



Osmorespiratory compromise in an elasmobranch: oxygen consumption, ventilation and nitrogen metabolism during recovery from exhaustive exercise in dogfish sharks (*Squalus suckleyi*)

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Received: 11 January 2022 / Revised: 30 May 2022 / Accepted: 15 June 2022 / Published online: 15 July 2022
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Abstract

The functional trade-off between respiratory gas exchange versus osmolyte and water balance that occurs at the thin, highly vascularized gills of fishes has been termed the osmorespiratory compromise. Increases in gas exchange capacity for meeting elevated oxygen demands can end up favoring the passive movement of osmolytes and water, potentially causing a disturbance in osmotic balance. This phenomenon has been studied only sparsely in marine elasmobranchs. Our goal was to evaluate the effects of exhaustive exercise (as a modulator of oxygen demand) on oxygen consumption (MO_2), branchial losses of nitrogenous products (ammonia and urea-N), diffusive water exchange rates, and gill ventilation (frequency and amplitude), in the Pacific spiny dogfish (*Squalus suckleyi*). To that end, MO_2 , osmolyte fluxes, diffusive water exchange rate, and ventilation dynamics were first measured under resting control conditions, then sharks were exercised until exhaustion (20 min), and the same parameters were monitored for the subsequent 4 h of recovery. While MO_2 nearly doubled immediately after exercise and remained elevated for 2 h, ventilation dynamics did not change, suggesting that fish were increasing oxygen extraction efficiency at the gills. Diffusive water flux rates (measured over 0–2 h of recovery) were not affected. Ammonia losses were elevated by 7.6-fold immediately after exercise and remained elevated for 3 h into recovery, while urea-N losses were elevated only 1.75-fold and returned to control levels after 1 h. These results are consistent with previous investigations using different challenges (hypoxia, high temperature) and point to a tighter regulation of urea-N conservation mechanisms at the gills, likely due to the use of urea as a prized osmolyte in elasmobranchs. Environmental hyperoxia offered no relief from the osmorespiratory compromise, as there were no effects on any of the parameters measured during recovery from exhaustive exercise.

Keywords Gill permeability · Metabolic rate · Urea · Ammonia · Hyperoxia · Trade-offs

Communicated by B. Pelster.

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Introduction

The osmorespiratory compromise represents the functional conflict between respiratory gas exchange versus osmolyte and water balance that occurs at the thin, highly vascularized gill of fishes (Gilmour and Perry 2018; Wood and Eom 2021). This physiological trade-off arises when the demand for increasing gas exchange during exercise or exposure to low environmental oxygen (hypoxia) favors the passive movement of osmolytes and water due to the high functional surface area and small diffusion distance at the gills (Randall et al. 1972; Gonzalez and McDonald 1992). This phenomenon can lead to a potential disturbance in the ionic and osmotic balance, and in the case of elasmobranchs, that use nitrogenous-based molecules as osmolytes (particularly urea), a disruption in nitrogen (N) metabolism. This

phenomenon has been well characterized in teleost fishes and recently has been reviewed in detail (Gilmour and Perry 2018; Wood and Eom 2021).

A few commonly identified knowledge gaps are studies performed in seawater and/or isosmotic conditions (Damsgaard et al. 2020; Giacomini et al. 2020) and also in non-teleost model species (Giacomini et al. 2018). In freshwater (where the fish is hyperosmotic), a typical response to an increased need for oxygen consumption (MO_2) is an exacerbation of the passive loss of ions and uptake of water (Wood and Randall 1973a,b,c; Gonzalez and McDonald 1992; Iftikar et al. 2010; Robertson et al. 2015). It is expected that the opposite would happen in seawater, where fish would face an increase in water loss and ion loading (Wood and Eom 2021) due to being hypoosmotic. Recently, Damsgaard et al. (2020) showed that coho salmon (*Oncorhynchus kisutch*) acclimated to seawater were more affected by hypoxia exposure than their freshwater-acclimated counterparts, displaying a large increase in plasma osmolality.

To date, even fewer studies have looked at the osmorepiratory compromise in elasmobranchs (Zimmer and Wood 2014; Giacomini et al. 2017). Elasmobranchs are approximate osmoconformers (i.e. they maintain the internal osmolality slightly greater than that of seawater), which promotes the osmotic influx of water, eliminating the need for drinking (Smith 1931; 1936; Wright and Wood 2016). The high osmolality of the internal fluids is achieved through the synthesis and retention of organic osmolytes, such as urea, which is retained in tissues and plasma at high levels (300–400 mmol/L), supplemented by lower concentrations of counteracting methylamines such as trimethylamine N-oxide (TMAO) which stabilize protein function (Yancey 2016). This osmoregulatory strategy is energetically expensive as urea is synthesized from ammonia through the ornithine-urea cycle, “costing” 5 mol of ATP per mole of urea produced (Ballantyne 1997). Therefore, several mechanisms of urea conservation have been identified in elasmobranchs (Wright and Wood 2016). These include very low permeability to urea at the gills (Boylan 1967; Wood et al. 1995) owing to a fundamentally different lipid composition of the branchial cell membranes (Fines et al. 2001), and active urea back-transporters located at the basolateral (Pärt et al. 1998; Fines et al. 2001) and/or apical (Wood et al. 2013) cell membranes. These mechanisms ensure that elasmobranchs are able to maintain a high plasma urea concentration despite an extremely high outwardly directed gradient (350 mmol/L in the plasma versus virtually zero in the water). With urea being such a highly prized osmolyte, any potential disruptions of urea homeostasis are of great concern, and yet, few studies have focused on this subject.

