

Brain and gills as internal and external ammonia sensing organs for ventilatory control in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Ammonia is both a respiratory gas and a toxicant in teleost fish. Hyperventilation is a well-known response to elevations of both external and internal ammonia levels. Branchial neuroepithelial cells (NECs) are thought to serve as internal sensors of plasma ammonia (peripheral chemoreceptors), but little is known about other possible ammonia-sensors. Here, we investigated whether trout possess external sensors and/or internal central chemoreceptors for ammonia. For external sensors, we analyzed the time course of ventilatory changes at the start of exposure to high environmental ammonia (HEA, 1 mM). Hyperventilation developed gradually over 20 min, suggesting that it was a response to internal ammonia elevation. We also directly perfused ammonia solutions (0.01–1 mM) to the external surfaces of the first gill arches. Immediate hypoventilation occurred. For central chemoreceptors, we injected ammonia solutions (0.5–1.0 mM) directly onto the surface of the hindbrain of anesthetized trout. Immediate hyperventilation occurred. This is the first evidence of central chemoreception in teleost fish. We conclude that trout possess both external ammonia sensors, and dual internal ammonia sensors (perhaps for redundancy), but their roles differ. External sensors cause short term hypoventilation, which would help limit toxic waterborne ammonia uptake. When fish cannot avoid HEA, the diffusion of waterborne ammonia into the blood will stimulate both peripheral (NECs) and central (brain) chemoreceptors, resulting in hyperventilation. This hyperventilation will be beneficial in increasing ammonia excretion via the Rh metabolon system in the gills not only after HEA exposure, but also after endogenous ammonia loading from feeding or exercise.

1. Introduction

Fish are known to hyperventilate in response to elevations in environmental ammonia (Smart, 1978; Lang et al., 1987; Fivelstad and Binde, 1994; Knoph and Thorud, 1996). While this is often interpreted as a general stress response to a toxicant, it may in fact reflect activation of a key mechanism for controlling ventilation. As teleost fish are ammoniotelic, they metabolically produce and excrete large amounts of ammonia across the gills at a rate amounting to 10–20% of O₂ consumption and CO₂ production. Ammonia, like carbon dioxide (CO₂ + HCO₃⁻) exists in both unionized (NH₃) and ionized (NH₄⁺) forms. (Randall and Ip, 2006). Rh glycoproteins, as well as aquaporins, are widely distributed in fish tissues, especially the gills (Nawata et al., 2007; Nakada et al., 2007; Wright and Wood, 2009), serving to facilitate ammonia and CO₂ movement across membranes (Perry et al., 2010;

Talbot et al., 2015). Using the rainbow trout as a model system, it has been established that increases in blood ammonia can stimulate ventilation (McKenzie et al., 1993), that this effect is independent of changes in the other respiratory gases (Zhang and Wood, 2009), and that the hyperventilation is effective in increasing ammonia excretion across the gills, once diffusive limitation (Randall and Ip, 2006) has been removed by activation of the Rh metabolon system (Eom et al., 2020). For all these reasons, there is growing recognition that ammonia is a respiratory gas in fish (e.g. Perry and Tzaneva, 2016; Jonz, 2018), an idea first articulated by Randall and Ip (2006).

The neuro-epithelial cells (NECs) on the first and second gill arches, which are known to be polymodal receptors for the other respiratory gases (reviewed by Milsom, 2012; Perry and Tzaneva, 2016; Jonz, 2018) appear to play a key role in sensing blood ammonia levels (Zhang et al., 2011; Zhang et al., 2015). In mammals, it has long been known that

Abbreviations: ANOVA, analysis of variance; EDF, extradural fluid; HEA, high environmental ammonia; I.D., inner diameter; MS-222, tricaine methanesulfonate; NEC, neuroepithelial cell; O.D., outer diameter; PO₂, partial pressure of oxygen; Rh, Rhesus glycoprotein; Vw, ventilatory flow.

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elevations in blood ammonia can stimulate ventilation (Poppell et al., 1956; Roberts et al., 1956; Warren, 1958; Campbell et al., 1973) probably by actions on central chemo-sensors in the brain (Wichser and Kazemi, 1974). Detection in the brain may also play a role in fish, as Zhang et al. (2013) showed a close association between increases in ventilation and increases in brain total ammonia levels in trout during various experimental treatments. However, the evidence was only correlational. It is less clear how elevations in external ammonia are sensed. The hyperventilatory response to high environmental ammonia (HEA) seems to develop gradually over time (Zhang et al., 2013), in contrast to the almost instantaneous response to injections of ammonia into the blood stream (McKenzie et al., 1993; Zhang and Wood, 2009; Zhang et al., 2011). This could suggest that ammonia is not detected externally, and that ventilation rises during HEA exposure only once blood ammonia increases by inward diffusion across the gills to a level sufficient to stimulate internal receptors (NECs and/or brain). Indeed, this appears to be the case in the hagfish, a primitive ancestral vertebrate (Eom et al., 2019) and in the elasmobranch dogfish, a representative of another ancient lineage (De Boeck and Wood, 2015).

The present study focuses on short-term ventilatory responses to ammonia in trout, in order to understand whether ammonia is detected externally, and whether the brain serves as an ammonia sensor. In Series I, we exposed the fish to HEA, and closely followed the initial time course of the ventilatory responses. As previous studies had made only indirect measurements (buccal pressure amplitude and frequency) of ventilatory flow (Vw), we additionally measured Vw directly using a flow-meter system. In Series II, we measured ventilation changes in response to direct perfusion of ammonia onto the external surfaces of the gill arches. In Series III, we measured ventilation changes in response to injections of ammonia in mock extradural fluid (EDF) directly onto the surface of the brain. Our overall hypothesis was that fish would have both external and multiple (gill NECs and brain) internal ammonia receptors so as to make appropriate ventilatory responses to this respiratory gas and widespread toxicant.

2. Materials and methods

2.1. Experimental animals

Rainbow trout (100–300 g) were obtained from a hatchery (Miracle Springs Inc., Fraser Valley, BC, Canada) and held in 900-L fibreglass tanks, 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 CaCO₃; pH 7.0; temperature, 6.5–9.0 °C) at the University of British Columbia. The fish were regularly fed with commercial food pellets (EWOS, Surrey, BC, Canada) three times per week but they were fasted for a week prior to experiments in order to minimize the internally increased ammonia metabolism after feeding. The experiments were approved by the University of British Columbia under animal care protocol # A17-0301 following the guidelines of the Canadian Council on Animal Care. After completion of the experiments, the fish were humanely euthanized using an over-dose (360 mg L⁻¹) of tricaine methanesulfonate (MS-222, Western Chemicals Inc., Ferndale, WA, USA; pH was adjusted to 7.0 with 5 M NaOH).

2.2. Fish cannulations and analytical techniques

Three different series of experiments were performed. Series I ($N = 7$) used both flow and buccal pressure measurements to monitor the immediate ventilatory responses of the fish to high environmental ammonia (HEA) in the external water. Series II ($N = 5$) employed buccal pressure measurements to examine the ventilatory responses to a logarithmic series of increasing ammonia concentrations perfused directly onto the external surfaces of the gill arches. Series III ($N = 6$) explored the effects on ventilation (monitored by buccal pressure measurements) of injection of different concentrations of ammonia onto the surface of

the hindbrain (Fig. 1).

