



# Understanding ventilation and oxygen uptake of Pacific hagfish (*Eptatretus stoutii*), with particular emphasis on responses to ammonia and interactions with other respiratory gases

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## Abstract

The hagfishes are an ancient and evolutionarily important group, with breathing mechanisms and gills very different from those of other fishes. Hagfish inhale through a single nostril via a velum pump, and exhale through multiple separate gill pouches. We assessed respiratory performance in *E. stoutii* (31 ppt, 12 °C, 50–120 g) by measuring total ventilatory flow ( $\dot{V}_w$ ) at the nostril, velar (respiratory) frequency (fr), and inspired ( $P_{I}O_2$ ) and expired ( $P_{E}O_2$ ) oxygen tensions at all 12 gill pouch exits plus the pharyngo-cutaneous duct (PCD) on the left side, and calculated ventilatory stroke volume ( $S\dot{V}_w$ ), %  $O_2$  utilization, and oxygen consumption ( $\dot{M}O_2$ ). At rest under normoxia, spontaneous changes in  $\dot{V}_w$  ranged from apnea to  $>400 \text{ ml kg}^{-1} \text{ min}^{-1}$ , due to variations in both fr and  $S\dot{V}_w$ ; “normal”  $\dot{V}_w$  averaged  $137 \text{ ml kg}^{-1} \text{ min}^{-1}$ ,  $\dot{M}O_2$  was  $718 \mu\text{mol kg}^{-1} \text{ h}^{-1}$ , so the ventilatory convection requirement for  $O_2$  was about  $11 \text{ L mmol}^{-1}$ . Relative to anterior gill pouches, lower  $P_{E}O_2$  values (i.e. higher utilization) occurred in the more posterior pouches and PCD. Overall,  $O_2$  utilization was 34% and did not change during hyperventilation but increased to  $>90\%$  during hypoventilation. Environmental hypoxia ( $P_{I}O_2 \sim 8\%$  air saturation, 1.67 kPa, 13 Torr) caused hyperventilation, but neither acute hyperoxia ( $P_{I}O_2 \sim 275\%$  air saturation, 57.6 kPa, 430 Torr) nor hypercapnia ( $P_{I}CO_2 \sim 1\% \text{ CO}_2$ , 1.0 kPa, 7.5 Torr) significantly altered  $\dot{V}_w$ .  $\dot{M}O_2$  decreased in hypoxia and increased in hyperoxia but did not change in hypercapnia. Acute exposure to high environmental ammonia (HEA, 10 mM  $\text{NH}_4\text{HCO}_3$ ) caused an acute decrease in  $\dot{V}_w$ , in contrast to the hyperventilation of long-term HEA exposure described in a previous study. The hypoventilatory response to HEA still occurred during hypoxia and hyperoxia, but was blunted during hypercapnia. Under all treatments,  $\dot{M}O_2$  increased with increases in  $\dot{V}_w$ . Overall, there were lower convection requirements for  $O_2$  during hyperoxia, higher requirements during hypoxia and hypercapnia, but unchanged requirements during HEA. We conclude that this “primitive” fish operates a flexible respiratory system with considerable reserve capacity.

**Keywords** High environmental ammonia (HEA) · Hypercapnia · Hyperoxia · Hypoxia · Gill pouches · Oxygen utilization

## Abbreviations

$PCO_2$	Carbon dioxide tension
$P_{E}O_2$	Expired oxygen tension
HEA	High environmental ammonia
$P_{I}O_2$	Inspired oxygen tension
$\dot{M}O_2$	Oxygen consumption
PCD	Pharyngo-cutaneous duct
fr	Velar (respiratory) frequency

$\dot{V}_w$	Ventilatory flow
$S\dot{V}_w$	Ventilatory stroke volume

## Introduction

Modern agnathans such as the Pacific hagfish *Eptatretus stoutii* are extant representatives of what is arguably the most ancient vertebrate lineage (Goodrich 1930; Bardack 1998; Miyashita et al. 2019; Rasmussen et al. 1998; Heimberg et al. 2010; Oisi et al. 2013). Their breathing mechanism and branchial structure are fundamentally different from those of most other fishes (Bartels 1998; Johansen and Strahan 1963; Malte and Lomholt 1998; Eom and Wood 2019). Unlike teleosts, where the gills are grouped together on each side, covered by bilaterally symmetrical operculae and ventilated

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via the mouth by a buccal force—opercular suction pump (Hughes 1960; Hughes and Shelton 1962), or elasmobranchs which operate a similar system with separate gill slits (Piiper and Schumann 1968), the hagfish have multiple separate gill pouches on each side, each with a separate exit (Mallatt 1984; generally 2 rows  $\times$  12 gill pouches in *E. stoutii*). The gill pouches are internal structures surrounded by cartilaginous plates, muscle, and connective tissues (Marinelli and Strenger 1956). Water is inhaled through the single separate nostril, not the mouth, by the suction created by the up-and-down pumping action of a central velar scroll (Bartels 1998; Eom and Wood 2019). The velum chamber contracts rhythmically. This forces water down a long pharynx and into the 24 separate afferent branchial ducts leading to the separate gill pouches, as well as into an apparent bypass shunting system, the pharyngo-cutaneous duct (PCD) which has a single exit on the left side in *E. stoutii*. The gill pouches contract at a much lower rate than the velum chamber and seem to play a role in driving exhalation through the 24 separate efferent branchial ducts, as well as through the PCD. A similar expiratory pumping role for the gill pouches has been proposed for the Atlantic hagfish, *Myxine glutinosa* (Goodrich 1930; Johansen and Hol 1960). We recently characterized the breathing mechanism in *E. stoutii* as a two-phase unidirectional pumping system with a fast suction pump (velum) for inhalation through the single nostril and a much slower force pump (gill pouches and PCD) for exhalation (Eom and Wood 2019). Given this apparent disconnect of the two pumps, and the fact that ventilatory flow ( $\dot{V}_w$ ) can vary spontaneously from zero (apnea) to very high levels (Eom and Wood 2019), the volume and residence time of water in the system may vary greatly, which would likely affect the extent of respiratory gas exchange.

Unlike the holobranchs of other fish, the hagfish has lens-shaped bi-lobed pouches with internal radial folds that in turn bear filaments and respiratory lamellae. These have been thoroughly described in *E. stoutii* by Mallatt and Paulsen (1986). While their orientation relative to the body axis is very different from other fish, these respiratory structures are surprisingly similar in architecture, blood channels, and cellular composition, and organized so as to provide a largely countercurrent flow of blood against the water. The mean effective diffusion distance is about 5–10  $\mu\text{m}$ , typical of many fish (Hughes 1984). In *E. stoutii*, the gills appear to be the major sites of respiratory gas exchange, accounting for about 80% of  $\text{O}_2$  uptake and 70% of ammonia excretion, based on divided chamber studies (Clifford et al. 2014, 2016), though the skin may play a larger role in Atlantic hagfish such as *Myxine glutinosa* (Steffensen et al. 1984; Lesser et al. 1997). At present, we know very little about the process of  $\text{O}_2$  uptake ( $\dot{M}\text{O}_2$ ) from the water, and how it might vary with flow ( $\dot{V}_w$ ), or with the gas composition of the water. Indeed, we are aware of no measurements of

expired  $\text{O}_2$  tensions (i.e.  $P_{\text{E}}\text{O}_2$ ), how they may vary with inspired  $\text{O}_2$  tension ( $P_{\text{I}}\text{O}_2$ ), or the % utilization of  $\text{O}_2$  at the gills in hagfish, either overall, or on an individual gill pouch basis. However, there is general agreement that under resting normoxic conditions,  $\text{M}\text{O}_2$  is very low in *E. stoutii*, (Munz and Morris 1965; Clifford et al. 2016; Eom et al. 2019) but some disagreement as to how well it is maintained under hypoxic conditions (Perry et al. 2009b; Drazen et al. 2011; Giacomini et al. 2019b). Nevertheless, both Perry et al. (2009b) and Giacomini et al. (2019b) reported that hyperventilation occurred during environmental hypoxia, and Giacomini et al. (2019a) reported a non-significant increase in ventilation during environmental hyperoxia, the latter unlike the hypoventilatory response seen in most fish. Perry et al. (2009b) also found hyperventilation during environmental hypercapnia, and indirect evidence for the presence of both external and internal chemo-receptors based on cyanide injection. Eom et al. (2019) found that elevated environmental ammonia (HEA) caused a marked short-term (minutes) hypoventilation followed by longer term hyperventilation after several hours when blood ammonia levels increased.

In teleosts, the respiratory gases ( $\text{O}_2$ ,  $\text{CO}_2$ , and ammonia) are known to be detected by neuroepithelial cells (NECs) on the gills (Perry et al. 2009a). These cells are thought to be polymodal chemoreceptors responding to water and/or blood levels of these gases, as well as to surrogate stimuli such as cyanide (reviewed by Perry and Tzaneva 2016; Jonz 2018). At present, it is not known whether these peripheral chemoreceptors are present in agnathans, but the documented hyperventilatory responses to hypoxia (Perry et al. 2009b; Giacomini et al. 2019a), hypercapnia (Perry et al. 2009b), cyanide (Perry et al. 2009b) and ammonia treatments (Eom et al. 2019) all suggest that NECs, or functional analogues of NECs, must be present in hagfish.

With this background in mind, in the present study, we directly measured ventilatory flow ( $\dot{V}_w$ ) using an electromagnetic flowmeter (Perry et al. 2009b; Eom and Wood 2019) and  $\text{O}_2$  tensions at various sites using needle-tip optodes. We first characterized the variations in  $P_{\text{E}}\text{O}_2$  and, therefore, % utilization of  $\text{O}_2$  amongst the 12 gill pouches and PCD on one side. Our hypothesis was that the more posterior pouches would exhibit lower  $P_{\text{E}}\text{O}_2$  and higher % utilization because of likely recycling of water from front-to-back, but that the PCD would show the highest  $P_{\text{E}}\text{O}_2$  and lowest % utilization because of its role as a bypass shunt. We also hypothesized that  $P_{\text{E}}\text{O}_2$  would be low and % utilization high during spontaneous hypoventilation, and vice versa during spontaneous hyperventilation, in light of the well-known inverse relationship between  $\dot{V}_w$  and % utilization in teleost fish (e.g. Davis and Cameron 1971; Randall 1970; Bushnell and Brill 1992). We also characterized the effects of these spontaneous changes in  $\dot{V}_w$  on  $\text{M}\text{O}_2$ , as a comparator for examining  $\dot{V}_w$  versus  $\text{M}\text{O}_2$  relationships

during experimental manipulations of respiratory gases in the ambient water. Next, we examined the impact of acute 10 mM HEA exposure on the same parameters under normoxia. Our focus here was on the short-term hypo-ventilatory response as the longer term hyperventilatory response to HEA (Eom et al. 2019) was too time-variable for analysis (see “Results”), and probably also less relevant to the day-to-day life of the animal. Finally, we examined these same parameters in hagfish acutely exposed to environmental hypercapnia (1% CO<sub>2</sub>, PCO<sub>2</sub> = 7.5 Torr, 1.0 kPa), to severe environmental hypoxia (~8% air saturation, PO<sub>2</sub> = 13.0 Torr, 1.7 kPa), and to environmental hyperoxia (~275% air saturation, PO<sub>2</sub> = 430 Torr, 57.6 kPa), followed by simultaneous exposure to 10 mM HEA to evaluate possible interactive effects. We hypothesized that the initial responses would be similar to those documented by previous workers as outlined above—i.e. marked hyperventilation in response to hypercapnia and hypoxia, and moderate hyperventilation in response to hyperoxia. We also predicted that because it is an important defensive response to delay and therefore minimize ammonia uptake (Eom et al. 2019), the hypoventilation caused by HEA would persist regardless of the presence of the other respiratory gas treatments.

