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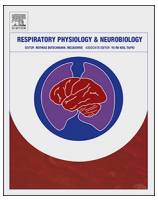

Is ammonia excretion affected by gill ventilation in the rainbow trout *Oncorhynchus mykiss*?

Junho Eom*, Sandra Fehsenfeld[1], Chris M. Wood

Department of Zoology, University of British Columbia, 6270 University Blvd., Vancouver, BC, V6T 1Z4 Canada

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ABSTRACT

Ammonia (NH_3 + NH_4^+) is the major nitrogenous waste in teleost fish. NH_3 is also the third respiratory gas, playing a role in ventilatory control. However it is also highly toxic. Normally, ammonia excretion through the gills occurs at about the same rate as its metabolic production, but the branchial transport mechanisms have long been controversial. An influential review in this journal has claimed that ammonia excretion in fish is probably limited by diffusion rather than by convection, so that increases in ventilation would have negligible effect on the rate of ammonia excretion. Why then should elevated plasma ammonia stimulate ventilation? The diffusion-limitation argument was made before the discovery of Rhesus (Rh) glycoproteins and the associated metabolon in the gills, which serve to greatly increase branchial ammonia permeability under conditions of ammonia loading. Therefore, we hypothesized here that (i) in accord with the diffusion-limitation concept, changes in ventilation would not affect the rate of ammonia excretion under conditions where branchial Rh metabolon function would be low (resting trout with low plasma ammonia levels). However, we also hypothesized that (ii) in accord with convective limitation, changes in ventilation would influence the rate of ammonia excretion under conditions where diffusion limitation was removed because branchial Rh metabolon function would be high (ammonia-loaded trout with high plasma ammonia levels). We used variations in environmental O_2 levels to manipulate ventilation in trout under control or ammonia-loaded conditions – i.e. hyperventilation in moderate hypoxia or hypoventilation in moderate hyperoxia. In accord with hypothesis (i), under resting conditions, ammonia excretion was insensitive to experimentally induced changes in ventilation. Ammonia-loading by NH_4HCO_3 infusion for 30h + increased the gill mRNA expressions of two key metabolon components (Rhcg2, V-H^+-ATPase or HAT), together with a 7.5-fold increase in plasma ammonia concentration and a 3-fold increase in ammonia excretion rate. In accord with hypothesis (ii), in these fish, hypoxia-induced increases in ventilation elevated the ammonia excretion rate and lowered plasma ammonia, while hyperoxia-induced decreases in ventilation reduced the ammonia excretion rate, and elevated plasma ammonia concentration. We conclude that under conditions of natural ammonia loading (e.g. meal digestion, post-exercise recovery), diffusion-limitation is removed by Rh metabolon upregulation, such that the stimulation of ventilation by elevated plasma ammonia can play an important role in clearing the potentially toxic ammonia load.

1. Introduction

Since ammonia excretion through the gills of fish was first measured by Homer Smith (1929), the transport mechanisms have been controversial. Ammonia movement has been variously explained by simple diffusion of NH_3 along P_{NH3} gradients, by NH_4^+ diffusion along electrochemical gradients, by direct exchange of Na^+ for NH_4^+ (first suggested by August Krogh, 1938), or NH_4^+ movement via K^+ channels and transporters (for reviews see Evans and Cameron, 1986; Wood, 1993; Wilkie, 1997, 2002; Evans et al., 2005; Fehsenfeld and Weihrauch, 2016). However, since 2000, the paradigm has shifted with the discovery that in mammals, Rh glycoproteins can serve as selective channels which greatly facilitate the diffusion of ammonia across cell membranes (Marini et al., 2000; Westhoff et al., 2002; Ripoche et al., 2004). Weihrauch et al. (2004) first showed that homologues of these Rh proteins are expressed in the gills of crabs, then Nakada et al. (2007a, b), Hung et al. (2007), and Nawata et al. (2007) demonstrated their presence in the gills of teleost fish. Their importance in ammonia excretion was reinforced by the discovery that the mRNA expression levels of Rh proteins in the gills were upregulated by external (Nawata

* Corresponding author.
E-mail addresses: june@zoology.ubc.ca (J. Eom), Sandra.Fehsenfeld@uqar.ca (S. Fehsenfeld), woodcm@zoology.ubc.ca (C.M. Wood).
[1] Sandra Fehsenfeld's current address: Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, Rimouski, Canada G5L 3A1.

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et al., 2007; Wood and Nawata, 2011) or internal ammonia loading (Nawata and Wood, 2009) in trout, and that molecular knockdown of Rh protein expression severely reduced ammonia excretion in larval zebrafish (Braun et al., 2009). The ammonia transport function of trout Rh proteins was conclusively proven by in vitro oocyte expression studies (Nawata et al., 2010b).

The Rh proteins now play a key role in current models for branchial ammonia excretion in teleost fish (Tsui et al., 2009; Wright and Wood, 2009, 2012; Weihrauch et al., 2009; Ito et al., 2013). While some ammonia may pass by simple diffusion through the lipoprotein cell membranes, a significant portion may also pass by facilitated diffusion through Rh protein channels, and this fraction is thought to increase when Rh protein expression is induced by ammonia loading. Indeed active outward transport of ammonia by an Rh-mediated metabolon linked to Na^+ uptake and acid excretion (Wright and Wood, 2009, 2012) has been proposed to explain the increased ammonia excretion after feeding, exercise, or ammonia infusion, as well as the restoration of ammonia excretion when the normal outwardly directed P_{NH3} and electrochemical NH_4^+ gradients are reversed by high external ammonia (Nawata et al., 2007; Nawata and Wood, 2009; Zimmer et al., 2010; Wood and Nawata, 2011; Sinha et al., 2013; Zhang et al., 2015).

Hyperventilation in response to high external ammonia has been frequently observed in fish, and often interpreted as a general stress response to a toxicant (e.g. Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph, 1996). However, there is evidence that ventilation is also stimulated by elevation of internal ammonia levels (Hillaby and Randall, 1979; McKenzie et al., 1993). More recent studies on trout have proven that this effect can occur independently of changes in blood O_2, CO_2, and acid-base status (Zhang and Wood, 2009), and that it is mediated at least in part by neuroepithelial cells (NECs) on the 1st and 2nd gill arches (Zhang et al., 2011, 2015). Indeed, as ammonia is produced at about 10–20 % the rate of CO_2, it is essentially the third respiratory gas in ammoniotelic teleost fish (Randall and Ip, 2006). Therefore, it makes sense that ammonia should be detected by the same receptor systems as for O_2 and CO_2 (Zhang et al., 2015; Perry and Tzaneva, 2016). Central detection by sensing of brain ammonia buildup may also be involved (Zhang et al., 2013). Blood ammonia levels are markedly elevated after feeding (Bucking and Wood, 2008; Wicks and Randall, 2002; Zimmer et al., 2010) and exhaustive exercise (Wood, 1988; Mommsen and Hochachka, 1988; Wang et al., 1994), so ammonia-stimulated hyperventilation would be a very appropriate response if it helped to increase ammonia excretion.

