

# Modulation of Rh glycoproteins, ammonia excretion and Na<sup>+</sup> fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia

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**Short title:** *Chronic ammonia exposure in 3 teleosts*

## SUMMARY

We investigated relationships among branchial unidirectional  $\text{Na}^+$  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  $\text{NH}_4\text{HCO}_3$ ; pH 7.9). During HEA, carp and goldfish elevated ammonia excretion ( $J_{\text{amm}}$ ) and  $\text{Na}^+$  influx rates ( $J_{\text{in}}^{\text{Na}}$ ) while trout experienced higher plasma ammonia ( $T_{\text{amm}}$ ) and were only able to restore control rates of  $J_{\text{amm}}$  and  $J_{\text{in}}^{\text{Na}}$ . All three species exhibited increases in  $\text{Na}^+$  efflux rate ( $J_{\text{out}}^{\text{Na}}$ ). At the molecular level, there was evidence for activation of a “ $\text{Na}^+/\text{NH}_4^+$  exchange metabolon” likely in response to elevated plasma cortisol and  $T_{\text{amm}}$ , though surprisingly, some compensatory responses preceded molecular responses in all three species. Rhbg, Rhcg (Rhcg-a and Rhcg-b),  $\text{H}^+$ -ATPase (V-type, B-subunit) and  $\text{Na}^+/\text{K}^+$ -ATPase (NKA) mRNA expressions were up-regulated in goldfish, Rhcg-a and NKA in carp, and Rhcg2, NHE-2 ( $\text{Na}^+/\text{H}^+$  exchanger) and  $\text{H}^+$ -ATPase in trout. Branchial  $\text{H}^+$ -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  $\text{Na}^+/\text{NH}_4^+$ -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,  $\text{Na}^+/\text{NH}_4^+$  exchange metabolon, Rainbow trout, Common carp, Goldfish.

## 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  $\text{NH}_3$ , diffusion and/or as  $\text{NH}_4^+$  transport linked in some manner to  $\text{Na}^+$  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  $\text{Na}^+$  uptake, the scheme first proposed by Krogh (1939). Current hypotheses include a direct coupling through  $\text{Na}^+/\text{NH}_4^+$  exchangers, coupling with  $\text{H}^+$  excretion by an apical  $\text{Na}^+/\text{H}^+$  exchanger (NHE), and coupling with  $\text{H}^+$  excretion via an apical  $\text{H}^+$ - pump which energizes  $\text{Na}^+$  uptake through a putative  $\text{Na}^+$  channel. The latter two mechanisms would acidify the gill boundary layer, thereby enhancing the “diffusion-trapping” of  $\text{NH}_3$  as  $\text{NH}_4^+$  and maintaining the partial pressure gradient for diffusive  $\text{NH}_3$  efflux, thereby manifesting as an apparent  $\text{Na}^+/\text{NH}_4^+$  exchange (Clarke and Potts, 1998; Wilson et al., 1994).

Recently, the key involvement of Rhesus (Rh) glycoproteins in this diffusive  $\text{NH}_3$  efflux has been recognized (Nakada et al., 2007a; Nawata et al. 2007). These appear to function as ammonia channels, binding  $\text{NH}_4^+$  but facilitating the diffusion of  $\text{NH}_3$  (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  $\text{NH}_4^+$  at the cytoplasmic side of the Rhcg channel may provide a source of  $\text{H}^+$  ions to drive  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  influx (*via* NHE and/or  $\text{H}^+$ -ATPase). As the coupling appears to be indirect and loose, involving several interacting transporters, Wright and Wood (2009) have described it as a ‘ $\text{Na}^+/\text{NH}_4^+$  exchange complex or metabolon’.

