fig.4.6), ammonia transport (as NH_4^+) across the branchial basolateral membranes might occur *via* Na^+/K^+-ATPase enzyme functioning as a Na^+/NH_4^+ -ATPase (Evans et al., 2005; Wilkie, 2002) since similarities in the hydration radius of K^+ and NH_4^+ might allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999). The potential ability of NH_4^+ to substitute for K^+ and/or augment the activity of branchial NKA proved negligible in all the three species (Fig.4.11A) and therefore demonstrated that NH_4^+ is not a preferred substrate for this enzyme. Our result is in tune with earlier report on trout gills (Salama et al., 1999; Wood and Nawata, 2011). In none of the species was the NH_4^+ activation equal to or greater than the K^+ activation in contrast to toadfish (Mallery, 1983), tilapia (Balm et al., 1988), mudskippers (Randall et al., 1999) and pufferfish (Nawata et al., 2010b), where active NH_4^+ movement across the gill basolateral membranes on the 'K^+ site' of the NKA molecule may be very important during HEA exposure.

4.6.4. Cortisol response

Cortisol is the principal corticosteroid in teleost fish and plays a crucial role in the stress response and in osmoregulatory processes (McCormick, 2001; Wendelaar Bonga, 1999). Plasma cortisol levels increased during HEA in all three species (Table 4.3), a commonly observed response in cyprinids and salmonids (Ortega et al., 2005; Sinha et al., 2012b; Tsui et al., 2009; Wood and Nawata, 2011). Cortisol has been shown to regulate the expression of many ion-regulatory genes (e.g. NKA, NHEs) in fish gills (Ivanis et al., 2008; Kiilerich et al., 2007; McCormick et al., 2008) and, in combination with ammonia, may also play a key role in regulating the expression of Rh glycoproteins, thereby activating the "Na^+/NH_4^+ exchange metabolon" (Tsui et al., 2009). However, in the present and previous studies (Nawata and Wood, 2008; Wood and Nawata, 2011) there was no clear-cut relationship between circulating cortisol levels and Rh mRNA expression or ammonia flux. This may be because plasma T_{amm} and cortisol act more effectively in combination than alone, at least in an *in vitro* gill cell culture system (Tsui et al., 2009). Nevertheless, it is important to mention that in present study the control value for plasma $T_{amm,}$ particularly for trout, was relatively high (Fig.4.4) compared to other published data (Nawata and Wood, 2009; Nawata et al., 2007; Wilkie et al., 2011; Wood and Nawata, 2011; Zimmer et al., 2010). It may have been due to the confinement and/or sampling stress, as also evident from our cortisol results which were also high. Moreover, these data

were consistent across the treatments for all the studied fish species. Recently, Kolarevic et al. (2012) reported a very high resting plasma T_{amm} level (up to 1.5 mmol l^{-1}) in Atlantic salmon (*Salmo salar*) which sustained chronically (up to many days) without ill effects, as evidenced by unchanged growth rate. Moreover, a relatively high basal level of plasma T_{amm} was also evident in the goldfish (0.8-1.4 mmol l^{-1}; Liew et al., 2012; Sinha et al., 2012a; Smith et al., 2012) as well as in the carp (0.6-1.1 mmol l^{-1}; De Boeck et al., 2006; Liew et al., 2012).

There is some evidence that cortisol may also contribute to the regulation of urea production in fish (Hopkins et al., 1995; Mommsen et al., 1999; Vijayan et al., 1996b) and experimentally elevated plasma cortisol increased urea-N excretion rates (J_{urea}) in trout (McDonald and Wood, 2004b). Cortisol seemed to exert a significant action on urea metabolism in goldfish, which displayed a significant rise in J_{urea} (Fig.4.3) coinciding with the increment in cortisol levels (Table 4.3). In future, it would be interesting to investigate the receptor or sensor that links HEA to cortisol release and whether the responses of Rh glycoproteins reflect the direct or indirect impacts of cortisol. Moreover, future studies are also warranted to investigate whether the size differences between trout and the much smaller cyprinids could have influenced the physiological and molecular responses.

4.6.5. Urea excretion: role in ammonia detoxification during HEA

Most freshwater fish are ammoniotelic. However, some teleosts (e.g. mudskippers, Indian catfish, Lake Magadi tilapia, gulf toadfish etc.) expend energy to detoxify ammonia and may become partially or totally ureotelic (Iwata et al., 2000; Randall et al., 1989; Saha and Ratha, 1998; Walsh et al., 1990; Wood et al., 1989a; Wood et al., 1989b). Our study indicates that one way by which goldfish are able to cope with HEA more effectively than the other two species is by substantially increasing J_{urea}; indeed urea-N excretion became almost equal to ammonia-N excretion at 12 h HEA (Figs.4.2, 4.3). Similarly, Olson and Fromm (1971) reported an increased J_{urea} in goldfish subjected to HEA. In a recent study on goldfish, Wilkie et al. (2011) also noted a significant rise in J_{urea} over 5 days of HEA (5mmol l^{-1} NH_4Cl) exposure. Potential pathways of the increased urea production in goldfish (e.g. uricolysis, arginolysis, ornithine-urea cycle) are worthy of future investigation. However, the key enzymes of the ornithine-urea cycle were reported to be absent in goldfish tissues (Felskie et al., 1998), making it improbable that the fish would be inducing this pathway during HEA. Previously, urea was thought to permeate cell membranes solely by simple diffusion through cell membranes (Wood, 1993; Wright et al., 1995b) but abundant evidence now suggests the occurrence of a specialized facilitated diffusion-type urea transporter (UT) in teleost gills (see McDonald et al., 2012 for a recent review). We found a marked increase in branchial mRNA expression of UT during HEA, occurring only in goldfish (Fig.4.10). This likely facilitated urea diffusion across the basolateral membranes of the gill cells, accelerating J_{urea} in goldfish (Fig.4.3). UT expression also

increased in the gills of zebrafish during HEA exposure, together with a transient rise in J_{urea} (Braun et al., 2009b). Contrary to our data in trout, Wood and Nawata (2011) reported an increased mRNA expression of UT in the gills of this species during HEA, but again there was no significant increase in urea-N excretion. The reason for this discrepancy is unknown, but it may relate to water chemistry or strain differences.

4.7. CONCLUSIONS

The results of the present study suggest that exposure to same level of HEA (1mM) induced differential physiological and molecular responses among the three freshwater teleosts. In goldfish and carp J_{amm} was elevated significantly during HEA exposure while in trout J_{amm} could only be restored to basal level. Na^+ uptake (J^{Na}_{in}) increased in carp and goldfish coincident with elevated J_{amm}, but not in trout. Carp and goldfish were able to maintain Na^+ homeostasis during HEA as they exhibited persistent, simultaneous augmentations in both J^{Na}_{in} and J^{Na}_{out}. At the molecular level, there was evidence for activation of a "Na^+/NH_4^+ exchange metabolon" in all three species, likely in response to elevations in plasma cortisol and T_{amm}. Goldfish appear to rely on H^+-ATPase and trout on H^+-ATPase as well as on NHE-2, likely for boundary layer acidification and ammonia trapping. In all three species, up-regulation of Rhcg (Rhcg-a and Rhcg-b in goldfish, Rhcg-a in carp, and Rhcg2 in trout) may play a key role in ammonia transport and Na^+ uptake during HEA exposure. Moreover, Rhbg was upregulated only in goldfish highlighting their additional ability to deal with ammonia challenge. The activity and expression of the basolateral enzyme, NKA was increased in goldfish and carp which might offer another mechanism for driving higher rates of Na^+ uptake in these species. In future studies it will be of interest to see if apical NHE transporters are also upregulated in these two cyprinids as a mechanism to increase both Na^+ uptake and ammonia excretion during HEA exposure. However none of the three species appeared to use NKA as an NH_4^+ transporter. Surprisingly in all the species, J_{amm} and J^{Na}_{in} were restored or increased before elevations in mRNA levels of Rh glycoproteins and transporters/exchangers were seen, suggesting non-genomic activation and/or post-translational regulation of existing transport protein function. Furthermore, during HEA exposure the transcript level of UT in the gills were markedly elevated only in goldfish, together with a rise in urea-N excretion rate. Strategies to cope with HEA are diverse among freshwater teleosts but goldfish have more effective ways to deal with ammonia exposure than do carp, and trout appear to be least effective. In the present study, most gill transporters were investigated only at the mRNA level. Changes in gene expression do not always translate into comparable changes in protein function, although there was a fairly good correlation in the present ATPase results. In future studies, investigation of the HEA-induced responses of gill transporters at the translational level, particularly those of the Rh glycoproteins, will be crucial.

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Part II

Comparative detoxification and defense against ammonia toxicity

Chapter 5

Regulation of amino acid metabolism as a defensive strategy in the brain of three freshwater teleosts in response to high environmental ammonia exposure

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Abstract

Many teleosts have evolved mechanisms to cope with ammonia toxicity in the brain when confronted with high environmental ammonia (HEA). In the present study, the possible role of conversion of accumulated ammonia to glutamine and other free amino acids in the brain of three freshwater teleosts differing in their sensitivities to ammonia was investigated. The detoxification mode of ammonia in brain is suggested to be through amination of glutamate to glutamine by the coupled activities of glutamate dehydrogenase (GDH), transaminase (aspartate aminotransaminase 'AST' and alanine aminotransaminase 'ALT') and glutamine synthetase (GSase). We investigated the metabolic response of amino acids in the brain of highly sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less sensitive cyprinid *Cyprinus carpio* (common carp) and the highly resistant cyprinid *Carassius auratus* (goldfish) when exposed to 1 mM ammonia (as NH_4HCO_3; pH 7.9) for 0 h (control), 3 h, 12 h, 24 h, 48 h, 84 h and 180 h. Results show that HEA exposure increased ammonia accumulation significantly in the brain of all the three species from 12 h onwards. Unlike in trout, ammonia accumulation in carp and goldfish was restored to control levels (48 h-84 h); which was accompanied with a significant increase in glutamine content as well as GSase activity. In trout, glutamine levels also increased (84 h-180 h) but GSase was not activated. The elevated glutamine level in trout was accompanied by a significant depletion of the glutamate pool in contrast to the stable glutamate levels seen in carp and goldfish. This suggests a simultaneous increase in the rate of glutamate formation to match with the demand of glutamine formation in cyprinids. The activity of GDH was elevated significantly in carp and goldfish but remained unaltered in trout. Also, the transaminase enzymes (AST and ALT) were elevated significantly in exposed carp and goldfish while only ALT was up-regulated in trout. Consequently, in carp and goldfish both aspartate and alanine were utilized under HEA, whereas only alanine was consumed in trout. With ammonia treatment, significant changes in concentrations of other amino acids also occurred. None of the species could detoxify brain ammonia into urea. This study suggests that protective strategies to combat ammonia toxicity in brain are more pronounced in carp and goldfish than in trout.

Keywords: High environmental ammonia (HEA), Glutamine, Free amino acids, Glutamine synthetase (GSase), Glutamate dehydrogenase (GDH), Rainbow trout, Common carp, Goldfish.

5.1. INTRODUCTION

In teleosts, ammonia is produced as the major end product of amino acid catabolism and is excreted across the gills in the surrounded environment providing there is a concentration gradient. Moreover, ammonia also enters the water bodies arising from sources such as sewage effluents, industrial wastes, agricultural run-off and decomposition of biological wastes (Randall and Tsui, 2002). As exogenous ammonia builds up in the environment and reaches the levels that reverse the normal NH_3 partial pressure gradient, ammonia excretion in fish is hindered and/or net uptake of ammonia from the environment occurs. Hence, at high environmental ammonia (HEA), fish are confronted simultaneously with accumulation of endogenous ammonia and uptake of exogenous ammonia, resulting in both chronic and acute toxic affects (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002). Some noted effects include decrease in growth rate (Dosdat et al., 2003; Foss et al., 2004; Lemarie et al., 2004; Pinto et al., 2007), alteration in energy metabolism (Arillo et al., 1981b), disruption of ionic balance (Soderberg and Meade, 1992; Wilkie, 1997), increased vulnerability to disease, and pathological changes in gill structure (Wilkie,1997). At the ecological level, high ammonia load in the water bodies or in aquaculture system can contribute to the eutrophication, acidification of the soil and fertilization of the available vegetation. Therefore, in long term, it can be speculated that ammonia pollution may hamper the stability of the aquatic ecosystem.

Ammonia is produced and also accumulated within the cell; therefore, fish have evolved a number of different strategies to defend against ammonia toxicity at the cellular and the subcellular level (Ip et al., 2001a, b, 2004a, 2005). The conversion of accumulated ammonia to various free amino acids (FAAs) in the brain tissue is one of these stratagems. It supports the suppression of ammonia production rate in fish by diminishing the rates of FAAs catabolism (Ip et al., 2001a, Lim et al., 2001; Tsui et al., 2004). As such it changes the FAA metabolic equilibrium towards their synthesis, leading to FAAs accumulation under HEA as previously shown in numerous teleosts (Anderson et al., 2002; Chew et al., 2001; Dabrowska and Wlasow, 1986; Ip et al., 2001b; Iwata, 1988; Iwata et al., 1981; Peng et al., 1998; Tsui et al., 2002; Wilson et al., 1998). In addition, some teleosts have the capacity to detoxify ammonia to the less toxic urea possibly through the ornithine urea cycle (OUC). Ureogenesis in fish is energetically expensive, therefore, majority of teleosts do not rely on ureogenesis as a major strategy to detoxify exogenous and endogenous ammonia when confronted with HEA. Moreover, several recent studies have also confirmed that OUC is not functional among most of the freshwater teleosts (Anderson, 2001; Ip et al., 2004c; Sanderson et al., 2010), although there are exceptions (Wood et al., 1989b).

Additionally, glutamine formation is an important ammonia detoxification strategy reported in the brain of many freshwater teleosts (Hernández et al., 1999; Ip et al., 2001a; Mommsen and Walsh, 1992; Saha et al., 2002; Wee et al., 2007; Wicks and Randall, 2002a). Glutamine is formed from

glutamate and NH_4^+, the reaction is catalyzed by the enzyme glutamine synthetase (GSase). Glutamate is produced from α-ketoglutarate (α-KG) and NH_4^+ by glutamate dehydrogenase (GDH) or from α-KG and other amino acids catalyzed by various transaminases. Therefore, starting with α-KG, formation of 1 mole of glutamine will detoxify 2 moles of NH_4^+. Upon exposure to ammonia, cerebral glutamine levels have been reported to increase many fold in Lake Magadi tilapia, *Oreochromis grahamii* (Mommsen and Walsh, 1992), goldfish, *Carassius auratus* (Levi et al., 1974), mudskippers, *Periophthalmus schlosseri* and *Boleophthalmus boddaerti* (Ip et al., 2001a; 2005; Peng et al., 1998), common carp, *Cyprinus carpio* (Dabrowska and Wlasow, 1986), Nile tilapia, *Oreochromis niloticus* (Hegazi et al., 2010) and rainbow trout, *Oncorhynchus mykiss* (Sanderson et al., 2010; Wicks and Randall, 2002a). The brain is the organ most sensitive to the ammonia toxicity, virtually all fish brain possess higher activity of GSase (Cooper and Plum, 1987; Wang and Walsh, 2000; Wright et al., 2007) which is thought to provide protection against ammonia toxicity and also maintain homeostasis of the excitatory neurotransmitter glutamate (Sanderson et al., 2010; Suárez et al., 2002). Glutamine oxoglutarate aminotransferase (GOGAT) generates glutamate from glutamine, and thus along with GSase plays a central role in the regulation of nitrogen assimilation.

Moreover, the levels of glutamine and other amino acids in brain are also regulated by two key transaminase enzymes, aspartate aminotransaminase (AST) and alanine aminotransaminase (ALT). AST catalyzes the reversible transfer of an α-amino group between aspartate and glutamate while ALT catalyzes the reversible transamination reaction which transfers the amino group of alanine to α-KG, resulting in the formation of pyruvate and glutamate. Increased total activity of AST and ALT has been demonstrated in the brain of the fish species exposed to HEA (Hernández et al., 1999; Jeney et al., 1992; Mommsen and Walsh, 1992; Wee et al., 2007; Wicks and Randall, 2002a).

Nevertheless, the ability to detoxify ammonia by above mentioned cellular and subcellular mechanisms varies considerably between species (Iwata, 1988; Vedel et al., 1998). In spite of the recent advances in research on the strategies used by fish species to cope with a high ammonia load, there has been limited knowledge with regard to freshwater teleosts which have different tolerance limits to ammonia toxicity. Understanding these differences might help to identify underlying mechanisms involved in ammonia sensitivity. Therefore, the focus of the present comparative study was to elucidate the compensatory responses mediated by amino acid metabolism in three commercially important freshwater fish: a sensitive salmonid, the rainbow trout *Oncorhynchus mykiss*, a less sensitive cyprinid, the common carp, *Cyprinus carpio*, and the very resistant cyprinid, goldfish, *Carassius auratus* when exposed acutely (3 h) and chronically (up to 180 h) to high environmental ammonia (1 mM; pH 7.9).

In brief, the purpose of this study was 3-fold. The first goal was to investigate whether three fish species under ammonia threat could detoxify ammonia into glutamine by measuring glutamine

content in the brain and the changes in the activities of key enzymes associated with the metabolic pathway of glutamine synthesis. The second was to determine the content of various other FAA in the brain. The last was to examine if these species could convert ammonia into less toxic urea form as an alternative detoxification strategy. These findings will help us to assess if differences in physiological defence mechanisms mainly related to amino acid metabolism in brain of these fish contribute to the differences in the sensitivity to HEA.

5.2. MATERIALS AND METHODS

5.2.1. Experimental system and animals

Rainbow trout, *Oncorhynchus mykiss*, were obtained from a fish farm - Pisciculture Collette, Bonlez, Belgium; goldfish, *Carassius auratus*, were obtained from Aqua Hobby, Heist op den Berg, Belgium; common carp, *Cyprinus carpio,* were obtained from the fish hatchery at Wageningen University, The Netherlands. Fish were kept at the University of Antwerp in aquaria (200 L) for at least a month before the exposure started. A total of 96 goldfish, 96 common carp and 96 rainbow trout were distributed species wise into four 200 L tanks (n=24 per tank). Each of these tanks was equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17±1°C and photoperiod was 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Water parameters were: pH 7.4 ± 0.2, dissolved oxygen 6.9-7.4 mg/L, total NH_3 0.006-0.009 mM, nitrite 0.0015- 0.0021 mM, nitrate 0.015-0.042 mM, Ca^{2+} 0.8–1.0 mM, Mg^{2+} 0.19-0.21 mM, Na^{+} 1.2-1.4 mM, K^{+} 0.09-0.10 mM, Cl^{-} 0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 226 mg $CaCO_3$/L. Average mass (mean ± standard deviation) of rainbow trout was 15 ± 2 g (4–4.5 months old), of common carp 18 ± 3 g (6–6.5 months old), and of goldfish 17 ± 3 g (6–7 months old). Fish were acclimated for 2 weeks prior to the experiment and were fed ad libitum once a day with either commercial pellets ('Hikari Staple', Kyorin Food Ind. Ltd., Japan) for common carp and goldfish, or 'Trouvit' (Trouw Nutrition, Fontaine-les-Vervins, France) for rainbow trout. Feeding was suspended 2 days before experimentation.

5.2.2. Exposure and sampling intervals

The experimental set up consists of exposing the goldfish, carp and trout to 1mM ammonia for a period of 3 h, 12 h, 24 h, 48 h, 84 h and 180 h. The exposure was conducted in 8 L glass aquaria (water volume set to 6 L). Control groups (no HEA) were setup in parallel to 12 h, 48 h, 84 h and 180 h exposure groups. The experimental aquaria were shielded with black plastic to minimize visual disturbance and fitted with individual air-stones.

Fish (n=2) were placed in an individual glass aquaria the evening before an experiment and left overnight to settle with continuous aeration. The experimental protocols consisted of exposing 8 fish (in 4 aquaria) per experiment to HEA. Each exposure aquaria was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 1.08 ± 0.06 mM of ammonia was maintained throughout the experiment. Exposure ammonia concentrations were measured (using the salicylate-hypochlorite method, Verdouw et al., 1978) 6 h after the onset of treatment and the concentration of ammonia in the tanks was maintained by adding calculated amount of the NH_4HCO_3 solution. Moreover, to avoid the microbial breakdown of test chemical and build-up of other waste products, 60–80% of the water was discarded after each 2 days and replaced with fresh water containing the respective amount of ammonia. Water pH was maintained at 7.8-8.0 throughout the experimental period using dilute HCl and/or KOH.

5.2.3. Sampling procedure and sample analysis

For sampling, fish were removed from aquaria (n=8 from each treatment and control group), anesthetized using an overdose of neutralized MS222 (ethyl 3-aminobenzoate methane-sulfonic acid, 1 g/L, Acros Organics, Geel, Belgium). Fish were dissected on ice; brain was removed and divided (sagittally) into two parts, frozen in liquid nitrogen, and stored at -80°C for analysis of FAAs, ammonia and urea content and for enzymatic assays.

5.2.3.1. Determination of ammonia and free amino acids (FAAs)

One part of the frozen brain sample was weighed and ground in liquid nitrogen. Five volumes (w/v) of HPLC-grade water were added and the mixture was homogenised using a Teflon-coated mechanical homogenizer (Ultra-Turrax T- 25 basic, IKA®-Werke). The samples were centrifuged for 30 min at 14000 rpm at 4°C .The resultant supernatant was carefully collected and aliquoted for the determination of ammonia, urea and FAA content.

For the analysis of FAAs, a fixed volume of aliquot was diluted with the internal standard deuterium labelled *L*-Glutamine-2,3,3,4,4-d5 (C/D/N Isotopes INC, Pointe-Claire, Quebec at a concentration: 94.5 μmol) in a 1:1 ratio. The internal standard was used to compensate for matrix effects and to increase the accuracy of quantitation as well as to correct for different mass spectrometry responses. The diluted aliquots were centrifuged at 14000 rpm for 30 min at 4°C. The supernatant was collected and was passed through two Millipore micro filters (0.2µM pore size) before assaying FAAs level, such as glycine, alanine, serine, proline, ornithine, asparatate, glutamine, glutamate, histidine, arginine. FAA content was determined by using a Waters Acquity UPLC-tqd system (Milford, Massachusetts) equipped with a BEH amide 2.1 X 50 column.

Ammonia content was determined according to Wright et al. (1995a) using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Urea concentration was analyzed by the diacetyl monoxime assay (Rahmatullah and Boyde, 1980).

5.2.3.2. Enzyme assay

5.2.3.2.1. Extraction procedure and analysis

For the enzymatic assays, the remaining frozen brain samples were homogenized in 100 mM phosphate buffer (pH 7.9) supplemented with 1% (w/v) polyvinylpolypyrrolidone, 1 mM EDTA, 15% glycerol, 2 mM phenylmethanesulfonylfluoride and 10 mM 2-mercaptoethanol. All steps for the preparation of tissue extract were carried out at 4°C. The homogenates were then centrifuged at 14000 rpm for 30 min at 4°C. The resultant supernatant was carefully collected for the assay.

Glutamine synthetase (GSase) activity was determined according to the method of Temple et al. (1996). It was measured in a reaction mixture of 200 mM Tris-acetate (pH 6.4) supplemented with 35 mM L-glutamine, 8.75 mM hydroxylamine, 0.75 mM ADP, 2.25 mM $MnCl_2$, 17.5 mM sodium arsenate, and 1 mM EDTA. The mixture was incubated at 37°C for 60 min followed by the addition of $FeCl_3$ reagent (10g trichloro-acetic acid and 8g ferric chloride in 250 mL 0.5 N hydrochloric acid). γ-glutamyl-hydroxamate produced was determined at 500 nm. One unit of GSase activity is defined as 1 pmol of γ -glutamyl hydroxamate formed per minute.

Glutamate dehydrogenase (GDH) activity was assayed by following the oxidation of NADH at 340 nm at 30°C (Groat and Vance 1981). The reaction mixture contained 100 mM phosphate buffer (pH 7.6), 2.5 mM 2-oxoglutarate, 0.1 mM NADH, 200 mM ammonium chloride, 1mM EDTA. One unit of GDH activity is defined as oxidation of 1 µmol NADH per minute.

Glutamine oxoglutarate aminotransferase (GOGAT) activity was assayed following the methods of Groat and Vance (1981) and Singh and Srivastava (1986) in 100 mM phosphate buffer (pH 7.6) consisted of 1 mM EDTA, 1 mM 2-oxoglutarate, 1.0 mM aminooxyacetate, 0.1 mM NADH, 10.0 mM L-glutamine, and 0.1% (v/v) 2-mercaptoethanol. The activity was monitored at 340 nm. One activity unit of GOGAT is defined as oxidation of 1 µmol NADH per minute.

Alanine aminotransaminase (ALT) activity in the direction of alanine degradation was assayed in homogenates as described by Wootton (1964) with micro-modifications for use in a 96-well microplate. The substrate comprised of 0.2 M DL-alanine and 2 mM α-KG in 0.05 M phosphate buffer (pH 7.4). In the experimental and control tubes, 0.5 ml of substrate was added. The reaction was started by adding 0.1 ml of tissue homogenate. The assay mixture was incubated at 37°C for 60 min. The reaction was terminated by adding 0.5 ml of 1 mM 2,4 dinitrophenyl hydrazine (DNPH). In the control tubes, the enzyme source was added after DNPH solution. The tubes were held at room

temperature for 20 min with occasional shaking. Then 5 ml of 0.4 N NaOH solution was added and the contents were thoroughly mixed. After 10 min, the OD was recorded at 540 nm against a blank.

Aspartate aminotransaminase (AST) activity in the direction of aspartate degradation was assayed by same procedure as for ALT activity except the substrate comprised of 0.2 M DL aspartic acid instead of alanine.

All the enzyme activities were expressed per mg of soluble protein content. The protein content of the homogenate was determined by Lowry et al. (1951).

5.2.4. STATISTICAL ANALYSIS

All data have been presented as mean values ± standard error (S.E.). For comparisons between different experimental groups a one-way analysis of variance (ANOVA) was performed followed by Duncan's multiple range test (Duncan, 1955). Student's two-tailed t-test was used for single comparisons. The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 16.0. A probability level of 0.05 was used for rejection of the null hypothesis.

Within species, no significant differences were found between any of the control values at different sampling times. Therefore, pooled controls for each experimental group are shown for clarity of the figure.

5.3. RESULTS

5.3.1. Ammonia and urea level

Exposure to 1 mM HEA resulted in a net influx of ammonia from the medium into the tissue which was evident from a dramatic increase in ammonia concentration in the brain tissue of all three species studied (Fig.5.1). The level of ammonia increased significantly in all three species after 12 h exposure which persisted until the end of the exposure period (180 h) in trout. Ammonia content in carp remained considerably higher ($P < 0.05$) till 48 h and thereafter returned to a level which was not significantly different from the control. Goldfish followed the same pattern as carp, but the ammonia accumulation reduced to control level a bit sooner (at 48 h) than in carp.

Despite the remarkable increase in ammonia level during HEA exposure, little change ($P > 0.05$) in the urea concentration in brain tissue was observed for both salmonid and cyprinids (Fig.5.2).

5.3.2. Free amino acids

On exposure to HEA, the level of glutamine was enhanced in all the three species (Fig.5.3). The most prominent effect was noticed in goldfish; the level increased with the exposure periods and was consistently higher ($P < 0.05$) relative to the control from 24 h onwards. Such an increment was delayed in carp and trout and occurred after 48 h and 84 h respectively. The relative augmentations (*P*

< 0.05) in carp after 48 h, 84 h and 180 h exposure were 60%, 72% and 68% higher than the control respectively. In trout, the level increased by 62% ($P < 0.01$) and 72% ($P < 0.01$) correspondingly after 84 h and 180 h exposure.

Glutamate level in trout reduced gradually with exposure time especially ($P < 0.05$) during the last two exposure time points (Fig.5.4). The concentration lowered by 35% ($P < 0.05$) and 41% ($P < 0.01$) respectively after 84 h and 180 h of HEA exposure. In contrast, carp and goldfish exhibited no significant change in glutamate content when confronted with HEA.

Exposure to 1 mM ammonia tended to reduce the aspartate level in both carp and goldfish brain with significant effects from 48 h exposure onwards (Fig.5.5). The reductions in carp after 48 h, 84 h and 180 h were 42% ($P < 0.05$), 40% ($P < 0.05$) and 43% ($P < 0.05$) while goldfish displayed a corresponding reduction of 45% ($P < 0.05$), 52% ($P < 0.01$) and 50% ($P < 0.05$). Though the aspartate level in trout also reduced in response to HEA but remained statistically insignificant compared to the control.

Ammonia exposure induced a declining pattern in alanine content in all the studied fish species (Fig.5.6). In trout we observed a reduction of 42% ($P < 0.05$) and 44% ($P < 0.05$) relative to control after 84 h and 180 h exposure respectively. Among cyprinids, the effect occurred sooner and the reduction became significant ($P < 0.05$) from 24 h and 48 h onwards for carp and the goldfish respectively. The changes in serine content (Table 1) in each species followed almost the same pattern as seen for alanine.

On the other hand, upon exposure to HEA proline levels rose substantially in all three fish (Table 1), an effect that was significant from 48 h onwards and the elevations remained significant ($P < 0.05$) till the end of exposure period.

Exposure to HEA had no effect ($P > 0.05$) on arginine content in cyprinids (Table 5.1). In contrast, the level raised drastically in trout, compared to control, when exposed for 48 h (82% increment, $P < 0.01$), 84 h (75% increment, $P < 0.05$) and 180 h (72% increment, $P < 0.05$).

Ornithine content was not altered by HEA exposure in carp while a temporary reduction ($P < 0.05$) was seen in trout during the 24 h only, with recovery thereafter (Table 5.1). In goldfish, levels were significantly lower than the corresponding values of the control after 24 h and 48 h. Analogous to trout, the decline in goldfish was followed by a subsequent recovery to a value not significantly different from the control group.

No significant alteration in brain glycine and histidine levels was observed throughout the 180 h exposure period in all the three experimental fish species (Table 5.1).

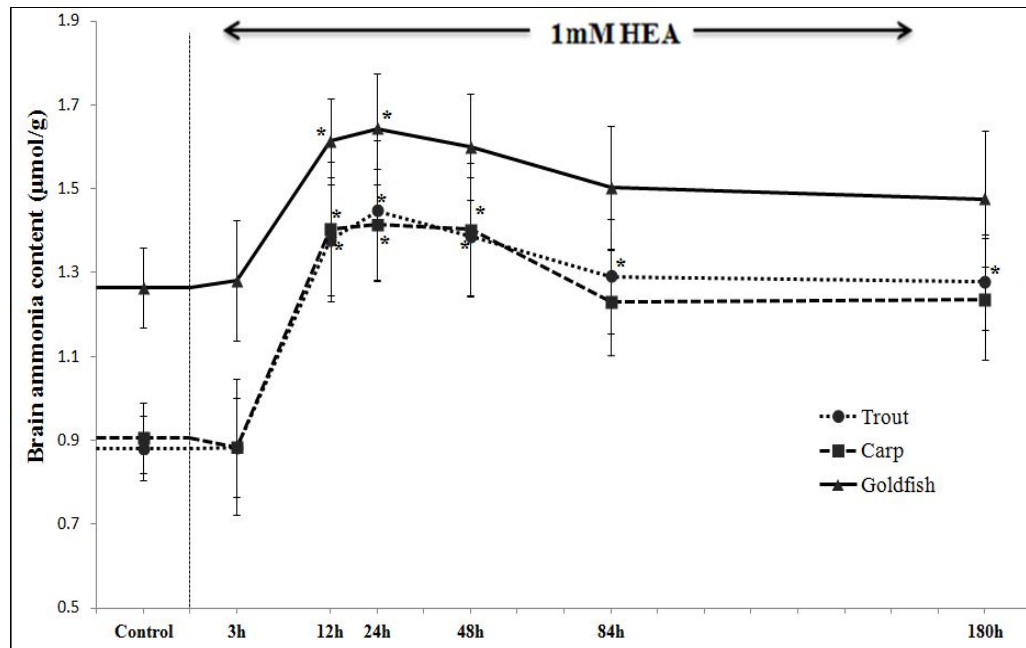

Figure. 5.1. Ammonia accumulation (µmol/g) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$).

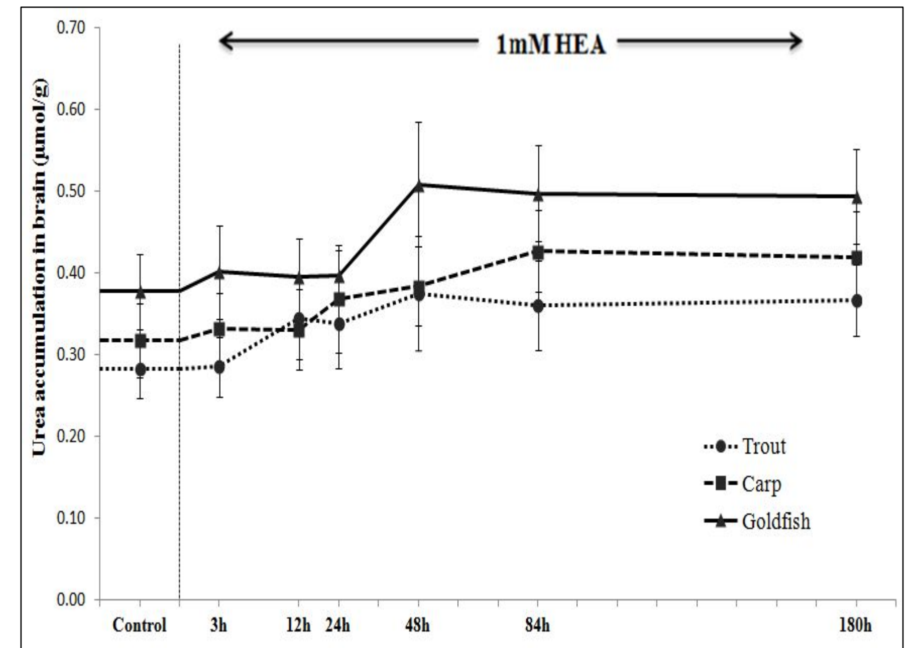

Figure. 5.2. Accumulation (µmol/g) of urea in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E.

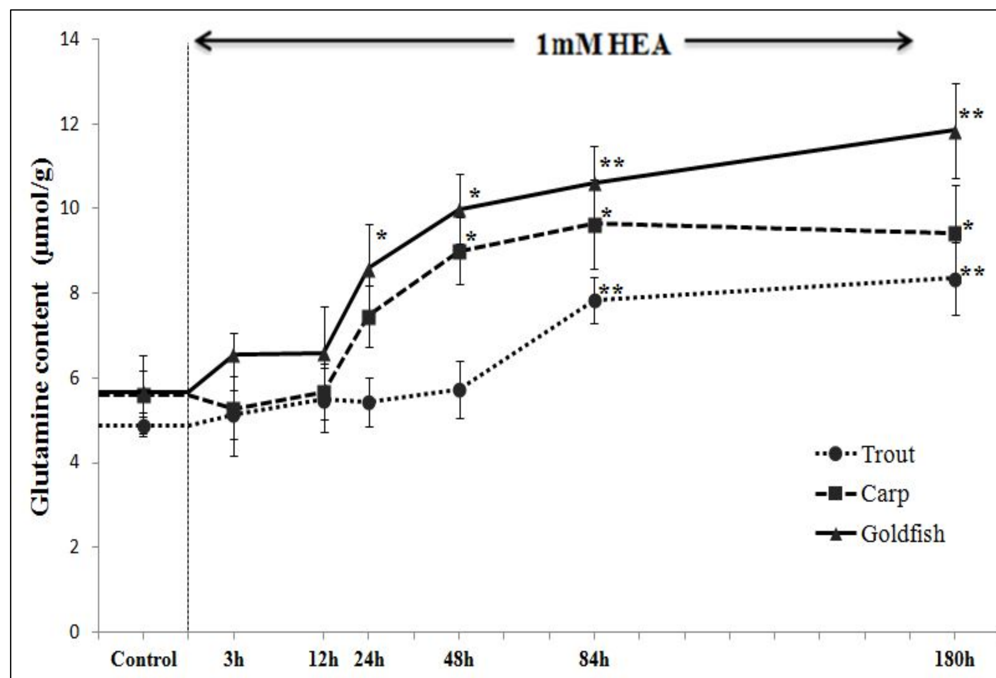

Figure. 5.3. Glutamine content (μmol/g) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

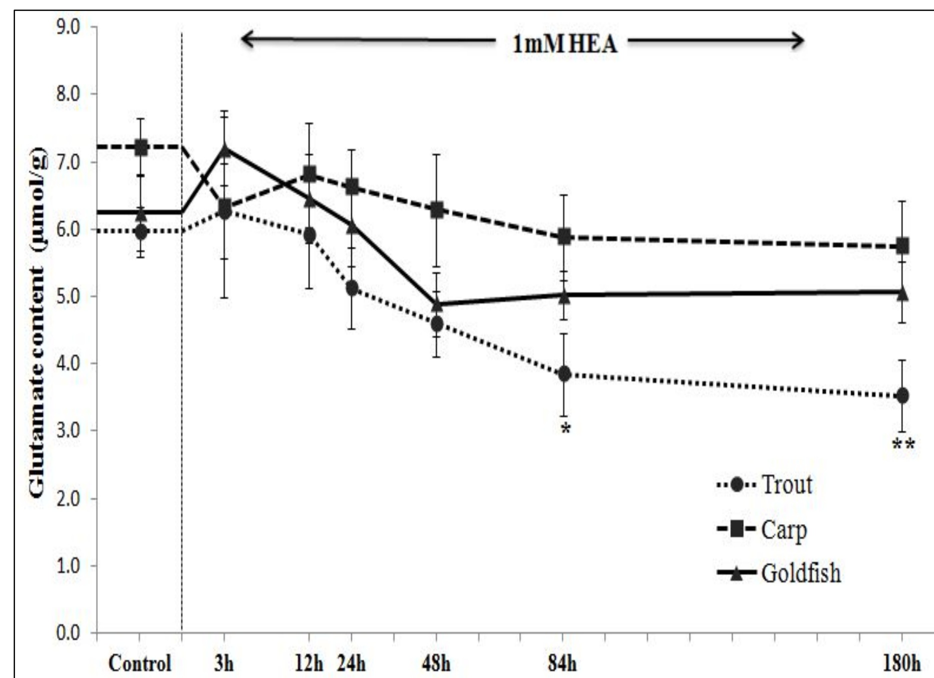

Figure. 5.4. Glutamate content (μmol/g) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

5.3.3. Enzyme activity

5.3.3.1. GSase, GDH and GOGAT

GSase activity in all the three fish species showed a tendency to increase in response to HEA (Fig.5.7), but the increments in trout were not as pronounced as seen for the goldfish and the carp. GSase activity in goldfish brain augmented by 61% ($P < 0.05$), 63% ($P < 0.05$), 66% ($P < 0.05$) and 68% ($P < 0.01$) respectively after 24 h, 48 h, 84 h and 180 h of exposure compared to the control. The stimulation was postponed in carp and increment was prominent from 48 h onwards. The relative increase in carp brain was 73% ($P < 0.05$), 88% ($P < 0.05$) and 93% ($P < 0.01$) respectively after 48 h, 84 h and 180 h.

Surprisingly, for cyprinids the activity of GDH (Fig.5.8) demonstrated an almost similar trend as GSase activity. The relative elevations of GDH activity in carp brain at 48 h, 84 h and 180 h were 69% ($P < 0.05$), 67% ($P < 0.05$) and 80% ($P < 0.01$) to the control. A parallel increment of 62% ($P < 0.05$), 73% ($P < 0.05$) and 79% ($P < 0.05$) was recorded for goldfish. On the contrary, HEA did not appear to exert any remarkable effect ($P > 0.05$) on GDH activity in trout. In addition, the activity of GOGAT remained unchanged in both salmonid and cyprinids when subjected to HEA (Fig.5.9).

5.3.3.2. Transaminase enzymes

Ammonia exposure induced differential changes in AST activity among cyprinids and trout (Fig.5.10). Our results showed that AST activity in goldfish and carp elevated ($P < 0.05$) from 24 h onwards. The relative increment in goldfish as compared to control was about 43%, 46%, 48% and 50% after 24 h, 48 h, 84 h and 180 h respectively, while in carp the average elevation was 40% compared to its control value. In contrast, AST activity did not increase considerably ($P > 0.05$) in trout on HEA exposure but the values (from 24 h) tended to be numerically higher than the control.

A remarkable effect of HEA on ALT activity was observed in all fish species (Fig.5.11). In both goldfish and the carp, the activity was augmented ($P < 0.05$) from 24 h onwards and remained significantly elevated till the end. However, such an increment was delayed in trout and became significant only after 48 h of exposure.

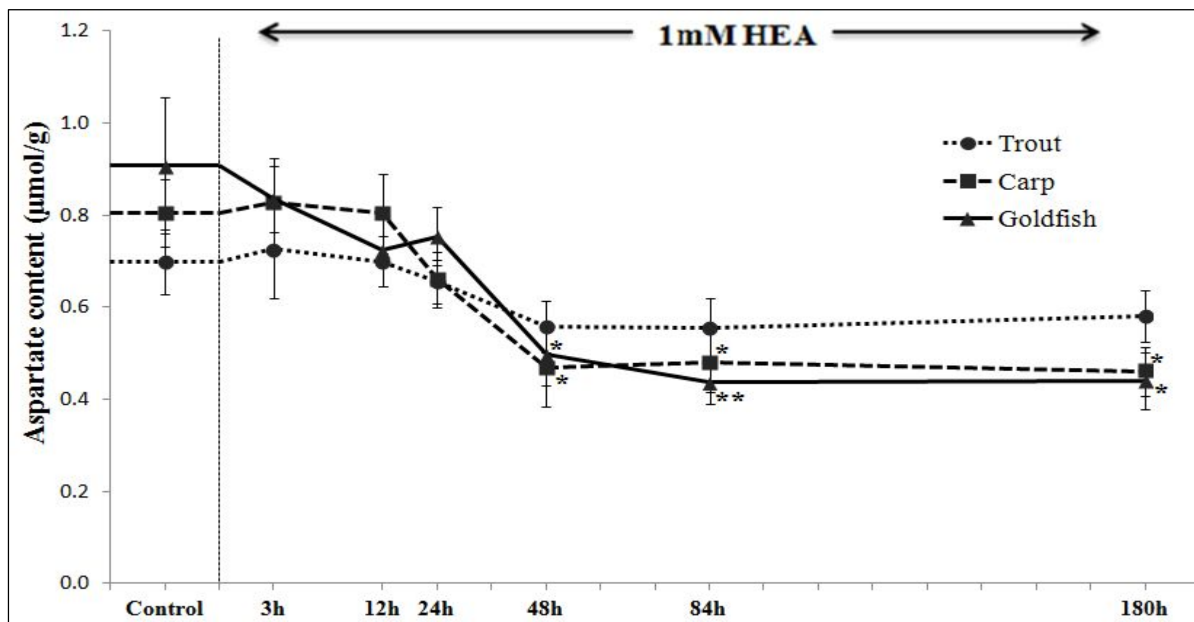

Figure. 5.5. Aspartate concentration (µmol/g) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

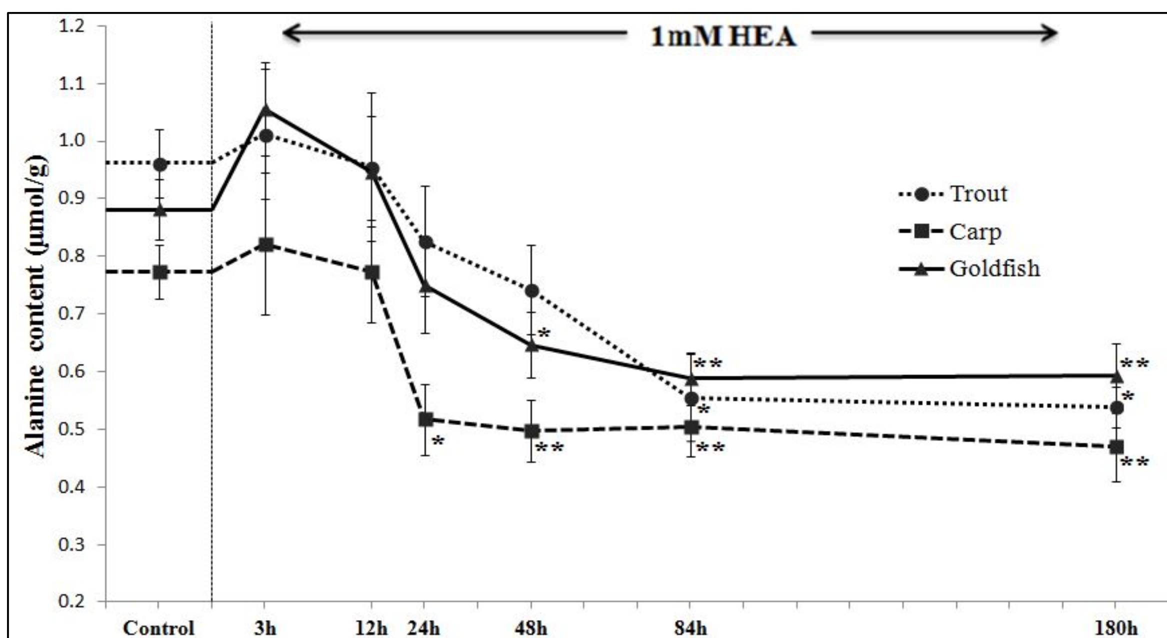

Figure. 5.6. Alanine concentration (µmol/g) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

5.4. DISCUSSION

5.4.1. Glutamine metabolic pathways

Ammonia exerts its toxic effects in the brain by disrupting cerebral blood flow (Andersson et al., 1981), causing astrocyte swelling (Gregorios et al., 1985), and by interfering with amino acid transport (Mans et al., 1983) as well as excitatory amino acid neurotransmitter metabolism (Hindfelt et al., 1977). Therefore, it is essential to have mechanisms to protect the brain against ammonia toxicity. The results of the present study demonstrate that exposure to 1 mM ammonia led to drastic increase in ammonia content in the brain of goldfish during 12-24 h exposure and up to 48 h in carp. Elevated ammonia levels in trout brain persisted even longer (till 180 h) and failed to re-establish to control levels as seen for cyprinids. The reduction in ammonia level among cyprinids was accompanied by an increase in glutamine content. This suggests that the strategies which detoxify ammonia to glutamine were initiated in goldfish and carp, and therefore, appear to remediate build up of ammonia in brain more efficiently than in trout. Glutamine formation in the brain has been proposed as one of the classic strategies for fish to deal with increasing concentrations of ammonia (Ip et al., 2004b,c; Korsgaard et al., 1995; Peng et al., 1998). In many teleosts, under hyperammonia conditions, the synthesis of glutamine is activated in the brain which is then released to the blood stream and may serve as a non-toxic carrier of ammonia from different tissues to the liver (Suárez et al., 2002). Trout exposed to HEA also had higher levels of glutamine, mostly evident during the end of the experimental periods, but these increments were not sufficient to reduce ammonia accumulation significantly. Also, the increase in brain glutamine was faster and more prolonged in cyprinids than in the trout. These elevations in glutamine could explain in part the restoration of ammonia to basal levels in cyprinids.

In general, glutamine is formed from glutamate and NH_4^+ catalysed by the enzyme glutamine synthetase (GSase). In the present study, a greater activity of GSase was evident in all the three fish species during HEA exposure. Carp and goldfish showed a significant elevations in GSase activity which were accompanied by a corresponding increase in glutamine synthesis. Moreover, a positive and significant correlation between GSase activity and glutamine accumulation was observed for carp ($Y = 6.22X + 5.17$, $R^2 = 0.172$, $P < 0.01$) and goldfish ($Y = 5.93X + 5.80$, $R^2 = 0.115$, $P < 0.05$). Increases in brain GSase activity and glutamine concentration have been reported for a number of fish species in response to ammonia exposure (Hegazi et al., 2010; Peng et al., 1998; Wicks and Randall, 2002a). Surprisingly, in trout the glutamine content was elevated considerably during HEA (80 h-184 h) despite the fact that GSase activities were not activated significantly, though an increasing trend was observed in response to HEA. It is possible that the higher resting brain GSase activity in trout provided more than enough glutamine synthetic capacity. It can also be speculated that although GSase appears to be the key enzyme that catalyses glutamine synthesis, it is not the only effective

mechanism (in trout) to regulate brain ammonia and glutamine concentrations. Likewise, Sanderson et al. (2010) also reported no change GSase activity in brain of rainbow trout when subjected to 1 mM ammonia while the glutamine level rose considerably, reinforcing an alternative detoxification pathway in trout brain. However, these results are contrasting with the findings of Wicks and Randall (2002a) who reported that activity of GSase in brain of fed rainbow trout was increased correspondingly to an increase in glutamine concentrations when fish were exposed to high ammonia level. Such discrepancy may be likely due to the feeding effect. In present study and the experiment conducted by Sanderson et al. (2010), fish were fasted for 48 h prior to commencement of the exposure while in experiment of Wicks and Randall (2002a) ammonia treatment was done just 1 h after feeding. Nevertheless, how the feeding and/or protein level in diet regulate the activity of GSase in fish remains unexplained.

