Comparative Biochemistry and Physiology, Part A 232 (2019) 47–59

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

The effects of salinity and hypoxia exposure on oxygen consumption, ventilation, diffusive water exchange and ionoregulation in the Pacific hagfish (*Eptatretus stoutii*)

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A B S T R A C T

Hagfishes (Class: Myxini) are marine jawless craniate fishes that are widely considered to be osmoconformers whose plasma [Na^+], [Cl^-] and osmolality closely resemble that of sea water, although they have the ability to regulate plasma [Ca^{2+}] and [Mg^{2+}] below seawater levels. We investigated the responses of Pacific hagfish to changes in respiratory and ionoregulatory demands imposed by a 48-h exposure to altered salinity (25 ppt, 30 ppt (control) and 35 ppt) and by an acute hypoxia exposure (30 Torr; 4 kPa). When hagfish were exposed to 25 ppt, oxygen consumption rate (MO_2), ammonia excretion rate (J_{amm}) and unidirectional diffusive water flux rate (J_{H2O}, measured with ^3H_2O) were all reduced, pointing to an interaction between ionoregulation and gas exchange. At 35 ppt, J_{H2O} was reduced, though MO_2 and J_{amm} did not change. As salinity increased, so did the difference between plasma and external water [Ca^{2+}] and [Mg^{2+}]. Notably, the same pattern was seen for plasma Cl^-, which was kept below seawater [Cl^-] at all salinities, while plasma [Na^+] was regulated well above seawater [Na^+], but plasma osmolality matched seawater values. MO_2 was reduced by 49% and J_{H2O} by 36% during hypoxia, despite a small elevation in overall ventilation. Our results depart from the "classical" osmorespiratory compromise but are in accord with responses in other hypoxia-tolerant fish; instead of an exacerbation of gill fluxes when gas transfer is upregulated, the opposite happens.

1. Introduction

Gas exchange across the gill can be modulated under conditions that require increased O_2 uptake and CO_2 excretion, such as exercise or environmental hypoxia. The osmorespiratory compromise is the trade-off between the need for high gill permeability to promote respiratory gas exchange and low gill permeability to limit ion and water diffusion (Nilsson and Sundin, 1998; Randall et al., 1972). The majority of the studies on the osmorespiratory compromise thus far have focused on how freshwater teleosts (e.g. the rainbow trout) deal with the permeability trade-off at the gills following increases in metabolic demand caused by exercise (Gonzalez and McDonald, 1992; Randall et al., 1972; Wood and Randall, 1973) or a reduction in environmental oxygen supply, i.e. hypoxia (Iftikar et al., 2010; Robertson et al., 2015a; Wood et al., 2009). The classic response is an increase in the fluxes of ions and osmolytes as a consequence of the increases in functional branchial surface area, ventilation and perfusion rates which are needed to elicit increases in oxygen consumption (Gonzalez and McDonald, 1992; Gonzalez, 2011). However, there are a few examples in hypoxia-tolerant fish where hypoxia causes the opposite response – a decrease in the fluxes of ions and water across the gills despite increases in effective O_2 permeability (Robertson et al., 2015a; Scott et al., 2008; Wood et al., 2009; Wood et al., 2007). To date, investigations on the osmorespiratory compromise have been focused largely on freshwater species, mostly for technical and practical reasons, even though seawater fishes are also susceptible to such phenomena (Stevens, 1972).

More recently, it has been shown that the osmorespiratory compromise also occurs in two non-teleost marine species. These include sharks (Class Chondrichthyes), where a failure of the urea retention mechanism occurs, evidenced by exacerbated urea efflux rates when dogfish (*Squalus acanthias suckleyi*) are exposed to either hypoxia (Zimmer and Wood, 2014) or increased temperature (Giacomin et al., 2017). Hagfishes (Class: Myxini), which are widely accepted as osmoconformers, are benthic scavengers that feed on decaying carrion and often burrow themselves into the coelomic cavities of their prey (Martini, 1998) where they can be exposed through long periods of time to severe hypoxia or even anoxia. Pacific hagfish (*Eptatretus stoutii*) inherently possess extremely low oxygen consumption rates (Giacomin

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https://doi.org/10.1016/j.cbpa.2019.03.007
Received 6 December 2018; Received in revised form 30 January 2019; Accepted 6 March 2019
Available online 13 March 2019

et al., 2018; Munz and Morris, 1965). When exposed to higher temperatures (as a modulator of oxygen demand), these animals show a high sensitivity of MO_2 (Q_{10} = 3.22), accompanied by marked increases in the diffusive fluxes of ammonia and water (Giacomin et al., 2018). Therefore, these animals also appear to suffer from an osmorespiratory interaction. The present study aims to follow up on the osmorespiratory compromise responses of Pacific hagfish using two experimental treatments: short term salinity alteration and an acute hypoxia exposure, in order to elicit changes in gill respiratory and ionoregulatory demands.

One may question whether an ionoregulatory challenge is relevant for a hagfish, since for a long time these animals have been considered the only extant craniates that are both osmo- and ionoconformers, with plasma [Na^+], [Cl^-] and osmolality closely resembling those of sea water (Robertson, 1976; Smith, 1932). However, it is now known that divalent cations, such as [Ca^{2+}] and [Mg^{2+}] are kept well below seawater levels (Giacomin et al., 2018; Sardella et al., 2009). Very recently, it has been shown that there is a small offset between plasma and seawater levels of Na^+ and Cl^-, where the former is regulated slightly above sea water, while the latter is regulated slightly below sea water (Clifford et al., 2015; Giacomin et al., 2018). A second goal of the present study was to delve deeper into this phenomenon of apparent ionoregulation, using this same two challenges in order to elicit plasma ion concentration changes.

Our first hypothesis was that as salinity is altered away from the control (seawater) condition, MO_2 would be depressed so that permeability could be reduced to preserve osmo/ionoregulatory status. Secondly, if plasma ions really are actively regulated to maintain constant internal concentrations, we predicted that exposure to different external ion concentrations would elicit compensatory changes; that is, the plasma-to-water gradients would become smaller with low salinity and greater with high salinity. Thirdly, we hypothesized that as hypoxia-tolerant animals, hagfish would decrease gill permeability and ventilation when exposed to hypoxia, thus preventing the unfavourable exacerbation in the fluxes that arise due to the osmorespiratory compromise. Our approach involved measurements of the rates of oxygen uptake (MO_2), ammonia excretion (J_{amm}), and diffusive water flux, as well as plasma ions, ammonia, acid-base status, and osmolality. Ventilatory rates were also recorded during the hypoxia challenge.

2. Material and methods

2.1. Animal collection and housing

Pacific hagfish (*Eptatretus stoutii*; average mass = 48.5 ± 2.34 g; total N number = 66) were caught in two batches from Trevor Channel, near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada, under Fisheries and Oceans Canada collecting permit XR1942017, using bottom-dwelling traps baited with rotting hake (*Merluccius productus*). Fish were immediately transferred to BMSC, where they held in 200-L darkened tanks, served with flow-through fully aerated sea water (12–13 °C, 30 ppt) for a period of 1–4 weeks. Two collections were performed, and the second batch of fish were held for a lesser period (2–3 weeks) than the first (4–5 weeks). For both, fish were fasted for at least 1 week prior to experimentation. At the end of all experimentation and sampling, fish were euthanized by an overdose of anesthetic (MS-222, Syndel Labs, Parksville, BC, Canada; 5 g/L neutralized to pH 7.8 with 5 M NaOH), followed by evisceration to ensure death. All experiments were performed following the guidelines of the Canada Council for Animal Care, under joint approval of the animal care committees at BMSC and the University of British Columbia (AUPs A14–0251 and RS–17-20).

2.2. The effects of salinity on oxygen consumption, ammonia excretion, and diffusive water exchange

Hagfish from the first collected batch were transferred in groups of n = 16 from the main holding tanks to 80-L darkened plastic containers where they were exposed for 48 h to 25 ppt (obtained by diluting seawater with dechlorinated BMSC tap water), 30 ppt (plain filtered seawater), or 35 ppt (filtered seawater where salinity was raised by the addition of Instant Ocean artificial salts; Spectrum Brands, Blacksburg, VA, USA), all under normoxia. Salinity was monitored using a portable conductivity meter (Cond 3110, WTW, Weilheim, Germany). Water in the exposure tanks was renewed at 24 h. The fish were then transferred to separate individual containers for experimental measurements.

All flux experiments were carried out using 1-L glass jars as the experimental containers, covered with dark plastic, topped with a fine mesh, and fitted with fine tubing for aeration. At 42 h of salinity exposure, fish were transferred to the flux containers filled with water at the appropriate salinity and were allowed to settle in for a minimum of 6 h before the start of the experiment. Water temperature was maintained by placing the jars in a water bath kept at 12 °C and at the target salinity. Throughout all experimentation hagfish were left undisturbed for several hours prior to any measurements, as only the jars were touched. This protocol prevented the production of slime. For measurements of ammonia concentration in the water, samples (5 mL) were taken at 0, 3, and 6 h and immediately frozen at −20 °C for analysis within 1 week. During the water sampling intervals (0, 3 and 6 h), the jars were sealed with gas-impermeable dental dam, immediately after an initial PO_2 value was taken using an Accumet AP84 handheld O_2 meter (Fisher Scientific, Toronto, ON, Canada). A final PO_2 measurement was obtained simultaneous with water sampling at the end of each 3-h interval, and then the jars were lightly bubbled with air for re-oxygenation, and the procedure was repeated once until the end of the experimental period. Oxygen consumption (MO_2) and ammonia excretion (J_{amm}) rates did not vary between the two periods, so averages of the two measurements have been reported. Chamber sterilization and blank measurements were performed as described by Giacomin et al. (2018).