## Materials and methods

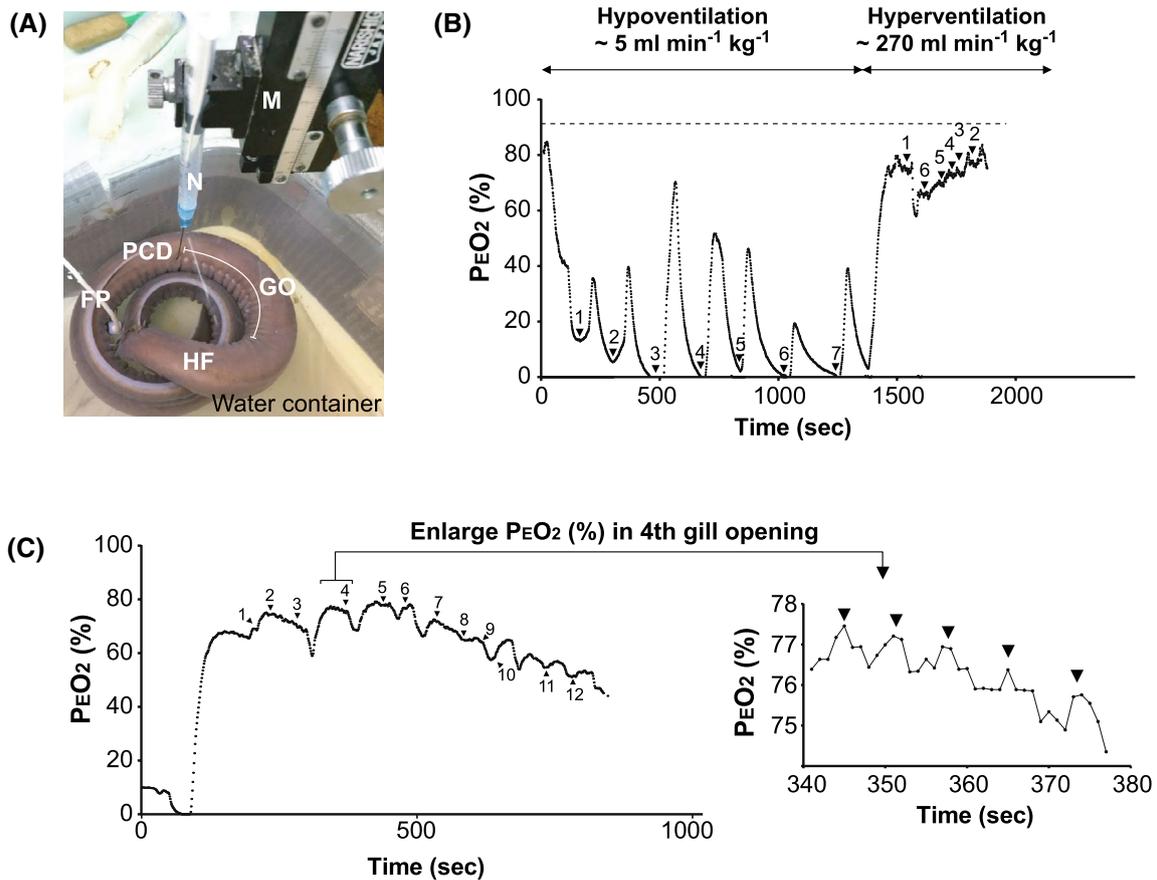
### Experimental animals

Under permits (XR 202 2016, XR 194 2017, XR 204 2018, and XR 212 2019) from the Department of Fisheries and Oceans Canada (DFO), Pacific hagfish (*Eptatretus stoutii*, 50–120 g) were captured in Trevor channel (48° 50.8440' N, 125° 08.3210' W) close to Bamfield Marine Sciences Centre (BMSC) located on the southwest coast of Vancouver Island, BC, Canada. Bottom-dwelling traps baited with strips of Pacific hake (*Merluccius productus*) were used. At BMSC, captured hagfish were placed in fiberglass tanks served with flowing sea water (temperature 11–13 °C, salinity 30–31 ppt) and short pieces of pipe for shelter. The animals were regularly provided with hake strips but rarely fed in captivity. Experiments were performed under animal usage permits (AUP) approved by the University of British Columbia (A14-0251, A18-0271) and BMSC Animal Care Committees (AUP RS-17–20, RS-18–20, RS -19–15), and followed the guidelines of the Canadian Council of Animal Care. All experiments reported here were performed on hagfish that were fasted for at least 1 week. After experiments, the hagfish were anesthetized by an overdose of tricaine methane sulfonate (MS-222, 5 g L<sup>-1</sup> neutralized to pH 7.8 with 5 M NaOH; Syndel laboratories, Parksville, Canada) and euthanized by evisceration to ensure death.

### Ventilation flow recording from the nostril duct

The hagfish are nocturnal so mostly active after sunset, therefore, surgical operations were performed during the day-time and most experiments were performed during the night-time in an area that was well shielded from disturbance. Prior to operation, the hagfish were anesthetized in MS-222 (0.6 g L<sup>-1</sup>, neutralized to pH 7.8 with 5 M NaOH) for 1–2 min, placed on an operating table without gill irrigation as they are very hypoxic tolerant, and moistened with sea water wet tissue during air exposure. As previously described (Eom and Wood 2019; Eom et al. 2019), a 3-cm length of transparent silicone tubing (6.35 mm O.D. and 4.32 mm I.D.) was inserted into the nostril duct of the hagfish, secured by two stitches (26 mm 1/2C taper, Perma-Hand Silk, Ethicon, Somerville, NJ, USA) laterally to the skin. After the operation, the hagfish were allowed to recover in flowing sea water. Upon recovery, they almost always adopted a typical coiled posture.

Similar to the approach of Perry et al. (2009b) and Eom et al. (2019), the ventilatory flow rate of hagfish ( $\dot{V}_w$ ) was measured using an ultrasonic microcirculation blood flow probe (V-series, Transonic Systems Inc., Ithaca, NY, USA) attached to a dual-channel small animal blood flowmeter (T106 series, Transonic Systems Inc.) (Fig. 1A). The hagfish often exhibited anti-predation responses such as generating slime and knotting behavior in response to any attachments on their body including the flow probe, therefore, it was connected onto the nostril tubing only during periods of ventilation recording. The measured analogue signal was amplified (LCA-RTC, Transducer Techniques, Temecula, CA, USA), converted into digital signals in a PowerLab data integrity system (ADInstruments, Colorado Springs, CO, USA), and visualized and analyzed into ventilation flow ( $\dot{V}_w$ , ml min<sup>-1</sup>) and frequency (fr, min<sup>-1</sup>) in LabChart software version 7.0 (ADInstruments). The analyzed ventilation flow (ml min<sup>-1</sup>) data were averaged per 30 s intervals and divided by fish body weight to yield mass-specific  $\dot{V}_w$  (ml kg<sup>-1</sup> min<sup>-1</sup>). As first noted by Perry et al. (2009b), the intrinsic calibration of the flow probe is altered in sea water, so the probe was manually recalibrated by flowing salinity 30 ppt sea water at 12 °C at known rates (determined gravimetrically) through the probe attached to the 3-cm length of silicone tubing, by means of an aqua lifter vacuum pump (Cheng Gao Plastic and Hardware Electricity, Dongguan, Guangdong, China). The flow correction was incorporated by the LabChart software. The standardized  $\dot{V}_w$  was then divided by fr (frequency of velum contraction as detected in the flow trace; Eom and Wood 2019) to calculate mean stroke volume ( $S\dot{V}_w$ , ml kg<sup>-1</sup>) for the period. During or after recording, electrical and/or physical non-specific noise signals were removed by a low-pass type filter in a range between 1 – 10 Hz which was incorporated in the LabChart



**Fig. 1** **a** On the flipped over hagfish (HF), which was submerged in water, expired oxygen tension ( $P_{E}O_2$ ) was measured at the gill openings (GO) including the pharyngo-cutaneous duct (PCD) by a needle-type oxygen probe (N) attached on a micro-manipulator (M), which was magnetically mounted on an iron plate. The oxygen probe could be moved between gill openings by moving the micro-manipulator. Simultaneously, ventilation flow ( $\dot{V}_w$ ) was also measured using a flow probe (FP) attached on silicone tubing in the nostril duct. **b** In this example of  $P_{E}O_2$  measurements, the hagfish was initially exhibiting hypoventilation. The needle-type oxygen probe was moved sequentially between different numbered gill openings, with 1 being the most anterior, and subsequent numbers being more and more posterior openings. The points marked by arrow heads represent the

true  $P_{E}O_2$  measurements at each numbered gill pouch opening. Note the lower  $P_{E}O_2$  values as the probe was moved to more posterior gill openings. At approximately 1400 s, the hagfish spontaneously changed to hyperventilation.  $P_{E}O_2$  values increased markedly, but the trend whereby more posterior openings exhibited lower  $P_{E}O_2$  values continued. **c** In this example, the trend for lower  $P_{E}O_2$  in posterior gill pouches is clearly seen; again the arrowheads represent the points where the probe was correctly positioned to measure true  $P_{E}O_2$  values at the respective numbered gill openings. In the time scale expansion of recording at the 4<sup>th</sup> gill opening, the measured  $P_{E}O_2$  fluctuated slightly, probably reflecting the slow rhythmic contractions of the gill pouches

software. The flow probe (Transonic Systems Inc.) detects the direction of flow so correct orientation of flow probe was essential.

### Series I. Measurement of oxygen partial pressure ( $PO_2$ ) from gill openings including the pharyngo-cutaneous duct (PCD)

Each individual hagfish was placed in a 2-L plastic container served with flowing normoxic sea water ( $PO_2 > 75\%$  air saturation,  $> 118$  Torr,  $> 15.7$  kPa). Pacific hagfish generally have 12 gill pouch openings on the ventral surface on each side ( $\sim 24$  in total) plus the opening of the larger

pharyngo-cutaneous duct (PCD) on the left side (Eom and Wood 2019). Prior to measurements under normoxia, the coiled hagfish ( $N = 14$  for control measurements) were gently flipped over to expose the ventrally placed gill openings (Fig. 1a). After that, an oxygen sensor (micro-optode) mounted in a #23 hypodermic needle and attached to a micro-fiber optic oxygen meter (PreSens, Microx TX 3, Precision Sensing GmbH, Regensburg, Germany) was carefully placed within 5 mm of the respective gill openings by adjusting the model M33301R three-axis micro-manipulator (Narishige, Tokyo, Japan), which was magnetically mounted on an iron plate. To move the probe between gill openings, the micro-manipulator

was moved on the plate, then the micro-optode was correctly repositioned by fine adjustments with the micro-manipulator. The oxygen partial pressures (expressed as % air saturation) of the exhaled water ( $P_{E}O_2$ ) at each of the 12 gill openings on one side including the PCD, as well as the inspired  $P_1O_2$  of water inhaled *via* the nostril duct, were measured in control normoxia initially. Ventilation is very variable in hagfish, and based on prior experience (Eom and Wood 2019; Eom et al. 2019), we recognized three types of spontaneous ventilation in resting animals: normal ventilation ( $75\text{--}175\text{ ml kg}^{-1}\text{ min}^{-1}$ ), hypoventilation ( $< 75\text{ ml kg}^{-1}\text{ min}^{-1}$ ) and hyperventilation ( $> 175\text{ ml kg}^{-1}\text{ min}^{-1}$ ), in addition to apnea. Collected data were assigned to these three categories; the apnea was not included.

