A product of its environment: the epaulette shark (*Hemiscyllium ocellatum*) exhibits physiological tolerance to elevated environmental CO_2

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Ocean acidification, resulting from increasing anthropogenic CO_2 emissions, is predicted to affect the physiological performance of many marine species. Recent studies have shown substantial reductions in aerobic performance in some teleost fish species, but no change or even enhanced performance in others. Notably lacking, however, are studies on the effects of near-future CO_2 conditions on larger meso and apex predators, such as elasmobranchs. The epaulette shark (*Hemiscyllium ocellatum*) lives on shallow coral reef flats and in lagoons, where it may frequently encounter short-term periods of environmental hypoxia and elevated CO_2, especially during nocturnal low tides. Indeed, *H. ocellatum* is remarkably tolerant to short periods (hours) of hypoxia, and possibly hypercapnia, but nothing is known about its response to prolonged exposure. We exposed *H. ocellatum* individuals to control (390 µatm) or one of two near-future CO_2 treatments (600 or 880 µatm) for a minimum of 60 days and then measured key aspects of their respiratory physiology, namely the resting oxygen consumption rate, which is used to estimate resting metabolic rate, and critical oxygen tension, a proxy for hypoxia sensitivity. Neither of these respiratory attributes was affected by the long-term exposure to elevated CO_2. Furthermore, there was no change in citrate synthase activity, a cellular indicator of aerobic energy production. Plasma bicarbonate concentrations were significantly elevated in sharks exposed to 600 and 880 µatm CO_2 treatments, indicating that acidosis was probably prevented by regulatory changes in acid–base relevant ions. Epaulette sharks may therefore possess adaptations that confer tolerance to CO_2 levels projected to occur in the ocean by the end of this century. It remains uncertain whether other elasmobranchs, especially pelagic species that do not experience such diurnal fluctuations in their environment, will be equally tolerant.

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Introduction

Anthropogenic CO_2 emissions have caused an increase in atmospheric CO_2 by almost 40% over the past 250 years (IPCC, 2013). The resulting rise from pre-industrialization levels (~280 ppm) to 400 ppm in 2014 has occurred at a rate unprecedented for the past 800 000–1 000 000 years (Raven et al., 2005; Doney and Schimel, 2007; Lüthi et al., 2008). The oceans have absorbed more than 30% of the additional CO_2 released by human activities, thus tempering the atmospheric rise in CO_2 (Sabine et al., 2004; Sabine and Feely, 2007). However, the resulting rise in seawater CO_2 partial pressure (PCO_2) and the associated reduction in pH, called ocean acidification, is a significant threat to marine organisms and ecosystems (Hoegh-Guldberg et al., 2007; Fabry et al., 2008).

The reduced carbonate saturation state that accompanies lower seawater pH affects the ability of calcifying marine organisms to form carbonate shells and skeletons (Orr et al., 2005; Doney et al., 2009), but rising oceanic CO_2 may also impact the respiratory physiology of many water-breathing organisms. Acid–base disturbances related to elevated environmental CO_2 can reduce oxygen uptake and delivery, which could directly impact metabolic performance. Reductions in an organism's scope for aerobic metabolic performance can result in less energy being available for crucial life-history processes, such as growth and reproduction (Pörtner, 2008; Pörtner and Farrell, 2008). For instance, Humboldt squid (Dosidicus gigas) exhibit a 30% reduction in resting metabolic rate and a 45% decrease in activity upon exposure to projected near-future CO_2 levels, owing to an impaired oxygen transport system, which would be predicted to reduce overall performance and compress their habitable depth range (Rosa and Seibel, 2008). In contrast, teleost fishes are expected to be physiologically well equipped to compensate pH and ion disturbances caused by high CO_2 (Ishimatsu et al., 2008; Brauner and Baker, 2009). Nevertheless, interspecific variation is evident in the physiological responses of teleost fish to elevated CO_2; for example, some fishes exhibit no change in aerobic scope in high-CO_2 environments (Ishimatsu et al., 2008; Melzner et al., 2009a; Couturier et al., 2013), whereas others reduce aerobic scope (Munday et al., 2009) and some even increase aerobic scope (Couturier et al., 2013; Rummer et al., 2013a) when exposed to near-future CO_2 levels. Consequently, the effects of ocean acidification on a broad range of species, including vulnerable and tolerant species, should be investigated in order to identify traits that will be important for individual performance and success in near-future oceans and predict changes in community structure (Melzner et al., 2009b).