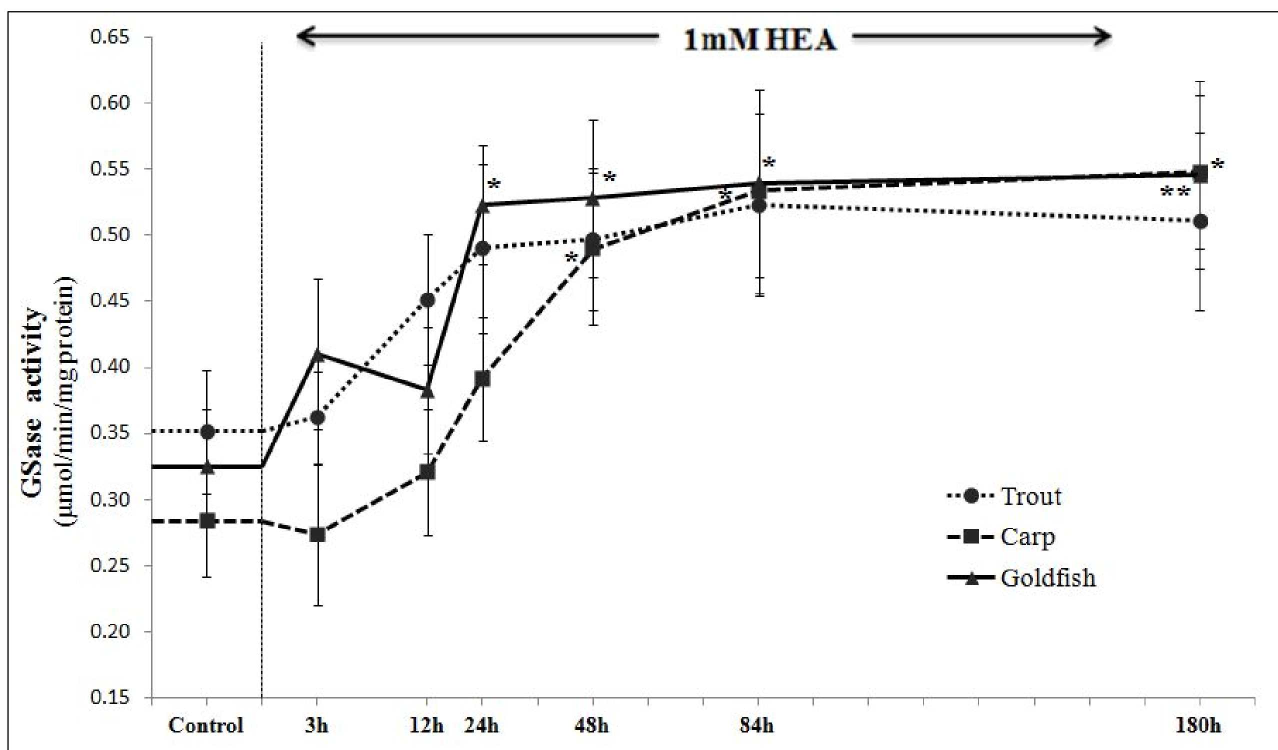

Figure.5.7. Glutamine synthetase (GSase) activity (µmol/min/mg protein) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

Table 5.1
Concentration (μmol/g) of free amino acids in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure.

	Control	3 h	12 h	24 h	48 h	84 h	180 h
Rainbow trout							
Arginine	0.045± 0.005	0.041± 0.004	0.050± 0.004	0.052 ± 0.007	0.083± 0.006**	0.079±0.011*	0.078 ± 0.006*
Glycine	0.687 ± 0.065	0.691± 0.081	0.686 ± 0.100	0.702 ± 0.099	0.741± 0.093	0.768± 0.056	0.817± 0.085
Histidine	0.275 ± 0.044	0.312 ± 0.034	0.283 ± 0.028	0.308 ± 0.044	0.365 ± 0.053	0.345 ± 0.018	0.379 ± 0.048
Ornithine	0.042 ± 0.003	0.043 ± 0.005	0.029 ± 0.003	0.024 ± 0.003*	0.033 ± 0.005	0.037 ± 0.003	0.038 ± 0.005
Proline	0.0048 ± 0.0003	0.0040 ± 0.0004	0.004 ± 0.0005	0.006 ± 0.0008	0.0086 ± 0.0007**	0.0104 ±0.0009***	0.0108 ±0.0007***
Serine	0.669 ± 0.031	0.673± 0.069	0.682± 0.092	0.590± 0.069	0.530 ± 0.056	0.397± 0.054*	0.392 ± 0.030*
Common carp							
Arginine	0.092 ± 0.011	0.096 ± 0.013	0.079 ± 0.012	0.091 ± 0.012	0.079± 0.012	0.088± 0.008	0.087 ± 0.007
Glycine	0.820 ± 0.071	0.885 ± 0.078	0.879 ± 0.107	0.868 ± 0.096	0.940 ± 0.110	0.960 ± 0.094	0.962 ± 0.121
Histidine	0.321 ± 0.051	0.302 ± 0.037	0.375 ± 0.039	0.386 ± 0.046	0.353 ± 0.057	0.375 ± 0.078	0.389 ± 0.077
Ornithine	0.051± 0.008	0.048 ± 0.007	0.047 ± 0.009	0.037 ± 0.008	0.035 ± 0.005	0.043 ± 0.006	0.042 ± 0.008
Proline	0.0068 ± 0.0007	0.0078 ± 0.0008	0.0082 ± 0.0006	0.008 ± 0.0011	0.0093 ± 0.0006*	0.0098 ± 0.0009*	0.0099 ± 0.0008*
Serine	0.511 ± 0.050	0.560 ± 0.068	0.515 ± 0.077	0.327± 0.053*	0.357 ± 0.047	0.315 ± 0.055*	0.285 ± 0.032**
Goldfish							
Arginine	0.065 ± 0.011	0.070 ± 0.013	0.057 ± 0.014	0.054 ± 0.009	0.048 ± 0.010	0.052 ± 0.009	0.060 ± 0.006
Glycine	0.598 ± 0.072	0.592 ± 0.131	0.662 ± 0.082	0.647 ± 0.092	0.743 ± 0.067	0.783 ± 0.080	0.799 ± 0.077
Histidine	0.299 ± 0.058	0.321 ± 0.057	0.289 ± 0.055	0.373 ± 0.095	0.369 ± 0.029	0.365 ± 0.068	0.383 ± 0.093
Ornithine	0.064 ± 0.007	0.053 ± 0.012	0.056 ± 0.008	0.038 ± 0.005*	0.037 ± 0.004**	0.045 ± 0.007	0.047 ± 0.008
Proline	0.008 ± 0.0009	0.0078 ± 0.0008	0.0082 ± 0.0012	0.009 ± 0.0010	0.0122 ± 0.0008*	0.0124 ±0.0012*	0.0127 ± 0.0010*
Serine	0.384 ± 0.043	0.367 ± 0.088	0.384 ± 0.069	0.286 ± 0.040	0.229 ± 0.036*	0.240 ± 0.028*	0.235 ± 0.038*

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$;***$P < 0.001$).

5.4.2. Glutamate pool and related enzymes

Glutamine synthesis in the brain can be followed by a decrease in glutamate levels (Hegazi et al., 2010) and can apparently vary among species. In many teleosts, the increase in brain glutamine is accompanied by a decrease in glutamate (Arillo et al., 1981b; Hegazi et al., 2010; Iwata, 1988; Veauvy et al., 2005; Vedel et al., 1998; Wicks and Randall, 2002a) as was also observed (84 h-180 h) for trout in the present study, implying that under HEA the supply of glutamate was not perfectly matched with the demand. Interestingly, the accumulation of glutamine was not associated by a concomitant depletion of glutamate in brains of both carp and goldfish throughout the 180 h exposure period, suggesting an efficient replenishment of glutamate used for glutamine synthesis, possibly through the amination of α-KG catalyzed by glutamate dehydrogenase (GDH). The activity of GDH was up-regulated in carp and goldfish and therefore subsequently used to maintain glutamate homeostasis whereas the absence of an induction of GDH in trout might explain the diminution of glutamate content. The accumulation of NADH and α-KG could also contribute to ammonia toxicity (Cooper and Plum, 1987; Ip et al., 2005), it is apparent from our study that carp and goldfish were adapted to overcome these problems by fueling them for glutamate formation. A significant increase of GDH activity in the largemouth bass (*Micropterus salmoides*) after exposing the fish to 0.25 mM NH_4Cl for 12 days was also reported (Kong et al., 1998). Likewise, Iwata et al. (1981) and Peng et al. (1998) also confirmed a significant augmentation of GDH activity during ammonia exposure in the mudskipper *Periophthalmus cantonensis* and *Periophthalmus schlosseri* respectively. The activity of glutamine oxoglutarate aminotransferase (GOGAT) remained unaltered in all the species signifying that glutamine was not deaminated to glutamate.

Glutamate is a neurotransmitter in the brain (Eckert et al., 1988) and is needed for other important biochemical reactions including the formation of GABA (gamma-aminobutyric acid), an inhibitory neurotransmitter, by the decarboxylation of glutamate in a reaction catalyzed by glutamate decarboxylase (Stryer, 1988). Since glutamate is excitatory and GABA is inhibitory, both neurotransmitters work together to control many processes. Additionally, glutamate is an essential component of the 'malate–aspartate shuttle', and the shuttle system might be inhibited by reduced glutamate levels, and consequently can affect the brain energy metabolism adversely (McKenna et al., 2006). Therefore, the decrease in brain glutamate as observed for trout in the present study might be a serious problem. We observed that carp and goldfish were able to maintain the glutamate pool during ammonia exposure despite the fact that the glutamine concentration rose to very high levels. This reflects their advanced ammonia detoxifying capacity over trout. Nevertheless, the limitations of this study need to be mentioned, as the concentration of GABA and kinetics of malate–aspartate shuttle were not measured.

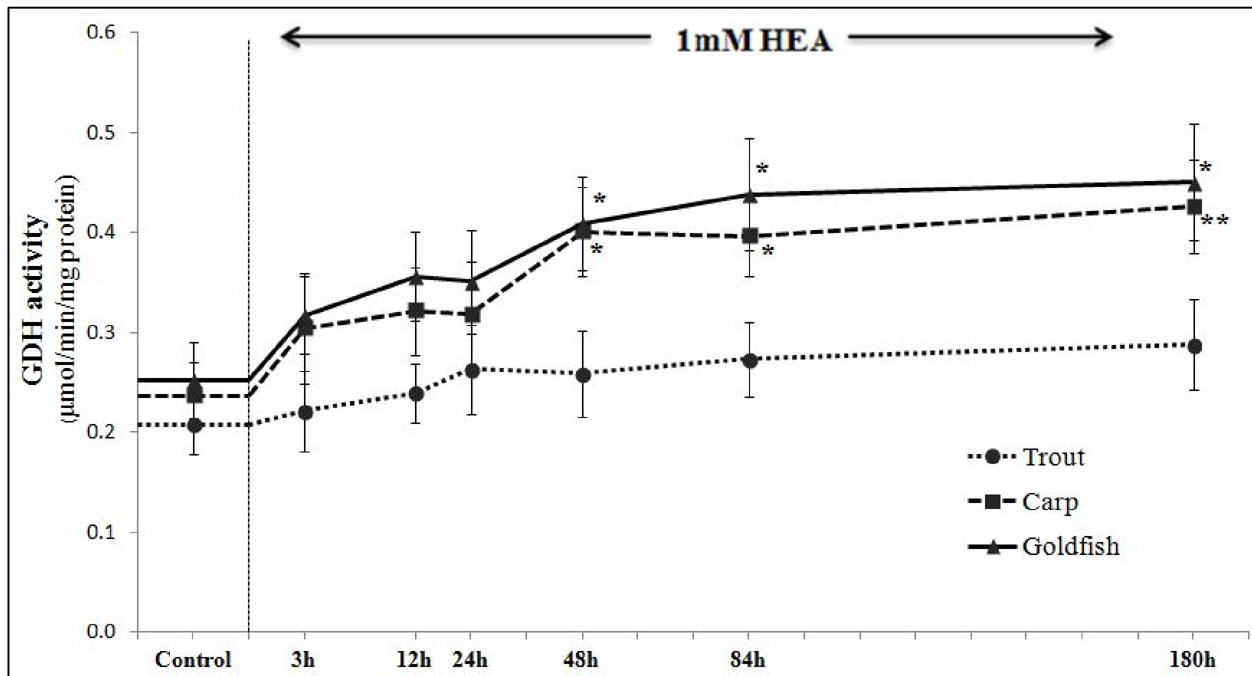

Figure. 5.8. Glutamate dehydrogenase (GDH) activity (µmol/min/mg protein) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

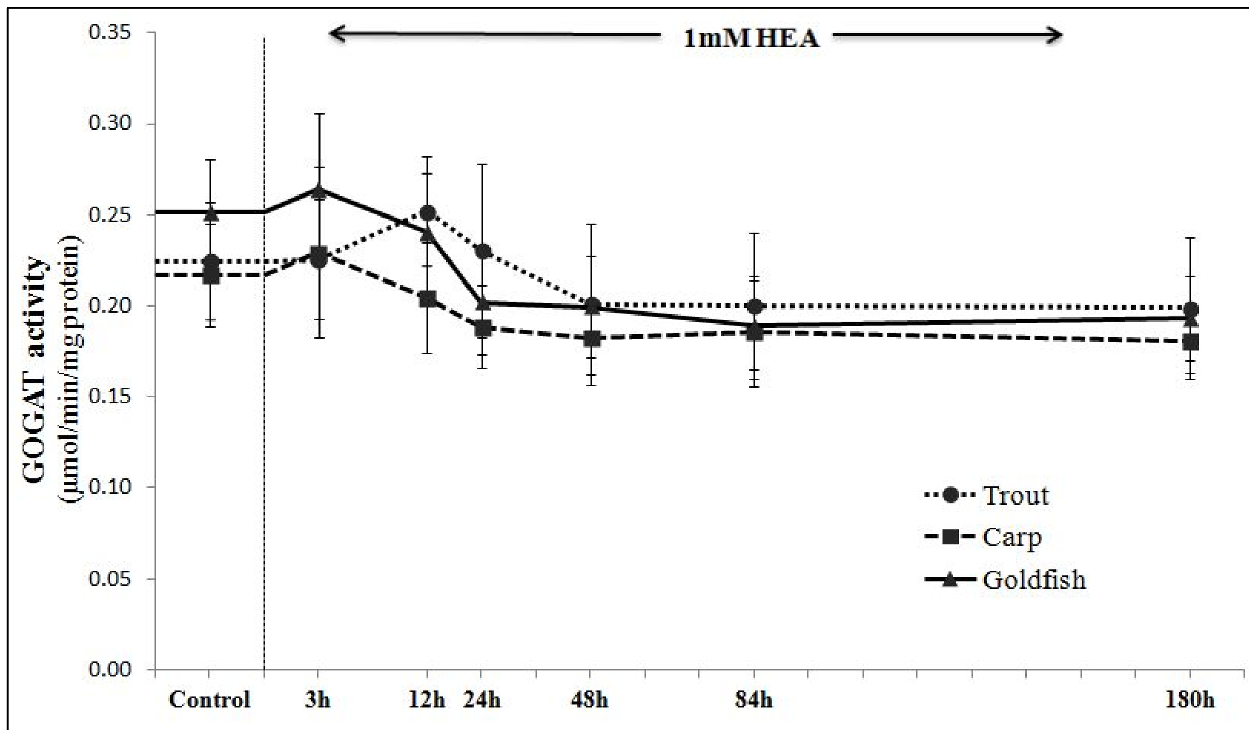

Figure. 5.9. Glutamine oxoglutarate aminotransferase (GOGAT) activity (µmol/min/mg protein) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E.

5.4.3. Transaminase enzymes and associated amino acids

A decrease in alanine concentration in response to ammonia exposure was observed in all the three fish species. Along with the GDH reaction, alanine also acts as a precursor for synthesis of glutamate via the transaminase reaction, and thus serves to maintain glutamate balance in the brain. The reaction is catalyzed by ALT, producing glutamate and pyruvate. We observed an increase in the activity of the ALT coinciding with a decline in the alanine level in the brain of all studied species. Nonetheless, the ALT mediated transaminase and GDH reactions deplete α-KG which is a tricarboxylic acid cycle (TCA) intermediate, and reduce the provision of ATP required for glutamine synthesis (via GSase). The exhaustion of α-KG must be addressed by delivering TCA cycle intermediates, such as acetyl-CoA which can be supplied via the transaminases that have pyruvate as a product. The amino acids alanine, glycine and serine, all have a common endpoint, the metabolic intermediate pyruvate. Therefore, the reduction in brain alanine and serine observed in present study might have been a consequence of acetyl-CoA biosynthesis and thus replenishment of α-KG. Elevated ammonia is also reported to significantly reduce brain alanine and serine level in rainbow trout (Sanderson et al., 2010). On the contrary, in Atlantic salmon, *Salmo salar* (Kolarevic et al., 2012), giant mudskipper (Ip et al., 2001a) and Nile tilapia (Hegazi et al., 2010) an increase in alanine brain concentration was observed during ammonia exposure. Perhaps, alanine production in these fish species could be a strategy to limit ATP expenditure by minimizing the usage of glutamate for energetically expensive glutamine synthesis (Ip et al., 2001a).

Furthermore, HEA had a profound effect on the aspartate level in cyprinids only; the content in brain was reduced considerably from 48 h onwards. Our result corroborates with earlier reports in Nile tilapia (Hegazi et al., 2010), giant mudskipper (*Periophthalmodon schlosseri*) (Peng et al., 1998), rainbow trout (Sanderson et al., 2010) and Atlantic salmon (Kolarevic et al., 2012) that showed lowering of aspartate level on ammonia exposure. Along with alanine, aspartate can also provide glutamate *via* transaminase reactions mediated by AST. Among cyprinids, the increment in AST activity was followed by the reduction in aspartate content. However, AST activity and aspartate level remained statistically unaltered in trout highlighting their incompetency to fuel aspartate as a substrate for the synthesis of glutamate. This might also explicate the depletion of glutamate content in exposed trout beside the limited induction of GDH activity.

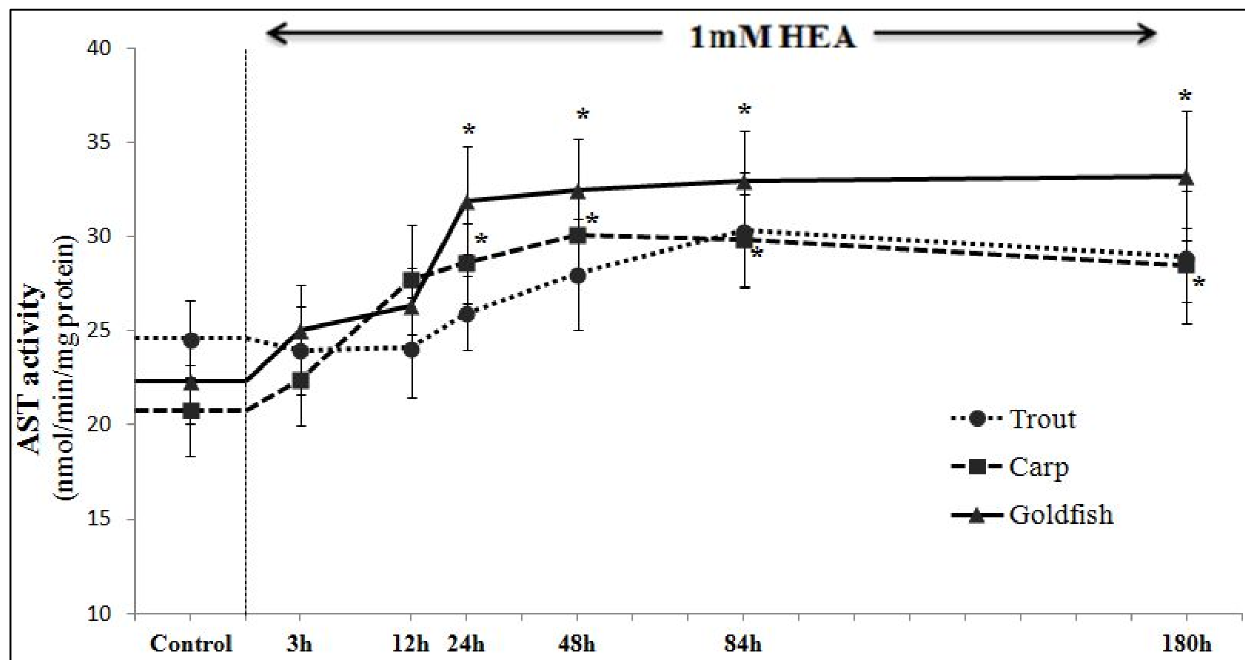

Figure.5.10. Aspartate aminotransaminase (AST) activity (nmol/min/mg protein) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$).

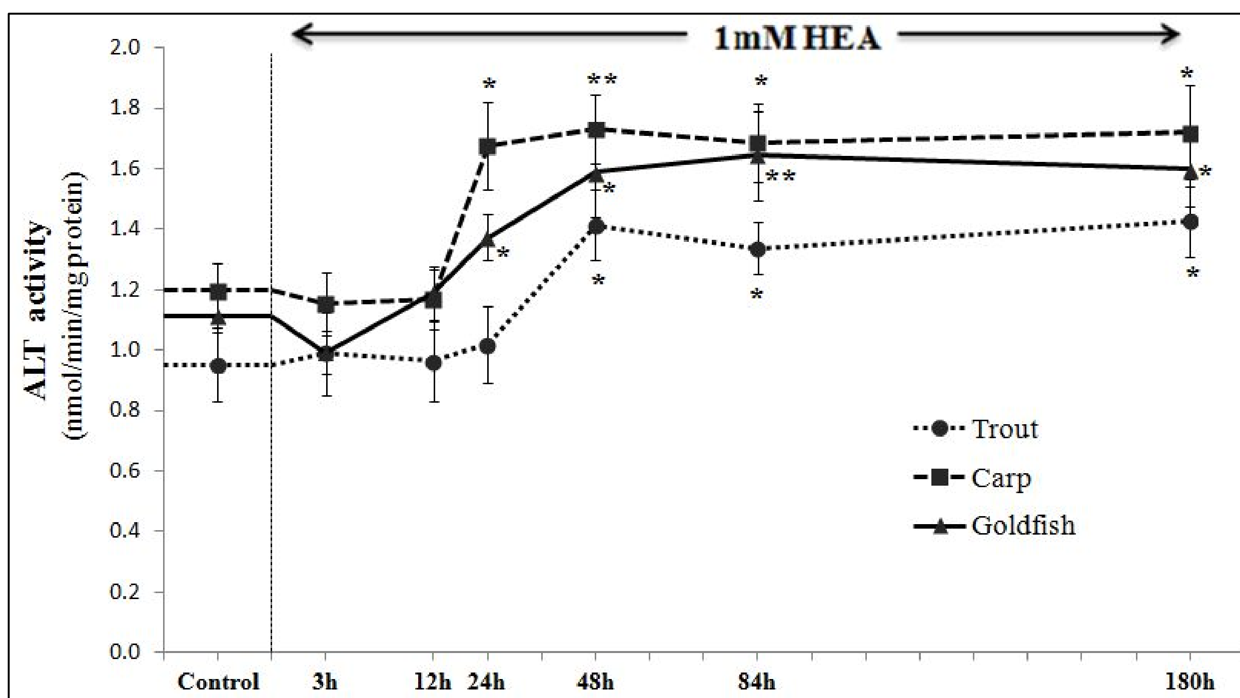

Figure.5.11. Alanine aminotransaminase (ALT) activity (nmol/min/mg protein) in the brain of rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$).

5.4.4. Detoxification of ammonia to urea: a strategy not active in brain of studied fish species

The majority of teleost fishes are ammoniotelic but urea also constitutes about 10–30% of the total nitrogenous wastes in most of them (Saha and Ratha, 1998). To date, only a few teleosts (e.g. Lake Magadi tilapia *Oreochromis grahami*, African lungfish *Protopterus dolloi*, Gulf toadfish *Opsanus beta*) are known to synthesis urea as a major strategy in detoxifying ammonia. Ureogenesis in fishes is energetically expensive; the detoxification of the net influx of exogenous ammonia to urea and its subsequent excretion would result in a high expenditure of energy and the loss of carbon. Probably, because of these drawbacks ureogenesis is not a common mechanism adopted by teleosts to cope with ammonia loading. In the present study both cyprinids and trout did not detoxify ammonia to urea during 180 h of exposure as evident from Fig.5.2. Alternatively, glutamine formation is energetically more efficient than ureogenesis where every mole of ammonia detoxification consumes 2 mol of ATP while a total of 5 mol of ATP are hydrolyzed for each mole of urea synthesized (Ip et al., 2004c). Therefore it is apparent from present study that trout, carp and goldfish adopted the strategy of detoxifying ammonia to glutamine rather than switching to energetically more expensive ureogenesis pathways. An extra advantage of following glutamine metabolic pathways over urea formation is that glutamine can be stored in the tissues and is readily utilized for other anabolic processes (e.g. syntheses of purine, pyrimidine, mucopolysaccharides) upon return to normal conditions (Ip et al., 2004a,c).

Some reports suggest that urea is synthesized via the degradation of dietary arginine (Cvancara, 1969; Wright et al., 1993) and also produces ornithine as one of the by-products. In the present study, an increment (in trout) or no alteration (in carp and goldfish) in arginine content is likely to be independent of urea cycle. Likewise, the amendments in the ornithine content do not fit with the pattern of arginine, suggesting that the urea cycle in the brain of studied species may not be very functional. Beside arginine, proline can also act as a source of ornithine synthesis. The production of proline during HEA exposure was significantly high, presumably because they can be produced as by-products of pyruvate synthesis. Nevertheless, the source of urea production in teleosts and the involvement of the ornithine urea cycle are still under debate.

5.5. CONCLUSION

Ammonia detoxifying strategies in brain which employ glutamine synthesis and associated metabolic pathways were induced differentially among the three freshwater teleosts following exposure to 1 mM ammonia. In carp and goldfish, the increment in glutamine was accompanied by a decline in brain ammonia level and a significant increment in GSase activity. Unlike cyprinids, trout failed to re-store the brain ammonia to basal level. Activities of GDH and transaminase enzymes (ALT and AST) were elevated notably in both carp and goldfish, facilitating them to replenish glutamate pool. It

signifies that under HEA the supply of glutamate was in parallel with the demand for glutamine synthesis, thereby promoting glutamine mediated ammonia detoxification pathways very efficiently in cyprinids. However, exposure to HEA could not activate GDH and AST enzymes in trout resulting in momentous depletion in brain glutamate content during the course of detoxification process. Significant alteration in the concentration of other amino acids occurred in all the three species during ammonia exposure suggesting that multiple enzymatic pathways can supply glutamate for the production of glutamine. Furthermore, the present study also indicates that neither cyprinids nor trout uses ureogenesis as a strategy to deal with accumulated ammonia in brain; presumably because urea synthesis is energetically expensive. In summary, the physiological mechanisms related to amino acid metabolism to avoid ammonia toxicity are endowed very effectively in carp and the goldfish, enabling them to tolerate high ambient ammonia more efficiently than the trout. However, the possible signalling mechanisms inducing different amino acid metabolic enzymes and changes in the concentration of various effector molecules, and/or the phosphorylation status of the pre-existing enzymes during high ammonia load need to be investigated. Nevertheless, the regulation of these enzymes at the transcriptional level and ammonia transporting function of Rhesus (Rh) glycoproteins might be crucial in further examinations.

Acknowledgments

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Part III

Interaction of ammonia with additional stressors- starvation and exhaustive swimming

MATERIALS AND METHODS

Experimental system and animals

Gold fish (*C. auratus*) juveniles (10-15 g) were obtained from Aqua Hobby, Heist op den Berg, Belgium. Common carp (*C. carpio*) juveniles (12-16 g) were obtained from the University of Wageningen, The Netherlands.Fish were kept at the University of Antwerp in tanks (200L) for at least a month before the exposure started. A total of 600 goldfish and 600 carp were distributed species wise into ten 200 L tanks (n= 60 per tank) equipped with flow through in a climate chamber where temperature was adjusted at 17±1 °C and photoperiod was 12 h light and 12 h dark. Fish were acclimated for 2 weeks prior to experiment and were fed *ad libitum* once a day. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. All the water parameters were in the optimum range (pH 7.0-7.5, dissolved oxygen 6.9-7.4 mg/L, total NH_3 0.1-0.2 mg/L, nitrite 0.07-0.1 mg/L and nitrate 1-3mg/L). During the actual exposure, charcoal and lava stones were removed from the filter to prevent ammonia absorption in the filter.

The concentration of ammonia used in this part of PhD work (chapter 6, 7, 8 and 9) was 1mg/L which represents the legal average quality for surface waters in Flanders (Decision Flemish Government. 1 June 1995: (B.S. 31 July 1995)).

Experimental group and exposure

The experimental set up for each fish species consists of four experimental groups: 1) control fed fish (CF) (2 tanks), 2) ammonia exposed fed (AF) fish (3 tanks), 3) control starved fish (CS) (2 tanks) and, 4) ammonia exposed starved (AS) fish (3 tanks). Starved groups were fasted for 1 week prior to sampling. Each unfed tank of fish was paired with a tank of fed fish. Fish were hand fed with commercial diet (Trouvit, Fontaine-lès-Vervins, France) every morning at a rate of 2% on their wet body weight/day. Feeding was adjusted based on the weight and the number of fish remaining in the tank after each sampling periods. Moreover, on the days of measurement, remaining fish were fed after fish used for swimming performance and respiration measurements were removed.

Each exposure tank was spiked with the required amount of an NH_4Cl stock solution (Merck, Darmstadt, Germany). A constant concentration of 1.07±0.10 mg/L of ammonia was maintained throughout the experiment. Ammonia concentrations were measured each 6 hrs after the onset of treatment and the concentration of ammonia in tank was maintained by adding calculated amount of the NH_4Cl solution. Moreover, to avoid the microbial breakdown of test chemical and build-up of other waste products, 40%–60% of the water was discarded twice a week and replaced with fresh water containing the respective amount of ammonia.

Sampling intervals

Control groups (for both goldfish and carp) were measured on day 0, 4, 10, 21 and 28. Four fish (species wise) for each group were collected from each of their respective two tanks. Exposed groups were measured after 3h, 12 h, and 1, 4, 10, 21, 28 days. During each exposure time, three fish from first two tanks and two fish from third tank were collected. All the groups were sampled for biochemical analysis from respirometers after O_2 consumption (MO_2) and/or after U_{crit} measurements were taken (fish were forced to swim at increasing speed intervals until they became exhausted and were swept against the mesh at the end of the respirometer flume). Fish sampled after MO_2 were called routine-swimming (RS) (swum at minimum water speed) while fish sampled after U_{crit} were designated as exhaustive-swimming (ES) as these fish were forced swum continuously until they become exhausted.

Blazka-style swimming respirometers of known volume (≈3.9 L) were used for the present experiment. The fish were placed in the respirometers 12 h prior to sampling (the evening before measurement) to minimize handling stress. Exposure with ammonia was carried out within the respirometers for those groups of fish which were sampled at 3h and 12 h. Water composition in the recirculation system containing the respirometers was identical to the water composition of the exposure tanks. Water speed was set at 5 cm/s which allowed the fish to orient themselves against the water current and swim gently overnight. The fish were not fed within the respirometers and therefore even the fed fish had remained unfed for about 24 h prior measurements and sampling. The details of exposure regimes and sampling schedule is illustrated in fig.6.1 (refer below)

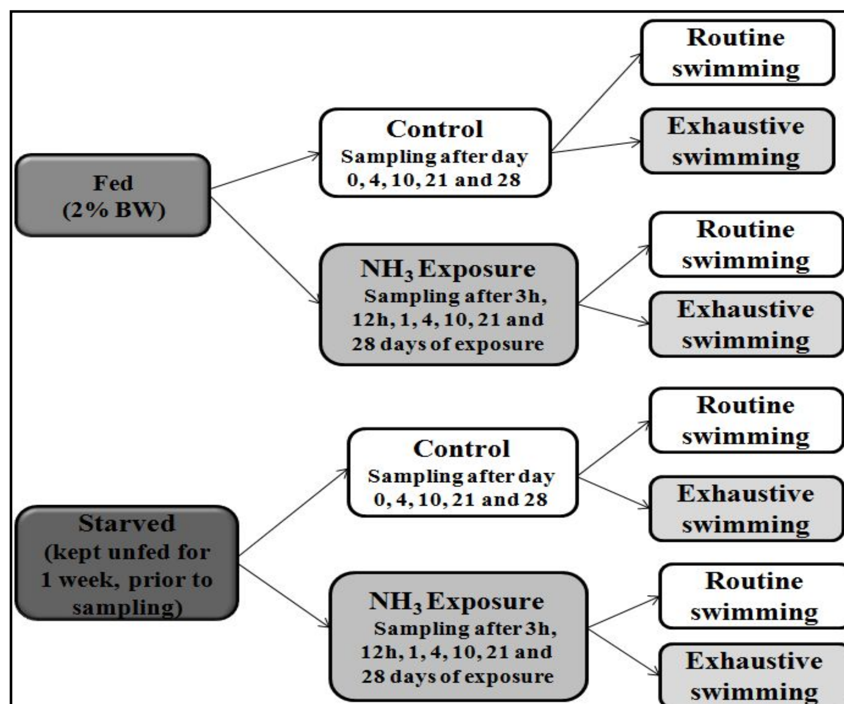

Figure. 6.1. Schematic diagram of experimental set up for chapter 6, 7, 8 and 9

Determination of water ammonia, U_{Crit} and oxygen consumption at routine-swimming (MO_2) and at exhaustive-swimming (MO_{2UCrit})

At the start of the MO_2 measurements, initial water samples were collected from 8 respirometers containing one fish each. Water circulation through the respirometers was cut off, air bubbles were removed through the outlets without stressing the fish, the oxygen electrodes (WTW, Weilheim, Germany), one in each respirometer, were inserted and the system was made airtight so that water leakage from the respirometers was negligible. For routine swimming fish, final water samples were collected to measure ammonia excretion by fish after an interval of 3 h swimming at 10cm/sec, during which oxygen concentration (mg/L) was continuously recorded using Windmill Logger (Windmill Software Limited, Manchester, UK). For exhaustive exercising, fish were allowed to swim for 1 h at the speed equivalent to 3/4 U_{crit} (see below) for measurement of MO_{2UCrit} and ammonia excretion.

Water total ammonia levels were determined using the salicylate–hypochlorite method (Verdouw et al., 1978). Ammonia excretion was calculated as µmol ammonia excreted per gram of fish per hour. Oxygen consumption (MO_2 and MO_{2UCrit}) was calculated as µmol oxygen consumed per gram of fish per hour.

For *Ucrit* determination, another set of 8 fish was placed in the respirometer tunnels, and water velocity was then increased in increments of 5 cm/s at 20-min intervals, until fish become fatigued. Fish were considered exhausted once they impinged on the rear screen and would not swim after the water velocity was temporarily lowered and then returned to the speed at which exhaustion had occurred. *U*crit was calculated according to the equation given by (Brett, 1964)

$$U\text{crit} = Ui + [Uii(Ti/Tii)]$$

where *Ui* is the highest velocity maintained for the whole interval, *Uii* is the velocity increment (5 cm/s), *Ti* is the time elapsed at fatigue velocity, and *Tii* is the interval time (20 min). The absolute values (cm/s) were converted to relative swimming speeds in body lengths per second (BL/s) by factoring the absolute values by the fork length of the fish.

Sampling procedure and sample analysis

Fish from each experimental group for both goldfish and carp were bulk weighed at the beginning of the experiment and on day 4, day 10, day 21 and day 28. Growth performance of juveniles was evaluated in terms of weight gain based on following standard formulae:

Weight gain (%) = (Final weight−Initial weight) × 100/ Initial weight.

For sampling, fish were removed from respirometers (8 from RS and 8 from ES experiments), anaesthetized using an overdose of neutralized MS222 (Ethyl 3-aminobenzoate methanesulfonic acid, 1 g/l, Acros Organics, Geel, Belgium), blotted dry, weighed and fork length was measured. A blood

sample was collected from the caudal blood vessel using a heparinized syringe. Blood was immediately centrifuged (for 1min at the speed of 16,000 rpm at 4 °C), and aliquots of plasma were frozen in liquid nitrogen and stored at -80°C until later analysis. Fish were dissected on ice, gills, liver, pituitary and muscle tissue was removed, frozen in liquid nitrogen, and stored at -80°C for for further analysis.

Plasma and muscle ammonia was determined according to Wright et al. (1995a) using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Plasma lactate concentrations were analysed enzymatically using a commercially available kit (R-Biopharm AG, Darmstadt, Germany). Samples of liver and muscle were analyzed for protein content by Bradford's method (Bradford, 1976), glycogen content by using the anthrone reagent (Roe and Dailey, 1966) and lipid content were measured following Bligh and Dyer (1959).

Determination of Plasma ions and gill Na^+/K^+-ATPase activity

Plasma [Na^+], [Cl^-], [K^+] and [Ca^{2+}] were analyzed using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium).

Na^+/K^+-ATPase activity in gill samples was determined using the method of McCormick (1993). Protein content was measured using Bradford reagent (Bradford, 1976) with bovine serum albumin as a standard, at 595 nm.

Determination of plasma hormones

Radioimmunoassay (RIA) to determine total plasma [T3] and [T4] were performed as described by Van der Geyten et al. (2001).

A commercial Cortisol RIA kit (MP Biomedicals, New York, U.S.A) and a Gamma counter (Wallac wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Science) was used for RIA determination of plasma cortisol.

RNA Extraction and real time RT-PCR

Total RNA was isolated from liver, gill and pituitary gland samples using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. The size of pituitary gland of the individual common carp fish was considerably small, therefore, glands from two individual fish during each sampling time was pooled together. The extracted RNA samples were subjected to DNA-free (DNase) treatment to avoid genomic DNA contamination. The quantity of the RNA was evaluated by using Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and purity by OD_{260}/OD_{280} nm absorption ratio > 1.95. A starting amount of 1 µg RNA was transcribed to First

strand cDNA according to “Revert Aid H minus First strand cDNA synthesis kit” (Fermentas, Cambridgeshire). For the real-time PCR reaction, the final volume of 20 µL was adjusted to 100 µL to achieve a working amount (5 µL) of approximately 50 ng RNA-equivalent for each reaction.
Highly purified salt-free ‘OliGold’ primers (Eurogentec, Seraing, Belgium) for the quantification of the target genes and reference genes were designed using the Lightcycler probe design software, version 1.0 (Roche Diagnostics, Vilvoorde, Belgium) (refer Table 7.1 in chapter 7 for goldfish; Table 9.1 in chapter 9 for common carp). Real time PCR mastermix was prepared as follows: 5.5µL nuclease free water, 1 µL forward and 1 µL reverse primer and 12.5µL Maxima SYBR Green qPCR Master mix (Fermentas, Cambridgeshire). Mastermix (20 µL) was mixed with 5 µL of cDNA template in the glass capillaries. A four-step experimental run protocol was used in the light cycler (Roche version 3.5): a denaturation program (10min at 95 °C); an amplification and quantification program repeated 40 times (15 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C); a melting curve program (55-95 °C with a heating rate of 0.10 °C/s and a continuous fluorescence measurement) and finally a cooling step (4°C) (Sinha et al., 2010).

Confirmation of primer specificity and efficiency

Melting curve of the target genes and reference genes for each fish species were performed which resulted in single product specific melting temperatures.

Relative Quantification

Relative quantification of the target gene-transcript with a chosen reference gene transcript was done following the Pfaffl method with the Relative Expression Software tool (REST©) (Pfaffl, 2001; Pfaffl et al., 2002) by using the formula:

$$\text{Ratio} = (E_{\text{target}})^{\Delta CT_{\text{target(control – sample)}}} / (E_{\text{ref}})^{\Delta CT_{\text{ref(control – sample)}}}$$

where C_T (cycle threshold) value corresponds to the number of cycles at which the fluorescence emission monitored in real time exceeded the threshold limit. E is PCR efficiency determined by standard curve using serial dilution of cDNA (refer Table 7.1 in chapter 7 for goldfish; Table 9.1 in chapter 9 for common carp). The value of E is calculated according to equation $E = 10^{(-1/\text{slope})}$. C_T value of dilution series (1x, 2x, 4x, 8x etc) were used to calculate the slope for target and reference genes. Each point of dilution was tested in 3 replicates. ΔC_T is the crossing point deviation of the sample versus a control.

For goldfish: Comparison of several reference genes (β-actin, 18S rRNA and GAPDH) favoured β-actin because of its stable expression level in these test conditions. The calculated value (from C_T value of 20 random samples) of coefficient of variation for β-actin, 18s rRNA, and GAPDH was 0.02, 0.09 and 0.15 respectively.

For common carp: Comparison of several reference genes (β-actin, EF1α and GAPDH) favoured β-actin because of its stable expression level in these test conditions. The calculated value (from C_T value of 20 random samples) of coefficient of variation (%) for β-actin, EF1α, and GAPDH was 1.39, 5.08 and 3.11 respectively.

STATISTICAL ANALYSIS

Data are presented as mean ± S.E (standard error). Comparison within treatment was done by one-way ANOVA at 5% level of significance. Main effects of feeding, exercising, and ammonia and their interactions were analyzed by three-way ANOVA. Linear regression analysis was performed to show the relationship between plasma ammonia levels and plasma lactate with U_{crit}. Significant difference between two means was measured using Duncan's multiple range test (Duncan, 1955). The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 16.0.

Results for gene quantification (for both goldfish and common carp) are expressed as fold expression relative to β-actin. The expression level at the control (0 h) condition has been designated value '1' and thereby the expression ratio of treatments was expressed in relation to the control. Significant differences in expression between control and treatments were analyzed by Relative Expression Software tool-Multiple condition solver (REST-MCS) Version 2 using Pair Wise Fixed Reallocation Randomisation Test©. A probability level of 0.05 was used for rejection of the null hypothesis.

No significant differences were found between any of the control values at different sampling times (for chapters 6, 7, 8 and 9). Therefore, pooled controls for each experimental group are shown for clarity of the figures.

Chapter 6

The interactive effects of ammonia exposure, nutritional status and exercise on metabolic and physiological responses in goldfish (*Carassius auratus* L.)

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ABSTRACT

This study aimed to elucidate the physiological effects of high environmental ammonia (HEA) following periods of feeding (2% body weight) and starvation (unfed for 7 days prior to sampling) in gold fish (*Carassius auratus*). Both groups of fish were exposed to HEA (1mg/L; Flemish water quality guideline) for 0 h (control), 3 h, 12 h, 1 day, 4 days, 10 days, 21days and 28 days. Measurements of weight gain (%), oxygen consumption (MO_2), ammonia excretion rate, ammonia quotient (AQ), critical swimming speeds (U_{crit}), plasma and muscle ammonia accumulation, plasma lactate, liver and muscle glycogen, lipid and protein content was done at various time intervals during the experimental periods. Overall, ammonia excretion rates, plasma ammonia accumulation and AQ were significantly affected by food regime in ammonia free water. HEA, the additional challenge in the present study, significantly altered all the studied parameters among fed and starved groups in days-dependent manner. Results shows that weight gain (%), MO_2, U_{crit}, protein content in liver and muscle, and glycogen content in muscle among starved fish under HEA were considerably reduced compared to control and fed fish. Additionally a remarkable increase in plasma ammonia level, muscle ammonia, lactate accumulation and AQ was seen. However in fed fish, MO_2, ammonia excretion rate, AQ and lactate level augmented after exposure to HEA. These results indicate that starved fish appeared more sensitive to HEA than fed fish. Furthermore, as expected, the toxic effect of ammonia exposure in both feeding treatments were exacerbated when imposed to exhaustive swimming (swum at $3/4^{th}$ U_{crit}). Such effects were more pronounced in starved fish. This suggests that starvation can instigate fish more vulnerable to external ammonia during exercise. Therefore, it was evident from our study that feeding ameliorates ammonia handling and reduces its toxicity during both routine and exhaustive swimming. Moreover, recovery was observed for some physiological parameters (e.g. MO_2, ammonia excretion, U_{crit}, plasma ammonia) during the last exposure periods (21-28 days) while for others (e.g. growth, tissue glycogen and protein content, muscle ammonia) effects only became apparent at this time. In the future, these results need to be considered in ecological context as fish in ammonia polluted may experience different phenomenon (starvation and exercise) simultaneously.

Keywords: Ammonia toxicity, Gold fish (*Carassius auratus*), Routine swimming, Critical swimming speeds (U_{crit}), Exercise, Starvation, Plasma lactate, Ammonia excretion.

6.1. INTRODUCTION

Waterborne ammonia has become a persistent pollutant of aquatic habitats, and enters the water bodies from several sources such as sewage effluents, industrial wastes, agricultural run-off and decomposition of biologic wastes (Randall and Tsui, 2002). In aqueous solution, ammonia exists as unionised ammonia (NH_3) and ionized ammonium (NH_4^+) (Randall and Tsui, 2002; Wajsbrot et al., 1993). The toxicity of ammonia is attributed to its unionised form (NH_3) which can readily diffuse across the gill membranes due to its lipid solubility and lack of charge. Fish can excrete ammonia as NH_3 across the gill membranes into water providing there is a concentration gradient and this process is facilitated by Rhesus (Rh) glycoprotein (Nawata et al., 2007). In addition, the potential roles of Na^+/H^+ exchanger, proton pump and Na^+/K^+-ATPase for ammonia transport in fish gills are well documented (Evans et al., 2005; Wilkie, 2002). However, high environmental ammonia (HEA) reduces the outward flux of ammonia excretion through the gills, so that the outward flux is blocked and a reversed inward ammonia flux occurs. As a result blood and tissue ammonia levels increase and fish experience both chronic and acute toxic affects (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002). Some noted effects include decrease in growth rate (Dosdat et al., 2003; Foss et al., 2004; Lemarie et al., 2004; Pinto et al., 2007), alteration in energy metabolism (Arillo et al.,1981b), disruption of ionic balance (Soderberg and Meade, 1992; Wilkie, 1997), increased vulnerability to disease, and pathological changes in gill structure (Wilkie, 1997). Numerous studies on different fish species concerning acute and chronic ammonia toxicity alone already exist (Benli et al., 2008; Dosdat et al., 2003; Knoph and Olsen, 1994; Knoph and Thorud, 1996; Lemarie et al., 2004; Person-Le Ruyet et al., 1997, 1998, 2003; Tomasso, 1994; Weinstein and Kimmel, 1998; Wicks and Randall, 2002a).

An issue that is almost neglected in ecotoxicological studies is the nutritional status, which can have pronounced impacts on the performance and physiological condition of fish under ammonia threat. Feeding not only induces an increased energy demand as part of the specific dynamic action (SDA), but it also leads to an increased endogenous production and excretion of ammonia (Randall and Tsui, 2002; Wicks and Randall, 2002b). In toxicological and physiological studies, however, fish are often starved or fed below a normal level to avoid build-up of waste products which may allow fish to maintain internal ammonia concentrations below toxic levels (Wicks and Randall, 2002b). Although reduction in feeding rates may be convenient and increase survival, it will undoubtedly hamper growth rate (Person-Le Ruyet et al., 1997). On the other hand, extensive non-toxicological oriented reports are available on ammonia production and excretion rates in feeding fish under normal circumstances (Dosdat et al., 1996; Gelineau et al., 1998; Leung et al., 1999). Surprisingly, the combined effect of ammonia exposure and feeding status on internal ammonia homeostasis has not been widely studied. In this scenario, it can be expected that feed deprivation may not be a

problem but high endogenous ammonia production as a consequence of feeding can create undesirable effect.

In nature, fish must swim in order to capture prey, avoid predators and reproduce, and maximum sustainable (critical) swimming speed (U_{crit}) (Brett, 1964) is often used to assess the impact of environmental factors such as temperature, hypoxia, diseases or contaminants on fish performance (Brett and Glass, 1973; Hammer, 1995; Nikl and Farrell, 1993; Plaut, 2001; Thomas and Rice, 1987; Waiwood and Beamish, 1978). Swimming has been shown to induce endogenous ammonia production and increases internal ammonia levels when compared with resting (Knoph and Thorud, 1996). Ammonia accumulation in the plasma has been implicated in reduced critical swimming speeds in salmonids (Shingles et al., 2001; Wicks et al., 2002). Beaumont et al. (2000b) suggested that increased NH_4^+ levels alter the metabolic status within the fish, arising from the effects on a number of metabolic pathways which may lead to premature muscle fatigue. Besides, depolarisation of muscle membrane potential due to the substitution of K^+ with NH_4^+ has also been hypothesized for impairment of muscle contraction (Beaumont et al., 1995a,b,2000a; Cooper and Plum, 1987; Raabe and Lin, 1985). Furthermore, when fish are forced to swim at higher speed the ammonia induced toxic responses in fish are amplified (Wicks et al., 2002). Various metabolic and physiological responses may differ among swimming fed and fasting fish (Knoph and Thorud, 1996; McKenzie et al., 2003; Wicks et al., 2002). Therefore, swimming in fish may act as an additional stressor in combination with HEA and feeding/food deprivation. To date, however, there is little information on how the interactions of these three processes influence physiological performance in fish.

In the present study, the ornamental gold fish (*Carassius auratus*) was exposed at 1mg/L ammonia. It is more tolerant to ammonia (96 h LC50 is 9mM) (Dowden and Bennett, 1965; Thurston et al., 1981) compared to other cyprinids and salmonids, which allows a prolonged and in depth study of the occurring physiological alterations. Therefore, the purpose of this study was to investigate the metabolic and physiological changes that occur in goldfish as compensatory mechanisms (1) to deal with HEA (2) to deal with high load of endogenous ammonium production which is expected to be high under HEA, feeding and swimming conditions (3) to test the hypothesis that fed fish with higher endogenous ammonia production are more susceptible to HEA and to exhaustive swimming than starved fish.

To achieve these goals we measured (i) swimming performance, measured as critical swimming speed (U_{crit}), (ii) oxygen consumption rates under routine and exhaustive swimming conditions (MO_2 and MO_{2UCrit}), (iii) plasma lactate as an indicator of anaerobic metabolism, (iv) muscle and liver energy budgets, (v) plasma and tissue ammonia accumulation and (vi) ammonia excretion rate at various sampling intervals up to 4 weeks. These results will link nutritional status, ammonia exposure and exercise with ecologically relevant fitness parameters of the fish and

furthermore will offer a better understanding of how fish in ammonia polluted water can be affected by periods of starvation and exhaustive swimming.

6.2. RESULTS

6.2.1. Non-invasion measurements

Growth

After 4 weeks of ammonia exposure weight gain (%) in exposed fed fish was 22.5% lower ($P < 0.05$) compared to control fed fish (Table 6.1). This reduction in growth was more pronounced in starved fish where a significant difference was already seen after 3 and 4 weeks of ammonia administration, with values that were 35.8% and 35.3% lower than their control respectively. Furthermore, weight gain (%) in starved fish after 3 and 4 weeks of ammonia exposure were significantly lower than their corresponding values in exposed fed fish. However, no remarkable difference was noted among control groups at different sampling points.