In addition,  $\text{Na}^+/\text{K}^+$ -ATPase (NKA), present in the basolateral membrane of branchial cells, also plays a major role in the transport of  $\text{Na}^+$  ions across gill membranes (Evans et al.,

2005; Kultz and Somero, 1995; Wilkie, 1997). Since  $\text{NH}_3$  or  $\text{NH}_4^+$  are moved across the apical membrane in loose exchange for apical  $\text{Na}^+$  uptake, NKA would contribute to the driving force to uptake  $\text{Na}^+$  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  $\text{NH}_4^+$  has also been implicated since similarities in the hydration radius of  $\text{K}^+$  and  $\text{NH}_4^+$  may allow substitution at the  $\text{K}^+$  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  $\text{NH}_4^+$  substitution for  $\text{K}^+$  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  $\text{Na}^+/\text{NH}_4^+$  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  $\text{Na}^+/\text{NH}_4^+$  exchange metabolon was not examined at a molecular level, trout clearly exhibited the largest disturbances in ammonia excretion,  $\text{Na}^+$  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  $\text{Na}^+/\text{NH}_4^+$  exchange metabolon.

Specifically, we investigated the combined physiological and molecular compensatory responses and the linkages between branchial ammonia fluxes and unidirectional  $\text{Na}^+$  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). The reported ammonia 96h  $\text{LC}_{50}$  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).

To achieve our goals we measured net ammonia ( $J_{\text{amm}}$ ) and urea ( $J_{\text{urea}}$ ) flux rates as well as plasma ammonia and urea concentrations, plasma cortisol levels, unidirectional ( $J_{\text{in}}^{\text{Na}}$ ,  $J_{\text{out}}^{\text{Na}}$ )

and net ( $J_{\text{net}}^{\text{Na}}$ ) sodium flux rates and mRNA expression of Rh glycoproteins (Rhbg and Rhcg),  $\text{H}^+$ -ATPase (V-type, B subunit), NHE-2, NKA (alpha subunit) and urea transporter (UT) during HEA challenge. The sequence information for Rh glycoproteins,  $\text{H}^+$ -ATPase, NKA, and UT was not available for the carp and goldfish, so we cloned partial sequences of these cDNAs.  $\text{H}^+$ -ATPase and NKA enzyme activities were measured and the ability of  $\text{NH}_4^+$  to activate gill NKA was evaluated *in vitro* for all the three species. Moreover, we also assessed net  $\text{K}^+$  flux rates ( $J_{\text{net}}^{\text{K}}$ ) 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 “ $\text{Na}^+/\text{NH}_4^+$  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.

## MATERIALS AND METHODS

### Experimental system and animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) were obtained from a fish farm - Bijmens, 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  $17 \pm 1^\circ\text{C}$  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  $7.4 \pm 0.2$ , dissolved oxygen 6.9-7.4 mg  $\text{l}^{-1}$ , total  $\text{NH}_3$  0.006-0.009 mM, nitrite 0.0015- 0.0021 mM, nitrate 0.015-0.042 mM,  $\text{Ca}^{2+}$  0.8–1.0 mM,  $\text{Mg}^{2+}$  0.19-0.21 mM,  $\text{Na}^+$  1.2-1.4 mM,  $\text{K}^+$  0.09-0.10 mM,  $\text{Cl}^-$  0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 226 mg  $\text{CaCO}_3 \text{ l}^{-1}$ .

Average mass (mean  $\pm$  standard deviation) of rainbow trout was  $132 \pm 22$  g, of carp  $16 \pm 4$  g, and of goldfish  $15 \pm 5$  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.

### **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. This concentration represents 11%, 38% and 59% of the 96h LC<sub>50</sub> values respectively for goldfish, carp and trout (see Introduction for references). 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<sub>4</sub>HCO<sub>3</sub> stock solution (Sigma, Germany). A constant concentration of  $1.09 \pm 0.08$  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<sub>4</sub>HCO<sub>3</sub> 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.