Zimmer and Wood (2014) investigated the effects of hypoxia exposure in the Pacific spiny dogfish shark, also referred to as *Squalus acanthias suckleyi*, and here referred

to as *Squalus suckleyi* (Ebert et al. 2010), a common model elasmobranch in physiological research. Hypoxia led to elevated rates of urea and ammonia losses to the water, effects that persisted for hours post exposure. The authors suggested that physiological mechanisms that improve oxygen consumption and survival in hypoxia, were accompanied by an impairment of the branchial urea retention mechanisms (Zimmer and Wood 2014). Giacomini et al. (2017) exposed this same species to acute changes in water temperatures. At high temperature, diffusive water fluxes and MO_2 increased in parallel, but urea and ammonia excretion were elevated to a much greater extent, again suggesting selective inhibition of retention mechanisms (e.g. lipid phase changes or impairment of back-transport mechanisms for ammonia and urea-N in gill cell membranes). Indeed, the best correlation was between approximately equivalent changes in MO_2 and diffusive water flux rates at different temperatures. While the authors also hypothesized that increasing the environmental supply of oxygen (hyperoxia) would help mitigate the effects of the osmorepiratory compromise, exposure of the animals to hyperoxia resulted in even higher urea and ammonia losses at the gills, whereas diffusive water fluxes were unchanged (Giacomini et al. 2017). These two previous studies have started to showcase some unexpected consequences of the osmorepiratory compromise in elasmobranch fishes.

The goal of the present study was to evaluate the effects of exhaustive exercise on MO_2 , branchial losses of nitrogenous products (ammonia and urea), diffusive water exchange rates and gill ventilation, again using the Pacific spiny dogfish as a model. Exhaustive exercise is a well-documented stressor for elasmobranch fishes, as it can lead to severe metabolic, ionic, acid–base, and hematological disturbances (Wood 1991; Brill et al. 2008; Richards et al. 2003; Skomal and Mandelman 2012; Schwieterman et al. 2021). We hypothesized that exhaustive exercise would trigger an increase in MO_2 accompanied by elevated ventilation of the gills, thereby leading to increases in passive branchial losses of osmolytes (urea and ammonia) and increased diffusive water fluxes. At the same time, we postulated that this treatment, in contrast to high temperature or hypoxia, would be less likely to impact selective protein-mediated retention mechanisms at the gills, thereby revealing more clearly the simple diffusive components of the osmorepiratory compromise.

To that end, after control measurements, animals were chased in a circular tank until exhaustion, then quickly transferred to experimental chambers, where MO_2 , branchial losses of highly prized osmolytes (ammonia and urea), diffusive water exchange rate, and ventilation dynamics (breathing frequency and amplitude of breath) were monitored concomitantly as the animals recovered for the subsequent 4 h. These measurements allowed us to quantify both the nitrogen quotient (NQ) as an indicator of fuel use in oxidative metabolism (Lauff and Wood 1996) and excess post-exercise

oxygen consumption (EPOC), an indicator of non-steady-state anaerobic metabolic costs repaid after exercise (Scarbello et al. 1991; Zhang et al. 2018). Little is known about the magnitudes of these two phenomena in elasmobranchs. Additionally, as part of an ongoing effort to investigate the potential alleviating effects of hyperoxia on the osmorepiratory compromise in fishes, another experimental series was performed, where the animals were allowed to recover from post-exercise exhaustion in hyperoxic (> 310 Torr; 41.3 kPa) water. We predicted that by elevating the availability of oxygen in the surrounding water, the animals would not need to perform cardio-respiratory and branchial adjustments to increase MO_2 , thereby preventing the detrimental effects associated with the osmorepiratory compromise.

Material and methods

Animal collection and acclimation

Dogfish (*S. suckleyi*; average mass = 1.81 ± 0.11 kg, $n = 19$) were caught by angling near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, under Fisheries and Oceans Canada collecting permits XR239-2015 and XR32-2016, where they were held for approximately 2 weeks of acclimation prior to experiments. At BMSC, they were kept in a large indoors concrete tank (150 m^3), with flowing sea water ($12\text{--}13^\circ\text{C}$, 30 ppt salinity, dissolved $\text{O}_2 > 130$ Torr, 17.3 kPa), and a 10 h dim light:14 h dark photoperiod. Sharks were fed twice a week with commercially purchased frozen hake (*Merluccius productus*). Feeding was stopped at least 48 h before any experimentation began. All experiments were performed according to the guidelines of the Canada Council for Animal Care, under joint approval (AUP A14-0251) of the animal care committees at BMSC and the University of British Columbia.

Experimental design

The experimental design consisted of measuring gill ventilation (breathing frequency and amplitude), ammonia, urea-N and diffusive water fluxes in dogfish at rest (i.e. prior to) and after exhaustive exercise. The experiments were performed under either normoxia ($\text{PO}_2 > 130$ Torr, 17.3 kPa) or hyperoxia ($\text{PO}_2 > 310$ Torr, 41.3 kPa). In a separate series, dogfish were put through the same rest and exhaustive exercise protocol for the measurement of oxygen consumption (MO_2). This was only done under normoxia.

Animals were removed from the main holding tank and anaesthetized (MS-222, Syndel Labs., Parksville, BC, Canada; 0.6 g/L neutralized to pH 7.8 with 5 M NaOH). They were placed on an operating table while irrigation of the gills with a diluted anesthetic solution (0.1 g/L) was maintained.

A short (1 cm) PE160 cannula (BD, Intramedic, Franklin Lakes, NJ, USA) that had been heat-flared at the inner end was inserted through the second gill slit, and secured in place by a simple stitch with suture silk (3–0, Perma-Hand Silk, Ethicon, Somerville, NJ, USA). A longer (~ 15 cm) heat-flared PE90 catheter was fitted through the PE160 and both cannulae were glued together with Acrylic glue (Vetbond; 3 M, Saint Paul, MN, USA). When needed for ventilation recordings, this water-filled catheter was bridged to a connecting PE50 using a blunt #21-gauge needle. The entire surgical procedure was performed in less than 5 min. Quickly after surgery, animals were transferred from the main holding tank to individual 40 L polyurethane-coated wooden boxes, previously used for studies with dogfish (Zimmer and Wood 2014; Giacomini et al. 2017; Wood and Giacomini 2016). Each box was served with perimeter aeration and flow-through seawater. Temperature was maintained by placing the wooden box in a water-bath served with flow-through ambient sea water at 12°C . Animals were allowed to recover in the boxes overnight for a minimum of 12 h.

