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Ammonia as a stimulant to ventilation in rainbow trout *Oncorhynchus mykiss*

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ABSTRACT

Ammonia is the third most important respiratory gas in ammoniotelic fish after oxygen and carbon dioxide. We here investigated the effects of elevated plasma ammonia on ventilation in freshwater rainbow trout. Intact trout fitted with indwelling dorsal aortic catheters were given injections (over 5 min) of Cortland saline, isotonic high ammonia solutions (NH_4HCO_3, $(NH_4)_2SO_4$, NH_4OH at pH 8.0, and NH_4OH at pH 9.0), and other solutions as controls for acid–base effects, while ventilatory rate (VR) and buccal pressure amplitude (ΔP_{buccal}) were recorded. All high ammonia solutions resulted in immediate elevations of plasma $Tamm_a$, Pa_{NH_3}, and $[NH_4^+]_a$, and increases in ventilatory ΔP_{buccal} and VR to different degrees. However, while Pa_{O_2} remained constant, in every case there was a confounding change in one or more components of acid–base status (decreases in pH_a or increases in $[HCO_3^-]_a$ or Pa_{CO_2} in different treatments), although the ventilatory responses to ammonia injections were generally larger than could be explained by changes in acid–base status. Therefore a series was performed in which normal blood perfusion of the gills was replaced by ventral aortic perfusion with either Cortland saline or Cortland saline plus high ammonia in which pH, $[HCO_3^-]$, P_{CO_2}, and P_{O_2} remained unchanged. Although ventilation was depressed in these anaesthetized, spontaneously ventilating preparations, perfusion with high ammonia saline increased ΔP_{buccal}. In a final series, trout were infused for 24 h with Cortland saline, isotonic NH_4HCO_3, or isotonic $(NH_4)_2SO_4$ solutions. The two ammonia solutions both caused persistent elevations in VR and ΔP_{buccal}, together with similar large increases in plasma $Tamm_a$, Pa_{NH_3}, and $[NH_4^+]_a$. As there was no changes in Pa_{O_2}, pH_a, Pa_{CO_2}, or $[HCO_3^-]_a$ in the $(NH_4)_2SO_4$ infusion series, this, together with the ventral aortic perfusion experiment, provides the most convincing evidence that ammonia stimulates ventilation. 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, and propose that ammonia-induced hyperventilation may also facilitate ammonia excretion in rainbow trout.

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1. Introduction

Most fish ventilate continuously to meet metabolic requirements for oxygen, as well as the excretion of carbon dioxide and ammonia. [Note: throughout this paper, the term 'ammonia' is used to refer to total $NH_3 + NH_4^+$ (total ammonia = Tamm), whereas these chemical symbols refer to the individual components of ammonia gas (NH_3) and ammonium ion (NH_4^+).] As respiratory gas tensions are highly variable in different water environments, it is essential for fish to sense and respond to these changes. It has been widely accepted for many years that O_2 provides the primary drive to ventilation in fish (see Shelton, 1970; Randall, 1982, 1990; Randall and Daxboeck, 1984; Perry and Wood, 1989 for reviews of early seminal studies). Many more recent investigations have provided additional evidence that environmental hypoxia and/or arterial hypoxaemia cause substantial increases in ventilation amplitude and moderate increases in ventilation rate (e.g. Kinkead et al., 1991; Gilmour and Perry, 1994; Maxime et al., 1995; Perry and Gilmour, 1996; McKenzie et al., 1997; Sundin et al., 1999). In contrast, hypoventilation is induced by elevated external and internal O_2 tensions (e.g. Wood and Jackson, 1980; Wilkes et al., 1981; Gilmour and Perry, 1994). Although there is marked interspecific diversity and some controversy in the area, P_{CO_2} and/or blood pH have also been reported to influence ventilation in several teleost species such as rainbow trout, common carp, and zebrafish (reviewed by Gilmour, 2001). Increases in environmental PI_{CO_2}, Pa_{CO_2} and/or decreases in pH_a appeared to cause substantial increases in ventilation independent from secondary effects on blood O_2 status in some species, including rainbow trout (e.g. Janssen and Randall, 1975; Neville, 1979; Graham et al., 1990; Gilmour and Perry, 1994; Wood and Munger, 1994; Burleson and Smatresk, 2000; McKendry et al., 2001).

However there is a third respiratory gas in fish, ammonia, which is produced and excreted at a rate of about 10% of MCO_2 in ammoniotelic teleosts (Randall, 1990; Randall and Ip, 2006). Ammonia is produced in the liver, muscle, and other tissues as a waste from the

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catabolism of dietary and structural proteins. Because of its highly toxic effects, ammonia must be continually removed from the body mainly by excretion across the gills to the water, but blood ammonia levels are known to rise considerably after feeding (e.g. Kaushik and Teles, 1985; Wicks and Randall, 2002a,b; Bucking and Wood, 2008), exhaustive exercise (e.g. Mommsen and Hochachka, 1988; Wood, 1988; Wang et al., 1994), and of course exposure to high environmental ammonia (e.g. Wilson and Taylor, 1992; Wilson et al., 1994; Wright et al., 2007; Nawata et al., 2007). The first two of these conditions are also known to cause hyperventilation even though there is no evidence that arterial O_2 status is perturbed, and there are many descriptive reports that fish exposed to high environmental ammonia also exhibit hyperventilation (e.g. Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph, 1996). Is it possible that ammonia is serving as a signal to drive ventilation in these circumstances?

In mammals, it has long been known that ammonia, acting centrally, can serve as a ventilatory stimulant, although the exact mechanism and the adaptive value of the response remain unclear (Roberts et al., 1956; Warren, 1958; Campbell et al., 1973; Wichser and Kazemi, 1974). As with sensitivity to the other two respiratory gases, O_2 and CO_2, the ventilatory response to ammonia may have originated in ammoniotelic fish and may have been retained throughout evolution so as to manifest in mammals. Curiously, in ammoniotelic fish, this ventilatory sensitivity to ammonia has been largely over-looked. Hillaby and Randall (1979) noted that ventilation appeared to increase after injection of various doses of NH_4HCO_3 and NH_4Cl into the dorsal aorta of trout. However, to our knowledge, the study of McKenzie et al. (1993) is the only one to directly investigate whether ammonia can act as a ventilatory stimulant in teleosts. These workers quantified ventilation and reported that the injection of a 200 mmol l^{-1} NH_4HCO_3 solution into the dorsal aorta of trout caused a marked hyperventilation, but as blood HCO_3^- and Pa_{CO_2} levels also increased, and as injections of 200 mmol l^{-1} $NaHCO_3$ caused similar hyperventilatory effects, the responses could not be attributed specifically to ammonia.

The objective of the present study was therefore to investigate the effects of elevated plasma ammonia on ventilation in freshwater rainbow trout. Three experimental approaches were employed. In the first, extending the approach of McKenzie et al. (1993), ventilation was recorded in intact, unanaesthetized trout fitted with chronic indwelling catheters. The fish were given acute injections of isotonic saline, various isotonic ammonium salts, and various other salts as controls for acid–base effects, directly into the dorsal aorta. All of the ammonium injections caused hyperventilation, but in every case, interpretation was confounded by simultaneous changes in one or more acid–base variables (pH_a, Pa_{CO_2}, or $[HCO_3^-]_a$). Therefore a second series employed an anaesthetized but spontaneously ventilating trout preparation where solutions of exactly matched acid–base status, with or without elevated ammonia, could be perfused directly into the ventral aorta. A third series employed prolonged infusion (24 h) with isotonic saline or isotonic ammonium salts into intact, unanaesthetized trout to examine the effects of chronic elevation of plasma ammonia on ventilation.

2. Materials and methods

2.1. Fish husbandry

Rainbow trout (*Oncorhynchus mykiss* Walbaum, 250–380 g) were obtained from Humber Springs Trout Hatchery (Orangeville, ON, Canada) and then acclimated to laboratory conditions for 2 weeks or longer before experimentation. Acclimation and experimental temperature was 12 ± 1 °C. The trout were held in flowing dechlorinated Hamilton (ON, Canada) tap water ($[Na^+]$ 0.6 mmol l^{-1}, $[Cl^-]$ 0.7 mmol l^{-1}, $[K^+]$ 0.05 mmol l^{-1}, $[Ca^{2+}]$ 1.0 mmol l^{-1}, $[Mg^{2+}]$ 0.1 mmol l^{-1}; titration alkalinity 1.9 mequiv. l^{-1}; hardness 140 mg l^{-1} as $CaCO_3$ equivalents; pH 8.0, flow rate about 500 ml min^{-1}) at a density of approximately 40 fish per 500 l water. The trout were fed a 2% body ration commercial trout food (crude protein 41%; carbohydrates 30%; crude fat 11%; Martin Mills; Elmira, ON, Canada) every 48 h. All the fish were fasted at least 5 days prior to experimentation, to minimize the influence of feeding on ammonia metabolism. All procedures were approved by the McMaster University Animal Research Ethics Board and are in accordance with the Guidelines of the Canadian Council on Animal Care.