However in a seminal review, Randall and Ip (2006) argued that ammonia excretion in fish is probably limited by diffusion rather than by blood perfusion or water convection, so "*that increases in ventilation would have negligible effect on the rate of ammonia excretion, and therefore represent a non-physiological response on the part of the fish*". This seems counterintuitive. Diffusion limitation would mean that only the ammonia gradients (PNH_3 and/or NH_4^+ electrochemical gradients) and the effective permeability of the gills to ammonia would affect excretion rate; convective flow of blood or water would not have any influence. However, it is important to note that this was a theoretical prediction formulated in the absence of any evidence whether (or not) changes in ventilation affected ammonia excretion, and it was made just one year before the discovery of Rh proteins in fish gills (Nakada et al., 2007a, b; Hung et al., 2007; Nawata et al., 2007). If Rh proteins serve to facilitate ammonia movement through the gill epithelium, diffusion limitation may not apply, and this argument may be invalid. However, to our knowledge, this idea has never been experimentally tested.

In the present study, we evaluated two hypotheses in adult rainbow trout. The first was that in accord with the diffusion-limitation concept of Randall and Ip (2006), changes in ventilation would not affect the rate of ammonia excretion under conditions where branchial Rh protein expression would be minimal (fasted, resting fish with low plasma ammonia levels). The second hypothesis we tested was the convection-limitation hypothesis where changes in ventilation would influence the rate of ammonia excretion under conditions where branchial Rh protein expression would be high (ammonia-loaded fish with high plasma ammonia levels), i.e. no diffusion limitation for ammonia.

Ammonia loading was created by chronic intravascular infusion of ammonia (140 mM NH_4HCO_3), a treatment previously shown to up-regulate branchial Rh protein expression in trout (Nawata and Wood, 2009), and ventilation was experimentally manipulated using moderate hypoxia (50 % air saturation) to achieve hyperventilation, and hyperoxia (> 300 %) to induce hypoventilation. Cortisol levels were measured, as there is circumstantial evidence that they contribute to regulation of the branchial Rh metabolon (Tsui et al., 2009; Nawata and Wood, 2009). Ammonia excretion was monitored throughout, plasma ammonia concentrations were measured, and mRNA abundances of three key genes of the Rh metabolon (Rhcg2, NHE2, and V-H^+-ATPase or HAT) were quantified in the gills using quantitative real-time PCR (qPCR).

2. Materials and methods

2.1. Experimental animals

Rainbow trout (*Oncorhynchus mykiss,* 203.4 ± 6.5 g, 24.9 ± 0.3 cm) were transported from Miracle Springs Inc. Trout Hatchery (Fraser Valley, BC, Canada) to aquatic facilities at the University of British Columbia. There, the fish were held in 90-L glass aquaria served with flow-through charcoal-filtered dechlorinated Vancouver City tap water ($[Na^+]$, 0.17 mmol L^{-1}; $[Cl^-]$, 0.21 mmol L^{-1}; hardness, 30 mg L^{-1} as $CaCO_3$; pH 7.0; temperature, 6.5–9.0 °C). The fish were fed with commercial pellets (EWOS, Surrey, BC, Canada) but were fasted for a week prior to experiment to minimize the influence of feeding on ammonia metabolism. The fish were treated in accordance with the University of British Columbia animal care protocol #A14-0251, and the guidelines of the Canadian Council on Animal Care. At the end of experiments, the fish were euthanized by an overdose (120 mg L^{-1}) of tricaine methanesulfonate (MS-222, Western Chemicals Inc., Ferndale, WA, USA; pH was adjusted to ~7.0 with 1 mmol L^{-1} of NaOH).

2.2. Fish operations

In order to monitor ventilatory pressure amplitude and frequency in both Series I and Series II, trout were implanted with buccal catheters as described by Holeton and Randall (1967), with minor modifications. In brief, fish were anesthetized in 60 mg L^{-1} MS-222 (pH neutralized to ~7.0 as described above), and irrigated on an operating table. A hole was drilled in the roof of the mouth using an 18 G needle, taking care to avoid the nares, and a 3-cm sleeve of PE160 polyethylene tubing (Clay-Adams, Sparks, MD, USA; OD 1.57 x ID 1.14 mm), flared at the mouth end, was inserted. A 30-cm length of PE50 tubing (OD 0.97 x ID 0.58 mm), again flared at the mouth end, was fitted through the PE 160 sleeve and cemented to it using cyanoacrylate glue (Krazy Glue, High Point, NC, USA). Silk suture was used to hold the catheter in place by knots at the outside and stitches through the dorsal fin of the fish.

In both Series I and II, a pair of enameled copper wires was implanted externally, one on each side of the pericardium, for measurement of the electro-cardiogram (ECG) as an indicator of heart rate, to give a bipolar recording as described by Roberts (1975). The wires were anchored in place by a suture placed on the dorsal fin of the fish.

In Series II, trout were additionally fitted with arterial catheters for ammonia infusion, using a modification of the method of Soivio et al. (1972). A second PE160 sleeve was fitted through another hole in the roof of the mouth next to buccal catheter. The dorsal aorta was cannulated between the 2nd and 3rd gill arches using a 30-cm length of PE50 tubing, cut sharp at the insertion end, and filled with Cortland's saline (124 mmol L^{-1} NaCl, 5.1 mmol L^{-1} KCl, 1.6 mmol L^{-1} $CaCl_2$, 0.9 mmol L^{-1} $MgSO_4$, 11.9 mmol L^{-1} $NaHCO_3$, 3.0 mmol L^{-1}

NaH_2PO_4, 5.6 mmol L^{-1} glucose; Wolf, 1963) which was heparinized at 50 international units ml^{-1} with sodium heparin (Sigma-Aldrich, St. Louis, MO, USA). After penetration of the artery was confirmed by blood appearance in the tubing, the catheter was advanced ~5 cm deep into the dorsal aorta at a ~10° angle, almost parallel to roof of the mouth, and secured in place by a silk suture stitch. Then, the other end of the PE50 tubing was passed through the PE160 sleeve, drawn taught, sealed with a pin, and secured in place with cyanoacrylate glue and silk suture as described for the buccal catheter. After operation, fish were recovered overnight in individual black plexiglass chambers (length 38 x width 10 x height 18 cm) served with 500 ml min^{-1} of aerated flowing water.

2.3. Experimental series

In both series, the experiments were performed in the same individual black plexiglass chambers in which the fish had been allowed to recover from surgery. To allow rapid manipulation of PO_2 levels in the water, the bottoms of the chambers were fitted with multi-pored airline tubing which facilitated bubbling of air for normoxia, pure nitrogen for hypoxia, or pure oxygen for hyperoxia. During experiments, the chambers were aerated but water flow-through was suspended, and the water volume was set to 3.4 L. The individual chambers were submerged in a flowing reservoir to maintain water temperature between 6.5–9.0 °C.

2.3.1. Series I – Relationship between ventilation and ammonia excretion in fish under baseline conditions

This experiment was performed over two days (9 h per day) following recovery from surgery, and fish were randomly treated with either hypoxia on day 1 and hyperoxia on day 2, or hyperoxia on day 1 and hypoxia on day 2. Within each day, a 3-h control period under normoxia (bubbling with air), was followed by a 3-h experimental period (bubbling with nitrogen for hypoxia, or oxygen for hyperoxia), and then a 3-h recovery period under normoxia (bubbling with air again). Within each period, water PO_2, ventilation, and the rate of ammonia excretion were monitored, and between each period, a 0.5-h interval was used to flush the chambers to keep ammonia concentrations low, and to reset water PO_2 to the desired levels which were 100 % air saturation in normoxia and recovery, 50 % saturation in hypoxia, and > 300 % saturation in hyperoxia. These PO_2 levels were selected based on our preliminary experiments which demonstrated that they caused the desired changes in ventilation and could be tolerated indefinitely by the fish at this temperature (6.5–9.0 °C). Between days 1 and 2, the flow-through (500 ml min^{-1}) of normoxic water was restored.

