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RESEARCH ARTICLE

Ventilatory sensitivity to ammonia in the Pacific hagfish (*Eptatretus stoutii*), a representative of the oldest extant connection to the ancestral vertebrates

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ABSTRACT

Ventilatory sensitivity to ammonia occurs in teleosts, elasmobranchs and mammals. Here, we investigated whether the response is also present in hagfish. Ventilatory parameters (nostril flow, pressure amplitude, velar frequency and ventilatory index, the last representing the product of pressure amplitude and frequency), together with blood and water chemistry, were measured in hagfish exposed to either high environmental ammonia (HEA) in the external sea water or internal ammonia loading by intra-vascular injection. HEA exposure (10 mmol l^{-1} NH_4HCO_3 or 10 mmol l^{-1} NH_4Cl) caused a persistent hyperventilation by 3 h, but further detailed analysis of the NH_4HCO_3 response showed that initially (within 5 min) there was a marked decrease in ventilation (80% reduction in ventilatory index and nostril flow), followed by a later 3-fold increase, by which time plasma total ammonia concentration had increased 11-fold. Thus, hyperventilation in HEA appeared to be an indirect response to internal ammonia elevation, rather than a direct response to external ammonia. HEA-mediated increases in oxygen consumption also occurred. Responses to NH_4HCO_3 were greater than those to NH_4Cl, reflecting greater increases over time in water pH and P_{NH_3} in the former. Hagfish also exhibited hyperventilation in response to direct injection of isotonic NH_4HCO_3 or NH_4Cl solutions into the caudal sinus. In all cases where hyperventilation occurred, plasma total ammonia and P_{NH_3} levels increased significantly, while blood acid–base status remained unchanged, indicating specific responses to internal ammonia elevation. The sensitivity of breathing to ammonia arose very early in vertebrate evolution.

KEY WORDS: Blood acid–base status, High environmental ammonia, HEA, Internal ammonia loading, Plasma ammonia levels, Ventilation

INTRODUCTION

Ammonia, the major nitrogenous waste in ammoniotelic fish, can exist in solution as both the dissolved gas (NH_3) and the dissociated ion (NH_4^+), analogous to CO_2 and HCO_3^-, and it can act like a third respiratory gas (Randall and Ip, 2006). Here, we use the term ammonia to refer to the total of these two forms. Ammonia is produced and excreted at a rate of about 10–20% of carbon dioxide excretion ($\dot{M}_{CO_2}$) in ammoniotelic teleost fish (Wood, 2001). Building on pioneering studies by Hillaby and Randall (1979) and McKenzie et al. (1993), Zhang and co-workers have shown that ammonia plays an important role in stimulating ventilation in the freshwater rainbow trout (*Oncorhynchus mykiss*; Zhang and Wood, 2009; Zhang et al., 2015), and the same appears to be true in the zebrafish (*Danio rerio*; Perry and Tzaneva, 2016). Somewhat surprisingly, breathing is also stimulated by ammonia in a ureotelic elasmobranch, the Pacific spiny dogfish (*Squalus acanthias suckleyi*; De Boeck and Wood, 2015). These studies demonstrated that the hyperventilatory response is specific to elevations in blood ammonia, and not confounded by changes in blood acid–base status that often accompany experimental treatments with ammonia in fish. Hyperventilation occurs in response to both high external ammonia in the environment (HEA) and internal elevation of blood ammonia, but the bulk of the evidence indicates that the latter is the major proximate cause, i.e. HEA acts by causing ammonia to diffuse across the body surface, predominantly the gills, into the bloodstream where it acts on internal receptors. At least in part, these internal ammonia receptors are the neuroepithelial cells (NECs) in the gills (Zhang et al., 2011), which are now believed to be trimodal oxygen, carbon dioxide and ammonia detectors for respiratory sensing (Zhang et al., 2015; Perry and Tzaneva, 2016), although there is also some evidence that ammonia may also act centrally on the brain (Wilkie et al., 2011; Zhang et al., 2013; Lisser et al., 2017). Ammonia can also act as a ventilatory stimulant in mammals, which are ureotelic like elasmobranchs, and this stimulation is thought to be important in exercise-induced hyperventilation (Mutch and Banister, 1983), as well as in supporting breathing during severe respiratory acidosis and hepatic coma (Roberts et al., 1956; Warren, 1958; Felipo and Butterworth, 2002). The detection mechanism is unclear, but the best correlation appears to be with ammonia concentrations in the brain, suggesting a central site of chemo-detection (Wichser and Kazemi, 1974).

These findings in groups as diverse as teleosts, elasmobranchs and mammals, with very different strategies for handling nitrogenous wastes, raise the prospect that ventilatory sensitivity to ammonia arose very early in vertebrate evolution. To evaluate this hypothesis, we examined whether breathing was sensitive to ammonia in the Pacific hagfish, *Eptatretus stoutii*. The exact phylogenetic position of the hagfishes (Class Myxini) is controversial, but there is general agreement that present-day hagfishes represent the oldest extant connection to the ancestral vertebrates (e.g. Bardack, 1998; Rasmussen et al., 1998; Heimberg et al., 2010; Oisi et al., 2013), though they may be highly derived, evolving on a separate trajectory from jawed vertebrates (Miyashita et al., 2019). Nevertheless, they are important for understanding the evolutionary origin of vertebrate traits. Hagfishes are marine jawless fish possessing a notochord but lacking a proper vertebral column and have a very different breathing

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List of abbreviations

$[HCO_3^-]$	bicarbonate concentration
HEA	high environmental ammonia
$\dot{M}_{CO_2}$	carbon dioxide production rate
$\dot{M}_{O_2}$	oxygen consumption rate
N	number of fish
NaCl	sodium chloride (chloride control)
$NaHCO_3$	sodium bicarbonate (bicarbonate control)
NECs	neuroepithelial cells
NH_4Cl	ammonium chloride
NH_4HCO_3	ammonium bicarbonate
P_{CO_2}	partial pressure of carbon dioxide
P_{NH_3}	partial pressure of ammonia
P_{O_2}	partial pressure of oxygen
T_{Amm}	total ammonia concentration
T_{CO_2}	total carbon dioxide concentration
Urea-N	urea nitrogen

mechanism from other fishes, inhaling water through a single nostril by a velar pump, and exhaling it through 18–24 separate gill pouches (Kardong, 2012). Hagfish are ammoniotelic (Walsh et al., 2001; Giacomin et al., 2019), and feed by burrowing into dead and dying animals (Martini, 1998), so are probably exposed periodically to very high levels of environmental ammonia (i.e. HEA) in nature. They are extremely tolerant of HEA in the laboratory (Clifford et al., 2015, 2017). Their behaviour of writhing and coiling, and releasing vast amounts of slime when disturbed, makes them difficult to work with, but recently we have developed methods for measuring ventilation in the Pacific hagfish (Eom and Wood, 2019). In the present study, we applied these methods, together with measurements of blood ammonia levels and acid–base chemistry, to examine whether breathing is sensitive to internal and/or external elevations in ammonia, and whether the response is specific to ammonia.

MATERIALS AND METHODS

Experimental animals and chemicals

Pacific hagfish (*Eptatretus stoutii*, 80.9±4.1 g, N=129) were captured under permits (XR 202 2016 and XR 194 2017) from the Department of Fisheries and Oceans Canada in July–September of 2016 and 2017. Bottom-dwelling traps baited with strips of Pacific hake (*Merluccius productus*) were set in Trevor channel (48 50.844 N, 125 08.321 W, depth 100 m). The captured fish were transferred to Bamfield Marine Sciences Centre (BMSC) located on the southwest coast of Vancouver Island, BC, Canada. Hagfish were housed together in fibreglass tanks (20 m^3), furnished with PVC pipes for shelter, and served with flowing sea water (temperature 11–13°C, salinity 30–31 ppt). While hake strips were offered as food, the animals generally did not eat during the holding period, which was up to 2 months. The animal usage permits (AUP) for experiments were approved by the University of British Columbia (A14-0251) and BMSC Animal Care Committees (AUP RS-17-20) and followed guidelines of the Canadian Council of Animal Care. After experiments, the fish were killed by an overdose of tricaine methanesulfonate (MS-222, 5 g l^{-1} neutralized to pH 7.8 with 5 mol l^{-1} NaOH; Syndel Laboratories, Parksville, BC, Canada) followed by evisceration to ensure death. All other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Physiological recording from the nostril duct in the respiratory system

Generally, surgical operations were performed in the late afternoon and experiments were carried out mostly during the night-time, as hagfish are nocturnally active. Procedures were similar to those described by Eom and Wood (2019). In preparation for ventilatory pressure or ventilatory flow measurements, hagfish were anaesthetized in MS-222 (0.6 g l^{-1}, neutralized with NaOH) and placed on an operating table. Gill irrigation was not necessary because the hagfish are hypoxia tolerant (Sidell et al., 1984; Forster et al., 1992; Perry et al., 2009), but the fish body was kept moist with wet tissue paper during air exposure. A 3 cm length of transparent silicone tubing (6.35 mm o.d. and 4.32 mm i.d.) was snugly fitted into the single nostril duct in the anterior midline and secured by two stitches (26 mm 1/2C taper, Perma-Hand Silk, Ethicon, Somerville, NJ, USA) which were made laterally to the skin around the nostril entrance. Hagfish were allowed to recover in flowing sea water for several hours before experiments started.

