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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: 1 mM for a period of 12 h, identical for all species, and 5 mM for the cyprinids and 1.4 mM for the trout for 3 h. Elevation of plasma cortisol at both levels of HEA was apparent in all species. At 1 mM, 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 1 mM HEA, Na^+ imbalance became progressively more severe in trout and carp due to a stimulation of unidirectional Na^+ efflux (J_{out}^{Na}) without a concomitant increase in unidirectional Na^+ influx (J_{in}^{Na}). Additionally, a transient reduction of J_{in}^{Na} was evident in trout. Goldfish showed an opposite trend for J_{out}^{Na} with reduced efflux rates and a positive Na^+ balance during the first few hours of HEA. However, after 12 h of exposure, both J_{in}^{Na} and J_{out}^{Na} were also increased in both carp and goldfish, whereas only J_{out}^{Na} 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 1.4 mM ammonia for 3 h: J_{in}^{Na} was significantly inhibited while considerable activation of J_{out}^{Na} 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.

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

Abbreviations: HEA, high environmental ammonia; J_{amm}, net ammonia flux rate; J_{urea}, net urea flux rate; J_{in}^{Na}, sodium influx rate; J_{out}^{Na}, sodium efflux rate; J_{net}^{Na}, sodium net flux rate; T_{amm}, total ammonia concentration; TEP, transepithelial potential; J_{net}^{K}, net potassium flux rate.

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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., 2007; 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., 1981), 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 1–5 mM, 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 96 h 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 osmoregulatory 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_{in}^{Na}, J_{out}^{Na}) and net (J_{net}^{Na}) 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_{out}^{Na}) measured during the Na^+ balance experiments, we also assessed net K^+ flux rates (J_{net}^{K}), diffusive water exchange rates (using ^3H_2O), and transepithelial potential (TEP). J_{net}^{K} (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 (1 mM for 12 h for all species, and 5 mM for 3 h for carp and goldfish or 1.4 mM 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_{in}^{Na}, J_{out}^{Na} and J_{net}^{Na}. 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_{net}^{K}. Overall, the results indicate that these fish species show differential compensatory responses toward 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.

2. Materials and methods

2.1. Experimental system and animals

Rainbow trout, *O. mykiss*, were obtained from a fish farm – Bijnens, Zonhoven, Belgium; goldfish, *C. auratus*, were obtained from Aqua Hobby, Heist op den Berg, Belgium; common carp, *C. 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 $17 \pm 1\,^{\circ}C$ 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 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.

2.2. 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 PerkinElmer 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 1 mM HEA for a period of 3 h and 12 h. This concentration is sublethal for all 3 species (11%, 38% and 59% of the 96 h 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 5 mM HEA for a period of 0 h (control) and 3 h. Rainbow trout were exposed for the same periods to 1.4 mM. These concentrations were close to 96 h 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 (4×5 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.

2.3. 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 1 mM HEA for 3 h and 12 h, with all recordings being made on the same fish.

TEP was measured by means of 3 mol/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.

2.4. 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 PerkinElmer, Boston, MA, USA). The protocol was modeled 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 (10 μCi ^3H_2O in 200 μL Cortland saline for trout, 2 μCi ^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 1 mM 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.

2.5. Analytical techniques and calculations

Plasma cortisol levels were determined by radioimmunoassay (RIA) using a kit from MP Biomedicals (New York, USA) 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. (1995) 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, PerkinElmer Life Science, Turku, Finland). Na^+ and K^+ concentrations in water were measured using flame atomic absorption spectrophotometry (AAnalyst 800, PerkinElmer). 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 (PerkinElmer, Wellesley, MA). Tests demonstrated that quenching was constant, so no correction was necessary.

Net flux rates (in μmol/kg/h) of Na^+ (J_{net}^{Na}) 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_{net}^{Na}).

Na^+ influx rates (J_{in}^{Na}, by convention positive) were calculated from the mean external specific activity, and the disappearance of counts from the external water.

Calculation of influx (J_{in}^{Na}) was done by the formulae

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

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

$$J_{out}^{Na} = J_{net}^{Na} - J_{in}^{Na}$$

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):

$$k = \frac{(\ln CPM_1 - \ln CPM_2) \times 100}{(t_1 - t_2)}$$

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).

2.6. 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. Results

3.1. Ammonia and urea flux

Ammonia excretion rate (J_{amm}) invariably declined in carp and trout during the first 3 h of 1 mM ammonia exposure (Fig. 1A). This response was more pronounced ($P < 0.05$) in trout which displayed a reversal of ammonia flux from a control value of −372 μmol/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 1.4 mM ammonia, with all three species experiencing a reversal of J_{amm} (Fig. 1B).

Urea excretion rate (J_{urea}) also exhibited some changes in the face of HEA. At 1 mM a gradual increment with exposure time was seen in goldfish and the same trend was seen in carp but not in trout (Fig. 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 1.4 mM), J_{urea} was elevated considerably ($P < 0.01$) only in goldfish, with a 2.2-fold increase compared to control (Fig. 2B).

