Exposure to Acute Severe Hypoxia Leads to Increased Urea Loss and Disruptions in Acid-Base and Ionoregulatory Balance in Dogfish Sharks (*Squalus acanthias*)

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ABSTRACT

The effects of acute moderate (20% air O_2 saturation; 6-h exposure) and severe (5% air O_2 saturation; 4-h exposure) hypoxia on N-waste, acid-base, and ion balance in dogfish sharks (*Squalus acanthias suckleyi*) were evaluated. We predicted that the synthesis and/or retention of urea, which are active processes, would be inhibited by hypoxia. Exposure to moderate hypoxia had negligible effects on N-waste fluxes or systemic physiology, except for a modest rise in plasma lactate. Exposure to severe hypoxia led to a significant increase in urea excretion (J_{urea}), while plasma, liver, and muscle urea concentrations were unchanged, suggesting a loss of urea retention. Ammonia excretion (J_{amm}) was elevated during normoxic recovery. Moreover, severe hypoxia led to disruptions in acid-base balance, indicated by a large increase in plasma [lactate] and substantial decreases in arterial pHa and plasma [HCO_3^-]a, as well as loss of ionic homeostasis, indicated by increases in plasma [Mg^{2+}], [Ca^{2+}], and [Na^+]. We suggest that severe hypoxia in dogfish sharks leads to a reduction in active gill homeostatic processes, such as urea retention, acid-base regulation and ionoregulation, and/or an osmoregulatory compromise due to increased functional gill surface area. Overall, the results provide a comprehensive picture of the physiological responses to a severe degree of hypoxia in an ancient fish species.

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Introduction

The Pacific spiny dogfish (*Squalus acanthias suckleyi*), like most seawater elasmobranchs, is an osmoconformer, maintaining internal osmolarity equal to or greater than that of the surrounding seawater. This is achieved by the synthesis and retention of high levels of urea (200–400 mmol/L) as an osmolyte in tissues and plasma (for review see Ballantyne 1997; Hazon et al. 2003). This osmoregulatory strategy, like ionoregulation in hyporegulating seawater teleosts, is energetically expensive. The synthesis of urea occurs via the ornithine-urea cycle (OUC), which, overall, consumes 5 mol ATP/1 mol urea synthesized (see Ballantyne 1997). The two most important sites for urea synthesis are the liver and white muscle, based on the activity of OUC enzymes in these tissues and their large contribution to overall body mass (Kajimura et al. 2006). In addition to the costly synthesis of urea, the retention of these high levels of urea is also an energy-dependent process. While an unusual cholesterol-rich composition of the gill basolateral membranes is thought to help reduce diffusive urea losses (Fines et al. 2001), elasmobranchs also possess an active branchial back-transport system for urea; this may be located either basolaterally (Fines et al. 2001) or apically (Part et al. 1998; Hill et al. 2004; Wood et al. 2013). The system appears to operate via a Na^+/K^+-ATPase-driven Na^+/urea-antiporter so as to effectively limit diffusive urea losses by reabsorbing urea leaking across the gills (for review see McDonald et al. 2006). In general, dogfish have very tight regulation over nitrogen homeostasis and, moreover, appear to have a very effective mechanism for the retention and conservation of nitrogen (e.g., Wood et al. 2005, 2007; Kajimura et al. 2008), an important feature of the osmoregulatory strategy of these fish.

Exposure to hypoxia in dogfish and other elasmobranchs leads to a variety of cardiovascular and hematological responses in addition to metabolic and acid-base disturbances (Butler and Taylor 1975; Butler et al. 1979; Chapman and Renshaw 2009; Speers-Roesch et al. 2012). However, to our knowledge, no study to date has assessed the effects of hypoxia on nitrogen homeostasis in an elasmobranch species. As both the synthesis and the retention of urea in elasmobranchs are energetically costly, it would be expected that the energy limitations imposed by hypoxia would lead to a reduction in either or both of these processes. Moreover, in teleost fish, ammonia production by white muscle increases under anaerobic conditions as seen during recovery from exhaustive exercise (Mommsen and Hochachka 1988; Wang et al. 1994) and, potentially, during hypoxia (for review see Van Waarde 1983). The anaerobic production of ammonia is believed to occur as a result of

adenylate depletion and may act to buffer intracellular pH in white muscle (Mommsen and Hochachka 1988). In dogfish, exhaustive exercise also leads to an increase in ammonia levels in the plasma and white muscle, the latter occurring with a concomitant decrease in white muscle [ATP] (Richards et al. 2003). A shift to anaerobic metabolism in response to hypoxia in sharks would potentially have the same effects on ammonia production, and potentially N-waste handling in general, though this has not been studied to date. As the strategy of these fish is to conserve nitrogen (e.g., Wood et al. 2005, 2007), it is of interest to understand the fate of this potential anaerobic ammonia load.

The goal of this study was to evaluate the effects of two levels of acute hypoxia (moderate = 20% air O_2 saturation; severe = 5% air O_2 saturation) on N-waste handling and general systemic physiology in dogfish sharks. These levels were chosen to bracket the critical oxygen tension (P_{crit}) of dogfish sharks, which is approximately 10% of air O_2 saturation (G. De Boeck, personal communication). Dogfish were fitted with indwelling caudal arterial cannulas. Ammonia and urea-N excretion (J_{amm} and J_{urea}, respectively) and plasma total ammonia and urea-N (T_{amm} and T_{urea}, respectively) were assessed during exposure to 4 or 6 h of moderate and severe hypoxia, respectively, followed by 12 h of normoxic recovery. In an additional experiment, tissue-specific responses of the gills, liver, and white muscle following 2 h of severe hypoxia were also assessed. We hypothesized that exposure to hypoxia would reduce the active synthesis of urea by the liver and muscle and/or the active retention of urea by the gills. Furthermore, we hypothesized that the active retention mechanism at the gills, which are bathed in a much more oxic environment than the internal tissues, would be the more hypoxia-sensitive process. As such, we predicted that J_{urea} would increase in response to hypoxia, as a result of decreased active retention, and that this would occur in conjunction with little change in plasma T_{urea} and tissue T_{urea}. On the other hand, if urea synthesis were more severely affected by hypoxic exposure, a decrease in J_{urea}, plasma T_{urea}, and/or tissue T_{urea} would be expected. We further predicted that this would occur with increases in ammonia production and/or excretion as less ammonia is converted to urea due to impaired synthesis. Hypoxia may also lead to an increase in ammonia production as a result of adenylate breakdown, which would be reflected by increases in tissue and plasma T_{amm}. A number of other blood, plasma, and tissue hematological (hematocrit, [hemoglobin], [protein]), acid-base (arterial blood pHa, $PaCO_2$, $[HCO_3^-]$a, tissue pHi), metabolic ([lactate], [pyruvate], [glucose]), and ionoregulatory (plasma $[Na^+]$, $[Cl^-]$, $[K^+]$, $[Mg^{2+}]$, $[Ca^{2+}]$) parameters were also measured in response to hypoxia to obtain a comprehensive understanding of the hypoxic response in these fish.

Methods and Material

Experimental Animals

Experiments were performed in two separate field seasons in August of 2012 (series 1 and 2) and 2013 (series 3 and 4) on adult male dogfish sharks (1–2 kg). Note that we retain the name *Squalus acanthias suckleyi* while recognizing that Ebert et al. (2010) have recently proposed that these northeast Pacific elasmobranchs are a separate species (*Squalus suckleyi*) distinct from *Squalus acanthias*. In series 1 and 2, sharks were caught off the west coast of Vancouver Island, near Bamfield, British Columbia, Canada, and were held in a large indoor tank containing approximately 150,000 L of circulating, well-aerated Bamfield Marine Station seawater (12°C, 30 ppt salinity) for approximately 1 mo prior to experimentation. Fish were fed twice a week (5% body weight ration) with hake. Prior to experimentation, fish were fasted for a minimum of 5 d. In series 3 and 4, due to difficulties in obtaining experimental animals and resulting time restrictions, dogfish were caught and held for a period of only 1 wk under the same conditions as series 1 and 2; sharks were fasted over this period. All husbandry and experimental protocols were conducted in accordance with the guidelines of the Canada Council for Animal Care at Bamfield Marine Sciences Centre and McMaster University, and fish were collected under permits from the Department of Fisheries and Oceans Canada.

Series 1: Hypoxic Exposures of Cannulated Animals

In the field season of August 2012, a series of experiments was conducted to assess the effects of acute moderate- and severe-hypoxic exposures (described below) on N-waste regulation and a number of blood and plasma parameters in cannulated dogfish.

Cannulation Technique. Dogfish were fitted with an indwelling caudal artery cannula, similar to the procedure described by Richards et al. (2003). Fish were anesthetized with a 300 mg/L neutralized MS-222 solution and weighed, and, while the gills were constantly irrigated by this anesthetic solution, a 5-cm incision was made along the lateral line, just anterior to the second dorsal fin. The cartilaginous hemal canal was revealed and a small nick was made with a needle, through which a PE50 cannula containing a heparinized isotonic saline solution was inserted. Successful insertion into the caudal artery was marked by a generation of pressure in the cannula. The wound was then sutured closed tightly and the cannula was secured into place by a PE160 sheath, flared at both ends, which was glued to the cannula and then ligated to the side of the fish with two sutures. The cannula was completely flushed free of blood using lithium-heparinized (50 i.u./mL) saline and then sealed with a pin. Fish were allowed to recover for a minimum of 24 h prior to any experimentation. Recovery took place in individual 40-L wooden, polyurethane-coated flux boxes identical to those used in previous studies (e.g., Wood et al. 1995, 2005). During recovery, boxes were well aerated and received flow-through seawater.