On the next day, without disturbing the fish, the water-filled PE50 tubing was connected to a pressure transducer (DPT-100, Utah Medical Products, Midvale, UT, USA) and pressure amplitude (cm H_2O) and breathing frequency (breaths/min) were recorded for 4 h, in the undisturbed resting fish. The pressure transducer was calibrated against a 2 cm water column. The analog signal was amplified by an amplifier (LCA-RTC, Transducer Techniques, Temecula, CA, USA), digitalized in a PowerLab Data Integrity system (ADInstruments, Colorado Springs, CO, USA), and visualized and analyzed using LabChart v. 7.0 (ADInstruments). After the recording, the water flow to the box was stopped, and the volume set to 35 L by removing a rubber stopper located near the bottom of the box. Pre-exercise ammonia and urea-N fluxes were measured by appearance of these substances in the water. Water samples (5 mL) were taken at 0 h and 2 h (duration of the flux period), and immediately frozen at -20°C for later analyses of ammonia and urea concentrations. For the duration of the “flux measurement period”, water flow was interrupted and aeration remained constant. While the box was gently flushed with new sea water, the fish were quickly removed and injected intraperitoneally with $20 \mu\text{Ci}$ of $^3\text{H}_2\text{O}$ (Perkin-Elmer Wellesley, MA, USA) added to 10 mL of isotonic 500 mM NaCl solution. This was done for the measurement of diffusive water flux during the post-exercise period. Pilot experiments shown that a stable $^3\text{H}_2\text{O}$ efflux rate could be measured from 0.5 to 2.5 h after injection, before radioisotope recycling back to the animal became a problem, when the external specific activity exceeded 10% of the internal specific activity (Kirschner 1970; Giacomini et al. 2017). The fish were then quickly transported in a water-filled tub to a 250 L circular tank, set up with flow-through sea water at 12°C , where

they were exercised until exhaustion. The exercise protocol consisted of constantly prodding the fish's tail, chasing and motivating it to swim against a moderate water current generated by the inflow of water in the circular tank. This was repeated until the fish did not respond to stimuli anymore (routinely 20 min). Upon exhaustion, dogfish were returned to their individual boxes, with the volume set to 35 L. The water-filled PE50 cannula was immediately reconnected to the pressure transducer, and ventilation was recorded for 4 h as the dogfish recovered from exercise. Water samples (5 mL for $^3\text{H}_2\text{O}$ radioactivity measurements) were taken at 0.25 h intervals for 4 h, with a final sample at 6 h, after which water flow-through was re-established to the box. Additional 5 mL samples taken at 0, 2 and 4 h post-exercise were immediately frozen at $-20\text{ }^\circ\text{C}$ for later analyses of ammonia and urea-N concentrations. During this 6 h experimentation period, aeration in the box was kept constant and PO_2 was measured and kept at normoxia (140 Torr and above).

The number of available experimental animals was limited. Therefore, as this was a companion study to the temperature effects investigation of Giacomini et al. (2017) performed on the same batch of dogfish with identical methods, diffusive water flux data for animals at rest were taken from that investigation. The measurements in animals acclimated to $12\text{ }^\circ\text{C}$ and tested under either normoxia or hyperoxia in that study were used as comparisons for this investigation.

To evaluate the effects of hyperoxia on resting ventilation (breathing frequency and amplitude), ammonia, and urea-N flux rates and these same parameters during post-exercise recovery, the experimental protocol and timeline were followed exactly as described above. Pre-exercise ventilation was recorded for 4 h in normoxia, when normal aeration was stopped, and pure oxygen gas was bubbled into the perimeter aerator of the box until PO_2 reached >310 Torr, 41.3 kPa (hyperoxia). The PO_2 was checked every 0.25 h with a WTW Oxi 3205 oxygen meter (WTW, Weilheim, Germany). Fish were exposed to hyperoxia for 2 h prior to exercise, while ventilation was recorded, and water samples were taken for measurements of ammonia and urea concentrations. The procedures for IP injection of $^3\text{H}_2\text{O}$ and exhaustive exercise were identical to those described above. Note that the 20 min period of chasing was performed under normoxia, as we wished to ensure that the extent of exercise was the same in the two treatment groups. Once fish were returned to their individual boxes, hyperoxia was reinstated and water samples were collected over 4 h, as described above, with a final sample at 6 h for $^3\text{H}_2\text{O}$ radioactivity measurement.

MO_2 measurements were performed on a separate set of fish using the same procedures as described above, and the same methods as Giacomini et al. (2017). Briefly, water flow to the box was interrupted, the volume was set to 35 L and an initial PO_2 value was taken using a WTW Oxi 3205 oxygen meter. The box was then sealed with a floating lid to prevent

O_2 diffusion from the atmosphere to the water, and after 0.25–0.5 h a final PO_2 value was measured. The difference between the initial and final PO_2 values was used to calculate the MO_2 . This procedure was performed before exercise, and at hourly intervals during the 4 h post-exercise period, with gentle re-aeration in between measurement periods. PO_2 inside the box never fell below 120 Torr, 16.0 kPa.

Analytical techniques and calculations

For all calculations involving volume (V), the weight of the fish was subtracted from the wooden box volume, assuming 1 g fish = 1 mL. Oxygen consumption rates (MO_2 ; $\mu\text{mol O}_2/\text{kg/h}$) were calculated using the following equation:

$$\text{MO}_2 = [((\text{PO}_{2(i)} - \text{PO}_{2(f)}) \times \alpha_{\text{O}_2}) \times V] / (W \times T) \quad (1)$$

where $\text{PO}_{2(i)}$ and $\text{PO}_{2(f)}$ are the partial pressures of oxygen in the water (Torr) at the start and at the end of the experiment. α_{O_2} is the O_2 solubility coefficient ($\mu\text{mol/Torr/L}$) obtained from Boutilier et al. (1984) for $12\text{ }^\circ\text{C}$ and 30 ppt. V is volume (L), W is weight (kg) and T is the measurement period (h). Excess post-exercise oxygen consumption (EPOC) was calculated for each individual shark by fitting a polynomial curve to the hourly MO_2 values post-exercise (up to 4 h in recovery), and integrating the area under the curve using an individuals' shark pre-exercise MO_2 as the curve baseline intercept (Bouyoucos et al. 2017).