Oxygen Consumption

Overall, we observed little effect of the feeding regime alone on M_{O2} when fish were swum either at low (M_{O2}, Fig.6.2A) or high speeds ($M_{O2Ucrit}$, Fig. 6.2B). A significant effect of feeding regime and ammonia exposure together was evident in oxygen consumption of fish when swum at lowest speed (Fig. 6.2A). Routine-swum ammonia exposed fed (RSAF) fish had a 46% higher M_{O2} ($P < 0.05$) than CF after 10 days of exposure, after which M_{O2} values returned to normal. In contrast, M_{O2} was drastically reduced in routine-swum ammonia exposed starved (RSAS) fish ($P < 0.05$) after 4 and 10 days of exposure. Reduction in M_{O2} corresponded to approximately 65% and 70% of the M_{O2} measured for control starved fish.

As expected, MO_{2UCrit} at high swimming speeds was elevated above M_{O2} for all groups and exposure times ($P < 0.05$, Fig.6.2B). At high speeds, oxygen consumption rates for both fed and starved fish increased till 10 days of exposure and subsequently returned to normal. These differences were significant compared to control after 4 days of exposure in exhaustive-swum ammonia exposed fed (ESAF) fish, after 4 and 10 days for exhaustive-swum ammonia exposed starved (ESAS) fish.

Table 6.1

Weight gain (%) at different sampling points.

Sampling intervals	Fed		Starved	
	Control	Exposed	Control	Exposed
4 days	3.90 ± 1.05	4.21± 1.75	3.68± 2.02	3.65± 2.41
10 days	17.9 ± 3.05	16.23± 4.05	16.22± 3.06	14.06 ± 4.13
21 days	34.20 ± 4.05	32.55± 3.85	30.11± 3.16	22.18± 3.52*†
28 days	41.92± 3.02	34.22± 3.15*	34.23± 4.14	25.31± 3.21*†

Values are mean ± S.E. An Asterisk (*) represents the significant difference between exposed group and its control at the same sampling time. Dagger (†) denotes the significant difference between exposed treatments of fed and starved groups.

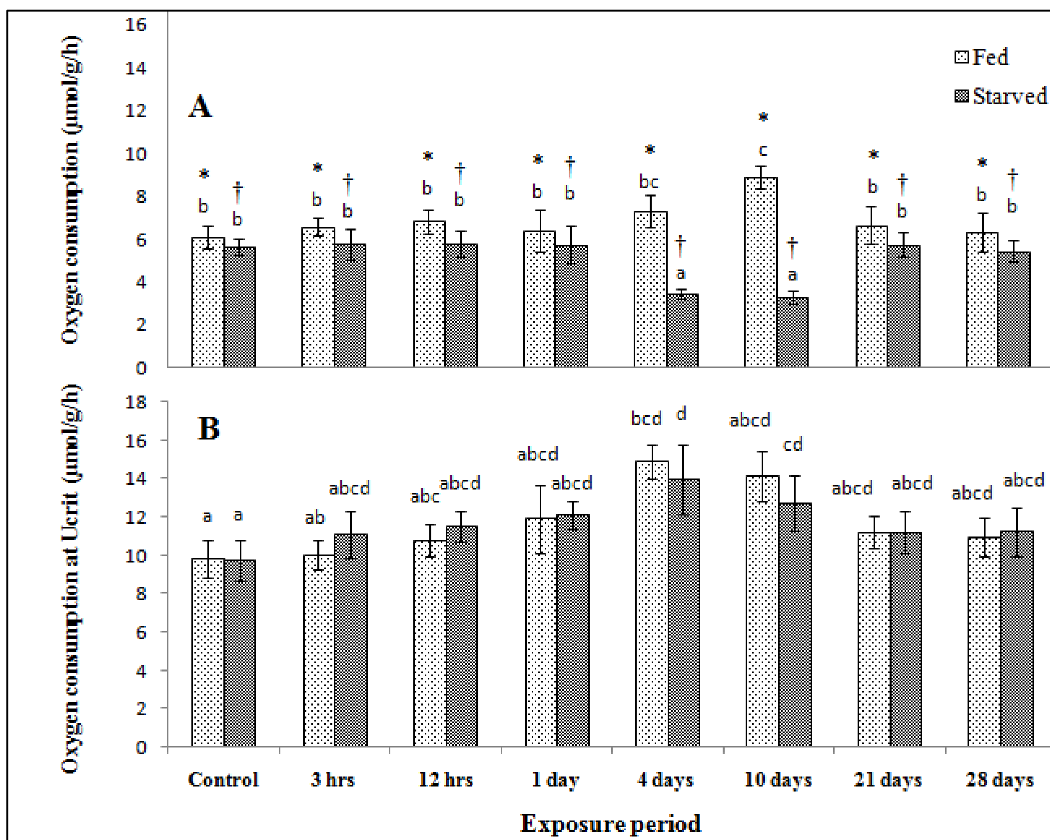

Figure. 6.2. Oxygen consumption rates in fed and starved fish at **(A)** minimum speed (M_{O2}) **(B)** *U*crit ($M_{O2Ucrit}$). Values are mean ± S.E. Different superscript letters denote significant differences (P < 0.05). Asterisk (*) and dagger (†) denotes the significant differences between M_{O2} and $M_{O2Ucrit}$ for fed and starved groups respectively.

Critical swimming speeds (U_{crit})

Under control conditions fed and starved fish swam equally well. But critical swimming speeds in AS fish were significantly affected after 10 and 21 days of ammonia exposure both compared to CS and AF (Fig. 6.3). The U_{crit} was reduced by 47.7 % and 59.6% respectively against control and 33.2% and 33.5% compared to the fed fish. No effects were seen in fed fish. Consequently, a significant combined effect of ammonia and food on U_{crit} was observed in the present experiment.

Ammonia excretion

The exposure of ammonia to RSAF induced a gradual increase in ammonia excretion which became significant ($P < 0.01$) at 4, 10 and 21 days of exposure, respectively 86%, 75.8% and 49% higher than the control (Fig. 6.4A). However, in RSAS no remarkable change was observed on ammonia exposure during any of the experimental periods. A difference ($P < 0.01$) between fed and starved fish during RS was noted in control condition and at day 4, day 10 and day 21 of HEA, with values for fed fish 55.1%, 54%, 48.6% and 73.7% higher than the starved fish respectively. The excretion rates in fed fish slowly recovered at the fourth week of exposure. Exercising the fish at high speed had a profound effect on ammonia excretion rates: the values for all experimental groups augmented considerably ($P < 0.05$) (Fig.6.4B). ESAF fish eliminated 89% and 98% more ($P < 0.05$) ammonia than control after 4 days and 10 days of exposure. Also, ESAS fish the excretion level was increased significantly after 4, 10 and 21 days of HEA. Analogous to RS, the distinct effect of feeding on ammonia excretion rate was also seen in ES fish, the value for fed fish under control and HEA conditions were significantly higher ($P < 0.05$) than starved fish. Overall, a significant interaction (Table 6.2) was observed between feeding, ammonia and exercise on ammonia excretion rate.

Ammonia quotient

The result of AQ shows that after 4 days and 10 days of exposure the value in RSAS fish increased considerable (P <0.05) as compared to control, clearly indicating exposure effect of ammonia (Fig.6.5A). Moreover, for the fed fish the highest value ($P < 0.05$) was noted after 4 days of exposure. No effect of food on AQ among RS fish was reported. On the contrary, when swum at higher speed, the differences among fed and starved fish became prominent ($P < 0.05$) (Fig.6.5B) and fed fish had a higher AQ throughout the exposure period. Since AQ in ESAS fish remained remarkably stable at control levels, the differences seen in RSAS fish also was significantly different from ESAS fish at day 4 and 10. In ESAF fish, AQ was increased at day 10. Overall, significant effects of food, ammonia exposure and exercising were observed in ammonia quotient of fish (Table 6.2).

Maximal AQ in fish for 100% aerobic protein degradation is 0.33 (Kutty, 1978), and this value becomes lower when other substances are used. Based on this, we found that AQ approached this

value or slightly overshooted 0.33 in RS fish that were exposed to ammonia for 4 and 10 days. At ES we found the AQ was lower than 0.33 in starved fish while in fed fish these values became quite high over the entire experimental period (Fig.6.5B) as fed fish excreted immense amounts of ammonia when swum at high speed (Fig.6.4B).

6.2.2. Plasma metabolites

Ammonia

Under control conditions fed fish have higher ($P < 0.05$) plasma ammonia than starved fish. But this trend was reversed under HEA and that by day 10 and 21 the starved fish have significantly higher plasma ammonia.

In RSAS fish, significantly elevated plasma ammonia levels were observed at 4, 10, 21 and 28 days of exposure which was 93.6%, 166.4%, 162.8% and 107% respectively higher than the control (Fig.6.6A). In ESAS fish, substantially ($P < 0.05$) elevated plasma ammonia levels as compared to control were observed at day 4 (101.1 %), day 10 (170.3%), day 21 (142.2 %) and day 28 (91.2 %) (Fig. 6.6B). RSAS showed ($P < 0.05$) higher plasma ammonia levels at 10 and 21 days of exposure as compared to RSAF (Fig.6.6A). A similar trend ($P < 0.01$) was seen in ES group from at 10 and 21 days of exposure (Fig.6.6B). In all groups, ES fish showed significantly higher plasma ammonia levels than the fish that were swum at low speed.

Overall effect of food ($P < 0.05$), exercise ($P < 0.001$) and ammonia exposure ($P < 0.001$) in individual as well as in combined form ($P < 0.001$) were noteworthy in plasma ammonia levels of fish (Table 6.2). Analyzing the RS and ES fish apart, overall effects of food and ammonia exposure were apparent in plasma ammonia levels.

Lactate

Ammonia exposure induced plasma lactate accumulation in RS fed and starved fish, which was significant from 10 days of exposure onwards compared to the controls (Fig.6.7A). No obvious differences ($P > 0.05$) between fed and starved fish were reported in any of the sampling periods. Overall significant effect of feeding treatments and ammonia exposure was observed in ES fish. A distinct ($P < 0.05$) elevation in plasma lactate was detected in starved fish after 4 days of exposure which continued till the end. The same effect was observed in fed fish, but only at day 21 and 28.Unlike RS group, a significant difference in plasma lactate was seen between ES fed and starved fish after 4 days and 10 days of exposure (Fig.6.7B). ES fish accumulated higher plasma lactate compared to fish swum at low speed.

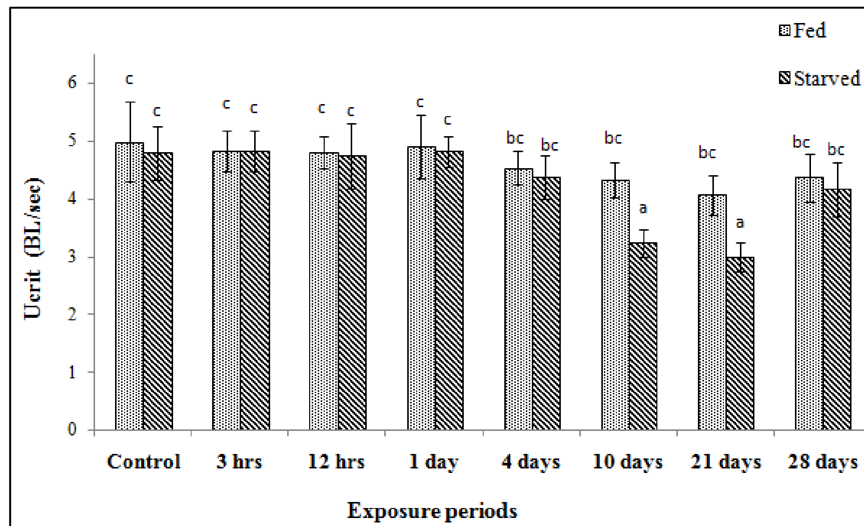

Figure. 6.3. Critical swimming speeds in fed and starved fish exposed to ammonia. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$).

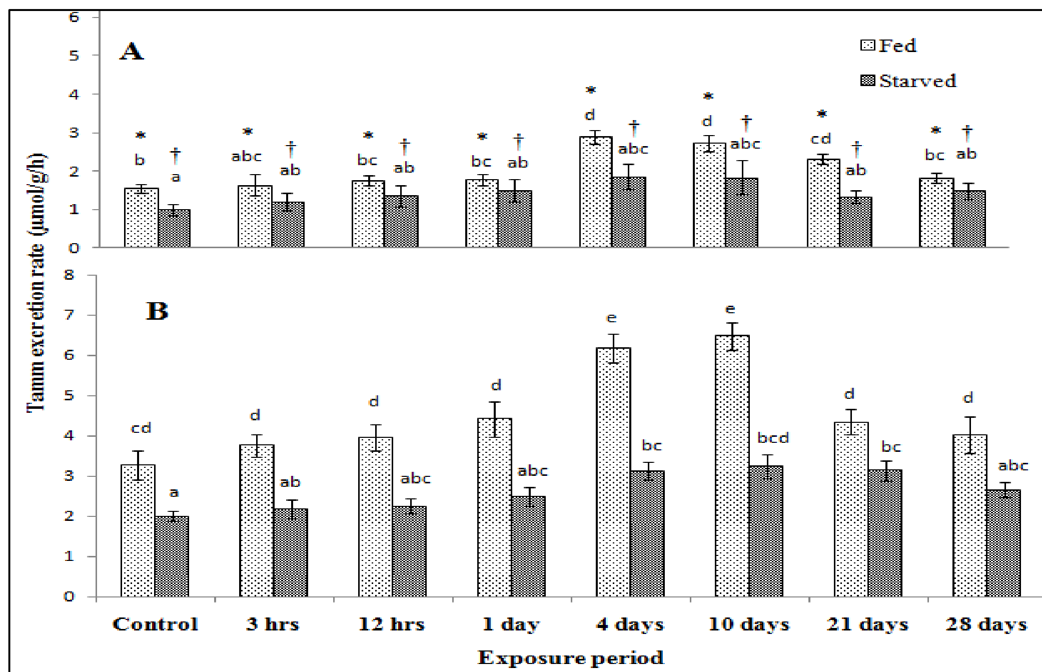

Figure. 6.4. Ammonia excretion rates in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for fed and starved groups respectively.

6.2.3. Tissue metabolites

Muscle ammonia

Muscle ammonia content for fed fish increased significantly compared to control after 28 days of exposure in both RS and ES group (Fig. 6.8A, 6.8B). In RSAS fish, a considerable ammonia accumulation was noted at 21 ($P < 0.01$) and 28 days ($P < 0.001$). A similar trend was seen for ESAS fish. In RS fish, no substantial difference was noted between fed and starved fish in control, 3 h, 12 h, 1 day, 4 days and 10 days of exposure. After 21 and 28 days of exposure the ammonia accumulation in starved fish was 35.5% and 21.9% times higher ($P < 0.05$) than in fed fish. The ES group of fed and starved fish revealed the similar trend as RS fish except at the end of exposure period when muscle ammonia for fed and starved fish was not statistically different.

Fish swum at higher speed accumulated significantly higher muscle ammonia compared to low speed swum fish. Feeding regime and ammonia exposure have a remarkable effect ($P < 0.05$) on muscle ammonia level.

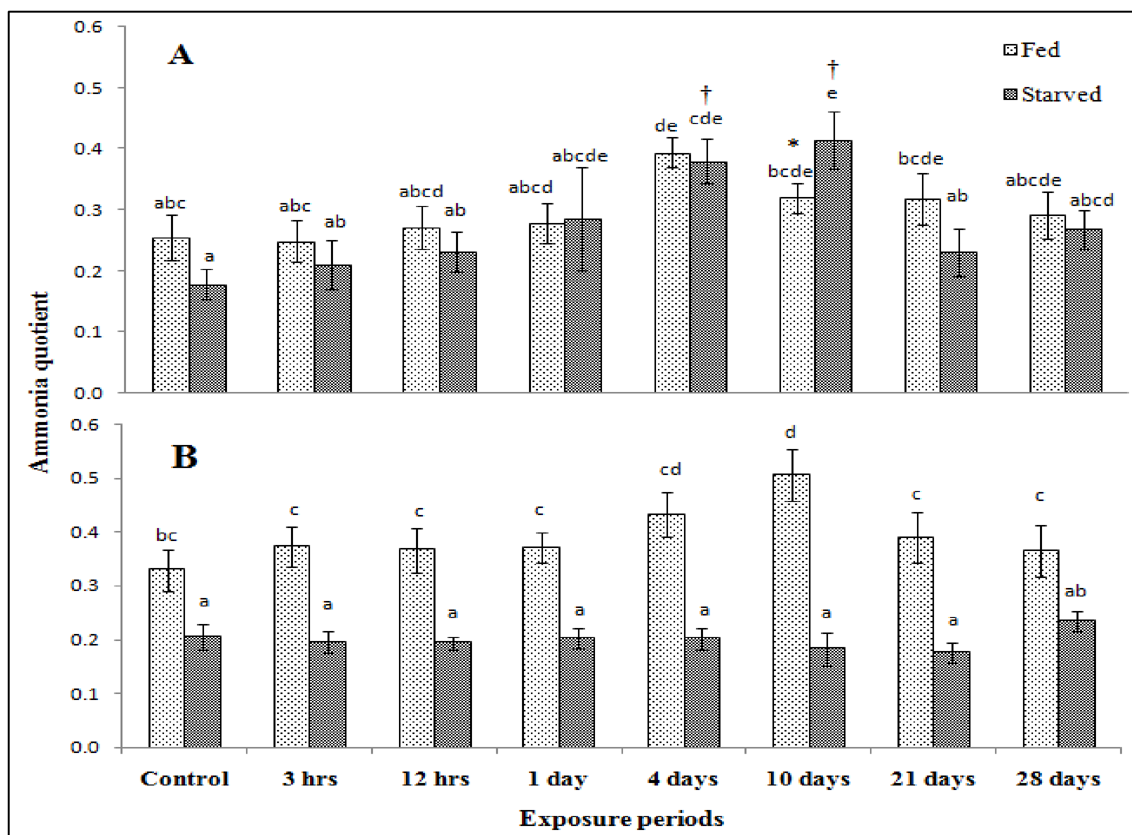

Figure. 6.5. Ammonia quotient values in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for fed and starved groups respectively.

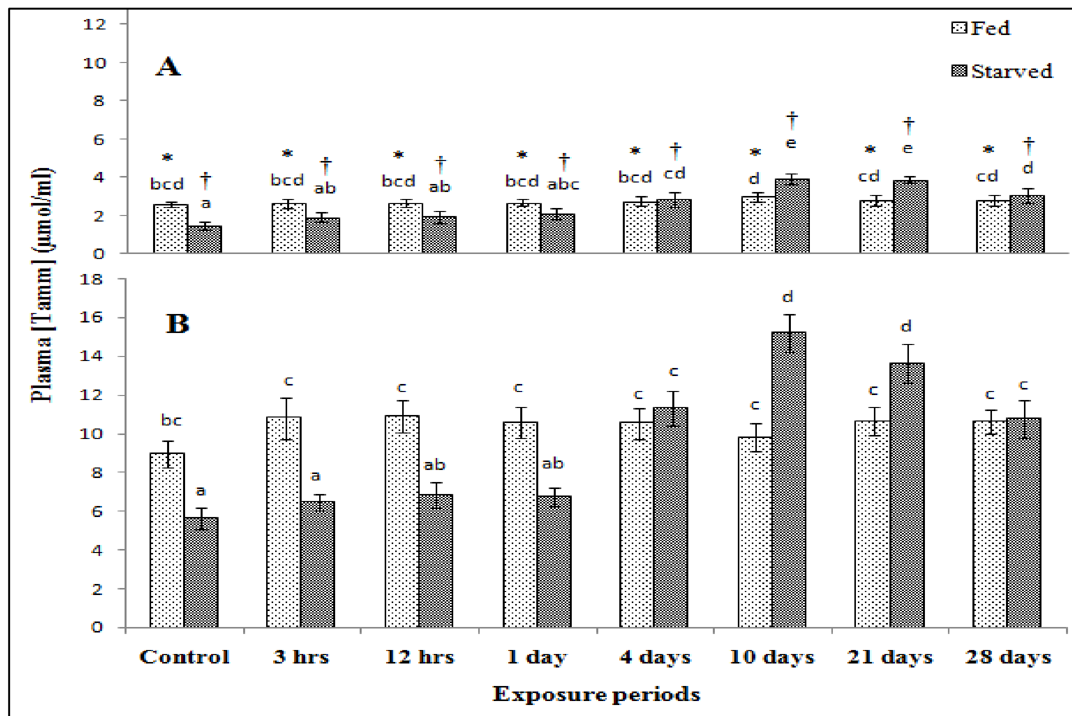

Figure.6.6. Plasma ammonia accumulation in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for fed and starved groups respectively.

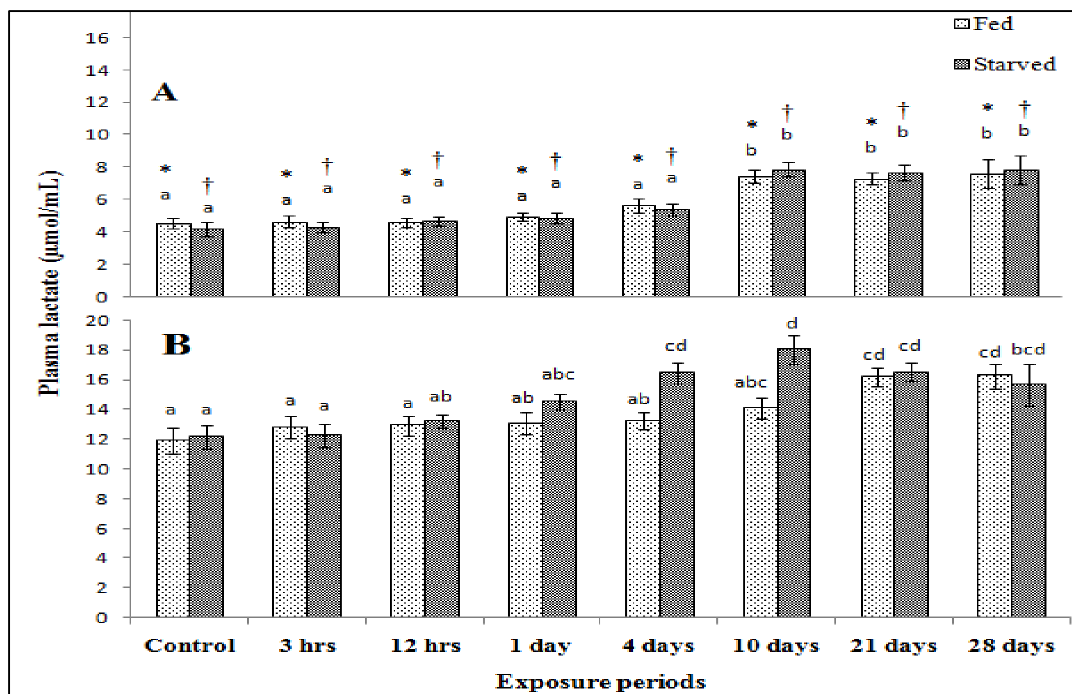

Figure. 6.7. Plasma lactate accumulation in fed and starved fish during (A) Routine swimming (B) Exhaustive swimming. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for fed and starved groups respectively.

6.2.4. Glycogen, protein and lipid content

Glycogen content in liver of RS fed fish remained stable during the first 10 days of exposure, but after 3 and 4 weeks of exposure glycogen content increased compared to control (Table 6.3). In starved fish the glycogen level remained uniform through the exposure period. A difference ($P < 0.05$) between the two feeding regimes was seen after 21 days of exposure. In contrast to RS fish, no remarkable difference was observed between control and exposed groups or between starved and fed fish in ES fish (Table 6.3). A significant increase in glycogen content of RS fed fish was observed as compared to ES fed fish when exposed for 4 weeks.

In muscle glycogen, however, a completely different pattern was seen (Table 6.3). In RS group, a steady reduction was evident in fed fish up to 21 days followed by an abrupt (77 %) decrease ($P < 0.05$) in the fourth week. In starved fish, the effect of ammonia became prominent ($P < 0.05$) a bit sooner with a significant reduction at 21 days of exposure and a further reduction at the fourth week. Toxicity effect became more pronounced when starved fish were subjected to swim at higher speed; muscle glycogen level diminished from 4 days onwards. The lowest value was seen on day 21when the glycogen content decreased ($P < 0.01$) 165.3 % compared to control. A significant difference among feeding groups was seen on day 21 in low speed swum fish while for ES fish it started at day 4 and continued till the end of experimental period. Table 6.2 shows that a significant interaction exists between all the three experimental factors on muscle glycogen content.

In liver and muscle tissue, a drop ($P < 0.05$) in protein level was occurred at fourth week of exposure among RS fed fish. Comparable reduction was evident among RS starved fish in contrast to control and fed fish after three ($P < 0.05$) and four week ($P < 0.01$) of exposure. A similar trend among feeding treatments was noted when fish were forced to swim at higher speed. Feeding regime or exercise alone did not have a considerable effect on protein content. Lipid stores in muscle and liver tissue did not change under the experimental circumstances.

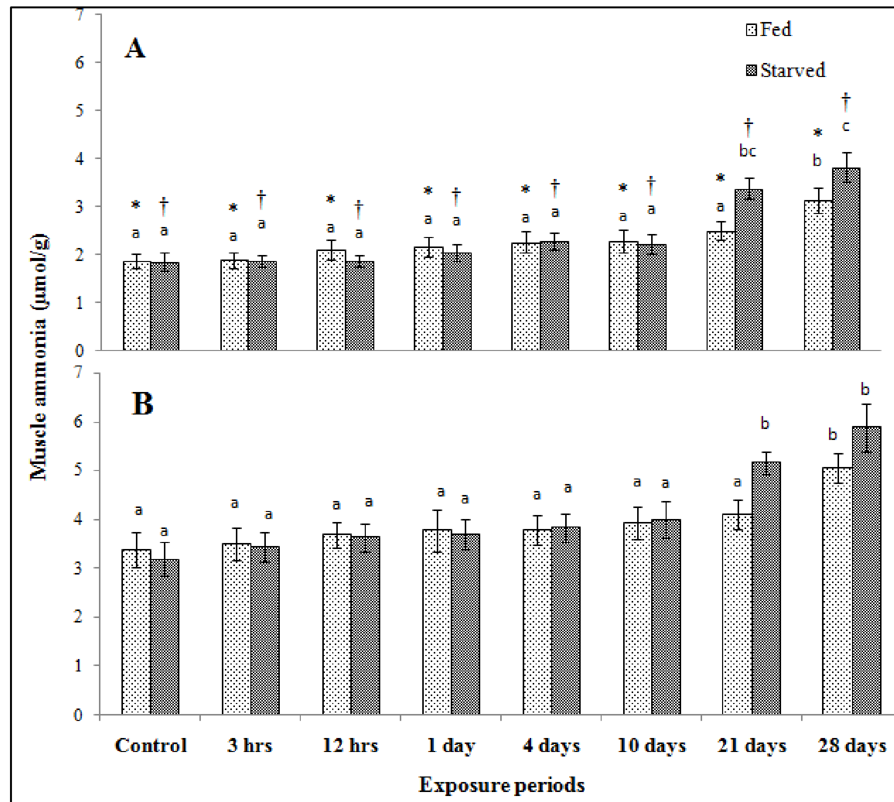

Figure. 6.8. Muscle ammonia accumulation in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Different superscript letters denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for fed and starved groups respectively.

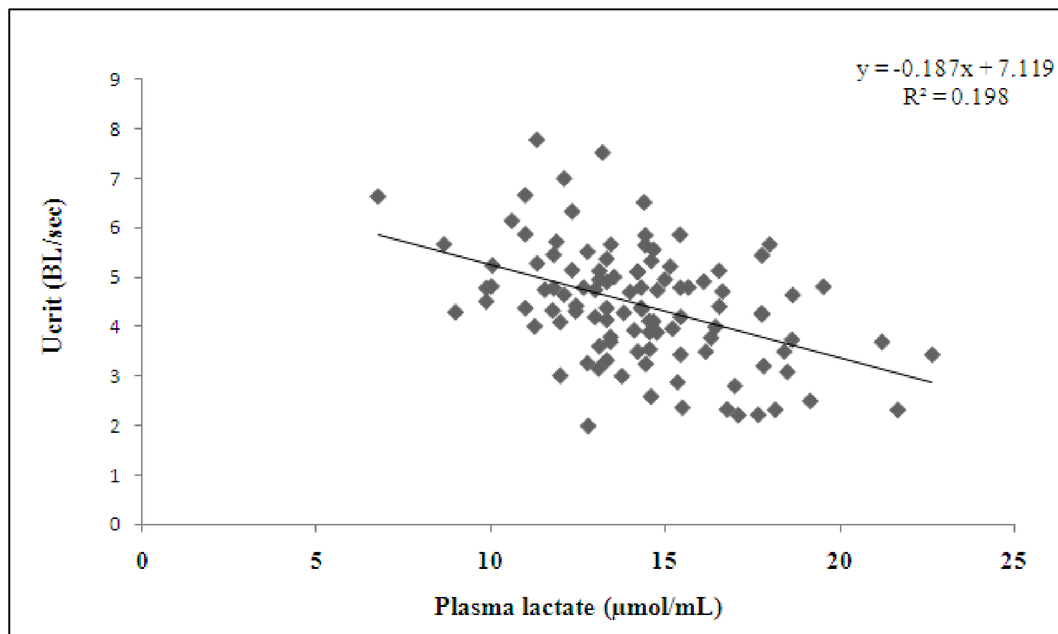

Figure. 6.9. Relationship between critical swimming speed (U_{crit}) of gold fish and plasma lactate.

6.3. DISCUSSION

6.3.1. Growth

Our results demonstrate that 3 to 4 weeks exposure to HEA reduced growth rate in gold fish. Reduction in weight gain under chronic ammonia exposure has been reported in juvenile seabass, (*Dicentrarchus labrax*) (Dosdat et al., 2003; Lemarie et al., 2004), Atlantic cod (*Gadus morhua*), (Foss et al., 2004) and Senegalese sole (*Solea senegalensis*) (Pinto et al., 2007). In current study, decrease in growth rate was seen both in fed and starved fish under HEA. It indicates that coping with HEA requires extra use of energy. Fed fish obviously can acquire some of this energy from the food and therefore, reduction in weight gain appeared at later period of exposure than starved fish. This consequence was reflected in the energy stores; muscle glycogen and protein store (in RS) were reduced at more or less the same time scale (week 4 for fed, week 3 and 4 for starved). Surprisingly, lipid was not used by the gold fish to cope with the toxicity of HEA.

6.3.2. Aerobic-anaerobic metabolism

Fed fish exposed for 10 days had an M_{O2} considerably higher than control (Fig. 6.2A), indicating that HEA can alter routine metabolic rate. Compared to starved fish, fed fish shows an increase in M_{O2} at 4 and 10 days of exposure, indicating an increase in aerobic metabolism. This coincides or is followed by anaerobic metabolism as evident from plasma lactate accumulation (Fig. 6.7A). Plasma lactate concentration has long been considered a useful indicator of aerobic limitations and anaerobic capabilities in exercise studies. Lactate concentration in plasma of RS fed and starved fish were elevated remarkably after 10 days of ammonia exposure (Fig.6.7A) which highlights the onset of anaerobic metabolism. This also indicates that lactate was produced faster than it was been metabolized and it showed that exposed fish were at anaerobic threshold condition. The elevation in M_{O2} among fed fish as compared to fasting fish may reflect the SDA component (Beamish and Trippel, 1990; Brown and Cameron, 1991; Jobling, 1981; Lyndon et al., 1992) but SDA was not perceptible from our M_{O2} measurements, most likely because even in the fed fish, the last meal was 24 hrs beforehand. Furthermore, the reduction in M_{O2} among feed deprived (at 4-10 days) fish might indicate a metabolic depression as these fish might have attempted to save energy stores and minimize energy investment under HEA (Scarabello et al., 1991; Wieser et al., 1992; Yang and Somero, 1993). Likewise, feed deprivation in juvenile rainbow trout and largemouth bass (*Micropterus salmoides*) were reported to have respectively 68% and 75% lower M_{O2} than fed fish (Alsop and Wood, 1997; Gingerich et al., 2010). In the same treatment (RSAS), anaerobic metabolism was switched on (Fig. 6.7A) and when swimming at higher speed (ESAS), this switch happened a lot sooner (from day 4) than in the fed fish (Fig. 6.7B).The lactate level in ESAS started to decline from day 21 onwards (but remained higher than in control) while in ESAF the level

increased till the end of the exposure period. Possibly, starved fish might have started to metabolize lactate as an energy substrate while fed fish did not (Sangiao-Alvarellos et al., 2005; Soengas et al., 1996,1998; Vijayan et al., 1996a). Moreover, a negative and significant correlation between U_{crit} and plasma lactate accumulation (Y= -0.187X+7.119, R^2= 0.198, $P < 0.0001$) was observed in present study (Fig. 6.9). Likewise, a significant reduction in U_{crit} was reported in rainbow trout and sockeye salmon (*Oncorhynchus nerka*) when plasma lactate concentration augmented more than the threshold limits (Farrell et al., 1998; Jain and Farrell, 2003; Stevens and Black, 1966).

Furthermore, elevated ammonia and also feed withdrawal have demonstrated to deplete glycogen stores (Arillo et al., 1981a,b; Gingerich et al., 2010; Kieffer, 2000; Wang et al., 1994). The reduced muscle glycogen stores in ammonia-exposed starved fish might have hampered continued burst swimming, resulting in lower U_{crit} than fed fish. Glycogen store in liver showed a completely different trend. In RSAF fish the increase in anaerobic metabolism precedes the increase in liver glycogen. One possible explanation is that gold fish exposed for long term ammonia were experiencing some form of hypoxia because ammonia has been shown to disrupt gill epithelia in fish (Hillaby and Randall, 1979; Wilkie, 1997). Increased liver glycogen levels could therefore be a consequence of a defense mechanism against this ammonia induced hypoxic condition, also reported by De Boeck et al. (1997) in common carp exposed to sublethal level of copper. We do not know if the fish in present study were hypoxic, but the fact that anaerobic metabolism was switch on might be a sufficient signal. RSAF might be the only group that had enough energy to cope with ammonia induced oxygen demand.

As expected, $M_{O2Ucrit}$ values for fed and starved fish in control and ammonia exposure were higher than M_{O2}. During exercise, fish require more energy which therefore increases the oxygen consumption. Moreover, aerobic exercise increases vasodilation in the gills, increases blood flow through the gills, and increases in the flow rate of water passing over the gills, and thus facilitates oxygen intake.

6.3.3. Metabolic dynamics of ammonia production- excretion

Among control groups, fed fish shows higher plasma ammonia levels and excretion rates than starved fish, reflecting the effect of SDA (Fig.6.6A). Under HEA (from day 4 onwards), this trend reversed and starved fish had higher plasma values, most likely because they were not able to excrete sufficiently. Our results demonstrate that under RS, fed fish increased excretion while in starved fish excretion remained low (Fig.6.4A). This corresponds with the lack of increase in M_{O2} in starved fish, possibly because these fish were not ventilating as much compared to fed fish. Under ES, fish have to swim and thus were forced to increase ventilation, which increased M_{O2} and ammonia excretion (Alsop and Wood, 1997; McKenzie et al., 2003; Wicks et al., 2002). However, in present study, the

increase in M_{O2} and ammonia excretion in starved fish was not sufficient, as there was still an increase in plasma ammonia level.

The low ammonia- excretion rates of the starved fish represent the *endogenous* or maintenance fraction (Alsop and Wood, 1997; Wood, 1993). The spectacular differences between the fed and starved fish are due to the *exogenous* fraction, or the portion not retained from the absorbed food. Ingested proteins are hydrolyzed into amino acids which are then absorbed and utilized for protein synthesis. Excess amino acids in protein metabolism are broken down, leading to high ammonia production. In addition to increased ammonia excretion rates, fed fish retained significantly higher protein stores during the third and fourth week of exposure in the present study (Table 6.3).

There is now growing evidence about Rhesus (Rh) protein involvement in ammonia transport processes in gills of freshwater fish (Hung et al., 2007; Nakada et al., 2007a; Nawata et al., 2007; Nawata et al., 2010a; Weihrauch et al., 2009; Wright and Wood, 2009). Studies have shown that high external ammonia exposure in fish triggers an upregulation of Rh (*Rhbg* and *Rhcg2*) mRNA expression in gills and tissues and thus enhances the ammonia excretion rate (Braun et al., 2009b; Hung et al., 2007, Nawata et al., 2007; Nawata and Wood, 2009; Tsui et al., 2009; Zimmer et al., 2010). Similarly, feeding has been shown to increase *Rhcg2* transcript levels in juvenile trout gills under high water ammonia level (Zimmer et al., 2010) resulting in higher ammonia excretion rate in fed fish. Both these effects seem to be confirmed by our ammonia excretion data, and this can explain the fact that fed fish are better equipped to tolerate HEA compared to starved fish. Molecular evidence of increased Rh protein expression in gold fish is at present still lacking. Alternatively, the increased excretion rate in ammonia-exposed gold fish may have been a consequence of gill damage, as ammonia is known to cause gill damage at lethal concentrations (Benli et al., 2008; Hillaby and Randall, 1979; Randall and Tsui, 2002; Smart, 1976; Wilkie, 1997). Although this effect cannot be ruled out in the present study, but water ammonia concentrations were sub-lethal and hence are dubious to have caused significant gill damage. In general, HEA in the present study induced increases in ammonia excretion rate in both feeding treatments (Fig.6.4A, 6.4B). This could be related to stress induced by chronic exposure of ammonia. Under these circumstances the production of ammonia is stimulated by an elevation of cortisol (Knoph and Olsen, 1994) and the consequent alteration of hepatic metabolism (Beaumont et al., 2003; Hogstrand et al., 1999).

It was noted that the ammonia excretion rate recovered to pre-exposure rates at the end of the exposure period, as did the plasma ammonia levels (although plasma ammonia levels remained significantly higher in starved fish). At this moment muscle ammonia levels increased, and more prominently in starved fish (Fig.6.8). This indicates a switch from excretion to accumulation or perhaps local use of protein in muscle, as this accumulation coincided with the significant drop in muscle protein. The rise of muscle plasma accumulation during last exposure period (3-4 weeks)

indicates a toxicity effect in long run (De Boeck et al., 2006; Shingles et al., 2001). Additionally, effect on plasma and muscle ammonia content in control and exposed fish were amplified when imposed to exhaustive swimming, (Fig.6.6B, 6.8B). Similar effects have previously been reported for numerous fish species following exercise treatments (Kieffer, 2000; Milligan, 1996; Suski et al., 2006; Wood, 1991). The increment likely occurred as adenylates were broken down to inosine-monophosphate (IMP) and NH_4^+ as part of anaerobic energy generation and has been well documented in rainbow trout (Driedzic and Hochachka, 1976; Mommsen and Hochachka, 1988; Suski et al., 2007; Wang et al., 1994).

6.3.4. Swimming performance

The swimming performance (U_{crit}) was reduced when gold fish were subjected to 10 days and 21 days of ammonia exposure, with a more pronounced effect in starved fish. Likewise, both rainbow trout and coho salmon (*Oncorhynchus kisutch*) showed a significant decrease in critical swimming velocity under HEA (Shingles et al., 2001; Wicks et al., 2002). Various theories have been proposed for ammonia induced reduction in $U_{crit.}$.The depolarization of muscle membrane potential by the replacement of K^+ with NH_4^+ in higher environmental ammonia load has been hypothesized to limit swimming performance (Beaumont et al., 1995a, 2000a,b; McKenzie et al., 2003; Randall and Tsui, 2002; Wicks et al., 2002). Many studies have reported a negative effect of plasma and tissue ammonia accumulation on swimming performance of fish (Beaumont et al., 1995b; Day and Butler, 1996; Shingles et al., 2001; Wicks et al., 2002). In the present work we noted considerable high plasma ammonia in fasted fish after 10 and 21 days of exposure, exactly when swimming capacity in these fish was reduced most. It connotes that decreases in swimming performance correlate better with plasma than muscle ammonia since muscle ammonia increased much slower and plateaus after 3 weeks of exposure. Moreover, we found negative and significant correlations between U_{crit} and plasma ammonia accumulation ($Y= -0.071X + 5.136$, $R^2= 0.038$, $P < 0.05$). Thus, our findings are in tune with the various hypotheses proposed above and subsequently confirmed that swimming performance not only influenced by ammonia but also by feed deprivation. In brief, it can be said that reduction in U_{crit} among exposed-starved fish was either individual or combined consequence of plasma lactate, plasma ammonia and muscle glycogen stores dynamics.

Table 6.2

The effects of feeding status, ammonia exposure and exercise and their interactions on physiological parameters in gold fish.

Treatment	**Feed**		**Ammonia**		**Exercise**		**Feed* Ammonia**		**Feed* Exercise**		**Ammonia* Exercise**		**Feed*Ammonia* Exercise**	
	F value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value	*F* value	*P* value
Non-invasive measurements														
MO_2	99.05	0.000	0.768	0.600	65.76	0.000	0.319	0.945	262.1	0.000	1.574	0.144	5.11	0.000
Ammonia excretion	184.22	0.000	19.67	0.000	317.54	0.000	3.21	0.003	54.31	0.000	3.26	0.003	2.082	0.047
Ammonia Quotient	65.12	0.000	5.078	0.000	0.443	0.507	0.373	0.917	40.99	0.000	1.222	0.292	2.190	0.036
Plasma metabolities														
Ammonia	4.617	0.033	16.57	0.000	1208.9	0.000	14.25	0.000	2.654	0.105	5.237	0.000	6.137	0.000
Lactate	22.35	0.000	15.67	0.000	1355.5	0.000	5.134	0.000	22.804	0.000	0.692	0.678	3.241	0.003
Liver metabolities														
Glycogen	7.77	0.006	3.074	0.004	13.78	0.000	0.646	0.718	0.240	0.625	0.201	0.985	0.427	0.885
Protein	13.69	0.000	8.70	0.000	7.33	0.007	2.23	0.0468	0.463	0.497	0.340	0.935	0.082	0.99
Lipid	4.085	0.048	7.48	0.07	2.21	0.36	0.246	0.973	0.157	0.692	0.102	0.998	0.081	0.99
Muscle metabolities														
Ammonia	4.086	0.048	21.46	0.000	281.8	0.000	2.450	0.019	0.092	0.762	0.352	0.929	0.47	0.989
Glycogen	51.07	0.000	25.37	0.000	81.73	0.000	2.078	0.048	10.25	0.003	2.077	0.048	2.23	0.032
Protein	14.65	0.000	7.63	0.000	8.40	0.004	2.077	0.048	0.269	0.605	0.481	0.848	0.061	1.00
Lipid	12.15	0.001	2.69	0.12	8.35	0.004	0.257	0.969	0.151	0.698	0.261	0.968	0.04	1.00

6.3.5. Ammonia quotient

In general AQ was higher in fed than starved fish (Fig.6.5) due to the higher ammonia excretion among fed fish. Moreover, in starved fish AQ remained quite stable and increase at day 4 and 10 in RS fish, is caused by the drop in M_{O2} and not because of an increase in ammonia excretion rate. Despite this it seems that they did not turn down N-use, AQ was around or slightly over 0.33 meaning that some anaerobic protein degradation occurred. This is confirmed by the use of their protein stores, with reduction in protein stores occurring sooner in starved fish because they lack the supply from food. In ESAF fish, AQ was around or above 0.33 during all the periods. It indicates that they heavily rely on protein use, both aerobically and anaerobically. Thereby, it suggests that protein metabolism is stimulated by HEA and is also controlled by food availability. In this context, it has been reported that the activity of glutamine synthetase is up-regulated during feeding, most likely as a means to convert the potentially toxic ammonia (accumulated internally as a consequence of HEA) into glutamine. The non-toxic glutamine acts as storage of ammonia until circumstances allow the fish to excrete excess ammonia by diffusion across the gills or the glutamine is utilized in other metabolic pathways (Vedel et al., 1998; Wicks and Randall, 2002a,b). Feeding also up-regulate pathways that utilize NH_4^+ directly in the production of amino acids. These include alanine, aspartate, glutamate and glutamine. All four of these amino acids could act as temporary depots of toxic NH_4^+(Wicks and Randall, 2002b). Once this pathway is up-regulated, it is possible that it will help protect fish from both self-intoxification and/or ammonia toxicity milieu. Similar consequence was evident from the study of Vedel et al. (1998) on rainbow trout exposed to HEA (500µM). Perhaps, the above mentioned mechanism could also be the reason for higher plasma ammonia level in starved fish.

Thus, our hypothesis that fed fish are more susceptible to HEA and to exhaustive swimming than starved fish, is rejected. From our findings we can conclude that fish fed have developed better ways to deal with ammonia exposure and exhaustive exercise than starved fish.

Table 6.3

Glycogen, protein and lipid content in liver (wet tissue) and muscle (wet tissue) in gold fish under different treatments.

Treatments			Glycogen (mg/g)		Protein (mg/g)		Lipid (mg/g)	
			Liver	Muscle	Liver	Muscle	Liver	Muscle
Control	Routine swimming	Fed	309.2 ± 22.51^{a}	7.15 ± 0.43^{b}	200.4 ± 11.7^{d}	227.8 ±16.1^{c}	47.1 ± 4.58^{a}	28.5 ± 3.22^{abc}
		Starved	282.1 ± 28.3^{a}	6.49 ± 0.44^{b}	188.2 ± 8.9^{cd}	208.1 ± 12.2^{bc}	44.6 ± 4.99a	25.7 ± 3.75^{abc}
	Exhaustive swimming	Fed	281.9 ± 24.1^{ab}	6.13 ± 0.47^{e}	182.4 ± 12.1^{cd}	204.9 ± 11.8^{c}	44.1 ± 4.77^{a}	25.6 ± 3.50^{a}
		Starved	258.6 ± 26.3^{ab}	5.59 ± 0.43^{cde}	180.1 ± 10.8^{cd}	192.6 ± 10.5^{bc}	41.1 ± 4.4^{a}	22.8 ± 3.22^{a}
3 hrs	Routine swimming	Fed	305.4 ± 25.5^{ab}	7.10 ± 0.37^{b}	186.3 ± 8.7^{cd}	215.0 ± 17.9^{bc}	50.1 ± 6.65^{a}	32.6 ± 3.17^{bc}
		Starved	296.9 ± 25^{a}	6.40 ± 0.33^{b}	192.6 ± 10.43^{cd}	202.6 ± 15.2^{bc}	45.4 ± 4.50^{a}	25.7 ± 2.94^{abc}
	Exhaustive swimming	Fed	274.0 ± 23.4^{ab}	5.92 ± 0.35^{de}	183.0 ± 10.7^{cd}	208.0 ± 8.4^{c}	48.9 ± 4.50a	27.2 ± 2.49^{a}
		Starved	245.9 ± 20.5^{a}	5.49 ± 0.39^{cde}	189.9 ± 13.3^{d}	195.6 ± 13.4^{bc}	41.9 ± 4.1^{a}	21.0 ± 2.25^{a}
12hrs	Routine swimming	Fed	294.7 ± 17.4^{a}	6.92 ± 0.42^{b}	189.6 ± 12.6^{cd}	217.6 ± 16.8^{bc}	50.7 ± 6.53^{a}	30.7 ± 4.37^{abc}
		Starved	302.5 ± 32^{a}	6.42 ± 0.41^{b}	176.8 ± 15.4^{bcd}	213.4 ± 14.8^{c}	47.0 ± 3.59a	23.6 ± 3.17^{abc}
	Exhaustive swimming	Fed	270.1 ± 15^{ab}	6.08 ± 0.4^{e}	193.0 ± 10.4^{d}	196.2 ± 14.0^{bc}	48.9 ± 4.52^{a}	25.6 ± 2.69^{a}
		Starved	258.0 ± 19.1^{ab}	5.51 ± 0.41^{cde}	175.7 ± 17.4^{cd}	184.5 ±19.0^{abc}	39.4 ± 3.7^{a}	22.1 ± 3.33^{a}
1 day	Routine swimming	Fed	322.0 ± 21.5^{abc}	6.85 ± 0.39^{b}	196.4 ± 9.3^{cd}	220.2 ± 13.9^{b}	49.9 ± 4.56^{a}	33.5 ± 3.62^{c}
		Starved	318.4 ± 22.7^{ab}	6.27 ± 0.32^{b†}	173.4 ± 11.2^{bcd}	197.0 ± 16.9^{b}	47.6 ± 3.50^{a}	26.0 ± 1.93^{abc}
	Exhaustive swimming	Fed	302.0 ± 25.4^{ab}	5.97 ± 0.45^{de}	176.8 ± 7.3^{cd}	200.1 ± 9.7^{c}	44.6 ± 2.85^{a}	24.2 ± 3.31^{a}
		Starved	277.8 ± 15.4^{ab}	4.87 ± 0.41^{cde}	165.8 ± 10.8^{bcd}	187.0 ± 12.9^{bc}	40.8 ± 4.8^{a}	19.6 ± 3.54^{a}
4 days	Routine swimming	Fed	338.7 ± 20.8^{abc}	6.60 ± 0.37^{b*}	188.1 ± 16.0^{cd}	225.0 ± 14.4^{bc}	48.7 ± 3.11^{a}	27.1 ± 4.28^{abc}
		Starved	309.7 ± 18.3^{ab}	6.02 ± 0.61^{b†}	167.4 ± 7.07^{abcd}	203.9 ± 17.8^{bc}	43.6 ± 4.47^{a}	21.8 ± 3.20^{abc}
	Exhaustive swimming	Fed	300.6 ± 22.5^{ab}	5.08 ± 0.44^{cde}	168.3 ± 7.0^{bcd}	186.3 ± 14.1^{c}	41.4 ± 3.91^{a}	24.7 ± 1.72^{a}
		Starved	282.7 ± 29.2^{ab}	3.34 ± 0.27^{ab}	160.4 ±12.9^{bcd}	177.1 ± 19.5^{abc}	36.3 ± 4.1^{a}	19.3 ± 3.21^{a}
10 days	Routine swimming	Fed	334.0 ± 28.3^{abc}	6.39 ± 0.35^{b}	182.1 ± 8.42^{bcd}	198.7 ± 16.0^{bc}	41.7 ± 4.50^{a}	26.0 ± 3.23^{abc}
		Starved	327.2 ± 19.7^{ab}	5.85 ± 0.33^{b†}	159.2 ± 8.44^{abc}	181.1 ± 11.1^{bc}	44.5 ± 3.39^{a}	20.2 ± 3.15^{ab}
	Exhaustive	Fed	322.6 ± 22.8^{ab}	4.92 ± 0.47^{cde}	165.8 ± 5.48^{bcd}	185.8 ± 11.0^{bc}	39.8 ± 4.50^{a}	23.0 ± 3.39^{a}

	swimming	Starved	313.9 ± 31^{ab}	2.48 ± 0.24^{a}	153.8 ± 14.8^{abcd}	166.8 ± 13.2^{abc}	38.4 ± 4.9^{a}	18.4 ± 2.96^{a}
21days	Routine swimming	Fed	402.4 ± 20.0^{c}	5.87 ± 0.41^{b}	176.6 ± 6.43^{bcd}	185.4 ± 13.8^{bc}	42.3 ± 4.66^{a}	22.3 ± 2.31^{abc}
		Starved	312.7 ± 20.5^{ab}	4.00 ± 0.32^{a†}	148.3 ± 7.1^{ab}	143.3 ± 9.6^{a}	39.0 ± 4.03^{a}	18.9 ± 3.88^{a}
	Exhaustive swimming	Fed	335.3 ± 33.5^{b}	4.64 ± 0.58^{cde}	156.9 ± 5.5^{bcd}	178.6 ± 8.2^{bc}	37.0 ± 4.64^{a}	19.9 ± 3.53^{a}
		Starved	299.3 ± 21.7^{ab}	2.11 ± 0.23^{a}	130.3 ± 5.5^{ab}	150.2 ± 7.35^{a}	33.5 ± 3.3^{a}	16.4 ± 3.61^{a}
28 days	Routine swimming	Fed	387.3 ± 18.2^{bc*}	4.04 ± 0.29^{a}	164.4 ± 8.54^{abc}	179.5 ± 12.1^{b}	41.4 ± 3.90^{a}	21.4 ± 2.49^{abc}
		Starved	308.8 ± 23.2^{ab}	3.88 ± 0.3^{a†}	135.1 ± 7.34^{a}	136.8 ± 10.6^{a}	38.6 ± 3.90^{a}	20.3 ± 2.74^{ab}
	Exhaustive swimming	Fed	305.4 ± 27.8^{ab}	4.33 ± 0.53^{bc}	147.3 ± 7.77^{ab}	165.6 ± 9.98^{b}	34.7 ± 3.80^{a}	18.1 ± 2.17^{a}
		Starved	290.6 ± 20^{ab}	2.16 ± 0.29^{a}	118.9 ± 6.9^{a}	132.2 ± 9.95^{a}	35.2 ± 2.3^{a}	16.9 ± 3.75^{a}

Values are mean ± S.E. Different superscript letter denote significant differences ($P < 0.05$). Asterisk (*) and dagger (†) denotes the significant differences between routine and exhaustive swimming conditions for feeding and starved groups respectively.