At the end of the experiment, fish were quickly transferred to a neutralized MS-222 (0.6 g/L) solution, prepared at the appropriate salinity, and a blood sample (~ 500 μL) was drawn from the posterior sinus using a heparinized gas-tight syringe (Hamilton, Reno, NV, USA). The sample was immediately transferred to a tube placed in a temperature-controlled water bath, and pH was measured using an MI-4156 Micro-Combination pH probe (Microelectrodes Inc., Bedford, NW, USA) and Accumet pH meter (Fisher Scientific, Toronto, ON, Canada). After that, plasma was separated by centrifugation (12,000 *g* for 3 min) aliquoted, flash frozen in liquid N_2 and stored at −80 °C for later analyses. Fish were then immediately weighed and euthanized as described above (Section 2.1).

Diffusive water exchange rates were measured using radiolabelled tritiated water (^3H_2O) in different groups of fish. After 42 h of exposure to each salinity, hagfish (n = 8) were incubated as a group in 20 μCi/L of ^3H_2O (Amersham Pharmacia Biotech, Little Chalfont, UK) in sea water at the target salinity under normoxia for 6 h in a shielded glass container placed in a water bath for temperature control. At the end of the incubation period, duplicate water samples (2 × 5 mL) were taken to measure the water specific activity. Preliminary experiments showed that complete equilibration of ^3H_2O between the internal compartment of the hagfish and the external sea water occurred within 6 h (Giacomin et al., 2018).

After incubation, the fish were rinsed with water free of radioactivity, and then placed in individual experimental chambers (darkened 1-L glass jars identical to those described above) with water at the appropriate salinity. Water samples (5 mL) were taken every 5 min from 0 to 30 min and at 10 min interval thereafter from 30 to 70 min. Final samples were taken at approximately 12 h to assess specific

activity of the system (hagfish plus external water) once equilibrium had been reached. Ten mL of scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) was added to the samples, and they were stored in the dark for a minimum of 12 h prior to counting for beta emissions on a Triathler portable counter (Hidex, Helsinki, Finland). Tests showed that quench was constant. After the final water sample was taken, fish were immediately weighed and euthanized as described above (Section 2.1).

2.3. The effect of hypoxia on oxygen consumption, ammonia excretion, diffusive water exchange and ventilation

Hagfish from the second collected batch, acclimated to normoxia, 30 ppt and 12 °C, were used to examine the effects of hypoxia on MO_2, J_{amm} and diffusive water flux rates. All flux experiments were carried out using the experimental chambers described above. Fish were transferred to and allowed to settle in the chambers for 6 h. After that, water was gently siphoned from all the jars and replaced with either normoxic or hypoxic sea water at 35 Torr (4.7 kPa). Water samples (5 mL) were taken at 0, 3, and 6 h and frozen at −20 °C for later analysis of ammonia concentration. Similarly to the procedures described above, the jars were sealed with gas-impermeable dental dam immediately after a PO_2 value had been measured. At the end of the 3-h flux period, the jars were opened, a final PO_2 value and a water sample were collected, the PO_2 was reset to 35 Torr through light N_2 bubbling, and the procedure was repeated once more until 6 h. The same experimental procedures were carried out for normoxic fish in order to mimic the manipulations of the jars. The average PO_2 during hypoxia was about 30 Torr (4 kPa) and during normoxia about 130 Torr (17.3 kPa).

For the diffusive water flux measurements, fish were loaded for 6 h with ^3H_2O under normoxic conditions at 12 °C and duplicate water samples (2 × 5 mL) were taken to determine specific activity (cpm/mL) of the external water at the end of the loading period. The fish were rinsed, and then acutely transferred to the experimental chambers containing either normoxic water (120–150 Torr) or hypoxic water at 25–35 Torr. PO_2 was monitored throughout the duration of the experiment, and if necessary, water was bubbled with N_2 to reduce PO_2 or with plain air to increase PO_2. After 70 min, full aeration was restored and final samples were taken at approximately 12 h to assess specific activity of the system at equilibrium.

In order to measure ventilation, fish were anesthetized (MS-222; 0.6 g/L neutralized to pH 7.8 with 5 M NaOH), fitted with a device in the single nostril for measurements of ventilatory pressure and frequency, and allowed to recover overnight with flow-through control sea water before experiments were performed. The procedures used here were the same as those employed by (Giacomin et al., 2018) and described in detail by Eom and Wood (2019). Briefly, a flexible plastic tube was inserted snugly into the nostril, and stitched to the skin (Ethicon, Somerville, NJ, USA). PE160 tubing (BD, Intramedic, Franklin Lakes, NJ, USA) was inserted through the tubing and then bridged using a blunt needle to an extra piece of PE 160 tubing (both water-filled) when recordings were performed. Frequency (breaths/min) and pressure amplitude (cm H_2O) were recorded at the nostril duct using a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA), which had been previously calibrated against a 2-cm water column. Signals were amplified (LCA-RTC, Transducer Techniques, Temecula, CA, USA) and digitized using a PowerLab Data Integrity system (ADInstruments, Colorado Springs, CO, USA), visualized and analyzed using LabChart v. 7.0 (ADInstruments).

Fish were then transferred to the same experimental containers described above and allowed to settle for several hours. Ventilation was recorded for 5 min and then the water was replaced with water that had been pre-equilibrated with N_2 until the total volume (1 L) reached a stable PO_2 of 30 Torr (4 kPa). Animals were kept in hypoxia for 6 h and then ventilation was recorded for 5 min. During the experiment, water PO_2 was monitored and, if needed, adjusted by lightly bubbling with either N_2 or pure air.

2.4. Analytical techniques and calculations

For all calculations involving volume (V), the weight of the fish was subtracted from the 1-L respirometer volume, assuming 1 g = 1 mL. Since closed-system respirometry was used, oxygen consumption rates (MO_2: μmol O_2/kg/h) were calculated with the following equation:

$$MO_2 = [((PO_{2(i)} - PO_{2(f)}) \times \alpha O_2) \times V]/(W \times t) \quad (1)$$

where $PO_{2(i)}$ and $PO_{2(f)}$ are the initial and final water PO_2 (Torr) respectively; αO_2 is the oxygen solubility coefficient (Boutilier et al., 1984) at 12 °C at the appropriate salinity; V is the water volume (L); W is the body mass of the fish (kg); t is the duration of the measurement period (h).

Ammonia concentrations in sea water were measured spectrophotometrically following the method described in Verdouw et al. (1978) using NH_4Cl standards made up at the appropriate salinity. Ammonia flux rate (J_{amm}, μmol/kg/h) was calculated using the following equation:

$$J_{amm} = [(Amm_{(i)} - Amm_{(f)}) \times V]/(W \times t) \quad (2)$$

where $Amm_{(f)}$ and $Amm_{(i)}$ are the final and initial water ammonia (μmol/L) concentrations respectively, and the other parameters are the same as in Eq. 1. The ammonia quotient was calculated from the simultaneous measurements of MO_2 and J_{amm} using the following equation:

$$AQ = J_{amm}/MO_2 \quad (3)$$

For the diffusive water flux measurements, the total amount of ^3H_2O radioactivity (R_{total}, cpm) that the fish had originally taken up during the loading period (i.e. the total present at the start of the washout period) was calculated. R_{total} was calculated as the sum of total radioactivity in the system (water + fish) after complete equilibration between fish and water had occurred at 12 h, plus all radioactivity removed during water sampling.

Using R_{total} and values of ^3H_2O radioactivity appearance in the water at each time interval, radioactivity left in the fish (R_{time}) was back-calculated for each time during the experiment and used in a regression of natural log of R_{time} against time. Therefore, the rate constant of ^3H_2O efflux (k) was calculated from the exponential rate of decline in total ^3H_2O radioactivity in the fish (Evans, 1967):

$$k = (\ln R_{time1} - \ln R_{time2})/(t_1 - t_2) \quad (4)$$

where k is the rate constant of the efflux (in h^{-1}), and R_{time1} and R_{time2} are total ^3H_2O radioactivity (in cpm) in the fish at times t_1 and t_2 (h). The product of k x 100 yields the percent of the total body water pool turned over per hour. The rate constant (k) for ^3H_2O efflux was only calculated over the range where efflux was linear (usually up to 30 min after the start of the experiments).

It was also possible to calculate the exchangeable internal water volume of each fish (V_{H2O}; ml/kg) as:

$$V_{H2O} = R_{total}/SA_{H2O} \times W \quad (5)$$

where SA_{H2O} is the specific activity (cpm/ml) of the external water at the end of the original ^3H_2O loading period, and W is body mass in kg. The unidirectional diffusive water flux rate (J_{H2O}; mL/kg/h) could then be calculated as:

$$J_{H2O} = k * V_{H2O} \quad (6)$$

where k was calculated using Eq. 4 and V_{H2O} was calculated using Eq. 5.