Ammonia-N (J_{amm} ; $\mu\text{mol N/kg/h}$) and urea-N ($J_{\text{urea-N}}$; $\mu\text{mol N/kg/h}$) net flux rates were calculated using the following equations:

$$J_{\text{amm}} = [(\text{Amm}_{(f)} - \text{Amm}_{(i)}) \times V] / (W \times T) \quad (2)$$

$$J_{\text{urea-N}} = \{[(\text{Urea-N}_{(f)} - \text{Urea-N}_{(i)}) \times V] / (W \times T)\} * 2 \quad (3)$$

where $\text{Amm}_{(f)}$ and $\text{Urea-N}_{(f)}$ are the final water ammonia ($\mu\text{mol/L}$) and urea-N ($\mu\text{mol/L}$) concentrations; $\text{Amm}_{(i)}$ and $\text{Urea-N}_{(i)}$ are the initial water ammonia ($\mu\text{mol/L}$) and urea-N ($\mu\text{mol/L}$) concentrations; V is volume (L), W is weight (g) and t is the measurement period (min). Ammonia and urea-N concentrations in the water ($\mu\text{mol/L}$) were measured colorimetrically using the methods described in Verdouw et al. (1978) and Rahmatullah and Boyde (1980) respectively. The nitrogen quotient (NQ) was calculated as follows:

$$\text{NQ} = J_{\text{N total}} / \text{MO}_2 \quad (4)$$

where $J_{\text{N total}}$ is the total nitrogen excretion rate obtained by the sum of the ammonia and urea-N excretion rates ($J_{\text{amm}} + J_{\text{urea-N}}$; Eq. 2 and 3 respectively), divided by the MO_2 (obtained in Eq. 1).

The ventilatory index (cmH₂O/sec) was calculated by multiplying the ventilation frequency (breaths/sec) by the ventilation amplitude (cmH₂O/breath).

For measuring ³H₂O radioactivity in water samples, scintillation fluor (Optiphase, PerkinElmer) was immediately added to the 5 mL samples in a 2:1 ratio (fluor:water). Samples rested for 12 h in the dark to minimize chemiluminescence before being counted for beta-emissions (Tri-Carb 2900TR Liquid Scintillation Analyzer; PerkinElmer). Tests showed that quench was constant.

The diffusive water flux calculations, based on the time-dependent efflux of ³H₂O cpm from the dogfish, were performed as described in detail by Giacomini et al. (2017). The original total amount of ³H₂O cpm in the fish at the end of exercise (start of post-exercise recovery) was calculated from the 6 h sample when washout was complete. The rate constant of ³H₂O turnover was calculated from the rate of ³H₂O radioactivity washout from the fish to the water, which was approximately exponential with time (Evans, 1967; Giacomini et al. 2017) using the following equation:

$$k = (\ln \text{CPM}_{(t_1)} - \ln \text{CPM}_{(t_2)}) / (t_1 - t_2) \quad (5)$$

where k is the rate constant of the ³H₂O efflux (in h⁻¹), CPM_(t₁) is the total ³H₂O radioactivity (cpm) in the fish at time t_1 (in h), and CPM_(t₂) is the total ³H₂O radioactivity (cpm) in the fish at time t_2 (in h). In practice, k was taken from linear regression slope of Eq. 5 over the first 2 h of washout. The product of $k \times 100\%$ yields the percent of body water turned over per hour.

Statistical analysis

All data are shown as means \pm 1 SEM. Parametric assumptions (normality and homoscedasticity) were checked before all parametric statistical analyses. Data from experiments where the same animal was measured throughout different time points were analyzed by a repeated-measures one or two-way ANOVA, depending on the number of factors being tested. Details of the statistical analyses performed for each data set, including post-hoc tests, are stated in the figure captions.

Results

The oxygen consumption rate (MO₂; Fig. 1) in pre-exercise fish was 2116 $\mu\text{mol O}_2/\text{kg/h}$ and significantly increased by \sim 1.8-fold in the first hour of post-exercise recovery, remaining significantly elevated until 2 h post-exercise (2663 $\mu\text{mol O}_2/\text{kg/h}$). At 3 h post-exercise, MO₂ returned to control levels. Exhaustive exercise resulted in an EPOC of $2739 \pm 223 \mu\text{mol O}_2/\text{kg}$.

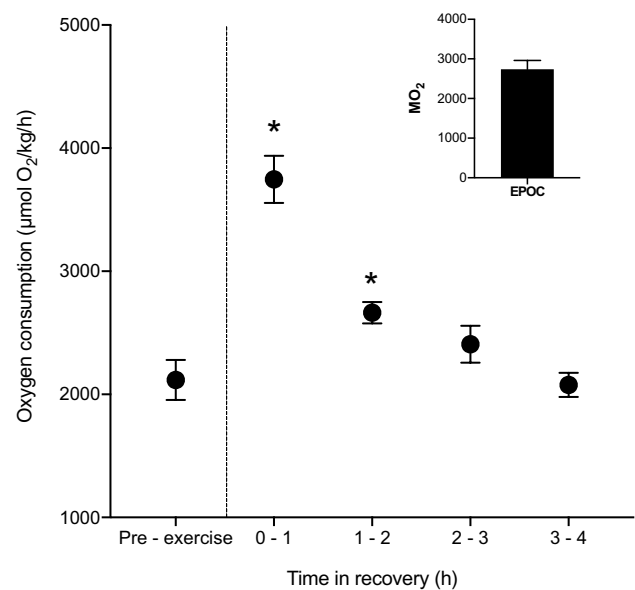


Fig. 1 Effect of exhaustive exercise on oxygen consumption rate (MO₂). MO₂ ($\mu\text{mol O}_2/\text{kg/h}$) in control (pre-exercise) and post-exhaustive exercise dogfish (*Squalus suckleyi*). Asterisks represent a significant difference between MO₂ in post-exercised and control (pre-exercise) as determined by a repeated-measures one-way ANOVA, followed by a Dunnett's post-hoc test. Excess post-exercise oxygen consumption (EPOC) is shown in the inset graph. Data are means \pm SEM, ($n=6$)

Oxygen level did not have a significant overall effect on ammonia efflux rates (J_{amm}) at any time period, though there were significant effects of time (Fig. 2A). J_{amm} (15–20 $\mu\text{mol}/\text{kg/h}$) did not differ between normoxia and hyperoxia in pre-exercise fish. In normoxia, J_{amm} increased by \sim 7.6-fold in the first hour post-exercise and stayed significantly elevated until 3 h post-exercise. In hyperoxia, there was a tenfold increase in J_{amm} in the first hour of recovery from exercise. Oxygen level also did not have a significant overall effect on urea-N efflux rates ($J_{\text{urea-N}}$; Fig. 2B), while time had a significant effect (Fig. 2B). $J_{\text{urea-N}}$ was not different between normoxia and hyperoxia (550–720 $\mu\text{mol N}/\text{kg/h}$) in pre-exercise fish. $J_{\text{urea-N}}$ was significantly elevated immediately post-exercise in both conditions, by 1.74-fold in normoxia and 1.78-fold in hyperoxia (Fig. 2B). $J_{\text{urea-N}}$ returned to control levels at 2 h post-exercise in both oxygen conditions. As MO₂ was measured only in normoxia, the nitrogen quotient (NQ) was calculated for control and post-exhaustive exercise fish in normoxia only (Table 1). No significant changes were observed in NQ in post-exercised fish over time in comparison to control (pre-exercise).