6.4. CONCLUSIONS

We have determined the sub lethal effect of ammonia among fed and feed deprived goldfish under routine and exhaustive mode of swimming. The results of present study show that chronic ammonia exposure (28 days) altered various physiological and biochemical activities through interactive effects with feeding levels and swimming activities. Plasma ammonia, plasma lactate, tissue ammonia, oxygen consumption rate at U_{crit} were considerable elevated in ammonia exposed starved group of fish. Moreover, swimming performance, oxygen consumption rate at routine swimming, nitrogen excretion, weight gain (%) and energy store was reduced in starved fish compared to the fed and control group. These ammonia induced metabolic changes were further exacerbated when fish were forced to swim at high speed and more severe effects were noted in feed deprived fish. Exhaustive swum fish were more susceptible to high environmental ammonia than routine swum fish. Fed fish were less affected than fasted fish by elevated ambient ammonia as well as by higher swimming speeds. During last exposure periods (21-28 days) some physiological parameters (eg. MO_2, ammonia excretion, U_{crit}, plasma ammonia) showed signs of recovery while other parameters (growth, energy stores, anaerobic metabolism) started to show changes from that period onwards. It indicates, despite that these fish have ways to cope with ammonia exposure, at the end prolonged exposure exhausted them, and more prolonged exposure to this level (the Flemish water quality guideline) would probably be detrimental even for this very resistant species.

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Chapter 7

Combined effects of high environmental ammonia, starvation and exercise on hormonal and ionregulatory response in goldfish (*Carassius auratus* L.)

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Abstract

Due to eutrophication, high environmental ammonia (HEA) has become a frequent problem in aquatic environments, especially in agricultural or densely populated areas. During certain periods, e.g. winter, feed deprivation may occur simultaneously in natural waters. Additionally, under such stressful circumstances, fish may be enforced to swim at a high speed in order to catch prey, avoid predators and so on. Consequently, fish need to cope with all these stressors by altering physiological processes which in turn are controlled by genes expression. Therefore, in the present study, ammonia toxicity was tested in function of nutrient status (fed versus starved) and swimming performance activity (routine versus exhaustive). Goldfish, a relatively tolerant cyprinid, were exposed to HEA (1mg/L; Flemish water quality guideline for surface water) for a period of 3h, 12h, 1 day, 4 days, 10 days, 21 days and 28 days and were either fed (2% body weight) or starved (kept unfed for 7 days prior to sampling). Results showed that the activity of Na^+/K^+-ATPase in the gills was stimulated by HEA and disturbance in ion balance was obvious with increases in plasma [Na^+], [Cl^-] and [Ca^{2+}] after prolonged exposure. Additionally, osmoregulation and metabolism controlling hormones like cortisol and thyroid hormones (T3 and T4) were investigated to understand adaptive responses. The expression kinetics of growth, stress and osmo-regulatory representative genes such as Insulin-like growth factor 1(IGF-I), growth hormone receptor (GHR), thyroid hormone receptorβ (THRβ), prolactin receptor (PRLR), cortisol receptor (CR) and Na^+/K^+-ATPase α_3 were examined. Overall effect of HEA was evident since Na^+/K^+-ATPase activity, plasma cortisol, Na^+ and Ca^{2+} concentration, expression level of CR and Na^+/K^+-ATPase α_3 mRNA in fed and starved fish were increased. On the contrary, transcript level of PRLR was reduced after 4 days of HEA; additionally T3 level and expression of GHR, IGF-I and THRβ genes were decreased following 10-21 days of HEA. Starvation, the additional challenge in the present study, significantly increased plasma cortisol level and CR transcript level under HEA compared to the fed exposed and control fish. Furthermore, a remarkable reduction in T3 and mRNA levels of THRβ, IGF-I and GHR genes was observed under starvation. The toxic effects in both feeding treatments were exacerbated when imposed to exhaustive swimming with more pronounced effects in starved fish. This confirms that starvation makes fish more vulnerable to external ammonia, especially during exercise.

Keywords: Ammonia, Starvation, Exercise, Goldfish, Na^+/K^+-ATPase, Thyroid hormones, Cortisol, Insulin-like growth factor 1(IGF-I), Growth hormone, Real Time PCR.

7.1. INTRODUCTION

The accumulation of ammonia is a serious threat for fish culture, especially in aquaria and fish ponds. Toxicity induced by high environmental ammonia (HEA) can impair many physiological functions in fish (da Silva et al., 2009; Mommsen et al., 1999; Wendelaar Bonga, 1997). Alterations in acid-base regulation, ion exchange, thyroid hormones and cortisol levels are some of the physiological compensatory mechanisms that respond to the internal metabolic disorder in response to ammonia stress (Knoph and Thorud, 1996; Wilson and Taylor, 1992).

Gills play a crucial role in hydromineral homeostasis of a fish as chloride cells in branchial epithelium are involved in the uptake of ions from surrounding water (Flik et al., 1994; Perry 1997) and also contain a number of transporters and associated mechanisms that are involved in maintenance of ammonia homeostasis (Avella and Bornancin, 1989; Evans, 1980; Evans et al., 1999; Wilkie, 1997). The most important and extensively studied enzyme is Na^+/K^+-ATPase, present in the basolateral membrane of branchial cells, which has a major role in the transport of sodium ions across gill membranes (Evans et al., 2005; Kultz and Somero, 1995; Wilkie, 1997). Although, the function of Na^+/K^+-ATPase is primarily associated with sodium transport, its importance in ammonia excretion has been implicated since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999). NH_4^+ ions are moved across the apical membrane in exchange of sodium through Na^+/H^+ (NH_4^+) exchanger (Evans et al., 2005; Wilkie, 2002). Therefore, Na^+/K^+-ATPase also provides the driving force for the exchange of Na^+ from the water for ammonia in the fish (Avella and Bornancin, 1989; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997) suggesting that in fish this enzyme may play a major role in excretion of ammonia.

In this study we want to focus on the iono-and osmoregulatory disturbances that can be caused by HEA. Several hormones are involved in controlling ion- and osmoregulation such as cortisol, prolactin (PRL), growth hormone (GH) and thyroid hormones (THs), and are therefore followed during HEA exposure. Cortisol acts to mobilize and synthesize metabolites and, thus, directly as well as indirectly influences many physiological processes during stress in fish (Babitha and Peter, 2010; Vijayan et al., 1997; Wendelaar Bonga, 1997). PRL is produced in the pituitary gland, and is characterized by its function in water and electrolyte balance (Sangiao-Alvarellos et al., 2006). It is equally crucial for metabolism, growth, development, reproduction, behavior and immunoregulation (Power, 2005). In general, PRL initiates its actions through binding to a specific cell surface PRL receptor (PRLR). In fish, thyroid hormones are involved in development, metabolism and osmoregulation, and are synthesized, stored, and released from thyroid follicles (Peter et al., 2000; Peter and Peter, 2009). The key hormone synthesized in these follicles is L-thyroxin (T4), which is metabolized to the more biologically potent 3,5,3′-triiodo-L-thyronine (T3) via deiodinases, mostly in

peripheral tissues. Extensive evidence indicates that most thyroid hormone effects are mediated at the genomic level via binding to the nuclear thyroid hormone receptors (THRs), of which two forms (THRα and THRβ) exist. THRβ has been shown to have higher expression in the muscle and gill compared to THRα (Filby and Tyler, 2007; Power et al., 2001; Swapna and Senthilkumaran, 2007). Moreover, the expression levels of insulin like growth factor-1(IGF-I) and growth hormone receptor (GHR) which are involved in the control of growth, are regulated by circulating THs (Higgs et al., 1982).

Beside ammonia toxicity, food restriction is a natural phenomenon in wild populations of fish and also occurs regularly for cultured fish. Aquaculture species are often subjected to periods of restricted feeding and fasting as management tools for water quality and disease (Robinson and Li, 1999). It has already been demonstrated that nutritional inputs in fish can modulate numerous physiological systems such as metabolism, ionic balances and endocrine functions (Bucking and Wood 2006a, b; Gaylord et al., 2001, 2005; Peterson and Small, 2004; Small et al., 2002; Small, 2005; Small and Peterson, 2005).

Like feeding, swimming is another important physiological activity in fish. Exhaustive swimming has an important effect on the success of prey hunting and predator avoidance. It is largely supported by anaerobic glycolysis in the white muscle (Kieffer, 2000; Kieffer et al., 2001; Milligan, 1996). Under HEA, fish often need to undertake different activities simultaneously. Therefore, it is important to study the interactions between tasks because simultaneous demands may reduce the performance of two tasks or one function may receive higher priority (Andersen and Wang 2003; Fu et al., 2009; Hicks and Bennett, 2004; Li et al., 2010). Hence, it would be interesting to observe how the combined effects of fasting and exercise at HEA could alter hormonal and ion-regulatory responses in fish.

Goldfish (*Carassius auratus*), a cold water fish, is one of the most common ornamental species in the world. Therefore, in the present study, we used juveniles of goldfish as a test organism to examine how this species manipulates its hormones and iono-osmoregulatory process in order to retain body homeostasis when introduced to different stressors such as high ammonia (1mg/L), feed deprivation and exercise at the same time.

Table 7.1

PCR primer sequences, accession numbers, amplicon size, melting temperatures and calculated efficiency.

Gene	Accession no.	Sequence of Primer (5´→ 3´)	Amplicon size (bp)	Melting temperature (T_m)	Calculated efficiency
Target genes					
IGF-I	GU583648	F: CAGGGGCATTGGTGTGA	154	60.6	1.78
		R: GCAGCGTGTCTACAAGC		60.1	
Na^+/K^+-ATPaseα_3	AB062885	F: GGCAAGAGATGGGCCAA	162	60.4	1.89
		R: GCTGGCTCATCTTCGGT		60.0	
THRβ	AY973630.1	F: GATGATTCGGAGGTGGCA	176	60.2	1.85
		R: TTCATCAGCAGCTTGGGC		60.2	
PRLR	AF144012.1	F: AGACAGACCGGTTAGACG	160	59.3	1.82
		R: CGGCTACATTGAACTGGC		59.3	
GHR	AF293417	F: CAGGCAACTTTCCGCCA	207	60.8	2.08
		R: CCACTTCCTGCACCACA		60.6	
CR	EF042099	F: GTGAGACTGCAAGTGTCCAA	156	60	2.12
		R: CTCTCTCTTCACTATGGCCT		60.3	
Reference genes					
β-actin	AB039726	F: ATCCAGGCTGTGCTCTC	150	58.8	1.98
		R: CAGATCACGACCAGCCA		58.8	
18S rRNA	AF047349	F: GGGGCCCAGAGCATTTAC	178	60.2	1.88
		R: CCTCTAGCGGCGCAATAC		60.3	
GAPDH	AM701793	F: ACCAGGACAAGTATGACCC	156	60.1	1.92
		R: TCTTCTGTGTGGCGGTGTA		60.2	

The accession number refers to the registered sequence used from Genbank. F: forward, R: reverse.

7.2. RESULTS

7.2.1. Ion concentrations in plasma

Prolonged HEA led to increased plasma [Na^+] roughly from 10 days onwards in all exposed groups (Table 7.2). No pronounced difference between feeding treatments either for RS (routine swimming) or ES (exhaustive swimming) fish could be noted during any sampling periods, although ES tended to lead to a numerical rise in plasma [Na^+] in fed and starved fish compared to their respective RS groups, which became significant ($P < 0.05$) only in fed group during 21 days of exposure.

No prominent effect of HEA or feed deprivation was evident on [Cl^-] in plasma of RS fish (Table 7.2). These effects became prominent under ES conditions. A significant increment in plasma [Cl^-] among exhaustive-swimming ammonia exposed starved (ESAS) fish was seen after 21 and 28 days compared to routine-swimming ammonia exposed starved (RSAS), control ES and exhaustive-swimming ammonia exposed fed (ESAF) fish.

Plasma [K^+] did not change considerably under HEA or feed deprivation. Moreover, no effect of exercise was observed. Although, the concentration in ammonia exposed starved fish (in both RS and ES) was numerically higher than in fed ones, these differences were statistically insignificant (Table 7.2).

Plasma [Ca^{2+}] concentration was affected ($P < 0.05$) by 21 days of ammonia exposure among routine-swimming ammonia exposed fed (RSAF) fish, the value increased by 61.9% compared to control (Table 7.2). Similarly, in RSAS fish, an increment of 54.4% ($P < 0.05$) and 56.8% ($P < 0.05$) compared to control was seen after 10 and 21 days. ESAF fish had respectively 70.2% ($P < 0.05$) and 85.08% ($P < 0.01$) higher [Ca^{2+}] than the control after 10 and 21 days (Table 7.2). Moreover, in ESAS fish, the considerable increment ($P < 0.05$) was evident from 10 days of exposure onwards. Exhaustive exercising increased the plasma [Ca^{2+}] in both fed and starved fish compared to RS groups but the differences remained statistically insignificant. Also, no considerable difference could be noted among feeding treatments (either in RS or ES conditions) during any sampling period.

7.2.2. Activity and gene expression profile of Na^+/K^+-ATPase in gill tissue

In the present experiment, Na^+/K^+-ATPase activity in fish gills was affected by ammonia exposure (Fig.7.1). In RSAF and RSAS fish, the activity was temporary increased ($P < 0.05$) after 10 days of exposure compared to their respective controls. However, this increase was no longer significant after 21 and 28 days. During ES, this increase persisted longer: activity of Na^+/K^+-ATPase in fed fish augmented 45.6% ($P < 0.01$) and 41.8% ($P < 0.01$) respectively after 10 and 21days of exposure compared to control and during the same exposure periods an increment of 46.5% ($P < 0.05$) and 45.6% ($P < 0.05$) was evident among starved fish . No considerable effect of starvation on Na^+/K^+-ATPase activity was evident from our study although under ES conditions, the value for fed fish

tended to be numerically higher than for starved ones. A distinct increased ($P < 0.05$) activity among ESAF fish was detected compared to RSAF after 4 days of exposure.

Effect of HEA and exercise was evident on transcript level of Na^+/K^+-ATPase α_3 mRNA in gills tissue (Fig. 7.2). It increased in both RSAF and RSAS after 4 and 10 days HEA in contrast to their respective controls and then slowly recovered. The transcript level in both ESAF and ESAS reached a peak ($P < 0.001$; $P < 0.01$) after 4 days of exposure and remained significantly higher compared to their respective controls after 10 and 21 days of exposure. Exhausting exercise stimulated the expression level in both fed and starved fish during 4, 10, 21 and 28 days, the transcript levels were significantly higher than in their respective RS groups. Expression level did not appear to be affected by feed deprivation.

7.2.3. Plasma cortisol level and expression profile of the cortisol receptor gene

Overall, effects of HEA, starvation and exercise were noteworthy in plasma cortisol level of fish (Fig. 7.3). Starvation led to increased cortisol levels in ES fish, both compared to RS (over the entire period) and compared to the fed ES (control, 3h, 12h, 4days, 28days). This difference between feeding treatments was virtually absent in RS fish (except at day 4). Ammonia exposure itself only had moderate effects. Plasma cortisol concentration in RSAF fish at 10 days was significantly ($P < 0.05$) higher (30.4%) compared to its control. After 4, 10 and 21 days of exposure, the values became remarkably higher in RSAS compared to its control. Under ES, the level of cortisol in fed fish increased ($P < 0.05$) after 4 days and remained higher till 21 days while in starved fish an increment ($P < 0.05$) as compared to control was seen after 4 and 10 days of exposure.

mRNA concentration of the CR gene was effected by HEA, starvation and exercise (Fig.7.4). HEA induced an increment in CR mRNA at days 10 and 21 in fed fish under both swimming regimes ($P < 0.05$). This increment occurred earlier in starved fish, with increases from day 4 up to day 21 in RS fish and from day 4 up to day 28 in ES fish ($P < 0.05$ or $P < 0.01$). The difference between feeding treatments ($P < 0.05$) under RS was evident only at day 4 and under ES the difference ($P < 0.05$) was observed at day 4, 10 and 21. When forced to swim at higher speed, expression level amplified in both fed (during 4 and 10 days) and starved fish (from day 1 onwards) compared to respective RS groups.

Table 7.2

Ions concentration (mmol/L) in plasma of goldfish under different treatments.

Treatments			$[Na^+]$	$[Cl^-]$	$[K^+]$	$[Ca^{2+}]$
Control	Routine swimming	Fed	118 ± 2.36	109 ± 3.93	4.28 ± 0.39	1.11 ± 0.16
		Starved	121 ± 2.58	115 ± 3.79	4.15 ± 0.58	1.34 ± 0.19
	Exhaustive swimming	Fed	113 ± 4.86	108 ± 3.84	4.53 ± 0.36	1.12 ± 0.15
		Starved	120 ± 2.26	115 ± 4.58	4.59 ± 0.48	1.14 ± 0.15
3 hrs	Routine swimming	Fed	107 ± 12.12	107 ± 5.43	4.40 ± 0.43	1.00 ± 0.28
		Starved	112 ± 5.55	114 ± 4.31	4.44 ± 0.30	1.30 ± 0.21
	Exhaustive swimming	Fed	111 ± 7.39	109 ± 4.52	4.53 ± 0.38	1.15 ± 0.14
		Starved	115 ± 6.62	116 ± 4.28	4.58 ± 0.53	1.26 ± 0.12
12hrs	Routine swimming	Fed	115 ± 15.77	103 ± 3.73	4.45 ± 0.26	1.07 ± 0.13
		Starved	109 ± 5.89	105 ± 5.18	4.51 ± 0.28	1.29 ± 0.17
	Exhaustive swimming	Fed	113 ± 4.70	108 ± 5.46	4.72 ± 0.54	1.14 ± 0.16
		Starved	118 ± 5.38	113 ± 4.89	4.68 ± 0.64	1.23 ± 0.18
1 day	Routine swimming	Fed	114 ± 12.23	108 ± 5.77	4.77 ± 0.38	1.11 ± 0.08
		Starved	112 ± 4.80	107 ± 4.27	4.83 ± 0.38	1.41 ± 0.17
	Exhaustive swimming	Fed	119 ± 5.00	112 ± 5.09	4.96 ± 0.63	1.37 ± 0.14
		Starved	120 ± 4.50	114 ± 5.84	4.84 ± 0.61	1.42 ± 0.20
4 days	Routine swimming	Fed	121 ± 3.37	114 ± 4.59	4.71 ± 0.44	1.34 ± 0.22
		Starved	126 ± 4.22	113 ± 5.00	5.12 ± 0.38	1.35 ± 0.15
	Exhaustive swimming	Fed	124 ± 6.39	117 ± 4.48	5.18 ± 0.46	1.52 ± 0.20
		Starved	122 ± 4.63	121 ± 3.41	5.54 ± 0.48	1.70 ± 0.22
10 days	Routine swimming	Fed	133 ± 4.44*	111 ± 5.48	5.17 ± 0.51	1.48 ± 0.22
		Starved	131 ± 3.07*	115 ± 5.88	5.45 ± 0.53	2.07 ± 0.19*
	Exhaustive swimming	Fed	134 ± 3.65*	118 ± 6.74	5.70 ± 0.56	1.91 ± 0.21*
		Starved	132 ± 6.25	125 ± 3.07	6.00 ± 0.39	1.99 ± 0.22*
21days	Routine swimming	Fed	136 ± 4.85*†	115 ± 4.38	5.30 ± 0.47	1.80 ± 0.20*
		Starved	132 ± 3.31*	118 ± 3.35†	5.52 ± 0.59	2.10 ± 0.17*
	Exhaustive swimming	Fed	149 ± 3.34**	121 ± 3.35	6.10 ± 0.43	2.07 ± 0.19**
		Starved	143 ± 4.39*	132 ± 3.25*•	6.26 ± 0.21	2.07 ± 0.16*
28 days	Routine swimming	Fed	137 ± 4.79*	121 ± 5.01	5.23 ± 0.60	1.64 ± 0.23
		Starved	133 ± 3.59*	124 ± 3.28†	5.29 ± 0.52	1.82 ± 0.18
	Exhaustive swimming	Fed	145 ± 4.65*	123 ± 3.77	5.56 ± 0.39	1.76 ± 0.18
		Starved	140 ± 3.94*	136 ± 3.16*•	5.86 ± 0.39	1.97 ± 0.15*

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$).

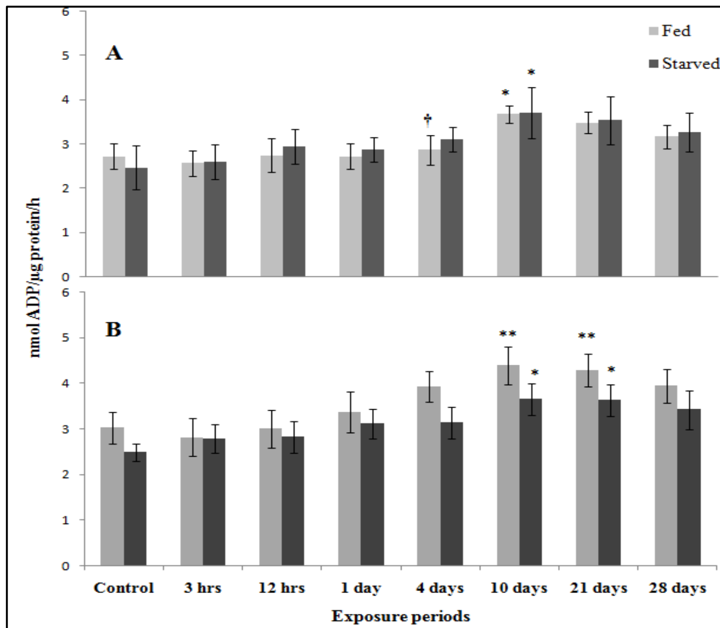

Figure.7.1. Na^+/K^+-ATPase activity (nmol ADP/µg protein/h) in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†P < 0.05).

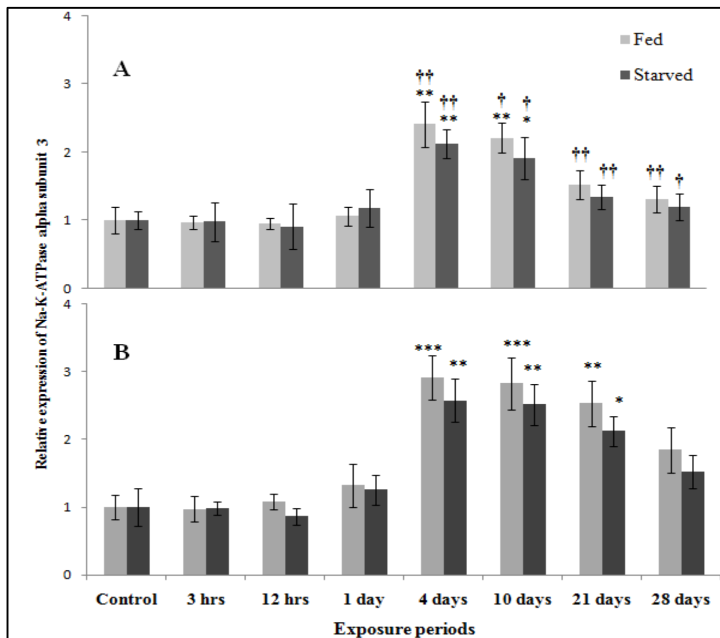

Figure.7.2. Relative expression of Na^+/K^+-ATPase α_3 gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01; ***P < 0.001), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†P < 0.05; †† P < 0.01).

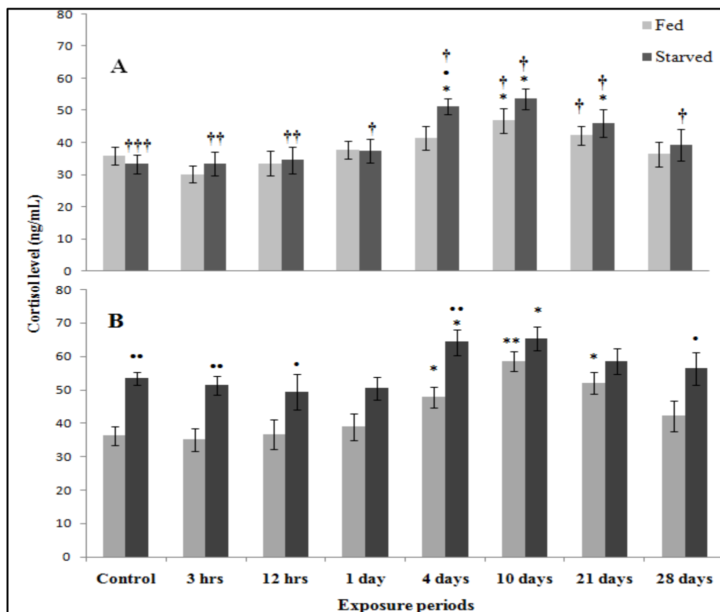

Figure. 7.3. Cortisol level (ng/mL) in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), bullet (•) indicates a significant difference between fed and starved fish at the same sampling period (•P < 0.05; ••P < 0.01), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†P < 0.05; †† P < 0.01; †††, P < 0.001).

7.2.4. Thyroid hormone concentration in plasma and expression of the thyroid hormone receptor β gene in gill tissue

Exhaustive swimming had a profound effect on plasma [T3], values were significantly reduced in control and exposed fish compared to their respective RS groups (Fig.7.5). Effects of HEA and feeding only become apparent after prolonged exposure, with more severe reductions in starved fish. In RSAF, ammonia exposure induced a significant reduction (32.44%) during the last exposure period (28 days) while in RSAS fish the reduction became prominent after 21 (31.11%; $P < 0.05$) and 28 days (42.51%; $P < 0.05$). Effect of feed deprivation in RS fish was seen after 21 days of exposure where [T3] was reduced by 34.41% ($P < 0.05$) in starved fish as compared to fed fish, in ES fish it was notable ($P < 0.05$) after 10 and 21 days.

Contrary to plasma [T3], no clear effect of HEA on plasma [T4] was observed either in fed or starved fish during RS (Fig.7.6). Only a significant reduction in ESAS fish compared to control and ESAF was noticed at 10 and 21 days. This lead to a decrease ($P < 0.05$) in ESAS T4 levels compared to RSAS during 10 and 21 days of exposure, although in general ES did not alter plasma T4 level compared to RS.

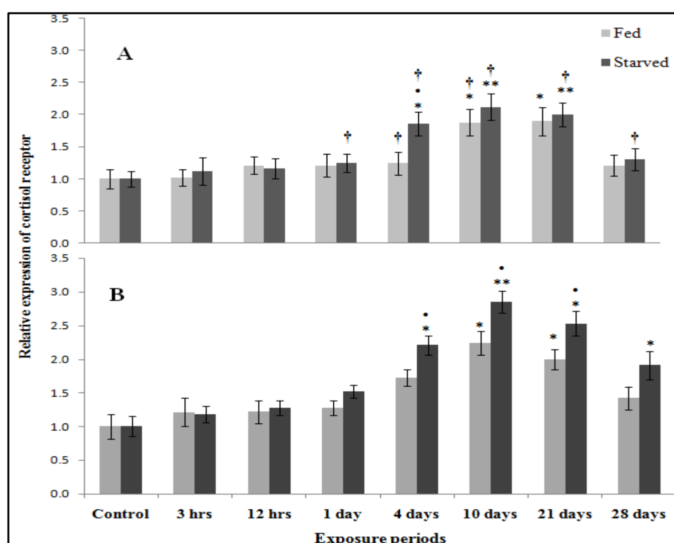

Figure. 7.4. Relative expression of cortisol receptor gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$), dagger (†) denotes the significant differences between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$).

THRβ mRNA quantity in gill tissue was altered in response to HEA, starvation and exercise (Fig.7.7). A strong reduction ($P < 0.05$) in RSAF and RSAS fish from10 days onwards compared to their respective controls was reported. In ESAF and ESAS fish a prominent reduction ($P < 0.05$) was observed from day 4 up to day 21. Recovery occurred at day 28 for RSAF, ESAF and ESAS. Effect of feed deprivation during RS was noted only at day 10, the THRβ mRNA expression level in starved fish was 43.33% lower ($P < 0.05$) than in fed fish. During ES, a similar feeding effect was evident after 10 and 21 days of exposure, where values in starved fish were 47.72% ($P < 0.01$) and 50% ($P < 0.01$) lower than in fed ones respectively. In general, ES tended to decrease the THRβ mRNA

transcript level in both ammonia exposed fed and starved fish compared to their respective RS groups.

7.2.5. Expression pattern of other hormone (receptor) genes

PRLR mRNA quantity in gill tissue was reduced during HEA at 10 days in RSAF and at 4 and 10 days in RSAS ($P < 0.05$, Fig.7.8). Exercising fed and starved fish at higher speed reduced the expression level compared to their respective controls from day 4 till day 10 for fed fish and from day 4 to day 21 for starved fish. Effect of ES compared to RS was observed only among starved fish, mRNA level was reduced considerably during 4, 10 and 21 days of exposure. No effect of feed deprivation was noted either during RS or ES.

Hepatic IGF-1 mRNA level was significantly affected by HEA and starvation. Significant reductions in RSAF compared to control was observed at the end of the exposure period (28 days) and occurred a bit earlier (from 21 days onwards) when fish were subjected to ES (Fig.7.9). Also in RSAS and ESAS hepatic IGF-1 mRNA declined significantly compared to their controls from day 21 and day 10 onwards respectively. A significant difference in IGF-1 mRNA level among feeding treatments was seen on day 21 during RS and on day 10 during ES. Swimming at exhaustion speed did not cause any significant change in IGF-1 mRNA transcript in both fed and starved ES fish compared to their respective RS groups.

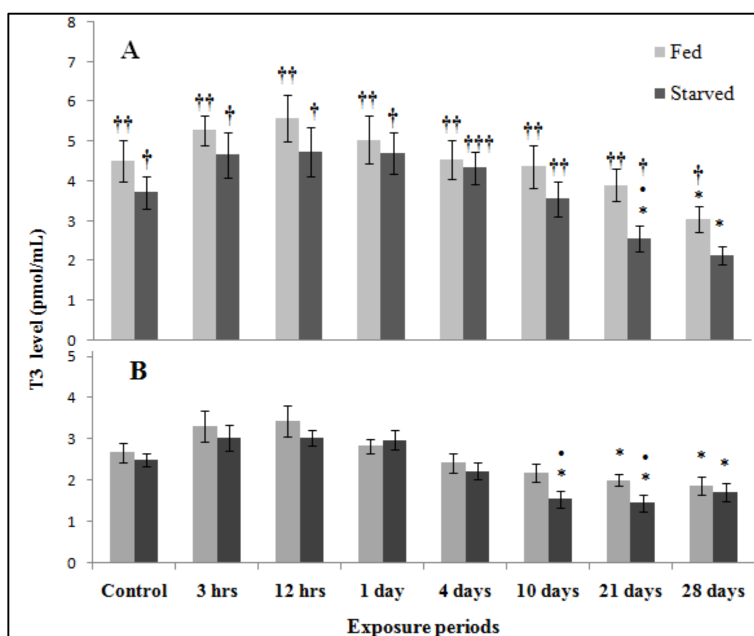

Figure. 7.5. T3 plasma concentrations (pmol/mL) in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$; †† $P < 0.01$; †††, $P < 0.001$).

Changes in hepatic GHR mRNA abundance are shown in Fig.7.10. HEA slowly decreased the transcript level in both RSAF and RSAS fish compared to their respective controls which resulted in a significant decrease at 28 days ($P < 0.05$). Under ES, considerable reduction ($P < 0.05$) as compared

to controls was noted at 28 days for fed and at 21 and 28 days for starved fish respectively. A difference ($P < 0.05$) between feeding treatments was noted on day 28 and from day 21 onwards for RS and ES fish respectively.

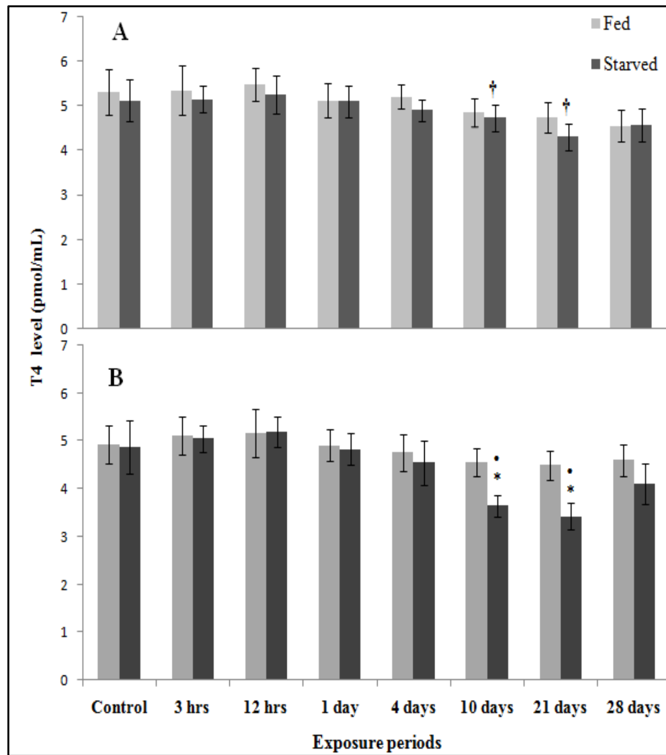

Figure. 7.6. T4 plasma concentrations (pmol/mL) in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$).

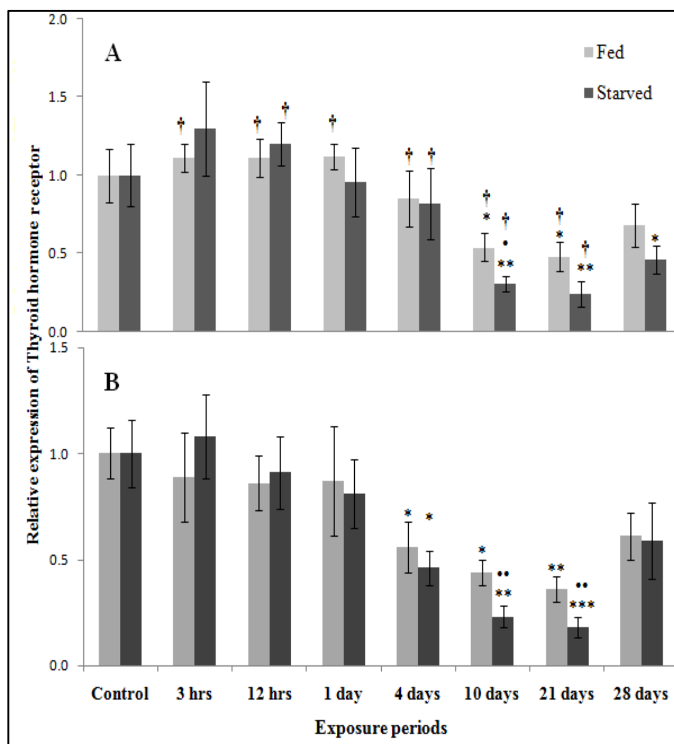

Figure. 7.7. Relative expression of thyroid hormone receptor β gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$; ***$P < 0.001$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$; ••$P < 0.01$), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$).

7.3. DISCUSSION

7.3.1. Ion-regulatory response

In the present study, goldfish showed an increase in plasma [Na^+] among all groups roughly from day 10 onwards (Table 7.2). Na^+ uptake across the gill epithelium is favoured by the electrochemical gradient assisted by Na^+/K^+-ATPase located in the basal membrane of gill epithelial cells, which work in series with apical membrane proton pumps (Avella and Bornancin, 1989). NH_4^+ is known to interfere with apical and basolateral transport of Na^+ by altering transport by the carrier mediated Na^+/ NH_4^+ exchanger (McDonald and Prior, 1988; Wilson and Taylor, 1992) or/and by substituting K^+ on the basolateral Na^+/K^+-ATPase (Lin and Randall, 1991; Randall et al., 1999; Walsh, 1998). The increase in plasma [Na^+] was accompanied by an increase in branchial Na^+/K^+-ATPase activity. Interestingly, only a transient increment in the Na^+/K^+-ATPase activities was observed after 10 to 21days of ammonia exposure in fed and starved fish (Fig.7.1). This increase in Na^+/K^+-ATPase activity was accompanied and preceded by an activation of the transcript level of Na^+/K^+-ATPase α_3, the predominant isoform in gills of teleosts (Bystriansky and Schulte, 2011; D'Cotta et al., 2000; Lee et al., 1998; Madsen et al., 2009; Pressley, 1992; Richards et al., 2003), from 4 days of HEA onwards until the activity of the transporter returned to normal levels (Fig.7.2). Similarly, Alam and Frankel (2006) reported increases in gill Na^+/K^+-ATPase activity of silver perch (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*) with increasing ammonia concentrations (0-5 mg/L). The activity in African catfish (*Clarias gariepinus*) was significantly increased when reared in high ammonia water (1084µM) for 34 days (Schram et al., 2010). Furthermore, the present study showed that the activation Na^+/K^+-ATPase is time dependent with signs of recovery after prolonged exposure. Therefore, time dependent and detailed studies on the interactions between Na^+ transport and HEA should be included in future work.

Despite the fact that we observed only a transient increment in Na^+/K^+-ATPase activity in ammonia exposed fed and starved fish, we noted a more continuous augmentation in plasma [Na^+] (Table 7.2). It indicates that Na^+/K^+-ATPase is not the sole ion-regulatory process responding to ammonia exposure. Perhaps different systems are also affected depending on the duration of HEA. H^+-ATPase present in the gill epithelium actively excludes H^+ across the membrane and generates a negative potential inside the apical membrane which drives Na^+ inwards via a sodium channel. Therefore, investigation of H^+-ATPase activity may be crucial for future experiments as well.

Concomitant with the increase in plasma [Na^+], an increase in [Cl^-] was only seen in starved fish swimming at high speed. After exposure, NH_3 would rapidly diffuse down its partial pressure gradient from water to fish (gill), followed by binding with proton. This then stimulates the hydration reaction of CO_2 resulting in proton and bicarbonate formation. This bicarbonate could than be exchanged for Cl^- at the apical side via Cl^-/HCO_3^- exchanger (Evans et al., 1999), leading to increased

uptake and increased plasma levels. Our finding suggests that branchial uptake mechanisms are perhaps activated by starvation, and increased CO_2 production due to the exercise may be a second prerequisite for the increase in plasma [Cl^-]. Exercise accounted to have a pronounced effect on Na^+/K^+-ATPase activity (Fig.7.1B): under ES, fed fish showed numerically higher activities than starved ones. As described in our previous work (Sinha et al., 2012), ESAF were able to maintain relatively low plasma ammonia concentrations in contrast to ESAS fish. In this condition, it is assumed that as the external ammonia concentration increases, the excretion of NH_4^+ among fed fish (during ES) is enhanced by an increased, energized Na^+/K^+-ATPase mediated export.

The temporary increase in plasma [Ca^{2+}] observed in the ammonia exposed fish corroborates with earlier findings in Atlantic salmon (*Salmo salar*) that showed significantly higher Ca^{2+} level in plasma when exposed to 1.2-6.3 µg/L NH_3-N for 2 weeks (Knoph and Olsen, 1994) and 225µg/L NH_3-N for 2 to 3 days (Knoph and Thorud, 1996). The observed effect might be a consequence of reduction in the capacity of Stannius corpuscles to secrete stanniocalcin, leading to a loss in the control of calcium exchange (Person-Le Ruyet et al., 2003). It was seen that in rivers with high NO_2^- and ammonia concentrations, the Stannius corpuscles of roach (*Rutilus rutilus*) showed signs of hyperactivity or even presented pathological features (Lopez et al., 1989).

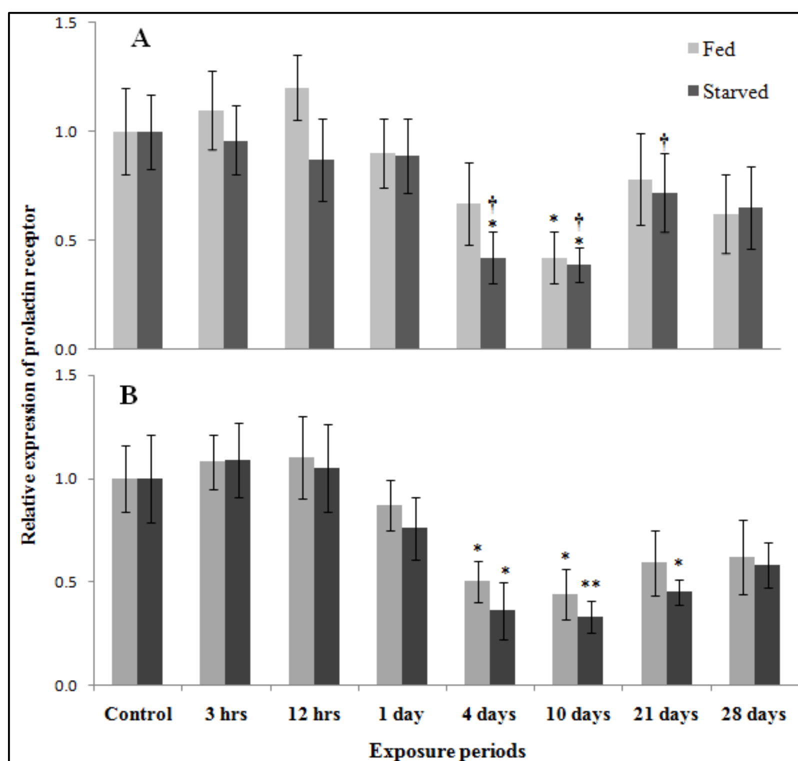

Figure. 7.8. Relative expression of Prolactin receptor gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$), dagger (†) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (†$P < 0.05$).

7.3.2. Hormonal control of iono-osmoregulation

In our study, the time course of the changes in cortisol and prolactin seem to respond primarily to the disturbances in ion-regulation by ammonia exposure. An increase in cortisol level was seen when goldfish were exposed to high ammonia (4 to 10 days) (Fig.7.3). This may correspond to the transitory increment in plasma [Ca^{2+}] as cortisol has been shown to stimulate calcium uptake (Flik and Perry, 1989). Moreover, cortisol has been shown to increase the ion-transporting capacity of the gills by the proliferation of chloride cells (Goss et al., 1992) and to increase the level of ion-transporting enzymes (Flik and Perry, 1989; Lin and Randall, 1993; McCormick and Bern, 1989; Shrimpton and McCormick, 1999; Wilson et al., 2002). In our study, we observed some, but not all, of the specific actions of increased cortisol levels: Na^+/K^+-ATPase activity and plasma [Na^+], [Ca^{2+}] increased in the exposed groups. The action of cortisol in the gills is probably mediated by the intracellular CR. In the current study, up-regulation of CR among ammonia exposed fish (from 4-10 days) corresponds with the increment of plasma cortisol level (Fig.7.3, 7.4). Extensive research efforts have been done on CR in aspects of smolting, salinity acclimation and reproduction (Shrimpton and McCormick, 1999; Stolte et al., 2008), but not on ammonia stress, nutritional status and exercise. Overall, our study confirms other reports suggesting that the number and mRNA quantity of gill CR is strongly correlated with the capacity of cortisol to stimulate gill Na^+/K^+-ATPase *in vitro* and *in vivo* (Shrimpton et al., 1994; Shrimpton and McCormick, 1999), indicating that the regulation of CR is physiologically relevant during disruption of ionic balance.

Besides its ion-regulatory function, cortisol is also involved in metabolic processes in fish as evident from our study; the level was strongly influenced by a period of starvation (Fig.7.3). Prior to and after exhaustive exercise, cortisol concentration in starved fish was higher than in fed fish suggesting that the starved fish under HEA are more stressed than fed fish. It corroborates with the finding in rainbow trout where starvation for 30 days induced increases in plasma cortisol (Vijayan and Moon, 1992). A similar effect was noted for gobies (*Gillichthys mirabilis*) (Kelley et al., 2001), channel catfish (Peterson and Small, 2004) and largemouth bass (*Micropterus salmoides*) (Gingerich et al., 2010). Therefore, it connotes that cortisol plays an important role in fish during periods of fasting, but its exact role and mechanism has yet to be clearly defined (Gingerich et al., 2010; Van der Boon et al. 1991).

The osmo-regulatory process in fish is also driven by PRL (Evans, 2002; Lee et al., 2006a; Seale et al., 2006), which initiates its actions through binding to the PRLR. It shows an inhibitory control over branchial Na^+/K^+-ATPase activity (Madsen and Bern, 1992; Pickford et al., 1970), limits branchial permeability to water and ions (Evans, 2002; Wendelaar Bonga et al., 1990), and reduces chloride cell numbers and activity (Foskett et al., 1982). Correspondingly, the reduction in PRLR

mRNA abundance during 4-10 days of ammonia threat might have resulted in high activity and expression level of Na^+/K^+-ATPase.

T4 and T3 are the principal thyroid hormones known for their metabolic and osmo-regulatory actions in fish. Effect of HEA on plasma T4 level was very little, suggesting that hormone release from the thyroid gland was not much altered. But a significant reduction in plasma [T3] in both feeding treatments under HEA suggests that ammonia-induced stress decreases the process of 5'-monodeiodination in peripheral tissues. Similarly, a reduction in plasma [T3] in European sea bass (*Dicentrarchus labrax*) was observed following ammonia threat (0.493 mg/L NH_3-N) over a period of 61days (Dosdat et al., 2003). Rearing turbot in high ammonia water (3.35-4.27 mmol/L) for 7 days resulted in significant reduction in plasma [T3] with a mild effect on [T4] (Person-Le Ruyet et al., 1998). In addition, we observed that the HEA induced reduction in plasma [T3] was intensified during starvation (Fig. 7.5), signifying that besides ammonia exposure, nutritional state is another factor affecting thyroid function in fish. Our results are in tune with the finding of Gaylord et al. (2001) who demonstrated that in channel catfish plasma thyroid hormones were modulated by feed deprivation. De Pedro et al. (2003) also demonstrated decreased circulating [T3] and [T4] in tench (*Tinca tinca*) in response to short-term food deprivation (7 days). Additionally, we investigated the expression of THRβ mRNA in gill tissue. Looking at the results of THRβ mRNA expression, ammonia exposure (after 4-10 days) tends to down-regulate the expression level and more severely among starved fish, corresponding to the levels of plasma [T3] (Fig.7.5, 7.7). The decline in plasma [T3] and gill THRβ mRNA concentration in goldfish (starved fish in particular) is clearly an adaptive response to reduce metabolism and to preserve nutritional reserves, as was also reported by Hemre et al. (1993) for cod (*Gadus morhua*). Reports have suggested that physiological levels of T4 and T3 increase chloride cell size and gill Na^+/ K^+-ATPase activity suggesting a role of THs in ion uptake in the fish (Ayson et al., 1995; Madsen and Korsgaard, 1989; McCormick, 1995; Peter et al., 2000; Trombetti et al., 1996). In contrast, in our experiment, we did not find a pertinent correlation of THs with the ion-regulatory response.

