



# Acute temperature effects on metabolic rate, ventilation, diffusive water exchange, osmoregulation, and acid–base status in the Pacific hagfish (*Eptatretus stoutii*)

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Received: 1 May 2018 / Revised: 30 October 2018 / Accepted: 5 November 2018 / Published online: 27 November 2018  
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## Abstract

The Pacific hagfish (*Eptatretus stoutii*) is a representative of the most basal extant craniates, and is a marine osmoconformer with an extremely low metabolic rate ( $MO_2 = 475 \mu\text{mol O}_2/\text{kg/h}$  at  $12^\circ\text{C}$ ). We investigated potential physiological trade-offs associated with compensatory changes in gill ventilation and perfusion when  $12^\circ\text{C}$ -acclimated hagfish were acutely exposed to  $7^\circ\text{C}$  or  $17^\circ\text{C}$ , as reflected in diffusive unidirectional water flux ( $J_{H_2O}$ , measured with tritiated water:  $^3\text{H}_2\text{O}$ ), net ammonia flux ( $J_{\text{amm}}$ ), and plasma ion and acid–base status.  $J_{H_2O}$  was high ( $\sim 1.4 \text{ L/kg/h}$  at  $12^\circ\text{C}$ ) in comparison to marine teleosts and elasmobranchs.  $MO_2$  increased linearly with temperature ( $R^2 = 0.991$ ), and was more sensitive ( $Q_{10} = 3.22$ ) in the  $12$ – $7^\circ\text{C}$  range than either  $J_{\text{amm}}$  ( $1.86$ ) or  $J_{H_2O}$  ( $1.35$ ), but the pattern reversed from  $12$  to  $17^\circ\text{C}$  ( $Q_{10}$ s:  $MO_2 = 2.77$ ,  $J_{\text{amm}} = 2.88$ ,  $J_{H_2O} = 4.01$ ). Heart rate, ventilatory index (a proxy for total ventilation), and coughing frequency also increased but with different patterns. At  $17^\circ\text{C}$ , plasma  $[\text{Ca}^{2+}]$  and  $[\text{Mg}^{2+}]$  decreased, although osmolality increased, associated with elevations in plasma  $[\text{Na}^+]$  and  $[\text{Cl}^-]$ . Blood pH and  $\text{PCO}_2$  were unaffected by acute temperature changes while  $[\text{HCO}_3^-]$  increased. Hyperoxia ( $\text{PO}_2 > 300 \text{ Torr}$ ) attenuated the increase in  $J_{H_2O}$  at  $17^\circ\text{C}$ , did not affect  $J_{\text{amm}}$ , and had diverse effects on plasma ion and acid–base status. Our results suggest a clear osmorepiratory compromise occurring for the diffusive water fluxes as a result of acute temperature changes in this osmoconformer.

**Keywords** Temperature · Gill permeability · Hagfish · Metabolic rate · Osmoconformer · Tritiated water · pH regulation

## Introduction

Hagfish are marine jawless vertebrates that are considered to be osmoconformers, maintaining plasma  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  at concentrations that closely resemble those of sea water (Robertson 1976), although they have the ability to regulate plasma  $[\text{Ca}^{2+}]$  and  $[\text{Mg}^{2+}]$  when acclimated to salinities above and below that of normal sea water (Sardella et al. 2009). They are also known to be excellent regulators of acid–base status (Clifford et al. 2014; Baker et al. 2015). In teleost fishes, which are osmoregulators, a phenomenon termed the osmorepiratory compromise occurs (Randall et al. 1972; Nilsson 1986; Gonzalez 2011). The osmorepiratory compromise represents a tradeoff between osmoregulation and oxygen uptake. Under conditions when energy demand increases, physiological mechanisms such as increased gill ventilation, perfusion, and effective gas permeability must be activated to increase oxygen uptake, but these changes may cause undesirable passive fluxes of ions, osmolytes and water. Although the osmorepiratory

Communicated by G. Heldmaier.

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compromise is well documented in teleost fishes, the extent to which this phenomenon would be expected to occur in osmoconformers remains unclear. There is some evidence for an osmorepiratory compromise in Chondrichthyan fishes, which are osmoconformers but ionoregulators (Wright and Wood 2016). For example, fluxes of ammonia, urea, and water (diffusive water flux) in the Pacific spiny dogfish shark (*Squalus acanthias suckleyi*) all increase to a greater extent than does  $\text{MO}_2$  in response to acute increases in temperature (Giacomin et al. 2017). However, the presence or extent of an osmorepiratory compromise has not previously been examined in species, such as hagfishes, whose plasma  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  are similar to those in seawater. In addition, hagfishes exhibit very low oxygen consumption rates ( $\text{MO}_2$ ; e.g., Munz and Morris 1965; Clifford et al. 2016), often less than 20% of those in comparably sized jawed fishes (Clarke and Johnston 1999), again opening the possibility of a minimal osmorepiratory compromise in these animals.

Here, we investigate the osmorepiratory compromise in Pacific hagfish *Eptatretus stoutii*, through increasing their metabolic rate by exposing them to acute temperature increases in the laboratory. Very little is known about the physiological responses of hagfishes to changes in temperature, although when acclimated to 10 °C, Pacific hagfish linearly increase  $\text{MO}_2$  when exposed acutely from 5 to 15 °C with a  $Q_{10}$  of 2.4 (Munz and Morris 1965), which is relatively high compared to that of most teleost fishes (Wood 2001; Lea et al. 2016). In nature, hagfish are thought to be found in thermally stable environments. For example, Pacific hagfish have been captured at depths where temperature varied only between 10 and 11 °C, and thus their tolerance to temperature fluctuations is thought to be low (McInerney and Evans 1970). However, *Eptatretus stoutii* tolerated short exposures to 29 °C when maintained at 22 °C for a few days (Worthington 1905). Hagfish also may swim near the surface (Adam and Strahan 1963) where they could potentially be exposed to sharp changes in temperature, as well as hyperoxic conditions during phytoplankton blooms. Recently a novel species (*Eptatretus strickrotti*) has been found near hydrothermal vents, raising the question of whether hagfishes are confined to low temperature environments (Moller and Jones 2007). In addition, the increased commercial demand for hagfish and the current use of fishing practices that result in hagfish being exposed to large acute changes in temperature (as well as in salinity and oxygen concentration; Olla et al. 1998) necessitate an understanding of how hagfish react to such challenging conditions.

Our initial goal was to evaluate the effects of acute temperature changes on the rates of oxygen consumption, ammonia excretion, diffusive water flux (measured with tritiated water), and on ventilation and heart rate in the Pacific hagfish *Eptatretus stoutii*, an animal which is known to be an

ionoconformer for major ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) but an excellent regulator of plasma  $[\text{Ca}^{2+}]$ ,  $[\text{Mg}^{2+}]$ , and acid–base status. We hypothesized that if there was a permeability trade off at the gills accompanying increases in ventilation or heart rate as  $\text{MO}_2$  increased with temperature, the fluxes of ammonia and water would also increase. We also hypothesized that unfavorable fluxes of ions and water would be reflected in perturbed plasma homeostasis. Additionally, we investigated the possible role of environmental hyperoxia ( $\text{PO}_2 > 300$  Torr) in attenuating the effects of exposure to high temperature. Our hypothesis here was that by increasing oxygen supply at the gills (through hyperoxia), the hypothesized trade-offs for ion regulation at high temperature would be minimized.

## Materials and methods

### Animal housing and acclimation

Pacific hagfish (*Eptatretus stoutii*; average mass =  $46.5 \pm 0.8$  g) were caught using bottom-dwelling traps (rotting hake *Merluccius productus* was used as bait), from Trevor Channel, near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada, under Fisheries and Oceans Canada collecting permit XR1942017. The traps consisted of 22 L plastic buckets, fitted with a modified lid and containing a conical entrance, which allowed the hagfish to enter the bucket but prevented exit. The traps were placed at approximately 80 m depth, and recovered after 8 h. At BMSC hagfish were held in 200 L darkened tanks, served with flow-through fully aerated sea water (12–13 °C, 30 ppt). During acclimation, food was offered in the form of hake strips, but the fish did not feed. Therefore, fish were fasted for at least 1 week, and for a maximum of 3 weeks prior to experimentation. 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). After completion of experiments, animals 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.

### Effects of acute temperature changes

#### Oxygen consumption, ammonia excretion, and blood parameters

All experiments were carried out using darkened, 1 L glass jars topped with a fine plastic mesh, fitted with fine tubing providing gentle aeration and served with flow-through sea water at 50 mL/min. The effect of acute temperature changes on hagfish  $\text{O}_2$  consumption rates ( $\text{MO}_2$ ) and ammonia flux

rates ( $J_{\text{amm}}$ ), as well as blood acid–base and ion status, were assessed at 12 °C (control=acclimation temperature), and after acute transfer to 7 and 17 °C. Fish were first allowed to settle in the experimental chambers for a minimum of 6 h before the start of the experiment. For the 12 °C experiments, the water flow to the jar was stopped and the volume of water was set to exactly 1 L. For the acute 7 or 17 °C exposure, the jars were gently tipped so that almost all the control water was drained, and new water at the prepared temperature was poured inside until 1 L of water at the exact temperature was reached. This procedure was chosen as it avoided touching the hagfish and prevented the formation of slime. Temperatures of 7, 12 and 17 °C were maintained by partially submerging the jars in a thermostatted water bath.

Experiments started 0.5 h after an adjustment period to the new temperature. Water samples (5 mL) were taken at 0, 3, and 6 h (for the 7 and 12 °C experiments), and at 0, 2, 4 and 6 h (for the 17 °C experiments), and immediately frozen at –20 °C for later analyses of ammonia concentration. During the water sampling intervals, the jars were sealed with gas-impermeable dental dam, right after an initial  $\text{PO}_2$  value was taken using an Accumet AP84 handheld  $\text{O}_2$  meter (Fisher Scientific, Toronto, ON, Canada). The detection limit of the oxygen probe was ~0.5 Torr. Simultaneous with water sampling at the end of an interval, a final  $\text{PO}_2$  was measured, and then the jars were lightly bubbled with air for re-oxygenation, the volume was replaced, and the procedure was repeated until the end of the experiment. During the periods where the chamber was sealed, the rate of  $\text{PO}_2$  decrease varied from 0.15 to 0.45 Torr/min, and the final value never fell below 75 Torr. The differences between the initial and final  $\text{PO}_2$  or total ammonia values in each interval were used to calculate  $\text{MO}_2$  and  $J_{\text{amm}}$  (see below), which are reported as an average of the multiple measurements (2 or 3 depending on temperature).  $\text{MO}_2$  was not measured in the hyperoxia series as we did not have an  $\text{O}_2$  probe that measured  $\text{PO}_2$  accurately at that high background. At the end of an experimental day, all jars were rinsed with 70% ethanol to prevent any bacterial build-up. A blank trial (respirometer with no fish in it) was run in the same fashion as the real experiments, every time a respirometry trial was run. No significant changes in  $\text{PO}_2$  or ammonia were observed throughout the duration of the blank trials.

At the end of the experiment, fish were quickly transferred to MS-222 (0.6 g/L neutralized with 5 M NaOH) solution, and once fish were anesthetized, a blood sample (~500  $\mu\text{L}$ ) was drawn from the posterior sinus using a heparinized gas-tight syringe (Hamilton, Reno, NV, USA). Fresh blood was immediately transferred to a temperature-controlled water bath, and blood pH was measured using an MI-4156 Micro-Combination pH probe (Microelectrodes Inc., Bedford, NH, USA) and Accumet pH meter (Fisher Scientific, Toronto, ON, Canada). After that, plasma was separated by

centrifugation (12,000g for 3 min), aliquoted, flash frozen in liquid  $\text{N}_2$  and stored at –80 °C for later analyses. Fish were then immediately weighed and euthanized as described above (“[Animal housing and acclimation](#)”). Sea water samples were also taken in all trials and similarly frozen for comparison to plasma ionic composition and osmolality.

