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RESEARCH ARTICLE

Sensitivity of ventilation and brain metabolism to ammonia exposure in rainbow trout, *Oncorhynchus mykiss*

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SUMMARY

Ammonia has been documented as a respiratory gas that stimulates ventilation, and is sensed by peripheral neuroepithelial cells (NECs) in the gills in ammoniotelic rainbow trout. However, the hyperventilatory response is abolished in trout chronically exposed (1+ months) to high environmental ammonia [HEA; 250 μmol l^{-1} $(NH_4)_2SO_4$]. This study investigates whether the brain is involved in the acute sensitivity of ventilation to ammonia, and whether changes in brain metabolism are related to the loss of hyperventilatory responses in trout chronically exposed to HEA ('HEA trout'). Hyperventilation (*via* increased ventilatory amplitude rather than rate) and increased total ammonia concentration ($[T_{Amm}]$) in brain tissue were induced in parallel by acute HEA exposure in control trout in a concentration-series experiment [500, 750 and 1000 μmol l^{-1} $(NH_4)_2SO_4$], but these inductions were abolished in HEA trout. Ventilation was correlated more closely to $[T_{Amm}]$ in brain rather than to $[T_{Amm}]$ in plasma or cerebrospinal fluid. The close correlation of hyperventilation and increased brain $[T_{Amm}]$ also occurred in control trout acutely exposed to HEA in a time-series analysis [500 μmol l^{-1} $(NH_4)_2SO_4$; 15, 30, 45 and 60 min], as well as in a methionine sulfoxamine (MSOX) pre-injection experiment [to inhibit glutamine synthetase (GSase)]. These correlations consistently suggest that brain $[T_{Amm}]$ is involved in the hyperventilatory responses to ammonia in trout. The MSOX treatments, together with measurements of GSase activity, T_{Amm}, glutamine and glutamate concentrations in brain tissue, were conducted in both the control and HEA trout. These experiments revealed that GSase plays an important role in transferring ammonia to glutamate to make glutamine in trout brain, thereby attenuating the elevation of brain $[T_{Amm}]$ following HEA exposure, and that glutamate concentration is reduced in HEA trout. The mRNAs for the ammonia channel proteins Rhbg, Rhcg1 and Rhcg2 were expressed in trout brain, and the expression of Rhbg and Rhcg2 increased in HEA trout, potentially as a mechanism to facilitate the efflux of ammonia. In summary, the brain appears to be involved in the sensitivity of ventilation to ammonia, and brain ammonia levels are regulated metabolically in trout.

Key words: Rh protein, ammonia, brain, fish, glutamine synthetase, ventilation.

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INTRODUCTION

Ammonia is the third respiratory gas in fish (Randall and Ip, 2006). (Note: we use the term 'ammonia' or the abbreviation T_{Amm} to refer to the sum of NH_3 and NH_4^+, whereas we use these formulae to refer to the individual components of ammonia gas and ammonium ion, respectively.) The control of ventilation in fish by both O_2 (Shelton, 1970; Randall, 1982) and CO_2/pH (Gilmour, 2001) is now well established, but only recently has an additional role for ammonia been clearly documented (Zhang and Wood, 2009). Building on earlier results of McKenzie et al. (McKenzie et al., 1993), we demonstrated that increases in blood ammonia itself stimulate hyperventilation in rainbow trout, separate from any confounding effects of accompanying O_2 or CO_2/pH changes in the arterial blood (Zhang and Wood, 2009). Our subsequent study (Zhang et al., 2011) provided evidence that neuroepithelial cells (NECs) in the gills played an important role in this response, acting as ammonia chemoreceptors. These cells are now thought to be the major sites of O_2 and CO_2 sensitivity in fish [see Milsom (Milsom, 2012), for a recent comprehensive review], so they may function as polymodal receptors and/or there may be several subtypes (Qin et al., 2010; Zhang et al., 2011). Interestingly, after chronic exposure to high environmental ammonia (HEA) in the external water, the ventilatory response to experimental elevations of ammonia in both blood and water disappeared, and this change was accompanied by both structural and functional changes in the NECs. The cell size and density of the NECs were reduced in HEA trout. Meanwhile, although the intercellular Ca^{2+} in the NECs was still elevated by high ammonia exposure, the response was attenuated in HEA trout (Zhang et al., 2011). Clearly the NECs are involved, but they are not necessarily the only site of ventilatory sensitivity to ammonia.

In higher vertebrates, the central chemoreceptors of the brain are a second site of ventilatory sensitivity to respiratory gases, and the more important control site for CO_2 detection. Whereas O_2 enters from the environment, CO_2 is produced internally by endogenous metabolism, so central control for CO_2 makes sense. Like CO_2, ammonia is also produced by endogenous metabolism, and in mammals, it has long been known that ammonia can serve as a ventilatory stimulant (Poppell et al., 1956; Warren, 1958; Campbell

et al., 1973; Wichser and Kazemi, 1974). The exact mechanism is unknown, but the best correlation appears to be with total ammonia concentrations ($[T_{Amm}]$) in the brain tissue, rather than with blood plasma or cerebrospinal fluid (CSF) concentrations (Wichser and Kazemi, 1974). In fish, there has been only minimal research on potential central chemoreceptivity to O_2 and CO_2/pH, and none on ammonia. However, a recent review (Milsom, 2012) noted that with respect to O_2, all efforts to evoke a ventilatory response have failed, and with respect to CO_2/pH, that there is firm evidence only in air-breathing fish, although at least one study has indicated a contribution from brain intracellular pH (pH_i) in a water-breathing elasmobranch (Wood et al., 1990).

Nevertheless, several lines of evidence suggest that central sensitivity to ammonia as a ventilatory stimulant is certainly a possibility in fish. Ammonia readily crosses the blood–brain barrier in teleost fish, because when blood ammonia levels increase during HEA, intracellular ammonia concentrations in the brain tissue also increase. While this has been widely documented in tropical fish (reviewed by Ip et al., 2001; Chew et al., 2005), it has also been seen in rainbow trout (Wright et al., 2007; Sanderson et al., 2010). Furthermore, mRNA expressions of the Rhbg and Rhcg1 glycoproteins have been detected in trout brain (Nawata et al., 2007); these appear to function as NH_3 channels (Nawata et al., 2010) and their expression decreases after 48 h of HEA exposure (Nawata et al., 2007). This could serve as an adaptive measure, decreasing brain permeability to ammonia during chronic exposure, thereby lessening the stimulus for hyperventilation. The enzyme glutamine synthetase (GSase), which detoxifies ammonia by adding it to glutamate, is high in brain tissue, and in trout increases in expression and/or activity of various GSase isoforms occur during HEA (Wicks and Randall, 2002; Wright et al., 2007); this could also decrease the central stimulus for hyperventilation during chronic HEA exposure.

The present study focuses on the possible central sensitivity to ammonia in the control of ventilation in the rainbow trout. Our overall hypothesis was that ventilatory changes would be closely associated with changes in brain tissue T_{Amm} concentrations. As a first step, the general approach used by Wichser and Kazemi (Wichser and Kazemi, 1974) in mammals was adopted, examining the relative correlations of ventilation with plasma, CSF and brain tissue $[T_{Amm}]$ during ammonia loading. Trout were challenged with HEA both acutely and after chronic (1+ month) exposure, the latter so as to examine how relationships changed after the hyperventilatory response accommodated. The mRNA expressions of Rh proteins in the brain were also examined in trout chronically exposed to HEA, to see whether the earlier reported downregulation (Nawata et al., 2007) persisted once the hyperventilatory response had abated. Blood acid-base and O_2 status were also monitored, together with CSF pH and brain pH_i, to assess whether possible changes in these parameters confounded interpretation. In a separate experiment, the time course of changes in brain T_{Amm} and ventilation were followed closely during acute HEA challenge. Methionine sulfoxamine (MSOX), a well-established inhibitor of GSase in previous fish studies (Ip et al., 2005; Veauvy et al., 2005; Wee et al., 2007; Sanderson et al., 2010) was also employed in both acute and chronic exposures, together with measurements of GSase activity, and concentrations of T_{Amm}, glutamine, and glutamate in brain tissue. These tests examined how ammonia detoxification in the brain interacts with the ventilatory responses to ammonia. While correlation does not prove causation, our overall results support the idea that the brain is involved in the sensitivity of ventilation to ammonia in trout.

MATERIALS AND METHODS

Fish husbandry and chronic HEA exposure

All procedures were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care. Rainbow trout [*Oncorhynchus mykiss* (Walbaum), 200–300 g] were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and then acclimated to laboratory conditions for more than 1 week before experimentation. The trout were held in flowing dechlorinated Hamilton (ON, Canada) tapwater (in $mmol\,l^{-1}$: 0.6 Na^+, 0.7 Cl^-, 0.05 K^+, 1.0 Ca^{2+}, 0.1 Mg^{2+}; titration alkalinity 1.9 $mequiv\,l^{-1}$; hardness 140 $mg\,l^{-1}$ as $CaCO_3$ equivalents; pH 7.8–8.0, 12±1°C). The fish were fed a commercial trout food (crude protein 41%; carbohydrates 30%; crude fat 11%; Martin Mills, Elmira, ON, Canada) at a ration of 2% body mass every 3 days. All the fish were fasted at least 5 days before experimentation, to minimize the influence of feeding on ammonia metabolism.