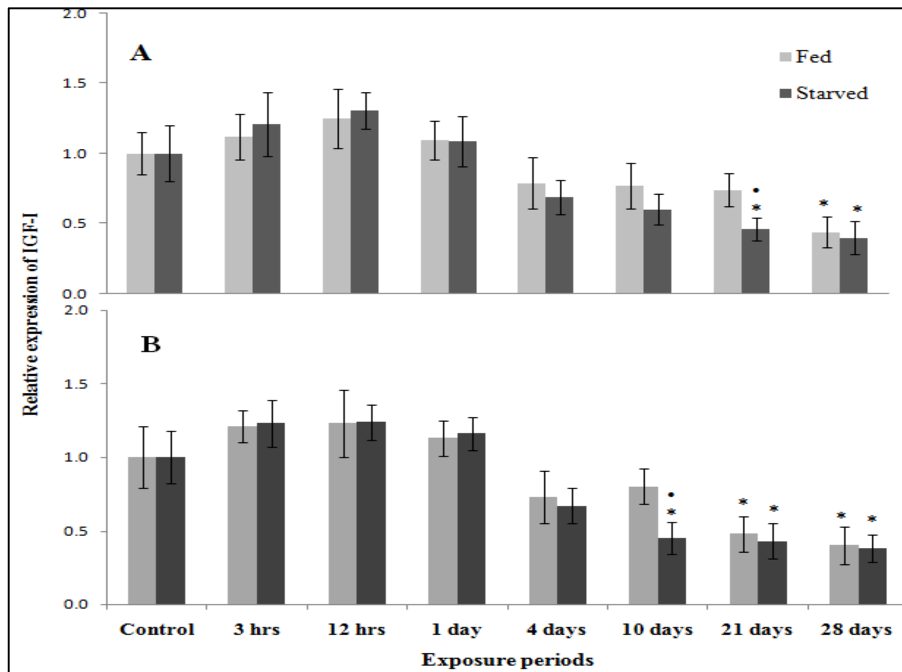

Figure. 7.9. Relative expression of IGF-I gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$).

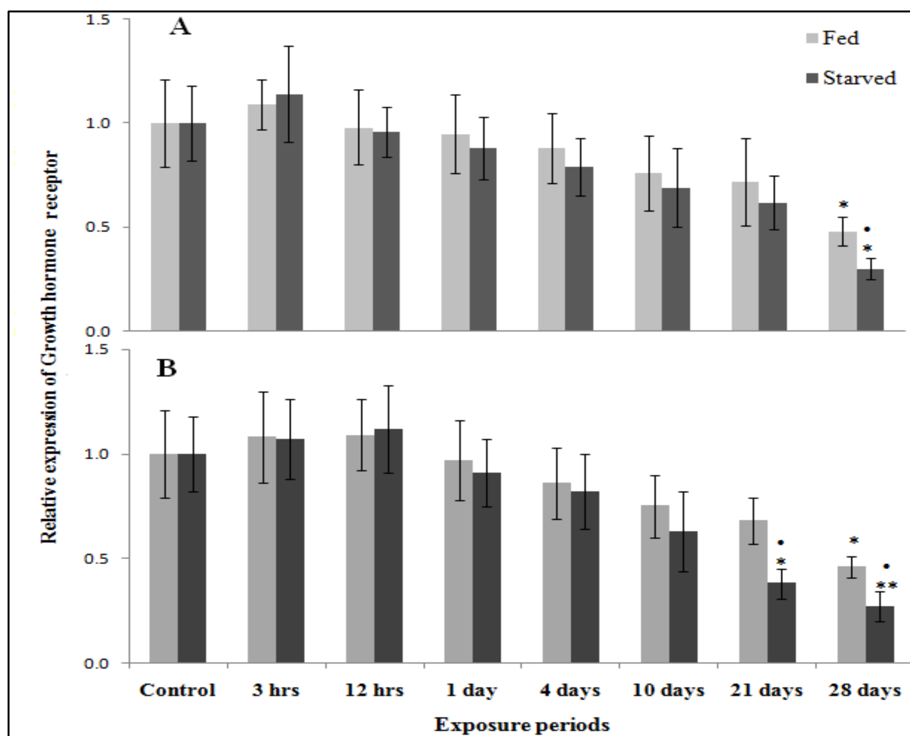

Figure. 7.10. Relative expression of Growth hormone receptor gene in fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$), bullet (•) indicates a significant difference between fed and starved fish for the same sampling period (•$P < 0.05$).

7.3.3. Hormonal control of growth: GH/IGF-I axis

THs regulate animal metabolism also indirectly through interaction with the GH/IGF-I axis (Evans, 2002; McCormick, 2001). Growth of fish is regulated in part by IGF-I, which is produced by the liver in response to GH secretion from the pituitary. It can only be expected that nutritional status is one of the factors that regulate GH/IGF-I axis in fish (Wood et al., 2005). The apparent paradox between increased concentrations of circulating GH and decreased concentrations and mRNA level of IGF-I during starvation has been explained for fish as tissue resistance to GH (Duan and Plisetskaya, 1993; Duan et al., 1995) and reduction in hepatic GH-receptors (GHR) (Ayson et al., 2007; Deng et al., 2004; Fukada et al., 2004; Gray et al., 1992; Pedroso et al., 2006; Perez-Sanchez et al., 1994, 1995, 2002). The interplay of GHR and IGF-I hepatic mRNA expression kinetics under ammonia threat and feed deprivation as seen in the present study is consistent with these reports. Thus, our finding suggests that the expression levels of these genes are not only altered by nutritional status but also by ammonia induced stress.

7.3.4. Implication of exercise

In addition, most of the studied hormones and/or their respective genes were found to be considerably influenced during ES, with more adverse effect in starved fish. This indicates that besides HEA and feed limitation, exhaustive swimming can be a crucial environmental factor manipulating functions of ion- and metabolism regulatory hormones. Overall T3, THRβ, IGF-1 and GHR levels seem to be related more to the changes in energy metabolism in response to ammonia exposure, starvation and/or exhaustive exercise. Previous research (Sinha et al., 2012) showed that long term ammonia exposure resulted in reduced growth and energy stores (protein and glycogen) in goldfish after 3-4 weeks and these effects were more severe in starved fish. This time course was also seen in this study with reduced levels of these respective hormones and receptors, with more pronounced effects in starved and/or exhaustively swimming fish. It is also clear from our study that some of the hormones are related both to changes in iono-regulation and in energy metabolism, as evident from cortisol which was elevated in starved fish over the entire exposure period. In brief it can be stated that, the respective increase or decreases in the level of hormones and their receptors observed in the present study facilitate/induce some of the compensatory responses in goldfish under ammonia threat, starvation and exercise.

7.4. Conclusion

From our experiment we can conclude that exposure to 1mg/L ammonia gives rise to a number of ionic and hormonal changes, and also alters the expression profile of studied genes in goldfish. Many of the iono-regulatory effects became prominent after 4 to 10 days of exposure while the impacts on energy metabolism and growth related hormones/genes became clear after a more prolonged 3 to 4 weeks of exposure. Most of these effects were exacerbated in starved fish. Under HEA, the increase in Na^+/K^+-ATPase activity, plasma cortisol, [Na^+] and [Ca^{2+}], and the expression level of CR and Na^+/K^+-ATPase α_3 genes with concomitant reduction in [T3] and transcript level of GHR, IGF-1, THRβ and PRLR genes could help to explain the tolerance mechanism of goldfish to ammonia stress. The present results also reveal that under ammonia threat fed fish have a physio-biochemical advantage over food deprived individuals since cortisol level, thyroid hormone level, expression level of growth and osmo-regulatory controlling genes (IGF-1,GHR, CR, THRβ) were remarkably influenced during starvation. Furthermore, exercising fish at higher speed only had a mild effect on ionic balance but the primary stress responses and portions of the secondary stress responses exerted by ammonia exposure were strongly influenced, with more spectacular effects on starved fish. The patterns of ionic, hormonal and gene-expression modulation among fed fish observed in the present study indicates that fed fish have developed better ways to deal with ammonia exposure and exhaustive exercise than starved fish.

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Chapter 8

Compensatory responses in Common carp (*Cyprinus carpio*) under ammonia exposure: additional effects of feeding and exercise

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Abstract

Ammonia is an environmental pollutant that is toxic to all aquatic animals. In the present study, the impact of ammonia toxicity was tested on common carp (*Cyprinus carpio*) with a different nutrient status (fed versus starved) and swimming activity (exhaustive swimming versus routine swimming). Fish were exposed acutely (up to 12 h) and chronically (up to 28 days) to high envornmental ammonia (1 mg/L as NH_4Cl) and were either fed (2% body weight) or starved (unfed for seven days prior to the experiment). Energy metabolism was followed by measurements of oxygen consumption and ammonia excretion, changes in muscle and liver energy budget (glycogen, lipid and protein content), critical swimming speed (U_{crit}), and ammonia and lactate accumulation in the plasma. Plasma ion (Na^+, Cl^-, K^+ and Ca^{2+}) concentrations were measured in order to understand the ion-osmo regulatory consequences of the the exposure. Cortisol plays an important role in both the regulation of the energy and of ion-osmoregulation, therefore plasma cortisol was measured. Results show that the excretion rate of ammonia was elevated in the fed fish during ammonia exposure and were able to excrete against the conentration gradient more efficiently than the starved fish. The buildup of ammonia in plasma of ammonia exposed fed fish was much slower than the ammonia exposed starved fish. Level of cortisol in plasma augmented in both the feeding regimes, effect of ammonia exposure and exercise on the cortsiol level was more pronounced in starved fish than the fed fish. Overall, fed fish were less sensitive to external ammonia than starved fish and the toxic effect in both feeding treatments seemed to increase with exercise with more pronounced effect on starved fish. This suggests that fish become more vulnerable to external ammonia during exercise and starvation further intensifies the adverse effect of high ammonia and exercise.

Keywords: Ammonia toxicity, common carp, Ammonia excretion, Feeding, Starvation, Plasma ammonia, Lactate, Exercise.

8.1. INTRODUCTION

The increase in the level of water borne ammonia in natural waters and in culture systems is becoming a serious health issue for aquatic animals. In general, ammonia is produced as a metabolic waste product of protein catabolism and/or generated from decomposition of organic material, industrial emission or produced by micro-organisms. High environmental ammonia (HEA) not only hinders ammonia excretion in fish but can also cause net uptake of ammonia from the environment. Hence, during HEA fish are confronted simultaneously with accumulation of endogenous ammonia and uptake of exogenous ammonia, causing adverse effects on the performance and welfare (Eddy, 2005; Randall and Tsui, 2002). Both acute and chronic ammonia toxicity is well documented for a number of fish species (Ip et al., 2001a; Twitchen and Eddy, 1994). Waterborne ammonia can exist in two forms, the unionized ammonia (NH_3) and the ionized form (NH_4^+) (Randall and Tsui, 2002; McKenzie et al., 2003) with the latter greatly predominating at normal water pHs (Randall and Tsui, 2002; Wajsbrot et al., 1993). The largest part of the ammonia toxicity is caused by the unionized form which can diffuse passively through the gill epithelium due to its lipophilic nature and lack of charge (Benli et al., 2008; Randall and Tsui, 2002). Moreover, it is apparent that fish have acquired ammonia excretory mechanisms that enable them to resist the impact of HEA. Fish can excrete ammonia as NH_3 across the gill into the water providing there is an outwardly directed gradient. Insights into mechanisms of ammonia excretion in fish have been broadened by the discovery of the ammonia transporting function of Rhesus (Rh) glycoproteins (Nakada et al., 2007). These finding are confirmed for a number of species (Nawata et al., 2007; Shih et al., 2008). A new model for ammonia excretion in freshwater fish was proposed by Wright and Wood (2009) suggesting an involvement of several ammonia transporters or exchangers working together as a 'metabolon' in facilitating branchial ammonia excretion and in maintaining ion-regulatory homeostasis.

These ammonia handling mechanisms are the characteristic of the species and are used by the fish to cope with high ammonia exposure. In nature, the presence of other influential environmental factors is very much expected and may modulate the toxicological response of HEA. Majority of the research have focused on the impact of HEA on physiological, metabolic and ion-regulatory responses in fish; examination of such responses when fish are subjected to an assortment of different stressful conditions along with high ammonia load is rather scarce.

In this context, food availability is an important factor in wild populations. It has already been demonstrated that nutritional inputs can modulate numerous physiological and biochemical processes, such as the ionic balance, the metabolism and endocrine functions. Therefore food could influence the coping ability of the fish under ammonia threat. Feeding not only leads to an increased oxygen demand but fish may also experience large fluxes of ammonia into the plasma (Brett and Zala, 1975; Wicks and Randall, 2002a, b; Wicks et al., 2002) as a consequence of ingested proteins catabolism.

Intracellular alkalosis due to production of large quantities of ammonia has been reported to cause many ill effects in fish leading to the reduction in overall performance (Dosdat et al., 2003; Lemarie et al., 2004; McKenzie et al., 2003; Randall and Tsui, 2002). In this scenario, it can be hypothesized that keeping the fish starved during ammonia exposure would be advantageous over feeding. However, this hypothesis was rejected in our previous study (Sinha et al., 2012 a,b) on a highly ammonia resistant fish species, the goldfish (*Carassius auratus*; 96 h LC_{50} value for ammonia is 9mM). We reported that fed goldfish have developed better ways to deal with ammonia exposure and that fed goldfish were less susceptible to ammonia toxicity than starved ones. This present study aimed to investigate if the similar consequences are also applicable for a moderately ammonia-tolerant fish species, the common carp (Cyprinus carpio; 96 h LC_{50} ammonia value is 2.6 mM).

Like feeding, exercise is another important physiological activity of fish. Fish swim constantly, either in the form of low-intensity routine swimming, or high-intensity exhaustive swimming as a means of coping with water currents and predatory, hunting and migratory challenges. During exhaustive swimming (or exercise) fish rely on their energy reserves, and metabolism is largely supported by anaerobic glycolysis in the white muscle (Kieffer, 2000; Kieffer et al., 2001; Milligan, 1996). Furthermore, exhaustive swimming has been shown to intensify endogenous ammonia production and increase internal ammonia levels (Knoph and Thorud, 1996), and is thereby expected to amplify ammonia induced toxic responses in fish. Ammonia accumulation in the plasma has also been implicated in reducing swimming capacity in salmonids (Shingles et al., 2001; Wicks et al., 2002).

Until this date, there is little information on how ammonia exposure, nutrition status and swimming activity influence the performance of the fish. Therefore, the present experiment was designed with two fold purpose (i) to study the different metabolic and physiological changes and (ii) to investigate ion and hormonal responses which occur as compensatory mechanisms to deal with HEA in combination with nutrition status and swimming activity. In this present study, we used juveniles of the common carp (*Cyprinus carpio*) which were exposed to 1 mg/L of ammonia and were subjected to periods of starvation and exhaustive swimming.

To achieve our goals we measured the ammonia excretion rate, plasma ammonia and lactate concentration, and ammonia quotient (AQ). We also examined critical swimming speed (U_{crit}), the oxygen consumption rates (MO_2) as well as cortisol levels. Glycogen, lipid and protein level was also measured in muscle and liver tissue as indicators of available energy stores. The result of present work will offer a better insight in how carp deploy their biological processes when subjected to different ecological stressors (HEA, starvation and exercise) at a time.

8.2. RESULTS

8.2.1. Oxygen consumption

After one day, a temporary increment in oxygen consumption of both exposed fed and exposed starved fish was observed when swum at lowest speed (Fig. 8.1A). Oxygen consumption at high swimming speeds was elevated above M_{O2} for all the groups during most of the exposure periods ($P <$ 0.05; Fig. 8.1B). At high speeds, oxygen consumption rates for fed fish increased ($P < 0.05$) at 12 h compared to their control while in starved fish such increments ($P < 0.05$) was noted on day 1 and day 10. The distinct effect of feeding became apparent during 3/4U_{crit} with higher values for fed fish compared to starved fish under control and HEA conditions ($P < 0.05$).

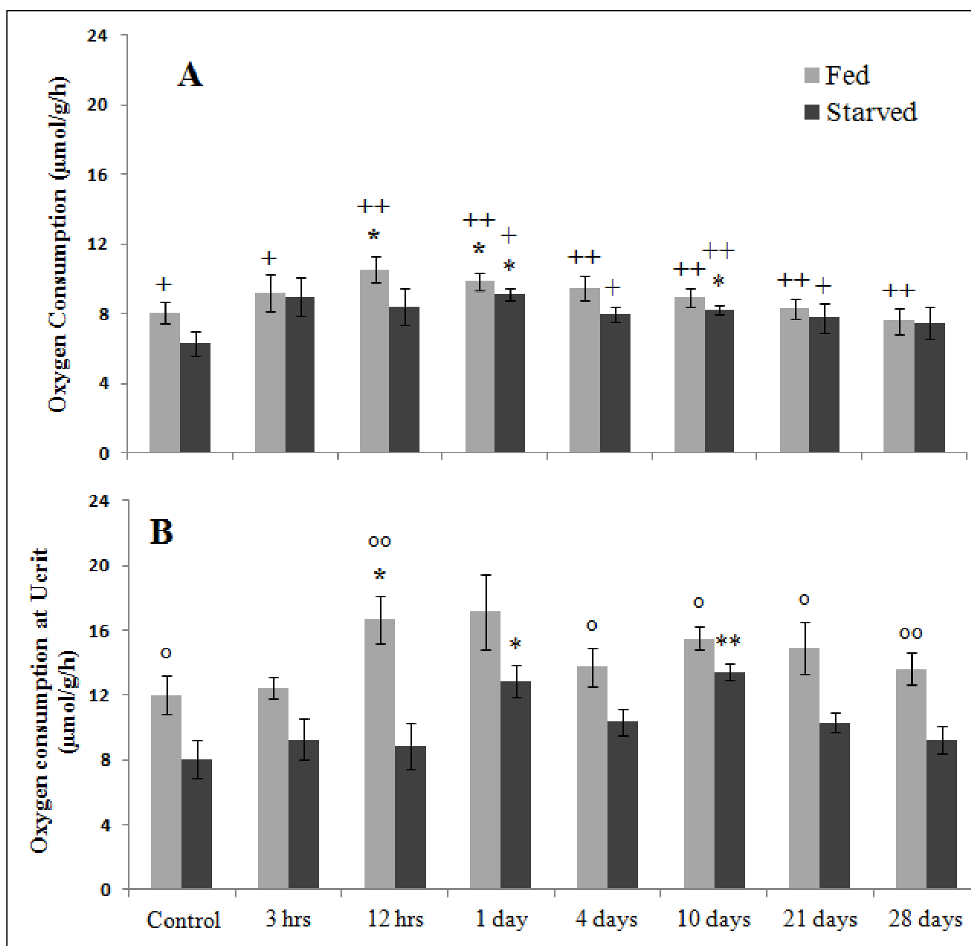

Figure 8.1. Oxygen consumption rates in fed and starved fish swum at **(A)** minimum speed (MO_2) **(B)** 3/4 U_{crit} (MO_{2Ucrit}). Values are means ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$, ^{oo} $P < 0.01$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart($^{+}P < 0.05$, $^{++}P < 0.01$).

8.2.2. Ammonia excretion

The ammonia excretion rate in the RSAF group was elevated ($P < 0.01$-0.001) from day 4 onwards and remained higher than the control untill the end of exposure period (Fig. 8.2). A slight increment was also seen among the RSAS group as compared to its respective control but it was not as prominent as for RSAF fish. An almost similar pattern was noticed when both feeding groups were subjected to ES. Exercising the fish at high speed had a profound effect on ammonia excretion rates: the values for all experimental groups augmented considerably ($P < 0.05$-0.001). The distinct effect of feeding on ammonia excretion rate was seen after 4 and 21 days during RS while during ES, fed fish excreted significantly higher ammonia than starved fish throughout the exposure period. Also under control conditions, excretion rate in fed fish was higher than starved fish.

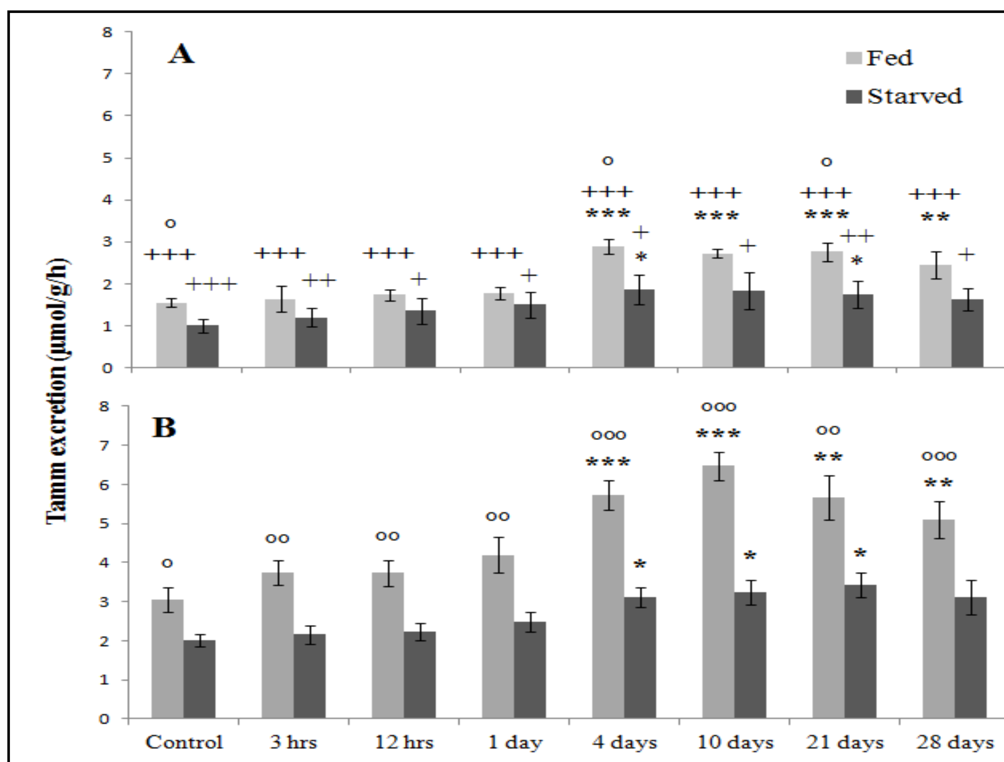

Figure. 8.2. The ammonia excretion rate of fed and fasted fish **(A)** at routine performance, **(B)** at exhaustive performance. Values are means ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$, ^{oo} $P < 0.01$ and $^{ooo}P < 0.001$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart($^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}$ $P < 0.001$).

8.2.3. Ammonia quotient

The AQ in RSAF fish increased considerably from day 4 day onwards ($P < 0.05$) compared to the control, clearly indicating the effect of HEA (Fig.8.3). Similarly, when swum at higher speed, AQ values were elevated in fed fish and were significantly higher than the control after 4 and 21 days of

exposure. The value for RSAS and ESAS fish increased numerically during 4-21 days but remained statistically insignificant compared to their respective controls. No prominent effect of feeding was reported on AQ among RS and ES fish except during 28 days of exposure in RS condition. Swimming at higher speed only had a very mild effect on the AQ.

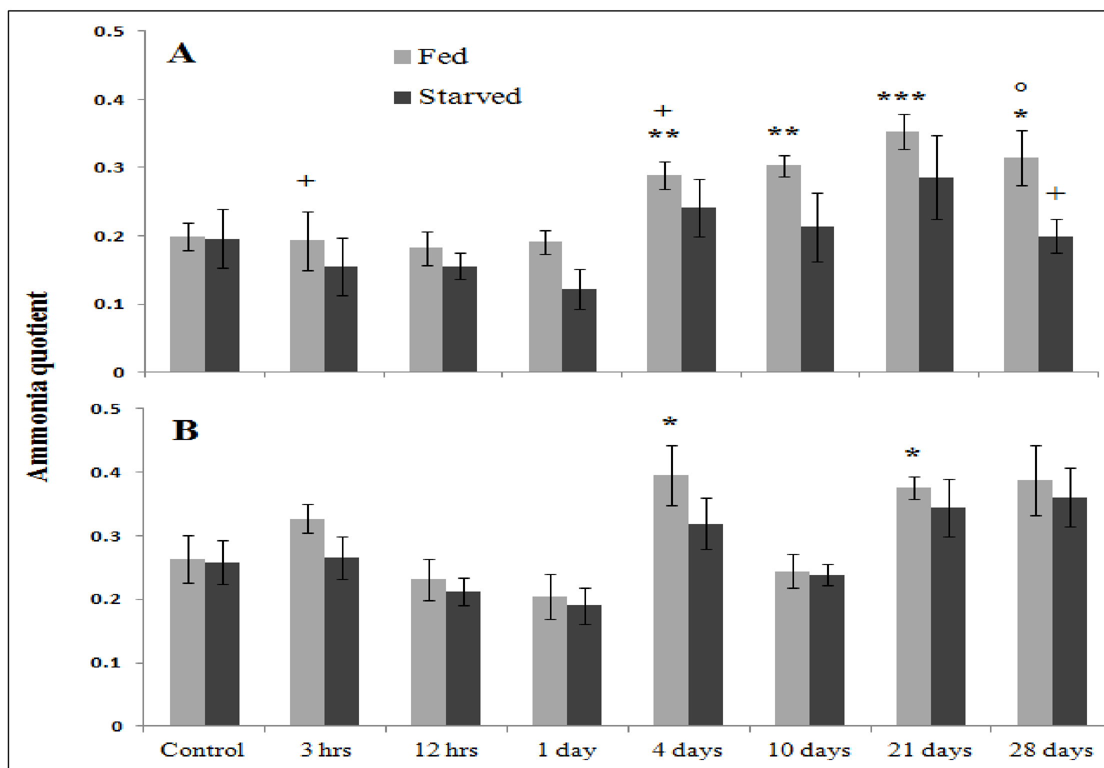

Figure 8. 3. Ammonia quotient values in fed and starved fish during **(A)** routine swimming and **(B)** exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart($^{+}P < 0.05$).

8.2.4. Critical swimming speed

It is apparent from the present study that HEA has no significant effect on the swimming performance of either fed fish or starved fish (Fig. 8.4). However, U_{crit} tended to reduce compared to their respective control in both feeding groups from day 4 onwards. This effects was more intense in starved exposed fish.

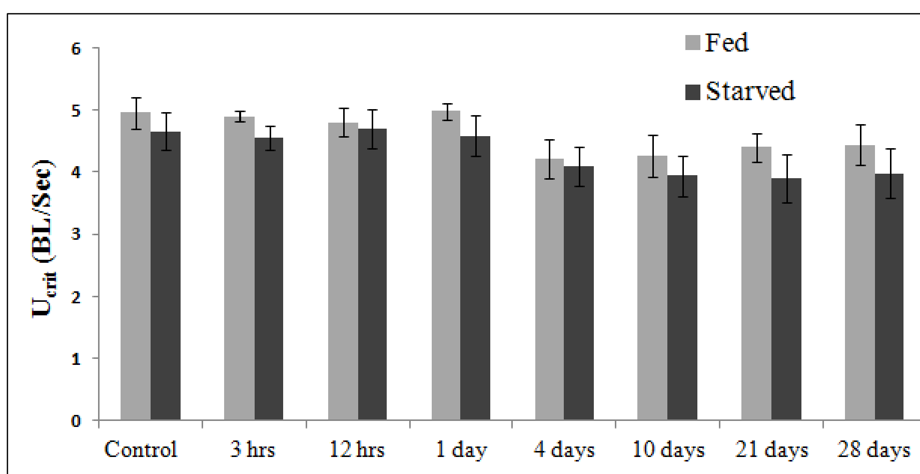

Figure 8.4. Critical swimming speeds in fed and starved fish exposed to ammonia. Values are mean ± S.E.

8.2.5. Plasma metabolities

Plasma ammonia

The level of ammonia in plasma of RSAF fish was significantly elevated after 4 and 10 days of exposure and was 78% and 75% above control levels respectively (Fig. 8.5). A similar trend was noted for the ESAF group where the increment ($P < 0.05$) continued untill 21 days of HEA. The effect of HEA on RSAS and ESAS fish was evident from day 4 onwards and persisted untill the end of experimental period ($P < 0.05$ or 0.01 or 0.001). The effect of feeding was also notice in both RS and ES groups. In the RS fish, starved fish accumulated substantially more ammonia ($P< 0.05$, 0.001) than fed ones from day 4 untill day 21. Such difference was evident from day 1 onwards in the ES group. Swimming at higher speed significantly elevated the ammonia level in fed fish at day 10 while in starved fish such effect was observed at day 21 and 28.

Plasma lactate

Lactate level in fish plasma was affected by ammonia exposure (Fig. 8.6). In RSAF fish the level was increased ($P < 0.05$) from 12 h exposure onwards relative to their control with a transient reduction at 1 day of exposure. A significant increment was also noted in ESAF and ESAS respectively at 10 and 28 days of exposure. The lactate content was not altered by feeding regime (except at day 1 in RS). However, a prominent effect of exercise was noted with higher lactate levels in both control and ammonia exposed groups under ES.

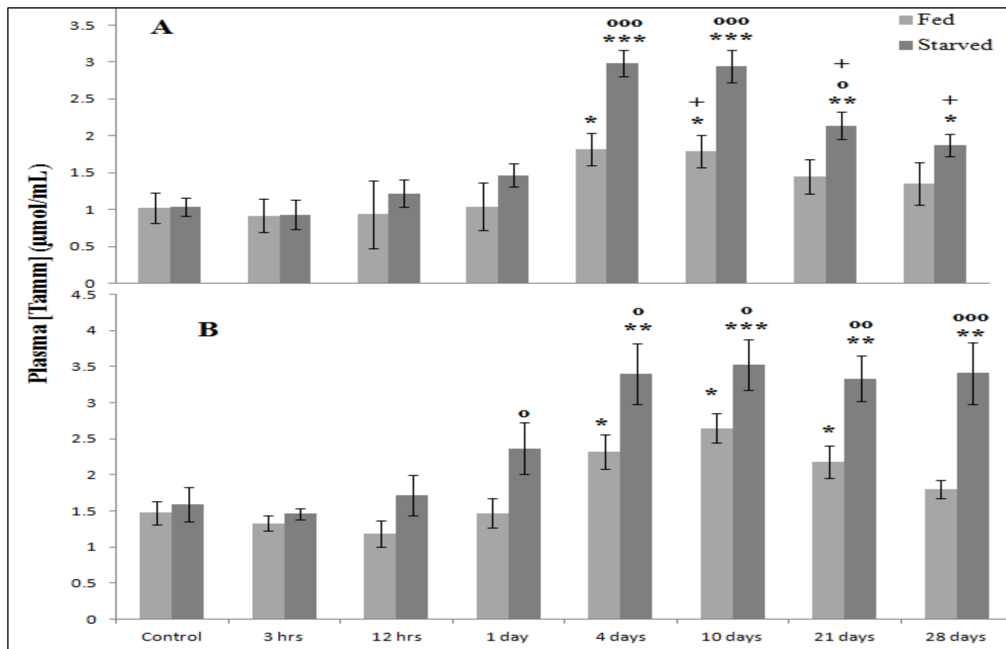

Figure 8.5. The accumulation of ammonia in the plasma of fed and fasted fish **(A)** at routine performance, **(B)** at exhaustive performance. Values are means ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$, ^{oo} $P < 0.01$ and $^{ooo}P < 0.001$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart($^{+}P < 0.05$).

Plasma cortisol

Overall, effects of HEA, starvation and exercise were present in plasma cortisol level of fish (Fig. 8.7). HEA induced a transient increment in cortisol level in RSAF fish after 4 and 10 days and in the ESAF group after 10 days. The effect of HEA was more prominent among starved fish; a significant increment was noted in RSAS fish from day 10 onwards which continued untill the end of exposure period. This increment occurred earlier in ESAS fish and became significant from 12 h onwards and remained elevated higher untill 28 days. The difference between feeding treatments ($P < 0.05$) under RS was evident at 12 h, 21 days and 28 days, and under ES the difference ($P < 0.05$) was observed only at day 1 with higher levels ($P < 0.05$) of cortisol in plasma of starved fish compared to the fed fish. When forced to swim at higher speed, the cortisol level augumented mainly in starved fish.

Plasma ions

HEA, feeding regime and swimming activities had only a very gentle effect on the conentration of different plasma ions. The concentration of Na^{+}, K^{+}, Ca^{2+} and Cl^{-} remained statistically similar between control and experimental groups except for a very few time points (see table 1).

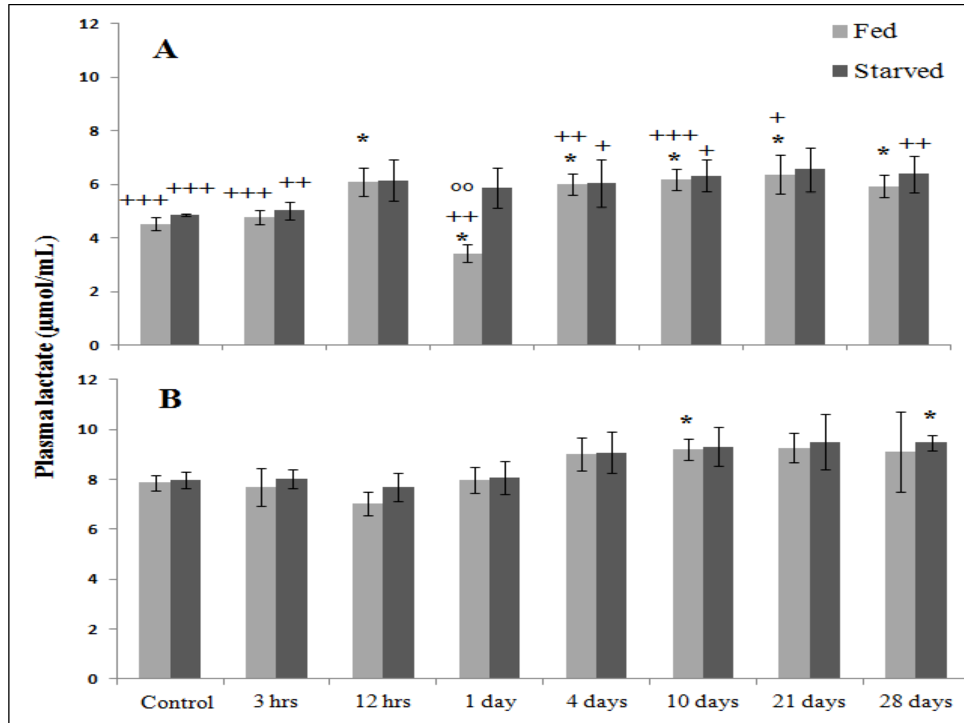

Figure 8.6. Lactate level in the plasma of fed and fasted fish **(A)** at routine performance, **(B)** at exhaustive performance. Values are means ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period(^{oo} $P < 0.01$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart ($^{+}P < 0.05$, $^{++}P < 0.01$ and $^{+++}$ $P<0.001$).

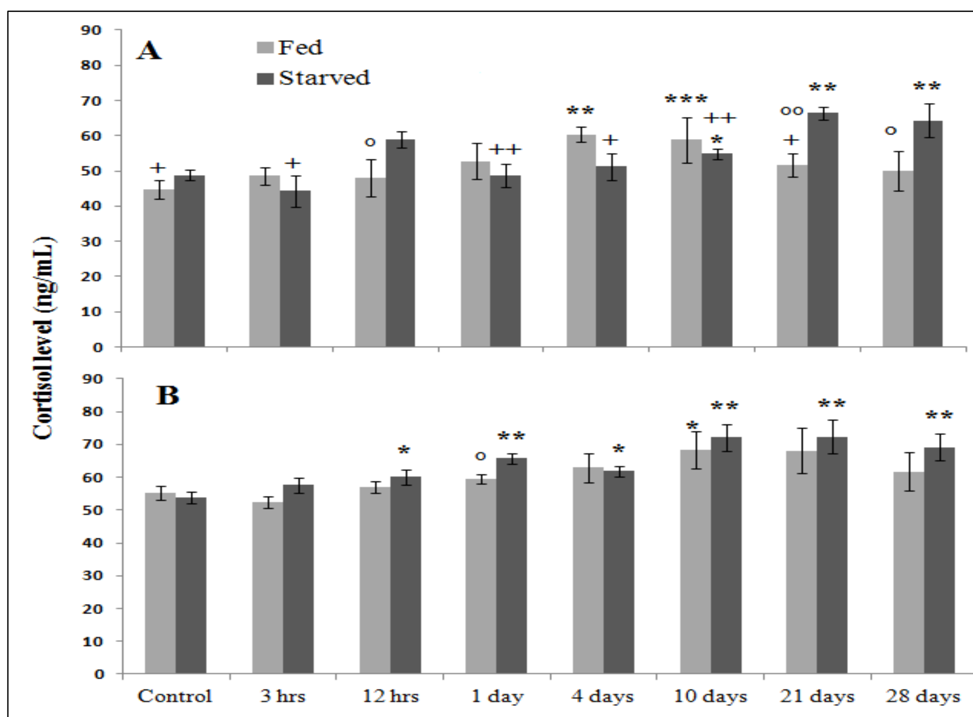

Figure 8.7. Plasma cortisol level in fed and fasted fish **(A)** at routine performance, **(B)** at exhaustive performance. Values are means ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$, **$P < 0.01$ and ***$P < 0.001$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$, ^{oo} $P < 0.01$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart($^{+}P < 0.05$, $^{++}P < 0.01$).

8.2.6. Energy stores

During RS, fed fish had numerically higher concentrations of all the energy parameters (glycogen, proteins and lipids) for both liver and muscle compared to the fasted fish (table 2). Both fed and starved groups showed a decreasing trend for the energy stores as exposure time progressed. In the RSAF and RSAS group a significant reduction ($P < 0.05$ or 0.01) in liver glycogen was evident from day 10 onwards. Such a reduction ($P < 0.05$ or 0.01) was noted in RSAS, ESAF and ESAS group for muscle tissue. An overall effect of HEA, feeding and swimming activities on glycogen content was observed only in muscle tissue.

HEA also exerted significant effect on the protein content in both feeding group under RS and ES conditions. Protein content in liver was reduced significantly in both the RSAF and RSAS group compared to their respective controls at 10-21 days of exposure while in ES condition such effect ($P < 0.05$) was noted after 21 days of exposure. The effect of HEA on protein content in muscle of both fed and starved fish under both swimming conditions showed more or less a similar patter as seen for liver tissue. Among RS fish, a significant reduction in lipid content was seen in liver of the RSAS group (after 21-28 days) while under ES conditions, a significant reduction was noted for both feeding groups after 10 days of exposure. Lipid stores in muscle did not change in any experimental group.

Table 1: Ions concentration (mmol/L) in plasma of common carp under different treatments.

			[Ca^{2+}]	[Cl^{-}]	[K^{+}]	[Na^{+}]
Control	RS	Fed	0.99 ± 0.06	118 ± 4	3.44 ± 0.17	120 ± 3
		Starved	1.07 ± 0.08	104.6 ± 1.9	4.2 ± 0.2	121.3 ± 1.3
	ES	Fed	0.94 ± 0.05 $^{\circ}$	107.0 ± 1.7	4.43 ± 0.2	126 ± 3
		Starved	1.15 ± 0.07	118.29 ± 2	3.68 ± 0.16	124 ± 3
3 hours	RS	Fed	0.84 ± 0.03$^{*\ \circ\circ\circ}$	101 ± 3	3.47 ± 0.12	128 ± 5
		Starved	1.3 ± 0.05 *	112 ± 3	3.7 ± 0.11	131 ± 3
	ES	Fed	0.97 ± 0.06 $^{\circ\circ}$	110 ± 3	3.68 ± 0.13	128 ± 3
		Starved	1.29 ± 0.05	111 ± 4	3.47 ± 0.09	131 ± 4
12 hours	RS	Fed	1.13 ± 0.04	109 ± 3	3.7 ± 0.2	125 ± 2
		Starved	1.19 ± 0.05	108.8 ± 1.9	4.03 ± 0.15	132 ± 1.3
	ES	Fed	1.07 ± 0.03	111 ± 4	4 ± 0.19	131 ± 4
		Starved	1.11 ± 0.05	110.6 ± 1.7	3.8 ± 0.4	136 ± 3
1 day	RS	Fed	1.13 ± 0.05	103.6 ± 1.2	3.2 ± 0.07	124.9 ± 0.7
		Starved	1.22 ± 0.06	110.8 ± 1.6	4.4 ± 0.2	133 ± 3
	ES	Fed	0.9 ± 0.12	97 ± 3	4.1 ± 0.3	131 ± 6
		Starved	0.95 ± 0.04	100 ± 2	3.57 ± 0.10	127 ± 9
4 days	RS	Fed	0.98 ± 0.03	107 ± 4	3.44 ± 0.15	131 ± 3
		Starved	1.02 ± 0.04	112 ± 3	3.9 ± 0.3	135 ± 6
	ES	Fed	1.06 ± 0.05	117 ± 9	4.4 ± 0.4	142 ± 8
		Starved	1.11 ± 0.07	103 ± 3	3.36 ± 0.18	141 ± 3
10 days	RS	Fed	0.91 ± 0.03	103 ± 2	3.5 ± 0.2	127 ± 4
		Starved	1.05 ± 0.08	109.3 ± 1.9	3.6 ± 0.2	128 ± 3
	ES	Fed	1.03 ± 0.05	102.33 ± 0.9	4.4 ± 0.4	138 ± 6
		Starved	1.07 ± 0.03	104 ± 5	3.8 ± 0.3	131 ± 5
21 days	RS	Fed	0.81 ± 0.11	105 ± 4	4 ± 0.3	123 ± 4
		Starved	0.98 ± 0.16	98 ± 5	4.27 ± 0.3	131 ± 5
	ES	Fed	0.91 ± 0.11	107 ± 3	4.5 ± 0.3	137 ± 4
		Starved	0.98 ± 0.03	106 ± 4	4.3 ± 0.3	130 ± 4
28 days	RS	Fed	0.8 ± 0.09	105 ± 5	4.1 ± 0.3	121 ± 3
		Starved	0.84 ± 0.11	101 ± 5	4.26 ±	124 ± 4
	ES	Fed	0.96 ± 0.13	108 ± 5	4.7 ± 0.4	129 ± 5
		Starved	0.94 ± 0.10	106 ± 4	4.4 ± 0.3	130 ± 3

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$; $^{oo}P < 0.01$ and $^{ooo}P < 0.001$).

Table 2: Glycogen, protein and lipid content in liver (wet tissue) and muscle (wet tissue) in gold fish under different treatments.

			Glycogen		Lipids		Proteins	
			Liver	**Muscle**	**Liver**	**Muscle**	**Liver**	**Muscle**
Control	RS	Fed	161 ± 13	7.5 ± 0.6	62 ± 4	33 ± 4	195 ± 10	197 ± 9
		Fasted	143 ± 11	6.8 ± 0.4	60 ± 6	31 ± 4	182 ± 10	182 ± 9
	ES	Fed	140 ± 11	7.1 ± 0.6	57 ± 5	33 ± 4	186 ± 9	181 ± 8
		Fasted	132 ± 18	6.5 ± 0.6	59 ± 4	30 ± 4	179 ± 8	178 ± 9
3 hours	RS	Fed	154 ± 10	7.4 ± 0.6	63 ± 6	34 ± 4	214 ±14	185 ± 12
		Fasted	143 ± 16	6.1 ± 0.7	63 ± 6	34 ± 3	185 ± 15	184 ± 12
	ES	Fed	134 ± 15	6.6 ± 0.6	51 ± 2	35 ± 3	188 ± 18	181 ± 12
		Fasted	130 ± 17	5.9 ± 0.6	69 ± 2	31 ± 6	180 ± 21	179 ± 11
12 hours	RS	Fed	149 ± 25	7.2 ± 0.6	65 ± 6	34 ± 4	198 ± 15	183 ± 12
		Fasted	129 ± 18	6.7 ± 0.4	59 ± 3	32 ± 4	186 ± 7	178 ± 13
	ES	Fed	132 ± 21	6.7 ± 0.5	52 ± 9	32 ± 3	174 ± 9	172 ± 11
		Fasted	128 ± 10	5.6 ± 0.4	61 ± 4	28 ± 4	178 ± 13	180 ± 16
1 Day	RS	Fed	151 ± 21	7.3 ± 0.4	61 ± 7	28 ± 2	186 ± 14	176 ± 12
		Fasted	132 ± 18	6.4 ± 0.4	58 ± 4	27 ± 3	174 ± 18	171 ± 11
	ES	Fed	134 ± 15	6.3 ± 0.4	49 ± 9	33 ± 4	179 ± 16	176 ± 20
		Fasted	124 ± 18	5.6 ± 0.7	59 ± 3	25 ± 4	180 ± 9	163 ± 10
4 days	RS	Fed	145 ± 16	7 ± 0.3 ° +	60 ± 5	27 ± 3	182 ± 19	174 ± 10
		Fasted	123 ± 11	5.7 ± 0.4	54 ± 4	27 ± 3	176 ± 12	163 ± 12
	ES	Fed	118 ± 16	6 ± 0.3	50 ± 9	33 ± 3	171 ± 8	161 ± 11
		Fasted	110 ± 11	4.9 ± 0.5	53 ± 4	28 ± 6	175 ± 24	162 ± 14
10 days	RS	Fed	116 ± 11 *	6.4 ± 0.6	50 ± 6	28 ± 3	165 ± 13 **	169 ± 11
		Fasted	98 ± 8 **	5.8 ± 0.6	50 ± 4	27 ± 4	168 ± 16	159 ± 12
	ES	Fed	111 ± 9	5.2 ± 0.4 *	44 ± 3 *	28 ± 4	162 ± 9	163 ± 13
		Fasted	98 ± 10	4.8 ± 0.6	43 ± 4 *	25 ± 5	157 ± 12	166 ± 17

			Glycogen		Lipids		Proteins	
			Liver	**Muscle**	**Liver**	**Muscle**	**Liver**	**Muscle**
21 days	RS	Fed	108 ± 12 *	6.3 ± 0.5 ++	43 ± 6	29 ± 4	152 ± 10 *	156 ± 11 *
		Fasted	86 ± 9 **	5 ± 0.5 *	41 ± 3 ***	25 ± 4	152 ± 6 *	149 ± 9 *
	ES	Fed	97 ± 7 **	4.3 ± 0.3 **	41 ± 3 *	26 ± 3	148 ± 12 *	152 ± 9 *
		Fasted	96 ± 10	3.9 ± 0.4 *	41 ± 3 **	22 ± 4	141 ± 11 *	143 ± 9 *
28 days	RS	Fed	122 ± 10 *	6.4 ± 0.6 ° ++	47 ± 5	29 ± 3	157 ± 10 *	153 ± 9 **
		Fasted	97 ± 7 **	4.3 ± 0.5 **	41 ± 3 **	26 ± 3	144 ± 9 *	148 ± 9 *
	ES	Fed	96 ± 9 *	4.3 ± 0.4 **	44 ± 4	26 ± 4	151 ± 8 *	150 ± 7 *
		Fasted	95 ± 9	4.1 ± 0.4 *	39 ± 4 **	22 ± 3	148 ± 9 *	142 ± 6 **

Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (* $P < 0.05$, **$P < 0.01$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period($^{o}P < 0.05$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart ($^{+}P < 0.05$, $^{++}P < 0.01$).

8.3. DISCUSSION

8.3.1. Ammonia dynamics

Under control conditions, ammonia excretion rate was higher in fed fish than starved fish, similar to our previous experiment with goldfish (Sinha et al., 2012a). This increment in fed fish might relate to the SDA (specific dynamic action). During HEA exposure the ammonia excretion rate increased considerably in fed fish from day 4 onwards and they continued to excrete against the concentration gradient untill the end of the experiment. Such increment was very limited in starved fish which resulted in a considerable difference in excretion rate between the fed and starved exposed group. This corresponds with the increase in M_{O2} in fed fish indicating a higher metabolic rate compared to starved fish. Interestingly, during ES conditions, though excretion rate also increased in exposed starved fish, the fed fish were able to excrete ammonia at a considerably higher rate throughout the exposure period. Under ES, fish had to swim fast and thus were forced to increase ventilation, which increased M_{O2} and intenfied the ammonia excretion rate (Alsop and Wood, 1997; McKenzie et al., 2003; Wicks et al., 2002). The combined and/or synergistic effect of SDA and elevated M_{O2} could have been the reason for the high excretion rate in the fed group. The increase in ammonia excretion rate among fed ammonia exposed fish may also be related to the Rh glycoprotein expression. Studies on juvenile trout gills showed that feeding could increase Rhcg2 transcript levels under HEA, resulting in higher ammonia excretion rate in fed fish. Future studies are warranted to investigate the effect of feeding on mRNA transcript of Rh glycoprotein in cyprinids.

Interestingly, despite the fact that ammonia excretion rate increased considerably (from day 4 onwards) in fed exposed fish, plasma ammonia still remained elevated (during 4-10 days). It signifies that excretion during the 4-10 days exposure period was not sufficient to get rid of internal ammonia. However, the increment in the excretion rate during days 21-28 corresponds well the reduction of plasma ammonia back to control level. Exposed starved fish were not able to excrete ammonia as efficiently as fed fish; only a limited increment in excretion rate was seen in starved fish. This explains the substantial plasma ammonia accumulation in starved fish. Furthermore, effect on plasma ammonia content in exposed fish (both fed and starved) was augmented when imposed to exhaustive swimming. Similar effects have previously been reported for numerous fish species following exercise treatments (Kieffer, 2000; Milligan, 1996; Suski et al., 2006; Wood, 1991). The increment likely occurred as adenylates were broken down to inosine-monophosphate (IMP) and NH_4^+ as part of anaerobic energy generation and has been well documented in rainbow trout (Driedzic and Hochachka, 1976; Mommsen and Hochachka, 1988; Suski et al., 2007; Wang et al., 1994). However, the concentration of IMP was not measured in this study.