Following completion of the resting measurements under normoxia, comparable measurements were made in 10 mM HEA, 1%  $CO_2$ , severe hypoxia, and hyperoxia sequentially. The methods used for creating these various respiratory gas treatments are explained in Series II and III below. In each of the treatments, the  $P_{E}O_2$  measurements from all the sites took about 0.5-h, and the order of the application of the different treatments was varied randomly among the fish, with 0.5-h between different gas treatments. It was not possible to test all gas treatments in all fish. As noted in “Results”, the measured  $P_{E}O_2$  from the respective gill openings typically fluctuated slightly in a rhythmic fashion over time at a much slower frequency than the velum movements, and this likely reflected ventilatory contraction of the gill pouches. The data were collected in TX3 software version 6.02 (PreSens) and the  $P_{E}O_2$  values were exported to Excel and averaged for further calculations. For example, in each hagfish, these averaged  $P_{E}O_2$  (% air saturation) values for each gill opening and the PCD were used for calculation of  $O_2\%$  utilization by Eq. 1:

$$O_2\% \text{ Utilization} = [P_1O_2(\%) - P_EO_2(\%)] / P_1O_2(\%) \times 100(\%) \quad (1)$$

The routine oxygen consumption rate ( $\dot{M}O_2$ ) was also calculated from these values using Eq. 2:

$$\dot{M}O_2 = [P_1O_2(\text{Torr}) - P_EO_2(\text{Torr})] \times \alpha_{O_2} \times \dot{V}w \quad (2)$$

As we could not determine the individual flow rates through each of the 24 gill openings and PCD, we used the average  $P_{E}O_2$  recorded from the 13 different sites on one side for each fish. The validity of this approach is discussed subsequently. This average  $P_{E}O_2$ , together with the measured  $P_1O_2$ , nostril ventilatory flow rate ( $\dot{V}w$  in  $L\text{ kg}^{-1}\text{ h}^{-1}$ ) and the appropriate  $O_2$  solubility coefficient ( $\alpha_{O_2}$ ,  $1.7747\text{ }\mu\text{mol Torr}^{-1}\text{ L}^{-1}$ ) for 30 ppt sea water at  $12^\circ$  (Boutilier et al. 1984) were used in the calculation. Prior

to  $\dot{M}O_2$  calculation,  $PO_2$  (% air saturation) values were converted into  $O_2$  tensions in Torr, taking into account the barometric pressure and the vapour pressure of water at  $12^\circ$ .

## Series II. Basic ventilatory responses to high environmental ammonia (HEA)

Each individual hagfish ( $N=9$ ) was placed in a plastic container which was filled with 2 L of normoxic sea water ( $PO_2 > 75\%$  air saturation,  $> 118\text{ Torr}$ ,  $> 15.7\text{ kPa}$ ). Animals exhibiting spontaneous apnea during the control period (see Eom and Wood 2019) were not used. After the hagfish ventilation was stable, the ventilatory parameters ( $\dot{V}w$ , fr) were measured (control) for about 5 min. Then, 40 ml of 500 mM of  $NH_4HCO_3$  (Sigma-Aldrich, St. Louis, MO, USA) was added into the air-bubbled sea water of the individual hagfish container to prepare 10 mM HEA ( $P_{NH_3}$ :  $2643\text{ }\mu\text{Torr}$  or  $0.000352\text{ kPa}$ ). The HEA treatment typically lasted 120 min. After HEA treatment, the hagfish were recovered by flushing normoxic sea water directly through the plastic container; the recovery period typically lasted 30 min. Ventilatory parameters ( $\dot{V}w$ , fr, and calculated  $S\dot{V}w$ ) were recorded throughout the HEA and recovery periods.

## Series III. Ventilatory responses to other respiratory gases and interactive effects of exposure to HEA

The same measurements of ventilatory parameters ( $\dot{V}w$ , fr, and calculated  $S\dot{V}w$ ) as in Series II were performed in individual hagfish under control normoxic conditions ( $PO_2 > 75\%$  air saturation,  $> 118\text{ Torr}$ ,  $> 15.7\text{ kPa}$ ) and then during three different experimental treatments, each on separate hagfish. Again, animals exhibiting spontaneous apnea during the control period were not used. These treatments involved exposure to alterations in environmental  $PCO_2$  or  $PO_2$  for at least 1 h to allow patterns to stabilize, followed by acute exposure to 10 mM HEA as in Series II, though it lasted only for 10 min. This was followed by a 30-min recovery period when the chamber was flushed with normoxic sea water. The goal was to understand first the basic responses to the respiratory gas treatments, and second whether the presence of these other respiratory gas treatments affected the acute response to HEA—i.e. possible interactive effects. The experimental treatments included severe hypoxia ( $\sim 8\%$  air saturation,  $PO_2 = 13.0\text{ Torr}$ ,  $1.67\text{ kPa}$ ) ( $N=4$ ), hyperoxia ( $\sim 275\%$  air saturation,  $PO_2 = 430\text{ Torr}$ ,  $57.6\text{ kPa}$ ) ( $N=5$ ), or hypercapnia (1%  $CO_2$ ,  $PCO_2 = 7.5\text{ Torr}$ ,  $1.0\text{ kPa}$ ) ( $N=7$ ). For hypercapnia, 1 part of 100%  $CO_2$  (Praxair, Delta, Canada) was mixed with 99 parts of air using a Wösthoff 301aF gas-mixing pump (Bochum, Germany) so as to generate 1%  $CO_2$ . This was directly bubbled into the hagfish container. Also, the hypoxic or hyperoxic environments were prepared

by bubbling pure N<sub>2</sub> or O<sub>2</sub> (Praxair) directly into the fish container. The decreasing or increasing oxygen concentration was continuously monitored by an O<sub>2</sub> macro-electrode and meter (YSI, Model 55, OH, USA). Similar to Series II, for acute HEA exposure, 40 ml of 500 mM NH<sub>4</sub>HCO<sub>3</sub> (Sigma-Aldrich) was added to the individual fish containers to administer 10 mM HEA in the continuing presence of the other gas treatment.

## Statistical analyses

The measured P<sub>I</sub>O<sub>2</sub>, P<sub>E</sub>O<sub>2</sub>,  $\dot{V}w$  and fr, and calculated S $\dot{V}w$ , % O<sub>2</sub> utilization, MO<sub>2</sub>, and convection requirement values have been reported as means  $\pm$  standard error of the mean (SEM) (*N*) where *N* represents the number of fish contributing to the mean. Multiple comparisons were performed by one-way ANOVA followed by Tukey's post hoc test to identify individual differences, with appropriate transformations to achieve normality and homogeneity of variances. Regression lines were compared by ANCOVA. Student's one-tailed *t* test was used to assess whether responses to individual respiratory gas stimuli were significant, where the direction of change was predicted. All tests were performed using GraphPad Prism software version 6.0 (La Jolla, CA, USA), and a threshold for statistical significance of *p* < 0.05 was used throughout.

## Results

### Series I—Measured expired O<sub>2</sub> tensions and calculated O<sub>2</sub> utilization under normoxia and in various respiratory gas treatments

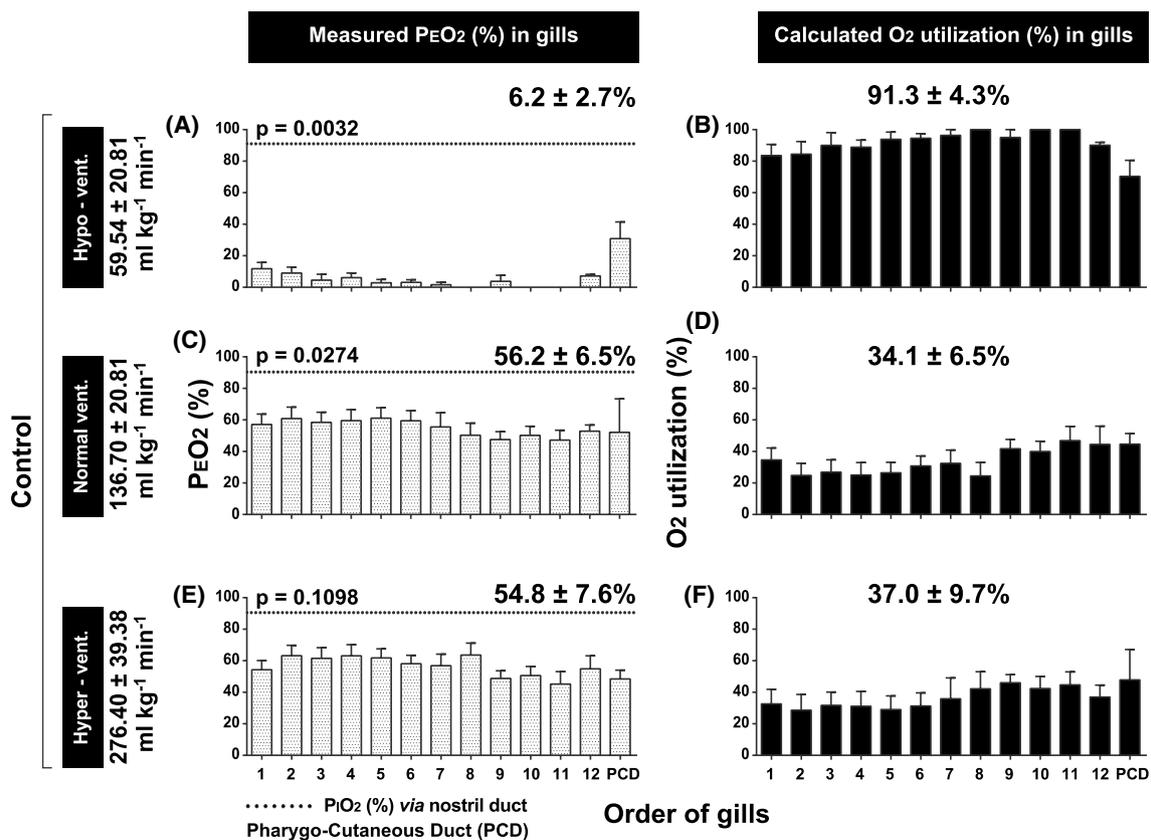
The hagfish inhaled external sea water (P<sub>I</sub>O<sub>2</sub>, 90.0  $\pm$  1.3% air saturation; 141.3  $\pm$  1.2 Torr; 18.8  $\pm$  0.2 kPa, *N* = 14) via the nostril and exhaled via the gill openings after exchanging respiratory gases in the gill pouches. P<sub>E</sub>O<sub>2</sub> in the gill openings was correlated to spontaneous ventilation changes. Figure 1b illustrates an experiment in which a hypoventilating hagfish with a  $\dot{V}w$  of  $\sim$ 5 ml kg<sup>-1</sup> min<sup>-1</sup> suddenly initiated spontaneous hyperventilation with a  $\dot{V}w$  of  $\sim$ 270 ml kg<sup>-1</sup> min<sup>-1</sup>. This caused marked changes in P<sub>E</sub>O<sub>2</sub> measured at the gill openings, and therefore in % O<sub>2</sub> utilization. Figure 1c shows an example of a P<sub>E</sub>O<sub>2</sub> recording at one particular gill pouch, displayed on a fine scale. Very small rhythmic fluctuations of P<sub>E</sub>O<sub>2</sub> are apparent, that were visually correlated to the gill pouch contraction rhythm (typically 3–6 min<sup>-1</sup>) that was much lower than the velar pumping rhythm.