Normoxia-Hypoxia: Normoxic Recovery Protocols. All hypoxic exposures followed the same protocol: 6 h normoxia; 4–6 h moderate or severe hypoxia; 12 h normoxic recovery. The hypoxic exposure period was 6 h for moderate hypoxia and only 4 h for severe

hypoxia, as preliminary tests showed that this was the maximum period of severe hypoxia that these fish could withstand, while moderate hypoxia could be withstood for at least 10 h.

For initial normoxia measurements, flow to the boxes was stopped and the volume of water in the box was lowered to 21 L by removing a rubber stopper from the bottom of the box and replacing it thereafter. An initial 10-mL water sample was taken for the determination of water ammonia and urea-N concentrations, marking the beginning of the initial 6-h normoxic period (normoxia; $Po_2 = 149.1 \pm 1.0$ torr). Following 3 h, a 1-mL blood sample was taken for analysis of the parameters described in detail below. After 6 h of normoxia, a final 10-mL water sample was taken and then the water was replaced by allowing the box to fill completely with seawater and then draining the box through the bottom hole in a cycle that was repeated three times while keeping the dogfish fully submerged throughout the process. After this water renewal, volume was again set to 21 L in each box. N_2 was then bubbled into the water (at a rate of approximately 7 L/min monitored by a flowmeter) to displace oxygen in the water. Over a period of 30 min, water O_2 levels were brought down to either 20% air O_2 saturation (moderate hypoxia; $Po_2 = 37.3 \pm 1.4$ torr) or 5% air O_2 saturation (severe hypoxia; $Po_2 = 9.8 \pm 0.3$ torr). Dissolved oxygen content in the water was monitored using a portable CellOx 325 dissolved oxygen probe and Oxi 330 handheld meter (WTW, Weilheim in Oberbayern, Germany). Once target O_2 saturation was attained, an initial 10-mL water sample was taken, marking the start of the hypoxic period, and boxes were bubbled with both air and nitrogen for the remainder of the 6-h (moderate) or 4-h (severe) hypoxic exposure to maintain water at the desired hypoxia level. Additional 10-mL water samples were taken every 2 h during this exposure. Blood samples were taken at 1, 3, and 5 h of moderate-hypoxia exposure and at 1 and 3 h of severe-hypoxia exposure to coincide with the midpoint of the flux periods. After the final water sample at 6 h (moderate hypoxia) or 4 h (severe hypoxia) was taken, N_2 bubbling was stopped and boxes were flushed in the same manner described above, restoring initial water O_2 saturation and removing accumulated ammonia and urea-N. With the box volume again set to 21 L, a 10-mL water sample was taken, marking the beginning of the 12-h normoxic recovery flux. Here, water samples were taken at 0, 2, 4, and 12 h, whereas blood samples were taken at 1, 3, and 11 h. This recovery protocol was the same for both degrees of hypoxia.

Handling and Processing of Blood Samples. To obtain blood and plasma samples, the arterial catheter was initially cleared by drawing the blood-saline mixture into a separate plastic syringe, and then a 1-mL blood sample was drawn using a gas-tight Hamilton syringe. Hematocrit, arterial blood pHa, blood PaO_2, and plasma total CO_2 (TCO_2) were determined immediately while aliquots for the later determination of blood hemoglobin (Hb) and plasma lactate, pyruvate, glucose, urea, and ion concentrations were also obtained. Blood PaO_2 was measured on a 400-μL aliquot of blood, using a polarographic blood oxygen electrode (Radiometer, Copenhagen, Denmark) thermostatted to 12°C and connected to a polarographic amplifier (model 1900, A-M Systems, Everett, WA). A separate aliquot (100 μL) was used to quickly fill two heparinized microhematocrit tubes (Fisher Scientific, Ottawa, Ontario), which were then plugged with Hemato-Seal tube sealant (Fisher Scientific) and spun down (2,000 *g* for 5 min) to measure hematocrit levels. Yet another aliquot (500 μL) was placed into a 1.0-mL centrifuge tube and pHa was measured at 12°C using a pH microelectrode (PerpHecT ROSS pH microelectode, Thermo Scientific, Waltham, MA) connected to a handheld pH meter (Accumet AP84 meter, Cole-Parmer, Vernon Hills, IL). Duplicate 20-μL blood samples were then removed from this sample and diluted in 5 mL Drabkin's reagent (Sigma-Aldrich, Burlington, Ontario) and kept in the dark at room temperature until later [Hb] determination. The remainder of this aliquot was then spun down (2,000 *g* for 5 min) and 50 μL plasma was removed for the determination of plasma TCO_2 content using a total CO_2 analyzer (965 CO_2 analyzer, Corning, Corning, NY). Following TCO_2 analysis, the remainder of the plasma was then flash frozen in liquid N_2 and kept at −80°C until further analysis. All remaining red blood cells were resuspended in nonheparinized saline and injected back into the fish through the caudal artery cannula in order to maintain hematocrit levels in the sharks. The cannula was then filled with heparinized saline and the pin was reinserted.

Series 2: Hypoxic Exposures of Noncannulated Animals

An additional control series of experiments was performed in the 2012 field season to assess the effects of the cannulation procedure on whole-body ammonia and urea-N excretion rates. A separate group of noncannulated dogfish were placed individually into the same flux boxes described above and exposed to the same moderate- and severe-hypoxia protocols. However, for these noncannulated fluxes, only initial and final water samples were taken for the initial normoxia, hypoxia, and normoxic recovery periods, and overall responses over the entire hypoxia and normoxic recovery were obtained.

Series 3: Additional Hypoxic Exposures of Cannulated Animals

In the field season of 2013, a second set of cannulation experiments was conducted for the determination of plasma total ammonia, as these samples from series 1 had been lost. These experiments followed the same exposure procedures and protocols described for series 1, except that the dogfish had been held at the marine station (and fasted) for only 1 wk prior to experimentation.

Series 4: Terminal Hypoxic Exposures of Noncannulated Animals for Tissue Analysis

An additional set of experiments in 2013 examined tissue-specific responses to severe hypoxia. Dogfish that had been held as in series 3 were placed individually in wooden flux boxes containing 25 L well-aerated, flow-through seawater and allowed to adjust

to this setup for 1 h prior to experimentation. Flow to the boxes was stopped and aeration was allowed to continue (normoxia) or was ceased (severe hypoxia) and N_2 was bubbled into the box over a period of 30 min to reach severe hypoxia (5% air O_2 saturation); thereafter, air and N_2 were bubbled lightly to maintain the appropriate O_2 level. Sharks were exposed to normoxia or severe hypoxia for 2 h, after which they were sacrificed by MS-222 anesthesia (300 mg/L) followed by spinal cord severance. Gill, liver, and white muscle samples were then harvested, freeze clamped immediately, flash frozen in liquid N_2, and stored at −80°C until later analysis.

Analytical Procedures and Calculations

Ammonia and Urea Excretion Rates. Total ammonia-N (T_{amm}) and total urea-N (T_{urea}) in water samples were measured using the methods described by Verdouw et al. (1978) and Rahmatullah and Boyde (1980), respectively. Ammonia-N (J_{amm}; μmol N/kg/h) and urea-N (J_{urea}; μmol N/kg/h) flux rates were calculated using the following equations:

$$J_{amm} = \frac{(T_{amm\,f} - T_{amm\,i}) \times V}{w \times t}, \quad (1)$$

$$J_{urea} = \frac{(T_{urea\,f} - T_{urea\,i}) \times V}{w \times t}, \quad (2)$$

where $T_{amm\,f}$ and $T_{amm\,i}$ and $T_{urea\,f}$ and $T_{urea\,i}$ are the final and initial water T_{amm} and T_{urea} (μmol N), respectively; V is volume of the flux box (L); w is weight of the shark (kg); and t is duration of the flux (h).

Plasma Analyses. Plasma total ammonia-N (T_{amm}; μmol N/L) was determined using the Raichem commercial kit (Cliniqa Corporation), and plasma total urea-N (T_{urea}; mmol-N/L) was determined using the method described above for water analyses. Arterial blood pHa and PaO_2 (torr) and plasma TCO_2 (mmol/L) were measured using the methods described above. Plasma $PaCO_2$ was determined using appropriate pK′ and CO_2 solubility coefficients (αCO_2) for dogfish plasma at 12°C from Boutilier et al. (1984) and the following equation:

$$PaCO_2 = \frac{TCO_2}{[1 + \text{antilog}(\text{pHA} - \text{pK}') \times \alpha CO_2]}. \quad (3)$$

From this, plasma $[HCO_3^-]$a (mmol/L) was calculated using the following equation:

$$[HCO_3^-]a = TCO_2(PaCO_2 \times \alpha CO_2). \quad (4)$$

Plasma [lactate] and [pyruvate] (mmol/L) were determined on deproteinized (see below for deproteinization procedure) plasma samples using the reversible lactate dehydrogenase method (Bergmeyer 1983). Plasma glucose was determined using a commercial kit (Infinity Glucose Kit, Thermo Scientific).

Plasma [protein] (g/dL) was measured using the Bradford reagent (Sigma-Aldrich) against a bovine serum albumin standard. Hematocrit (%) was measured using the methods described above, and [Hb] (g/dL) was determined by measuring absorbance at 540 nm following incubation with Drabkin's reagent (Sigma-Aldrich). Mean cell hemoglobin content (MCHC; g/mL) was calculated using the following equation:

$$\text{MCHC} = \frac{[\text{Hb}]}{\text{Hct}}, \quad (5)$$

where Hct is hematocrit.