2.3.2. Series II – Relationship between ventilation and ammonia excretion in ammonia-loaded fish

This series was also performed over two days following recovery from surgery, but each fish was subjected to only one experimental treatment (hypoxia or hyperoxia) on day 2. On day 1, control measurements of ventilation, and the rate of ammonia excretion were made on each fish over a 3-h period under baseline conditions in normoxia, as in Series 1, prior to the start of ammonia infusion. These values are designated as “control(-)” in Table 1 and Figs. 3 and 4, A–D. Immediately thereafter, the chamber was flushed, flow-through conditions were re-established, and a blood sample (200 μL, with saline replacement and re-infusion of red blood cells) was taken from the dorsal aortic catheter for the measurement of plasma total ammonia concentration. The sample was immediately centrifuged (5000 G x2 min), and the plasma was flash-frozen in liquid N_2 and stored at -80OC until analysis. Infusion with 140 mmol L^{-1} NH_4HCO_3 via the dorsal aorta was then started a rate of 2.98 ± 0.13 ml kg^{-1} h^{-1} using a Minipuls2 Peristaltic Pump (Gilson, Middleton, WI, USA) in order to raise the plasma ammonia concentration. This infusion was continued for 33 h – i.e. into day 2. Starting at 24 h, control measurements were made under normoxia in these ammonia-loaded fish. These values are designated “control(+)” in Table 1B and Figs. 3 and 4, A–D. The chambers were then flushed, a second blood sample was taken, and either hypoxic or hyperoxic conditions were established for a 3-h experimental period, and a final blood sample, followed by a 3-h normoxic recovery period. Ammonia loading was continued throughout, and measurements of water PO_2, ventilation, and ammonia excretion rate were made in all periods.

Table 1
Heart rates (beats sec^{-1}, via ECG) under baseline conditions (Series I) and ammonia-loading (Series II).

A. Heart rate in Series I (Baseline condition)

Control (-)	Treatment (-)	Recovery (-)
0.77 ± 0.02	Hypoxia: 0.65 ± 0.01*	0.76 ± 0.02
0.74 ± 0.03	Hyperoxia: 0.71 ± 0.04	0.74 ± 0.03

B. Heart rate in Series II (Ammonia-loaded)

Control (-)	Control (+)	Treatment (+)	Recovery (+)
0.87 ± 0.02	0.85 ± 0.05	Hypoxia: 0.60 ± 0.05*	0.98 ± 0.06
0.81 ± 0.04	0.75 ± 0.08	Hyperoxia: 0.70 ± 0.06	0.82 ± 0.04

Asterisks (*) indicate significant differences from A) control(-) in Series I or B) control(+) in Series II. Symbol of (-) represents baseline condition without ammonia-loading, while symbol of (-) represents baseline condition with ammonia-loading. Regardless of ammonia-loading, heart rate significantly decreased with hypoxia in both Series I (one-way ANOVA, $p = 0.0067$, $N = 6$) and Series II ($p = 0.0018$, $N = 7$). In hyperoxia, the heart rate did not change in either Series I ($N = 6$) or Series II ($N = 7$).

2.3.3. Series III – Influence of ammonia-loading on gene expression in the gills

The protocol was identical to that of Series II, except that the fish were kept in normoxia throughout. There were with two infusion treatments (3 ml kg^{-1} h^{-1}), 140 mmol L^{-1} NH_4HCO_3 and 140 mmol L^{-1} NaCl, ending at 33-h. At this time, the fish were rapidly euthanized as described above, the second gill arches were harvested, and the filaments were immediately cut off and transferred to ice-cold RNAlater™ (Ambion Inc., Austin, TX, USA) for storage at -20 °C.

2.4. Analytical techniques

For measurements of ventilatory frequency (sec^{-1}) and pressure amplitude (cm H_2O, as an indicator of stroke volume), the buccal catheter, filled with water, was connected to pressure transducer (Utah Medical Products, Midvale, UT, USA) which had been calibrated against a column of water. The analogue signal of the pressure transducer was amplified using an LCA-RTC amplifier (Transducer Techniques, Temecula, CA, USA) and converted into a digital signal via a PowerLab™ data integrity system (ADInstruments, Colorado Springs, CO, USA). The recorded digital signal was analyzed using LabChart™ version 7.0 software (ADInstruments). The ventilatory index (cm H_2O sec^{-1}, as an indicator of total ventilatory water flow) was calculated as the product of mean ventilatory pressure amplitude (cm H_2O) and mean frequency (sec^{-1}). The copper wires were connected to the same recording and analysis system for measurement of heart rate (sec^{-1}). Within each 3-h period, data taken from 0.5 to 3.0-h were generally stable and were averaged; the first 0.5-h was discarded as a period of transition.

Water PO_2 levels in the experimental chambers were continuously monitored using a Radiometer E-5036 polarographic electrode (Radiometer, Copenhagen, Denmark) connected to a polarographic amplifier (Model 1900, A–M Systems, Sequim, WA, USA) and captured by the PowerLab™ data integrity system (ADInstruments).

Water samples (5 ml) were taken at 0.5-h intervals throughout each 3-h period for total ammonia measurements by the colorimetric sodium salicylate method of Verdouw et al. (1978). The mean rate of ammonia excretion (μmol kg^{-1} h^{-1}) was calculated from the linear regression slope (μmol L^{-1} h^{-1}) of a plot of water ammonia concentration (μmol L^{-1}) versus time (0.5–3.0-h), factored by body mass (kg) and water volume (L). Plasma total ammonia concentration (μmol L^{-1}) was measured using an enzymatic reagent kit based on the glutamate dehydrogenase / NAD method (Raichem™ R85446, Cliniqua, San Marcos, CA, USA). The same standards used for the water ammonia assay were employed. Plasma cortisol concentrations were assayed with an ELISA Kit (07M-21602, MP Biomedicals, Solon, OH, USA), Ann Arbor, Michigan), according to the manufacturer's instructions.

For measurement of mRNA expression of V-type proton ATPase B subunit (HAT, AF14002), Na^+/H^+ exchanger-2 (NHE2, EF446605), and elongation factor 1-α total (EF1α, AF498320) in gills, forward and reverse primer sequences tabulated by Wood and Nawata (2011) for *Oncorhynchus mykiss* were used. For measurement of *Oncorhynchus mykiss* rhesus glycoprotein type c (Rhcg2, AY619986.1), forward: GGTAGTCTGCTTCGTCTGGC, reverse: TCATGGGCCTTGGTCTCTAC primers were used. RNA was extracted from the RNAlater™ fixed 2nd gill filaments with Trizol (Invitrogen, Burlington, ON, Canada). After removing potential DNA contamination with DNaseI (Invitrogen), RNA was quantified and quality assessed on a Nanodrop spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE, USA). Absence of DNA traces were verified by regular PCR on EF1α. 1 μg of purified total RNA was then reversely transcribed into cDNA with the iScript™ cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). Quantitative real-time PCR (qPCR) reactions were performed using SsoFast™ EvaGreen® Supermix (Bio-Rad) and the CFXConnect™ real-time system (Bio-Rad). The qPCR protocol consisted of an initial step at 98 °C for 2 min to activate the enzyme, and then 40 cycles of 5 s at 98 °C for denaturation and 20 s at 60 °C for annealing. Reactions were set up in a final volume of 15 μl using 2 μl of cDNA template and a final primer concentration of 0.4 μmol L^{-1}. Products were verified to result in a single specific amplicon by melt curve analysis (65–95 °C, steps of 0.5 °C for 1s). A standard curve using a 1:2 serial dilution of gill cDNA was included for quantification and to ensure performance of the reaction (efficiency = 97.6 %; R^2 = 0.995). Analyses were performed with the CFX manager software ver. 3.1 (Bio-Rad). EF1α expression, which did not vary with treatment, was used for normalization.