### **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  $\text{Na}^+$  fluxes with  $^{22}\text{Na}$  (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  $^{22}\text{Na}$  (typically  $2\ \mu\text{Ci l}^{-1}$ ) was added to each container and allowed to equilibrate for 30 min. Water samples ( $4 \times 5\ \text{ml}$  for  $^{22}\text{Na}$ , total  $\text{Na}^+$  and  $\text{K}^+$  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,  $1\ \text{g l}^{-1}$ , 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 ( $2500\ \text{units ml}^{-1}$  lithium heparin, Sigma, Munich, Germany) 1-ml syringes with 23-gauge needles. Blood was immediately centrifuged (for 1 min at 16,000 rpm at  $4^\circ\text{C}$ ), and aliquots of plasma were frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for later analysis. Fish were dissected; gills were removed, washed with saline and blotted. One portion was added to five volumes of RNeasy lysis buffer (Qiagen, GmbH, Hilden, Germany) and stored at  $4^\circ\text{C}$  for later molecular analysis, while the remaining gills were flash-frozen in liquid nitrogen and stored at  $-80^\circ\text{C}$  for enzymatic assays.

## Enzymatic analyses

NKA activity and  $\text{H}^+$ -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  $\text{NH}_4^+$  could activate NKA activity, the assay was performed under optimised conditions replacing the native concentration of KCl (10 mM) with same molar concentration of  $\text{NH}_4\text{Cl}$  in the reaction medium. Activities were then measured in the absence and presence of ouabain.



## **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<sub>260</sub>/OD<sub>280</sub> 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<sup>+</sup>-ATPase (vacuolar, B subunit), NKA (alpha subunit), and urea transporter (UT) were amplified using taq DNA polymerase (Invitrogen) and the primers listed in Table1. Basic Local Alignment Search Tool (BLAST) analyses were done on the National Center for Biotechnology Information (NCBI) database.

### **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 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-dimers 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-1 $\alpha$ , glyceraldehyde-3-phosphate dehydrogenase and 18S rRNA) favoured beta-actin ( $\beta$ -actin) for cyprinids (goldfish and carp) and elongation factor-1 $\alpha$  (EF-1 $\alpha$ ) 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.

### Analytical techniques and calculation

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 using the salicylate-hypochlorite method (Verdouw et al., 1978) and urea concentrations by the diacetyl monoxime assay (Rahmatullah and Boyde, 1980). Plasma ammonia levels were determined according to Wright et al. (1995a) using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

$^{22}\text{Na}$  activities in water samples were measured by a gamma counter (Wallac Wizard 3" 1480 Automatic Gamma Counter, Perkin Elmer Life Science, Turku, Finland).  $\text{Na}^+$  and  $\text{K}^+$  concentrations in water were measured using flame atomic absorption spectrophotometry (AAAnalyst 800, Perkin Elmer, New Jersey, USA). Net flux rates (in  $\mu\text{mol kg}^{-1}\text{h}^{-1}$ ) of  $\text{Na}^+$  ( $J_{\text{net}}^{\text{Na}}$ ) were calculated from changes in concentration (in  $\mu\text{mol l}^{-1}$ ), factored by the known fish mass (in kg), volume (in l), and time (in h). Net flux rates of  $\text{K}^+$  ( $J_{\text{net}}^{\text{K}}$ ), ammonia ( $J_{\text{amm}}$ ) and urea ( $J_{\text{urea}}$ ) were calculated as for net flux rates of  $\text{Na}^+$  ( $J_{\text{net}}^{\text{Na}}$ ).

$\text{Na}^+$  influx rates ( $J_{\text{in}}^{\text{Na}}$ , by convention positive) were calculated from the mean external specific activity, and the disappearance of counts from the external water as outlined in detail by Wood (1992):

$$J_{\text{in}}^{\text{Na}} = ([\text{CPM}_i] - [\text{CPM}_f]) (V) / (SA_{\text{ext}}) (t) (M)$$

where,  $\text{CPM}_i$  is the initial  $^{22}\text{Na}$  radioactivity in the water ( $\text{cpm ml}^{-1}$ ) at the start of the flux period;  $\text{CPM}_f$  is the final  $^{22}\text{Na}$  radioactivity in the water ( $\text{cpm ml}^{-1}$ ) at the end of the flux period;  $V$  is the volume of water (in ml);  $SA_{\text{ext}}$  is the mean external specific activity ( $^{22}\text{Na}$  per total  $\text{Na}^+$ ) in the water ( $\text{cpm nmol}^{-1}$ ), calculated from measurements of water  $^{22}\text{Na}$  radioactivity and total water  $[\text{Na}^+]_{\text{ext}}$  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).  $\text{Na}^+$  unidirectional efflux rates ( $J_{\text{out}}^{\text{Na}}$ , by convention negative) were calculated by difference:

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

## Statistical analysis

All data have been presented as mean values  $\pm$  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.