In chronic HEA treatments, trout were held in groups of 20 in tanks containing 800 l of dechlorinated Hamilton tapwater. $(NH_4)_2SO_4$ stock solution (adjusted to pH 7.80) was added to the tanks to achieve a nominal HEA concentration of 250 $\mu mol\,l^{-1}$ (i.e. 500 $\mu mol\,l^{-1}$ ammonia). Fish were fed (1% body mass) every 3 days. At 24 h after feeding, two-thirds of the water was renewed and an appropriate amount of $(NH_4)_2SO_4$ was added to maintain the correct HEA concentration. In the control treatment, the fish were held under the same conditions as for the ammonia-exposed fish, but without adding $(NH_4)_2SO_4$ to water. The acclimation lasted 1 to 2 months, and ammonia concentrations were checked regularly by assay (Verdouw et al., 1978) to ensure that they remained within ±15% of nominal values. $(NH_4)_2SO_4$ was used in acclimations and experimental tests because the sulfate ion was found to have no effect on ventilation in our previous study (Zhang and Wood, 2009). Nitrite concentrations were checked regularly in the HEA tanks using test strips (Aquarium Pharmaceuticals, Chalfont, PA, USA), and no concentrations were found above the detection limit of 0.5 $mg\,l^{-1}$.

Acute exposure to a series of $(NH_4)_2SO_4$ concentrations

Trout were anaesthetized and irrigated with 80 $mg\,l^{-1}$ tricaine methanesulphonate (MS-222; Syndel Laboratories, Vancouver, Canada; adjusted to pH 7.8 with NaOH) in tapwater on an operating table. Buccal catheters (flared tubing Clay-Adams PE90, Sparks, MD, USA) were implanted by passing through a hole drilled in the roof of the mouth in order to monitor ventilation, as described by Holeton and Randall (Holeton and Randall, 1967). Dorsal aortic catheters were implanted by the method of Soivio et al. (Soivio et al., 1975), and filled with Cortland saline [in $mmol\,l^{-1}$: 124 NaCl, 5.1 KCl, 1.6 $CaCl_2$, 0.9 $MgSO_4$, 11.9 $NaHCO_3$, 3.0 NaH_2PO_4, 5.6 glucose (Wolf, 1963)] in order to sample blood with minimal disturbance. Trout were then placed individually in darkened Plexiglas boxes (4.5 l volume) served with constant aeration and flowing water (0.4 $l\,min^{-1}$) and allowed to recover for 24 h before experimentation. The fish that had been chronically exposed to HEA were always kept in water containing 250 $\mu mol\,l^{-1}$ $(NH_4)_2SO_4$ (pH 7.80) during surgery and recovery. Each treatment group consisted of five fish.

During experimentation, control trout [i.e. not previously exposed to $(NH_4)_2SO_4$] were continuously exposed to clean water for 20 min, and then to clean water for another 60 min ('background' treatment), or to elevated concentrations of $(NH_4)_2SO_4$ (500, 750 or 1000 $\mu mol\,l^{-1}$) for 60 min in a flow exposure system (flow rate 0.5 $l\,min^{-1}$). The latter was achieved by adding appropriate amounts of $(NH_4)_2SO_4$ stock to the closed 4.5 l boxes at 20 min from the

start. Chronic HEA trout were continuously exposed to 250 μmol l^{-1} $(NH_4)_2SO_4$ for 20 min, and then to 250 μmol l^{-1} $(NH_4)_2SO_4$ for another 60 min (which constituted the 'background' treatment for the HEA trout), or to elevated concentrations of $(NH_4)_2SO_4$ (500 or 1000 μmol l^{-1}) for 60 min. Ventilation was measured immediately before and at 10 min intervals during the exposures (i.e. at 0, 10, 20, 30, 40, 50, 60, 70 and 80 min), except in the background treatments, where ventilation was measured at 0, 20, 50 and 80 min. Dorsal arterial blood samples (600 μl each, with saline replacement) were drawn into a 1 ml gas-tight Hamilton syringe (Reno, NV, USA) *via* the catheters immediately before the end of each exposure to a different $(NH_4)_2SO_4$ concentration. At the end of experiments, trout were killed by an overdose of pH-adjusted MS-222 (250 mg l^{-1}), and CSF and the brain were immediately sampled and frozen in liquid nitrogen.

CSF sampling was accomplished by inserting a 50 μl gas-tight Hamilton syringe through the roof of the cranium at a predetermined spot and to a predetermined depth, so as to puncture into the ventricular space of the optic lobe, and then applying very gentle suction. Then the cranial cavity was opened by a scalpel blade, and the whole brain tissue was removed, wrapped in foil, and flash frozen in liquid nitrogen. The whole tissue sampling was finished within 2 min.

Acute exposure to 500 μmol l^{-1} $(NH_4)_2SO_4$ in a time series

Control trout [i.e. not previously exposed to $(NH_4)_2SO_4$] were anaesthetized and buccal-catheterized in the same way as described above. During experimentation, five separate groups of trout (N=5 each) were continuously exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$ for 0, 15, 30, 45 or 60 min, respectively, after initial exposure to clean water for 20 min. Ventilation was measured immediately before the start and the end of these $(NH_4)_2SO_4$ exposures. Brains were immediately sampled and frozen in liquid nitrogen immediately after the final ventilation measurement – i.e. at each sample time.

MSOX pre-injection experiments

MSOX was dissolved in Cortland saline to yield a 60 mg ml^{-1} stock. Control and HEA-exposed trout were anaesthetized, weighed, buccal-catheterized and injected intraperitoneally with Cortland saline (100 μl kg^{-1}) or MSOX [100 μl kg^{-1} or 6 mg kg^{-1}, dosage derived from Sanderson et al. (Sanderson et al., 2010)]. They were then kept individually in darkened plexiglass boxes with aeration and flowing water for 48 h to allow the effects of MSOX to develop. Trout (N=5 in each treatment) were continuously exposed to the background water [clean water for control trout and 250 μmol l^{-1} $(NH_4)_2SO_4$ for HEA trout, respectively] for 60 min. An additional five trout in each treatment (control and HEA trout) were exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$ for 60 min after exposure to the background water. Ventilation was measured at the beginning and end of the high ammonia exposure. Brains were then sampled as above for later analysis of the tissue concentrations of ammonia, glutamine and glutamate, as well as GSase and glutaminase activities. Livers were also sampled for reference.

Measurement of ventilation

Ventilation was measured using the system described by Zhang and Wood (Zhang and Wood, 2009), recording ventilation rate (f_v, breaths min^{-1}) and buccal pressure amplitude (ΔP_{buccal}, cmH_2O), as an index of stroke volume. ΔP_{buccal} was calculated as the average value of 10 measurements of amplitude (randomly selected from periods of normal breathing, not using episodes of coughing or disturbance) and f_v was calculated as the frequency of breaths in 1 min. Ventilation index was calculated as the product of relative ΔP_{buccal} and relative f_v, where the reference values (equal to 1) were taken during the control period when the fish were exposed to only background conditions, prior to any exposures to elevated $(NH_4)_2SO_4$.

Analysis of blood and tissue samples

Methods for analysis of blood and plasma samples followed those detailed by Zhang and Wood (Zhang and Wood, 2009). In brief, arterial whole-blood pH_a (i.e. plasma pH) and P_aO_2 were measured in 12°C thermostatted chambers using a combination glass pH microelectrode (Biotrode, Hamilton, Reno, NV, USA) coupled to a PHM82 standard pH meter (Radiometer, Copenhagen, Denmark), and a Radiometer polarographic oxygen electrode coupled to a polarographic amplifier (Model 1900, A-M Systems, Everett, WA, USA), respectively. Whole-blood O_2 content (C_aO_2) was measured using a blood oxygen content analyzer (Oxycon, Cameron Instrument Company, Port Aransas, TX, USA). Whole-blood hemoglobin was measured colorimetrically using Drabkin's reagent (Sigma-Aldrich, St Louis, MO, USA). Plasma was separated by centrifugation at 9000 ***g*** for 30 s. Plasma and CSF T_{Amm} concentrations were measured using a commercial kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method. Plasma total CO_2 was measured using a Corning model 965 CO_2 analyzer (Lowell, MA, USA). Plasma CO_2 tension (P_aCO_2) and bicarbonate concentration ($[HCO_3^-]_a$) were calculated using the Henderson–Hasselbalch equation with plasma pK′ values and CO_2 solubility coefficients for trout plasma at 12°C from Boutilier et al. (Boutilier et al., 1984).

pH_i in brain was measured using a method described by Pörtner et al. (Pörtner et al., 1990). The frozen brain tissue was ground in liquid nitrogen using a mortar and pestle. Approximately 0.1 g of tissue powder was immediately transferred to a 1.5 ml bullet tube and resuspended in 1 ml ice-cold medium containing 150 mmol l^{-1} potassium fluoride and 6 mmol l^{-1} Na_2-NTA (nitrilotriacetic acid) on ice. The resuspension was then centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant was removed and kept on ice, until pH measurement using the same microelectrode and 12°C thermostatted chamber as for blood and CSF pH measurements.

T_{Amm}, glutamine and glutamate concentrations in brain tissue were measured using the method of Lund (Lund, 1986). Tissue powder was obtained as described above and a weighed amount (~0.1 g) was transferred to a 1.5 ml bullet tube. A 0.5 ml aliquot of ice-cold $HClO_3$ (8%, with 1 mmol l^{-1} EDTA) was added, mixed with the powder using a needle, and then the suspension was vortexed. Thereafter, the mixture was kept on ice for 5 min and centrifuged at 18,000 ***g*** at 4°C for 5 min. Then 250 μl of the supernatant was withdrawn to another tube and neutralized by adding 125 μl KOH solution (2 mol l^{-1}, supplemented with 0.4 mol l^{-1} imidazole and 0.4 mol l^{-1} KCl). After centrifuging again, the supernatant was removed and kept for T_{Amm} measurement by the same Raichem kit as used for plasma and CSF T_{Amm}. Glutamine and glutamate were measured using a glutamine/glutamate kit (Sigma-Aldrich) for spectrophotometric measurements *via* enzymatic deamination of L-glutamine and dehydrogenation of L-glutamate, with conversion of NAD^+ to NADH.

