

RESEARCH ARTICLE

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

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SUMMARY

We investigated relationships among branchial unidirectional Na⁺ fluxes, ammonia excretion, urea excretion, plasma ammonia, plasma cortisol, and gill transporter expression and function in three freshwater fish differing in their sensitivity to high environmental ammonia (HEA). The highly ammonia-sensitive salmonid *Oncorhynchus mykiss* (rainbow trout), the less ammonia-sensitive cyprinid *Cyprinus carpio* (common carp) and the highly ammonia-resistant cyprinid *Carassius auratus* (goldfish) were exposed chronically (12–168 h) to 1 mmol l⁻¹ ammonia (as NH₄HCO₃; pH 7.9). During HEA exposure, carp and goldfish elevated ammonia excretion (J_{Amm}) and Na⁺ influx rates ($J_{\text{in}}^{\text{Na}}$) while trout experienced higher plasma ammonia (T_{Amm}) and were only able to restore control rates of J_{Amm} and $J_{\text{in}}^{\text{Na}}$. All three species exhibited increases in Na⁺ efflux rate ($J_{\text{out}}^{\text{Na}}$). At the molecular level, there was evidence for activation of a 'Na⁺/NH₄⁺ exchange metabolon' probably in response to elevated plasma cortisol and T_{Amm} , though surprisingly, some compensatory responses preceded molecular responses in all three species. Expression of Rhbg, Rhcg (Rhcg-a and Rhcg-b), H⁺-ATPase (V-type, B-subunit) and Na⁺/K⁺-ATPase (NKA) mRNA was upregulated in goldfish, Rhcg-a and NKA in carp, and Rhcg2, NHE-2 (Na⁺/H⁺ exchanger) and H⁺-ATPase in trout. Branchial H⁺-ATPase activity was elevated in goldfish and trout, and NKA activity in goldfish and carp, but NKA did not appear to function preferentially as a Na⁺/NH₄⁺-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.

Key words: HEA, urea excretion, Na⁺/NH₄⁺ exchange metabolon, rainbow trout, common carp, goldfish.

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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 NH₃ diffusion and/or as NH₄⁺ transport linked in some manner to Na⁺ uptake (Wilkie, 1997; Wilkie, 2002; Wood, 1993). While the exact mechanisms are not yet fully revealed, the process often manifests as a coupling of ammonia excretion to Na⁺ uptake, the scheme first proposed by Krogh (Krogh, 1939). Current hypotheses include a direct coupling through Na⁺/NH₄⁺ exchangers, coupling with H⁺ excretion by an apical Na⁺/H⁺ exchanger (NHE), and coupling with H⁺ excretion *via* an apical H⁺ pump, which energizes Na⁺ uptake through a putative Na⁺ channel. The last two mechanisms would acidify the gill boundary layer, thereby enhancing the 'diffusion-trapping' of NH₃ as NH₄⁺ and maintaining the partial pressure gradient for diffusive NH₃ efflux, thus manifesting as an apparent Na⁺/NH₄⁺ exchange (Clarke and Potts, 1998; Wilson et al., 1994).

Recently, the key involvement of Rhesus (Rh) glycoproteins in this diffusive NH₃ efflux has been recognized (Nakada et al., 2007a; Nawata et al., 2007). These appear to function as ammonia channels, binding NH₄⁺ but facilitating the diffusion of NH₃ (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 NH₄⁺ at the cytoplasmic side of the Rhcg channel may provide a source of H⁺ ions to drive Na⁺ uptake under circumstances such as low external pH (Hirata et al., 2003; Kumai and Perry, 2011; Lin et al., 2012; Shih et al., 2012), which otherwise would seem thermodynamically challenging (Parks et al., 2008). In two studies, increased water Na⁺ concentration resulted in elevated ammonia excretion (Shih et al., 2012; Wood et al., 2007). Furthermore, ammonia loading by infusion (Nawata and Wood, 2009; Salama et al., 1999), feeding (Zimmer et al., 2010) or high environmental ammonia (HEA) exposure (Braun et al., 2009b; Hung et al., 2007; Nawata et al., 2007; Nawata et al., 2010b; Wood and Nawata, 2011) resulted in both increased Na⁺ uptake and increased mRNA expression of the gill Rh proteins, especially Rhcg, in a number of teleost species. In some freshwater teleosts the ammonia excretion

is initially inhibited by exposure to HEA but with later recovery (Liew et al., 2013; Nawata et al., 2007; Payan, 1978; Wilkie et al., 2011; Wilson et al., 1994; Zimmer et al., 2010). Moreover, cortisol increases greatly during HEA exposure (Ortega et al., 2005; Tsui et al., 2009) and may play a role. The combined stimulus of HEA and elevated cortisol augmented both Na^+ uptake and ammonia transport capacity in cultured freshwater rainbow trout gill epithelia (Tsui et al., 2009). Therefore, there is evidence for several potential linkages between ammonia efflux (*via* Rh proteins) and Na^+ influx (*via* NHE and/or H^+ -ATPase). As the coupling appears to be indirect and loose, involving several interacting transporters, Wright and Wood (Wright and Wood, 2009) have described it as a ' $\text{Na}^+/\text{NH}_4^+$ exchange complex or metabolon'.

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

While chronic toxicity during HEA exposure is well documented (Dosdat et al., 2003; Knoph and Thorud, 1996; Lemarié 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, 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 end points 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 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, the goldfish, *Carassius auratus*, when exposed chronically (up to 168 h) to HEA (1 mmol l^{-1} at pH 7.9). The reported ammonia 96 h LC_{50} values (expressed as total ammonia) for goldfish, common carp and trout are $\sim 9 \text{ mmol l}^{-1}$ (pH 8.0), 2.6 mmol l^{-1} (pH 7.5–7.8) and 1.7 mmol l^{-1} (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_{Amn}) and urea (J_{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), H^+ -ATPase (V-type, B-subunit), NHE-2, NKA (α -subunit) and urea transporter (UT) during HEA challenge. The sequence information for Rh glycoproteins, H^+ -ATPase, NKA and UT was not available for the carp and goldfish, so we cloned partial sequences of these cDNAs. H^+ -ATPase and NKA enzyme activities were measured and the ability of NH_4^+ to activate gill NKA was evaluated *in vitro* for all three species. Moreover, we also assessed net K^+ flux rates ($J_{\text{net}}^{\text{K}}$), which are interpreted as indices of gill transcellular permeability (Laurén 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 the $\text{Na}^+/\text{NH}_4^+$ exchange complex were more effective in dealing with the ammonia challenge in goldfish than 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 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 to $17 \pm 1^\circ\text{C}$ and photoperiod was 12 h light: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 $6.9\text{--}7.4 \text{ mg l}^{-1}$, total NH_3 $0.006\text{--}0.009 \text{ mmol l}^{-1}$, nitrite $0.0015\text{--}0.0021 \text{ mmol l}^{-1}$, nitrate $0.015\text{--}0.042 \text{ mmol l}^{-1}$, Ca^{2+} $0.8\text{--}1.0 \text{ mmol l}^{-1}$, Mg^{2+} $0.19\text{--}0.21 \text{ mmol l}^{-1}$, Na^+ $1.2\text{--}1.4 \text{ mmol l}^{-1}$, K^+ $0.09\text{--}0.10 \text{ mmol l}^{-1}$, Cl^- $0.9\text{--}1.2 \text{ mmol l}^{-1}$, titratable alkalinity $1.6\text{--}1.8 \text{ mmol l}^{-1}$ and hardness $226 \text{ mg CaCO}_3 \text{ l}^{-1}$.

