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RESEARCH ARTICLE

Ammonia sensing by neuroepithelial cells and ventilatory responses to ammonia in rainbow trout

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SUMMARY

Ammonia, the third respiratory gas in teleost fish, acts as an acute stimulant to ventilation in ammoniotelic rainbow trout. We investigated whether this sensitivity is maintained in trout chronically exposed (1+ months) to high environmental ammonia [HEA, 250 μmol l^{-1} $(NH_4)_2SO_4$] in the water, and whether gill neuroepithelial cells (NECs) are involved in ammonia sensing. Hyperventilation was induced both by acute external $(NH_4)_2SO_4$ exposure [250 or 500 μmol l^{-1} $(NH_4)_2SO_4$] and by intra-arterial $(NH_4)_2SO_4$ injection (580 μmol kg^{-1} of ammonia) in control trout, but these responses were abolished in chronic HEA animals. Hyperventilation in response to acute ammonia exposure persisted after bilateral removal of each of the four gill arch pairs separately or after combined removal of arches III and IV, but was delayed by removal of gill arch I, and eliminated by combined removal of arches I and II. NECs, identified by immunolabeling against 5-HT, were mainly organized in two lines along the filament epithelium in all four gill arches. In control trout, NECs were slightly smaller but more abundant on arches I and II than on arches III and IV. Chronic HEA exposure reduced the density of the NECs on all four arches, and their size on arches I and II only. Fura-2 fluorescence imaging was used to measure intracellular free calcium ion concentration ($[Ca^{2+}]_i$) responses in single NECs in short-term (24–48 h) culture *in vitro*. $[Ca^{2+}]_i$ was elevated to a comparable extent by perfusion of 30 mmol l^{-1} KCl and 1 mmol l^{-1} NH_4Cl, and these $[Ca^{2+}]_i$ responses presented in two different forms, suggesting that ammonia may be sensed by multiple mechanisms. The $[Ca^{2+}]_i$ responses to high ammonia were attenuated in NECs isolated from trout chronically exposed to HEA, especially in ones from gill arch I, but responses to high K^+ were unchanged. We conclude that the hyperventilatory response to ammonia is lost after chronic waterborne HEA exposure, and that NECs, especially the ones located in gill arches I and II, are probably ammonia chemoreceptors that participate in ventilatory modulation in trout.

Key words: ammonia, NH_3, NH_4^+, fish, neuroepithelial cell, chemoreceptors, ventilation, acclimation, acid–base status.

INTRODUCTION

Ammonia is the third important respiratory gas after oxygen and carbon dioxide in ammoniotelic teleosts. (Note: throughout this paper, the term 'ammonia' is used to refer to total NH_3 + NH_4^+, whereas NH_3 and NH_4^+ refer to the individual components of ammonia gas and ammonium ion, respectively.) Ammonia is the major nitrogenous waste (approximate 70%) produced from the catabolism of dietary and structural proteins in the liver, muscle and other tissues in ammoniotelic teleosts. To avoid highly toxic effects, ammonia is excreted continually from the gills to the water at a rate of about 10% of the rate of CO_2 excretion (Randall, 1990; Randall and Ip, 2006).

Unlike O_2 and CO_2, which have been widely accepted as the important drivers to ventilation in fish for many years (Perry and Wood, 1989; Gilmour, 2001), ammonia was not clearly revealed as a potential stimulant to ventilation until recently. McKenzie and colleagues reported that the injection of an NH_4HCO_3 solution into the dorsal aorta of trout caused a marked hyperventilation, but as blood $[HCO_3^-]$ and CO_2 tension (P_{aCO_2}) levels also increased, the responses could not be attributed specifically to ammonia (McKenzie et al., 1993). Our recent study (Zhang and Wood, 2009) followed McKenzie and colleagues' idea and elevated plasma ammonia by a number of different treatments to directly investigate whether ammonia can act as a ventilatory stimulant. Increases in plasma total ammonia concentration ($[T_{amm}]$) by a variety of methods always resulted in hyperventilation, and this occurred even in circumstances where there was no change in blood P_{aCO_2}, O_2 tension (P_{aO_2}) or acid–base status.

In fish, elevated internal $[T_{amm}]$ occurs after feeding (e.g. Wicks and Randall, 2002; Bucking and Wood, 2008), exhaustive exercise (e.g. Wood, 1988; Wang et al., 1994) or exposure to high environmental ammonia (HEA) (Wilson and Taylor, 1992; Knoph, 1996; Nawata et al., 2007), suggesting the possible function of ammonia to induce hyperventilation in these circumstances. For the first two circumstances (after feeding, after exercise), this stimulation could be adaptive to improve O_2 and CO_2 exchange (and possibly ammonia excretion) during specific dynamic action or excess post-exercise O_2 consumption, or even during the stress associated with short-term ammonia pulses in the water (Zhang and Wood, 2009). However, it is difficult to see how this hyperventilatory response would remain adaptive during chronic HEA, because ventilation in fish is a costly process (e.g. Jones, 1971). We therefore hypothesized that the response would be attenuated or lost during chronic HEA; the first objective of our study was to test this hypothesis, and to see whether the same attenuation occurred in response to intravascular ammonia injection.

The hyperventilatory response to ammonia in trout suggests the presence of ammonia-sensing chemoreceptors. There is now abundant evidence that O_2 and CO_2/pH sensors occur on the gills, with a particular focus on the first pair of gill arches (gill arch I) (Smith and Jones, 1978; Gilmour, 2001; Milsom and Burleson, 2007). Many recent studies indicate that the branchial neuroepithelial cells (NECs) are the actual chemoreceptors for these two respiratory gases in blood and/or water (Jonz et al., 2004; Jonz and Nurse, 2006; Vulesevic et al., 2006; Milsom and Burleson, 2007; Coolidge et al., 2008; Qin et al., 2010). Indeed, the receptors on gill arches I and II (embryonic arches III and IV) are thought to represent the phylogenetic antecedents of the mammalian carotid and aortic bodies, respectively (Milsom and Burleson, 2007). Our second objective was therefore to evaluate whether the hyperventilatory response to acute ammonia exposure was altered by selective removal (by ligation) of different pairs of arches. We hypothesized that if there was an effect, it would be most pronounced for the first and possibly second pair (gill arches I and II).

Assuming positive results with the first two hypotheses, our third objective was to investigate whether branchial NECs were involved in the observed responses to ammonia. NECs in fish resemble the type I glomus cells of the carotid body, which are recognized to act as both O_2 and CO_2 sensors in mammals (Gonzalez et al., 1994; Lahiri and Forster, 2003). NECs were immunofluorescently labeled by antisera against serotonin (5-HT). We hypothesized that the loss of the acute hyperventilatory response to ammonia after chronic exposure to HEA would be accompanied by changes in the size, density or distribution of NECs on gill arches if these cells were involved in ammonia sensing. Such changes have been seen in zebrafish subjected to chronic hypoxia (Jonz et al., 2004) and hyperoxia (Vulesevic et al., 2006).

By using patch-clamp techniques, Jonz et al. (Jonz et al., 2004) and Burleson et al. (Burleson et al., 2006) demonstrated that NECs of both zebrafish and catfish act as O_2 chemoreceptors, as hypoxia caused inhibition of their background K^+ currents. More recently, Qin et al. (Qin et al., 2010) showed that the NECs of the zebrafish gill respond to increasing CO_2 levels in the same way as to hypoxia, demonstrating that NECs are bimodal sensors of O_2 and CO_2. It was proposed that the demonstrated inhibition of background K^+ current and resulting partial cell depolarization caused by hypoxia or hypercapnia would lead to voltage-gated Ca^{2+} influx, subsequent neurotransmitter release and afferent nerve activation, though these steps have not yet been directly proven for teleost NECs. Furthermore, there has so far been no study on the responses of teleost NECs to ammonia. However, Randall and Ip (Randall and Ip, 2006) have suggested that the NECs could also mediate ammonia-induced hyperventilation because the K^+ channels are permeable to NH_4^+ (most values of the permeability ratio of NH_4^+/K^+ in K^+ channels are in the range 0.1–0.3) (Choe et al., 2000), and NECs therefore may sense ammonia when the background K^+ current is inhibited by NH_4^+.

With this background in mind, our final objective was to assess whether $[Ca^{2+}]_i$ in branchial NECs from trout gills is sensitive to ammonia at physiologically realistic levels. NECs were isolated from control trout and trout chronically exposed to HEA (chronic HEA trout), and their $[Ca^{2+}]_i$ responses to 1 mmol l^{-1} NH_4Cl and 30 mmol l^{-1} KCl (as a positive control) were examined using Fura-2 fluorescence imaging methods (Williams et al., 1985). We hypothesized that if NECs serve as ammonia sensors at realistic levels, their $[Ca^{2+}]_i$ would change, and this response would be attenuated in NECs isolated from trout chronically exposed to HEA.