As described in Methods, we classified ventilation and accompanying P<sub>E</sub>O<sub>2</sub> data collected under control normoxic conditions as either normal ventilation ( $\dot{V}w$ : 136.7  $\pm$  20.8 ml kg<sup>-1</sup> min<sup>-1</sup>,

fr: 12.2  $\pm$  1.1 min<sup>-1</sup>, S $\dot{V}w$ : 11.2  $\pm$  0.8 ml kg<sup>-1</sup>), hypoventilation ( $\dot{V}w$ : 59.5  $\pm$  1.5 ml kg<sup>-1</sup> min<sup>-1</sup>, fr: 9.3  $\pm$  2.2 min<sup>-1</sup>, S $\dot{V}w$ : 6.4  $\pm$  1.7 ml kg<sup>-1</sup>), or hyperventilation ( $\dot{V}w$ : 276.4  $\pm$  13.3 ml kg<sup>-1</sup> min<sup>-1</sup>, fr: 24.4  $\pm$  2.2 min<sup>-1</sup>, S $\dot{V}w$ : 11.3  $\pm$  3.9 ml kg<sup>-1</sup>). Thus, spontaneous changes in  $\dot{V}w$  involved changes in both fr and S $\dot{V}w$ . In general, the percentage utilization of O<sub>2</sub> was graded in an anterior-to-posterior direction, being higher when measured from water exiting the posterior pouches than from the anterior ones (Fig. 2b, d, f). This was confirmed by significance in one-way ANOVA for normal (*p* = 0.0274) and hypo-ventilation (*p* = 0.0032) but not for hyper-ventilation (*p* = 0.1098).

Note that the utilization calculation is based on comparing the measured P<sub>E</sub>O<sub>2</sub> of water exiting by each of the 13 pathways (12 gill openings, plus the opening of the PCD) with the single P<sub>I</sub>O<sub>2</sub> value of all entering water, inhaled through the only entrance, the nostril. P<sub>E</sub>O<sub>2</sub> at the PCD was similar to those at the posterior pouches, except under hypoventilation when it tended to be higher (Fig. 2a, c, e). However, when averaged over all hagfish, the differences were not particularly marked. For example, in normally ventilating hagfish, the greatest variation was from a minimum mean value of 25.0% utilization at the 4th gill opening to 46.9% at the 11th gill opening (Fig. 2b). During normal ventilation, average P<sub>E</sub>O<sub>2</sub> from exhaled water at the 12 gill openings and PCD was 56.2  $\pm$  6.5 (7) % air saturation (Fig. 2a). As a result, the fish utilized 34.1  $\pm$  6.5 (7) % of the inspired O<sub>2</sub> on an overall basis (Fig. 2B). Compared to normally ventilating hagfish, the hyperventilating hagfish exhibited similar overall O<sub>2</sub> utilization (37.0  $\pm$  7.0 (6) %, Fig. 2C) in their gill pouches, so the fish exhaled sea water that contained a similar mean O<sub>2</sub> tension (P<sub>E</sub>O<sub>2</sub>: 54.8  $\pm$  7.6 (6) % air saturation (Fig. 2d). However, these patterns were very different in spontaneously hypoventilating hagfish; the fish exhaled severely hypoxic water due to significantly increased O<sub>2</sub> utilization (O<sub>2</sub> utilization = 91.3  $\pm$  4.3 (6) %, P<sub>E</sub>O<sub>2</sub>: 6.2  $\pm$  2.7 (6) % air saturation, Fig. 2e, f).

Except for the severe hypoxia treatment (Fig. 3e, f), the other three respiratory gas treatments all resulted in similar mean O<sub>2</sub> utilizations ranging from 25.2% in hyperoxia (Fig. 3h) to 30.6% in 1% CO<sub>2</sub> hypercapnia (Fig. 3d) and 32.4% in 10 mM HEA (Fig. 3b); none of these were significantly different from the 34.1% in normally ventilating hagfish under normoxic control conditions (Fig. 2b). However, it should be noted that both mean P<sub>I</sub>O<sub>2</sub> (280% air saturation) and mean P<sub>E</sub>O<sub>2</sub> (197%) were much higher in the hyperoxic hagfish (Fig. 3g) than in the hagfish treated with 10 mM HEA (Fig. 3a) or 1% CO<sub>2</sub> hypercapnia (Fig. 3c), where mean P<sub>I</sub>O<sub>2</sub> and P<sub>E</sub>O<sub>2</sub> were about 90% and 62% air saturation, respectively. Overall, similar to the situation in normoxia (Fig. 2), posterior gill pouches



**Fig. 2** Expired O<sub>2</sub> tensions (P<sub>E</sub>O<sub>2</sub>, % air saturation) and % utilization of O<sub>2</sub> (calculated by Eq. 1) from the respective gill openings including the pharyngo-cutaneous duct (PCD) in control normoxic hagfish, which showed three different ventilation magnitudes (<75, 75–175, and >175 ml kg<sup>-1</sup> min<sup>-1</sup>, see “Methods”): **a, b** hypoventilation ( $\dot{V}_w$ : 59.5 ± 20.8 ml kg<sup>-1</sup> min<sup>-1</sup>); **c, d** normal ventilation

( $\dot{V}_w$ : 136.7 ± 20.8 ml kg<sup>-1</sup> min<sup>-1</sup>); and **e, f** hyperventilation ( $\dot{V}_w$ : 276.4 ± 39.4 ml kg<sup>-1</sup> min<sup>-1</sup>). These values are also shown on the left-hand axis. In Panels **a, c**, and **e**, the dashed line illustrates the mean P<sub>E</sub>O<sub>2</sub>. Means ± 1 SEM (N = 6–7). The significance of anterior-to-posterior differences for P<sub>E</sub>O<sub>2</sub> was confirmed by one-way ANOVA for panels **a** (p = 0.0032), and **c** (p = 0.0274), but not **e** (p = 0.1098)

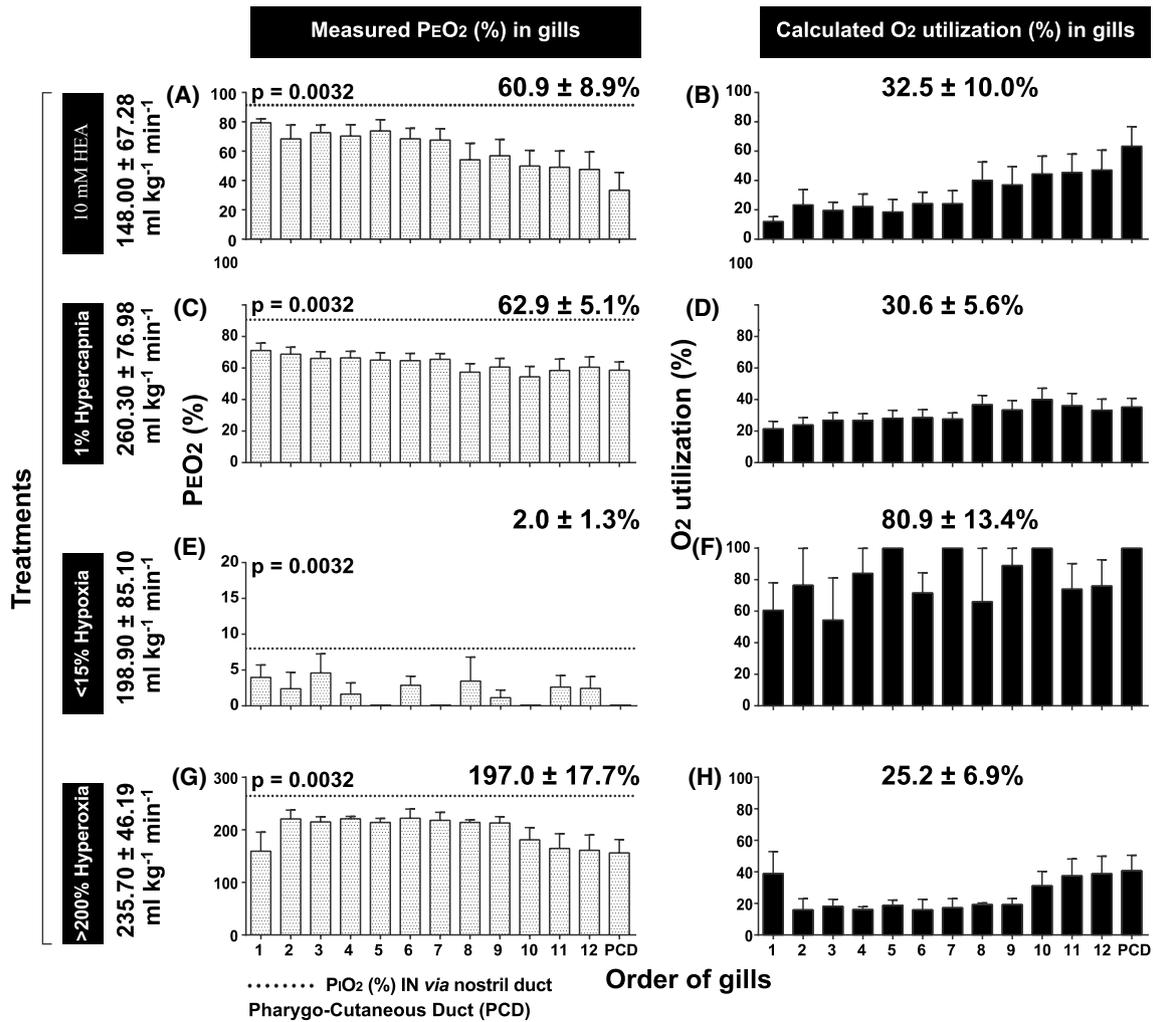
and PCD consumed more O<sub>2</sub> than anterior ones (as confirmed by one-way ANOVA (Fig. 3a: p = 0.0403, Fig. 3c: p = 0.0061, Fig. 3g: p = 0.0243), except in the hypoxia treatment (Fig. 3e: p = 0.4965), and hypoventilating hagfish utilized more O<sub>2</sub> than hyperventilating hagfish.

In light of the fairly uniform values of P<sub>E</sub>O<sub>2</sub> and O<sub>2</sub> utilization among the different gill pouches and PCD seen within the various treatments, we proceeded to calculate routine  $\dot{M}O_2$  by Eq. 2 using the P<sub>I</sub>O<sub>2</sub>, mean  $\dot{V}_w$ , and overall mean P<sub>E</sub>O<sub>2</sub> values (from the 13 measurement sites) for each fish under each condition. The spontaneous differences in ventilation seen among normally ventilating, hypoventilating, and hyperventilating hagfish under control normoxic conditions resulted in significant alterations in  $\dot{M}O_2$  (Fig. 4a). Compared to the  $\dot{M}O_2$  during normal ventilation (718.0 μmol kg<sup>-1</sup> h<sup>-1</sup>),  $\dot{M}O_2$  was decreased by 71% in hypoventilating hagfish but increased by 87% in hyperventilating animals (Fig. 4a). The 10 mM HEA treatment resulted in a 64% reduction in  $\dot{M}O_2$ , and severe hypoxia caused a significant 85% fall, whereas hyperoxia resulted in

a significant 204% elevation in  $\dot{M}O_2$  (Fig. 4b). Hypercapnia had no significant effect on routine  $\dot{M}O_2$  (Fig. 4b).

