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Short communication

# A lack of red blood cell swelling in five elasmobranch fishes following air exposure and exhaustive exercise

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ABSTRACT

In teleost fishes, catecholamine-induced increases in the activity of cation exchangers compensate for decreases in hemoglobin oxygen affinity and maximum blood oxygen carrying capacity caused by decreases in plasma pH (i.e., metabolic acidosis). The resultant red blood cell (RBC) swelling has been documented in sandbar (*Carcharhinus plumbeus*) and epaulette (*Hemiscyllium ocellatum*) sharks following capture by rod-and-reel or after a 1.5 h exposure to anoxia (respectively), although the underlying mechanisms remain unknown. To determine if RBC swelling could be documented in other elasmobranch fishes, we collected blood samples from clearnose skate (*Rostroraja eglanteria*), blacktip reef shark (*Carcharhinus melanopterus*), and sicklefin lemon shark (*Negaprion acutidens*) subjected to exhaustive exercise or air exposure (or both) and measured hematocrit, hemoglobin concentration, RBC count, RBC volume, and mean corpuscular hemoglobin content. We did likewise with sandbar and epaulette sharks to further explore the mechanisms driving swelling when present. We could not document RBC swelling in any species; although hematocrit increased in all species (presumably due to RBC ejection from the spleen or fluid shifts out of the vascular compartment) except epaulette shark. Our results indicate RBC swelling and associated ion shifts in elasmobranch fishes is not inducible by exercise or hypoxia, thus implying this response maybe of lesser importance for maintaining oxygen delivery during acute acidosis than in teleost fishes.

Metabolic acidosis (i.e., reductions in plasma pH, $pH_e$) occurs in both teleost and elasmobranch fishes following exhaustive exercise, air exposure, severe hypoxia, or exposure to elevated carbon dioxide ($CO_2$) levels (Wood et al., 1983; Farrell and Richards, 2009). The concomitant reduction in red blood cell (RBC) intracellular pH ($pH_i$) can reduce blood oxygen ($O_2$) affinity and maximum $O_2$ carrying capacity if fixed-acid Bohr and Root effects are present (Wood and Perry, 1985). These, in turn, reduce the ability of blood to bind $O_2$ at the gills and maximum blood $O_2$ carrying capacity, and thus maximum rates of $O_2$ delivery to the tissues during recovery when high rates of $O_2$ delivery are especially needed (Hladky and Rink, 1977; Nikinmaa, 1983; Waser, 2011). There are at least eight mechanisms participating in control of $pH_i$ and RBC volume regulation that involve either a sodium pump, ion cotransport, or active ion exchange mechanisms. The last includes sodium-proton exchangers (NHEs) which can be stimulated by cell shrinkage, decreases in $pH_i$, and increases in plasma catecholamine levels (Motais et al., 1989; Nikinmaa et al., 1990). Catecholamine activation of RBC NHEs has been documented in teleost fishes through measurable increases in RBC volume (i.e., RBC swelling) and $pH_i$ (e.g., Baroin et al., 1983; Nikinmaa, 1983; Borgese et al., 1987; Fievet et al., 1987; Lowe et al., 1998). The former results from exchange of an osmotically inactive particle ($H^+$) for an osmotically active ion ($Na^+$), but both increase blood-$O_2$ affinity (the latter through decreases in red cell organic phosphate and mean cell hemoglobin (Hb) concentrations; e.g., Nikinmaa, 1983; Nikinmaa, 2011; Nikinmaa et al., 2019).

There is, however, yet no direct evidence of catecholamine activation of NHEs in RBC of elasmobranch fishes; although, multiple investigators have tried to induce this response in vitro (Lowe et al., 1995;