## RESULTS

### Flux measurements

#### Sodium uptake and exchange

Trout exposed to 1mM ammonia (HEA) exhibited an initial 3.5 fold stimulation of  $J_{\text{out}}^{\text{Na}}$  ( $P < 0.01$ ) at 12 h, but this had returned to control values by 84 h (Fig. 1).  $J_{\text{in}}^{\text{Na}}$  remained statistically similar in exposed trout in comparison to the control group. In carp, both  $J_{\text{in}}^{\text{Na}}$  and  $J_{\text{out}}^{\text{Na}}$  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  $J_{\text{in}}^{\text{Na}}$  and  $J_{\text{out}}^{\text{Na}}$ , 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,  $J_{\text{net}}^{\text{Na}}$  was affected to a lesser extent (values fluctuated within the range of control values) than in trout though  $J_{\text{net}}^{\text{Na}}$  values remained negative throughout in these fasted fish. In contrast,  $\text{Na}^+$  balance was severely altered in trout at 12 h HEA, as  $J_{\text{net}}^{\text{Na}}$  decreased to  $-533 \mu\text{mol kg}^{-1}\text{h}^{-1}$  from a control value of  $-1.06 \mu\text{mol kg}^{-1}\text{h}^{-1}$  ( $P < 0.05$ ), but beyond 12 h this species also was able to retain  $J_{\text{net}}^{\text{Na}}$  at the control level as seen for cyprinids.

#### Net $\text{K}^+$ flux rates

In all three species, net  $\text{K}^+$  flux rates ( $J_{\text{net}}^{\text{K}}$ ) were negative (i.e. net losses) under control conditions (Table 3). After 12 h of exposure, the  $\text{K}^+$  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.  $J_{\text{net}}^{\text{K}}$  returned to control rates in goldfish at 84 h while in the other two at 168 h.

### Ammonia and urea excretion rate

In contrast to cyprinids, trout exposed to HEA displayed no significant changes in ammonia excretion rates ( $J_{\text{amm}}$ ) during any of the sampling periods, which remained at approximately  $-300 \mu\text{mol kg}^{-1}\text{h}^{-1}$  throughout despite the unfavourable gradient (Fig. 2).  $J_{\text{amm}}$  in carp was  $-402 \mu\text{mol kg}^{-1}\text{h}^{-1}$  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  $J_{\text{amm}}$  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  $J_{\text{amm}}$  was  $-250 \mu\text{mol kg}^{-1}\text{h}^{-1}$  and there was no change at 12 h of HEA exposure. However, after 40 h,  $J_{\text{amm}}$  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 ( $J_{\text{urea}}$ ) was notable only in goldfish (Fig. 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  $J_{\text{urea}}$  was seen either in carp or in trout.

### Plasma metabolites

#### Ammonia and urea accumulation

Plasma total ammonia ( $T_{\text{amm}}$ ) 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). In the cyprinids at 84 h and 168 h, plasma  $T_{\text{amm}}$  levels were only slightly higher than the mean exposure level ( $1.09 \text{ mmol l}^{-1}$ ) while in trout  $T_{\text{amm}}$  was about two- fold higher than the ammonia level in the water.

Under control conditions, plasma urea-N levels were considerably higher than plasma  $T_{\text{amm}}$  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. 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 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% ( $P < 0.05$ ).

## 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. 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. 7A). In goldfish, a 5.1 fold ( $P < 0.001$ ) and 2.4 fold ( $P < 0.01$ ) increase in transcript level was observed 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. 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. 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. 8B). NHE-2 expression displayed a gradual rise with exposure time which became significant after 84 h exposure (Fig. 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<sup>+</sup>-ATPase**

Significant effects of HEA exposure were seen on NKA mRNA expression levels in gills of cyprinids only (Fig. 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<sup>+</sup>-ATPase in gills of trout and goldfish while no significant effects ( $P > 0.05$ ) were noted for carp (Fig. 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<sup>+</sup>-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 from 12 h onwards (Fig. 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.