Mean (\pm s.d.) mass of rainbow trout was $132 \pm 22 \text{ g}$, of carp $16 \pm 4 \text{ g}$, and of goldfish $15 \pm 5 \text{ g}$. 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 Industries, Kasai City, 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 mmol l^{-1} HEA for a period of 12, 84 and 168 h for all measurements. This concentration represents 11%, 38% and 59% of the 96 h LC_{50} 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_4HCO_3 stock solution (Sigma, Munich, Germany).

A constant concentration of $1.09 \pm 0.08 \text{ mmol l}^{-1}$ of ammonia was maintained throughout the experiment. Control groups (no HEA) were set up in parallel to these exposure groups. Ammonia concentration was measured (using the salicylate–hypochlorite method) (Verdouw et al., 1978) every 6 h after the onset of treatment and the concentration of ammonia in the tanks was maintained by adding a calculated amount of the NH_4HCO_3 solution. Moreover, to avoid the microbial removal of ammonia and the accumulation of other waste products, 60–80% of the water was discarded every 2 days and replaced with fresh water containing the respective amount of ammonia. Water pH was maintained at 7.8–8.0 throughout the experimental period using dilute HCl and/or KOH.

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 experiments was performed in another climate chamber, maintaining the same temperature and photoperiod to which fish had been 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 (four from each of two tanks for cyprinids, two from each of four tanks for trout) were sampled and transferred individually into their respective experimental containers. The experimental protocols consisted of exposing the fish ($N=8$) to HEA while simultaneously measuring unidirectional Na^+ fluxes with ^{22}Na (Perkin Elmer, Boston, MA, USA) and net ammonia and urea fluxes. Tests on control groups were conducted in parallel. Exposure to HEA started within the container for those groups of fish that were sampled at 12 h.

At the start of each flux period, an aliquot of ^{22}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}Na , total Na^+ and K^+ measurements) were taken at the start of the experiment and at subsequent 1 h intervals up to 4 h after the onset of ammonia exposure. For the ammonia and urea flux measurements, initial and final water samples were also taken. Following the last (i.e. 4 h) water sampling, the animals were terminally anaesthetized with a lethal dose of neutralized (with 2 parts NaOH) MS-222 (ethyl 3-aminobenzoate methane-sulphonic acid, 1 g l^{-1} ; Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, ~ 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) 1 ml syringes with 23-gauge needles. Blood was immediately centrifuged (for 1 min at $16,000 \text{ r.p.m.}$ at 4°C), and aliquots of plasma

were frozen in liquid nitrogen and stored at -80°C for later analysis. Fish were dissected; gills were removed, washed with saline and blotted. One portion was added to five volumes of RNeasy lysis reagent (Qiagen, Hilden, Germany) and stored at 4°C for later molecular analysis, while the remaining gills were flash-frozen in liquid nitrogen and stored at -80°C for enzymatic assays.

Enzymatic analyses

NKA activity and H^+ -ATPase activity were measured in crude gill homogenates of control fish and fish exposed to HEA using previously published methods (McCormick, 1993; Lin and Randall, 1993), as modified by Nawata et al. (Nawata et al., 2007). Protein concentrations were measured with Bradford reagent and BSA standards (Sigma). In order to assess whether NH_4^+ could activate NKA activity, the assay was performed under optimized conditions replacing the native concentration of KCl (10 mmol l^{-1}) with the same molar concentration of NH_4Cl in the reaction medium. Activity was 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 using Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and the purity by measuring the $\text{OD}_{260}/\text{OD}_{280}$ absorption ratio (>1.95).

For cloning, first-strand cDNA was synthesized with Superscript II (Invitrogen). Partial sequences for carp and goldfish Rhb, Rhcg, H^+ -ATPase (vacuolar, B-subunit), NKA (α -subunit) and urea transporter (UT) were amplified using Taq DNA polymerase (Invitrogen) and the primers listed in Table 1. Basic Local Alignment Search Tool (BLAST) analyses were carried out on the National Center for Biotechnology Information (NCBI) database.

Real-time PCR

For quantitative real-time PCR (qPCR), a starting amount of $1 \mu\text{g}$ RNA was transcribed into first-strand cDNA using the Revert Aid H minus First strand cDNA synthesis kit (Fermentas, Cambridge, UK). mRNA expression in the gills of fish exposed to HEA for 12, 84 and 168 h was compared with 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 (Wood and Nawata, 2011).

qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). Reactions ($20 \mu\text{l}$) containing $4 \mu\text{l}$ of $5 \times$ diluted cDNA, 4 pmol each of forward and reverse primers, $0.8 \mu\text{l}$ ROX dye (1:10 dilution) and $10 \mu\text{l}$ Platinum SYBR

Table 1. Cloning primers

Primer	Primer sequence (5'→3')
Rhb (carp/goldfish)	F: catcctcatcatcctcttggc; R: cgtctacctgtggatgtctg
Rhcg-a (carp/goldfish)	F: tgggctcttctcatgcaaggctgg; R: ctgctgatcatctcactctggat
Rhcg-b (carp/goldfish)	F: gcacactgttctctggtgatg; R: tctggggagatcctgctg
H^+ -ATPase (carp)	F: cccgtggacagaagatccccatc; R: ggatacatcacagagggacagctg
H^+ -ATPase (goldfish)	F: ctaccccaggagatgatccag; R: cagctgcacaacagacagatct
NKA (carp/goldfish)	F: aagggtggacaactcctccctgactg; R: gcgtatggccaagaagaactgctggt
UT (carp/goldfish)	F: gatggtggcactcaatggtttgttg; R: caggccactacaatcaccactccc

F, forward; R, reverse.

Table 2. Accession numbers, qPCR primer list and efficiencies of the PCR reactions

Gene	Accession no.	Primer sequence (5'→3')	Efficiency (%)
Trout			
Na ⁺ /K ⁺ -ATPase (α1a-subunit)	AY319391	F: ttgacctggatgaccacaag R: ggatcctctagcccgaac	87.2%
H ⁺ -ATPase (V-type, B-subunit)	AF100042	F: tcagccttggttgagatg R: caacattgggggaaacagg	103.7%
Rhbg	EF051113	F: cgacaacgactttactaccgc R: gacgaagcctgcatgagag	75.3%
Rhcg1	DQ431244	F: catcctcagcctcatacatgc R: tgaatgacagacggagccaatc	80.1%
Rhcg2	AY619986	F: cctctcggagtctctac R: ctatgctgctggtgatgtg	88.7%
NHE-2	EF446605	F: tatggccattgtgacctgtg R: caggcctctccactaagg	92.8%
UT	EF688013	F: gtataggccagggtatggg R: gatcgctcaaatggagctg	106.0%
EF-1α	AF498320	F: ggaaagtcaaccaccacag R: gataccacgctccctctcag	88.6%
Common carp			
Na ⁺ /K ⁺ -ATPase	JX570881	F: agtgggacaactcctccctg R: atacgacctgacagtagc	153%
H ⁺ -ATPase	JX570880	F: ctatgggggtcaacatggag R: ccaacacgtgctctcacac	102.5%
Rhbg	JX570877	F: tcccagttccaggatgttc R: tggaaaaagccctgcataag	93.7%
Rhcg-a	JX570878	F: atcctgaacatcctccatgc R: aactggccagaacatccac	113.2%
Rhcg-b	JX570879	F: caciaagccacacacagtcc R: tctttctcgcggtctctg	93.8%
UT	JX570882	F: agtgcactctggtggtctc R: aggactgtgggaagtggg	129.8%
β-actin	M24113.1	F: cgtgatggactctggtgatg R: tcacggacaattccctctc	110.9%
Goldfish			
Na ⁺ /K ⁺ -ATPase	JX570887	F: gtcatgggtcgattgcatc R: gttacagtggcaggagacc	89.1%
H ⁺ -ATPase	JX570886	F: ctatgggggtcaacatggag R: ccaacacgtgctctcacac	79.1%
Rhbg	JX570883	F: atgatgaaacggatgccaag R: tctggaaactgggataacg	95.1%
Rhcg-a	JX570884	F: gctggttcattctctggac R: atctcggcatggaggacag	120.2%
Rhcg-b	JX570885	F: attgtgggctctctgtgg R: ggcacacgtttctcaaaagc	90.1%
UT	JX570888	F: tgttaaagggcagggtgaag R: cggatataacggcatcttg	111.4%
β-actin	AB039726	F: ggccctcctgtctatctcc R: ttgagaggttgggtggtc	95.0%