In all series, fish were surgically prepared under deep anaesthesia (180 mg L⁻¹ MS-222, pH neutralized to ~7.0 with 5.0 M NaOH), and in Series I and II, during surgery, Vaseline™-impregnated cotton balls were inserted into both nares to block the nostril entrance so as to remove olfactory complications (Wisby and Hasler, 1954). After completion of surgery in these first two series, the fish were allowed to recover overnight in individual boxes (black plexiglass chambers, 380 × 100 × 180 mm, length × width × height) served with 500 ml min⁻¹ of aerated flowing dechlorinated water at the acclimation temperature, (6.5–9.0 °C). In Series I, as described below, after overnight recovery, the fish were transferred to a newly developed system for the direct measurement of ventilation flow (Vw) (Eom and Wood, 2020). In Series II, the fish were maintained in their individual boxes. In Series III, the fish were again maintained in their individual boxes for experimentation. However, surgery was performed under deep anaesthesia, and then the fish were switched to a lighter level of anaesthesia (60 mg L⁻¹ MS-222, pH neutralized) for 2–3 h of recovery, and this lighter anaesthesia was maintained during the subsequent experiments. All experiments were performed in aerated flowing dechlorinated Vancouver city water (6.5–9.0 °C).

In Series I, the fish were anesthetized and placed on an operating table while neutralized MS-222 solution was irrigated over the gills via the opercular openings. Using a #18G needle, a hole was drilled through the roof of mouth cavity, taking care to avoid the nares, and a 3-cm sleeve of PE160 polyethylene tubing (Clay-Adams, Sparks, MD, USA; O.D. 1.57 × I.D. 1.14 mm), heat-flared at the buccal side, was inserted through the hole. An additional 30-cm length of PE50 tubing (Clay-Adams, O.D. 0.97 × I.D. 0.58 mm), again heat-flared at the buccal side, was inserted through the PE160 which was already fitted into mouth cavity. The cannulae were cemented together using cyanoacrylate glue (Krazy Glue, High Point, NC, USA). In order to prevent movement of the cemented tubing, the outside of cemented tubing was tightened with a large silk suture knot. The catheter was used to monitor the frequency and buccal pressure amplitude of ventilation (Holeton and Randall, 1967). The overnight recovered fish were then transferred into a newly developed flow-measuring device which has been described in detail by Eom and Wood (2020); see Fig. 1 in that paper. The system is designed for measuring ventilatory flow (Vw) without any further surgical operation, and is submerged in an 8-L temperature-controlled, recirculating reservoir. In brief, the apparatus is based on the original mask-system of Van Dam (1938). However, instead of a constant-level overflow, the system incorporates an electromagnetic blood flow probe (Transonic Systems Inc., Ithaca, NY, USA) coupled to a flow-meter (T106 series, Transonic Systems Inc.) into the front of the form-fitting mask for the continuous recording of ventilatory water flow. As in Van Dam (1938), no stitches or gluing are used to attach the mask. However, the fish is restricted inside a hollow polyvinyl chloride tube which minimizes side-to-side and back-and-forth movement.

In Series II, the first buccal catheter was placed as in Series I, and a second buccal catheter was placed for perfusing ammonia directly onto the external surfaces of the gill arches, using a technique similar to that of Daxboeck and Holeton (1978). For the latter, a second hole was drilled through the other side of the roof of the mouth cavity using the #18G needle, again avoiding the nares. Another heat-flared 3-cm PE160 sleeve was placed, and a 30-cm length of PE50 tubing was inserted through the PE160 sleeve. A Y-shaped connector (I.D. 1.59 mm, Cole-Parmer, St. Montreal, QC, Canada) was joined to the PE50 tubing at the buccal cavity end; the size gap between the PE50 tubing and Y-connector was filled by wrapping the parafilm around the PE50 so as to make a tight seal. The device was positioned so that the open Y-ends targeted the 1st pair of gill arches, and was tightly sutured to the roof of the mouth. The other end of the tubing was connected to a Minipuls 2 peristaltic pump (Gilson, Middleton, WI, USA) in order to apply a series of increasing ammonia concentrations directly to the external surfaces of the two gill arches simultaneously. The buccal pressure changes

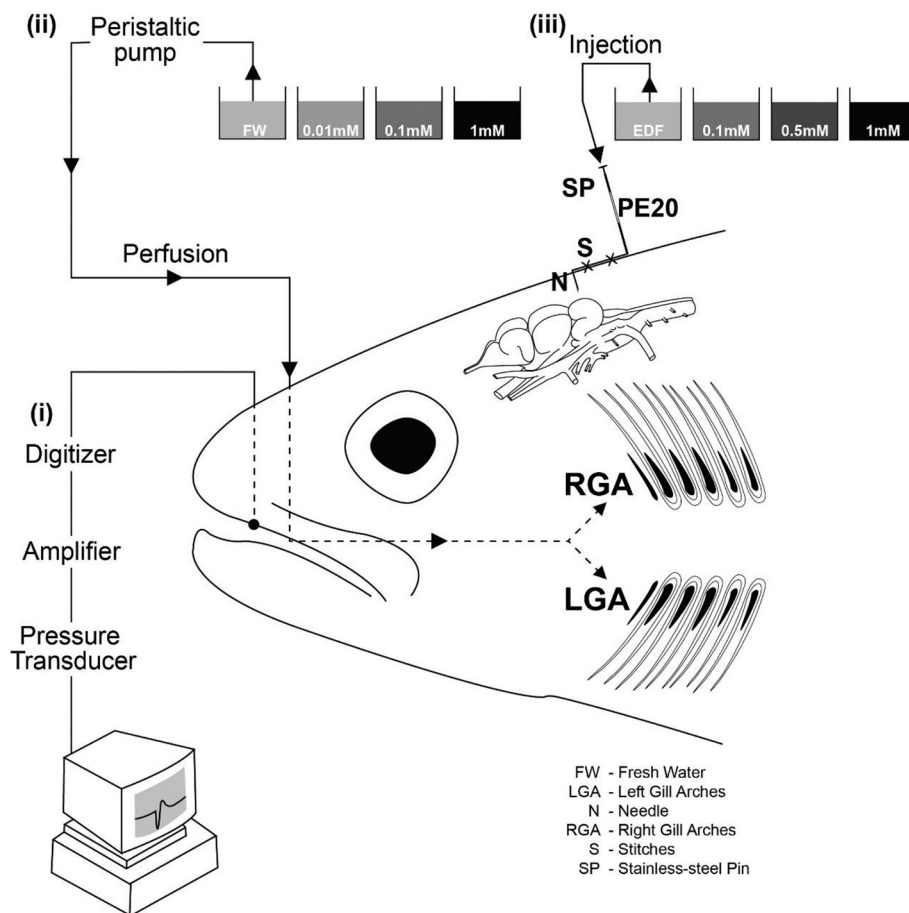


Fig. 1. Schematic diagram of the experimental setups. Two cannulations were placed in the buccal cavity: (i) for measuring buccal ventilation in Series I, II, and III, and (ii) for additionally perfusing a logarithmic series of increasing ammonia concentration between 0.01 mM and 1 mM directly to external surfaces of the 1st gill arches in Series II. In Series III, (iii) another cannulation was performed, placing a needle-tip into the cranial cavity over the hind-brain for injecting ammonia concentrations between 0.1 mM and 1 mM prepared in mock EDF directly onto the brain surface.

associated with breathing could be monitored using the first buccal catheter, as in Series I.