### Ventilation and heart rate

A separate group of fish was used for this experimental series. The surgical procedures used here are the same as those outlined in detail in Eom and Wood (2018). Briefly, hagfish were anesthetized in neutralized MS-222 (0.6 g/L sea water) for <5 min and transferred to an operating table. A small piece of flexible plastic tubing was inserted snugly into the duct of the single nostril and stitched to the skin (26 mm 1/2C taper, Perma-Hand Silk, Ethicon, Somerville, NJ, USA). A ~2 cm PE160 tubing (BD, Intramedic, Franklin Lakes, NJ, USA) was secured inside the plastic tubing and this was then bridged to a connecting PE 160 catheter using a blunt #18-gauge needle when necessary for recording. The water-filled PE 160 tubing was connected to a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA) so that pressure amplitude (cm  $\text{H}_2\text{O}$ ) and frequency (breaths/min) could be recorded at the nostril duct. The pressure transducer was calibrated against a 4-cm water column. Two laminated copper wires (AWG #32, Belden, Chicago, IL, USA) were stripped of their insulation for about 1 cm at the recording end, and placed slightly under the skin (as “fish hook” electrodes), in the abdomen, posterior to the 12th gill pouch so as to monitor the branchial heart rate. The wires were stitched to the skin as described above. Heart beat frequency was collected by connecting the wires to an impedance converter (Model 2991, UFI, Morro Bay, CA, USA). Both analog signals were amplified by an amplifier (LCA-RTC, Transducer Techniques, Temecula, CA, USA), and digitalized in a PowerLab Data Integrity system (ADInstruments, Colorado Springs, CO, USA), and visualized and analyzed using LabChart v. 7.0 (ADInstruments).

Following surgery, fish were allowed to recover overnight in darkened plastic containers supplied with flow-through control seawater. Two different series of experiments were conducted. First, ventilation and heart rate were recorded for 5 min at 12 °C, then the water was gently replaced with flowing water at either 7 or 17 °C, until the total volume (1 L) of the containers had reached a stable temperature. Animals were allowed to adjust to the new temperature for 30 min, and ventilation and heart rate were recorded for additional 5 min.

In the second experimental series, ventilation alone was recorded only at 12 °C and 17 °C, in normoxic and hyperoxic conditions, and a longer term (6 h) exposure was employed to match the time of blood sampling. Similar protocols as

stated above were used to change the water and adjust temperature. For the hyperoxia experiments, water that had been bubbled with pure O<sub>2</sub> to a PO<sub>2</sub> > 300 Torr was introduced at the proper temperature. Animals were allowed to adjust to the new conditions for 6 h, then ventilation was recorded for 5 min. Note that in this series, heart rate could not be recorded as the animals invariably displaced the impedance leads over this longer (6-h) period.

The ventilation frequency (breaths/min) reported here represents the frequency of the beat of the velum, and the pressure amplitude (cm H<sub>2</sub>O) represents the difference between average maximum and average minimum pressure in the ventilatory cycle. Eom and Wood (2018) have shown that there is a direct correlation between pressure amplitude and ventilatory stroke volume, and that ventilatory index (cm H<sub>2</sub>O/min), calculated as the product of frequency and pressure amplitude, is directly correlated with the rate of total ventilatory flow. The software measured frequency and pressure amplitude in the short periods in between coughs, and the frequency of coughing (coughs/min) was tabulated separately.

### Diffusive water exchange

A separate group of fish was used for the assessment of diffusive water exchange. Water exchange rates were measured using radiolabelled tritiated water (<sup>3</sup>H<sub>2</sub>O). Preliminary experiments demonstrated that water exchange rates were high, such that complete equilibration of <sup>3</sup>H<sub>2</sub>O between the internal compartment of the hagfish and the external sea water occurred within 6 h. In each experimental treatment, hagfish (*N* = 8) were incubated as a group in 20 µCi/L of <sup>3</sup>H<sub>2</sub>O (Amersham Pharmacia Biotech, Little Chalfont, UK) in control sea water at 12 °C under normoxia for 6 h in a darkened 4 L glass container placed in a water bath for temperature control. At the end of the loading period, duplicate 5 mL water samples were taken to assess water specific activity at equilibrium. The fish were removed from the incubation container, quickly placed in an intermediate basin containing sea water free of radioactivity for a cold displacement rinse, and then transferred to individual experimental chambers (darkened 1 L glass jars identical to those described above). The jars contained normoxic sea water at the desired water temperature (7, 12 or 17 °C) according to the trial being performed. Temperature control was achieved as described above. Water samples (5 mL) were taken at 5-min intervals from 0 to 30 min, and 10-min intervals thereafter from 30 to 70 min. Final samples were taken at the next day at 9–14 h to assess specific activity of the system (hagfish plus external water) at equilibrium after complete <sup>3</sup>H<sub>2</sub>O washout. Scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) was immediately mixed to the water samples in a 2:1 ratio (fluor:water), and samples were stored

in the dark for a minimum of 12 h to eliminate chemiluminescence prior to counting for beta emissions on a Triathler portable counter (Hidex, Helsinki, Finland). Tests showed that quench was constant.

### Interactive effects of temperature changes and hyperoxia

A separate group of hagfish were used for these tests. In the first set of experiments, the effects of acute hyperoxia exposure (PO<sub>2</sub> > 300 Torr) on *J*<sub>amm</sub> and blood parameters were assessed at 12 °C and after acute exposure to 17 °C. In a second set, the effects of the same treatments on diffusive water exchange rates were determined. The experimental apparatus and procedures used to reach and maintain temperature were the same as those described earlier, the only difference being that the sea water was bubbled with pure O<sub>2</sub> until it reached a PO<sub>2</sub> > 300 Torr in the hyperoxia tests. PO<sub>2</sub> was monitored throughout the experiments using the same handheld O<sub>2</sub> meter and the container was reoxygenated as necessary.

In the first set of experiments, procedures were identical to those described earlier, though it proved to be impractical to measure O<sub>2</sub> consumption under hyperoxia. In the second set, procedures were again similar. Note that the fish were loaded with <sup>3</sup>H<sub>2</sub>O under normoxic conditions at 12 °C, and then acutely transferred to the experimental treatments (12 or 17 °C, with or without hyperoxia).

### 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.

Ammonia concentration in the water was measured colorimetrically according to Verdouw et al. (1978). Ammonia (*J*<sub>amm</sub>, µmol/kg/h) flux rate was calculated using the following equation:

$$J_{\text{amm}} = [(A_{\text{mm}(f)} - A_{\text{mm}(i)}) \times V] / (W \times t) \quad (1)$$

where *A*<sub>mm(*f*)</sub> and *A*<sub>mm(*i*)</sub> are the final and initial water ammonia (µmol/L) concentrations, respectively; *V* is the volume of water used (L); *W* is the body mass of the fish (kg); *t* is the duration of the flux period (h).

Oxygen consumption rates (MO<sub>2</sub>; µmol O<sub>2</sub>/kg/h) were calculated with the following equation:

$$\text{MO}_2 = [((\text{PO}_{2(i)} - \text{PO}_{2(f)}) \times \alpha_{\text{O}_2}) \times V] / (W \times t) \quad (2)$$

where PO<sub>2(*i*)</sub> and PO<sub>2(*f*)</sub> are the initial and final water PO<sub>2</sub> (Torr), respectively; α<sub>O<sub>2</sub></sub> (µmol/Torr/L) is the oxygen solubility constant from Boutilier et al. (1984) at the appropriate temperature and salinity; *W* is the body mass of the fish (kg); *t* is the duration of the measurement period (h).



Since ammonia excretion rate ( $J_{\text{amm}}$ ) and oxygen consumption ( $\text{MO}_2$ ) were measured concomitantly, the ammonia quotient (AQ) was calculated:

$$\text{AQ} = J_{\text{amm}}/\text{MO}_2 \quad (3)$$

where  $J_{\text{amm}}$  was calculated using Eq. 1 and  $\text{MO}_2$  calculated using Eq. 2.

The temperature coefficient ( $Q_{10}$ ) was calculated using the average values for the physiological rate of interest at each experimental increment as:

$$Q_{10} = (R_2/R_1)^{10/(T_2-T_1)} \quad (4)$$

where  $R_1$  and  $R_2$  are the rates measured at temperature 1 ( $T_1$ , °C) and temperature 2 ( $T_2$ , °C).

The ventilatory index ( $\text{cm H}_2\text{O}/\text{min}$ ) was calculated using the following equation:

$$\text{ventilatory index} = \text{frequency} \times \text{pressure amplitude} \quad (5)$$

where frequency represents velar breaths/min and amplitude is in  $\text{cm H}_2\text{O}$ .

Plasma total ammonia ( $T_{\text{amm}}$ ;  $\mu\text{mol}/\text{L}$ ) was determined using a commercial kit (Raichem, Clinica, San Marcos, CA, USA). Due to background interference by hagfish plasma, a standard curve was created using hagfish plasma as a matrix spiked with increasing concentrations of  $\text{NH}_4\text{Cl}$ , to compensate for the matrix effect. The concentration of ammonia in the plasma was calculated by the standard addition method. Plasma  $\text{TCO}_2$  content was measured using a total  $\text{CO}_2$  analyzer (Corning 965  $\text{CO}_2$  analyzer, Ciba Corning Diagnostic, Halstead, Essex, UK). Plasma ion concentrations ( $\text{mmol}/\text{L}$ ) were determined by a chloridometer (Radiometer CMT10, Copenhagen, Denmark) for  $[\text{Cl}^-]$ , or by atomic absorption (1275 Atomic Absorption Spectrophotometer, Varian, Mulgrave, Victoria, Australia) for  $[\text{Na}^+]$ ,  $[\text{Ca}^{2+}]$ , and  $[\text{Mg}^{2+}]$  using certified commercial solutions as standards (Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). Plasma osmolality was measured using a Wescor vapor pressure osmometer and standards (Wescor 5100C, Logan, UT, USA).

The solubility coefficient of carbon dioxide in hagfish ( $\alpha\text{CO}_2$ ;  $\text{mmol}/\text{L}/\text{Torr}$ ) plasma was calculated for the three different temperatures using the following equation from Heisler (1984):

$$\begin{aligned} \alpha\text{CO}_2 = & 0.1008 - 9.8 \times 10^{-3}(M) + (1.218 \times 10^{-3}(M) - 3.639 \times 10^{-3}) \times T \\ & - (19.57 \times 10^{-6}(M) - 69.59 \times 10^{-6}) \times T^2 \\ & + (71.71 \times 10^{-9}(M) - 559.6 \times 10^{-9}) \times T^3 \end{aligned} \quad (6)$$

where  $M$  is the measured osmolality ( $\text{mOsm}/\text{kg}$ ) and  $T$  is temperature (°C).

The apparent pK of  $\text{CO}_2$  ( $\text{pK}_{\text{app}}$ ) in hagfish plasma was calculated using the following equation from Heisler (1984):

$$\begin{aligned} \text{pK}_{\text{app}} = & 6.583 - 13.41 \times 10^{-3}(T) + 228.2 \times 10^{-6}(T^2) \\ & - 1.516 \times 10^{-6}(T^3) - 0.341M^{0.323} \\ & - \log\{1 + 0.00039[\text{Pr}] + 10^{\text{pH}} \\ & - 10.64 + 0.011T + (0.737M^{0.323}) \\ & \times (1 + 10^{1.92 - 0.01T - (0.737M^{0.323})} + \log[\text{Na}^+] \\ & + (-0.494M + 0.651)(1 + 0.0065[\text{Pr}]))\} \end{aligned} \quad (7)$$

where  $M$  is the measured osmolality ( $\text{mOsm}/\text{kg}$ ),  $T$  is temperature (°C),  $[\text{Na}^+]$  is the measured sodium concentration ( $\text{mol}/\text{L}$ ) and  $[\text{Pr}]$  is a constant amino acid concentration of 1 g/L (Cholette et al. 1970; Currie and Edwards 2010). With those two calculated parameters, plasma  $\text{PCO}_2$  (Torr) was calculated from measured  $\text{TCO}_2$  and pH values using a modified Henderson–Hasselbalch equation:

$$\text{PCO}_2 = \text{TCO}_2 / [\alpha\text{CO}_2 \times (1 + \text{antilog}(\text{pH} - \text{pK}_{\text{app}}))] \quad (8)$$

where  $\alpha\text{CO}_2$  ( $\text{mmol}/\text{L}/\text{Torr}$ ) was calculated using Eq. 6, and  $\text{pK}_{\text{app}}$  was calculated using Eq. 7.

Plasma  $[\text{HCO}_3^-]$  ( $\text{mmol}/\text{L}$ ) was calculated as:

$$[\text{HCO}_3^-] = \text{TCO}_2 - (\alpha\text{CO}_2 \times \text{PCO}_2) \quad (9)$$

where  $\text{PCO}_2$  was calculated using Eq. 8.