### **NKA and H<sup>+</sup>-ATPase enzyme activity**

In general, enzyme activity responses (Figs. 11A,C) paralleled mRNA expression data (Figs. 9A,B) quite well for these two enzymes. Branchial NKA activity in carp and goldfish increased considerably at 84 h HEA exposure (Fig. 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  $\text{NH}_4^+$  to activate gill NKA activities in these three species was examined (Fig. 11B). In the course of these trials, we found that NKA activities were always lower when  $\text{NH}_4^+$  was substituted for  $\text{K}^+$ , regardless of the treatment, time, or species, although carp appeared to take  $\text{NH}_4^+$  slightly better than goldfish and trout. Thus while  $\text{NH}_4^+$  can support some  $\text{Na}^+/\text{NH}_4^+$ -ATPase activity, it is not as effective as  $\text{K}^+$  (i.e.  $\text{Na}^+/\text{K}^+$ -ATPase activity) on an equimolar (10 mM) basis in any of the three species. In carp and goldfish, the increases in  $\text{Na}^+/\text{K}^+$ -ATPase activities occurring at 84 h and 168 h were tracked by smaller increases in  $\text{Na}^+/\text{NH}_4^+$ -ATPase activities (Fig. 11A,B).

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<sup>+</sup>-ATPase activities were observed for this species after 84 h and 168 h of HEA exposure (Fig. 11C). 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<sup>+</sup>-ATPase activity in carp following HEA exposure were not significant.

## **DISCUSSION**

### **Ammonia flux and Rh glycoproteins**

In our earlier study on acute exposure to the same level of HEA, we found that ammonia excretion ( $J_{\text{amm}}$ ) 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  $J_{\text{amm}}$  by 12 h (Liew et al., 2013). The current results confirm the restoration of  $J_{\text{amm}}$  by 12 h in all three species, and indeed show a significant increase in the carp at this time (Fig. 2). This sequence of initial  $J_{\text{amm}}$  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  $J_{\text{amm}}$  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  $P_{\text{NH}_3}$  gradient from the blood to the bulk water as  $T_{\text{amm}}$  concentrations became virtually identical in the two media by 84-168 h (Fig. 4). In contrast, trout exposed to HEA were not able to increase the excretion rate above control levels and had plasma  $T_{\text{amm}}$  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.

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  $\text{Na}^+$  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. 6). These increases in goldfish were accompanied by restored levels or significant increases in  $J_{\text{amm}}$  at these times (Fig. 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 appreciable 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. 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. 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).

### **$Na^+$ 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. 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. 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. 8C), along with increases in  $H^+$ -ATPase expression (Fig. 9B),  $H^+$ -ATPase activity (Fig. 11C), and Rhcg2 expression (Fig. 8B), accompanied by restoration of  $J_{amm}$  to the control level (Fig. 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. 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. 9B) and activity (Fig. 11C), accompanied by parallel up-regulation of Rhcg-a and Rhcg-b (Fig. 7), elevated  $J_{amm}$  (Fig. 2), and elevated ( $J^{Na}_{in}$ ) (Fig. 1). In contrast, in carp neither the activity nor the expression level of  $H^+$ -ATPase was affected by HEA (Figs. 11C, 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. 7A).