MATERIALS AND METHODS

Fish husbandry and 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; 5–10 or 250–500 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and then acclimated to laboratory conditions for more than 4 weeks before experimentation. The trout were held in flowing dechlorinated Hamilton (ON, Canada) tap water (concentration in mmol l^{-1}: Na^+ 0.6, Cl^- 0.7, K^+ 0.05, Ca^{2+} 1.0, Mg^{2+} 0.1; 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, 20 large trout (250–500 g) and 50 small trout (5–10 g) were held in separate tanks containing 800 and 200 l dechlorinated Hamilton tap water, respectively. $(NH_4)_2SO_4$ stock solution (adjusted to pH 7.80) was added to the tanks to achieve a nominal HEA concentration of 250 μmol l^{-1} (i.e. 500 μmol l^{-1} ammonia). Fish were fed (1% body mass) every 3 days. Two-thirds of the water was renewed 24 h after feeding and an appropriate amount of $(NH_4)_2SO_4$ was added to maintain the correct HEA concentration. In the control treatment, fish were held under the same conditions as for the ammonia-exposed ones, but without the addition of $(NH_4)_2SO_4$ to the water. The acclimation lasted 1–3 months, and ammonia concentrations were checked regularly by assay (Verdouw et al., 1978) to ensure that they remained within ±15% of nominal values. Nitrite concentrations were checked by test strips (Aquarium Pharmaceuticals, Chalfont, PA, USA) during chronic HEA exposure, and no elevation was found (lower limit of detection, 0.5 mg l^{-1}).

Ventilatory responses to waterborne ammonia

Experiments were performed on large trout that had been held under control conditions or chronically exposed to HEA. Trout were anesthetized and irrigated with 80 mg l^{-1} tricaine methanesulfonate (MS-222, Syndel Laboratories Ltd, Vancouver, BC, Canada; adjusted to pH 7.8 with NaOH) in tap water on an operating table. Buccal catheters (flared tubing, Clay-Adams PE90, Sparks, MD, USA) were implanted through a hole drilled in the roof of the mouth (see Holeton and Randall, 1967), in order to monitor ventilation. Dorsal aortic catheters were implanted as described elsewhere (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), for blood sampling 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 μmol l^{-1} $(NH_4)_2SO_4$ (pH 7.80) during surgery and recovery.

During experimentation, control trout were continuously exposed to clean water for 30 min, 250 μmol l^{-1} $(NH_4)_2SO_4$ (i.e. 500 μmol l^{-1} ammonia) for 60 min, and 500 μmol l^{-1} $(NH_4)_2SO_4$ (i.e. 1000 μmol l^{-1} ammonia) for 60 min by adding appropriate amounts of $(NH_4)_2SO_4$ stock to the closed 4.5 l boxes at 30 and 90 min from the start. Chronic HEA trout were continuously exposed to 250 μmol l^{-1} $(NH_4)_2SO_4$ for 30 min and 500 μmol l^{-1} $(NH_4)_2SO_4$ for 60 min. $(NH_4)_2SO_4$ was used because the sulfate ion was found to have no effect on ventilation in our previous study (Zhang and Wood, 2009).

Ventilation was measured immediately before and at 10 min intervals during exposure. After exposure, trout were kept in their original water for 20 h of recovery. Then the procedures were repeated, with the addition of blood sampling. Dorsal arterial blood samples (600 µl each, with saline replacement) were drawn immediately *via* the catheters before the end of each exposure to a different $(NH_4)_2SO_4$ concentration. At the end of the experiments, trout were killed by an overdose of pH-adjusted MS-222.

Ventilatory response to intravascular ammonia injection

Control and chronic HEA trout were implanted with dorsal aortic and buccal catheters using the same methods as above. After 24 h recovery in the darkened Plexiglas® boxes, the fish were injected with 4.13±0.01 ml kg^{-1} of either Cortland saline or 70 mmol l^{-1} $(NH_4)_2SO_4$ (pH 7.8) *via* the dorsal aortic cannula over a 5 min period. The injected dose was therefore about 580 µmol kg^{-1} of ammonia. This injection rate did not cause any behavioral response or irritation. Ventilation was measured immediately before and 2 min after the 5 min injection period.

The role of different gill arches in the ventilatory responses to waterborne ammonia

Individual pairs of arches (arches I, II, III and IV separately, as well as arches I and II simultaneously, and arches III and IV simultaneously) were functionally removed from control trout before experimentation. Fish were anesthetized and irrigated with 80 mg l^{-1} MS-222 on an operating table. The two ends of both arches (i.e. bilaterally) in a pair were then tightly tied by no. 2-0 surgical silk sutures (Ethicon Inc., Somerville, NJ, USA). After recovery in the holding tank for 24 h, trout in which the ligations were bilaterally successful, as demonstrated by obvious coagulation or complete loss of blood in the gill filaments of both arches, were chosen for experimentation. After the recovery period, the behavior of trout appeared normal. These fish were then anesthetized again, fitted with buccal catheters only, and allowed to recover for a further 24 h in flowing clean water. Thereafter, ventilation was monitored for 30 min in clean water, followed by 60 min in the presence of HEA [250 µmol l^{-1} $(NH_4)_2SO_4$]. A separate group of fish, with all gill arches intact, were put through an identical protocol and served as controls.

Identification and quantification of NECs by immunofluorescence

Control and chronic HEA small trout were killed by an overdose of pH-adjusted MS-222. Gill tissues were prepared and immunolabeled as described previously (Jonz and Nurse, 2003). In brief, gill arches were immediately removed, washed in cold PBS (in mmol l^{-1}: 137 NaCl, 15.2 Na_2HPO_4, 2.7 KCl, 1.5 KH_2PO_4; pH 7.8) to remove mucus, and fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. Arches were then rinsed with PBS 3 times and permeabilized in 0.5% Triton X-100 in PBS (PBS-TX) at 4°C for 48–72 h. NECs of the gill filaments were identified by immunolabeling with antisera against 5-HT. Arches were incubated in the primary antibody, 1:250 rabbit anti-5-HT antibody, at 4°C overnight. After a rinse with PBS-TX, arches were incubated in the secondary antibody, 1:50 goat anti-rabbit antiserum, conjugated with fluorescein isothiocyanate (FITC; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at room temperature in the dark for 1 h. Arches were rinsed in PBS and the gill filaments were removed and mounted on glass slides in Vectashield (Vector Laboratories Inc., Burlingame, CA, USA) to reduce photo-bleaching. The slides were examined under an upright Olympus BX 60 microscope equipped with epifluorescence (Carson Group Inc., Markham, ON, Canada) and the images were captured using a CCD camera and analyzed by imaging analysis software (Northern Eclipse, Empix Imaging Inc., Cheektowaga, NY, USA).

Four filaments were selected randomly from specific arches in each of eight trout. The densities of NECs in the filaments were calculated by counting NECs at different planes of focus in the segments of the filaments near the tips (excluding the tips themselves) and were described as the number per mm of filament length. The sizes of five individual NECs in each of the four filaments were analyzed by converting the pixels of their projection area to μm^2 using Adobe Photoshop CS (Adobe Systems Incorporated, San Jose, CA, USA).

$[Ca^{2+}]_i$ responses of NECs

NECs were isolated from small trout that had been held under control conditions or chronically exposed to HEA. Methods were modified from previous studies (Kelly et al., 2000; Jonz et al., 2004). Specific gill arches were excised, then rinsed with PBS (composition as above). The gill filaments were cut off the gill bars and placed into a 15 ml centrifuge tube containing 2 ml of wash solution (sterilized penicillin 200 i.u. l^{-1}, streptomycin 200 µg ml^{-1} and gentamicin 400 µg ml^{-1} in sterilized PBS) on ice for 15 min, twice. Then the tissues were placed in a plastic culture dish filled with 2 ml of 0.25% trypsin/EDTA at room temperature. After digestion in trypsin/EDTA for 45 min at room temperature, the filament tissues were torn apart by two pairs of flame-sterilized forceps until few whole filament tips could be seen. The tissue suspension was transferred to a 15 ml centrifuge tube and triturated rapidly 200 times by a plastic pipet to continue dissociation. Fetal calf serum (0.2 ml, FCS; Invitrogen, Grand Island, NY, USA) was added and triturated briefly to stop the trypsin reaction. After removal of the undissociated tissue by passage through a 100 µm cell strainer (BD Falcon, Bedford, MA, USA), the cell suspension was centrifuged at 500 ***g*** for 5 min at 4°C. The supernatant was aspirated and the pellet was resuspended in 2 ml of rinse solution (5% FCS in PBS) and centrifuged again, as above. The pellet was then resuspended in 2 ml L-15 media (Invitrogen; containing sterilized penicillin 200 i.u. ml^{-1} and streptomycin 200 µg ml^{-1}) by triturating gently. A few drops of the cell suspension were layered on the central wells of modified 35 mm polystyrene dishes (Nunc, Roskilde, Denmark). The dishes had been modified by drilling a 10 mm diameter round central hole in the bottom, and pasting a glass coverslip (VMR, Radnor, PA, USA) to the underside with Sylgard (Paisley Products Inc., Scarborough, ON, Canada). Before use, the modified dishes were UV sterilized, and coated with poly L-lysine (0.1 mg ml^{-1}, Sigma, St Louis, MO, USA) and Matri-Gel (Collaborative Research, Bedford, MA, USA). Cells were then kept in an 18°C incubator for 24–48 h. L-15 media (2 ml) was added to the dishes 15 h after seeding. The majority of cells in the dishes were pavement cells (PVCs), because the major proportion of gill cells isolated in this manner are PVCs (~80%) and mitochondria-rich cells (~10%) but mitochondria-rich cells cannot be seeded using this method (Kelly et al., 2000). NECs were identified by staining with 2 mg ml^{-1} Neutral Red (Sigma) as in previous studies (Jonz et al., 2004), and verified by immunolabeling with 5-HT antiserum (Jonz and Nurse, 2003). PVCs were identified as the predominant unstained cells.

