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Reference:

Zhang Li, Nawata C. Michele, De Boeck Gudrun, Wood Chris M..- *Rh protein expression in branchial neuroepithelial cells, and the role of ammonia in ventilatory control in fish*

Comparative biochemistry and physiology : A : molecular & integrative physiology - ISSN 1095-6433 - (2014), p. 1-13

DOI: http://dx.doi.org/doi:10.1016/j.cbpa.2014.10.004

Rh protein expression in branchial neuroepithelial cells, and the role of ammonia in ventilatory control in fish

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Running Head: Ventilation, ammonia, Rh proteins, and NECs in fish

Abstract

Bill Milsom has made seminal contributions to our understanding of ventilatory control in a wide range of vertebrates. Teleosts are particularly interesting, because they produce a 3rd, potentially toxic respiratory gas (ammonia) in large amounts. Fish are well known to hyperventilate under high environmental ammonia (HEA), but only recently has the potential role of ammonia in normal ventilatory control been investigated. It is now clear that ammonia can act directly as a ventilatory stimulant in trout, independent of its effects on acid-base balance. Even in ureotelic dogfish sharks, acute elevations in ammonia cause increases in ventilation. Peripherally, the detection of elevated ammonia resides in gill arches I and II in trout, and *in vitro*, neuroepithelial cells (NECs) from these arches are sensitive to ammonia, responding with elevations in intracellular Ca^{2+} ($[Ca^{2+}]_i$). Centrally, hyperventilatory responses to ammonia correlate more closely with concentrations of ammonia in the brain than in plasma or CSF. After chronic HEA exposure, ventilatory responsiveness to ammonia is lost, associated with both an attenuation of the $[Ca^{2+}]_i$ response in NECs, and the absence of elevation in brain ammonia concentration. Chronic exposure to HEA also causes increases in the mRNA expression of several Rh proteins (ammonia-conductive channels) in both brain and gills. "Single cell" PCR techniques have been used to isolate the individual responses of NECs versus other gill cell types. We suggest several circumstances (post-feeding, post-exercise) where the role of ammonia as a ventilatory stimulant may have adaptive benefits for O_2 uptake in fish.

Key Words: ammonia, teleost fish, elasmobranchs, chemoreceptors, Rhesus glycoproteins, neuroepithelial cells, ventilation, brain, 1st gill arch, serotonin

Abbreviations:

5-HT 5-hydroxytryptamine = serotonin
$[Ca^{2+}]_i$ intracellular calcium ion concentration
$CaCO_3$ calcium carbonate
CO_2 carbon dioxide
CO6a cytochrome c oxidase subunit Via
CSF cerebrospinal fluid
EDTA ethylenediaminetetraacetic acid
GS glutamine synthetase
H-ATP v-type proton adenosine triphosphatase
$[HCO_3^-]$a arterial plasma bicarbonate ion concentration
$[HCO_3^-]$v venous plasma bicarbonate ion concentration
HEA high environmental ammonia
K^+ potassium ion
K_{2P} two-pore domain potassium channel
M_{Amm} total ammonia excretion rate
M_{CO2} carbon dioxide excretion rate
M_{O2} oxygen consumption rate
Mg^{2+} magnesium ion
MSOX methionine sulfoxamine
MRC mitochondria-rich cell
mRNA messenger ribonucleic acid
MS-222 tricaine methane sulphonate
Na^+ sodium ion
NaCl sodium chloride
$NaHCO_3$ sodium bicarbonate
NH_3 ammonia gas
NH_4^+ ammonium ion
NH_4Cl ammonium chloride
NH_4HCO_3 ammonium bicarbonate
NH_4OH ammonium hydroxide
$(NH_4)_2SO_4$ ammonium sulphate
NHE2 sodium hydrogen exchanger-2
NKA sodium potassium adenosine triphosphatase
NEC neuroepithelial cell
O_2 oxygen
$PaCO_2$ arterial carbon dioxide partial pressure
$PvCO_2$ venous carbon dioxide partial pressure
PaO_2 arterial oxygen partial pressure
PvO_2 venous oxygen partial pressure

P_{NH3} partial pressure of ammonia
PBS phosphate buffered saline
pHa arterial pH
pHv venous pH
pK negative base 10 logarithm of the dissociation constant
PVC pavement cell
qPCR quantitative real time polymerase chain reaction analysis
Rh Rhesus
SDA specific dynamic action
SEM standard error of the mean
$[SO_4^{2-}]$ sulphate ion concentration
ST serotonin transporter
T_{Amm} total ammonia, the sum of NH_3 and NH_4^+
TASK-1 TWIK-related acid-sensitive potassium channel-1

1. Introduction

In contrast to most other vertebrates, ammoniotelic teleost fish must regulate three respiratory gases – oxygen, carbon dioxide, and ammonia (Randall and Ip, 2006). This third gas is excreted at rates (M_{Amm}) about 10-20% those of oxygen consumption (M_{O2}) or carbon dioxide production (M_{CO2}). Ammonia is particularly interesting because it exists in physical solution in two forms – the dissolved gas (NH_3) and the protonated ammonium cation (NH_4^+). In this article we use NH_3 and NH_4^+ to refer to the gas and the cation respectively, and the term total ammonia (T_{Amm}) to refer to the sum of the two. NH_3 and NH_4^+ are interconvertible with a pK of approximately 9.5, such that the latter dominates quantitatively (> 95%) at physiological pHs. In this regard, ammonia is analogous to carbon dioxide which exists in solution as the dissolved gas (CO_2) and the hydrated bicarbonate anion (HCO_3^-) which are interconvertible with an effective pK of about 6.1, such that the latter dominates quantitatively at physiological pHs. Ammonia is also similar to carbon dioxide in that both are quite toxic, both affect physiological pH, and both play intimate roles in ionoregulation such that blood plasma concentrations and body stores must be tightly regulated. In most situations, the goal of the regulatory systems appears to be to match the rate of excretion across the gills with the rate of production by the tissues, while maintaining blood plasma levels of T_{Amm} in an optimal concentration range. This appears to be about 50 – 300 μmol L^{-1} T_{Amm} in resting, non-fed teleosts of various species (reviewed by Wood, 1993), though levels in the 1000 - 2500 μmol L^{-1} range have been measured in salmonids which are actively feeding and/or surviving in high environmental ammonia (Tsui et al., 2009; Zimmer et al., 2010).

There has been a vast amount of research on ammonia as a toxicant in fish (reviewed by Randall and Tsui, 2002; Eddy 2005; Ip et al., 2010), as well as on its mechanisms of branchial excretion and role(s) in ionoregulation (reviewed by Wright and Wood, 2009, 2012, and Weihrauch et al., 2009). Perhaps the most important finding of the last decade in these areas is that ammonia movement through the branchial epithelium is facilitated by specific channels, the Rh glycoproteins (Nakada et al., 2007; Nawata et al. 2007). While some ammonia may pass by simple diffusion through the lipoprotein cell membranes of the gills, a significant fraction moves by channel-mediated facilitated diffusion. Even though NH_4^+ dominates at physiological pH, and is the moiety which binds at the channel gate, the actual form moving through the Rh channel appears to be NH_3 (Nawata et al., 2010b), so the H^+ removed from NH_4^+ must be shuttled by another mechanism (Na^+/H^+ exchanger or v-type H^+-ATPase linked to a Na^+-selective channel) if the fish is to excrete NH_4^+ on a net basis. Therefore, these Rh channels appear to play an integral role in a "Na^+/NH_4^+ exchange complex" consisting of several transporters working together as a metabolon which provides a loose coupling of Na^+ uptake with branchial ammonia excretion under normal circumstances (Wright and Wood, 2009; Ito et al., 2013). This modern model is rather close to the original ideas of August Krogh (1938) for linkage of Na^+ uptake with NH_4^+ excretion at the gills of aquatic animals, yet again illustrating the prescience of the father of comparative physiology!

Under external ammonia loading, elements of the metabolon may be involved in the active excretion of ammonia against a gradient, energized by Na^+,K^+-ATPase (NKA - e.g. Hung et al., 2007; Nawata et al., 2007, 2010a; Tsui et al., 2009; Braun et al., 2009; Zimmer et al. 2010; Wood and Nawata, 2011; Wood et al., 2013; Sinha et al., 2013), with indications that NH_4^+ can effectively substitute for K^+ on Na^+,K^+-ATPase in at least some species (Mallery, 1983; Balm et al., 1988; Randall et al., 1999; Nawata et al. 2010a; Wood et al., 2013). Additionally, elevated ammonia excretion through the metabolon is now thought to drive active Na^+ uptake in fish chronically exposed to low pH and/or ion-poor water (Kumai and Perry, 2011; Shih et al., 2012; Lin et al., 2012), circumstances in which earlier models predicted that Na^+ uptake would become impossible (Avella and Bornancin, 1989: Randall et al., 1996; Parks et al., 2008). mRNA expression data indicate that the system is also activated in response to internal loading by ammonia infusion (Nawata et al., 2009), exhaustive exercise (endogenous ammonia production by adenylate breakdown; Mommsen and Hochachka, 1988; Wood, 1988; Wang et al., 1994; cf. Fig. 1A) and feeding (endogenous ammonia production by deamination of amino acids; Wicks and Randall, 2002a,b; Bucking and Wood, 2008; Zimmer et al., 2010; cf. Fig. 1B)

There has been far less research on ammonia's possible "respiratory" role. However, it is well known that fish hyperventilate in the latter two circumstances of internal ammonia loading. Increased breathing is needed to support the elevated O_2 demands of post-prandial specific dynamic action (SDA; Jobling, 1994; Secor, 2009), and to pay off the anaerobic component of exhaustive exercise (Scarabello et al., 2001). Fish also exhibit marked hyperventilation during high environmental ammonia (HEA) exposure (e.g. Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph, 1996). Is it possible that elevated internal and/or external ammonia levels provide the proximate stimulus for hyperventilation under these circumstances? This might be particularly important after feeding if ammonia could serve as a ventilatory stimulant to counteract any depression of ventilation caused by the post-prandial 'alkaline tide' (Wood et al., 2005; Bucking and Wood, 2008; Cooper and Wilson, 2008, Wright and Wood, 2012)? And if so, since rapid penetration of ammonia to the ammonia-sensing sites would presumably be needed, is it possible that Rh proteins play a role?

Indeed in mammals, it has long been known that under certain pathological conditions (e.g. liver failure), internal ammonia buildup can serve to stimulate ventilation (Roberts et al., 1956; Vanamee et al., 1956; Poppell et al., 1956; Warren, 1958; Campbell et al., 1973; Wichser and Kazemi, 1974; Felipo and Butterworth, 2002). Ammonia-induced hyperventilation (causing respiratory alkalosis) may help to offset the lactacidosis that often accompanies hepatotoxicity. Is it possible that this emergency response had its evolutionary roots in the normal ventilatory responsiveness of fish to ammonia?