Plasma  $[\text{NH}_4^+]$  was calculated using a modified Henderson–Hasselbalch equation and  $\text{pK}'$  values obtained from Cameron and Heisler (1983) adjusted to hagfish plasma NaCl concentration:

$$[\text{NH}_4^+] = T_{\text{amm}} / [1 + \text{antilog}(\text{pH} - \text{pK}')] \quad (10)$$

where  $T_{\text{amm}}$  is the measured total plasma ammonia ( $\mu\text{mol}/\text{L}$ ). The partial pressure of  $\text{NH}_3$  ( $\text{PNH}_3$ ;  $\mu\text{Torr}$ ) was calculated using the following equation:

$$\text{PNH}_3 = (T_{\text{amm}} - [\text{NH}_4^+]) / \alpha\text{NH}_3 \quad (11)$$

where  $\alpha\text{NH}_3$  is the solubility coefficient of  $\text{NH}_3$  in hagfish plasma ( $\mu\text{mol}/\text{L}/\text{Torr}$ ) plasma, was obtained from Cameron and Heisler (1983) for the three temperatures tested in the present study.

The rate constant of  $^3\text{H}_2\text{O}$  turnover ( $k$ ) was calculated from the exponential rate of decline in total  $^3\text{H}_2\text{O}$  radioactivity in the fish (Evans 1967):

$$k = (\ln R_1 - \ln R_2) / (t_1 - t_2) \quad (12)$$

where  $k$  is the rate constant of the efflux ( $\text{in h}^{-1}$ ), and  $R_1$  and  $R_2$  are total  $^3\text{H}_2\text{O}$  radioactivity ( $\text{in cpm}$ ) in the fish at times  $t_1$  and  $t_2$  ( $\text{in h}$ ). The product of  $k \times 100$  yields the percent of body water turned over per hour. In practice, the rate

constant  $k$  for  $^3\text{H}_2\text{O}$  efflux was calculated by regression of the natural logarithm of  $R$  against time over the range of linearity (generally 5–30 min after transfer to the experimental chambers). By measuring the radioactivity in the water after 9–14 h, when complete  $^3\text{H}_2\text{O}$  washout and equilibration between the fish and the water had occurred, multiplying by the known volume of the system (fish plus water), and then adding radioactivity removed in sampling, it was possible to accurately calculate the total amount of  $^3\text{H}_2\text{O}$  radioactivity ( $R_{\text{total}}$ , cpm) that the fish had originally taken up during the loading period. Therefore, from  $R_{\text{total}}$  and from measurements of  $^3\text{H}_2\text{O}$  radioactivity appearance in the water at each time interval, it was possible to back-calculate  $R$  in the fish at each time during the experiment for use in the regression of  $\ln R$  against time in Eq. 12. It was also possible to calculate

the exchangeable internal water volume of each fish ( $V_{\text{H}_2\text{O}}$ , mL/kg) as:

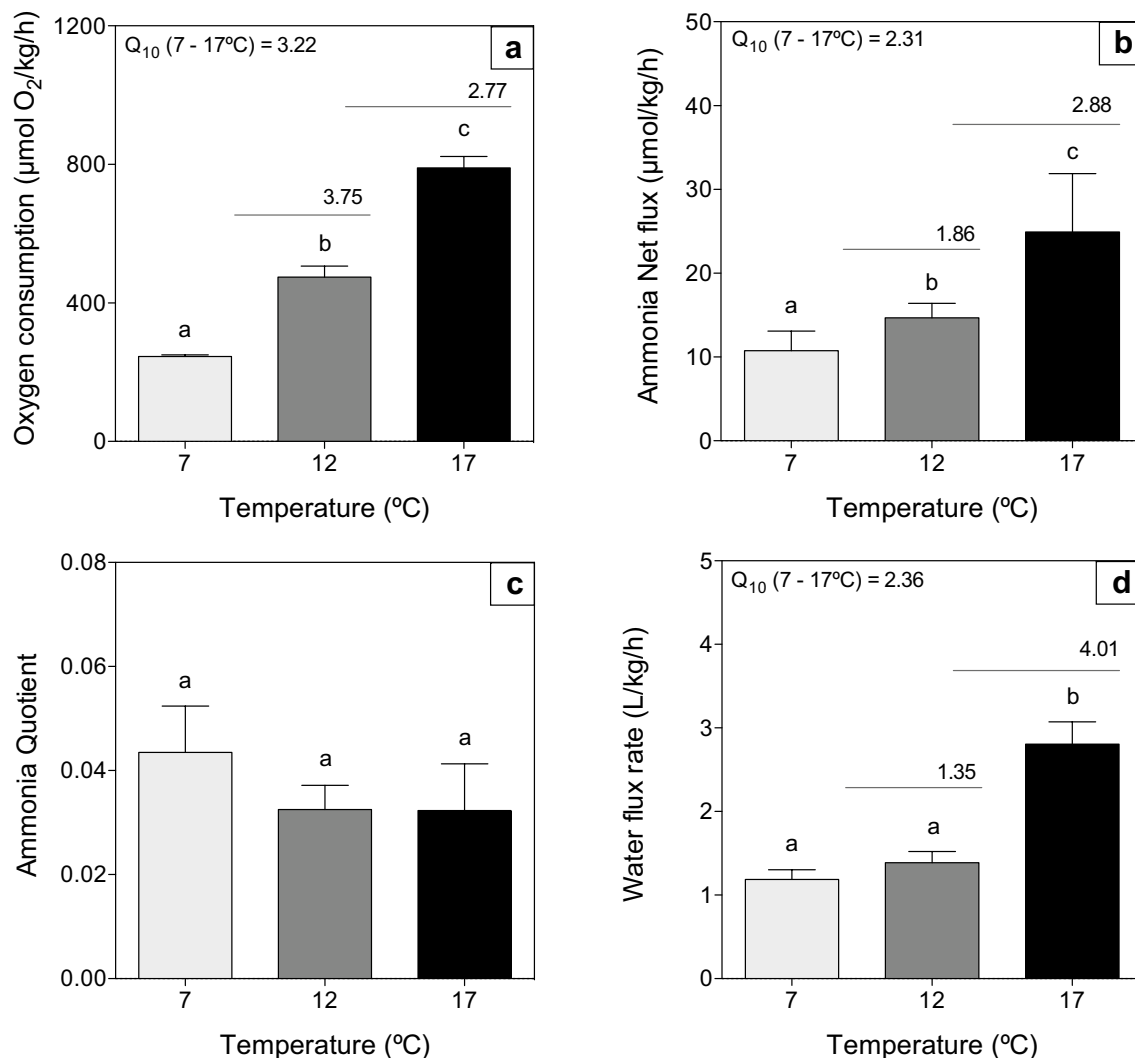
$$V_{\text{H}_2\text{O}} = R_{\text{total}} / \text{SA}_{\text{H}_2\text{O}} \times W \quad (13)$$

where  $\text{SA}_{\text{H}_2\text{O}}$  is the specific activity (cpm/mL) of the external water at the end of the original  $^3\text{H}_2\text{O}$  loading period, and  $W$  is body mass in kg. The unidirectional diffusive flux rate of water ( $J_{\text{H}_2\text{O}}$ , mL/kg/h) could then be calculated as:

$$J_{\text{H}_2\text{O}} = k \times V_{\text{H}_2\text{O}}. \quad (14)$$

### Statistical analysis

All data are shown as means  $\pm$  1 SEM ( $N$  = number of animals). Requirements for using parametric statistics (normality and homogeneity of variances) were tested, and in case



**Fig. 1** **a** Oxygen consumption rate ( $\text{MO}_2$ ), **b** ammonia net flux rate ( $J_{\text{amm}}$ ), **c** calculated ammonia quotient (AQ) and **d** diffusive water flux rate ( $J_{\text{H}_2\text{O}}$ ) in *Epiplatys stoutii* acutely exposed to 7, 12 (control) and 17 °C. The  $Q_{10}$  values over the entire range (7–17 °C) are displayed on the upper left corner. The lower range (7–12 °C)  $Q_{10}$  are

shown above the 12 °C bar and the higher range (12–17 °C)  $Q_{10}$  are shown above the 17 °C bar. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey's post hoc test). Data are shown as means  $\pm$  1 SEM ( $n$  = 8–16)

**Table 1** Rate constant of  $^3\text{H}_2\text{O}$  turnover ( $k$ ) in Pacific hagfish exposed to 7, 12 and 17 °C in normoxia (series 1), and to 12 and 17 °C in both normoxia and hyperoxia (series 2)

Temperature (°C)	Rate constant ( $k$ )		
	Normoxia	Normoxia	Hyperoxia
7	$1.92 \pm 0.17^a$	–	–
12	$2.28 \pm 0.15^a$	$2.04 \pm 0.21^A$	$2.20 \pm 0.22^A$
17	$3.38 \pm 0.28^b$	$3.38 \pm 0.28^{X*}$	$2.55 \pm 0.19^X$

Series 1 data were compared through a one-way ANOVA. Different lower case letters indicate statistical significance (Tukey's post hoc test). Series 2 data were compared through a two-way ANOVA. Similar upper case letters indicate lack of significant differences between normoxia and hyperoxia at the same temperature. [Two-way ANOVA  $p$  values ( $k$ :  $p_{\text{interaction}}=0.058$ ,  $p_{\text{oxygen}}=0.002$ ,  $p_{\text{temperature}}=0.194$ )]. Asterisks indicate significant differences between 12 and 17 °C at the same oxygen level (Bonferroni post hoc test) (series 2 only). Data are means  $\pm$  1 SEM ( $n=7$ )

of failure, data were transformed either using a square root or log transformation. Data from series 2.2 were compared using one-way analysis of variance (ANOVA). Data from series 2.3, where the effects of temperature and hyperoxia were tested in combination, were evaluated using two-way ANOVA. Detailed results of the statistical tests and post-hoc analysis are shown in specific figure captions. For the one-way ANOVA, mean values were considered significantly different when  $p < 0.05$ , while for the two-way ANOVA, the Bonferroni correction for multiple comparisons was applied to define the alpha values.

## Results

### Acute temperature exposures

$\text{MO}_2$  at the control temperature of 12 °C was  $475 \mu\text{mol O}_2/\text{kg/h}$  and increased by about 65% after an acute warming to 17 °C, and decreased by about 48% after an acute cooling to 7 °C (Fig. 1a). The  $Q_{10}$ s for both 5 °C changes were high, being greater from 7 to 12 °C ( $Q_{10}=3.75$ ), and slightly lower at 12–17 °C ( $Q_{10}=2.77$ ), while the overall 10 °C  $Q_{10}$  was 3.22. The  $J_{\text{amm}}$  at the control temperature was only about  $15 \mu\text{mol/kg/h}$ .  $J_{\text{amm}}$  showed a similar pattern of temperature response as  $\text{MO}_2$ , but the  $Q_{10}$ s were on average, lower (Fig. 1b). Differently from  $\text{MO}_2$ , the  $J_{\text{amm}}$   $Q_{10}$  from 7 to 12 °C (1.86) was lower than the  $Q_{10}$  from 12 to 17 °C (2.88). The overall 10 °C  $Q_{10}$  for  $J_{\text{amm}}$  was 2.31. We were not able to measure the urea-N excretion rate, and previous studies have shown that in Pacific hagfish it is anywhere between 6–60% of the ammonia excretion (Walsh et al. 2001; Braun and Perry 2010; Wilkie et al. 2017). Therefore, we calculated the ammonia quotient (AQ) using only ammonia excretion

rate as a proxy for total nitrogen ( $\text{N}_2$ ) excretion. The AQ (0.03 at control 12 °C) was very low and did not vary over the temperature range tested (Fig. 1c). Over the temperature range from 7 to 12 °C,  $J_{\text{H}_2\text{O}}$  was the most conserved of the three rates measured, ( $Q_{10}=1.35$ ); this increase was not significant (Fig. 1d). However, the  $Q_{10}$  from 12 to 17 °C was the highest of all ( $Q_{10}=4.01$ ). At the acclimation temperature, diffusive water flux rate was about 1.4 L/kg of water per hour, and when hagfish were exposed to 17 °C, the diffusive water flux rate increased to almost 3 L/kg per hour. The full range  $Q_{10}$  (7–17 °C) was 2.36. The mean exchangeable internal water pool ( $V_{\text{H}_2\text{O}}$ ; Eq. 13) of all the fish used in this study was  $621.5 \pm 14.3 \text{ mL/kg}$  ( $n=55$ ), obtained from all fish loaded at 12 °C. Table 1 shows the rate constants of  $^3\text{H}_2\text{O}$  turnover (as calculated in Eq. 12).