In fish maximal AQ for 100% aerobic protein degradation is 0.33 (Kutty, 1978), and this value becomes lower when other substances are used. Based on this, we found that AQ approached

this value or slightly overshooted 0.33 in RS fed fish during 4-28 days of ammonia exposure and in RS starved fish during 21 days. This signifies anaerobic protein degradation, confirmed by the use of their protein stores, with a reduction in protein content. At ES the AQ value for fed and starved fish was higher than 0.33 during 4 days, 21 days and 28 days indicating that they rely heavily on protein use, both aerobically and anaerobically. This suggests that protein metabolism is stimulated by both HEA and food availability.

8.3.2. Swimming performance

During the exposure to HEA, no significant difference in the U_{crit} was seen in fed and starved fish, although a small decline was notable over time. However, Wicks et al. (2002) demonstrated a significant and linear decrease in the U_{crit} in rainbow trout (*Oncorhynchus kisutch*) in response to HEA. Some other studies also stated that ammonia exposure induces a reduction in the critical swimming speed of the fish (Farrel et al., 1998; Jain and Farrel, 2003). A negative effect of plasma accumulation on swimming performance of fish has been reported in many toxicological studies (Beaumont et al., 1995b; Day and Butler, 1996; Shingles et al., 2001; Wicks et al., 2002). De Boeck et al. (2006) noted that during an exposure to copper, critical swimming speed in carp reduced along with an elevation of the ammonia concentration in plasma, and not muscle, suggesting ammonia accumulation in the plasma as a key factor affecting swimming performance in fish. However, plasma ammonia increases in this study were far larger than in the present study. We did find a negative correlation between U_{crit} and plasma ammonia levels of the fish in the present study but the ammonia increase was possibly not sufficient to cause a significant effect on the swimming performance.

Also the accumulation of lactate in plasma has been related to the reduction in swimming performance of sockeye salmon, *Oncorhynchus nerka* (Farrell et al., 1998; Jain and Farrell, 2003; Stevens and Black, 1966). In our study, the increment in plasma lactate did not correlated with the numerical reduction in U_{crit}. Additionally, the depletion of glycogen stores during HEA observed in present study might be related to the reduction of U_{crit} as well. Muscle glycogen stores have long been considered as an important indicator the capacity for burst swimming (Gingerich et al., 2010; Kieffer, 2000; Wang et al., 1994). We noted that the muscle glycogen store in ammonia-exposed starved fish reduced to a much greater extent than ammonia-exposed fed fish (table 1). This might explain the larger numerical reduction in U_{crit} among starved-exposed fish compared to fed-exposed groups.

8.3.3. Cortisol and ion-regulation

Cortisol is the principal corticosteroid in teleost fish and plays a crucial role in the stress response and in osmoregulatory processes (McCormick, 2001; Wendelaar Bonga, 1999). Cortisol acts to mobilize

and synthesize metabolites, and thus influences many physiological processes during stress in fish directly as well as indirectly (Babitha and Peter, 2010; Vijayan et al., 1997; Wendelaar Bonga, 1997). Furthermore, elevated cortisol levels are reported to stimulate ammonia excretion in fish (Knoph and Olsen, 1994; Tsui et al. (2009). Plasma cortisol levels increased during HEA in all the feeding treatments, a commonly observed response in cyprinids and salmonids (Ortega et al., 2005; Sinha et al., 2012b; Tsui et al., 2009; Wood and Nawata, 2011). Ortega et al. (2005) reported a linear relationship between plasma cortisol levels and ammonia levels in rainbow trout exposed to HEA. In vitro studies on cultured trout gill epithelia by Tsui et al. (2009) indicated that cortisol can play a role in augmenting ammonia transport capacity of gills. However, in the present studies there was no clear-cut relationship between circulating cortisol levels and ammonia excretion rate which is in tune with findings of Nawata and Wood (2008) and Wood and Nawata (2011). Moreover, cortisol has been shown to increase the ion-transporting capacity of the gills by the proliferation of chloride cells (Goss et al., 1992) and to increase the level of ion-transporting enzymes (Flik and Perry, 1989; Lin and Randall, 1993; McCormick and Bern, 1989; Shrimpton and McCormick, 1999; Wilson et al., 2002). In our study, the time course of the changes in cortisol corresponds to the transitory increment in plasma [Ca^{2+}] favouring the fact that cortisol stimulates calcium uptake (Flik and Perry, 1989). However, the concentration of other ions (Na^+, Cl^-, and K^+) remained almost unaltered in all experimental groups even though a considerable increment of cortisol level was noted. It indicates that cortisol is not the sole ion-regulatory process responding to ammonia exposure. Perhaps different hormones (e.g. prolactin and thyroid hormone) are also the important factors controlling ion balance during stressful conditions. Therefore, investigation of these hormones and the dynamics of other ion-regulatory enzymes may be crucial for future experiments.

The level of cortisol was strongly influenced by a period of starvation; prior to and after exhaustive exercise, cortisol concentration in starved fish was higher than in fed fish (during 12h, 1 day, 21days and 28 days) suggesting that the starved fish under HEA are more stressed than the fed fish. Also in rainbow trout (Vijayan and Moon, 1992), gobies (*Gillichthys mirabilis*) (Kelley et al., 2001), channel catfish (Peterson and Small, 2004) and largemouth bass (*Micropterus salmoides*) (Gingerich et al., 2010) starvation was reported to induce increases in plasma cortisol level. The exact role and mechanism for cortisol in providing metabolic and ion-homeostasis during ammonia exposure and feed limitation still remains elusive (Gingerich et al., 2010; Van der Boon et al., 1991).

In brief, most of the studied parameters under HEA were found to be considerably influenced during exhaustive swimming, with more adverse effect in starved fish. This indicates that besides HEA and exhaustive swimming, feed limitation can be a crucial environmental factor manipulating the compensatory responses of common carp. Especially fed common carp were able to excrete

ammonia against a concentration gradient; however this occurred at a metabolic cost leading to increased MO_2, decreased energy stores, and both aerobic and anaerobic use of protein.

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Chapter 9

Expression pattern of potential biomarker genes related to growth, ion-regulation and stress in response to ammonia exposure, food deprivation and exercise in common carp (*Cyprinus carpio*)

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Abstract

Waterborne ammonia has become a persistent pollutant of aquatic habitats. During certain periods (e.g. winter), food deprivation may occur simultaneously in natural water. Additionally, under such stressful circumstances, fish may be enforced to swim at a high speed in order to catch prey, avoid predators and so on. Consequently, fish need to cope with all these stressors by altering physiological processes which in turn are controlled by their genes. In this present study, toxicogenomic analyses using real time PCR was used to characterize expression patterns of potential biomarker genes controlling growth, ion regulation and stress responses in common carp subjected to elevated ammonia (1mg/L; Flemish water quality guideline for surface water) following periods of feeding (2% body weight) and fasting (unfed for 7 days prior to sampling). Both feeding groups of fish were exposed to high environment ammonia (HEA) for 0 h (control), 3 h, 12 h, 1 day, 4 days, 10 days, 21days and 28 days, and were sampled after performing swimming at different speeds (routine versus exhaustive). Results show that the activity and expression of Na^+/K^+-ATPase, an important branchial ion regulatory enzyme, was increased after 4-10 days of exposure. Effect of HEA was also evident on expression patterns of other ion-regulatory hormone and receptor genes; prolactin and cortisol receptor mRNA level(s) were down-regulated and up-regulated respectively after 4, 10 and 21 days. Starvation and exhaustive swimming, the additional challenges in present study significantly further enhanced the HEA effect on the expression of these two genes. mRNA transcript of growth regulating hormone and receptor genes such as Insulin-like growth factor I, growth hormone receptor, and the thyroid hormone receptor were reduced in response to HEA and the effect of ammonia was exacerbated in starved fish, with levels that were remarkably reduced compared to fed exposed fish. However, the expression of the growth hormone gene itself was up-regulated under the same conditions. Expression of somatolactin remained unaltered. Stress representative genes, cytochrome oxidase subunit 1 showed an up-regulation in response to HEA and starvation while the mRNA level of heat shock protein 70 was increased in response to all the three stressors. The expression kinetics of the studied genes could permit to develop a “molecular biomarker system” to identify the underlying physiological processes and impact of these stressors before effects at population level occur.

Keywords: Ammonia, Starvation, Exercise, Na^+/K^+-ATPAse, Real time PCR, Gene-expression, Cortisol receptor, Prolactine, HSP70, Insulin-like growth factor I, Growth hormone, Thyroid hormone receptor.

9.1. INTRODUCTION

Ammonia toxicity has become a universal problem for aquatic animals, especially in fish. Toxicity induced by high environmental ammonia (HEA) may affect the performance of fish in several ways. It reduces growth rate (Atwood et al., 2000; El-Shafai et al., 2004; Hegazi and Hasanein, 2010), causes gill hyperplasia (Benli et al., 2008), disrupts ion-osmo homeostasis (Knoph and Thorud, 1996; Person-Le Ruyet et al., 1998; 2003), alters hormone regulation (Dosdat et al., 2003; Knoph and Olsen, 1994), and at high levels even induces hyperexcitability, coma, convulsions and death (Ip et al., 2001a).

Fish can excrete ammonia across the gill membranes into water providing there is a concentration gradient and this process is facilitated by Rhesus (Rh) glycoprotein (Nawata et al., 2007). However, under HEA the outward flux of ammonia excretion through the gills is reduced, and a reversed inward ammonia flux occurs. The branchial cells of teleosts and other aquatic animals contain a number of transporters and associated mechanisms that are involved in maintenance of ammonia homeostasis by transporting the ammonium ions (Evans et al., 1999; 2005; Wilkie, 1997). The branchial enzyme Na^+/K^+-ATPase, primarily associated with sodium transport, also plays an important role in ammonia excretion since similarities in the hydration radius of K^+ and NH_4^+ allow substitution at the basolateral transport sites of branchial epithelia (Alam and Frankel, 2006; Randall et al., 1999; Wilkie, 1997). As a result NH_4^+ is transported into the intracellular compartment and from there moves across the apical membrane in exchange of sodium through Na^+/NH_4^+ exchanger (Evans et al., 2005; Wilkie, 2002). Therefore, Na^+/K^+-ATPase provides a driving force for the exchange of Na^+ from the water for ammonia from the fish. Consequently, alterations in the activity and mRNA transcript level of Na^+/K^+-ATPase may be an important indicator of ammonia pollution.

Beside ammonia toxicity, food restriction is a natural occurrence in wild populations of fish and also occurs regularly for cultured fish. In order to maintain water quality in fish farming system the animals are often subjected to periods of restricted feeding and fasting (Robinson and Li, 1999). It has already been demonstrated that nutritional inputs can modulate numerous physiological and biochemical systems through gene-associated process (Ayson et al., 2007; Deng et al., 2004; Pedroso et al., 2006; Pierce et al., 2005; Small et al., 2006). Like feeding, exercise is another important physiological activity of fish. Fish swim constantly, either in the form of low-intensity routine swimming (RS), or high-intense exhaustive swimming (ES) as a means of coping with water currents and predatory, prey hunting and migratory challenges. Exhaustive exercise is known to elevate metabolic processes and induce endogenous ammonia production (Kieffer, 2000; Knoph and Thorud, 1996; Li et al., 2010). If food deprivation and exercise, which both have negative effect on growth performance and ion-regulation occur simultaneously with ammonia exposure, synergistic effect can be expected and become a source of stress for fish. It has already been illustrated that environmental

challenges may induce the expression of genes associated with short term and/or long term stress responses which have profound effect on overall performance of fish (Lee et al., 2006a,b). Hence, it is advisable to evaluate how the combined effects of fasting and exercise at HEA could alter the expression profiles of various key genes controlling growth, ion regulation and stress responses in fish. As such, they do not only give insight in the physiological responses of the fish, but consequently could also be a sensitive 'early warning tool' of the ecotoxicological impact (Carajaville et al., 2000; Chevre et al., 2003; de la Torre et al., 2005). Although researchers have looked into toxicant- or starvation-induced gene expression alterations in fish, the implementation of "biomarker genes" to evaluate performance of fish under multiple stressors is rather scarce. Therefore, the goal of present study is to evaluate, in conditions simulating natural environment, the *in vivo* effects of HEA (1mg/L), nutritional restriction and exhaustive exercise on well known genes regulating growth, ion-balance and stress response in common carp (*Cyprinus carpio*), an important freshwater culture species throughout the world.

To achieve our goals we measured expression dynamics of thyroid hormone receptor (TR), insulin-like growth factor I (IGF-I), somatolactin (SL), growth hormone (GH) and growth hormone receptor (GHR) genes as representatives of fish growth rate. Cortisol receptor (CR), Na^+/K^+-ATPase and prolactin (PRL) genes were selected as key genes for studying changes in ion-regulatory process. Actually, some of these genes (PRL, TR, CR) may have both growth and ion-regulatory effects. Heat shock protein70 (HSP70) and cytochrome oxidase subunit 1 (COI) genes were selected as candidate genes for stress identification and sentinels of contaminant exposure. Moreover, it is well understood that no single biomarker gene has emerged as a widely used indicator for toxicological studies without some limitation, which made us implement a multibiomarker gene approach in present work.

9.2. RESULTS

9.2.1. Activity of Na^+/K^+-ATPase in gill

Na^+/K^+-ATPase activity in fish gill was affected by ammonia exposure (Fig.9.1). In routine-swum ammonia exposed fed (RSAF) and routine-swum ammonia exposed starved (RSAS) fish, the activity was temporary increased ($P < 0.05$) after 10 and 21 days of exposure relative to their control. A similar pattern was seen for exhaustive-swum ammonia exposed fed (ESAF) and exhaustive-swum ammonia exposed starved (ESAS). An effect of exercise was observed after 4 days of exposure, the enzymatic activity was significantly increased in ESAF and ESAS fish compared to their respective RS fed and starved fish.

9.2.2. mRNA expression of enzyme, hormone and hormone receptor genes involved in ion regulation

The effect of HEA was seen on Na^+/K^+-ATPase α_3 mRNA expression in gill tissue of all groups, with significant increment starting after 4 days of exposure, which continued for RSAF and RSAS till 21 days and then recovered to control level (Fig.9.2). In ES fish, augmentation of the transcript level in both fed and starved fish continued till the end of exposure period. Similar to the Na^+/K^+-ATPase activity, the gene expression was not altered by feed restriction, but in general activity and mRNA level in fed fish was numerically higher than starved fish, especially in ES fish.

Significant reduction in pituitary PRL mRNA level was observed after 10 and 21days, and after 4, 10 and 21 days in RSAF and RSAS fish respectively compared to the controls (Fig.9.3). When subjected to higher swimming speeds the reduction in both feeding treatments became prominent ($P < 0.05$ or $P < 0.01$) from day 4 onwards and continued till day 21. Moreover, after 4, 10 and 21 days of exposure period, starved fish in both swimming conditions had lower mRNA level of PRL than the respective fed groups. Exercising fish at higher speed significantly decreased the expression level in fed (after 21 days) and starved (after 10 and 21 days) fish compared to their routine swimming counterparts respectively.

CR mRNA expression in gill was considerably influenced by ammonia exposure, feed deprivation and exercise (Fig.9.4). Levels in RSAS and RSAF fish increased significantly starting after 4 and 10 days respectively compared to their control and this increased level of expression continued till 21 days of exposure. This increment ($P < 0.05$ or $P < 0.01$) occurred a bit earlier (from day 1 onwards for ESAS and from day 4 for ESAF) when fish were subjected ES. Starved fish in both swimming conditions had higher expression levels than fed fish which became prominent ($P < 0.05$) after 4, 10 and 21 days of exposure. Swimming at higher speed augmented mRNA level in both fed (day 10 and 21) and starved fish (from day 1 to day 28) compared to their respective RS groups.

In addition, we also measured the level of ammonia accumulation in plasma (according to Wright et al., 1995a). The level was significantly elevated from day 4 onwards in both fed and starved fish (under both RS and ES conditions). After 21-28 days plasma ammonia returned to control levels in fed fish only (refer chapter 8, fig. 8.5).

Table 9.1

PCR primer sequences, accession numbers, melting temperatures and calculated efficiency.

Gene	Accession no.	Sequence of Primer (5´→ 3´)	Melting temperature (T_m)	Calculated efficiency
Ion-regulatory genes				
Na^+/K^+-ATPaseα_3	AB062885	F: GGCAAGAGATGGGCCAA	60.4	1.91
		R: GCTGGCTCATCTTCGGT	60.0	
Cortisol receptor	EF042099	F: GTGAGACTGCAAGTGTCCAA	60.0	1.87
		R: CTCTCTCTTCACTATGGCCT	60.3	
Prolactin	X52881.1	F: GTAAGCACTCCTCCATTCC	59.7	1.94
		R: GGCTCGCTCCAGTAAATC	60.2	
Growth controlling genes				
Growth hormone	M27000	F: TAACGACTCCTTGCCGC	60.9	1.82
		R: TCTACAGGGTGCAGTTGG	60.0	
Insulin-like growth factor-I	AF465830	F: GATGGCAAGTCACCTCC	59.8	1.94
		R: GACAAGAGCCAAGCCTG	60.4	
Growth hormone receptor	AY691176	F: GAGCAGGGGTACCAAAC	60.2	1.98
		R: GCTGTGAGGGCATATCG	60.2	
Thyroid hormone receptorβ	BC163106.1	F: GACATTGGATCGGCACCT	60.4	2.08
		R: ACTGCTGCTCGAAGAGAC	60.3	
Somatolactin	GU434163.1	F: TCGTTACGATGACGCCC	60.4	1.84
		R: GGCGTCCTTCTTGAAGC	60.6	
Stress representative genes				
Heat shock protein 70	AY120894	F: GGCAGAAAGTTTGATGACCCA	60.4	2.00
		R: GCAATCTCCTTCATCTTCACC	60.3	

Cytochrome oxidase subunit1	HQ536347	F: GGAACTTAGCCCACGCA	60.2	1.78
		R: AAGCACGGATCAGACGA	60.2	
Reference genes				
Beta actin	M24113.1	F: AGCTAGGCCTTGAGCTAT	58.8	2.01
		R: CCTGCTTGCTAATCCACA	58.8	
Elongation factor 1α	AF485331.1	F: TGGAGATGCTGCCATTGT	60.1	1.88
		R: TGCAGACTTCGTGACCTT	60.2	
Glyceraldehyde-3-phosphate dehydrogenase	AJ870982.1	F: ATCTGACGGTCCGTCT	60.2	1.91
		R: CCAGCACCGGCATCAAA	59.8	

The accession number refers to the registered sequence used from Genbank. F: forward, R: reverse.

Table 9.2

Weight gain (%) at different sampling points.

Sampling intervals	Fed		Starved	
	Control	Exposed	Control	Exposed
1 day	1.10 ± 2.05	1.22 ± 1.88	1.06 ± 2.01	1.11± 1.99
4 days	3.32 ± 1.16	3.57 ± 2.89	3.14 ± 1.88	3.17 ± 2.43
10 days	14.23 ± 2.89	12.16 ± 1.88	13.34± 1.66	8.23 ± 1.43*
21 days	32.77 ± 3.97	23.55 ± 2.96*	26.58 ± 2.33	18.67± 3.08*
28 days	38.61 ± 4.38	28.72 ± 3.01*	30.11 ± 4.07	21.27 ± 2.56*+

Values are mean ± S.E. An Asterisk (*) represents the significant difference between exposed group and its control at the same sampling time. Plus (+) denotes the significant difference between exposed treatments of fed and starved groups.

9.2.3. Growth rate and expression pattern of growth controlling hormone and receptor genes

The 7 day starvation period did not induce a significant reduction in growth as % weight gain, but exposure to HEA did (Table 9.2). The reduction in growth under HEA was more pronounced in starved fish where a significant reduction was observed from 10 days onwards, while in fed fish this was only obvious from 21 days onwards, and at the end of the exposure period (28 days) the difference between the two feeding treatments was significant with a 25.94% ($P < 0.05$) lower weight gain in starved fish.

In fed fish, HEA did not affect GH mRNA level in the pituitary gland, but in starved fish GH mRNA levels were significantly higher compared to both control and fed fish after 21 and 28 days of exposure under RS and from day 10 onwards when subjected to ES (Fig.9.5).

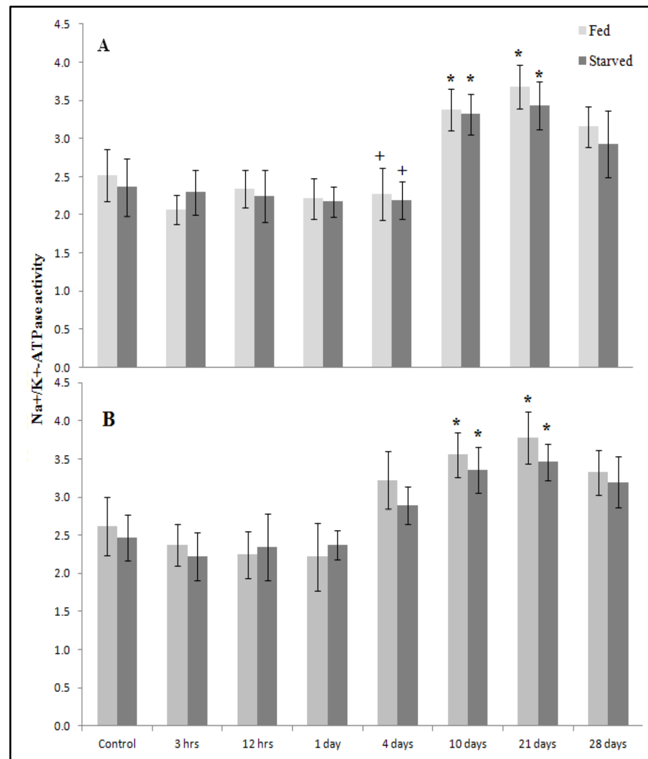
Hepatic IGF-1 expression was altered both by HEA and feed deprivation (Fig. 9.6). A reduction in mRNA level ($P < 0.05$) relative to the control (in both RS and ES fish) was evidenced from day 10 onwards in starved fish and from day 21 onwards in fed fish respectively. Overall, HEA affected IGF-1 mRNA level more severely in starved fish compared to fed fish, the difference being significant ($P < 0.05$) from day 21 under RS and from day 10 under ES conditions respectively.

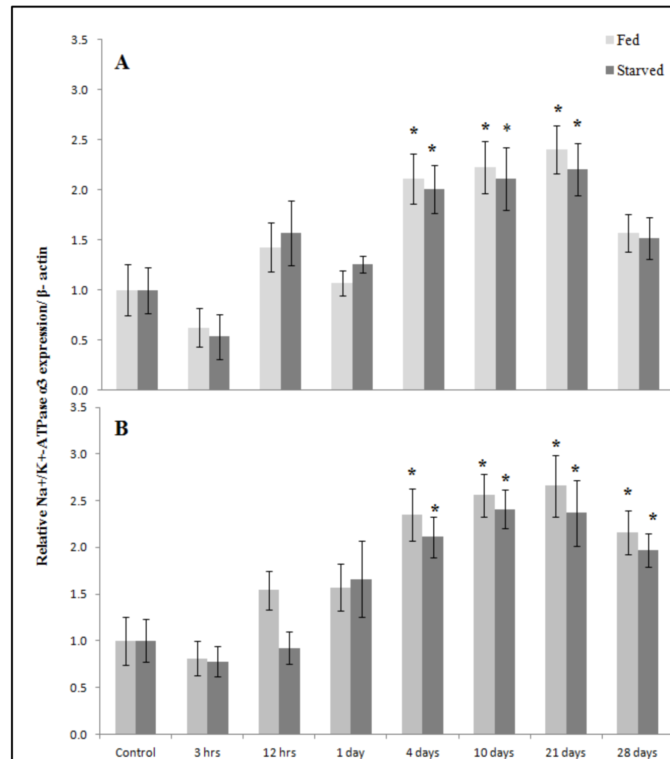
In contrast to the mRNA levels for GH in starved fish, the mRNA level of hepatic GHR was drastically ($P < 0.05$ or $P < 0.01$) reduced in both RSAS and ESAS fish compared to control from day 21 onwards (Fig.9.7). These reductions were also significant compared to their fed counterparts. Effect of HEA in RSAF fish became significant only at the end of exposure period (28 days) when level decreased by 44% compared to the control. Such reduction ($P < 0.05$ or $P < 0.01$) occurred slightly earlier (from day 21 onwards) in ESAF fish. Exercising fish at higher speed did not alter GHR mRNA transcript in any of the feeding treatments compared to their respective RS groups.

In order to investigate the additional osmoregulatory role of the GH/IGF-I axis, we also measured the expression of these hormone genes in gill tissue. No remarkable ($P > 0.05$) effect of HEA or starvation or exercise was noted on expression profile of these genes (data not shown).

The expression pattern of SL mRNA in pituitary gland of carp was irregular and no significant effect of HEA, starvation or exercise could be noted (Fig.9.8).

A pronounced effect of ammonia exposure was seen on the transcript level of TRβ in liver, which was aggravated by both feed limitation and exercise (Fig.9.9). Ammonia exposure significantly reduced the expression in both fed and starved fish from day 10 onwards in RS conditions ($P < 0.01$ or 0.05), and in ES fish such a reduction ($P < 0.05$ or 0.01) was evident from day 4 onwards. Effect of HEA was more pronounced among starved fish compared to fed ones, and difference between feeding treatments became prominent ($P < 0.05$) after 10 and 21 days in both swimming conditions. Swimming at exhaustive speed further reduced ($P < 0.05$) the mRNA level in both fed and starved fish from day 4 onwards compared to their respective RS groups.





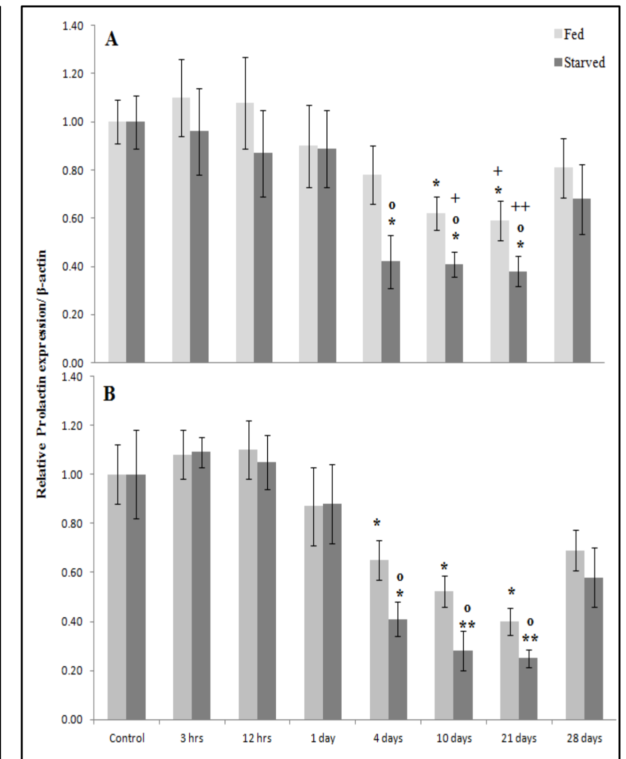


Figure. 9.1. Na^+/K^+-ATPase activity (nmol ADP/µg protein/h) in gill tissue of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (+P < 0.05).

Figure. 9.2. Relative expression of Na^+/K^+-ATPase α_3 mRNA in gill tissue of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05).

Figure. 9.3. Relative expression of prolactin mRNA in pituitary gland of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} P < 0.05), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (+P < 0.05; ++ P < 0.01).

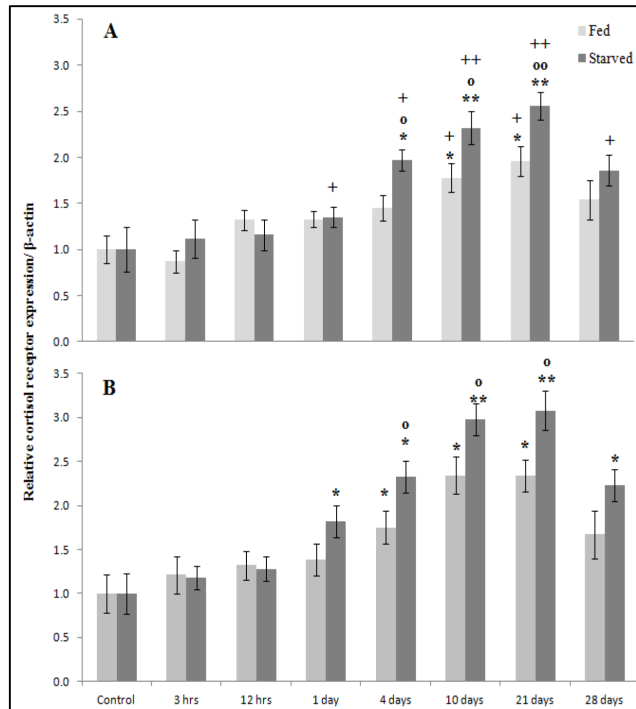


Figure. 9.4. Relative expression of cortisol receptor mRNA in gill tissue of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$; ^{oo} $P < 0.01$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (+$P < 0.05$; ++ $P < 0.01$).

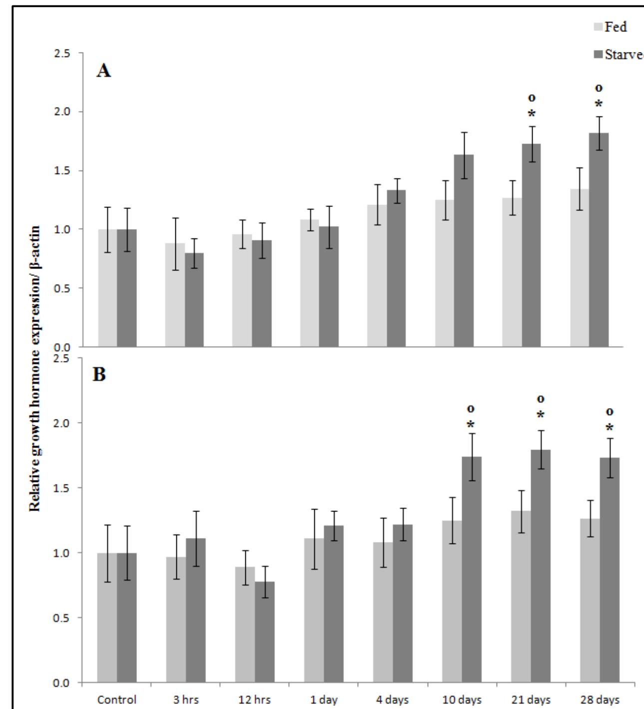


Figure. 9. 5. Relative expression of growth hormone mRNA in pituitary gland of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$).

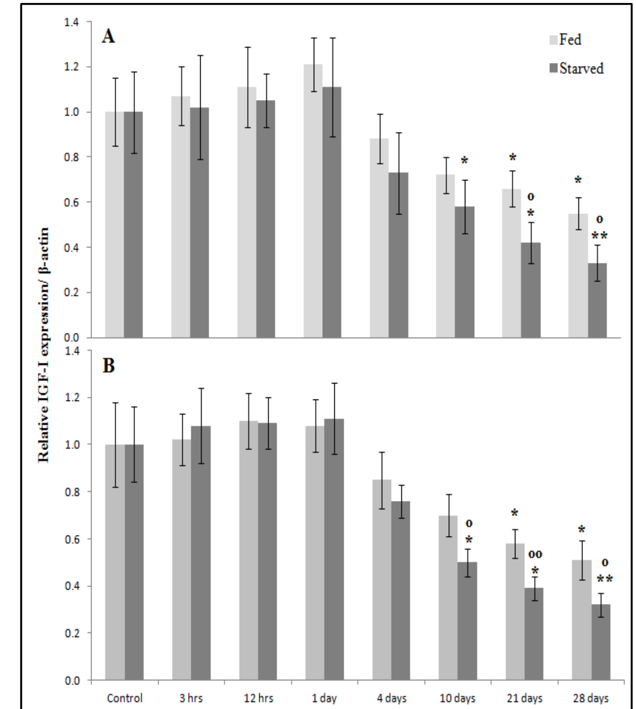


Figure. 9.6. Relative expression of IGF-I mRNA in liver of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$; ^{oo} $P < 0.01$).

9.2.4. Expression pattern of genes controlling stress responses

HSP70 mRNA in hepatic tissue of RSAF and RSAS fish increased gradually with the exposure period and became significantly higher than control respectively from day 10 and day 4 onwards (Fig.9.10) and reached a peak ($P < 0.05$ or $P < 0.01$) with roughly a 3 to 4 fold increase in expression after 21 days of exposure. Subsequently, the levels suddenly dropped at the end of exposure period, but with transcript levels remaining significantly higher than controls. Almost the same pattern was observed for ESAF and ESAS fish: the mRNA levels in both feeding treatments were significantly elevated from day 4 onwards. We also observed an effect of feed restriction in the present study, the expression was significantly higher in starved fish after 10 and 21 days in RS condition, and after 4, 10 and 21 days under ES. The expression level was amplified when fish were subjected to exercise as well, with a significant increment in ESAF (after 10 and 21 days) and ESAS (from day 4 onwards) fish compared to their respective RS groups.

The effect of HEA on COI gene expression in liver of both fed and starved fish was apparent ($P < 0.05$) after 21 and 28 days of exposure in both RS as well as ES (Fig.9.11). The difference ($P < 0.05$) between feeding treatments under RS was evident only at day 28 while under ES the difference ($P < 0.05$) was observed at day 21 and 28.

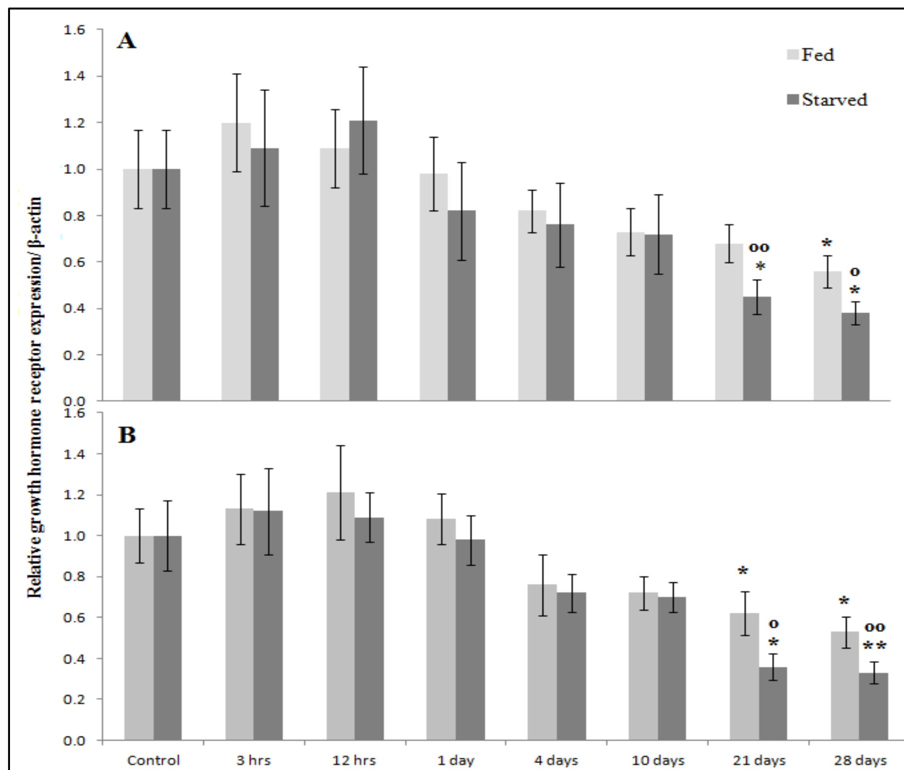


Figure. 9.7. Relative expression of growth hormone receptor mRNA in liver of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$; ^{oo} $P < 0.01$).

9.3. DISCUSSION

9.3.1. Control of ion-regulatory processes at the gills

Waterborne ammonia induces intricate molecular and cellular changes in the gills of freshwater teleosts (Alam and Frankel , 2006). As a fundamental part of the response, a change in the activity of Na^+/K^+-ATPase, a key enzyme in maintaining ion homeostasis was observed. The activity of Na^+/K^+-ATPase was significantly activated by HEA, probably as a compensatory mechanism to counteract HEA induced disturbance in ion balance. This increase in Na^+/K^+-ATPase activity was preceded and accompanied by an up-regulation of the Na^+/K^+-ATPase α_3 gene, the predominant isoform in gills of teleosts (Bystriansky and Schulte, 2011; D'Cotta et al., 2000), from 4 days of HEA onwards. Our result corroborates the finding of Alam and Frankel (2006) in silver perch (*Bidyanus bidyanus*) and golden perch (*Macquaria ambigua*). They reported an increase in gill Na^+/K^+-ATPase activity with increases in ammonia concentrations (0-5 mg/L),and also the activity in African catfish (*Clarias gariepinus*) was significantly increased when cultured in high ammonia water (1084μM) for 34 days (Schram et al., 2010). Significantly higher Na^+/K^+-ATPase activity in the gills of scale-less carp (*Gymnocypris przewalskii*) was reported as well when transferred to water containing 0.4-1.0 mmol/L total ammonia (Wang et al., 2003). In our experiment, the Na^+/K^+-ATPase activity and expression of the Na^+/K^+-ATPase α_3 gene was reduced again approximately to control levels at the end of exposure period which might indicate an acclimatization response. Also in goldfish we found a tendency towards recovery in some, but not all, metabolic and ion-regulatory parameters at the end of a similar exposure (Sinha et al., 2012a,b). Regrettably, the quantitation of transcript levels of the ammonium transporters (Rhesus glycoproteins), the Na^+/H^+ exchanger and the H^+-ATPase pump , all possibly involved in ammonia transport and acclimation processes to HEA, was not possible since these genes remain to be sequenced in common carp.

Ion- and osmoregulation is also controlled by the hormone PRL, produced in the pituitary gland. Regulation of branchial permeability to water and ions is one of the main functions of PRL in fish, therefore its involvement in maintaining ionic homeostasis under ammonia threat is expected. Moreover, it is equally crucial for metabolism, growth, development, reproduction, behavior and immunoregulation (Cavaco et al., 2003; Power, 2005; Yada et al., 2002). The down-regulation of PRL expression in response to HEA (from 4-10 days) as observed in present study supports the finding in goby (*Mugilogobius abei*), mRNA expression of PRL was suppressed when exposed to 2 mM NH_4Cl (Iwata et al., 2005). In contrast to Na^+/K^+-ATPase, PRL mRNA expression followed an opposite trend under HEA. This inverse pattern of PRL and Na^+/K^+-ATPase expression in response to HEA is not surprising because they are known to exert opposite effects in controlling ion-regulatory mechanism in fish (Madsen and Bern, 1992). Interestingly, PRL expression levels also recover by day 28, emphasising this interactive effect between PRL and Na^+/K^+-ATPase expression.

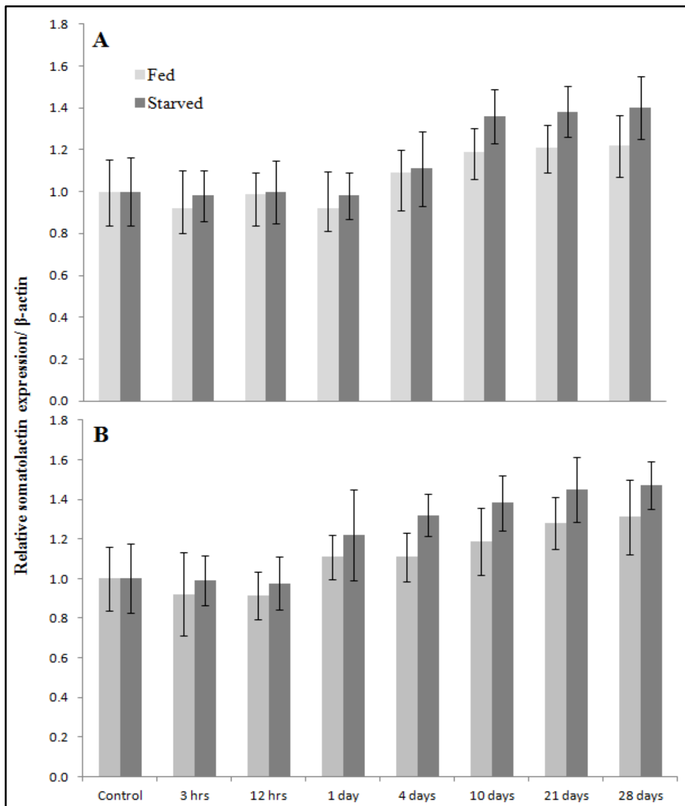


Figure. 9.8. Relative expression of somatolactin mRNA in pituitary gland of fed and starved fish during (A) Routine swimming (B) Exhaustive swimming. Values are mean ratio ± S.E.

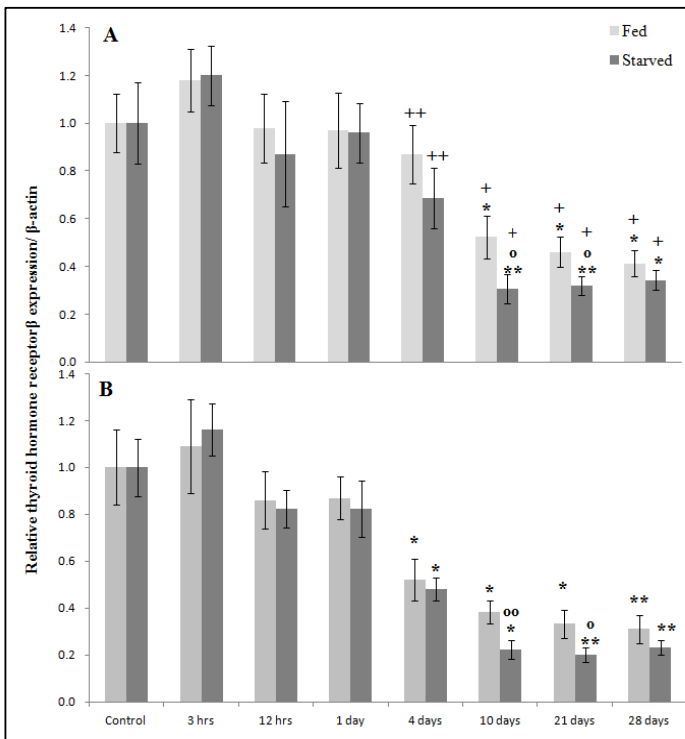


Figure. 9.9. Relative expression of thyroid hormone receptorβ mRNA in liver of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05;**P < 0.01), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (o P < 0.05; oo P < 0.01), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (+P < 0.05; ++ P < 0.01).

The effect of starvation on the mRNA levels of PRL became prominent when fish were confronted with ammonia toxicity for 4 days. The expression levels in starved fish were reduced to a larger extend compared to fed fish in both RS and ES conditions. Although there is the possibility that this is related to its role in ion-regulation, since starved fish are much more challenged (their only possible ion uptake route comes from the water), this hypothesis is not supported by the data on the Na^+/K^+-ATPase activity and expression of the Na^+/K^+-ATPase α_3 which did not differ significantly between fed and starved fish. It might indicate PRL's involvement in metabolism in common carp as this is one of the many other functions of PRL (Ben-Jonathan et al., 2006). In fish, it has been shown that PRL limits the activities of enzymes involved in generating NAPDH for fatty acid biosynthesis in the liver (Leena et al., 2001). Hence, in order to supply the energy requirement during starvation and exercise, it is obvious that PRL level will be reduced to allow mobilisation of fat reserves. Consequently, an even more profound reduction was noted among the starved group subjected to exhaustive exercise (after 10 and 21 days of exposure). Conversely, it is not clear that whether the effect of starvation on PRL mRNA level is direct or mediated by some other factors. The secretion of PRL in teleosts is under inhibitory control of dopamine (Ben-Jonathan, 1985; Nishioka et al., 1988), and in tench (*Tinca tinca*) it has been reported that the dopamine level was increased after short-term food deprivation (de Pedro et al., 2003). Subsequently, it will be interesting to examine whether the reduction in PRL mRNA levels in response to starvation observed in present study is mediated by the action of dopamine. Surprisingly, PRL's involvement during period of starvation is largely unknown in fish; therefore, it could be a potential area for future research.

Cortisol is the end product of the hypothalamic-pituitary-interrenal axis. It plays a crucial role in the stress response and in osmoregulatory processes as well as in energy metabolism (McCormick, 2001; Wendelaar Bonga, 1997). The action of cortisol is likely mediated by high-affinity cortisol receptors (CR) in the gills (Chakraborti et al., 1987; Maule and Schreck, 1990). The number and expression of hormone receptors is an important parameter in the control of physiological mechanisms as the responsiveness of cells is dependent on the receptor concentration (Danielsen and Stallcup, 1984; Vanderbilt et al., 1987). The expression of CR was considerably up-regulated after 4-10 days of ammonia exposure, suggesting that HEA caused a stress response in the fish. At the same time, it would allow for CR to play an important role in maintaining ion homeostasis in ammonia polluted water, as it was noticed that expression kinetics of Na^+/K^+-ATPase and CR followed the same pattern favouring the hypothesis that the number of gill CR is strongly correlated with the capacity of cortisol to stimulate gill Na^+/K^+-ATPase (Shrimpton et al., 1994; Shrimpton and McCormick, 1999). A similar relationship has been reported in many freshwater fish including cichlids (Dang et al., 2000), salmonids (Shrimpton and McCormick, 1999; Wilson et al., 2002) and cyprinids (Abo Hegab and Hanke, 1984). The decline in the level of CR mRNA during the last

exposure period could therefore actually be the cause of the corresponding decline in the activity and expression of Na^+/K^+-ATPase. A possible explanation for the reduced CR expression could be a negative feedback on cortisol production or exhaustion of the pituitary-inter-renal-axis, as is known to occur after a prolonged hyperactivity of this system in response to chronic exposures (Hontela et al., 1992). However, the possibility of mere acclimatization with Na^+/K^+-ATPase activity, expression of the Na^+/K^+-ATPase α_3 gene, expression of CR and expression of PRL returning to control levels definitely deserves further attention. Similar to PRL, CR expression was strongly influenced by a period of starvation. Prior to and after exhaustive exercise, expression in starved fish was many fold up-regulated relative to fed fish suggesting that the starved fish under HEA are more stressed than fed fish. As for PRL, our result suggests that besides its ion-regulatory function, the CR gene is also involved in metabolic processes in fish and plays an important role during periods of fasting, but its exact role and mechanism has yet to be clearly defined.

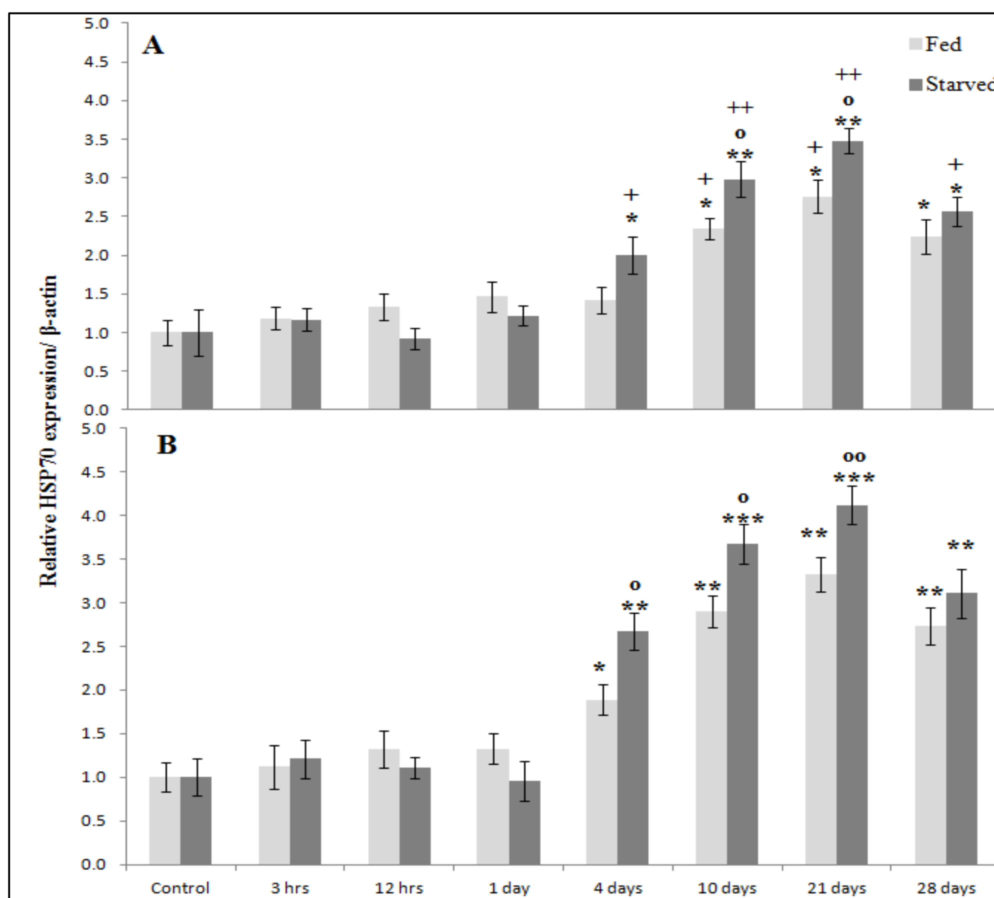


Figure. 9.10. Relative expression of HSP70 mRNA in liver of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$;**$P < 0.01$; ***$P < 0.001$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$; ^{oo} $P < 0.01$), plus (+) denotes the significant difference between the routine swimming fish and its respective exhaustive swimming counterpart (+$P < 0.05$; ++ $P < 0.01$).

Overall, most of the ion-regulatory responses were seen after 4 (or 10 days) of exposure. This consequence may be related to the elevated accumulation of ammonia in plasma which became significantly higher from day 4 onwards. Elevated plasma ammonia level profoundly affects the ionic balance by reducing Na^+ influx and K^+ loss through substitution of NH_4^+ for K^+ in Na^+/K^+-ATPase (Wilkie, 1997). Therefore subsequent activation of these responses may be the defence mechanisms to attain homeostasis under ammonia toxicity.