Much of what we know about the control of breathing in fish, and indeed in vertebrates in general, comes from the fundamental contributions of Bill Milsom and his colleagues over the past 30 years (e.g. Jones and Milsom, 1982; Milsom and Jones, 1985; Shelton et al., 1986; Milsom, 1990, 2002, 2012; Milsom et al., 2002; Milsom and Burleson, 2007). This formidable

body of research has focused on the roles of O_2, CO_2, pH, and proprioceptive feedback in controlling ventilation. In the present report, we integrate our recent research, both published and unpublished, into this framework, to show that the third respiratory gas ammonia, also plays a significant role in the control of breathing in fish. While these studies have focused on a model ammoniotelic teleost, the rainbow trout, our experiments on a model ureotelic elasmobranch, the dogfish shark, suggest that similar processes may occur in this ancient group. We also present new data, obtained using "single cell" qPCR techniques, on the molecular expression of Rh proteins in the putative chemoreceptive cells of the rainbow trout, and their responses to chronic HEA exposure.

2. Materials and Methods

All procedures were approved by the Animal Research Ethics Boards of the appropriate institutions (McMaster University, Bamfield Marine Sciences Centre) and were in accordance with the Guidelines of the Canadian Council on Animal Care.

In general, apart from the new studies with yearling trout detailed below (Section 2.1), the freshwater rainbow trout (*Oncorhynchus mykiss*) were adults (200-400 g) of mixed sex, acclimated to flowing dechlorinated Hamilton (Ontario, Canada) tapwater ($[Na^+]$ 0.6 mmol l^{-1}, $[Cl^-]$ 0.7 mmol l^{-1}, $[K^+]$ 0.05 mmol l^{-1}, $[Ca^{2+}]$ 1.0 mmol l^{-1}, $[Mg^{2+}]$ 0.1mmol l^{-1}; titration alkalinity 1.9 mequiv l^{-1}; hardness 140 mg l^{-1} as $CaCO_3$ equivalents; pH 7.8 - 8.0, temperature 11 – 13^{O}C). Juvenile trout (5-10g) were used in the cell isolation experiments of Zhang et al. (2011). The marine dogfish sharks (*Squalus acanthias suckleyi*) were adult males (1.0 – 2.5 kg), acclimated to seawater (12-14 ℃, salinity 30 – 32 ‰) from Bamfield Inlet (British Columbia, Canada) in a flow-through system. All fish were fasted for at least 3 days prior to experimentation to minimize the influence of feeding on ammonia metabolism. For methodological details, the reader is referred to our published studies on rainbow trout (Zhang and Wood, 2009; Zhang et al., 2011, 2013) and dogfish sharks (DeBoeck and Wood, 2014). Detailed methods are described below only for the new studies reported in the present paper.

2.1 Fish husbandry and chronic high environmental ammonia (HEA) exposure

Yearling rainbow trout (*Oncorhynchus mykiss* Walbaum, 70-100 g., mixed sex) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and then acclimated to flowing dechlorinated Hamilton tapwater (see above) for more than 1 week before experiments. In the chronic HEA treatment, trout were held in a tank containing 800 liters of dechlorinated Hamilton tapwater. When necessary in control and experimental tanks, water pH was adjusted to 7.8 using NaOH and HCl. An $(NH_4)_2SO_4$ stock solution (adjusted to pH 7.80 with NaOH) 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 weight) every 2 days with a commercial trout food (crude protein 41%; carbohydrates 30%; crude fat 11%; Martin Mills; Elmira, ON, Canada). At 8 h after every feeding, 65% 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 ones, but without the addition of $(NH_4)_2SO_4$ to water. The acclimation lasted 1 to 2 months, and total ammonia concentrations were checked regularly by assay (Verdouw et al., 1978) to ensure that they remained within ±15% of nominal values. The fish were fasted for 3 days prior to sampling to minimize the influence of feeding on the mRNA expression of Rh proteins (Zimmer et al., 2010).

2.2 Total branchial cDNA synthesis

Fresh filaments of the 1st gill arches were dissected from control and HEA-exposed trout (N = 6 per treatment) which had been euthanized by overdose with pH-adjusted MS-222 (250 mg l^{-1}) and perfused with physiological phosphate buffered saline (PBS) to remove blood from gill tissues. The filaments of the 1st gill arches were dissected and placed into 5 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. 1 μg 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 ℃ overnight for quantitative real-time PCR (qPCR).

2.3 Cell isolation and cDNA synthesis for qPCR on pooled samples of individually-identified cell types

Different types of the branchial cells were isolated from the 1st gill arch using the methods of Jonz et al. (2004) followed by those of Galvez et al. (2002). In brief, fresh filaments of the 1st gill arches were removed from control and HEA-exposed fish (N = 6 per treatment) after gill perfusion with ice-cold Cortland salmonid saline (Wolf, 1963) to remove RBCs. Then the tissues were placed in 2 ml of 0.25% trypsin/EDTA at room temperature for digestion for 45 min. The filament tissues were torn apart by 2 pairs of flame-sterilized forceps and the tissue suspension was transferred to a 15-ml centrifuge tube and triturated rapidly 200 times by a plastic pipette to continue dissociation. The trypsin reaction was stopped by adding fetal calf serum (0.2 ml, FCS, Invitrogen, Grand Island, NY, USA). 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 3,000 rpm for 5 min at 4°C to remove the reaction media. 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 supernatant was aspirated, and the pellet was resuspended again in 2 ml of PBS, which was then layered onto a discontinuous Percoll density gradient.

Cells were harvested separately from the 1.03–1.05 g ml^{-1} and 1.05–1.09 g ml^{-1} interface. Neuroepithelial cells (NECs) were identified as cells which stained partially with neutral red (2 mg L^{-1}, see Jonz et al. , 2004) in the 1.03–1.05 g ml^{-1} interface; pavement cells (PVCs) were identified as small unstained cells in the 1.03–1.05 g ml^{-1} interface; and mitochondrial-rich cells (MRCs) were identified as large unstained cells in the1.05–1.09 g ml^{-1} interface.

Fabricated borosilicate glass electrodes were pulled on a vertical pipette puller (PP-83, Narishige, Japan) to create tips of 10-20 μm diameter (revised from Jonz et al., 2004 for NEC patch-clamp recording). Approximately 100 of each of the three kinds of cell from each fish were collected individually using a micro-manipulator mounted on the stage of an inverted microscope (Axiovert S 100, Zeiss). The identified cells were drawn one by one by negative pressure onto the glass electrodes filled with Cortland saline. The collected cells were transferred to 0.5-ml conical plastic centrifuge tubes, so that each contained a pool of about 100 cells. The tubes were centrifuged to remove the saline. The cell pellets were then lysed by using a cell lysis buffer (Signosis, Sunnyvale, CA) to isolate total RNA, and the cDNA was synthesized by using Superscript II reverse transcriptase (Invitrogen) as described above. Samples were stored at -20 ℃

2.4 Quantitative real time PCR

The qPCR primers for Rhbg (forward: cgacaacgacttttactaccgc, reverse: gacgaagccctgcatgagag), Rhcg1 (forward: catcctcagcctcatacatgc, reverse: tgaatgacagacggagccaatc), Rhcg2 (forward: cctcttcggagtcttcatc, reverse: ctatgtcgctggtgatgttg), v-type H^+-ATPase (H-ATP, forward: tcagccttggttgtgagatg, reverse: caacattggtgggaaacagg), Na^+,K^+-ATPase- isoform α1a (NKA, forward: ttgacctggatgaccacaag, reverse: ggatctccttagcccgaac), Na^+/H^+ exchanger-2 (NHE2, forward: tatggccattgtgacctgtg, reverse: caggcctctccacactaagg) were described and validated in rainbow trout by Nawata et al. (2007). The primers for cytochrome c oxidase subunit VIa (CO6a, forward: catcacacgcaggccatgaggga, reverse: gtggttgccatcaccccagggc) were designed from the *O. mykiss* cytochrome c oxidase subunit VIa sequence (Genbank **NM_001171897**). The primers for the neuroepithelial cell marker gene serotonin transporter (ST, forward: gctacaacccctttcacaacaact, reverse: ttggcgatggcttcagcatagatgat) were designed from *Danio rerio* solute carrier family 6 (neurotransmitter transporter, serotonin) (Genbank **BC163766**). The primers for a background potassium channel (TASK-1, forward: ggcaaggtgttctgcatgctctac, reverse: acacagcgtgctcatgcaggag) were designed from the sequence of potassium channel subfamily K in *Danio rerio* (Genbank **NC_007128**) and *Oryzias latipes* (Genbank **XM_004082808**). Primers were validated by sequencing of products (ABI 3100 Gene Analyzer at the MOBIX Lab, McMaster University, Hamilton, ON, Canada).

qPCR analyses were performed on an Mx3000P QPCR System (Stratagene, Cedar Creek, TX). 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 ℃ (2 min), 95 ℃ (2 min), followed by 40 cycles of 95 ℃ (15 s) and 60 ℃ (30 s). Melt-curve analysis confirmed production of a unique product, and gel electrophoresis verified the presence of a single band. For the whole gill mRNA, expressions of elongation factor-1α (EF-1α, GenBank **AF498320**) were constant, and were used as endogenous standards to calculate relative mRNA expressions by the standard curve method. For the "single cell" mRNA expression, the relative gene expressions in each cell sample (about 100 cells) were

calculated by the standard curve method, and then the "single cell" expressions of each gene were normalized by the cell number of each sample, yielding relative RNA expression per cell.

2.5 Statistical analysis

Data have been expressed as means ± 1 SEM (N) where N = number of fish. A one-way ANOVA followed by Tukey's test was applied to compare mRNA expression of individual genes among PVCs, NECs, and MRCs under control conditions. Student's unpaired two tailed t-test was used to evaluate the effect of chronic HEA exposure on the expression of individual genes in the whole gill and in individual cell types, relative to the control situation. A significance level of $p < 0.05$ was employed throughout. All statistical tests were run using SigmaStat (ver. 3.1; Systat Software, San Jose, CA, USA).