### Series II—Ventilation changes in 10 mM high environmental ammonia (HEA)

In exposures to 10 mM HEA alone, hagfish initially decreased  $\dot{V}_w$  by 33% (p = 0.0201) (Fig. 5b). This decrease in  $\dot{V}_w$  was achieved by a significant decrease in fr (Fig. 5a, p = 0.0361), while  $S\dot{V}_w$  did not change (Fig. 5c) This effect persisted through 10 min of HEA exposure. However, when HEA was removed by the addition of fresh seawater, overall ventilatory parameters quickly returned to the original control levels (Fig. 5, p = 0.0418). The HEA exposures were typically maintained for about 2 h, but the responses during this longer time period were very variable. Some hagfish either maintained or intensified the ventilatory inhibition, whereas in others ventilation gradually rose back to or above controls with differing time courses. This is in accord with the pattern reported by Eom et al. (2019) where HEA acutely



**Fig. 3** Expired oxygen tension ( $P_{E}O_2$ ) and % utilization of  $O_2$  (calculated by Eq. 1) from the respective gill openings including pharyngo-cutaneous duct (PCD) of hagfish treated with **a**, **b** high environmental ammonia (10 mM HEA), **c**, **d** hypercapnia (1%  $CO_2$ ), **e**, **f** severe hypoxia < 15% air saturation, and **g**, **h** hyperoxia > 200% air saturation. The  $\dot{V}w$  (means  $\pm$  1 SEM) during the measurements is shown on

the left-hand axis. In Panels A, C, E, and G, the dashed line illustrates the mean  $P_1O_2$ . Means  $\pm$  SEM  $N=5-8$ . The significance of anterior-to-posterior differences for  $P_{E}O_2$  was confirmed by one-way ANOVA for panels A ( $p=0.0403$ ), C ( $p=0.0061$ ), and G ( $p=0.0243$ ), but not for E ( $p=0.4965$ )

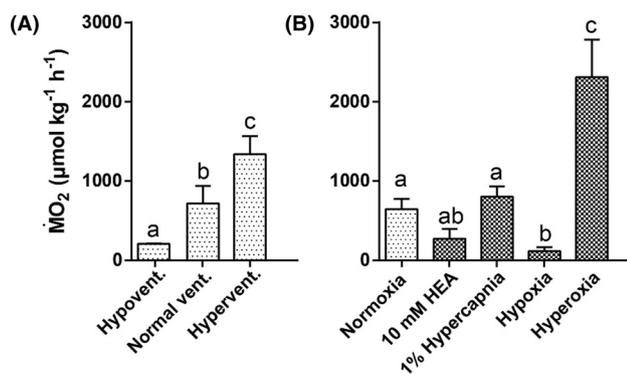
inhibits ventilation, and then ventilation later recovers and eventually increases above the control level as HEA is maintained up to 3 h. For this reason, in the subsequent tests with the other respiratory gases, the 10 mM HEA exposure period was limited to 10 min (with measurements at 2 min and 10 min), so as to evaluate only whether the acute inhibitory effect of 10 mM HEA on ventilation was affected by the simultaneous presence of the other respiratory gas treatment.

### Series III—Ventilation changes in 1% hypercapnia, hypoxia, hyperoxia, and 10 mM HEA

Exposure to hypercapnia alone had minimal overall effects. In general, ventilatory responses to 1%  $CO_2$  were variable,

with some fish increasing and some decreasing  $\dot{V}w$ ; none of the changes in this series were significant (Fig. 6). After the addition of 10 mM HEA in the continuing presence of 1%  $CO_2$ , the hagfish showed a pattern of decreased  $\dot{V}w$  due to lowered  $S\dot{V}w$  that was maintained for 10 min. These decreased ventilatory parameters gradually recovered after the addition of fresh seawater. Average frequency levels (fr) were barely changed regardless of the different treatments (Fig. 6).

During the severe hypoxia treatment (mean  $P_1O_2 \sim 8\%$  air saturation, 1.67 kPa, 13 Torr), the hagfish increased  $\dot{V}w$  by 122% ( $p=0.0272$ ) but the increased flow was greatly decreased below the control level ( $p=0.0058$ ) when acute



**Fig. 4** Routine  $\dot{M}O_2$  calculated by Eq. 2 using the  $P_{I}O_2$ , mean  $\dot{V}w$ , and overall mean  $P_{E}O_2$  values (from the 13 measurement sites) for each fish under each experimental condition. **a** In control normoxic conditions, with data classified according to whether they were recorded during normal ventilation, hypoventilation, or hyperventilation (<75, 75–175, and >175 ml kg<sup>-1</sup> min<sup>-1</sup>, see “Methods”). **b** In various respiratory gas treatments—control normoxia, high environmental ammonia (10 mM HEA), hypercapnia (1% CO<sub>2</sub>), severe hypoxia <15% air saturation, and hyperoxia >200% air saturation. Within a panel, means not sharing the same letter are significantly different from one another. Means  $\pm$  SEM ( $N=9$ )

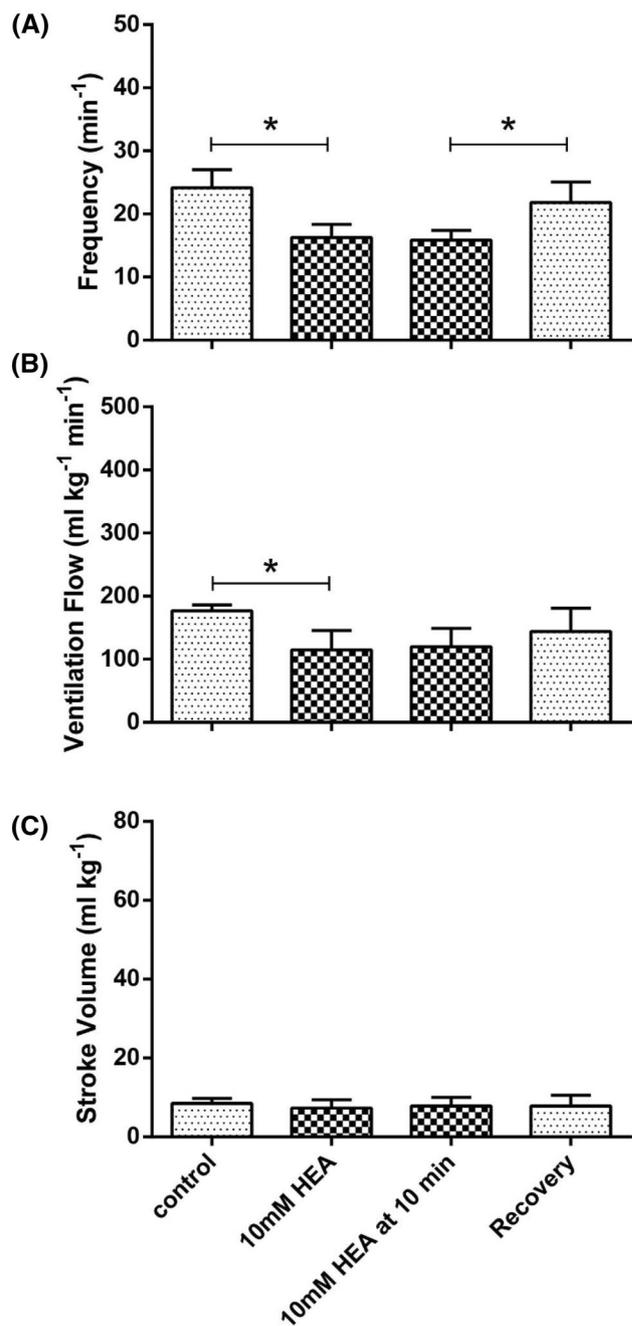
10 mM HEA was added in the continuing presence of hypoxia. However, thereafter,  $\dot{V}w$  tended to increase again so this hypoventilatory effect did not persist for 10 min, and there was complete recovery back to control levels after the addition of fresh seawater (Fig. 7b). The pattern of changes in  $f_r$  paralleled that of  $\dot{V}w$ . The initially increased  $f_r$  accompanying hypoxia was decreased to 76% of control levels by HEA ( $p=0.0360$ ) and lasted for 10 min (Fig. 7a). Changes in  $S\dot{V}w$  were variable and non-significant (Fig. 7c).

During the hyperoxia treatment (mean  $P_{I}O_2 \sim 275\%$  air saturation, 57.6 kPa, 430 Torr), hagfish barely changed overall ventilatory parameters but when HEA was added in the continuing presence of hyperoxia, the parameters of  $f_r$  and  $\dot{V}w$  were immediately decreased by 40% (Fig. 8a,  $p=0.0483$ ) and 55% (Fig. 8b,  $p=0.0313$ ), respectively. A slight decrease in  $S\dot{V}w$  was not significant (Fig. 8c). After 10 min in 10 mM HEA plus hyperoxia, the suppressed ventilatory parameters recovered toward control levels so there was little further change upon the addition of fresh seawater.

## Discussion

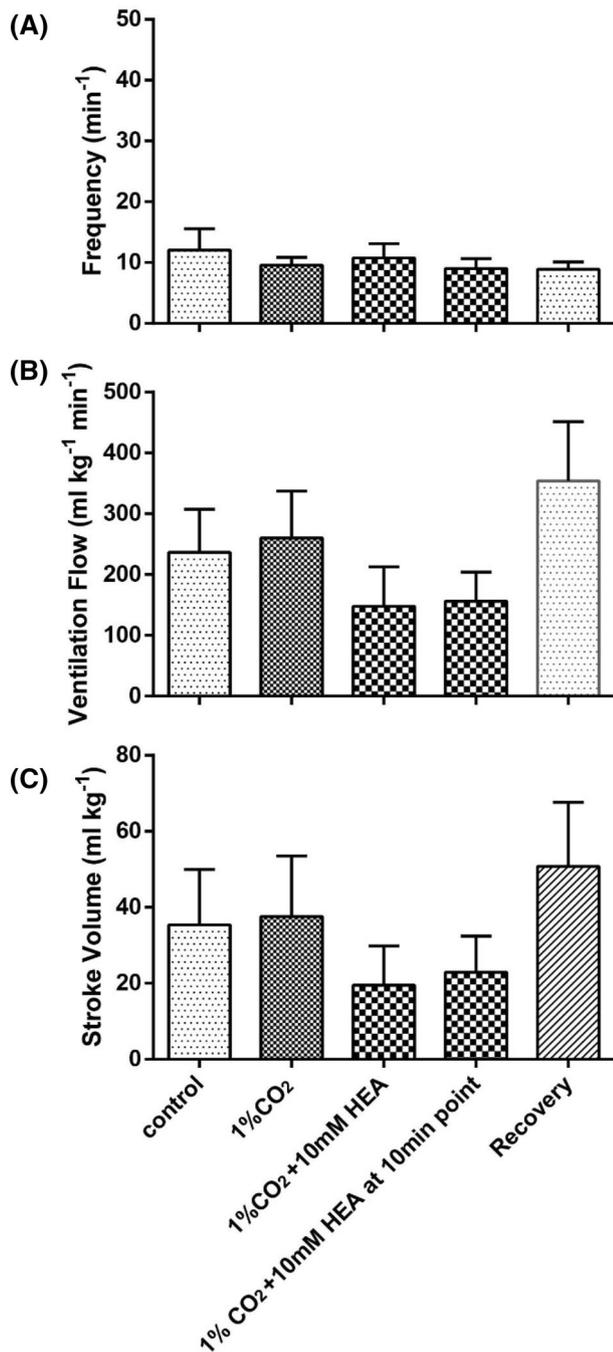
### Overview

An important overall conclusion, based on this and our two preceding studies (Eom et al. 2019; Eom and Wood 2019) is that when hagfish are non-stressed and allowed to exhibit normal behaviour, their respiratory physiology is very variable.  $\dot{V}w$  can range from 0 ml kg<sup>-1</sup> min<sup>-1</sup> (during



