Differential Effects of Temperature on Oxygen Consumption and Branchial Fluxes of Urea, Ammonia, and Water in the Dogfish Shark (*Squalus acanthias suckleyi*)

Marina Giacomin[1,2,*]
Patricia M. Schulte[1]
Chris M. Wood[1,2,3,†]
[1]Department of Zoology, University of British Columbia, Vancouver, British Columbia V6T 1Z4, Canada; [2]Bamfield Marine Sciences Centre, Bamfield, British Columbia V0R 1B0, Canada; [3]Department of Biology, McMaster University, Hamilton, Ontario L8S 4K1, Canada

Accepted 6/28/2017; Electronically Published 10/3/2017

ABSTRACT

Environmental temperature can greatly influence the homeostasis of ectotherms through its effects on biochemical reactions and whole-animal physiology. Elasmobranchs tend to be N limited and are osmoconformers, retaining ammonia and urea-N at the gills and using the latter as a key osmolyte to maintain high blood osmolality. However, the effects of temperature on these key processes remain largely unknown. We evaluated the effects of acute exposure to different temperatures (7°, 12°, 15°, 18°, 22°C) on oxygen consumption, ammonia, urea-N, and diffusive water fluxes at the gills of *Squalus acanthias suckleyi*. We hypothesized that as metabolic demand for oxygen increased with temperature, the fluxes of ammonia, urea-N, and ^3H_2O at the gills would increase in parallel with those of oxygen. Oxygen consumption (overall $Q_{10} = 1.76$ from 7.5° to 22°C) and water fluxes (overall $Q_{10} = 1.96$) responded to increases in temperature in a similar, almost linear, manner. Ammonia-N efflux rates varied the most, increasing almost 15-fold from 7.5° to 22°C ($Q_{10} = 5.15$). Urea-N efflux was tightly conserved over the 7.5°–15°C range ($Q_{10} \sim 1.0$) but increased greatly at higher temperatures, yielding an overall $Q_{10} = 1.45$. These differences likely reflect differences in the transport pathways for the four moieties. They also suggest the failure of urea-N- and ammonia-N-conserving mechanisms at the gill above 15°C. Hyperoxia did not alleviate the effects of high temperature. Indeed, urea-N and ammonia-N effluxes were dramatically increased when animals were exposed to high temperatures in the presence of hyperoxia, suggesting that high partial pressure of oxygen may have caused oxidative damage to gill epithelial membranes.

Keywords: temperature, elasmobranch, gill permeability, urea, ammonia, tritiated water, hyperoxia, osmorespiratory compromise.

Introduction

Environmental temperature has profound effects on multiple levels of biological organization and can greatly affect organismal homeostasis (Hochachka and Somero 2002). In a classic study on the lesser-spotted dogfish (*Scyliorhinus canicula*), Butler and Taylor (1975) showed that oxygen uptake (M_{O_2}), cardiac output, and heart rate all increased with water temperature, with Q_{10} between 2.1 and 2.5 (Butler and Taylor 1975). More recent studies on metabolic rate in other elasmobranchs have confirmed that they exhibit a temperature sensitivity that is typical of ectotherms, with Q_{10} usually ranging from 2 to 3 (e.g., Miklos et al. 2003; Di Santo and Bennett 2011), although Di Santo (2016) recently reported much higher values (5 to 11.5) in a species of skate. However, few studies have focused on the effects of temperature on the excretion of nitrogenous-waste (N-waste) products. N-waste handling is particularly important in this group, because of their ureotelism and their unique urea-based osmoregulatory strategy, which requires some retention of N-waste. In contrast, this topic has been well studied in ammoniotelic teleost fish where Q_{10} for metabolic rate is somewhat lower (usually below 2.0), and there is general agreement that N-waste excretion is much more sensitive than M_{O_2} to increases in temperature (i.e., higher Q_{10}; for review see Wood 2000). For example, in three model teleosts (goldfish [Maetz 1972], trout [Kieffer et al. 1998], and tilapia [Alsop et al. 1999]), Q_{10} for ammonia excretion was well above the value for M_{O_2}. The one elasmobranch study of which we are aware is that of Boylan (1967) on the spiny dogfish (*Squalus acanthias*). Using an unusual artificially ventilated whole-animal preparation, Boylan reported a surprising conclusion that has never been confirmed: branchial urea-N excretion was independent of temperature up to 15°C and then increased sharply at higher temperatures, suggesting the breakdown of some urea-N conservation mechanism.

Urea-N conservation is important in seawater elasmobranchs because they are osmoconformers and utilize organic osmolytes in order to maintain internal osmolality slightly greater than that of their environment, thereby promoting the osmotic influx of wa-

*Corresponding author; e-mail: mahhgiacomin@gmail.com.
†ORCID: http://orcid.org/0000-0002-9542-2219.

Physiological and Biochemical Zoology 90(6):627–637. 2017. 1522-2152/2017/9006-16112$15.00. DOI: 10.1086/694296

ter and eliminating the need for drinking seawater (Smith 1931, 1936). The major organic osmolyte retained is urea, supplemented by much lower concentrations of counteracting methylamines, such as trimethylamine N-oxide, which stabilize protein function (see Yancey 2016). Urea is synthesized from ammonia through the ornithine-urea cycle (OUC), at the expense of 5 mol of ATP per mol of urea (2 mol of urea-N) produced (for review see Ballantyne 1997), accounting for approximately 10% of the overall M_{O_2} of elasmobranchs (Kirschner 1993). This urea-retaining strategy results in a massive urea gradient across the gills (300–400 mmol/L inside vs. 0 mmol/L outside).

The mechanism of urea-N retention at the gills against this enormous gradient remains controversial. Dogfish (*S. acanthias*) gill basolateral membranes possess unusually high cholesterol-to-phospholipid and phosphatidylcholine-to-phosphatidylethanolamine ratios that are thought to contribute to low urea permeability (Fines et al. 2001). The apical cell membrane surface area is very low relative to the basolateral cell membrane surface area, and apical membrane permeability to urea is even lower than basolateral membrane permeability (Pärt et al. 1998; Hill et al. 2004). A "back-transport" mechanism appears to trap urea that leaks through the gills (Wood et al. 1995; Pärt et al. 1998). Although no urea-transporting protein has ever been isolated in the elasmobranch gill (Wright and Wood 2016), there is physiological evidence for an active urea back-transporter located basolaterally (Fines et al. 2001) and/or apically (Wood et al. 2013).

The elasmobranch gill is also even less impermeable to ammonia than it is to urea, keeping ammonia losses at just a few percent of urea-N losses (Wood et al. 1995, 2005). Recently, Nawata et al. (2015) and Wood and Giacomin (2016) showed that nitrogen-limited *S. acanthias* are capable of actively scavenging ammonia-N from the water by a branchial transporter exhibiting saturation kinetics, resulting in increased synthesis of urea. The mechanism of ammonia uptake is unknown but appears to involve Rh proteins.

Another important aspect of the elasmobranch osmoregulatory strategy is the branchial transport of water. Water permeability at the elasmobranch gill has to be kept relatively high, given the usually low osmotic gradient from seawater to plasma (10–70 mOsmol/L) and the need to take up water to form urine and rectal gland secretions (Wright and Wood 2016). The few available measurements of diffusive water turnover rates (with ^3H_2O; Payan and Maetz 1971; Payan et al. 1973; Hayward 1975) indicate branchial permeabilities about fivefold greater than in marine teleosts.