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Berenbrink et al., 2005). The general absence of Bohr and Root effects in elasmobranch fish blood could explain the absence of catecholamine activation of NHEs. The presence and extent of either effect in elasmobranch fish blood is, however, species-specific (reviewed by Morrison et al., 2015). For example, blood from Arctic skate (*Amblyraja hyperborea*) and Eaton's skate (*Bathyraja eatonii*) (Verde et al., 2005) exhibits no Bohr effect, while blood from porbeagle (*Lamna nassus*) and blacktip reef (*Carcharhinus melanopterus*) sharks do, both of which is of a magnitude similar to teleost fishes (Larsen et al., 2003; Bouyoucos et al., 2020). There are, however, several known differences between the oxygen offloading mechanisms between teleost and elasmobranch fishes, primarily the absence of plasma-accessible carbonic anhydrase (CA) in teleost gills (Rummer and Brauner, 2011; Rummer et al., 2013; Randall et al., 2014). Oxygen delivery in elasmobranch fishes may thus rely more on other mechanisms of Hb-oxygen affinity modulation, such as intracellular adenosine triphosphate (ATP) and glutamine triphosphate (GTP) concentrations (Pennelly et al., 1975; Leray, 1979; Weber, 1983; Tetens and Wells, 1984), intracellular chloride concentrations ($[Cl^-]$) (Fievet et al., 1987; Nikinmaa and Salama, 1998), plasma trimethylamine oxide (TMAO) and urea concentrations (Weber, 1983; Weber et al., 1983a; Weber et al., 1983b; Tetens and Wells, 1984), or $O_2$ availability (Bushnell et al., 1982).

Elasmobranch fishes also generally do not display large increases in hematocrit (Hct) following exhaustive exercise (Piiper et al., 1970; Lowe et al., 1995) that would result from RBC swelling, fluid shifts out of the vascular compartment, or release of RBC into the circulation due to splenic contraction, or some combination thereof (Olson et al., 2003; Hedrick et al., 2020). The presence of the latter in elasmobranch fishes is still debated (Nilsson et al., 1975; Brill and Lai, 2016). RBC swelling or alkalization of $pH_i$ (or both) have been documented in blood sampled from juvenile sandbar and mako sharks (*Carcharhinus plumbeus* and *Isurus oxyrinchus,* respectively) following rod-and-reel capture and epaulette shark (*Hemiscyllium ocellatum*) following exposure to anoxic water for 1.5 h (Brill et al., 2008; Chapman and Renshaw, 2009). In contrast, RBCs from little skate (*Leucoraja ocellata*) and shovelnose ray (*Rhinobatos typus*) do not exhibit RBC swelling or $pH_i$ regulation following 24–48 h of exposure to normoxic hypercapnia or 10 min of enforced exercise, respectively (Graham et al., 1990; Lowe et al., 1995). The inconsistent observations of RBC swelling or $pH_i$ regulation in elasmobranch fishes led us to try to document their presence in three phylogenetically disparate elasmobranch species: clearnose skate (*Rostroraja eglanteria*), blacktip reef (*Carcharhinus melanopterus*), sicklefin lemon (*Negaprion acutidens*) shark. Previous reports on RBC swelling in sandbar and epaulette sharks are surprising and were revisited here. We hypothesized that, if this response was common in elasmobranch fishes, all species would show RBC swelling following stressors that induce acidosis.

All work was approved by the William & Mary Institutional Animal Care and Use Committee (IACUC-2019-03-18-13,539-rwbril), the James Cook University Animal Ethics Committee (A2588 and A2394), and the French Polynesian Ministère de la Promotion des Langues, de la Culture, de la Communication, et de l'Environnement (Arrêté 9524). Clearnose skate ($n = 10$; $61 \pm 0.7$ cm total length, mean ± SE) were collected by otter trawl in the Chesapeake Bay and maintained in recirculating indoor holding tanks ($20 \pm 1$ °C) at the Virginia Institute of Marine Science Eastern Shore Laboratory (VIMS-ESL). Sandbar shark ($n = 10$; $83 \pm 6$ cm TL) were caught by rod and reel in the coastal lagoons near Wachapreague, Virginia and maintained in a semi-flow-through outdoor holding tank ($28 \pm 1$ °C) at the VIMS-ESL. Blacktip reef ($n = 10$; $57 \pm 5$ cm TL) and sicklefin lemon ($n = 8$; $67 \pm 9$ cm TL) sharks were caught via gillnet in shallow reef areas of Moorea, French Polynesia and maintained in semi-flow-through outdoor holding tanks ($29 \pm 1$ °C) at the Centre de Recherches Insulaires et Observatoire de l'Environnement. Epaulette sharks ($n = 10$; $60 \pm 1$ cm TL) were collected by hand near Orpheus Island, Australia and maintained in recirculating holding tanks ($26 \pm 1$ °C) at the Marine and Aquaculture Research Facilities Unit at James Cook University. We maintained all subjects in captivity for at least two weeks prior to use in experiments, except for epaulette shark, which had been in captivity for eight months.