$[Ca^{2+}]_i$ was monitored by the fluorescent Ca^{2+} indicator Fura-2/AM (Invitrogen). Ratiometric Ca^{2+} imaging was performed using a Nikon Eclipse TE2000-U inverted microscope (Nikon, Mississauga, ON, Canada) equipped with a Lambda DG-4 ultra high-speed wavelength changer (Sutter Instrument Co., Novato, CA,

USA; exposure time 100 ms), a Nikon S-Fluor ×40 oil-immersion objective lens with a numerical aperture of 1.3, and a Hamamatsu OCRCA-ET digital CCD camera (Hamamatsu, Sewickley, PA, USA). Dual images (340 and 380 nm excitation and 510 nm emission) were collected, and pseudocolor ratiometric data were obtained using Simple PCI software version 5.3 (Hamamatsu). The imaging system was standardized with a Fura-2 calcium-imaging calibration kit (Invitrogen) through a procedure described previously (Buttigieg et al., 2008).

Cells attached to the dish were loaded with 10 μmol l^{-1} Fura-2/AM (in Cortland saline) for 40 min at room temperature and subsequently washed in Cortland saline aerated with 0.3% CO_2 and 99.7% O_2 gas mixture for 15 min to remove free dye. During the experiment, cells were continuously perfused with the gas-equilibrated saline. High K^+ (30 mmol l^{-1}) and high NH_4^+ saline (1 mmol l^{-1}), which were made by equimolar substitution of KCl or NH_4Cl for NaCl in the Cortland saline so as not to change the chloride concentration, were substituted for control Cortland saline for 15 s intervals at certain time points.

Analytical techniques

Ventilation was measured as described before (Zhang and Wood, 2009). This system recorded ventilation rate (f_V, breaths min^{-1}) and the buccal pressure amplitude (ΔP_{buccal}, mmHg), as an index of stroke volume. f_V was calculated as the frequency of breaths in 1 min at the designated time. ΔP_{buccal} was calculated as the mean value of 10 measurements of amplitude (randomly selected from periods of normal breathing, omitting episodes of coughing or disturbance) at the designated time.

Arterial blood samples (300 μl) were analyzed immediately for arterial blood pH (pHa), O_2 tension (P_{aO_2}) and O_2 content (C_{aO_2}), and frozen for later hemoglobin (Hb) analysis. The remaining blood (300 μl) was centrifuged at 9000 ***g*** for 30 s to the separate plasma; 100 μl plasma was used for the analysis of total CO_2 and the remainder was frozen in liquid nitrogen for later analysis of plasma [T_{amm}], [Na^+] and [Cl^-]. All these steps were finished within 2 min of blood sampling.

The whole blood pHa and P_{aO_2} were measured in 12°C thermostatically controlled chambers using a Radiometer GK2401C glass combination electrode coupled to a PHM82 standard pH meter (Radiometer Ltd, Copenhagen, Denmark), and a polarographic oxygen electrode coupled to a polarographic amplifier (Model 1900, A-M Systems, Everett, WA, USA), respectively. C_{aO_2} was measured in duplicate on 20 μl samples using a blood oxygen content analyzer (Oxycon^{TM}, Cameron Instrument Company, Port Aransas, TX, USA). Blood (50 μl) was transferred to a bullet tube with a small amount of lithium heparin (Sigma) and stored at –20°C for Hb measurement. Whole blood Hb was later assayed using Drabkin's reagent (Sigma) and an LKB 4054 UV/visible spectrophotometer (LKB-Biochrom, Cambridge, UK), and measured against a calibration series of Hb standards from bovine blood (Sigma). Plasma [T_{amm}] was measured using the same spectrophotometer with a commercial kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method. Plasma [Na^+] and [Cl^-] were measured using a Varian Spectra-220FS flame atomic absorption spectrometer (Varian, Mulgrave, Australia) and a coulometric chloride titrator (CMT10, Radiometer), respectively. Plasma total CO_2 was measured in duplicate on 50 μl samples using a Corning model 965 CO_2 analyzer (Lowell, MA, USA). Plasma P_{aCO_2} and [HCO_3^-] 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). Plasma [NH_3] was calculated using the Henderson–Hasselbalch equation with pK′ values for trout plasma at 12°C from Cameron and Heisler (Cameron and Heisler, 1983). Water ammonia levels were checked by the colorimetric assay of Verdouw et al. (Verdouw et al., 1978).

Statistics

Data are routinely expressed as means ± 1 s.e.m. (N), where N is the number of fish in a treatment mean (except for NEC $[Ca^{2+}]_i$ measurements, where N is the number of NEC cells). A one-way ANOVA followed by Tukey's test was applied to compare: (1) the density and size of NECs among the four gill arches; (2) $[Ca^{2+}]_i$ in NECs before and after high K^+ or high NH_4^+ perfusion; and (3) f_V, ΔP_{buccal}, and blood and plasma variables in different $(NH_4)_2SO_4$ exposures in both control fish and those chronically exposed to HEA. A one-way repeated measures (RM)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 Student's two-tailed paired t-test was applied to compare f_V and ΔP_{buccal} means before and after $(NH_4)_2SO_4$ exposures in the fish with specific gill arches removed, and to compare f_V and ΔP_{buccal} before and after intravascular $(NH_4)_2SO_4$ injections in both control and chronic HEA trout. A significance level of $P<0.05$ was employed throughout. All statistical tests were run using SigmaStat (v. 3.1; Systat Software, San Jose, CA, USA).

RESULTS

Ventilatory responses to waterborne ammonia

In the absence of blood sampling

The ΔP_{buccal} and f_V remained constant at 1.5±0.2 mmHg and 66±6 breaths min^{-1} in control trout exposed to clean water during the initial 30 min (Fig. 1A,C). After acute exposure to 250 and 500 μmol l^{-1} $(NH_4)_2SO_4$, ΔP_{buccal} increased continuously throughout the exposure periods, up to 170% of the initial amplitude at the end (Fig. 1A), whereas f_V kept constant (Fig. 1C). After pooling ΔP_{buccal} and f_V at different times in each $(NH_4)_2SO_4$ exposure, the three ΔP_{buccal} means were significantly different from each other (Fig. 1B), but the three f_V means were similar (Fig. 1D).

In chronic HEA trout, ΔP_{buccal} and f_V were kept constant at 1.3±0.2 mmHg and 60±3 breaths min^{-1} during exposure to 250 μmol l^{-1} $(NH_4)_2SO_4$ (Fig. 1A,C). This was in fact the chronic HEA medium, and the values were not significantly different from those of the control group in clean water, so after 1+ month continuous HEA exposure, the hyperventilatory response had been lost. After pooling values at different times in each $(NH_4)_2SO_4$ exposure, ΔP_{buccal} was comparable to the value in control trout exposed to clean water and significantly less than that in control trout exposed to 250 μmol l^{-1} $(NH_4)_2SO_4$ (Fig. 1B). The f_V was not significantly different from the mean rate in control trout in clean water or in 250 μmol l^{-1} $(NH_4)_2SO_4$ (Fig. 1D). After 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure, ΔP_{buccal} in chronic HEA trout remained constant (Fig. 1A) but f_V was reduced gradually, to 52±2 breaths min^{-1} at the end (Fig. 1C). After pooling, this decrease was again significant relative to control trout. The means of ΔP_{buccal} and f_V between 250 and 500 μmol l^{-1} $(NH_4)_2SO_4$ exposure treatments were not significantly different in chronic HEA trout (Fig. 1B,D), again emphasizing the loss of the hyperventilatory response as a result of long-term exposure.