Against this background, the goal of this study was to evaluate the effects of acute exposure to different temperatures on M_{O_2}, on the excretion of N-wastes (ammonia and urea-N), and on diffusive water transport at the gills of the Pacific spiny dogfish (*Squalus acanthias suckleyi*). *Squalus acanthias* was chosen as our experimental model due to the extensive body of literature on the physiology of this species, which is a migratory, long-lived, very important demersal resource that is under threat due to overfishing and global climate change (Taylor and Galluci 2009), like many other marine elasmobranchs (Dulvy et al. 2014). The present investigation was part of a larger ongoing study on the osmorespiratory compromise and gill function in this species (Zimmer and Wood 2014; Nawata et al. 2015; Wood and Giacomin 2016). The osmorespiratory compromise is the functional trade-off between respiratory gas exchange versus osmolyte and water exchange that has been well documented in freshwater teleosts (Randall et al. 1972; Nilsson 1986) but never examined in marine elasmobranchs. The osmorespiratory compromise predicts that permeability changes at the gills that favor increased O_2 consumption may cause unfavorable passive fluxes of osmolytes and water. A particular focus was to replicate the unusual results obtained by Boylan (1967) while using a less invasive experimental technique. Ammonia, urea-N, and tritiated water (^3H_2O) fluxes were assessed following exposure to 7.5°, 12° (acclimation temperature, used as control), 15°, 18°, and 22°C. Based on fishery data and capture records, this temperature range is environmentally relevant for this species that occurs between extremes of 4° and 24°C in the wild (Bangley and Rulifson 2014; Sagarese et al. 2014). Our general hypothesis was that as metabolic demand for oxygen increased with temperature, the fluxes of ammonia, urea-N, and ^3H_2O at the gill would increase in parallel with that of oxygen. We performed follow-up experiments where the animals were exposed to control (12°C) and high (22°C) temperature in the presence of hyperoxia. We hypothesized that increasing oxygen availability would minimize or prevent the associated effects of rising temperature on gill permeability.

Material and Methods

Animal Housing and Acclimation

Spiny dogfish (*Squalus acanthias suckleyi*) were caught by angling near Bamfield Marine Sciences Centre (BMSC), Bamfield, British Columbia, Canada (48.8355°N, 125.1355°W) under Fisheries and Oceans Canada collecting permit XR2392015. At BMSC, they were held for approximately 20 d before experimentation in a large (150,000-L) indoor concrete tank. The tank was served with flowing seawater (12°–13°C, 30 ppt salinity) pumped directly from the nearby ocean, and dissolved O_2 was kept at >80% saturation. Dim lighting was maintained in the room for approximately 10 h/d. During this period, fish were fed twice a week with a 3% ration of commercially purchased frozen hake (*Merluccius roductus*). Feeding was stopped at least 1 wk before experimentation began. All animal experimentation was carried out under the guidelines of the Canada Council for Animal Care under approval of animal care committees at BMSC and the University of British Columbia (joint AUP A14-0251). In total, 20 adult male dogfish weighing 1.62 ± 0.05 kg were used in this study. Animals were not fed during the experimentation period (maximum of 2 wk).

Experimental Setup

Animals were transferred from the main holding tank to individual 40-L wooden boxes coated with polyurethane, which have been previously used for studies with dogfish (Wood et al. 1995; Zimmer et al. 2014). Each box was served with perimeter aeration and fed with flow-through seawater. All animals were acclimated

to the boxes for at least 12 h before experiments were conducted. In all experiments, O_2 and temperature levels were monitored using a YSI 55 handheld oxygen meter fitted with a temperature probe (YSI Incorporated, Yellow Springs, OH), which was referenced to a precision thermometer, serial 210620, traceable to National Institute of Standards and Technology standards (H-B Instrument, Trappe, PA).

Experimental Series

Series 1: Acute Temperature Exposure. The effect of acute temperature changes on dogfish O_2 consumption rates, ammonia, and urea-N fluxes was assessed using temperatures of 7.5°, 12° (control = acclimation temperature), 15°, 18°, and 22°C. The same dogfish ($n = 8$) were tested at 12°, 15°, 18°, and 22°C on different days, with a minimum 24-h interval back at 12°C between each experiment; the order of temperature testing was sequential rather than random in these animals. A different set of animals ($n = 6$) was tested at 7.5°C.

For flux measurements, the water flow to the box was stopped, and the volume of water was set to 35 L by the removal of a rubber stopper located near the bottom of the box. The control temperature of 12°C was maintained by placing the wooden box in a water bath served with flow-through ambient seawater at 12°C. The 7.5°C temperature was achieved by the addition of several 250-mL, –80°C-frozen seawater blocks, which were mixed into the water in the box. After the target temperature of 7.5°C was reached, ice chips were added to the bath surrounding the box, in order to maintain the 7.5°C inside without altering or diluting the box volume. The increased temperature treatments of 15°, 18°, and 22°C were performed by gradually mixing heated seawater with the water in the box, until the desired temperature was reached, and thereafter the experimental temperature was maintained using immersed aquarium heaters. Water mixing inside the boxes was ensured by constant, well-spread perimeter aeration. The temperature in each box was monitored every 0.25 h and never differed from the target temperature by more than ±0.25°C. The desired temperatures (7.5°, 15°, 18°, and 22°C) were always achieved within 0.5 h, and all animals were left to adjust to the new temperature for an additional 0.5 h before the start of the experiment. Water samples (5 mL) were taken at 0, 2, 4, and 6 h and then frozen at −20°C for later analyses of ammonia and urea concentrations. During the final 2 h of the experiment, shortly after the 4-h water sample, air flow inside the box was stopped, and an initial oxygen partial pressure (P_{O_2}) value was taken using the YSI 55 handheld O_2 meter. After that, the box was sealed with a floating lid to prevent O_2 diffusion from the air into the water, and after 0.25–0.5 h, depending on the temperature, a final P_{O_2} value was measured. O_2 saturation never fell below 120 Torr. The difference between the initial and final P_{O_2} values was used to calculate the O_2 consumption rate. This procedure was repeated once after a 1-h reaeration period. At the end of the 6-h experiment, a final 5-mL water sample was taken, ending the experimental period. After that, the seawater flow-through and aeration were reestablished to the box, and the temperature was gradually brought back to control levels.

The effect of acute temperature changes on tritiated water (^3H_2O) turnover rates in dogfish was measured in separate experiments, using some of the same animals from the preceding trials ($n = 6$). Animals used for this series remained in the experimental chambers for no longer than 2 wk. The same experimental protocol as described above for changing and maintaining the temperature was used. Pilot experiments demonstrated that stable ^3H_2O efflux rates could be measured from about 0.5 to 1.5–2.5 h after injection, depending on temperature, after which recycling of the radioisotope became a problem, because external specific activity exceeded 10% of internal specific activity (Kirschner 1970).

Once the fish had settled at the experimental temperature for 0.25 h, they were injected intraperitoneally with 20 μCi of ^3H_2O (PerkinElmer, Wellesley, MA) diluted in 10 mL of isotonic NaCl (500 mmol/L). For the injection, the fish were not anesthetized and spent less than 1 min out of water; this protocol was adopted to avoid a long postanesthesia recovery time, which would have compromised the measurement window for tritiated water efflux. The fish was allowed to settle for 0.25 h before an initial 5-mL water sample was taken, marking the start of the experiment. Water samples were taken at 0.25-h intervals for 2.5 h, with a final sample at 4–6 h, after which seawater flow-through was reestablished to the box, bringing the temperature gradually back to control levels. The final sample was taken to ascertain the exact dose of ^3H_2O administered to each fish, because by this time the radioisotope had completely equilibrated between the fish and the water (see "Analytical Procedures and Calculations"). The experiment was repeated at different temperatures on subsequent days after at least 24 h recovery back at 12°C. Water samples were immediately dosed with scintillation fluor (Optiphase, PerkinElmer, Waltham, MA) in a 2 : 1 ratio (fluor : water). Samples rested for 12 h in the dark to eliminate chemiluminescence before being counted for beta emissions (Tri-Carb 2900TR Liquid Scintillation Analyzer; PerkinElmer). Tests showed that quench was constant.

Series 2: Hyperoxia Experiments. The effects of hyperoxia ($P_{O_2} > 310$ Torr) on the responses of ammonia and urea-N fluxes, as well as on ^3H_2O turnover rates, to acute temperature changes were assessed. A new set of fish ($n = 6$) was tested at 12°C (control) and 22°C under hyperoxia. The protocol used to reach and maintain the targeted temperature was similar to that of series 1. Once the fish had settled in experimental temperature for approximately 30 min, aeration was stopped and pure oxygen gas (O_2) was bubbled in the box until the P_{O_2} reached >310 Torr (hyperoxia). The P_{O_2} and temperature were checked every 0.25 h with a YSI 55 handheld meter. For ammonia and urea-N flux measurements, the fish were allowed to settle at the experimental temperature for 0.5 h, and an initial 5-mL water sample was then taken to start the experiment (0 h), with additional samples at 2 and 4 h. Water samples were frozen at −20°C for later analyses of ammonia and urea concentrations. After 4 h, normoxic seawater flow-through was reestablished, and fish were allowed to recover for at least 24 h. Data from these fish were compared with data from normoxic fish of series 1, measured over the same 4-h period. The same two sets of fish (normoxia

and hyperoxia exposed) were used subsequently for the ^3H_2O turnover measurements at the two temperatures, using a protocol identical to that outlined in series 1.