The ventilatory index (cm H_2O/min) was calculated using the following equation:

$$\text{Ventilatory Index} = \text{frequency} \times \text{pressure amplitude} \quad (7)$$

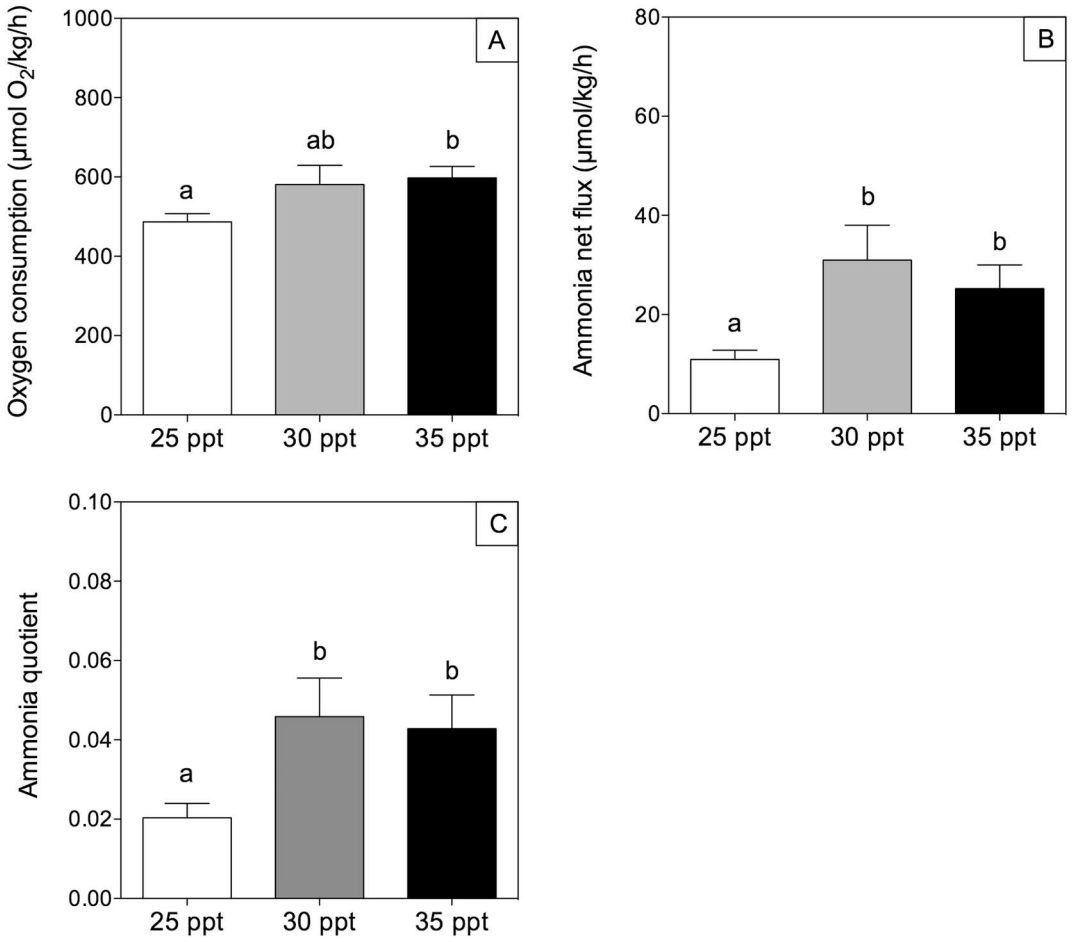

Fig. 1. (A) Oxygen consumption rate (MO_2, μmol O_2/kg/h), (B) ammonia net flux rate (J_{amm}, μmol/kg/h) and (C) ammonia quotient in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48 h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey's post hoc test. Data are shown as means ± 1 SEM (n = 7–8).

where frequency represents velar breaths/min and amplitude is in cm H_2O.

Plasma total ammonia (T_{amm}; μmol/L) was determined using a commercial kit (Raichem, Cliniqa, San Marcos, CA, USA), and background correction was done as described in Giacomin et al. (2018). Plasma chloride concentration (mmol/L) was determined using a chloridometer (Radiometer CMT10, Copenhagen, Denmark). Plasma cations (sodium, calcium and magnesium) were measured by atomic absorption spectroscopy (Varian, Mulgrave, Victoria, Australia) using certified commercial solutions as standards (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Lanthanum chloride ($LaCl_3$) at a final concentration of 1% was added to the samples and standards in order to measure plasma Ca^{2+} and Mg^{2+}. Plasma osmolality was determined using a Wescor vapor pressure osmometer and standards (Wescor 5100C, Logan, UT, USA).

Plasma TCO_2 content was measured using a total CO_2 analyzer (Corning 965 CO_2 analyzer, Ciba Corning Diagnostic, Halstead, Essex, UK). The solubility coefficient of carbon dioxide (αCO_2: mmol/L/Torr) in hagfish plasma was calculated using an equation from Heisler (1984), where measured values for plasma osmolality (mOsm/kg) were used. The apparent pK of CO_2 (pK_{app}) in hagfish plasma was calculated using a second equation from Heisler (1984). Both equations are described in Giacomin et al. (2018).

With those two calculated parameters (αCO_2 and pK_{app}), plasma PCO_2 (Torr) was calculated from measured TCO_2 and pH values using a modified Henderson-Hasselbalch equation:

$$PCO_2 = TCO_2/[\alpha CO_2 \times (1 + \text{antilog}(pH - pK_{app}))] \quad (8)$$

Plasma $[HCO_3^-]$ (mmol/L) was calculated as:

$$[HCO_3^-] = TCO_2 - (\alpha CO_2 \times PCO_2) \quad (9)$$

where PCO_2 was calculated using Eq. 8.

Plasma $[NH_4^+]$ was calculated using a modified Henderson-Hasselbalch equation and pK' values obtained from (Cameron and Heisler, 1983) adjusted to hagfish plasma NaCl concentration:

$$[NH_4^+] = T_{amm}/[1 + \text{antilog}(pH - pK')] \quad (10)$$

where T_{amm} is the measured total plasma ammonia (μmol/L). Plasma $[NH_4^+]$ data have not been reported as they were quantitatively similar to plasma T_{amm} data, but were required for the calculation of the partial pressure of NH_3 (PNH_3; μTorr) using the following equation:

$$PNH_3 = (T_{amm} - [NH_4^+])/\alpha NH_3 \quad (11)$$

where αNH_3, the solubility coefficient of NH_3 in hagfish plasma (μmol/L/μTorr), was obtained from Cameron and Heisler (1983), and NH_4^+ was calculated in Eq. 10.

2.5. Statistical analysis

All data were tested for normality and homogeneity of variances (D'Agostino-Person and Bartlett's test respectively) and in case of failure were transformed either using a log transformation, or a square root transformation. Data from Section 2.2 (effect of salinity) were compared using one-way analysis of variance (ANOVA) followed by a Tukey's post-hoc test, while data from Section 2.3 (effect of hypoxia) were evaluated using Student's two-tailed *t*-test, paired or unpaired as

appropriate (details are shown in figure captions). Mean values were considered significantly different when $p < .05$. All data are shown as means ± 1 SEM (n = number of animals). All statistical analyses were performed using Graph Pad Prism version 5.0b.

3. Results

3.1. Responses to salinity exposure

These experiments were performed with hagfish from the first collected batch. Oxygen consumption rate (MO_2) and ammonia excretion rates (J_{amm}) did not differ significantly (i.e. $p > .05$) between the two experimental intervals in the various series, and therefore averaged values have been reported. There was an overall increase in MO_2 with increasing salinity exposure (Fig. 1A). While MO_2 of hagfish exposed to 25 ppt was 16% lower than at the control salinity (581 μmol O_2/kg/h), MO_2 did not vary significantly between 30 and 35 ppt salinity (Fig. 1A). J_{amm} was highest at the control salinity (31 μmol/kg/h) and decreased significantly by 64% in fish exposed to 25 ppt (Fig. 1B). Similar to MO_2, there was no significant differences between J_{amm} at 30 and 35 ppt (Fig. 1B). The ammonia quotient (AQ) was ~ 0.04 in control fish at 30 ppt and decreased significantly by 44% in fish acclimated to 25 ppt (Fig. 1C). AQ did not differ significantly between 30 and 35 ppt exposed fish (Fig. 1C). Diffusive water flux rate (J_{H2O}) was 1580 mL/kg/h at the control salinity and significantly decreased in fish exposed to both to 25 and 35 ppt (Fig. 2A). As expected, the exchangeable water pool, which was 62% of body weight under control conditions, increased by 11% at 25 ppt and decreased by 11% at 35 ppt. Although the pool at 25 ppt was significantly different than that at 35 ppt, both values were not significantly different than the control (Fig. 2B).