With blood sampling

When the fish were blood sampled, there were some differences in absolute values, but basic patterns remained qualitatively similar. Pooled values at different times in each $(NH_4)_2SO_4$ exposure are

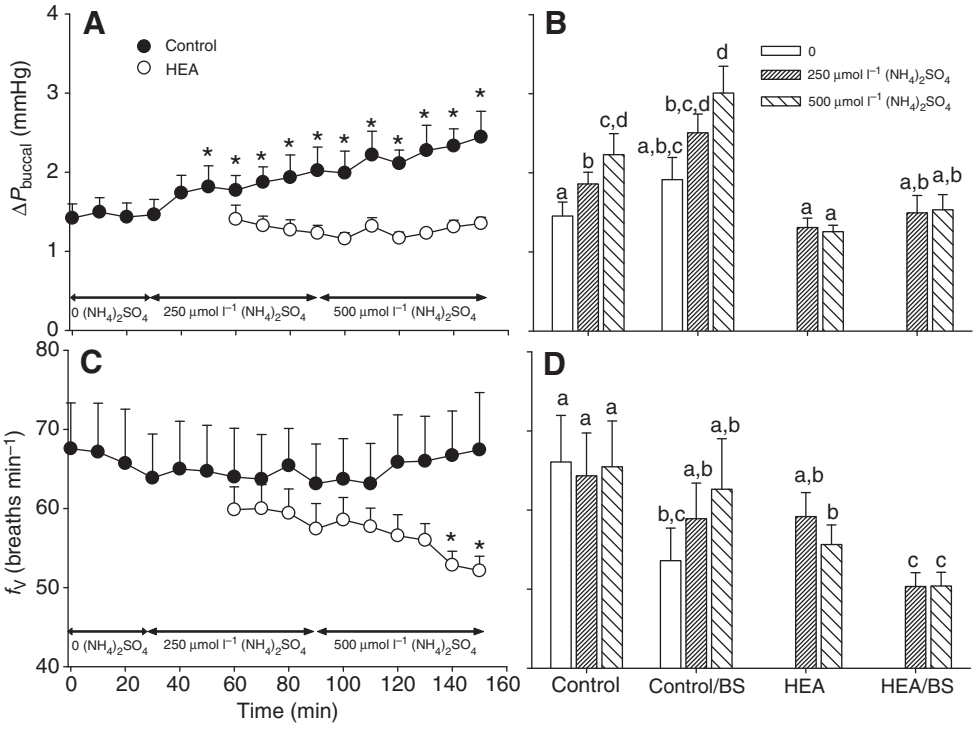

Fig. 1. Ventilatory responses to elevated waterborne ammonia $(NH_4)_2SO_4$ exposure in control trout and in trout chronically exposed to high environmental ammonia (HEA) [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. A and C show the changes of ventilatory amplitude (ΔP_{buccal}) and frequency (f_V) over time during the exposure periods. B and D show the mean values after pooling ΔP_{buccal} and f_V measurements at different times in each $(NH_4)_2SO_4$ exposure period from the data reported in A and C. In addition, B and D also summarize comparable results in trout subjected to blood sampling during the experimental periods, as control with blood sampling (Control/BS) and chronic HEA exposure with blood sampling (HEA/BS). *N*=5. In A and C, asterisks indicated significant increases relative to the data in the 0 $(NH_4)_2SO_4$ exposure period (P<0.05). In B and D, means not sharing the same letter are significantly different from one another (P<0.05).

shown (Fig. 1B,D). In control trout, ΔP_{buccal} was higher and f_V was lower than in undisturbed fish. As in non-blood-sampled fish, ΔP_{buccal} further increased after high $(NH_4)_2SO_4$ exposure, but the increase in f_V was not significant. In chronic HEA trout subjected to blood sampling, the baseline ΔP_{buccal} at 250 μmol l^{-1} $(NH_4)_2SO_4$ was unchanged, but f_V was reduced relative to non-blood-sampled fish (Fig. 1D). After acute exposure to 500 μmol l^{-1} $(NH_4)_2SO_4$, neither ΔP_{buccal} nor f_V changed, again demonstrating the loss of the hyperventilatory response after chronic HEA exposure (Fig. 1D).

Some arterial blood variables were also changed significantly after acute exposure of control fish to 250 and 500 μmol l^{-1} $(NH_4)_2SO_4$ (Table 1). In particular, pHa, [T_{amm}] and [NH_3] all increased progressively. Although plasma [HCO_3^-] did not change, Pa_{CO_2} decreased significantly coincident with the hyperventilation. However, contrary to expectations, both blood Ca_{O_2} and Pa_{O_2} also decreased progressively. As there was a small but significant fall in Hb, probably due to repetitive blood sampling, this could have caused the decrease in Ca_{O_2}. However, when the data were expressed as Ca_{O_2}/Hb, the decrease remained significant, indicating a true drop in Hb O_2 saturation (Table 1). Plasma [Na^+] and [Cl^-] remained constant.

In chronic HEA trout, many arterial blood variables were significantly different from those in control trout (Table 1). In particular, in chronic HEA trout sampled under long-term exposure conditions [250 μmol l^{-1} $(NH_4)_2SO_4$] relative to control trout sampled under control conditions, arterial pHa, [T_{amm}] and [NH_3] were all significantly higher, Pa_{CO_2} was significantly lower, while Ca_{O_2}, Pa_{O_2} and Ca_{O_2}/Hb were comparable, indicating that these last variables were restored during chronic HEA exposure. [Cl^-] were comparable but [Na^+] were significantly lower. Notably, [T_{amm}] and [NH_3] were 3- to 4-fold higher in these chronic HEA fish than in control trout exposed to the same ammonia level [250 μmol l^{-1} $(NH_4)_2SO_4$]. Also notable is the fact that in chronic HEA fish, none of the measured variables changed significantly when the ammonia exposure was acutely changed from 250 to 500 μmol l^{-1} $(NH_4)_2SO_4$ (Table 1).

Ventilatory response to intravascular ammonia injection

In control trout, the initial baselines of ΔP_{buccal} and f_V were 1.5±0.2 mmHg and 53±2 breaths min^{-1} (Fig. 2). f_V and ΔP_{buccal} did not change after injection of saline, but both increased significantly (by paired *t*-test) after intravascular injection of about 580 μmol kg^{-1} of ammonia [as 70 mmol l^{-1} $(NH_4)_2SO_4$]. The response was immediate. In trout chronically exposed to HEA, the initial baselines of ΔP_{buccal} and f_V were 1.6±0.2 mmHg and 51±2 breaths min^{-1}, comparable to values in control trout. However, there were no significant ventilatory changes after injection of $(NH_4)_2SO_4$ or saline (Fig. 2). Thus, the loss of the ventilatory response as a result of chronic waterborne HEA exposure is also seen when the hyperventilatory stimulus is presented only to the bloodstream.

The role of different gill arches in the ventilatory response to waterborne ammonia

All trout with ligated gill arches appeared healthy and breathed in a normal fashion. ΔP_{buccal} and f_V in control fish without gill ligations were 1.5±0.2 mmHg and 64±5 breaths min^{-1} (Fig. 3A,C). ΔP_{buccal} and f_V in trout with the various pairs of gill arches removed were comparable to control, except for the f_V in fish where both arches I and II had been ligated, which was 71±3 breaths min^{-1}. After acute exposure to waterborne ammonia [250 μmol l^{-1} $(NH_4)_2SO_4$], ΔP_{buccal} increased gradually and significantly over the ensuing 60 min in all control animals, with the first significant increase at about 10 min, whereas f_V did not change (Fig. 3A,C). Hyperventilation also occurred in response to 250 μmol l^{-1} $(NH_4)_2SO_4$ in trout subjected to separate removal of gill arches I, II, III or IV, or combined removal of gill arches III and IV. However, in trout lacking gill arch I, the increase in ΔP_{buccal} was delayed until 40 min (Fig. 3A). Furthermore, in trout in which both arches I and II were removed, there was no hyperventilatory response to acute waterborne ammonia exposure, with both ΔP_{buccal} and f_V remaining constant throughout the 60 min experimental period. This difference

Table 1. Arterial blood variables in control and chronic high environmental ammonia (HEA) trout

	Water $(NH_4)_2SO_4$ (μmol l^{-1})	Control	Chronic HEA
pHa	0	7.83±0.03[a]	
	250	7.85±0.03[a,b]	7.89±0.02[b]
	500	7.90±0.02[b]	7.92±0.02[b]
$[T_{amm}]$ (μmol l^{-1})	0	78.1±8.9[a]	
	250	210.8±17.0[b]	684.1±97.1[d]
	500	390.8±50.3[c]	816.3±73.3[d]
$[NH_3]$ (μmol l^{-1})	0	0.84±0.11[a]	
	250	2.41±0.29[b]	8.53±1.28[d]
	500	5.07±0.86[c]	10.73±0.95[d]
$[HCO_3^-]$ (mmol l^{-1})	0	5.56±0.79[a]	
	250	5.38±0.74[a,b]	4.31±0.48[a,b]
	500	4.54±0.71[a,b]	3.76±0.48[b]
Pa_{O_2} (mmHg)	0	1.82±0.18[a]	
	250	1.67±0.15[a]	1.22±0.11[b]
	500	1.27±0.17[b]	1.02±0.13[b]
Ca_{O_2} (ml 100 ml^{-1})	0	11.0±1.3[a]	
	250	7.0±1.4[b]	10.6±0.7[a]
	500	6.1±1.6[b]	8.5±1.2[a,b]
Pa_{O_2} (mmHg)	0	75.8±3.3[a]	
	250	69.6±6.9[a,b]	68.9±3.5[a,b]
	500	58.6±8.4[b]	67.1±4.2[a,b]
Hb (g 100 ml^{-1})	0	8.95±0.71[a]	
	250	8.48±0.59[a,b]	7.89±0.63[a,b]
	500	7.42±0.72[b]	7.22±0.47[b]
Ca_{O_2}/Hb (ml g^{-1})	0	1.27±0.11[a]	
	250	0.80±0.12[b]	1.24±0.09[a]
	500	0.79±0.11[b]	1.07±0.12[a]
$[Cl^-]$ (mmol l^{-1})	0	125.4±2.2[a]	
	250	122.6±3.1[a]	128.3±1.4[a,b]
	500	123.8±3.1[a]	130.3±1.9[b]
$[Na^+]$ (mmol l^{-1})	0	162.4±6.4[a]	
	250	153.2±7.1[a]	140.8±3.8[b]
	500	152.6±4.9[a]	138.6±2.9[b]