The ventilation frequency at the control temperature was ~25 breaths/min and it did not differ at the three temperatures tested (Fig. 2a). This was reflected in very low  $Q_{10}$ s, where the full range  $Q_{10}$  was only 1.09 (Fig. 2a). Pressure amplitude showed a different response pattern with temperature (Fig. 2b), where there was a steady increase with temperature, however due to high variability, there was no statistical significance detected. Since this measurement is not a rate,  $Q_{10}$ s were not calculated (Fig. 2b). The ventilatory index, a proxy for total ventilatory flow (Eom and Wood 2018), is the product of ventilation rate and pressure amplitude (Fig. 2c). Over 7–12 °C, the ventilatory index was very temperature sensitive, showing a  $Q_{10}$  of 6.53. There was no significant change in the ventilatory index between 12 and 17 °C ( $Q_{10}=1.29$ ), but the ventilatory indices at both those temperatures were significantly higher than at 7 °C. The  $Q_{10}$  over the full range was 2.90. The coughing frequency (data not shown) seemed to be the most temperature sensitive metric with an overall  $Q_{10}$  of 49.5 (7–17 °C). It decreased by 92% from 12 to 7 °C, and increased by 75% from 12 to 17 °C. The heart rate (~15 beats/min at 12 °C; Fig. 2d) showed a very different pattern from the various ventilatory measurements. It did not change significantly from 7 to 12 °C ( $Q_{10}=1.38$ ), but it was significantly elevated (about 1.6-fold) at 17 °C, with a  $Q_{10}$  of 2.58 (Fig. 2d).

Plasma  $[\text{Na}^+]$  tended to rise but did not change significantly with temperature (Fig. 3a), while plasma  $[\text{Cl}^-]$  was significantly elevated at 17 °C (Fig. 3b). While mean plasma  $[\text{Na}^+]$  values (551–600 mmol/L) at all three temperatures were significantly above the mean concentration measured in SW ( $465 \pm 4 \text{ mmol/L}$ ), all plasma  $[\text{Cl}^-]$  values (452–486 mmol/L) were significantly below the mean SW concentration ( $515 \pm 7 \text{ mmol/L}$ ). In contrast to  $[\text{Na}^+]$  and  $[\text{Cl}^-]$ , plasma  $[\text{Mg}^{2+}]$  (Fig. 3c) and  $[\text{Ca}^{2+}]$  (Fig. 3d) decreased at 17 °C. Both plasma Mg (8–10 mmol/L) and  $[\text{Ca}^{2+}]$  (3–4 mmol/L) showed values well below those of seawater ( $[\text{Mg}^{2+}]=51.2 \pm 0.2$  and  $[\text{Ca}^{2+}]=8.8 \pm 0.1 \text{ mmol/L}$ ), highly significant differences. Plasma osmolality decreased

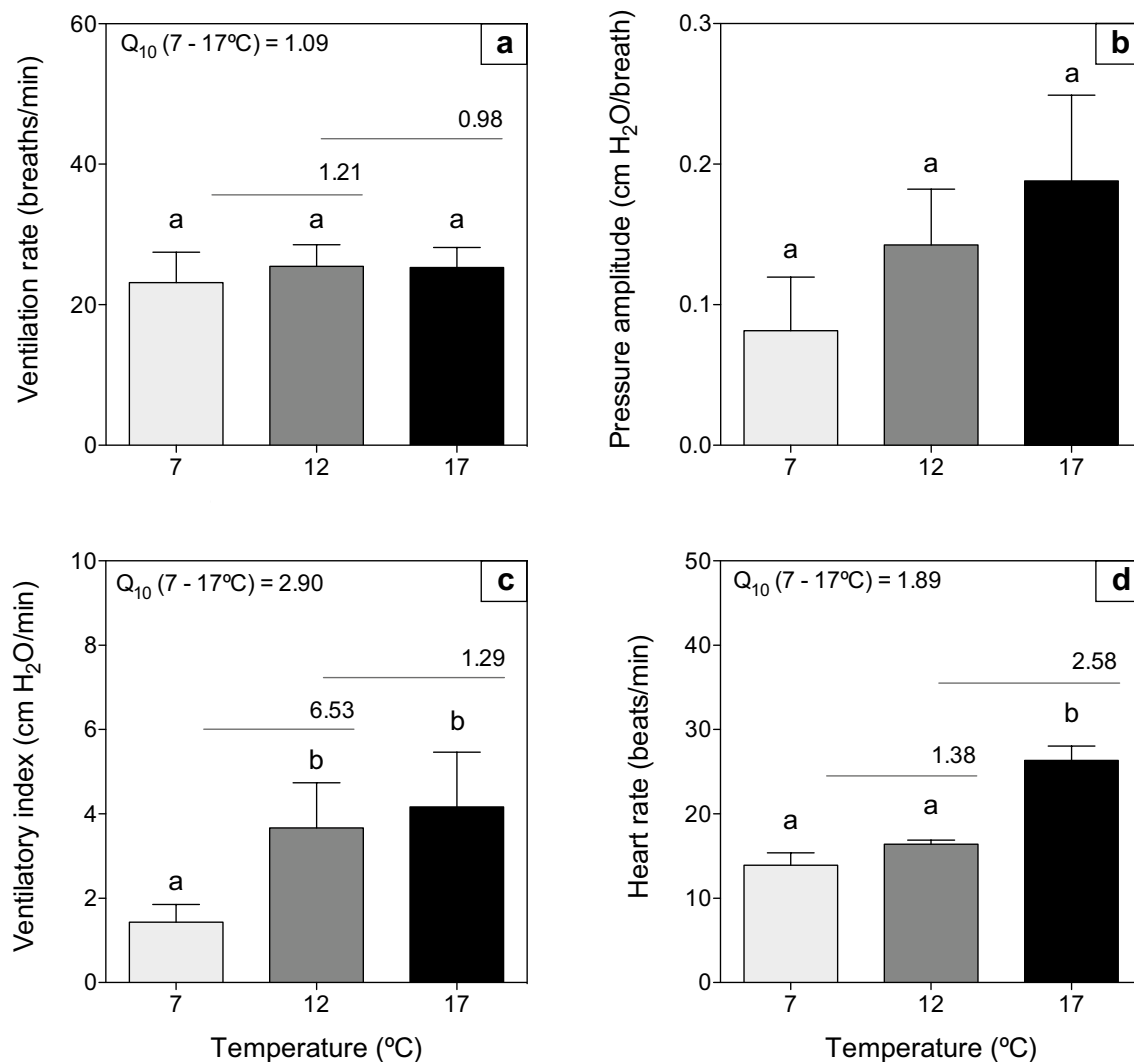
by about 50 mOsm/kg with an acute exposure to 7 °C, but did not change significantly with a 5 °C increase in temperature (Fig. 3e). With the exception of the 7 °C treatment, mean plasma osmolalities (890–948 mmol/L) in all treatments (Figs. 3e, 8e) were not significantly different from the seawater osmolality ( $958 \pm 4$  mOsm/kg).

Plasma total ammonia concentration decreased by about 60% from 12 to 7 °C but it was not altered at 17 °C (Fig. 4a) despite the 1.7-fold increase in  $J_{\text{amm}}$  at this temperature (Fig. 1b). However the calculated partial pressure of  $\text{NH}_3$  ( $\text{PNH}_3$ ) increased with temperature, and was significantly different between 7 and 17 °C (Fig. 4b). Plasma  $[\text{NH}_4^+]$  did not statistically change at the three temperatures tested (data not shown), however the data pattern resembled that of total plasma ammonia. Blood pH and plasma  $\text{PCO}_2$  were not

altered at the three temperatures tested (Fig. 5a, b). However, there was a significant elevation in plasma  $\text{HCO}_3^-$  between 7 and 17 °C (Fig. 5c).

### Interactive effects of temperature and hyperoxia

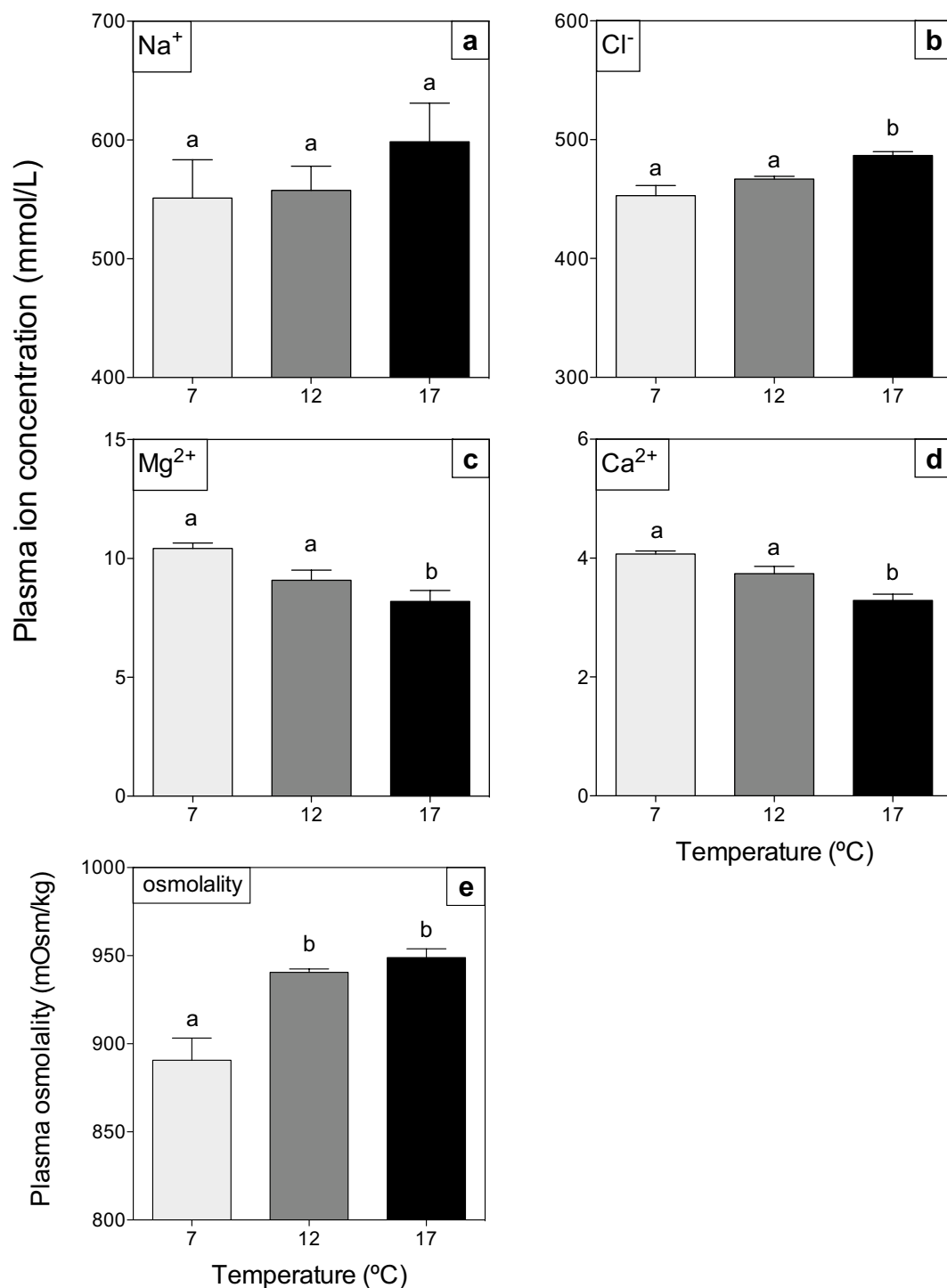
Animals exposed to hyperoxia at 12 °C showed no significant change in  $J_{\text{amm}}$  relative to normoxia at the control temperature (Fig. 6a). At 17 °C,  $J_{\text{amm}}$  in normoxia was also not different than in hyperoxia. However the increase in  $J_{\text{amm}}$  in hyperoxia (12–17 °C  $Q_{10}=4.94$ ) was greater than the one seen in normoxia ( $Q_{10}=2.14$ ). Although temperature had a significant overall effect on  $J_{\text{amm}}$  ( $p=0.03$ ), oxygen did not ( $p=0.44$ ), and temperature and oxygen did not have a significant interaction ( $p=0.60$ ) (Fig. 6a).