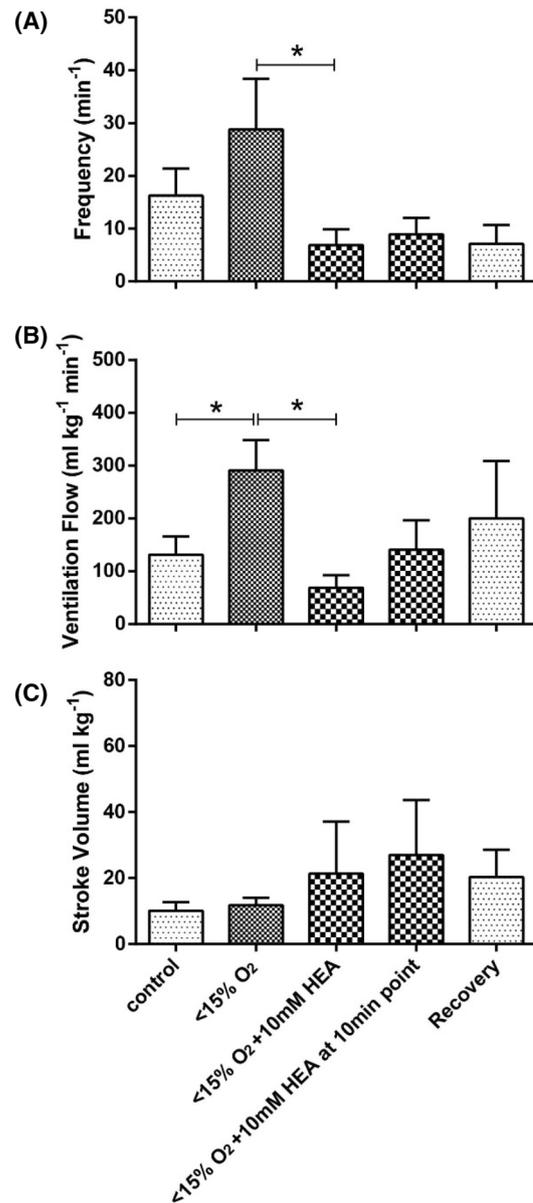
**Fig. 5** Changes in ventilatory parameters **a** frequency ( $f_r$ ), **b** total ventilatory flow ( $\dot{V}w$ ), and **c** ventilatory stroke volume ( $S\dot{V}w$ ) in hagfish acutely exposed to 10 mM high environmental ammonia (HEA) in normoxia. The chamber was flushed with fresh normoxic seawater for recovery. Means  $\pm$  SEM ( $N=9$ ). \*Signifies significant change from the preceding condition ( $P < 0.05$ )

periods of prolonged apnea) to >400 ml kg<sup>-1</sup> min<sup>-1</sup> (during extreme spontaneous hyperventilation). Our impression is that much of this variability disappears when the animal becomes stressed, accompanied by high continuous  $\dot{V}w$ . The responses of resting hagfish to standardized respiratory gas



**Fig. 6** Changes in ventilatory parameters **a** frequency (fr), **b** total ventilatory flow ( $\dot{V}_w$ ), and **c** ventilatory stroke volume ( $S\dot{V}_w$ ) in hagfish exposed to hypercapnia (1% CO<sub>2</sub>) and then subsequently acutely exposed to 10 mM high environmental ammonia (HEA) in the continuing presence of hypercapnia. The chamber was flushed with fresh normoxic seawater for recovery. Means  $\pm$  SEM ( $N=7$ ). None of the changes were statistically significant (i.e.  $P > 0.05$ )

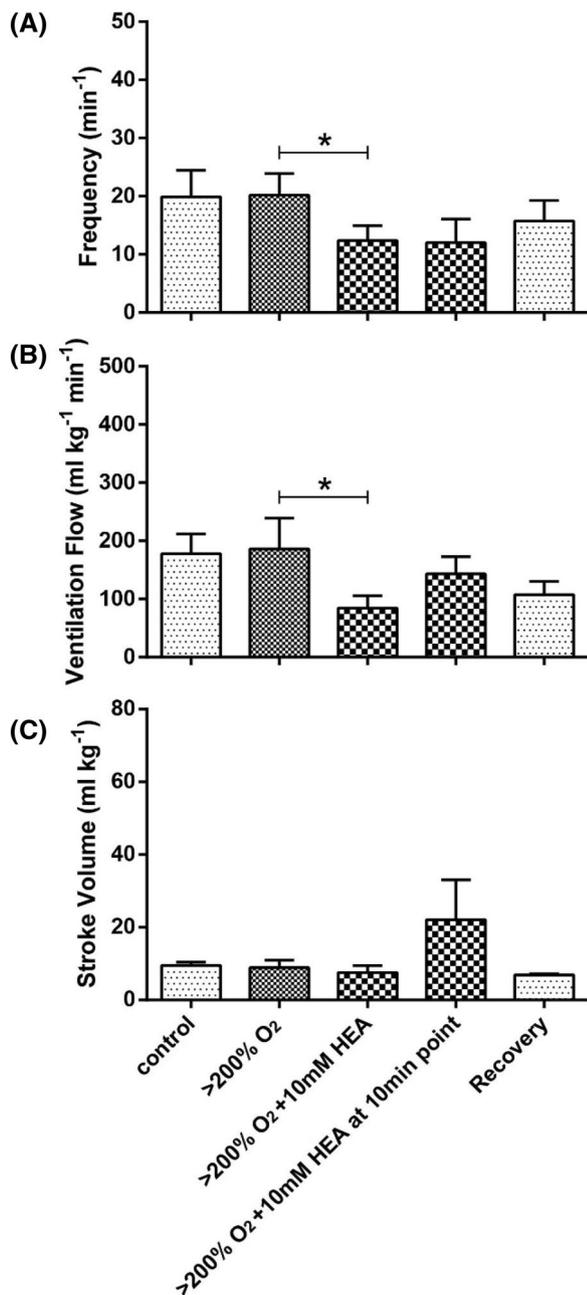
stimuli are also highly variable, ranging from no or small responses to very large responses on variable time scales, and sometimes opposite responses in different animals, as



**Fig. 7** Changes in ventilatory parameters **a** frequency (fr), **b** total ventilatory flow ( $\dot{V}_w$ ), and **c** ventilatory stroke volume ( $S\dot{V}_w$ ) in hagfish exposed to severe hypoxia ( $PO_2 < 15\%$  air saturation) and then subsequently acutely exposed to 10 mM high environmental ammonia (HEA) in the continuing presence of hypoxia. The chamber was flushed with fresh normoxic seawater for recovery. Means  $\pm$  SEM ( $N=4$ ). \*Signifies significant change from the preceding condition ( $P < 0.05$ )

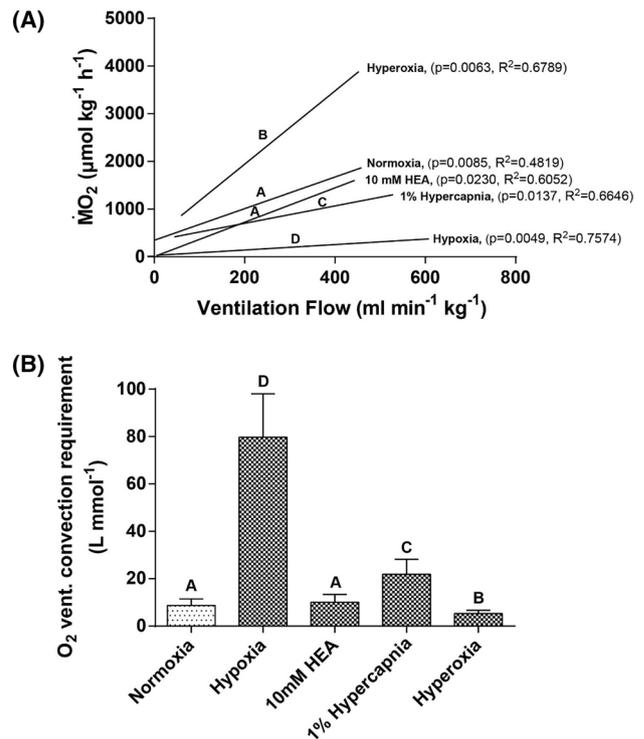
seen with the 1% CO<sub>2</sub> treatment. In the present study, our focus was on unstressed animals, so it is unclear how highly stressed animals respond to respiratory gas stimuli.

With respect to our original goals, we found a general trend, moving from anterior to posterior gill pouches, for  $P_{E}O_2$  to decrease, and therefore, % utilization to increase, supporting the idea that some water may be recycled in the



**Fig. 8** Changes in ventilatory parameters **a** frequency (fr), **b** total ventilatory flow ( $\dot{V}_w$ ), and **c** ventilatory stroke volume ( $S\dot{V}_w$ ) in hagfish exposed to hyperoxia ( $PO_2 > 200\%$  air saturation) and then subsequently acutely exposed to 10 mM high environmental ammonia (HEA) in the continuing presence of hyperoxia. The chamber was flushed with fresh normoxic seawater for recovery. Means  $\pm$  SEM ( $N=5$ ). \*Signifies significant change from the preceding condition ( $P < 0.05$ )

pharynx as it moves from front-to-back before being exhaled (Figs. 1b, c, 2, 3). However, as  $P_{E}O_2$  was also low at the PCD, we did not confirm our idea that % utilization would be low at this bypass shunt. We also confirmed the well-known inverse relationship between  $\dot{V}_w$  and % utilization seen in



**Fig. 9 a** Regressions between routine oxygen consumption rate ( $\dot{M}O_2$ , Y axis) and total ventilatory flow ( $\dot{V}_w$ , X axis) in hagfish under different respiratory gas treatments. The  $R^2$  and  $P$  values are shown. The equations are: Normoxia:  $\dot{M}O_2 = 3.315 \dot{V}_w + 337.4$  ( $N = 13$ ); 10 mM HEA:  $\dot{M}O_2 = 3.602 \dot{V}_w - 4.26$  ( $N = 8$ ); 1%  $CO_2$ ; Hypercapnia:  $\dot{M}O_2 = 1.825 \dot{V}_w + 329.23$  ( $N = 8$ ); Hypoxia:  $\dot{M}O_2 = 0.570 \dot{V}_w + 22.15$  ( $N = 8$ ); Hyperoxia:  $\dot{M}O_2 = 7.683 \dot{V}_w + 391.9$  ( $N = 9$ ). By ANCOVA, none of the intercepts are significantly different from one another, whereas letters indicate significant differences in slope ( $P < 0.05$ ). **b** The convection requirement for O<sub>2</sub>, (L mmol<sup>-1</sup>) calculated from the slopes of regressions between total ventilatory flow ( $\dot{V}_w$ , Y axis) and routine oxygen consumption rate ( $\dot{M}O_2$ , X axis) in the same hagfish under different respiratory gas treatments. Means  $\pm$  SEM ( $N$ ), where  $N$  is the same as in panel (a). Letters indicate significant differences ( $P < 0.05$ )

teleost fish. However, we were surprised that % utilization could exceed 90% in spontaneously hypoventilating hagfish under normoxia (Fig. 2b), and 80% in hyperventilating hagfish under severe hypoxia (Fig. 3f), and also that utilization could be maintained at a constant level between normal ventilation and hyperventilation under normoxia (Fig. 2d, f). Very clearly,  $\dot{M}O_2$  depends on  $\dot{V}_w$  (Figs. 4, 9). In accord with previous studies, we found that acute exposure to HEA inhibited  $\dot{V}_w$  (Fig. 5), that acute hypoxia stimulated  $\dot{V}_w$  (Fig. 7), and confirmed the unusual lack of a hypoventilatory response to hyperoxia (Fig. 8). However, there was no overall effect of hypercapnia on  $\dot{V}_w$  (Fig. 6), in contrast to a previous report (Perry et al. 2009a, b). By exploiting the wide range of individual  $\dot{V}_w$  versus  $\dot{M}O_2$  measurements, we uncovered effects of respiratory gas treatments superimposed on the effects of