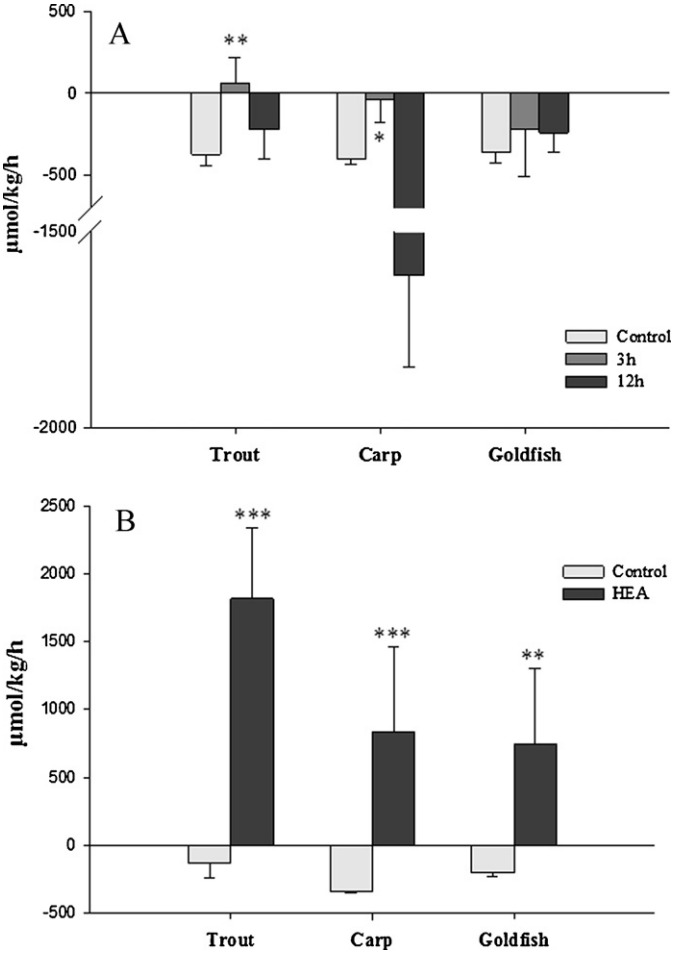

Fig. 1. Net excretion rate of ammonia (J_{amm}) in rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and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

3.2. Plasma metabolites

3.2.1. Plasma ammonia

Plasma total ammonia (T_{amm}) level was significantly elevated by 30% in trout after 3 h exposure at 1 mM ammonia (Fig. 3A). This increase was followed by a subsequent recovery at 12 h to a value not significantly different from the control group. A trend toward 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 5 mM (or 1.4 mM), they showed much higher (many fold increments, $P < 0.01$ or 0.001) accumulations of T_{amm} in comparison to the control groups (Fig. 3B). The respective increments in trout, carp and goldfish were 270, 225% and 320% of control levels.

3.2.2. 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 1 mM HEA exposure (Fig. 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

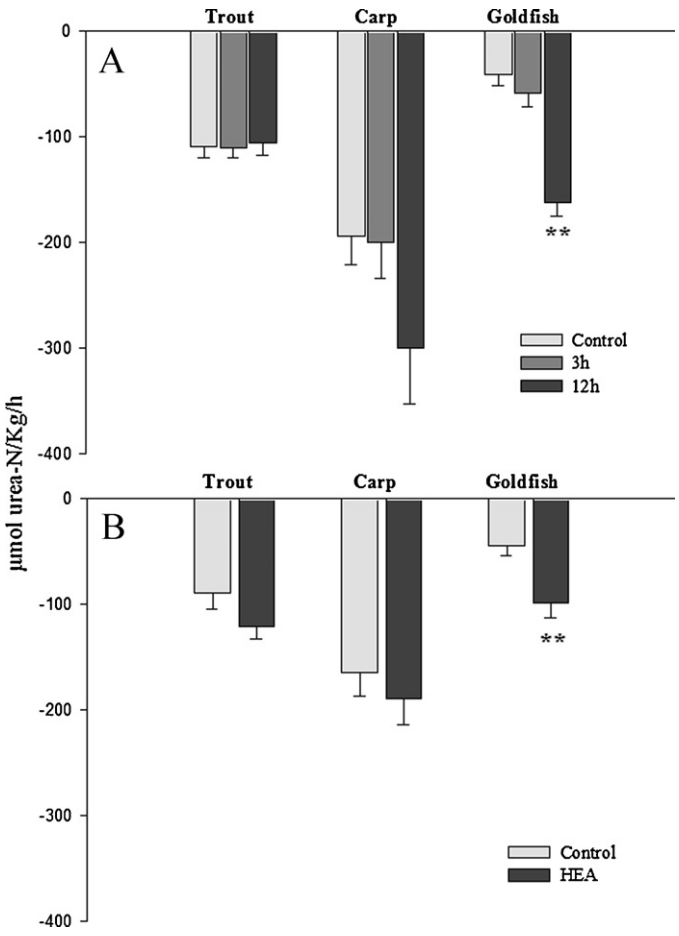

Fig. 2. Net excretion rate of urea (J_{urea}) in rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its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

a divergent pattern of urea–N accumulation between the two cyprinids upon exposure to 1 mM HEA. Very similar patterns in plasma urea were seen at 5 mM (or 1.4 mM) HEA, confirming these inter-specific differences (Fig. 4B).