In contrast to the growing body of knowledge about the effects of ocean acidification on teleost fishes, little is known about the impacts of rising levels of oceanic CO_2 on elasmobranchs. Elasmobranchs buffer a pH disturbance, such as that associated with exposure to high CO_2, in a manner similar to teleosts. Bicarbonate is accumulated in the blood, but

in addition, elasmobranchs may also increase branchial ammonia excretion rates to ameliorate the acidosis further (Evans, 1982; King and Goldstein, 1983; Claiborne and Evans, 1992; Brauner and Baker, 2009; Tresguerres et al., 2010). The haemoglobin of elasmobranchs also has a much higher buffering capacity compared with that of most teleosts, and thus, O_2 transport and aerobic performance may be less sensitive to pH disturbances (Berenbrink et al., 2005). Yet, it is thought that the resilience of elasmobranchs to acid–base disturbances is related largely to their sophisticated acid excretion processes at the gill (Wood et al., 1995). If elasmobranchs are notably tolerant to near-future CO_2 conditions, this could potentially increase predation pressure and alter species compositions of marine environments.

The epaulette shark (Hemiscyllium ocellatum) exhibits exceptionally high tolerance to the severe hypoxia (low oxygen) that it routinely experiences while inhabiting shallow coral reef flats (Routley et al., 2002; Nilsson and Renshaw, 2004), and thus, it may not be surprising if this species is also tolerant to near-future CO_2. However, acute responses may differ dramatically from the responses to long-term exposure; studies on H. ocellatum in response to anoxia or hypoxia have been following only minutes to hours of exposure (Wise et al., 1998; Renshaw et al., 2002; Routley et al., 2002; Chapman and Renshaw, 2009; Dowd et al., 2010; Speers-Roesch et al., 2012a). No study, to date, has examined how H. ocellatum responds to prolonged exposure to elevated CO_2. Given that increased uptake of CO_2 by the ocean will affect both the average CO_2 levels and the magnitude of extreme CO_2 levels (Shaw et al., 2013), it is important to consider longer-term responses to elevated CO_2 beyond those that would be experienced on a diurnal basis (e.g. hours; Ohde and van Woesik, 1999; Compagno, 2002; Last and Stevens, 2009; Shaw et al., 2013). Thus, both the physiological sensitivity of the organism and the variations it may already be experiencing in its habitat are important when considering which species will exhibit positive or negative responses to rising ocean CO_2 levels. However, it is also important to consider the relationship between environmental cues and other traits, such as behaviour—which is especially relevant to species like H. ocellatum—when considering the importance of phenotypic plasticity, because this could influence selection over the longer term (Marais and Chown, 2008).

We exposed H. ocellatum to near-future CO_2 conditions for a minimum of 60 days and measured resting oxygen consumption rates and critical oxygen tensions as proxies for resting metabolic rate and sensitivity to hypoxia, respectively. In addition to whole-organism responses, we also measured or calculated haematological and tissue parameters, including plasma ionic (HCO_3^-, Cl^-, Na^+ and K^+) and urea concentrations, haemoglobin (Hb), mean cell haemoglobin concentration (MCHC), haematocrit (Hct), spleen–somatic index (SSI) and citrate synthase activity in heart, brain and red muscle. The aim was to provide insight into the physiological parameters that may underpin changes in metabolic performance and sensitivity to hypoxia in this species.

We hypothesized that *H. ocellatum* can physiologically tolerate elevated CO_2 because it routinely experiences daily reductions in environmental O_2 (Routley *et al.*, 2002; Nilsson and Renshaw, 2004) and probably elevations in CO_2. However, if CO_2 tolerance is related to the diurnal patterns this species already experiences in their natural habitat, prolonged exposure (60 days) to elevated CO_2 may negatively affect metabolic rate and hypoxia tolerance.

Materials and methods

Experimental animals

Epaulette sharks (*Hemiscyllium ocelatum*) were collected from the northern Great Barrier Reef by Northern Barrier and Cairns Marine (Cairns, Queensland, Australia) and transported to James Cook University (JCU). Five to six individuals were placed in each of six 700 l tanks in a recirculating seawater system. Individuals were measured [standard length, 33.38 ± 7.29 cm (mean ± SD)] to ensure an equal distribution of sizes among tanks. Unique fin clips along the margins of pectoral, pelvic and dorsal fins were used for individual identification. Shelter was provided in the form of PVC pipe sections placed within each tank. Food was provided once every 24 h and consisted of 4% of shark biomass per tank in raw prawn meat. There was no indication that any individuals or treatment groups were eating less than this amount throughout the duration of the study. Sharks were acclimated to laboratory conditions for at least 4 weeks prior to commencing CO_2 treatments.