3.0 Results and Discussion

3.1 Evidence that ammonia directly stimulates ventilation in teleost fish

There has long been a general awareness from toxicological studies that exposure to HEA causes hyperventilation in teleosts (see Introduction and Sections 3.3 and 3.4). However, the first real evidence that ammonia might be involved in the control of breathing was the work of Hillaby and Randall (1979) who reported that ventilation increased after arterial injection of various doses of NH_4HCO_3 and NH_4Cl into trout. Later, McKenzie et al. (1993) monitored arterial blood O_2 and acid-base status as well as breathing after injection of NH_4HCO_3 into the dorsal aorta of trout. While ventilation increased markedly, the response could not be attributed solely to ammonia because blood HCO_3^- and $PaCO_2$ levels also increased, and injections of $NaHCO_3$ also caused similar effects. Zhang and Wood (2009) carried out a more extensive series of ammonia and control injection experiments in trout, but with a similar outcome. All injections of ammonia solutions (NH_4HCO_3, $(NH_4)_2SO_4$, NH_4OH) raised plasma T_{Amm} concentrations (to the range of 700-2200 μmol L^{-1}) and caused immediate increases in ventilation, with more pronounced effects on amplitude (ventilatory stroke volume) than on frequency (Fig. 2A, B). However, in every case there was a confounding change in one or more components of acid–base status (decreases in pHa and/or increases in $[HCO_3^-]a$ or $PaCO_2$). Nevertheless, the ventilatory responses to ammonia injections were generally larger than could be explained by changes in acid–base status alone (note the difference in equimolar responses to NH_4HCO_3 versus $NaHCO_3$ injections in Fig. 2A, B). Two subsequent experimental series sought to clarify whether the hyperventilatory response could be attributed to ammonia alone (Zhang and Wood, 2009).

In the first, an anaesthetized, spontaneously ventilating preparation was perfused with Cortland saline via the ventral aorta using a peristaltic pump. When the ammonia-free perfusion saline was changed to one containing a high but physiologically relevant level of T_{Amm} (1900 μmol L^{-1} as NH_4Cl) but unchanged pHv, $PvCO_2$, $[HCO_3^-]v$, and PvO_2, ventilation amplitude increased immediately by about 25%, and the effect was reversible upon return to control

perfusion. In the second, unanaesthetized resting trout were chronically infused for 24 h via the dorsal aorta with Cortland saline or isotonic NH_4HCO_3 or $(NH_4)_2SO_4$ solutions so as to achieve stable plasma T_{Amm} concentrations of about 1500 μmol L^{-1}. While saline infusion had no effect, both ammonia infusions caused persistent elevations in ventilation, with large increases in amplitude and small increases in frequency (Fig. 2C,D). The NH_4HCO_3 infusion caused small changes in acid-base status. However, in the case of $(NH_4)_2SO_4$ infusion, the hyperventilatory responses occurred in the absence of any changes in pHa, $PaCO_2$, $[HCO_3^-]a$, or PaO_2. Together, these two experimental series provided strong evidence that physiologically relevant levels of T_{Amm} in the blood plasma can directly stimulate ventilation in a teleost fish, and that internal receptors mediate this response.

3.2 Evidence that ammonia also stimulates ventilation in elasmobranch fish

Elasmobranchs are ureotelic, and excrete only minimal amounts of ammonia (Wood et al., 1995), even after feeding (Wood et al., 2005, 2007, 2010; Kajimura et al., 2006, 2008). Blood plasma T_{Amm} levels are extremely low in fasted animals (< 50 μmol L^{-1}). Nevertheless, like teleosts, they too experience marked increases in plasma $[T_{Amm}]$, both after exhaustive exercise (Richards et al., 2003), and after a meal (Wood et al., 2005; Kajimura et al., 2008). Furthermore, O_2 consumption increases greatly after exercise (Piiper et al., 1977; Richards et al., 2003), as well as after feeding (SDA; Sims and Davies, 1994; Wood et al., 2007) so there is a need for increased ventilation at these times. Is it possible that internal ammonia accumulation serves as a stimulus in these situations?

De Boeck and Wood (2014) evaluated this idea by injecting dogfish sharks via caudal artery cannulae with isotonic solutions of $(NH_4)_2SO_4$ or NH_4HCO_3, raising plasma $[T_{Amm}]$ to the 400 – 800 μmol L^{-1} range, and eliciting increases in ventilation within 2 min (Fig. 3A,B). As in trout, the relative increases in ventilatory amplitude were much greater than those in frequency. However, interpretation was confounded by accompanying changes in acid-base status, as with earlier work in trout (see Section 3.1). These probably played some role, because the hyperventilatory response was larger with $(NH_4)_2SO_4$ injections, which also caused a marked metabolic acidosis (decreases in pHa and $[HCO_3^-]a$), than with NH_4HCO_3, which caused only minor increases in $PaCO_2$ and $[HCO_3^-]a$ at unchanged pHa. Control injections of NaCl, Na_2SO_4, and $NaHCO_3$ provided some clarification, showing that the hyperventilatory responses were not associated with the injection itself, or with increases in $[SO_4^{2-}]$, $PaCO_2$, or $[HCO_3^-]a$ (Fig. 3A,B), suggesting that at least part of the response was specific to internal ammonia elevation.

More convincingly, De Boeck and Wood (2014) found that exposure to waterborne HEA (1500 μmol L^{-1} NH_4HCO_3), elicited a slowly developing (not significant until 4 h) and progressive increase in ventilatory amplitude over 24 h (Fig. 3D). This treatment caused no changes in arterial blood gases or acid-base status, and the time course of ventilatory increase was well correlated with the time course of plasma $[T_{Amm}]$ elevation which eventually rose close to environmental concentrations (Fig. 3C). Overall, these results indicate that the chemosensing

of ammonia is internal in dogfish; external HEA only stimulates ventilation after ammonia diffuses into the bloodstream.

3.3 Are there external ammonia receptors controlling ventilation?

The elasmobranch data summarized in Section 3.2 suggest that ventilation responds only to internal ammonia in elasmobranchs. In teleosts, the hyperventilatory response to waterborne HEA is much faster, so it remains an open question whether the hyperventilatory response to external (waterborne) HEA is mediated only through internal receptors (due to blood loading), or whether there are additional water-facing ammonia sensors. At present, we favour the idea that internal receptors are responsible for the bulk of the responsiveness, for two reasons. Firstly, the hyperventilatory response to internal ammonia injections is essentially immediate (McKenzie et al., 1993; Zhang and Wood, 2009), whereas the response to waterborne HEA takes 5-10 min and increases progressively thereafter for some time (Zhang et al., 2011, 2013). Secondly, as discussed in Section 3.4, it is difficult to see how ventilatory responsiveness to waterborne ammonia alone would be adaptive.

One possibility is that external receptors for ammonia serve primarily for olfaction, and that any chemosensory role for ventilatory control is secondary or indirect. It has long been known that fish exhibit olfactory sensitivity to ammonia, avoiding high, acutely toxic concentrations of HEA (millimolar range), with either avoidance or attraction being reported at lower concentrations (micromolar range) (Jones, 1948), perhaps because they represent a signal given off by a highly stressed fish (avoidance) or a potential prey, food, or mate signal (attraction). Amazingly, avoidance has been reported in arctic charr at the submicromolar range of ammonia (Olsen, 1986). HEA can also alter the normal olfactory-based responses to other cues (Weber et al., 2012). Future studies should examine the ventilatory response to waterborne ammonia in fish made experimentally anosmic.

3.4 Desensitization of the ventilatory response to ammonia by chronic HEA exposure

It also remains an open question whether hyperventilation actually aids ammonia excretion in fish. Randall and Ip (2006) argued that branchial ammonia excretion should not be subject to limitations by ventilation (or blood perfusion), but rather by diffusion because of ammonia's low solubility in lipid membranes (Evans and Cameron, 1986). However this conclusion was drawn only one year before the discovery that Rh glycoproteins are present in fish gills (Nakada et al., 2007; Nawata et al., 2007; Hung et al., 2007) and facilitate ammonia diffusion across the cell membrane when expressed in *Xenopus* oocytes (Nakada et al., 2007; Nawata et al, 2010b), and across branchial cell epithelia cultured on filter supports *in vitro* (Tsui et al., 2009). Future investigations should therefore test whether experimental increases in ventilatory water flow actually increase ammonia excretion in intact fish.

Regardless of the outcome, it is difficult to see how hyperventilation would be adaptive during HEA exposure, except during the brief period after the fish has left the HEA area when it may be useful to accelerate the excretion of the accumulated ammonia load. Certainly, during

chronic HEA exposure, hyperventilation would seem useless and perhaps even maladaptive, because it is metabolically costly (Jones, 1971), and might even exacerbate ammonia loading. It was therefore not surprising that acute exposure of trout to waterborne HEA (500 μmol L^{-1} as 250 μmol L^{-1} $(NH_4)_2SO_4$) caused a marked and progressive hyperventilation (Fig. 4A,B), but after 1+ month exposure to this same level of waterborne HEA, ventilation had returned to normal, even though plasma [T_{Amm}] remained 8-fold elevated (Zhang et al., 2011). Furthermore, acute additional elevations of waterborne ammonia to as high as 1000 μmol L^{-1}, which further raised plasma [T_{Amm}], failed to elicit a hyperventilatory response (Fig. 4A) in these chronic HEA fish (indeed frequency actually declined, Fig. 4B) , and they were also unresponsive to dorsal aortic injections of $(NH_4)_2SO_4$ solutions. Thus the internal receptors had become desensitized, and responsiveness to external ammonia was affected in a similar manner. This desensitization phenomenon has proven to be a powerful tool in probing the nature of ammonia chemoreception in fish (see Sections 3.5 and 3.6). Notably, the situation appears to differ from O_2 chemoreception, where prolonged acclimation to environmental hypoxia does not blunt sensitivity to acute hypoxic challenges (Jonz et al., 2004; Vulesevic et al., 2006).

3.5 The location and nature of the peripheral ammonia chemoreceptors

Neuroepithelial cells (NECs) and their associated afferent nerves on the 1st and 2nd gill arches (embryonic arches III and IV) are thought to represent the phylogenetic antecedents of the mammalian carotid bodies (innervated by cranial nerve IX = vagus) and aortic bodies (innervated by cranial nerve X = glossopharyngeal) respectively (Milsom and Burleson, 2007). These are the major sites of peripheral O_2 and CO_2/pH sensing in mammals, so the same might be expected in fish. Indeed, there is now abundant evidence that O_2 and CO_2/pH sensors occur on the gills, especially but not exclusively on the 1st pair of gill arches (Smith and Jones, 1978; Gilmour, 2001; Milsom and Burleson, 2007; Milsom 2012). Branchial NECs of several different types, with either water-facing, blood-facing, or dual orientation appear to be the actual chemoreceptors, with many similarities to mammalian Type I glomus cells (Jonz et al., 2004; Jonz and Nurse, 2006; Vulesevic et al., 2006; Milsom and Burleson, 2007; Coolidge et al., 2008; Qin et al., 2010). Therefore, investigations to date on peripheral ammonia-sensing in fish have focussed on the gill arches and NECs.