2.2. Injection and infusion

In the injection and infusion experiments (first and third series, respectively), the trout were anaesthetized and irrigated with ventilatory water on an operating table in 80 mg l^{-1} tricaine methanesulphonate (MS-222, Syndel Laboratories Ltd., Vancouver, Canada; pH adjusted with NaOH) and fitted with indwelling dorsal aortic catheters (Clay-Adams PE50 tubing, Sparks, MD, USA) by the method of Soivio et al. (1975). These catheters were filled with heparinized saline (lithium heparin salt, 50 unit ml^{-1}, Sigma–Aldrich, St. Louis, MO, USA) and served for injections, infusions, and blood sampling without disturbance to the fish. In order to monitor ventilation, buccal catheters, consisting of flared tubing (PE90) passed through a hole drilled on the roof of the mouth, were implanted as described by Holeton and Randall (1967). The trout were then placed in individual darkened plexiglass boxes (4 l volume) served with constant aeration and flowing water (400 ml min^{-1}) and allowed to recover for 24 h before experimentation.

In injection experiments, the trout were injected with 3.91 ± 0.01 ml kg^{-1} of various isotonic solutions via the dorsal aortic cannula over a 5 min period. At this injection rate, about 0.8 ml kg^{-1} min^{-1}, no behavioral response or irritation was observed. The solutions included Cortland 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) and various isotonic solutions including 135 mmol l^{-1} NaCl + 5 mmol l^{-1} HCl, 140 mmol l^{-1} $NaHCO_3$, 140 mmol l^{-1} NH_4HCO_3, 70 mmol l^{-1} $(NH_4)_2SO_4$, 70 mmol l^{-1} Na_2SO_4, and 140 mmol l^{-1} NH_4OH at either pH 9.0 or pH 8.0 (adjusted by adding HCl). In effect, these latter two therefore represented mixtures of NH_4OH and NH_4Cl in which the total NH_4^+ ion concentration was 140 mmol l^{-1}, the same as in the other ammonium salt injections. Ventilation was measured immediately before and at 0, 1, 2, 3, 4, 10, 15, and 30 min after the 5 min injection period in all treatments, as well as at 45 and 60 min after injection in the Cortland saline, NH_4HCO_3, and $(NH_4)_2SO_4$ treatments. Immediately following the pre-injection ventilation measurements and at 2 min after the end of the injection period, 0.6 ml blood was sampled using a 1 ml gas-tight Hamilton syringe and replaced with 0.6 ml Cortland saline. In the fish that were injected with Cortland saline, NH_4HCO_3, and $(NH_4)_2SO_4$, another 0.6 ml blood was sampled at 50 min after injection.

In infusion experiments, the trout were chronically infused at 3.24 ± 0.08 ml kg^{-1} h^{-1} with Cortland saline, 140 mmol l^{-1} NH_4HCO_3, or 70 mmol l^{-1} $(NH_4)_2SO_4$ through the dorsal aortic cannula by using a Gilson Minipuls 3 peristaltic pump (Middleton, WI, USA) for 24 h. Fish were quietly resting in the dark boxes with flowing aerated dechlorinated tap water during the infusion process. Immediately before infusion and at 3, 6, 12, and 24 h during infusion, ventilation was measured and 0.6 ml blood sam-

ples were taken sequentially and replaced with 0.6 ml Cortland saline.

2.3. Ventral aortic perfusion

Responses to two perfusion solutions were compared in this second series: control Cortland saline and a high ammonia saline consisting of Cortland saline plus 1.9 mmol l^{-1} NH_4Cl. Both solutions were kept at 12 °C in separate water-jacketed reservoirs and gassed with 0.3% CO_2 and 99.7% O_2 gas mixture for 3 h prior to and during the experiments. The hyperoxic condition was used to ensure adequate O_2 delivery to the preparation in the absence of red blood cells. Heparin (lithium salt, 10 i.u. ml^{-1}) and epinephrine bitartrate (10^{-7} mol; Sigma–Aldrich, St. Louis, MO, USA) were added to the solutions which were adjusted to pH 8.0 by adding NaOH and filtered through a 0.22 μm Millipore filter (Millipore, Billerica, MA, USA) immediately prior to use. Epinephrine was used to maintain the gills in a vasodilated state (Wood, 1974). A peristaltic pump (Buchler Instruments, Fort Lee, NJ, USA) was used to provide a constant perfusion flow at 18.8 ml kg^{-1} min^{-1}. Immediately before every perfusion, pH, $[HCO_3^-]$, P_{O_2}, and the flow rates of the perfusion solutions were measured, and samples were collected and frozen in liquid nitrogen for later measurement of Tamm.

A series of preliminary experiments was performed to find an anaesthetic regime in which the fish would remain unresponsive to external stimuli yet continue ventilating spontaneously throughout a ~30 min period during which the normal ventral aortic blood flow to the gills was replaced by perfusion with oxygenated saline. In the final protocol, the trout was initially anaesthetized in 100 mg l^{-1} MS-222 and then moved to an operating table where it was irrigated with a 60 mg l^{-1} MS-222 solution. A dose of 5000 i.u. kg^{-1} heparin in 1 ml kg^{-1} Cortland saline was injected via caudal puncture and allowed to circulate for 5 min to prevent blood clotting. A buccal catheter was implanted and the fish was then moved to a chamber containing well-aerated freshwater with 60 mg l^{-1} MS-222. A pre-perfusion ventilation measurement was made at this time. The fish was then returned to irrigation on the operating table, the pericardial cavity was opened and a flared, saline-filled PE60 catheter was inserted and secured in the ventral aorta through a trans-section of the ventricle. Care was taken to avoid the introduction of any air bubbles. Perfusion with control saline was started immediately after the cannulation, and the fish was then moved back to the chamber containing well-aerated freshwater plus 60 mg l^{-1} MS-222. A ventilation measurement was made at this time (time 0). In one treatment, the trout were then continuously perfused via the ventral aorta with control saline for 30 min, and in the other treatment, the perfusion reservoir was switched at 5 min after the time 0 measurement, from the control saline to the high ammonia saline for a 5 min period. Ventilation was measured again after this 5 min perfusion with high ammonia saline. Perfusion with the control saline was then re-instated to test whether the effects observed were reversible. Ventilation was therefore measured before perfusion and at 0, 10, and 20 min during perfusion in both treatment groups.

2.4. Analytical techniques

Blood was analyzed for pH (pH_a) and O_2 tension (Pa_{O_2}) in whole arterial blood immediately after sampling. Remaining blood (0.3 ml) was centrifuged at room temperature to separate plasma. 0.1 ml plasma was used for analyzing total CO_2 and the remainder was frozen in liquid nitrogen for later analysis of total ammonia (Tamm). All these steps were finished within 2 min after blood sampling. The whole blood pH_a and Pa_{O_2} was measured in 12 °C thermostated chambers using a Radiometer GK2401C glass combination electrode coupled to a PHM82 standard pH meter (Radiometer Ltd., Copenhagen, Denmark), and a polarographic oxygen electrode coupled to a polarographic amplifier (Model 1900, A-M System, Everett, WA), respectively. Plasma total CO_2 was measured in duplicate on 50 μl samples using a Corning model 965 CO_2 analyzer (Lowell, MA, USA). Tamm was measured using a commercial kit (Raichem, San Diego, CA, USA) based on the glutamate dehydrogenase/NAD method and assayed using a 4054 UV/visible spectrophotometer (LKB-Biochrom, Cambridge, England). Plasma CO_2 tension (Pa_{CO_2}) and bicarbonate concentration ($[HCO_3^-]_a$) were calculated using the Henderson–Hasselbalch equation with plasma pK′ values and CO_2 solubility coefficients for trout plasma at 12 °C from Boutilier et al. (1984). Plasma $[NH_3]_a$, $[NH_4^+]_a$, and NH_3 tension (Pa_{NH_3}) were calculated using the Henderson–Hasselbalch equation with plasma pK′ values and NH_3 solubility coefficients for trout plasma at 12 °C from Cameron and Heisler (1983). Identical techniques were used for perfusate samples in the second series.