In HEA exposed trout,  $Na^+$  influx was maintained at the control level (Fig. 1), while  $H^+$ -ATPase activity (and expression) increased at 84 h and 168 h (Figs. 11C, 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. 1) and  $J_{amm}$  (Fig. 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. 11C) or genomic up-regulation of  $H^+$ -ATPase (Fig. 9B), Rhcg2 (Fig. 8B), and NHE-2 (Fig. 8C). A similar discrepancy was noted for cyprinids where  $J^{Na}_{in}$  (Fig. 1) and  $J_{amm}$  (Fig. 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. 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). 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<sup>+</sup> efflux rate ( $J_{\text{out}}^{\text{Na}}$ ) increased when trout were exposed to HEA for 12 h and similar increases were also evident in carp and goldfish (Fig. 1). These are likely due to the increased diffusive leakage of Na<sup>+</sup> during HEA - i.e. enhanced Na<sup>+</sup> permeability (transcellular and/or paracellular) of the gills (Gonzalez and McDonald, 1992). K<sup>+</sup> loss rate ( $J_{\text{net}}^{\text{K}}$ ), an indicator of transcellular leakage, was measured in the present study (Table 3) but did not show a consistent pattern in any of the fish species analogous with their  $J_{\text{out}}^{\text{Na}}$ . 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<sup>+</sup> loss ( $J_{\text{out}}^{\text{Na}}$ ) was stimulated and exceeded influx rate ( $J_{\text{in}}^{\text{Na}}$ ) in trout and in carp, resulting in net Na<sup>+</sup> loss in these two species. However, goldfish displayed an opposite trend and were able to maintain a positive net Na<sup>+</sup> 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;  $J_{\text{in}}^{\text{Na}}$  was stimulated precisely at the same time when there was an increase in  $J_{\text{out}}^{\text{Na}}$  (Fig. 1). Also, from 84 h onwards, trout were able to maintain net Na<sup>+</sup> flux closer to zero. These consequences may indicate some sort of recovery response in these experimental animals towards normal Na<sup>+</sup> balance as part of the ammonia excretion mechanism, and/or that an increase in  $J_{\text{out}}^{\text{Na}}$  among cyprinids might be coupled directly to an increase in  $J_{\text{in}}^{\text{Na}}$  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.

### NKA response

Basolaterally situated NKA is believed to provide the major source of energy driving Na<sup>+</sup> 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. 9A) and activity (Fig. 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  $J_{\text{in}}^{\text{Na}}$  in these species (Fig. 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. 6), ammonia transport (as  $\text{NH}_4^+$ ) across the branchial basolateral membranes might occur *via*  $\text{Na}^+/\text{K}^+$ -ATPase enzyme functioning as a  $\text{Na}^+/\text{NH}_4^+$ -ATPase (Evans et al., 2005; Wilkie, 2002) since similarities in the hydration radius of  $\text{K}^+$  and  $\text{NH}_4^+$  might allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999). The potential ability of  $\text{NH}_4^+$  to substitute for  $\text{K}^+$  and/or augment the activity of branchial NKA proved negligible in all the three species (Fig. 11A,B) and therefore demonstrated that  $\text{NH}_4^+$  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  $\text{NH}_4^+$  activation equal to or greater than the  $\text{K}^+$  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  $\text{NH}_4^+$  movement across the gill basolateral membranes on the 'K<sup>+</sup> site' of the NKA molecule may be very important during HEA exposure.

### 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 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 " $\text{Na}^+/\text{NH}_4^+$  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  $T_{\text{amm}}$  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  $T_{\text{amm}}$ , particularly for trout, was relatively high (Fig. 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  $T_{\text{amm}}$  level (up to 1.5 mmol l<sup>-1</sup>) 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  $T_{\text{amm}}$  was also evident in the goldfish (0.8-1.4 mmol l<sup>-1</sup>; Liew et al., 2012; Sinha et al., 2012a; Smith et al., 2012) as well as in the carp (0.6-1.1 mmol l<sup>-1</sup>; 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., 1996) and experimentally elevated plasma cortisol increased urea-N excretion rates ( $J_{\text{urea}}$ ) in trout (McDonald and Wood, 2004). Cortisol seemed to exert a significant action on urea metabolism in goldfish, which displayed a significant rise in  $J_{\text{urea}}$  (Fig. 3) coinciding with the increment in cortisol levels (Table 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.