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

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 were also run to ensure no contamination of reagents, no primer-dimer formation, etc.

In the 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 without RT as controls was not considered.

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

the samples (20 random samples were tested) and these 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, NY, USA) as described elsewhere (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. (Wright et al., 1995a) using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany).

^{22}Na activity in water samples was measured by a gamma counter (Wallac Wizard 3 in 1480 Automatic Gamma Counter, Perkin Elmer, Turku, Finland). Na^+ and K^+ concentrations in water were measured using flame atomic absorption spectrophotometry (AAAnalyst 800, Perkin Elmer). Net flux rates (in $\mu\text{mol kg}^{-1} \text{h}^{-1}$) of 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 K^+ ($J_{\text{net}}^{\text{K}}$), ammonia (J_{Amm}) and urea (J_{urea}) were calculated as for net flux rates of Na^+ ($J_{\text{net}}^{\text{Na}}$).

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 previously (Wood, 1992):

$$J_{\text{in}}^{\text{Na}} = [(CPM_i - CPM_f) V] / (SA_{\text{ext}} \times t \times M), \quad (1)$$

where CPM_i is the initial ^{22}Na radioactivity in the water (c.p.m. ml^{-1}) at the start of the flux period, CPM_f is the final ^{22}Na radioactivity in the water (c.p.m. ml^{-1}) 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 (c.p.m. nmol^{-1}), calculated from measurements of water ^{22}Na radioactivity and total water $[\text{Na}^+]_0$ at the start and end of the flux period; t is the duration of the flux period (h) and M is the mass of the fish (g). Na^+ unidirectional efflux rates ($J_{\text{out}}^{\text{Na}}$, by convention negative) were calculated by difference:

$$J_{\text{out}}^{\text{Na}} = J_{\text{net}}^{\text{Na}} - J_{\text{in}}^{\text{Na}}. \quad (2)$$

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 1 mmol l^{-1} ammonia (HEA) exhibited an initial 3.5-fold stimulation of $J_{\text{out}}^{\text{Na}}$ ($P < 0.01$) at 12h, but this had returned to control values by 84h (Fig. 1). $J_{\text{in}}^{\text{Na}}$ remained statistically similar in HEA-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 12h exposure, by 2.6- and 2.4-fold, respectively, restored after 84h and then increased again ($P < 0.05$) at the end of the exposure period. In goldfish, 12h 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 with the control level. These elevations were followed by a decline (at 84 and 168h) but they 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, Na^+ balance was severely altered in trout at 12h 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 12h this species also was able to retain $J_{\text{net}}^{\text{Na}}$ at the control level, as seen for cyprinids.

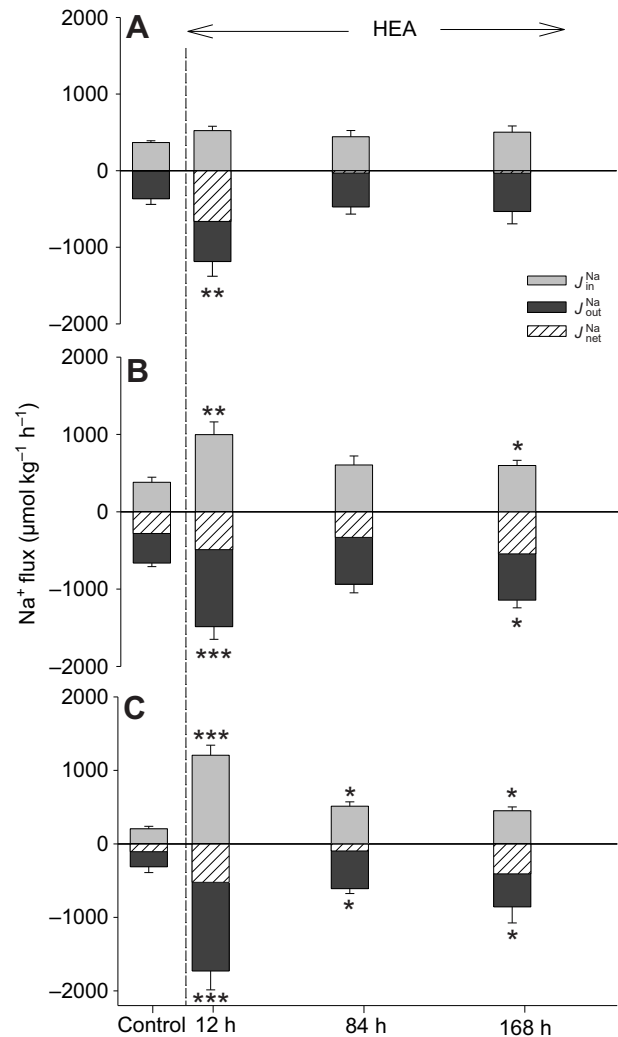


Fig. 1. Na^+ unidirectional influx ($J_{\text{in}}^{\text{Na}}$), Na^+ efflux ($J_{\text{out}}^{\text{Na}}$) and Na^+ net flux ($J_{\text{net}}^{\text{Na}}$) rates in (A) rainbow trout, (B) common carp and (C) goldfish during exposure to high environmental ammonia (HEA; 1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Net K^+ flux rates

In all three species, $J_{\text{net}}^{\text{K}}$ was negative (i.e. net losses) under control conditions (Table 3). After 12h of exposure, the K^+ flux was reversed in both cyprinids, resulting in a net uptake ($P < 0.05$ or 0.01). However, the net loss rates increased significantly in both trout (by 228%, $P < 0.01$) and carp (by 148%, $P < 0.01$) after 84h HEA. $J_{\text{net}}^{\text{K}}$ returned to control rates in goldfish at 84h while in the other two at 168h.

Ammonia and urea excretion rate

In contrast to cyprinids, trout exposed to HEA displayed no significant changes in ammonia excretion rate (J_{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_{Amm} in carp was $-402 \mu\text{mol kg}^{-1} \text{h}^{-1}$ under control conditions and increased significantly after 12h HEA. The significant elevation persisted till the end of the exposure period except at 40h, where a slight decline was observed. The relative J_{Amm} increments at 12, 84 and 168h were 150% ($P < 0.01$), 70% ($P < 0.05$) and 186% ($P < 0.01$) higher than the

Table 3. Net flux rates of K^+ and plasma cortisol level in rainbow trout, common carp and goldfish during exposure to 1 mmol l^{-1} ammonia

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

Values are expressed as means \pm s.e.m.