Two different physiological recording methods were applied to the hagfish nostril to measure their ventilatory parameters. In most of the studies, a pressure transducer was used to record the fluctuations in pressure created by velar pumping, in a tube connected to the nostril. In some of the studies (those shown in Figs 3 and 7), a blood flow meter was attached to the tube to measure total ventilatory flow. In both methods, analogue ventilatory parameters were amplified (LCA-RTC, Transducer Techniques, Temecula, CA, USA), converted to digital signals in a PowerLab data integrity system (ADInstruments, Colorado Springs, CO, USA), and then visualized and analysed in LabChart software version 7.0 (ADInstruments). In order to remove non-specific noise, a low-pass type filter was incorporated in the LabChart software while the ventilatory parameters were recorded.

For the ventilatory pressure measurements, a 3 cm non-flared length of polyethylene tubing (PE160, 1.57 mm o.d., 1.14 mm, i.d., Clay-Adams, Sparks, MD, USA) was inserted 1 cm deep into the transparent silicone tubing and secured to it by two stitches. This secured PE160 tubing could then be periodically connected via an 18-gauge needle shaft to another ∼30 cm piece of water-filled PE160 tubing which was attached to a medical pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA). The pressure transducer was zeroed to the water surface, calibrated with a column of water in the range 0–4 cm, and used for monitoring ventilatory pressure amplitude (cm H_2O) and frequency (min^{-1}) in the nostril. The product of pressure amplitude (cm H_2O) and frequency (min^{-1}) yielded the ventilatory index (cm H_2O min^{-1}). Eom and Wood (2019) demonstrated that there was a strong correlation between the ventilatory index recorded in this manner and the total ventilatory flow measured directly with a flowmeter.

For the direct measurement of total ventilatory flow, an ultrasonic microcirculation blood flow probe (V-series, Transonic Systems Inc., Ithaca, NY, USA) connected to a dual-channel small-animal blood flowmeter (T106 series, Transonic Systems Inc.) was fitted onto the transparent silicone tubing. The pulsatile signal allowed the measurement of velar frequency (min^{-1}) and total water flow rate (ml min^{-1}), from which ventilatory stroke volume (ml $stroke^{-1}$) could be calculated. Correct orientation of the flow probe was essential; the probe detects both the magnitude and direction of flow, so in our recordings, negative values (i.e. below zero flow) represent inhalation through the nostril, and positive values represent exhalation as, for example, occurs during coughing. Intrinsic calibration and zero of the system were checked by flowing sea water (12°C, 30–31 ppt) through the probe at known rates using an aqua lifter vacuum pump (Cheng Gao Plastic and Hardware Electricity, Dongguan, Guangdong, China). Flow was determined gravimetrically. As noted by Perry et al. (2009), the intrinsic calibration overestimated true sea water flow. Therefore, voltage

Journal of Experimental Biology (2019) 222, jeb199794. doi:10.1242/jeb.199794

outputs were converted into corrected flow units (ml min^{-1}) by the LabChart software.

Blood sampling

It is not possible to reliably sample blood by catheterization in hagfish, so blood samples (~300 µl) were taken as described by Clifford et al. (2017) by rapidly anaesthetizing the fish in MS-222 (0.6 g l^{-1}, neutralized with NaOH), holding it vertically and puncturing the caudal blood pool in the subcutaneous venous sinus using a 23-gauge needle attached to a 1 ml gas-tight syringe (Hamilton, Reno, NV, USA). The whole procedure took about 1–2 min, including anaesthesia, and the fish was air exposed for <0.5 min. Upon return to anaesthetic-free sea water, the hagfish resumed its normal coiled behaviour within 5 min. The whole-blood samples were immediately used for measurement of pH and then centrifuged (5000 ***g***, 1 min) for collection of plasma. The plasma was decanted and flash-frozen in liquid N_2, then stored at −80°C until analysis for total ammonia (T_{Amm}) and total CO_2 (T_{CO_2}).

Series I: ventilation changes in hagfish loaded externally with ammonia (HEA)

Prior to recording nostril ventilation, the hagfish were anaesthetized in neutralized MS-222 (0.6 g l^{-1}) for cannulation of the nostril duct. After recovery from anaesthesia, the hagfish were isolated in individual tanks of aerated sea water at 12°C and their nostril ventilation was measured (internal control treatment), then the fish were exposed to 5, 10 or 20 mmol l^{-1} ammonium bicarbonate (NH_4HCO_3, N=6 per treatment) or 10 mmol l^{-1} ammonium chloride (NH_4Cl, N=6) over 15 h. Nostril ventilatory parameters (frequency, pressure amplitude and ventilatory index) were measured immediately after the start of exposure (0 h) and every 3 h thereafter up to 15 h. For controls, a series with no addition (N=6) and one with the addition of 10 mmol l^{-1} sodium bicarbonate ($NaHCO_3$, N=6) were also performed. The standard protocol was to set the volume of the animal's chamber to 980 ml of aerated sea water for the control measurements, and then add 20 ml of the appropriate salt stock (in sea water) for the experimental measurements. We have previously shown that Pacific hagfish commonly exhibit long periods of spontaneous apnoea (Eom and Wood, 2019), so exposures were only performed on animals that were breathing during the pre-treatment control period. The effects of the addition of the various salts on water pH were also measured (N=6).

Series II: the effect of HEA on oxygen consumption rate

Hagfish routine oxygen consumption rate ($\dot{M}_{O_2}$) was measured over 15 h in 3 h intervals in HEA environments created with 10 mmol l^{-1} NH_4HCO_3 (N=6) or 10 mmol l^{-1} NH_4Cl (N=6), or with 10 mmol l^{-1} $NaHCO_3$ (control, N=4). Briefly, each hagfish was placed individually in an air-tight plastic container filled with 1 litre of normoxic sea water and containing a magnetic spin-bar to allow for appropriate mixing. Oxygen tension (Torr) was measured intermittently using fibre optic oxygen sensors connected to a WITROX 4 oxygen meter (Loligo, Viborg, Denmark). At the conclusion of each flux period, the chambers were unsealed, refreshed with air-saturated treatment water of the appropriate composition, then resealed to begin the next flux period. At the conclusion of the 15 h experiment, hagfish were anaesthetized and weighed. Oxygen tension in Torr was converted to µmol l^{-1} using the oxygen solubility constants described in Boutilier et al. (1984), and $\dot{M}_{O_2}$ was calculated as previously described (Clifford et al., 2016).

Series III: relationship between ventilation and altered blood chemistry in hagfish loaded externally with ammonia (HEA)

This series examined ventilation and blood chemistry before and during exposure to HEA in the external water for 3 h. Based on the results of the previous series, an external concentration of 10 mmol l^{-1} NH_4HCO_3 was chosen. Two groups of hagfish were used, one (N=6) for measurement of total ventilatory flow and the other (N=6) for blood collection prior to and during exposure to 10 mmol l^{-1} HEA. The first group of hagfish had been fitted with nostril tubing for recording of total ventilatory flow and pressure amplitude, and were placed in 980 ml of aerated sea water in a plastic chamber. After control recordings were taken in the absence of added ammonia, the animals were acutely exposed to 10 mmol l^{-1} HEA (by the addition of 20 ml of 500 mmol l^{-1} reagent grade NH_4HCO_3), and the nostril ventilatory parameters were continuously measured up to 180 min.

The second group of hagfish were used only for collecting blood for analysis of pH, and plasma T_{CO_2} and T_{Amm}. A blood sample was taken under control conditions initially, and then after recovery, the hagfish were exposed to 10 mmol l^{-1} HEA prepared with NH_4HCO_3 in the same manner as the first group. Another blood sample was taken after 5 min of HEA exposure, and a final terminal sample after 180 min of HEA exposure.

Series IV: relationship between ventilation and altered blood chemistry in hagfish loaded internally with ammonia

This series examined ventilation and blood chemistry before (0 h) and after (0.5 and 1 h) injection of the following ammonium salts into the venous sinus in the tail (at a volume load of 2 µl g^{-1}) at a dose of either 70 µmol kg^{-1} (low dose) or 1000 µmol kg^{-1} (high dose): 35 mmol l^{-1} NH_4HCO_3 (N=6), 500 mmol l^{-1} NH_4HCO_3 (N=18), 35 mmol l^{-1} NH_4Cl (N=6) and 500 mmol l^{-1} NH_4Cl (N=6). Additionally, high doses (1000 µmol kg^{-1}) of 500 mmol l^{-1} NaCl (N=6, as a chloride control) and 500 mmol l^{-1} $NaHCO_3$ (N=11, as a bicarbonate control) were also injected. The low doses using 35 mmol l^{-1} ammonium salts were made up in a background concentration of 500 mmol l^{-1} NaCl to minimize osmotic disturbance. The predicted initial concentrations (T_{Amm}) in hagfish plasma (14% of body mass) or extracellular fluid (ECF, 30% of body mass; Forster et al., 2001) ranged between 500 µmol l^{-1} (low dose) and 7142 µmol l^{-1} (high dose) in plasma, or 233 µmol l^{-1} (low dose) and 3330 µmol l^{-1} (high dose) in ECF, respectively; these predicted concentrations cover the T_{Amm} range reported in plasma of Pacific hagfish after natural feeding (Wilkie et al., 2017) for the low dose and after exposure to 20 mmol l^{-1} HEA for the high dose (Clifford et al., 2015).