**Fig. 2** **a** Ventilation rate (frequency), **b** ventilation pressure amplitude, **c** calculated ventilatory index and **d** heart rate in *Eptatretus stoutii* acutely exposed (30 min) to 7, 12 (control) and 17 °C. The  $Q_{10}$  values over the entire range (7–17 °C) are displayed on the upper left corner. The lower range (7–12 °C)  $Q_{10}$  are shown above the 12 °C

bar and the higher range (12–17 °C)  $Q_{10}$  are shown above the 17 °C bar. Means sharing the same lower case letters are not statistically different (repeated measures one-way ANOVA; Tukey's post hoc test). Data are shown as means  $\pm 1$  SEM ( $n=6-8$ )



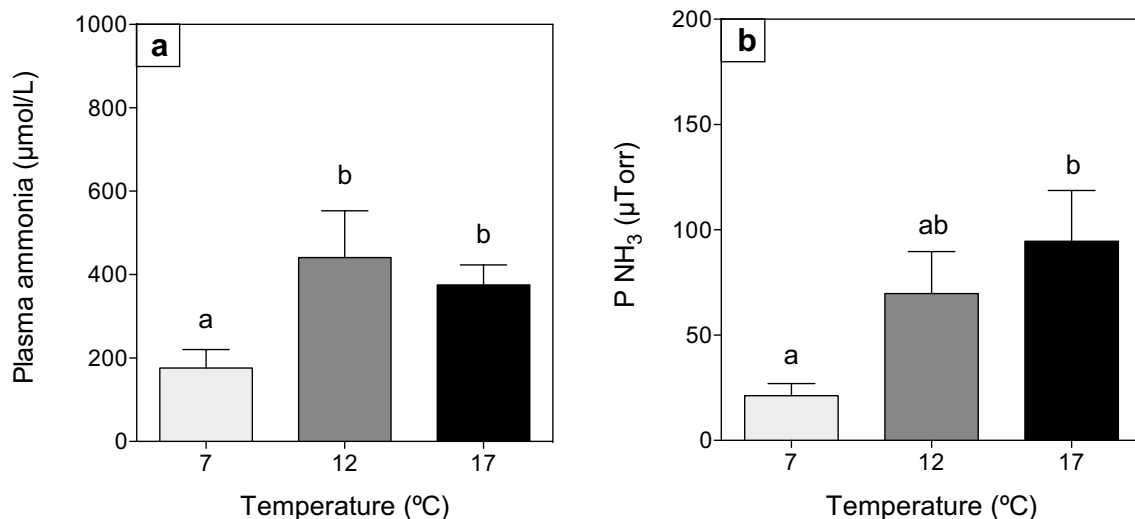


**Fig. 3** Plasma ion concentration **a** Na<sup>+</sup>, **b** Cl<sup>-</sup>, **c** Mg<sup>2+</sup>, **d** Ca<sup>2+</sup> and **e** osmolality in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17 °C. Means sharing the same lower case letters are not statistically

different (one-way ANOVA; Tukey's post hoc test). Data are shown as means  $\pm$  1 SEM ( $n = 7-8$ )

However in contrast to the pattern in  $J_{\text{amm}}$ , hyperoxia had no effect on  $J_{\text{H}_2\text{O}}$  at 12 °C but greatly attenuated the large increase in  $J_{\text{H}_2\text{O}}$  which occurred in normoxia at 17 °C (Fig. 6b). As a result, the 12–17 °C  $Q_{10}$  in hyperoxia was

also much smaller than the one in normoxia (1.86 and 4.00, respectively). There was a significant overall effect of oxygen ( $p = 0.01$ ) and temperature ( $p < 0.001$ ), as well as significant interaction between temperature and oxygen ( $p = 0.01$ ).



**Fig. 4** **a** Total plasma total ammonia, **b** and partial pressure of NH<sub>3</sub> in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17 °C. Means sharing the same lower case letters are not statistically different (one-

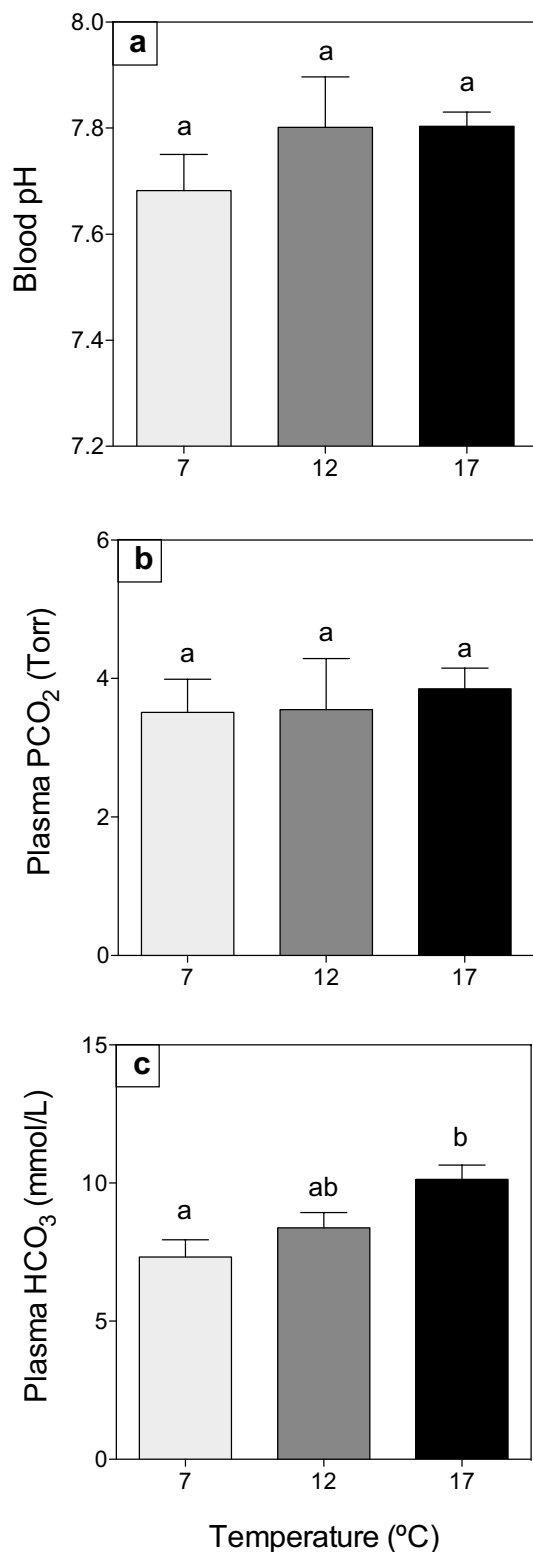
way ANOVA; Tukey's post hoc test). Data are shown as means  $\pm$  1 SEM ( $n=6-8$ )

There were significant main effects of both oxygen ( $p < 0.0001$ ) and temperature ( $p = 0.004$ ) on the ventilation rate (velar frequency) of longer term (6 h) exposed hagfish (Fig. 7a), and there was no significant interaction ( $p = 0.35$ ). Longer term exposure to 17 °C tended to increase ventilation rate (Fig. 7a), although this difference was not statistically significant in post hoc test. At 12 °C, hyperoxia significantly increased ventilation rate by more than twofold, and similarly, at 17 °C, exposure to hyperoxia significantly increased velar rate (Fig. 7a). There was no significant overall effect of either oxygen ( $p = 0.17$ ) or temperature ( $p = 0.22$ ) on ventilation pressure amplitude (Fig. 7b). Exposure to hyperoxia slightly decreased ventilation amplitude at both 12 and 17 °C; however, these changes were not statistically significant. Oxygen had a significant overall effect ( $p = 0.04$ ) increasing the calculated ventilatory index (Fig. 7c), while the overall stimulatory effect of temperature was not significant ( $p = 0.30$ ). In normoxia, longer term ventilatory index did not increase with temperature (Fig. 7c), similarly to short term measurements (Fig. 2c). Longer term measurements in hyperoxia suggest that while the pattern of ventilatory increases is reversed in comparison to short term acute measurements, its absolute rates did not change.

There was no significant main effect ( $p = 0.59$ ) of oxygen or interaction ( $p = 0.47$ ) between temperature and oxygen on plasma [Na<sup>+</sup>] (Fig. 8a). At 12 °C there was a small, but significant increase in plasma [Cl<sup>-</sup>] with hyperoxia, but this same increase was not seen at 17 °C (Fig. 8b). Only the

interaction term was significant ( $p = 0.04$ ). Hyperoxia did not alter plasma [Mg<sup>2+</sup>] at 12 °C, but significantly increased it at 17 °C (Fig. 8c). Overall, there was no main effect of temperature ( $p = 0.18$ ), but the overall effect of oxygen ( $p < 0.001$ ) and the interaction term ( $p = 0.01$ ) were both significant. At both temperatures tested, plasma [Ca<sup>2+</sup>] was significantly higher in hyperoxia than in normoxia (Fig. 8c). In normoxia only there was a significant decrease in plasma [Ca<sup>2+</sup>] at 17 °C in comparison to 12 °C. Overall, there was a significant main effect of oxygen ( $p < 0.001$ ) on plasma [Ca<sup>2+</sup>], but no significant main effect of temperature ( $p = 0.12$ ) or interaction term ( $p = 0.07$ ). Plasma osmolality was not significantly affected by either temperature or oxygen, and there was no significant interaction (Fig. 8e).

At 12 °C, plasma total ammonia concentration was not different between animals in normoxia, or acutely exposed to hyperoxia (Fig. 9a). However, at 17 °C, there was a significant decrease in plasma ammonia between normoxia and hyperoxia. There was a significant main effect of oxygen ( $p = 0.04$ ) but there was no significant overall effect of temperature ( $p = 0.34$ ), and the interaction term ( $p = 0.26$ ) was also not significant (Fig. 9a). Plasma [NH<sub>4</sub><sup>+</sup>] (data not shown) exhibited the same trends as plasma total ammonia concentration. There was no significant effect of either of the main factors, temperature or oxygen, on the partial pressure of ammonia, and the interaction term was also not significant (Fig. 9b). PNH<sub>3</sub> seemed to be reduced with exposure to hyperoxia at 17 °C, however the effect was not significant.



**Fig. 5** **a** Blood pH, **b** plasma PCO<sub>2</sub> and **c** plasma HCO<sub>3</sub><sup>-</sup> in *Eptatretus stoutii* acutely exposed to 7, 12 (control) and 17 °C. Means sharing the same lower case letters are not statistically different (one-way ANOVA; Tukey's post hoc test). Data are shown as means ± 1 SEM ( $n=6-8$ )

Blood pH increased with exposure to hyperoxia at 12 °C (Fig. 10a), while at 17 °C, although it was elevated, it was not significantly different from the normoxic value. The overall effect of oxygen ( $p=0.005$ ) was significant, while those of temperature ( $p=0.94$ ) and the interaction term ( $p=0.40$ ) were not. Plasma PCO<sub>2</sub> decreased markedly with exposure to hyperoxia at 12 °C, but it was not altered by hyperoxia at 17 °C (Fig. 10b). However, there was a significant increase in PCO<sub>2</sub> at 17 °C in hyperoxia only (Fig. 10b). There was a significant overall effect of oxygen ( $p=0.03$ ) but not temperature ( $p=0.13$ ), and the interaction term was significant ( $p=0.01$ ). Like PCO<sub>2</sub>, plasma [HCO<sub>3</sub><sup>-</sup>] also tended to fall with hyperoxia at 12 °C, and it also tended to rise with hyperoxia at 17 °C but neither difference was significant. However, plasma [HCO<sub>3</sub><sup>-</sup>] was significantly higher in animals exposed to hyperoxia at 17 °C in comparison to hyperoxic hagfish at 12 °C (Fig. 10c). The overall effects of oxygen ( $p=0.55$ ) and temperature ( $p=0.09$ ) were not significant, but there was a significant interaction ( $p=0.01$ ) between the two factors.