Analytical Procedures and Calculations. Ammonia-N (J_{amm} [μmol N/kg/h]) and urea-N ($J_{urea\text{-}N}$ [μmol N/kg/h]) flux rates were calculated using the following equations:

$$J_{amm} = \frac{(amm_f - amm_i) \times V}{W \times T}, \quad (1)$$

$$J_{urea\text{-}N} = \frac{(urea_f - urea_i) \times V}{W \times T} \times 2, \quad (2)$$

where amm_f and $urea_f$ are the final water ammonia (μmol/L) and urea (μmol urea/L) concentrations; amm_i and $urea_i$ are the initial water ammonia (μmol/L) and urea (μmol urea/L) concentrations; V is the volume of water during the experiment (L); W is the weight of the animal (kg); and T is the duration of the flux period (h). Ammonia and urea concentrations in the water were measured colorimetrically according to Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. The total nitrogen excretion rate ($J_{N\ total}$ [μmol N/kg/h]) was then calculated as

$$J_{N\ total} = J_{amm} + J_{urea\text{-}N}. \quad (3)$$

Note that the molar urea excretion rate is multiplied by 2 in equation (2) so the two nitrogen atoms of urea are taken into account; therefore, $J_{N\ total}$ is expressed in units of N.

The rate constant of ^3H_2O turnover was calculated from the rate of decline in total ^3H_2O radioactivity in the fish, which was approximately exponential with time (Evans 1967):

$$k = \frac{\ln CPM_1 - \ln CPM_2}{T_1 - T_2}, \quad (4)$$

where k is the rate constant of the efflux (in h^{-1}), CPM_1 = total ^3H_2O radioactivity (in cpm) in the fish at time T_1 (in h), and CPM_2 = total ^3H_2O radioactivity (in cpm) in the fish at time T_2 (in h). The product of $k \times 100\%$ yields the percent of body water turned over per hour.

In practice, ^3H_2O efflux rates were calculated by regressing the natural logarithm of CPM measurements against time over the range of linearity (generally 0.5 to 1.5–2.5 h after injection), depending on temperature, to yield the slope k. By measuring the ^3H_2O radioactivity in the water after 4–6 h, when complete equilibration between the fish and the water had occurred, it was possible to calculate accurately the total amount of radioactivity (CPM_{total}) in the system. The volume of the system was taken as the measured volume of external water plus the volume of the fish. Therefore, from CPM_{total} and from measurements of ^3H_2O radioactivity appearance in the water at each time interval, it was possible to back-calculate the CPM in the fish at each time during the experiment.

O_2 concentrations in the water were obtained by converting P_{O_2} values using solubility constants from Boutilier et al. (1984). Oxygen consumption rates (M_{O_2} [μmol O_2/kg/h]) were calculated using the following equation:

$$M_{O_2} = \frac{(O_{2i} - O_{2f}) \times V}{W \times T}, \quad (5)$$

where O_{2i} and O_{2f} are oxygen concentrations in the water (μmol/L) at the start and end of the experiment, respectively, and the other variables are as defined above.

The temperature coefficient (Q_{10}) for every 10°C increment in temperature was calculated using the mean values for each physiological parameter at each experimental increment as

$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{10/(T_2 - T_1)}, \quad (6)$$

where R_1 and R_2 are the rates of interest measured at temperature 1 (T_1 [°C]) and temperature 2 (T_2 [°C]).

The relative protein use as a fuel for aerobic metabolism was calculated according to Lauff and Wood (1996). First, the nitrogen quotient (NQ) was calculated as

$$NQ = \frac{J_{N\ total}}{M_{O_2}}. \quad (7)$$

The percentage of metabolism fueled by the oxidation of protein was then determined by

$$\%protein = \frac{NQ}{0.27} \quad (8)$$

because 0.27 represents the maximum theoretical value when protein is the only fuel being metabolized (Van Den Thillart and Kesbeke 1978).

Statistical Analyses

All data are represented as means ± 1 SEM. Normality and homoscedasticity were tested through Shapiro's and Bartlett's tests, respectively, before all parametric statistical analyses. All data reported here passed the two criteria for using parametric tests. Data from experiments where measurements were made on the same animals under different experimental treatments were analyzed by either a repeated-measures one-way ANOVA followed by a Tukey's post hoc test (for multiple comparisons) or a paired Student's two-tailed *t*-test (for binary comparisons). Means originating from independent experiments were compared through a one-way ANOVA followed by a Tukey's post hoc test (for multiple comparisons) or an unpaired Student's two-tailed *t*-test (for binary comparisons). Where data were used in two comparisons, the Bonferroni correction was applied. Significance was accepted at the level of 0.05. The specific statistical analyses employed for each data set are detailed in the figure legends.

Results

The oxygen consumption rate (M_{O_2}) at the acclimation temperature of 12°C was approximately 2,140 μmol O_2/kg/h and varied with acute changes in temperature in a close to linear fashion (fig. 1*A*). M_{O_2} fell significantly at 7.5°C and increased significantly