Figure 3 shows the effect of exhaustive exercise and oxygen concentration on the ventilation dynamics of dogfish. Oxygen level did not have a significant overall effect on any of the ventilation parameters measured (Fig. 3A: frequency; B: amplitude; C: index). Ventilation frequency

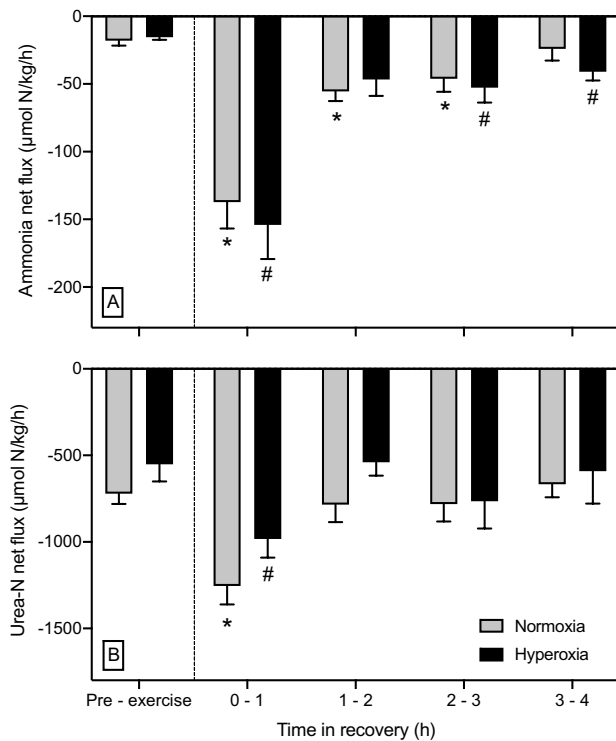


Fig. 2 Effect of hyperoxia on ammonia and urea-N flux rate in recovery from exhaustive exercise. **A** Ammonia efflux rate ($\mu\text{mol N/kg/h}$) and **B** Urea-N efflux rate ($\mu\text{mol N/kg/h}$) in control (pre-exercise) and post-exhaustive exercise dogfish (*Squalus suckleyi*), in normoxia (gray bars) and hyperoxia (black bars). Data were analyzed through a repeated-measures two-way ANOVA with time and oxygen level as factors. Asterisks and hashtags represent a significant difference between time post-exercise against control (pre-exercise) in normoxia and hyperoxia respectively, as determined by a Dunnett's multiple comparisons test. [Two-way ANOVA p values: **A** Ammonia efflux: $p_{\text{time}} < 0.001$, $p_{\text{oxygen}} = 0.6363$, $p_{\text{time} \times \text{oxygen}} = 0.6862$; **B** Urea-N efflux: $p_{\text{time}} < 0.001$, $p_{\text{oxygen}} = 0.1781$, $p_{\text{time} \times \text{oxygen}} = 0.5465$]. Data are means \pm SEM, ($n = 15$ for normoxia, $n = 8$ for hyperoxia)

Table 1 Effect of exhaustive exercise on Nitrogen quotient (NQ) of pre-exercise (control) and post-exhaustive exercise dogfish (*Squalus suckleyi*) in normoxia

	Nitrogen quotient (NQ)
Pre-exercise	0.432 ± 0.05
0–1 h post-exercise	0.418 ± 0.05
1–2 h post-exercise	0.375 ± 0.06
2–3 h post-exercise	0.386 ± 0.06
3–4 h post-exercise	0.373 ± 0.08

Data were not significantly different by a one-way ANOVA. Means \pm SEM, ($n = 8$)

was ~ 0.7 breaths/sec in control fish in normoxia and ~ 0.6 breaths/sec in control fish in hyperoxia. In normoxia, post-exercise ventilation frequency did not differ from controls, while in hyperoxia, post-exercise ventilation frequency