The fish were fitted with nostril tubing for recording of ventilatory pressure and flow, allowed to recover, then placed in a plastic chamber containing 1 litre of aerated sea water. After recording of control ventilatory parameters, an initial blood sample was taken (0 h) and then 2 µl g^{-1} of the respective salt solution was injected into the venous sinus. The fish was then returned to the 1 litre sea water chamber. Additional ventilation measurements, followed immediately by blood collection, were taken at 0.5 and 1 h. In the high dose NH_4HCO_3 and 500 mmol l^{-1} $NaHCO_3$ injection experiments, 5 ml water samples were also taken at 0, 0.5 and 1 h for measurement of ammonia and urea-nitrogen (urea-N) excretion rates. The collected water samples were stored in plastic bottles at −20°C until later analysis.

Blood and water chemistry analyses

Freshly collected blood (in a gas-tight Hamilton syringe) and water were transferred to 0.5 ml micro-centrifuge tubes and placed in a

12°C water bath, and pH was immediately (~2 min) measured using a thermo-jacketed Orion ROSS micro pH electrode (Fisher Scientific, Ottawa, ON, Canada), taking care to insert the pH probe to the bottom of the sample so as to minimize any impact of air exposure at the surface on the sample. The blood sample was then centrifuged (2 min at 12,000 ***g***; Eppendorf, Model 5140C, Hamburg, Germany) and the plasma drawn off and transferred to a second 0.5 ml micro-centrifuge tube and flash-frozen in liquid N_2. The entire blood processing procedure was completed in ~5 min. Deep-frozen plasma was later thawed on ice for measurement of plasma T_{CO_2} and T_{Amm}, using a Corning 965 CO_2 analyser (Ciba Corning Diagnostic, Halstead, Essex, UK) and an enzymatic reagent kit based on the glutamate dehydrogenase/NAD method (Raichem™ R85446, Cliniqua, San Marcos, CA, USA), respectively. In our experience, these procedures yield values identical to those obtained from freshly collected samples, and our measured control plasma ammonia and acid–base data were very close to those reported in previous studies on the same species (Clifford et al., 2015, 2017; Giacomin et al., 2019). Because of background interference by hagfish plasma in the T_{Amm} measurement, a standard curve was created using the plasma as a matrix spiked with increasing concentrations of NH_4Cl, in order to compensate for the matrix effect. The concentration of T_{Amm} in the plasma was calculated by the standard additions method. The Henderson–Hasselbalch equation was applied to T_{CO_2} and pH values to calculate hagfish plasma CO_2 tension (P_{CO_2}) and bicarbonate concentration ($[HCO_3^-]$), using plasma pK' values and CO_2 solubility coefficients from Boutilier et al. (1984). Plasma ammonia tension (P_{NH_3}) and ionic ammonium concentration ($[NH_4^+]$) were similarly calculated from T_{Amm} and pH values by the Henderson–Hasselbalch equation using plasma pK' values and NH_3 solubility coefficients from Cameron and Heisler (1983). Full equations and the assumptions made for adjusting the CO_2 and ammonia constants for the ionic strength of hagfish plasma are given in Giacomin et al. (2019).

Freshly thawed water samples were employed for measurement of ammonia concentration using the colorimetric assay of Verdouw et al. (1978) and urea-N concentrations by the colorimetric assay of Rahmatullah and Boyde (1980). Flux rates (μmol N kg^{-1} h^{-1}) were calculated by factoring changes in concentration (μmol N l^{-1}) by water volume (l), body mass (kg) and time (h).

Statistical analyses

Data are reported as means±s.e.m. (N) where N represents the number of fish used in each respective experiment. One-way repeated-measures ANOVA followed by Dunnett's test was used in series I, III and IV, and two-way ANOVA followed by Tukey's multiple comparisons test in series II. Where necessary, data were log-transformed in order to pass normalization and homogeneity of variance tests. The statistical analyses were performed in GraphPad Prism software v.6.0 (La Jolla, CA, USA) and in the R project for statistical computing program. The threshold for statistical significance was $P<0.05$.

RESULTS

Series I: ventilation changes in hagfish loaded externally with ammonia (HEA)

In this series, ventilation was measured from the nostril by a pressure transducer. There was some variation in the control ventilatory parameters amongst the various treatment groups (Fig. 1), reflecting the high variability of hagfish breathing patterns, so the responses in each treatment were compared against the respective pre-treatment controls in the individual animals.

For simplicity, responses to only three of the experimental treatments are shown in Fig. 1, up to 6 h of exposure: 10 mmol l^{-1} $NaHCO_3$ (bicarbonate control), 10 mmol l^{-1} NH_4HCO_3 and 10 mmol l^{-1} NH_4Cl. By 3 h of exposure, both 10 mmol l^{-1} NH_4HCO_3 and 10 mmol l^{-1} NH_4Cl caused significant increases in ventilatory index, which persisted at 6 h in the latter (Fig. 1C). These were due to significant increases in pressure amplitude for both salts (Fig. 1B), and in frequency for NH_4Cl only (Fig. 1A). Over the same time frame, there were no changes in ventilation in response to 10 mmol l^{-1} $NaHCO_3$ (Fig. 1).

The full dataset of all six treatments up to 15 h of exposure, including 0 mmol l^{-1} NH_4HCO_3 as a 'no addition' control and 5 mmol l^{-1} NH_4HCO_3 (both of which had no effects) and 20 mmol l^{-1} NH_4HCO_3 (which significantly stimulated both pressure amplitude and frequency), are included in Fig. S1. Notably, the 10 and 20 mmol l^{-1} NH_4HCO_3 treatments proved to be toxic, resulting in mortality which started by 9 h of exposure in the 10 mmol l^{-1} NH_4HCO_3 group (50% mortality) and by 6 h of exposure in the 20 mmol l^{-1} NH_4HCO_3 group (100% mortality). Prior to these times, the fish appeared to be healthy and were not moribund, suggesting that a time-dependent toxic threshold was surpassed, as explained below. There were no mortalities in the other treatments.

The control pH in 30 ppt aerated sea water at 12°C was 8.31±0.02 (N=6). Addition of the various salts lowered the pH as follows: 5 mmol l^{-1} NH_4HCO_3, pH 7.95±0.01; 10 mmol l^{-1} NH_4HCO_3, pH 7.86±0.01; 20 mmol l^{-1} NH_4HCO_3, pH 7.78±0.01; 10 mmol l^{-1} NH_4Cl, pH 7.98±0.01; and 10 mmol l^{-1} $NaHCO_3$, pH 7.97±0.01

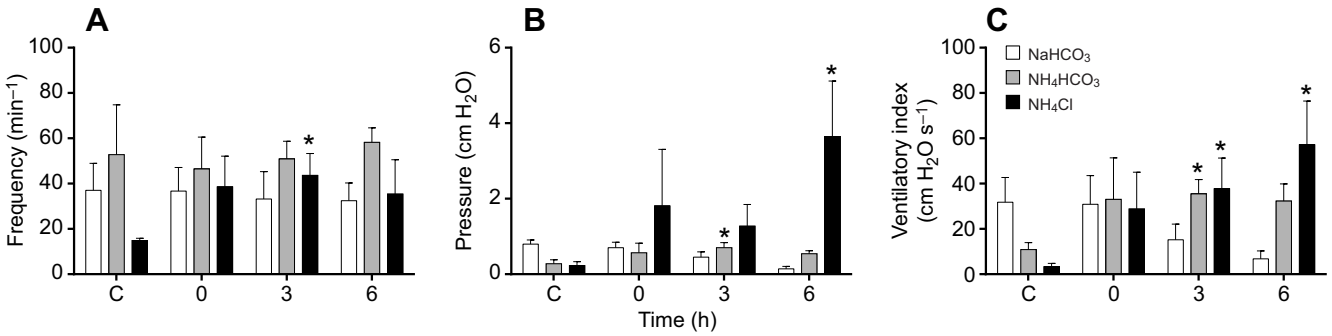

Fig. 1. Ventilatory responses of hagfish loaded externally with ammonia. (A) Ventilatory frequency, (B) ventilatory pressure amplitude and (C) ventilatory index (pressure amplitude×frequency) of hagfish (N=6 per each treatment) exposed to 10 mmol l^{-1} $NaHCO_3$ (bicarbonate control) and two high environmental ammonia (HEA) treatments: 10 mmol l^{-1} NH_4HCO_3 and 10 mmol l^{-1} NH_4Cl (series I). Data are means±1 s.e.m. Asterisks indicate a significant difference (P<0.05) from pre-exposure control value (C).