In rainbow trout, Zhang et al. (2011) selectively removed (by ligation) each pair of gill arches individually. Removal of any individual pair alone, as well as the 3rd and 4th in combination, did not prevent the typical hyperventilation seen in response to an acute HEA challenge (250 μmol L^{-1} $(NH_4)_2SO_4$, although the response was considerably delayed (40 min) after ligation of the 1st pair. However, when the 1st and 2nd gill arches were removed in combination, the response was eliminated. These findings support the hypothesis that the phylogenetic antecedents of the mammalian carotid and aortic bodies are involved in ammonia sensing in fish. Furthermore they suggest but do not prove that the receptors on the 2nd gill arch detect only internal ammonia (very slow hyperventilatory response), whereas those on the 1st arch (faster response) may additionally sense external ammonia. Notably, only the 1st arch

receives innervations from cranial nerve IX, whereas branches of nerve X innervate all four arches (Milsom and Burleson, 2007). In trout, as in zebrafish (Jonz and Nurse, 2003), NECs are distributed on all four of the gill arches, though NECs are slightly smaller but more abundant on the 1st and 2nd than on the 3rd and 4th arches. However, the reason why only the 1st and 2nd, and not the 3rd and 4th pair of arches appear to function in ammonia sensing is unclear, because NECs from all four arches showed similar responses to acute and chronic ammonia challenges, as discussed below.

Current models for hypoxia and hypercapnia chemoreception by mammalian glomus cells involve inhibition of the currents through background K^+ channels and resulting partial cell depolarization leading to voltage-gated Ca^{2+} influx, a transient rise in intracellular calcium ($[Ca^{2+}]_i$), subsequent neurotransmitter release, and afferent nerve activation (Lahiri and DeLaney, 1975; Peers, 1990a, 1990b; Buckler and Vaughan-Jones, 1994a, 1994b; Dasso et al., 2000; Zhang and Nurse, 2004; Nurse, 2010). A similar scheme, though not yet proven, is thought to apply to fish NECs (Jonz et al., 2004; Burleson et al., 2006; Qin et al., 2010; Abdallah et al., 2012; Perry and Abdallah, 2012). Therefore Zhang et al. (2011) used Fura-2 imaging (Williams et al., 1985) to evaluate whether $[Ca^{2+}]_i$ in branchial NECs of trout gills is sensitive to ammonia at physiologically realistic levels.

NECs were isolated from the gills of juvenile trout and maintained in short term tissue culture (24-48h). Notably, NECs are rich in 5-HT (Laurent, 1984), so they could be detected by vital staining with Neutral Red dye (Jonz et al., 2004), and verified by immunolabeling with 5-HT antiserum (Jonz and Nurse, 2003). NECs from all four gill arches responded to a brief (15 sec) ammonia challenge in the bathing saline (1000 μmol L^{-1} as NH_4Cl, with unchanged pH or Cl^- concentration) with two types of responses, either a slow increment in $[Ca^{2+}]_i$ (Type A response; Fig. 5A) or a sharp short-lasting rise and recovery ('spike') in $[Ca^{2+}]_i$, followed by a similar slow increment (Type B response; Fig. 5C). The Type B response was comparable to that caused by a 30-fold higher K^+ challenge (30 mmol L^{-1}) in the bathing saline, a positive control treatment designed to cause partial depolarization (Fig. 5B, D). Pavement cells, tested as a negative control, showed no response at all to either stimulus (Fig. 5A). The Type A (Fig. 5A) and the slow phase of the Type B responses (Fig. 5C) appear similar, and might result from intracellular acidosis, a response seen in most cells in the classic 'ammonium prepulse' technique (Roos and Boron, 1981), with an immediate alkalosis during the ammonia loading period, and a slower acidosis after ammonia washout. Indeed, intracellular acidosis is one of the mechanisms for sensing elevated $PaCO_2$ in mammalian Type I glomus cells (Putnam et al., 2004; Lahiri and Forster, 2003; Qin et al., 2010) and is thought to contribute in zebrafish NECs (Quin et al., 2010; Abdallah et al., 2012; Perry and Abdallah, 2012), so CO_2 and ammonia may have a common signalling pathway in some fish NECs. However the 'spike' of the Type B response (Fig. 5C) suggests a direct effect. While NH_4^+ is well known to enter through K^+ channels, 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; Randall and Ip, 2006). Yet the 1 mmol l^{-1} NH_4^+ challenge caused comparable or larger $[Ca^{2+}]_i$ responses than did the 30 mmol l^{-1} K^+ challenge (Fig. 5B,D). This raises the possibility

that ammonia can enter fish gill NECs in more efficient ways, perhaps via Rh proteins as discussed in Section 3.7.

In this same study (Zhang et al., 2011), the loss of ventilatory sensitivity to ammonia as a result of chronic HEA exposure was accompanied by both structural and functional changes in the NECs, providing further evidence of their involvement. The abundance of NECs was significantly reduced (by 9%) only on the 1st and 2nd arches, but their size was reduced by about 15% on all four arches, in HEA trout. This was clearly different from the increases in NEC size seen in zebrafish after prolonged exposure to hypoxia, a treatment that did not change ventilatory sensitivity to acute hypoxic challenge (Jonz et al., 2004; Vulesevic et al., 2006). Moreover, although the $[Ca^{2+}]_i$ in the trout NECs was still elevated by the high ammonia stimulus, both Type A and Type B responses were attenuated in the NECs from the chronic HEA trout, and this decrement was particularly prominent in NECs from the 1st gill arch (Zhang et al., 2011). Notably, the spike responses to the 30 mmol L^{-1} K^+ stimulus were not attenuated, so this effect was specific to the high ammonia stimulus.

3.6 Evidence for central ammonia chemoreceptors

Clearly the branchial NECs are involved, but they are not necessarily the only site of ventilatory sensitivity to ammonia. Like CO_2 (and unlike O_2), ammonia is normally generated internally by metabolism rather than entering from the environment, and it is the response to internal rather than external ammonia loading that appears to be adaptive, as argued in Sections 3.1 and 3.3. In fish, there is abundant evidence of peripheral sensitivity to CO_2, while there has been only minimal research on potential central chemoreceptivity to CO_2 (reviewed by Gilmour, 2001; Perry and Abdallah, 2012; Milsom, 2012). Nevertheless, there are studies suggesting a role for central CO_2/pH chemoreception in an elasmobranch (Wood et al., 1990) and a primitive actinopterygian fish (Wilson et al., 2000), and in higher vertebrates, the central chemoreceptors of the brain are the more important site for CO_2 detection. In mammals, ammonia readily crosses the blood-brain barrier with resultant diverse actions on brain metabolism (Cooper and Plum 1987; Felipo and Butterworth, 2002). All existing evidence points to ammonia acting centrally to stimulate ventilation when it builds up under pathological circumstances (see Introduction). This raises the question whether the same might be true for ammonia in fish under normal as well as abnormal circumstances, because ammonia readily crosses the blood-brain barrier, and builds up in cerebral tissue in response to internal or external loading (Wright et al., 1988; Ip et al., 2001; Chew et al., 2005; Wright et al., 2007; Sanderson et al., 2010).

Zhang et al. (2013) addressed this question in the rainbow trout using several different approaches. After acute exposure to HEA (1000 or 2000 μmol l^{-1} as $(NH_4)SO_4$), $[T_{Amm}]$ in arterial blood plasma, cerebrospinal fluid (CSF), and brain tissue were measured; all increased, but by far the strongest correlation of the increased ventilation was with brain $[T_{Amm}]$ (Fig. 6A,B). Indeed, over the time course of progressive hyperventilation, there was an extremely strong relationship between ventilation and brain $[T_{Amm}]$ (Fig. 6C,D). Furthermore, in chronic HEA trout which had lost ventilatory sensitivity, brain $[T_{Amm}]$ no longer increased during ammonia

challenge, whereas plasma [T_{Amm}] and CSF [T_{Amm}] still did so. While all this evidence is correlational, it fits well with the mammalian situation, where ventilatory stimulation during ammonia intoxication correlates best with [T_{Amm}] in the brain tissue, rather than with blood plasma or CSF concentrations (Wichser and Kazemi, 1974).

A somewhat different approach to this same question employed methionine sulfoxamine (MSOX), a specific inhibitor of glutamine synthetase (GS), to manipulate brain [T_{Amm}] levels (Zhang et al., 2013). In fish, GS is present at many fold higher activity levels in brain than in other tissues, and normally reacts ammonia with glutamate to form glutamine, thereby preventing excessive ammonia buildup in brain tissue. Indeed, GS is thought to play a key role in preventing hyperexcitability, coma, and eventual death under ammonia-loading circumstances (Arillo et al., 1981; Schenone et al., 1982; Wicks and Randall, 2002a; Eddy, 2005; Walsh et al., 2007; Wright et al., 2007; Sanderson et al., 2010). When MSOX was injected into trout, brain [T_{Amm}] levels and ventilation increased in parallel, with the largest elevations in both occurring in the chronic HEA animals. Furthermore ventilatory increases in response to acute ammonia challenges were greater than in saline-injected control animals. Notably, plasma [T_{Amm}] levels did not change. Again, the evidence is circumstantial but it points to central chemosensitivity to ammonia residing at the level of the brain tissue itself. Clearly, the next step should be to apply ammonia directly to the brain using *in situ* or *in vitro* preparations while monitoring real or fictive breathing (e.g. Wilson et al., 2000).

Zhang et al. (2013) also investigated the potential involvement of cerebral Rh proteins in these phenomena, because earlier, Nawata et al. (2007) had demonstrated that Rhbg and Rhcg1 mRNAs were expressed in trout brain, and that their expression levels decreased after 48 h of HEA exposure. Presumably, the normal presence of Rh channels allows the brain to quickly detect plasma ammonia levels, while their down-regulation could serve as an adaptive measure, decreasing brain permeability to ammonia during chronic HEA exposure, thereby lessening the stimulus for hyperventilation. However, somewhat surprisingly, in trout chronically exposed to HEA for 30+ days, the mRNA expression levels of two of the Rh genes (Rhbg and Rhcg2) were upregulated (1.7-2.1 fold), while a similar change in a third one (Rhcg1) was not significant. However, by taking brain and plasma pH and [T_{Amm}] gradients into account, Zhang et al. (2013) calculated that there is normally a positive P_{NH3} gradient from brain to plasma which facilitates ammonia washout. After long term HEA exposure, this gradient disappears, even though plasma [T_{Amm}] levels have been lowered through active ammonia excretion across the gills by activation of the branchial Rh-mediated "Na^+/NH_4^+ exchange complex" (see Introduction; Tsui et al., 2009; Kolarevic et al., 2012; Sinha et al., 2013). Possibly, the upregulation of Rh channels in the brain at this time may reflect a similar response to that in the gills so as to achieve active ammonia export from brain to blood plasma. Future studies should investigate whether such a system is present in the blood-brain barrier.