Plasma ion concentrations (mmol/L) were determined either by the use of a digital chloridometer (Labconco, Kansas City, MO; for $[Cl^-]$) or by atomic absorption (1275 Atomic Absorption Spectrophotometer, Varian, Mulgrave, Victoria, Australia; for $[Na^+]$, $[Ca^{2+}]$, $[Mg^{2+}]$, and $[K^+]$) using commercial standards.

Tissue Analyses. Tissue samples for the determination of T_{amm}, T_{urea}, and [lactate] were first ground under liquid N_2 with a liquid N_2–cooled mortar and pestle and were deproteinized by adding 1 vol of ice-cold 8% perchloric acid and incubating on ice for 5 min. The samples were then spun down (12,000 *g* for 5 min) and the resulting supernatant was neutralized by the addition of 1 M KOH. Determination of T_{amm}, T_{urea}, and [lactate] in these deproteinized and neutralized tissue samples followed the same protocols used for plasma samples described above. Unfortunately, samples for muscle [lactate] were lost due to a procedural error. Tissue intracellular pH (pHi) was measured using the methods described by Portner et al. (1990). Briefly, tissues were ground to a fine powder under liquid N_2 using a liquid N_2–cooled mortar and pestle. Approximately 100 mg of this powder was added to a 1.5-mL buffer containing 150 mmol/L KF and 6 mmol/L nitrilotriacetic acid (Na_2-NTA). Tissue pH was then determined in this buffer at 12°C using an Accumet AccuTupH electrode and Accumet AB15 meter (Fisher Scientific).

Statistics

All data are represented as means ± 1 SEM, and n is sample size. Statistical analyses between different time points within one hypoxic treatment in the cannulation experiments were performed using a repeated-measures one-way ANOVA as each individual fish acted as its own control during the initial 6-h normoxic period. Statistical analyses between different tissues within a treatment (normoxia or hypoxia) in the terminal experiments were performed using a one-way ANOVA. Comparisons between the two hypoxia protocols at the same time points and comparisons between tissues from normoxia and severe-hypoxia terminal exposures were performed using Student's unpaired two-tailed *t*-tests. Specific descriptions of statistical analyses and post hoc tests are stated within corresponding figure captions. Significance was accepted at the $P < 0.05$ level.

Results

N-Waste Homeostasis

All ammonia (J_{amm}) and urea (J_{urea}) flux rates and concentrations (T_{amm}, T_{urea}) are reported as ammonia-N and urea-N in "Results" and "Discussion."

In series 1, during the normoxic period preceding moderate

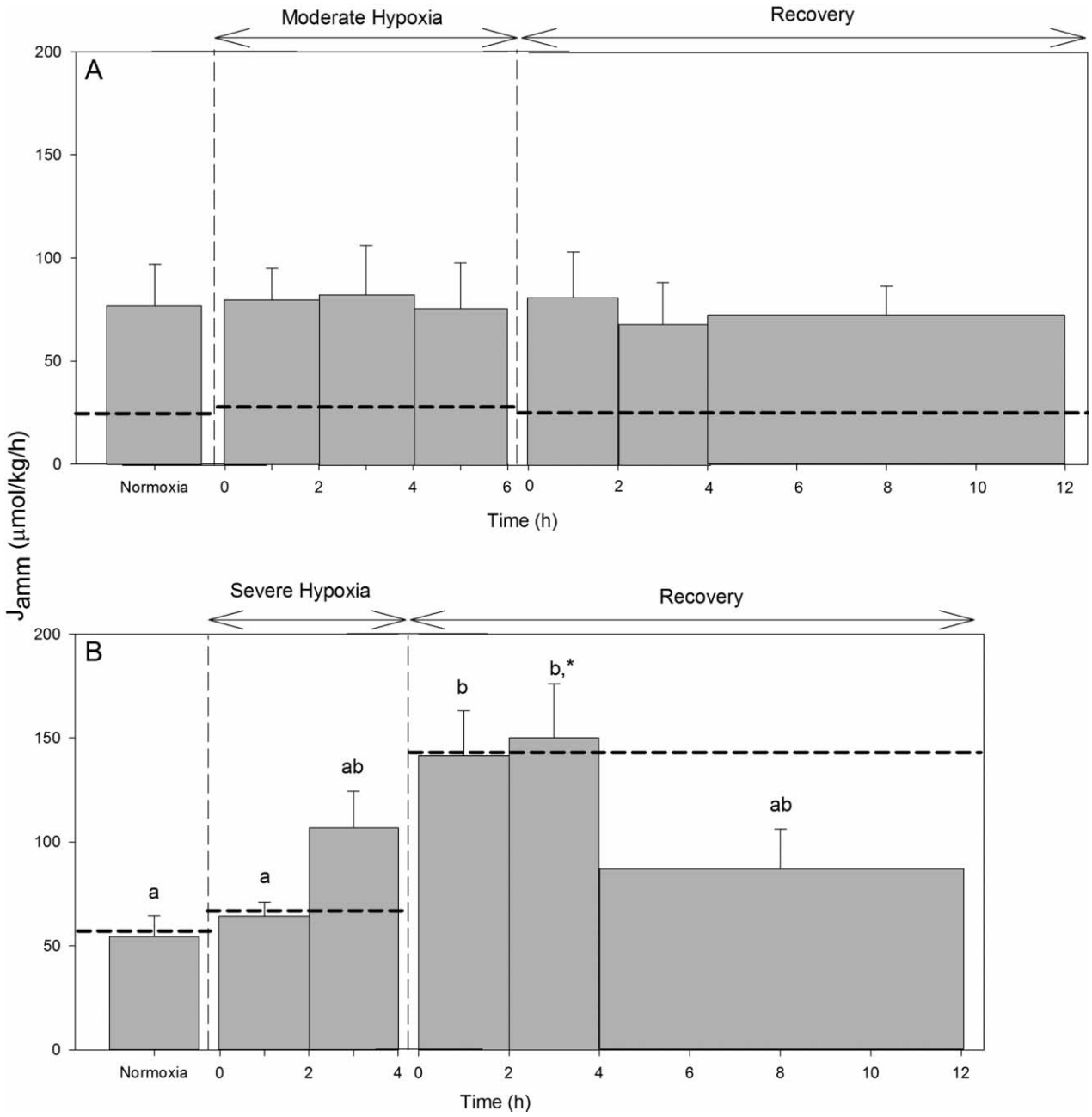

Figure 1. Ammonia-N excretion rates (J_{amm}) in dogfish during normoxia followed by moderate (*A*) and severe (*B*) hypoxia and subsequent recovery in normoxic water in series 1. Dashed horizontal lines represent overall means from a noncannulated control series 2 ($n = 8$). Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test. Means ± 1 SEM ($n = 8$).

hypoxia, J_{amm} and J_{urea} in cannulated dogfish were 78.3 ± 18.6 and 738.7 ± 54.1 μmol N/kg/h, respectively (figs. 1, 2). Exposure to 6-h moderate hypoxia (20% air O_2 saturation) had no significant effects on either J_{amm} or J_{urea}, and subsequent recovery in normoxic water also had no significant effects (figs. 1*A*, 2*A*). This same trend was observed in the series 2 experiments performed on noncannulated dogfish, the means of which are shown as dotted lines in figures 1 and 2.

In the severe-hypoxia tests, initial normoxic J_{amm} and J_{urea} values (55.0 ± 9.6 and 651.3 ± 57.9 μmol N/kg/h, respectively) were similar to the previous values. Exposure to severe hypoxia led to a nearly twofold increase in J_{amm} (fig. 1*B*), though this was not