Journal of Experimental Biology (2019) 222, jeb199794. doi:10.1242/jeb.199794

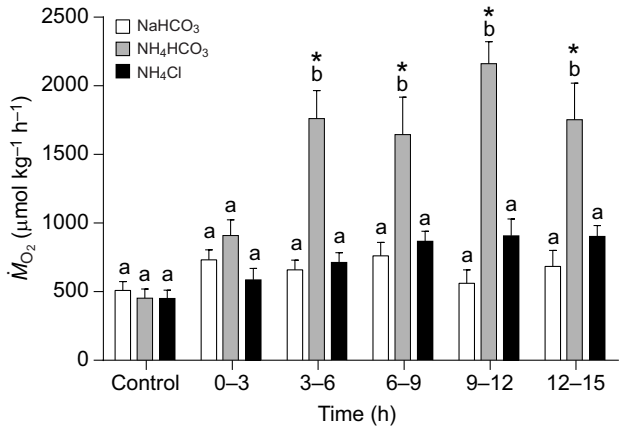

Fig. 2. Changes in oxygen consumption rate ($\dot{M}_{O_2}$) of hagfish in response to HEA. Hagfish were exposed to one of two HEA treatments (10 mmol l^{-1} NH_4Cl, N=6, or 10 mmol l^{-1} NH_4HCO_3, N=6) or control treatment (10 mmol l^{-1} $NaHCO_3$, N=4) (series II). Data are means±1 s.e.m. Asterisks indicate a significant difference (P<0.05) from pre-exposure control value. Different letters indicate significant differences (P<0.05) among treatments within each time interval.

(N=6). In view of the mortalities seen in the 10 mmol l^{-1} NH_4HCO_3 treatments but not in the 10 mmol l^{-1} NH_4Cl treatment, an additional experiment was performed to assess the possible impact of aeration on seawater pH over time (Fig. S2A). This experiment demonstrated that seawater pH gradually rose with time in both treatments, as well as in the 10 mmol l^{-1} $NaHCO_3$ control. However, while pH in both the 10 mmol l^{-1} NH_4Cl and 10 mmol l^{-1} $NaHCO_3$ treatments stabilized at about 8.2 (i.e. slightly below the pH of control sea water) after 5 h of aeration, pH plateaued at about 8.6 over the same time frame in the 10 mmol l^{-1} NH_4HCO_3 treatment. As a result, by the time mortalities started to occur in the 10 mmol l^{-1} NH_4HCO_3 treatment of series I, environmental P_{NH_3} levels (Fig. S2B) would have been approximately 3-fold higher than in the NH_4Cl treatment.

Series II: the effect of HEA on $\dot{M}_{O_2}$

There were no mortalities in these treatments, and the animals remained healthy throughout the 15 h experiment. Notably, in this series, the water was partially replaced at 3 h intervals, and aeration was not continuous, so increases in water pH and more importantly in P_{NH_3} in the NH_4HCO_3 exposure would have been attenuated. $\dot{M}_{O_2}$ tended to increase with exposure to HEA in the form of both 10 mmol l^{-1} NH_4Cl (doubling from 6–9 h onwards) and 10 mmol l^{-1} NH_4HCO_3 (doubling by 0–3 h, and reaching a 4-fold stimulation by 3–6 h onwards); only the response to 10 mmol l^{-1} NH_4HCO_3 was significant (3–6 h onwards). In the 10 mmol l^{-1} $NaHCO_3$ control treatment, the fish did not significantly change their $\dot{M}_{O_2}$ (Fig. 2).

Series III: relationship between ventilation and changes in blood chemistry in hagfish loaded externally with ammonia (HEA)

In this series, which was designed to investigate the early response of ventilation and blood chemistry to HEA in the form of 10 mmol l^{-1} NH_4HCO_3, breathing was recorded simultaneously from the nostril by both the flowmeter and pressure transducer. For simplicity, only the flowmeter results are shown (Fig. 3). The typical response was a rapid (i.e. within 5 min) decrease in ventilatory flow to close to zero (e.g. Fig. 3A), an effect that was sustained from 5.2 min (minimum) to 94.2 min (maximum), with a mean period of decreased flow of 38.7±17.2 min (N=6). Thereafter, flow increased greatly above the pre-treatment control levels (Fig. 3A). Two other examples are shown in Fig. S3. Overall, these changes in total ventilatory flow were significant, with the mean decrease in flow rate being about 80%, and the subsequent mean increase in flow rate being about 3-fold relative to the original pre-treatment control values (Fig. 3B). Interestingly, these changes were achieved entirely by changes in ventilatory stroke volume (Fig. 3D). Although breathing became very shallow, velar frequency was not reduced (Fig. 3C), and this was confirmed by the pressure transducer recording, which showed unchanged frequency but marked reductions in ventilatory pressure amplitude and ventilatory index, similar to those in stroke volume and total nostril flow, respectively. Note that the initial response of greatly decreased ventilation would not have been seen in series II, where the first measurements were taken only after 3 h.

In the parallel blood-sampling experiment, the control plasma T_{Amm} of approximately 150 μmol l^{-1} had increased by about 2.3-fold by 5 min of exposure to HEA (Fig. 4A), a blood-sampling time that would correspond to close to the start of the period of ventilatory flow depression. However, by 180 min when breathing had rebounded and increased above control levels (Fig. 3), plasma T_{Amm} had increased to about 1630 μmol l^{-1} i.e. 11-fold control levels (Fig. 4A). These changes in T_{Amm} were quantitatively reflected in parallel increases in P_{NH_3} from the control level of about 50 μTorr to about 650 μTorr at 180 min (Fig. 4B). There were no significant changes in either blood pH from a control level of about 8.05 (Fig. 4C) or P_{CO_2} from a control level of about 1.3 Torr (Fig. 4D), although plasma [HCO_3^-] did increase significantly at 180 min by about 55% from a control level of 6.8 mmol l^{-1} (Fig. 4E).

Series IV: relationship between ventilation and changes in blood chemistry in hagfish loaded internally with ammonia

Increased plasma ammonia levels appeared to be associated with hyperventilation in series I and III, so we hypothesized that the

Table 1. Blood acid–base chemistry of hagfish before (Control) and 0.5 and 1 h after (Post) injection of the respective doses of salts into the venous sinus (series IV)

	pH			P_{CO_2} (Torr)			[HCO_3^-] (mmol l^{-1})		
	Control	0.5 h Post	1 h Post	Control	0.5 h Post	1 h Post	Control	0.5 h Post	1 h Post
1000 μmol kg^{-1} $NaHCO_3$ (N=11)	7.96±0.02	8.14±0.03*	8.12±0.02*	1.49±0.17	1.97±0.20*	0.87±0.09*	6.74±0.79	12.75±0.95*	5.64±0.70
1000 μmol kg^{-1} NH_4HCO_3 (N=18)	8.01±0.05	8.05±0.02	8.03±0.02	1.30±0.15	1.65±0.19	1.18±0.10	7.96±1.40	9.19±1.11	6.17±0.61
70 μmol kg^{-1} NH_4HCO_3 (N=6)	8.14±0.02	8.19±0.02	8.19±0.02	0.90±0.06	0.88±0.07	0.81±0.10	7.99±0.78	8.58±0.67	8.01±0.96
1000 μmol kg^{-1} NaCl (N=6)	8.17±0.03	8.11±0.04	8.18±0.05	0.89±0.11	1.03±0.22	0.70±0.10	8.40±1.11	7.94±1.02	6.82±0.97
1000 μmol kg^{-1} NH_4Cl (N=6)	8.12±0.05	8.11±0.04	8.13±0.04	0.96±0.08	0.84±0.11	0.85±0.12	8.01±1.00	6.57±0.72	7.29±1.07
70 μmol kg^{-1} NH_4Cl (N=6)	8.12±0.05	8.13±0.05	8.13±0.03	0.92±0.06	0.87±0.08	0.93±0.06	7.68±0.75	7.44±0.66	7.89±0.35

Data are means±s.e.m. Asterisks indicate a significant difference (P<0.05) from pre-injection control value.