In Series III, a buccal catheter was placed as in Series I for monitoring ventilation. Another cannula was placed in order to precisely inject ammonia solutions onto the hind brain. A #26G needle was cut off at 2-cm length, and bent at two points: 90° and -90° at 1/3 and 2/3 of the distance along the cut needle. The sharp-end of cut needle was gently inserted through the translucent cartilage of the cranium, using pre-determined co-ordinates based on the dissection of dead trout, so as to penetrate into the cranial cavity and terminate directly over the hind-brain. The blunt-end of the needle was connected to 5-cm length of PE20 tubing (Clay-Adams, O.D. 1.09 x I.D. 0.38 mm), facilitating the injection of ammonia solutions prepared in mock extracellular fluid (EDF; 120 mmol L⁻¹ NaCl, 4 mmol L⁻¹ KCl, 1 mmol L⁻¹ MgSO₄, 1 mmol L⁻¹ CaCl₂, 10 mmol L⁻¹ NaHCO₃) (Hedrick et al., 1991) into the hindbrain cavity. The PE20 and connected needle were always filled with mock EDF, and plugged with a stainless-steel pin to prevent brain damage from external freshwater contamination. After completion of the experiment, the fish were humanely euthanized by an overdose of pH-neutralized MS-222 (0.5 g L⁻¹) and the position of needle was confirmed at necropsy.

In all series, the extended PE50 tubing from the buccal catheter was filled with freshwater and connected to a medical pressure transducer (Utah Medical Products, Midvale, UT, USA) which had been two-point calibrated against the experimental water surface (0 cmH₂O) and 2 cm above the water surface (2 cmH₂O). The buccal pressure fluctuations were amplified by an LCA-RTC amplifier (Transducer Techniques, Temecula, CA, USA) and converted into a digital signal by a Powerlab™ data integrity system (ADInstruments, Colorado, Springs, CO, USA). Using LabChart™ version 7.0 (ADInstruments), the converted pressure signals were analyzed into pressure amplitude (cmH₂O) and frequency

(min⁻¹). These ventilation parameters were then exported to Excel (Microsoft, Redmond, WA, USA) for further calculation of ventilatory index (cmH₂O min⁻¹) as the product of buccal pressure amplitude times frequency, which is a commonly used indicator of total ventilatory flow in fish (e.g. Zhang et al., 2013; Eom and Wood, 2020).

In Series I, the ventilatory flow (V_w) was also digitized into the above Powerlab™ data integrity system (ADInstruments) and the recorded data were exported to Excel (Microsoft) for further calculation of stroke volume (ml kg⁻¹) as the product of buccal V_w divided by frequency. However, at the time these experiments were performed, the ventilatory flow-measuring device was only a prototype still under development, and we are not confident that the actual flow calibration was correct, whereas we are confident in the relative changes. For this reason, the experimental flow (V_w) and stroke volume measurements during the experimental HEA treatment have been normalized as a percentage of the preceding control measurements (100%) for each fish.

2.3. Experimental series

2.3.1. Series I – Acute responses to high environmental ammonia (HEA) in the external water

After overnight recovery from surgery, the fish were gently transferred to the ventilatory flow-measuring device, while aeration continued in the water reservoir. The buccal catheter and flow probe were connected to the pressure transducer and flow-meter respectively, simultaneously measuring buccal pressure and ventilation flow (V_w) until stable recordings were achieved for a control period of 10–20 min. Then 16 ml of freshwater were removed and 16 ml of 500 mmol L⁻¹ NH₄HCO₃ (Sigma-Aldrich, St. Louis, MO, USA) were added to the air bubbling site to prepare 1 mM of high environmental ammonia (HEA, pH 7.0, N = 7); buccal pressure and V_w were measured continuously for

the first 20 min of the HEA treatment.

2.3.2. Series II – Ammonia perfusion to the external surfaces of the gill arches

The black plexiglass boxes were maintained on flow-through. Prior to experiments ($N = 5$), each concentrated NH_4HCO_3 ammonia solution was prepared in a 500-ml glass beaker and placed in the same flow-through water reservoir as for the fish chambers in order to keep the temperature of the solution equal to that of the fish. Also, the correct distribution of solutions pumped through the Y-tube system was checked using commercial food dye (McCormick & Company, Inc., Hunt Valley, MD, USA). Ventilatory parameters were monitored throughout the experiment by pressure recordings from the first buccal catheter, as in Series I. For control measurements, the intake end of the PE50 tubing connected to the Y-tube system was submerged in the fish box so that freshwater was continually pumped to left and right gill 1st arches via the peristaltic pump at a rate of 2.28 ml min^{-1} . By subsequently shifting the intake of the tubing from 0.01, 0.1, and finally 1 mM NH_4HCO_3 , all at pH 7.0, the external surfaces of the gills of fish were exposed to progressively higher ammonia solutions. Ammonia perfusions were for 5 min for each concentration, separated by approximately 5 min of freshwater perfusion, so as to completely remove the previous solution from the gill region.

2.3.3. Series III– Ammonia injection onto the hindbrain

After completion of surgery in Series III, the fish were transitioned to lighter anaesthesia (60 mg L^{-1} neutralized MS-222), representing Stage 4 of McFarland (1959), and allowed to stabilize for several hours, with the flow-through maintained by a recirculating system. This represented: “total loss of equilibrium, reaction only to strong stimuli, opercular movements slightly depressed”, and was maintained throughout the experiments. The fish were breathing continuously, generally unresponsive, but still showed strong ventilatory responses to stimuli injected into the cranial cavity. Prior to experiments ($N = 6$), injection solutions were equilibrated to the experimental temperature and atmospheric PO_2 . Using a gas-tight glass syringe (Hamilton, Reno, NV, USA), $15 \mu\text{l}$ of mock EDF (control), or NH_4HCO_3 solutions (0.1 mmol L^{-1} , 0.5 mmol L^{-1} , and 1 mmol L^{-1} NH_4HCO_3 in mock EDF, pH 7.8) were gently injected to the hindbrain over 1 s through the PE20 cannula. Injections were administered in random order at intervals $>5 \text{ min}$. Meanwhile, the ventilatory parameters were simultaneously measured from the buccal catheter as in the other two series. The ventilation tracings from each fish were analyzed in twelve successive 3-s “bins”, starting at the time of injection, resulting in 12 sets of mean data which covered the first 36 s of the response. These were then averaged to produce a mean response for each fish for each injection. In turn, these were then averaged ($N = 6$) to show the mean responses.