In addition, exhaustive exercise represents both an ionregulatory and metabolic stress for fish. Indeed, circulating levels of stress hormones such as cortisol are well known to increase with exercise in fish in part to aid in oxygenation of tissues and mobilization of energy stores (Gamperl et al., 1994; Milligan, 1996). Therefore, exercise-induced augmentation in the concentration of cortisol might have accounted for increased in CR mRNA level among ES fish compared to their RS counterparts. In general, the mRNA transcript level of all ion-regulatory associated genes investigated in present study gradually recovered to normal level at the end of exposure period, which depicts that common carp have the ability to overcome the ionic disturbance induced by ammonia exposure.

9.3.2. The GH/IGF-I axis and related hormones

Growth in fish is a complex process; various factors such as environment, nutrition, physiological activity and gene-interplay are involved in this process. We found that prolonged exposure (10-21 days) to an ammonia level of 1 mg/L hampered the growth performance of common carp. The negative effect of HEA on the growth performance in the present study is consistent with the findings in juvenile seabass, (*Dicentrarchus labrax*) (Dosdat et al., 2003; Lemarie et al., 2004), turbot (*Scophthalmus maximus*) (Rasmussen and Korsgaard, 1996), Atlantic cod (*Gadus morhua*), (Foss et al., 2004), Senegalese sole (*Solea senegalensis*) (Pinto et al., 2007), African catfish (*Clarias gariepinus*) (Schram et al., 2010) and goldfish (*Carassius auratus*) (Sinha et al., 2012a). Furthermore, this reduction in growth was aggravated by starvation. Therefore, we investigated whether the gene expression patterns of growth regulating hormones and receptors (GH, SL, IGF-I, GHR, PRL, TR) changed with HEA and with food restriction when subjected to HEA. In general, these hormones act in concert to affect growth and their secretion patterns are influenced by a given set of environmental and physiological factors. The biological activity of thyroid hormone (TH) is predominantly mediated by binding to thyroid hormone receptors, of which two forms (TRα and TRβ) exist in fish (Filby and Tyler, 2007; Swapna and Senthilkumaran, 2007). The expression of TRβ isoform is found to be predominant in many fish species (Kawakami et al., 2003; Marchand et al., 2004). Several environmental contaminants interfere with the regulatory network of TH by binding to the TR (Schriks et al., 2007; Ulrich, 2003). In the present study, ammonia exposure for 10 days tends to down-regulate the expression level of TRβ mRNA and more severely so among starved fish. This

reduction is clearly an adaptive response to reduce metabolism and to preserve nutritional reserves, as was also reported by Hemre et al. (1993) for cod (*Gadus morhua*). Likewise, Raine et al. (2005) reported a decline in mRNA accumulation of TRβ in intestinal tract of fasted late-stage embryos of rainbow trout (*Oncorhynchus mykiss*).

Exercise to exhaustion is reported to have no consistent effect on plasma TH levels in rainbow trout (*Oncorhynchus mykiss*) (Pagnotta et al., 1994) but in our study swimming activity seems to interact with HEA as reflected by decrease in TRβ expression (from day 4 onwards) among exhaustive swimmers compared to routine swimmers. However, little is known on the effect of environmentally relevant stressors on the concentration and expression of TR in fish.

GH and IGF-I have metabolic functions so it can only be expected that nutritional status is the principal environmental regulator of GH/IGF-I axis in fish (Duan, 1998; Moriyama et al., 2000; Thissen et al., 1994, 1999). However, in control groups no difference between feeding treatments was observed on the level of GH and IGF-I mRNA. This indicates that the 7 days of food deprivation used in the present study is too short to bring considerable adjustments in GH/IGF axis hormones. Several studies have reported that the effect of fasting on GH and IGF-I level becomes prominent only after 2 week of starvation (Duan and Plisetskaya, 1993; Moriyama et al., 1999; Perez-Sanchez et al., 1995; Pierce et al., 2004, 2005; Uchida et al., 2003). In the present study, the effect of feed deprivation on the level of GH and IGF-I mRNA was observed when fasted fish were exposed to 21 day of HEA in RS condition. The expression of GH gene was significantly up-regulated in starved fish relative to control and fed fish after 21 days of ammonia exposure. On the other hand, IGF-1 and GHR expression was decreased more or less in the same time frame (at the same time or slightly earlier), which fits with the decreased weight gain (%). There seems to be a delicate interplay between GH expression, GHR expression and IGF-I expression. It is likely that cessation in growth rate reported in our study under stressful condition is due to the decline in the binding capacity of GH to the hepatic GH receptors concomitant with the decrease in GHR mRNA level. This leads to a reduction of IGF-I synthesis (or RNA) since there is a reduced signal from the GHR. As a consequence, IGF-I can then no longer perform its negative feedback on GH expression. Hence GH levels can rise, but at the same time cannot exert their effect due to the lack of receptors.

The opposing picture of GH and IGF-I mRNAs in the present study is consistent with what is observed in rabbit fish (*Siganus guttatus*) and groupers (*Epinephelus coioides*); GH mRNA increased whereas IGF-I mRNA decreased with starvation (Ayson et al., 2007; Pedroso et al., 2006). Our study confirms that expression kinetics of these mentioned genes are not only altered by nutritional status but also by ammonia induced stress. The increment in GH mRNA on a high ammonia dose (2 mM) was also reported in ureogenic goby (*Mugilogobius abei*) (Iwata et al., 2005). Furthermore, when subjected to exercise, the prominent change in GH and IGF-I gene expression occurred earlier (from

day 10) as compared to RS condition (from day 21). This suggests that exercising the fish may acts as an additional environmental stimulus influencing the expression of GH and IGF-I.

Although only mRNA levels of IGF-I, GH and GHR were measured in the present study, it would be reasonable to examine correlation between mRNA levels and their corresponding protein. In addition, investigation on the production of IGF binding proteins and their regulatory role for functioning of IGF-I will also be vital for understanding the genetic basis of physiological alterations in growth studies.

Furthermore, evidence indicates that the GH/IGF-I axis has osmoregulatory function in salmonids and some species of non salmonids teleosts (Mancera and McCormick, 1998a; McCormick, 2001). GH/IGF-I axis stimulate the number and/or size of gill chloride cells (Sakamoto et al.,1993; Xu et al., 1997) and increase gill Na^{+}/K^{+}-ATPase activity and/or mRNA levels in salmonids, killifish (*Fundulus heteroclitus*), striped bass (*Morone saxatilis*) (Madsen et al., 1995; Mancera and McCormick, 1998b; Xu et al., 1997; Sakamoto et al., 1997). We did not observe a distinct response of GH and IGF-I gene in gill tissue under any exposure conditions, suggesting that GH/IGF-I axis may not play a osmoregulatory role in common carp. Evidence on the osmoregulatory role of the GH/IGF-I axis is derived from a small number of teleost species particularly seawater fish. Therefore, intense study is necessary to confirm the osmoregulatory role of GH/IGF-I axis in freshwater fish.

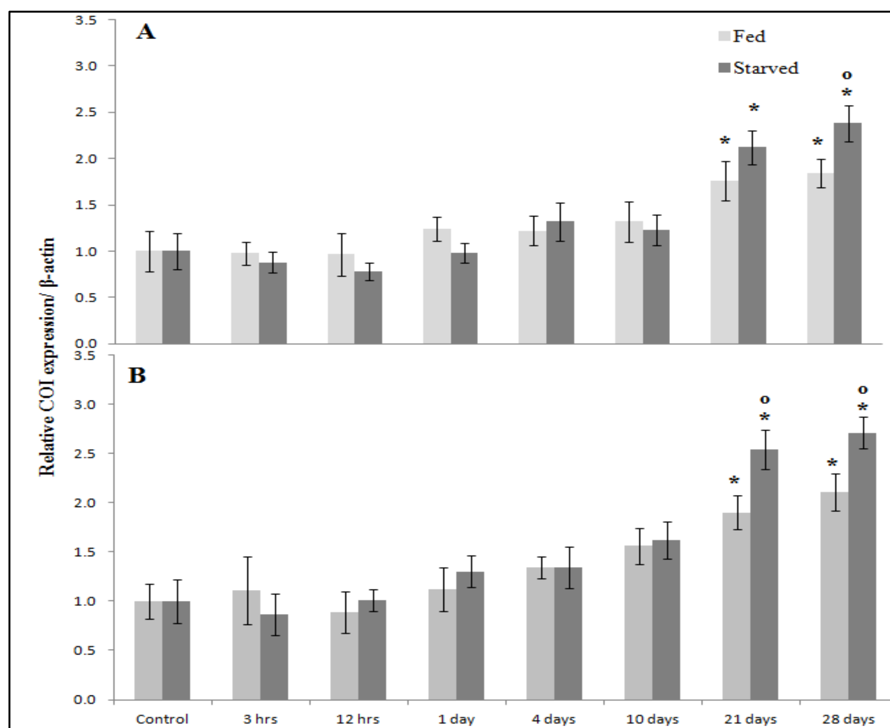


Figure. 9.11. Relative expression of COI mRNA in liver of fed and starved fish during **(A)** Routine swimming **(B)** Exhaustive swimming. Values are mean ratio ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$), circle (o) indicates a significant difference between fed and starved fish at the same sampling period (^{o} $P < 0.05$).

9.3.3. The expression of stress related biomarker genes

The generalized stress response at the cellular level is in part mediated by the actions of a family of proteins known as HSPs. They are distinguished by their molecular weight and the most extensively characterized of the heat shock proteins is a 70 kDa protein (HSP70) and also the most commonly induced stress protein in response to suboptimal physiological conditions (Hutchinson et al., 1994; Iwama et al., 1998). The expression level of HSP70 increased with exposure time except at the end of exposure period. This may reflect that stress due to HEA increased in fish in parallel with the exposure time with a slight acclimatization at the end, as was also seen in ionregulatory processes. The rationale for increased expression of HSP70 in response to HEA is increased amounts of misfolded or denatured intracellular proteins (Hightower, 1991; McDuffee et al., 1997). Ammonia has been shown to interfere with cellular protein and amino acid homeostasis (Cooper and Plum, 1987). As a consequence, the amount of protein fragments may increase and induce proteotoxicity. Our study demonstrates that common carp are able to cope with HEA induced proteotoxicity by the stimulation of HSP70 expression, which can repair partly denatured proteins. A similar effect was seen in brown trout (*Salmo trutta* f. *fario*) under chronic exposure to sub-lethal concentrations of ammonia (Luckenbach et al., 2003). It was noted that the stressful effect of ammonia was exacerbated when fish were subjected to starvation; HSP70 was highly up-regulated in starved fish in contrast to fed fish. When fish are deprived of food, the endogenous tissue protein will be broken down to supply the required amino acids leading to tissue breakdown, therefore affecting cellular protein homeostasis. Subsequently, our result suggests that HSP70 is expressed as a protective strategy against starvation provoked stress in common carp. Correspondingly, Cara et al. (2005) also demonstrated that food-deprivation enhanced HSP70 and HSP90 expression in larval gilthead sea bream (*Sparus aurata*) and rainbow trout (*Oncorhynchus mykiss*). Similarly, liver of *Labeo rohita* fingerlings was found to be highly sensitive to feed deprivation which is evident from the increase in HSP70 induction with the increase in duration of starvation (Yengkokpam et al., 2008). Eventually, it can be acknowledged that the expression of HSP70 as indicators of stress and animal health status holds promise for ecotoxicologal studies.

Furthermore, induction of HSP70 protein under HEA was concomitant with increase in swimming speed. It is very likely that intense exercise introduces several factors (such as energy depletion, oxygen deprivation, generation of reactive oxygen species, lactate production and so on) which could result in cellular damage (Armstrong et al., 1991; Noble, 2002) exemplified by an increase in the production of unfolded or misfolded proteins. Given the liable importance of denatured proteins in HSP induction, it is apparent that protein damage incurred during exercise may signal the up-regulation of HSP as a protective mechanism against subsequent bouts of potential damage to tissues. However, patterns of HSP induction under exercise is not well studied in fish

unlike in mammals where exercise is a well-known physiological inducer of HSP70 expression (Gonzalez et al., 2000; Salo et al., 1991; Skidmore et al., 1995). In contrast to our result, no induction of HSPs (HSP70, HSP30, or HSP90) in response to exhaustive exercise could be noted in rainbow trout (*Oncorhynchus mykiss*) (Clarkson et al., 2005). It may be that high-performance fish like trout are perpetually exercising and are adapted for burst-type exercise.

HEA also induces oxidative stress in fish (Ching et al., 2009; Hegazi and Hasanein, 2010) by generating reactive oxygen species (ROS) and reactive nitrogen species (Murthy et al., 2001), resulting in extensive destruction of proteins (Kosenko et al.,1999) and oxidation of RNA (Görg et al., 2008). In these contexts, COI function as an antioxidant by either preventing the reduction in electron flow (Benzi et al., 1992) or by uncoupling electron transport from proton transfer (Richter, 1997). In the present study, COI gene expression in liver was up-regulated after 3 weeks of ammonia exposure demonstrating an induced adaptive response in attempting to neutralize the oxidative effect of ammonia. Since COI is considered as the rate-limiting step for mitochondrial respiration (Villani and Attardi, 2000), elevated expression of the COI gene could also be a compensating mechanism to restore the decrease in mitochondrial activity and to efficiently consume oxygen (Achard-Joris et al., 2006), thus limiting HEA associated damage in the cell. We also noticed that the ammonia induced COI gene response (during a 3-4 week exposure time) was more pronounced in starved fish compared to fed fish. It may be that starvation leads to depletion of antioxidant stores and increases the generation of oxygen free radicals, particularly in liver (Domenicali et al., 2001; Robinson et al., 1997). Furthermore, many reports confirm that dietary restriction induces oxidative stress in fish (Dou et al., 2002; Meton et al., 2003; Morales et al., 2004; Pascual et al., 2003). Therefore, an increment in COI mRNA level in response to starvation may signify an adaptive feedback in common carp to minimise oxidative damage. However, to our knowledge, the direct relationship between COI expression and ammonia exposure and/or nutritional restriction in fish has never been reported before. Besides, the precise mechanisms by which ammonia exposure and periods of starvation results in free radical production leading to oxidative stress is not completely understood at present. Nevertheless, an advantage of using COI to study the impact of environmental stressors is that the COI gene sequence is highly conserved between lower and higher eukaryotes (Capaldi, 1990). Thus a similar COI gene-expression patterns between different species of fish can be expected.

9.4. CONCLUSIONS

We have determined the expression kinetics of various candidate genes responsible for ion-regulation, growth and stress responses in common carp under the combined effect of ammonia exposure (1mg/L), feed deprivation and exercise. The increased activity and expression of Na^+/K^+-ATPase suggest that it has a function in ammonia homeostasis in common carp. Expression of other

ion-regulatory genes, CR and PRL were not only altered by HEA but were intensified during starvation and exercise. During the last exposure period the expression level of all ion-regulatory genes showed an acclimatization response to HEA. This illustrates that common carp have the ability to overcome the ionic disturbance induced by ammonia exposure. Furthermore, the expression kinetics of growth regulating hormone and receptor genes such as GH, IGF-I, GHR and TR were profoundly influenced by ammonia exposure and severe effects were seen among starved fish. Correspondingly, these changes were reflected by decrease in weight gain (%) in exposed fish with a momentous effect among starved fish. Among stress indicating genes, the interactive effect of HEA, starvation and exercise was seen on the HSP70 mRNA level while expression of COI was influenced by only HEA and starvation. Conclusively, the responses measured at gene level assure the application of molecular biomarker as a monitoring tool for assessing ecotoxicological impact of environmental stressors. Nevertheless, the importance of these studied genes in terms of both functionality and isoform variation and receptor-ligand interaction might be crucial in further examinations.

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Chapter 10

CONCLUSION AND FUTURE PERSPECTIVE

OVERALL CONCLUSION

Ammonia is an environmental toxicant that is especially problematic for aquatic organisms. Its level in aquatic systems can rise due to agricultural run-off and decomposition of biological waste. Moreover, during the past five decades, anthropogenic activities have substantially altered the global nitrogen cycle, increasing both the availability and the mobility of nitrogen (Vitousek et al., 1997; Carpenter et al., 1998; Howarth et al., 2000; Galloway and Cowling, 2002) in water bodies. Consequently, in addition to natural sources, ammonia also enters aquatic ecosystems via point and nonpoint sources derived from human activities. To prevent the situation from deteriorating any further, federal and state governments have introduced environmental regulations and guidelines to protect the quality of surface and ground water from ammonia pollutants and to protect the aquatic organisms. As exposed organisms try to cope with ammonia pollution, they develop species-specific defense mechanisms, but the exact defensive mechanism or startegies for ammonia excretion or handling in (freshwater) fish are not yet fully revealed. Although the diversity of mechanisms of ammonia (and urea) excretion by the gills of aquatic organisms has been studied for decades, such studies are limited to very few fish species. Mechanistic variations are expected among various groups of fish species which differ in the sensitivity (or resistivity) to ammonia. Even within species that are ammonoteles, the process of ammonia excretion is not purely ‘passive’, often relying on the energizing effects of ATPase pumps and transporters. In addition, since the recent discovery that Rh glycoproteins also transport ammonia and are analogs of MEP and Amt proteins (Marini et al., 2000), it became apparent that ammonia is not simply permeable through lipid membranes, but in fact could move through specific membrane Rh proteins. These findings have led to the resurgence of studies on ammonia transport mechanisms in fish. The current hypothesis includes the diffusion of NH_3, most likely facilitated by Rhesus (Rh) proteins, followed by trapping as NH_4^+ in the acidified gill boundary layer as well as interactions between the transport of Na^+ and NH_4^+ through Na^+/NH_4^+ exchangers and the substitution of K^+ by NH_4^+ in basolateral Na^+/K^+-ATPase transport.

The first part of this doctoral thesis aimed at revealing the dynamics of different functional components of gill epithelia and various ionregulatory hormones in regulating ammonia excretion in three freshwater teleosts- a sensitive salmonid, the rainbow trout, a less sensitive cyprinid, the common carp, and the very resistant cyprinid, goldfish. Moreover, we also investigated various indices of gill permeability which play a role in determining the movement of ammonia and ions across the gill. This comparative study provided an insight in how these commercially important freshwater fish species manipulated their biological processes to maintain homeostasis when exposed to ammonia threat.

In our experiment with 1mM HEA we found that trout and carp suffered an initial inhibition (at 3 h) in ammonia excretion rate which was then (from 12 h onwards) followed by either restoration

to control levels (in trout) or an increment above control levels (in carp). In contrast, goldfish showed no inhibition during 168 h HEA exposure and were able to excrete ammonia against a concentration gradient. Na^+ homeostasis was disrupted in trout, partially maintained in carp and fully retained by goldfish. These results suggest that during 1mM HEA goldfish and carp could regulate ammonia and Na^+ homeostasis more efficiently than the trout. We hypothesized that such differences may be due to the differential physiological, biochemical and molecular compensatory responses. Subsequently, we observed that goldfish were able to implement all these responses more effectively in response to HEA without compromising net Na^+ balance whereas these countervailing responses were weakest in trout.

The main obstacle in this part of the research was to study the molecular responses because of the non-availability of Rhbg and Rhcg isoform sequence for goldfish and carp in NCBI data base. Therefore, we identified partial sequences of Rh cDNAs in these two cyprinids by sequencing the PCR products followed by homology comparison with related species such as rainbow trout and zebra fish. During HEA exposure, up-regulation of Rhbg was noted only in goldfish. It was accompanied by restored levels or significant increases in ammonia excretion rate, signifying that unlike carp and trout, Rhbg is involved in the maintenance or regulation of ammonia transport in goldfish. Under HEA excretion ability in goldfish was further enhanced by the up-regulation of Rhcg-a and Rhcg-b while only Rhcg-a transcript level was elevated in carp. In trout, the restoration of ammonia excretion after an initial inhibition could be explained by an increment in Rhcg2 expression level. Furthermore, it was apparent from our studies that goldfish could also utilise the other components of "Na^+/NH_4^+ exchange metabolon" very efficiently since H^+-ATPase activity and expression was augmented in response to HEA, implementing boundary layer acidification and ammonia trapping mechanism effectively. The improved Na^+ homeostasis maintaining capacity in carp and goldfish was revealed by an increment in Na^+/K^+-ATPase activity and expression during HEA. Unlike in trout where regulation of gill permeability was disturbed during ammonia exposure, these cyprinid species exhibited smaller changes in diffusive water efflux and net K^+ loss rate and were able to repress their Na^+ loss rate.

Our study illustrates that it was only goldfish which could substantially increase urea excretion and thus were able to cope with high ammonia more effectively. This was further confirmed at the gene level: a marked increase in branchial mRNA expression of urea transporter occurred only in goldfish. Besides high ammonia, cortisol also regulates the expression of many ion-regulatory genes and Rh glycoproteins in fish gills. However, we did not find a clear-cut relationship between circulating cortisol levels and Rh mRNA expression in any of the experimental species. This may be because plasma ammonia and cortisol act more effectively in combination than alone, also supported by Tsui et al. (2009) in an *in vitro* gill cell culture system.

Surprisingly, ammonia excretion rate and Na^+ intake in all the species were restored or increased before elevations in mRNA levels of Rh glycoproteins and transporters/exchangers were seen. This delayed up-regulation of these associated genes in all three species occurred in conjunction with the elevated plasma ammonia suggesting that internal ammonia levels may act as an inducer for the activate of ammonia transporter genes. This hypothesis was also supported by Nawata and Wood (2009) and Tsui et al. (2009). This temporary disconnect between the changes recorded in mRNA expression and those in functional activity can also be due to post-translational modifications (PTMs) as proposed by Nawata et al. (2010b) for pufferfish.

In future we will investigate the receptor or sensor that links ammonia loading to cortisol release and whether the responses of Rh glycoproteins are connected to cortisol. Moreover, the investigation of these Rh glycoprotein expressions at the protein (functional) level might be crucial in further examinations. An alternative experiment will be conducted to offer a better understanding of non-genomic activation and/or post-translational regulations of existing transport protein function in response to exogenous/endogenous ammonia load.

In a parallel study we also examined the comparative physiological and biochemical responses in these three fish species after subjection to an acutely toxic dose of 5 mM HEA for the cyprinids and 1.4 mM for the trout for a period of 3 h. The experimental animals (especially carp and trout) could not survive the high ammonia dose (5 or 1.4 mM) when exposed for 12 h. Therefore, it prevented us to consider 12 h as another time point for this series of experiment. At this high exposure dose, Na^+ homeostasis was not only disrupted in sensitive trout and moderately resistant carp but was also severely affected in ammonia tolerant goldfish. Moreover, many of the ammonia extenuating abilities in these experimental animals were also suppressed at the high dose of ammonia exposure suggesting that these high concentrations (1.4/5 mM HEA) were close to 96h LC_{50} values for all species.

In the second part of this doctoral thesis we investigated the comparative detoxification and defense mechanism in these three fish species when subjected to the same ammonia level (1mM) as applied in early experiments (part one). In general, ammonia is produced as a by-product of protein catabolism and also accumulated within the cell. Therefore, it is obvious that fish might have evolved a number of different strategies to defend against ammonia toxicity at the cellular and the subcellular level. Such mechanisms vary considerably between species which might determine their sensitivity or resistivity to high ammonia (Iwata, 1988; Vedel et al., 1998). Virtually, the brain is the organ most sensitive to the ammonia toxicity (Cooper and Plum, 1987; Wang and Walsh, 2000; Wright et al., 2007) and could disrupt blood flow in brain, cause astrocyte swelling and interfere with excitatory amino acid neurotransmitter metabolism. Therefore, in this section we tried to evaluate various potential detoxifying strategies meant to remediate build up of ammonia in brain. Conversion of

ammonia into various free amino acids (FAAs) (particularly glutamine) and detoxification of ammonia to the less toxic urea were examined as key protective strategies in the brain of these three fish species. This study confirmed that goldfish could implement these cellular and sub-cellular defensive mechanisms very efficiently, offering this fish species a better resistance against high ammonia. Similar conclusions were also evident from the first part of our study at physiological and molecular level illustrating that the excretion ability against an ammonia gradient is more pronounced in goldfish followed by carp. As such it was interesting to see that under hyperammonia the synthesis of glutamine was substantially activated in the brain of cyprinids (compared to trout), corresponding with a decline in accumulated brain ammonia level. This response of glutamine accumulation in cyprinids correlated positively and significantly with GSase activity. Surprisingly, increment in glutamine level was not sufficient in trout to substantially reduce ammonia accumulation to basal level, making trout more prone to ammonia toxicity than cyprinids. In addition, an efficient replenishment of glutamate (a neurotransmitter) via GDH and transaminase reactions allowed goldfish and carp to retain homeostasis of the excitatory neurotransmitter glutamate and thereby possessed advanced ammonia detoxifying capacity over the ammonia sensitive trout. Furthermore, in our previous experiments (chapter 3, 4) we reported that during HEA exposure only goldfish were able to elevate urea excretion by up-regulating the UT gene. We concluded that this is an extra ability in goldfish compared to carp and trout by which this very resistant fish could tolerate high ammonia. Despite of this ability in goldfish to facilitate urea diffusion through gill, they were not able to utilize ureogenesis in the brain as a potential pathway to convert accumulated brain ammonia into urea, and neither could carp and trout. We hypothesized that it may be related to the fact that the detoxification of ammonia to urea is energetically 2.5 times expensive than glutamine synthesis. Apparently, these species adopted glutamine synthesis rather than switching to energetically more expensive ureogenesis pathways.

In this study, we measured the activity of enzyme at translational level, however, regulation of these enzymes need to be confirmed at the transcriptional level. Also, the possible mechanism(s) by which high ammonia induces metabolic pathways of amino acid was not explained in our study; this issue demands a future attention. In our study, fish were fasted for 48 h prior to commencement of the exposure, nevertheless, reports suggests that feeding could affect the response of amino acid metabolism based defensive mechanisms (Sanderson et al., 2010; Wicks and Randall, 2002). Consequently, it would be advisable to observe how feeding could regulate the activity of the glutamate-glutamine allied ammonia detoxification pathway. In brief, based on the data mining of chapter 3, 4 and 5, we can say that goldfish are endowed with a very efficient physiological-biochemical and gene associated ammonia excretory mechanisms and they can implement ammonia detoxifying strategies very effectually, allowing them to maintain ammonia homeostasis better than

the other two species. Carp was found to utilise these processes moderately while trout was the weakest among all. Moreover, our results based on the molecular to physiological approaches provided a better insight in to why goldfish is very resistant to ammonia while carp and trout are respectively fairly and weakly tolerant.

To investigate the effects of lower levels of HEA under more natural conditions, we conducted other series of experiments which added a few other biological parameters such as feeding and swimming. In these experiments, we first examined the effect of nutritional status on the response to HEA. In general, it can be speculated that during the postprandial period (a few hours after feeding) fish may experience large fluxes of ammonia into the plasma (Brett and Zala, 1975; Wicks and Randall, 2002a, b; Wicks et al., 2002) as a consequence of catabolism of ingested proteins. Intracellular alkalosis due to production of large quantities of ammonia has been reported to cause many ill effects in fish leading to the reduction in overall performance (for details see 'General Introduction'), thereby, it could be hypothised that keeping the fish unfed rather than providing food would be more beneficial to deal with ammonia toxicity.This strategy of keeping fish fasted could also be recommended to aqua-culturists when the level of ammonia is high in the rearing water. Although reduction in feeding rates or starvation may be a handy measure, it will undoubtedly hinder growth rate. We did find that growth rate was reduced significantly when ammonia exposed (1mg/L) goldfish and carp were subjected to additional starvation (refer chapter 6 and 9). These findings led us to re-think 'if feeding amplifies internal ammonia load (affecting physiological performance) then HEA exposure should have also induced a reduction in growth rate among fed fish.' The significant reduction in growth rate among starved fish compared to fed fish during ammonia exposure emphasizes that feeding could indeed ameliorate the internal load of ammonia, presumably because of elevated ammonia excretion rates. To test this hypothesis we made a comparison at physiological, metabolic, hormonal, iono-regulatory and mRNA transcriptional level for these two groups: fed and starved fish during ammonia exposure. In these experiments we used 1mg/L ammonia which is 17 times lower than the concentration applied in chapter 3, 4 and 5 (1mM or 17mg/L). 1 mg/L ammonia represents the legal average quality for surface waters in Flanders. The most interesting result evidenced from our study was that under control conditions (no HEA) unfed goldfish acquired lower plasma ammonia levels compared to fed ones, but surprisingly this was reversed after 4 days of HEA (1mg/L); accumulation was considerably higher in starved fish compared to fed fish, meaning that fed fish were able to remove ammonia more efficiently than the starved fish. This outcome prompted us to check for the ammonia excretion rate, and we observed that fed fish increased excretion while in starved fish excretion remained low. This was found to be associated with the lack of increase in oxygen consumption rate in starved fish and moreover, it corresponded very well with the time frame when fed fish started reducing plasma ammonia level. From our study it was also apparent that fed

exposed goldfish retained significantly higher protein stores compared to the unfed exposed group, presumably because the fed group could utilize hydrolyzed ingested amino acids for protein synthesis. Protein synthesis also provides another possibility for low plasma ammonia in the exposed fed fish. Furthermore, the results of chapter 4 validated that transcript levels of Rh glycoproteins were elevated considerably in goldfish on HEA exposure, thereby facilitating an efficient ammonia excretion. Similarly, Zimmer et al., (2010) reported that feeding could increase Rhcg2 transcript levels in juvenile trout gills under HEA resulting in higher ammonia excretion rates in fed fish. The involvement of Rh glycoprotein seems to be a crucial factor to explain the fact that fed fish are better equipped to tolerate HEA compared to starved fish. However, the sequence details of Rh gene for goldfish (and common carp) were not available at the time we conducted this experiment, restricting us from confirming the consequence of nutritional status on Rh expression dynamics. This perhaps could be an interesting issue which certainly needs to be verified in future experiments.

Differential regulation of amino acid metabolisms (mentioned in chapter 5) could also be the reason for lower plasma ammonia level in ammonia exposed fed fish compared to their starved counterpart. In this context, HEA and feeding has been reported to up-regulate the activity of glutamine synthetase in goldfish which convert accumulated ammonia into non-toxic glutamine (Sinha et al., 2013; Vedel et al., 1998; Wicks and Randall, 2002a,b). In addition, Wicks and Randall, (2002b) also confirmed that feeding also up-regulated pathways that utilize NH_4^+ directly in the production of certain amino acids such as alanine, aspartate, glutamate and glutamine. All four of these amino acids could act as temporary depots of toxic NH_4^+. Once this pathway is up-regulated, it will protect fish from ammonia toxicity. Thus, it is very clear that fed fish have developed better ways to make them less vulnerable to ammonia toxicity than the starved fish, highlighting the significance of 'feeding' as a key factor to combat the problems of HEA.

Exposure with ammonia did not hamper swimming performance (U_{crit}) in fed fish while starved fish suffered. The U_{crit} reduction in starved exposed fish was associated with a remarkable increase in plasma ammonia and lactate accumulation, and considerable depletion in muscle glycogen stores. In salmonids, ammonia accumulation in the plasma has been implicated in reduced critical swimming speeds (Shingles et al., 2001; Wicks et al., 2002). Increased NH_4^+ levels alter the metabolic status within the fish, and may lead to premature muscle fatigue (Beaumont et al., 2000b). Besides, depolarization of muscle membrane potential due to the substitution of K^+ with NH_4^+ has also been proposed for impairment of muscle contraction (Beaumont et al., 1995a,b, 2000a; Cooper and Plum, 1987; Raabe and Lin, 1985). Subsequently, we propose that swimming performance is not only influenced by ammonia exposure but also by feed deprivation.

Thereafter, we were eager to examine if feeding could also be advantageous over starvation in maintaining ion-regulation and hormonal homeostasis during HEA. Investigating the relationships

between plasma ions, plasma T3/T4, plasma cortisol, gill $Na^{+/}K^{+}$-ATPase activity and gill mRNA expression level of growth and osmo-regulatory controlling genes (IGF-1, growth hormone receptor 'GHR' cortisol receptor 'CR', thyroid hormone receptor 'THRβ', prolactin receptor 'PRLR') gave us an insight in how fed and starved goldfish manipulated their important iono-regulatory hormones and ion osmo-regulatory processes when exposed to HEA. Our study (Chapter 7) further confirmed that under ammonia threat fed goldfish have a physio-biochemical advantage over food deprived individuals since cortisol level, thyroid (T3) hormone level, expression level of growth and osmo-regulatory controlling genes (IGF-1,GHR, CR, THRβ) were adversely influenced during starvation while fed fish were able to maintain many of these parameters at basal level. This reduction in transcript level of growth controlling genes explains the growth rate cessation in ammonia exposed starved fish (refer chapter 6). Interestingly, the increase in plasma [Na^{+}] in both fed and starved exposed group was accompanied by an increase in branchial Na^{+}/K^{+}-ATPase activity. Our experiment with substitution of K^{+} with NH_4^{+} (refer chapter 4) showed no activation in the activity of branchial Na^{+}/K^{+}-ATPase in goldfish (and carp), suggesting that NH_4^{+} is not a preferred substrate for this enzyme. Consequently, the increment in plasma [Na^{+}] was not solemnly associated with Na^{+}/K^{+}-ATPase but apparently due to increment of H^{+}-ATPase activity (in goldfish, evident from chapter 4) which eliminates H^{+} across the membrane and generates a negative potential inside the apical membrane which drives Na^{+} inwards via a sodium channel. In brief, the patterns of ionic, hormonal and gene-expression modulation along with metabolic and physiological responses in starved exposed cyprinids indicates that starvation amplifies the ill-effect of ammonia toxicity.

In continuation, we investigated how swimming can influence the combined effect of HEA and nutritional status in cyprinids. Swimming at higher speed has been shown to induce endogenous ammonia production and increases internal ammonia levels when compared with resting (Knoph and Thorud, 1996). Likewise, in our experiment when goldfish were forced to swim at higher speed the ammonia induced physiological and metabolic responses in fish were amplified, suggesting that exhaustively swimming fish were more susceptible to HEA than routinely swimming fish. Fed fish were less affected (than fasted fish) by elevated ambient ammonia as well as by higher swimming speeds while severe effects were noted in fasted fish. In addition, most of the ion and metabolism regulatory hormones and/or their respective genes studied (refer above) were found to be considerably influenced during exhaustive swimming, with more adverse effect in starved fish.
These outcomes indicate that besides HEA and feed limitation, exhaustive swimming can be a crucial environmental factor manipulating biological processes in fish, and further recommend that starvation can render fish more vulnerable to external ammonia during exercise.

We did a parallel experiment to observe if the above mentioned consequence of combined effect of HEA, starvation and exercise on the fitness of goldfish were also true for a moderately

resistant fish, the common carp. Interestingly, this fish species (carp) revealed almost the same pattern as seen in goldfish for most of the parameters studied (refer chapter 8 and 9) but the response time varied from the goldfish, probably because of the difference in ammonia tolerance ability between these two cyprinids. Furthermore, it would have been interesting to conduct such experiments also with ammonia-sensitive fish such as trout. Non-availability of the right sized trout (that can fit within the respirometer) restricted us to conduct this experiment within the time frame of this PhD project.

FUTURE PERSPECTIVE

Compared to the overwhelming research in freshwater fish addressing the mechanism of ammonia excretion and ammonia toxicity, the information on marine fish is relatively scarce. The recent model by Wright and Wood (2009) for describing ammonia excretion that emphasizes the involvement of Rhesus (Rh) glycoprotein in branchial ammonia excretion and the linkage of ammonia removal with Na^+ uptake is mainly limited to freshwater teleosts but may not be applicable for marine fish because of their different iono–osmoregulatory mechanism strategies. This could influence the physiology, hormonal activity and gene expression of these marine fishes in different ways compared to freshwater fish when exposed to high ammonia. Seawater fish have enhanced permeability to NH_4^+ unlike freshwater fish but other potential modes of excretion such as apical $Na^+/H^+(NH_4^+)$ exchange and NH_3 trapping mechanisms are less active and also the existence of apical proton pumps is doubtful, therefore ammonia appears to be more toxic in seawater compared to freshwater (Randall and Tsui, 2002; Wilkie, 1997; Wilson and Taylor, 1992). However, more studies need to be done in the future to reveal the ammonia excretory mechanisms in marine fish and also to make a direct comparison of ammonia induced toxicity with freshwater.

The ability of some fishes to reversibly remodel their gill morphology has become a focus of research after the discovery of extreme morphological gill plasticity in crucian carp and goldfish. Therefore, electron microscopic based gill remodeling examination should be consider in future ammonia toxicity studies to provide a better insight of iono-osmo-respiratory and ammonia excretory compromise. Some preliminary results on this issue have been presented in chapter 11 (refer below).

Chapter 11

Some preliminary results on macro-and ultrastructural modifications in the gills of three freshwater teleosts in response of high environmental ammonia

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Abstract

The present study aimed to investigate the modification in gill morphology of three freshwater fish differing in their sensitivity to high environmental ammonia (HEA). The highly ammonia-sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less ammonia-sensitive cyprinid *Cyprinus carpio* (common carp) and the highly ammonia-resistant cyprinid *Carassius auratus* (goldfish) were used as test species and were exposed for 0 h (control), 3 h, 12 h, 24 h, 48 h, 84 h and 180 h to 1 mM ammonia (as NH_4HCO_3; pH 7.9). In trout, only the lamellae thickened and shortened during the course of the ammonia exposure but the filaments remained intact while in both cyprinids filaments thickened as well. These changes in cellular morphology during ammonia exposure may be an attempt to reduce the surface area, which was more pronounced in goldfish where complete remodelling of the gill lamellae took place. Excessive mucus production was obvious in carp and goldfish. While goldfish and carp showed shrinkage of apical crypts of mitochondrion rich cells (MRCs), probably aiding to control ion fluxes, trout showed enlarged apical crypts of MRCs. All species displayed changes in the microridges covering the pavement cells (PVCs). Overall, the present study illustrate that under ammonia threat the goldfish revealed a 'classic' mode of gill reorganization (or gill remodeling) which was 'modest' in carp and absent in trout where only lamellae reacted. In brief, our results suggest that the goldfish with its minimal surface area of lamellae and a large population of the MRCs with small apical crypts located on the edge of interlamellar cell mass is better prepared for survival in ammonia polluted water than the carp which maintain larger lamellae and especially the trout that did not show're-design'.

Keywords: High environmental ammonia (HEA), Gill remodeling, Scanning Electron Microscope, Mucous cells, Pavements cells Mitochondrion rich cells, Freshwater teleosts.

1. INTRODUCTION

The fish gills are the primary site for gas exchange, iono-regulation, acid–base balance and nitrogenous waste excretion. In teleosts, each branchial arch is divided into multiple filaments, which are further subdivided into thousands of lamellae that are the main sites of gas exchange. The epithelium covering the gill filaments and lamellae provides a distinct boundary between a fish's external environment and extracellular fluids and allows the branchial tissue to be exposed to variations of the medium. The epithelium is typically one to three cell layers, is composed of several distinct cell types such as pavement cells (PVCs), mitochondrion rich cells (MRCs) (often termed as 'chloride cells'), accessory cells (ACs; only in marine fish), and mucous cells (MCs). PVCs are the most abundant cell type covering the epithelium (> 90% of the surface area) and are thin squamous to cuboid-shaped cell also referred as respiratory cell. The apical surface of PVCs consists of microvilli and/or microplicae (microridges) projections likely increase the functional surface area of the epithelium and may also play a role in anchoring mucous to the epithelial surface. Cytoplasm composition of PVC includes few mitochondria, well-developed rough endoplasmic reticulum, and Golgi apparatus that produce microvesicles (Laurent and Perry, 1991; Wilson and Laurent, 2002). When these vesicles fuse with the apical cell membrane, glycoproteins are released to the cell surface and become a component of the mucous layer. The mucous granules containing glycoproteins, mucopolysacharides and carbohydrates, are produced by the specialized MCs. When these granules are discharged and rupture onto the epithelial surface, their content forms a mucous layer. This has been proposed as a part of the stress response in fish, or may participate in ion and water balance (Shephard, 1994; Wendelaar Bonga, 1997). MRCs are found interspersed with the PVCs, especially in the interlamellar epithelium and often on the trailing edge of the filament (Laurent, 1984; Van Der Heijden et al., 1997). The MRCs are characterized by a relatively high metabolic activity compared to PVCs (Perry and Walsh, 1989). Therefore, PVCs are largely considered to play a passive role in the gill physiology and their major function is gas exchange while MRCs are considered to be the primary sites of active physiological processes in the gills and play a central role in ion-regulation (Evans et al., 2005; Perry 1997; Marshall 2002; Kaneko et al., 2008). In addition, MRCs are characterized by the presence of an extensive tubular system emanating from the basolateral membrane. This is associated with the localization of the transporter enzyme Na^+/K^+-ATPase, which plays a crucial role in osmo-regulation (Karnaky et al., 1976a, b). Moreover, the importance of MRCs or chloride cells for ion-regulation in freshwater teleosts is well-established (Laurent et al., 1985; Perry and Laurent, 1989; Laurent and Perry, 1990, Perry et al., 1992), and alterations in the number and distribution of MRCs within the gill epithelium of freshwater fish have been observed in response to a variety of environmental stressors (Laurent and Perry 1991; Goss et al. 1995).

Furthermore, a number of studies have shown that in response to stressful environments, some teleosts have the ability to modify their gill morphology through plasticity of the branchial components (Sollid et al., 2003; Brauner et al., 2004; Ong et al., 2007) and can be greatly altered within hours to days (van der Meer et al., 2005; Sollid and Nilsson, 2006). This ability of some fish species to undergo modifications in their gill morphology in an attempt to attain homeostasis is often termed as 'gill remodeling'. The morphological changes in the gills and the changes in specialized cells in the gill epithelia associated with the gill remodeling have been well documented in Crucian carp (*Carassius carassius*) (Sollid et al. 2003, 2005; Sollid and Nilsson, 2006; Nilsson 2007), goldfish (*Carassius auratus*) (Sollid et al. 2005; Mitrovic and Perry 2009, Mitrovic et al., 2009), Amazonian oscar (*Astronotus ocellatus*) (Matey et al., 2011), rainbow trout (*Oncorhynchus mykiss*) (Matey et al., 2011) and scaleless carp (*Gymnocypris przewalskii*) (Matey et al., 2008) in response to hypoxia. It was seen that under normoxic conditions, in order to prevent diffusive ion loss to the water, the crucian carp (*Carassius carassius*) maintains a cell mass enveloping the gill lamellae, deemed the interlamellar cell mass (ILCM) (Sollid et al., 2003; Sollid and Nilsson, 2006). However, during hypoxia, fish gradually lose the ILCM through a combination of a lowered rate of mitosis and heightened rate of apoptosis (Sollid and Nilsson, 2006), thereby increasing surface area by up to many folds and effectively increases the gas exchange area (Sollid et al., 2003). Temperature and salinity changes seems to induce similar effects (Sollid et al., 2005; Rissanen et al., 2006; Pisam et al., 1988; Franklin, 1990; Cioni et al., 1991; Brown, 1992; Kultz et al., 1995). Metal exposure is also known to induce morphological changes in the gills, including reversible thickening of the epithelium. It will increase the diffusion distances (Lappivaara et al., 1995) between ambient water and gill epithelium, thus repress the rate of toxicant influx. Thickening of gill epithelium also tends to reduce the respiratory surface area which might be a protective response since small respiratory surface makes fish less accessible for toxic substances (ammonia, for example). Though a reduced respiratory surface area undoubtly depresses respiratory functions of the gills, it benefits ion-regulation and acid-base balance by reducing the costs of osmo-regulation by lowering the amount of ions that has to be replenished. Thus, gill morphology is likely to be a compromise between opposing demands and often experience osmo-respiratory compromise under stressful conditions.

Despite of these extensive arrays of studies on gill morphological dynamics in response to a number of unfavourable environments, little information is available on this aspect when fish are subjected to high environmental ammonia (HEA). We postulated that the ability to undergo gill remodeling in response to HEA may vary among fish species which have different tolerance limits to ammonia toxicity; a closer look into the changes in the cellular composition and/or morphology of gill lamellae and filament will offer a better insight in determining the ammonia sensitivity. In our previous experiments (Liew et al., 2013, Sinha et al., 2012a,b, 2013, Sinha et al., 2013 unpublished

data, refer ch. 4) we revealed differential physiological, biochemical and molecular compensatory responses in trout, common crap and goldfish to determine their sensitivity to ammonia, however, a morphological approach was still lacking.

Therefore, the aim of the present study was to determine, using scanning electron microscopy, the morphological and structural characteristics of mucous, chloride and pavement cells of the gill epithelia and to analyse the variations at the macro and ultrastructure level when three freshwater teleosts differing in their sensitivities to ammonia were exposed chronically (up to 180 h) to HEA (1mM at pH 7.9). In the present study we used the highly ammonia-sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less sensitive cyprinid *Cyprinus carpio* (common carp) and the highly resistant cyprinid *Carassius auratus* (goldfish) as test species. We hypothesize a differential response of (i) gill filament and lamellae macrostructure (ii) shape and structure of MRCs, PVCs and MCs and (iii) surface structure of MRCs and PVCs in these experimental fish species during ammonia threat.

2. MATERIALS AND METHODS

2.1. Experimental system and animals

Rainbow trout, *Oncorhynchus mykiss*, were obtained from a fish farm - Pisciculture Collette, Bonlez, Belgium; goldfish, *Carassius auratus*, were obtained from Aqua Hobby, Heist op den Berg, Belgium; common carp, *Cyprinus carpio,* were obtained from the fish hatchery at Wageningen University, The Netherlands. Fish were kept at the University of Antwerp in aquaria (200 L) for at least a month before the exposure started. A total of 96 goldfish, 96 common carp and 96 rainbow trout were distributed species wise into four 200 L tanks (n=24 per tank). Each of these tanks was equipped with a recirculating water supply in a climate chamber where temperature was adjusted at 17±1°C and photoperiod was 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Water parameters were: pH 7.4 ± 0.2, dissolved oxygen 6.9-7.4 mg/L, total NH_3 0.006-0.009 mM, nitrite 0.0015- 0.0021 mM, nitrate 0.015-0.042 mM, Ca^{2+} 0.8–1.0 mM, Mg^{2+} 0.19-0.21 mM, Na^+ 1.2-1.4 mM, K^+ 0.09-0.10 mM, Cl^- 0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 226 mg $CaCO_3$/L. Average mass (mean ± standard deviation) of rainbow trout was 15 ± 2 g, of common carp 18 ± 3 g, and of goldfish 17 ± 3 g. Fish were acclimated for 2 weeks prior to the experiment and were fed ad libitum once a day with either commercial pellets (‘Hikari Staple’, Kyorin Food Ind. Ltd., Japan) for common carp and goldfish, or ‘Trouvit’ (Trouw Nutrition, Fontaine-les-Vervins, France) for rainbow trout. Feeding was suspended 2 days before experimentation.

2.2. Exposure and sampling intervals

The experimental set up consists of exposing the goldfish, carp and trout to 1mM ammonia for a period of 3 h, 12 h, 24 h, 48 h, 84 h and 180 h. The exposure was conducted in 8 L glass aquaria (water volume set to 6 L). Control groups (no HEA) were setup in parallel to 0 h, 84 h and 180 h exposure groups. The experimental aquaria were shielded with black plastic to minimize visual disturbance and fitted with individual air-stones.

Fish (n=2) were placed in an individual glass aquaria the evening before an experiment and left overnight to settle with continuous aeration. The experimental protocols consisted of exposing 8 fish (in 4 aquaria) per experiment to HEA. Each exposure aquaria was spiked with the required amount of an NH_4HCO_3 stock solution (Sigma, Germany). A constant concentration of 1.08 ± 0.06 mM of ammonia was maintained throughout the experiment. Exposure ammonia concentrations were measured (using the salicylate-hypochlorite method, Verdouw et al., 1978) 6 h after the onset of treatment and the concentration of ammonia in the tanks was maintained by adding calculated amount of the NH_4HCO_3 solution. Moreover, to avoid the microbial breakdown of test chemical and build-up of other waste products, 60–80% of the water was discarded after each 2 days and replaced with fresh water containing the respective amount of ammonia. Water pH was maintained at 7.8-8.0 throughout the experimental period using dilute HCl and/or KOH.

2.3. Sampling procedure

For sampling, fish were removed from aquaria (n=8 from each treatment and control group) and anesthetized using an overdose of neutralized MS222 (ethyl 3-aminobenzoate methane-sulfonic acid, 1 g/L, Acros Organics, Geel, Belgium). Fish were dissected on ice. The second gill arches of each species were dissected out, rinsed and immediately fixed in cold Karnovsky's fixative. The fixed samples were stored at 4°C and transported to San Diego State University (SDSU, USA) for morphological examination using scanning electron microscopy (SEM) as described below.

2.4. Morphological analyses

AT SDSU the middle section of each fixed gill arch were cut into 2 equal pieces each of them bearing 5-6 filaments in both anterior and posterior rows. All fixed gill tissue were rinsed in phosphate-buffer saline (PBS) and post fixed in 1% osmium tetroxide for 1 h. The samples were dehydrated in ascending concentrations of ethanol from 30% to 100%, critical-point dried with liquid CO_2, mounted on the stubs, sputter-coated with platinum and examined with a Quanta 450 scanning electron microscope (FEI) (Hillboro, OR, USA) at the accelerating voltage of 20 kV.

3. RESULTS AND DISCUSSION

3.1. Gill morphology during control conditions

In control condition (0 h) the macrostructure examinations of goldfish and common carp gills revealed a typical teleost gill structure with long filaments, well-defined long and thin respiratory lamellae separated by regular interlamellar spaces (Fig.1A.Cc,Gr). Rainbow trout at 0 h illustrated the same pattern of gill macrostructure as seen for cyprinids but their lamellae were supported by very stout filaments (Fig.1A.Tr).