Journal of Experimental Biology (2019) 222, jeb199794. doi:10.1242/jeb.199794

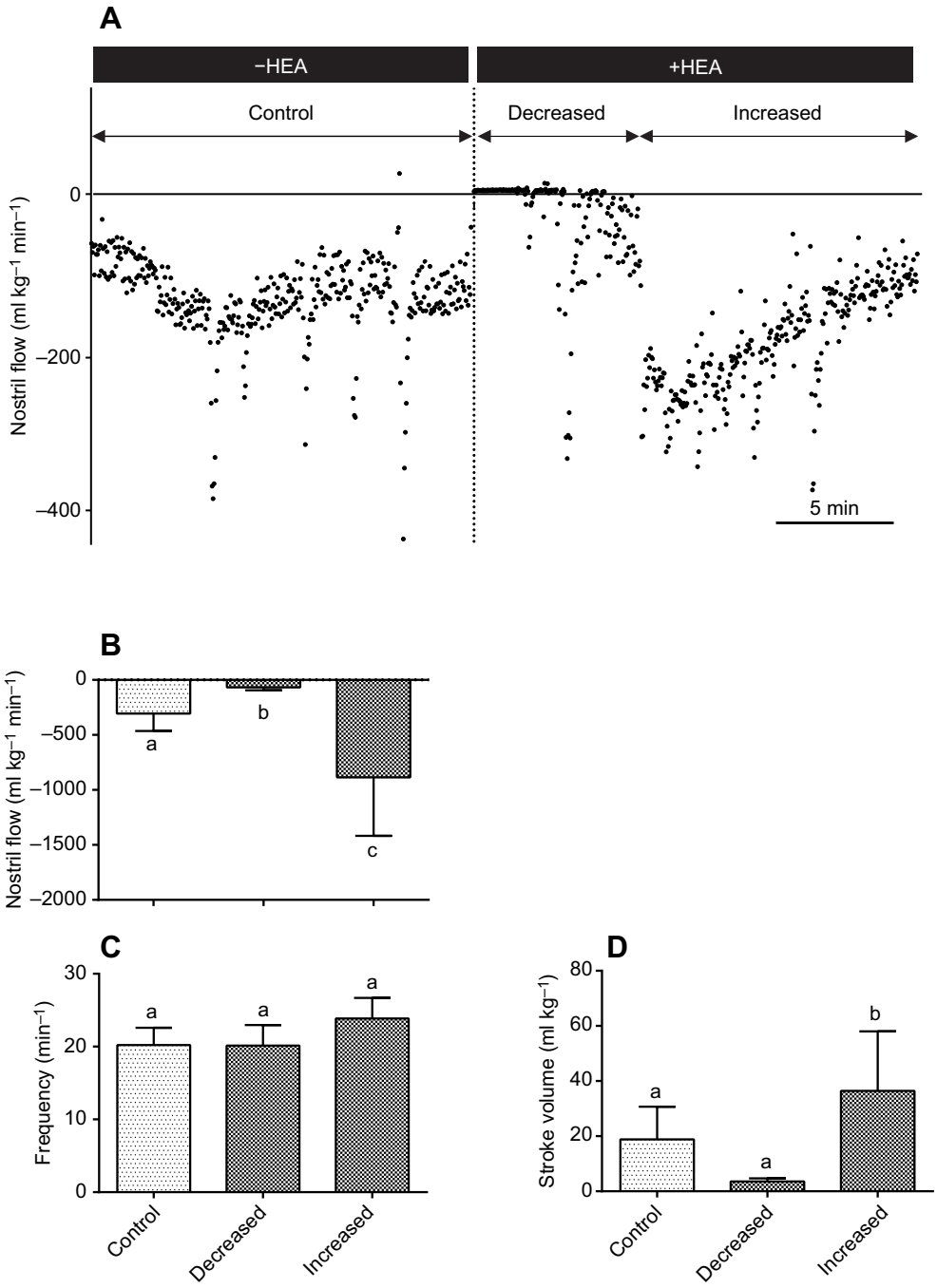

Fig. 3. Ventilatory changes in hagfish exposed to HEA. (A) Example flowmeter recording of nostril ventilatory parameters (measured directly via a flow probe placed in the nostril entrance) in hagfish exposed to HEA (10 mmol l^{-1} NH_4HCO_3) (series III). Note that these are flow recordings, in contrast to the pressure recordings of Figs 1 and 5. (B) Total flow rate, (C) frequency and (D) stroke volume. Data in B–D were sampled during the pre-exposure control period and over 5 min periods representing the times of greatest initial decrease and greatest subsequent increase in nostril ventilatory flow during the HEA treatment for each animal. Data are means±1 s.e.m. (*N*=6). Different letters indicate significant differences (P<0.05) among the three sampling periods. In HEA, hagfish quickly decreased nostril ventilatory flow for an average of 35.6 min (range: 5.2–94.2 min), after which ventilatory flow increased above the original pre-exposure control level. All these changes were achieved by changes in ventilatory stroke volume rather than velar frequency.

increases in plasma ammonia levels were a proximate stimulus for elevated ventilation in hagfish. In order to test this hypothesis, we injected low doses of 70 µmol kg^{-1} or high doses of 1000 µmol kg^{-1} ammonium salts (both NH_4HCO_3 and NH_4Cl) into the caudal venous sinus in order to increase plasma ammonia concentrations, and measured associated responses in blood chemistry and ventilatory parameters, with high-dose injections of $NaHCO_3$ and NaCl as parallel controls.

After injection of both low and high doses of NH_4HCO_3, the hagfish significantly increased ventilatory index within 1 h (Fig. 5C), mostly by increasing pressure amplitude (Fig. 5B); ventilatory frequency did not change (Fig. 5A). The fish exhibited simultaneous elevations in plasma T_{Amm} (Fig. 6A) and P_{NH_3} (Fig. 6B), which were much greater in the high-dose treatment, but plasma pH, $[HCO_3^-]$ and P_{CO_2} remained unchanged in both treatments (Table 1). These increases in plasma T_{Amm} (Fig. 6A) and P_{NH_3} (Fig. 6B) at 0.5 h in response to the high-dose NH_4HCO_3 injections were comparable to those seen at 180 min in the 10 mmol l^{-1} NH_4HCO_3 HEA exposures of series III (cf. Fig. 4). After injection of high-dose NH_4Cl, hagfish also showed significant hyperventilation (Fig. 5F), again mostly due to increased pressure amplitude (Fig. 5E). However, there were no significant ventilatory changes in response to injection of low-dose NH_4Cl (Fig. 5D–F). Plasma T_{Amm} (Fig. 6C) and P_{NH_3} (Fig. 6D) increased proportionately in both treatments, but there were no significant changes in acid–base status (plasma pH, $\dot{P}_{CO_2}$, $[HCO_3^-]$; Table 1).

With respect to the control injections, high-dose $NaHCO_3$ caused significantly increased frequency (Fig. 5A) while the pressure

Journal of Experimental Biology (2019) 222, jeb199794. doi:10.1242/jeb.199794

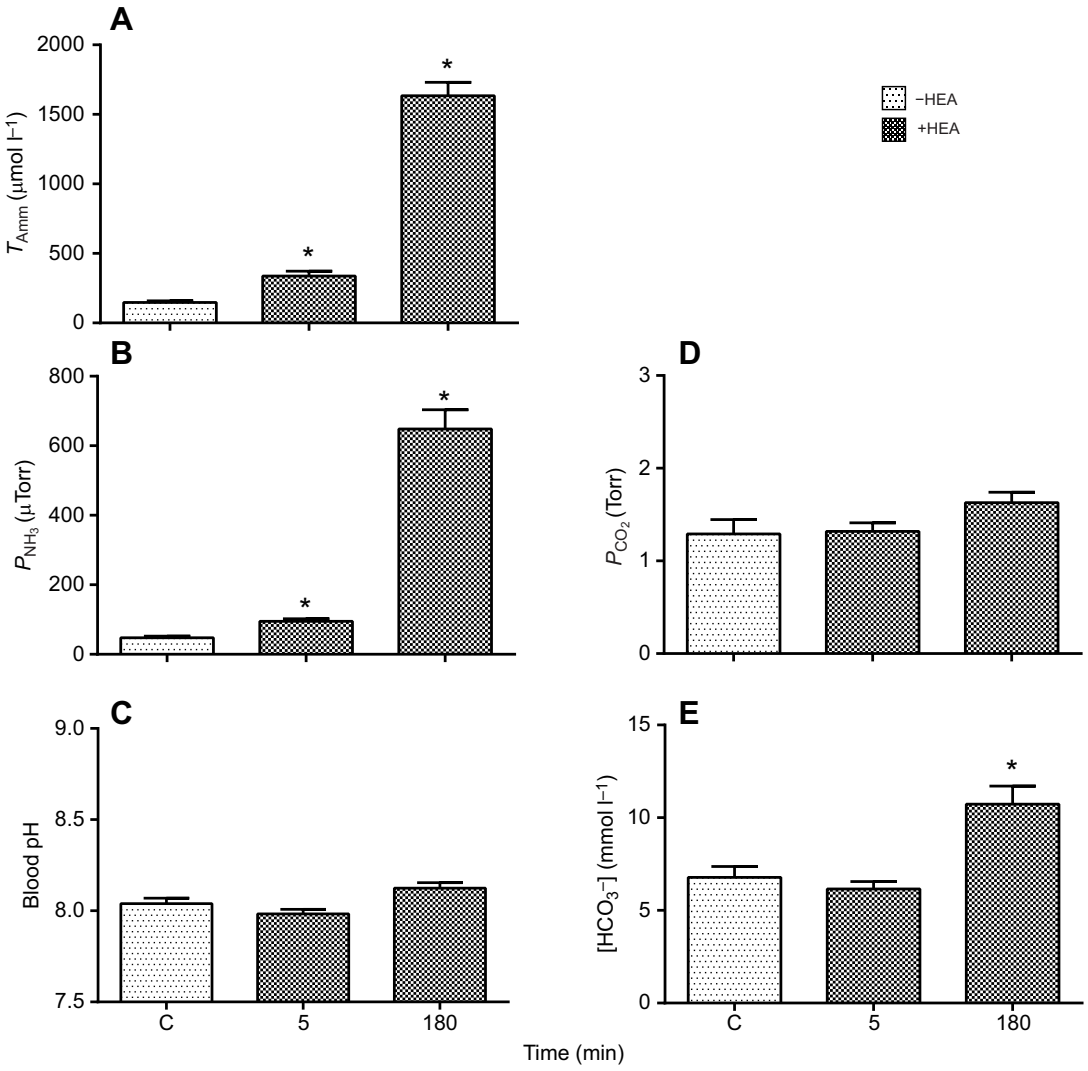

Fig. 4. Changes in blood chemistry in hagfish exposed to HEA. (A) Total ammonia concentration (T_{Amm}), (B) ammonia partial pressure (P_{NH_3}), (C) pH, (D) carbon dioxide partial pressure (P_{CO_2}) and (E) bicarbonate concentration ([HCO_3^-]) in plasma of hagfish (N=6) exposed to 10 mmol l^{-1} NH_4HCO_3 (HEA) for 5 or 180 min (series III). Data are means±1 s.e.m. The 5 min samples were taken just before the greatest ventilation decrease, and the 180 min samples just after the greatest subsequent increase in ventilation (see Fig. 3). Asterisks indicate a significant difference (P<0.05) from the pre-exposure control value (C).