3.2.3. Plasma cortisol

Exposure to 1 mM ammonia elevated plasma cortisol level ($P < 0.05$) in carp and goldfish from 3 h onwards (Fig. 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 1.4 mM) (Fig. 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.

3.3. Na^+ flux response to HEA

Unidirectional sodium influx rates (J_{in}^{Na}) did not change significantly relative to controls in common carp and goldfish during the first 3 h of exposure to 1 mM HEA (Fig. 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_{in}^{Na} was seen in trout during the first hour only, with recovery thereafter.

During the first 3 h, the response of unidirectional Na^+ efflux (J_{out}^{Na}) in goldfish displayed an opposite trend to carp and trout. In goldfish, J_{out}^{Na} was inhibited ($P < 0.05$) in the first and third hours of exposure to 1 mM 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_{out}^{Na} ($P < 0.001$ 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_{out}^{Na}, resulting in highly negative values of J_{net}^{Na} (Fig. 6).

At the higher exposure level (5 or 1.4 mM), J_{in}^{Na} was significantly inhibited in all species (Fig. 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_{in}^{Na} in trout at 1.4 mM followed the same trend as when subjected to 1 mM. J_{out}^{Na} 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. 7).

3.4. Indices of gill permeability

3.4.1. 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. 8). The rate increased significantly in trout after 3 h when exposed to HEA (1 mM) 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 1 mM HEA.

3.4.2. Transepithelial potential (TEP)

Under control conditions TEP was negative in carp and goldfish but slightly positive in trout (Fig. 9). Upon exposure to HEA (1 mM), 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.

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

In all three species, net K^+ flux rates (J_{net}^{K}) were negative (i.e. net losses) under control conditions. During exposure to 1 mM 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. 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 (5 mM or 1.4 mM), an increment in K^+ loss rate was observed all the three species (Fig. 10B). In exposed trout, the loss