2.3.4. Statistical analysis

Data have been reported as means $\pm$ S.E.M (N). One-way repeated-measures ANOVA followed by Dunnett’s post hoc test was applied for comparing ventilatory parameters back to reference or control levels in the three experimental series (GraphPad Prism 6.0, San Diego, CA, USA). In Series III, a two-tailed paired Student’s test was used to evaluate if there were any significant ventilatory changes to injections of mock EDF alone, and as the small increases were not significant, the response to mock EDF alone was used as the reference control level. The threshold for statistical significance was $p < 0.05$.

3. Results

During 20 min of acute 1 mM HEA treatment in Series I, the fish gradually increased ventilatory flow (V_w) and buccal ventilatory index. After perfusion of ammonia to the external surfaces of the gill arches in Series II, there was an immediate dose-dependent hypoventilation, whereas in Series III, internal injections of ammonia into the hindbrain

cavity elicited immediate dose-dependent hyperventilation.

3.1. Series I - Acute responses over time to high environmental ammonia (HEA) in the external water

In response to acute 1 mM HEA exposure, the fish gradually increased V_w over 20 min (Fig. 2A). By repeated measures one-way ANOVA, the overall response was significant ($p = 0.0045$), and by Dunnett’s test, the change became significant at 12–16 min where it peaked at about a 34% increase. The ventilatory stroke volume exhibited a similar increasing pattern (Fig. 2B), but the overall response was not significant. During 1 mM HEA exposure, the fish also increased buccal ventilatory index (Fig. 2C; control: $307.4 \pm 66.2 \text{ cmH}_2\text{O min}^{-1}$), a response which was significant overall by repeated measures one-way ANOVA ($p = 0.0003$) and significant by Dunnett’s test at 8 min. The maximum increase was 34%. This was mainly due to gradually increasing buccal pressure amplitude (Fig. 2D; control: $4.1 \pm 0.9 \text{ cmH}_2\text{O}$). However, the increase in buccal pressure was not significant by repeated measures one-way ANOVA ($p = 0.2017$). At 2 min, the mean elevation in buccal pressure amplitude was only 3%, but this gradually increased to 25%. Regardless, the ventilation frequency was virtually stable during 1 mM HEA (Fig. 2E, control: $71.9 \pm 2.7 \text{ min}^{-1}$). The overall decline at 18–20 min in some of the mean responses (Fig. 2A,B) reflected decreases in 2 of the 7 fish, whereas ventilation remained elevated in the other 5 trout.

3.2. Series II – Ammonia perfusion to the external surfaces of the gill arches

During the control period, the fish exhibited the following mean ventilatory parameters: ventilation pressure amplitude of $1.2 \pm 0.1 \text{ cmH}_2\text{O}$, frequency of $66.2 \pm 7.1 \text{ min}^{-1}$, and ventilatory index of $77.2 \pm 13.3 \text{ cmH}_2\text{O min}^{-1}$. Representative responses of the ventilatory index in a single trout to perfusion of the external surfaces of the gill arches with ammonia are shown in Fig. 3, and mean responses of all ventilatory parameters in Fig. 4. In response to 0.01 mM ammonia perfusion directly to the external surfaces of the first pair of gill arches, the fish decreased the pressure amplitude by 17% (Fig. 4B, repeated measures one-way ANOVA and Dunnett’s test, $p = 0.0109$) and the ventilatory index by 18% (Fig. 4C, $p = 0.0007$). When the external ammonia concentration was increased to 0.1 mM, the hypoventilatory responses increased and the trout decreased the buccal pressure amplitude by 34% (Fig. 4B, $p = 0.0109$) and the ventilatory index by 35% (Fig. 4C, $p = 0.0007$). At 1 mM ammonia, the response slightly attenuated but remained significant, averaging about 29% inhibitions for both pressure amplitude (Fig. 4B) and ventilatory index (Fig. 4C, $p = 0.0230$). Regardless of the concentration of ammonia perfused to the external surfaces of the gill arches, the fish barely changed the breathing frequency (Fig. 4A, control: $66.2 \pm 7.1 \text{ min}^{-1}$).

3.3. Series III – Ammonia injection into the hind-brain cavity

Overall, the fish increased ventilation in response to injections of ammonia (prepared in mock EDF) onto the hindbrain. Original recordings illustrating the range of responses observed are shown in Fig. 5, and mean responses are summarized in Fig. 6. Responses typically increased over about 30–40 s, then attenuated over the following 30 s. As explained in Methods, the mean responses over the first 36 s after injection were calculated for each fish, then averaged for all 6 fish.

Although trout slightly increased mean ventilatory parameters after injections of mock EDF alone relative to the pre-injection control values, the responses were not significant ($p = 0.5183$, Fig. 6). After injections of mock EDF alone, the fish showed the following mean ventilatory parameters: ventilation pressure amplitude of $1.6 \pm 0.2 \text{ cmH}_2\text{O}$, frequency of $54.6 \pm 4.0 \text{ min}^{-1}$, and ventilatory index of $82.6 \pm 17.4 \text{ cmH}_2\text{O min}^{-1}$. Responses to ammonia injections were compared to these values. Trout