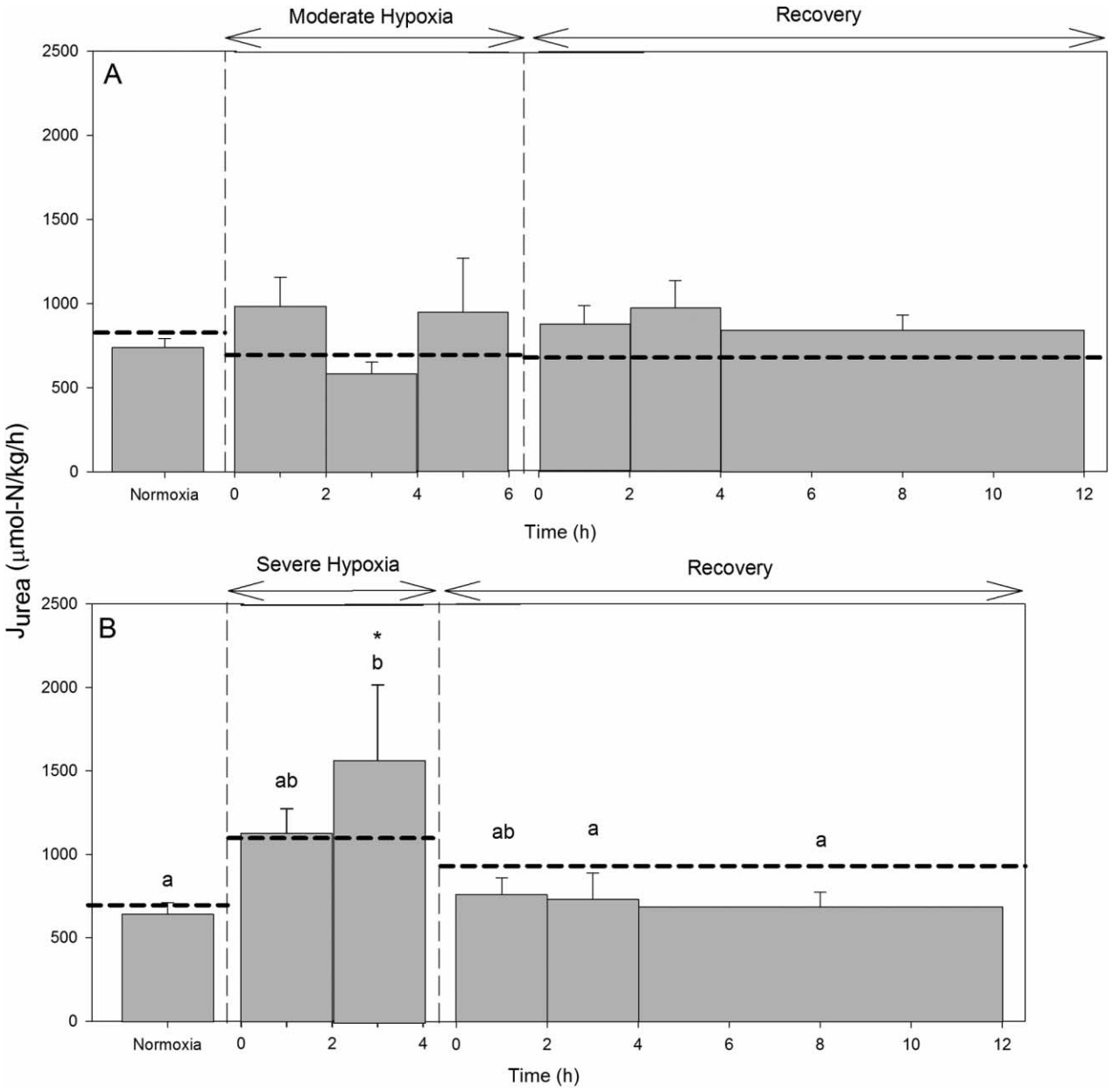

Figure 2. Urea-N excretion rates (J_{urea}) in dogfish during normoxia followed by moderate (*A*) and severe (*B*) hypoxia and subsequent recovery in normoxic water in series 1. Dashed horizontal lines represent overall means from a noncannulated control series 2 ($n = 8$). Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test. Means ± 1 SEM ($n = 8$).

statistically significant, and a significant 2.4-fold increase in J_{urea} (fig. 2*B*) by 2–4 h of exposure. Return to normoxic conditions led to a significant 2.6-fold increase in J_{amm} relative to initial normoxic rates over the first 4 h of recovery, whereas over the last 8 h of recovery, J_{amm} was restored to baseline rates (fig. 1*B*). J_{urea}, on the other hand, was immediately restored back to initial normoxic rates in response to normoxic recovery (fig. 2*B*).

In series 2, the additional hypoxic exposure in noncannulated fish (means represented by horizontal dashed lines in figs. 1, 2) revealed that cannulation had a marked effect (~65% increase) on control J_{amm} but no effect on J_{urea}. The same general effects of moderate and severe hypoxia on J_{amm} and J_{urea}, however, were seen in both cannulated and noncannulated dogfish, suggesting that cannulation did not alter the response to hypoxia.

In series 3, plasma T_{amm}, overall, was extremely variable, and no significant differences between normoxic and hypoxic values

Table 1: Plasma total ammonia (T_{amm}; μmol N/L; $n = 4$–8; series 3) and total urea-N (T_{urea}; mmol N/L; $n = 7$–8; series 1) during normoxia, moderate and severe hypoxia, and normoxic recovery

		Hypoxia (h)			Normoxic recovery (h)		
	Normoxia	1	3	5	1	3	11
Plasma T_{amm}:							
Moderate	94.5 ± 47.9	91.8 ± 38.5	99.9 ± 33.2	104.1 ± 32.5	108.1 ± 41.1	104.3 ± 42.6	176.6 ± 60.6
Severe	135.3 ± 92.4	146.1 ± 83.0	137.5 ± 101.8	...	144.3 ± 63.7	223.5 ± 109.0	157.2 ± 84.7
Plasma T_{urea}:							
Moderate	546.6 ± 33.5	556.7 ± 31.3	593.2 ± 31.8	564.7 ± 39.3	580.9 ± 44.0	561.1 ± 21.6	556.6 ± 31.2
Severe	592.3 ± 51.7	549.3 ± 31.7	535.4 ± 38.1	...	539.6 ± 38.9	556.7 ± 42.5	574.3 ± 35.4

Note. Values are representative of means ± 1 SEM, with no significant differences.

were observed (table 1*A*). When expressed relative to control values (% control), no significant differences were seen despite plasma T_{amm} reaching values exceeding 1,400% that of control (fig. 3*A*), demonstrating the high degree of variability in plasma T_{amm} in these fish. Plasma T_{urea}, however, was much more stable (table 1*B*) and was always within 10% of control values, being unresponsive to either hypoxic regime (fig. 3*B*).

Respiratory and Acid-Base Responses

In series 1, arterial blood PaO_2 (approximately 100 torr under normoxic conditions) decreased significantly in response to both moderate hypoxia (80% decrease) and severe hypoxia (90% decrease; fig. 4). During severe hypoxia, PaO_2 dropped to 6.7 ± 0.4 torr, quite similar to water Po_2 (9.8 ± 0.3 torr) at this level of hypoxia (fig. 4). In both exposures, PaO_2 was recovered to normoxic control values after just 1 h of return to normoxia (fig. 4). Arterial pHa and plasma $PaCO_2$ and $[HCO_3^-]$a were unaffected by moderate hypoxia (fig. 5), despite the observed significant drop in PaO_2. Blood pHa and plasma $[HCO_3^-]$a dropped significantly during severe hypoxia (fig. 4*A*, 4*B*), while plasma $PaCO_2$ was variable but not significantly changed (fig. 4*C*). Upon return to normoxia, blood pHa was recovered to control levels after 1 h, while plasma $[HCO_3^-]$a was not restored to control values until 11 h of normoxic recovery (fig. 4). Arterial PaO_2, pHa, $PaCO_2$, and $[HCO_3^-]$a were the same in both initial control periods (figs. 4, 5).

Metabolic and Hematological Responses

In series 1, plasma [lactate] increased 3.4- and 8.6-fold following 5 and 3 h of exposure to moderate and severe hypoxia, respectively, relative to normoxic values, which were, on average, 3.3 mmol/L (fig. 6*A*). While fish exposed to moderate hypoxia were able to reestablish normoxic levels by 11 h of normoxic recovery, those exposed to severe hypoxia were not (fig. 6*A*). Plasma [lactate] was the only parameter measured in this study that was significantly altered by moderate hypoxia relative to the initial normoxic period. Plasma [pyruvate] (0.16 mmol/L under normoxic conditions) was not affected by moderate hypoxia (fig. 6*B*). Similarly, no changes in plasma [pyruvate] were observed during severe hypoxia; however, a significant 3.2-fold increase was observed after 1 h of normoxic recovery, which was sustained until at least after 3 h recovery, but initial normoxic levels were reestablished by 11 h (fig. 6*B*). Again, plasma [lactate] and [pyruvate] were not different between the initial normoxic periods in the two exposures (fig. 6). Plasma [glucose] (about 8 mmol/L in normoxia) was unaffected by either hypoxic regime (table 2).

Plasma [protein] was not significantly affected by either hypoxic exposure, though there was a marked difference between the two normoxic groups (table 2). Under normoxic conditions, hematocrit was approximately 15% (not different between both initial normoxic periods) and was significantly higher during severe hypoxia (16.1%–16.2%) compared to the value observed following 11 h of subsequent normoxic recovery (12.6%; table 2). Blood [Hb] was also the same between both initial normoxic periods and ranged from 3.1 to 3.7 g/dL; neither hypoxic treatment led to a change in [Hb] relative to the initial normoxic period (table 2). In moderate hypoxia, however, [Hb] was consistently higher relative to [Hb] after 3 h of normoxic recovery (table 2). MCHC was also unaffected by either hypoxic treatment and did not differ between initial normoxic periods, ranging from 0.20 to 0.28 g/mL (table 2).

