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Effects of anaerobic exercise accompanying catch-and-release fishing on blood-oxygen affinity of the sandbar shark (*Carcharhinus plumbeus*, Nardo)

Richard Brill [a,*], Peter Bushnell [b], Stuart Schroff [c], Rebecca Seifert [b], Megan Galvin [b]

[a] *Cooperative Marine Education and Research Program, Northeast Fisheries Science Center-National Marine Fisheries Service, NOAA, Woods Hole, Massachusetts, USA*

[b] *Department of Biological Sciences, Indiana University South Bend, South Bend, Indiana, USA*

[c] *Department of Biology, Pomona College, Pomona, California, USA*

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Abstract

Recovery from anaerobic exercise is thought to be prolonged in elasmobranchs because they lack several mechanisms for maintaining or increasing oxygen delivery that are present in teleosts. For example, teleosts increase hematocrit and maximal blood-oxygen carrying capacity through red cell ejection from the spleen. Teleosts also counteract the reduction in hemoglobin oxygen affinity resulting from metabolic acidosis through an adrenergic-mediated increase in red cell Na^+–H^+ exchanger activity. To begin to assess the consequences of anaerobic exercise accompanying catch-and-release fishing occurring within the estuarine nursery habitats of juvenile sandbar sharks (*Carcharhinus plumbeus*, Nardo), we constructed blood-oxygen equilibrium curves using samples from individuals 1 h after capture by hook and line (exercise-stressed) and samples from fully-recovered animals maintained in a shore-side tank (control sharks). We also compared exercise-stressed and control sharks for hemoglobin concentration, hematocrit, red cell count, intracellular pH, and nucleoside triphosphate concentration ([NTP]). In contrast to results from previous studies on elasmobranchs, we found an elevation in both hematocrit (≈21%) and blood hemoglobin concentration (≈10%) in exercise-stressed sharks. There was also clear evidence of red cell swelling. Mean red cell volume was ≈28% higher and mean cell hemoglobin concentration was ≈10% lower in exercise-stressed sharks. Most important, in spite of significant metabolic acidosis (0.3 pH units), blood from exercise-stressed sharks had an oxygen affinity equivalent to that of blood from control sharks. This was a direct consequence of intracellular pH being alkalinized by approximately 0.15 pH units relative to plasma pH in exercise-stressed sharks. Our results using isolated hemoglobin solutions showed that the observed reduction (≈15%) in intracellular [NTP] also contributed to the leftward shift in the oxygen equilibrium curves. As expected, we found sandbar shark red cells to be unresponsive to exogenous catecholamines. Regardless, sandbar sharks appear able to prevent the decrease in blood-oxygen affinity resulting from anaerobic exercise (and the concomitant decreases in plasma pH), as has been

* Corresponding author. Virginia Institute of Marine Science, PO Box 1346 (mail), Route 1208 Greate Rd. (shipping), Gloucester Point, Virginia 23062, USA Tel.: +1 804 684 7875; fax: +1 804 687 7045.

E-mail address: rbrill@vims.edu (R. Brill).

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well-documented in teleosts. Our results suggest, therefore, that oxygen delivery following exhaustive exercise is not necessarily compromised in juvenile sandbar sharks, and that hook and line capture and subsequent release do not increase rates of mortality, although both are yet to be directly confirmed.
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1. Introduction

With the possible exception of the high-energy-demand, thermoconserving lamnid sharks (Bernal et al., 2001), the cardio-respiratory system of elasmobranchs is generally considered to be not completely effective at meeting oxygen demands during strenuous exercise (Piiper et al., 1977; Butler and Metcalfe, 1988; Baldwin and Wells, 1990; Lowe et al., 1995). Consequently, a considerable fraction of the energy demand is met anaerobically, which results in elevated muscle and blood lactate levels and metabolic acidosis (e.g., Cliff and Thurman, 1984). Recovery from anaerobic exercise is likewise thought to be problematic for elasmobranchs as it takes considerable time (>12 h) for blood acid–base status and lactate levels to return to pre-exercise values (e.g., Heisler, 1993; Richards et al., 2003). Prolonged recovery times result from elasmobranchs' lack of several mechanisms for maintaining or increasing oxygen delivery that are present in teleosts (Bushnell et al., 1982; Lowe et al., 1995; Opdyke and Opdyke, 1971). These mechanisms include increases in hematocrit and maximal blood-oxygen carrying capacity due to red cell ejection from the spleen (Yamamoto et al., 1980; Yamamoto and Itazawa, 1989; Wells and Weber, 1990; Bushnell et al., 1982; Piiper et al., 1977; Gallaugher and Farrell, 1998), and catecholamine activation of red cell Na^+–H^+ exchangers (Nikinmaa, 1982, 1983). The latter return intracellular pH to near pre-exercise levels and promote red cell swelling (Nikinmaa, 1992). Red cell swelling, in turn, decreases red cell nucleoside triphosphate concentration ([NTP]), a hemoglobin allosteric modifier (Nikinmaa, 1992). The overall result is the rapid return of hemoglobin–oxygen affinity to near pre-exercise levels in teleosts in spite of significant metabolic acidosis (Nikinmaa, 1990, 1992; Randall and Perry, 1992; Tufts and Randall, 1989).