Significant difference between the exposed fish ($N=7-8$) and its respective pooled control ($N=22-24$) ($P<0.05$, ** $P<0.01$, *** $P<0.001$).

control. In contrast, in goldfish, the control J_{Ammonia} 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_{Ammonia} 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 $\sim 25\%$, 30% and 13% of ammonia-N excretion rates in trout, carp and goldfish, respectively. The effect of HEA on urea excretion rate (J_{urea}) was

notable only in goldfish (Fig. 3). A significant rise was observed from 12 h onwards throughout the entire exposure course. The relative increments after 12, 40, 84 and 168 h compared with 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 exposure, the urea-N excretion rate became almost equal to the ammonia-N excretion rate in the goldfish. In contrast, no significant effect ($P>0.05$) of HEA on J_{urea} was seen either in carp or in trout.

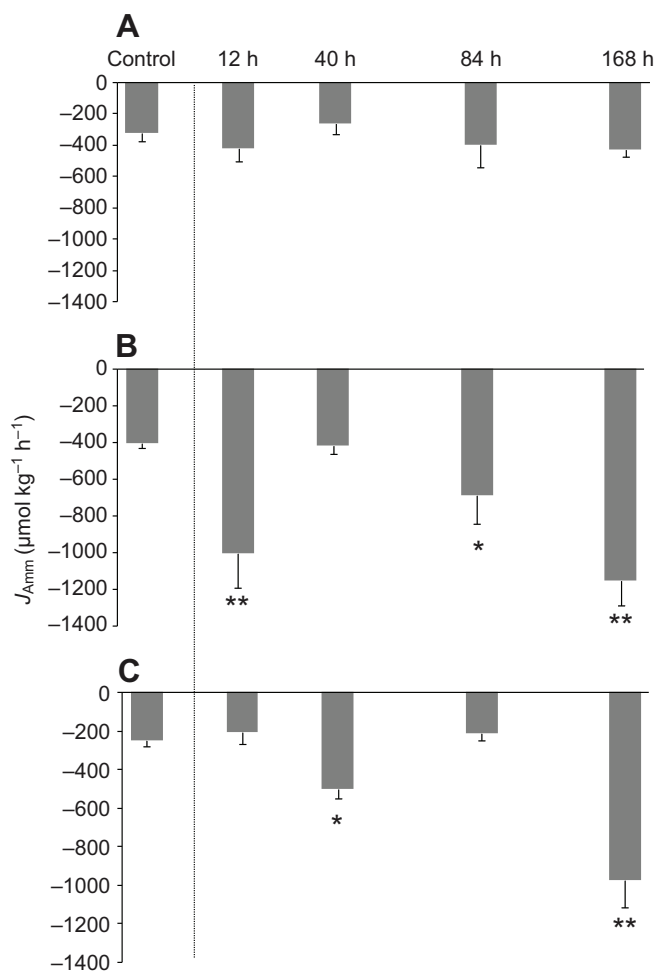


Fig. 2. Net excretion rate of ammonia (J_{Ammonia}) in (A) rainbow trout, (B) common carp and (C) goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=32$) (* $P<0.05$, ** $P<0.01$).

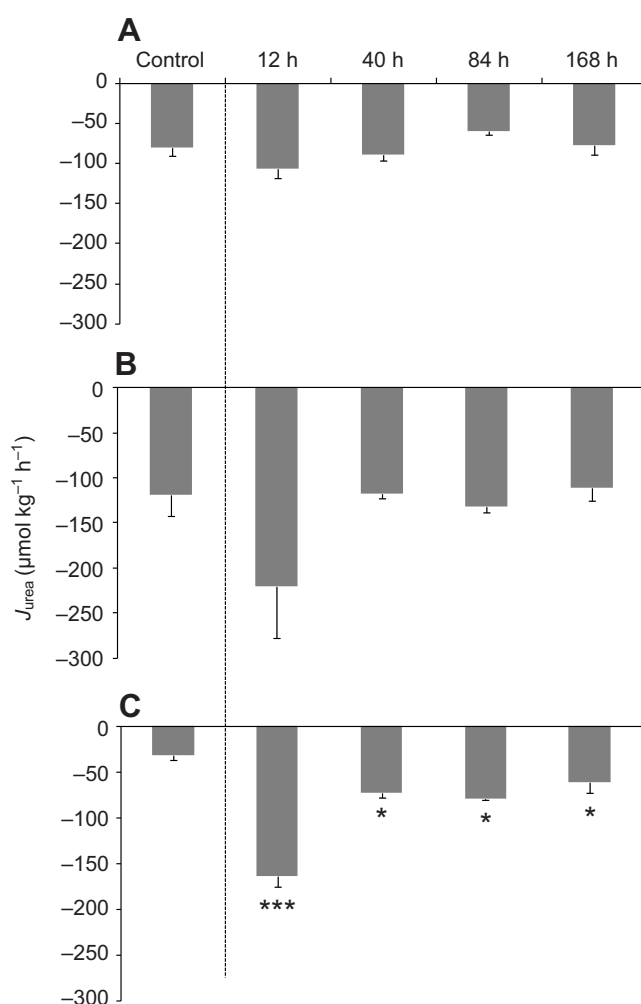


Fig. 3. Net excretion rate of urea (J_{urea}) in (A) rainbow trout, (B) common carp and (C) goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. *Significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=32$) (* $P<0.05$, *** $P<0.001$).

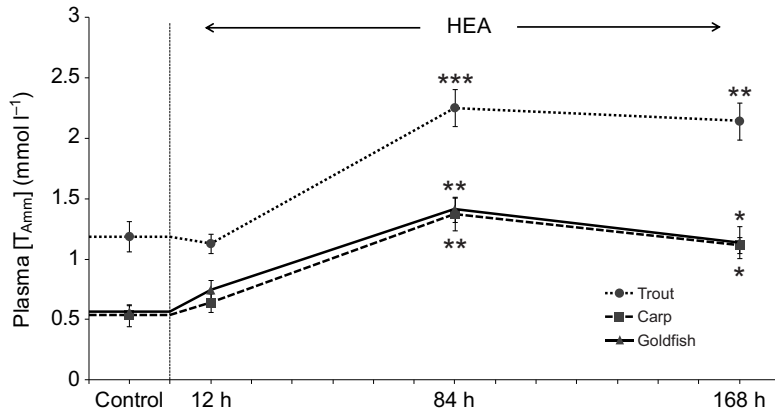


Fig. 4. Plasma total ammonia concentration (T_{Amm}) in rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means \pm s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Plasma metabolites

Ammonia and urea accumulation

Plasma total ammonia (T_{Amm}) was significantly elevated in both cyprinids and trout from 84h onwards and remained higher until the end of the HEA exposure period (168h) (Fig. 4). In the cyprinids at 84 and 168h, plasma T_{Amm} levels were only slightly higher than the mean exposure level (1.09 mmol l^{-1}), while in trout, T_{Amm} was about 2-fold higher than the ammonia level in the water.