amplitude (Fig. 5B) was significantly decreased, so the overall ventilatory index was not changed (Fig. 5C). Hagfish injected with high-dose $NaHCO_3$ exhibited significant elevations in plasma pH and P_{CO_2} at both sample times, and a large increase in [HCO_3^-] at 1 h (Table 1), with no changes in plasma T_{Amm} (Fig. 6A) and P_{NH_3} (Fig. 6B). There were no responses in ventilation (Fig. 5A–C), plasma ammonia parameters (Fig. 6A,B), or acid–base status (Table 1) to the other control treatment (high-dose NaCl injection).

Two of the trials (high-dose $NaHCO_3$ and NH_4HCO_3 injections) were repeated with measurements of ventilatory flow rather than pressure (Fig. 7A,B). These trials confirmed that the ventilatory index gave a reliable measure of the flow responses (cf. Fig. 5C); although, in this experiment there was a significant increase in frequency in response to the injection of ammonia.

Compared with animals injected with high-dose $NaHCO_3$, hagfish injected with high-dose NH_4HCO_3 exhibited a significant 2.7-fold higher rate of ammonia excretion at 0–0.5 h, and a 4.1-fold higher flux at 0.5–1 h. These fish also significantly increased their urea-N excretion rate by 2.1-fold at 0.5–1 h, but thereafter urea-N flux decreased to zero at 0.5–1 h post-injection (Table 2). The net elevation in N excretion over 1 h therefore amounted to about 30% of the high dose (1000 μmol kg^{-1}) of the ammonia-N injected in the form of NH_4HCO_3.

DISCUSSION

Overview

The present study demonstrated that ammonia stimulates ventilation in the Pacific hagfish, an animal with a deeply rooted evolutionary history in the vertebrate lineage. Ventilation in hagfish responds to both external elevations (i.e. HEA) and internal elevations of ammonia, but the hyperventilatory action of HEA appears to be through its effects on internal ammonia levels, as in other fish (see Introduction). Indeed, the initial response to HEA is a marked hypoventilation, perhaps mediated by external nociceptors or chemoreceptors, followed by a much later elevation of breathing when plasma levels of ammonia are greatly increased. The injection experiments demonstrated that hyperventilation in response to internal ammonia elevations is due specifically to ammonia and is not confounded by changes in blood acid–base status, again similar to the situation in other fish.

Journal of Experimental Biology (2019) 222, jeb199794. doi:10.1242/jeb.199794

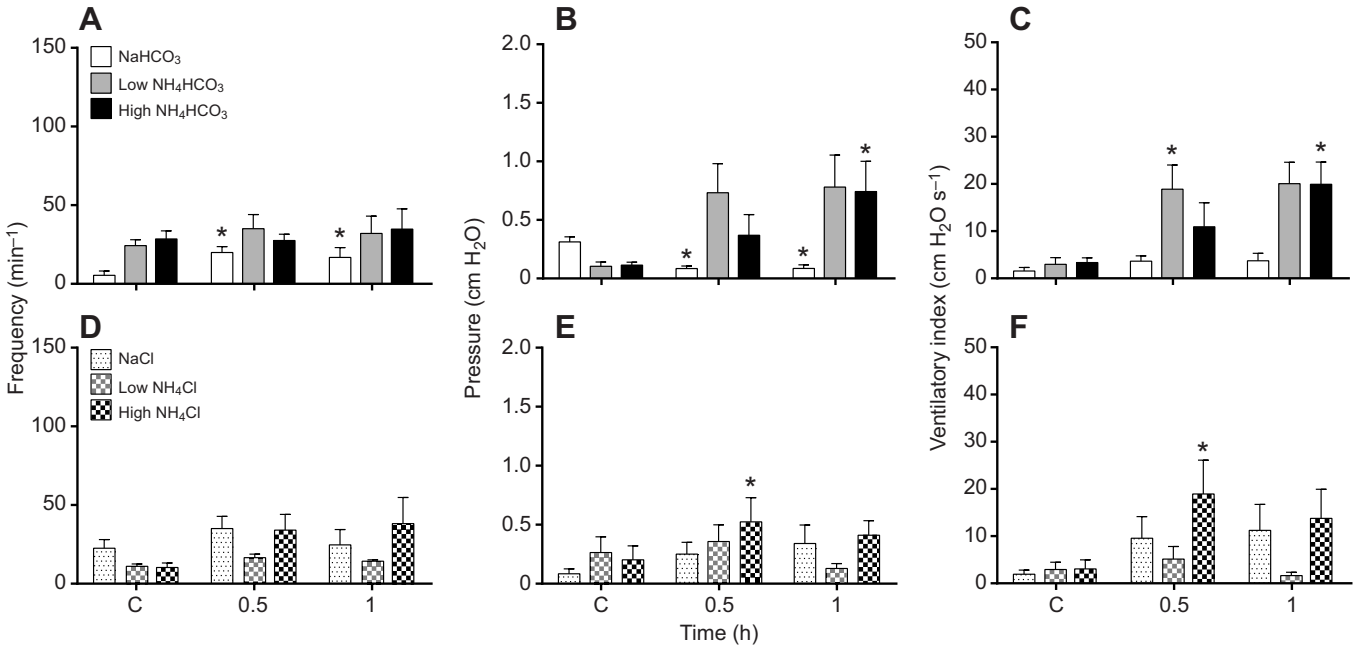

Fig. 5. Changes in ventilatory parameters of hagfish in response to injection of ammonium salts into the venous sinus. (A) Frequency, (B) pressure amplitude and (C) ventilatory index in response to 1000 µmol kg^{-1} $NaHCO_3$ (sodium salt control, *N*=11) and 70 µmol kg^{-1} (low, *N*=6) or 1000 µmol kg^{-1} (high, *N*=18) NH_4HCO_3 (series IV). (D) Frequency, (E) pressure amplitude and (F) ventilatory index in response to 1000 µmol kg^{-1} NaCl (sodium salt control, *N*=6) and 70 µmol kg^{-1} (low, *N*=6) or 1000 µmol kg^{-1} (high, *N*=6) NH_4Cl (series IV). Data are means±1 s.e.m. Asterisks indicate a significant difference (P<0.05) from pre-injection control value (C).