It should be noted, however, that changes in extracellular pH alone can cause changes in red blood cell volume. Decreases in extracellular pH are rapidly transferred to the intracellular compartment via the Jacobs–Stewart cycle (Obaid et al., 1979; Gilmour, 1998; Nikinmaa, 1992). This, in turn, decreases the net negative change on the hemoglobin and organic phosphate molecules, net movement of osmotically active anions and water into the cell, and red cell swelling. The change in intracellular and extracellular pH accompanying anaerobic exercise is, in turn, a function of the buffer capacities of red blood cells and plasma. In teleosts, plasma proteins account for generally only 20–40% of the whole blood buffering capacity, and the red blood cells (more specifically hemoglobin and organic phosphate molecules) for the remainder (Tufts and Perry, 1998). In elasmobranchs, in contrast, the situation is reversed and plasma proteins can be responsible for over 70% of the whole blood buffering capacity (Tufts and Perry, 1998). The high buffering capacity of plasma proteins may thus explain why red cell swelling is not routinely observed following exhaustive exercise in elasmobranchs (e.g., Lowe et al., 1995).

The sandbar shark (*Carcharhinus plumbeus*) is a cosmopolitan species found in coast waters over the continental shelves and adjacent deep waters, as well as in bays, river mouths and harbors from temperate to tropical areas (Compagno, 1984; Compagno and Niem, 1998). On the east coast of the United States, adult females use shallow inshore areas to pup (e.g., Chesapeake and Delaware Bays, and southeastern seaboard estuaries behind barrier islands). Juveniles use these same areas as nursery grounds where they benefit from high food availability and a reduced risk of predation by larger sharks (e.g., dusky sharks, *Carcharhinus obscurus*) (Medved and Marshall, 1981; Carlson, 1999; Wetherbee et al., 2001; Grubbs et al., 2007; Grubbs and Musick, 2007). The accessibility of sandbar sharks on the eastern shore of Virginia also provides the opportunity to conduct physiological studies of an active, obligate ram-ventilating Carcharhinid shark.

Western Atlantic sandbar shark populations have shown large decreases in recent decades due to heavy commercial fishing pressure (Casey et al., 1985; Casey and Natanson, 1992; Sminkey and Musick, 1995), and slow rates of stock recovery are predicted because of this species' slow growth rate and late maturity (Brewster-Geisz and Miller, 2000). Juvenile survivorship is considered the most significant stage of the life history with respect to population recovery (Sminkey and Musick, 1996; Brewster-Geisz and Miller, 2000). During their inshore residence periods juvenile sandbar sharks are, however, released bycatch in both

commercial and recreational fisheries, as well as a target species for recreational catch-and-release fisheries. Our immediate objective for undertaking this study is to quantify changes in blood-oxygen binding characteristics occurring in juvenile sandbar sharks following strenuous activity accompanying hook and line capture. Our long term objectives are to understand the ability of juvenile sandbar shark's cardio-respiratory system to function under these conditions, the consequences of capture-and-release fishing on juvenile sandbar sharks (including any increases in mortality), and ultimately the integration of information on the physiological abilities and tolerances of this shark species into fisheries management and conservation decisions (Young et al., 2006).

2. Materials and methods

2.1. Animal collection, maintenance, and handling procedures

Experiments involving live sharks were conducted during the summer months (June through September) at the Virginia Institute of Marine Science Eastern Shore Laboratory in Wachapreague, VA (USA). Juvenile sandbar sharks (≈1 to 5 kg, neonates to approximately 5 years old; Casey et al., 1985) were captured using standard recreational hook-and-line fishing gear in the surrounding tidal lagoon system. Sharks were taken from the water once they were alongside the fishing vessel; the hook was removed, and the sharks immediately placed in a 2.4 m diameter holding tank containing vigorously aerated seawater. In order to minimize the chances of injury during capture, no attempts were made to prolong nor to standardize fight times (which were generally less than 10 min).

To ensure complete recovery from the initial capture event, control sharks were transported to the laboratory and held in a shore-side circular fiberglass tank (7 m diameter, 1.8 m deep) for 4–7 days before being sampled. The tank was supplied with water pumped from the adjacent tidal lagoon, as a result water temperature ranged from 22–29 °C and salinity from 30–33‰ over the course of the study. Although 28 animals were used in the control group, the study was conducted over a three year period (2003–2005) and generally less than half that number were held in the tank at anytime to minimize stress due to crowding. The fish were offered food every other day, but food was withheld for at least 24 h prior to blood sampling.

In contrast, exercise-stressed sharks were left to swim in the holding tank aboard the fishing vessel for 60 min before blood samples were withdrawn. (In order to prevent additional stress due to crowding, no more than two individuals were maintained in the tank at the same time.) Because lactate and the concomitantly produced proton load generated by white muscle during anaerobic exercise are not immediately released into the blood stream, this procedure helped ensure that blood pH and bicarbonate levels were at or near their minimum, and blood lactate concentration was at or near its maximum (Cliff and Thurman, 1984; Richards et al., 2003).

Blood samples (≈10 –20 ml) were obtained by caudal peduncle puncture while individuals were gently restrained ventral side up on a V-board with wet cloth towels. This technique poses minimal risk to the animal and, more importantly, it does not perturb blood chemistry as does the anesthesia and manipulations required to place indwelling catheters (Cooper and Morris, 1998). Blood was drawn into heparinized syringes and placed on ice until use in an experiment. A 0.5 ml sample was also taken with an unheparinized syringe for measurement of intracellular [NTP]. Control fish were returned to the large holding tank for several days to several weeks and most were eventually released. Exercise-stressed fish were released following blood sampling, usually near the site of capture.