Under control conditions, plasma urea-N levels were considerably higher than plasma T_{Amm} in all species. During HEA exposure, plasma urea-N concentration in trout was elevated significantly compared with control by 68% ($P<0.001$) and 58% ($P<0.05$), respectively, after 12 and 168h (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 and 168h, respectively. Curiously, urea-N concentrations at 84h 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 12h HEA onwards (Table 3). The rise in trout after 12, 84 and 168h were 30% ($P<0.05$), 115% ($P<0.001$) and 104% ($P<0.01$), respectively. 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% ($P<0.05$).

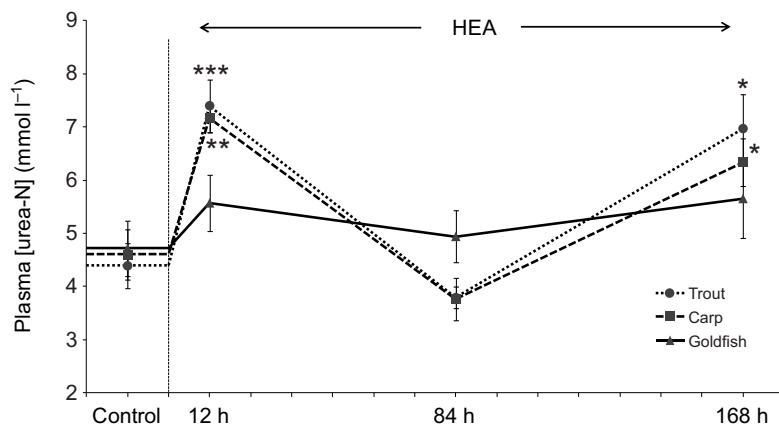


Fig. 5. Plasma urea-N concentration of rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means \pm s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Gill mRNA expression

BLAST results show that the carp and goldfish Rhb g partial sequences are most similar to that of *Danio rerio* Rhb g (AAH49405) as *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 Rhb g sequences are, respectively, 88% and 90% identical to the *D. rerio* Rhb g amino acid sequence and they are both 83% identical (E-value of $1e-116$) to *O. mykiss* Rhb g (ACF70599). Rhb g -a is most similar to *D. rerio* Rhb g a (NP_001083046) (89% amino acid identity and E-value of $3e-127$ for both carp and goldfish). Also, Rhb g -a is more similar to *O. mykiss* Rhb g 1 (NP_001118049) than to *O. mykiss* Rhb g 2 (NP_001117995) (86% and 88% identity and E-values of $1e-121$ and $2e-123$ for carp and goldfish, respectively, versus 85% identity and an E-value of $1e-120$ for both carp and goldfish). Rhb g -b is most similar to *D. rerio* Rhb g -like2 (Q8JI14) with an E-value of $4e-110$ and 86% identity to carp and an E-value of $3e-108$ and 85% identity to goldfish. Rhb g -b is more similar to *O. mykiss* Rhb g 2 than to *O. mykiss* Rhb g 1 (E-value of $3e-100$ with 78% identity to carp and an E-value of $1e-100$ with 79% identity to goldfish versus 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 Rhb g increased significantly at 12 and 84h 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 168h. In carp and trout, small increments in Rhb g transcript level were not significantly different from their respective control levels.

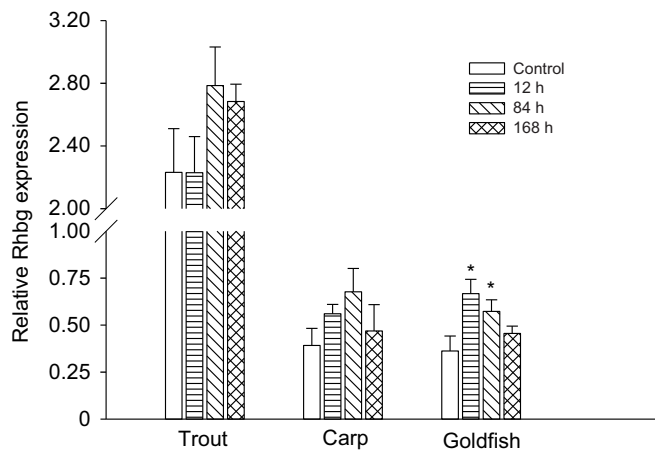


Fig. 6. Expression of Rhbg mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) ($*P<0.05$).

Rhcg-a expression level was significantly upregulated 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 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 the 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 and 168 h were 40% ($P<0.05$) and 50% ($P<0.05$), respectively.

NKA and H^+ -ATPase

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

Pronounced effects ($P<0.01$) of HEA were evident on the transcript levels of H^+ -ATPase in gills of trout and goldfish while no significant effects ($P>0.05$) were noted for carp (Fig. 9B). In trout, 84 and 168 h HEA exposure resulted in 1.5-fold ($P<0.01$) and 1.7-fold ($P<0.01$) elevations over the control. Likewise, H^+ -ATPase expression level in goldfish increased gradually and became significant (2.2-fold, $P<0.01$) at 84 h but returned to control values ($P>0.05$) after 168 h of HEA exposure.

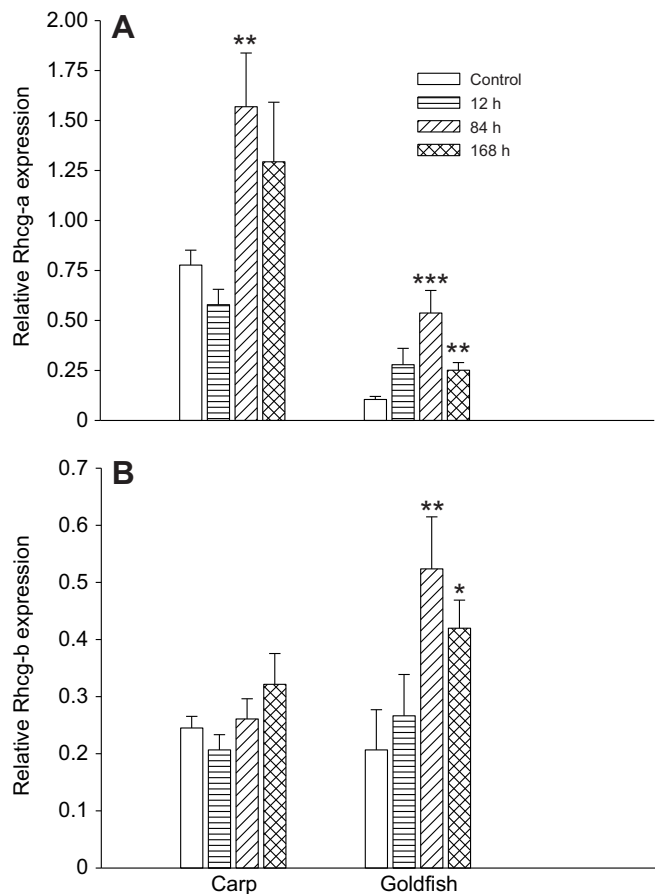


Fig. 7. Expression of (A) Rhcg-a and (B) Rhcg-b mRNA in the gills of common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) ($*P<0.05$, $**P<0.01$, $***P<0.001$).

Urea transporter

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, 84 and 168 h were 12-fold ($P<0.001$), 11-fold ($P<0.001$) and 6-fold ($P<0.01$), respectively. In contrast, HEA did not appear to exert any notable effect ($P>0.05$) on UT mRNA expression level in carp or trout.