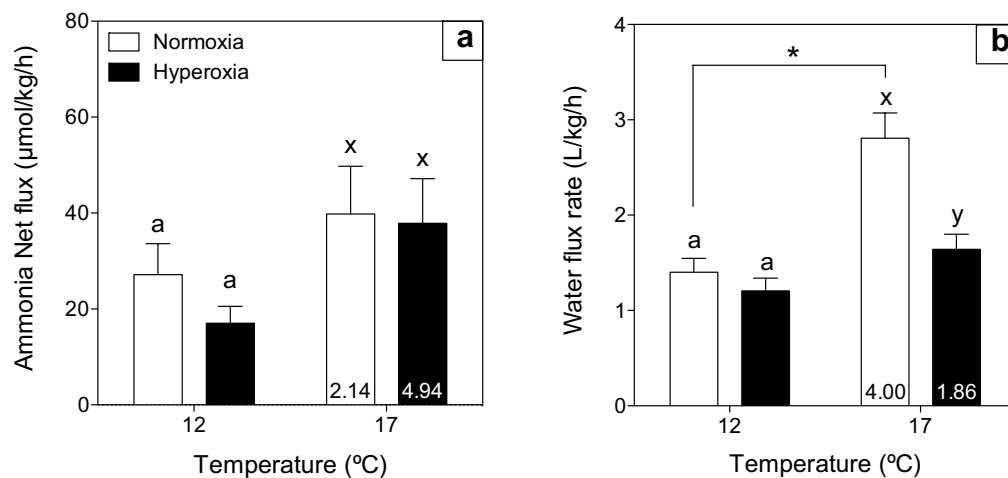
## Discussion

### Overview

Inasmuch as both ammonia and diffusive water flux rates increased as MO<sub>2</sub> and ventilation increased with temperature, and the water flux response was attenuated by hyperoxia, there was clear evidence of an osmorepiratory compromise in the Pacific hagfish. The differing  $Q_{10}$ s observed for MO<sub>2</sub>,  $J_{\text{amm}}$  and  $J_{\text{H}_2\text{O}}$  indicate that these are independently regulated, and this is likely because they move through different pathways at the gills and skin. Plasma ions and osmolality were also affected, whereas there was little change in plasma acid–base status. These data clearly demonstrate that even an animal commonly considered to be an osmoconformer shows interactions between osmoregulation and respiration.

### Temperature effects on the rates of oxygen consumption, ventilation, ammonia and water fluxes

The osmorepiratory compromise is a phenomenon that can occur at any gas exchange surface, and while we usually think of the gills, it could also happen at the skin. The debate on the major site of oxygen uptake in hagfish spans almost 30 years, and recently it has been shown, in *E. stoutii*, that the bulk of the animal's oxygen uptake occurs through their gills (Clifford et al. 2016). Our results confirm previous reports (Munz and Morris 1965; Steffensen et al. 1984; Perry et al. 2009) that hagfish MO<sub>2</sub> values are amongst the lowest reported for vertebrates. As metabolic demand for



**Fig. 6** **a** Ammonia net flux rate ( $J_{\text{amm}}$ ), **b** diffusive water flux rate ( $J_{\text{H}_2\text{O}}$ ) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17 °C. The normoxia  $Q_{10}$  are shown in the 17 °C normoxia bar while the hyperoxia  $Q_{10}$  are shown in the 17 °C bar. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same

temperature [Two-way ANOVA  $p$  values ( $J_{\text{amm}}$ :  $p_{\text{interaction}}=0.601$ ,  $p_{\text{oxygen}}=0.443$ ,  $p_{\text{temperature}}=0.039$ ;  $J_{\text{H}_2\text{O}}$ :  $p_{\text{interaction}}=0.014$ ,  $p_{\text{oxygen}}=0.001$ ,  $p_{\text{temperature}}<0.001$ ]. Asterisks indicate significant differences between 12 and 17 °C at the same oxygen level (Bonferroni post hoc test). Data are shown as means  $\pm$  1 SEM ( $n=6-8$ )

oxygen increases with temperature, oxygen uptake from the environment must be elevated and/or increased anaerobic metabolism must occur. In our study,  $\text{MO}_2$  increased linearly with temperature ( $R^2=0.991$ ; data not shown), with an overall  $Q_{10}$  of 3.22 (Fig. 1a), which is greater than in marine elasmobranchs (Butler and Taylor 1975; Giacomini et al. 2017) and teleosts where the  $Q_{10}$ s are usually around 2.0 or lower (Clarke and Johnston 1999; Clark et al. 2011; Lea et al. 2016). Munz and Morris (1965) reported  $Q_{10}$ s of 2.4–2.8 for  $\text{MO}_2$  in *E. stoutii*, similar to the results shown here, further confirming the temperature sensitivity of this species. In nature, hagfish generally inhabit the ocean floors, known to be nutrient-poor but very stable environments (Martini 1998). Similarly, *E. stoutii* is found in deep areas where temperature varies only between 10 and 11 °C (McInerney and Evans 1970). Importantly, the high temperature sensitivity  $\text{MO}_2$  of this species ( $Q_{10}$  7–12 °C = 3.75) at the low range tested (close to temperature range of their natural habitat) suggests that the impacts of temperature challenges on these fishes (as occurs during commercial fishing) may be of special concern.

Hagfish extract  $\text{O}_2$  from the water through a counter-current mechanism, resulting in high diffusive conductance of the gill (Malte and Lomholt 1998). There are very few reported measurements of hagfish ventilatory dynamics, mostly due to the technical difficulties involved in measuring ventilation on these complex animals (Steffensen et al. 1984; Perry et al. 2009). Eom and Wood (2018) have

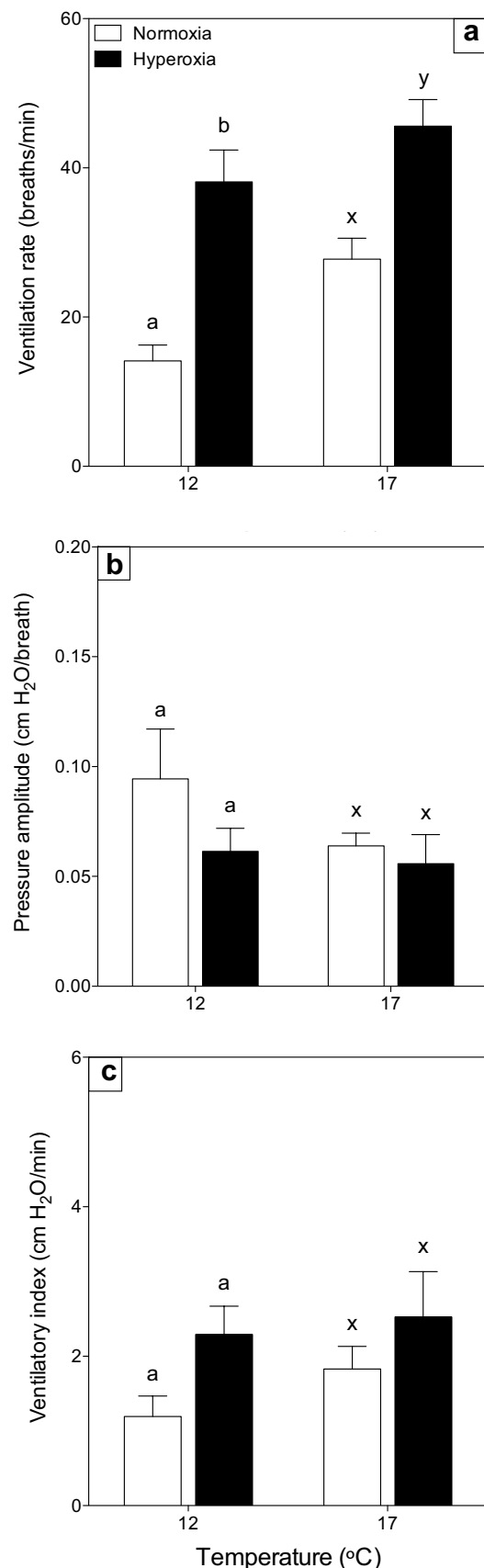
recently developed methods for measuring ventilation and heart rate in hagfish, which were employed here, and have shown that increases in both ventilatory pressure amplitude (reflecting velar stroke volume) and ventilatory frequency (velar rate) lead to increases in ventilatory water flow. In our study, ventilation frequency did not vary with temperature when the challenges were done acutely (Fig. 2a). In contrast, ventilation pressure amplitude increased linearly, resulting in a ventilatory index (a proxy for total ventilatory water flow, Eom and Wood 2018) that decreased substantially between 12 and 7 °C, but exhibited only a slight increase between 12 and 17 °C (Fig. 2c). The greatly increased coughing frequency at 17 °C suggests pathological impacts on ventilation at 17 °C; at high flows, perhaps more particles are encountered, resulting in more coughing. The function of the coughs in the ventilatory dynamics has yet to be elucidated and the possible contributions of these flow reversals to  $\text{MO}_2$  are unknown. The constancy of ventilatory frequency suggests that immediate temperature-induced changes in ventilatory water flow are achieved mainly by changes in the stroke volume of the velar apparatus. This contrasts with the effects of hypoxia and hypercapnia, where most of the reported changes resulted from alterations in respiratory frequency in this same species (Perry et al. 2009). However it is notable that our results differ greatly from the report of Coxon and Davison (2011) on the congeneric New Zealand hagfish (*Eptatretus cirrhatus*) where velar frequency increased with a  $Q_{10}$  of 3.2 during exposure to temperatures

**Fig. 7** **a** Ventilation rate (frequency), **b** ventilation pressure amplitude, and **c** calculated ventilatory index in hagfish exposed to experimental conditions for 6 h. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA  $p$  values (**rate**:  $p_{\text{interaction}}=0.3597$ ,  $p_{\text{oxygen}}<0.0001$ ,  $p_{\text{temperature}}=0.0045$ ; **pressure**:  $p_{\text{interaction}}=0.3994$ ,  $p_{\text{oxygen}}=0.1707$ ,  $p_{\text{temperature}}=0.2259$ ; **index**:  $p_{\text{interaction}}=0.6268$ ,  $p_{\text{oxygen}}=0.0421$ ,  $p_{\text{temperature}}=0.3045$ )]. Data are shown as means  $\pm$  1 SEM ( $n=6-8$ )

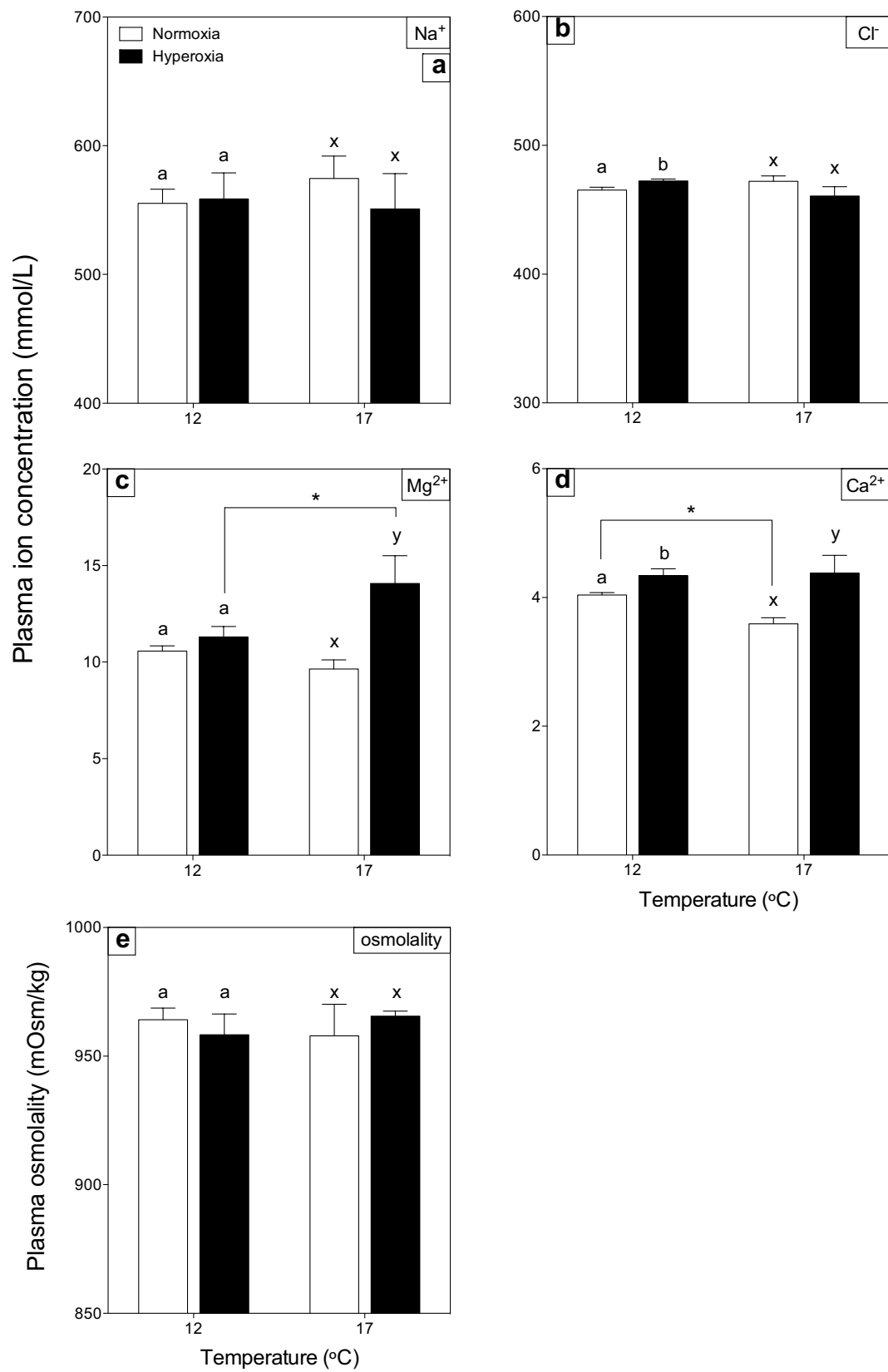
between 7 and 19 °C. However the temperature protocols were very different in the two studies, and Coxon and Davison (2011) did not measure ventilation, but rather velar frequency by electromyography.