2.2. Whole blood-oxygen equilibrium curves

Carbon dioxide and temperature conditions established in the tonometers were chosen to mimic those experienced by blood *in vivo*. The equilibration temperature (25 °C) is approximately in the mid-range of water temperatures experienced by juvenile sandbar sharks during the spring and summer months in the U.S. east coast estuaries (Grubbs et al., 2007; Grubbs and Musick, 2007), and the two elevated carbon dioxide levels employed (0.20 kPa and 0.37 kPa) cover the range of arterial and venous blood $P\text{CO}_2$ (≈0.3–0.4 kPa) reported in elasmobranchs (Tufts and Perry, 1998). A third carbon dioxide level ($P\text{CO}_2$=0.03 kPa, that found in air) was used in an attempt to achieve some equivalency of blood pH values between the two groups. Blood from exercise-stressed sharks was not exposed to a $P\text{CO}_2$ of 0.37 kPa as preliminary experiments showed that this procedure resulted in blood pH values (≈7.3) that we considered to be outside the physiological range. Blood pH below 7 has, however, been reported in juvenile dusky sharks following capture and transport (Cliff and Thurman, 1984).

To construct oxygen equilibrium curves, blood samples (≈3 ml) were placed in temperature equilibrated, glass tonometers continually flushed with water-vapor-saturated gas mixtures of six different nominal $P\text{O}_2$ levels: 0.66, 1.33, 2.67, 5.33, 10.7, 20.0 kPa (5, 10, 20, 40, 80, and 150 mm Hg). The gas mixtures (air, nitrogen, and carbon dioxide combined in the desired proportions) were

produced immediately prior to each experiment using electronic gas-flow controllers (MKS Instruments, Andover, MA, U.S.A.), and stored in automobile tire inner tubes. PCO_2 was continuously monitored during mixing with a Hewlett-Packard 47210A Capnometer (Hewlett-Packard Germany, D-7030, Böblingen, Germany). To ensure complete equilibration, the tonometers were flushed with the gas mixtures for at least 1 h prior to the addition of blood, and the blood was left in the tonometers (while the tonometers were continuously swirled) for an additional hour before samples were removed for analysis.

Our measurement techniques followed those described in Brill and Bushnell (1991) and Lowe et al. (2000). In brief, we determined both blood-oxygen content ($[O_2]$) and oxygen partial pressure (PO_2) in ≈0.3 ml blood samples removed from each tonometer using glass syringes, the former following procedures described by Tucker (1967) and the latter using a Radiometer PO_2 electrode installed in MKS Mark 2 blood gas analyzer (Radiometer America, Westlake, OH, U.S.A.). Blood pH was assessed using the associated capillary pH electrode (Radiometer analyzer). Blood-oxygen equilibrium curves were constructed by fitting the PO_2 and $[O_2]$ data (expressed as % saturation) to a logistics function using a least squares regression procedure (Sigmaplot, SPSS Inc., Chicago, IL, U.S.A.). During preliminary experiments we found that sandbar shark blood does not possess a Root effect, as is the case for most elasmobranchs (Pennelly et al., 1977; Brittain, 1991; Pelster and Randall, 1998). For each individual, we therefore assigned the $[O_2]$ of the blood equilibrated to the highest PO_2 as 100% saturation and calculated all the intermediate % saturation values relative to this $[O_2]$. We calculated the pH of whole blood samples (pH_e) at P_{50} ($pH_{e\ 50}$) by linear regression of % saturation and pH_e data, and then interpolating. Hill numbers were calculated by linear regression of log PO_2 and log [% saturation · $(100 - \%\ \text{saturation})^{-1}$]. Because Hill plots are nonlinear when data from 0% to 100% saturation are employed (Ikeda-Saito et al., 1983), we included only the mid-range values (from ≈20% to 80% saturation). The PO_2 at which the blood would be 50% saturated (P_{50}) was calculated using the regression parameters from the Hill plots.

2.3. Plasma and red cell parameters

Whole blood hemoglobin concentrations ([Hb]) were measured using the cyanmethemoglobin method (Dacie and Lewis, 1984). To remove the coagulated plasma proteins and cellular debris that would otherwise interfere with the photometric assay, samples were centrifuged at 14,000 g for approximately 1–2 min after the blood was added to the potassium cyanide–potassium ferricyanide solution (Drabkin's reagent, Sigma Chemical, St. Louis, MO, U.S.A.). Hematocrit was determined following centrifugation of blood samples (≈6000 g for ≈5 min) in microhematocrit tubes. Red cell counts were obtained using a Neubauer hemocytometer using blood samples diluted 100:1 with shark plasma. Five microscope fields were counted for each sample and averaged. Mean cell volumes were calculated as hematocrit · red cell count^{-1}, and mean cell hemoglobin concentrations as measured hemoglobin concentration · hematocrit^{-1}. Plasma bicarbonate concentrations ($[HCO_3^-]$) were determined using the procedure described in Cameron (1971), although no corrections were made for the small quantity of CO_2 in solution. Plasma lactate and urea levels were measured photometrically using commercial assay kits (Trinity Biotech, St. Louis, MO, U.S.A.; and QuantiChrom Urea Assay Kit, BioAssay Systems, Hayward, CA, U.S.A., respectively). Intracellular pH (pH_i) was measured using the freeze–thaw method to produce a hemolysate from packed red blood cells (Zeidler and Kim, 1977; Nikinmaa, 1983). We estimated pH_i at P_{50} ($pH_{i\ 50}$) by interpolation using a linear regression of % saturation and pH_i.

Red cell nucleoside triphosphate concentrations ([NTP]) were measured by following the production of NADH from NAD spectrophotometrically (at 340 nm) using a hexokinase-catalyzed phosphorylation of glucose by ATP, coupled by the glucose-6-phosphate dehydrogenase reaction (Kornberg, 1950; Bergmeyer, 1983). For this assay, blood samples (500 µl, not heparinized) were mixed with an equal volume of 8% perchloric acid and immediately centrifuged (14,400 g for ≈5 min). A 500 µl sample of the supernatant was mixed with 50 µl of saturated Trizma base (Sigma Chemical Company, St. Louis, MO, U.S.A.) to neutralize the perchloric acid, plus 2 M potassium chloride. The pH adjusted to 6.5–7.5 with 1 M potassium hydroxide and the solution placed on ice for 30 min. The mixture was centrifuged as above. The supernatant was collected and stored in liquid nitrogen until assayed.