$\dot{V}w$  alone (Fig. 9). Finally, in accord with our original prediction, the acute inhibitory influence of HEA persisted in the presence of hypoxia (Fig. 7) and hyperoxia (Fig. 8), showing its importance as a protective measure to minimize the uptake of toxic waterborne ammonia. However, the response was blunted during hypercapnia (Fig. 6) likely due to a water chemistry effect, as explained below. We conclude that the hagfish possesses a flexible respiratory system with considerable reserve capacity.

### Ventilation, utilization, and $\dot{M}O_2$ under normoxia

In general,  $\dot{M}O_2$  in resting hagfish was several-fold lower than in teleosts (Clarke and Johnston 1999) and somewhat lower than in elasmobranchs (Carlson et al. 2004). Our resting  $\dot{M}O_2$  measurements ( $\sim 700 \mu\text{mol kg}^{-1} \text{h}^{-1}$ ) obtained by direct measurement of  $\dot{V}w$  and  $P_{\text{E}O_2}$ , and using an average of all 13 measurements of  $P_{\text{E}O_2}$  at the different exits, would presumably have missed the small fraction ( $< 20\%$ ) of  $\dot{M}O_2$  thought to occur across the skin in this species (Clifford et al. 2016). Nevertheless, this resting  $\dot{M}O_2$  agreed well with some previous measurements obtained by classic closed system respirometry of the whole animal (Weinrauch et al. 2018; Eom et al. 2019; Giacomini et al. 2019a, b), but was 1.5–twofold higher than in three other respirometry studies at comparable temperature (Muniz and Morris 1965; Perry et al. 2009b; Giacomini et al. 2019b). From Fig. 4b, it is clear that the extent of spontaneous hypoventilation or hyper-ventilation can have a large effect on resting  $\dot{M}O_2$ , as can periods of spontaneous apnea (Eom and Wood 2019). In addition to flow effects on gas exchange in the gill pouches, it is possible that differences in  $\dot{M}O_2$  associated with the differences in ventilation are also being influenced by regional  $O_2$  consumption along the pharynx that may vary with increasing or decreasing ventilation as transit times are altered. Steffensen et al. (1984) reported much higher  $\dot{M}O_2$  in non-buried *Myxine glutinosa*, approximately equal to that in hyperventilating *E. stoutii* in our experiments (Fig. 4b). Unlike *E. stoutii* which have a broad range of surface substrate types in the benthic deep-sea environment (McInerney and Evans 1970), *M. glutinosa* are known to bury themselves in patches of mud between rocks (Tambs-Lyche 1969) and, therefore, may have been stressed by the lack of immersion.

Spontaneous changes in  $\dot{V}w$  at rest (Fig. 2b) involved changes in both  $fr$  and  $S\dot{V}w$ , in accord with our previous investigations (Eom and Wood 2019; Eom et al. 2019). However, changes in  $\dot{V}w$  in response to respiratory gas stimuli involved mainly changes in  $fr$  (Figs. 5, 6, 7, 8), in agreement with previous studies that reported that only velar frequency ( $fr$ ) changed (Perry et al. 2009b; Coxon and Davison 2011). Under resting conditions in normoxia, “normal”  $\dot{V}w$  was about  $137 \text{ ml kg}^{-1} \text{ min}^{-1}$

(Fig. 2) which agrees well with our previous study ( $125 \text{ ml kg}^{-1} \text{ min}^{-1}$ ; Eom and Wood 2019) but is lower than the  $235 \text{ ml kg}^{-1} \text{ min}^{-1}$  reported by Perry et al. (2009b) for *E. stoutii* at comparable temperature. In *Myxine glutinosa*, buried in sand,  $\dot{V}w$  was  $45 \text{ ml kg}^{-1} \text{ min}^{-1}$  at  $15^\circ\text{C}$ , but this increased to  $140 \text{ ml kg}^{-1} \text{ min}^{-1}$  when the animals were temperature-stressed at  $20^\circ\text{C}$  (Steffensen et al. 1984). Taking differences in mass and temperature into account, the present value for *E. stoutii* appears to be on the low end of  $\dot{V}w$  values recorded in teleosts with similar benthic, inactive lifestyles—e.g. tench (*Tinca tinca*; Eddy 1974), carp (*Cyprinus carpio* Lomholt and Johansen 1979;), and flounder (*Platichthys stellatus*; Wood et al. 1979), that have very different ventilatory mechanisms and gill structure. This, coupled with generally lower % utilization ( $\sim 35\%$ ; Fig. 2) versus higher values in these teleosts (55–80%) explains the lower  $\dot{M}O_2$  in the hagfish. However,  $\dot{V}w$ , % utilization, and  $\dot{M}O_2$  in the big skate (*Raja ocellata*; Graham et al. 1990), an elasmobranch that also has a very different respiratory system from the hagfish, were all very similar to values in *E. stoutii*.

The utilization calculations quantify the  $O_2$  extraction of each of the 13 pathways, comparing the measured  $P_{\text{E}O_2}$  of the exiting water of each to the common single  $P_{\text{I}O_2}$  value of the water inhaled through the only entrance, the nostril. While the general organization of the 13 pathways is in parallel, not in series, the general trend for increasing % utilization from anterior to posterior gill pouches (Figs. 2, 3) would be in accord with some recycling of water as it flowed posteriorly via the pharynx. Alternate or additional explanations could simply be that the posterior gills are more efficient at gas exchange, and/or that this reflects the cumulative removal of  $O_2$  by the bordering tissues of the pharynx as the water passes posteriorly along the respiratory tract. Nevertheless, it is easy to speculate that the inhaled water moves very slowly through the pouches or may even be briefly stored in the pouch structure for respiratory gas exchange. The afferent and efferent branchial ducts are guarded by sphincters (Malte and Lomholt 1998). The gill pouches contract at a low rhythm (Fig. 1D; Eom and Wood 2019) and in a peristaltic anterior-to-posterior manner as described in *Myxine glutinosa* (Johansen and Hol 1960; Johansen and Strahan 1963), which was visually observed in the present study. While this aids exhalation through the efferent branchial ducts, perhaps the sphincters allow some of the water to instead be forced back into the pharynx through the afferent branchial ducts, and therefore, rebreathed (“recycled”) by more posterior gill pouches. The generally high % utilization at the PCD exit suggests that the PCD does not act as a by-pass shunt under resting conditions, but rather serves to collect some of this sequentially rebreathed water. It could still assume a bypass shunt function when sediment or food particles need to be cleared.

The ability of *E. stoutii* to maintain % utilization unchanged (~35%) between “normal” and spontaneous hyperventilation at rest, and to increase it above 90% during spontaneous hypoventilation (Fig. 2b, d, f) indicates a respiratory system with considerable reserve capacity. In addition to the complex gill structure and moderate blood-to-water diffusion distance (Mallatt and Paulsen 1986), other key adaptations may be the counter-current flow of blood and water (Bartels 1998; Johansen and Strahan 1963; Malte and Lomholt 1998), an ability to vary blood flow distribution by opening or closing arterio-venous pathways in the gills (Bartels 1998), and a blood O<sub>2</sub>-dissociation curve with a fairly high O<sub>2</sub> affinity, though to our knowledge, this has only been measured in the related *Eptatretus cirrhatus* (P50~12 Torr; Wells et al. 1986). In addition, hypoventilating hagfish may store inhaled water in their gill pouches much longer, thereby decreasing physiological dead-space and greatly increasing oxygen utilization (Hofbauer 1934).

### Ventilation, utilization, and MO<sub>2</sub> under respiratory gas treatments

The hagfish are mostly scavengers, browsing on carrion-dead falls (Smith 1985). They penetrate into cavities via soft tissues or opercular regions, and/or rasp or engulf pieces of carcass (Eom and Wood 2019). In this feeding environment, the hagfish would likely experience a mixture of respiratory gas stimuli, such as depleted oxygen (hypoxia), and highly increased carbon dioxide (hypercapnia) and ammonia (HEA) emanating from the dead items. The short-term response to HEA is hypoventilation and/or apnea occurring over a time scale of minutes (Eom et al. 2019), which is the response studied here (Figs. 5, 6, 7, 8). This short-term decrease in breathing would likely be an ideal defensive mechanism to reduce inhalation of noxious water, and thereby minimize ammonia uptake across the gills, for the amount of time the hagfish would spend either immersed in or close to a rotting carcass while feeding in such an HEA environment. From our personal observations on hagfish feeding in captivity (Eom and Wood unpublished), this species does not often spend long periods inside dead prey, but rather makes short forays, dipping its head into or onto the surface of the prey, rasping off pieces that it ingests by engulfment. After ingesting at most 20 g of food, the hagfish (~100 g BW) stops feeding and leaves the prey, then coils on the bottom of the tank where it stays digesting, more or less motionless, for several days. Therefore, we believe that bouts of fairly short-term acute exposures may be more relevant to the day-to-day life of the species. Not surprisingly, as % utilization was unaltered (Fig. 3b), MO<sub>2</sub> also tended to fall (Fig. 4b) in accord with the fall in  $\dot{V}_w$  (Fig. 5). Interestingly, the anterior-to-posterior gradient in % utilization among the gill pouches

became particularly marked during HEA exposure, suggesting some re-organization of water flow (Fig. 3b). The acute reduction in  $\dot{V}_w$  is thought to be initiated by external receptors as a defensive response (Eom et al. 2019). While these could be chemoreceptors responsive to other respiratory gases, it is more likely that they are the well-developed olfactory rosettes (Theisen 1976; Holmes et al. 2011) or the taste-bud-like receptors of the Schreiner organs in the epidermis of the head, trunk and along the respiratory tracts (Braun 1998; Braun and Northcutt 1998). We predicted that as a defensive response, the inhibition of  $\dot{V}_w$  by HEA would persist in the presence of other respiratory stimuli, and this indeed was observed, though its duration may have been shortened by hypoxia (Fig. 7) and hyperoxia (Fig. 8), with these drives resuming precedence over time. The response was not significant in hypercapnia (Fig. 6) where it may have been blunted for physico-chemical reasons. Because of the acidifying effect of CO<sub>2</sub>, the water pH of the 10 mM HEA exposure in the presence of 1% CO<sub>2</sub> was approximately 7.95 and this would have lowered the P<sub>NH<sub>3</sub></sub> from 5948 μTorr (0.000793 kPa) in the normoxic situation (pH=8.3) to 2643 μTorr (0.000352 kPa) in the hypercapnic treatment.