For ventilation measurements, the buccal cannula was filled with distilled water and connected to a pressure recording system which consisted of a pressure transducer (Statham P23BB, Statham Instruments, Oxnard, CA, USA), a transducer amplifier (Harvard Apparatus, Holliston, MA, USA), and an oscillograph (Harvard Apparatus, Holliston, MA, USA). The pressure transducer was calibrated directly by connection to a column containing different heights of water. This system allowed recording of ventilation rate (VR, breaths min^{-1}) and the buccal pressure amplitude (ΔP_{buccal}, cmH_2O), as an index of stroke volume. VR was calculated as the frequency of breaths in one minute at the designated time. ΔP_{buccal} was calculated as the average value of ten measurements of amplitude (randomly selected from periods of normal breathing, not using episodes of coughing or disturbance) at the designated time.

2.5. Statistics

Data have been routinely expressed as means ± 1 SEM (N) where N is the number of fish in a treatment mean. Both VR and ΔP_{buccal} were normalized as relative values (%) to control (before injection, infusion, or perfusion) in respective fish. These data were subjected to arc-sine transformation prior to statistical tests. One-way repeated measures (RM) ANOVA followed by Dunnett's test was applied to compare the relative values of VR and ΔP_{buccal} after injection or infusion to initial control values in injection and infusion experiments. In the perfusion experiments, the same approach was used to compare ventilation values during treatments back to values prior to the start of perfusion in each treatment. A Student's two-tailed unpaired t-test was applied to compare ventilation values between the two treatments at each time point during the perfusion procedure. The blood and plasma variables before and after injection or infusion were compared by one-way RM ANOVA followed by Dunnett's test in the case of multiple comparisons, or by Student's two-tailed paired t-test in the case of single comparisons. A significance level of $P < 0.05$ was employed throughout.

3. Results

3.1. Responses to acute dorsal aortic injections

Prior to injections, mean overall values for VR and ΔP_{buccal} were 71 ± 2 breaths min^{-1} ($N = 55$) and 2.5 ± 0.2 cmH_2O ($N = 55$), respectively, and did not differ amongst treatment groups. Mean Pa_{O_2} values were 93.2 ± 3.0 mmHg ($N = 55$) and similarly invariant.

Injection of Cortland saline had no significant effects on ventilation within 60 min after injection (Figs. 1 and 2), and all the blood and plasma variables (ammonia, pH_a, $[HCO_3^-]_a$, Pa_{CO_2},

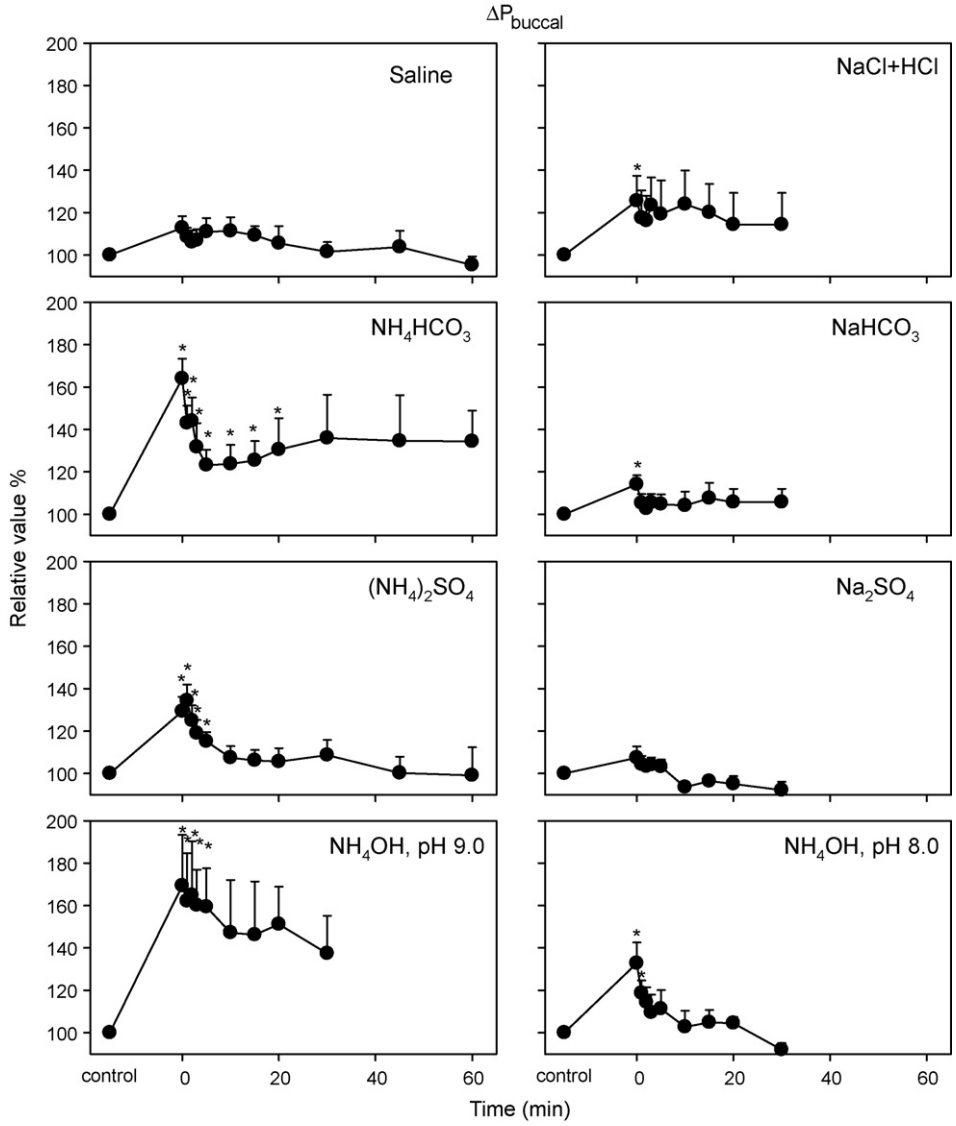

Fig. 1. The relative value (pre-injection control = 100%) of buccal pressure amplitude (ΔP_{buccal}) of rainbow trout following dorsal aortic injection of Cortland saline, 135 mmol l^{-1} NaCl + 5 mmol l^{-1} HCl, 140 mmol l^{-1} NH_4HCO_3, 140 mmol l^{-1} $NaHCO_3$, 70 mmol l^{-1} $(NH_4)_2SO_4$, 70 mmol l^{-1} Na_2SO_4, 140 mmol l^{-1} NH_4OH at pH 9.0, and 140 mmol l^{-1} NH_4OH at 8.0. Values are presented as means + SEM. N = 19 in $(NH_4)_2SO_4$ treatment and N = 6 in other treatments. Asterisks indicate that means are significantly different from control values by one-way RM ANOVA.

Pa_{O_2}) remained unchanged (Table 1). Therefore the experimental regime did not perturb any of the measured variables. In contrast, injection of all the other solutions (except $(Na)_2SO_4$, see below) increased ΔP_{buccal} or VR to differing degrees (Figs. 1 and 2). These changes were associated with various alterations of blood and plasma variables (Table 1). The one exception was Pa_{O_2} which did not change significantly at any time in any treatment (Table 1).

Injection of 140 mmol l^{-1} NH_4HCO_3 resulted in an immediate increase in ΔP_{buccal} and VR. ΔP_{buccal} increased significantly to 164% of the original value immediately after injection and then stabilized around 130% within 5–60 min after injection (Fig. 1). VR increased to 111% immediately after injection and stabilized at about 106% afterwards. At 2 min after injection, there were significant increases in $Tamm_a$, $[NH_4^+]_a$, $[NH_3]_a$, Pa_{NH_3}, $[HCO_3^-]_a$, and Pa_{CO_2} (Table 1). The decrease in pH_a was not significant (Table 1). At 50 min after injection, all of these variables had recovered.

Injection of 140 mmol l^{-1} $NaHCO_3$ caused a small, transitory increase (to 116%) of ΔP_{buccal} (Fig. 1), significant only in the first minute but not of VR (Fig. 2). $[HCO_3^-]_a$ and Pa_{CO_2} were elevated after injection, whereas none of the ammonia variables were affected (Table 1). The rise in pH_a was not significant (Table 1).

Injection of 70 mmol l^{-1} $(NH_4)_2SO_4$ resulted in significant increases in ΔP_{buccal} (to 134%; Fig. 1) and VR (to 106%; Fig. 2) during the initial several minutes after injection, followed by a quick recovery thereafter. Interestingly, the increase in ΔP_{buccal} varied greatly among different fish, less than 10% in 5 (no increase), 10–20% in 4, 20–50% in 7, and >50% in 3 fish. At 2 min after injection, there were significant increases of $Tamm_a$, $[NH_4^+]_a$, $[NH_3]_a$, Pa_{NH_3}, and significant decreases in pH_a, whereas all had recovered by 50 min after injection. $[HCO_3^-]_a$, and Pa_{CO_2} did not change during this treatment (Table 1).