Blood ion levels were measured using iStat System clinical blood analyzers (Abbott Laboratories, East Windsor, NJ, U.S.A.). Plasma protein levels were measured with a calibrated clinical refractometer, a method that yields results equivalent to colorimetric assays (Hunn and Greer, 1990).

2.4. Effects of catecholamines

The sensitivities of sandbar shark red blood cells to catecholamines were assessed following procedures described by Lowe et al. (1998). In brief, after being

exposed to bright room light for 2 h to degrade any endogenous catecholamines released during blood sampling, 2 ml blood samples were equilibrated in light-tight tonometers maintained at 25 °C and supplied with water-vapor saturated air containing 0.20 kPa PCO_2. Noradrenaline (norepinephrine bitartrate; Sigma Chemical Co., St Louis, MO, U.S.A.) and adrenaline (epinephrine bitartrate salt, Sigma Chemical Co.) solutions (100 µl each) were added separately to the tonometers to produce a final nominal concentration of 10^{-5} M. The solutions were prepared fresh daily in saline (0.9% sodium chloride solution) and stored at 4 °C in the dark. The time course of the response was determined by measuring the pH_e prior to and approximately 5, 10, 20 and 30 min after the addition of either catecholamine solution or an equal volume of saline.

2.5. Preparation of isolated hemoglobin and measurement of oxygen affinity

Red blood cells were separated by centrifugation, immediately frozen in liquid nitrogen, and stored at −80 °C until use. Thawed cells were mixed with three volumes of 0.1 M Tris buffer (pH 7.5, a mixture of Trizma HCl and Trizma Base, Sigma Chemical Co., St. Louis, MO, USA), vortexed to ensure complete hemolysis, centrifuged (9000 *g* for 15 min) to remove cellular debris, and the supernatant stored at 4 °C until use. HiPrep™ 16/60 Sephacryl™ S-200 high resolution ion elusion columns were used to remove organic phosphates. The columns were equilibrated overnight with 0.22 µm filtered 0.1 M sodium chloride solution (pH 7.5), the hemoglobin solution put through the column (flow rate 0.5 ml min^{-1}), and fractions collected in 3 ml aliquots. Purity of the isolated hemoglobin was confirmed by SDS-PAGE. Preliminary experiments demonstrated that the oxygen affinity of purified (P_{50}=0.77±0.01 kPa, 5.8±0.1 mm Hg; mean±SEM; n=4) and un-purified (P_{50}=0.75±0.04 kPa, 5.6±0.3 mm Hg; mean±SEM; n=7) hemoglobin solutions were not different (t=−0.51, P=0.62), so experiments were performed using unpurified samples.

The mixing method (Edwards and Martin, 1966; Wells and Weber, 1989) was used to determine the effect of two ligands on shark hemoglobin–oxygen affinity: ATP (2.5 mmol and 5.0 mmol) and urea (133 mmol). Hemoglobin solutions (6 ml) were equilibrated to humidified pure nitrogen or oxygen at 25 °C for 1 h. PO_2 was measured at the equilibration temperature with a Cameron Instruments (Port Arkansas, TX, U.S.A.) water-jacketed E101 oxygen electrode and a M2000 oxygen meter. P_{50} was calculated by linear regression using data from the 30%–80% oxygen saturation range.

2.6. Statistical treatments

All results in the text, tables, and graphs are presented as mean±standard error of the mean (SEM). A *t*-test was used to determine statistically significance differences in normally distributed data; non-normally distributed data were compared using the Mann–Whitney rank sum test. One-way analysis of variance (Holm–Sidak method) was used for comparison of slopes and intercepts of regression lines. All statistical analyses were performed using either the

Table 1
Blood parameters (mean±SEM) in control and exercise-stressed sandbar sharks sampled by caudal puncture

	Control sharks	Exercise-stressed	% change
Hematocrit (%)	17.7±0.4 (n=28)	21.4±0.5 (n=24)[a]	20.9
Hemoglobin (g l^{-1})	40.1±1.1 (n=25)	44.0±1.0 (n=24)[a]	9.7
Mean cell hemoglobin concentration (g l^{-1})	228±2 (n=25)	206±3 (n=24)[a]	−9.6
Red cell count 10^3 (cells µl^{-1})	402±18 (n=10)	444±24 (n=10)	
Red cell volume (fl)	406±15 (n=10)	521±14 (n=10)[a]	28.3
Red cell [NTP] (mM l RBC^{-1})	568±22 (n=16)	484±22 (n=26)[a]	−14.8
Hill number[b]	2.4±0.4 (n=20)	2.4±0.4 (n=22)	
Plasma [HCO_3^-] (mM l^{-1})[c]	4.0±0.3 (n=12)	3.3±0.2 (n=9)	
Plasma lactate (mg l^{-1})	27±5 (n=10)	349±57 (n=10)[a, d]	1192
Plasma urea (g l^{-1})	24.2±0.4 (n=10)	24.8±0.6 (n=14)	
Plasma protein (g l^{-1})	57.9±1.0 (n=11)	67.0±2.2 (n=11)[a, d]	15.7
Plasma sodium (mM l^{-1})	257±6 (n=11)	278±4 (n=16)[a, d]	8.2
Plasma potassium (mM l^{-1})	5.3±0.2 (n=11)	4.6±0.2 (n=16)[a]	13.2
Plasma chloride (mM l^{-1})	210±2 (n=11)	216±2 (n=16)	
Glucose (mM l^{-1})	550±24 (n=11)	553±20 (n=16)	
Ionized calcium (mM l^{-1})	1.8±0.2 (n=11)	2.2±0.1 (n=13)[a, d]	22.2

Fractional changes are shown only where differences are significant.
[a] Significant differences between control and exercise-stressed sharks.
[b] We found no significant difference in Hill numbers in control shark blood exposed to 0.20 kPa or 0.37 kPa PCO_2, therefore data were combined.
[c] Data have not been corrected for the nominal amount of physically dissolved CO_2.
[d] Data not normally distributed.