Experimental CO_2 conditions

The experimental system comprised three 8000 l recirculating seawater systems, each set to simulate one of the following three CO_2 treatments: control (~390 µatm); medium (~600 µatm); and high (~880 µatm). Carbon dioxide levels were achieved and maintained by CO_2 infusion of seawater in 3000 l sumps attached to each recirculating seawater system. The pH_{NBS} (National Bureau of Standards scale) levels were set to match target CO_2 concentrations and maintained using a CO_2-infusing system (Aqua Medic GmbH, Bissendorf, Germany). If the pH rose above the set point, an electronic solenoid initiated the system to deliver a steady stream of CO_2 into a diffuser within the corresponding sump. Carbon dioxide-equilibrated seawater from each system was delivered to two replicate 700 l tanks (~25 l min^{-1}) per treatment. Each tank contained five or six sharks, as described above. This central approach of pH manipulation allowed for stability in seawater pH and PCO_2 within the holding tanks. Tanks were covered with transparent plastic sheeting to reduce CO_2 loss to the atmosphere.

The pH_{NBS} was measured daily (Hach, model #HQ40d) in each tank to ensure that it remained within ±0.05 of desired levels. Temperatures were also measured daily and maintained at 28.5°C by automated heater/chillers attached to each seawater system. Salinity and alkalinity were measured on a weekly basis. Total alkalinity (TA) was estimated using Gran titrations and certified reference materials (Dr A. G. Dickson, Scripps Institution of Oceanography). Average seawater PCO_2 was calculated using these parameters in CO2SYS (Pierrot *et al.*, 2006) and using constants from Dickson and Millero (1987) (Table 1).

Sharks were introduced to the CO_2 treatments following 30 days acclimation to laboratory holding conditions and were then maintained in their respective CO_2 treatment conditions for a minimum of 60 days prior to physiological experimentation.

Experimental protocol

Resting oxygen consumption rates

Resting O_2 consumption rates ($\dot{M}O_{2Rest}$) were determined for sharks following 60–68 days of exposure to control ($n = 10$), medium ($n = 12$) and high ($n = 11$) CO_2 conditions and a 48 h fasting period using an intermittent-flow respirometry system with purpose-built respirometry chambers. Animals were transferred individually into the cylindrical 11 or 15 l respirometry chambers (depending on animal body size) submerged in a temperature-controlled aquarium (28.5°C) within each animal's respective experimental CO_2 conditions and habituated to the chamber for 12 h before oxygen consumption measurements commenced. Submersible pumps were fitted to each chamber to supply a continuous water flow (1300 l h^{-1}; WEIPRO WH-2000; Yongcheng Aquarium Co., Ltd, Guangdong, China) from the surrounding water bath through the chambers. During respirometry trials, a digital relay timer (MFRT-1 Multi Function Recycling Timer; Xiamen SUPERPRO Technology Co., Ltd, Xiamen, Fujian, China) was used to stop water flow for 15 or 20 min and then resume flushing for 15 min over a total period of 12 h. The intervals of interrupted water flow were short enough to ensure that oxygen within the chambers did not fall below 80% saturation at any time, while flush periods were long enough to eliminate accumulation of metabolic CO_2 and allow water oxygen levels to return to 100% saturation (Steffensen *et al.*, 1984; Steffensen, 1989). A second pump (1300 l h^{-1}; WEIPRO WH-2000) was connected to each respirometry chamber to recirculate water continuously within the chamber, thus ensuring complete mixing and homogeneous water PO_2 (P_WO_2). Contactless spots (2 mm) with oxygen-sensitive REDFLASH dye were adhered to the inside of glass tubes connected to the recirculating pumps on each respirometer. These spots were then linked to a Firesting Optical Oxygen Meter (Pyro Science e. K., Aachen, Germany) via 5 m fibre-optic cables to record continuously (0.5 Hz) the temperature-compensated O_2 concentration (in milligrams per litre) of the water within each chamber over the 12 h period of time. The 0 and 100% oxygen levels of the Firesting oxygen meter were calibrated using 0 and 100% air-saturated seawater. At the end of each trial, the wet mass was taken for each shark [232.47 ± 117.98 g (mean ± SD)] prior to release back to experimental holding conditions.