2.5. Data analysis

Data have been reported as means ± S.E.M. (N) where N represents the number of fish. One-way repeated-measures ANOVA followed by Dunnett's test was applied to compare the respiratory parameters, heart rates, and ammonia excretion rates, against control values within Series I and II experiments. Relationships between parameters were examined by linear and non-linear regression. Student's t - tests two-tailed t-tests, with Bonferroni correction when required, were employed to compare the plasma total ammonia and cortisol concentrations among treatments (Series II) as well as gene expression (Series III). GraphPad Prism 6.0 (La Jolla, CA, USA) was used for all analyses. The threshold for statistical significance was $p < 0.05$.

3. Results

3.1. Series I – Relationship between ventilation and ammonia excretion in fish under baseline conditions

When water oxygen concentration was experimentally lowered from 97 % air saturation (control(-)) to 48 % for a 3-h period of hypoxia, trout (N = 6) significantly increased both ventilatory pressure amplitude by 28 % (Fig. 1A) and frequency by 15 % (Fig. 1B). Together, the increased pressure and frequency resulted in a 50 % elevation of

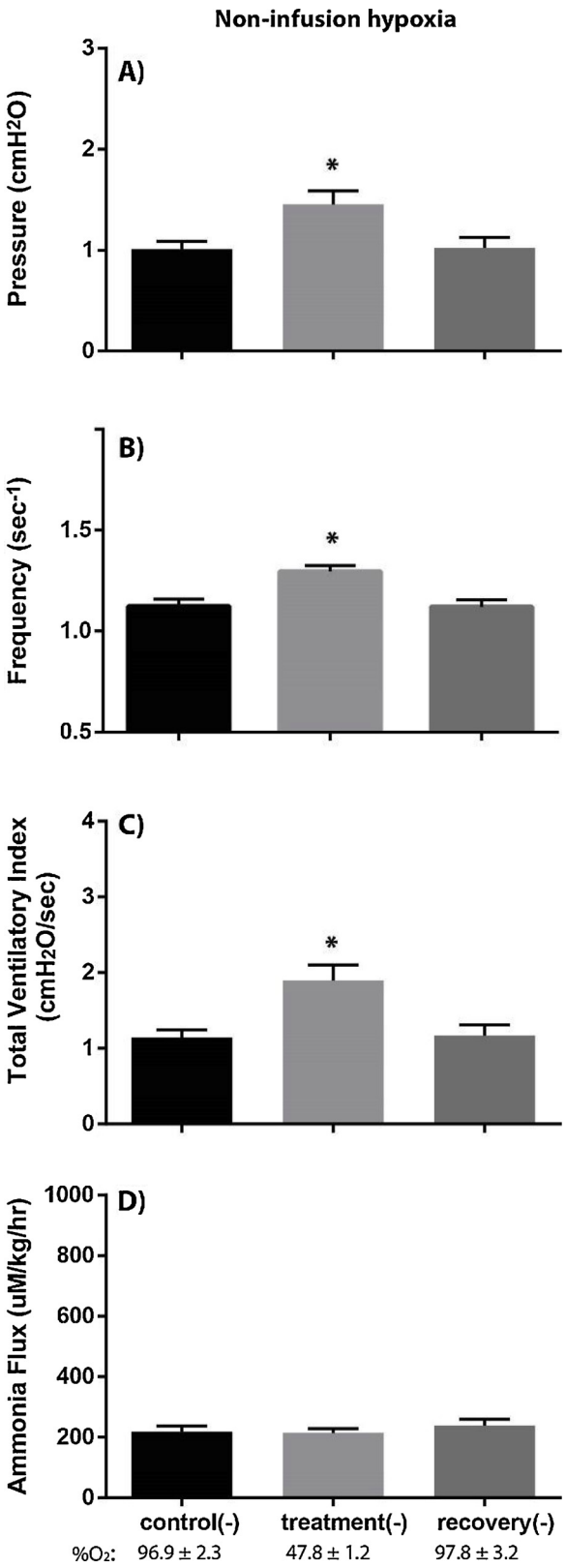

Fig. 1. Changes in A) ventilation pressure amplitude, B) frequency, C) total ventilatory index (frequency x pressure amplitude), and D) ammonia excretion flux rate in non-infused rainbow trout (N = 6) during hypoxia in Series I. Asterisks indicate means that are significantly different from control(-) value. Symbol of (-) represents non-infused control value. Although fish significantly increased ventilation (one way ANOVA, p = 0.0177) during hypoxia (48 % air saturation in water), ammonia flux rate was not affected (p = 0.3391). Ventilation recovered to control levels in 98 % air saturation water without changes in ammonia flux rate.