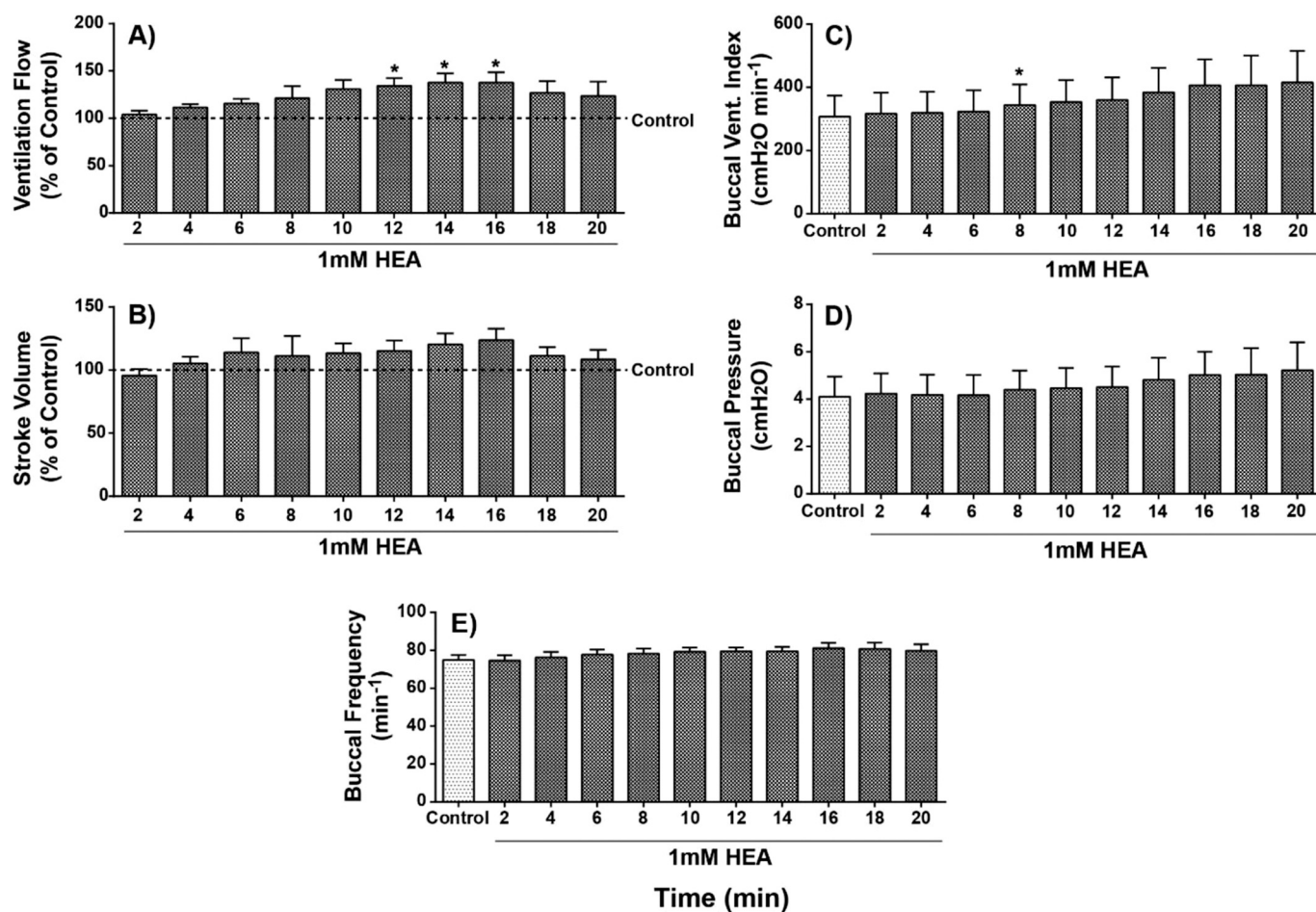


Fig. 2. The immediate time course of ventilatory responses to 1 mM HEA (NH₄HCO₃) exposure in rainbow trout in Series I. (A) relative ventilation flow (Vw); (B) relative ventilatory stroke volume (SVw); (C) buccal ventilatory index; (D) buccal pressure amplitude; and (E) buccal frequency. Means $\pm$ SEM ($N = 7$). The dashed line indicates the control level (100%) prior to HEA exposure in panels A and B. Asterisks indicate significant differences ($p < 0.05$) from control by repeated measures one-way ANOVA and Dunnett's test.

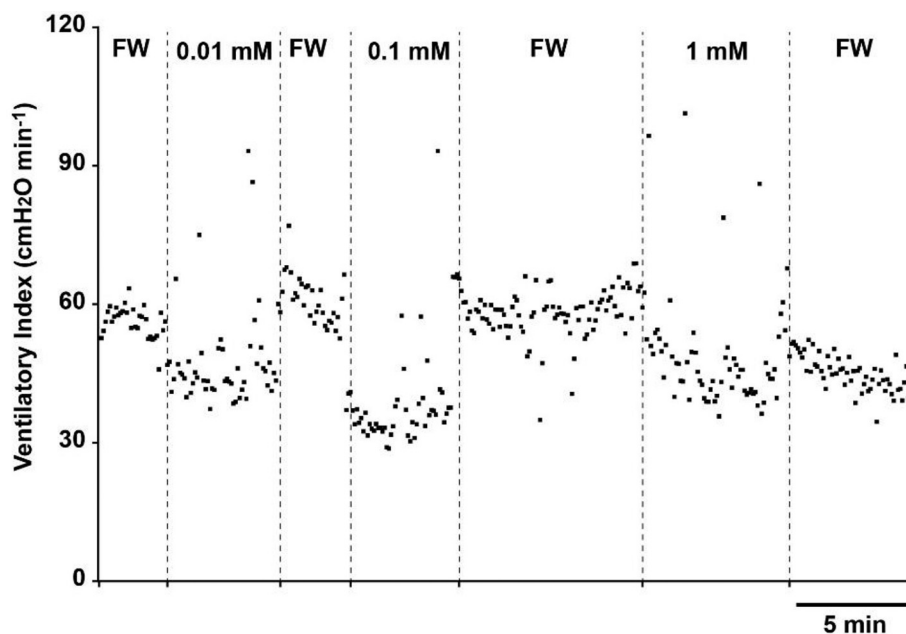


Fig. 3. Representative variations of buccal ventilatory index in a single trout of Series II in response to perfusion of a logarithmic series of increasing ammonia concentrations directly to the external surfaces of the 1st gill arches. Compared to control levels, the fish immediately decreased ventilatory index in response to 0.01 mM ammonia perfusion, and the extent of hypoventilation tended to increase with increased ammonia concentration. Each dot represents averaged ventilatory index per 3 s, whereas the bar below shows a 5-min time scale.

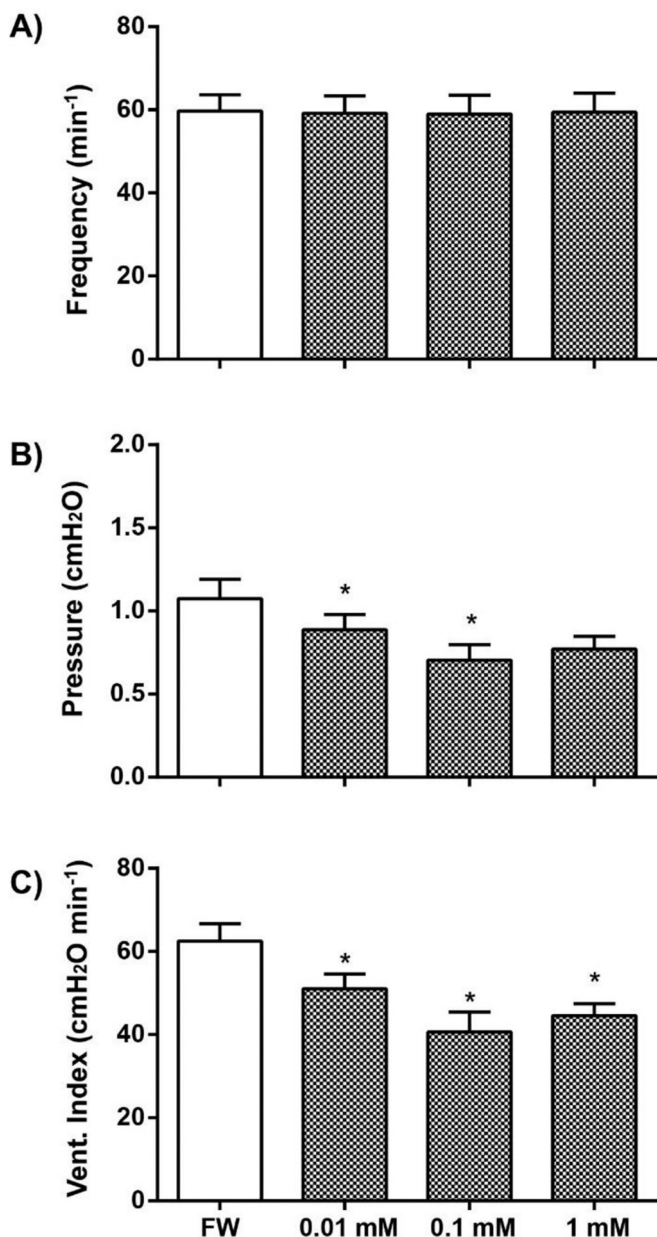


Fig. 4. Mean ventilatory parameters in Series II in response to perfusion of increasing ammonia concentrations to the external surfaces of the 1st gill arches. Means $\pm$ SEM ($N = 5$). The fish (A) barely changed overall frequency levels, but (B) significantly decreased buccal pressure amplitude and its level became lower as the perfused ammonia concentration increased (repeated measures one-way ANOVA, $p = 0.0047$). In (C), these responses in pressure amplitude were reflected in similar patterns in buccal ventilatory index ($p < 0.0001$). Asterisks indicate individual means significantly different from control by Dunnett's test.