Injection of the 135 mmol l^{-1} NaCl + 5 mmol l^{-1}HCl solution resulted in a transitory increase (to 125%) in ΔP_{buccal} immediately after injection, but no change of VR. This injection did not change any blood variables except for a significant decrease in pH_a (Table 1).

Injection of 70 mmol l^{-1} $(Na)_2SO_4$ did not cause any changes of ventilation or blood variables (Figs. 1 and 2, Table 1).

Injection of the NH_4OH solutions at both pH 8 and pH 9 caused significant increases in both ΔP_{buccal} (Fig. 1) and VR (Fig. 2). ΔP_{buccal}

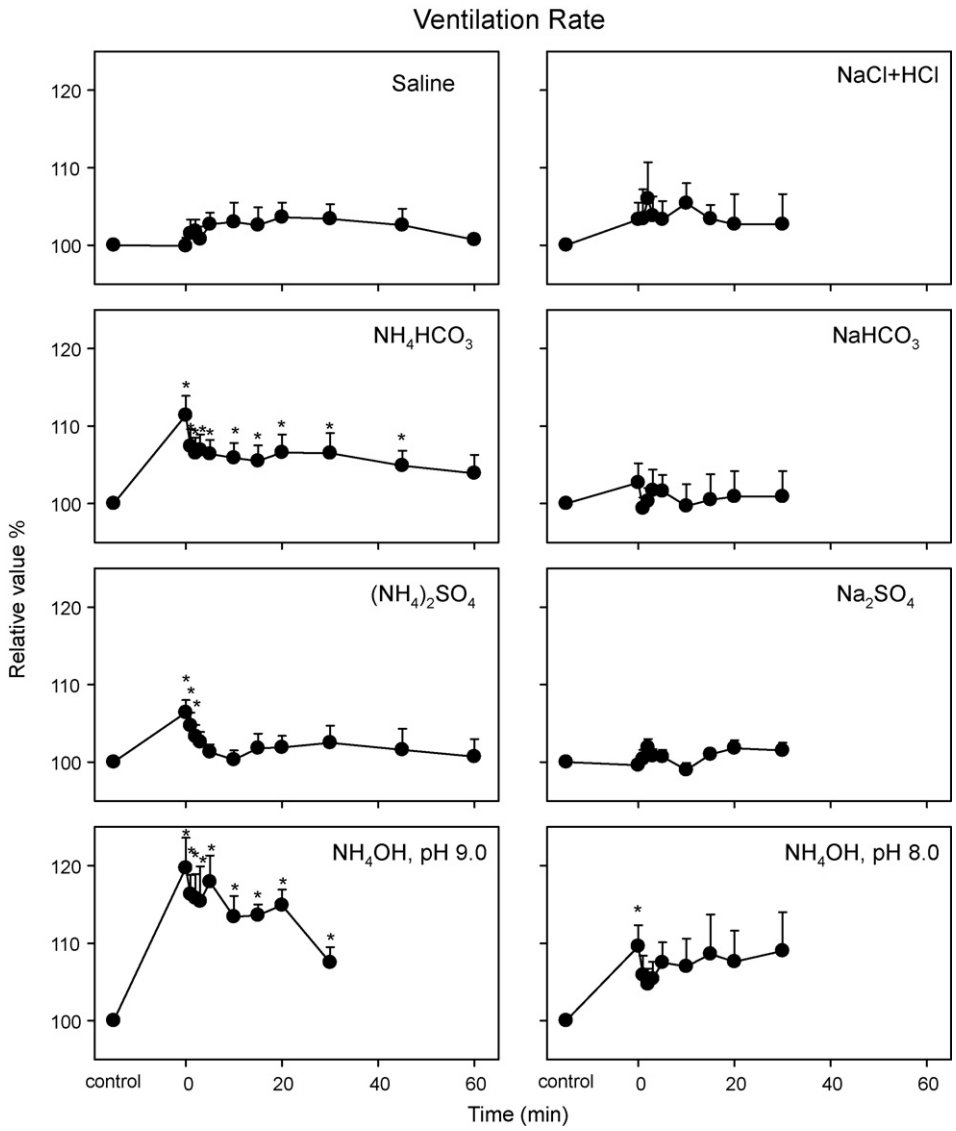

Fig. 2. The relative value (pre-injection control = 100%) of ventilation rate (VR) of rainbow trout following dorsal aortic injection of Cortland saline, 135 mmol l^{-1} NaCl + 5 mmol l^{-1} HCl, 140 mmol l^{-1} NH_4HCO_3, 140 mmol l^{-1} $NaHCO_3$, 70 mmol l^{-1} $(NH_4)_2SO_4$, 70 mmol l^{-1} Na_2SO_4, 140 mmol l^{-1} NH_4OH in pH 9.0, and 140 mmol l^{-1} NH_4OH at 8.0. Values are presented as means + SEM. N = 19 in $(NH_4)_2SO_4$ treatment and N = 6 in other treatments. Asterisks indicate that means are significantly different from control values by one-way RM ANOVA.

was increased to 133% by NH_4OH at pH 8 and to 169% by NH_4OH at pH 9; VR was increased to 110% by NH_4OH at pH 8 and to 120% by NH_4OH at pH 9. The effects of NH_4OH at pH 9 on ventilation were more powerful and lasted longer than those of NH_4OH at pH 8. Both of these two solutions caused significant increases in all plasma ammonia variables though they were less than half of those caused by 140 mmol l^{-1} NH_4HCO_3 or by 70 mmol l^{-1} $(NH_4)_2SO_4$ (Table 1). They also caused significant decreases in pH_a, moreover, NH_4OH at pH 9 also caused an increase in Pa_{CO_2}.

Therefore, all of the ammonium salt injections caused significant increases in both ΔP_{buccal} (Fig. 1) and VR (Fig. 2), and in general these were larger than could be explained by the accompanying changes in pH_a, Pa_{CO_2}, or $[HCO_3^-]_a$ (Table 1) when comparing these results versus responses to $NaHCO_3$ and NaCl + HCl injections, but in none of the treatments was there an effect that could be unequivocally attributed to ammonia alone. The ventral aortic perfusion experiment, where the exact levels of all variables could be precisely controlled, was designed to overcome this problem.

3.2. Responses to ventral aortic perfusion

The two perfusion solutions had essentially identical levels of pH, $[HCO_3^-]$, P_{CO_2}, and P_{O_2}, but very different Tamm, $[NH_4^+]$, $[NH_3]$, and P_{NH_3} values (Table 2). In the high ammonia saline Tamm was 1911 μmol l^{-1}, which was similar to the plasma Tamm after injection of 140 mmol l^{-1} NH_4HCO_3 or 70 mmol l^{-1} $(NH_4)_2SO_4$, although the $[NH_3]$ and P_{NH_3} levels were approximately 2-fold higher (Tables 1 and 2).

The combined effects of ventral aortic cannulation, perfusion, and/or ongoing anaesthesia caused decreases in ΔP_{buccal} and increases in VR, to 79% and 107% of the original pre-perfusion values, respectively (0.9 ± 0.1 cmH_2O, 70 ± 8 breaths min^{-1}, N = 12, in Fig. 3). Note that this mean pre-perfusion ΔP_{buccal}, obtained under anaesthesia, was already lower than seen in unanaesthetized fish of the other experimental series. With continuous perfusion of control saline, ΔP_{buccal} decreased further to 73% and 69% at 10 and 20 min (Fig. 3A), whereas VR remained constant (Fig. 3B). In the experi-

Table 1
Arterial blood pH (pH_a), plasma total ammonia ($Tamm_a$), $[NH_4^+]$ ($[NH_4^+]_a$), $[NH_3]$ ($[NH_3]_a$), NH_3 tension (Pa_{NH_3}), HCO_3^- ($[HCO_3^-]_a$), CO_2 tension (Pa_{CO_2}), and O_2 tension (Pa_{O_2}) in rainbow trout under control conditions and following dorsal arterial injections.