Brain and liver GSase activities were assayed based on the production of γ-glutamyl hydroxamate as described by Shankar and Anderson (Shankar and Anderson, 1985). Glutaminase activities were assayed based on the measurement of the production of ammonia (using the Raichem kit) *via* deamination

of glutamine. The protocol followed the Sigma quality control test procedure SSGLUT02 with modifications: specifically, the tissues were ground in liquid nitrogen and homogenized in PBS buffer, and the enzymatic reactions were performed over 15 min at 12°C.

Brain Rh gene expression

Fresh brain was dissected from control and chronically HEA-exposed trout killed by overdose with pH-adjusted MS-222 (250 mg l^{-1}) and placed into five volumes of pre-chilled TRIzol (Invitrogen, Burlington, ON, Canada) immediately after rinsing with PBS. Total RNA was extracted by a TRIzol protocol, quantified by spectrophotometry, and electrophoresed on 1% agarose gels stained with ethidium bromide to verify integrity. One microgram of total RNA of each sample was used to synthesize the first strand cDNA by employing an oligo (dT17) primer and Superscript II reverse transcriptase (Invitrogen). Samples were stored at –20°C overnight for quantitative real-time PCR (qPCR)

The primers of Rhbg (forward: cgacaacgacttttactaccgc, reverse: gacgaagccctgcatgagag), Rhcg1 (forward: catcctcagcctcatacatgc, reverse: tgaatgacagacggagccaatc) and Rhcg2 (forward: cctcttcggagtcttcatc, reverse: ctatgtcgctggtgatgttg) for qPCR were described and validated in rainbow trout by Nawata et al. (Nawata et al., 2007). qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX, USA). The 20 µl reactions containing 1 µl DNaseI-treated (Invitrogen) cDNA, 4 pmol of each primer, 10 µl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.8 µl of ROX (1:10 dilution) 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 analysis confirmed production of a unique product, and gel electrophoresis verified the presence of a single band. A non-reverse-transcribed sample controlled for possible genomic DNA contamination. Elongation factor-1α (EF-1α, GenBank AF498320) expressions were constant and were used as endogenous standards to calculate relative mRNA expressions by the standard curve method.

Statistical analysis

Data are expressed as means ± 1 s.e.m. A one-way repeated-measures ANOVA followed by Dunnett's test was applied to compare the f_v and ΔP_{buccal} during $(NH_4)_2SO_4$ exposures back to initial control values. A two-way or three-way ANOVA followed by Tukey's test was applied to compare ΔP_{buccal}, f_v, ventilation index, and variables in brain, CSF and blood in different experimental treatments in both control fish and those chronically exposed to HEA. A one-way ANOVA was applied to compare Rh mRNA expression means between control and HEA trout. A significance level of $P<0.05$ was employed throughout. All statistical tests were run using SigmaStat (version 3.1; Systat Software, San Jose, CA, USA).

RESULTS

Acute exposure to a series of $(NH_4)_2SO_4$ concentrations

In control trout prior to exposure to different waterborne ammonia concentrations, the ΔP_{buccal} and f_v were similar in all treatments at 1.5±0.2 mmHg and 55±4 breaths min^{-1}, respectively (Fig. 1). ΔP_{buccal} (Fig. 1A) and f_v (Fig. 1D) remained constant when control trout were kept in background clean water. After acute exposure to 500 or 1000 µmol l^{-1} $(NH_4)_2SO_4$, ΔP_{buccal} values were increased significantly after 30–40 min of the exposure, up to 147% (Fig. 1B) or 184% (Fig. 1C), respectively, of the initial amplitude at the end, whereas f_v did not change (Fig. 1E,F). Acute exposure to 750 µmol l^{-1} $(NH_4)_2SO_4$ caused changes of ΔP_{buccal} (and no change in f_v) similar to the other elevated $(NH_4)_2SO_4$ exposures, so the results are not shown. These results confirmed that acute exposure to elevated ammonia [>500 µmol l^{-1} $(NH_4)_2SO_4$] induced hyperventilation in trout.

In HEA trout, ΔP_{buccal} and f_v were 1.5±0.2 mmHg and 54±3 breaths min^{-1}, respectively, during the initial 20 min exposure to the background 250 µmol l^{-1} $(NH_4)_2SO_4$ for all treatments (Fig. 1), values that were comparable to those of the control group in clean water. ΔP_{buccal} (Fig. 1A) and f_v (Fig. 1D) remained constant when the HEA trout were kept in the background water [250 µmol l^{-1} $(NH_4)_2SO_4$] for the ensuing 60 min. After acute exposure to 500 or

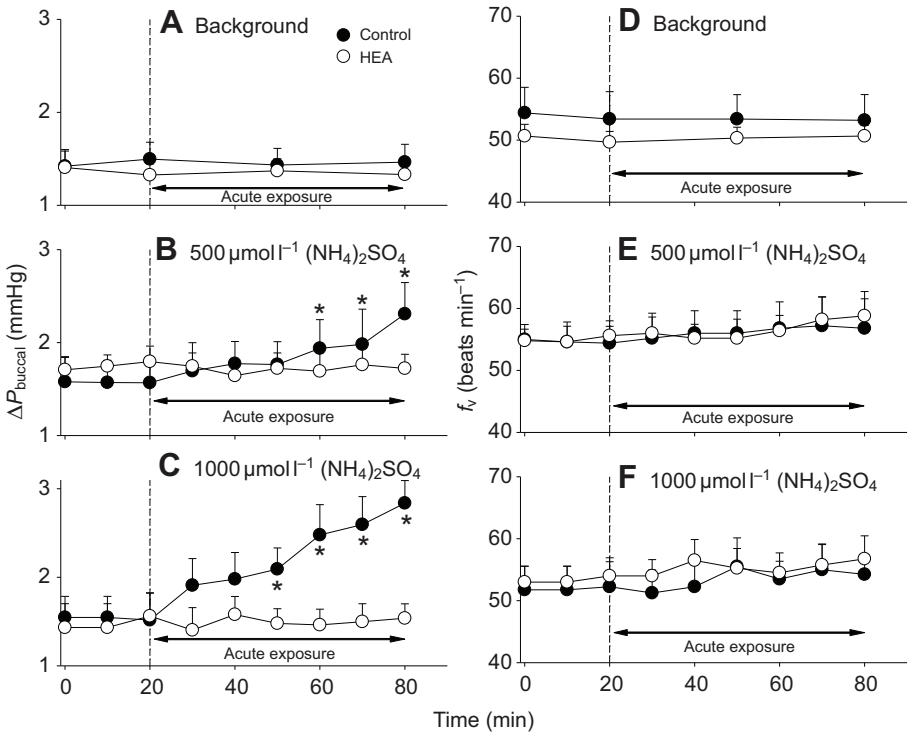

Fig. 1. Acute responses of (A–C) ventilatory amplitudes (ΔP_{buccal}) and (D–F) ventilatory frequencies (f_v) to various levels of elevated waterborne $(NH_4)_2SO_4$ in control trout and trout that had been chronically exposed to high environmental ammonia [HEA; 250 µmol l^{-1} $(NH_4)_2SO_4$] for 1+ month. Trout were exposed in the background water for 20 min and then exposed to the elevated HEA water for 60 min; the dashed vertical lines separate the two exposure periods. Asterisks indicate significant increases relative to the data at background concentrations prior to elevated $(NH_4)_2SO_4$ exposure ($P<0.05$). Data are means + s.e.m. ($N=5$).

1000 μmol l^{-1} $(NH_4)_2SO_4$, ΔP_{buccal} again remained constant at the same value as in trout exposed to background water and significantly lower than that in control trout exposed to the same $(NH_4)_2SO_4$ conditions (Fig. 1B,C); the f_v values were not significantly different from those in control trout in clean water or in 250 μmol l^{-1} $(NH_4)_2SO_4$ (Fig. 1E,F). The different ventilatory responses to acute elevated waterborne ammonia in the control (significantly enhanced ΔP_{buccal}) and HEA trout (constant ΔP_{buccal}) demonstrated the loss of the ammonia-caused hyperventilation as a result of chronic exposure.

In control trout, the $[T_{Amm}]$ values in plasma, CSF and brain, measured after 60 min acute exposure to elevated $(NH_4)_2SO_4$, were also increased significantly (Fig. 2). In the 1000 μmol l^{-1} $(NH_4)_2SO_4$ exposure, $[T_{Amm}]$ in plasma (Fig. 2A), CSF (Fig. 2B) and brain (Fig. 2C) increased fivefold, sixfold and twofold, respectively. When looking at the individual trout, the ventilatory indices (relative $\Delta P_{buccal} \times f_v$) were positively correlated to $[T_{Amm}]$ in plasma (r^2=0.62; Fig. 3A), CSF (r^2=0.26; Fig. 3B) and brain (r^2=0.78; Fig. 3C).