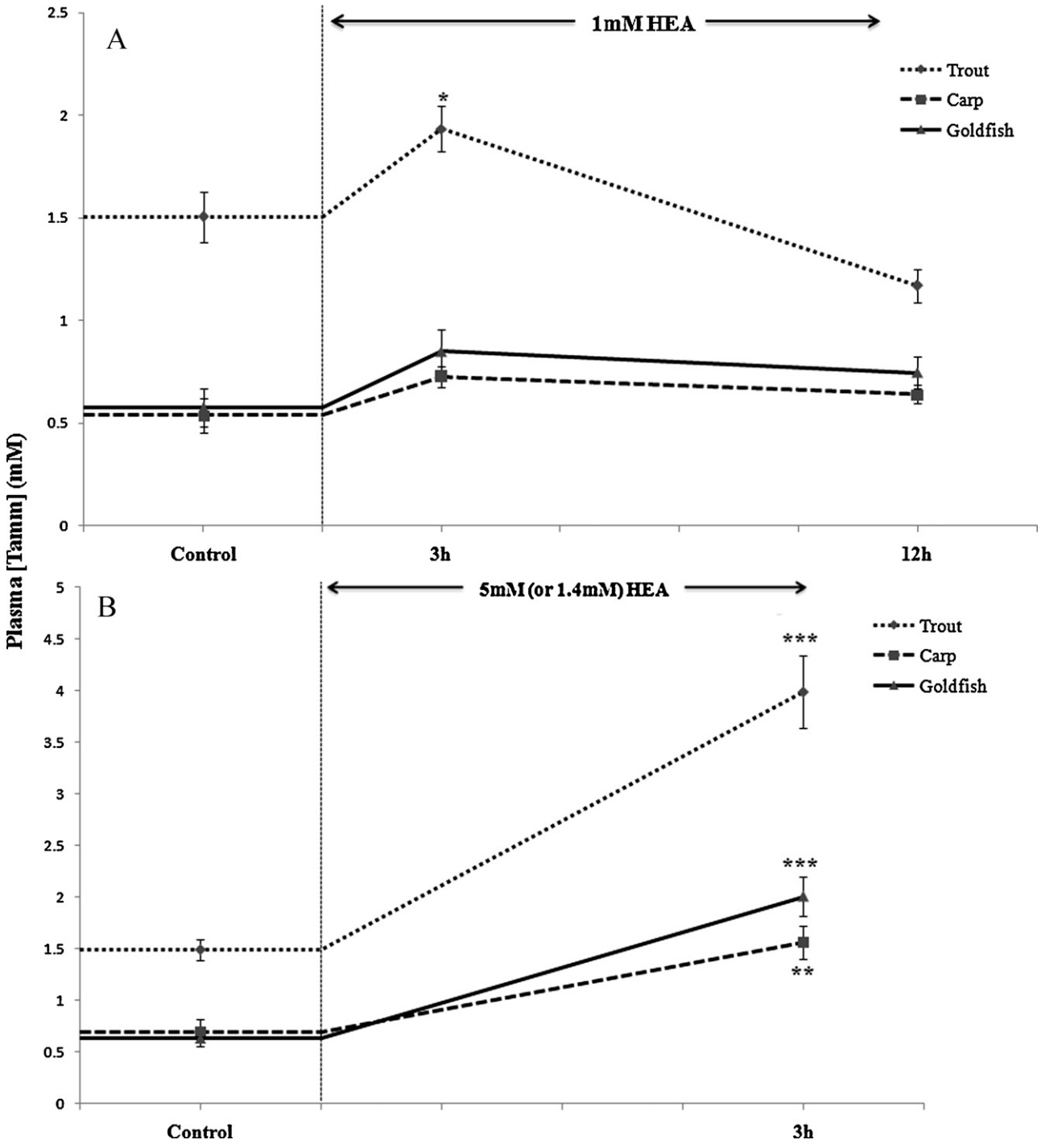

Fig. 3. Plasma ammonia accumulation in rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective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

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.

4. Discussion

4.1. Effects on ammonia metabolism

In trout and carp, ammonia excretion rate (J_{amm}) was depressed during the first 3 h of 1 mM 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

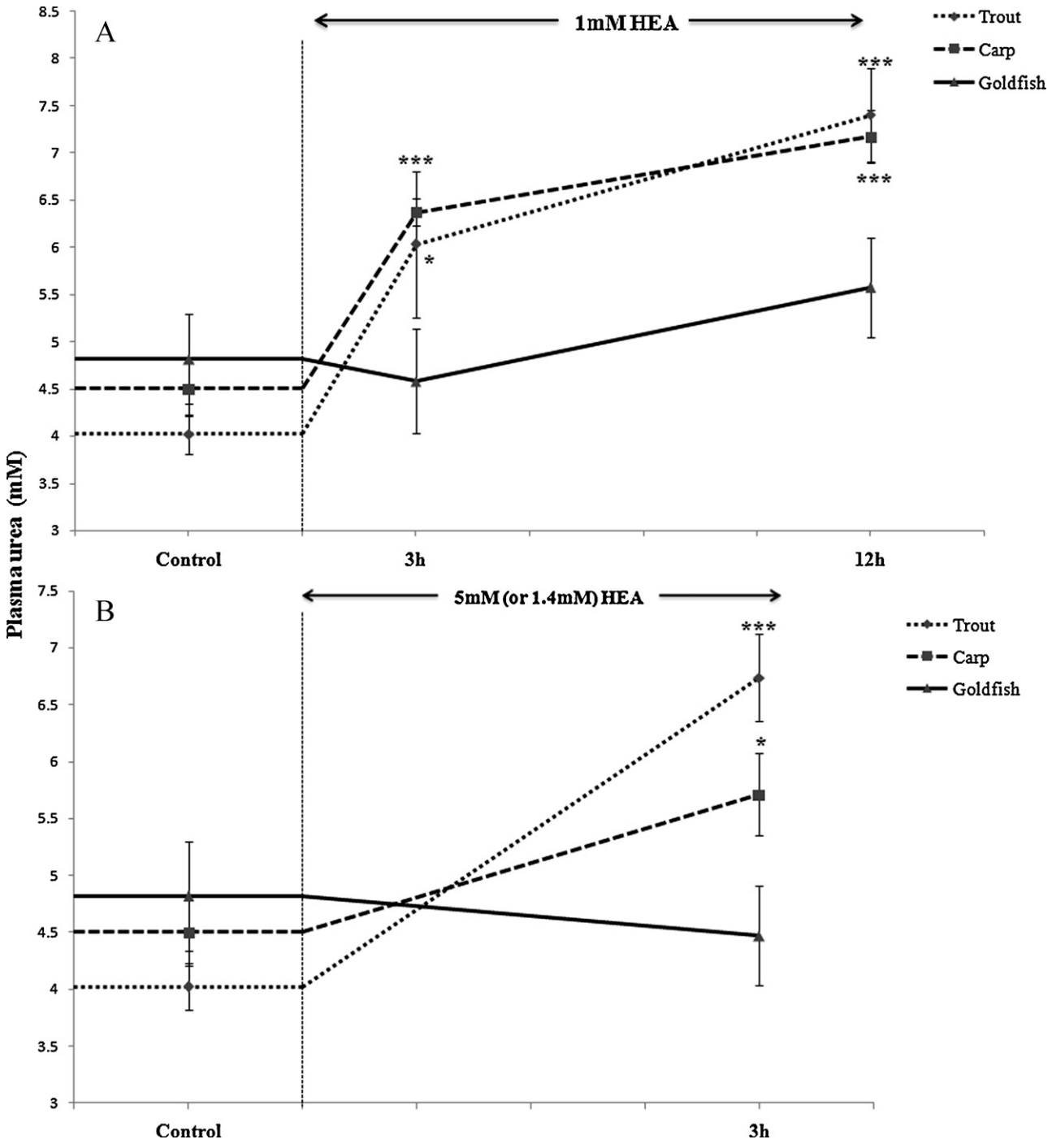

Fig. 4. Urea accumulation in plasma of rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$; ***$P < 0.001$).

restore ammonia excretion by 12 h exposure to 1 mM HEA. Recent findings (Nakada et al., 2007; 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_{in}^{Na}.