The measured seawater ion concentrations at each salinity are summarized in Supplementary Table S1. Plasma [Na^+] and [Cl^-] were 552 mmol/L and 463 mmol/L respectively under control conditions and varied greatly with salinity, being highest at 35 ppt (Fig. 3A, B). As seen in Fig. 4A, plasma [Na^+] was significantly higher than the seawater [Na^+] values at all three salinities, while plasma [Cl^-] was significantly lower than seawater [Cl^-] values at all salinities (Fig. 4B). Interestingly, for [Cl^-], the concentration differential between plasma and water increased as salinity increased (Fig. 4B). This pattern was not seen for [Na^+], but for both ions the differentials were greatest (+148 mmol/L for [Na^+], −73 mmol/L for [Cl^-]) though opposite in sign, at 35 ppt. While plasma [Mg^{2+}] was unaffected by salinity acclimation (Fig. 3C), the plasma-to-water concentration differential for [Mg^{2+}] increased as salinity increased, reaching −42 mmol/L at 35 ppt (Fig. 4C). Plasma [Ca^{2+}] was significantly reduced at 25 ppt and did not change between 30 and 35 ppt (Fig. 3D). Similar to [Mg^{2+}], the plasma to sea water difference for [Ca^{2+}] increased as salinity increased, reaching −5.4 mmol/L at 35 ppt (Fig. 4D). Plasma osmolality increased in proportion to salinity (Fig. 3E), with values that were not significantly different from seawater osmolalities, as seen by the plasma-to-water concentration difference ranging from −6 to +10 mOsm/kg (Fig. 4E).

At the control conditions, plasma pH was 7.77 and no changes were seen in fish exposed to higher or lower salinity. Plasma PCO_2 was 4.35 Torr at 30 ppt, while plasma HCO_3^- was 11.3 mmol/L. Overall, the measured blood acid-base parameters were mostly unaffected by salinity, with the exception of plasma [HCO_3^-], which significantly decreased at 35 ppt in comparison to 25 ppt (Fig. 5C). The total plasma ammonia concentration (Fig. 5D) and [NH_4^+] (data not shown), as well as plasma P_{NH3} (Fig. 5E), all tended to increase with salinity, but none of the differences were significant.

3.2. Responses to hypoxia exposure

Hagfish from the second collected batch, where the holding period was shorter, were used in these experiments. As all respirometry

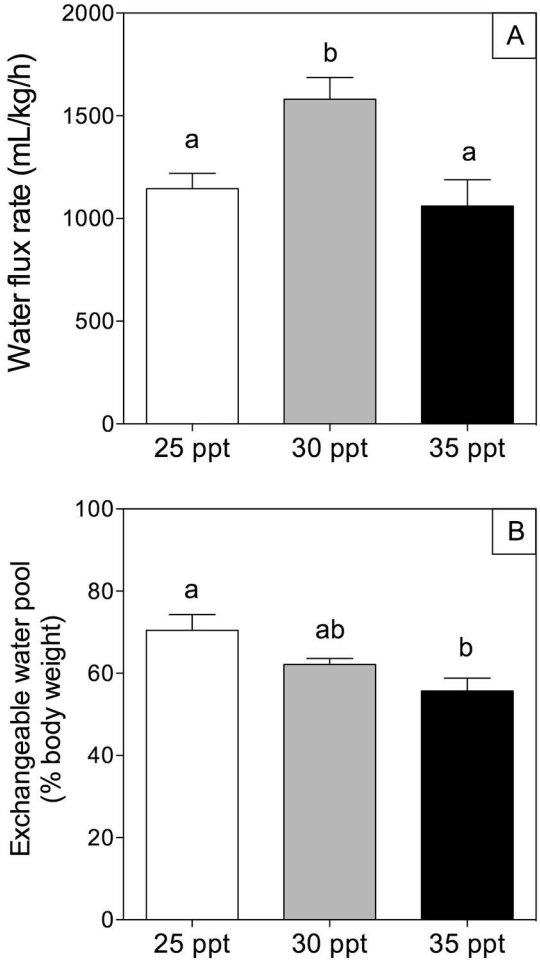

Fig. 2. (A) Diffusive water flux rate (mL/kg/h) and (B) exchangeable water pool (% body weight) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48 h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey's post hoc test. Data are shown as means ± 1 SEM (n = 7–8).

experiments were performed at the same time of day, the difference in holding period probably explained the approximately 50% higher MO_2 value under control conditions relative to the first batch (Fig. 6A versus Fig. 1A) though there were no significant differences in J_{amm} (Fig. 6B versus Fig. 1B) or J_{H2O} (Fig. 6C versus Fig. 2A).

Exposure to hypoxia for 6 h resulted in a 49% reduction in MO_2 from the control level of 872 μmol/kg/h (Fig. 6A). However, there was no change in J_{amm} from the control rate (31 μmol/kg/h) under hypoxia (Fig. 6B). Diffusive water flux rate (1338 mL/kg/h) under normoxia was significantly reduced by 36% with hypoxia exposure (Fig. 6C). In normoxia, the ammonia quotient (AQ) was 0.04 and despite an increase with exposure to hypoxia, it was not significantly different (Fig. 6D).

Ventilation rate was ~11 breaths/min under normoxia and increased 4.8-fold when hagfish were exposed to hypoxia (Fig. 7A), while ventilation pressure amplitude, which was 0.14 cmH_2O/breath under normoxia decreased by 75% (Fig. 7B). Thus, the ventilatory index, which in normoxia was 1.56 cmH_2O/min, increased in hypoxia by 24%, a small but significant increase on a paired basis (Fig. 7C).

Plasma pH did not change with hypoxia exposure (Fig. 8A), while plasma PCO_2 and HCO_3^- both decreased significantly by about 57 and 60% respectively (Fig. 8B, C). Plasma ion concentrations ([Na^+], [Cl^-], [Mg^{2+}] and [Ca^{2+}]), and osmolality were largely unaffected by hypoxia exposure, with no significant differences between normoxic control and hypoxia exposed fish (Table 1). Additionally, despite being reduced, total plasma ammonia, plasma [NH_4^+] (data not shown), and

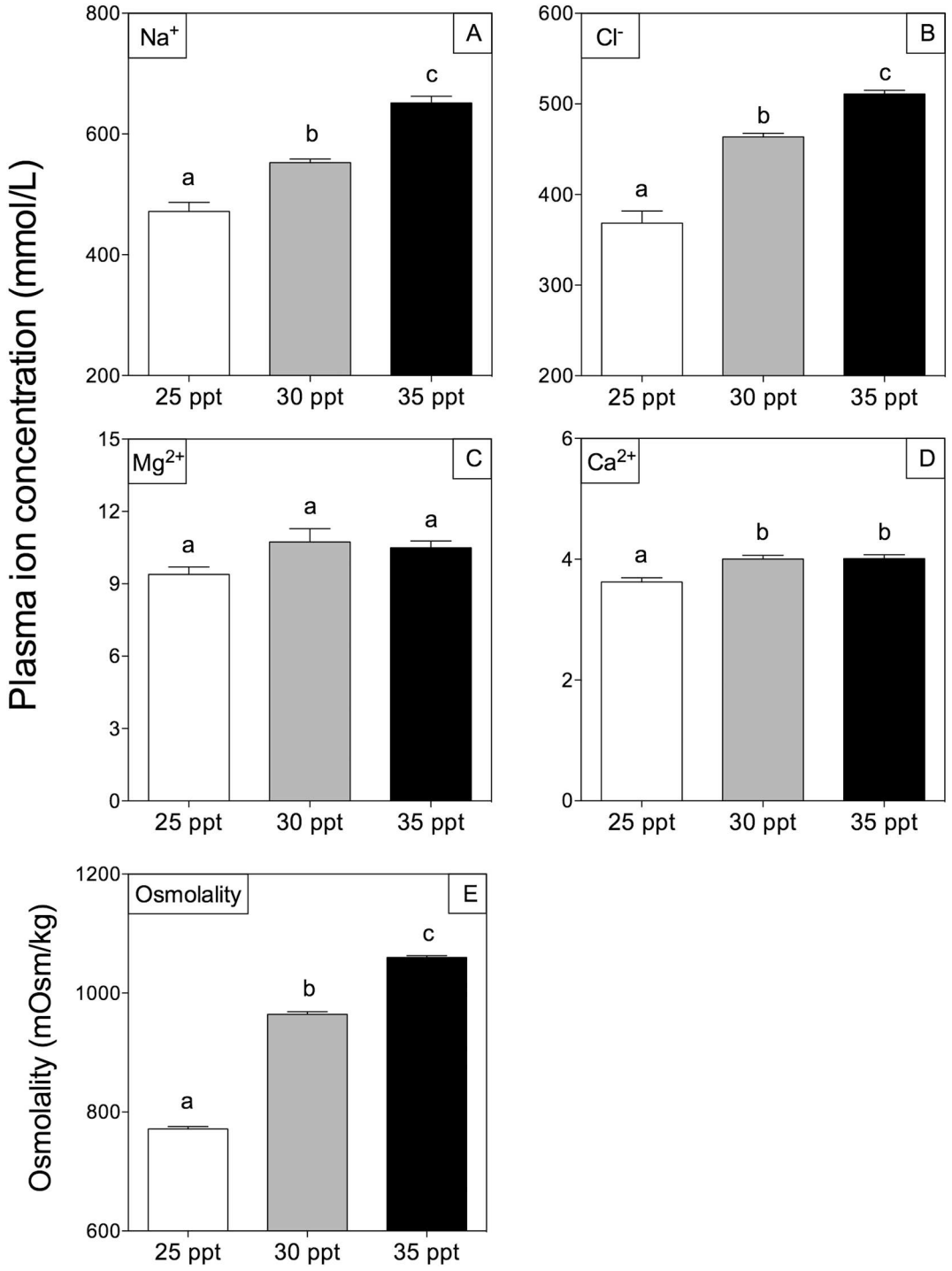

Fig. 3. Plasma ion concentration in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48 h. [(A) Sodium: Na^+; (B) Chloride: Cl^-; (C) Magnesium: Mg^{2+}; (D) Calcium: Ca^{2+} in mmol/L and (E) Osmolality (mOsm/kg)]. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey's post hoc test. Data are shown as means ± 1 SEM (n = 7–8).

the partial pressure of ammonia (P_{NH3}) were highly variable and did not statistically change with exposure to hypoxia (Table 2).