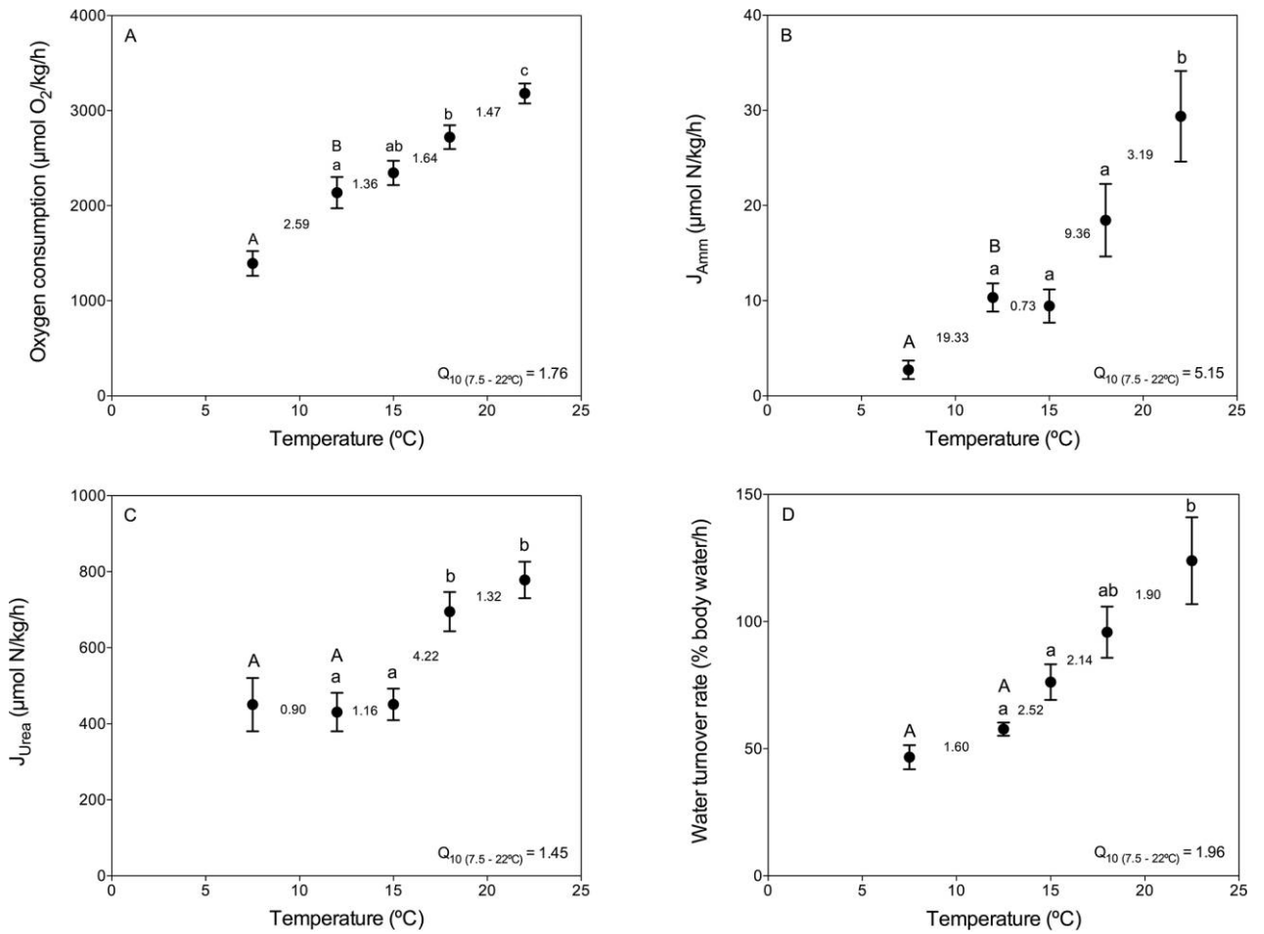

Figure 1. Oxygen consumption rates (M_{O_2}; *A*), ammonia excretion rates (J_{amm}; *B*), urea-N excretion rates ($J_{urea\text{-}N}$; *C*), and tritiated water turnover rates (*D*) in dogfish sharks (*Squalus acanthias suckleyi*) at five different temperatures. Numbers between data points are the Q_{10} values for each increment in temperature, whereas the Q_{10} values over the entire range from 7° to 22°C are tabulated in the lower right of each panel. The same eight animals were tested at 12°, 15°, 18°, and 22°C, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey's post hoc test. A different set of six fish was tested at 7°C, so an unpaired Student's two-tailed *t*-test was used to test the significance of differences between the 7° and 12°C treatments. Means sharing the same uppercase letters indicate the absence of statistical significance ($P < 0.05$) through an unpaired Student's *t*-test for 7.5° and 12°C data points. Means sharing the same lowercase letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12°–22°C. Data are shown as means ± 1 SEM ($n = 6$–8).

at 18° and 22°C. The Q_{10} values for each temperature increment were low (1.36–1.64), whereas the Q_{10} for the decrease from 12° to 7.5°C was somewhat higher (2.59). The overall Q_{10} (7.5°–22°C) was 1.76.

Ammonia excretion rates (J_{amm}; fig. 1*B*) exhibited a different pattern from M_{O_2}. At both 12° and 15°C, J_{amm} was approximately 10 μmol N/kg/h, thereafter increasing to a threefold higher value at 22°C. However, J_{amm} dropped by about 70% when temperature was acutely decreased to 7.5°C. In accord with this pattern, Q_{10} values were very high between 12° and 7.5°C (19.33), as well as between 15° and 18°C (9.36) and between 18° and 22°C (3.19). The overall Q_{10} (7.5°–22°C) was 5.15.

Urea-N excretion rate ($J_{urea\text{-}N}$) at 12°C was approximately 430 μmol N/kg/h (fig. 1*C*), i.e., 43-fold greater than J_{amm}. In contrast to J_{amm}, $J_{urea\text{-}N}$ was tightly conserved over the range from 7.5° to 15°C (i.e., Q_{10} values close to 1.0) but thereafter increased significantly at 18°C ($Q_{10} = 4.22$), with a further small increase at 22°C ($Q_{10} = 1.32$). The overall Q_{10} (7.5°–22°C) was 1.45, the lowest for the four flux parameters that were monitored.

At the acclimation temperature of 12°C, dogfish exchanged approximately 60% of their body water per hour (fig. 1*D*) based on tritiated water turnover rate, which would translate to about 450 mL/kg/h, assuming a body water content of 750 mL/kg. An acute decrease in temperature to 7.5°C resulted in only a small, nonsignificant reduction in water exchange rate ($Q_{10} = 1.60$), whereas acute elevations in temperature caused moderate, approximately linear increases (Q_{10} values = 1.90–2.52) over the range from 12° to 22°C. Only at the latter temperature was the water exchange rate significantly different from the value at 12°C. The overall Q_{10} (7.5°–22°C) was 1.96.

The simultaneous measurements of M_{O_2}, J_{amm}, and $J_{urea\text{-}N}$ allowed calculation of the NQ, which provides information on

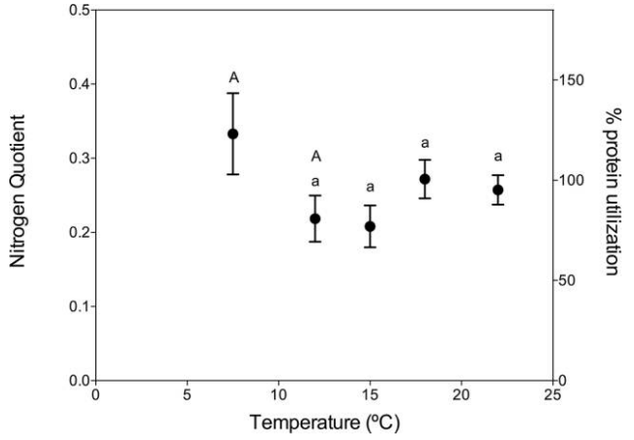

Figure 2. Nitrogen quotients in dogfish (*Squalus acanthias suckleyi*) at different temperatures (°C). The same eight animals were tested at 12°, 15°, 18°, and 22°C, so these data were analyzed by a repeated-measures one-way ANOVA followed by a Tukey's post hoc test. A different set of six fish was tested at 7°C, so an unpaired Student's two-tailed *t*-test was used to test the significance of differences between the 7° and 12°C treatments. Means sharing the same uppercase letters indicate the absence of statistical significance ($P < 0.05$) through an unpaired Student's *t*-test for 7.5° and 12°C data points. Means sharing the same lowercase letters indicate the absence of statistical significance by a repeated-measures one-way ANOVA for 12°–22°C. Data are shown as means ± 1 SEM (n = 6–8).

aerobic fuel usage (see "Material and Methods"). NQ values were not significantly different at any of the experimental temperatures, ranging from 0.20 to 0.33 (fig. 2). By standard metabolic theory, an aerobic metabolism based entirely (i.e., 100%) on protein oxidation would yield an NQ of 0.27 (see "Material and Methods"), and none of the experimental means were significantly different from this reference value. Therefore, at all temperatures, aerobic metabolism in these fasting dogfish was based largely on the oxidation of somatic protein.

A temperature of 22°C is likely close to the upper critical temperature ($C_{t\ \mathrm{max}}$) of *Squalus acanthias suckleyi*, at least without prior acclimation, because most of the present animals lost orientation, turning laterally or completely over during this treatment. Note, however, that they have been caught in the wild at 24°C (Bangley and Rulifson 2014). In order to test the hypothesis that gill permeability increases because dogfish become oxygen limited at 22°C, sharks were exposed to this high temperature (22°C) in the presence of hyperoxia ($P_{O_2} > 309$ Torr) and compared with fish maintained at the control temperature (12°C) but also exposed to hyperoxia. These results were also compared to data from the parallel normoxic treatments (data from series 1), which were calculated over a 4-h period to allow direct comparison with data from series 2. Surprisingly, exposure to hyperoxia significantly increased both J_{amm} (fig. 3*A*) and $J_{urea\text{-}N}$ (fig. 3*B*) by more than twofold at 12°C and to an even greater extent (by 2.7 to 4.2-fold) at 22°C. Most importantly, hyperoxia exacerbated rather than reduced the influence of high temperature on J_{amm} (fig. 3*A*) and $J_{urea\text{-}N}$ (fig. 3*B*). In contrast, small increments (6%–24%) in water exchange rates at both temperatures associated with the hyperoxic treatments were not significant (fig. 3*C*). The Q_{10} values under hyperoxia over the range from 12° to 22°C were 4.02 for J_{amm} (vs. 3.82 under normoxia), 3.77 for $J_{urea\text{-}N}$ (vs. 1.88 under normoxia), and 2.04 for water exchange rate (vs. 2.14 under normoxia).