In HEA trout, $[T_{Amm}]$ in plasma and CSF did not increase in response to acute high ammonia exposure at 500 μmol l^{-1}$(NH_4)_2SO_4$, but the increases became significant in the 1000 μmol l^{-1} $(NH_4)_2SO_4$ exposures (Fig. 2A,B). In contrast, brain $[T_{Amm}]$ did not change significantly in response to even this highest ammonia exposure, thereby paralleling the lack of change in ventilation (Fig. 2C). The relationships between ventilation index and tissue $[T_{Amm}]$ (in plasma, CSF or brain) seen in control trout were obliterated in HEA trout. However, in the combination of both control and HEA trout, ventilation indices remained positively correlated to brain $[T_{Amm}]$ (Fig. 3F, Fig. 5A), but not to $[T_{Amm}]$ in plasma or CSF (Fig. 3D,E).

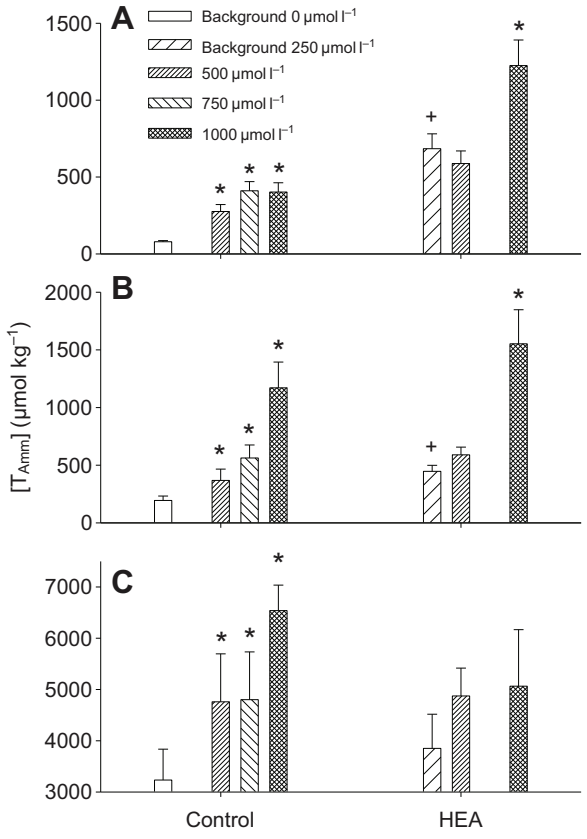

Fig. 2. Total ammonia concentration ($[T_{Amm}]$) in (A) blood plasma, (B) cerebrospinal fluid (CSF) and (C) brain tissue in response to a series of elevated waterborne $(NH_4)_2SO_4$ exposures in control trout and trout that had been chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ month. Asterisks indicate significant differences in trout between background and elevated $(NH_4)_2SO_4$ exposures; crosses indicate significant differences in trout between control and HEA trout exposed to background water (P<0.05). Data are means + s.e.m. (N=5).

In control trout, brain pH_i was approximately 0.6 units below arterial blood plasma pH_a, whereas CSF pH was approximately 0.2 units below pH_a (Table 1). Brain pH_i increased significantly in response to acute high $(NH_4)_2SO_4$ exposure, although pH in plasma and CSF remained constant (Table 1). In HEA trout, pH in plasma, CSF or brain did not change when comparing background and elevated $(NH_4)_2SO_4$ exposure. When exposed to the background water, HEA trout had higher plasma pH_a and brain pH_i but comparable CSF pH in comparison to control trout. The brain pH_i values in HEA trout were comparable to those in control trout exposed to elevated ammonia.

Most variables of arterial blood O_2 and CO_2 in control and HEA trout did not change in response to the acute elevations in $(NH_4)_2SO_4$ (Table 2). $[HCO_3^-]$ and P_aCO_2, which were calculated from plasma [total CO_2] and pH, were comparable in all the treatments. In control trout, P_aO_2, Hb, C_aO_2 and C_aO_2/Hb also remained constant, except for the reductions of C_aO_2 and C_aO_2/Hb in control trout acutely exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$. In HEA trout, P_aO_2, Hb, C_aO_2 and C_aO_2/Hb were constant at the elevated $(NH_4)_2SO_4$ exposures, whereas the values of Hb and C_aO_2 were significantly reduced relative to those in control trout exposed to the background clean water. Nevertheless, their C_aO_2/Hb ratios were comparable, indicating a similar Hb O_2 saturation.

Acute exposure to 500 μmol l^{-1} $(NH_4)_2SO_4$ in a time series

During the acute 60 min exposure to HEA [500 μmol l^{-1} $(NH_4)_2SO_4$], ΔP_{buccal} increased gradually while f_v remained constant, so the ventilation index also increased continuously over this period (Fig. 4A, Fig. 5B), in a pattern similar to that seen in the earlier experiments (Fig. 1, Fig. 5A). The ventilation was significantly elevated starting from 30 min exposure, to 123–199% of the initial value from 30 to 60 min. Fish were euthanized at each of these times; the brain $[T_{Amm}]$ increased slightly at 15 min, reaching 124% at 30 min, followed by significant increases to 140% (at 45 min) and 191% (at 60 min) (Fig. 4B). In general, the increases of ventilation and brain $[T_{Amm}]$ occurred simultaneously and well correlated (r^2=0.96, Fig. 5B). These results again suggest that brain $[T_{Amm}]$ directly induces hyperventilation.

Acute exposure to 500 μmol l^{-1} $(NH_4)_2SO_4$ after MSOX pre-injection

Saline-injected trout presented ventilatory patterns in response to acute exposure to HEA [500 μmol l^{-1} $(NH_4)_2SO_4$] (Fig. 6) similar to those of the trout without any injection described earlier (cf. Fig. 1) – i.e. marked hyperventilation in control trout, no changes in HEA trout. However, the ventilatory responses to elevated ammonia changed in both control and HEA trout after MSOX injection. In control trout, ΔP_{buccal} was significantly higher in MSOX-injected animals than in saline-injected animals when they were exposed to clean water; ΔP_{buccal} was even more elevated in MSOX-injected trout when they were acutely exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$, while f_v was constant in this treatment (Fig. 6).

In HEA trout, ΔP_{buccal} and f_v were again the same as in control trout under background conditions, and saline injections had no effect. ΔP_{buccal} was also significantly higher in MSOX-injected trout than in saline-injected trout when they were exposed to background 250 μmol l^{-1} $(NH_4)_2SO_4$, and ΔP_{buccal} was significantly elevated in

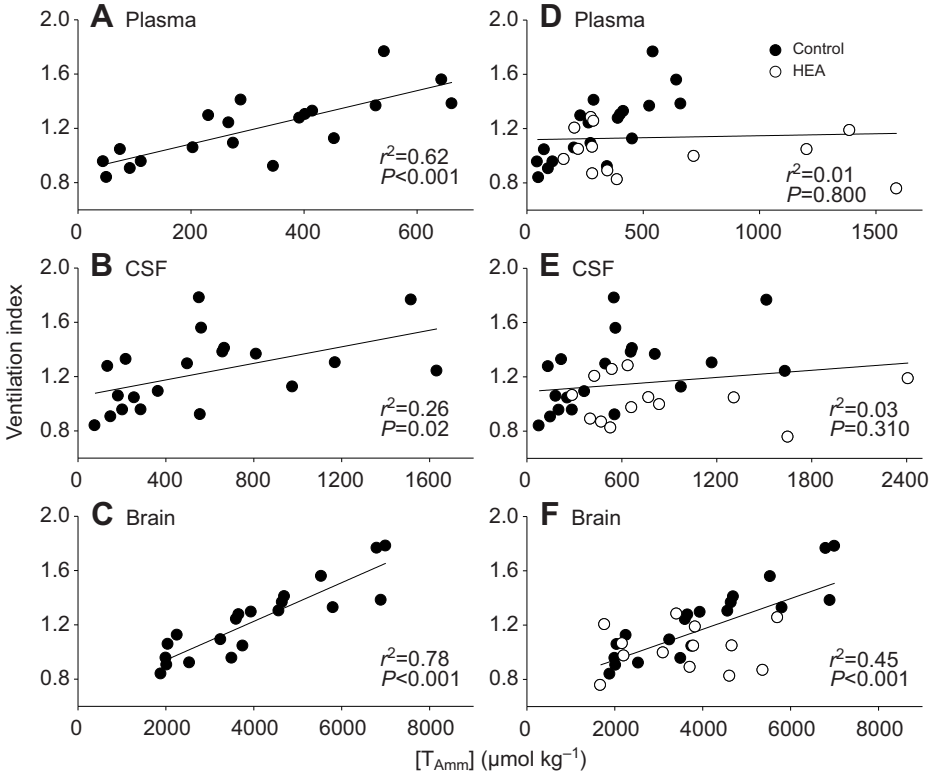

Fig. 3. Correlations between ventilation index and [T_{Amm}] in plasma, CSF and brain tissue in response to elevated waterborne ammonia $(NH_4)_2SO_4$ exposure in control trout (A–C) or the combination of control trout and trout that had been chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ month (D–F). Dots indicate individual trout.

response to 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure in MSOX-injected animals. In general, the increases in ΔP_{buccal} associated with MSOX treatments were twofold to threefold higher in HEA trout than in control trout (Fig. 6),

Plasma [T_{Amm}] values (Fig. 7A) were comparable in trout with or without saline injections (the latter results were shown earlier in Fig. 2), while brain [T_{Amm}] varied. However, brain [T_{Amm}] changed in both control and HEA trout after MSOX injection (Fig. 7B), while plasma [T_{Amm}] did not (Fig. 7A). In control trout, brain [T_{Amm}] was significantly higher in MSOX-injected animals than in saline-injected animals when they were exposed to background clean water; brain [T_{Amm}] was also elevated in both saline- and MSOX-injected animals when they were exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$ (Fig. 7B). In HEA trout, brain [T_{Amm}] was also significantly higher in MSOX-injected fish than in saline-injected fish when they were exposed to background 250 μmol l^{-1} $(NH_4)_2SO_4$, and brain [T_{Amm}] was significantly elevated in response to 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure in MSOX-injected fish, an effect that was not observed in saline-injected fish (Fig. 7B). As MSOX functioned in inhibiting GSase (Fig. 8), these increases of brain [T_{Amm}] in MSOX-injected trout indicated that GSase plays an important role in depressing brain [T_{Amm}] in control and HEA trout exposed to either background or elevated ambient ammonia. The accompanying increases in ventilation in the MSOX-injected fish (Fig. 6A), in the absence of increases in plasma [T_{Amm}] (Fig. 7A), again point to the importance of elevated brain [T_{Amm}] (Fig. 7B) in driving hyperventilation.