Individuals were subjected to three treatments conducted in random order, with at least 8 days between treatments. For the "control" treatment, we removed individuals from their holding tank via dipnet, manually restrained them, and obtained blood samples in less than 1 min. For the "air exposure" treatment, we removed individuals from their holding tank via dipnet, then held them out of the water for 5 min prior to taking a blood sample. For the "exhaustive exercise" treatment, we subjected individuals to 5 min of forced exercise using dipnets and plastic pipe to provide tactile stimulus following Crear et al. (2019). This was followed by 1 min of air exposure. Individuals subjected to the "exhaustive exercise" treatment were returned to their holding tanks for 1 h prior to blood sampling to mimic procedures used with rod-and-reel captured sharks by Brill et al. (2008). In all cases, we collected blood samples into heparinized syringes through direct caudal venipuncture, immediately transferred them to a cooler with ice packs and measured all hematological parameters within 15 min. Elasmobranch blood parameters are neither disturbed by gentle handling and restraint is associated with caudal venipuncture (Cooper and Morris, 1998), nor by blood storage durations (less than 3 h; Schwieterman et al., 2019). Indeed, our $pH_e$ range for all control individuals (Fig. 1) is similar to those from cannulated Port Jackson sharks (*Heterodontus portusjacksoni*; 7.70–7.88; Cooper and Morris, 1998). Following the blood draw, Hct, RBC counts, and blood hemoglobin concentration ([Hb]) were measured using standard procedures (e.g. Brill et al., 2008). Mean corpuscular hemoglobin content (MCHC) was calculated as $[Hb] \times (Hct/100)$ and mean cell volume (MCV) as RBC count $\times Hct^{-1}$. MCHC and MCV are the primary indicators of RBC swelling, but we include Hct, [Hb] and RBC count data to aid in interpretations of our results.

We measured $pH_e$ using the capillary pH electrode of a BMS3 Mk2 blood gas analyzer (Radiometer America, Westlake, OH, U.S.A.) maintained at the animal's holding tank temperature, or a Hanna Instruments 99161pH meter (Woonsocket, RI, USA) maintained at 25 °C (the latter following recommendations by Talwar et al., 2017). To measure intracellular pH ($pH_i$), we centrifuged whole blood and then removed the plasma and upper layer of RBCs. The remaining packed RBCs were frozen in liquid nitrogen and thawed twice before measurement of $pH_i$ using the capillary pH electrode of the BMS3 Mk2 blood gas analyzer maintained at the holding tank temperature (Baker et al., 2009). We were, however, able to measure $pH_i$ only in clearnose skate and sandbar shark blood due to the unavailability of required instrumentation in French Polynesia and Australia.

We conducted statistical analyses using R statistical software (R Team, 2019) and analyzed $pH_e$ data by species using an analysis of variance (ANOVA) procedure with treatment as a predictor. For instances of a significant effect of treatment, pairwise comparisons among the three treatments (control, air exposure, exhaustive exercise) were made using Tukey's *post-hoc* tests with the glht command in the multcomp package (Bretz et al., 2016). For the remaining metrics, we calculated relative change from the control treatment on the same individual and conducted *t*-tests on the resulting relative change data with each mean response tested for significant differences from zero. We also compared these results to the raw data from Brill et al. (2008) to using an analysis of variance (ANOVA) with Tukey's *post-hoc* tests to assess directly differences between our results and published values (statistical values presented in Supplemental Tables 1,2) All statistics were evaluated with a significance level of $\alpha = 0.05$.