Discussion

Overview

Our initial hypothesis was that as metabolic demand for O_2 increased with temperature, the fluxes of ammonia, urea-N, and

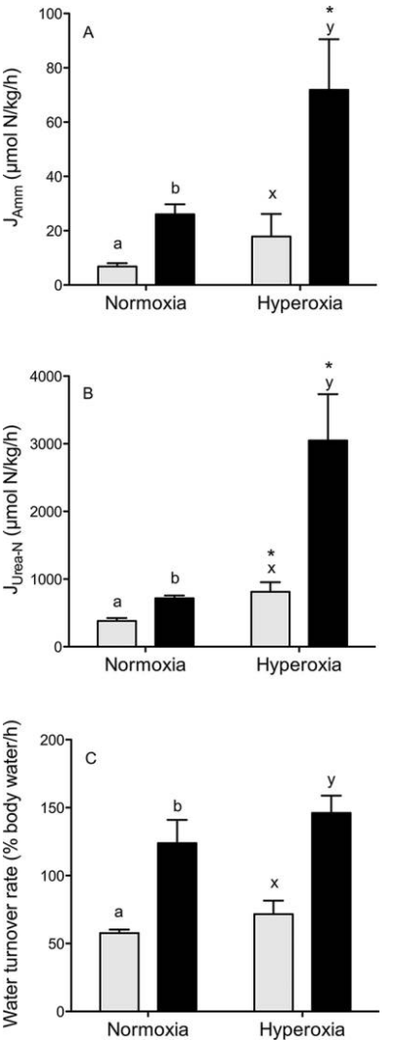

Figure 3. Ammonia excretion rates (J_{amm}; *A*), urea-N excretion rates ($J_{urea\text{-}N}$; *B*), and tritiated water turnover rates (*C*) in dogfish sharks (*Squalus acanthias suckleyi*) at 12°C (gray bars) and 22°C (black bars) measured under normoxia and hyperoxia. Different lowercase letters indicate statistical significance between 12° and 22°C tested at the same oxygen tension, as determined by a paired Student's two-tailed *t*-test ($P < 0.05$). Normoxia and hyperoxia data at a given temperature were compared through an unpaired Student's two-tailed *t*-test ($P < 0.05$), and significance is indicated by an asterisk. The Bonferroni correction for multiple comparisons was applied. Data are shown as means ± 1 SEM (n = 6–8).

water at the gill would increase in parallel with those of O_2. We thought that this would happen through the osmorespiratory compromise due to the normal physiological adjustments, such as increases in gill ventilation, perfusion, and effective branchial surface area, that promote an enhancement of O_2 transfer, thereby supplying the increased demand. Overall, the hypothesis was only partially supported. The fluxes of all four moieties (O_2, ammonia, urea, and ^3H_2O) increased with temperature, but only the changes in ^3H_2O exchange roughly paralleled those in M_{O_2}. The relative changes in $J_{urea\text{-}N}$ and, in particular, J_{amm} were much larger overall than those in M_{O_2} and displayed stepwise patterns indicative of temperature effects on specific N-conservation mechanisms, as discussed subsequently. In this regard, our results confirmed the surprising findings of Boylan (1967) that branchial urea-N excretion is independent of temperature up to 15°C and then increases sharply at higher temperatures. Finally, our hyperoxia treatments provided no support for the hypothesis that increased O_2 availability would minimize or prevent the effects of rising temperature on gill permeability. Rather, the results suggested that hyperoxia induced detrimental effects in the gills, thereby exacerbating the influence of temperature on gill permeability.

However, there are several important caveats in the interpretation of our data. The first is that animals were exposed to increased temperature for only 6.5 h; therefore, we cannot assess whether the animals were in long-term steady state or whether undershoot or overshoot phenomena may have occurred. Nevertheless, for urea, ammonia, and oxygen fluxes where two or three replicate measurements were made on each fish within this 6.5-h period, there was at least a short-term steady state as rates were constant over time. Therefore, while our data do not cast light on responses to global warming, they are very relevant to the daily life of this animal as it moves into warmer surface or inshore waters to feed or migrates through the thermocline. The second caveat is that no internal concentrations were measured and, therefore, we do not know whether the effects of temperature seen here were due to changes in internal concentrations, which would affect diffusion gradients. In addition, the different group of fish exposed to 7.5°C was not tested at 12°C; therefore, we cannot rule out the possibly of greater variability in the responses over this range. Finally, time controls were not performed to check the potential effects of prolonged fasting and confinement. Nevertheless, we believe that such effects would be negligible, because Wood et al. (2007) found that the rates of oxygen consumption did not change in comparably fasted dogfish kept in the boxes for 5 d, while Kajimura et al. (2008) reported that ammonia-N and urea-N flux rates were stable in dogfish kept under the same fasted conditions for up to 56 d.

Temperature Effects on O_2 Consumption

In ectotherms, metabolic demand for O_2 increases with temperature and then rapidly declines at higher temperatures (for review see Schulte 2015). In our study, M_{O_2} increased with increases in temperature in an almost linear fashion, with Q_{10} ranging from 1.36 to 2.59, with an overall value of 1.76 (fig. 1*A*). This temperature sensitivity lies at the lower end of the range reported in previous studies on both sharks and rays, where the temperature coefficient is usually around 2–3 (Butler and Taylor 1975; Hopkins and Cech 1994; Miklos et al. 2003; Di Santo and Bennet 2011) or occasionally higher (Di Santo 2016). Notably, in the current study, the Q_{10} was greatest (2.59) at the lower end of the temperature range (7.5°–12°C), which may be most representative of the normal annual range experienced by *Squalus acanthias suckleyi* in the north Pacific Ocean.

Temperature Effects on Urea-N Excretion

One of our initial goals was to replicate the study done by Boylan (1967) where, using an unusual ventilated whole-animal technique in which the head was air exposed, the author found that the urea-N excretion at the gills was independent of temperature from 1° to 15°C and then increased dramatically at temperatures higher than 15°C. Boylan (1967) apparently studied only three animals at a wide range of temperatures, whereas we elected to study a larger number of dogfish (six to eight) at only five fixed temperatures. Nevertheless, our results are very similar to those of Boylan (1967) and were obtained with a much less invasive and therefore less stressful experimental technique. Measurements of $J_{urea\text{-}N}$ did not vary from 7.5° to 15°C but increased greatly thereafter, reaching a twofold increase at 22°C (fig. 1*C*). This pattern was completely different from the linear increase in M_{O_2} over the entire range (fig. 1*A*). Even though urea-N production rates (via the OUC in liver and muscle) could have been upregulated due to increases in aerobic metabolic rate, it seems very unlikely that upregulation of the OUC alone could explain the increase in urea-N excretion, given that $J_{urea\text{-}N}$ was unchanged up to 15°C. Furthermore, in the study of Boylan (1967), it appears that only the temperature of the irrigated gills was manipulated and not the temperature of the whole animal.

Urea is retained at the elasmobranch gill mainly by two important factors: (1) an unusual gill membrane composition (Fines et al. 2001) and (2) the presence of an active back-transporter in the branchial epithelium (Wood et al. 1995, 2013; Pärt et al. 1998; Fines et al. 2001), the exact location of which is in debate (see "Introduction"). Is it possible that high temperatures may have affected one or both of the factors cited above? Boylan (1967) hypothesized that the breakpoint of urea-N excretion could be interpreted as a phase change in the ultrastructure of the gill cell membranes; i.e., increases in temperature may have influenced the lipids in the membranes, leading to an increase in membrane fluidity. This is an interesting area for future investigation. Additionally, it is also possible that the activity of the mechanism responsible for recapturing urea back to the plasma (Pärt et al. 1998; Fines et al. 2001) could have decreased drastically at temperatures higher than 15°C.