Plasma Ions

In series 1, means of all plasma ion concentrations measured did not differ between the initial normoxic periods in the two tests (fig. 7). Moreover, exposure to 6 h of moderate hypoxia and subsequent normoxic recovery did not result in any significant changes in the plasma concentrations of any of the ions (fig. 7). In response to severe hypoxia, plasma $[Na^+]$, approximately 260 mmol/L under normoxic conditions, was not altered but increased significantly following 1 h of normoxic recovery (fig. 7*A*). $[Cl^-]$ values were unchanged during severe hypoxia or subsequent recovery with control values of about 229 mmol/L (fig. 7*B*). Plasma $[K^+]$ also did not change relative to control values (3.4 mmol/L) during this hypoxic exposure, though plasma $[K^+]$ after 3 h of hypoxia was significantly greater than that following 11 h of normoxic recovery (fig. 7*C*). Plasma $[Mg^{2+}]$ and $[Ca^{2+}]$ demonstrated significant increases after 3 h of severe hypoxia exposure, from control values of 1.7 and 3.3 mmol/L, respectively (fig. 7*D*, 7*E*). After 1 h of normoxic recovery, both $[Mg^{2+}]$ and

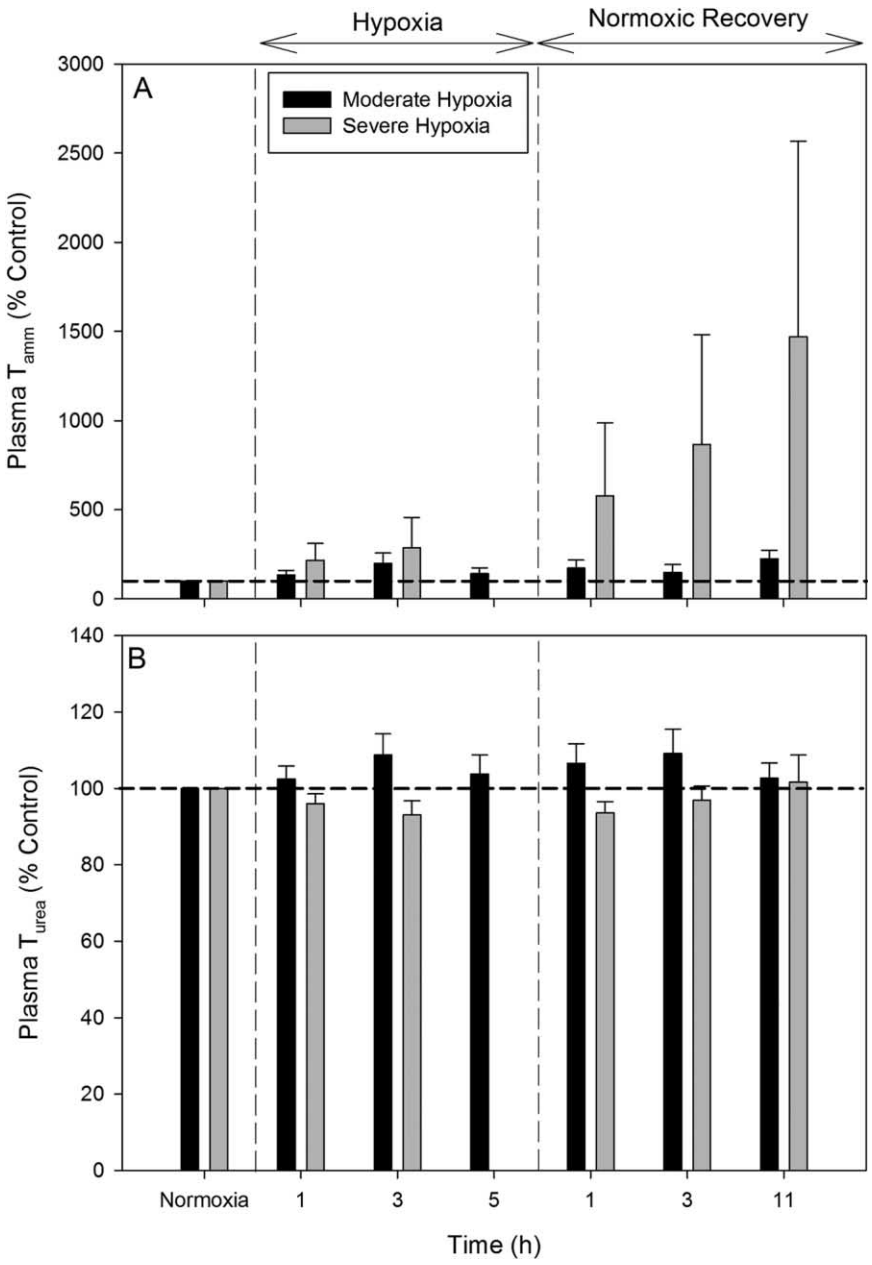

Figure 3. Plasma total ammonia-N (T_{amm}; *A*; series 3) and total urea-N (T_{urea}; *B*; series 1) expressed as percent of initial normoxic control during moderate (black bars) and severe (grey bars) hypoxia and subsequent recovery in normoxic water. No significant differences were observed within a given hypoxic treatment or between hypoxic treatments at a given time point as determined by a repeated-measures one-way ANOVA and Student's *t*-tests, respectively. Means ± 1 SEM (n = 4–8).

$[Ca^{2+}]$ reached their maximum levels, 30% and 12% greater, respectively, than those seen in normoxia (fig. 7*D*, 7*E*). By 11 h of normoxic recovery, all ions were not significantly different from initial normoxic levels (fig. 7).

Tissue Responses to Severe Hypoxia

In series 4, tissue T_{amm} was not altered by exposure to 2 h of severe hypoxia in the gill, liver, or white muscle (fig. 8*A*). Similarly, tissue T_{urea} was not affected by hypoxic exposure (fig. 8*B*). Gill and liver [lactate] increased 2.4- and 4.2-fold, respectively, in response to 2 h of severe hypoxia (data not shown). Unfortunately, samples for the measurement of muscle [lactate] were lost in processing. Tissue intracellular pHi in the gill, liver, and muscle decreased in response to hypoxia, though this was statistically significant only in the liver, narrowly escaping significance (P = 0.056) in the gill (fig. 8*C*).

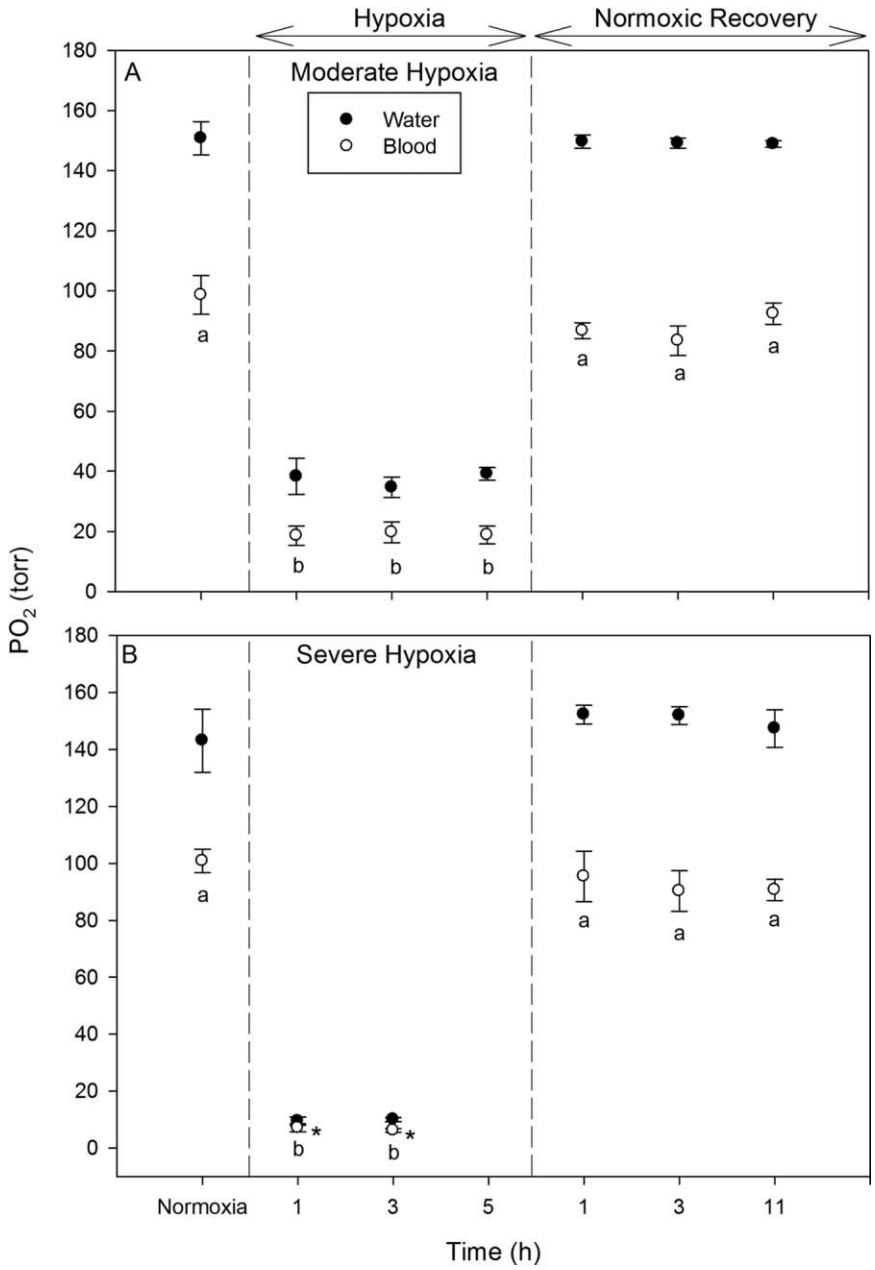

Figure 4. Water (filled circles) and arterial blood (open circles) oxygen partial pressure (Po_2) during normoxia followed by moderate (*A*) and severe (*B*) hypoxia and subsequent recovery in normoxic water in series 1. Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test. Means ± 1 SEM (n = 7–8).