NKA and H^+ -ATPase enzyme activity

In general, NKA and H^+ -ATPase activity responses (Fig. 11A,C) paralleled mRNA expression data (Fig. 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 NH_4^+ to activate gill NKA activity in these three species was examined (Fig. 11B). In the course of these trials, we found that NKA activity was always lower when NH_4^+ was substituted for K^+ , regardless of the treatment, time or species,

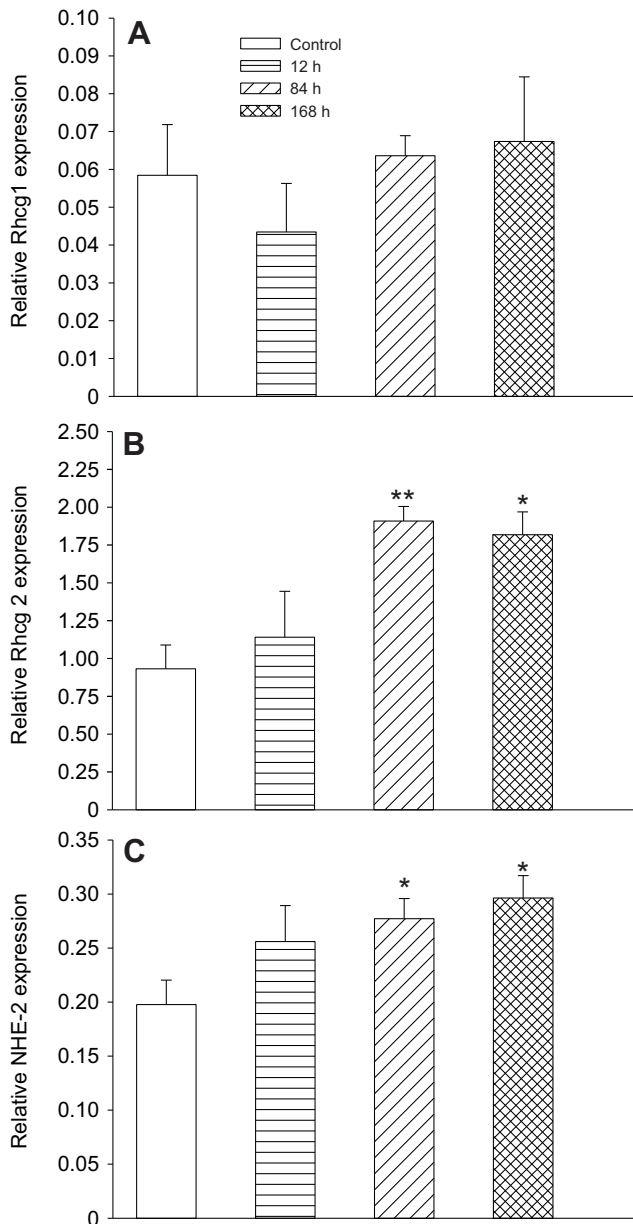


Fig. 8. Expression of (A) Rhcg1, (B) Rhcg2 and (C) Na⁺/H⁺ exchanger NHE-2 mRNA in the gills of rainbow trout during exposure to HEA (1 mmol l⁻¹ ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (**P*<0.05, ***P*<0.01).

although carp appeared to take NH₄⁺ slightly better than goldfish and trout. Thus, while NH₄⁺ can support some Na⁺/NH₄⁺-ATPase activity, it is not as effective as K⁺ (i.e. NKA activity) on an equimolar (10 mmol l⁻¹) basis in any of the three species. In carp and goldfish, the increases in NKA activity occurring at 84 and 168 h were tracked by smaller increases in Na⁺/NH₄⁺-ATPase activity (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⁺-ATPase activity were observed for this species after 84 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

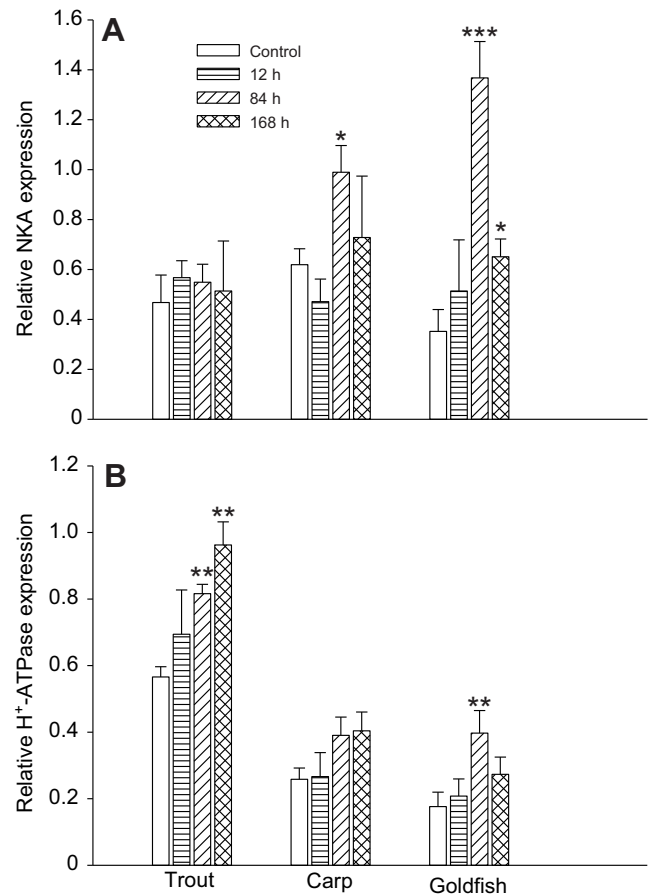


Fig. 9. Expression of (A) Na⁺/K⁺-ATPase (NKA) and (B) H⁺-ATPase mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l⁻¹ ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish (*N*=8) and its respective pooled control (*N*=24) (**P*<0.05, ***P*<0.01, ****P*<0.001).

goldfish, increases in branchial H⁺-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*_{Amm}) was strongly inhibited or reversed in carp and trout during the first few hours (3 h) of exposure, but remained statistically unchanged in goldfish, and that all three species had restored control rates of *J*_{Amm} by 12 h (Liew et al., 2013). The current results confirm the restoration of *J*_{Amm} by 12 h in all three species, and indeed show a significant increase in the carp at this time (Fig. 2). This sequence of initial *J*_{Amm} inhibition followed by re-establishment has already been reported for goldfish and trout (Nawata et al., 2007; Wilkie et al., 2011; Wilson et al., 1994; Zimmer et al., 2010). Furthermore, both cyprinids exhibited enhanced *J*_{Amm} at several time points, signifying their ability to excrete ammonia under unfavourable circumstances. Assuming that a similar pH is maintained inside (i.e. in the blood) as outside these cyprinids (7.8–8.0), there would have been virtually no *P*_{NH₃} gradient from the blood to the bulk water as *T*_{Amm} concentrations became virtually identical in the two media by 84–168 h (Fig. 4). In contrast, trout exposed to HEA were not able to increase the excretion rate above control levels and had plasma *T*_{Amm} elevated more than 2-fold over

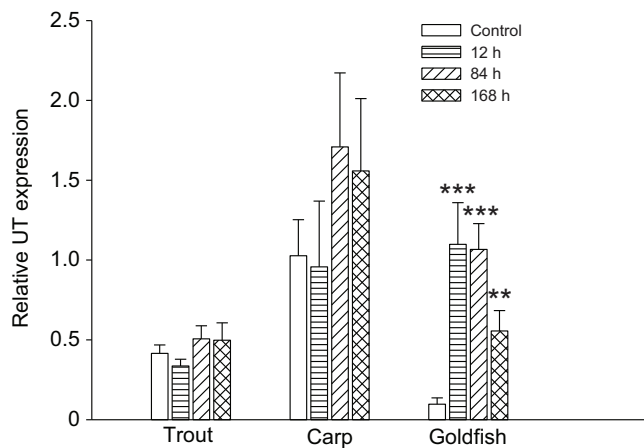


Fig. 10. Expression of urea transporter (UT) mRNA in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=24$) (** $P<0.01$, *** $P<0.001$).