4. Discussion

4.1. Overview

Our ultimate goal was to examine how Pacific hagfish respond to changes in respiratory and ionoregulatory demands elicited by a relatively short (48 h) exposure to altered salinity and by acute hypoxia challenge. We predicted that as salinity departed from the control condition, as a natural adaptive response, hagfish would depress aerobic metabolism and consequently reduce gill permeability. We were in part correct, since when kept for 48 h at a lower salinity (25 ppt), hagfish exhibited a reduction in MO_2, J_{amm} and J_{H2O}. Also, when exposed for 48 h to elevated salinity (35 ppt), J_{H2O} was again reduced, though MO_2 and J_{amm} did not change. Also, we hypothesized that as salinity was changed, hagfish would exhibit compensatory adjustments of plasma ions. We observed that the concentration difference between plasma and external water was directly modified with

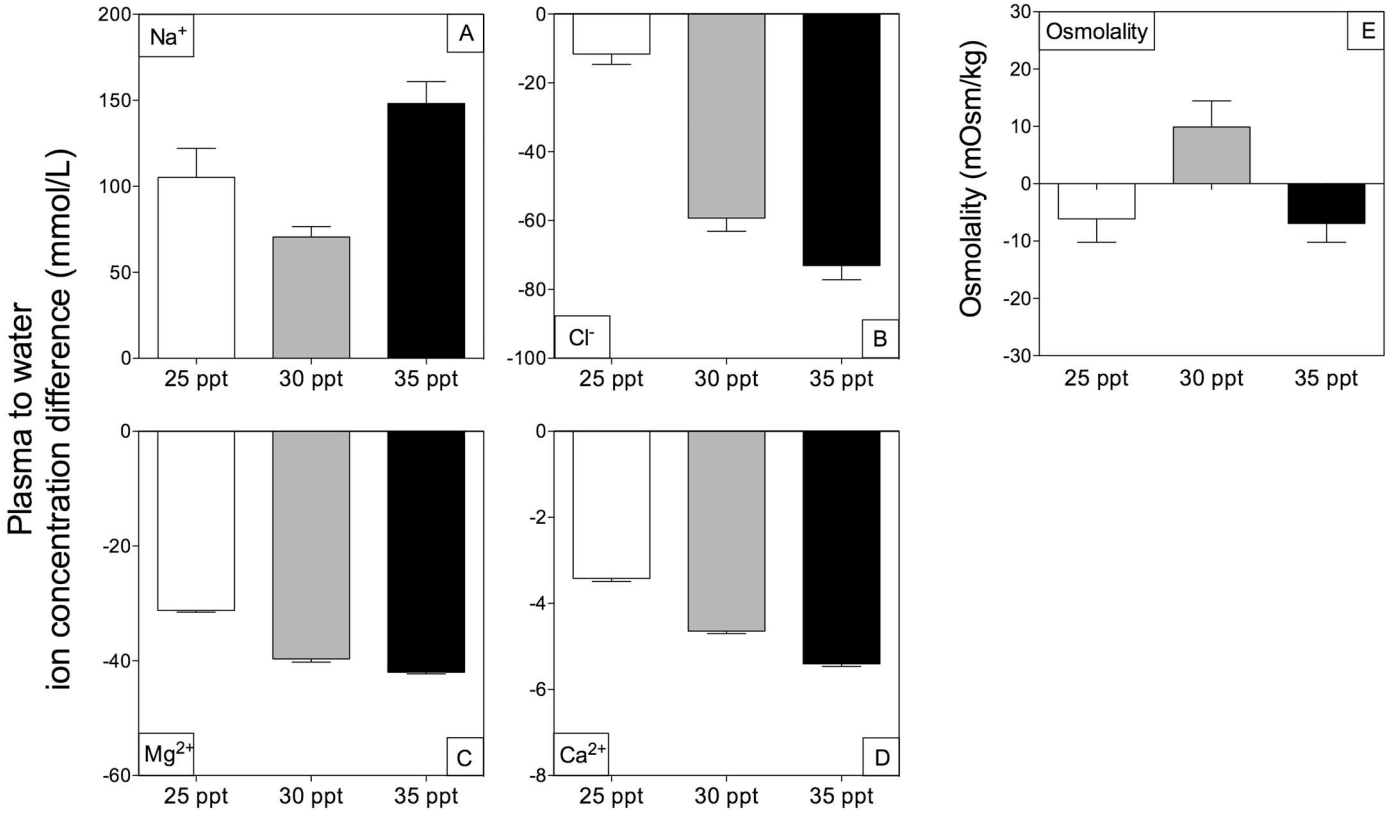

Fig. 4. Plasma ion to seawater concentration differential in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48 h. [(A) Sodium: Na^+; (B) Chloride: Cl^-; (C) Magnesium: Mg^{2+}; (D) Calcium: Ca^{2+} (in mmol/L) and (E) Osmolality (mOsm/kg)]. Data are shown as means ± 1 SEM (n = 7–8).

increasing salinity for the divalent cations ([Ca^{2+}] and [Mg^{2+}]) previously known to be actively regulated at levels well below those of sea water, and we identified a similar phenomenon for [Cl^-]. The salinity–dependent pattern was less clear for plasma [Na^+], but nevertheless, at all salinities, this cation was regulated at a level well above those in the external sea water. Finally, when exposed to hypoxia, hagfish decreased MO_2, even though overall ventilatory water flow, as represented by the ventilatory index, was slightly elevated. The observed reductions in J_{H2O} and J_{amm} at this time indicate that despite the fact that hagfish were attempting to improve MO_2, they were selectively reducing gill permeability. This points to a strategy of dealing with the osmorespiratory compromise that differs from the traditional model where gill permeability to respiratory gas exchange increases, and consequently gill permeability also increases to ions, water and nitrogenous compounds.

4.2. Effects of exposure to altered salinity on MO_2 and gill permeability

We had predicted that altered salinity exposure would elicit a metabolic depression, thereby avoiding associated compensatory costs, since nutrient acquisition for hagfish is thought to be difficult, giving the environment these fish inhabit. At the lower salinity (25 ppt), MO_2 was indeed moderately depressed (by 16%) in accord with our original hypothesis, in contrast, MO_2 was slightly elevated at 35 ppt (by 3%) contrary to our hypothesis. However it must be remembered that MO_2 was expressed per unit wet body mass (kg), measured at 48 h. According to Sardella et al. (2009) when *E. stoutii* were exposed for 48 h to a comparable salinity challenge, fish gained wet weight at lower salinity and lost wet weight at higher salinity. Based on the data reported by Sardella et al. (2009) we calculated that the fish in our study could have gained water amounting to approximately 3.5% body weight at 25 ppt and lost a comparable amount at 35 ppt. Therefore the changes in MO_2 seen at 25 and 35 ppt may have been overestimated, relative to rates expressed per unit original wet body mass. Indeed when we applied this correction factor on mass-specific MO_2, the effect of changed salinity was no longer statistically significant at either 25 or 35 ppt.

Ammonia excretion exhibited a pronounced (64%) decrease at 25 ppt (Fig. 1). This further downregulation of J_{amm} independently of the reduction in MO_2, with no change in plasma ammonia concentrations (Fig. 5D, E), could suggest a selective decrease in gill permeability for ammonia, while the system remained in balance. However, we cannot identify if this reduction occurred mostly at the gills or the skin. Using immunohistochemistry, Braun and Perry (2010) demonstrated that Pacific hagfish express ammonia transporting proteins (Rhbg and Rhcg1) at the gills. More recently, Clifford et al. (2017) have shown that the skin of hagfish expresses Rhcg proteins, providing a mechanisms by which ammonia can be excreted across the thick epidermal layer. Moreover, when exposed to high environmental ammonia, these fish are able to differentially regulate the ammonia permeability of the gills and the skin, revealing a wide-scope of ammonia handling strategies (Clifford et al., 2017). The ammonia quotient (Fig. 1C), or J_{amm}/O_2 ratio, is a proxy for the nitrogen quotient, which is an index of the use of protein/amino acids as an aerobic fuel (Lauff and Wood, 1996). In control hagfish, this index indicates that only ~16% of aerobic metabolism is fueled by proteins/amino acids, a conclusion consistent with previous findings that hagfish mostly utilize carbohydrates (Hansen and Sidell, 1983; Sidell et al., 1984). At 25 ppt, the AQ was significantly reduced, indicating that during this metabolic depression, protein oxidation was further decreased from the already low numbers.