barely changed their ventilatory parameters in response to the lowest concentration of ammonia (0.1 mM) in mock EDF. However, in response to a 5-fold higher concentration (0.5 mM) of injected ammonia, both ventilatory pressure amplitude and ventilatory index increased significantly by 21% (Fig. 6B, repeated measures one-way ANOVA plus Dunnett's test, $p = 0.0442$) and 25% (Fig. 6C, $p = 0.0342$). After 1 mM ammonia injection, there were further increases in both parameters, reaching about 28% ($p = 0.0246$) and 45% ($p = 0.0332$) in buccal pressure and ventilatory index respectively (Fig. 6B, C). Regardless of the concentration, the fish barely changed the breathing frequency (Fig. 6A).

4. Discussion

4.1. Overview

The present results provide support for our overall hypothesis that trout would have both external and multiple internal receptors (gill NECs and brain) for ammonia, so as to make appropriate ventilatory responses to a substance that is both a respiratory gas and a toxicant. Previous studies (summarized in the Introduction) have shown that the NECs on the first two gill arches play a key role in sensing elevated blood (i.e. internal) ammonia levels, thereby triggering hyperventilation. Using direct Vw measurements and a fine time course analysis, Series I supplied confirmation that the hyperventilation in response to exposure of the whole animal to elevated external ammonia (HEA) is not immediate, but develops gradually over time, suggesting that it is driven by internal ammonia receptors responding to elevated blood ammonia, rather than by external ammonia receptors. Indeed, Series II showed for the first time that the immediate effect of ammonia exposure, when limited to the external surfaces of the first gill arches, is hypoventilation rather than hyperventilation. This would be an appropriate response to a short-term exposure to a waterborne toxicant, so as to minimize uptake while behavioral escape occurs. Series III provided evidence that elevated internal ammonia can be sensed directly by the brain, thereby eliciting hyperventilation. To our knowledge, this is the first demonstration of central chemo-sensing of ammonia in a teleost fish, but is in accord with central chemo-sensing of ammonia in mammals (see Introduction). These findings indicate that there are both peripheral (gill NECs) and central chemoreceptors for ammonia in fish.

4.2. External and internal ammonia sensing in gills

In both Series I and II, the fish were exposed to high environmental ammonia (HEA) but showed hyperventilation in Series I (Fig. 2) but hypoventilation in Series II (Fig. 4). However, the natures of the HEA exposures were different – only to the external surfaces of the first gill arches for a short period in Series II, but to the whole gill and body surface for a longer period in Series I. The time courses of the responses also differed – virtually immediate in Series II (Fig. 3), but slowly developing in Series I (Fig. 2).

As the fish of Series I could not avoid the HEA, the entire surface area of the external surfaces of the gill arches and body would have been evenly exposed to 1 mM ammonia. Therefore, it would diffuse along PNH_3 and NH_4^+ electrochemical gradients (Wood and Nawata, 2011) into the blood plasma across the respiratory lamellae and perhaps also across the skin (Zimmer et al., 2010), resulting in elevated blood ammonia levels that have been documented in many similar HEA exposures of trout (e.g. Nawata et al., 2007; Wood and Nawata, 2011; Zhang et al., 2011). In turn, high internal ammonia levels would be sensed by gill NECs (peripheral chemoreceptors; Zhang and Wood, 2009; Zhang et al., 2011, Zhang et al., 2015) and/or by the brain (central chemoreceptors; Zhang et al., 2013, and present study, Figs. 5, 6), resulting in a gradually increasing ventilation (Fig. 2). The situation appears to be similar in elasmobranch dogfish (De Boeck and Wood, 2015) and agnathan hagfish (Eom et al., 2019), though the internal receptor sites have not been identified in these ancient fishes.

In contrast, in Series II, the ammonia perfused onto the surfaces of the first pair of gill arches would likely have been diluted by ventilated water which would quickly flush it away. As the normal Vw in these 100–300 g trout would have been about 30 ml min⁻¹ (Eom and Wood, 2020) and the ammonia perfusion rate was only 2.28 ml min⁻¹, there would have been little chance of uptake into the bloodstream. Yet hypoventilation occurred, even at a very low ammonia concentration (0.01 mM). The present technique was similar to that used by Daxboeck and Holetton (1978) to identify external O₂ sensors on the first gill arches responsible for the bradycardia response to environmental hypoxia. Today, it is generally believed that these O₂ sensors affecting heart rate