Over the longer term, ammonia may build up internally after feeding, taken up from the HEA environment and/or produced metabolically by the exercise accompanying feeding and processing of the protein-rich meal (Wilkie et al. 2017). As in teleosts (Zhang et al. 2011) and elasmobranchs (De Boeck et al. 2015), the long-term response in *E. stoutii* is hyperventilation occurring over a time scale of hours (Eom et al. 2019); in both hagfish and these other fish, this response can be elicited by ammonia injection and appears to be mediated by internal chemoreceptors. At least in teleosts, these ammonia-sensitive chemoreceptors appear to be the neuro-epithelial cells (NECs) in the gills which are likely polymodal receptors responsive to O<sub>2</sub>, CO<sub>2</sub>, ammonia and other stimuli (Perry and Tzaneva 2016; Jonz 2018), though the brain may also be involved (Zhang et al. 2013). To our knowledge, NECs have not been described in hagfish, but the cyanide injection experiments of Perry et al. (2009b) suggest that either they, or functional analogues, must be present. At least in teleosts, ammonia excretion is sensitive to convective water flow when branchial Rh proteins are activated by elevated plasma ammonia (Eom et al. 2020), so long-term hyperventilation would serve to flush out the excess ammonia via the Rh proteins (Braun and Perry 2010; Edwards et al. 2015; Clifford et al. 2017). By increasing  $\dot{V}_w$ , it would also facilitate greater O<sub>2</sub> uptake (Fig. 4a) and CO<sub>2</sub> excretion during the post-feeding period of high metabolic demand (“Specific Dynamic Action”; Weinrauch et al. 2018).

The mean level of acute hypoxia in our experiments was a P<sub>1</sub>O<sub>2</sub> of about 8% air saturation (1.67 kPa, 13 Torr; Fig. 3e); hagfish responded by increasing  $\dot{V}_w$  by 122%

(Fig. 7b), but appeared to remain very calm, keeping their normal coiled posture. Despite a marked increase in % utilization (Fig. 3f),  $\dot{M}O_2$  fell by 85% (Fig. 4a). The hyperventilatory response was qualitatively similar to that reported in *E. stoutii* by Perry et al. (2009b) using a similar level of hypoxia and similar measurement methods. Exact comparison is difficult, as Perry et al. (2009b) normalized their data to  $\Delta\dot{V}_w$  above baseline, but the present response would appear much smaller. These same authors, using a more moderate level of hypoxia (33% air saturation) in separate closed-system respirometry experiments, reported that  $\dot{M}O_2$  was maintained constant, but Giacomini et al. (2019a), using an intermediate level of hypoxia (20% air saturation), reported a significant rise in the ventilatory index (a proxy for  $\dot{V}_w$ ) and a 49% fall in  $\dot{M}O_2$  by closed-system respirometry. Overall, these data are congruent with recent findings that under identical conditions, the  $P_{crit}$  of *E. stoutii*, where  $\dot{M}O_2$  becomes dependent upon  $P_{IO_2}$  is about 11.2% air saturation (2.33 kPa, 17.5 Torr; A. Clifford and C.M. Wood, unpublished data), and the report of Drazen et al. (2011) that it is about 6.8% air saturation (1.43 kPa, 11 Torr) at a lower temperature (5 °C). Therefore, the differences in the  $\dot{M}O_2$  responses among Perry et al. (2009a, b), Giacomini et al. (2019a), and the present study (Fig. 4a) reflect the differences in the levels of hypoxia employed.

Note that the hagfish in all these studies were acclimated to normoxia, and this may not be representative of the deep-sea benthic environment in which these animals normally live, as noted by Drazen et al. (2011). Indeed, the hagfish used in the present experiments were captured at depth of ~100 m (10.4 °C) in Trevor channel close to Bamfield Marine Science Centre. The mean  $PO_2$  measured in the water next to the traps was 14.5% air saturation (23 Torr, 3.0 kPa) (CellOx325, WTW GmbH & Co., Weilheim, Germany) (personal communication, Ora Johannsson).

The mean level of acute hyperoxia in our experiments was a  $P_{IO_2}$  of about 275% air saturation, (57.6 kPa, 430 Torr). Contrary to the pattern of hypoventilation seen in most other fish (e.g. Wood and Jackson 1980; Heisler et al. 1988),  $\dot{V}_w$  did not change during hyperoxia in the hagfish (Fig. 8), in agreement with Giacomini et al. (2019b) who reported a non-significant rise in the ventilatory index, and a significant increase in fr. Indeed, we noticed that the hagfish became restless during hyperoxia exposure and often uncoiled, as though it were an irritant, while Giacomini et al. (2019b) reported associated disturbances in ion and acid–base status. With unchanged mean ventilatory flow and unchanged % utilization despite the greatly increased  $P_{IO_2}$  (Fig. 3f),  $\dot{M}O_2$  was elevated threefold in the present study (Fig. 4b). Again, all these unusual responses may reflect the fact that *E. stoutii* normally lives in a very hypoxic benthic environment and

rarely encounter high  $O_2$  levels. However, live hagfish are commonly sold in Asian wet markets (Honma 1998) where they are held in oxygen-bubbled seawater; this may be a mistake.

Environmental hypercapnia (1%  $CO_2$ , 1.0 kPa, 7.5 Torr) had no consistent effect on ventilation, such that  $\dot{V}_w$ , fr,  $S\dot{V}_w$  (Fig. 6) and  $\dot{M}O_2$  (Fig. 4b) all remained statistically unchanged. However, this disguises great variability in responses with some animals hyperventilating, interspersed with short periods of apnea, while others tended to reduce  $\dot{V}_w$ , so that when averaged, there were no changes. This contrasts with the report of Perry et al. (2009b) where exposure to 1%  $CO_2$  elicited a consistent increase in  $\dot{V}_w$  in *E. stoutii*, though the response appeared to be smaller than that elicited by hypoxia. One experimental difference of our study from that of Perry et al. (2009a, b) is that we randomized the order of the various respiratory gas treatments. It is possible that this contributed to the different results. Our results also contrast with the responses of most other fish, where high external  $PCO_2$  elicits hyperventilation (Gilmour 2001). Note, however, that the hagfish were acclimated to normocapnia; it would be interesting to measure the  $PCO_2$  in their hypoxic benthic environment. *E. stoutii* is known to be very tolerant of high  $PCO_2$  (Baker et al. 2015; Clifford et al. 2018).

### The relationship between $\dot{M}O_2$ and ventilation under different respiratory gas treatments

Given the ability of *E. stoutii* to maintain % utilization unchanged during spontaneous hyperventilation under normoxia, it is not surprising that  $\dot{M}O_2$  increased with  $\dot{V}_w$  (Fig. 4a). However, by just looking at the means (Figs. 4b, 5, 6, 7, and 8) from different experimental series, it is difficult to see how the relationship changed under the different respiratory gas treatments. Therefore, in Fig. 9, we have exploited the wide range of simultaneous individual  $\dot{V}_w$  versus  $\dot{M}O_2$  measurement pairs made in Series 1 and 2, to examine possible effects of the respiratory gas treatments superimposed on the effects of changing  $\dot{V}_w$  alone.

When  $\dot{M}O_2$  ( $Y$ -axis) was regressed against  $\dot{V}_w$  ( $X$ -axis), there were significant positive linear relationships in all of the respiratory gas treatments, and none of the intercepts at  $\dot{V}_w = 0$  were significantly different from each other or from zero (Fig. 9a). However, the slope of the hyperoxia relationship was about 2.3-fold greater than that of the normoxia relationship, whereas the slope of the hypoxia relationship was only about 17% of that of the normoxia relationship; both differences were significant. This analysis clearly demonstrated the effects of the elevated  $P_{IO_2}$  in hyperoxia and reduced  $P_{IO_2}$  in hypoxia (and comparably affected driving  $PO_2$  gradients) on  $\dot{M}O_2$ , separate from

the effects of differences in  $\dot{V}_w$  (Figs. 7, 8). Furthermore, although 10 M HEA inhibited  $\dot{V}_w$  (Fig. 5), the analysis also revealed that it did not affect the efficiency of  $\dot{M}O_2$  per unit flow, as the slope was not significantly different from that of the normoxic control. A simple interpretation is that HEA did not affect the ability of the gill to transfer  $O_2$  or the ability of the blood to take it up. This contrasts with the 1%  $CO_2$  hypercapnia treatment, where the slope was significantly depressed by about 45% relative to that of the normoxic control. Thus the efficiency of  $\dot{M}O_2$  per unit flow was reduced. One possible explanation would be decreased  $O_2$  affinity of the blood due to the Bohr effect, which has been observed in the whole blood of the congeneric *E. cirrhatus* (Wells et al. 1986).

Using mean values of  $\dot{V}_w$  ( $137 \text{ ml kg}^{-1} \text{ min}^{-1}$ ) and  $\dot{M}O_2$  ( $718 \text{ } \mu\text{mol kg}^{-1} \text{ h}^{-1}$ ) for hagfish exhibiting “normal ventilation” under normoxic control conditions, the ventilatory convection requirement for  $O_2$  ( $\dot{V}_w$  per unit  $\dot{M}O_2$ ) was about  $11 \text{ L mmol}^{-1}$ . This value is less than one-third of that ( $35 \text{ L mmol}^{-1}$ ) reported by Perry et al. (2019b) for *E. stoutii* under normoxia. Certainly, the present data suggest a more efficient system, comparable to that ( $10 \text{ L mmol L}^{-1}$ ) in the skate (Graham et al. 1990), but still less efficient than those ( $5\text{--}6 \text{ L mmol}^{-1}$ ) in tench (Eddy, 1973), common carp (Lomholt and Johansen 1979), and starry flounder (Wood et al. 1979). By reversing the axes of Fig. 9a and rationalizing the units to a common basis, it is possible to calculate the mean ventilatory convection requirement for  $O_2$  ( $\text{L mmol}^{-1}$ ) across a wide range of ventilatory flows, as the slopes of regressions of  $\dot{V}_w$  (Y-axis) against  $\dot{M}O_2$  (X-axis)—i.e.  $\Delta \dot{V}_w$  per  $\Delta \dot{M}O_2$  (Fig. 9b). Under normoxia, this overall convection requirement was about  $9 \text{ L mmol}^{-1}$ , very similar to the single point estimate above. This decreased significantly during hyperoxia to about  $5 \text{ L mmol}^{-1}$ . However, the overall convection requirement increased significantly by ninefold during hypoxia to about  $80 \text{ L mmol}^{-1}$ , and by 2.5-fold during 1%  $CO_2$  hypercapnia to about  $22 \text{ L mmol}^{-1}$ , but did not change during 10 mM HEA exposure, in accord with our earlier explanations. In future, studies on the effects of both  $CO_2$  and ammonia on blood  $O_2$ -binding and transport in *E. stoutii*, as well as the influence of all three respiratory gases on the distribution of ventilatory flow among gill pouches, would be illuminating.

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## Compliance with ethical standards

**Conflict of interest** The authors declare no competing or financial interests.

**Ethical approval** The animal usage permits (AUP) were approved by the University of British Columbia (A14-0251, A18-0271) and Bamfield Marine Science Centre (BMSC) animal care committees (AUP RS-17-20, RS-18-20, RS-19-15).

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