Discussion

Overview

This study demonstrates the first evidence, to our knowledge, that exposure to environmental hypoxia can lead to alterations in nitrogen handling in dogfish sharks and is in general agreement with our initial hypotheses. This is an interesting finding as it well documented that dogfish demonstrate tight regulation of nitrogen homeostasis (e.g., Wood et al. 2005, 2007), maintaining constant plasma T_{urea} and osmolarity, even up to 56 d of starvation (Kajimura et al. 2008). In accordance with our initial prediction, exposure to hypoxia (severe hypoxia only) led to an increase in urea-N loss (fig. 2*B*), which was apparently elicited via a disruption in gill retention. In contrast, urea-N concentrations of the internal production tissues (white muscle and liver), which likely function under less oxic conditions, were not significantly affected (fig. 8*B*). This, together with unchanged plasma urea-N concentrations (table 1*B*; fig. 3*B*), suggests that

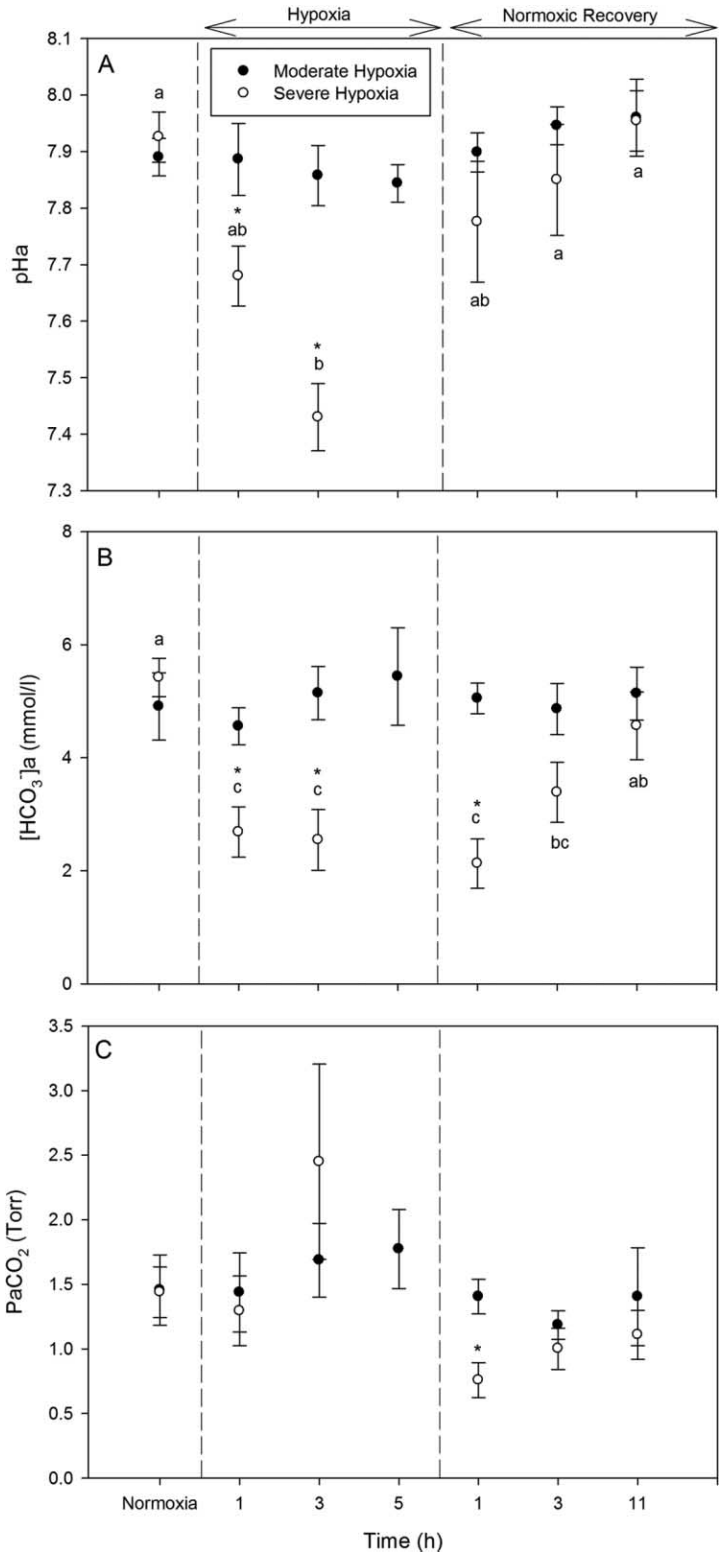

Figure 5. Arterial blood pHa (*A*) and plasma $[HCO_3^-]a$ (*B*) and carbon dioxide partial pressure ($PaCO_2$; *C*) during normoxia followed by moderate (filled circles) and severe (open circles) hypoxia and subsequent recovery in normoxic water in series 1. Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a Friedman repeated-measures ANOVA on ranks followed by a Tukey post hoc test for *A* and a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test for *B*. Means ± 1 SEM (n = 7–8).

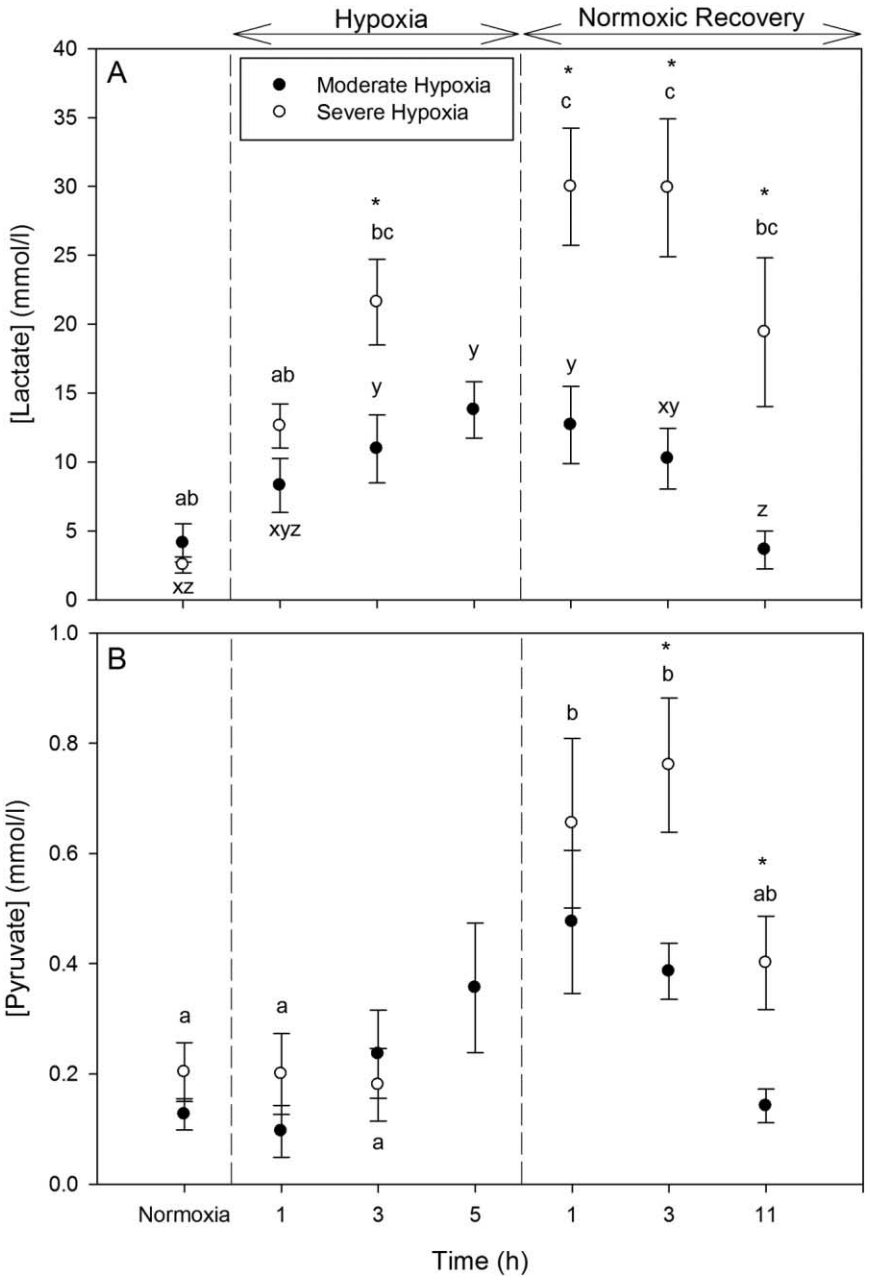

Figure 6. Plasma [lactate] (*A*) and [pyruvate] (*B*) during normoxia followed by moderate (filled circles) and severe (open circles) hypoxia and subsequent recovery in normoxic water in series 1. Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters (xyz for moderate hypoxia; abc for severe hypoxia) indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test. Means ± 1 SEM (n = 4–8).

urea-N synthesis rates were not impacted. Exposure to severe hypoxia also resulted in an acidosis of metabolic origin, as indicated by decreases in blood pHa (fig. 5*A*) and [HCO_3^-]a (fig. 5*B*) and increases in plasma lactate (fig. 6*A*) and a disruption in ionoregulatory balance, as indicated by increases in plasma [Mg^{2+}] and [Ca^{2+}] during severe hypoxia (fig. 7*D*, 7*E*). We propose that these findings are likely a result, at least in part, of a decrease in active homeostatic functions of the gill such as urea retention, acid-base regulation, and ionoregulation and/or an increase in functional gill surface area indicative of an osmorespiratory compromise in hypoxic dogfish.