Critical oxygen tension

Upon completion of $\dot{M}O_{2Rest}$ measurements, sharks were permitted to recover in their respective CO_2 treatment conditions for ~3 weeks. Then, the same respirometers used to determine $\dot{M}O_{2Rest}$ were used to determine the critical oxygen tension (P_{crit}) for the same sharks exposed to control ($n = 9$), medium ($n = 12$) and high CO_2 ($n = 10$). By this point, sharks would have been exposed to their respective experimental conditions for 85–92 days. Prior to measurements, sharks were fasted for 48 h before being introduced to the cylindrical 11–15 l respirometry chambers. Then, the $\dot{M}O_2$ of each animal was monitored for a minimum of 4 h using an intermittent flush cycle (15 min flush–15 min closed) so that stable $\dot{M}O_{2Rest}$ was achieved prior to commencing the hypoxia experiment. The respirometers were then sealed by turning off flush pumps and closing previously installed ball-valves downstream of the flush pumps. Oxygen levels in the chamber were monitored continuously (0.5 Hz) and allowed to decrease to at least 0.8 mg l^{-1} to ensure that the critical oxygen tension for each individual was recorded (based on estimates from Routley et al., 2002). The changes in water pH and PCO_2 that occur when using closed respirometry for a short period of time have been shown previously to have no effect on P_{crit} in fish (Henriksson et al., 2008). After this oxygen concentration was achieved, the aforementioned flush cycle was reinstated such that O_2 levels within each respirometer could quickly return to normoxic conditions (100% air-saturated seawater).

Haematological and tissue analyses

Following P_{crit} measurements, animals were returned to their treatment tanks to recover for ~1 week. After this time, blood was sampled from sharks exposed to control ($n = 8$), medium ($n = 10$) and high ($n = 8$) CO_2 conditions by inserting a 23 gauge needle posterior to the cloaca into the caudal vein and collecting the blood (<1% of body volume) into heparinized syringes. Animals were then euthanized by severing the spinal cord using the method described by Speers-Roesch et al. (2012b) so that tissues could be sampled. Whole blood [Hb] was determined using the HemoCue® (Hb 201 System,

Australia Pty Ltd) with 10 µl of whole blood and was reported as grams per 100 ml using a calibration curve according to Clark et al. (2008) corrected for tropical reef species by Rummer et al. (2013b). The Hct was determined by centrifuging 60 µl of whole blood in heparinized microcapillary tubes for 3 min at 17 000*g* and calculated as the ratio of packed red blood cells to total blood volume (as a percentage). Both [Hb] and Hct were used to calculate the MCHC. The spleen was dissected from each shark and weighed to the nearest 0.001 g. The SSI was calculated as the ratio of the spleen to body mass (as a percentage). Plasma was flash frozen immediately in liquid nitrogen and then stored at −80°C until analysis for [HCO_3^-] via colorometrically linked enzyme assay and for [Na^+], [K^+], [Cl^-] (1:1 dilution with deionized water) and [urea] (1:19 dilution with deionized water) via ion-specific electrodes (ISE; Beckman Coulter System AU480). Heart, brain and red muscle samples were also collected and frozen in liquid N_2 for citrate synthase enzyme analysis according to McClelland et al. (2005). Briefly, frozen tissues were homogenized in a standard buffer solution containing 5 mM EDTA, 0.1% Triton X-100, 0.2 mM dithiothreitol and 50 mM Hepes (adjusted to pH 7.4) and stored at −80°C. The citrate synthase assay buffer contained (mM): 20 Tris (pH 8.0), 0.1 5,5-dithiobis (2-nitrobenzoic acid) and 0.3 acetyl-CoA. The reaction was initiated by the addition of 0.5 mM oxaloacetate, and absorbance was measured for 5 min at 412 nm. Control samples were assayed without oxaloacetate to control for background hydrolase activity.