Recently, using radioisotopic techniques, Glover et al. (2017) have confirmed that Pacific hagfish do not drink sea water in order to maintain hydromineral balance, yet surprisingly, they possess one of the highest water exchange rates ever reported for any fish (Glover et al., 2017; Rudy and Wagner, 1970). This was confirmed by the present study (Fig. 1B) and earlier studies on the same species

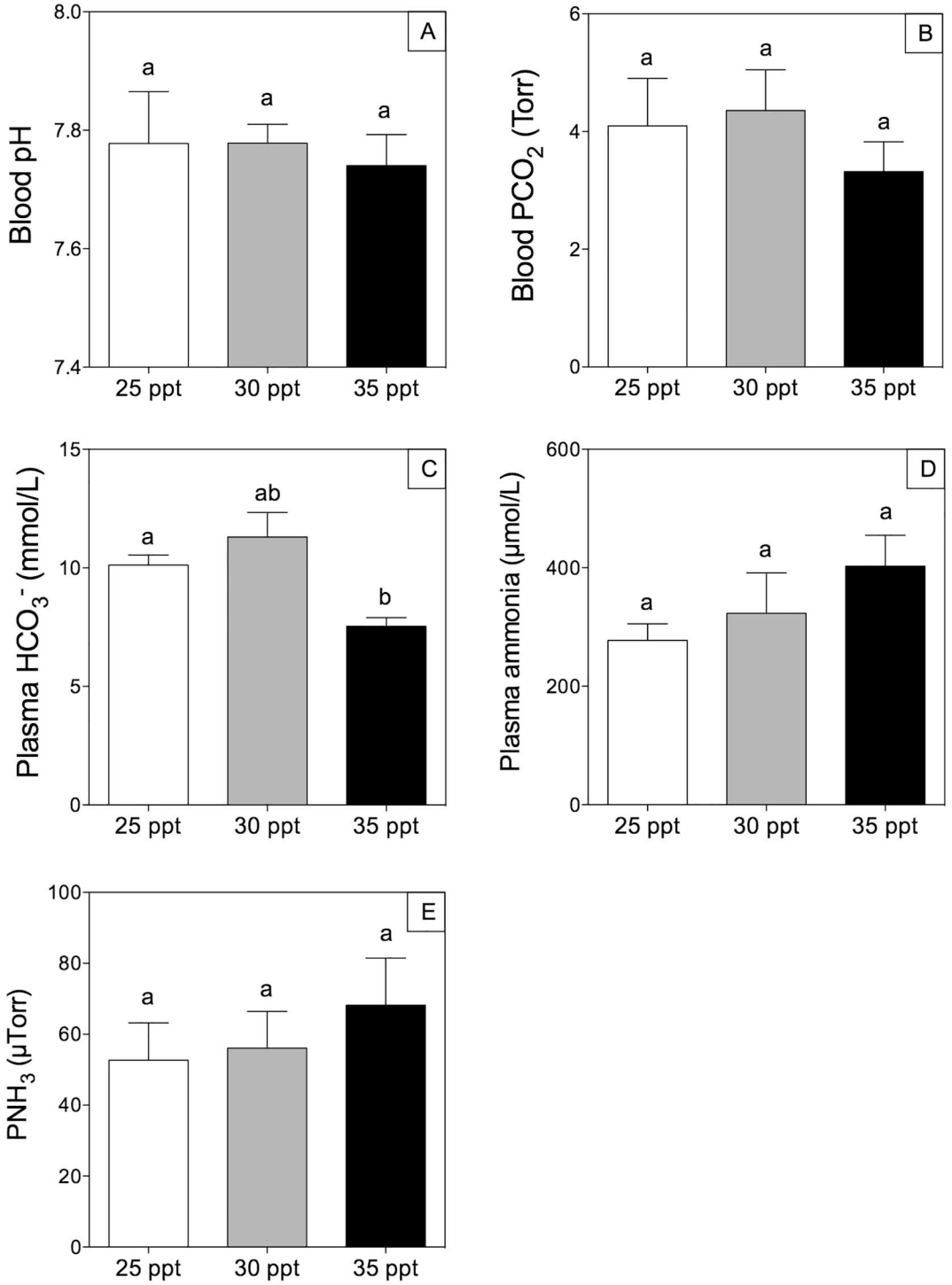

Fig. 5. (A) Blood pH, (B) plasma PCO_2 (Torr) and (C) plasma HCO_3^- (mmol/L), (D) plasma total ammonia in µmol/L and (E) partial pressure of ammonia (PNH_3 – µTorr) in *Eptatretus stoutii* acclimated to 30 ppt or exposed to 25 and 35 ppt for 48 h. Different lower case letters represent statistically different means detected by one-way ANOVA and a Tukey's post hoc test. Data are shown as means ± 1 SEM (n = 7–8).

(Giacomin et al., 2018). This high water permeability is probably a reflection of their osmoconforming strategy. Although the contribution of the skin to the high water permeability remains to be elucidated, the presence of two aquaporin homologs (AQP3 and AQP4) has been identified throughout the skin and on the slime glands of Pacific hagfish (Herr et al., 2014). AQP4 was also identified at the gills of another Pacific hagfish (*Eptatretus burgeri*), possibly located at the basolateral membrane of pavement cells (Nishimoto et al., 2007). Several earlier studies where environmental salinity has been manipulated have pointed to the fact that hagfish in general are better able to cope with a transfer to a hypotonic medium rather than to a hypertonic one, as evidenced by weight changes, tissue water content and blood pressure (Foster and Forster, 2007; McFarland and Munz, 1965; McFarland and Munz, 1958; Toop and Evans, 1993). However, no investigation so far has looked at aquaporin expression and distribution in relation to environmental salinity changes.

Diffusive water fluxes (measured as ^3H_2O fluxes) are unidirectional and are known to be about 100-fold higher than osmotic water fluxes

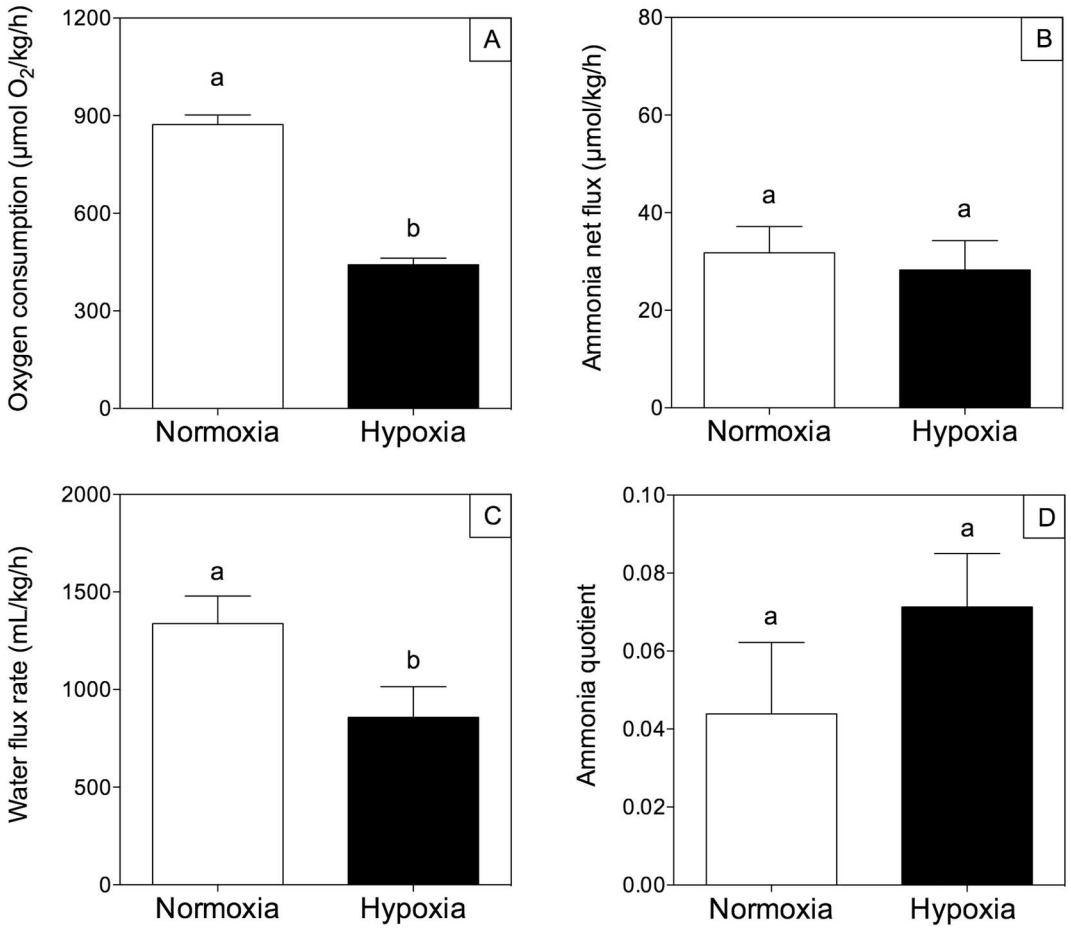

Fig. 6. (A) Oxygen consumption rate (MO_2, μmol O_2/kg/h), (B) ammonia net flux rate (J_{amm}, μmol/kg/h), (C) diffusive water flux rate (mL/kg/h) and (D) ammonia quotient in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12 °C). Different lower case letters represent statistically different means detected by Student's *t*-test. Data are shown as means ± 1 SEM (n = 7–8).

which are net fluxes. The latter are usually measured indirectly through body weight changes, drinking rate and urine flow. It is argued that such measurements underestimate osmotic water fluxes (Isaia, 1984). When hagfish are exposed to low salinity for 48 h, the decreased external osmotic pressure would drive the osmotic influx of water, and this likely led to the observed elevation in the internal exchangeable water pool (Fig. 2B) in the present study. Conversely, exposure to higher salinity would create an outwardly directed gradient for osmotic water flux, explaining the lower internal exchangeable water pool seen at 35 ppt (Fig. 2B). In control conditions, we measured an exchangeable water pool of 62% of body weight (Fig. 2B), which is low in comparison with the recent study by Glover et al. (2017), who obtained a value around 78% of body weight. The techniques used in (Glover et al., 2017) to determine the exchangeable water pool differ substantially from the ones employed here, and we believe this could be largely responsible for the difference in the results.