Measured heart rate values in control conditions ( $\sim 15$  beats/min) were somewhat higher than the ones reported by Cox et al. (2010) ( $\sim 10$  beats/min). In contrast to the ventilatory index, heart rate did not change between 12 and 7 °C but was elevated at 17 °C (Fig. 2e), suggesting that at the lower temperature range, rather than solely relying on changes in heart rate, modulation of cardiac stroke volume could be responsible for maintaining the  $\text{MO}_2$  at the gills. This response was observed when *E. stoutii* was exposed to anoxia, as cardiac stroke volume increased to compensate for the bradycardia that resulted from anoxia (Cox et al. 2010).

Ammonia transporting proteins Rhbg and Rhcg have been identified in both the gills and the skin of the Atlantic hagfish *Myxine glutinosa* (Edwards et al. 2015) and the Pacific hagfish *E. stoutii* (Clifford et al. 2017). They were likely an important route of ammonia excretion in the present study. In *E. stoutii*, 30% of the animal's resting  $J_{\text{amm}}$  can occur through the skin (Clifford et al. 2016), therefore, rates of  $J_{\text{amm}}$  reported here should be interpreted as a summation of branchial and cutaneous contributions. The present control rates of  $J_{\text{amm}}$  ( $\sim 15 \mu\text{mol/kg/h}$ ; Fig. 1b) were generally lower than those previously reported in the literature (30–50  $\mu\text{mol/kg/h}$ ) (Walsh et al. 2001; Braun and Perry 2010; Clifford et al. 2015; Wilkie et al. 2017). Overall,  $J_{\text{amm}}$  seemed to be less sensitive to temperature than  $\text{MO}_2$ , but this was only apparent in the 12–7 °C range where the  $J_{\text{amm}} Q_{10}=1.86$  was much lower than the  $\text{MO}_2 Q_{10}=3.75$  (Fig. 1a). In ammonotelic teleosts,  $J_{\text{amm}}$  is more sensitive to temperature than  $\text{MO}_2$  (Wood 2001), and the same was seen for the ureotelic spiny dogfish (Giacomin et al. 2017). The elevation in plasma ammonia at 12 °C and 17 °C in comparison to 7 °C was possibly a result of the general increase in metabolic rate (reflected in  $\text{MO}_2$ ) and the accompanying breakdown of amino acids (Fig. 4a). This would lead to an elevation in the gradient for ammonia diffusion ( $\text{PNH}_3$ ; Fig. 4b). Since  $\text{MO}_2$  and  $J_{\text{amm}}$  were measured simultaneously in the same fish, we have calculated the ammonia quotient (AQ) to estimate the metabolic fuel used in aerobic metabolism (Lauff and Wood 1996). AQ values ranged from 0.03 to 0.04, which are very low numbers, indicating that these fasting hagfish







**Fig. 8** Plasma ion concentrations **a**  $\text{Na}^+$ , **b**  $\text{Cl}^-$ , **c**  $\text{Mg}^{2+}$ , **d**  $\text{Ca}^{2+}$  and **e** osmolality in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17 °C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA  $p$  values ( $\text{Na}^+$ :  $p_{\text{interaction}}=0.479$ ,  $p_{\text{oxygen}}=0.595$ ,  $p_{\text{temperature}}=0.762$ ;  $\text{Cl}^-$ :  $p_{\text{interaction}}=0.037$ ,  $p_{\text{oxygen}}=0.626$ ,  $p_{\text{temperature}}=0.559$ ;  $\text{Mg}^{2+}$ :  $p_{\text{interaction}}=0.012$ ,  $p_{\text{oxygen}}=0.000$ ,  $p_{\text{temperature}}=0.184$ ;  $\text{Ca}^{2+}$ :  $p_{\text{interaction}}=0.067$ ,  $p_{\text{oxygen}}=0.000$ ,  $p_{\text{temperature}}=0.125$ ; **osmolality**:  $p_{\text{interaction}}=0.387$ ,  $p_{\text{oxygen}}=0.906$ ,  $p_{\text{temperature}}=0.948$ )]. Asterisks indicate significant differences between 12 and 17 °C at the same oxygen level (Bonferroni post hoc test). Data are shown as means  $\pm$  1 SEM ( $n=6-8$ )

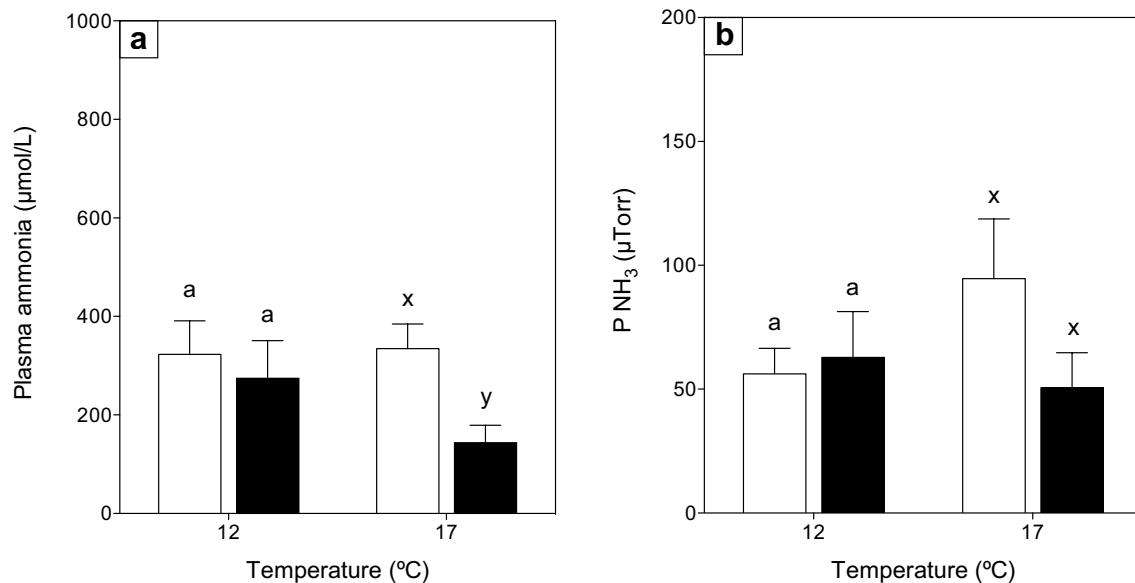
were utilizing only 13% protein to fuel aerobic metabolism (Fig. 1c). This may be an important adaptation for protein conservation in an animal that lives in a nutrient-poor environment where feeding opportunities are scarce. The AQ did not vary over the range of temperatures tested, similar to results seen with NQ in the ureotelic spiny dogfish shark (Giacomin et al. 2017), but in that animal, aerobic metabolism was fueled by approximately 100% somatic protein.

Pacific hagfish have very high permeability to water, with a rate constant ( $k$ ) of  $2.28 \text{ h}^{-1}$ , exchanging about 1.4 L water/kg/h at 12 °C, and that nearly doubled after a 5 °C increase in temperature ( $Q_{10}=4.01$ ) (Fig. 1d). The high diffusive water flux rates measured here are in close proximity to values measured in a recent study by Glover et al. (2017) on *E. stoutii* and to those of Rudy and Wagner (1970) on the same species (previously known as *Polioptremus stoutii*). Both were performed at the same control temperature (12 °C). Diffusive water flux rates are significantly lower in seawater teleosts compared to freshwater teleosts (Motais et al. 1969). Results from the current study indicate that hagfish possesses water flux rates that are higher than even those in freshwater fishes (e.g., Evans 1969; Onukwufor and Wood 2018) and substantially greater than those in marine teleosts (Motais et al. 1969) and elasmobranchs (Carrier and Evans 1972; Wright and Wood 2016; Giacomin et al. 2017). It is thought that this high water permeability is a trait linked to the primitive characteristics of these fishes, and likely a reflection of their osmoconforming strategy, where maintenance of the isosmotic balance is a key requirement (Rudy and Wagner 1970). To our knowledge, this is the first study that looked at the effects of temperature on the diffusive water flux rates in hagfishes. When fish were exposed to a 5 °C decrease in temperature,  $J_{\text{H}_2\text{O}}$  was mildly affected ( $Q_{10}$  of 1.35), differing from  $\text{MO}_2$  and  $J_{\text{amm}}$ , which were greatly affected. However, a 5 °C increase in temperature led to a significant increase in  $J_{\text{H}_2\text{O}}$ , a much more marked response than that seen with  $\text{MO}_2$  ( $J_{\text{H}_2\text{O}}$   $Q_{10}$  of 4.01 versus  $\text{MO}_2$   $Q_{10}$  of 2.77). A comparable response was also seen in the spiny dogfish (Giacomin et al. 2017). In hagfish gills, the expression of at least one

aquaporin (AQP4) has been identified, and localized in the basolateral membrane of pavement cells (Nishimoto et al. 2007). Two aquaporin homologs (AQP3 and AQP4) have also been identified at the slime glands throughout the skin (Herr et al. 2014). Although the functional role of aquaporins in the hagfish is yet to be characterized (Nishimoto et al. 2007; Madsen et al. 2015), it is very likely that they played a role in the changes in water permeability shown in the current study.