Arterial blood pH (pHa), O_2 content (Ca_{O_2}), O_2 tension (Pa_{O_2}), hemoglobin (Hb), Ca_{O_2}/Hb ratio, plasma total ammonia ($[T_{amm}]$), non-ionized ammonia ($[NH_3]$), $[HCO_3^-]$, CO_2 tension (Pa_{CO_2}), $[Na^+]$ and $[Cl^-]$ in control trout and trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. Values were recorded from fish in their acclimation water [either 0 or 250 μmol l^{-1} $(NH_4)_2SO_4$] and after 60 min exposure to higher waterborne ammonia concentrations [250 and 500 μmol l^{-1} $(NH_4)_2SO_4$]. Values are presented as means ± s.e.m. (N=5). Means not sharing the same letters are significantly different (P<0.05) from one another.

was also clearly seen when the data were pooled across the period (Fig. 3B,D).

Identification and quantification of NECs by immunofluorescence

There were no obvious differences in arrangement or staining in 5-HT-immunoreactive NECs among different gill arches. Therefore, the immunofluorescence results of arch I are shown as representatives in Fig. 4. In both control and chronic HEA trout, NECs were dispersed within the primary filament epithelium but not in the secondary respiratory lamellae. NECs were mainly organized in two lines in the filament epithelium. Although the arrangement of NECs did not change in trout chronically exposed to HEA, the density and size of NECs were both reduced.

In control trout, the density of NECs was comparable among the four gill arches, but slightly higher in arches I and II (109±3 and 106±5 cells mm^{-1} filament) than in arches III and IV (101±4 and 103±3 cells mm^{-1} filament) (Fig. 5A). Chronic exposure to HEA caused a significant 9% reduction in NEC density in arches I and II, but not in arches III and IV. Furthermore, in control fish, NECs were smaller in arches I and II than in arches III and IV (76±2 *versus* 87±3 μm^2), whereas in chronic HEA fish, the NECs were comparable in size in the four arches (69±2 μm^2) but significantly smaller by about 15% than in control trout (Fig. 5B).

$[Ca^{2+}]_i$ responses of NECs

NECs in culture were identified as cells that were partially colored red, as a result of the staining of intracellular vesicles with Neutral Red, as described previously (Jonz et al., 2004). Baseline $[Ca^{2+}]_i$ in NECs was about 100 nmol l^{-1}. Of the 43 NECs that responded to high K^+ (30 mmol l^{-1}) with a rise in $[Ca^{2+}]_i$, 39 also responded to high NH_4^+ (1 mmol l^{-1}) perfusion. In contrast, the Neutral Red-negative PVCs, which were predominant among the dispersed cell population, never exhibited any obvious $[Ca^{2+}]_i$ responses to either high K^+ or high NH_4^+ perfusion (Fig. 6A).

In NECs, $[Ca^{2+}]_i$ always increased and recovered rapidly after the high K^+ perfusion whereas $[Ca^{2+}]_i$ responses to high NH_4^+ perfusion presented two patterns (Fig. 6A,C). There were 19 cells (11 from control and 8 from chronic HEA trout) that presented a slow increase followed by a slow recovery of $[Ca^{2+}]_i$ (type A, in Fig. 6A). And there were 20 cells (15 from control and 5 from chronic HEA fish) that presented a rapid increase and recovery followed by a subsequent long-term elevation of $[Ca^{2+}]_i$ (type B, in Fig. 6C). Because the second elevation of $[Ca^{2+}]_i$ in type B responses was usually prolonged and sometimes persisted beyond the end of the recording period, the first rapid elevations of $[Ca^{2+}]_i$ were chosen to quantify responses in type B cells.

In NECs from both control and chronic HEA fish, the background $[Ca^{2+}]_i$ was similar, at 100±6 nmol l^{-1}, regardless of whether the

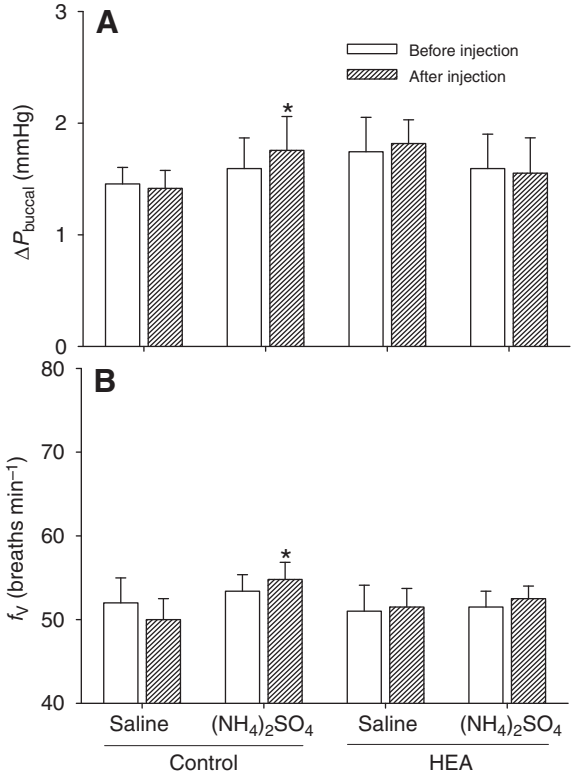

Fig. 2. Ventilatory responses to dorsal aortic injection of saline or 580 μmol kg^{-1} of ammonia [as 70 mmol l^{-1} $(NH_4)_2SO_4$] in control trout and in trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. A and B show the changes of ventilatory amplitude (ΔP_{buccal}) and frequency (f_V) before and after injection, respectively. *N*=5. Asterisks indicated significant increases (Student's paired *t*-test) in response to injection (P<0.05).

NECs exhibited type A or type B responses (Fig. 6B,D). After high K^+ perfusion, the $[Ca^{2+}]_i$ increased to comparable levels in NECs from control and chronic HEA fish. However, after high NH_4^+ perfusion, the $[Ca^{2+}]_i$ increased to significantly higher levels in NECs from control trout than in those from chronic HEA trout. This pattern of a differential effect on the responses to high NH_4^+ *versus* high K^+ was seen in NECs exhibiting both type A (Fig. 6B) and type B $[Ca^{2+}]_i$ responses (Fig. 6D).

There were no obvious differences in the frequency of type A *versus* type B responses when the data were analyzed according to the origins of the NECs from different gill arches, or different chronic exposure conditions. However, it was notable that the $[Ca^{2+}]_i$ response to high NH_4^+ perfusion was significantly lower in NECs from gill arch I of HEA fish (Fig. 7).

DISCUSSION

Overview

After acute exposure to elevated waterborne ammonia [either 250 or 500 μmol l^{-1} $(NH_4)_2SO_4$], rainbow trout exhibited a hyperventilatory response consisting of marked increases in amplitude (ΔP_{buccal}) but no change in breathing frequency (f_V). However, after chronic exposure to waterborne HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months, ventilation had returned to normal and there was no longer any acute stimulatory effect of higher waterborne levels of ammonia [500 μmol l^{-1} $(NH_4)_2SO_4$]. Thus, in confirmation of our first hypothesis, the fish had acclimated physiologically, and the stimulatory effect of ammonia on ventilation was attenuated or abolished, a phenomenon that is likely adaptive in the light of the high cost of ventilation in fish. The same attenuation occurred in the responses to intravascular ammonia injections (580 μmol kg^{-1}), suggesting that sensitivity to internal and external ammonia was affected in a similar manner. The acute hyperventilatory response to waterborne ammonia was delayed by removal of gill arch I, and eliminated by removal of both gill arches I and II. These findings support our second hypothesis that the phylogenetic antecedents of the mammalian carotid and aortic bodies are involved in ammonia sensing in fish. In accord with our third hypothesis, chronic HEA exposure reduced the density of NECs on gill arches I and II, and their size on all four gill arches, thereby providing indirect evidence of their involvement in ammonia sensing. More direct evidence and confirmation of our final hypothesis was provided by Fura-2 imaging of isolated NECs. These experiments demonstrated that their $[Ca^{2+}]_i$ surges in response to physiological levels of ammonia, and that these responses are attenuated in NECs isolated from fish that have been chronically exposed to HEA.