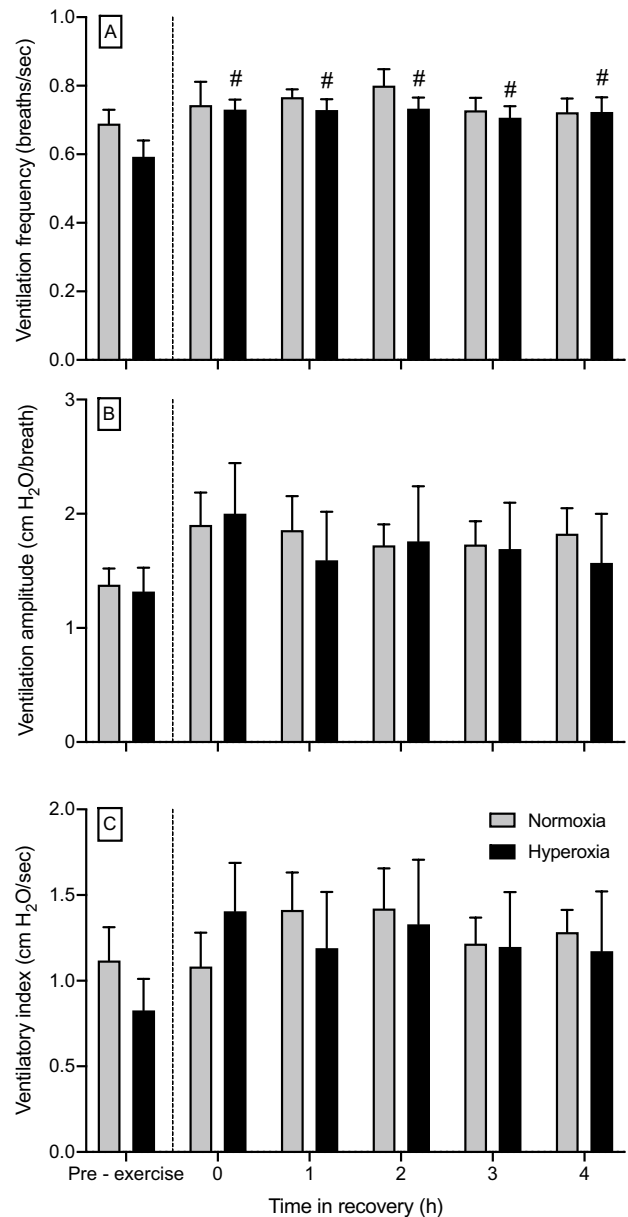


Fig. 3 Effect of hyperoxia on ventilation dynamics in recovery from exhaustive exercise. **A** Ventilation frequency (breaths/sec) and **B** Ventilation amplitude ($\text{cm H}_2\text{O/breath}$) and **C** Ventilatory index ($\text{cm H}_2\text{O/sec}$) in control (pre-exercise) and post-exhaustive exercise dogfish (*Squalus suckleyi*), in normoxia (gray bars) and hyperoxia (black bars). Data were analyzed through a repeated-measures two-way ANOVA with time and oxygen level as factors. Asterisks and hashtags represent a significant difference between time post-exercise against control (pre-exercise) in normoxia and hyperoxia respectively, as determined by a Dunnett's multiple comparisons test. [Two-way ANOVA p values: **A** ventilation frequency: $p_{\text{time}} = 0.0059$, $p_{\text{oxygen}} = 0.9078$, $p_{\text{time} \times \text{oxygen}} = 0.2293$; **B** ventilation amplitude: $p_{\text{time}} = 0.2561$, $p_{\text{oxygen}} = 0.8289$, $p_{\text{time} \times \text{oxygen}} = 0.8481$; **C** ventilatory index: $p_{\text{time}} = 0.0749$, $p_{\text{oxygen}} = 0.7794$, $p_{\text{time} \times \text{oxygen}} = 0.6304$]. Data are means \pm SEM, ($n = 8$)

significantly increased relative to the pre-exercise hyperoxia controls and remained elevated until 4 h post-exercise (Fig. 3A). There were no significant differences between normoxia and hyperoxia fish in ventilation amplitude (Fig. 3B). Time had no significant overall effect on ventilation amplitude, as no changes were seen in post-exercise fish in comparison to control, at both oxygen levels tested (Fig. 3B). The ventilatory index (i.e. the product of frequency times amplitude) was 1.12 cmH₂O/sec in control fish in normoxia, and 0.82 cmH₂O/sec in control fish in hyperoxia (Fig. 3C). The overall effect of time was close to significance ($p < 0.0749$), but the ventilatory index did not change significantly in post-exercise fish at any of the time points evaluated (Fig. 3C).

Oxygen level and time did not have significant overall effects on diffusive water flux rate (Fig. 4). In normoxia, water flux rate in control fish was 57.7% body water/h and it did not differ from post-exercise fish, (measured during the first 2 h of recovery) which had a water flux rate of 61% body water/h (Fig. 4). In hyperoxia, control fish had a higher water flux rate of 71.6% body water/h although not different from control normoxic fish. Post-exercise fish in hyperoxia had a water flux rate of 67.5% body water/h.

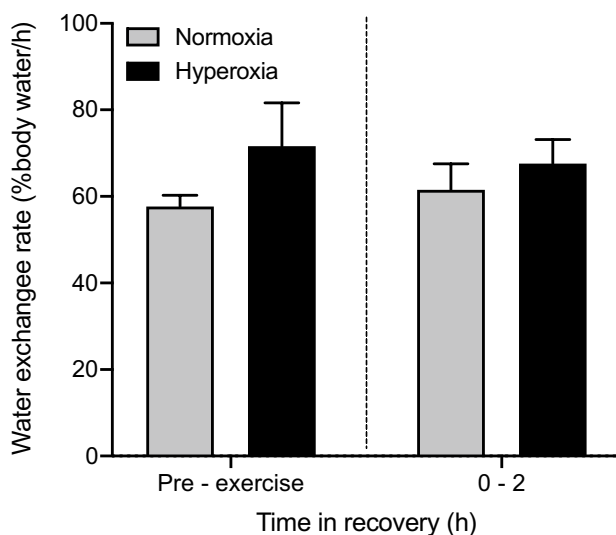


Fig. 4 Effect of hyperoxia on water flux rate in control and recovery from exhaustive exercise. Water flux rate (% body water/h) in control (pre-exercise) and post-exhaustive exercise in dogfish (*Squalus suckleyi*) in normoxia (gray bars) and hyperoxia (black bars). Data were analyzed through a two-way ANOVA with time and oxygen concentration as factors. [Two-way ANOVA p values: $p_{\text{time}} = 0.5508$, $p_{\text{oxygen}} = 0.9920$, $p_{\text{time} \times \text{oxygen}} = 0.1378$]. Data are means \pm SEM, ($n = 8$)

Discussion

As part of an ongoing effort to investigate the osmorepiratory compromise in fish species that are outside of the typical freshwater teleost model, our goal was to evaluate this phenomenon in dogfish sharks, using strenuous exercise as a way to trigger the physiological disturbances (increased metabolic demand resulting in elevated ventilation and MO_2) that are known to lead to the osmorepiratory compromise. Immediately after exhaustive exercise, MO_2 rose nearly two-fold on average, and was restored to control levels by 3 h in recovery (Fig. 1). These findings are consistent with an early study by Brett and Blackburn (1978) on the same species, who found that MO_2 was restored to resting rates after approximately 2.5 h of recovery from fatiguing exercise. We had hypothesized that to supply the elevated demand for oxygen during aerobic exercise, gill ventilation would be elevated and in general, our results did not agree with our hypothesis. We saw no significant differences in gill ventilation dynamics (frequency and amplitude of breathing) between control (rest) and recovery from exhaustive exercise (Fig. 4). Notably, in the larger spotted dogfish *Scyliorhinus stellaris*, Piiper et al. (1977) reported that ventilation declined rapidly after the end of exercise, returning to pre-exercise levels before the end of the period of EPOC. Increases in ventilation amplitude, representing increases in ventilatory stroke volume, appear to be more important contributor than changes in frequency in elevating ventilatory flow in elasmobranchs (Piiper et al. 1977; Milsom and Taylor 2016).