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 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 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 upregulated only in goldfish upon HEA (12–84 h) exposure (Fig. 6). These increases in goldfish were accompanied by restored levels or significant increases in J_{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, be broadly distributed and play an appreciable role in ammonia excretion (Braun et al., 2009a). In a previous study on rainbow trout, Nawata and colleagues (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 mmol l^{-1} HEA. However, their study showed that a significant Rhbg induction occurred in pavement cells but not in mitochondrial rich cells. As 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 exposure, and of Rhcg-b expression only in goldfish (Fig. 7). As J_{Amm} was restored or increased at these same

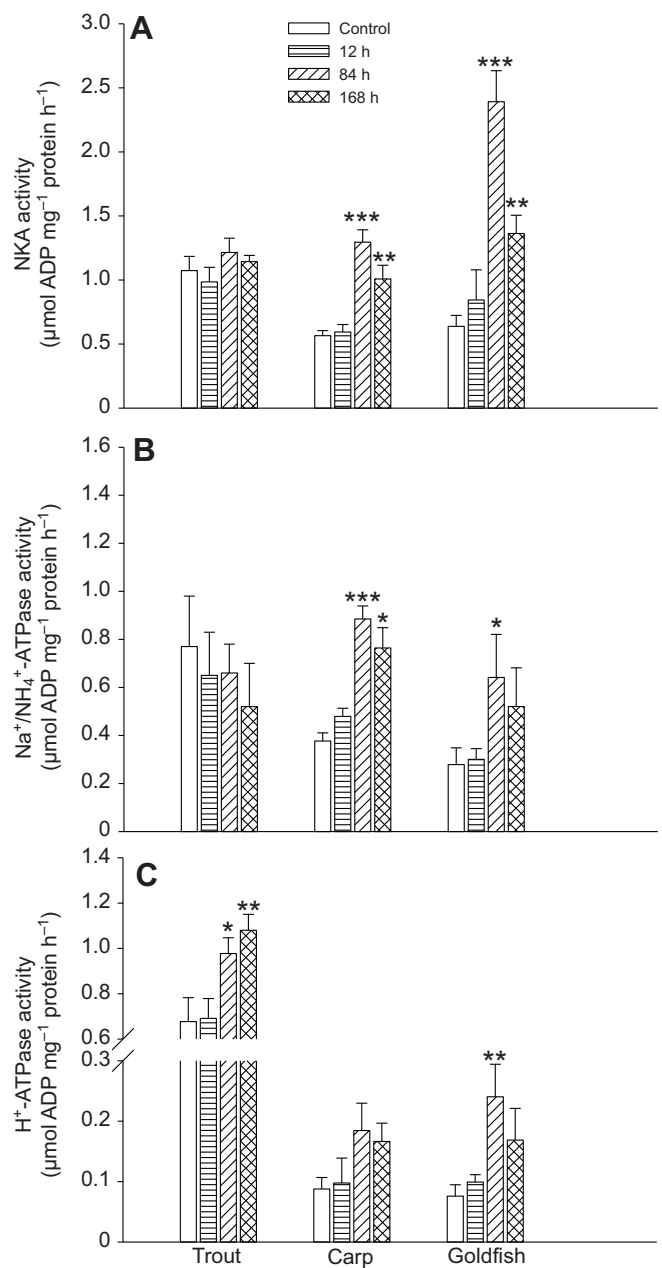


Fig. 11. Enzyme activity of (A) NKA, (B) $\text{Na}^+/\text{NH}_4^+$ -ATPase (K^+ replaced by NH_4^+ in the reaction media) and (C) H^+ -ATPase in the gills of rainbow trout, common carp and goldfish during exposure to HEA (1 mmol l^{-1} ammonia). Values are expressed as means and s.e.m. Asterisks indicate a significant difference between the exposed fish ($N=8$) and its respective pooled control ($N=22-24$) (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

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 upregulated at 84–168 h, in accord with previous observations (Nawata et al., 2007; 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 exposure 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⁺/NH₄⁺ exchange complex

The restoration of J_{in}^{Na} 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⁺/NH₄⁺ 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_{in}^{Na} (Fig. 1) after an initial inhibition in the first few hours of HEA exposure (Liew et al., 2013). Increased NHE-2 and H⁺-ATPase expression and/or activity, restored or increased J_{Amm} , and increased J_{in}^{Na} 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 upregulation of Rhcg-a and Rhcg-b (Fig. 7), elevated J_{Amm} (Fig. 2), and elevated J_{in}^{Na} (Fig. 1). In contrast, in carp neither the activity nor the expression level of H⁺-ATPase was affected by HEA (Fig. 11C, Fig. 9B), but carp may utilize other components of the Na⁺/NH₄⁺ exchange metabolon as suggested by a significant upregulation 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 and 168 h (Fig. 11C, Fig. 9B). If these changes signify an increased H⁺ efflux, this scenario predicts 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, NH₄⁺ 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 NH₃ 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_{in}^{Na} (Fig. 1) and J_{Amm} (Fig. 2) in trout were restored by 12 h of HEA exposure onwards, long before any significant increment (at 84–168 h) in H⁺-ATPase activity (Fig. 11C) or genomic upregulation of H⁺-ATPase (Fig. 9B), Rhcg2 (Fig. 8B) and NHE-2 (Fig. 8C). A similar discrepancy was noted for cyprinids, where J_{in}^{Na} (Fig. 1) and J_{Amm} (Fig. 2) increased by 12 h and 12–40 h, respectively, while the responses of Rhcg ammonia transporters became significant only at 84–168 h HEA (Fig. 7A,B). Notably, this delayed upregulation of these transporters in all three species occurred in conjunction with the elevated plasma T_{Amm} , which was apparent at 84 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⁺/NH₄⁺ exchange complex (Nawata and Wood, 2009; Tsui et al., 2009). Other factors such as post-translational modifications of the Rh proteins and other associated transporters during HEA exposure may be responsible for the temporary disconnect between the changes recorded in mRNA expression and those in functional activity. In this regard, Nawata and colleagues (Nawata et al., 2010b) reported that the molecular mass of Rh proteins increased by ~8 kDa in pufferfish (*Takifugu rubripes*) when exposed to 1 mmol l⁻¹ NH₄HCO₃ for 48 h. Perhaps under normal conditions, the majority of these transporters are in a dormant state, requiring stimuli such as high ammonia (and perhaps cortisol, as argued subsequently) to instigate the changes necessary for full functionality.