In control trout with saline injection acutely exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$, brain [glutamine] increased from 8.3±1.1 to 14.2±1.1 μmol g^{-1} (Fig. 8A), while [glutamate] decreased from 6.2±0.6 to 4.1±0.7 μmol g^{-1} (Fig. 8B). However, in control trout with MSOX injection, brain [glutamine] and [glutamate] did not change in response to elevated ambient ammonia, and remained at similar levels to those in the saline injected trout in background water (Fig. 8A,B). In HEA trout with saline injection, brain [glutamine] and [glutamate] did not change obviously in response to elevated ambient ammonia, from 7.0±0.9 to 8.2±0.8 μmol g^{-1} and 3.7±0.6 to 3.5±0.5 μmol g^{-1}, respectively. Furthermore, glutamine levels were comparable but glutamate concentration was significantly lower than those in the saline-injected control trout in background water. In HEA trout treated with MSOX injection and exposed to 500 μmol l^{-1} $(NH_4)_2SO_4$, brain [glutamine] still increased significantly (from 6.1±0.9 to 8.4±0.4 μmol g^{-1}) but only to the same levels as in saline-

Table 1. Measured pH values in plasma (pH_a), cerebrospinal fluid (CSF) and brain tissue (pH_i) at background and elevated waterborne ammonia $(NH_4)_2SO_4$ exposures in control trout and trout chronically esposed to high environmental ammonia (HEA; 250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months

	Water $(NH_4)_2SO_4$ (μmol l^{-1})	Control	HEA
Plasma	0	7.83±0.03	
	250		7.89±0.02#
	500	7.82±0.03	7.84±0.01*
	750	7.82±0.02	
	1000	7.81±0.03	7.89±0.03
CSF	0	7.65±0.05	
	250		7.60±0.02
	500	7.61±0.03	7.58±0.03
	750	7.62±0.04	
	1000	7.59±0.02	7.62±0.01
Brain	0	7.13±0.01	
	250		7.18±0.01#
	500	7.19±0.02*	7.17±0.01
	750	7.19±0.03*	
	1000	7.18±0.03*	7.16±0.02

Asterisks indicate significant differences (P<0.05) between background and elevated $(NH_4)_2SO_4$ exposures; pound marks indicate significant differences between control and HEA trout exposed to background water (P<0.05). Data are means ± s.e.m. (N=5).

Table 2. Arterial blood HCO_3^- ($[HCO_3^-]_a$), CO_2 tension (P_aCO_2), blood O_2 content (C_aO_2), O_2 tension (P_aO_2), hemoglobin (Hb) and C_aO_2/Hb ratio in control trout and trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months

	Water $(NH_4)_2SO_4$ (μmol l^{-1})	Control	HEA
$[HCO_3^-]_a$ (mmol l^{-1})	0	7.49±0.63	
	250		7.20±0.68
	500	7.85±0.92	7.70±0.44
	750	7.05±0.67	
	1000	7.60±0.78	7.50±0.98
P_aCO_2 (mmHg)	0	2.43±0.14	
	250		2.20±0.17
	500	2.63±0.18	2.52±0.19
	750	2.10±0.22	
	1000	2.62±0.39	2.28±0.37
C_aO_2 (ml O_2 100 ml^{-1})	0	10.8±1.5	
	250		5.6±0.5#
	500	6.9±1.1*	7.3±1.0
	750	9.8±2.1	
	1000	9.6±2.3	5.5±1.1
P_aO_2 (mmHg)	0	79.7±3.1	
	250		85.0±7.7
	500	84.4±7.1	75.5±8.0
	750	75.5±4.6	
	1000	79.5±3.6	64.0±11.3
Hb (g 100 ml^{-1})	0	7.31±0.79	
	250		5.32±0.71#
	500	6.99±0.58	5.55±0.44
	750	8.09±1.44	
	1000	7.82±0.53	4.94±1.11
C_aO_2/Hb (ml O_2 g^{-1})	0	1.32±0.20	
	250		1.27±0.23
	500	0.93±0.11*	1.05±0.10
	750	1.21±0.26	
	1000	1.23±0.19	1.11±0.09

Values were recorded from fish after 60 min exposure to the background [either 0 or 250 μmol l^{-1} $(NH_4)_2SO_4$] and elevated $(NH_4)_2SO_4$ concentrations. Asterisks indicate significant differences (P<0.05) between background and elevated $(NH_4)_2SO_4$ exposures; pound marks indicate significant differences between control and HEA trout exposed to background water (P<0.05). Data are means ± s.e.m. (N=5).

injected animals, and glutamate levels were comparable (~3.9 μmol g^{-1}). In general, the total concentration of brain glutamine plus glutamate was significantly lower in HEA trout (10.4±1.5 μmol g^{-1}) than in control trout (14.5±1.5 μmol g^{-1}), and these sums were not altered by acute 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure.

The activities of both brain GSase (Fig. 8C) and glutaminase (Fig. 8D) were comparable in control and HEA trout treated with saline injection, indicating no long-term effects of HEA exposure. GSase activities were significantly depressed by MSOX injection to a comparable extent (~75%) in both control and HEA trout, and glutaminase activities were enhanced by MSOX injection, but the latter effect was significant only in HEA trout. In comparison, liver GSase and glutaminase activity were also comparable in control and HEA trout (5.7±0.4 and 0.15±0.02 μmol g^{-1} min^{-1}), and the GSase activity was significantly depressed by MSOX injection (~15%).

Rh genes expression in trout brain

Rhbg and Rhcg2 were expressed at significantly higher levels in HEA trout than in control trout, by 1.7- and 2.1-fold, respectively (Fig. 9). Rhcg1 expression was also elevated by 1.7-fold, but the change was not significant (Fig. 9).

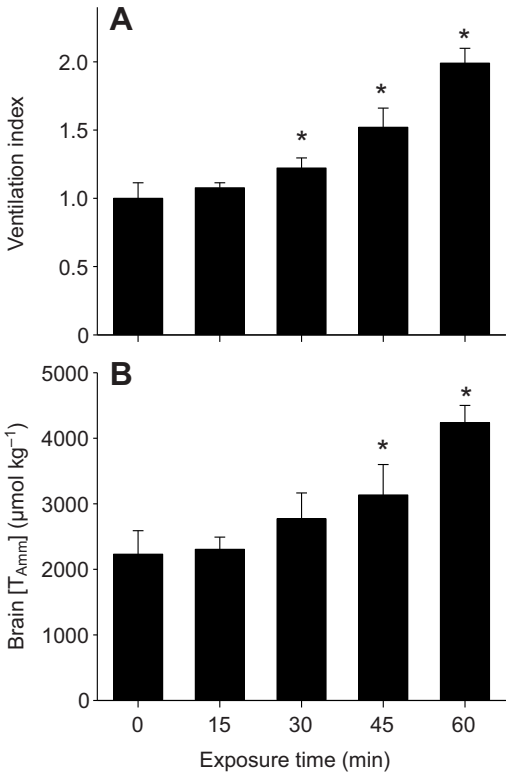

Fig. 4. Responses of ventilation and brain [T_{Amm}] to waterborne 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure in control trout in a time series over 60 min. Asterisks indicate significant increases relative to the data before the $(NH_4)_2SO_4$ exposure period (P<0.05). Data are means + s.e.m. (N=5).

DISCUSSION

Brain involvement in the sensitivity of ventilation to ammonia

In general, these correlations between brain [T_{Amm}] and ventilatory responses to ammonia occurred consistently in three independent experiments: an acute HEA $(NH_4)_2SO_4$ concentration series, an acute HEA time series and a MSOX pre-injection series. These results strongly suggest that brain [T_{Amm}] is involved in the sensitivity of ventilation to ammonia in trout.