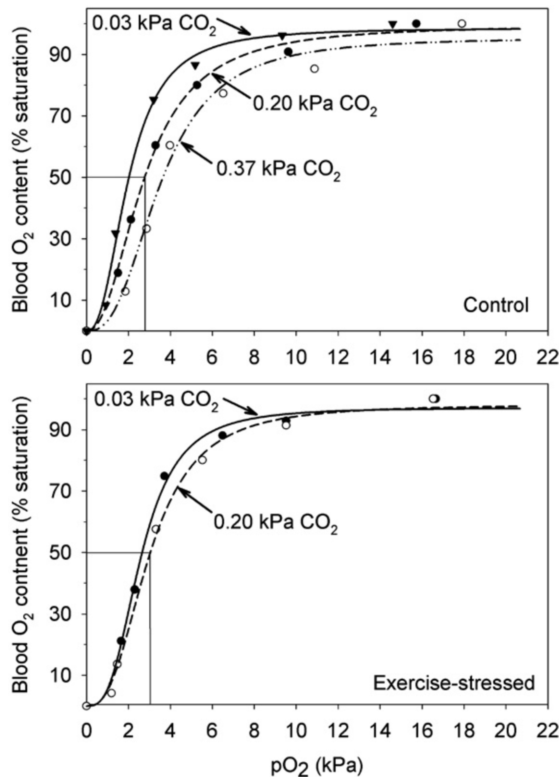

Fig. 1. Exemplary oxygen equilibrium curves, based on blood samples taken from four different individual sharks, showing the influence of PCO_2 and exercise on blood-oxygen affinity. Increasing levels of CO_2 cause significant reductions in blood-oxygen affinity (i.e., rightward-shift in the curves), whereas the influence of strenuous exercise (and the concomitant reduction in pH_e) is more modest. Note that the P_{50} of blood from normal and exercise-stressed sharks are nearly identical, in spite of significant metabolic acidosis in the latter.

Sigma Stat (Ver. 3.0.1, Systat Software, Inc., San Jose, CA, U.S.A.) or Statgraphics Plus (ver. 5.1, CorpStatPoint, Inc., Herndon, VA, U.S.A.) software packages. P values < 0.05 were taken to indicate significant differences.

3. Results

Exercise-stressed sharks have significantly higher hematocrit, hemoglobin and plasma protein concentrations than control sharks (Table 1). Plasma urea levels are, however, not different in the two groups. Compared to control sharks, exercised-stressed animals also have blood lactate levels that are over an order of magnitude higher (Table 1). Plasma sodium and ionized calcium levels are elevated in exercise-stressed sharks; whereas plasma potassium levels are lower (Table 1). Red cell volume is significantly higher and mean cell hemoglobin

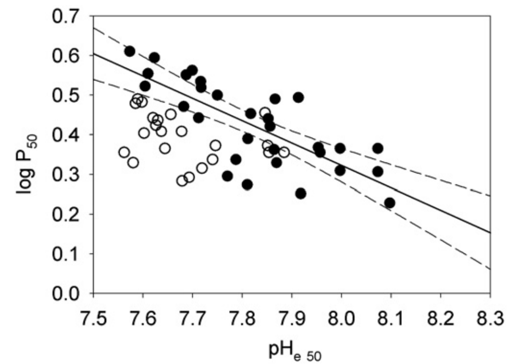

Fig. 2. Relationship of $pH_{e\ 50}$ and oxygen affinity (expressed as P_{50}, in kPa) of blood sampled from control (solid circles) and exercise-stressed (open circles) sandbar sharks. The solid line is the regression based on the data from control sharks, the dashed lines are the 95% confidence intervals.

concentration significantly lower in exercise-stressed sharks (Table 1).

Exemplary blood-oxygen equilibrium curves (based on blood samples taken from four different individuals) demonstrating the influence of increasing PCO_2 and the metabolic acidosis resulting from strenuous exercise are shown in Fig. 1. As expected, increasing PCO_2 causes a significant rightward-shift in the curves (i.e., reduction in oxygen affinity) in blood samples from control sharks. The influence of strenuous exercise and the concomitant reduction in pH_e is, in contrast, far more modest. At the same PCO_2, the P_{50} of blood from the normal and exercise-stressed sharks are nearly identical, in spite of significant metabolic acidosis in the latter.

The diminished influence of metabolic acidosis on the oxygen affinity of blood from exercise-stressed sharks can be clearly seen in the P_{50} and $pH_{e\ 50}$ data presented Fig. 2. In all instances but one, the P_{50} values of blood from exercise-stressed sharks are below and outside the 95% confidence interval of the regression line relating P_{50} and

Table 2
Effect of pH_e on the oxygen affinity of blood sampled from control and exercise-stressed sharks, expressed as the Bohr coefficient ($\Delta \log P_{50} \cdot \Delta pH_{50}^{-1}$), where P_{50} is expressed in kPa

	Control sharks (n=30)	Exercised-stressed (n=23)
Bohr coefficient ($\Delta \log P_{50} \cdot \Delta pH_e^{-1}$)	−0.56±0.09	−0.37±0.12
Intercept	4.8±0.7	3.2±0.9 [a]
r^2 (adjusted for degrees of freedom)	0.57	0.28

[a] Significant differences between control and exercise-stressed sharks.