### Temperature effects on ionoregulation and acid–base status

Hagfishes are unusual among aquatic vertebrates exhibiting a unique strategy of both ionic and osmoconformation (Wright 2007). More recently, two different studies have shown that although they appear to conform major plasma ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ), they actually maintain plasma  $[\text{Na}^+]$  slightly higher and plasma  $[\text{Cl}^-]$  slightly lower than sea water (Sardella et al. 2009; Clifford et al. 2018). The results presented here confirm that finding (Fig. 3a, b). Despite the significant evolutionary importance of hagfishes, little is known about the mechanisms that are involved in maintaining hydro-mineral balance in these animals (Currie and Edwards 2010). Interestingly, early findings by Bellamy and Chester Jones (1961), more recently confirmed by Sardella et al. (2009) and by the present study, show that Pacific hagfish have the capacity to regulate divalent ions ( $[\text{Ca}^{2+}]$  and  $[\text{Mg}^{2+}]$ ) in plasma well below environmental levels. In our study, while plasma  $[\text{Na}^+]$  did not statistically vary within the three temperatures tested (Fig. 3a), there was a tendency to rise with temperature, and there was a significant elevation in plasma  $[\text{Cl}^-]$  at 17 °C, bringing the level closer to that of sea water (Fig. 3b). The absolute increases in mean plasma  $[\text{Na}^+]$  and  $[\text{Cl}^-]$  were sufficient to account for the absolute increase in plasma osmolality, since  $\text{Na}^+$  and  $\text{Cl}^-$  are the major ions constituting the blood plasma. We are aware of only a single study (unpublished) that investigated the plasma ion status of *E. stoutii* after acute exposure to different temperatures (Hastey 2011). After 6 h of exposure to 6 and 12 °C increases in temperature, plasma  $\text{Na}^+$  did not change, while plasma  $\text{Cl}^-$  seemed to be reduced, a result opposite to the ones reported here. However, Hastey (2011) reported that plasma osmolality did tend to increase at high temperatures (Fig. 3e), similar to what we found. It is somewhat curious that both cations ( $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ ) that hagfish regulate at concentrations far lower than in sea water were maintained further away from the seawater levels as temperature and  $\text{MO}_2$  increased (Fig. 3c,d), contrary to the results seen by Hastey (2011), where plasma  $[\text{Mg}^{2+}]$  slightly increased with temperature. Our results support the previous



**Fig. 9** **a** Total plasma total ammonia, **b** and partial pressure of  $\text{NH}_3$  ( $\text{PNH}_3$ ) in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17 °C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA  $p$ -values (**total ammo-**

**nia**:  $p_{\text{interaction}}=0.256$ ,  $p_{\text{oxygen}}=0.040$ ,  $p_{\text{temperature}}=0.340$ ;  **$\text{PNH}_3$** :  $p_{\text{interaction}}=0.165$ ,  $p_{\text{oxygen}}=0.303$ ,  $p_{\text{temperature}}=0.466$ ]. Asterisks indicate significant differences between 12 and 17 °C at the same oxygen level (Bonferroni post hoc test). Data are shown as means  $\pm 1$  SEM ( $n=6-8$ )

data showing regulation of divalent cations, but the explanation for a higher excretion rate and/or lower entry rate for these ions at higher temperature remains unknown and is an interesting avenue for future research.

Baker et al. (2015) demonstrated the remarkable ability of hagfish in correcting blood pH disturbances, through an unprecedented capacity to accumulate  $\text{HCO}_3^-$  in the blood plasma, likely due to exchanges with  $\text{Cl}^-$  at the gills (Evans 1984). The skin may also play a role in acid–base exchange (Clifford et al. 2014). Intriguingly, in our study, at 17 °C we observed an elevation in both plasma  $\text{HCO}_3^-$  and plasma  $\text{Cl}^-$ , while plasma pH was not altered (Fig. 5a,b,c). According to the relative alkalinity (Rahn and Baumgardner 1972) or alphastat (Reeves 1977) hypotheses, blood pH falls with increases in temperature, while in our study, pH remained unchanged through the range of temperatures tested. At present, it is unclear whether the present data represent an alternative strategy to maintain blood pH constant, independent of temperature, or whether it is because the time frame of the temperature challenge (6 h) was too short to see the classic pattern typical of most fish. In future, longer-term exposures to altered temperatures would be informative.

### Does hyperoxia alleviate the effects of temperature on Pacific hagfish?

We had hypothesized that by increasing the oxygen supply at the gills through environmental hyperoxia, the disturbances

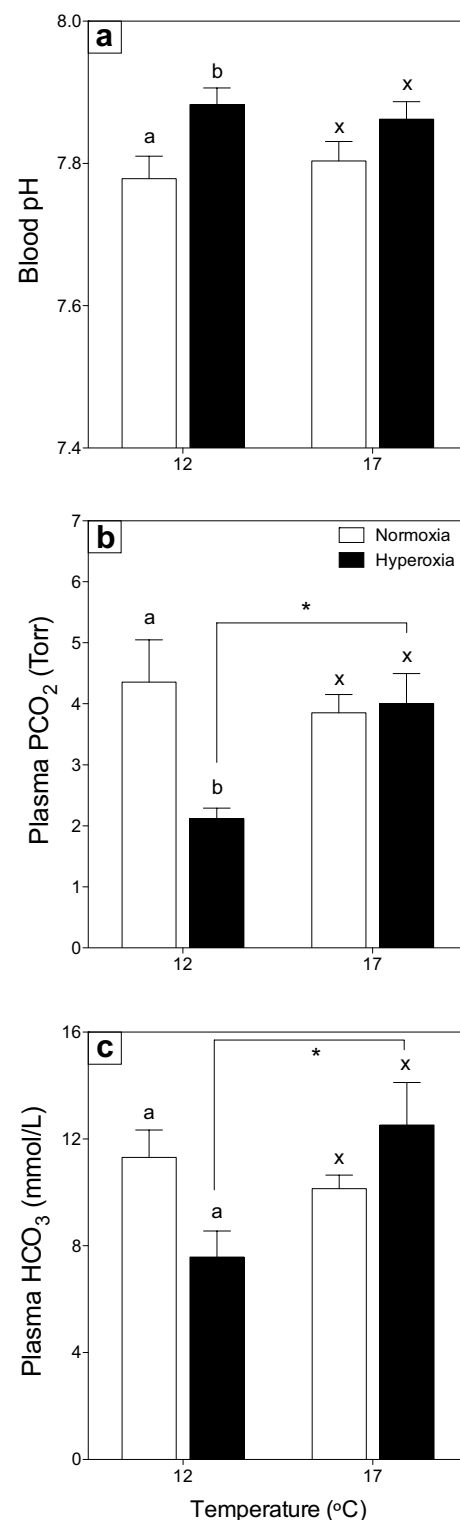
caused by elevated temperature in the fluxes of other moieties with the surrounding environment would be minimized. Our predictions were only partially supported. Certainly, at 17 °C, exposure to hyperoxia significantly reduced the diffusive flux of water ( $J_{\text{H}_2\text{O}}$ ) to almost control normoxic levels (Fig. 6a), whereas at 12 °C (control), hyperoxia did not have any effect on  $J_{\text{H}_2\text{O}}$  or  $J_{\text{amm}}$  (Fig. 6a,b). Surprisingly, contrary to our predictions, hyperoxia did not depress ventilation (Fig. 7). In fact, at both 12 °C and 17 °C, hyperoxia caused an increase in ventilation frequency (Fig. 7b), but since pressure amplitude was reduced (Fig. 7b), total ventilation did not change (Fig. 7c). While the two-way ANOVA showed no significant differences on the ventilatory index between normoxia and hyperoxia at 12 °C, a pairwise comparison demonstrated that hyperoxia significantly increased ventilatory index. Hyperoxia also did not alleviate the increase in  $J_{\text{amm}}$  at 17 °C, despite a decrease in  $\text{PNH}_3$  and a significant decrease in total plasma ammonia (Fig. 9a, b). This could indicate that hyperoxia is playing a role in decreasing the concentration gradient driving ammonia diffusion at the gills, but not affecting the metabolic production rate of ammonia. Our results differ from those of Giacomini et al. (2017) in the spiny dogfish, where the fluxes of ammonia and urea-N were actually exacerbated by hyperoxia at high temperature, whereas the diffusive water flux ( $J_{\text{H}_2\text{O}}$ ) was not affected. In the present study, plasma  $[\text{Na}^+]$ ,  $[\text{Cl}^-]$  and osmolality were largely unaffected by hyperoxia at the two temperatures tested (Fig. 8a, b). However, hyperoxia increased plasma  $[\text{Mg}^{2+}]$  and  $[\text{Ca}^{2+}]$  at the two temperatures tested (Fig. 8c,

d). As these ions are normally regulated substantially below concentrations in sea water, then the increases caused by hyperoxia could be detrimental rather than beneficial.

In most fish, hyperoxia usually depresses ventilation, so the increased  $\text{CO}_2$  retention in the blood causes a respiratory acidosis, and consequently, a decrease in blood pH (Wood 1991; Heisler 1993; Heisler et al. 1988). This hyperoxia-induced hypercarbia usually elicits slower compensatory responses than environmental hypercapnia (Heisler 1993). In our study, hyperoxia tended to increase rather than decrease ventilation, so the accompanying decrease in plasma  $\text{PCO}_2$  and elevation in pH at 12 °C (Fig. 10a,b) could be a reflection of this effect. Additionally, there was no change in plasma  $\text{Cl}^-$  during hyperoxia at either 12 or 17 °C (Fig. 8b), which could indicate that the  $\text{HCO}_3^-/\text{Cl}^-$  exchanger mechanisms had not been activated yet. Work by Perry et al. (2009) has shown that hagfish gills likely possess both internal and external  $\text{O}_2$  chemoreceptors involved in the control of breathing. A more detailed study of ventilatory control, oxygen transfer capabilities, and the influence of hyperoxia on blood  $\text{O}_2$  and  $\text{CO}_2$  in hagfish is required.

### Future directions and perspectives

Hagfish populations all over the world have been commercially exploited for about 30 years now. More recently, the international demand for hagfish skin for the production of leather, as well as a growing market for live hagfish in Korea, has added pressure for the harvest of these animals in both Pacific and Atlantic coasts of North America (Barss 1993; Grant 2006). Additionally, giving their demersal habitat, hagfish are common by-catch items resulting from bottom trawls which target other species. Current targeted fishing practices involve pulling baited traps full of hagfish rapidly to the ocean surface, where they can be exposed to quick changes in pressure, salinity and temperature (Olla et al. 1998). Hagfish that do not match size requirements are discarded. Very little is known about the general ecology and physiology of hagfish, knowledge which is usually a basic requirement for the establishment of a sustainable commercial fishery. Our results indicate that hagfish are quite susceptible to quick changes in ambient temperature. The higher temperature characteristic of surface waters and “on-deck” conditions will lead to elevations in the diffusive permeability of the gills to water, as well as marked increases in metabolic rate that likely result in increases in the rate of production of nitrogenous waste. Additionally, plasma ions and osmolality are disturbed at high temperature. We were able to show that hyperoxia can have some beneficial effects counteracting the temperature effects (reduced diffusive water fluxes), but for the majority of parameters analyzed here, hyperoxia led to adverse effects,



**Fig. 10** **a** Blood pH, **b** plasma  $\text{PCO}_2$  and **c** plasma  $\text{HCO}_3^-$  in *Eptatretus stoutii* acutely exposed to hyperoxia (black bars) or normoxia (white bars) at 12 and 17 °C. Normoxia and hyperoxia bars sharing the same lower case letters are not statistically different at the same temperature [Two-way ANOVA  $p$ -values ( $\text{pH}$ :  $p_{\text{interaction}}=0.403$ ,  $p_{\text{oxygen}}=0.005$ ,  $p_{\text{temperature}}=0.937$ ;  $\text{PCO}_2$ :  $p_{\text{interaction}}=0.011$ ,  $p_{\text{oxygen}}=0.025$ ,  $p_{\text{temperature}}=0.128$ ;  $\text{HCO}_3^-$ :  $p_{\text{interaction}}=0.010$ ,  $p_{\text{oxygen}}=0.548$ ,  $p_{\text{temperature}}=0.090$ )]. Asterisks indicate significant differences between 12 and 17 °C at the same oxygen level (Bonferroni post hoc test). Data are shown as means  $\pm$  1 SEM ( $n=7-8$ )

such as a disruption of plasma ion and acid–base balance. Future studies should specifically focus on the mechanisms employed to regulate divalent cations and blood acid–base status in hagfishes facing temperature challenges. In addition, since reactive oxygen species (ROS) can be generated both by temperature and hyperoxia, it is of great importance to characterize the responses of these fishes to ROS, as well as the potential for oxidative damage, and their antioxidant defense mechanisms.

The present results are similar to those reported by Giacomini et al. (2017) in the dogfish shark, another marine osmoconformer, where  $\text{MO}_2$  and the fluxes of ammonia, urea-N and water were differentially affected by temperature. These response patterns were likely due to the different transport pathways of these moieties. In summary, a complex osmorepiratory interaction occurs as a result of acute temperature changes, even in the most primitive extant craniate which is commonly believed to be an osmoconformer.

**Acknowledgements** This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to CMW and PMS. MG is supported by a 4-year graduate fellowship from the University of British Columbia. We would like to thank Dr. Eric Clelland (Bamfield Marine Sciences Centre research coordinator) for excellent logistic support, Dr. Alex Clifford for providing helpful advice with calculations, and three anonymous reviewers whose comments and suggestions have greatly improved the manuscript.

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