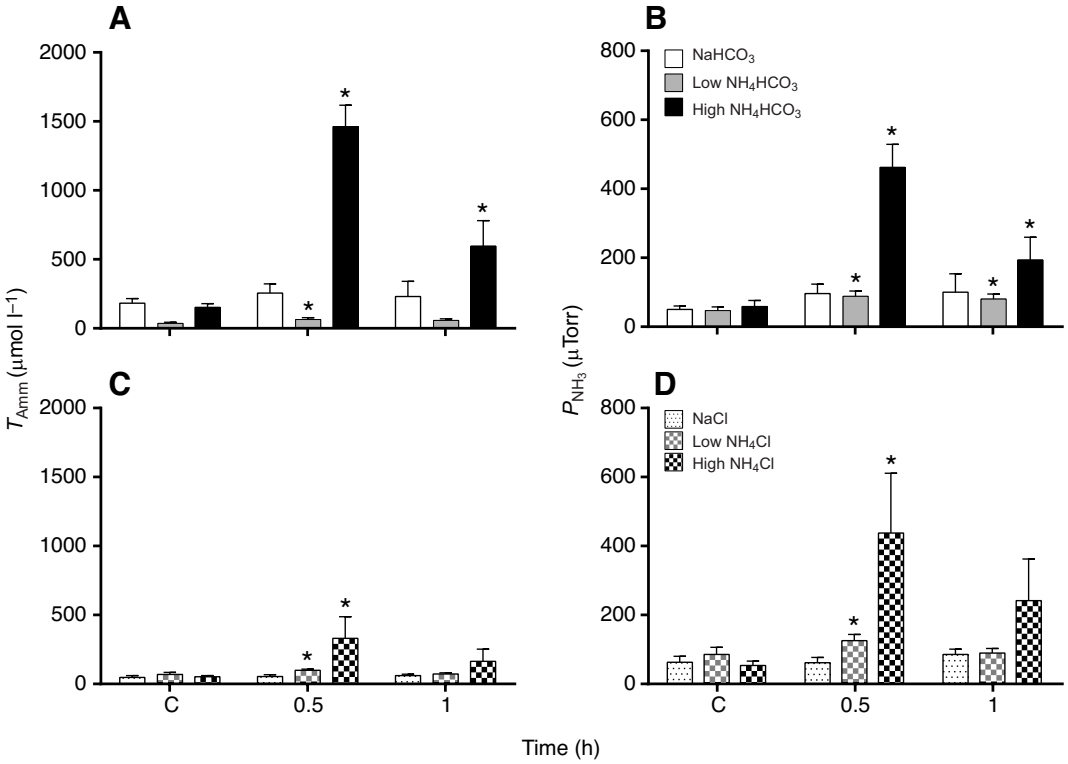

Fig. 6. Changes in plasma T_{Amm} and P_{NH_3} of hagfish in response to injection of ammonium salts into the venous sinus. (A) T_{Amm} and (B) P_{NH_3} in response to 1000 µmol kg^{-1} $NaHCO_3$ (sodium salt control, *N*=11) and 70 µmol kg^{-1} (low, *N*=6) or 1000 µmol kg^{-1} (high, *N*=18) NH_4HCO_3 (series IV). (C) T_{Amm} and (D) P_{NH3} in response to 1000 µmol kg^{-1} NaCl (sodium salt control, *N*=6) and 70 µmol kg^{-1} (low, *N*=6) or 1000 µmol kg^{-1} (high, *N*=6) NH_4Cl (series IV). Data are means±1 s.e.m. Asterisks indicate a significant difference (P<0.05) from pre-injection control value (C).

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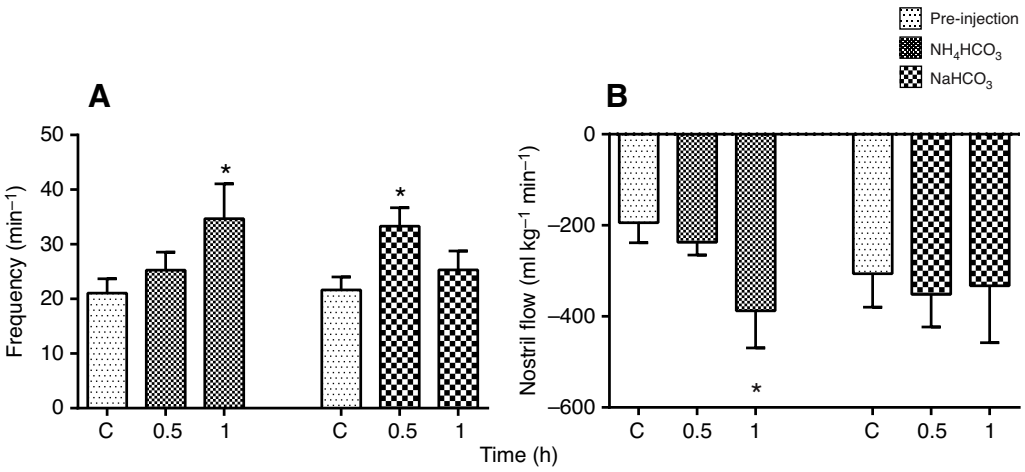

Fig. 7. Ventilatory changes in hagfish in response to injection of ammonium salts into the venous sinus. (A) Ventilatory frequency and (B) ventilatory flow (measured directly via a flow probe placed in the nostril entrance) in response to 1000 µmol kg^{-1} NH_4HCO_3 (*N*=6) and 1000 µmol kg^{-1} $NaHCO_3$ (bicarbonate control, *N*=6) (series IV). Data are means±1 s.e.m. Asterisks indicate a significant difference (*P*<0.05) from pre-injection control value (C).

Ventilation changes in hagfish loaded externally with ammonia

In our previous studies on responses to HEA in seawater fish, we had found that NH_4HCO_3 was preferable to NH_4Cl because, unlike the latter, it did not acidify the water (Wood and Nawata, 2011; Nawata et al., 2015; De Boeck and Wood, 2015). However, these studies had involved concentrations only up to 1.5 mmol l^{-1}, whereas in the present study we were using much higher concentrations, so we thought it prudent to investigate both salts. Indeed, both treatments significantly stimulated ventilation to a comparable extent by 3 h (Fig. 1C). In our initial tests with the two salts at 10 mmol l^{-1}, they had yielded virtually identical seawater pH values, as reported in the Results, so we were surprised at the mortalities occurring in the longer term only in the NH_4HCO_3 exposures (Fig. S1). This result was initially puzzling because the 10 mmol l^{-1} $NaHCO_3$ control treatment was without lethality. However, the mystery was solved by the subsequent finding that continued aeration of the seawater solution resulted in markedly higher water pH values, and therefore much greater P_{NH_3} levels in the 10 mmol l^{-1} NH_4HCO_3 treatment (Fig. S1). Presumably, NH_4HCO_3 tends to dissociate to NH_3, H_2O and CO_2, and the loss of CO_2 and persistence of NH_3 raises pH to a greater extent than with $NaHCO_3^-$- or NH_4Cl-supplemented sea water, where the more moderate increases in pH are due only to the loss of CO_2 from the sea water. The higher seawater pH in the presence of 10 mmol l^{-1} NH_4HCO_3 relative to 10 mmol l^{-1} NH_4Cl, created by continuous aeration, results in a greater P_{NH_3} gradient for ammonia entry, and therefore greater internal ammonia loading. Interestingly, mortality in the 10 mmol l^{-1} NH_4HCO_3 exposures did not occur in the $\dot{M}_{O_2}$ experiments of series II, where the seawater solution was refreshed at 3 h intervals, minimizing the increases in water pH and P_{NH_3}. Our findings with the two salts are instructive in implicating NH_3 rather than NH_4^+ as the principal form of ammonia entering across the body surface during HEA exposure. This concurs with previous reports that ammonia loading in hagfish during HEA exposure occurs in association with an internal alkalosis, suggesting that ammonia enters the hagfish as NH_3 rather than as ionized NH_4^+ (Clifford et al., 2015). Furthermore, cutaneous ammonia conductance appears be the prominent contributor to ammonia loading over branchial entry (Clifford et al., 2017).

Table 2. Ammonia and urea-N flux rates of hagfish after injection of 1000 µmol kg^{-1} NH_4HCO_3 (*N*=18) or 1000 µmol kg^{-1} $NaHCO_3$ (*N*=11) into the venous sinus (series IV)

	Time post-injection	$NaHCO_3$	NH_4HCO_3
Ammonia flux	0–0.5 h	−132.50±19.28	−364.20±54.71*
(µmol kg^{-1} h^{-1})	0.5–1 h	−107.40±17.86	−435.30±85.87*
Urea-N flux	0–0.5 h	−25.30±6.31	−52.23±12.78*
(µmol kg^{-1} h^{-1})	0.5–1 h	−22.40±9.67	2.77±22.66

Data are means±s.e.m. Negative values indicate excretion to the water. Asterisks indicate a significant difference (*P*<0.05) between the two treatments in the same time period.

Our initial experiments (series I) demonstrated that the hagfish increased ventilation at 3 h during exposure to 10 mmol l^{-1} HEA (or greater, as either NH_4HCO_3 or NH_4Cl) but there was no immediate response at 0 h (Fig. 1). However, a more detailed examination of the time course of the response (series III) revealed that the hagfish quickly (i.e. within about 5 min) decreased ventilatory stroke volume, pressure amplitude and ventilatory index during HEA treatment, reducing the total ventilatory flow by about 80%; velar frequency remained unchanged (Fig. 3). This reduction was maintained for a variable time period before ventilation finally increased significantly by 3 h (Fig. 3), the latter in accord with the series I results (Fig. 1). In essence, the hagfish appeared to be 'holding their breath' initially, possibly sensing toxic external ammonia with external chemoreceptors or nociceptors. These could be the well-developed olfactory organ with neural connections to the central nervous system (Theisen, 1976; Holmes et al., 2011) or Schreiner organs which contain taste bud-like external sensory structures in the epidermis of the head, trunk and along the respiratory tracts (Braun, 1998; Braun and Northcutt, 1998). This result suggests that elevated external ammonia in itself is not a direct stimulant of ventilation.