At both lower and higher salinity, we observed a decreased diffusive water flux rate (Fig. 2A) despite opposing changes in the size of the exchangeable water pool (Fig. 2B). At the lower salinity, it is possible that this decrease is a result of lowered MO_2, which would be in accord with our previous study (Giacomin et al., 2018), where high temperature and hyperoxia treatments indicated parallel changes in O_2, ammonia, and water permeability, reflective of an osmorespiratory compromise. Additionally, an independent down-regulation of water permeability may have occurred, through possible post-translational modifications affecting membrane aquaporins. Certainly, a down-regulation of aquaporin function may be a more likely explanation for the decrease in diffusive water flux at 35 ppt (Fig. 2A), where MO_2 (Fig. 1A) and J_{amm} (Fig. 1B) remained unchanged.

4.3. Effects of exposure to altered salinity on plasma ion concentrations

Preceding studies on the Pacific hagfish have suggested that these fish are stenohaline, seeming to lack the capacity to regulate either plasma [Na^+] or [Cl^-] upon acute salinity changes (McFarland and Munz, 1965; Sardella et al., 2009). However, several previous investigations have indicated that plasma [Na^+] is kept slightly below the levels in external sea water while plasma [Cl^-] is maintained slightly above the concentrations in sea water (Clifford et al., 2018; Giacomin et al., 2018; McFarland and Munz, 1965; Sardella et al., 2009). Furthermore, when exposed to 80% SW, hagfish were able to elevate plasma [Na^+] to seawater levels after 2 days (McFarland and Munz, 1965). There is clear evidence that divalent cations (Mg^{2+} and Ca^{2+}) are maintained at levels much lower than in the sea water, even when hagfish are acclimated and/or exposed to salinities above and below that of normal sea water (Hastey, 2011; Sardella et al., 2009). The present results (Figs. 3 and 4) confirm all these previous observations and additionally reveal that the differences between plasma and water for Cl^-, Mg^{2+}, and Ca^{2+} (and possibly Na^+) all increase as salinity increases (Fig. 4). These results indicate that not only do hagfish posses some ability to regulate all four ions, but also that this regulation becomes greater as environmental salinity increases. Giacomin et al. (2018) have shown that increases in environmental temperature induced an increase in both plasma [Na^+] and [Cl^-], while plasma [Ca^{2+}] and [Mg^{2+}] significantly decreased. Thus, there was increased internal regulation of [Na^+], [Ca^{2+}] and [Mg^{2+}] but decreased

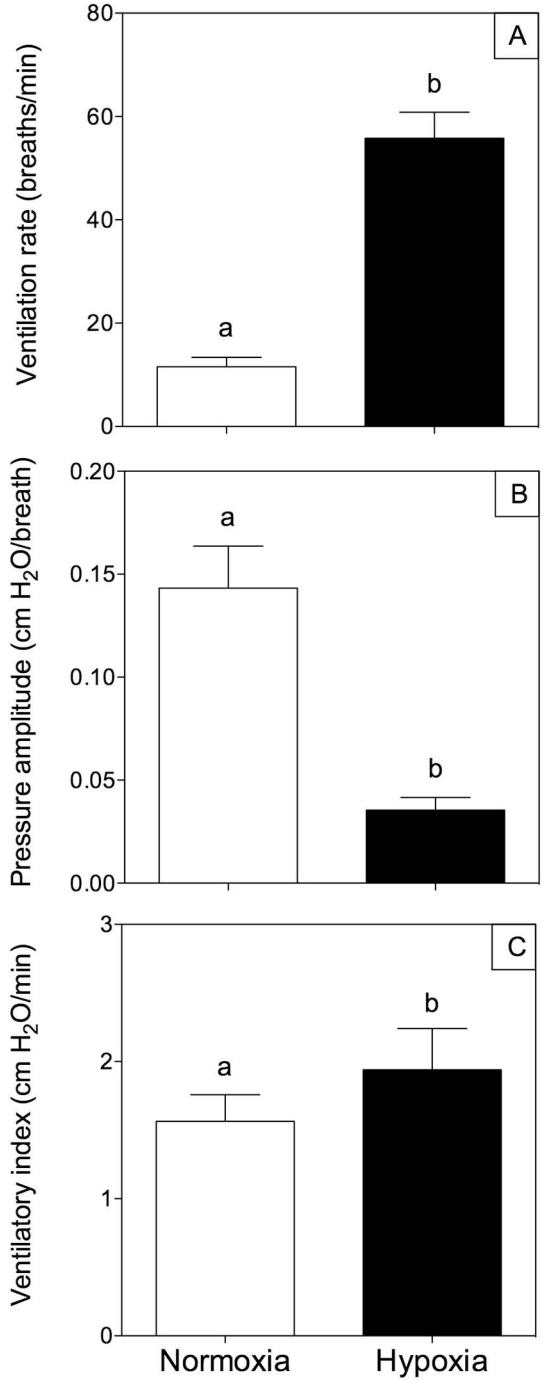

Fig. 7. (A) Ventilation rate (breaths/min), (B) ventilation pressure amplitude (cmH_2O/breath) and (C) ventilatory index (cmH_2O/min) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12 °C). Different lower case letters represent statistically different means detected by a paired Student's t-test. Data are shown as means ± 1 SEM (n = 7–8).

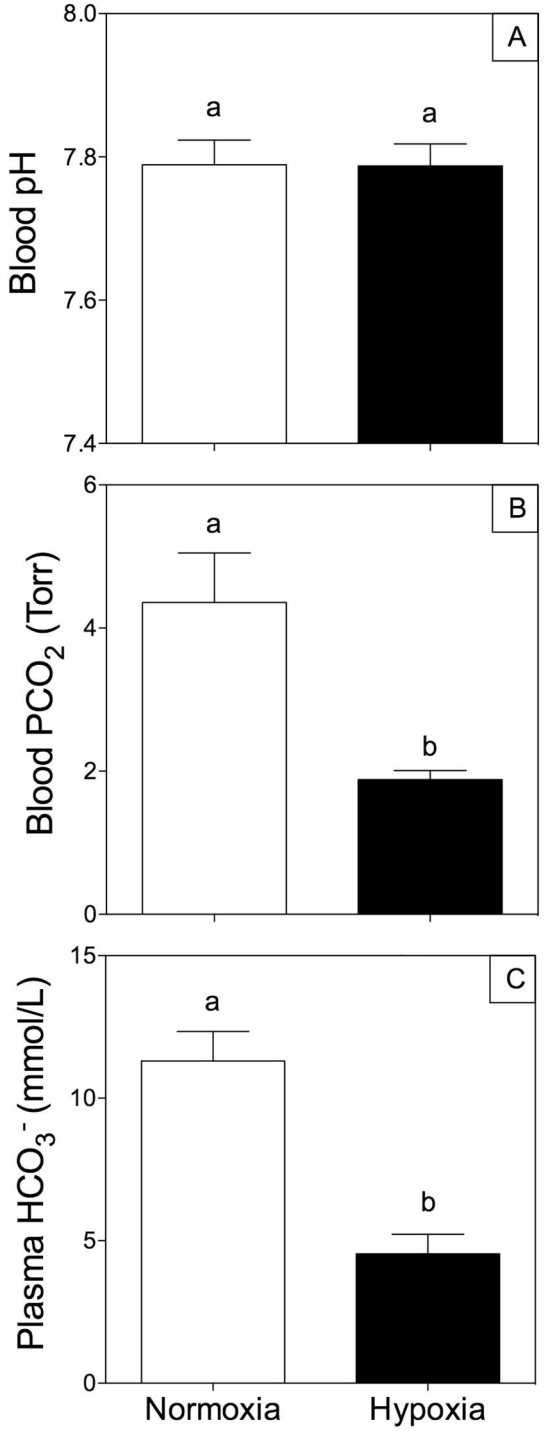

Fig. 8. (A) Blood pH, (B) plasma PCO_2 and (C) plasma HCO_3^- (mmol/L) in *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12 °C). Different lower case letters represent statistically different means detected by Student's t-test. Data are shown as means ± 1 SEM (n = 7–8).

regulation of $[Cl^-]$ at high temperature. The presence of ionocytes on both the gills and skin of hagfish is well established (Choe et al., 1999; Herr et al., 2014; Mallatt et al., 1987; Tresguerres et al., 2006); future studies should address their potential roles in the ionoregulatory phenomena identified here and the adaptive significance of this regulation.