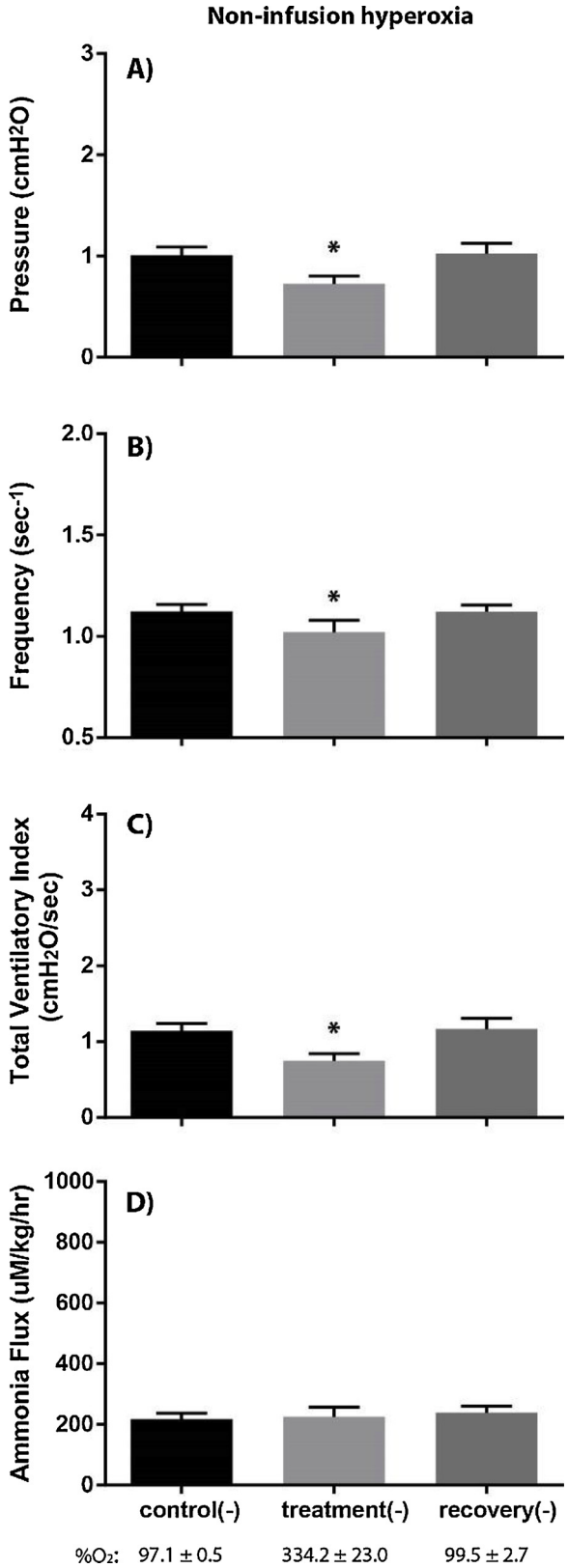

Fig. 2. Changes in A) ventilation pressure amplitude, B) frequency, C) total ventilatory index (frequency x pressure amplitude), and D) ammonia excretion flux rate in non-infused rainbow trout (N = 6) during hyperoxia in Series I. Asterisks indicate means that are significantly different from control(-) value. Symbol of (-) represents non-infused control value. Although fish significantly decreased ventilation (one way ANOVA, p = 0.0033) during hyperoxia (334 % air saturation in water), ammonia flux rate was not affected (p = 0.5166). Ventilation recovered to control levels in 98 % air saturation water without changes in ammonia flux rate.

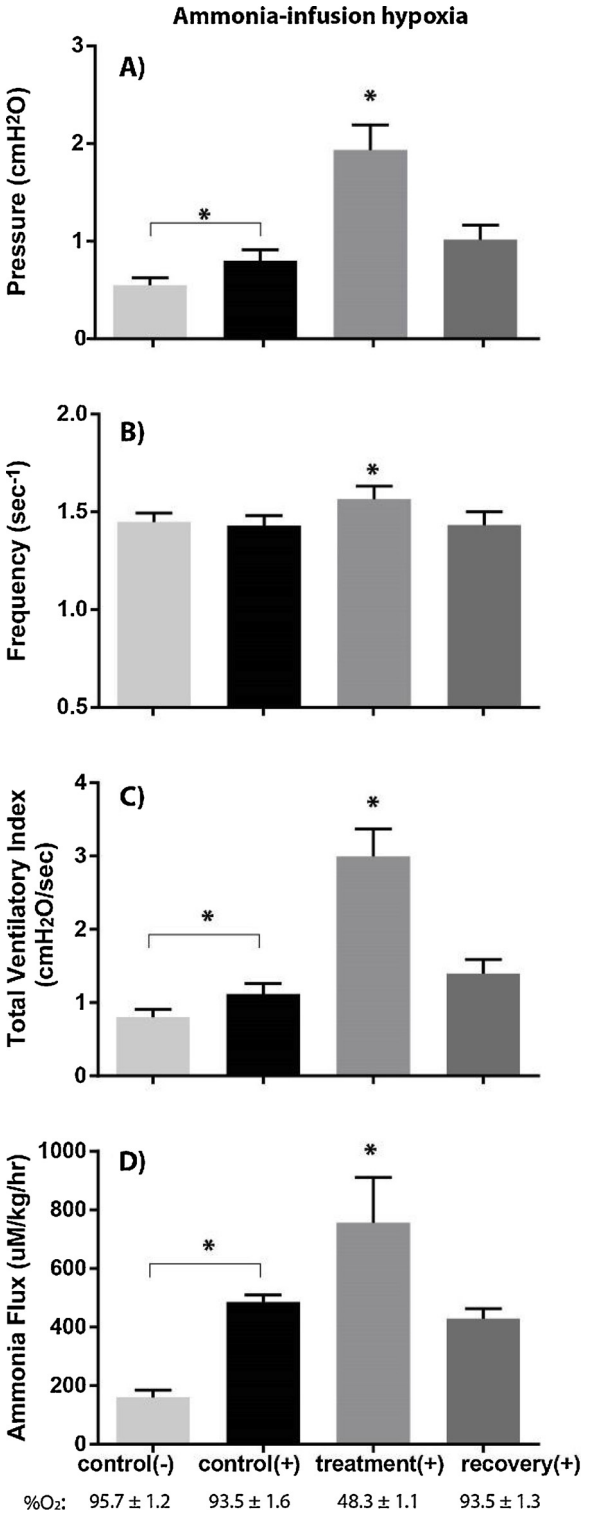

Fig. 3. Changes in A) ventilation pressure amplitude, B) frequency, C) total ventilatory index (frequency x pressure amplitude), and D) ammonia excretion flux rate in non-infused rainbow trout (N = 10) in response to ammonia infusion and subsequent treatment with hypoxia during continuing ammonia infusion in Series II. Asterisks indicate significant differences from respective controls. Symbol of (-) represents non-infusion control and symbol of (+) indicates infusion control. With ammonia infusion, fish significantly increased ammonia flux rate and ventilation relative to control(-) values. During hypoxia, fish increased ventilation further (one way ANOVA, p < 0.0001) with an accompanying elevation in ammonia flux rate (p = 0.0005). Ventilation and ammonia flux rate recovered to control(+) levels in 94 % air saturation water.