Temperature Effects on Ammonia Excretion

The gills of elasmobranchs are thought to be very impermeable to ammonia leakage relative to teleosts, thereby contributing to N conservation (Wood et al. 1995; Wright and Wood 2016). Nevertheless, among the variables tested, ammonia excretion rate

seemed to be the most temperature sensitive, with overall Q_{10} from 7.5° to 22°C equal to 5.15 (fig. 1*B*). Different from the results obtained for urea-N, it seems that ammonia excretion exhibits two breakpoints. We observed an increase in ammonia excretion from 7° to 12°C, no change from 12° to 15°C, and then a marked increase from 15° to 22°C. These results are not in agreement with previous data on the lesser-spotted dogfish (*Scyliorhinus stellaris*) by Heisler (1978), where a 10°C increase in temperature was reported to cause no changes in ammonia excretion rates. It is important to note that even though we recorded a 15-fold increase in ammonia excretion rates over the whole temperature range, our absolute values ranged only from 2 to ~30 μmol N/kg/h. Heisler (1978) did not report any absolute values of ammonia excretion rates because they were thought to be negligible, so it is possible that his animals could have experienced similar large-fold changes in very small rates. Nawata et al. (2015) reported that *Squalus acanthias* are N limited and, when presented with high concentrations of ammonia in the water, they have the ability to scavenge ammonia-N, convert it into urea, and elevate urea-N excretion rates. This uptake of ammonia exhibits classic Michaelis-Menten saturation kinetics, resulting in a net retention of nitrogen (Wood and Giacomin 2016). Along with other transformations at the gill, it is possible that at high temperatures the ammonia-conserving uptake mechanism could have been impaired, leading to the high temperature sensitivity seen. In contrast to the scarcity of data on the N-waste excretion sensitivity to temperature of elasmobranchs, this topic has been thoroughly explored in ammoniotelic teleosts, where it is well established that N-waste excretion is far more sensitive than M_{O_2} to increases in temperature. However, in this case, the explanation appears to be that as temperature increases, a greater percentage of aerobic metabolism is fueled by protein oxidation (Wood 2000).

Temperature Effects on the Nitrogen Quotient

We measured oxygen consumption rates, as well as urea-N and ammonia-N excretion rates, in the same experimental animals, and therefore we were able to calculate the N/O_2 ratio, or the nitrogen quotient (NQ), which is an index of fuel utilization supplying aerobic metabolism (Lauff and Wood 1996). Despite a slight decrease from 7.5° to 12°C, the NQ did not vary significantly across all temperatures tested. It appears that these fasted dogfish are metabolizing almost entirely protein (amino acids) in aerobic respiration. This finding is in accord with one previous study on fasted members of the same species, in which measurements were made at only $12° \pm 1°C$ (Wood et al. 2007). Interestingly, in that study, the NQ tended to fall after feeding as M_{O_2} increased and the OUC was activated, yet branchial urea-N excretion fell as part of the animal's postprandial N-retention strategy. We are aware of no other elasmobranch studies where the NQ has been measured, but in teleosts, NQ values tend to be much lower as fuels other than protein are burned during fasting (Lauff and Wood 1996; Wood 2000).

The implications of temperature increases for energy budgets are of interest in an organism that supports aerobic respiration almost entirely by the oxidation of protein. Total N-efflux rates (i.e., urea-N + ammonia-N losses) approximately doubled between 12° and 22°C (fig. 1*B*, 1*C*). Based on measurements made during feeding and fasting in this species and the protein content of a typical meal of teleost fish (Kajimura et al. 2006, 2008), this would double the need for food consumption, or reduce the time that a single meal would last from about 5 to 2.5 d so as to maintain body mass. Alternately, in a fasting dogfish, it would double the rate at which muscle protein was broken down so as to support this elevated rate of N loss while maintaining constant blood urea-N concentrations for osmoregulatory homeostasis.

Temperature Effects on Tritiated Water Fluxes

Using tritiated water (^3H_2O) we measured the diffusive water exchange rates at the gills, which are thought to be about 100-fold greater than the net osmotic flux of water, which depends on the low osmotic gradient from seawater to plasma (10–70 mOsmol/L; Wright and Wood 2016). At the control temperature, 12°C, the fish exchanged approximately 60% of their body water per hour (fig. 1*D*). To our knowledge, this is the first report of diffusive water exchange rates for *S. acanthias suckleyi*, and our results fall at the low end of the range of the very few available measurements for seawater elasmobranchs, where rates can vary from 81% to 157% body water per hour in sharks (Payan and Maetz 1971; Carrier and Evans 1972; Hayward 1975) and from 64% to 167% body water per hour in rays (Payan and Maetz 1971; Payan et al. 1973), values that are about fivefold higher than those in marine teleosts (Wright and Wood 2016). Water exchange rates increased with temperature in a linear fashion (fig. 1*D*), similar to the response pattern seen for M_{O_2} (fig. 1*A*), with an overall Q_{10} (7.5°–22°C) of 1.96. ^3H_2O is the only moiety for which the measured flux pattern fit clearly with our overall hypothesis based on the osmorespiratory compromise. This might suggest that the pathway of diffusive water flux across the gills is the same as that of diffusive O_2 flux. The role, if any, played by aquaporins in facilitating the diffusion of water across the gills of elasmobranchs is not yet fully understood. Initial research on isolated gill cell membranes of *S. acanthias* ruled out the participation of aquaporins (Hill et al. 2004). However, more recently Cutler et al. (2012*b*) have detected the presence of aquaporin AQP4 mRNA in the gills of *S. acanthias*, and AQP4 protein expression was localized to branchial ionocytes (Cutler et al. 2012*a*). Furthermore, after acclimation to 120% seawater, branchial mRNA expression of AQP4 declined, perhaps indicating an important role for aquaporins in salinity tolerance. The time frame of our acute temperature change experiments was short, and it is unclear whether aquaporin message and protein function could have been adjusted in that period.

The Impact of Hyperoxia on Temperature Effects

In our study, we hypothesized that by supplying excess amounts of oxygen in the water (hyperoxia), the animals would not need to upregulate gill ventilation, perfusion, and functional area to

the same extent in order to obtain the same amount of O_2 or even more O_2, thereby minimizing increases in nitrogen and water fluxes. Hyperoxia is an important and relevant environmental circumstance to dogfish sharks, which are well known for making foraging and breeding incursions into estuaries and river mouths (Ulrich et al. 2007). Estuaries are very dynamic ecosystems, exhibiting large daily variations in water parameters such as salinity, temperature, and dissolved oxygen, the latter due to photosynthesis and respiration rates (Kennish 1986).

Our data clearly do not support this hypothesis. Rather than attenuating N-waste fluxes, exposure to hyperoxia at 22°C led to a dramatic increase in ammonia (fig. 3*A*) and urea-N fluxes (fig. 3*B*) in comparison to animals that were exposed to high temperatures in normoxic conditions. To our knowledge, this is the first report of increased N-waste effluxes during exposure to hyperoxia in any elasmobranch species. Surprisingly enough, the same response was also seen at 12°C. The fluxes of ^3H_2O were not significantly elevated by hyperoxia; nevertheless, the response to temperature was clearly not reduced (fig. 3*C*).

Hyperoxia is known to cause an elevation of blood P_{CO_2}, leading to a decrease in blood pH and consequently to a respiratory acidosis. This phenomenon can be rapidly compensated by retention of bicarbonate (HCO_3^-) in the blood. In the larger spotted dogfish (*Scyliorhinus stellaris*), Heisler et al. (1998) reported that acute hyperoxia exposure caused a decrease in gill ventilation, increased arterial blood P_{O_2} (Pa_{O_2}), and hypercapnia, which was compensated by an increased net uptake of HCO_3^--equivalent ions at the gill epithelia. Although an acid-base disturbance could have exacerbated the N-compound effluxes, there are not enough available data to support such inferences.