Table 3
The pH of sandbar shark blood at P_{50} ($pH_{e\ 50}$)

PCO_2 (kPa)	Control sharks	Exercised-stressed
0.03	7.96±0.03 (n=12)	7.74±0.04 (n=9) [a]
0.20	7.92±0.09 (n=9)	7.64±0.01 (n=13) [a]
0.37	7.65±0.02 (n=6)	–

Values are mean±SEM.
[a] Significant differences between control and exercise-stressed sharks.

$pH_{e\ 50}$ in blood from control sharks. The oxygen affinity of blood from exercise-stressed sharks is, therefore, clearly higher than that of blood from control sharks at comparable $pH_{e\ 50}$. Conversely, exercise-stressed sharks have blood with P_{50} values generally comparable to those of blood from control sharks even though $pH_{e\ 50}$ values are lower. The Bohr coefficients (i.e., $\Delta \log P_{50} \cdot \Delta pH_e^{-1}$), calculated by linear regression from the data for exercise-stressed and control sharks (Fig. 2), are given in Table 2. The intercepts are significantly lower ($P<0.01$) in exercise-stressed fish (Table 2), although the slopes (i.e., Bohr coefficients) are not significantly different.

The data presented in Table 3 also show that the blood from exercise-stressed sharks is significantly acidotic (i.e., has lower $pH_{e\ 50}$ at the comparable PCO_2 level) relative to that of blood from control sharks. As a result the range of values for $pH_{e\ 50}$ and $pH_{i\ 50}$ in control and exercise-stressed sharks generally overlap only in those instances where the blood from exercise-stressed sharks was exposed to gas mixtures with PCO_2=0.03 kPa (Fig. 3A). The intercepts of the regression lines (Fig. 3) relating $pH_{e\ 50}$ and $pH_{i\ 50}$ are significantly different ($P<0.01$), even though the slopes are not. Therefore, at comparable $pH_{e\ 50}$ values, the $pH_{i\ 50}$ of red blood cells from exercise-stressed sharks are higher than those of control sharks. More important, as shown in Fig. 3B, the mean $pH_{e\ 50}-pH_{i\ 50}$ difference in blood from exercise-stressed sharks is significantly smaller (by approximately 0.15 pH unit) than the mean $pH_{e\ 50}-pH_{i\ 50}$ difference in blood from control sharks ($P<0.001$).

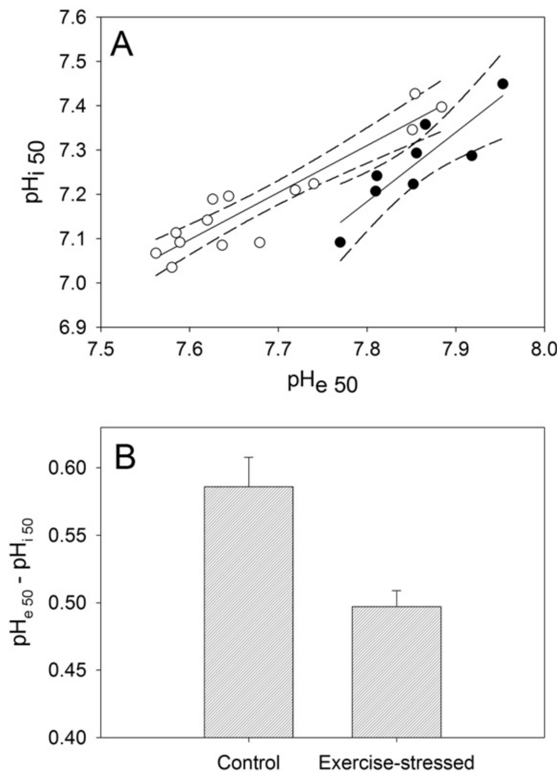

Fig. 3. (A) The relationship between $pH_{e\ 50}$ and $pH_{i\ 50}$ in samples from control (solid circles) and exercise-stressed (open circles) sandbar sharks. The regression equation for blood from control sharks is: $pH_{i\ 50}=1.56\ (\pm 0.35)\cdot pH_{e\ 50}-5.02\ (\pm 2.75)$ and the regression equation for blood from exercise-stressed sharks is: $pH_{i\ 50}=1.05\ (\pm 0.12)\cdot pH_{e\ 50}-0.97\ (\pm 0.89)$. The dashed lines indicate the 95% confidence intervals for the regression lines. (B) The mean (±SEM) $pH_{e\ 50}-pH_{i\ 50}$ differences in red blood cells from control and exercise-stressed sharks.

3.1. Effects of catecholamines

In two trials, the red blood cells of sandbar sharks did not respond to either catecholamine. There are no measurable changes in pH_e or hematocrit that would be indicative of either proton extrusion from the red blood cells or cell swelling.

3.2. Isolated hemoglobin solutions

The influences of ATP and urea on the oxygen affinity of sandbar shark hemoglobin are summarized in Table 4. The P_{50} of sandbar shark hemoglobin is unaffected by the addition of urea alone, but increases dramatically (i.e., hemoglobin–oxygen affinity decreases) in the presence of 5 mM ATP. The reduction in oxygen affinity with the addition of ATP is unaffected by urea.

Table 4
Influence of urea and ATP on the oxygen affinity of hemoglobin prepared from sandbar shark blood in 0.1 M Tris buffer (pH≈7.5) at 25 °C

Treatment	P_{50} kPa (mm Hg)
Control (no added urea or ATP)	0.75±0.04 (5.6±0.3), n=7
133 mM urea	0.76±0.03 (5.7±0.2), n=3
5.0 mM ATP	3.53±0.29 (26.5±2.2), n=7
5.0 mM ATP+133 mM urea	3.49±0.17 (26.2±1.3), n=3

Values are mean±SEM.