We also observed a decrease in plasma $[HCO_3^-]$ in fish exposed to 35 ppt for 48 h (Fig. 5C), though there was no change in plasma pH (Fig. 5A) because of the accompanying non-significant fall in PCO_2 (Fig. 5B). In marine fishes, HCO_3^- is secreted into the intestine of fishes and forms complexes with imbibed Ca^{2+} and Mg^{2+}, precipitating these salts and providing protection from potential divalent cation toxicity, and this process is elevated at higher salinities [see Wilson et al., 2002 for a review]. Sardella et al. (2009) suggested that this mechanism is also present in hagfishes, though there appears to be no direct evidence, and the lack of drinking (Glover et al., 2017) in these animals would seem to make this process unnecessary.

Table 1
Ion concentrations in blood plasma of *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12 °C). Sodium: Na^+; Chloride: Cl^-; Magnesium: Mg^{2+}; Calcium: Ca^{2+} (mmol/L) and Osmolality (mOsm/kg). No statistical differences were found between animals in normoxia versus hypoxia exposure. Data are shown as means ± 1 SEM (n = 7–8).

	Plasma ion concentration (mmol/L)	
	Normoxia	Hypoxia
Na^+	555 ± 11	558 ± 14
Cl^-	465 ± 2	462 ± 7
Mg^{2+}	10.6 ± 0.3	10.2 ± 0.3
Ca^{2+}	4.0 ± 0.1	4.2 ± 0.1
Osmolality (mOsm/kg)	964 ± 4	960 ± 5

Table 2
Plasma total ammonia (T_{amm} – μmol/L) and partial pressure of ammonia (PNH_3 – μTorr) of *Eptatretus stoutii* exposed to hypoxia (30 Torr for 6 h; 30 ppt at 12 °C). No statistical differences were found between animals in normoxia versus hypoxia exposure. Data are shown as means ± 1 SEM (n = 7–8).

	Normoxia	Hypoxia
T_{amm} (μmol/L)	323 ± 68	159 ± 61
PNH_3 (μTorr)	56 ± 11	31 ± 14

4.4. Effects of hypoxia exposure on MO_2, ventilation, gill permeability and plasma homeostasis

MO_2 decreased by 49% in hagfish during hypoxia exposure (Fig. 6A), similar to previous observations in both the Pacific hagfish (Clifford et al., 2016) and the New Zealand hagfish (Forster, 1990). While the critical oxygen tension (Pcrit) of *E. cirrhatus* was measured as 45 Torr at 11 °C by Forster (1990), the Pcrit of *E. stoutii* has been reported as only 11 Torr at 5 °C by Drazen et al. (2011). Our hypoxia exposures, which were performed at 12 °C, showed that MO_2 was significantly depressed at a PO_2 of 30 Torr (4 kPa). Therefore, the transition between regulating oxygen consumption and oxyconformation at 12 °C may have happened at a higher, as yet unknown, PO_2 in *E. stoutii*.

In hypoxia, the pattern of ventilation changed to higher frequency (Fig. 7A) and lower pressure amplitude [Fig. 7B, i.e. lower stroke volume, Eom and Wood, 2019], and total ventilatory water flow, as indicated by the ventilatory index (Fig. 7C), was moderately elevated in hypoxia. Thus, contrary to part of our initial hypothesis, the animals were not reducing ventilation, but rather attempting to increase O_2 uptake under hypoxia by increasing ventilation. It appears that hagfish utilize a strategy to increase breathing under low O_2 supply that differs from that of most teleosts, where ventilation amplitude is elevated and frequency is decreased, an adjustment that is thought to conserve energy (Perry, 2011). Our results are in agreement with Perry et al. (2009) where during hypoxia, hagfish markedly increased ventilation frequency. However, in their study, MO_2 was not altered by hypoxia exposure, whereas in our study, we see a significant reduction in MO_2 (Fig. 6A). As the authors mentioned, it is possible that the metabolic cost of hyperventilation in hagfish, where flow is generated through the velum pump, is lower than in species where ventilation is powered by both buccal and opercular pumps (Perry et al., 2009). Hagfishes often encounter hypoxia in the wild, either by inhabiting oxygen-depleted environments, such as anoxic sediments, or through their feeding style of burrowing their heads into the coelomic cavities of decaying carrion (Axelsson et al., 1990; Clifford et al., 2015; Forster et al., 1992). Likely due to their life history, hagfishes are extremely tolerant to O_2 starvation, exhibiting periods of spontaneous apnea under normoxia (Eom and Wood, 2019) and being able to maintain up to 70% of normoxic cardiac performance even during complete anoxia (Cox et al., 2010).

We had also hypothesized that hagfish, when exposed to hypoxia, would reduce gill permeability, differing from the prediction (increased permeability) of the "classic" osmorespiratory compromise (see Introduction). Our hypothesis was confirmed inasmuch as J_{H2O} was again reduced (Fig. 6C), as it had been during the salinity challenges, and there were no disturbances in plasma ions or osmolality (Table 1). Recent studies have identified several teleost species that also exhibit responses during hypoxia that differ from those of the "classic" osmorespiratory compromise. Robertson et al. (2015b) investigating 12 species of temperate and tropical teleosts have found that only 5 exhibit the typical predicted osmorespiratory compromise response of increased fluxes when exposed to hypoxia. Wood et al. (2007) and Wood et al. (2009) exposed the hypoxia-tolerant Amazonian oscar (*Astronotus ocellatus*) to hypoxia, and observed a downregulation of both transcellular and paracellular permeability of the gills to ions and ammonia, suggesting that this species attempts to maintain O_2 uptake at the gills without compromising ionoregulatory balance. Moreover, the oscar hyperventilated during hypoxia (Scott et al., 2008; Wood et al., 2009). Overall, these reports together with the present findings raise the interesting but as yet unanswered question of why hypoxia-intolerant, athletic species such as the rainbow trout do not adopt the same strategy. Gilmour and Perry (2018) discuss some of the newest discoveries on the diverging osmorespiratory compromise responses.

The present results indicate that the Pacific hagfish, an extant representative of the earliest craniates, can be added to the list of species that are capable of reducing gill permeability to ions and water during hypoxia, thereby avoiding the resulting effects of the classic osmorespiratory compromise. However, in a recent study by our group, we have identified that Pacific hagfish are very susceptible to increases in temperature, showing elevated MO_2 and increases in the fluxes of ammonia and water (Giacomin et al., 2018). We found that in response to an increase in metabolic demand, caused by rising temperatures, hagfish exhibited the "classic" osmorespiratory compromise (see Introduction), an opposite response to what has been shown in the present study. Exposure to hypoxia causes physiological responses that differ from the ones elicited by temperature. The subtle differences in the nature of the physiological adjustments that follow exposure to temperature and hypoxia could be responsible for the diverging patterns of osmorespiratory compromise between the present study and the results by (Giacomin et al., 2018). In future, it will be of interest to see if hagfish exhibit morphological responses of the branchial epithelium similar to those observed in other hypoxia-tolerant fish (De Boeck et al., 2013; Matey et al., 2011; Wood et al., 2009) under hypoxia.

5. Conclusions and future directions

We have provided new evidence supporting the idea that hagfish possess some ability to regulate plasma [Na^+] and [Cl^-], in addition to their previously known ability to regulate [Ca^{2+}] and [Mg^{2+}], when exposed to different salinities, although the mechanisms involved are still unknown. Future studies should focus on the role of already identified ion transporting proteins at the gills and skin in this ionoregulation, as well as its adaptive significance. We have also shown that exposure to altered salinity changes the metabolic demand of hagfish, with accompanying effects on the rates of ammonia excretion and diffusive water flux. Benthic habitats are often hypoxic, and hagfishes are additionally exposed to hypoxia in the wild due to their feeding style, where they burrow their heads into putrefying carcasses. Therefore, we evaluated the responses to acute hypoxia exposure within the context of the osmorespiratory compromise and found that despite an elevation of ventilation, permeability to water and ammonia excretion were reduced. Altogether, these results point to a strategy to deal with the osmorespiratory compromise that is typical of hypoxia-tolerant species. Since the commercial exploitation of hagfish has increased drastically in recent years, understanding of the basic physiology and general ecology are crucial for the development of sustainable fisheries practices.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2019.03.007.

Acknowledgements

This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to C.M.W. and P.M.S. M.G. was supported by a four-year graduate fellowship from the University of British Columbia. G.D.P was supported by the Brazilian Federal Agency for Support and Evaluation of Graduate Education (CAPES) with a visiting PhD scholarship (grant number 88881.134304/2016-01). We would like to thank Dr. Eric Clelland (Bamfield Marine Sciences Centre research coordinator) for excellent logistic support and Dr. Alex Clifford for providing helpful advice with calculations.

Conflict of interest declaration

The authors declare no conflict of interests in this submission either financial or otherwise.

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