EPOC, sometimes referred to as O_2 debt, can be used as an indirect quantification of the energy used to restore physiological disturbances and replenishment of substrates used during exhaustive exercise (Gaesser and Brooks 1984). Piiper et al. (1977) found that the duration of EPOC in the larger spotted dogfish was proportional to the duration of the exercise activity, and in our study we found that it took between 2 and 3 h for dogfish to recover from 20 min of exhaustive exercise. The magnitude of the EPOC (2739 $\mu\text{mol O}_2/\text{kg}$) would be sufficient to support the control resting MO_2 (2116 $\mu\text{mol O}_2/\text{kg/h}$) for only 1.3 h, very modest relative to exhausted salmonids where EPOC would be sufficient to support resting MO_2 for 5–7 h (Scarabello et al. 1991; Zhang et al. 2018). This is in accord with the findings of Piiper et al. (1977) on *S. stellaris* exhibiting prolonged spontaneous activity for 17 min, where the subsequent EPOC would support resting MO_2 for only about 0.5 h. These findings suggest that the anaerobic component of fatiguing exercise is much smaller in dogfish sharks.

Likely, an adjustment contributing to increased MO_2 during the period of EPOC was an increase in the oxygen

extraction efficiency of the gills (i.e. increased % utilization). Piiper and Schumann (1967) found that the efficiency of O_2 exchange in the gills of unstressed *S. stellaris* is highly variable, and likely due to the shunting of blood in the gills. Decreases in venous PO_2 (i.e. increased water-to-blood PO_2 gradient), increased cardiac output, gill vasodilation, and increased effective permeability of the branchial epithelium (decreased diffusion distance, increased surface area) could all make important contributions to this increased utilization (Brill and Lai 2016), and thereby evoke the osmorepiratory compromise. Additionally, increases in blood pressure during exercise could have made the gills more prone to the loss of osmolytes, as suggested in the seminal work by Gonzalez and McDonald (1992).

The second part of our hypothesis was that, as per the osmorepiratory compromise, elevations in MO_2 would be accompanied by increases in passive losses of nitrogenous-waste products, which in the case of elasmobranchs, also act as osmolytes (reviewed by Wright and Wood, 2016). Our results showed a marked elevation in both ammonia and urea losses (J_{amm} and J_{urea-N}) in recovery from exhaustive exercise (Fig. 2), with J_{amm} increasing by 7.6-fold and remaining elevated up until 3 h post-exercise. In a similar study, where dogfish were exercised until exhaustion, total ammonia in the plasma (T_{amm}) was significantly elevated by about two-fold, and remained high for 2 h post-exercise (Richards et al. 2003). In addition, Zimmer and Wood (2014) investigated the osmorepiratory compromise in dogfish sharks in response to exposure to different levels of hypoxia, and found marked increases in J_{amm} in severe hypoxia, which remained high for 4 h in recovery. However, plasma T_{amm} remained unchanged. Our results are consistent with previous investigations by Giacomini et al. (2017). In that study, dogfish were exposed to elevated water temperatures and while MO_2 rose in a near linear fashion with temperature, J_{amm} showed a biphasic response, increasing to a much greater extent at high temperature. In the present study, similar to that of Giacomini et al. (2017), ammonia flux was more affected than urea flux, as we observed a proportionally larger increase in J_{amm} than J_{urea-N} . It is possible that the elevated levels of total ammonia circulating in the plasma post-exercise (Richards et al. 2003) contributed to an elevation in J_{amm} , despite the extremely low effective permeability of the elasmobranch gill to ammonia (Wood et al. 1995; Wright and Wood 2016). However, differing from our study, Holeyton and Heisler (1983) reported that ammonia excretion remained unchanged for up to 30 h after exhaustive exercise in *S. stellaris*. Ammonia is used to fuel the ornithine-urea cycle (Zimmer et al. 2017; Ballantyne 1997) and recent studies have shown that dogfish sharks have the capacity to scavenge ammonia from the water against both a partial pressure and electrochemical gradients (Nawata

et al. 2015) with a transport system that exhibits Michaelis–Menten kinetics (Wood and Giacomini 2016). Although ammonia balance seems to be more sensitive to increases in MO_2 , urea plays a much larger overall role in the osmoconforming strategy of elasmobranchs, being accumulated in the plasma at very high concentrations (300–400 mmol/L) (Smith 1936; Wright and Wood 2016).

The earlier studies into the osmorepiratory compromise in freshwater teleosts reported a rise in Na^+ and Cl^- loss rates, which are the major osmolytes in teleost blood, in response to exercise (see Introduction). However, in elasmobranchs, urea is the major osmolyte in the blood plasma. Our results show that J_{urea-N} was significantly elevated immediately post-exercise by 1.74-fold in normoxia, and by 2 h of recovery, it was restored to control levels (Fig. 2B). To our knowledge, there are no other studies that have looked at urea metabolism and excretion during recovery from exhaustive exercise in elasmobranchs. In a previous investigation into the osmorepiratory compromise in elasmobranchs, we found that J_{urea-N} was affected by rising water temperatures, and like J_{amm} , it was decoupled from the rise in MO_2 (Giacomini et al. 2017). J_{urea-N} did not vary from 7.5 to 15 °C, but increased greatly reaching a two-fold increase at 22 °C. Similarly, Zimmer and Wood (2014) found that J_{urea-N} was elevated during exposure to hypoxia, and returned to pre-exposure levels as soon as normoxia was re-established. Although no urea-transporting protein has been identified in the elasmobranch gill (Wright and Wood 2016), there is mounting physiological evidence for several mechanisms of urea conservation at the gills that contribute to a very low effective permeability to urea (Wood et al. 1995, 2013; Pärt et al. 1998; Fines et al. 2001). Therefore, the increase in J_{urea-N} after exercise could be a result of a reduced effectiveness of the branchial urea retention mechanism, in the face of general gill permeability increases due to the osmorepiratory compromise. There seems to be now a foundational body of evidence supporting the susceptibility of both urea and ammonia regulation to various external (hypoxia and temperature) and internal (exercise) stressors. Future studies that couple measurements of plasma nitrogenous osmolytes with the monitoring of losses to the water would be useful in advancing our understanding of the osmoregulatory compromise in elasmobranchs.