Even though there were significant reductions in $pH_e$ in all species following both treatments (Fig. 1), we found no evidence of RBC swelling under either protocol except for sicklefin lemon shark, as indicated by the significant increase in MCV following exhaustive exercise (Fig. 2). This was, however, not accompanied by the expected decrease in MCHC (Fig. 2). In contrast, Brill et al. (2008) showed that similar reductions in $pH_e$ in sandbar shark following rod-and-reel

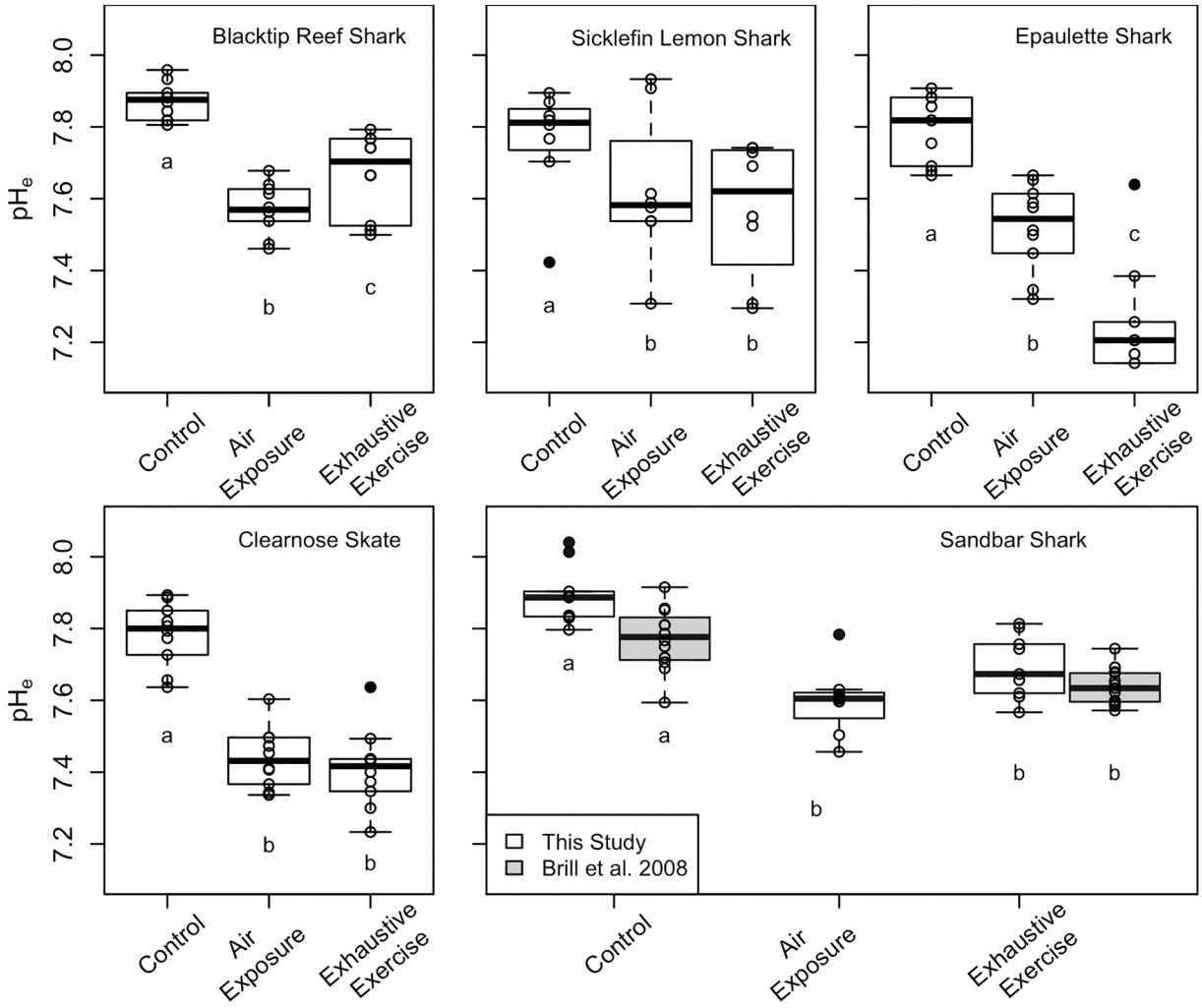


**Fig. 1.** The plasma pH ($pH_e$) in control individuals and those undergoing air exposure and exhaustive exercise protocols. Raw data from Brill et al., 2008 are shown in the shaded box plots for sandbar shark and represent individuals that were held in captivity ("control") and those captured with rod-and-reel. In all species, both treatments resulted in significant reductions in $pH_e$. Significant differences in mean $pH_e$ across treatments are denoted with different lowercase letters. The solid lines within the boxes mark median values, the boundaries of the box the 25th and 75th percentiles, and the whiskers (error bars) above and below the box represent the 90th and 10th percentiles. Data points between the 90th and 10th percentiles are shown as open circles and those outside this interval are shown as filled circles. N = 10 for all species for all treatments excluding sicklefin lemon shark, where N = 8 and the Brill et al., 2008 sandbar shark study, where N = 6.