Calculations and statistical analyses

Raw text files created for the Firesting recordings were imported offline into LabChart version 6.1.3 (ADInstruments, Colorado Springs, CO, USA), which was used to analyse data. A modified version of equations from Bushnell et al. (1994) and Schurmann and Steffensen (1997) was used to calculate $\dot{M}O_{2Rest}$ (in milligrams per kilogram per hour). To do this, the average of the shallowest 10% of slopes [change in O_2 concentration over a period of 15–20 min (in milligrams of O_2 per litre per second) in between flushing cycles] was determined for each individual shark. From this, the appropriate

Table 1: Mean values for PCO_2, pH, total alkalinity, salinity and temperature over the course of the CO_2 exposure period

Treatment	Tank number	PCO_2 (µatm)	pH	Total alkalinity (µmol kg^{-1})	Salinity (ppt)	Temperature (°C)
Control	1	397 ± 6.5	8.16 ± 0.006	2145 ± 4.7	35.6 ± 0.07	28.6 ± 0.05
Control	2	384 ± 6.8	8.18 ± 0.006	2145 ± 4.7	35.6 ± 0.07	28.4 ± 0.04
Medium	1	614 ± 16.6	8.00 ± 0.009	2095 ± 5.1	35.9 ± 0.07	28.7 ± 0.05
Medium	2	608 ± 16.5	8.00 ± 0.009	2095 ± 5.1	35.9 ± 0.07	28.6 ± 0.05
High	1	876 ± 14.6	7.86 ± 0.006	2079 ± 5.3	36.0 ± 0.03	28.7 ± 0.03
High	2	861 ± 14.4	7.87 ± 0.006	2079 ± 5.3	36.0 ± 0.03	28.7 ± 0.04

Total alkalinity was measured weekly for each treatment condition, and temperature was measured daily for each tank within each treatment. Means were calculated for each treatment over the entire experimental period and are given for each holding tank, ±SEM. Abbreviation: PCO_2, seawater carbon dioxide partial pressure.

proportion of background O_2 consumption, which was measured 2–3 h before and after each trial for each respirometer and assumed linear, was subtracted. This value was then multiplied by the volume of the respirometer (in litres; minus the volume of the fish), all of which was divided by the mass of the fish (in kilograms). Respirometers were cleaned daily to ensure that background (microbial) respiration did not exceed 10% of the $\dot{M}O_{2Rest}$ of the sharks. Means and SEM for $\dot{M}O_{2Rest}$ were calculated for each of the three CO_2 treatments.

A similar data extraction and calculation protocol was followed for determining P_{crit}. Again, $\dot{M}O_{2Rest}$ was calculated for each shark from the shallowest 10% of slopes that were recorded prior to sealing the respirometer. Then, the mean slope for every 5 min period of time while the respirometer was sealed was extracted (usually 20–30 slopes), and $\dot{M}O_2$ values were calculated from those slopes. To determine P_{crit}, all $\dot{M}O_2$ values were plotted against the oxygen concentration within the chamber for each shark. A horizontal (regression) line was fitted to the mean $\dot{M}O_{2Rest}$ prior to sealing the respirometer. Then, a linear regression was applied to all of the points that consecutively fell below $\dot{M}O_{2Rest}$ once the respirometer had been sealed. The point at which both regression lines intersected was reported as the critical oxygen tension or P_{crit} (in milligrams of O_2 per litre) for that individual (Fig. 1; Ott *et al.*, 1980; Nilsson *et al.*, 2004; Collins *et al.*, 2013). Means and SEM for P_{crit} were calculated for each CO_2 treatment.