The hyperventilatory response, its loss during chronic HEA exposure, and acclimation to ammonia

In agreement with many previous studies (see Introduction), acute elevation of water ammonia caused hyperventilation in trout. In our study, only ventilatory amplitude (ΔP_{buccal}) increased in response to acute waterborne ammonia, whereas f_V remained unchanged (Figs 1 and 3). Interestingly, in both our previous (Zhang and Wood, 2009) and present studies (Fig. 2), intravascular ammonia injections caused small elevations in f_V in addition to large elevations of ΔP_{buccal}, suggesting that either the threshold or the sensors for the breathing rate response may be different from those for the breathing amplitude response.

Estimates of the cost of ventilation in fish are generally high, ranging from 1% to 43% of resting metabolism, with many in the 5–15% range (see Jones, 1971). Therefore while hyperventilation in response to an acute ammonia signal may be beneficial for several reasons (see Introduction), chronically elevated ventilation in the face of persistent waterborne HEA would likely be maladaptive. Our finding that hyperventilation disappeared after chronic HEA exposure (1+ months) is in accord with one previous 28 day study, where trout appeared to hyperventilate (based on f_V counts only) during the initial 7 days and recovered afterwards (Lang et al., 1987). Notably, in the present study, the hyperventilatory responses to both further elevations in waterborne ammonia (Fig. 1) and intravascular injections of ammonia (Fig. 2) were also lost, suggesting a major blunting of both external and internal sensitivities.

In our previous study (Zhang and Wood, 2009), the ventilatory index ($\Delta P_{buccal} \times f_V$) was positively correlated with the arterial plasma ammonia parameters ($[T_{amm}]$ and $[NH_3]$) in 24 h intravascular ammonia infusion experiments. In the present study, the ventilatory index in chronic HEA trout exposed to 250 μmol l^{-1} $(NH_4)_2SO_4$ was only 81% of that in control trout exposed to clean water, or 65% of that in control trout exposed to the same media (Fig. 1). At 500 μmol l^{-1} $(NH_4)_2SO_4$, the ventilatory index of chronic HEA trout dropped to 48% of the comparable value in control trout. These differences occurred despite the fact that the arterial plasma ammonia parameters ($[T_{amm}]$ and $[NH_3]$) were significantly higher in chronic HEA trout than in control trout under all test conditions (Table 1). These results suggest that rainbow trout acclimate to chronic HEA in a physiological sense; toxicological acclimation to ammonia has been established previously (Render and Stickney, 1979; Alabaster et al., 1979).

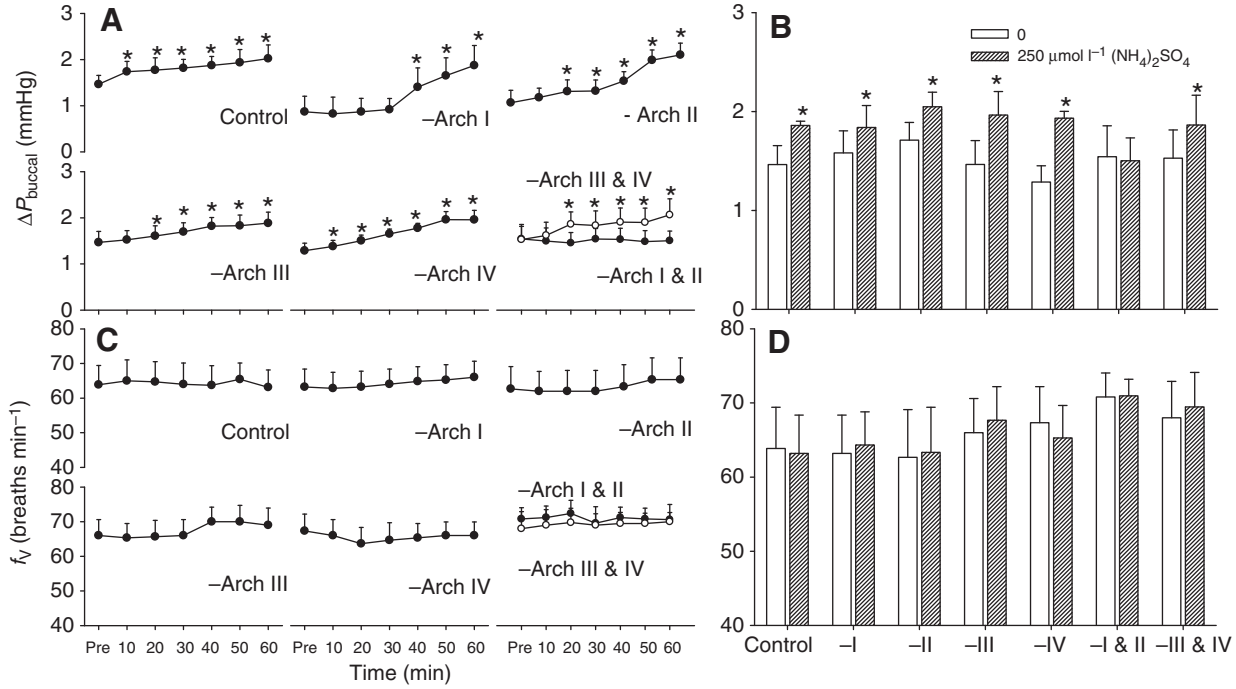

Fig. 3. Ventilatory responses to elevated waterborne ammonia [250 μmol l^{-1} $(NH_4)_2SO_4$] exposure in trout subjected to removal of specific gill arch pairs by ligation. Control trout had all gill arches intact. A and C show the changes of ventilatory amplitude (ΔP_{buccal}) and frequency (f_V) at different times in each exposure period. B and D show the mean values after pooling ΔP_{buccal} and f_V measurements at different times in each $(NH_4)_2SO_4$ exposure period from the data reported in A and C. N=5. Asterisks indicate significant increases (Student's paired t-test) relative to the data before (Pre) $(NH_4)_2SO_4$ exposure (P<0.05).

Ventilation was not the only parameter to change as a result of acclimation. Acute exposure to 250 μmol l^{-1} $(NH_4)_2SO_4$ in control trout raised plasma [NH_3] and [T_{amm}], and lowered Ca_{O_2} and Ca_{O_2}/Hb, with larger alterations after exposure to 500 μmol l^{-1} $(NH_4)_2SO_4$ (Table 1). After chronic HEA exposure, the changes in arterial Ca_{O_2} and Ca_{O_2}/Hb had been corrected despite even greater elevations in [T_{amm}] and [NH_3], as well as increases in pHa, and decreases in Pa_{CO_2} and plasma [Na^+] (Table 1). The changes of Ca_{O_2} and Ca_{O_2}/Hb are of particular interest, and raise the possibility that ammonia may act in part by reducing arterial Hb O_2 saturation, thereby eliciting the O_2 drive to ventilation (Smith and Jones, 1981). However, there was no indication of methemoglobinemia according to the color of the blood. The mechanism by which ammonia alters Hb O_2 binding is worthy of future study.

The location of ammonia sensors in trout gills

An important aim of this study was to find out the locations of the ammonia sensors involved in hyperventilation. The gill arch removal experiments provided an answer at a coarse level. Hyperventilation in response to $(NH_4)_2SO_4$ injections was immediate (see also Zhang and Wood, 2009), but took about 10 min in response to acute waterborne $(NH_4)_2SO_4$ exposure (Figs 1 and 3). The response was delayed until 30–40 min in trout lacking arch I, and eliminated in trout without arches I and II (Fig. 3), suggesting that the ammonia sensors are concentrated in these two arches. In the light of the 30–40 min delay in response seen after arch I removal, we speculate that arch I receptors may mainly sense the external waterborne ammonia (relatively fast response), whereas arch II receptors may sense the internal plasma ammonia (much slower response). In the former case, it will take a short but finite time for waterborne ammonia to diffuse across the epithelial and mucus barrier and raise the ammonia level at 'external' NECs sufficiently to trigger the hyperventilatory response. In the latter case, the much longer delay would be due to the time needed for the process of ammonia uptake from the water to raise plasma ammonia to the threshold level needed to trigger the 'internal' NECs that sense plasma ammonia levels.

At a finer level, the density of NECs decreased in arches I and II (Fig. 5A), and the $[Ca^{2+}]_i$ response to high NH_4^+ was clearly attenuated in NECs from arch I (Fig. 7) as a result of chronic HEA exposure, a treatment that also eliminated the hyperventilatory response to high ammonia (Figs 1 and 2). In teleosts, gill arch I is innervated by a branch of the glossopharyngeal nerve (IX) and all four arches are innervated by branches of the vagus nerve (X) (Milsom and Burleson, 2007). In phylogenetic terms, NECs in arch I (embryonic arch III) appear to be the antecedents of the mammalian carotid bodies, while the NECs of arch II (embryonic arch IV) are the antecedents of the aortic bodies. However, the reason why only gill arches I and II, and not arches III and IV, appear to function in ammonia sensing remains unclear, because 5-HT-positive NECs were distributed in all of the four gill arches without large differences in number or size (Fig. 5), which agrees with previous studies in zebrafish (Jonz and Nurse, 2003). Furthermore, NECs from all four arches exhibited $[Ca^{2+}]_i$ responses to high NH_4^+ (Fig. 7).