	pH_a	$Tamm_a$ (μmol l^{-1})	$[NH_4^+]_a$ (μmol l^{-1})	$[NH_3]_a$ (μmol l^{-1})	Pa_{NH_3} (10^{-6} mmHg)	$[HCO_3^-]_a$ (mmol l^{-1})	Pa_{CO_2} (mmHg)	Pa_{O_2} (mmHg)
Saline								
Control	7.79 ± 0.04	137.8 ± 14.1	136.4 ± 14.1	1.38 ± 0.42	22.0 ± 7.6	9.41 ± 0.52	3.49 ± 0.20	99.2 ± 8.0
2 min	7.76 ± 0.05	148.6 ± 19.8	147.1 ± 19.8	1.49 ± 0.32	24.2 ± 5.8	8.82 ± 1.06	3.30 ± 0.35	96.7 ± 9.0
50 min	7.74 ± 0.04	136.7 ± 13.1	135.3 ± 13.1	1.36 ± 0.16	22.0 ± 2.9	9.77 ± 1.32	4.29 ± 0.54	84.9 ± 8.7
140 mmol l^{-1} NH_4HCO_3								
Control	7.86 ± 0.06	94.1 ± 27.4	92.6 ± 27.4	1.47 ± 0.37	26.8 ± 6.7	9.37 ± 0.76	2.95 ± 0.14	97.5 ± 4.3
2 min	7.77 ± 0.06	2219 ± 11.9*	2203 ± 11.9*	16.55 ± 3.68*	301.2 ± 21.8*	12.64 ± 1.12*	5.89 ± 1.01*	104.9 ± 6.1
50 min	7.85 ± 0.05	70.9 ± 15.3	69.7 ± 15.3	1.20 ± 0.29	21.8 ± 5.3	10.78 ± 1.11	3.41 ± 0.45	105.3 ± 6.3
70 mmol $^{-1}$ $(NH_4)_2SO_4$								
Control	7.81 ± 0.04	110.6 ± 15.1	109.5 ± 15.1	1.06 ± 0.15	19.3 ± 2.7	10.11 ± 1.26	3.18 ± 0.48	92.2 ± 6.2
2 min	7.65 ± 0.03*	2152 ± 47.1*	2136 ± 47.1*	15.54 ± 0.94*	282.8 ± 17.1*	8.13 ± 0.94	3.44 ± 0.62	97.3 ± 5.8
50 min	7.82 ± 0.04	164.4 ± 17.9*	162.6 ± 17.9*	1.82 ± 0.11*	41.7 ± 4.5*	8.38 ± 1.28	2.63 ± 0.51	99.8 ± 5.7
140 mmol $^{-1}$ NH_4OH pH 9.0								
Control	7.89 ± 0.03	63.9 ± 13.1	63.0 ± 13.1	0.93 ± 0.21	18.9 ± 4.3	9.63 ± 0.86	2.80 ± 0.29	105.3 ± 5.7
2 min	7.75 ± 0.04*	717.6 ± 227.9*	712.0 ± 227.9*	5.94 ± 1.35*	120.8 ± 27.5*	9.33 ± 1.10	3.72 ± 0.34*	95.3 ± 5.4
140 mmol $^{-1}$ NH_4OH pH 8.0								
Control	7.89 ± 0.03	39.2 ± 7.1	38.7 ± 7.1	0.48 ± 0.07	13.8 ± 1.4	10.73 ± 1.80	3.09 ± 0.33	89.0 ± 4.1
2 min	7.74 ± 0.04*	785.6 ± 169.7*	778.1 ± 169.7*	7.05 ± 1.26*	143.4 ± 25.6*	8.74 ± 1.72	3.36 ± 0.50	96.4 ± 7.1
140 mmol $^{-1}$ $NaHCO_3$								
Control	7.82 ± 0.02	42.6 ± 12.6	41.9 ± 12.6	0.69 ± 0.20	14.0 ± 4.1	9.01 ± 0.51	2.92 ± 0.20	83.0 ± 5.2
2 min	7.87 ± 0.02	34.5 ± 3.7	33.9 ± 3.7	0.57 ± 0.07	11.6 ± 1.4	15.29 ± 0.79*	4.64 ± 0.22*	79.8 ± 7.4
70 mmol $^{-1}$ Na_2SO_4								
Control	7.80 ± 0.03	92.4 ± 10.3	91.5 ± 10.3	0.94 ± 0.12	17.1 ± 2.2	8.95 ± 0.56	3.03 ± 0.22	90.0 ± 5.3
2 min	7.82 ± 0.04	86.1 ± 6.8	85.2 ± 6.8	0.88 ± 0.14	16.0 ± 1.1	8.76 ± 0.63	2.84 ± 0.28	89.8 ± 6.9
135 mmol $^{-1}$ NaCl + 5 mmol $^{-1}$ HCl								
Control	7.86 ± 0.01	56.5 ± 4.9	55.8 ± 4.9	0.65 ± 0.08	13.2 ± 1.6	8.84 ± 0.61	2.76 ± 0.22	79.0 ± 5.6
2 min	7.76 ± 0.02*	52.3 ± 8.9	51.7 ± 8.9	0.58 ± 0.08	11.8 ± 1.6	8.42 ± 0.92	3.25 ± 0.17	75.2 ± 6.0

Values are presented as mean ± SEM. N = 19 in $(NH_4)_2SO_4$ treatment and N = 6 in other treatments. Data with asterisks indicate significant difference from control by Student's two-tailed paired t-test.

Table 2
pH, total ammonia (Tamm), $[NH_4^+]$, $[NH_3]$, NH_3 tension (P_{NH_3}), $[HCO_3^-]$, CO_2 tension (P_{CO_2}), and O_2 tension (P_{O_2}) values in the gill-perfusion solutions.

	pH	Tamm (μmol l^{-1})	$[NH_4^+]$ (μmol l^{-1})	$[NH_3]$ (μmol l^{-1})	P_{NH_3} (10^{-6} mmHg)	$[HCO_3^-]$ (mmol l^{-1})	P_{CO_2} (mmHg)	P_{O_2} (mmHg)
Saline	8.03 ± 0.01	13 ± 3	13 ± 3	0.22 ± 0.02	4.7 ± 0.4	8.02 ± 0.55	1.70 ± 0.11	310 ± 5
Saline + NH_4Cl	8.02 ± 0.01	1911 ± 14	1879 ± 14	31.9 ± 0.2	671.5 ± 5.2	7.96 ± 0.30	1.68 ± 0.07	310 ± 6

Values are presented as mean ± SEM. N = 6.

mental treatment, ΔP_{buccal} increased significantly to 96% at 5 min after the switchover to high ammonia saline (10 min data point) and thereafter, when perfusion of control saline was re-instituted, ΔP_{buccal} dropped back to the same level as the control at 20 min. In contrast, VR merely tracked the control response. Thus although the baseline was clearly changing, high perfusate ammonia caused a significant stimulation of ΔP_{buccal}, which was not confounded by changes in pH, P_{CO_2}, or $[HCO_3^-]$ (Table 2).

3.3. Responses to chronic dorsal aortic infusions

Prior to the start of infusion, mean overall values for VR and ΔP_{buccal} were 77 ± 3 breaths min^{-1} (N = 18) and 3.1 ± 0.3 cmH_2O (N = 18), respectively and did not differ amongst groups. Mean Pa_{O_2} values were 85.3 ± 3.9 mmHg (N = 18) and again were the same in all treatments.

Continuous infusion of Cortland saline did not change ventilation significantly during the 24-h period (Figs. 4 and 5). All the blood and plasma variables examined remained constant throughout the infusion period (Fig. 6). Therefore again, the experimental regime itself did not perturb any of the measured variables.

Chronic infusion of 140 mmol l^{-1} NH_4HCO_3 resulted in significant increases in ΔP_{buccal} (Fig. 4) and VR (Fig. 5). ΔP_{buccal} increased significantly by 3 h and stayed elevated throughout the infusion period, at 125–155% of the original value (Fig. 4). VR increased significantly by 3 h and kept constant subsequently, at about 108% of the original value (Fig. 5). Plasma $Tamm_a$, Pa_{NH_3} (Fig. 6) and $[NH_4^+]_a$ and $[NH_3]_a$ (not shown) increased greatly during the infusion process to levels midway between those seen in the injection series (Table 1) and the perfusion series (Table 2). These increases were in the range of 50–70-fold above control levels (Fig. 6). However, the responses were complicated by the fact that both $[HCO_3^-]_a$ and Pa_{CO_2} also increased significantly (Fig. 6). $[HCO_3^-]_a$ remained elevated throughout the infusion, whereas Pa_{CO_2} recovered after 12 h. pH_a remained constant during infusion, although it increased non-significantly after 6 h (Fig. 6). Pa_{O_2} (not shown) did not change.