Nested ANOVA, with holding tanks nested within CO_2 treatments, was first used to test whether there was a significant effect of holding tank on mean $\dot{M}O_{2Rest}$ (in milligrams per kilogram per hour) or mean P_{crit} (in milligrams of O_2 per litre). As there was no significant effect of tanks on either parameter, data from the two tanks within treatments were pooled for further analyses. ANCOVA was used to compare $\dot{M}O_{2Rest}$ among the three CO_2 treatments, with standard length as a covariate. To compare P_{crit} among treatment groups, a robust regression analysis was performed with standard length as a covariate. Robust regression analysis was chosen over ANCOVA for P_{crit} analysis due to potential outliers that could otherwise be solely responsible for significant outcomes. The removal of such outliers was rejected owing to the relatively small sample size. Instead, robust regression weighs values differently based on their chance of being an outlier. Hence, the further away a single data point was from the mean, the less influential it became for the statistical outcome of the analysis. There was no interaction between the main effect (CO_2) and the covariate in either analysis; therefore, to increase statistical power, the analyses were run again without this term included. Standard length was not included in haematological and tissue data analyses because it had no significant effect on the outcomes. ANOVAs were then used together with Holm–Sidak *post hoc* tests to compare haematological and tissue parameters between animals acclimated to control, medium and high CO_2 conditions. Statistical significance was accepted when $P < 0.05$. All analyses were carried out using S-Plus (TIBCO Software Inc., Palo Alto, CA, USA).
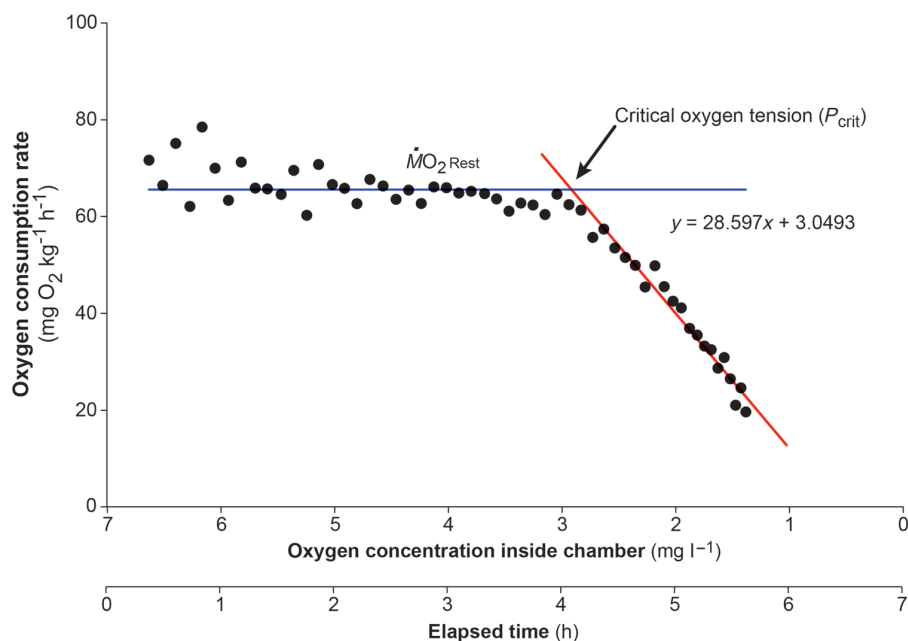
Figure 1: Representative trace illustrating the changes in oxygen consumption rate of an individual epaulette shark (*Hemiscyllium ocellatum*) as the oxygen concentration of the water decreased and the time over which this occurred. The parallel line represents the resting oxygen consumption rate. After the respirometry chamber was sealed, the oxygen consumption rate began to decrease below resting levels. The diagonal line is a trend line, with the intersection of both lines demarcating the critical oxygen tension (P_{crit}).

Results

Resting oxygen consumption rates

There were no significant differences in $\dot{M}O_{2Rest}$ values between CO_2 treatment groups ($F_{2,28} = 0.578$; $P = 0.568$). However, $\dot{M}O_{2Rest}$ depended on the standard length of the individuals, with larger animals having a higher $\dot{M}O_{2Rest}$ than smaller animals ($F_{2,28} = 6.70$; $P = 0.0151$; Fig. 2A). Values for $\dot{M}O_{2Rest}$ ranged from 46.8 to 95.4 mg O_2 kg^{-1} h^{-1} with a mean of 65.2 ± 2.13 mg O_2 kg^{-1} h^{-1} across all treatments.