Dogfish sharks were quite resistant to moderate hypoxia (20% air O_2 saturation), and, as such, the remainder of the discussion will focus mostly on the response to severe hypoxia (5% air O_2

Table 2: Plasma glucose concentration ([glucose]; $n = 7–8$), protein concentration ([protein]; $n = 7–8$), blood hematocrit (Hct; $n = 7–8$), hemoglobin concentration ([Hb]; $n = 7–8$), and mean cell hemoglobin content (MCHC; $n = 7–8$) during normoxia, moderate and severe hypoxia, and normoxic recovery in series 1

		Hypoxia (h)			Normoxic recovery (h)		
	Normoxia	1	3	5	1	3	11
[glucose] (mmol/L):							
Moderate	7.6 ± .8	6.8 ± .8	7.1 ± .8	7.2 ± 1.5	6.3 ± 1.0	7.5 ± 1.1	8.3 ± 1.1
Severe	8.5 ± 1.1	7.8 ± .9	6.8 ± 1.1	...	7.1 ± 1.0	7.3 ± 1.1	7.4 ± 1.2
[protein] (g/dL):							
Moderate	1.36 ± .18	1.00 ± .16	1.03 ± .22	1.02 ± .15	.9 ± .25	1.03 ± .15	1.09 ± .13
Severe	.89 ± .11	.99 ± .23	1.07 ± .17	...	.8 ± .13	1.03 ± .18	.71 ± .11
Hct (%):							
Moderate	15.8 ± 1.5	17.2 ± 1.8	16.1 ± 1.6	15.8 ± 1.4	15.9 ± 1.9	15.7 ± 2.2	14.9 ± 1.7
Severe	14.9^{AB} ± 2.4	16.1^{B} ± 2.1	16.2^{B} ± 2.4	...	14.7^{AB} ± 2.0	15.0^{AB} ± 1.8	12.6^{A} ± 1.7
[Hb] (g/dL):							
Moderate	3.72^{AB} ± .46	4.15^{B} ± .52	4.58^{B} ± .24	4.43^{B} ± .57	3.99^{AB} ± .52	3.17^{A} ± .47	3.76^{AB} ± .50
Severe	3.11 ± .50	3.97 ± .64	4.26 ± .75	...	4.72 ± 1.09	4.03 ± .70	4.11 ± .86
MCHC (g/mL):							
Moderate	.23 ± .02	.24 ± .02	.28 ± .01	.27 ± .02	.24 ± .03	.20 ± .02	.25 ± .03
Severe	.20 ± .01	.21 ± .02	.23 ± .03	...	.27 ± .06	.23 ± .03	.27 ± .05

Note. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test.

saturation). Indeed, in the cannulation experiments, moderate hypoxia had little effect on any of the parameters assessed, except for plasma [lactate], which increased significantly (fig. 6*A*). However, this did not result in acid-base disturbance, in contrast to severe hypoxia (fig. 5). This general lack of response may be expected as arterial blood PaO_2 during moderate hypoxia dropped to approximately 18 torr (fig. 4*A*), slightly higher than the expected P50 for dogfish blood, which is approximately 13–17 torr (Lenfant and Johansen 1966; Wells and Weber 1983). Contrastingly, PaO_2 during severe hypoxia fell to approximately 7 torr (fig. 4*B*), well below the P50 of dogfish blood. The level of the P50 relative to the two hypoxic challenges probably accounts for the large disparity in responses to moderate- and severe-hypoxic exposures in these fish and is consistent with a P_{crit} for dogfish of approximately 14 torr (G. De Boeck, personal communication). It appears that in dogfish held at dissolved O_2 levels above P_{crit}, there are no adverse physiological effects, while at dissolved O_2 levels below P_{crit}, disruptions in nitrogen, acid-base, and ion balance are observed. Exposure to either degree of hypoxia generally had no effects on any hematological parameter measured in this study (table 2).

N-Waste Handling in Response to Severe Hypoxia

The synthesis and retention of urea are both energetically expensive processes (for review see Ballantyne 1997; McDonald et al. 2006). As such, we predicted that either or both would be altered in response to hypoxic exposure. Exposure to 6 h of moderate hypoxia had no significant effects on J_{amm}, J_{urea}, plasma T_{amm}, or plasma T_{urea} (figs. 1*A*, 2*A*, 3). During severe hypoxia, there were also no significant increases in J_{amm} or plasma or tissue T_{amm} (figs. 1*B*, 3*A*, 8*A*). During recovery from severe hypoxia, however, J_{amm} increased significantly (fig. 1*B*) while plasma T_{amm} increased up to 15-fold relative to the controls, although this was not a statistically significant difference (fig. 3*A*). This lack of significance was likely due to the highly variable nature of plasma T_{amm} in dogfish (table 1*A*), which has been observed previously (e.g., Kajimura et al. 2008; Wood et al. 2010; M. J. Lawrence, P. A. Wright, and C. M. Wood, unpublished results). Nonetheless, the increase in J_{amm}, and potentially plasma T_{amm}, may have been due to an increase in the anaerobic breakdown of adenylates, as observed during recovery from exhaustive exercise in dogfish (Richards et al. 2003). Alternatively, ammonia production may have occurred in order to compensate for intracellular acidosis, which occurs by 2 h of severe hypoxia, at least in the liver (fig. 8*D*). Interestingly, the apparent increase in ammonia production occurred only upon normoxic recovery. It is not clear whether this is a function of reoxygenation in recovery or whether there is a temporal delay in hypoxia-induced ammonia production. Indeed, tissue T_{amm} was unchanged following 2 h of severe hypoxia (fig. 8*A*).

Severe hypoxia also led to a significant increase in J_{urea} by 2–4 h of exposure (fig. 2*B*), which, however, was not accompanied by any significant changes in plasma T_{urea} (fig. 3; table 1*B*). Moreover, T_{urea} in white muscle and liver was not significantly affected by 2 h of severe hypoxia (fig. 8*B*), suggesting that the production of urea-N was not affected by hypoxia. This may be due to the fact that these internal tissues are likely bathed in a less oxic environment and thus may have a higher capacity for anaerobic function.

Urea-N loss, for which the gill accounts >90% under normal conditions (Wood et al. 1995), increased in response to severe

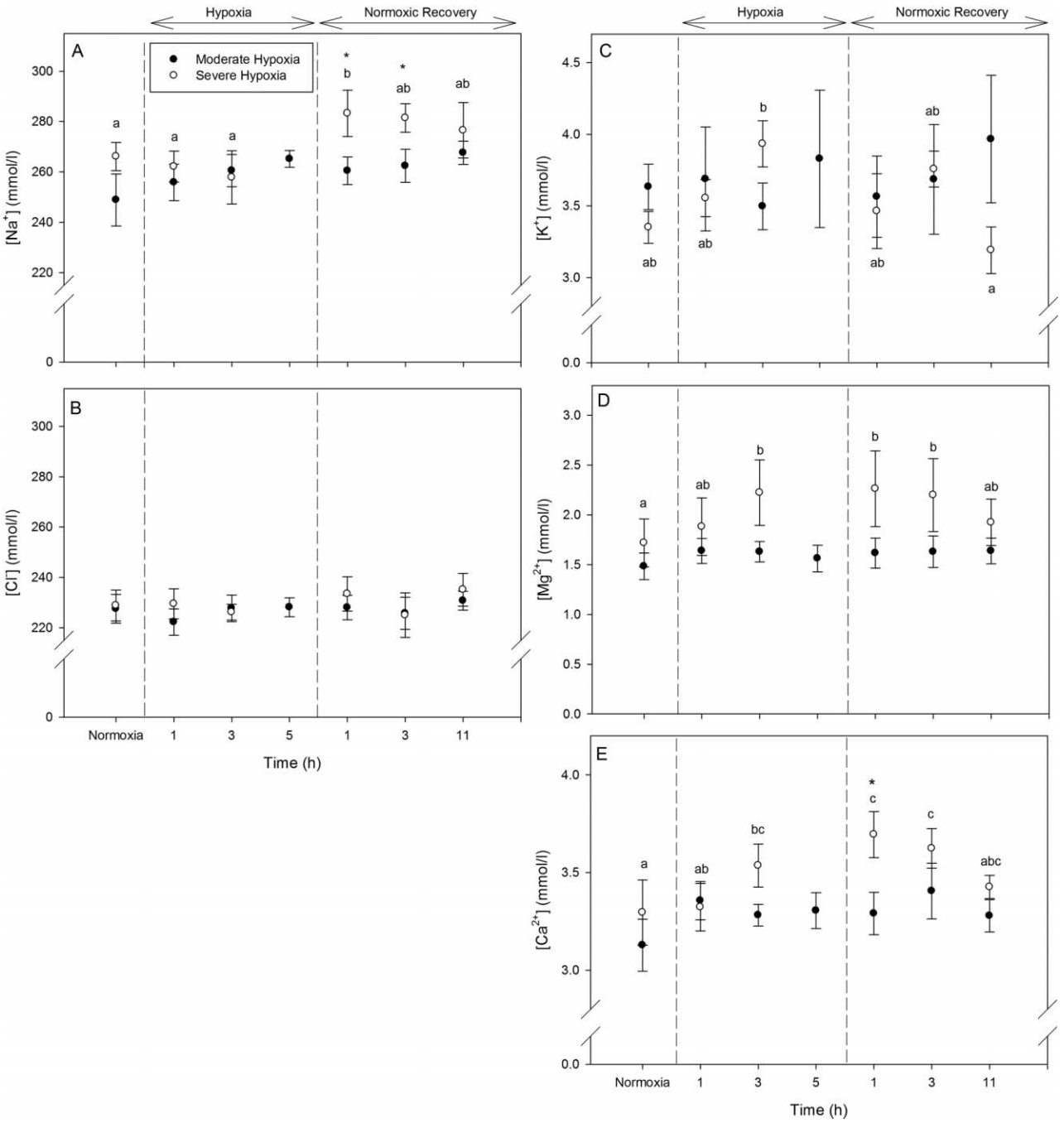

Figure 7. Plasma $[Na^+]$ (*A*), $[Cl^-]$ (*B*), $[K^+]$ (*C*), $[Mg^{2+}]$ (*D*), and $[Ca^{2+}]$ (*E*) during normoxia followed by moderate (filled circles) and severe (open circles) hypoxia and subsequent recovery in normoxic water in series 1. Asterisks represent means from the severe-hypoxia series at a given time point that were significantly different from the corresponding moderate-hypoxia series as determined by a Student's *t*-test. Means not sharing the same letters indicate statistically significant differences between normoxia, hypoxia, and recovery time points within a given hypoxic series as determined by a repeated-measures one-way ANOVA followed by a Holm-Sidak post hoc test or Fisher LSD post hoc test ($[Na^+]$ only). Means ± 1 SEM (n = 7–8).