Chronic infusion of 70 mmol l^{-1} $(NH_4)_2SO_4$ resulted in generally similar increases in ventilation and plasma ammonia values, but without the confounding influence of changes in acid–base status. ΔP_{buccal} increased significantly from 3 to 12 h, up to 156%; although it dropped to 127% at 24 h, it was still significantly higher than the original value (Fig. 4). VR increased to 108% at 3–6 h and 115% at 12–24 h (Fig. 5). Plasma $Tamm_a$, Pa_{NH_3} (Fig. 6), and $[NH_4^+]_a$ and $[NH_3]_a$ (not shown) all increased significantly, by 50–70-fold, at 3–24 h of infusion (Fig. 6). There were no significant changes in pH_a $[HCO_3^-]_a$, Pa_{CO_2} (Fig. 6) or Pa_{O_2} (not shown) during 70 mmol l^{-1} $(NH_4)_2SO_4$ infusion.

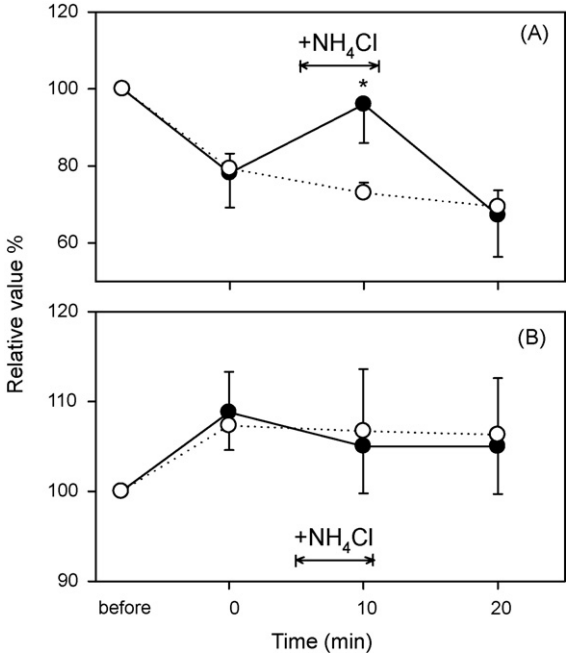

Fig. 3. The relative value (pre-perfusion control = 100%) of (A) buccal pressure amplitude and (B) ventilation rate of rainbow trout following ventral aortic perfusion. In the control treatment (open circles), fish were continuously perfused with Cortland saline; and in the high ammonia treatment (closed circles), fish were perfused with Cortland saline at 0–5 min, which was changed to Cortland saline + 1.9 mmol l^{-1} NH_4Cl at 5–11 min, then back to Cortland saline at 11–20 min. Values are presented as means ± SEM. $N = 6$. Asterisk indicates that the mean in the high ammonia treatment is significantly different from the control treatment by Student's two-tailed unpaired *t*-test.

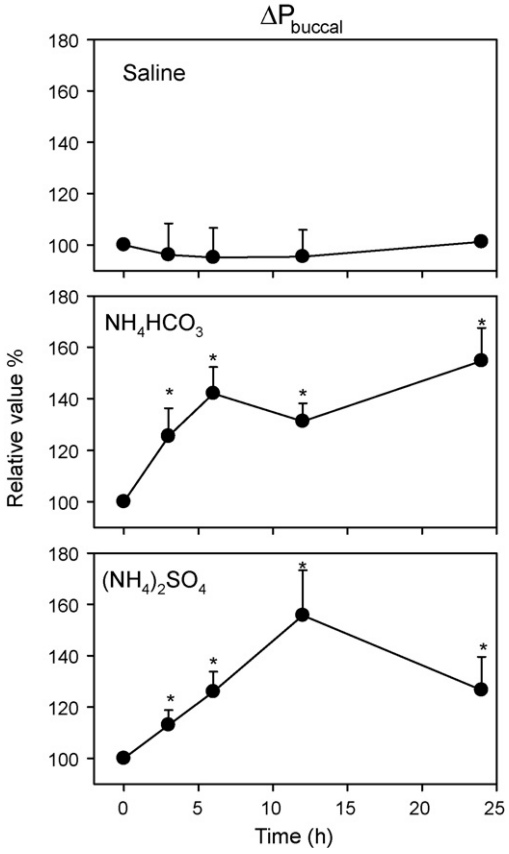

Fig. 4. The relative value (pre-infusion 0 h control = 100%) of buccal pressure amplitude (ΔP_{buccal}) of rainbow trout following dorsal aortic infusion of Cortland saline, 140 mmol l^{-1} NH_4HCO_3, or 70 mmol l^{-1} $(NH_4)_2SO_4$ for 24 h. Values are presented as means + SEM. $N = 6$. Asterisks indicate that means are significantly different from control values by one-way RM ANOVA.

4. Discussion

4.1. Ammonia stimulates ventilation

Intravascular administration of high ammonia solutions resulted in hyperventilation in all the experiments of this study. These treatments included acute dorsal aortic injection of NH_4HCO_3, $(NH_4)_2SO_4$, NH_4OH at pH 8.0, and NH_4OH at pH 9.0, and chronic dorsal aortic infusions of NH_4HCO_3, and $(NH_4)_2SO_4$ in intact, conscious animals. In most treatments, both ventilatory amplitudes (ΔP_{buccal}) and rates (VR) were stimulated, though the former to a much greater extent. This was in accord with the well-established pattern that changes in ventilation are generally achieved by small changes in rate but large changes in stroke volume in trout and most other teleosts (e.g. Janssen and Randall, 1975; McKenzie et al., 1993; Gilmour, 2001).

As in the study of McKenzie et al. (1993), the acute injections of various ammonium salts could not demonstrate that the hyperventilation is a direct consequence of the elevation of ammonia, because of the concurrent changes of one or more of pH_a, Pa_{CO_2}, or $[HCO_3^-]_a$. However, a comparison of the responses between 140 mmol l^{-1} NH_4HCO_3 and 140 mmol l^{-1} $NaHCO_3$ injections (the latter caused similar increases in Pa_{CO_2} and $[HCO_3^-]_a$, but no plasma ammonia elevation), and between 70 mmol l^{-1} $(NH_4)_2SO_4$ and 135 mmol l^{-1} NaCl plus 5 mmol l^{-1} HCl injections (the latter caused a similar drop of pH_a but no plasma ammonia elevation) is instructive. In both cases, the elevation of plasma ammonia resulted in much more intense hyperventilations. Therefore, we suggest that ammonia is able to stimulate ventilation additionally to the effects of arterial CO_2 and pH in rainbow trout.

None of these treatments caused any change in Pa_{O_2}, suggesting that hyperventilation to ammonia in these experiments was not a result of altering blood O_2 status. This is in agreement with the experiments of McKenzie et al. (1993), but not with those of Smart (1978) who recorded a marked decrease in Pa_{O_2}. However the latter used an extremely high environmental ammonia exposure which killed the fish within a few hours, so gill damage likely occurred in that study. However, we cannot eliminate the possibility that high plasma ammonia may have altered haemoglobin O_2 binding, and therefore altered blood O_2 content. This should be examined in the future studies.

Another important piece of evidence that ammonia stimulates ventilation was provided by the ventral aortic perfusion experiment. Ventral aortic perfusion with a high ammonia saline of identical pH, P_{CO_2}, $[HCO_3^-]$, and P_{O_2} caused a stimulation of ventilation in anaesthetized trout, despite the fact that ventilation was depressed in these anaesthetized preparations.

Chronic infusion with 70 mmol l^{-1} $(NH_4)_2SO_4$ provided the most convincing evidence that ammonia stimulates ventilation. In this treatment, the hyperventilation was coupled only with the elevated plasma ammonia during the 24-h infusion period. Simultaneously, there were no changes in blood/plasma O_2, CO_2 tensions or pH status. Indeed, the fact that chronic infusion with 140 mmol l^{-1} NH_4HCO_3 produced very similar elevations in ventilation and plasma ammonia levels, despite differences in acid–base status (elevated Pa_{CO_2} and $[HCO_3^-]_a$) suggests that ammonia was the principal driver in these experiments.