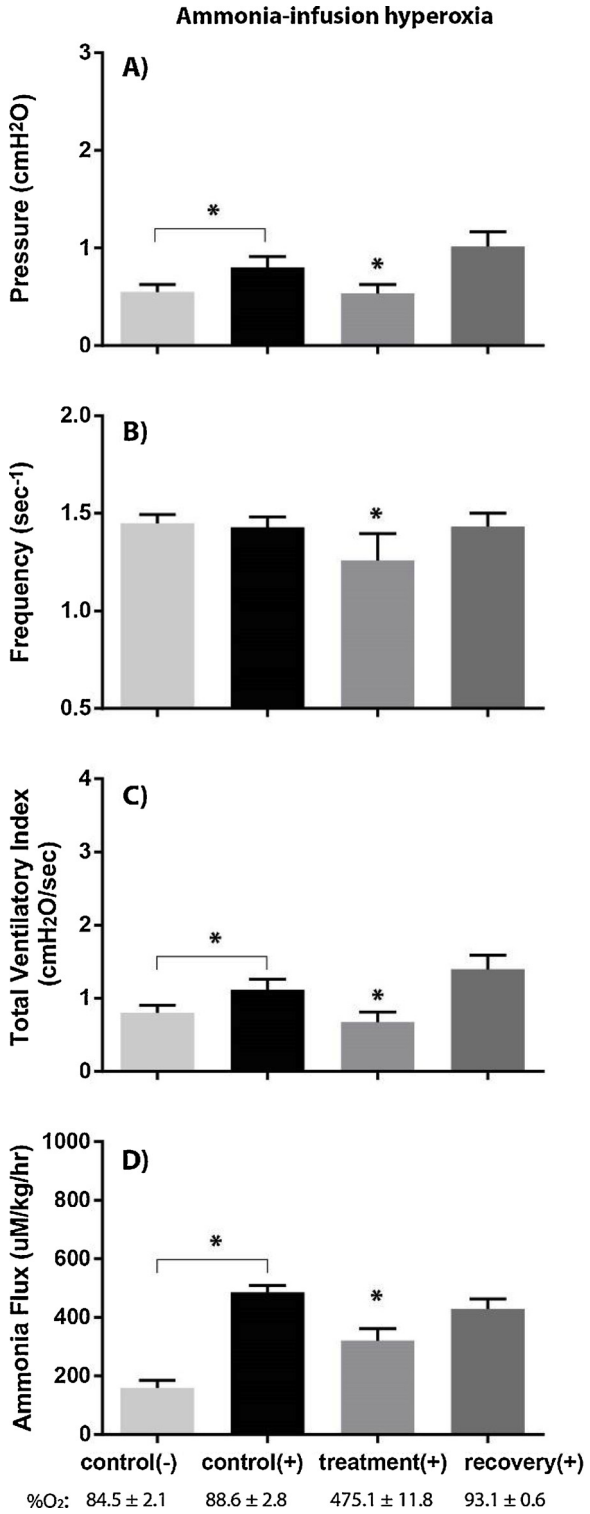

Fig. 4. Changes in A) ventilation pressure amplitude, B) frequency, C) total ventilatory index (frequency x pressure amplitude), and D) ammonia excretion flux rate in non-infused rainbow trout (N = 10) in response to ammonia infusion and subsequent treatment with hyperoxia during continuing ammonia infusion in Series II. Asterisks indicate significant differences from respective controls. Symbol of (-) represents non-infusion control and symbol of (+) indicates infusion control. With ammonia infusion, fish significantly increased ammonia flux rate and ventilation relative to control(-) values. During hyperoxia, fish decreased ventilation (one way ANOVA, $p < 0.0084$) with an accompanying decrease in ammonia flux rate ($p = 0.0005$) relative to control(+) values. Ventilation and ammonia flux rate recovered to control(+) levels in 94 % air saturation water.

total ventilatory index (Fig. 1C). However, there was no significant change in ammonia flux rate (Fig. 1D). After restoration of normoxia (98 % saturation), all ventilatory parameters returned to control(-) levels and there was no change in ammonia flux rate. During hypoxia, heart rate decreased by 16 %, but recovered to control(-) levels after restoration of normoxia (Table 1A).

When water oxygen concentration was experimentally raised from 97 % saturation ((control(-)) to 334 % for a 3-h period of hyperoxia, trout (N = 6) significantly decreased both ventilatory pressure amplitude by 18 % (Fig. 2A) and ventilatory frequency by 9 % (Fig. 2B). In combination, these resulted in a 26 % reduction of total ventilatory index (Fig. 2C). After restoration of normoxia (100 % saturation) there was no change in ammonia excretion rate, but all ventilatory parameters returned to control(-) levels. During hyperoxia, heart rate did not change (Table 1A).

Overall, under baseline conditions, trout were responsive to low and high oxygen levels in water by increasing and decreasing ventilation respectively, but these changes did not affect their rates of ammonia excretion, as summarized in Fig. 5A.

3.2. Series II – Relationship between ventilation and ammonia excretion in ammonia-loaded fish

Infusion with 140 mmol L^{-1} of NH_4HCO_3 at a rate of 2.98 ± 0.13 ml kg^{-1} h^{-1} for 30-h raised the plasma ammonia concentration by about 7.5-fold from the control(-) level of 267 μmol L^{-1} to a control(+) level close to 2000 μmol L^{-1} (Fig. 6A). This was accompanied by a 3-fold elevation in the rate of ammonia excretion as indicated by the significant increases from control(-) to control(+) values (Figs. 3 D, 4D). Ammonia infusion also resulted in significant increases in total ventilatory index by about 40 % which were achieved solely by elevations in ventilatory pressure without significant changes in frequency (Figs. 3 and 4). Ammonia infusion had no effect on heart rate (Table 1B). These infused fish (control (+)) with chronically elevated levels of plasma total ammonia, ammonia excretion, and ventilation were then subjected to the same experimental treatments as in Series I, during which time infusion was continued.

Reductions in water oxygen concentration from 94 % saturation (control(+)) to 48 % (hypoxia) for 3-h in these ammonia-infused trout (N = 10) resulted in hyperventilation, with significant increases of all ventilatory parameters amounting to 113 % in pressure amplitude (Fig. 3A), 5 % in frequency (Fig. 3B), and 135 % in total ventilatory index (Fig. 3C). This elevation in ventilation was accompanied by a highly significant 53 % increase in ammonia flux rate (Fig. 3D). After hypoxia, fish were returned to normoxic water (94 % saturation) which resulted in decreases of all ventilatory parameters (Figs. 3A,B,C), which returned to levels not significantly different from control(+) values. Notably, ammonia excretion also decreased to a rate close to control (+) levels (Fig. 3D), in parallel to the decrease in ventilation. During hypoxia in these ammonia-loaded fish, heart rate decreased by 29 % but recovered to control(+) levels after restoration of normoxia (Table 1B).

Elevations in water oxygen concentrations from 88 % saturation (control(+)) to 475 % (hyperoxia) in ammonia-infused trout (N = 7) resulted in hypoventilation with significant decreases in all ventilatory parameters, amounting to 17 % in pressure amplitude (Fig. 4A), 7 % in frequency (Fig. 4B), and 24 % in total ventilatory index (Fig. 4C). This reduction in ventilation was accompanied by a highly significant 33 % reduction in ammonia flux rate (Fig. 4D). After hyperoxia, fish were returned to normoxic water (93 % saturation) which resulted in increases in ventilatory parameters, all of which returned to levels which were not different from control(+) values. Ammonia excretion also increased to a rate close to control(+) levels, in parallel to the increase in ventilation. Hyperoxia had no effect on heart rate in these ammonia-loaded fish (Table 1B).

Overall, chronic ammonia loading by infusion caused increased ventilation, increased plasma ammonia concentrations, and increased

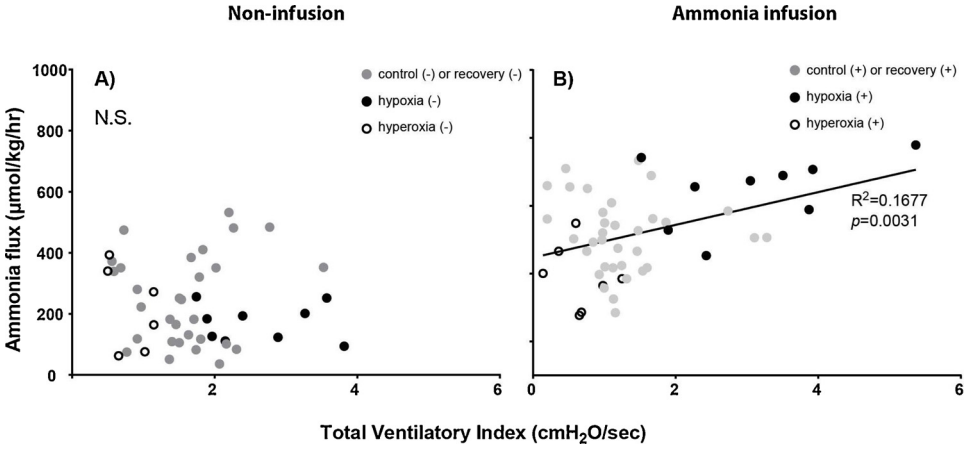

Fig. 5. A) Hyperventilation (during hypoxia) or hypoventilation (during hyperoxia) in Series I did not affect ammonia excretion flux rate in non-infused fish that were not loaded with ammonia. Therefore there was no significant relationship between ammonia excretion rate and ventilatory index. B) However, when fish were loaded with ammonia by infusion in Series II, hyperventilation (during hypoxia) increased ammonia excretion rate, and hypoventilation (during hyperoxia) decreased ammonia excretion rate, resulting in a significant relationship between ammonia excretion rate and ventilatory index. Symbol of (-) represents non-infused fish and symbol of (+) represents ammonia-infused fish. N.S. represents "not significant.".

rates of ammonia excretion. When ventilation was subsequently manipulated by hypoxia and hyperoxia treatments, the rate of ammonia excretion became sensitive to ventilation, increasing with hyperventilation, and decreasing with hypoventilation, as summarized in Fig. 5B. Furthermore, measurements of plasma total ammonia revealed significantly lower concentrations in the trout subjected to hypoxia-induced hyperventilation (hypoxia) relative to those subjected to hyperoxia-induced hypoventilation (Fig. 6 B). Interestingly, the hyperoxia-treated trout also had significantly lower plasma cortisol concentrations (5.45 ± 1.72 ng dl^{-1}) than the hypoxia–treated animals (13.85 ± 4.30 ng dl^{-1}). The plasma cortisol concentration in control(+) fish was 16.33 ± 4.61 ng dl^{-1}.