hypoxia (fig. 2*B*). As outlined in the "Introduction," branchial urea retention occurs in part due to an active urea back-transport mechanism(s) in the gills (Wood et al. 1995, 2013; Part et al. 1998; Fines et al. 2001; Hill et al. 2004). Inhibition of this retention system may have occurred during severe hypoxia as J_{urea} increased significantly by 2–4 h exposure, yet plasma, gill, liver, and muscle T_{urea} were unchanged. Alternatively or additionally, increased J_{urea} may have occurred in response to a hypoxia-induced increase in functional gill surface area, indicating an osmorespiratory compromise in these fish. Overall,

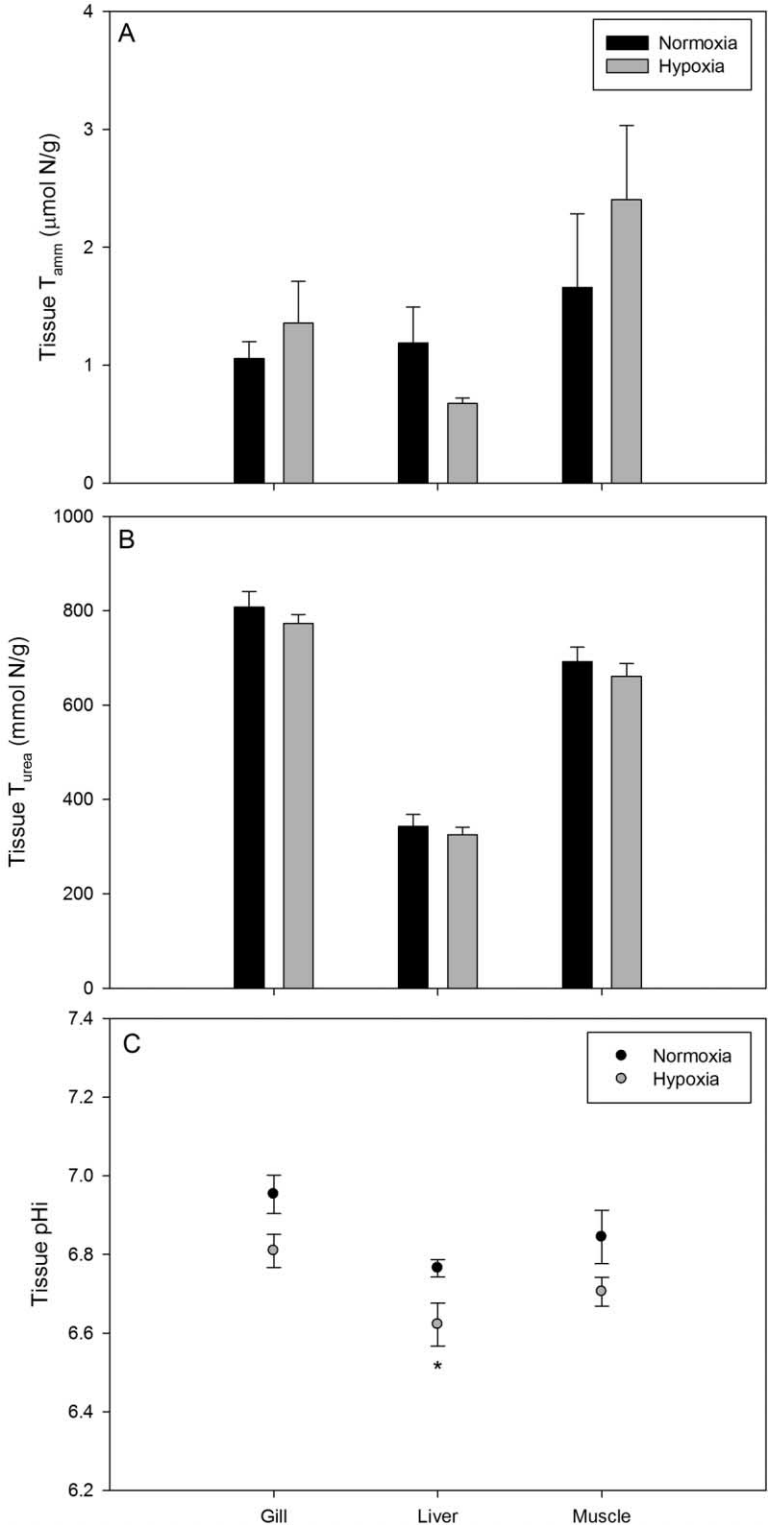

Figure 8. Gill, liver, and white muscle tissue total ammonia-N (T_{amm}; *A*) and urea-N (T_{urea}; *B*) and intracellular pHi (*C*) following 2 h of normoxia (black bars, black circles) and severe hypoxia (gray bars, gray circles) in series 4. Asterisks represent means from the hypoxia-exposed series that differed significantly from respective normoxia-exposed means by a Student's *t*-test. Means ± 1 SEM (n = 5–6).

severe hypoxia in dogfish leads to a loss of metabolic nitrogen via decreased urea-N retention and increased J_{amm}.

Metabolic Acidosis in Response to Severe Hypoxia

During severe hypoxia, plasma [lactate] increased more than eightfold (fig. 6*A*), which was associated with significant decreases in blood pHa and plasma $[HCO_3^-]$a (fig. 5*A*, 5*B*), suggesting that acute severe hypoxia leads to a metabolic acidosis. The response to acute severe hypoxia in dogfish is comparable to that seen following recovery from exhaustive exercise, with increases in plasma [lactate], [pyruvate], and T_{amm} and decreases in pHa and $[HCO_3^-]$a, also indicative of a metabolic acidosis (Piiper et al. 1972; Holeton and Heisler 1983; Richards et al. 2003). In this study, $PaCO_2$ did not change significantly (fig. 5*C*), so there was no respiratory component. Following severe exercise in teleost fish, the accumulation of lactate in the extracellular fluid is accompanied by a decrease in extracellular levels of Cl^- as this ion is shuttled into the intracellular space (e.g., Wood 1991; Wang et al. 1994). Interestingly, plasma $[Cl^-]$ was not altered during severe hypoxia or during recovery (fig. 7*B*), despite large increases in plasma [lactate]. It is possible that any loss of Cl^- from the plasma into the intracellular fluid is countered by branchial influx from the external environment, accompanied by even greater Na^+ influx (fig. 7*A*). Note that the difference between plasma $[Na^+]$ and $[Cl^-]$ (fig. 7*A*, 7*B*) increased during recovery, which would balance the large rise in [lactate], thereby maintaining electroneutrality.

Plasma [lactate] also increased during moderate hypoxia, though only by 3.4-fold (fig. 6*A*), yet there was no associated acid-base disturbance, indicating that these fish are able to maintain acid-base balance despite significant increases in plasma [lactate]. An excess of lactate versus metabolic acid accumulation in the blood has been commonly observed in active fish species, including elasmobranchs (reviewed by Wood and Perry 1985) and is explained, in part, by the ability of such fish to rapidly excrete acid equivalents across the gills into the surrounding environment while retaining lactate in the extracellular fluid. However, in severe hypoxia, sharks suffered significant decreases in plasma pHa and $[HCO_3^-]$a (fig. 5*A*, 5*B*), suggesting that these fish may also lose some capacity to acid-base regulate at the gills. Indeed, upon reoxygenation in normoxic recovery, pHa was recovered to initial normoxic levels by 1 h (fig. 5*A*), potentially via Na^+/H^+ exchange (fig. 7*A*), despite plasma [lactate] remaining significantly greater than normoxic levels throughout 11 h of normoxic recovery (fig. 6*A*).

Conclusions

In our initial hypotheses, we predicted that the active retention of urea-N at the gills would be sensitive to hypoxic exposure. Indeed, the observed increase in J_{urea} during severe hypoxia suggests that active retention in these fish may be impaired. However, an alternate or additional possibility is that the overall diffusive permeability of the gills to urea-N is increased as the fish makes changes in branchial area, diffusion distance, and/or blood flow pattern in an attempt to maximize O_2 uptake—the classic "osmorespiratory compromise" (Randall et al. 1972; Nilsson 1986; Iftikar et al. 2010). Even if the rate of active urea-N back-transport continues unimpaired during severe hypoxia, greater diffusive permeability to urea-N would result in greater rates of loss. The significant increases in plasma Mg^{2+} and Ca^{2+} concentrations (fig. 7*C*, 7*D*) could also be interpreted in this context; however, it is also possible that the active excretion of Ca^{2+} and Mg^{2+} at the gills is impaired by ATP limitation during hypoxia in a similar manner to the impairment of active urea-N back-transport and acid-base regulation.

Overall, results presented here suggest that branchial function is inhibited under severely hypoxic conditions in a model elasmobranch and that this can result in disruptions in N-waste, ionoregulatory, and acid-base homeostasis. Understanding the response to severe levels of hypoxia in marine organisms may be particularly important given the spread of hypoxic and anoxic "dead zones" in the marine environment (Diaz and Rosenberg 2008).

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