### **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  $J_{\text{urea}}$ ; indeed urea-N excretion became almost equal to ammonia-N excretion at 12 h HEA (Figs. 2,3). Similarly, Olson and Fromm (1971) reported an increased  $J_{\text{urea}}$  in goldfish subjected to HEA. In a recent study on goldfish, Wilkie et al. (2011) also noted a significant rise in  $J_{\text{urea}}$  over 5 days of HEA (5mmol l<sup>-1</sup> NH<sub>4</sub>Cl) 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. 10). This likely facilitated urea diffusion across the basolateral membranes of the gill cells, accelerating  $J_{\text{urea}}$  in goldfish (Fig. 3). UT expression also increased in the gills of zebrafish during HEA exposure, together with a transient rise in  $J_{\text{urea}}$  (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.

## 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  $J_{\text{amm}}$  was elevated significantly during HEA exposure while in trout  $J_{\text{amm}}$  could only be restored to basal level.  $\text{Na}^+$  uptake ( $J_{\text{in}}^{\text{Na}}$ ) increased in carp and goldfish coincident with elevated  $J_{\text{amm}}$ , but not in trout. Carp and goldfish were able to maintain  $\text{Na}^+$  homeostasis during HEA as they exhibited persistent, simultaneous augmentations in both  $J_{\text{in}}^{\text{Na}}$  and  $J_{\text{out}}^{\text{Na}}$ . At the molecular level, there was evidence for activation of a “ $\text{Na}^+/\text{NH}_4^+$  exchange metabolon” in all three species, likely in response to elevations in plasma cortisol and  $T_{\text{amm}}$ . Goldfish appear to rely on  $\text{H}^+$ -ATPase and trout on  $\text{H}^+$ -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  $\text{Na}^+$  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  $\text{Na}^+$  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  $\text{Na}^+$  uptake and ammonia excretion during HEA exposure. However none of the three species appeared to use NKA as an  $\text{NH}_4^+$  transporter. Surprisingly in all the species,  $J_{\text{amm}}$  and  $J_{\text{in}}^{\text{Na}}$  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.

## LIST OF ABBREVIATIONS

HEA: High environmental ammonia

$J_{\text{amm}}$ : Net ammonia flux rate

$J_{\text{urea}}$ : Net urea flux rate

$J_{\text{in}}^{\text{Na}}$ : Sodium influx rate

$J_{\text{out}}^{\text{Na}}$ : Sodium efflux rate

$J_{\text{net}}^{\text{Na}}$ : Sodium net flux rate

$T_{\text{amm}}$ : Total ammonia concentration

$J_{\text{net}}^{\text{K}}$ : Net potassium flux rate

NKA:  $\text{Na}^+/\text{K}^+$ -ATPase

NHE:  $\text{Na}^+/\text{H}^+$  exchanger

Rh: Rhesus

UT: Urea transporter



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## Figure Captions

**Figure. 1.**  $\text{Na}^+$  unidirectional influx ( $J_{\text{in}}^{\text{Na}}$ , upward bars),  $\text{Na}^+$  efflux ( $J_{\text{out}}^{\text{Na}}$ , downward bars) and  $\text{Na}^+$  net flux ( $J_{\text{net}}^{\text{Na}}$ , hatched bars) rates in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  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. 2.** Net excretion rate of ammonia ( $J_{\text{amm}}$ ) in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  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. 3.** Net excretion rate of urea ( $J_{\text{urea}}$ ) in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  s.e.m. Asterisk (\*) indicates a significant difference between the exposed fish ( $N=8$ ) and its respective pooled control ( $N=32$ ) (\* $P < 0.05$ ; \*\*\* $P < 0.001$ ).

**Figure. 4.** Plasma total ammonia concentration ( $T_{\text{amm}}$ ) in rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  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. 5.** Plasma urea-N concentration of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  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. 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  $\pm$  s.e.m. Asterisk (\*) indicates a significant difference between the exposed fish ( $N=8$ ) and its respective pooled control ( $N=24$ ) (\* $P < 0.05$ ).