By measuring MO_2 as well as urea-N and ammonia-N excretion rates concomitantly, we were able to calculate the N/O_2 ratio, also known as the nitrogen quotient (NQ) (Table 1). The NQ is an index of fuel utilization for the maintenance of aerobic metabolism (Lauff and Wood 1996). There was no change between the NQ of fish in recovery (at all times tested) and control fish, and the relatively high absolute values indicate that protein (amino acids) seem to be the primary metabolic fuel used by fasting dogfish, at rest and during recovery from exhaustive exercise. This finding

is in accord with previous studies on resting dogfish (Wood et al. 2007; Giacomini et al. 2017).

Diffusive water fluxes are another important, yet overlooked, component of the osmorepiratory compromise (Wood and Eom 2021). In most elasmobranchs, reported water exchange rates can vary from 80% to nearly 170% of the total body water pool per hour (Payan et al. 1971; Carrier and Evans 1972), which are notably higher than in marine teleosts (Wright and Wood 2016). Our rates were slightly lower (~60–70% per hour; Fig. 4), but still much higher than in marine teleosts, as noted previously (Giacomini et al. 2017). In that study, water exchange rates varied almost linearly with MO_2 , but increased far less than J_{amm} or J_{urea-N} at high temperature. This pattern is somewhat similar to that seen in the present study, where there was actually no significant change in water flux rates, but substantial elevations in MO_2 , J_{urea-N} , and especially J_{amm} during recovery from exhaustive exercise. Nevertheless, it was initially surprising that diffusive water flux rates, measured over 0–2 h of recovery, did not increase at all (Fig. 4), despite significant elevations of MO_2 at both 0–1 h, and 1–2 h (Fig. 1). However, a possible explanation lies in the discovery of several types of aquaporins (diffusive water flux channels) in the gills of this species (Cutler et al. 2012), including aquaporin AQP3 (Cutler et al. 2022). Modulation of the protein expression of AQP3 has recently been implicated in diffusive water flux responses to hypoxia in the killifish (Ruhr et al. 2020), and Cutler et al. (2022) have demonstrated that the water-permeability function of AQP3 is strongly inhibited by extracellular acidosis, as occurs after exhaustive exercise in the Pacific spiny dogfish shark (Richards et al. 2003). While we had initially speculated (see Introduction) that exhaustive exercise, in contrast to hypoxia (Zimmer and Wood 2014) or temperature elevations (Giacomini et al. 2017) would be less likely to impact selective protein-mediated fluxes at the gills, thereby revealing more clearly the simple diffusive components of the osmorepiratory compromise, this now seems overly simplistic. Regardless, it is now clear that ammonia losses are the most affected, urea-N losses are intermediate, and diffusive water fluxes are the least affected by the osmorepiratory compromise in this species.

Hyperoxia is a relevant environmental condition for dogfish sharks, which are known for migrations and for making foraging and breeding movements into estuaries and river mouths (Ulrich et al. 2007; Bouyoucos et al. 2021). Estuaries on their own are highly variable environments, exhibiting large daily variations in water temperature, salinity and dissolved oxygen, mostly due to variations in photosynthesis and respiration rates (Kennish 1986). To investigate the potential alleviating effects of an improved oxygen availability (hyperoxia) on the osmorepiratory compromise, we allowed the animals to recover from exhaustive exercise in hyperoxia. Contrary to our

prediction that elevated oxygen availability would alleviate the osmorepiratory compromise, we saw no effect of hyperoxia on any of the parameters we investigated. In general, environmental hyperoxia decreases gill ventilation in fish (Dejours et al. 1977), and it has been shown to have a profound hypoventilatory effect in *S. stellaris*, which was attributed to increased arterial partial pressure of oxygen (PaO_2) (Heisler et al. 1998). However, a recent assessment on *S. suckleyi* reported only small, non-significant effects of hyperoxia on breathing frequency and amplitude (Acharya-Patel et al. 2018). This is consistent with our findings, where we saw only a small, non-significant depression in ventilation during hyperoxia exposure in control (at rest) animals (Fig. 3). Note that the significant increase in ventilation frequency after exercise seen only in our hyperoxia-exposed dogfish reflected not a higher absolute frequency than in normoxia-exposed fish, but rather their lowered control frequency (Fig. 3A). We saw no protective effect of hyperoxia on recovery from exercise as fluxes of osmolytes were elevated in a similar magnitude as for normoxic animals (Fig. 2). Indeed, Giacomini et al. (2017) found a further exacerbation in osmolyte fluxes when dogfish were exposed to high temperature in combination with hyperoxia, re-enforcing this lack of benefit. Thus, marine hyperoxic zones will not offer relief for refuge-seeking tired or temperature-stressed dogfish.

In summary, exhaustive exercise seems to elicit physiological disturbances that can be attributed to the osmorepiratory compromise, and while they are of significant nature at the start of the recovery period, these disturbances are resolved within the time frame of our experiments (4 h). Dogfish are often by-catch in recreational angling or commercial fisheries practices in the west coast of North America. The ability to re-establish osmolyte fluxes within a short time frame can be of great benefit for angled or trawled sharks that are released back to the environment (Brill et al. 2008; Schwieterman et al. 2021). Similar to responses to severe hypoxia (Zimmer and Wood 2014) and high temperatures (Giacomini et al. 2017), urea-N retention seems to be more greatly impacted than ammonia retention by the osmorepiratory compromise after exhaustive exercise, in accord with the critical role of urea in the osmoregulatory strategy of marine elasmobranchs (Smith 1936).

Acknowledgements This study was supported by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grants to CMW (RGPIN-2017-03843 and RGPIN/473-2012) and PMS (RGPIN-203189). M.G. was 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 at the time) and the BMSC research and animal care supporting staff for excellent and invaluable support. Also, a whole-hearted thanks to Dr. Junho Eom for training and assistance with the ventilation set up. Finally, the authors would like to acknowledge the incredible women who assisted with shark handling and support with experiments: Dr.

Gudrun De Boeck, Dr. Lygia Nogueira, Dr. Roberta D. Klein and Olivia J.L. McMillan.

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