3.3. Series III – the influence of ammonia loading on gene expression in the gills

Chronic infusion with 140 mM NH_4HCO_3 for 33-h, parallel to the ammonia-loading treatment in Series II, was compared to chronic infusion with 140 mM NaCl for the same time period. Ammonia-loading resulted in significant increases in the expression of Rhcg2 (Fig. 7A) in the gills by 2.29-fold and HAT (Fig. 7B) by 1.26-fold respectively, relative to the NaCl treatment. The expression level of NHE2 was not significantly affected (Fig. 7C).

4. Discussion

4.1. Overview

The present results have confirmed our two hypotheses, showing that the prediction of Randall and Ip (2006) ("diffusion limitation") was correct for resting trout with low concentrations of plasma ammonia (Series I), but became incorrect when diffusion limitation was removed in trout chronically loaded with ammonia (Series II). Thus, experimental manipulations of ventilation using moderate hypoxia and hyperoxia had no effect on ammonia excretion rate in resting trout, but these same treatments caused large changes in ammonia excretion when plasma ammonia concentrations were greatly elevated. The probable explanation is that diffusion limitation was removed because elevated plasma ammonia activated the Rh protein metabolon in the gills, thereby effectively increasing the ammonia permeability of the branchial epithelium. This would involve opening up facilitated diffusion channels and increasing the provision of protons for diffusion trapping of ammonia in the gill water boundary layer (Wright and Wood, 2009, 2012). Thus, greater convection of water could carry away more ammonia. It is also possible that under these circumstances, ammonia excretion would become sensitive to the convective flow of blood through the gills, though this was not evaluated in the present study. Heart rate did not change significantly in response to either ammonia loading or hyperoxia, but it fell during hypoxia. This classic bradycardia would change the temporal pattern of blood flow through the gills, but not necessarily the absolute flow rate. Regardless, it is now clear that the ability of elevated plasma ammonia concentrations to stimulate ventilation can play a functional role in augmenting the excretion rate of potentially toxic ammonia under conditions of natural ammonia loading such as during digestion of a meal (Bucking and Wood, 2008; Wicks and Randall, 2002; Zimmer et al., 2010) and during recovery from exhaustive exercise (Wood, 1988; Mommsen and Hochachka, 1988; Wang et al., 1994). Gene expression data indicate that the Rh metabolon is likely activated in trout in both circumstances, though at present confirmatory protein expression data are lacking (Zimmer et al., 2010; Zhang et al., 2015).

4.2. Ammonia permeability in gills

In both Series I and Series II experiments, the fish changed ventilation in response to hypoxia and hyperoxia, but the ammonia excretion rates were only affected in fish infused with ammonia (Series II). This means that the internally elevated ammonia increased the permeability of the gills to ammonia. There is circumstantial evidence that cortisol may also play a role in activating the branchial Rh metabolon (Tsui

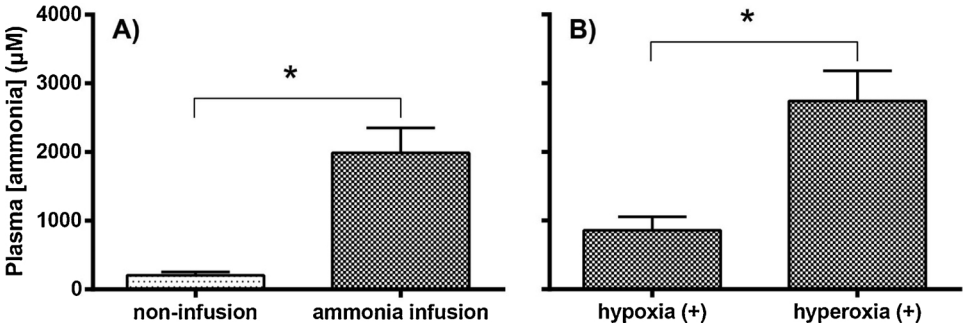

Fig. 6. A) After ammonia infusion in Series II, plasma [ammonia] significantly increased in rainbow trout (N = 17) (Student's t-test, $p < 0.0001$). With continued ammonia infusion, B) hyperventilating fish in hypoxia (N = 8) showed significantly lower plasma [ammonia] than hypoventilating fish in hyperoxia (N = 9) ($p = 0.0058$). Symbol of (+) represents ammonia-infused fish.

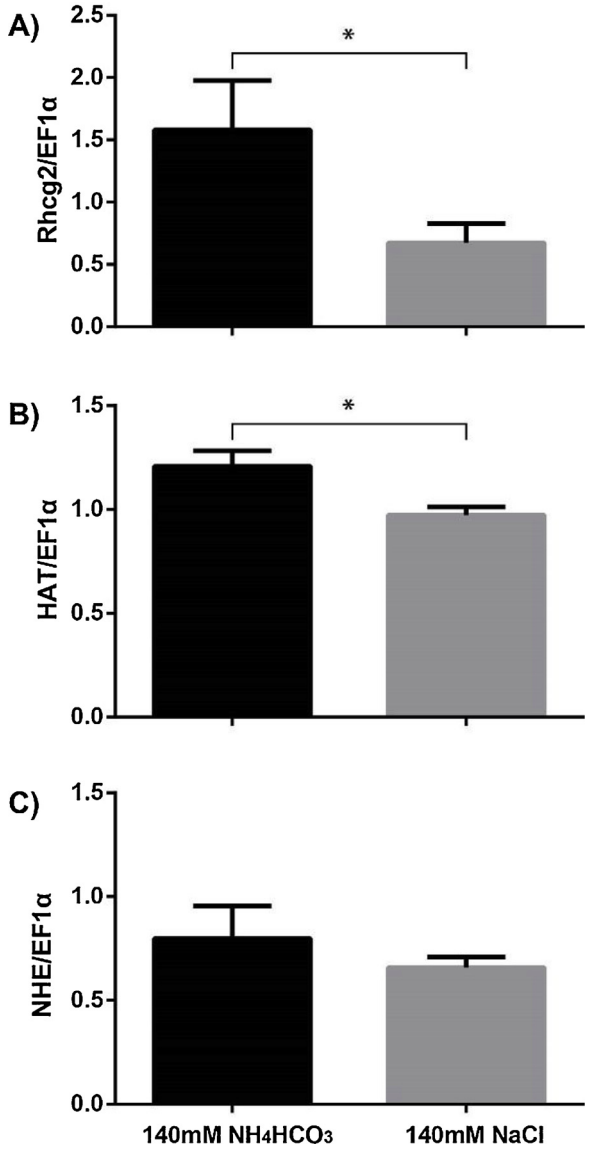

Fig. 7. Expression levels of Rhcg2, HAT, and NHE2 genes in the gills of rainbow trout infused with 140 mM NH_4HCO_3 or 140 mM NaCl for 33 h in Series III.

et al., 2009; Nawata and Wood, 2009). In this regard, it is interesting that in the ammonia-loaded fish of Series II, plasma cortisol levels in the hypoxia-treated group were significantly greater than in the hyperoxia-treated group, a difference which paralleled the greater ammonia excretion rate in the former.