It was notable that all injections of ammonium salts tended to have immediate acidifying effects on the blood plasma (at the 2 min sampling point), although the change was not always significant (Table 1). This is likely because NH_3 was rapidly removed by

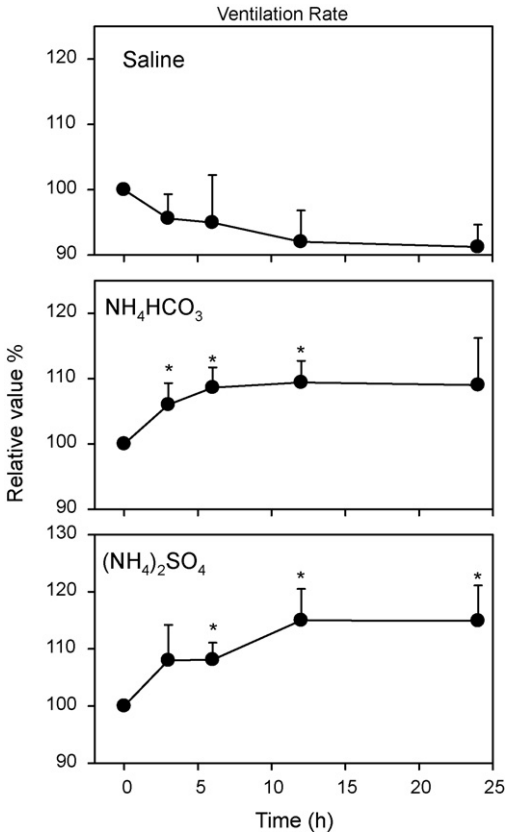

Fig. 5. The relative value (pre-infusion 0 h control = 100%) of ventilation rate (VR) of rainbow trout following dorsal aortic infusion of Cortland saline, 140 mmol l^{-1} NH_4HCO_3, or 70 mmol l^{-1} $(NH_4)_2SO_4$ for 24 h. Values are presented as means + SEM. $N = 6$. Asterisks indicate that means are significantly different from control values by one-way RM ANOVA.

either diffusion across the gills (Hillaby and Randall, 1979), or into the tissues, or by metabolic uptake (e.g. via glutamine synthetase or glutamate dehydrogenase, Duda and Handler, 1958), leaving H^+ behind. Remarkably, this occurred even in cases where some or all of the accompanying anion was a base (i.e. NH_4OH, NH_4HCO_3 injections), suggesting that the *in vivo* situation is dynamic, and re-equilibration reactions take some time. During chronic infusions, with the first sample taken at 3 h, this acidifying effect was no longer seen (Fig. 6), as there was sufficient time for re-equilibration and acid–base homeostatic mechanisms to occur. Very similar pH homeostasis was reported by Salama et al. (1999) for 24-h infusions of NH_4HCO_3 and $(NH_4)_2SO_4$ into trout at a similar rate to that used in the present study. These workers noted a small but significant increase in pH_a with chronic NH_4HCO_3 loading, whereas this was not significant in the present study. Furthermore, by 24 h, the rate of ammonia excretion to the water matched or exceeded the rate of ammonia infusion for both ammonium salts (Salama et al., 1999).

It was surprising that ammonia variables were not significantly different between the 140 mmol l^{-1} NH_4OH treatments at pH 9.0 versus pH 8.0, in spite of the large difference in pH of the injections (Table 1). This may reflect the large buffering capacity of the blood. Because the injection amounts and rates were small, the injection solutions, no matter NH_4OH at pH 9.0 or NH_4OH at pH 8.0, were rapidly mixed with blood and rapidly equilibrated to a similar extracellular pH inside the animal. Therefore, at 2 min after the injection, similar total amounts of ammonia were removed from blood, and remaining plasma ammonia variables were similar.

4.2. The mechanism by which ammonia stimulates ventilation

In mammals, the mechanism by which ammonia stimulates ventilation is unclear (Roberts et al., 1956; Warren, 1958; Campbell et al., 1973; Wichser and Kazemi, 1974). However, the best correlation appears to be with intracellular ammonia concentrations in the brain, suggesting a central site of chemo-detection (Wichser and Kazemi, 1974). In fish, this is certainly a possibility worthy of future study. For example, a recent report has shown that both high environmental ammonia exposure and hypoxia stimulate the corticotrophin-releasing factor system of the pre-optic area and caudal neurosecretory system of trout (Bernier et al., 2008). High intracellular ammonia concentration in central neurons (Wright et al., 2007) may interrupt the normal glutamine–glutamate cycling, inducing increased serotonin production and neurotransmission (Winberg et al., 1997). Another possibility that should be investigated in the future is that ammonia may act on neuroepithelial cells ("NECs") in the gills, which are situated so as to respond to respiratory gases in the blood and/or water (Jonz et al., 2004; Jonz and Nurse, 2006; Milsom and Burleson, 2007; Coolidge et al., 2008). Specifically, these NECs have been proposed as the chemoreceptors responsive to O_2 (and possibly other stimuli) in fish because of their similar structure to mammalian O_2 chemoreceptors and their probable involvement in hypoxic hyperventilation caused by inhibition of the resting K^+ current (Jonz et al., 2004). Randall and Ip (2006) have suggested that these NECs could also mediate NH_4^+-induced hyperventilation if the K^+ channels are permeable to NH_4^+; most values of the permeability ratio of NH_4^+/K^+ in K^+ channels are in the range of 0.1–0.3 (Choe et al., 2000), and therefore, ammonia-induced hyperventilation may occur because of an activation of neuroepithelial O_2 chemoreceptors by NH_4^+ ions.

Another important question is whether the ventilatory sensitivity is due to NH_3 (P_{NH_3}), NH_4^+, or both. Because the distribution of ammonia between NH_3 and NH_4^+ depends on pH, it is not possible to experimentally manipulate the relative concentrations of each without altering acid–base status. A further difficulty in resolving this question lies in the fact that Pa_{NH_3} and $[NH_4^+]_a$ tended to co-vary in most experimental series of the present study. For example, in Fig. 7, we have regressed relative ventilation [relative rate (VR) × relative amplitude (ΔP_{buccal})] (Figs. 4 and 5) against Pa_{NH_3} (Fig. 7A) and $[NH_4^+]_a$ (Fig. 7B) for individual measurements in the chronic infusion series with 70 mmol l^{-1} $(NH_4)_2SO_4$, the one *in vivo* series where there were no complicating changes in Pa_{CO_2}, pH_a, or $[HCO_3^-]_a$ (Table 1). While both relationships are highly significant, variability is large and the r^2 values virtually identical.

It is also possible that ammonia influences ventilation by altering intracellular pH (pH_i). Mammalian glomus cells respond to changes in pH_i elicited by either changes in extracellular pH or the intracellular conversion of CO_2 to H^+ catalyzed by carbonic anhydrase (Putnam et al., 2004; Lahiri and Forster, 2003). If the high ammonia solutions lead to changes in pH_i in chemoreceptor cells, it may be pH_i rather than ammonia per se that triggers ventilatory responses. In the classic ammonium prepulse technique (Roos and Boron, 1981), cells are first exposed to a solution containing elevated Tamm. As NH_3 enters the cell and is converted to NH_4^+, pH_i rises, with the rise gradually tapering off as NH_4^+ also, but more slowly, enters the cell. The cells are then returned to a solution free of Tamm, resulting in intracellular acidification as NH_3 leaves the cells, triggering the dissociation of intracellular NH_4^+ into NH_3 and H^+. The changes in plasma ammonia in the injection series of the present study followed this kind of pattern, i.e. a transient exposure of the tissues to elevated Tamm, raising the possibility of changes in pH_i upon injection of high ammonia solutions. Whether the ventilatory response resulted from the change of pH_i in some specific cells, especially in putative ammonia sensing cells such as the NECs

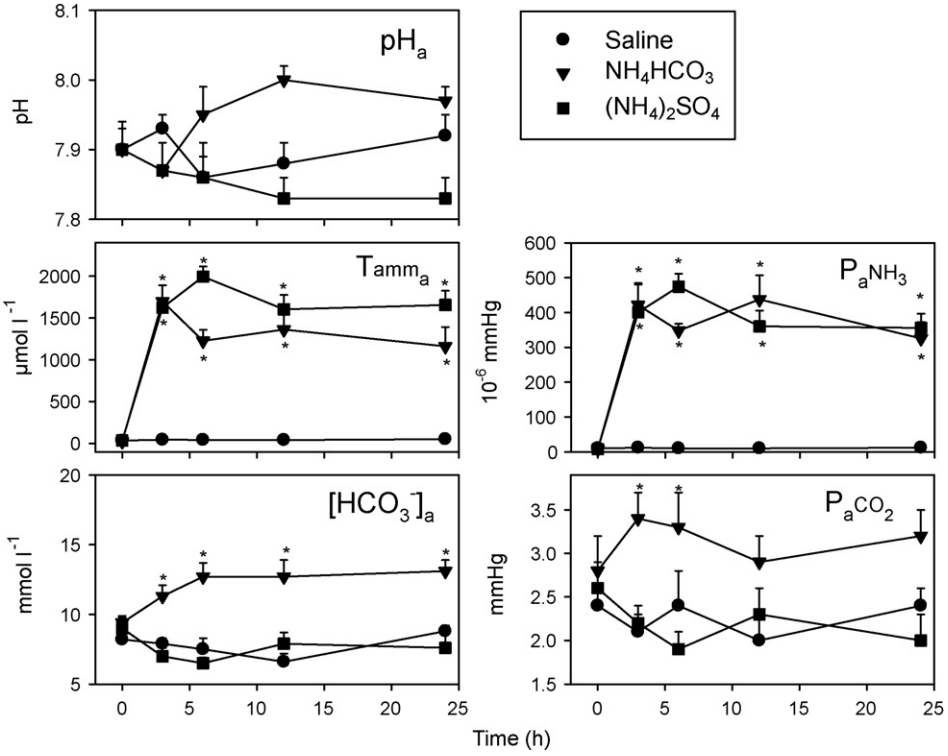

Fig. 6. The changes in arterial blood pH (pH_a), plasma total ammonia ($Tamm_a$), NH_3 tension (Pa_{NH_3}), HCO_3^- ($[HCO_3^-]_a$) and CO_2 tension (Pa_{CO_2}) in rainbow trout following dorsal aortic infusion of Cortland saline, 140 mmol l^{-1} NH_4HCO_3, or 70 mmol l^{-1} $(NH_4)_2SO_4$ for 24 h. Values are presented as means + SEM. $N = 6$. Asterisks indicate that means are significantly different from control values by one-way RM ANOVA.