4. Discussion

The hematological values measured in control sandbar sharks (Table 1) are within the range of values reported previously for this species (Emery, 1986; Arnold, 2005) and other active elasmobranchs (Bushnell et al., 1982; Wilhelm Filho et al., 1992a; Butler and Metcalfe, 1988; Cooper and Morris, 1998; Gallaugher and Farrell, 1998; Tufts and Perry, 1998). Hematocrit and hemoglobin concentrations are, however, below those reported for high-energy-demand thermoconserving sharks (Emery, 1986; Graham et al., 1990). This is as expected because blood parameters relevant to oxygen transport generally reflect energy demand in fishes (Emery, 1986; Wilhelm Filho et al., 1992a; Lay and Baldwin, 1999). Both the active and standard metabolic rates of juvenile sandbar sharks are similar to those of other active shark species, but well below those of thermoconserving sharks (Dowd et al., 2006). The oxygen affinity of sandbar shark blood (P_{50}=2.7 kPa) under circumstances approximating those *in vivo* (25 °C, pH=7.9, and PCO_2=0.20 kPa) is somewhat higher than the 1.6 kPa of lemon shark (*Negaprion brevirostris*) (Bushnell et al., 1982) and 2.0 kPa of Port Jackson shark (*Heterodontus portusjacksoni*) (Cooper and Morris, 2004) blood measured under similar conditions. The Bohr coefficients (Table 3) for blood from sandbar sharks are, however, at the upper range of values reported for other elasmobranchs (−0.49 to −0.25 $\Delta\log P_{50}\cdot\Delta pH_e^{-1}$) (Butler and Metcalfe, 1988; Baldwin and Wells, 1990).

The fractional increase in plasma lactate levels (≈12×, Table 1) recorded in exercise-stressed sandbar sharks is similar to that in dusky sharks following capture and 70 min confinement (≈15× increase) (Cliff and Thurman, 1984). Pacific spiny dogfish (*Squalus acanthias*) chased to exhaustion showed a ≈8× in plasma lactate above resting levels 4 h post-exercise (Richards et al., 2003), and giant shoveled nose ray (*Rhinobatos typus*) a ≈10× increase (Lowe et al., 1995). Peak plasma lactate levels in the latter (1.3 mmol l^{-1}) are, however, well below those of sandbar sharks (Table 1). Because the sharks used in our study were only exercised during hook-and-line capture with recreational fishing gear, their level of exertion may not have resulted in maximum anaerobic activity and lactate levels may be below those of fish purposely chased to exhaustion. There is a small decrease in K^+ and a substantial increase in Na^+ (Table 1) in exercise-stressed sandbar sharks. Lowe et al. (1995) also recorded an increase in plasma K^+ levels, but found no change in plasma Na^+ or Cl^- concentrations in giant shovelnosed ray following strenuous exercise.

Strenuous exercise in elasmobranchs generally does not result in significant increases in maximum blood-oxygen carrying capacity because there is no adrenergic-mediated splenic contraction (Opdyke and Opdyke, 1971), and usually only minor (10% or less) increases in hematocrit (Bushnell et al., 1982; Piiper et al., 1977). This contrasts with the approximate 50–100% increases in hematocrit accompanying strenuous activity in teleosts (Yamamoto et al., 1980; Wells and Weber, 1990; Gallaugher and Farrell, 1998). There is also no catecholamine activation of red cell Na^+–H^+ exchangers in elasmobranchs (Lowe et al., 1995). As a result, anaerobic exercise in elasmobranchs is not accompanied by the typical teleostean responses: red cell swelling, decreases in red cell organic phosphate concentration, increases in Hb oxygen affinity, and the return of pH_i to pre-exercise levels countering the effects of metabolic acidosis (Tufts and Randall, 1989; Lowe et al., 1995; Nikinmaa and Salama, 1998). Blood-oxygen delivery is, therefore, thought be more compromised following anaerobic exercise in elasmobranchs than in teleosts (Wells et al., 1992; Lowe et al., 1995).

Our results suggest for the first time, however, that the consequences of anaerobic exercise on blood-oxygen transport are not necessarily the same in all elasmobranchs. In sandbar sharks, there is a ≈21% increase in hematocrit which appears to be due in part to a ≈28% increase in mean red cell volume (Table 1). This increase in red cell volume is approximately double that recorded in rainbow trout (*Onchorynchus mykiss*) following adrenaline injection (Nikinmaa, 1982). Although there is an increase in the hemoglobin concentration (≈10%, Table 1) in exercise-stressed sharks, there is no significant difference in red cell count. The latter finding may be due to high standard errors inherent in red blood cell counts in sharks (Arnold, 2005). Nonetheless, our data suggest that either red cell ejection from the spleen, net movement of fluid from the intravascular to the extravascular compartment, or both accompany anaerobic activity in sandbar sharks (Gallaugher and Farrell, 1998).

Probably of the most functional importance, however, is that the oxygen affinity of blood from exercise-stressed sharks is equivalent to that of blood from control sharks in spite of the ≈0.2–0.3 unit reduction in $pH_{e\ 50}$ occurring post capture (Fig. 2). There are several possible explanations. One, there is clear evidence in exercise-stressed sharks that pH_i is alkalinized relative to pH_e (Fig. 3B), as the change in $pH_{i\ 50}$ following anaerobic exercise is lower than predicted based on the change in $pH_{e\ 50}$ (Fig. 3A). Given the large observed Bohr coefficients (Table 3), changes in the pH_i–pH_e relationship