Na⁺ efflux rate (J_{out}^{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 probably due to the increased diffusive leakage of Na⁺ during HEA, i.e. enhanced Na⁺ permeability (transcellular and/or paracellular) of the gills (Gonzalez and McDonald, 1992). K⁺ loss rate (J_{net}^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_{out}^{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⁺ loss (J_{out}^{Na}) was stimulated and exceeded influx rate (J_{in}^{Na}) in trout and in carp, resulting in net Na⁺ loss in these two species. However, goldfish displayed the opposite trend and were able to maintain a positive net Na⁺ balance, illustrating a divergent pattern between the two cyprinids during the early exposure period (<12 h). In the present experiment, an interesting pattern was noticed among the cyprinids; J_{in}^{Na} was stimulated precisely at the same time that there was an increase in J_{out}^{Na} (Fig. 1). Also, from 84 h onwards, trout were able to maintain net Na⁺ flux closer to zero. These consequences may indicate some sort of recovery response in these experimental animals towards normal Na⁺ balance as part of the ammonia excretion mechanism, and/or that an increase in J_{out}^{Na} among cyprinids might be coupled directly to an increase in J_{in}^{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⁺ 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_{in}^{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 NH₄⁺) across the branchial basolateral membranes might occur *via* NKA enzyme functioning as a Na⁺/NH₄⁺-ATPase (Evans et al., 2005; Wilkie, 2002) as

similarities in the hydration radius of K^+ and NH_4^+ might allow substitution at transport sites (Alam and Frankel, 2006; Randall et al., 1999). The potential ability of NH_4^+ to substitute for K^+ and/or augment the activity of branchial NKA proved negligible in all the three species (Fig. 11A,B) and therefore demonstrated that NH_4^+ is not a preferred substrate for this enzyme. Our result is in tune with earlier reports on trout gills (Salama et al., 1999; Wood and Nawata, 2011). In none of the species was the NH_4^+ activation equal to or greater than the K^+ activation in contrast to toadfish (Mallery, 1983), tilapia (Balm et al., 1988), mudskippers (Randall et al., 1999) and pufferfish (Nawata et al., 2010b), where active NH_4^+ movement across the gill basolateral membranes on the 'K⁺ site' of the NKA molecule may be very important during HEA exposure.

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, 1997). Plasma cortisol levels increased during HEA exposure 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 Na^+/NH_4^+ exchange metabolon (Tsui et al., 2009). However, in the present and previous studies (Nawata and Wood, 2008; Wood and Nawata, 2011) there was no clear-cut relationship between circulating cortisol levels and Rh mRNA expression or ammonia flux. This may be because plasma T_{Amm} and cortisol act more effectively in combination than alone, at least in an *in vitro* gill cell culture system (Tsui et al., 2009). Nevertheless, it is important to mention that in the present study the control value for plasma T_{Amm} , particularly for trout, was relatively high (Fig. 4) compared with 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 and colleagues (Kolarevic et al., 2012) reported a very high resting plasma T_{Amm} level (up to 1.5 mmol l^{-1}) in Atlantic salmon (*Salmo salar*), which was sustained chronically (up to many days) without ill effects, as evidenced by an unchanged growth rate. Moreover, a relatively high basal level of plasma T_{Amm} was also evident in the goldfish ($0.8\text{--}1.4 \text{ mmol l}^{-1}$) (Liew et al., 2012; Sinha et al., 2012a; Smith et al., 2012) as well as in the carp ($0.6\text{--}1.1 \text{ mmol l}^{-1}$) (De Boeck et al., 2006; Liew et al., 2012).

There is some evidence that cortisol may also contribute to the regulation of urea production in fish (Hopkins et al., 1995; Mommsen et al., 1999; Vijayan et al., 1996) and experimentally elevated plasma cortisol increased urea-N excretion rates (J_{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_{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 impact 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_{urea} ; indeed, urea-N excretion became almost equal to ammonia-N excretion at 12 h HEA exposure (Figs 2, 3). Similarly, Olson and Fromm (Olson and Fromm, 1971) reported an increased J_{urea} in goldfish subjected to HEA. In a recent study on goldfish, Wilkie and colleagues (Wilkie et al., 2011) also noted a significant rise in J_{urea} over 5 days of HEA ($5 \text{ mmol l}^{-1} \text{ NH}_4\text{Cl}$) exposure. Potential pathways for 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 (for review, see McDonald et al., 2012). We found a marked increase in branchial mRNA expression of UT during HEA, occurring only in goldfish (Fig. 10). This probably facilitated urea diffusion across the basolateral membranes of the gill cells, accelerating J_{urea} in goldfish (Fig. 3). UT expression also increased in the gills of zebrafish during HEA exposure, together with a transient rise in J_{urea} (Braun et al., 2009b). In contrast to our data in trout, Wood and Nawata (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 the same level of HEA (1 mmol l^{-1}) induced differential physiological and molecular responses among the three freshwater teleosts. In goldfish and carp, J_{Amm} was elevated significantly during HEA exposure, while in trout, J_{Amm} could only be restored to basal level. Na^+ uptake (J_{in}^{Na}) increased in carp and goldfish coincident with elevated J_{Amm} , but not in trout. Carp and goldfish were able to maintain Na^+ homeostasis during HEA exposure as they exhibited persistent, simultaneous augmentations in both J_{in}^{Na} and J_{out}^{Na} . At the molecular level, there was evidence for activation of a Na^+/NH_4^+ exchange metabolon in all three species, probably in response to elevations in plasma cortisol and T_{Amm} . Goldfish appear to rely on H^+ -ATPase and trout on H^+ -ATPase as well as NHE-2, probably for boundary layer acidification and ammonia trapping. In all three species, upregulation of Rhcg (Rhcg-a and Rhcg-b in goldfish, Rhcg-a in carp, and Rhcg2 in trout) may play a key role in ammonia transport and Na^+ uptake during HEA exposure. Moreover, Rhbg was upregulated only in goldfish, highlighting their additional ability to deal with ammonia challenge. The activity and expression of the basolateral enzyme NKA was increased in goldfish and carp, which might offer another mechanism for driving higher rates of Na^+ uptake in these species. In future studies it will be of interest to see whether apical NHE transporters are also upregulated in these two cyprinids as a mechanism to increase both Na^+ uptake and ammonia excretion

during HEA exposure. However, none of the three species appeared to use NKA as an NH_4^+ transporter. Surprisingly in all the species, J_{Amm} and $J_{\text{in}}^{\text{Na}}$ 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 was 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 SYMBOLS AND ABBREVIATIONS

HEA	high environmental ammonia
J_{Amm}	net ammonia flux rate
$J_{\text{net}}^{\text{K}}$	net potassium flux rate
$J_{\text{in}}^{\text{Na}}$	sodium influx rate
$J_{\text{net}}^{\text{Na}}$	sodium net flux rate
$J_{\text{out}}^{\text{Na}}$	sodium efflux rate
J_{urea}	net urea flux rate
NHE	Na^+/H^+ exchanger
NKA	Na^+/K^+ -ATPase
Rh	Rhesus
T_{Amm}	total ammonia concentration
UT	urea transporter

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AUTHOR CONTRIBUTIONS

A.K.S. performed the experiments, analyzed the data and wrote the paper. H.J.L. performed the experiments and analysed the data. C.M.N. performed the experiments, interpreted the results and corrected the manuscript. R.B. contributed reagents/materials/analysis tools and corrected the manuscript. C.M.W. designed the experiments, performed the experiments, analysed the data and corrected the manuscript. G.D.B. contributed to the experiments, provided reagents/materials/analysis tools and corrected the manuscript.

COMPETING INTERESTS

No competing interests declared.

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