The ultra-structure inspection of filament epithelium during 0 h demonstrates that surface pattern of the pavement cells (PVCs) was slightly different in the goldfish and carp but in both species it was a pattern composed with straight or slightly branched microridges and well-defined cell boundaries (Fig.1B.Cc; Fig.1B.Gr). In trout, PVCs showed a complex surface pattern composed by long and branched microridges (Fig.1B.Tr). Mitochondria- rich cells (MRCs) in cyprinids opened on the epithelial surface with flat or slightly concave apical crypts ornamented by short and straight microvilli (Fig.1B.Cc; Fig.1B.Gr). In trout, the apical crypts of MRCs have flat or slightly convex surface and "carpet-like" appearance due to highly interdigitated microvilli (Fig.1B.Tr). Interestingly, abundant mucous cells (MCs) were seen in goldfish which were discharging mucus on the epithelial surface while in carp few MCs were evident which were distributed randomly between PVCs. At 0 h the MCs in trout were roughly triangular or had elongated openings and showed mucus matter within while the openings of the MCs.

In our experiment feeding was suspended 48 h before the experimentation, so it can be anticipated that keeping the fish unfed chronically up to 132 h (in 84 h exposure group) and 228 h (in 180 h exposure group) may have an effect on gill morphology. Therefore, to observe the effect of starvation, we also run control group for a period 84 h and 180 h in a similar way as it was done for exposure period. In both cyprinids and the salmonid, no difference in the ultrastructure of their filament epithelial surface as well as macrostructure of their gills was recorder between 0 h (Fig.1A, 1B), 84 h and/or 180 h control groups (Fig.1C, 1D) (except for a slight difference in MCs for trout, evident by enlargement of MCs during 84 h-180 h), signifying that periods of starvation have little impact on gill morphology in studied fish species.

3.2. Changes in the macrostructure during ammonia exposure

No difference in the macrostructure (filaments and lamellae) was seen in any of the three fish species within first 3 h of HEA exposure compared to their respective control (Fig. 2A). After 12 h HEA, an adjustment was noted only in goldfish as lamellae became slightly shorter and thicker in contrast to the slim and long lamellae observed in control group (Fig. 2C.Gf). However, the structure of gill filaments and lamellae in 12 h exposed carp (Fig. 2C.Cc) and trout (Fig. 2C.Tr) remained unaltered

compare to their respective controls as well as to 3 h exposed groups (Fig. 2A.Cc,Tr). In goldfish, progressive shortening and thickening of lamellae continued, their focal fusions and thickening of filaments were seen after 24 h exposure (Fig.3A.Gf) and were strengthened after 48 h of exposure (Fig.3C.Gf). On the contrary, first signs of alterations in the macrostructure of the trout and carp gills were only seen after 48 h of exposure; thickening and slight shortening of the lamellae was observed in trout (Fig.3C.Tr) while in carp (Fig.3C.Cc) length of gill lamellae remained similar to control lamellae, but they were very thickened compared to the control group. A dramatic change in the general structure of goldfish gill filaments and lamellae was observed after 84 h exposure (Fig.4A.Gf), gill filaments became enormously enlarged and lamellae became extremely short and thick although still separated by interlamellar spaces. The thickening of filaments and lamellae continued thereafter, marked by the presence of rudimental and almost fused lamellae after 180 h which completely faded along the distal one/ fifth part of filaments (Fig.4C,D.Gf). In contrast to the goldfish, macrostructure of the carp and trout gills did not show such dramatic alterations during 84-180 h exposure (Fig.4A, 4C), no fusion of lamellae was seen along filaments and distribution of lamellae to the distal part of filaments was the same as in their respective controls. Though the gill lamellae in carp became shorter and thicker during 84-180 h HEA (Fig.4A, 4C.Cc), they remained well-shaped and were separated by distinctive interlamellar spaces. It is important to note that the filaments also were thickened in carp as was seen for goldfish. In 84-180 h exposed trout, shape and size of filaments remained the same as for control, but lamellae were thickened (Fig.4A, 4C.Tr) and differed from carp by the fact that interlamellar spaces were reduced and lamellae were separated by the narrowest gap-like spaces.

These results suggest that goldfish and carp were able to manipulate the macrostructure of gills in response to ammonia through the reorganization in filaments as well as in lamellae. However, trout could only rely on lamellae manipulation. The thickening of gill filaments and lamellae seen in cyprinids may be a protective strategy by increasing the water-blood barrier (refer Introduction) and may mitigate the effect of ammonia. This compensatory approach which incites a reduction of the respiratory surface, is beneficial for reducing passive losses of salts across the gill and thus cut-down the energetic costs of ionic regulation (Sollid et al., 2003; Sollid and Nilsson, 2006; Nilsson, 2007).
The alterations at the gill macrostructure in the goldfish during 180 h exposure was more pronounced than for the carp and the trout; and revealed a picture of a "classic" complete reorganization (or gill remodeling) in contrast to the very closely related carp. The results of previous studies have demonstrated a remarkable structural remodelling of the gills of goldfish in response to changes in ambient temperature or O_2 levels (Sollid et al., 2005; Mitrovic et al., 2009; Mitrovic and Perry, 2009). In consent to the present results, functional lamellar surface area was also decreased and water-to-blood diffusion distances increased in goldfish when acclimatized to low water temperature (<15°C;

Sollid et al., 2003) owing to the formation of a mass of cells between adjacent lamellae. However, further examination of gill filaments with light microscopy will be helpful in describing the morphology of interlamellar spaces and/or the significance of ILCM during ammonia exposure.

3.3. Changes in the ultrastructure of gill filament under exposure conditions

3.3.1. Mucous cells (MCs)

In the present study, a common response in all the studied fish species to ammonia exposure was production and deposition of mucus on the gill surface. However a species specific difference was apparent as the copiousness of MCs and intensity of mucous production varied among trout, carp and goldfish during HEA exposure. Following 3 h-12 h HEA exposure, numerous openings of MCs appeared on the epithelial surface in the goldfish and carp (Fig.2B, 2D.Gf, Cc). These cells released globules to the nearby MRCs, and that may facilitate concentration of mucus on the surface of MRCs. During this time frame excessive discharge of mucus was exhibited in goldfish as huge globules were released by goldfish MCs (Fig.2B, 2D.Gf) compared to the small solitary globules in the carp (Fig.2B, 2D.Cc). The overproduction of mucous in goldfish continued thereafter which filled microgroves separating microridges and by 48 h it covered almost all smooth surface of PVCs (Fig.3B, 3D.Gf). During the last exposure periods (84 h- 180 h) the epithelial surface was also masked by a film of mucus, and huge mucus globules were seen on epithelial surface (Fig.4B, 4E.Gf). Compared to goldfish, the production of mucus was moderate in carp even after 84-184 h exposure (Fig.4B, 4D.Cc).

Similar to goldfish, trout also produced excessive mucus from their MCs during 3 h- 24 h HEA (Fig.2B, 2D.Tr) and its deposition on the epithelial surface as globules formed a thin film or patches that masked cells located beneath and complicated their identification. In trout the active precipitation of huge amount of mucus on the epithelial surface reached its peak after 48 h of exposure (Fig.3D.Tr), filled microgroves separating microridges on the PVCs surface, covered apical crypts of MRCs and completely masked surface of a number of cells. However, a divergent pattern was observed between trout and cyprinids during last two exposure periods (84 h and 180 h). In cyprinids MCs continued to actively produce mucous throughout the exposure periods; while a decline in quantity and functionality of mucous cells was noticed in trout during 84-180 h HEA exposure (Fig.4B, 4D.Tr). It was evident by sparsely distributed MCs, the epithelial surface which was not covered by mucus and the number of MRCs that were easily traceable.

Overall, HEA clearly induced MCs that led to the overproduction and deposition of mucous on the gill surface in cyprinids, with immense increments in goldfish; while the initial deposition in trout was not sustained at 84-180 h HEA. It is well known that stress caused by variations in the environment and by toxicants induces the proliferation of mucous cells (Perry and Laurent, 1989)

and subsequent increases in mucus secretion (Wipfli et al., 1994).This is a generalized response of fish gills to a range of environmental stressors (Mallatt, 1985; Wendelaar Bonga, 1997). Mucus protects fish gill epithelium from viral and bacterial infections, abrasive action of particles in suspension (Lewis, 1979), desiccation and mechanical injuries. Overproduction of mucus in response to water contamination plays an important role by holding in control toxic substances and thus preventing entrance of toxicant into gills (Perry and Laurent, 1993). Consequently, it can be anticipated that augmentation in mucus production among cyprinids might provide a defense against ammonia toxicity. Moreover, polyanionic mucous on the surface of ionocytes (or MRCs) has been proposed to create a microenvironment rich in ions, increasing local ion concentrations at the gill surface, facilitating ion exchange and limit water influx (Handy et al., 1980; Shephard, 1994). Thus, mucous may actually restrict the magnitude of the iono-regulatory disturbance. This consequence was reflected by an increase in plasma level of Na^+ and K^+ ions in cyprinids (data not shown) at the end of the exposure period which corresponds with copious production of mucous in this time frame. However, in trout no such elevation in plasma ions was noted which might associate with the decline in mucous production during last two exposure periods. Moreover, in our previous similar experiments (Liew et al., 2013, Sinha et al., 2013 unpublished data, refer ch. 4) we reported that cyprinids were able to regulate ion-flux better than trout; it might corroborates with the present finding of mucous production beside the potential involvement of ammonia transporters or exchangers illustrated in previous experiments.

Furthermore, MCs are predominantly responsible for the mucous production but granule secretion from other cells such as rodlet cells may also contribute to mucous yield. Laurent and Perry (1991) verified that PVCs possess numerous glycoprotein-containing microvesicles that can fuse to the apical membrane and can release their content on the gill surface, and therefore can also administer mucous production. The relative contribution of rodlet cells and PVCs to mucous production during ammonia threat requires further investigation.

3.3.2. Pavement cells (PVCs) and mitochondrial rich cells (MRCs)

Following 3 h HEA exposure we observed that macrostructure of goldfish and carp gills did not differ from control fish but at the same time, in both fish species surface ultra-structure of gill filaments altered (Fig.2B.Gf, Cc). Different responses were registered in the MRCs and PVCs of all species examined. In the goldfish, apical crypts of MRCs became very large and highly convex and contained only rudimentary microvilli that were used to provide stretching of the apical membrane of these cells (Fig.2B.Gf). In contrast, apical crypts of MRCs in the carp became small, flat or slightly concave and sometimes contained small particle of mucus attached to straight microvilli (Fig.2B.Cc). In the goldfish (Fig.2B.Gf), surface relief of the PVCs did not change significantly from their control, but in

the carp (Fig.2B.Cc), surface relief of the cells was formed completely by tightly packed microridges. The ultra-structural adjustments in cyprinids gills continued thereafter and manifested itself by a noticeable change after 12 h HEA. In the goldfish, MRCs gathered into clusters (located close or side-by-side to MCs releasing enormously huge globules of mucus) demonstrating smaller and less convex apical surfaces equipped with more distinctive microvilli (Fig.2D.Gf). In carp, apical crypts of the MRCs were narrow, elongated and flat with short microvilli (Fig.2D.Cc). Surface structure of PVCs in goldfish was not altered while in carp it underwent a definite reduction compared to 3 h of exposure. The ultra-structural modifications in epithelial cells of trout gill filaments during 3h- 12 h exposure (Fig. 2B, 2D.Tr) also demonstrated some changes but these were not as intense as compared to cyprinids. In trout, apical crypts of MRCs were enlarged and lost their “carpet-like” appearance becoming almost smooth or having short microvilli. Changes in the filament epithelium further progressed in both goldfish and carp. Apical crypts of goldfish MRCs underwent further transformations becoming flat or shallow with knob-like microvilli after 24 h of exposure, and concave, partially or completely filled with mucus after 48 h of exposure (Fig.3B, 3D.Gf). Moreover, gradual reduction of the PVC’s surface pattern was notable especially after 48 h (Fig. 3D.Gf). In carp during 24 h HEA, MRCs opened on the epithelial surface with small and flat apical crypts (Fig. 3B.Cc). These crypts became enlarged and slightly convex after 48 h of exposure (Fig. 3D.Cc), and during the same exposure period a reduced pattern of PVCs was noticed. In trout, apical crypts of MRCs were enlarged further during 24 h-48 h and almost completely masked by mucus patch (Fig. 3B, 3D.Tr). Few MRC’s crypts could be seen without mucus deposition on the top, and those which were seen displayed a definite reduction of their microvilli surface pattern. Mucus also filled the groves between microridges on the PVCs and also covered the surface area. During the last two exposure periods, the apical surface of trout MRCs were more or less convex and contained short (or vestigial) microvilli and were mucus-free (Fig.4B, 4D.Tr). Solitary and clustered MRCs were noticed particularly during the last exposure period, and surface pattern of PVCs were highly reduced because the microridges ornamenting PVC surface were shrink to short microvilli (Fig.4D.Tr).

In the goldfish the epithelial surface showed signs of the severe transformation during last two exposure periods. After 84 h of exposure, the surface of PVCs was decorated with a few short microridges (Fig.4B.Gf) which were completely lost after 180 h and the surface of PVCs became smooth (Fig.4E.Gf). Following 84 h of exposure, the apical crypts of MRCs still remained large and were flat or slightly convex with a smooth surface while concave crypts were filled with mucus (Fig.4B.Gf). Only solitary and very small apical crypts of MRCs were seen on the epithelial surface when subjected to 180 h HEA (Fig.4E.Gf).

Surface pattern of PVCs in 84 h-180 h exposed carp (Fig.4B, 4D.Cc) showed the restoration of well shaped cellular borders that were lost during 12- 48 h of exposure. During these exposure

time points, the microridge pattern looked different from control (and other treatment groups) and was composed by very short microvilli. Moreover, apical crypts of MRCs became shallow and filled with mucous content, also manifested by numerous openings of MCs on the epithelial surface (Fig.4B, 4D.Cc).

In general, a marked difference was noted during ammonia exposure in MRCs morphology between cyprinids and trout; the apical surface area in cyprinids was reduced while in trout an enlargement was perceived. The findings in trout are in consent with the results obtained during metabolic alkalosis in which rainbow trout also experienced a marked proliferation of lamellar and filamental MRCs (Goss and Perry, 1994; Goss et al. 1994a, b; Perry and Goss, 1994). As a response to an alkaline environment, American eel (*Anguilla rostrata*) possesses an extremely low surface area of branchial MRCs (Goss and Perry, 1994) similar to the response evident in the present study in cyprinids when exposed to HEA.

Freshwater teleosts continually lose ions across their permeable body surfaces of which the gill, owing to its large surface area, is the most significant. Ionic equilibrium is achieved as a result of a more-or-less equivalent absorption of ions from the water. However, there is much uncertainty surrounding the role of the MRCs in ionic regulation in freshwater teleosts, although it has been widely assumed that these cells are responsible for the uptake of Na^+, K^+ and Cl^- from the water.
Moreover, the apical crypt commonly contains a polyanionic mucosubstance (Philpott, 1968; Handy and Eddy, 1991; Powell et al., 1994) and has been proposed to play a role in the concentration of cations and the maintenance of an ionic concentration gradient. Therefore, given the fact that apical membrane of MRCs is the site for ion uptake from the water, a reduction in apical surface area may contribute to the progressive reduction in plasma ions. This however, was not true in present study as plasma Na^+ ion level was elevated in exposed goldfish and carp despite the reduction in the apical surface area in these cyprinids. Moreover, in similar experiments (Liew et al, 2013; Sinha et al., 2013 unpublished data, refer ch. 4) we reported that under same ammonia exposure dose (1mM) Na^+ influx in cyprinids were higher than in trout. This suggest an alternate model for Na^+ uptake in freshwater fish emphasizing the potential role of Na^+/H^+ present on the apical membrane of epithelial cell and Na^+/K^+-ATPase located in basolateral membrane. We also reported (Sinha et al., 2012b,c, 2013 unpublished data, refer ch. 4) an increase in expression and activity of Na^+/K^+-ATPase in goldfish and carp during ammonia exposure. Thus, it is very clear from our study that there is no correlation between MRCs apical surface area and Na^+ uptake in goldfish and carp, at least during ammonia exposure, indicating that not the surface, but the micro-environment in the deep apical crypt might be of the uttermost importance in the efficiency of ion uptake. This is also valid for trout where we reported an enlargement of MRCs surface area but no increment in Na^+ uptake (refer Liew et al, 2013; Sinha et al., 2013 unpublished data, ch. 4) was registered. Similarly, in brown bullheads

(*Ictalurus Nebulosus*) experiencing hypercapnic acidosis, the surface area of MRCs apical membranes was reduced by as much as 90% without any effect on the rate of Na^+ uptake (Goss et al., 1994b). Likewise, a study on brown trout (*Salmo trutta*) has shown that changes in the environmental Na^+ concentration did not alter the surface pattern of MRCs in the gills (Morgan et al., 1994). Nevertheless, our results also indicate that MRCs may regulate their apical surface in response not only to the ionic composition of the water, which has been well documented (Lee et al., 1996; Lee et al., 2000; Chang et al., 2001; Chang et al., 2002; Shieh et al., 2003) but also in response to the elevated level of ammonia in water.

PVCs have been shown to be involved in the covering and uncovering of MRCs under certain conditions (Goss et al., 1994a; Bartels et al., 1996; Daborn et al., 2001), but the role of the PVCs in the ion-regulation process is somewhat secondary to that of the MRCs (see Introduction). The role of PVCs has been implicated in gas exchange (Perry, 1997; Goss et al., 1998). The apical microridges may mechanically enhance the adhesion of water molecules, thus favouring the diffusion of respiratory gases from water to blood and vice versa (Rajbanshi, 1977). Consequently, completely loss of microridges in exposed goldfish and reduced sized microridges in exposed carp may be a protective response to reduce the diffusion rate of ammonia from external environment, although it is very likely that by switching to this strategy these cyprinids have to compromise with ion and O_2 intake. Furthermore, PVCs are also suggested to involve in osmotic regulation (Wilson et al., 2000) as the site of Na^+ uptake via channels electrically associated with the H^+-ATPase of the apical membrane. However, physiological significance of microridges transformation on the PVC surface of the experimental animals remains unclear.

In the near future, more extensive examination of MRCs and PVCs fine structure in the gills of ammonia exposed fish, using light microscopy and transmission electron microscopy will be performed.

4. CONCLUSION

It was apparent from our study that HEA affected the macrostructure of gills in all the studied species but with different intensity. The first (and non-specific) response recorded in all the fish species was the overproduction of mucus which continued through the exposure period in cyprinids while trout suffered a decline during the last two end points. The second, more slowly developed response was reorganization of the gill structure, also referred as gill remodeling. It was “classic” in the goldfish, “modest” in carp, and almost not present in trout where only lamellae reacted. We reported that gill alterations in carp were not as extensive as in the goldfish, but whether the remodeling in carp could develop further if exposure will be prolonged needs to be investigated in future experiment. We can conclude that the most severely affected gill structure of the exposed goldfish were better prepared

for survival in the water containing ammonia than the less affected gills of the carp, while the hardly affected trout gills might actually indicate an incapacity to deal with environmental changes.

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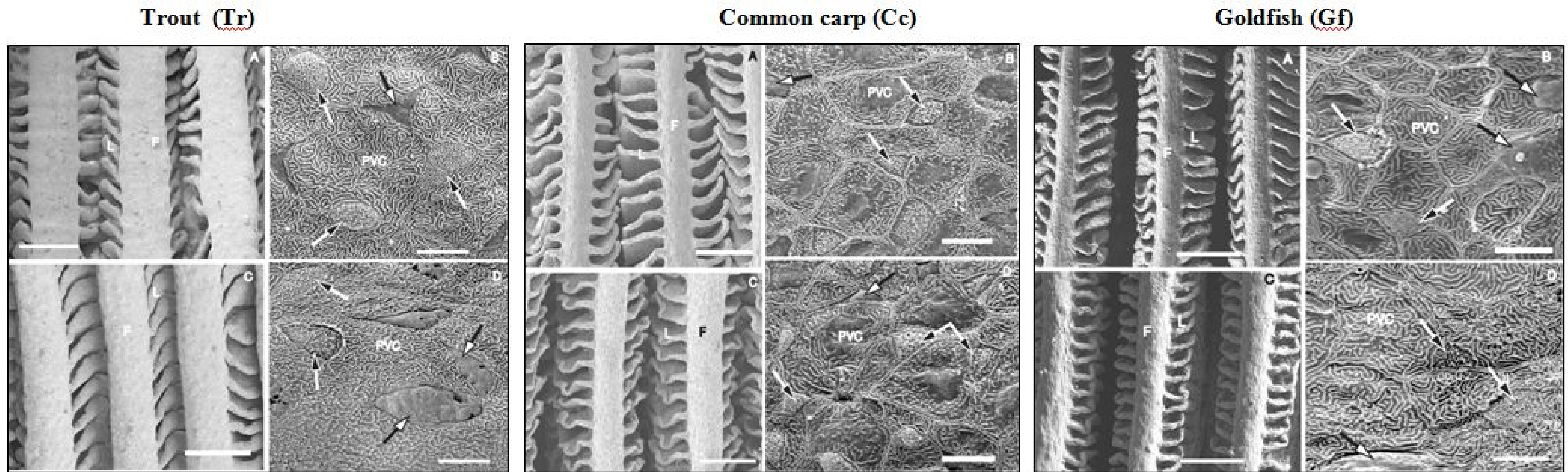

Fig.1.Tr. SEM micrographs of the gills of trout in control conditions. A,B-0 h (control); C,D- 84 h or 180 h (control).
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Fig.1.Cc. SEM micrographs of the gills of common carp in control conditions. A,B-0 h(control); C,D- 84 h or 180 h (control).
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Fig.1.Gf. SEM micrographs of the gills of goldfish in control conditions. A,B-0 h (control); C,D- 84 h or 180 h (control).
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

F : filament, L: lamella; PVC : pavement cell.
Here and rest of the places:
-Blackhead arrows designated apical crypts of mitochondria-rich cells
-Whitehead arrows designated mucous cells.
-Scale bars: A, C- 100 µm; B, D- 5 µm.

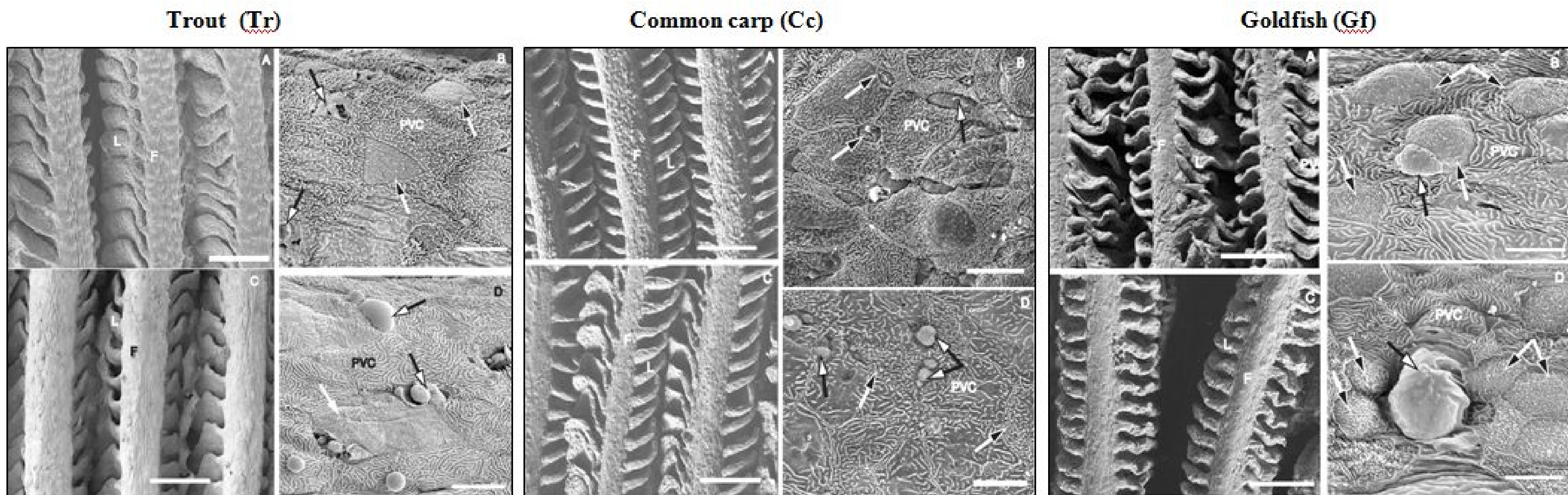

Fig.2.Tr. SEM micrographs of the gills of trout during ammonia (1mM) exposure.
A,B-3 h; C,D- 12 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.
In fig. D. White arrow designates patch of mucus which are deposited on the epithelial surface masking a cell located beneath.

Fig.2. Cc. SEM micrographs of the gills of common carp during ammonia (1mM) exposure.
A,B-3 h; C,D- 12 h
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Fig.2. Gf. SEM micrographs of the gills of goldfish during ammonia (1mM) exposure.
A,B-3 h; C,D- 12 h
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Trout (Tr)

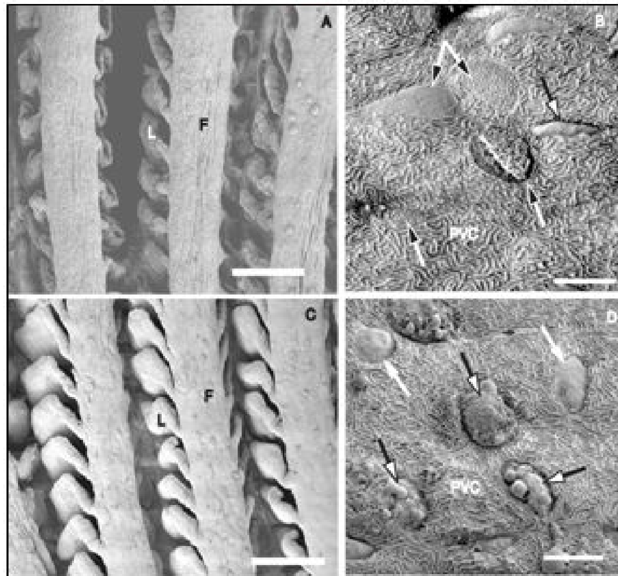

Fig.3.Tr. SEM micrographs of the gills of trout during ammonia (1mM) exposure.
A,B-24 h; C,D- 48 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.
In fig. D. mucus fills groves between microridges and covers cells making them unidentified.
White arrows indicate those cells whose attribution could not be done.

Common carp (Cc)

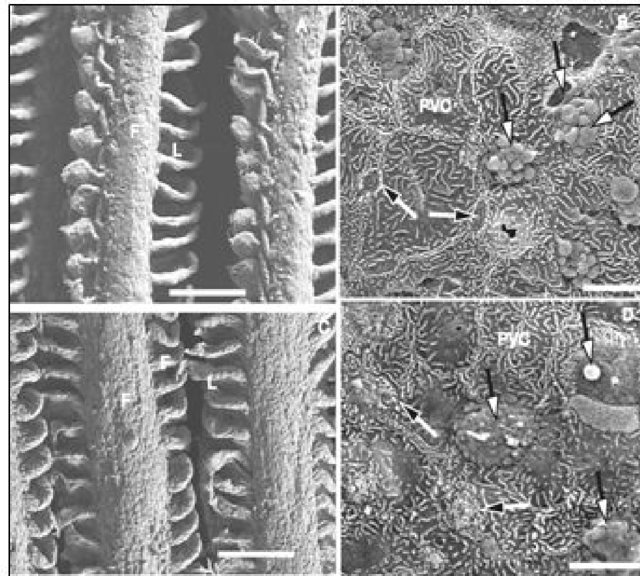

Fig.3.Cc. SEM micrographs of the gills of common carp during ammonia (1mM) exposure.
A,B-24 h; C,D- 48 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Goldfish (Gf)

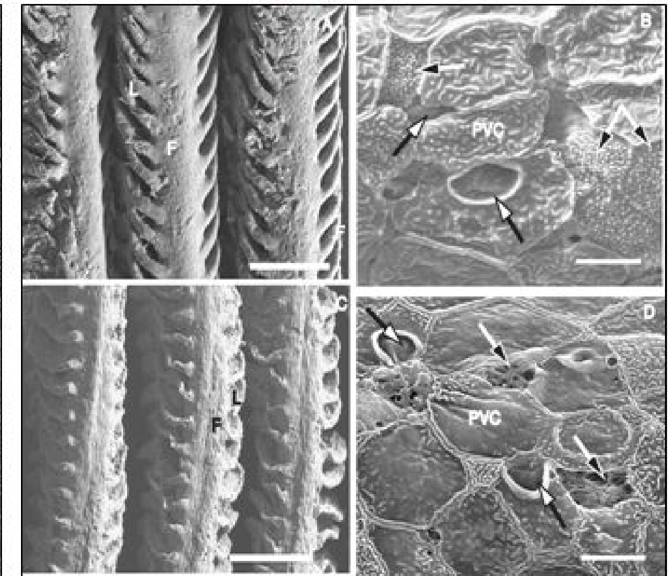

Fig.3.Gf. SEM micrographs of the gills of goldfish during ammonia (1mM) exposure.
A,B-24 h; C,D- 48 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Trout (Tr)

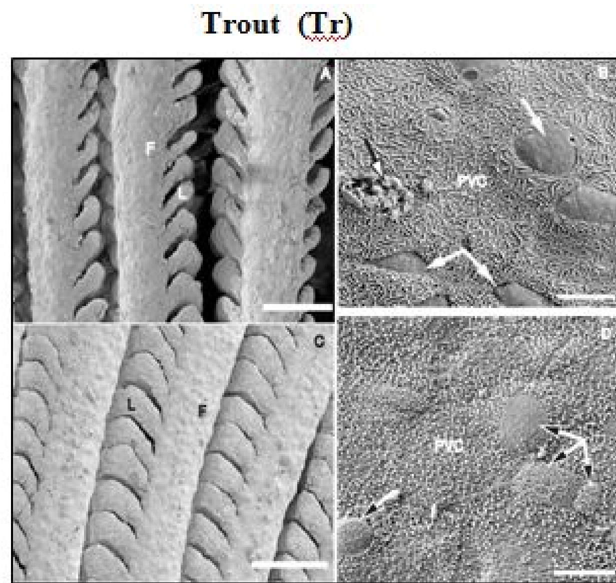

Fig.4.Tr. SEM micrographs of the gills of trout during ammonia (1mM) exposure.
A,B-84 h; C,D- 180 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.
In fig. B. MCs with globules of secretion and cells with apical surface that looks like surface of MRCs but cannot be identified properly due to overlayed mucus (indicated by white arrows).

Common carp (Cc)

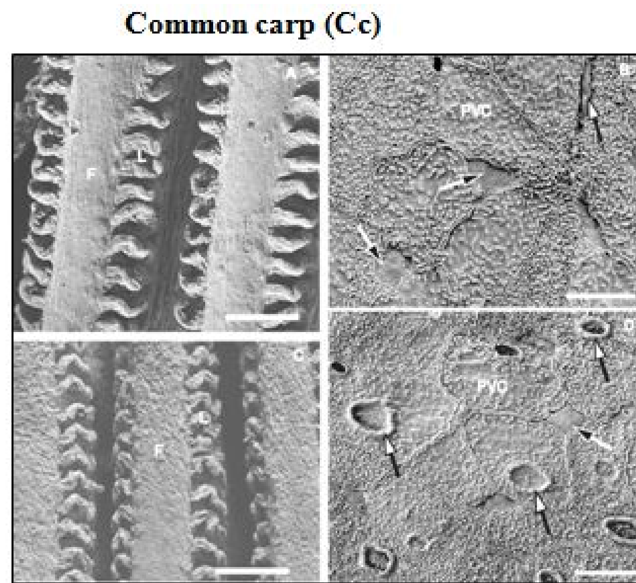

Fig.4.Cc. SEM micrographs of the gills of common carp during ammonia (1mM) exposure.
A,B-84 h; C,D- 180 h.
A and C represents the middle part of the gill filaments. B and D show the trailing edge of the gill filament.

Goldfish (Gf)

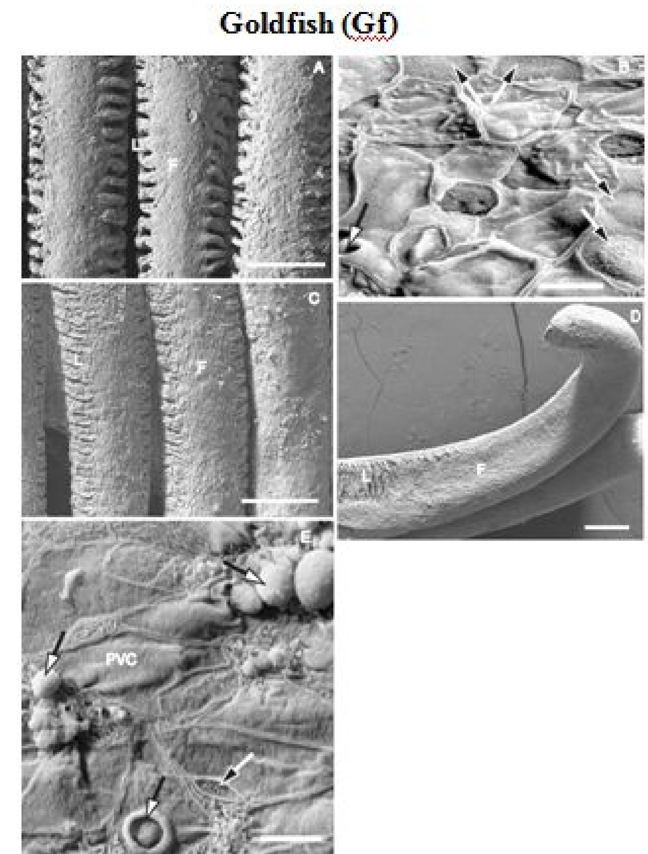

Fig.4.Gf. SEM micrographs of the gills of goldfish during ammonia (1mM) exposure.
A,B-84 h; C,D,E- 180 h.
A and C represents the middle part of the gill filaments. B and E show the trailing edge of the gill filament. D represents distal part of filament

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Z

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Curriculum vitae

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Nationality: Indian
Sex: Male
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EDUCATION

Degree	University	Session	Percentage	Major
Ph.D.	University of Antwerpen, Belgium	2009–2013 (May.)		Fish Physiology
M.F.Sc.	Gent University, Belgium	2006-2008	80.2#	Aquaculture
B.F.Sc.	CAU, Imphal, India	2001-2005	86.6*	Fisheries Sciences

#Greater Distinction
*** GOLD MEDALIST**

M.F.Sc. AND Ph.D. DISSERTATION

M.F.Sc. : "The Effect of poly-β-hydroxybutyrate (PHB) on the growth performance of European sea bass (*Dicentrarchus labrax*)"

Ph.D. : "Ammonia handling in freshwater teleosts: a comparative study"

FELLOWSHIPS AND AWARDS

- Secured 15th rank in All India Combined Examination for Junior Research Fellowship (JRF) conducted by Indian Council of Agricultural Research (ICAR), New Delhi (2005).
- Selected as 3rd* Best Fisheries Graduate of India in 2005 conducted by PFGF (Post fisheries graduate forum). * Gold medal
- Awarded by Vlaamse Interuniversitaire Raad (VLIR) scholarship from Flemish Interuniversity council, to

pursue Master degree in Aquaculture in Gent University, Belgium for year 2006-2008.

- Awarded with Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO) scholarship – to pursue PhD in Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerpen for year 2009-2013.
- Consolation prize "Alltech Young Scientist Award 2010" for the paper entitled "The bacterial storage compound poly-beta-hydroxybutyrate (PHB) increases growth performance and intestinal microbial diversity in juvenile European sea bass"
- Best poster award: P. De Schryver, A. Kumar Sinha, P. Singh Kunwar, K. Baruah, N. Boon, W. Verstraete, G. De Boeck, and P. Bossier. The bacterial storage compound Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal microbial diversity in juvenile european sea bass (*Dicentrarchus labrax*). Poster presentation: Larvi 2009, 5th fish & shellfish larviculture symposium, Ghent university, Belgium, 7 - 10 Sept, 2009
- "Johnson & Johnson Pharmaceutical Research and Development (J&J PRD) Award": For exclusive VIP entrance pass to attend European Science Open Forum (ESOF 2010), Torino, Italy. Based on Paper entitled "My view on life -Cancer free world by 2020.

REVIEWER OF PEER REVIEW JOURNALS

- Aquaculture Nutrition
- Animal Feed Science and Technology
- Aquaculture Research
- Fish Physiology and Biochemistry
- Fish and Fisheries
- Journal of Experimental Pathology
- Environmental Science and Pollution Research
- Journal of Comparative Physiology
- Journal of Animal Physiology and Animal Nutrition
- Anatomia, Histologia, Embryologia
- Ecotoxicology and Environmental Safety

WORK EXPERIENCE/SHORT TERM TRAINING

- Visiting Scientist (2008-2009) in Laboratory for Ecophysiology, Biochemistry and Toxicology, University of Antwerpen, Belgium, for a project entitled "Analytical and biological methods in support of sustainable aquaculture practices in Vietnam (A Joint Vietnamese – Belgian Project Funded By SPO)"
- Six-month internship training experience in various aspects of Aquaculture, Processing technology, Gear technology and Fishing.
- Six months research experience in fish physiology and molecular biology at Department of Biology (Under supervision of Prof. Dr. Chris Wood), McMaster University, Canada.
- Feed formulation for Finfishes and Shellfishes, seed production and hatchery management
- Nursery and grow-out management of shrimp and carp farms.
- Training on Fish farm Construction & designing
- Since 2009, supervising practical class at Laboratory for Ecophysiology, Biochemistry and Toxicology, Department of Biology, University of Antwerp, Belgium.
- Supervised three Master students (thesis) and two Bachelor (thesis) students in Department of Biology, University of Antwerp, Belgium.

PUBLICATIONS

Research and review papers

1. Hon Jung Liew*, **Amit Kumar Sinha***, Nathalie Mauro, Marjan Diricx, Veerle M. Darras, Ronny Blust, De Boeck G. (2013). Feeding and swimming modulate iono-and-hormonal regulation differently in goldfish, *Carassius auratus* and common carp, *Cyprinus carpio*. *Comparative Biochemistry and Physiology A – Molecular & Integrative Physiology* 165: 13-21 (IF: 2.24) *contributed equally

2. **Amit Kumar Sinha**, Terri Giblen, Hamada AbdElgawad, Michelle De Rop, Han Asard, Ronny Blust, Gudrun De Boeck. (2013). Regulation of amino acid metabolism as a defensive strategy in the brain of three freshwater teleosts in response to high environmental ammonia exposure. *Aquatic toxicology* 130-131, 86- 96 (IF: 3.76).

3. Hon Jung Liew*, **Amit Kumar Sinha*,[1]**, C. Michele Nawata, Ronny Blust, Chris M. Wood, Gudrun De Boeck (2013). Differential responses in ammonia excretion, sodium fluxes and gill permeability explain different sensitivities to acute high environmental ammonia in three freshwater teleosts. *Aquatic toxicology 126: 63-76.* (IF: 3.76) *contributed equally; [1]correspondence author

4. **Amit Kumar Sinha**, Marjan Diricx, Lai Pong Chan, Hon Jung Liew, Vikas Kumar, Ronny Blust, Gudrun De Boeck (2012). Expression pattern of potential biomarker genes related to growth, ion regulation and stress in response to ammonia exposure, food deprivation and exercise in common carp (*Cyprinus carpio*). *Aquatic toxicology 122-123:93-105.* (IF: 3.76)

5. **Amit Kumar Sinha**, Hon Jung Liew, Marjan Diricx, Vikas Kumar, Veerle M. Darras, Ronny Blust, Gudrun De Boeck (2012). Combined effects of high environmental ammonia, starvation and exercise on hormonal and ionregulatory response in goldfish (*Carassius auratus* L.). *Aquatic toxicology* 114-115, 153-164. (IF: 3.76)

6. Hon Jung Liew*, **Amit Kumar Sinha***, Nathalie Mauro, Marjan Diricx, Ronny Blust, De Boeck G. 2012. Fasting goldfish, *Carassius auratus* and common carp, *Cyprinus carpio*, use different metabolic strategies when swimming. *Comparative Biochemistry and Physiology A – Molecular & Integrative Physiology* 163: 327-335. (IF: 2.24) *contributed equally.

7. **Amit Kumar Sinha**, Hon Jung Liew, Marjan Diricx, Ronny Blust, Gudrun De Boeck (2012). The interactive effects of ammonia exposure, nutritional status and exercise on metabolic and physiological responses in gold fish (*Carassius auratus* L.). *Aquatic toxicology* 109, 33-46. (IF: 3.76)

8. Vikas Kumar*, **Amit Kumar Sinha***, Harinder P.S. Makkar, Gudrun De Boeck, Klaus Becker. (2012). Nonstarch Polysaccharide in human nutrition- A Review. *Critical Reviews in Food Science and Nutrition* 52: 899-935. (IF: 4.79) *contributed equally

9. **Amit Kumar Sinha**, Vikas Kumar, Harinder P.S. Makkar, Gudrun De Boeck, Klaus Becker (2011). Non-starch polysaccharide in Fish Nutrition: An overview. *Food Chemistry* 127:1409-1426. (IF: 3.66)

10. Vikas Kumar*, **Amit Kumar Sinha*,** H.P.S. Makkar, G. De Boeck, K. Becker.(2011). Phytate and phytase in fish nutrition: A Review. *Journal of Animal Physiology and Animal Nutrition* 96: 335-364 (IF 0.86) *contributed equally

11. **Amit Kumar Sinha**, Caroline Vanparys, Gudrun De Boeck, Patrick Kestemont, Nguyen Thanh Phuong, Neil Wang, Marie-Louise Scippo, Wim De Coen, Johan Robbens. (2010). Expression characteristics of potential biomarker genes in Tra catfish, *Pangasianodon hypophthalmus,* exposed to Trichlorfon. *Comparative Biochemistry and Physiology, Part D 5:207–216.* (IF: 1.72)

12. Vikas Kumar, **Amit Kumar Sinha**, Harinder P.S. Makkar, Klaus Becker. (2010). Dietary roles of phytate and phytase in human nutrition: A review. *Food Chemistry 120, 945–959.* (IF: 3.66)

13. Peter De Schryver*, **Amit Kumar Sinha***, Prabesh Singh Kunwar, Kartik Baruah, Willy Verstraete, Nico Boon, Gudrun De Boeck and Peter Bossier (2010). Poly-β-hydroxybutyrate (PHB) increases growth performance and intestinal bacterial range-weighted richness in juvenile European sea bass, *Dicentrarchus*

labrax. *Applied Microbiology and Biotechnology* 86:1535-1541. (IF: 3.43) *contributed equally

14. G. De Boeck, M. Eyckmans, I. Lardon, R. Bobbaers, **Amit Kumar Sinha**, R. Blust.(2010). Metal accumulation and metallothionein induction in the spotted dogfish *Scyliorhinus canicula*. *Comparative Biochemistry and Physiology, Part A Molecular & Integrative Physiology* 155:503-508. (IF: 2.24)

15. Vikas Kumar, N.P. Sahu, A.K. Pal, Shivendra Kumar, Vidya Sagar, **Amit Kumar Sinha**, Jayant Ranjan (2010). Nucleic acid content changes of a tropical freshwater fish *Labeo rohita* fed gelatinized and non-gelatinized starch diet. *Journal of the World Aquaculture Society*. Vol. 41, No. S2, 270-277. (IF: 0.71)

16. Vikas Kumar, N.P. Sahu, A.K. Pal, Shivendra Kumar, **Amit Kumar Sinha,** Jayant Ranjan and Kartik Baruah (2010). Modulation of key enzymes of glycolysis, gluconeogenic amino acid catabolism, and TCA cycle of the tropical freshwater fish, *Labeo rohita* fed with gelatinized and nongelatinized starch diet. *Fish Physiology and Biochemistry* 36:491–499. (IF: 1.53)

17. Prakash Sharma, Vikas Kumar, **Amit Kumar Sinha**, Jayant Ranjan, H.M.P.Kithsiri and Gudipati Venkateshwarlu (2009). Comparative fatty acid profiles of wild and farmed tropical freshwater fish rohu *(Labeo rohita)*. *Fish Physiology and Biochemistry* 36:411–417. (IF: 1.53)

18. Vikas Kumar, N. P. Sahu, A. K. Pal, Shivendra Kumar, Prakash Sharma, JK Chettri and **Amit Kumar Sinha (2009).** Non-gelatinized starch influences the deposition of n-3 fatty acids in the muscle of a tropical freshwater fish *Labeo rohita*. *Journal of Animal Physiology and Animal Nutrition* 93:659-668. (IF: 0.86)

19. Parisa Norouzitallab, Mehrdad Farhangi, Mohammad Babapour, Rouhollah Rahimi, **Amit Kumar Sinha**, Kartik Baruah (2009). Comparing the Efficacy of Dietary α-Tocopherol to DL-α-Tocopheryl Acetate Alone or in Combination with Ascorbic acid on Growth and Stress Resistance of Angelfish, *Pterophylum scalare*, juveniles. *Aquaculture International* 17:207-216. (IF: 0.91)

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21. Kumar Vikas, Sahu N.P., Pal A.K., Jain K.K., Kumar Shivendra, Sagar Vidya, **Amit Kumar Sinha** , Ranjan Jayant (2011). Gelatinized and non-gelatinized corn starch based diet influence the fatty acid profile in the liver of tropical freshwater fish, *Labeo rohita*. *Journal of aquaculture research and development,*2: 106-112. (I.F. 0.62)

Scientific Popular articles

1. Jiwan Kumar Chettri, Vikas Kumar, **Amit Kumar Sinha**, Rathod Ramesh, 2006. Antibiotic Residue and concept of Organic Fish Farming, Aqua international monthly magazine, Vol.14 (2): 33- 35.

2. Tejpal C. S., Kumar V., Chettri J. K., **Amit Kumar Sinha** (2006). Non-antibiotic growth promoters in fish and shellfishes. Aqua Internationl. Volume 14, (4): 32-36.

3. **Amit Kumar Sinha**, Kartik Baruah, Dipesh Debnath, A.K. Pal. (2007) Nutrizymes, Ideal nutraceuticals in Aquafeed: Potential and Limitation. Aquaculture Europe. Vol 32 (4): 11-14.

4. **Amit Kumar Sinha**, Kartik Baruah, Dipesh Debnath, A.K. Pal. (2007) Nutrizymes, Ideal nutraceuticals in Aquafeed: Potential and Limitation. Aquaculture Health International. Issue 11: 4-6.

5. **Amit Kumar Sinha**, K. Baruah, Peter Bossier (2008). Horizon scanning: The potential use of Bioflocs as an anti-infective strategy in aquaculture- An overview. Aquaculture Health International. Issue 13: 8-10.

6. **Amit Kumar Sinha**, Parisa Norouzitallab, Kartik Baruah.(2009). Antimicrobial Peptide: Possible therapeutics in Aquaculture. World Aquaculture Magazine. Vol. 40 (4). 50-53.

7. Vikas Kumar, Olesia Gavryliuk, **Amit Kumar Sinha**, Debtanu Barman, Eef De Clercq, Apu Das, Sagar C.

Mandal (2012). Jatropha meal, a promising plant protein source in aquafeed development. World Aquaculture Magazine, Vol. 43 (2), 38-44.

Congress abstract

1. Giblen T, De Rop M, **Sinha AK**, Blust R, De Boeck G. 2012. Compensatory strategies in goldfish and common carp in response to water-borne ammonia. SEB Annual Main meeting, Salzburg, Austria. June 29 – July 02, 2012.
2. **Sinha AK**, Liew HJ, Nawata CM, Blust R, Wood CM, De Boeck G. 2012. Salmonids respond differently to high external ammonia than Cyprinids: a comparative study on the regulation of Na^+ flux, ammonia transporters and associated mechanisms. SEB Annual Main meeting, Salzburg, Austria. June 29 – July 02, 2012.
3. Wood CM, Nawata CM, **Sinha AK**, Liew HJ, De Boeck G. 2012. Ammonia excretion, sodium uptake, cortisol and intracellular signalling during high environmental ammonia exposure in fish. SEB Annual Main meeting, Salzburg, Austria. June 29 – July 02, 2012.
4. Chan L, Diricx M, Liew HJ, **Sinha AK**, De Boeck G. 2011. Influence of ammonia exposure, nutritional status and exercise on the expression pattern of potential biomarker genes in the common carp (*Cyprinus carpio*). Annual Main Meeting 2011 Society of Experimental Biology, Glasgow, UK. July 1-4. p. 104.
5. De Boeck G, Liew HJ, **Sinha AK**, Nawata MC, Wood CM. 2011. Consequences of elevated environmental ammonia for Na transport and ammonia/urea excretion in freshwater fish. International Fisheries Symposium 2011, Kuala Terengganu, Malaysia. October 03-05, 2011: 50.
6. Liew HJ, **Sinha AK**, Darras VM, Blust R, De Boeck G. 2011. Combined impact of feeding and exhaustive exercise on ion- and hormonal regulation of Goldfish,*Carassius auratus*. International Fisheries Symposium 2011, Kuala Terengganu,Malaysia. October 03-05, 2011: 39-42.
7. Liew HJ, **Sinha AK**, De Boeck G. 2011. Effects of food depreviation and exhaustive swimming impacts on swimming performance, metabolic responses and the energy budget of goldfish, Carassius auratus. Annual Main Meeting 2011 Society of Experimental Biology, Glasgow, UK. July 1-4. p. 113.
8. **Sinha AK**, De Boeck G, Blust R, Diricx M, Liew HJ. 2011. The interactive effects of ammonia exposure, nutritional status and exercise on the ecological fitness of gold fish (Carassius auratus L.). Annual Main Meeting 2011 Society of Experimental Biology, Glasgow, UK. July 1-4. p.99.
9. De Boeck G, Eyckmans M, Lardon I, Bobbaers R, **Sinha AK**, Blust R. 2009. Metal accumulation and metallothionein induction in the spotted dogfish Scyliorhinus canicula. Abstracts of the 26th Congress of the newEuropean Society of Comparative Biochemistry and Physiology, Innsbruck, Austria. 6-9 September. Comparative Biochemistry and Physiology, Part A 154(1): S13-S22: pS14.
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