could be the prime contributor to the return of blood-oxygen affinity to that of control sharks (Fig. 2). Two, the decrease in mean cell hemoglobin concentration (Table 1) resulting from red cell swelling may in itself increase hemoglobin–oxygen affinity due to changes in aggregation state of hemoglobin (Nikinmaa, 1992). Three, based on our data from stripped hemoglobin (Table 4), the reduction of intracellular [NTP] (most likely either ATP or GTP) resulting from the observed cell volume increase also probably contributes to the increase in blood-oxygen affinity. This modulation of oxygen affinity by NTP has been observed in the stripped hemoglobin of other shark species (Borgese et sal., 1978; Weber et al., 1983a; Wilhelm Filho et al., 1992b). Furthermore, as has been reported in several ray species (Scholnick and Magnum, 1991), urea does not alter the effect of ATP on the oxygen affinity of stripped hemoglobin prepared from sandbar shark blood (Table 4). An interactive effect of urea and ATP on the oxygen affinity of stripped hemoglobin has, however, been seen in other elasmobranchs (e.g., dogfish, *Squalus acanthias*, and carpet shark, *Cephaloscyllium isabella*; Weber et al., 1983a,b; Tetens and Wells, 1984).

The obvious question that arises is what mechanism(s) and signal(s) are responsible for the apparent alkalization of pH_i and red cell swelling? There are two possibilities which could explain our observations and that can operate even in the absence of an active Na^+–H^+ exchanger. The first is that the potential decrease in pH_i is buffered by hemoglobin and organic phosphates. Indeed, elasmobranch hemoglobins do have higher buffering capacity than teleost hemoglobins (Jensen et al., 1998). Buffering of intracellular H^+, however, necessarily reduces the net negative charge on both hemoglobin and organic phosphate molecules. Anions must then enter or cations must leave the cell to maintain electroneutrality. Because red cell membranes in elasmobranchs (and teleosts) are more permeable to Cl^- than Na^+ or K^+, a decrease in the net negative charge of intracellular polyions results in a net influx of Cl^-, a decrease in the pH gradient across the erythrocyte (which we observed, Fig. 3), and cell swelling (due to the net influx of the osmotically active Cl^- ions) (Fuchs and Albers, 1988; Nikinmaa, 1992; Nikinmaa and Salama, 1998). The second possibility is that the decrease in red cell [NTP] in exercise-stressed sharks (Table 1) is the cause of red cell swelling rather than its result (Nikinmaa, 2006). A reduction in the total pool of intracellular NTP would likewise result in a net influx of Cl^- (and probably HCO_3^-) ions to preserve electroneutrality, a decrease in the pH gradient across the cell, the net influx of water, and red cell swelling.

Although the two mechanisms mentioned above could induce swelling in the absence of a Na^+–H^+ exchanger, there is preliminary histochemical evidence for the presence of this ion exchanger in sandbar shark red blood cells (C. Watson, Central Connecticut State University, unpublished observations). If the mechanism is an increase in activity in the Na^+–H^+ exchanger (discussed in detail in Hoffmann and Simonsen, 1989), then there are several possible signals: β-adrenergic hormones, hypertonic shrinkage, increases in extracellular calcium (iCa^{2+}), and decreases in pH_i (Virkki and Nikinmaa, 1998; Weaver et al., 1999; Nikinmaa, 2006). Of these, the first (catecholamine activation) can be discounted. This effect is well-documented teleost red cells (reviewed by Nikinmaa 1992, 1997; Nikinmaa and Salama, 1998) but, like previous investigators, we found exogenous adrenaline and noradrenaline levels sufficient to cause red cell swelling in teleosts (Lowe et al., 1998) to have no apparent effect on sandbar shark red cells. Of the second two possibilities (hypertonic shrinkage, increases in iCa^{2+}), the latter is more plausible. It is unlikely that the relatively small increase in plasma Na^+ (Table 1) would result in osmotic shrinkage of red cells and the concomitant stimulation of the Na^+–H^+ exchanger. Moreover, osmotically shrunk red blood cells generally only return to their normal volume (Weaver et al., 1999). Increases in iCa^{2+} have been reported to increase activity in the Na^+–H^+ exchange (Hoffmann and Simonsen, 1989) and we did measure an increase in iCa^{2+} in exercise-stressed sharks (Table 1).

At this point, however, it seems to us that decreases in pH_i are most likely the proximate signal. Decreases in pH_e (Table 2) result in initial decreases in pH_i because H^+ are rapidly transferred to the intracellular compartment through the Jacobs–Stewart cycle (Hlakdy and Rink, 1997; Obaid et al., 1979; Nikinmaa, 1992). The decrease in pH_i would then directly stimulate the Na^+–H^+ exchanger (especially if blood-oxygen levels are reduced during recovery from anaerobic exercise) (Virkki et al., 1998). It is also possible that hormones or other chemical stimuli are causing an upward displacement of the pH_i set point of the Na^+–H^+ exchanger resulting in alkalinization and red cell swelling (Demaurex and Grinstein, 1994). The increase in plasma Na^+ *per se* (Table 1) is unlikely to be responsible for increase in Na^+–H^+ exchanger activity because, as far as we can determine, this has never been observed. Future studies employing appropriate blockers (described by Hoffmann and Simonsen, 1989) to define possible cellular mechanism(s) and signal(s) operating in sandbar shark blood are clearly warranted.

In conclusion, our data show that anaerobic exercise has less of an impact on blood-oxygen delivery in juvenile sandbar sharks than other in elasmobranchs. This is because, like teleosts, sandbar sharks have mechanisms which minimize disruption of blood-oxygen transport

accompanying metabolic acidosis. Our results imply that juvenile sandbar sharks captured by hook and line and subsequently released do not suffer increased rates of mortality, although this is yet to be confirmed. Relatively high rates of survival following capture and release have been directly documented in several pelagic shark species (Moyes et al., 2006), and similar studies on juvenile sandbar sharks are needed.

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