In the freshwater rainbow trout, components of the Rh metabolon include Rhcg2 (the apical ammonia channel), an as yet uncharacterized apical Na^+ channel, apical NHE2, intracellular carbonic anhydrase and HAT (the apical H^+ pump) (Tsui et al., 2009; Wright and Wood, 2009, 2012). When the metabolon is activated, Na^+ uptake and ammonia excretion increase, and ammonia can be excreted even against prevailing gradients (Nawata et al., 2007; Nawata and Wood, 2009; Zimmer et al., 2010; Wood and Nawata, 2011; Nawata et al., 2010a; Sinha et al., 2013). In the present study, the mRNA abundance of two key genes (Rhcg2 and HAT) in the gills were elevated by chronic ammonia loading, in accord with the earlier findings of Nawata and Wood (2009), who used a similar 140 mM NH_4HCO_3 infusion protocol. The lack of significant change in NHE2 expression is in accord with previous studies showing that this generally has a less pronounced response than HAT to ammonia loading in freshwater trout (Nawata et al., 2007; Nawata and Wood, 2009; Wood and Nawata, 2011). However, an important cautionary note is that changes in gene expression do not necessarily indicate changes at the protein level, so in the future. protein expression levels of these transporters should be studied. The metabolon is thought to work by facilitating NH_3 diffusion through the apical Rh channel and increasing the export of H^+ across the apical membrane to allow diffusion trapping of NH_3 and its conversion to NH_4^+ in the gill water boundary layer. The H^+ ions are generated by intracellular carbonic anhydrase. At the same time, HAT creates an electrochemical gradient across the apical membrane to increase active Na^+ uptake from the water, thereby maintaining electroneutrality. The excreted NH_4^+ ions cannot diffuse back, and as long as the fish continues to hyperventilate, they will be washed away. The fish gain benefits in several different ways. These include increased ammonia excretion which becomes responsive to ventilation, increased H^+ excretion, and greater active Na^+ uptake at times (e.g. after feeding or exercise) when elevated metabolism is generating more ammonia and acidic equivalents, accompanied by greater diffusive Na^+ losses through the osmorespiratory compromise (Randall et al., 1972; Nilsson, 1986).

4.3. Possible ammonia sensing organs in fish

We employed experimental ammonia-loading to elevate plasma ammonia concentrations. The fish gills and/or brain are thought to be the organs involved in detecting elevated internal ammonia concentrations. Zhang et al. (2011) provided evidence that serotonin (5-HT)-immunoreactive neuroepithelial cells (NECs) on gills of arches 1 and 2, which are known to also be O_2 and CO_2 sensors (Burleson and Milsom, 1990, 1993; Jonz et al., 2004; Burleson et al., 2006; Coolidge et al., 2008; Perry et al., 2009; Abdallah et al., 2012: Zachar and Jonz, 2012; Perry and Tzaneva, 2016), serve as potential ammonia chemoreceptors, displaying intracellular Ca^{2+} signals in response to physiological levels of extracellular NH_4^+. This was reinforced by the discovery that Rh glycoproteins are expressed in these cells (Zhang et al., 2015), thereby providing a route for ammonia entry. Serotonergic neural pathways would transmit the signals back to respiratory control centers in the brain, and in turn motor outflow along cranial nerves to the breathing muscles would increase ventilation. Zhang and Wood (2009) and Zhang et al. (2011) also showed that the hyperventilatory response to elevated internal ammonia was almost immediate, while that to elevated external ammonia was somewhat slower in the trout, and this was later reinforced by parallel observations in an elasmobranch (De Boeck and Wood, 2015). These suggest that the NECs primarily sense internal plasma ammonia and that the response to high environmental ammonia (HEA) in the external water is indirect (i.e. after ammonia entry into the bloodstream), but further research is needed on this issue.

Zhang et al. (2013) also suggested the brain as a potential ammonia-sensing organ, because brain ammonia levels directly matched the hyperventilatory response to HEA in trout. This is in accord with earlier evidence that the brain in mammals plays a role in ammonia sensing (Vanamee et al., 1955; Warren, 1958; Cooper and Plum, 1987; Wichser and Kazemi, 1974). The evidence for central chemoreceptors for CO_2, pH, and O_2 in fish is not strong (Wilson et al., 2000; Hedrick et al., 1991; Milsom, 2012). However, there is abundant evidence that elevated plasma ammonia permeates the blood brain-barrier in fish, and for this reason there are powerful detoxifying mechanisms for ammonia in the brain which convert it to glutamine (Schenone et al., 1982; Wright et al., 2007; Sanderson et al., 2010; Zhang et al., 2013) so as to avoid central neurotoxicity (Randall and Tsui, 2002; Walsh et al., 2007). Rh glycoproteins are also expressed in trout brain tissue and respond to ammonia loading (Nawata et al., 2007; Zhang et al., 2013). In the future, we suggest that simple ammonia injection experiments into the brain while simultaneously measuring ventilation may be instructive.

4.4. Interaction of ammonia with O_2 in ventilatory control

The experimental design of Series II provides some indication of how ammonia and O_2 interact in ventilatory control. Clearly, the well-established ventilatory responses of fish to hypoxia and hyperoxia (Shelton et al., 1986; Perry et al., 2009), involving only small changes in frequency and larger changes in ventilatory pressure amplitude (i.e. stroke volume) persisted even when ventilation was stimulated by chronic ammonia loading. Ammonia similarly acted almost entirely by increasing amplitude, in accord with previous studies (Zhang and Wood, 2009; Zhang et al., 2011). It is interesting that the hyperventilatory response to hypoxia in the ammonia-loaded fish of Series II appeared to be greater than that in the non-infused fish of Series I (compare Figs. 3C versus 1C). This suggests that high ammonia and low O_2 may act synergistically to stimulate breathing, an interesting topic for future investigation.

4.5. Perspectives

We propose the following scenario. Elevated internal ammonia levels stimulate increased expression of components of the branchial Rh metabolon that can accelerate ammonia excretion at the gills, thereby effectively increasing their ammonia permeability. Meanwhile, increased plasma ammonia can be sensed by NECs in the gills and/or by the brain. These ammonia-stimulated signals would be transmitted to integrating centers in the central nervous system that control breathing, generating hyperventilation. Analogous to the model of Knepper and Agre (2004) which was originally proposed for more primitive ammonia channels, the excretion pathway for elevated plasma ammonia will involve conversion of ionized ammonium (NH_4^+, the dominant form in the plasma) into gaseous ammonia (NH_3) at the Rh channel entrance. Upon exit at the apical surface, it is immediately converted back to NH_4^+ by protons that acidify the boundary layer, in this case provided by increased activity of V-H^+-ATPase (HAT). The excreted ionic NH_4^+ is in turn eliminated by increased convective water flow in hyperventilating fish.

Author contributions

J. E. and C.M.W. designed the study, J. E. performed the experiments, S.F. helped with the mRNA expression analysis, J.E. analyzed the data, J.E. prepared the first draft of the MS, and all authors revised the MS.

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