An alternative, and perhaps more likely, explanation for the increased urea-N and ammonia fluxes at the gills of animals exposed to hyperoxia is the production of reactive oxygen species (ROS) in the water and subsequent accumulation in tissues, causing oxidative damage to gill cell membranes and leading to an elevated leakage of N-waste compounds. The presence of ROS in the water has been shown to increase with natural diel increases in the O_2 concentration in the water (Johannsson et al. 2014). Johannsson et al. (2014) and Pelster et al. (2016) have reported that two different species of facultative air-breathing teleost fishes increased the frequency of air-breathing events when exposed to aquatic hyperoxia. These behavioral alterations are thought to be an adaptation to prevent ROS damage caused by increased ROS concentration in hyperoxic waters (Johannsson et al. 2014; Pelster et al. 2016). The oxidative stress response in teleost fish exposed to hyperoxia has been well reported, with studies showing downregulation of both enzymatic and nonenzymatic antioxidant defenses, accumulation of oxyradicals in tissues, and oxidative damage to lipids and proteins (Lygren et al. 2000; Lushchak et al. 2005; Johannsson et al. 2014; for review see Lushchak and Bagnyukova 2006). Hypoxaemia at high temperatures may also result in internal ROS production (Lushchak 2006), which could be synergistic with ROS originating from exposure to hyperoxic water. In contrast to teleosts, studies investigating the hyperoxia-induced oxidative stress response in elasmobranchs are still lacking. This is an important area for future investigation.

Table 1: Ammonia excretion rate, urea-N excretion rate, oxygen consumption rate, water turnover rate, and nitrogen quotient at 12° and 15°C

	Temperature (°C)	
	12	15
Ammonia efflux (μmol N/kg/h):		
Average	10.3	9.4
SEM	1.5	1.8
Urea-N efflux (μmol N/kg/h):		
Average	430.8	451.2
SEM	50.5	41.5
Oxygen consumption (μmol O_2/kg/h):		
Average	2,137.5	2,344.9
SEM	164.2	128
Water turnover rate (% body water/h):		
Average	57.7	76.2*
SEM	2.6	7.1
Nitrogen quotient (NQ):		
Average	.21	.20
SEM	.02	.07

Note. Means are shown ± 1 SEM ($n = 6$–8).

*Significant differences between 12° and 15°C obtained through a paired Student's *t*-test ($P < 0.05$).

Implications

Based on the data in figure 1, above 15°C, the large temperature-dependent increases in ammonia and urea-N excretion will pose the greatest physiological challenges for the organism, especially the latter because urea-N losses comprise such a large component of the N budget of sharks. However, it is the 12°–15°C range that is the most relevant for the day-to-day movements of *S. acanthias suckleyi* during the summer. The temperature at depth close to Bamfield Marine Sciences Centre (from which the acclimation water was pumped and at which most of the dogfish were caught by angling) is about 12°C, and 15°C is very representative of normal sea-surface temperatures in the area (where some of the animals were caught by angling; C. M. Wood, personal observations). In light of this, we have compared our control data (12°C) with data on animals that were exposed to 15°C (table 1). The physiological aspect that seems to be most sensitive to an increase of 3°C in water temperature was the water exchange rate, increasing significantly by 32%; M_{O_2}, $J_{urea\text{-}N}$, and J_{amm} were not significantly altered. Clearly, future studies on water balance in this species will be valuable, especially since fluctuations in temperature are often coupled with fluctuations in salinity in inshore regions due to the influence of river inflows. Overall, the current data are useful in characterizing the thermal sensitivity of branchial processes that may be involved in setting thermal constraints to the whole organism.

Acknowledgments

Supported by Natural Sciences and Engineering Research Council of Canada Discovery Grants to C.M.W. and P.M.S. and by an

award from the International Development Research Centre (Ottawa, Canada) to C.M.W. and Dr. Adalto Bianchini. We thank Dr. Eric Clelland (Bamfield Marine Sciences Centre research coordinator) for excellent logistic support and three anonymous reviewers whose comments have greatly improved the article. M.G. is supported by a four-year graduate Fellowship from the University of British Columbia.

Literature Cited

Alsop D.H., J.D. Kieffer, and C.M. Wood. 1999. The effects of temperature and swimming speed on instantaneous fuel use and nitrogenous waste excretion of the Nile tilapia. Physiol Biochem Zool 72:474–483.

Ballantyne J.S. 1997. Jaws: the inside story—the metabolism of elasmobranch fishes. Comp Biochem Physiol B 118:703–742.

Bangley C. and R. Rulifson. 2014. Observations on spiny dogfish (*Squalus acanthias*) captured in late spring in a North Carolina estuary. F1000Research 3:189.

Boutilier R.G., T.A. Heming, and G.K. Iwama. 1984. Appendix: physicochemical parameters for use in fish respiratory physiology. Pp. 403–430 in W.S. Hoar and D.J. Randall, eds. Fish physiology: gills. Vol. 10. Academic Press, Orlando, FL.

Boylan J.W. 1967. Gill permeability in *Squalus acanthias*. Pp. 197–206 in P.W. Gilbert, R.F. Mathewson, and D.P. Rall, eds. Sharks, skates, and rays. John Hopkins University Press, Baltimore.

Butler P.J. and E.W. Taylor. 1975. The effect of progressive hypoxia on respiration in the dogfish (*Scyliorhinus canicula*) at different seasonal temperatures. J Exp Biol 63:117–130.

Carrier J.C. and D.H. Evans. 1972. Ion, water and urea turnover rates in the nurse shark, *Ginglymostoma cirratum*. Comp Biochem Physiol A 41:761–764.

Cutler C.P., S. Harmon, J. Walsh, and K. Burch. 2012*a*. Characterization of aquaporin 4 protein expression and localization in tissues of the dogfish (*Squalus acanthias*). Front Physiol 3:21.

Cutler C.P., B. MacIver, G. Cramb, and M. Zeidel. 2012*b*. Aquaporin 4 is a ubiquitously expressed isoform in the dogfish (*Squalus acanthias*) shark. Front Physiol 2:107.

Di Santo V. 2016. Intraspecific variation in physiological performance of a benthic elasmobranch challenged by ocean acidification and warming. J Exp Biol 219:1725–1733.

Di Santo V. and W.A. Bennett. 2011. Effect of rapid temperature change on resting routine metabolic rates of two benthic elasmobranchs. Fish Physiol Biochem 37:929–934.

Dulvy N.K., S.L. Fowler, J.A. Musick, R.D. Cavanagh, P.M. Kyne, L.R. Harrison, J.K. Carlson, et al. 2014. Extinction risk and conservation of the world's sharks and rays. Elife 3:e00590. doi: 10.7554/eLife.00590.

Evans D.H. 1967. Sodium, chloride, and water balance of the intertidal teleost, *Xiphister atropurpureus*. III. The roles of simple diffusion, exchange diffusion, osmosis and active transport. J Exp Biol 47:525–534.

Fines G.A., J.S. Ballantyne, and P.A. Wright. 2001. Active urea transport and an unusual basolateral membrane composition in the gills of a marine elasmobranch. Am J Physiol 280:R16–R24.

Haywood G.P. 1975. Indications of sodium, chloride, and water exchange across the gills of the striped dogfish *Poroderma africanum*. Mar Biol 29:267–276.

Heisler N. 1978. Bicarbonate exchange between body compartments after changes of temperature in the larger spotted dogfish (*Scyliorhinus stellaris*). Respir Physiol 33:145–160.

Heisler N., D.P. Toews, and G.F. Holeton. 1998. Regulation of ventilation and acid-base status in the elasmobranch (*Scyliorhinus stellaris*) during hyperoxia-induced hypercapnia. Respir Physiol 71:227–246.

Hill W.G., J.C. Mathai, R.H. Gensure, J.D. Zeidel, G. Apodaca, J.P. Saenz, E. Kinne-Saffran, et al. 2004. Permeabilities of teleost and elasmobranch gill apical membranes: evidence that lipid bilayers alone do not account for barrier function. Am J Physiol 287:C235–C242.

Hochachka P.W. and G.N. Somero. 2002. Biochemical adaptation. 1st ed. Oxford University Press, Oxford.

Hopkins T.E and J.J. Cech. 1994. Effect of temperature on oxygen consumption of the bat ray, *Myliobatis californica* (Chondrichthyes, Myliobatididae). Copeia 1994:529–532.

Johannsson O.E., H.L. Bergman, C.M. Wood, P. Laurent, D.G. Kavembe, A. Bianchini, J.N. Maina, et al. 2014. Air breathing in Magadi tilapia *Alcolapia grahami*, under normoxic and hyperoxic conditions, and the association with sunlight and reactive oxygen species. J Fish Biol 84:844–863.