We previously observed that hyperventilation occurred as plasma [T_{Amm}] increased; however, after chronic HEA exposure, the ventilatory response to experimental elevations of ammonia in both water and blood disappeared, and we attributed this change to structural and functional changes in the branchial NECs (Zhang and Wood, 2009; Zhang et al., 2011). This relationship seemed reasonable because NECs had been documented as bimodal receptors for the other two respiratory gases, O_2 and CO_2 (reviewed by Milsom, 2012). There was abundant evidence that NECs in gills, particularly on the first pair of gill arches, functioned as O_2 and CO_2/pH sensors (Smith and Jones, 1978; Gilmour, 2001; Milsom and Burleson, 2007). The receptors on this first pair of gill arches (embryonic arches III) are thought to represent the phylogenetic antecedents of the mammalian carotid bodies, which function in sensing blood O_2 and CO_2/pH and drive respiration in mammals (Milsom and Burleson, 2007). However, several findings of present study suggest that the plasma ammonia sensed by NECs might not be the only signal and pathway involved in ventilatory responses to ammonia. Firstly, it was again confirmed that the elevated plasma [T_{Amm}] failed to induce hyperventilation in response to acute higher ammonia exposure in HEA trout, as shown in a previous study

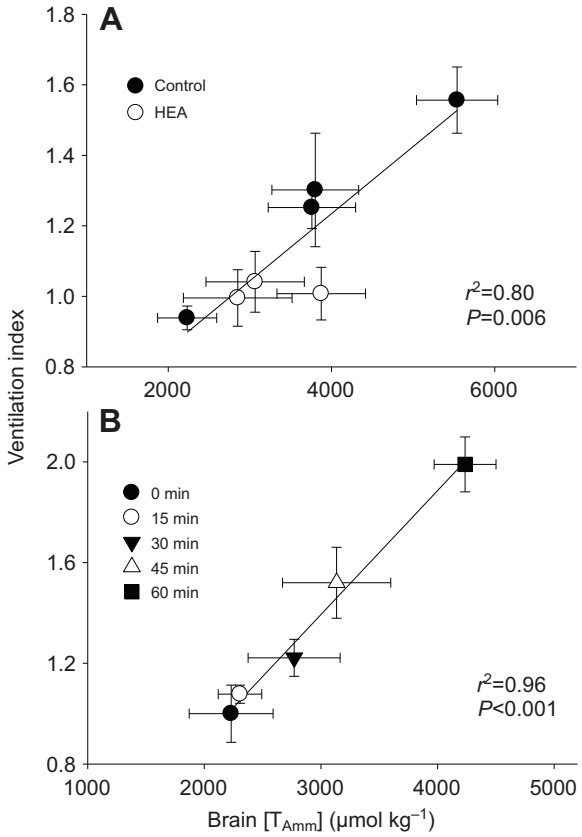

Fig. 5. Correlations between ventilation index and [T_{Amm}] in brain tissue in response to elevated waterborne ammonia $(NH_4)_2SO_4$ exposures. Data have been grouped as treatment means. Data in A are from both control trout and trout chronically exposed to HEA in the ammonia concentration series experiments [from background to 1000 μmol l^{-1} $(NH_4)_2SO_4$, for 60 min]. Data in B are from control trout in the time series experiment [500 μmol l^{-1} $(NH_4)_2SO_4$, from 0 to 60 min].

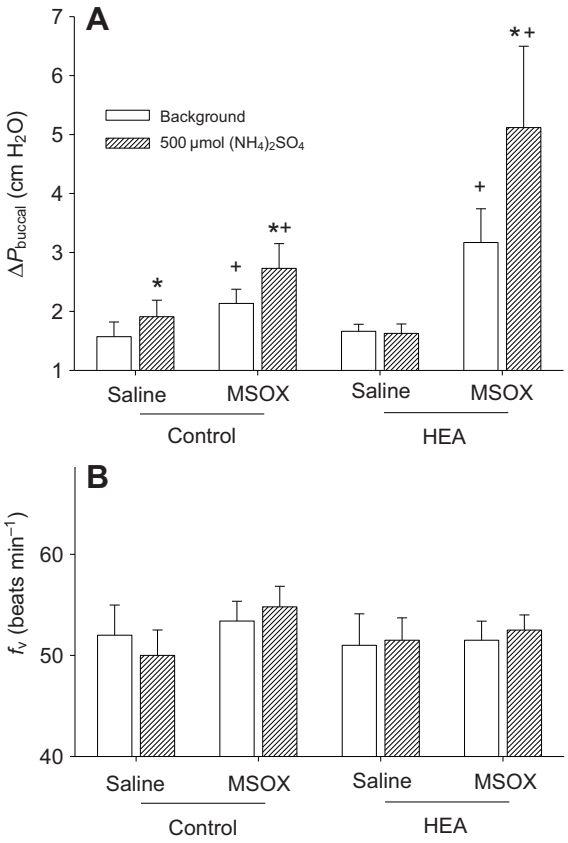

Fig. 6. The effect of glutamine synthetase (GSase) blockade on the responses in (A) ventilatory amplitude (ΔP_{buccal}) and (B) ventilatory frequency (f_v) in response to elevated waterborne $(NH_4)_2SO_4$ exposure in control trout and trout that had been chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ month. Trout were pre-injected with either saline or 6 mg kg^{-1} MSOX 48 h previously. Asterisks indicate significant differences between background and elevated $(NH_4)_2SO_4$ exposure; crosses indicate significant difference between saline and MSOX pre-injection treatments (P<0.05). Data are means + s.e.m. (N=5).

(Zhang et al., 2011). Instead, brain [T_{Amm}] (Fig. 2C) and ventilation (Fig. 1A–C) both remained unchanged in the response to this challenge in HEA trout. Secondly, even in the control trout, where ventilation was correlated with both plasma [T_{Amm}] and brain [T_{Amm}], the relationship with brain [T_{Amm}] was stronger than with plasma [T_{Amm}] (r^2=0.78 for brain *versus* 0.62 for plasma). Thirdly, the effect of MSOX on ventilation (Fig. 6) was coincident with brain [T_{Amm}] rather than plasma [T_{Amm}] (Fig. 7) in control trout exposed to clean water and in HEA trout exposed to either background or 500 μmol l^{-1} $(NH_4)_2SO_4$. Overall, these observations that brain [T_{Amm}] is more strongly associated with ventilation than is plasma [T_{Amm}] indicate that brain [T_{Amm}] could function in driving hyperventilation in trout.

To our knowledge, the effects of brain [T_{Amm}] on ventilation in fish have not been investigated previously. However, many studies about the toxicity of ammonia have provided some helpful but indirect information. On the one hand, hyperventilation has been widely reported as a symptom of the toxic effects of HEA exposure (Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph, 1996; Eddy, 2005). On the other hand, the brain is well known as the most vulnerable tissue to the toxicity of ammonia [i.e. 'central neurotoxicity' (reviewed by Randall and Tsui, 2002; Walsh et al., 2007)]. In mammals, it has long been known that ammonia-stimulated hyperventilation is of central origin in mammals (Wichser and Kazemi, 1974), and ammonia toxicity in fish has been characterized by a cascade of deleterious events resembling those associated with anoxic/ischemic injury (a well known driver to hyperventilation) in the central nervous system (Wilkie et al., 2011). Recent studies have demonstrated that fish exhibit symptoms to acutely toxic levels of ammonia similar to those of mammals, including hyperventilation and hyper-excitability, followed by convulsions, coma and death (reviewed by Eddy, 2005; Walsh et al., 2007). In the present study, in which trout were subjected to treatments to elevate plasma [T_{Amm}] and brain [T_{Amm}] within physiologically realistic limits, trout exhibited only hyperventilation without any obvious behavioral disturbances such as hyperexcitability, convulsions or coma. Taken together, the present results and previous reports indicate that brain [T_{Amm}] can influence ventilation under both physiological and excess conditions, and that toxicity occurs when physiological regulation fails.

Current knowledge on the pathways of neurotoxicity by ammonia and O_2 deprivation in the brain (Walsh et al., 2007) may cast light on the mechanism(s) by which brain [T_{Amm}] affects ventilation in fish. At least in mammals, a common feature of both causes of neurotoxicity is glutamate excitotoxicity due to localized excesses of glutamate causing over-stimulation of *N*-methyl-D-aspartate (NMDA) receptors. The proximate causes appear to be different – i.e. ATP deficit in O_2 deprivation causing increased synaptic

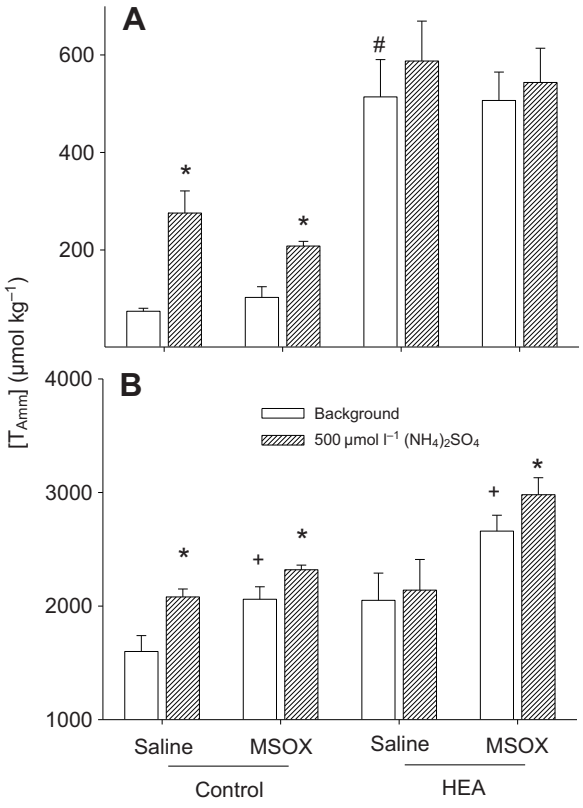

Fig. 7. [T_{Amm}] in plasma (A) and brain (B) in response to elevated waterborne $(NH_4)_2SO_4$ exposures in control trout and in trout that had been chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. Fish were pre-injected with either Cortland saline or 6 mg kg^{-1} MSOX 48 h previously. Asterisks indicate significant differences in trout between background and elevated $(NH_4)_2SO_4$ exposures; crosses indicate significant differences between saline and MSOX pre-injection treatments; pound marks indicate significant differences between control and HEA trout exposed to background water (P<0.05). Data are means + s.e.m. (N=5).

glutamate release *versus* excess ammonia inhibiting astrocytic glutamate transporters, thereby increasing synaptic glutamate concentration (Rao et al., 1992; Bosman et al., 1992; Schmidt et al., 1993). Nevertheless, the key event in neurotoxicity due to either O_2 deficit or hyperammonemia in vertebrates appears to be over-stimulation of glutamate receptors (Bickler et al., 2000; Lutz et al., 2003; McKenzie et al., 1993; Wilkie et al., 2011).