3.7 The potential roles of Rh proteins and Task-1 K^+ channels in ammonia chemosensitivity of NECs

Our most recent work has used "single cell" qPCR techniques (see Materials and Methods) to examine mRNA expression of Rh proteins in the NECs of the 1st gill arch. Comparisons have been made to the two other major (and more abundant) gill cell types, mitochondria rich cells (MRCs) and pavement cells (PVCs), and to the response of the whole gill. We hypothesized that if the NECs are to serve as rapidly responding peripheral chemoreceptors for ammonia, they would express Rh proteins to facilitate ammonia entry, and furthermore that these might be downregulated after chronic HEA exposure, coincident with the desensitization of the ventilatory response to ammonia (see Section 3.4). We also examined other transport genes (H-ATP, NKA, and NHE2 as components of the ammonia excretion mechanism, see Section 1) and selected marker genes (serotonin transporter (ST) as a potential marker gene for NECs because of their high serotonin content (see Section 3.5); cytochrome c oxidase 6a (CO6A) as a potential marker gene for MRCs because of their high mitochondria content; and TASK-1 as a background K^+ channel potentially involved in the chemoreception of respiratory gases - see Section 3.5). We hypothesized that ST and TASK-1 might also be down-regulated as part of the desensitization seen during chronic HEA exposure.

3.7.1 mRNA responses in the whole 1st gill arch

Three of these genes (H-ATP, NKA, and NHE2) could only be detected at the whole gill level (Fig. 7B), while the other six were detected in both the whole gill (1st arch) and "single cell" analyses (Figs. 7 and 8). Clearly, chronic HEA exposure had marked effects on mRNA expression in the whole 1st gill arch, with significant changes in 6 of the 9 selected genes (Fig. 7).

All of the three Rh genes (basolateral Rhbg, and apical Rhcg1 and Rhcg2) were expressed at significantly higher levels in trout chronically exposed to HEA than in control trout, by 3.0, 2.9 and 2.0 -fold respectively (Fig. 7A), a similar pattern to that in the brain (see Section 3.6). Similar increases in branchial Rhcg1 and Rhcg2 expression (Rhbg not measured) were seen in Atlantic salmon exposed to HEA for an even longer period (15 weeks; Kolarevic et al., 2012), whereas only Rhbg and Rhcg2 expression increased in rainbow trout exposed *in vivo* to HEA for 12 h – 168 h (Nawata et al., 2007; Wood and Nawata, 2011; Sinha et al., 2013) or in trout gill cells *in vitro* cultured in HEA (Tsui et al., 2009), though all three were upregulated in trout infused with 140 mmol L^{-1} NH_4HCO_3 for 12 h (Nawata and Wood, 2009) . There were no significant changes in NKA expression (Fig. 7B), in agreement with all of these previous reports except for the internal ammonia infusion study of Nawata and Wood (2009), where NKA was upregulated. Branchial expressions of two other components of the "Na^+/NH_4^+ exchange complex" (see Section 1) also did not change significantly in the present study (Fig. 7B), whereas variable responses ranging from upregulation (e.g. NHE2 in Tsui et al., 2009, Nawata and Wood, 2009, and Sinha et al., 2013 ; H-ATP in Nawata et al., 2007, Tsui et al., 2009, Wood and Nawata, 2011, and Sinha et al., 2013) to downregulation (H-ATP in Kolarevic et al., 2012) to no change (e.g. NHE2 in Nawata et al., 2007 and Wood and Nawata, 2011) have been reported in other studies on salmonids. Overall, these results support the model of active ammonia excretion against a gradient by the Rh system of the gills in fish subjected to chronic ammonia

626 loading, and discrepancies probably result from differences in the extent, duration, and nature
627 (internal versus external loading) of the treatments.
628 Chronic HEA exposure had no effect on the mRNA expression of the serotonin transporter
629 ST, but caused a significant doubling of expression of CO6A at the level of the whole gill (Fig.
630 7C). This suggests an increase in gill mitochondrial content and/or activity in chronically
631 exposed animals. In contrast, expression of the background K^+ channel TASK-1 was
632 significantly reduced by about 70% as a result of chronic HEA exposure (Fig. 7C), suggesting a
633 marked change in membrane conductive properties . To our knowledge, there are no previous
634 studies on how these three genes respond to environmental perturbations in fish gills.
635
636 *3.7.2 mRNA responses in specific gill cell types*
637 The gill is a complex organ consisting of multiple cell types, so the goal of the "single
638 cell" qPCR analyses (Fig. 8) was to see whether the gene expression changes seen at the whole
639 gill level could be localized to the three cell types of particular interest (NECs, MRCs, PVCs).
640 Looking first at expression levels under control conditions, neither of the potential
641 marker genes (ST for NECs, CO6A for MRCs) were differentially expressed among the three
642 cells, and the same was true of TASK-1. However, not surprisingly, absolute expression levels
643 per cell were about an order of magnitude higher for CO6A and TASK-1 than for ST, as might
644 be expected based on general versus specific functions of these proteins. In contrast, mRNA
645 expression of two of the three Rh proteins did differ among cell types under control conditions,
646 with a higher Rhcg1 signal per cell in NECs and a lower Rhcg2 signal per cell in MRCs.
647 Furthermore, absolute expression levels per cell of Rhbg and Rhcg2 were about an order of
648 magnitude higher than those of Rhcg1. Overall these data confirm our hypothesis that Rh
649 proteins are expressed in NECs. The cell-specific expression pattern fits with the conclusions of
650 Nawata et al. (2007) that while basolateral Rhbg is generally distributed, Rhcg2, rather than
651 Rhcg1, is the dominant apical isoform in trout gills, localized primarily in the highly abundant
652 PVCs rather than in the less abundant MRCs.
653 The present finding that the control expression level of Rhcg1 was higher in NECs
654 relative to the other two cell types (Fig. 8) is therefore of some interest. It is also notable that the
655 mRNA expression level of Rhcg1 fell below detection limits in the NECs after chronic HEA
656 exposure, while that of Rhbg increased about 6-fold (Fig. 8). While the decline in Rhcg1 was
657 common to all three cell types, the Rhbg increase occurred only in the NECs. Earlier, Nawata et
658 al. (2007) reported that Rhbg expression was increased in PVCs and remained constant in MRCs
659 in trout exposed to HEA for only 48 h. However, in that study, PVCs were obtained from the
660 1.03–1.05 g ml^{-1} interface of the discontinuous Percoll density gradient, whereas in the present
661 study we found that the majority of NECs were also distributed in this interface. Therefore, the
662 'PVCs' of Nawata et al. (2007) were mostly likely a combination of PVCs + NECs, and based on
663 this, the increased Rhbg expressions in shorter term (Nawata et al., 2007) and longer term HEA
664 (present study) were consistent, with the response occurring in the NECs. Overall, the unique
665 pattern of Rh gene expression in NECs suggests that Rh glycoproteins may have a different

function in these cells (channels for ammonia entry to permit chemoreception?) than in the other cell types, and the loss of Rhcg1 expression could contribute to the desensitization of the ventilatory response to ammonia after chronic HEA exposure (see Section 3.4).

The loss of Rhcg1 expression with HEA in all three cell types (Fig. 8) is difficult to reconcile with the whole gill data, where Rhcg1 expression increased significantly (Fig. 7A). This discrepancy suggests a need to examine Rhcg1 expression in other gill cell types (e.g. vascular endothelium, pillar cells, neurons, mucus cells). Earlier, Nawata et al. (2007) reported that Rhcg1 expression remained constant in PVCs (i.e. NECs + PVCs) but dropped to half in MRCs in trout exposed to short-term HEA. The increase in Rhcg2 expression in all three cell types, though significant only in MRCs (Fig. 8), was consistent with the significant increase seen in the whole of the 1st gill arch (Fig. 7A). Nawata et al. (2007) reported that during 12-48 h HEA exposure, Rhcg2 expression in PVCs (i.e. PVCs + NECs) increased over 10-fold, but remained constant in MRCs. As noted earlier, these differences with respect to the chronic HEA pattern may reflect time-dependent changes, as reported by Sinha et al. (2013) for ammonia excretion and Na^+ uptake.

As with Rhcg1, there was a general loss or severe down-regulation of the mRNA signals for ST and CO6A in all three cell types after chronic HEA exposure (Fig. 8), which did not agree with the constancy (ST) or up-regulation (CO6A) in the whole gill (Fig. 7C). The decrease in ST expression fits with the observation of smaller, less responsive NECs (see Section 3.5), but again, these discrepancies highlight the need to examine other cell types in the gills.

There were small, non-significant depressions in TASK-1 expression in PVCs and NECs, with a significant decrease in the MRCs (Fig. 8). Overall, this agrees qualitatively with the significant decline seen in TASK-1 expression at the whole gill level (Fig. 7C), and again could be consistent with a general desensitization of gill cell membranes as a result of chronic HEA exposure. TASK-1 was the first two pore-domain potassium channel (K_{2P}) to be identified that was able to produce a current presenting all background channel properties (Duprat et al. 1997). In mammals, TASK-1 channels are thought to be involved in numerous physiological processes, particularly in respiratory control due to their high pH sensitivity and their presence in carotid bodies (Buckler et al. 2000; Patel and Honore, 2001). There have been no direct reports on the specific function of TASK-1 in fish, though both O_2 (Jonz et al., 2004) and CO_2 sensing (Quin et al., 2010; Abdallah et al., 2012) in zebrafish gill NECs appear to be mediated by inhibition of a background K^+ conductance of unknown origin. The present results confirm that TASK-1 is expressed in trout NECs, the fish homologue of carotid body glomus Type 1 cells. However, it remains an open question whether TASK-1 channels are involved in ammonia sensing, because TASK-1 channels are also expressed in the other two cell types, yet *in vitro*, only NECs respond to high extracellular $[K^+]$ or high $[NH_4^+]$ with a surge in $[Ca^{2+}]_i$ (Section 3.5; Zhang et al., 2011).