capture (Fig. 1) are accompanied by decreases in $pH_e$ - $pH_i$ and swelling (the latter indicated by reductions in MCHC increases in MCV, Supplemental Fig. 1). We also measured increases in Hct in all species, except for epaulette shark, following the air exposure and exhaustive exercise protocols (Fig. 2). But surprisingly, increases in Hct were only accompanied by increases in [Hb] in blacktip reef sharks and clearnose skates. This contrast with the results from Brill et al. (2008), where increases in Hct were accompanied by increases in [Hb] (Supplemental Fig. 1). We were also unable to document changes in RBC count, with the exception of blacktip reef shark following air exposure. We have no immediate explanation for the incongruity of these observations. We observed a decrease in the $pH_e$ - $pH_i$ in both clearnose skate and sandbar shark blood, with the latter being comparable to the changes previously documented in sandbar shark blood following rod-and-reel capture by Brill et al. (2008) (Supplemental Fig. 2) and epaulette shark (*Hemiscyllium ocellatum*) following exposure to anoxic water for 1.5 h (Brill et al., 2008; Chapman and Renshaw, 2009). The stressors employed in these two studies obviously crossed a physiological threshold, or induced a suite of responses, that our treatments did not. Nikinmaa (1992) summarizes the stimuli that could induce RBC swelling and concomitant cell volume regulation responses, and our limited observations do not allow rule out any of these mechanism, with the exception of the Jacobs-Stewart cycle (Jacobs and Stewart, 1942), such that observed decreases in MCHC and increases in MCV could be entirely due to reductions in $pH_e$. The reductions in $pH_e$ values we measured were equivalent to those observed by Brill et al. (2008) (Fig. 2), and thus should have also elicited a swelling response if $pH_e$ changes were responsible for RBC swelling.

The reason we did not observe RBC swelling in the two species known to have this response (sandbar and epaulette sharks) under other stressful conditions may have also been related to the circumstances under which fish were held. Chapman and Renshaw (2009) found significant differences in the circulating lactate concentrations of unstressed wild-caught and captive epaulette sharks, implying a general stress response in the latter. But in the same study, grey carpet shark (*Chiloscyllium punctatum*) maintained in captivity for two weeks had smaller hematological responses (e.g., lower fractional changes in Hct) than wild-caught individuals following exposure to anoxia. This smaller treatment effect implies that diminution of hematological responses can occur during captivity. Likewise, Gilmour et al. (1994) observed down regulation of RBC adrenoreceptors in response to chronically increased plasma catecholamines although, as previously noted, there is yet no