Kajimura M., P.J. Walsh, T.P. Mommsen, and C.M. Wood. 2006. The dogfish shark (*Squalus acanthias*) increases both hepatic and extra-hepatic ornithine-urea cycle enzyme activities for nitrogen conservation after feeding. Physiol Biochem Zool 79: 602–613.

Kajimura M., P.J. Walsh, and C.M. Wood. 2008. The spiny dogfish (*Squalus acanthias* L.) maintains osmolyte balance during long-term starvation. J Fish Biol 72:656–670.

Kennish, M.J. 1986. Ecology of estuaries: physical and chemical aspects. Vol. 1. CRC, Boca Raton, FL.

Kieffer J.D., D. Alsop, and C.M. Wood. 1998. A respirometric analysis of fuel use during aerobic swimming at different temperatures in rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 201:3123–3133.

Kirschner L.B. 1970. The study of NaCl transport in aquatic animals. Am Zool 10:365–376.

———. 1993. The energetics of osmotic regulation in ureotelic and hypoosmotic fishes. J Exp Zool 267:19–26.

Lauff R.F. and C.M. Wood. 1996. Respiratory gas exchange, nitrogenous waste excretion, and fuel usage during starvation in juvenile rainbow trout, *Oncorhynchus mykiss*. J Comp Physiol B 165:542–551.

Lushchak V.I. 2006. Environmentally induced oxidative stress in aquatic animals. Aquat Toxicol 101:13–30.

Lushchak V.I. and T.V. Bagnyukova. 2006. Effects of different environmental oxygen levels on free radical processes in fish. Comp Biochem Physiol B 144:283–289.

Lushchak V.I., T.V. Bagnyukova, V.V. Husak, L.I. Luzhna, O.V. Lushchak, and K.B. Storey. 2005. Hyperoxia results in transient oxidative stress and an adaptive response by antioxidant enzymes in goldfish tissues. Int J Biochem Cell Biol 37:1670–1680.

Lygren B., K. Hamre, and R. Waagbø. 2000. Effect of induced hyperoxia on the antioxidant status of Atlantic salmon *Salmo salar* fed three different levels of dietary vitamin E. Aquat Res 31:401–407.

Maetz J. 1972. Branchial sodium exchange and ammonia excretion in the goldfish *Carassius auratus*: effects of ammonia-loading and temperature changes J Exp Biol 56:601–620.

Miklos P., S.M. Katzman, and J.J. Cech. 2003. Effects of temperature on oxygen consumption of the leopard shark, *Triakis semifasciata*. Environ Biol Fish 66:15–18.

Nawata C.M., P.J. Walsh, and C.M. Wood. 2015. Physiological and molecular responses of the spiny dogfish shark (*Squalus acanthias*) to high environmental ammonia: scavenging for nitrogen. J Exp Biol 218:238–248.

Nilsson S. 1986. Control of gill blood flow. Pp. 87–101 in S. Nilsson and S. Holmgren, eds. Fish physiology: recent advances. Croom Helm, London.

Pärt P., P.A. Wright, and C.M. Wood. 1998. Urea and water permeability in dogfish (*Squalus acanthias*) gills. Comp Biochem Physiol A 119:117–123.

Payan P., L. Goldstein, and R.P. Forster. 1973. Gills and kidneys in ureosmotic regulation in euryhaline skates. Am J Physiol 224:367–372.

Payan P. and J. Maetz. 1971. Balance hydrique chez les élasmobranches: arguments en faveur d'un controle endocrinien. Gen Comp Endocrinol 16:535–554.

Pelster B., M. Giacomin, C.M. Wood, and A.L. Val. 2016. Improved ROS defense in the swimbladder of a facultative air-breathing erythrinid fish, jeju, compared to a non air-breathing close relative, traira. J Comp Physiol B 186:615–624.

Rahmatullah M. and T.R.C. Boyde. 1980. Improvements in the determination of urea using diacetyl monoxime: methods with and without deproteinisation. Clin Chem Acta 107:3–9.

Randall D.J., D. Baumgarten, and M. Malyusz. 1972. The relationship between gas and ion transfer across the gills of fishes. Comp Biochem Physiol A 41:629–637.

Sagarese S.R., M.G. Frisk, T.J. Miller, K.A. Sosebee, J.A. Musick, and P.J. Rago. 2014. Influence of environmental, spatial, and ontogenetic variables on habitat selection and management of spiny dogfish in the Northeast (US) shelf large marine ecosystem. Can J Fish Aquat Sci 71:567–580.

Schulte P.S. 2015. The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. J Exp Biol 218:1856–1866.

Smith H. 1931. The absorption and excretion of water and salts by the elasmobranch fishes. II. Marine elasmobranchs. Am J Physiol 98:296–310.

———. 1936. The retention and physiological role of urea in the elasmobranchii. Biol Rev 11:48–82.

Taylor I.G. and V.F. Galluci. 2009. Uncounfounding the effects of climate and density dependence using 60 years of data on spiny dogfish (*Squalus acanthias*). Can J Aquat Sci 66:351–366.

Ulrich G.F., C.M. Jones, W.B. Driggers, J.M. Drymon, D. Oakley, and C. Riley. 2007. Habitat utilization, relative abundance, and seasonality of sharks in the estuarine and nearshore waters of South Carolina. Am Fish Soc Symp 50:125–139.

Van Den Thillart G. and F. Kesbeke. 1978. Anaerobic production of carbon dioxide and ammonia by goldfish, *Carassius auratus*. Comp Biochem Physiol A 59:393–400.

Verdouw H., C.J.A. Van Echteld, and E.M.J. Dekkers. 1978. Ammonia determination based on indophenols formation with sodium salicylate. Water Res 12:399–402.

Wood C.M. 2000. Influence of feeding, exercise, and temperature on nitrogen metabolism and excretion. Pp. 201–238 in P. Wright and P. Anderson, eds. Fish physiology: nitrogen excretion. Vol. 20. Academic Press, San Diego, CA.

Wood C.M., C. Bucking, J. Fitzpatrick, and S.R. Nadella. 2007. The alkaline tide goes out and the nitrogen stays in after feeding in the dogfish shark, *Squalus acanthias*. Respir Physiol Neurobiol 159:163–170.

Wood C.M. and M. Giacomin. 2016. Turning a toxicant into a resource: how the dogfish shark scavenges ammonia from its environment. J Exp Biol 219:3218–3226.

Wood C.M., M. Kajimura, T.P. Mommsen, and P.J. Walsh. 2005. Alkaline tideand nitrogen conservation after feeding in an elasmobranch (*Squalus acanthias*). J Exp Biol 208:2693–2705.

Wood C.M., H.J. Liew, G. De Boeck, and P.J. Walsh. 2013. A perfusion study of handling of urea and urea analogues by the gills of the dogfish shark (*Squalus acanthias*). PeerJ 1:e33. doi:10.7717/peerj.33.

Wood C.M., P. Part, and P.A. Wright. 1995. Ammonia and urea metabolism in relation to gill function and acid-base balance in a marine elasmobranch, the spiny dogfish (*Squalus acanthias*). J Exp Biol 198:1545–1558.

Wright P.A. and C.M. Wood. 2016. Regulation of ions, acid-base, and nitrogenous wastes in elasmobranchs. Pp. 279–345 in R.E. Shadwick, A.P. Farrell, and C.J. Brauner, eds. Fish physiology. Vol. 34B. Physiology of elasmobranch fishes: internal processes. Academic Press, San Diego, CA.

Yancey P.H. 2016. Organic osmolytes in elasmobranchs. Pp. 222–264 in R.E. Shadwick, A.P. Farrell, and C.J. Brauner, eds. Fish physiology. Vol. 34B. Physiology of elasmobranch fishes: internal processes. Academic Press, San Diego, CA.

Zimmer A. and C.M. Wood. 2014. Exposure to acute severe hypoxia leads to increased urea loss and disruptions in acid-base and ionoregulatory balance in dogfish sharks (*Squalus acanthias*). Physiol Biochem Zool 87:623–639.