4.0 Conclusions

Ammonia, the third respiratory gas, is a specific stimulus for ventilation in both teleost and elasmobranch fish, and this is probably adaptive in increasing breathing in circumstances

where internal ammonia levels are naturally elevated, such as after feeding and exhaustive exercise. Fish hyperventilate in response to internal elevations in ammonia, and in trout the detection system involves peripheral chemoreceptors in the 1st (primarily) and 2nd gill arches. These ammonia receptors are very probably the NECs, which exhibit a rise in intracellular $[Ca^{2+}]_i$ in response to a physiologically relevant extracellular ammonia signal. There is strong circumstantial evidence that central chemoreceptors in the brain are also involved. It remains unclear whether the gill chemoreceptors also respond directly to elevated waterborne ammonia (HEA), which would be of questionable adaptive significance. Certainly, the ventilatory response to external ammonia is much slower than that to internal ammonia, especially in elasmobranchs. Chronic HEA exposure causes a loss of ventilatory sensitivity to ammonia, and this is correlated with a loss of responsiveness of brain ammonia levels, increases in the expression of Rh proteins (ammonia channels) in the brain and whole 1st gill, and changes in the structure, physiology, and gene expression of the NECs. Like the other two major gill cell types (MRCs and PVCs), NECs of the 1st gill arch express TASK-1 background K^+ channels, and at least three Rh proteins, of which one (Rhcg1) is at a higher level than in the other cells. Rhcg1 expression falls below detection limits in the NECs after chronic HEA exposure, while that of Rhbg increases markedly. These relatively new data on ammonia effects open up a new aspect to an area, control of breathing, where Bill Milsom continues to lead the field.

5.0 Acknowledgements

We thank Drs. Colin Nurse (McMaster University) and Michael Jonz (University of Ottawa) for very helpful advice in the trout NEC work, and the staff of Bamfield Marine Sciences Centre for their assistance in the dogfish studies. Funded by an NSERC Discovery grant to CMW, who was supported by the Canada Research Chair Program, by an International Collaboration Grant (IWS-BOF) from the Research Council of the University of Antwerp to GDB and CMW, and by grants from the National Natural Science Foundation of China (41376161) and 100 Talents Program of the Chinese Academy of Sciences to LZ.

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Figure Legends

Figure 1. Responses of Rhcg2 mRNA expression in the gills of adult rainbow trout to treatments which elevate internal ammonia levels by increased endogenous generation. (A) Exhaustive exercise. Rainbow trout (163-330 g; n=6 for each group) were exercised to exhaustion by chasing them in a circular tank (800 L) for 6 min at 15^{o}C. Fish were sacrificed at 3, 6, 9, and 12 hours post-exercise and gills were analyzed for Rhcg2 mRNA expression. (B) Feeding. Rainbow trout (179-209 g; n=6 for each group) were fed to satiation at 15^{o}C and subsequently sacrificed at 6, 12, 24, and 36 hours. The gills were analyzed for Rhcg2 mRNA expression. Means+ 1 SEM. Asterisks represent a significant increase from the control values (unexercised or fasted fish respectively). Previously unpublished data of C.M. Nawata and C.M. Wood.

Figure 2. Responses of rainbow trout to ammonia. (A) Relative ventilatory amplitude and (B) relative ventilatory frequency measured before and at various times after intra-arterial injections (3.9 ml kg^{-1}) of $NaHCO_3$ (140 mmol l^{-1}) or NH_4HCO_3 (140 mmol l^{-1}) (C) Relative ventilatory amplitude and (D) relative ventilatory frequency measured before and at various times during 24-h ammonia infusion (3.2 ml kg^{-1} h^{-1}) with either Cortland saline, $(NH_4)_2SO_4$ (70 mmol l^{-1}) , or NH_4HCO_3 (140 mmol l^{-1}) Means ± 1 SEM. Asterisks represent a significant increase from the pre-treatment control values. Data from Zhang and Wood (2009) where methodological details are provided.

Figure 3. Responses of dogfish sharks to ammonia. (A) Ventilatory amplitude and (B) ventilatory frequency measured before and at 2 min after intra-arterial injections (1.1ml kg^{-1}) of NaCl, $NaHCO_3$, NH_4HCO_3, Na_2SO_4, and $(NH_4)_2SO_4$ (all 500 mmol l^{-1}). (C) Ventilatory amplitude and (D) arterial plasma [T_{Amm}] measured before and at various times during exposure to HEA (1500 μmol l^{-1}). Note, there were no changes in ventilatory frequency in this treatment. Means + 1 SEM. Asterisks represent a significant increase from the "before" values. Data from De Boeck and Wood (2014) where methodological details are provided.

Figure 4. The effect of chronic exposure to HEA (250 μmol l^{-1} $(NH_4)_2SO_4$ for 1+ months) on the responses of rainbow trout to acute challenges with HEA. (A) Ventilatory amplitude. (B) Ventilatory frequency. Data from control animals are shown as solid circles; data from chronic HEA animals are shown as open circles. Asterisks represent significant changes relative to the data in the 0 $(NH_4)_2SO_4$ exposure period. Means ±1 SEM. Data from Zhang et al. (2011) where methodological details are provided.

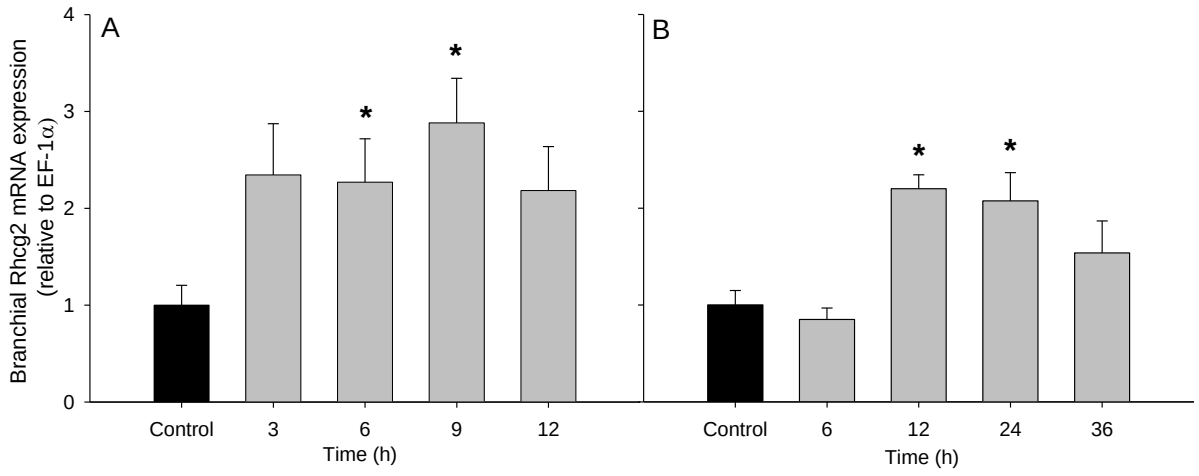
Figure 5. A comparison of two types of $[Ca^{2+}]_i$ response in NECs from rainbow trout gills to short term perfusion challenges (marked with horizontal lines) with high K^+ (30 mmol l^{-1} KCl) or high ammonia (1 mmol L^{-1} NH_4Cl). (A) A typical Type A slow change of $[Ca^{2+}]_i$ during and after the high ammonia perfusion. (C) A typical Type B fast change of $[Ca^{2+}]_i$ during and after the high ammonia perfusion. Note the rapid responses to high K^+ in both instances. PVCs never

responded as illustrated by the example in panel (A). (B) A summary of background and peak $[Ca^{2+}]_i$ in response to high K^+ and high ammonia in NECs showing Type A responses from control trout. (D) A comparable summary in NECs showing Type B responses from control trout. Means +1 SEM. Within each panel, means not sharing the same letter are significantly different from one another. Panels (A) and (C) show previously unpublished data of L. Zhang, M. Jonz, C.A., Nurse, and C.M. Wood. Panels (B) and (D) show data from Zhang et al. (2011) where methodological details are provided.

Figure 6. Various aspects of the relationship between ventilation index (= the product of relative ventilatory amplitude and relative ventilatory frequency), plasma [T_{Amm}], and brain [T_{Amm}] in control rainbow trout. (A) Correlations between ventilation index and plasma [T_{Amm}], and (B) between ventilation index and brain [T_{Amm}] in response to elevated waterborne HEA exposure. (C) Time-dependent responses of ventilatory index and (D) time-dependent responses of brain [T_{Amm}] to waterborne HEA (500 μmol L^{-1} $(NH_4)_2SO_4$) in control trout in a time series exposure over 60 min. In panels (A) and (B), dots indicate simultaneous measurements in different individual trout. In panels (C) and (D), data are means + 1 SEM, and asterisks indicate significant increases relative to the mean before the $(NH_4)_2SO_4$ exposure period. Data from Zhang et al. (2013), where methodological details are provided.

Figure 7. Gene expression in the filaments of the 1st gill arch in control trout and trout subjected chronic HEA exposure (250 μmol l^{-1} $(NH_4)_2SO_4$ for 1+ month). (A) Rh genes, (B) other genes possibly related to ammonia transport and (C) potential cell marker genes. Means + SEM (N=6). Asterisks indicate significant differences between control and chronic HEA means. Previously unpublished data of L. Zhang, C.M. Nawata and C.M. Wood.

Figure 8. Relative mRNA expression per single cell in isolated pavement (PVC), neuroepithelial (NEC), and mitochondrial-rich cells (MRC) from the filaments of the 1st gill arch in control trout and trout subjected to chronic HEA exposure (250 μmol l^{-1} $(NH_4)_2SO_4$ for 1+ month). Means + SEM (N=6 fish). Different letters above the solid bars indicate significant differences among the three types of cells in control trout. Asterisks above the open bars indicate significant differences between control and HEA trout. BDL is "below detection limit". Previously unpublished data of L. Zhang, C.M. Nawata and C.M. Wood.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Branchial Rhcg2 mRNA expression
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Fig. 1