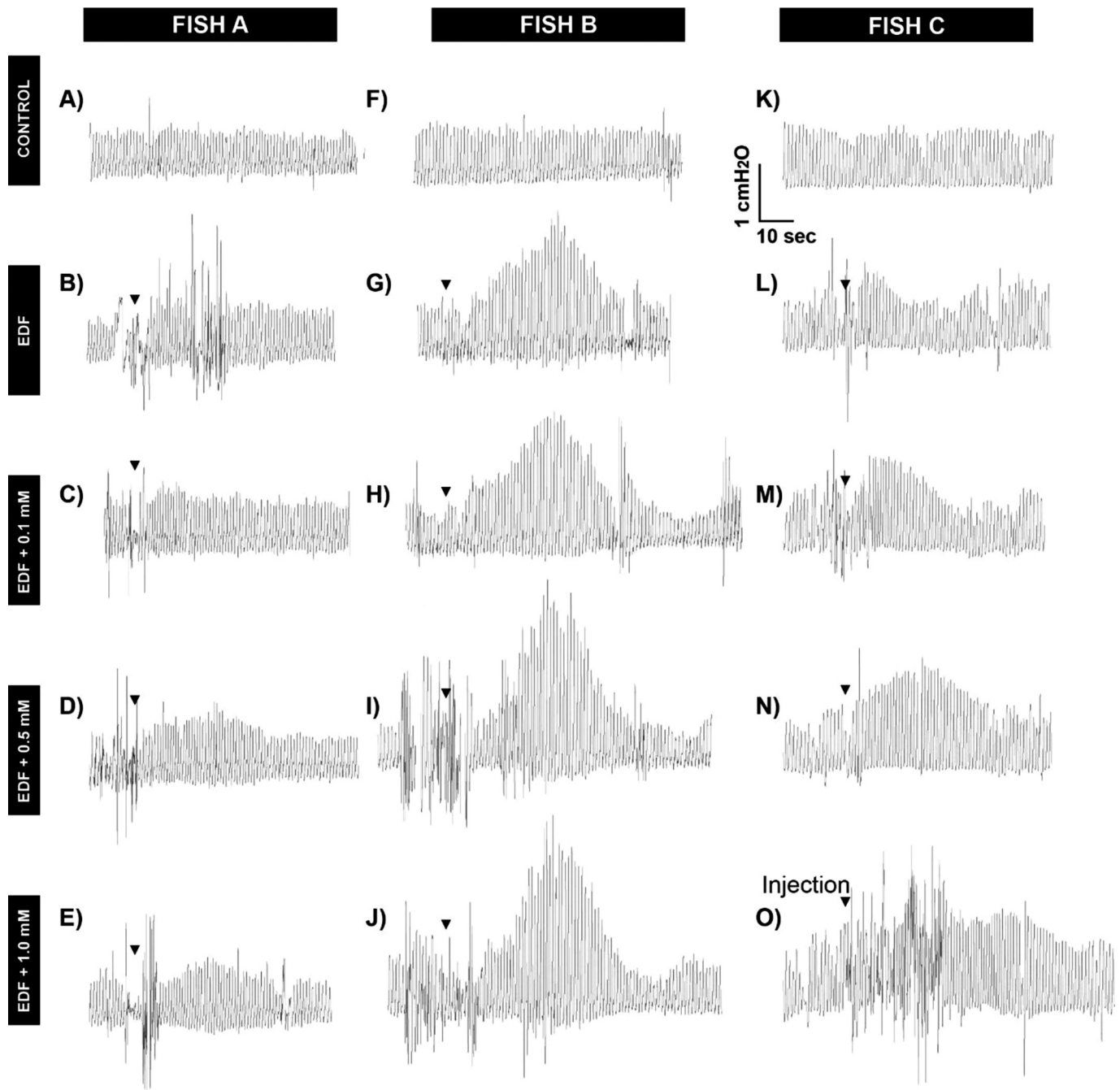


Fig. 5. Representative ventilation traces (buccal pressure recordings) in three trout of Series III after random injections (15 μ l) of mock EDF solutions, and ammonia concentrations in mock EDF, directly to the hind-brain cavity of rainbow trout. Arrowhead indicates time of injection. Increases in buccal pressure amplitude lasted for approximately 60 s, with progressive increases in first 30 s, followed by progressive decreases back to control levels over another 30 s.

in trout are externally-oriented NECs (Milsom, 2012; Perry and Tzaneva, 2016; Jonz, 2018). These may differ from the more widely distributed, internally-oriented NECs located on the basal membranes of gill filaments, facing the plasma. The latter appear to monitor blood O_2 levels, and elicit hyperventilation during environmental hypoxia, as first shown in trout by Smith and Jones (1982), though external O_2 receptors may also be involved in hypoxic hyperventilation (Milsom, 2012). Thus, there may be a parallel to peripheral O_2 chemoreception, with the external ammonia-sensing NECs responsible for ventilatory inhibition being analogous to those causing hypoxic bradycardia, while the internal ammonia-sensing NECs responsible for ventilatory stimulation being analogous to those causing hypoxic hyperventilation. However, further complicating interpretation is that CO_2 -sensing NECs involved in

hypercapnic hyperventilation appear to be exclusively external in orientation (Milsom, 2012), so by this scheme, externally oriented NECs could cause either hypoventilation (to ammonia) or hyperventilation (to CO_2) depending on the stimulus, or there could be two different types of external NECs.

This hypoventilatory response to HEA does not appear to have been reported previously. There are several likely explanations. Perhaps it is normally so transient, a sort of short-term breath-holding, that it has been overlooked. In this regard we have often seen hypoventilation in trout right at the start of HEA exposure, usually associated with aggressive struggling during which it is difficult to obtain ventilation recordings (J. Eom and C. M. Wood, personal observations). Presumably, the trout would have escaped to ammonia-free water if it had the

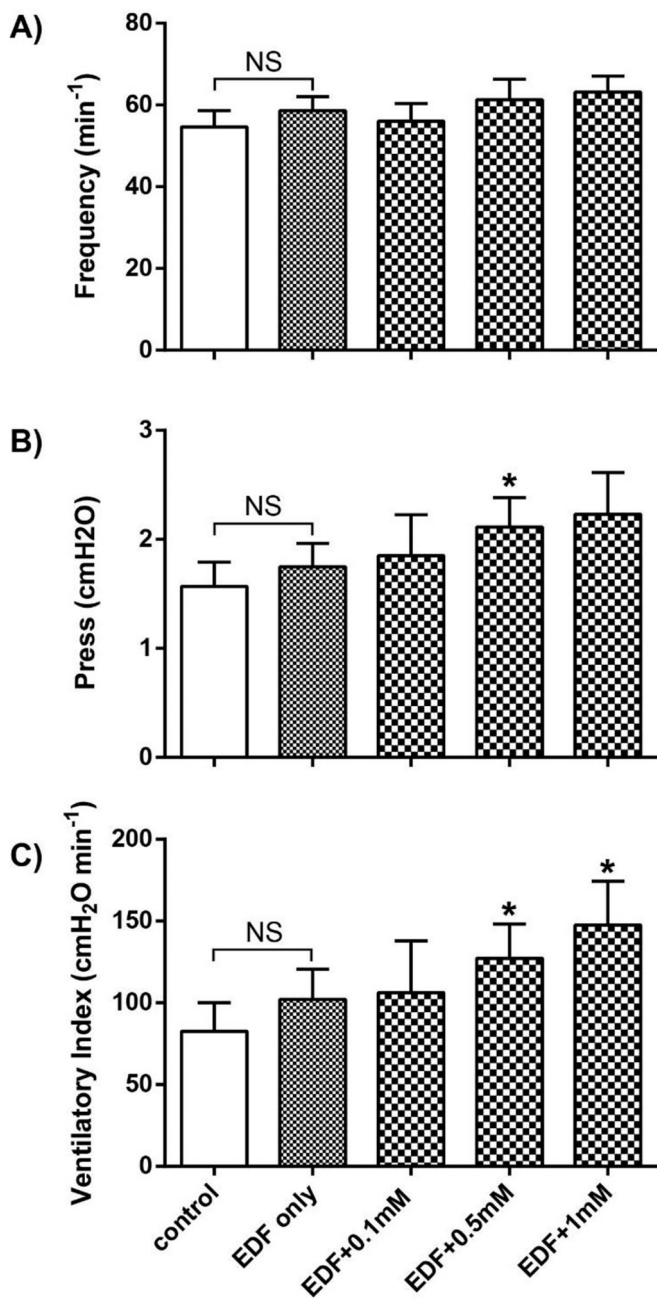


Fig. 6. Variations of ventilatory parameters after randomly ordered injections of ammonia concentrations (in mock EDF) to the hind-brain cavity in Series III (Means $\pm$ SEM, $N = 6$). As explained in Methods, the mean responses over the first 36 s after injection were calculated for each fish, then averaged for all 6 fish. The fish (A) barely changed overall frequency levels, but (B) there was a strong influence of injected ammonia concentration to increase buccal pressure amplitude (repeated measures one-way ANOVA, $p = 0.0525$) and (C) this resulted in a pattern of significantly increasing buccal ventilatory index ($p = 0.0325$). Asterisks indicate mean values significantly different from the injections of mock EDF alone by Dunnett's test. Although fish slightly increased mean ventilatory parameters in response to mock EDF injections alone, relative to the pre-injection control values, the responses were not significant (Student's t -test, $p = 0.5183$).

opportunity. The use of olfactory blockade with cotton balls prevents this struggling, facilitating the observations of hypoventilation in Series II. Other trout may exhibit no immediate response, followed by slowly developing hyperventilation. Additionally, when the whole animal, rather than just the first pair of gill arches is exposed to HEA in a

restrained situation, the ventilatory inhibition may be overwhelmed by the ventilatory stimulation elicited by gradually rising internal ammonia levels. In Series I, in our trout exposed to HEA while confined in the ventilation measuring system with olfactory blockade, hyperventilation first became apparent in Vw and buccal ventilatory index only at 8 min or later (Fig. 2).