Notably, after exposure to a higher ammonia level (5 or 1.4 mM), 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_{in}^{Na}. 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.

4.2. Effects on cortisol

Cortisol, produced as an end product of the hypothalamic–pituitary–interrenal axis, plays a crucial role in the stress response

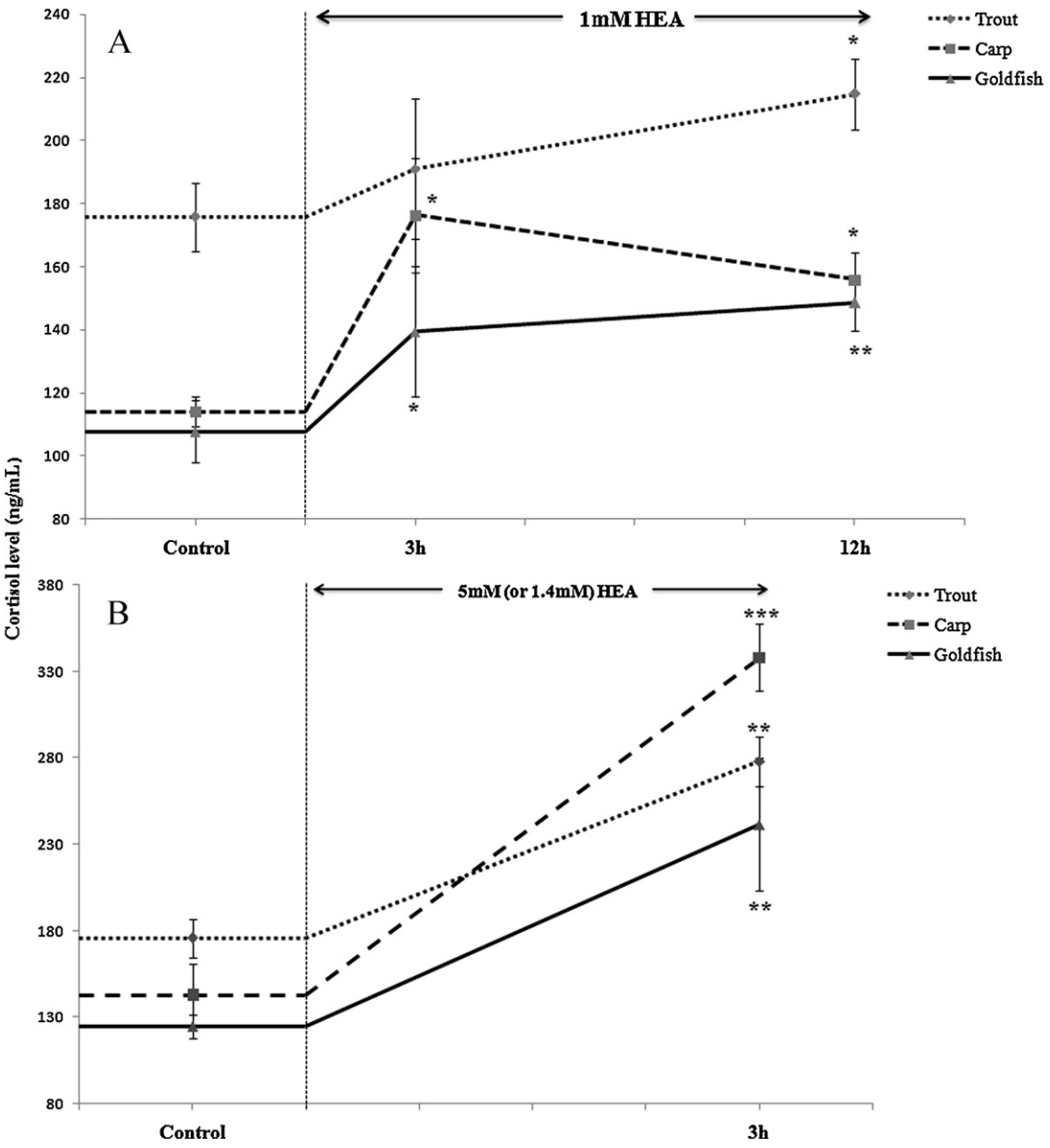

Fig. 5. Plasma cortisol level in rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control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

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., 2012b; 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.