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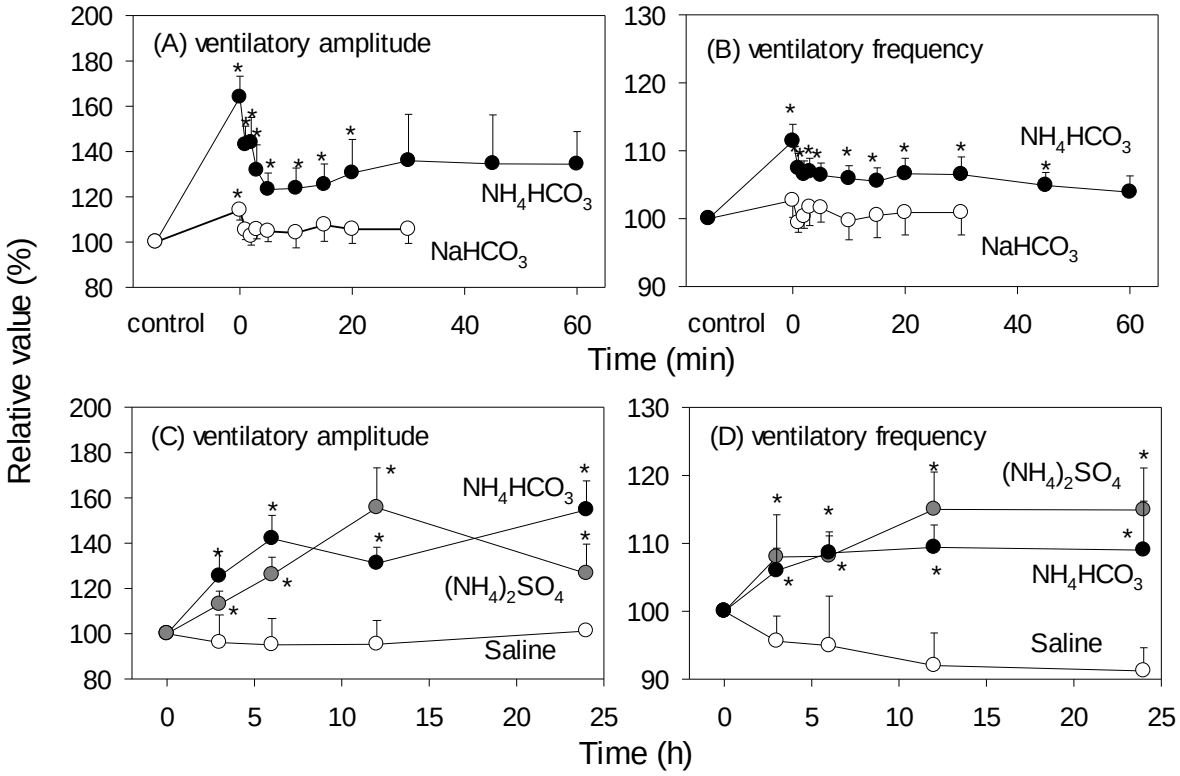

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1141 Fig. 2

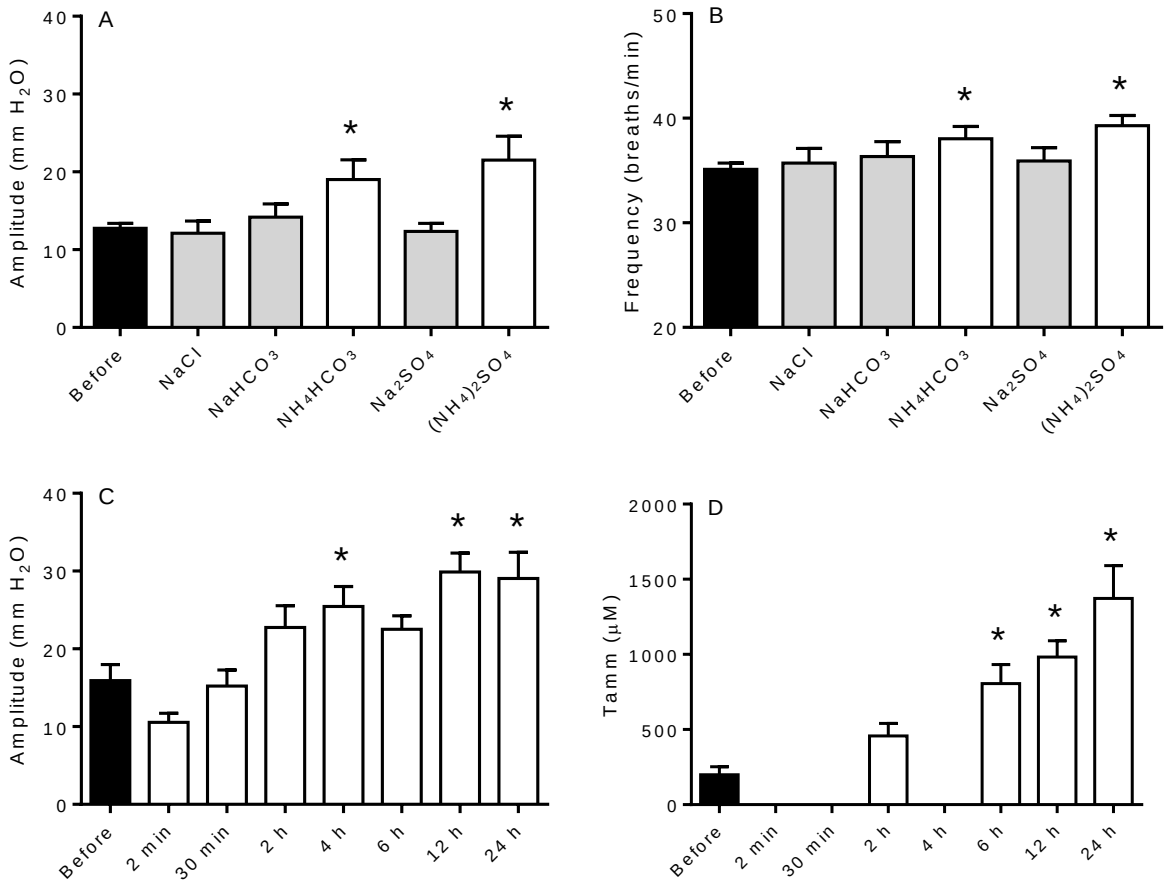
A
Amplitude (mm H_2O)
40
30
20
10
0
Before
NaCl
$NaHCO_3$
NH_4HCO_3
Na_2SO_4
$(NH_4)_2SO_4$
B
Frequency (breaths/min)
50
40
30
20
C
2 min
30 min
2 h
4 h
6 h
12 h
24 h
D
Tamm (μM)
2000
1500
1000
500

Fig. 3.

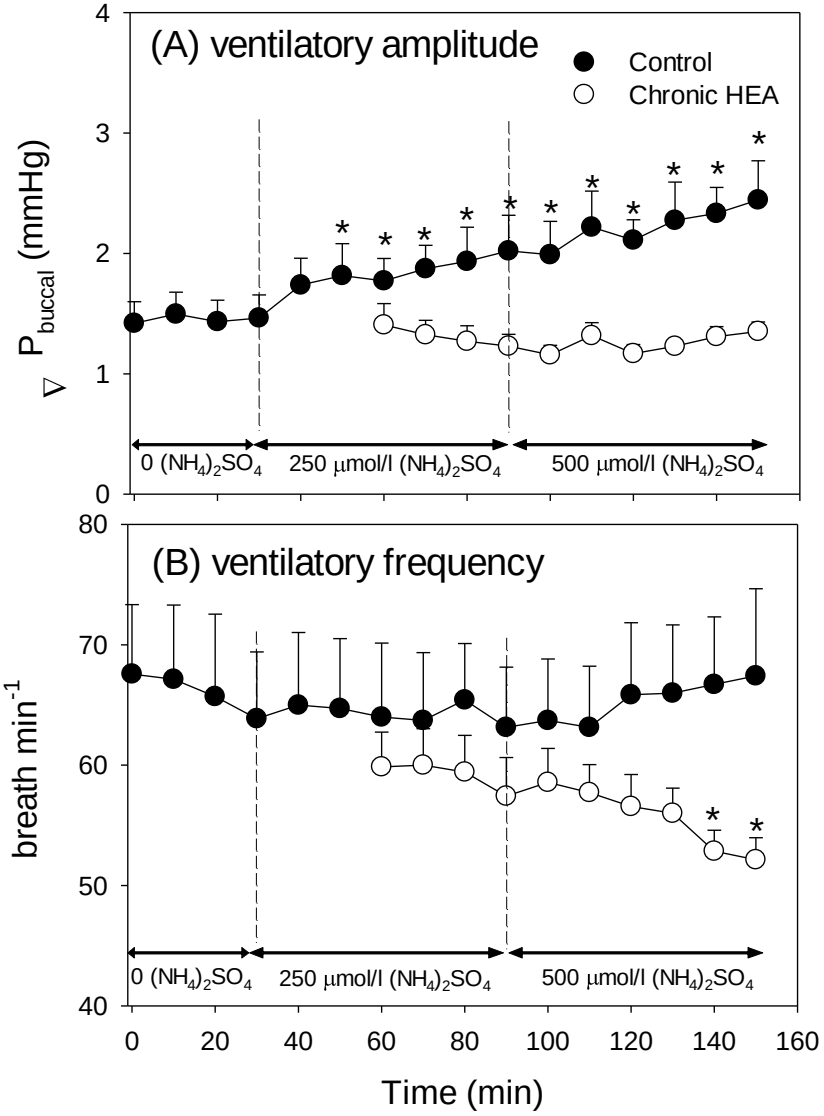
(A) ventilatory amplitude
Control
Chronic HEA
$\dot{V}$ P_{buccal} (mmHg)
0 $(NH_4)_2SO_4$
250 μmol/l $(NH_4)_2SO_4$
500 μmol/l $(NH_4)_2SO_4$
(B) ventilatory frequency
breath min^{-1}
Time (min)

Fig. 4.

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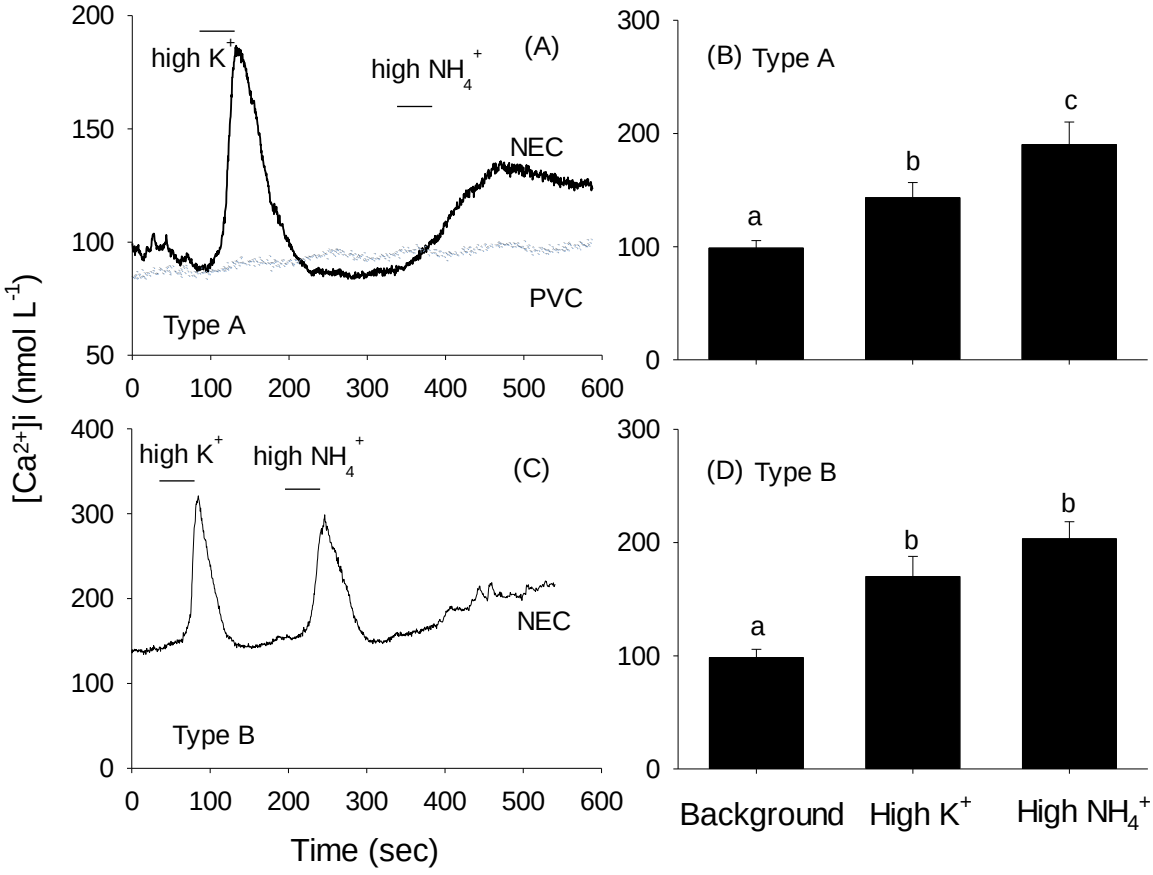