In fish, there is actually some evidence that hyperammonemia itself causes an ATP deficit in the brain (Arillo et al., 1981; Schenone et al., 1982), and there is also evidence that chronic HEA exposure downregulates NMDA receptors in goldfish, and that NMDA receptor blockade in trout reduces acute ammonia toxicity (Wilkie et al., 2011). Therefore, is it possible that activation of the glutamate–NMDA pathway by high brain [T_{Amm}] causes the acute hyperventilatory response? If so, then would later downregulation of NMDA receptors (Wilkie et al., 2011), improved ability to regulate brain [T_{Amm}] (Fig. 7B) and lower brain [glutamate] (Fig. 8) collectively result in the abolition of this response in trout chronically exposed to HEA? In support of these ideas, NMDA receptor blockade in rats resulted in depressed ventilation (Connelly et al., 1992) and attenuated the hyperventilatory response to acute hypoxia (Soto-Arape et al., 1995; Tarakanov et al., 2004), while central glutamate infusion stimulated ventilation in dogs (Chiang et al., 1986). At first glance, the fact that brain [glutamate] decreased significantly during acute HEA exposure in control trout (Fig. 8B) would seem to argue against this explanation, but as noted by Walsh et al. (Walsh et al., 2007), despite the global reductions in brain [glutamate] caused by the actions of GSase, there is also a localized excess of synaptic [glutamate] due, at least in part, to the inhibition of the astrocytic glutamate transporters by high ammonia. Therefore, unlike O_2 deprivation, ammonia-induced NMDA receptor over-activation does not appear to involve excess glutamate accumulation in the central nervous system on a global basis (Hermenegildo et al., 2000).

In mammals, two adaptive roles of elevated brain [T_{Amm}] have been identified. Through its buffer base effect as an alkalinizing agent, increased [NH_3] may help alleviate intracellular acidosis at times of both metabolic and respiratory acidosis, and through its direct effects in stimulating ventilation, it may help overcome acidosis (Wichser and Kazemi, 1974; Felipo and Butterworth, 2002). Notably, ammonia intoxication and lactacidosis often co-occur as a result of liver failure in mammals. Future studies might examine whether there are comparable effects in fish. In this regard, Wright et al. (Wright et al., 1988) noted a tendency for brain [T_{Amm}] to increase while brain pH_i fell only slightly after exhaustive exercise (combined metabolic and respiratory acidosis with increased ammonia production) in the lemon sole; both effects were not significant despite a large systemic acidosis. In the present study, no acidifying stimuli were administered, but it is notable that both acute and chronic exposure to HEA caused increases in brain pH_i (Table 1), illustrating the alkalinizing capacity of elevated intracellular [NH_3]. It is also notable that brain pH_i measurements in the present study were substantially lower than extracellular pH_a (Table 1), in accord with the situation in mammals (e.g. Nishimura et al., 1989), but in contrast to several earlier measurements on fish where brain pH_i was similar to pH_a (e.g. Wright et al., 1988; Wood et al., 1990). The difference may be of methodological origin, as the earlier fish studies used an indirect technique [the distribution of 5,5-dimethyl-2,4-oxazolidinedione (DMO)], while homogenization followed by direct measurement (Pörtner et al., 1990) was employed in the present study.

While we have argued for the importance of the brain in the ventilatory response to elevated ammonia, none of the current data negate our earlier findings on the involvement of the peripheral NECs (Zhang et al., 2011). It is likely that both sites are involved, as in the response to elevated CO_2 in higher vertebrates (see Introduction). Interestingly, our previous study suggested that ammonia might interact with both O_2 and CO_2/pH sensing in the peripheral branchial NECs (Zhang et al., 2011). NH_4^+ could substitute for K^+ at K^+ channels, and the differential flux rates of NH_3 *versus* NH_4^+ could lead to intracellular alkalosis during loading and to an intracellular acidosis during ammonia washout. Both of these aspects could result in changes in NEC membrane potential, with depolarization causing subsequent elevation of intracellular Ca^{2+} and release of neurotransmitters for ventilatory modulation. Indeed, in mammals, NMDA receptor activation plays an important role in the stimulation of peripheral chemoreceptors, which mediate ventilatory responses to hypoxia (Liu et al., 2009). Another investigation explored whether fish have glutamatergic mechanisms in the vagal sensory area (Xs) that could be involved in the generation of cardiorespiratory reflexes (including ventilation rate and amplitude), and showed that this glutamate-induced bradycardia was NMDA-receptor-dependent in shorthorn sculpin, suggesting that the peripheral [glutamate] has been demonstrated as a putative player in the central integration of the peripheral chemoreceptor and baroreceptor in fish (Sundin et al., 2003). Whether central and peripheral systems act in the same way at a cellular level in the response to ammonia is an important area for future study.

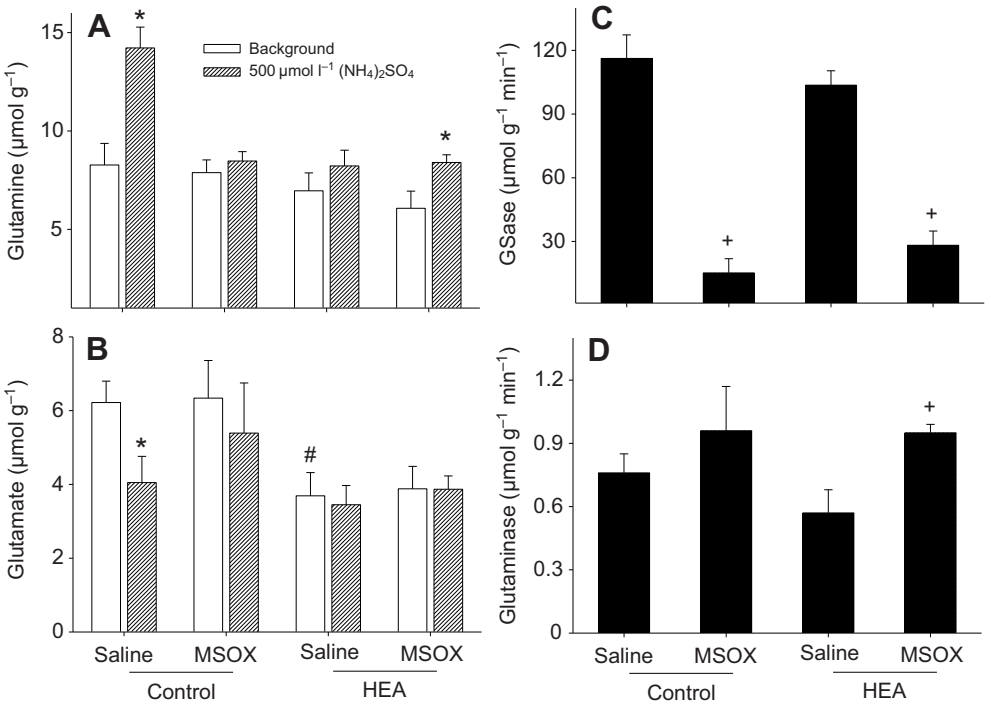

Fig. 8. Brain concentrations of glutamine (A) and glutamate (B), and enzymatic activities of glutamine synthetase (GSase) (C) and glutaminase (D) in control trout and trout that had been chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$]. Fish were pre-injected with either Cortland saline or 6 mg kg^{-1} MSOX 48 h previously and exposed to background water or 500 μmol l^{-1} $(NH_4)_2SO_4$ for 1 h. Asterisks indicate significant difference in trout between background and elevated $(NH_4)_2SO_4$ exposures; crosses indicate significant differences between saline and MSOX pre-injections; pound marks indicate significant differences between control and HEA trout exposed to background water (P<0.05). Data are means + s.e.m. (N=5).

The strategies of trout brain in dealing with ammonia toxicity

Ammonia is toxic to all vertebrates, causing damage to the central nervous system, and most fish have a variety of strategies to tolerate or avoid ammonia toxicity. As an adaptive response to acute HEA exposure, there are three widely accepted strategies including: (1) reducing proteolysis and amino acid catabolism, (2) converting ammonia to other less toxic substances such as glutamine or urea, and (3) maintaining/accelerating ammonia excretion (reviewed by Randall and Tsui, 2002). In the present study, the formation of glutamine (Fig. 8A) and increased expression of Rh proteins in the brain (Fig. 9) were found to deal with HEA exposure, suggesting that the latter two strategies work in trout. As argued subsequently, the upregulation of Rh proteins in the brain may serve to accelerate ammonia efflux from the central nervous system so that it can be excreted across the gills.

In a previous HEA study on rainbow trout, Nawata et al. (Nawata et al., 2007) reported that after an initial reversal, net ammonia excretion through the gills to the environment was re-established at rates equal to or above those in control fish for up to 48 h. A recent investigation extended this finding to a chronic HEA exposure of 7 days (Sinha et al., 2013). The HEA levels used by Nawata et al. (Nawata et al., 2007) and Sinha et al. (Sinha et al., 2013) were 1090–1500 μmol l^{-1} NH_4HCO_3, or approximately twofold to threefold higher than the total ammonia concentration [250 μmol l^{-1} $(NH_4)_2SO_4$] used in the current chronic HEA exposure for 1+ months. This ability to excrete ammonia in the face of HEA was correlated with an upregulation of the mRNA expressions of Rhcg2, NHE-2 and V-type H^+ATPase, as well as V-type H^+ATPase enzymatic activity in the gills in these two studies. These are all key components of the metabolon that is thought to be involved in active ammonia excretion at the gills (Wright and Wood, 2012). Indeed, Kolarevic et al. (Kolarevic et al., 2012) recently reported that Atlantic salmon chronically exposed to an even higher HEA level [900 μmol l^{-1} $(NH_4)_2SO_4$)] for 15 weeks were able to keep plasma T_{Amm} levels well below those in the water, and this response was again coincident with upregulation of Rhcg2 and V-type H^+ATPase mRNA expression in the gills.