4.3. 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 1 mM HEA, the interspecies difference between cyprinids and trout became apparent. In trout, HEA tended to depress J_{in}^{Na} transiently contrasting with the response of cyprinids, which showed no inhibition and increased J_{in}^{Na} 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_{in}^{Na} by

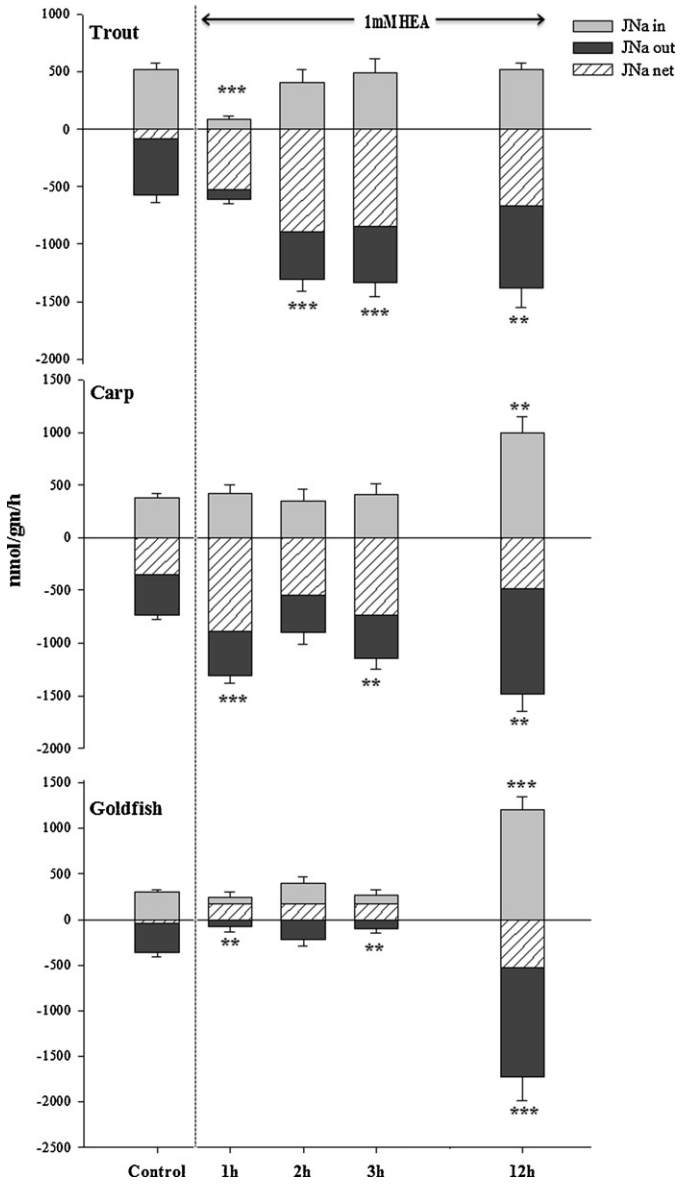

Fig. 6. Na^+ unidirectional influx (J_{in}^{Na}, upward bars), Na^+ efflux(J_{out}^{Na}, downward bars) and Na^+ net flux (J_{net}^{Na}, hatched bars) rates in rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant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