**Figure. 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  $\pm$  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. 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  $\pm$  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. 9.** Expression of (A) Na<sup>+</sup>/K<sup>+</sup>-ATPase and (B) H<sup>+</sup>-ATPase mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  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. 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  $\pm$  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. 11.** Enzyme activities of (A) Na<sup>+</sup>/K<sup>+</sup>-ATPase (B) Na<sup>+</sup>/NH<sub>4</sub><sup>+</sup>-ATPase (K<sup>+</sup> replaced by NH<sub>4</sub><sup>+</sup> in the reaction media) and (C) H<sup>+</sup>-ATPase in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mM ammonia). Values are expressed as mean  $\pm$  s.e.m. Asterisk (\*) indicates a significant difference between the exposed fish ( $N=8$ ) and its respective pooled control ( $N=22-24$ ) (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ).

## Tables

**Table 1**

Cloning primers

Primer	5'→3'
Rhbg (carp/goldfish)	F: catcctcatcatcctctttggc R: cgtctacctgtggatgttctg
Rhcg (carp/goldfish)	F: tgggctcttctcatgaaggctgg R: ctgcggtatcatctctacactgggat
Rhcb (carp/goldfish)	F: gcacactgttctgtggatg R: tctggggagatcctgctg
H <sup>+</sup> -ATPase (carp)	F: cccgtggacagaagatccccatc R: ggatacatcacagaggacaggtg
H <sup>+</sup> -ATPase (goldfish)	F: ctaccccgaggagatgatccag R: cagctgcacaacagacagatct
NKA (carp/goldfish)	F: aaggtggacaactcctccctgactg R: gcgtatggccaagaagaactgcctggt
UT (carp/goldfish)	F: gatgggtgggcactcaatggtttgttgg R: caggccactacaatcaccacttccc

**Table 2**

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

Gene	Accession no.	Sequence of Primer (5'→3')	Efficiency (%)
<b>Trout</b>			
Na <sup>+</sup> /K <sup>+</sup> -ATPase (α1a-subunit)	AY319391	F: ttgacctggatgaccacaag R: ggatctccttagcccgaac	87.2%
H <sup>+</sup> -ATPase (V-type, B-subunit)	AF100042	F: tcagccttggttgatgatg R: caacattggtgggaaacagg	103.7%
Rhbg	EF051113	F: cgacaacgacttttactaccgc R: gacgaagccctgcatgagag	75.3%
Rhcg1	DQ431244	F: catcctcagcctcatacatgc R: tgaatgacagacggagccaatc	80.1%
Rhcg2	AY619986	F: cctcttcggagtcttcac R: ctatgtcgtggtgatgttg	88.7%
NHE-2	EF446605	F: tatggccattgtgacctgtg R: caggcctctccacactaagg	92.8%
UT	EF688013	F: gtataggccaggtgtatggg R: gatgcctcaaattggagctg	106.0%
EF-1α	AF498320	F: ggaaagtcaaccaccacag R: gataccacgctccctctcag	88.6%
<b>Common carp</b>			
Na <sup>+</sup> /K <sup>+</sup> -ATPase	JX570881	F: aggtggacaactcctccctg R: atacgacctatgacagtacg	153%
H <sup>+</sup> -ATPase	JX570880	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	102.5%
Rhbg	JX570877	F: tcccagttccaggatgttc R: tggaaaaagccctgcataag	93.7%
Rhcg-a	JX570878	F: atcctgaacatcctccatgc R: aacttgccagaacatccac	113.2%
Rhcg-b	JX570879	F: caciaagccacacacagtcc	93.8%

UT	JX570882	R: tcttttctcgcggttcttg F: agtgcattcttggttggtctc R: aggacttggtggaagtgggtg	129.8%
$\beta$ -actin	M24113.1	F: cgtgatggactctggtgatg R: tcacggacaatttcctctc	110.9%
<b>Goldfish</b>			
Na <sup>+</sup> /K <sup>+</sup> -ATPase	JX570887	F: gtcattgggtcgtattgcatc R: gttacagtggcagggagacc	89.1%
H <sup>+</sup> -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: attgtgggcttctctgtgg R: ggcacacgtttctcaaaagc	90.1%
UT	JX570888	F: tgttaaagggcaggggtgaag R: cggatataacggcatcttgg	111.4%
$\beta$ -actin	AB039726	F: ggccctccctgtctatcttcc R: ttgagaggtttgggttggtc	95.0%

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



**Table 3**

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

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

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