Gill arch I is now widely recognized as an important site for sensors of aquatic respiratory gas tensions. For example, hypoxia and hypercapnia both produce a reflex bradycardia in fish; the receptors associated with these responses are restricted to gill arch I and appear to mainly sense water gas tensions in many species including rainbow trout (Daxboeck and Holeton, 1978; Smith and Jones, 1978; Smith and Jones, 1981; Perry and Reid, 2002). However, the sensors for the ventilatory response to hypoxia appear to be more diffuse and variable, sensing both external and internal

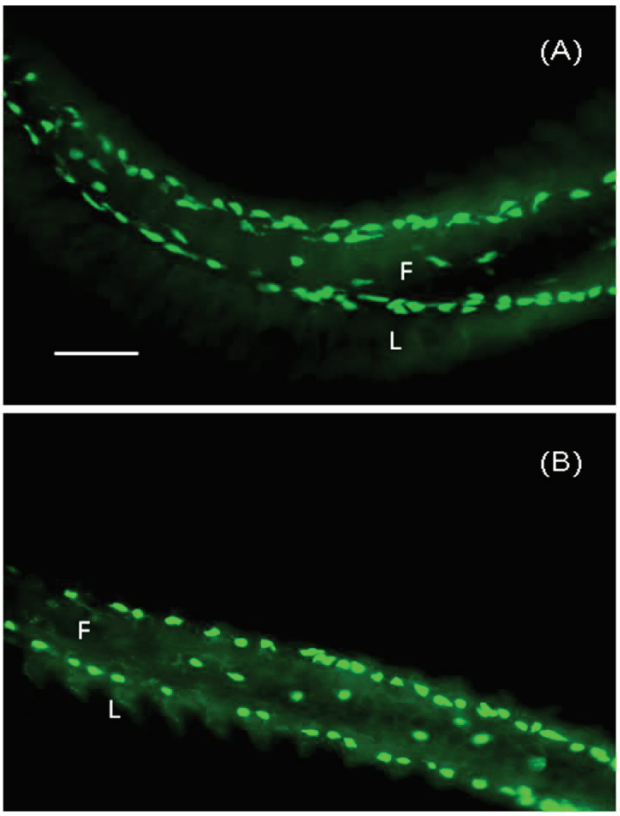

Fig. 4. Serotonin (5-HT)-immunoreactive neuroepithelial cells (NECs) in filaments of gill arch I from (A) a control trout and (B) a trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. F and L indicate the primary filament and the secondary respiratory lamellae, respectively. Bar indicates 100 μm.

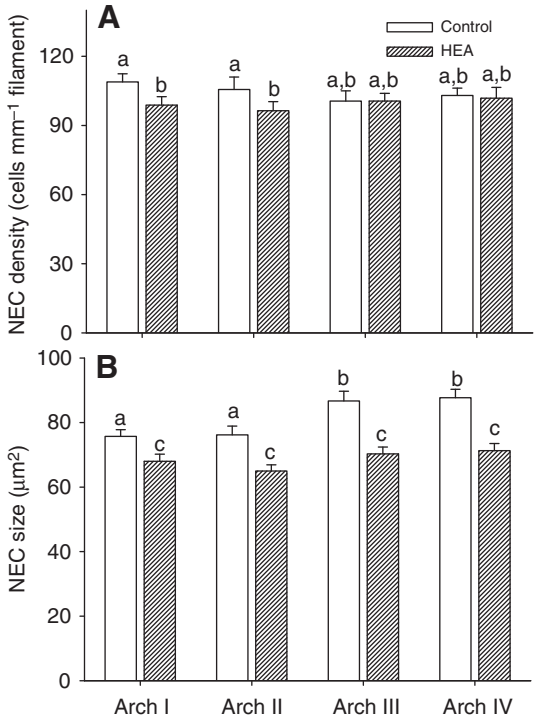

Fig. 5. The (A) density and (B) size of NECs in the filaments of different gill arches from control trout and from trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. N=8. Means not sharing the same letter are significantly different from one another (P<0.05).

gas levels (Daxboeck and Holeton, 1978). For example, in the catfish, the ventilatory response to hypoxia arises only from receptors confined to the gill arches (Burleson and Smatresk, 1990), but in many fish, total gill denervation fails to eliminate the hypoxic ventilatory response (Saunders and Sutterlin, 1971; Sundin et al., 1998) and the remaining receptors appear to occur at extrabranchial sites including the orobranchial cavity (Milsom et al., 2002).

It is possible but unlikely that the pseudobranch is involved in ammonia sensing. The pseudobranch is served with arterial blood from the first efferent gill artery but is not exposed to the external environment. Both older and more recent research suggests that the pseudobranch may not function in gas sensing. For example, bilateral de-afferentiation of the pseudobranch had no effect on the ventilatory responses to hypoxia or hyperoxia (Randall and Jones, 1973) and hypoxic release of catecholamines from chromaffin cells mediated by O_2 chemoreceptors did not involve the pseudobranch in trout (Reid and Perry, 2003). Indeed, only one or two 5-HT-containing cells resembling O_2-sensitive gill NECs were found in each pseudobranch filament in zebrafish (Jonz and Nurse, 2003).

Morphological changes in NECs during chronic HEA

While the detrimental effects of very high ammonia on gill histology (e.g. lamellar fusion, epithelial lifting and hemorrhage) have been well documented (Smart, 1976; Mallatt, 1985; Francis et al., 2000; Spencer et al., 2008), this appears to be the first study examining the effects of chronic sublethal HEA exposure on the branchial NECs. Both the acute ventilatory responses to ammonia (Figs 1 and 2) and the morphometry of the NECs were altered, with significant reductions in NEC number (density) in arches I and II (Fig. 5A), and reductions in their size (Fig. 5B) in all four arches. The response appears to be partially, but not completely, analogous to the findings of Vulesevic et al. (Vulesevic et al., 2006) on zebrafish, who showed a significant 35% decrease in NEC density after hyperoxia acclimation for 28 days, with a blunted sensitivity to subsequent acute hypoxic or hypercapnic challenge. Both studies suggest that the sensitivity of the ventilatory responses is influenced by the number of NECs. The difference, however, is that acute hyperoxia normally inhibits ventilation whereas acute ammonia normally stimulates ventilation. Further complicating the situation is the fact that Vulesevic et al. (Vulesevic et al., 2006) and Jonz et al. (Jonz et al., 2004) observed a 15% increase in size and no change in the number (density) of NECs in zebrafish after prolonged acclimation to hypoxia, but no change in the hyperventilatory response to acute hypoxia. Clearly there is much more to learn about the morphological and functional plasticity of branchial NECs.

NECs are probably ammonia chemoreceptors

An important finding of the present study is that not only do NECs respond to ammonia in fish but also they respond with a marked increase in $[Ca^{2+}]_i$, consistent with ammonia causing calcium influx. By analogy to investigations on glomus cells of the mammalian carotid body, previous studies on fish NECs subjected to hypoxic (Jonz et al., 2004) or hypercapnic stimuli (Qin et al., 2010) have speculated but not demonstrated that this $[Ca^{2+}]_i$ surge occurs. In this scheme, the appropriate stimulus inhibits a background K^+ current, causing membrane depolarization, which triggers voltage-