Despite the initial hypoventilation, ammonia progressively accumulated in the bloodstream during HEA exposure (Fig. 4). By the time of initiation of hypoventilation (i.e. 5 min), plasma T_{Amm} was already elevated by 2.3-fold, and by the time hyperventilation was clearly instituted (3 h), plasma T_{Amm} had increased by 11-fold. It is now well established that Rh proteins (bidirectional ammonia-conductive channels; Wright and Wood, 2009) are present in both the gills and skin of hagfish (Braun and Perry, 2010; Edwards et al., 2015; Clifford et al., 2017). Thus, even though water flow through the gill pouches was greatly reduced upon initial HEA exposure, ammonia could still permeate across the body surface along P_{NH_3} and/or NH_4^+ electrochemical gradients. Overall, we conclude that the stimulation of breathing by HEA in hagfish is probably due to its indirect action on internal chemoreceptors rather than a direct action on external

chemoreceptors. In this regard, the response parallels that of teleosts (Zhang et al., 2011) and elasmobranchs (De Boeck and Wood, 2015), though the absolute level of HEA needed to elicit the hyperventilatory effect is considerably higher in the hagfish, as 5 mmol l^{-1} NH_4CO_3 had no effect. Hagfish commonly feed by inserting their heads into decaying carrion (Martini, 1998), where HEA levels may be very high (Clifford et al., 2016), so the whole sequence of events (initial breath holding, followed by eventual hyperventilation when blood ammonia levels pass threshold values), as well as their exceptional tolerance of HEA (Clifford et al., 2015, 2017), may be adaptive for this trophic habit. The hyperventilation may be important in flushing out excess ammonia after feeding (Wilkie et al., 2017).

The experiments of series II showed that the increases in ventilatory flow occurring during HEA treatment were effective in increasing $\dot{M}_{O_2}$ (Fig. 2). Clifford et al. (2016) demonstrated that O_2 uptake occurs mainly at the gills in *E. stoutii*; the skin plays only a very minor role. While the metabolic cost of breathing in hagfish has yet to be elucidated, it is possible that some of the observed increase in $\dot{M}_{O_2}$ may have been consumed by the breathing mechanism. Breathing in hagfish consists of both contraction of the velar pump (i.e. velum and velar chamber) for inhalation and contraction of the gill pouches for exhalation, the latter at a much lower frequency than contraction of the velum (Eom and Wood, 2019). Total ventilatory flow in *E. stoutii* can be altered by changes in both velar frequency and stroke volume (Eom and Wood, 2019). As with the initial hypoventilation, the hyperventilatory effects of HEA were exerted almost exclusively on ventilatory stroke volume; velar frequency remained unchanged in most HEA exposures, while pressure amplitude and ventilatory index increased (Figs 1 and 3; Fig. S1). This response pattern is similar to that seen with internal ammonia loading (Fig. 5). Despite the fundamental differences in breathing mechanisms in hagfish versus other fish (Strahan, 1958; Malte and Lomholt, 1998; Kardong, 2012; Eom and Wood, 2019), this is also in accord with the HEA responses of both teleosts (Zhang et al., 2011, 2013) and elasmobranchs (De Boeck and Wood, 2015), where only ventilatory stroke volume increases. In Pacific hagfish, Perry et al. (2009) reported only increased velar frequency under external hypoxia and hypercapnia as ventilatory stimulants, but did not test HEA.

Ventilation changes in hagfish loaded internally with ammonia

After internal loading with ammonia in series IV, either as NH_4HCO_3 or as NH_4Cl, hagfish exhibited increased ventilation; the initial hypoventilation seen with external ammonia loading (HEA) never occurred. The hyperventilatory response was largely due to increased ventilatory stroke volume. Pressure amplitude and ventilatory index both increased while ventilatory frequency was largely unchanged (Fig. 5). This response is similar to the long-term response pattern to external ammonia loading (HEA; Figs 1 and 3; Fig. S1), and in qualitative accord with the patterns previously reported in teleosts (Zhang and Wood, 2009; Zhang et al., 2011) and elasmobranchs (De Boeck and Wood, 2015), where increases in amplitude predominated in the hyperventilatory response to internal ammonia loading. Importantly, these responses occurred in the absence of changes of blood acid–base status (Table 1), which have confounded interpretation in some previous studies on teleosts (McKenzie et al., 1993; Zhang and Wood, 2009). It is noteworthy that the one treatment showing a marked increase in frequency was the high-dose $NaHCO_3$ control injection (Fig. 5A), and this was the only treatment where blood P_{CO_2} increased (Table 1), in accord with the observation of Perry et al. (2009) that external hypercapnia elevated velar frequency in *E. stoutii*.

The blood plasma measurements demonstrated that in every injection treatment where significant hyperventilation occurred (Fig. 5), there were corresponding significant increases in mean blood T_{Amm} and P_{NH_3} levels (Fig. 6). These measurements give an indication of the approximate threshold values needed to initiate the hyperventilatory response. Hyperventilation was marginal with the low-dose injections of NH_4Cl and NH_4HCO_3 (Fig. 5) where mean blood plasma T_{Amm} and P_{NH_3} levels remained less than 100 µmol l^{-1} and 150 µTorr, respectively, but became prominent with the high-dose injections where values surpassed 350 µmol l^{-1} and 450 µTorr, respectively (Fig. 6). In the HEA exposures of series III, the significant hyperventilation at 3 h (Fig. 3) was associated with values (T_{Amm}>1500 µmol l^{-1}, P_{NH_3}>600 µTorr; Fig. 4) that clearly surpassed these thresholds. Overall, these plasma levels are similar to those reported to cause hyperventilation in teleosts (Zhang and Wood, 2009; Zhang et al., 2011, 2013), elasmobranchs (De Boeck and Wood, 2015) and mammals (e.g. Wichser and Kazemi, 1974; Mutch and Banister, 1983). Notably, the measured plasma T_{Amm} concentrations at 0.5 h after injection (Fig. 6) were less than 50% of those predicted if the injected ammonia load had distributed throughout the plasma volume, and less than 20% of those predicted if it had distributed throughout the extracellular volume (see Materials and Methods). We noted that only about 15% of the injected load was excreted into the external water over this time period (Table 2). Given that hagfish lack a complete complement of ornithine-urea cycle enzymes (Read, 1975; Braun and Perry, 2010), and previous studies demonstrated no increases in plasma glutamine and urea, as well as no increases in urea efflux following ammonia loading by HEA exposure (Clifford et al., 2015), biotransformation to alternative nitrogenous forms seems unlikely. Thus, we suggest that the missing ammonia partitioned into the tissues as previously demonstrated in lemon sole (*Parophrys vetulus*; Wright et al., 1988) and trout (Wright and Wood, 1988).

Our experiments were not designed to distinguish whether internal P_{NH_3} or T_{Amm} levels (or NH_4^+ concentration, which is very similar to T_{Amm} concentration) are the specific stimulants of hyperventilation in the hagfish. Regression analyses of all the paired breathing and plasma ammonia measurements in individual animals from series IV revealed significant positive relationships of ventilatory index with both plasma T_{Amm} (P=0.0037, r^2=0.1918) and P_{NH_3} (P=0.0022, r^2=0.1945), suggesting that either of these indicators of plasma ammonia could be the key stimulant (Fig. S4). However, we emphasize that correlation does not necessarily indicate causation. The experiments also did not localize the internal chemoreception sites for ammonia. In teleosts, there is strong evidence that NECs in the gills are involved, serving as trimodal receptors for O_2, CO_2 and ammonia (Zhang et al., 2011, 2015; Perry and Tzaneva, 2016), as well as correlative evidence that central chemoreception of elevated ammonia concentrations in the brain may also contribute to ammonia detection (Zhang et al., 2013). In mammals, the situation is unclear, but central chemoreception of elevated brain ammonia appears to be important in the hyperventilatory response (see Introduction). To our knowledge, there is no information on the presence or absence of NECs in hagfish. However, NECs are known to originate from neural crest cells, which are ubiquitously found during embryogenesis of all vertebrates, including hagfish (Baker, 2008; Ota et al., 2007). NECs are generally thought to be homologous to the glomus cells of the arterial (peripheral) chemoreceptors of higher vertebrates (though this idea has recently been challenged by Hockman et al., 2017) and

are widely distributed in respiratory epithelia ranging from fish gills to human lungs (Milsom, 2012; Milsom and Burleson, 2007). It would be surprising if they were not present in hagfish.

Summary and future directions

The present study has shown that ventilation can be specifically stimulated by internal ammonia in *E. stoutii*, a representative of the oldest extant connection to the ancestral vertebrates. From experiments on this same species, Perry et al. (2009) concluded that that ventilatory responses to environmental hypoxia and hypercapnia in vertebrates arose in the myxine lineage, and the same would appear to be true for the ventilatory responses to ammonia, the immediate product of amino acid metabolism in all animals. Therefore, the role of ammonia in stimulating breathing appears to be an ancient characteristic of vertebrates. In mammals, it remains important in post-exercise hyperventilation and in supporting breathing during respiratory acidosis and hepatic coma (see Introduction). Given the unique feeding habits of the hagfish discussed earlier, the adaptive value of ammonia-induced hyperventilation appears obvious in these animals. In teleosts, elevations in plasma ammonia after both feeding and exercise (Wood, 2001) have been implicated in adaptive hyperventilatory responses (Zhang et al., 2015). In *E. stoutii*, plasma T_{Amm} concentrations (100–200 µmol l^{-1}) reported after feeding (Wilkie et al., 2017) approached the threshold concentrations for ventilatory stimulation measured in the present study. It would be of interest to measure plasma ammonia levels after exercise in this species to see whether they too are in the range needed to stimulate ventilation. Clearly, additional physiological experiments and morphological analyses are now required to identify the sites of chemoreception for ammonia in hagfish, and to understand how ventilatory responses to ammonia are integrated with those to O_2 and CO_2.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

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Supplementary information

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