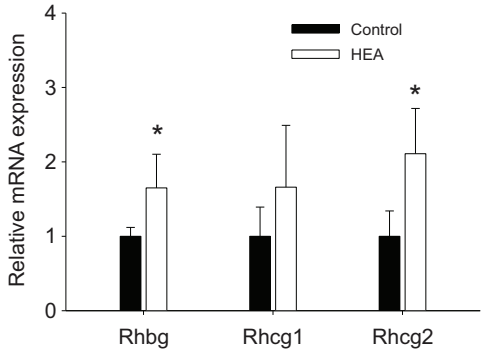

Fig. 9. mRNA expression of Rhbg, Rhcg1, Rhcg2 relative to elongation factor (EF)-1α expression in the brain of control trout and trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$]. Asterisks indicate significant difference between control and HEA trout (P<0.05). Data are means + s.e.m. (N=11).

As reported in many previous studies (e.g. Ip et al., 2004; Sanderson et al., 2010), brain [glutamine] increased and [glutamate] decreased after acute HEA exposure in control trout (Fig. 8A), revealing the formation of glutamine from glutamate and ammonia. Moreover, when brain GSase was inhibited by MSOX, brain ammonia increased in both control and HEA trout exposed to the background water (Fig. 7B), suggesting that GSase plays an important role in depressing endogenous brain [T_{Amm}] in both control and HEA trout. In fish, the activity of GSase is much higher in the brain than in other tissues (Cooper and Plum, 1987; Wright et al., 2007). In mammals (Suárez et al., 2002), it is suspected that the primary function of GSase in the brain is to maintain homeostasis of the excitatory neurotransmitter glutamate in the face of ammonia toxicity. As argued earlier, the same is probably true in fish because homeostasis of synaptic glutamate appears to be critically important (Walsh et al., 2007; Sanderson et al., 2010; Wilkie et al., 2011). In the present

Table 3. Calculated [NH_3] levels in in brain tissue and plasma, and the brain–plasma [NH_3] gradients at background and elevated waterborne ammonia $(NH_4)_2SO_4$ exposures in control trout and trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months

	Water $(NH_4)_2SO_4$ (μmol l^{-1})	Brain (μmol kg^{-1})	Plasma (μmol kg^{-1})	Gradient (μmol kg^{-1})
Control	0	7.0±1.3	0.9±0.1	6.1±1.4
	500	11.9±2.3	2.9±0.5	9.0±2.8
	750	12.1±2.3	3.7±0.6	8.4±2.9
	1000	16.0±1.2	4.2±0.6	11.8±1.8
HEA	250	9.5±1.6	8.5±1.2	1.0±2.8
	500	11.5±1.3	7.5±0.9	4.0±2.1
	1000	11.8±2.6	13.6±2.1	−1.8±4.7

Positive gradient values indicate a higher concentration in brain than in plasma. See Discussion for details. Data are means ± s.e.m. (*N*=5).

investigation, the brain GSase activity was not altered by either acute or chronic HEA exposure (Fig. 8C), which is consistent with the study by Sanderson et al. (Sanderson et al., 2010) but in contrast to other reports on the same species where HEA exposures of 9–96 h resulted in upregulated GSase activity in the brain (Wicks and Randall, 2002; Wright et al., 2007). As noted by Sanderson et al. (Sanderson et al., 2010), the difference may be of methodological origin: the present study and that of Sanderson et al. (Sanderson et al., 2010) both performed the GSase assay at the same temperature as the trout (12 and 16°C, respectively) but Sanderson and colleagues did so at much higher temperatures (around 26°C). Therefore, the present study likely reflected the real situation. Indeed, Wright et al. (Wright et al., 2007) reported that increased mRNA expression of all four brain GSase isoforms was induced after 9 h HEA exposure but returned to control levels after 48 h. This may reflect a maintenance function in light of increased enzyme turnover. Both the present study and that of Sanderson et al. (Sanderson et al., 2010) suggest that trout have a high control of brain GSase activity that provides more than enough glutamine synthetic capacity, so they do not confront chronic HEA by changing brain GSase activity, but rather by metabolizing glutamate to other fates.

In this regard, it is notable that the concentration of brain glutamate was significantly reduced in HEA trout (Fig. 8B). This is a global whole-tissue measurement, so one possible reason for this reduction is a long-term depletion of glutamate stores because of inhibited synaptic scavenging mechanisms (Rao et al., 1992; Bosman et al., 1992; Schmidt et al., 1993). In concert, glutamate levels in the brain tissue could be lowered by conversion to other amino acids, which could provide a reserve capacity to synthesize glutamate by transamination for times when ammonia challenge requires increased glutamine synthesis (Sanderson et al., 2010). Glutamate could also be depleted by glutamate dehydrogenase reactions to α-ketoglutarate, a tricarboxylic acid cycle intermediate, thereby providing ATP (Sanderson et al., 2010). Regardless, the fact that the HEA trout had less brain [glutamate] indicated that they could be more tolerant to the toxicity of elevated ammonia because less glutamate could be released to trigger the NMDA receptor over-activation.

Our finding that the mRNA expressions of both basolateral (Rhbg) and apical (Rhcg2) Rh proteins were increased in trout chronically exposed to HEA (Fig. 9) was initially surprising, as Nawata et al. (Nawata et al., 2007) had reported a downregulation of Rhbg1 and Rhcg1 after 48 h of HEA exposure. This had been interpreted as an adaptive mechanism to reduce ammonia permeability, and thereby limit ammonia loading of the brain, in accord with later data showing that these Rh proteins serve as ammonia channels, facilitating the movement of ammonia along NH_3 gradients (Nawata et al., 2010). If this were the case, then why should ammonia permeability increase during chronic HEA exposure? However, brain [T_{amm}] and acid-base status were not measured in the study of Nawata et al. (Nawata et al., 2007).

The present investigation provided another possible explanation. In Table 3, using the measured brain and plasma [T_{amm}] values (Fig. 2) and extracellular (plasma) pH_a and brain pH_i values (Table 1), we calculated the brain–plasma [NH_3] gradients using the Henderson–Hasselbalch equation, and dissociation constants for trout body fluids from Cameron and Heisler (Cameron and Heisler, 1983), as outlined by Wright et al. (Wright et al., 1988). These calculations should be interpreted cautiously, as they treat the whole brain and blood plasma as single compartments, and do not take into account regional heterogeneity or the possible confounding role of the intervening CSF compartment. Nevertheless, the calculations suggest that all the [NH_3] gradients were positive in the outward direction (i.e. from brain tissue to blood plasma) in the control trout under background conditions and also during acute HEA exposure, but in the chronic HEA trout they were not significantly different from zero in any of the treatments (Table 3). The only comparable previous study, on the lemon sole, also reported an outwardly directed [NH_3] gradient both at rest and after exhaustive exercise (Wright et al., 1988). Therefore, the function of the Rh proteins under control conditions may normally be to facilitate the efflux of endogenously produced ammonia from the brain. Given the high metabolic activity of nervous tissue, there may be a high catabolic production of ammonia, and indeed one of the functions of constitutively high brain GSase activity may be to deal with this endogenous ammonia load. However, during chronic HEA exposure, the brain to blood plasma [NH_3] gradient decreases, and there may be a need for increased Rh protein expression to facilitate the active efflux of ammonia from the brain in the absence of a driving gradient. This does not negate the explanation for the downregulation at 48 h of HEA offered by Nawata et al. (Nawata et al., 2007), because measured plasma [T_{amm}] in that study had reached a much higher value (~1100 μmol l^{-1}) by that time, more than twice the levels seen after 1 h HEA exposure in the present study (Fig. 2A). At 48 h, the animals were clearly not in steady-state, and there may have been a critical need to limit the permeability of the brain to ammonia. In light of the earlier caveats, the fact that mRNA expression may not reflect protein abundance or activity, and our current lack of knowledge on barrier functions in the fish brain, these arguments are clearly speculative. In future studies it will be important to localize and quantify the Rh proteins in the brain, and identify how they might change over time during HEA exposure.

In summary, this study demonstrated that the brain is involved in the ventilatory sensitivity to ammonia in trout. Brain [T_{Amm}], rather than plasma [T_{Amm}] or CSF [T_{Amm}], was the variable most directly related to ventilation. Both the hyperventilatory response to acute HEA exposure and its loss after chronic HEA exposure can be correlated to brain [T_{Amm}]. GSase plays an important role in

regulating brain [T_{Amm}] by converting it to glutamine, but other metabolic pathways and Rh proteins may also be involved. During chronic HEA exposure, decreases in brain [glutamate] may contribute to decreased ventilatory sensitivity to ammonia. In fish, ammonia appears to influence ventilation through effects on both peripheral NECs and central pathways.

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AUTHOR CONTRIBUTIONS

L.Z. made the greatest contribution to experiment design and execution, data interpretation, and drafting and revising the article. C.M.N. assisted with experimental execution, interpretation of the findings and manuscript revision. C.M.W. contributed to experiment conception and design, and drafting and revising the article.

COMPETING INTERESTS

No competing interests declared.

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