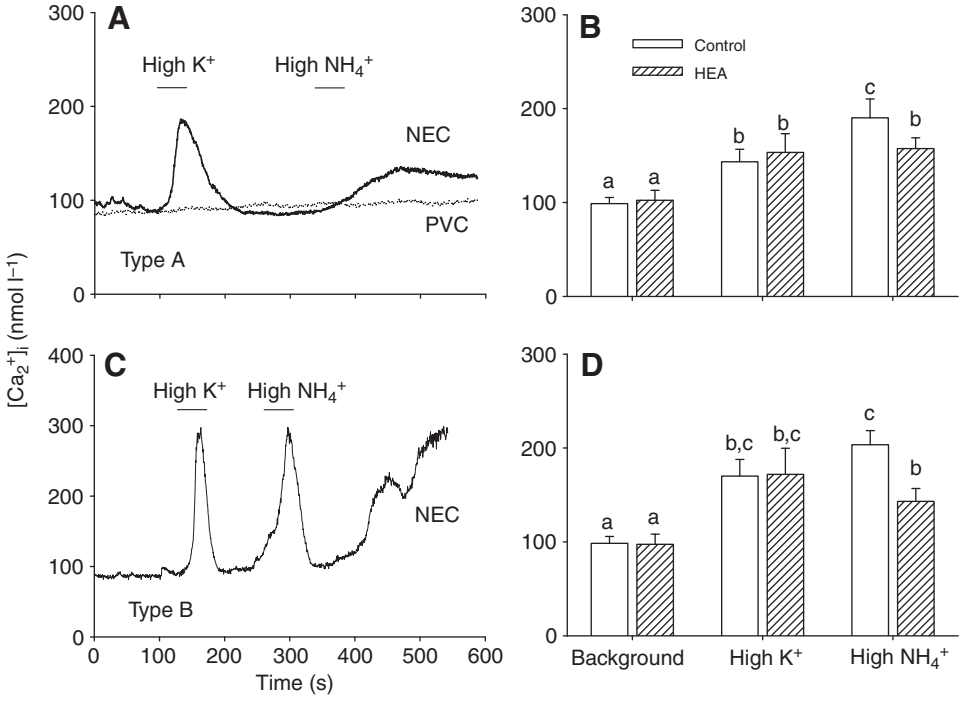

Fig. 6. Two types of $[Ca^{2+}]_i$ response in NECs to the perfusion of high K^+ (30 mmol l^{-1}) or high NH_4^+ (1 mmol l^{-1}) Cortland saline. (A) A typical type A slow change of $[Ca^{2+}]_i$ during and after the high NH_4^+ perfusion period in a representative NEC, as well as in a pavement cell (PVC). PVCs never responded. (C) A typical type B change (both fast and slow) of $[Ca^{2+}]_i$ during and after the high NH_4^+ perfusion period in a representative NEC. (B) A summary of background and peak $[Ca^{2+}]_i$ of type A responses in NECs from control trout (N=11) and from trout (N=8) chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. (D) A summary of background and peak $[Ca^{2+}]_i$ of type B responses in NECs from control trout (N=15) and from trout (N=5) chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ months. Means not sharing the same letter are significantly different from one another (P<0.05).

gated Ca^{2+} influx, subsequent neurotransmitter release and afferent nerve activation (Lahiri and DeLaney, 1975; Peers, 1990a; Peers, 1990b; Buckler and Vaughan-Jones, 1994a; Buckler and Vaughan-Jones, 1994b; Dasso et al., 2000; Zhang and Nurse, 2004). If these ideas are correct, the sensing of ammonia by fish NECs would intersect with the hypoxia- and hypercapnia-sensing pathways. Thus, the elevation of $[Ca^{2+}]_i$ revealed in the present study would be due to changes in the K^+ current in NECs. Two of the four known classes of K^+ channels (Hille, 2001), the background (or leak) K^+ channels and voltage-dependent K^+ channels, have been evaluated in fish NECs. Because the depolarizations induced by both hypoxia and hypercapnia are blocked by the presence of quinidine, a non-specific blocker of background K^+ channels, but not by several blockers of voltage-dependent K^+ channels, it has been proposed that these stimuli cause membrane depolarization by inhibiting background K^+ channels (Jonz et al., 2004; Qin et al., 2010). In turn, the elevation of $[Ca^{2+}]_i$ in NECs would lead to the subsequent release of neurotransmitter for the ventilatory modulation, with 5-HT being the prime candidate (Fritsche et al., 1992; Burleson and Milsom, 1995; Sundin et al., 1998; Jonz and Nurse, 2003; Coolidge et al., 2008). Catecholamines, ACh and ATP (co-stored with monoamines in vesicles) may also play important roles as likely neurotransmitters in NEC physiology (Burleson and Milsom, 1995; Coolidge et al., 2008; Nurse, 2010).

It was interesting that there were two types of $[Ca^{2+}]_i$ response to high NH_4^+ perfusion; type A showing only a 'slow' response, and type B showing a 'fast' plus a 'slow' response (Fig. 6). Although the slow responses in the two types were not the same (peaked increase in type A, prolonged increase in type B), both of them occurred after NH_4^+ had been removed from the perfusion media, and were long lasting. It is well known that ammonia exposure to cells results in pH_i disturbance. Indeed, this is the basis of the classic 'ammonium prepulse' technique (Roos and Boron, 1981), with an alkalosis during the ammonia loading period, and an acidosis after ammonia washout, reflecting the more rapid flux of NH_3 than of NH_4^+ or H^+. The recovery of cells from acidification is a long process. The temporal consistency of the slow $[Ca^{2+}]_i$ response and acidosis to NH_4^+ perfusion suggests that intracellular acidification may be a candidate mechanism for how some NECs sense ammonia. In a recent study on zebrafish NECs, carbonic anhydrase was shown to enhance the electrophysiological response to hypercapnia (Qin et al., 2010). This phenomenon suggests that intracellular acidification is one of the mechanisms for sensing CO_2, as in mammalian type I glomus cells (Putnam et al., 2004; Lahiri and Forster, 2003; Qin et al., 2010). In future investigations, it will be

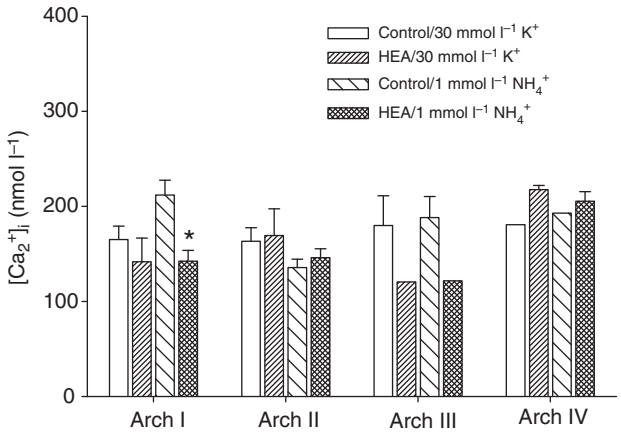

Fig. 7. $[Ca^{2+}]_i$ responses to perfusion of high K^+ (30 mmol l^{-1}) or high NH_4^+ (1 mmol l^{-1}) Cortland saline in NECs from different gill arches of control trout and of trout chronically exposed to HEA [250 μmol l^{-1} $(NH_4)_2SO_4$] for 1+ month. Type A and type B responses have been combined. N values for NECs in gill arches I, II, III and IV are 17, 3, 5 and 1 in the control trout and 5, 5, 1 and 2 in the HEA trout, respectively. Asterisk highlights a significant reduction in the response to high NH_4^+ (1 mmol l^{-1}) only on gill arch I (P<0.05).

worthwhile to monitor the change of pH_i of NECs in response to high NH_4^+ perfusion.

On the other hand, type B responses were fast $[Ca^{2+}]_i$ surges in response to high extracellular NH_4^+ perfusion, which were very similar to the responses to the depolarizing high extracellular K^+ stimulus. This phenomenon is not surprising inasmuch as K^+ channels are commonly permeable to NH_4^+, leading to similar downstream responses. However, it is not fully explained because most values of the permeability of NH_4^+ through K^+ channels are in the range of 10–30% relative to K^+ (Choe et al., 2000), yet in our study the 1 mmol l^{-1} NH_4^+ perfusion caused a comparable $[Ca^{2+}]_i$ response to 30 mmol l^{-1} K^+ perfusion. This raises the question of whether ammonia can enter NECs in more efficient ways, and cause membrane depolarization as well. An early study on squid giant neuron suggested that NH_4^+ could substitute for K^+ and affect the membrane potential (Binstock and Lecar, 1969). In frog sartorius muscle cells, NH_4^+ ions were also found to cause membrane depolarization which led to a reduction in the twitch tension generated (Heald, 1975). Furthermore, in brown trout exposed to HEA, the measured depolarization of white muscle cells (from –86.5 to –52.2 mV) was coupled with an increase of the ratio of intracellular to extracellular total ammonia (Beaumont et al., 2000). A group of Rh glycoproteins has been found to facilitate ammonia transport across gills in PVCs and mitochondria-rich cells in rainbow trout and other teleosts (Hung et al., 2007; Nakada et al., 2007; Nawata et al., 2007). These appear to bind NH_4^+ but transport NH_3 (Wright and Wood, 2009; Nawata et al., 2010). Although Rh glycoproteins have not been reported in NECs, this provides the possibility that ammonia may rapidly enter NECs through particular channels, thereby eliciting either direct or indirect actions. The lack of the fast response in type A cells suggests the existence of NECs lacking mechanisms of rapid ammonia influx. Investigation of Rh protein expression in NECs as well as the measurement of pH_i in NECs will be required to test these ideas in future studies.

In conclusion, the present study has demonstrated that the hyperventilatory response to ammonia is lost after chronic waterborne HEA exposure, that branchial NECs, especially the ones located in gill arches I and II, are probably ammonia chemoreceptors which participate in the ventilatory modulation in trout, and that $[Ca^{2+}]_i$ plays an important signaling role in the responses of the NECs. These results help advance our understanding of the ventilatory responses to a respiratory gas, ammonia, which to date has received only sparse experimental attention.

LIST OF ABBREVIATIONS

Ca_{O_2}	arterial O_2 content
f_V	ventilation rate
Hb	hemoglobin
HEA	high environmental ammonia
NEC	neuroepithelial cell
Pa_{CO_2}	arterial CO_2 partial pressure
Pa_{O_2}	arterial O_2 partial pressure
pHa	arterial blood pH
PVC	pavement cell
T_{amm}	total ammonia (NH_3 + NH_4^+)
ΔP_{buccal}	buccal pressure amplitude

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