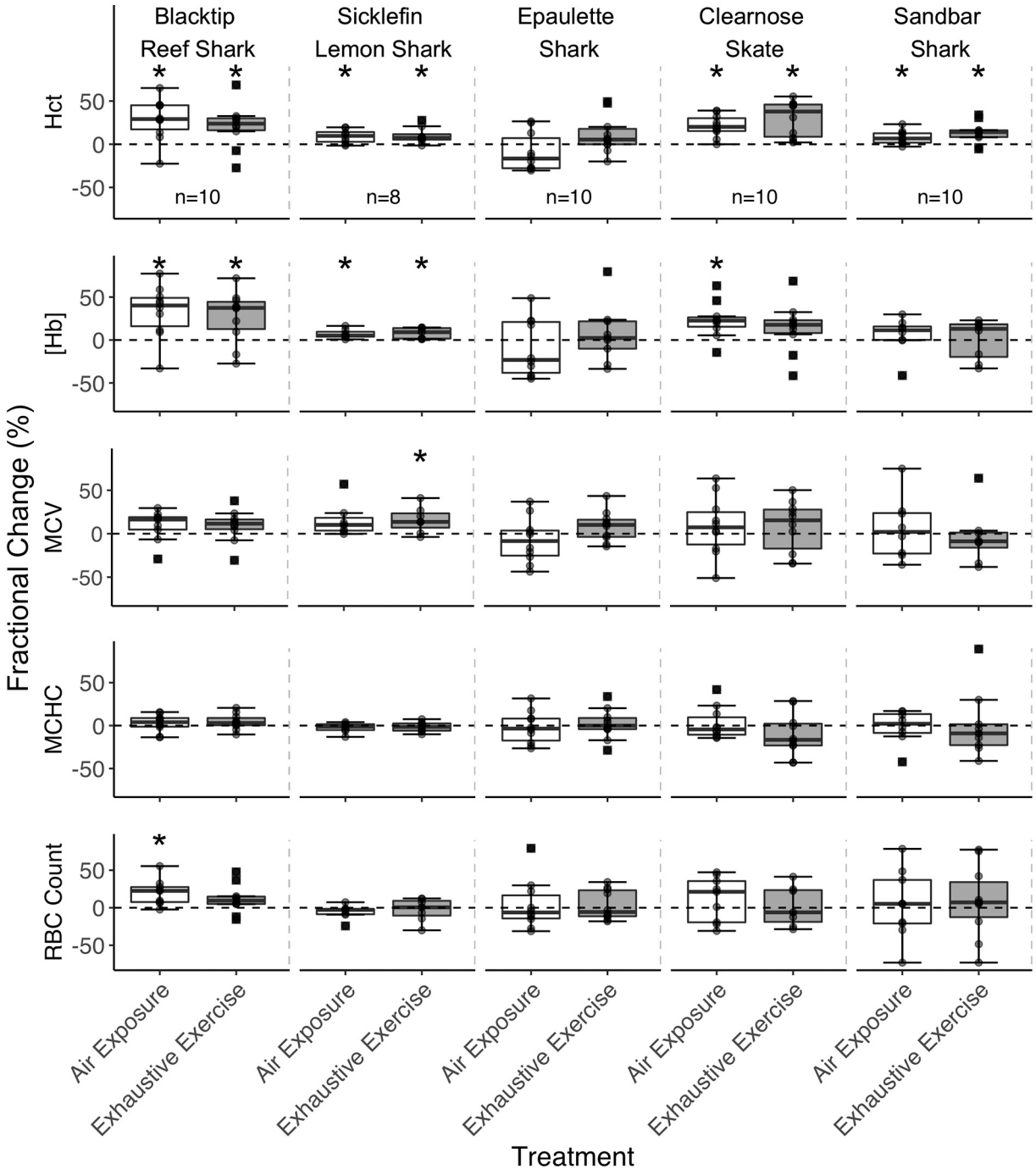


**Fig. 2.** The fractional changes (%) in hematological parameters in blood collected from animals exposed to air and exhaustive exercise treatments relative to control conditions. Significant differences from zero are denoted with asterisks. Box and whisker plots represent raw data, with whiskers representing maximum and minimum points within 1.5 times the interquartile range above the upper quartile and below the lower quartile. Black squares denote points outside of this range. Abbreviations: hematocrit (Hct), hemoglobin concentration ([Hb]), mean cell volume (MCV), mean corpuscular hemoglobin content (MCHC), red blood cell count (RBC count).

direct evidence of catecholamine activation of NHEs in RBC of elasmobranch fishes. The sandbar shark we used were dip-netted and transferred between tanks for use in another study. This may have led to a sustained stress response obscuring the responses to our acute stress treatments. Wise et al. (1998) found that the hypoxia tolerances of epaulette shark change with time in captivity, implying there may be some habituation. In contrast to the sandbar shark we studied, the epaulette shark had been in captivity for eight months, and we assume they would have habituated to captivity. Yet, our treatments could not induce RBC swelling responses in either; although, both had been previously shown to possess RBC swelling (Brill et al., 2008; Chapman and Renshaw, 2009). In summary, the RBC swelling response in elasmobranch fishes appears to be labile and not species-specific, and further research regarding the role of NHEs on elasmobranch $pH_i$ is warranted.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2021.110978.

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