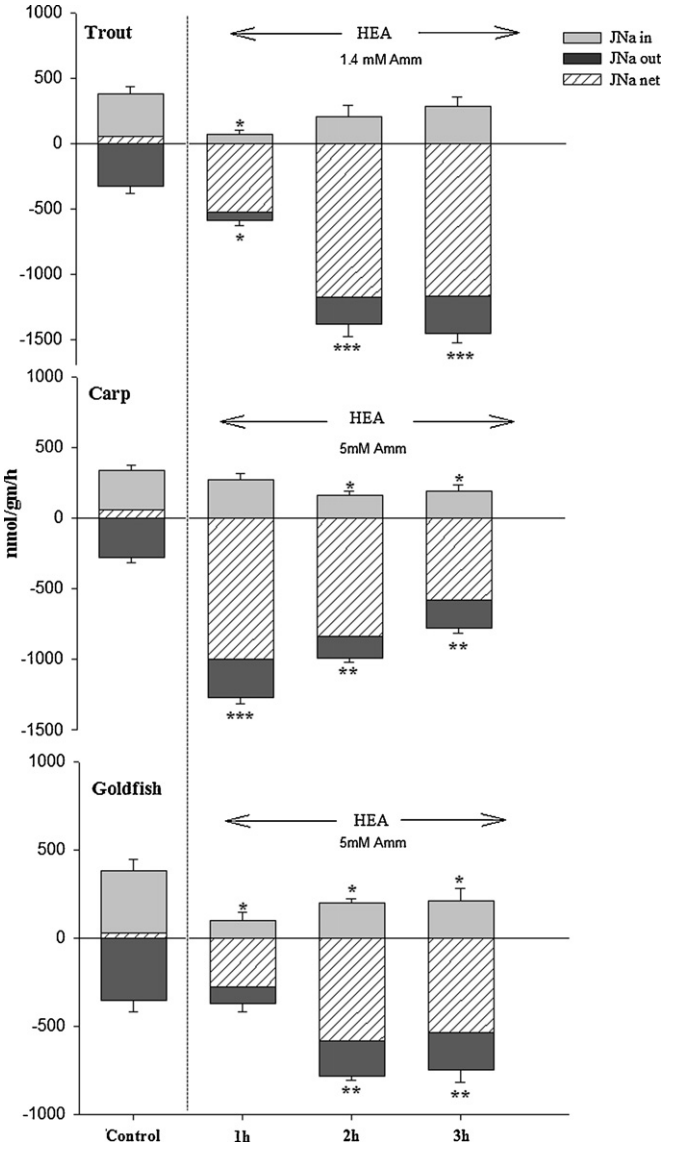

Fig. 7. Na^+ unidirectional influx (J_{in}^{Na}, upward bars), Na^+ efflux(J_{out}^{Na}, downward bars) and Na^+ net flux (J_{net}^{Na}, hatched bars) rates in rainbow trout, common carp and goldfish during 5 mM or 1.4 mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*P < 0.05; **P < 0.01; ***P < 0.001).

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_{in}^{Na} recovered to normal values. This recovery of J_{in}^{Na} 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_{in}^{Na} when subjected to more severe exposure (5 or 1.4 mM). 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_{out}^{Na}) was almost doubled in trout when exposed to 1 mM and 1.4 mM ammonia. Our result is in accordance with the findings of Twitchen and Eddy (1994) who reported that HEA increased the diffusive

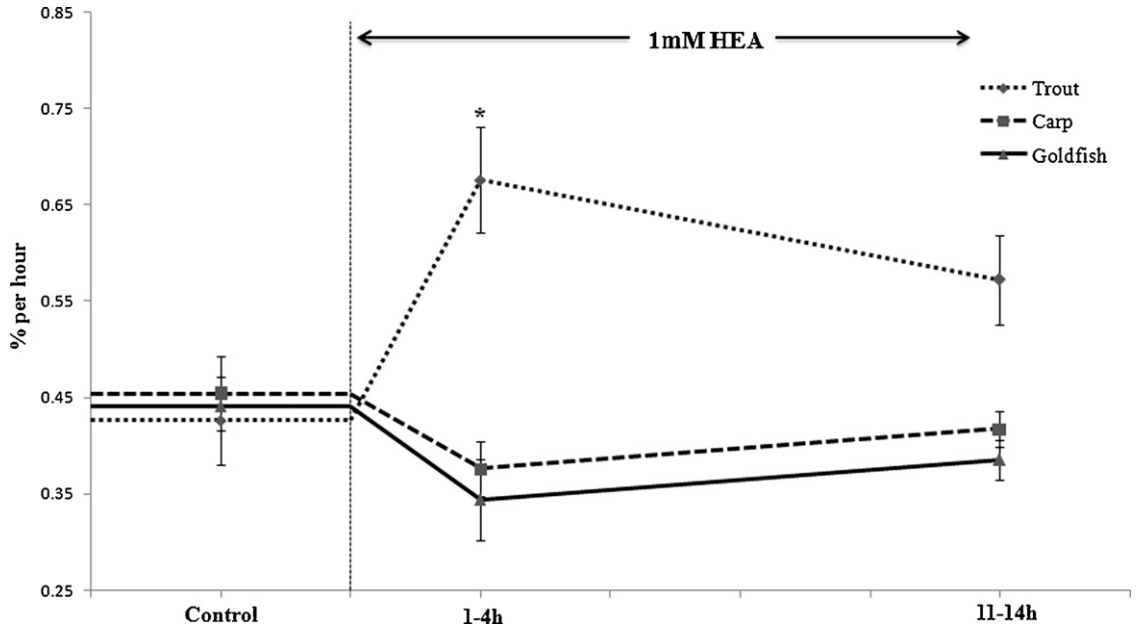

Fig. 8. Diffusive water efflux rates measured with ^3H_2O in rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$).

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_{out}^{Na} 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 1 mM 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_{net}^{K}) 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_{net}^{K} 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