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1164 Fig. 5

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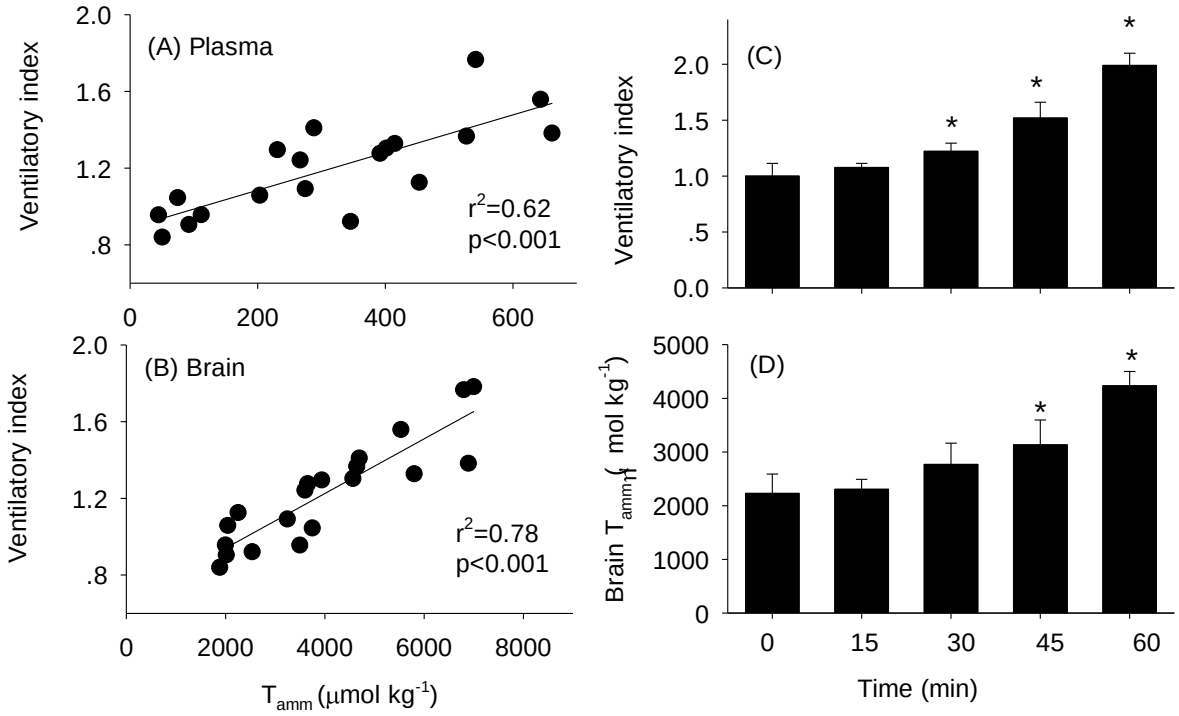

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1170 Fig. 6

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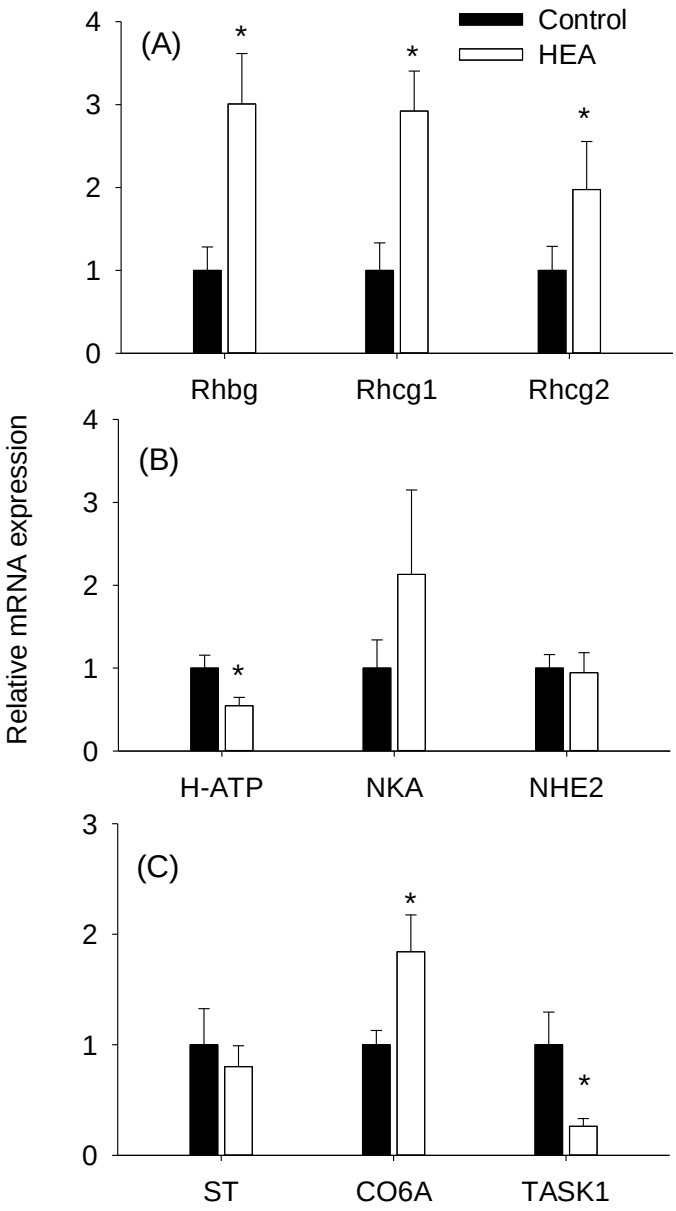

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1177 Fig. 7

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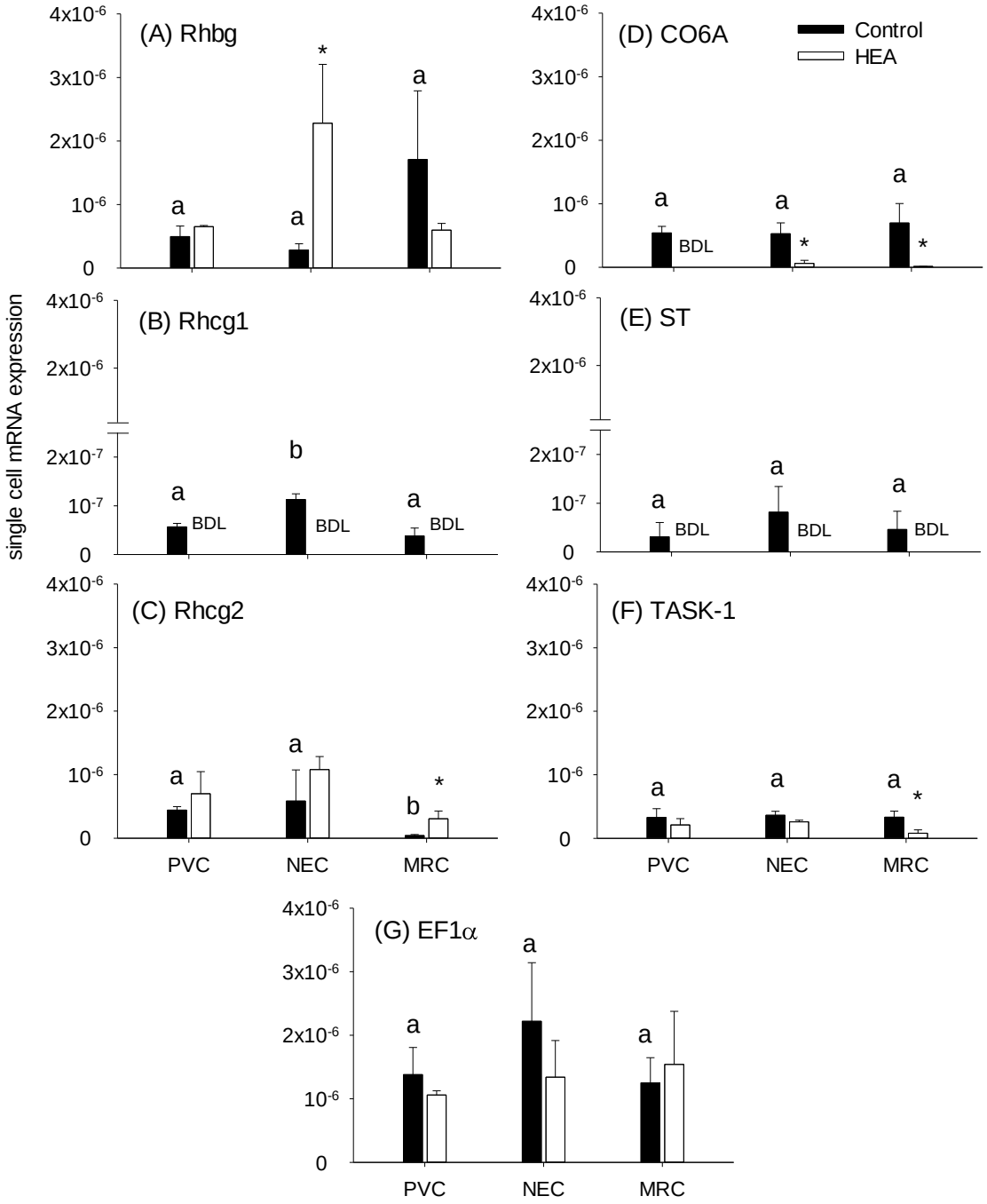

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1181 Fig. 8

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