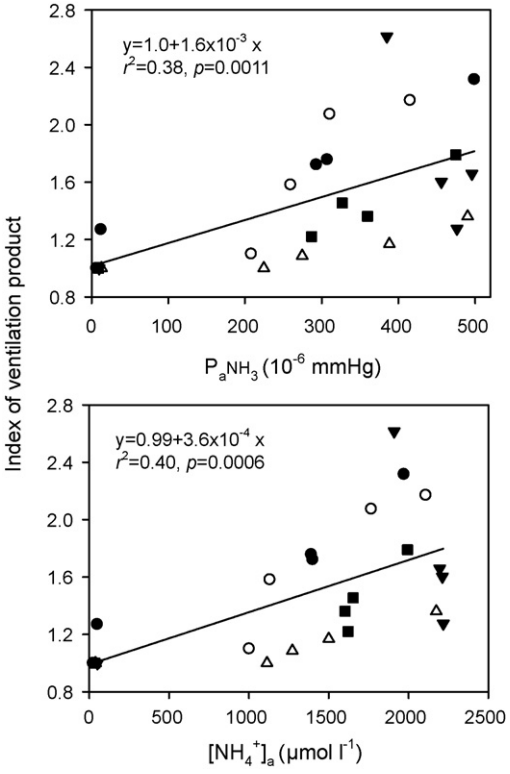

Fig. 7. Regression analyses of the relationship between index of ventilation product [relative rate (VR) × relative amplitude (ΔP_{buccal})] against simultaneous measurements of (A) Pa_{NH_3} and (B) $[NH_4^+]_a$ for individual measurements in the 24-h 70 mmol l^{-1} $(NH_4)_2SO_4$ infusion series. Data for each fish are indicated by a different symbol.

or central chemoreceptors, is an important topic for investigation in future studies.

4.3. The adaptive value of ammonia as a ventilatory control signal

As noted earlier, the adaptive value of the ventilatory response to ammonia in ureotelic mammals is unclear. This may be because it is an evolutionary remnant of a response which originated in fish. However, there are some possible beneficial features in mammals. It is well documented that brain ammonia levels are increased during respiratory acidosis as a buffer mechanism to minimize intracellular acidosis (Kazemi et al., 1967, 1973; Felipo and Butterworth, 2002). Therefore Wichser and Kazemi (1974) suggested that cerebral ammonia accumulation may provide an additional benefit by stimulating ventilation, thereby overcoming respiratory acidosis. A similar idea is that ammonia intoxication and lactacidosis often co-occur as a result of liver failure, so the stimulatory effects of ammonia buildup on ventilation may be beneficial at this time in terms of central acid–base homeostasis (Felipo and Butterworth, 2002). Both of these explanations may apply to teleosts. Fish are able to modify their acid–base status by altering ventilation, though the scope is very limited and probably much less than in mammals (Iwama et al., 1987; Gilmour, 2001; Perry and Gilmour, 2006).

However, there are several other circumstances, perhaps of greater day-to-day significance, where the stimulatory effects may be clearly beneficial in fish. For example, during recovery from exhaustive exercise, fish hyperventilate for a prolonged period even though arterial blood O_2 status is normal (reviewed by Perry and Wood, 1989; Wood, 1991). While this has been attributed to elevated Pa_{CO_2} and/or depressed pH_a (Wood and Munger, 1994; Gilmour, 2001), the persistent rise in $Tamm_a$ (Mommsen and Hochachka, 1988; Wood, 1988; Wang et al., 1994) may also play a stimulatory role. A second circumstance is the hyperventilation that accompanies the increased MO_2 after a meal—the post-prandial

"specific dynamic action" (SDA, reviewed by Jobling, 1994; Secor, 2009). $Tamm_a$ is known to rise greatly at this time (Kaushik and Teles, 1985; Wicks and Randall, 2002a,b; Bucking and Wood, 2008). The systemic "alkaline tide" which also occurs at this time (Bucking and Wood, 2008; Cooper and Wilson, 2008) might be expected to depress ventilation, so elevated $Tamm_a$ may play a key role in providing the increased ventilation needed to satisfy the SDA. A third possibility as to the adaptive value of ammonia-induced hyperventilation is explored in the next section.

4.4. Hyperventilation may facilitate ammonia excretion

As ammonia is highly toxic to fish, whether hyperventilation to high ammonia is a mechanism to facilitate the actual removal of ammonia itself becomes an interesting topic. Traditionally, ventilation is considered to be adequate to maintain ammonia excretion under most conditions because the ammonia excretion rate is much lower than the O_2 uptake or CO_2 excretion rates (Randall, 1990; Randall and Ip, 2006). However in this study, after various injection and infusion treatments, arterial plasma $Tamm_a$ increased to over 1 mmol l^{-1}, which appears to be the lethal threshold concentration in many fish (Wilkie, 2002). Under these circumstances, excretion was clearly limiting, and the same may normally occur *in vivo* after feeding (Kaushik and Teles, 1985; Wicks and Randall, 2002a,b; Bucking and Wood, 2008) or exhaustive exercise (Mommsen and Hochachka, 1988; Wood, 1988; Wang et al., 1994), where $Tamm_a$ concentrations rise to the 200–1000 μmol l^{-1} range even though ammonia excretion rates are greatly elevated.

Randall and Ip (2006) argued that ammonia excretion in teleost fish is diffusion-limited rather than ventilation-limited, so increases in ventilation would not alter ammonia excretion rates. However this statement was made just before the discovery that a group of Rh glycoproteins are involved in facilitating ammonia excretion across fish gills (Nakada et al., 2007; Hung et al., 2007), and that in trout they are induced, at least at the mRNA level, by both high environmental ammonia exposure (Nawata et al., 2007; Tsui et al., 2009) and NH_4HCO_3 infusions similar to those employed in the present study (Nawata and Wood, in press). Under these circumstances, it may be important to increase water flow, and perhaps also blood flow, to match increased diffusion capacity. Diffusion trapping of NH_3 in the water boundary layer at the gill surface, by H^+ ions excreted by apical H^+ATPases, excreted by apical Na^+/H^+ exchangers, or produced by the CO_2 hydration reaction, is an important component of most modern models of branchial ammonia excretion (e.g. Wilkie, 2002; Randall and Ip, 2006; Nawata et al., 2007; Tsui et al., 2009). External alkalinization (Wilkie and Wood, 1991; Wright et al., 1993; McGeer, 1998) and buffering (Wright et al., 1989; Wilson et al., 1994; Salama et al., 1999) both reduce branchial ammonia excretion, so increased ventilatory water flow may be very important to prevent stagnation in the boundary layer at times of increased diffusion capacity. In future studies it will be of interest to see if the Rh proteins are induced by circumstances of "natural" ammonia loading such as feeding or exhaustive exercise, and whether artificial manipulation of ventilation (e.g. Wood and Jackson, 1980; Iwama et al., 1987) can alter ammonia excretion rates. For example, trout could be anaesthetised and ventilated artificially to different extents after infusion of ammonia, to see if gill water flow influences plasma clearance rates.

4.5. Overview

In conclusion, this study provides convincing evidence that elevated internal ammonia stimulates ventilation in rainbow trout, but raises numerous questions as to the precise nature of the signal, the site(s) of ammonia chemo-detection, and the adaptive value of the response. While there are several obvious situations in which this response would appear to be beneficial for O_2 uptake, it remains to be demonstrated whether the resulting increase in ventilation will actually increase ammonia excretion. If hyperventilation does facilitate ammonia excretion, it is difficult to see how this would not also facilitate ammonia uptake during high environmental ammonia exposures, thereby increasing the risks of ammonia toxicity in contaminated environments.

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