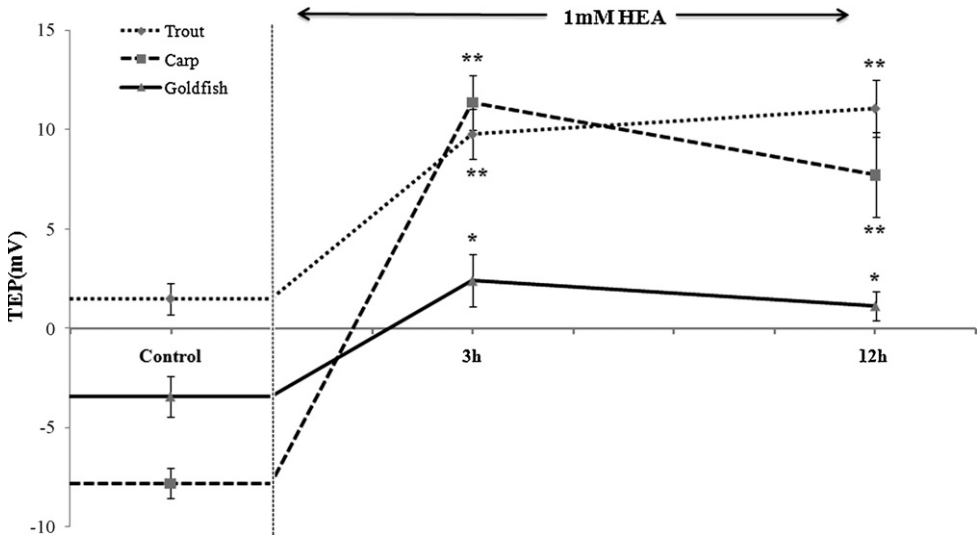

Fig. 9. Changes in transepithelial potential (TEP) in rainbow trout, common carp and goldfish during 1 mM ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$; **$P < 0.01$).

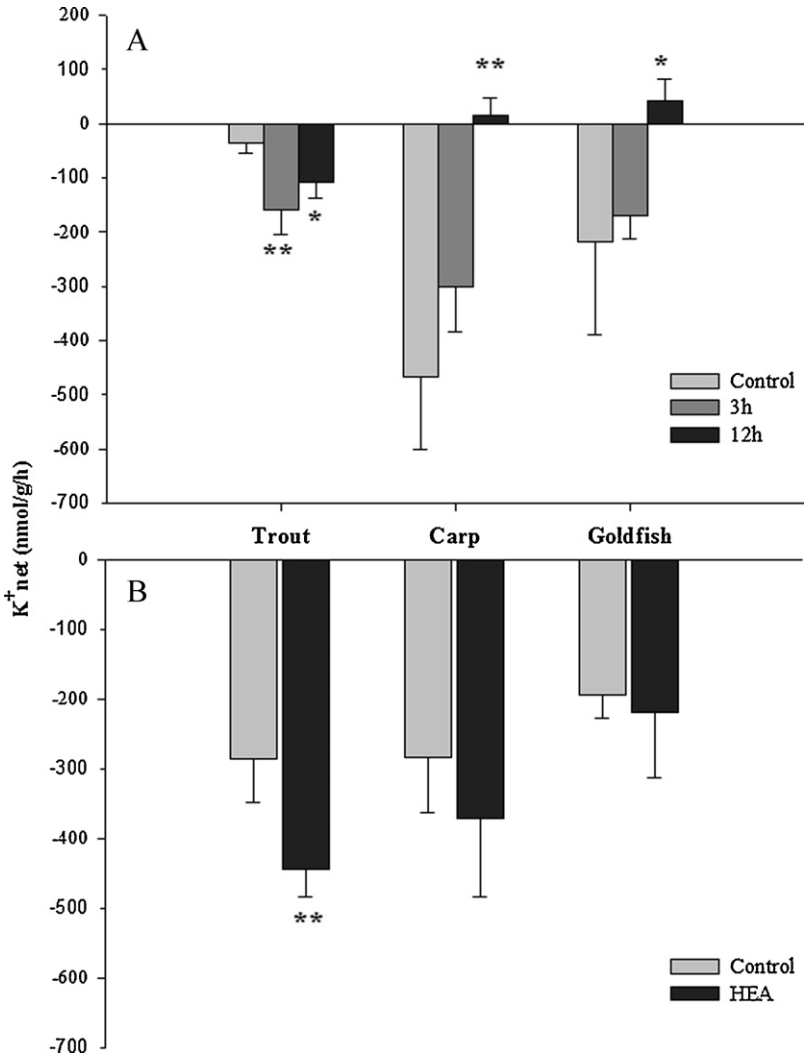

Fig. 10. Net flux rates of K^+ (nmol/g/h) in rainbow trout, common carp and goldfish during (A) 1 mM ammonia exposure (B) 5 mM (for cyprinids) or 1.4 mM (for trout) ammonia exposure. Values are expressed as mean ± S.E. Asterisk (*) indicates a significant difference between the exposed fish and its respective control (*$P < 0.05$; **$P < 0.01$).

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_{out}^{Na} in the initial 3 h period when exposed to 1 mM 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 remodeling 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 (5 mM), it appears that even goldfish could not regulate gill permeability effectively, resulting in higher J_{out}^{Na} as seen for carp and trout as well.

Furthermore, an analogous pattern was noticed after 12 h exposure among all these fish species; J_{out}^{Na} was elevated remarkably compared to respective controls. Interestingly, J_{in}^{Na} was also stimulated more or less in the same time frame among all species. It indicates some sort of recovery response toward normal Na^+ balance by enhancing Na^+ influx (Salama et al., 1999) as part of the ammonia excretion mechanism, and that an increase in J_{out}^{Na} might directly be coupled to an increase in J_{in}^{Na} 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.

4.4. 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 1 mM HEA for 12 h showed a nearly identical activation in J_{urea} as when placed in 5 mM 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. 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.

5. Conclusion

Exposure to 1 mM 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 5 mM 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.

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