4.3. Nociceptors as possible external ammonia-sensing receptors

In natural environments, as opposed to anthropogenically manipulated ones such as aquaculture or polluted waters, HEA is probably less common than hypoxia and hypercapnia, so it is possible that fish do not have specific external receptors for ammonia, but rather are using sensors responsive to a variety of waterborne toxicants, including ammonia. Nasal chemo-sensors could not have been involved in the responses of Series I or II as the nostrils were plugged. Nociceptors remain a possibility for the hypoventilation caused by ammonia application to the external surfaces of the first pair of gill arches in Series II (Figs. 3, 4). However, there are several reasons to discount their involvement. In general, increases (rather than decreases) in opercular movements coupled to ventilation (Hughes and Shelton, 1962) have been observed in response to nociceptor stimulation (Sneddon, 2003). Furthermore, the nociceptors on the surface of the head of the trout, which are innervated by the trigeminal nerve, are unresponsive to 0.02 mM ammonium chloride (Mettam et al., 2012). It is not clear whether there are nociceptors on the first pair of gill arches, and if present, whether they receive trigeminal innervation. More research is needed to clarify if nociceptors play a role in ventilatory control, such as ammonia-induced hypoventilation (Figs. 3, 4).

4.4. Internal ammonia sensing in the central nervous system

The results of Series III, where ammonia solutions were injected directly onto the surface of the hindbrain, provided direct evidence of central ammonia-sensing, resulting in hyperventilation (Figs. 5, 6). As reviewed by Milsom (2012) and Florindo et al. (2018), there is only sparse prior evidence (e.g. Wilson et al., 2000) for central chemoreception in fish other than sarcopterygians, some negative reports (Rovainen, 1977; Hedrick et al., 1991), and apparently no evidence at all on this possibility in teleost fish. However, these previous studies have not examined ammonia, which appears to be detected centrally in mammals (Wichser and Kazemi, 1974), eliciting a hyperventilatory response (Poppell et al., 1956; Roberts et al., 1956; Warren, 1958; Campbell et al., 1973). Earlier, Zhang et al. (2013) showed that ammonia readily permeates the blood-brain barrier in trout. Furthermore, based on correlation of brain intracellular ammonia concentrations with hyperventilation during HEA treatments, these workers proposed a role for central chemoreception of ammonia in fish. The present data confirm this idea. The ammonia concentrations (in mock EDF) applied to the brain surface that were effective in the present study were 0.5 and 1.0 mM (Fig. 6), and these were well within the ranges of both blood plasma and cerebrospinal fluid concentrations measured in HEA-exposed rainbow trout by Zhang et al. (2013).

At present we can only speculate on the detection mechanism(s) for ammonia in the central receptor cells, as this does not appear to be known in mammals. One possibility is that NH_3 diffuses into receptor cells, perhaps by Rh proteins, resulting in a rise in intracellular pH, as demonstrated in the whole brain of trout by Zhang et al. (2013). However, this seems to be an unlikely stimulus because intracellular alkalosis would generally be expected to inhibit rather than stimulate breathing (Nattie and Li, 2012). Another more likely possibility is that NH_4^+ , a well-known K^+ analogue that is very permeable through many K^+ channels (Yellen, 1987; Hille, 2001) causes an inhibition of K^+ currents, leading to partial depolarization, and ensuing effects on intracellular signaling. In this regard, it is notable that in isolated NECs (peripheral chemoreceptors) of trout, NH_4^+ was 30-fold more potent than K^+ in eliciting

intracellular Ca^{2+} mobilization (Zhang et al., 2011). The mechanisms and specific sites of ammonia-sensing, relative roles, time courses of response, and integration of afferent neural pathways of the brain versus branchial NECs need to be worked out, and there is a clear need for further research on these topics. Nevertheless, working in combination with peripheral chemoreceptors, central chemoreceptors would provide important redundancy to ensure that hyperventilation occurs when internal ammonia levels are elevated.

4.5. Why do fish regulate ventilation in response to elevated external or internal ammonia?

The regulation of ventilation through multiple detection sites would be beneficial for avoiding waterborne ammonia toxicity and for excreting increased plasma ammonia in fish. Very short-term hypoventilation as seen in Series II would help minimize ammonia uptake when the fish first encounters HEA, providing time for behavioral escape. This is similar to the marked reduction in V_w (sometimes complete apnea) seen in hagfish at the start of HEA exposure, prior to a later hyperventilatory response which takes over as blood ammonia levels eventually increase (Eom et al., 2019). Recently, we (Eom et al., 2020) have shown that in trout, hyperventilation is effective in increasing ammonia excretion only after plasma ammonia levels have been elevated for some time so as to activate the Rh glycoprotein metabolon system (Wright and Wood, 2009) in the gills, thereby removing diffusive limitations on ammonia flux (Randall and Ip, 2006). Prolonged elevations in plasma ammonia concentrations of endogenous origin occur after exercise (Wood, 1988; Mommsen and Hochachka, 1988; Wang et al., 1994) and feeding (Wicks and Randall, 2002; Bucking and Wood, 2008), which are times when elevated V_w is needed not only to excrete ammonia, but also to help provide increased O_2 uptake for the post-exercise “ O_2 debt” (Scarabello et al., 1991) and post-feeding “Specific Dynamic Action” (Seth et al., 2009) in trout. Elevated ventilation is also useful after chronic HEA exposure in order to get rid of the accumulated internal ammonia burden. In all these circumstances, the Rh metabolon system is upregulated in the gills (Nawata et al., 2007; Zimmer et al., 2010; Zhang et al., 2015) so the increased ventilation will help to excrete the excess ammonia. The fact that there are two receptor systems for elevated internal ammonia (gill NECs and brain) suggests that the homeostatic regulation of this respiratory gas, which is also a toxicant, is of critical importance.

Authors' contributions

J. E. and C.M.W. conceived the project, J.E. performed the experiments and generated the data, J. E. and C.M.W. analyzed the data together, J.E. wrote the first draft, and C.M.W. edited the manuscript.

Declaration of Competing Interest

The authors declare no competing or financial interests.

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