Critical oxygen tension

The P_{crit} values did not differ significantly between CO_2 treatment groups ($t_{4,26} = -0.170$; $P = 0.866$). However, standard length had a significant effect on the P_{crit} of individuals ($t_{4,26} = 2.26$; $P = 0.0323$; Fig. 2B), with larger animals reaching P_{crit} at a higher seawater O_2 concentration than smaller animals. The P_{crit} values ranged from 1.32 to 5.07 mg O_2 l^{-1} with a mean of 2.51 ± 0.122 mg O_2 l^{-1} across all treatment groups.
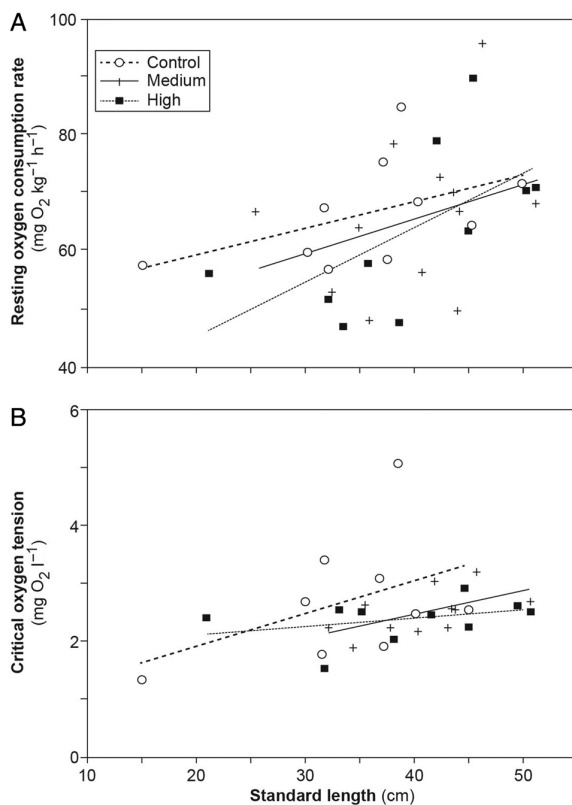
Figure 2: The relationship between resting oxygen consumption rate (**A**) and critical oxygen tension (P_{crit}; **B**) with the standard length of individual sharks from control, medium and high CO_2 treatment groups. Please refer to Materials and methods for further details regarding calculations.

Haematology and tissue samples

No significant differences were detected in Hct between CO_2 treatment groups ($F_{2,22} = 0.214$; $P = 0.809$; Fig. 3A). There was a significant increase in [Hb] between the control and the medium CO_2 treatment groups ($F_{2,23} = 3.447$; $P = 0.048$; Fig. 3B), an elevation that was maintained with the high CO_2 treatment group for MCHC values ($F_{2,21} = 5.067$; $P = 0.0160$; Fig. 3C). Although not significant, there was a trend toward decreased SSI with high CO_2 exposure ($F_{2,22} = 2.050$; $P = 0.153$; Fig. 3D). There was a significant increase in plasma [HCO$_3^-$] in both the medium and high CO_2 treatment groups ($F_{2,21} = 10.893$; $P < 0.001$; Fig. 4A). However, there was no difference in plasma [Na$^+$], [K$^+$], [Cl$^-$] or [urea] between control and CO_2 treatment groups ([Na$^+$], $F_{2,21} = 1.543$, $P = 0.237$, Fig. 4B; [K$^+$], $F_{2,21} = 0.247$, $P = 0.783$, Fig. 4C; [Cl$^-$], $F_{2,21} = 1.697$, $P = 0.207$, Fig. 4D; and [urea], $F_{2,21} = 2.907$, $P = 0.077$, Fig. 4E). Citrate synthase activity did not change significantly between control and CO_2 treatment groups in red muscle ($F_{2,16} = 0.371$; $P = 0.696$), heart ($F_{2,18} = 0.0238$; $P = 0.976$) or brain ($F_{2,19} = 0.131$; $P = 0.878$; Fig. 5).

Discussion

Long-term exposure to near-future CO_2 conditions did not significantly affect metabolic performance or hypoxia sensitivity of epaulette sharks. In contrast, changes in [Hb] and MCHC were evident after ~90 days of exposure to 600 μatm CO_2 levels, and plasma [HCO$_3^-$] was elevated in both the moderate and high CO_2 treatment groups, suggesting that physiological adjustments were being made to cope with elevated CO_2 at the level of oxygen transport and ion regulation. However, there was no increase in metabolic capacity at the level of the mitochondria, as indicated by the lack of change in citrate synthase activity. Our findings suggest that, for this reef-inhabiting benthic elasmobranch, neither the energetic costs of basic maintenance nor sensitivity to hypoxia may be compromised in the elevated CO_2 conditions projected for the end of this century.

The compensatory mechanisms used by *H. ocellatum* to maintain resting metabolic rates in normoxic and hypoxic conditions after prolonged exposure to elevated CO_2 may be linked to maintaining oxygen uptake and delivery and ion regulation. Following ~90 days of CO_2 exposure, epaulette sharks exhibited a significant increase in [Hb] and MCHC. Short-term changes in haematological parameters have been documented in teleosts and elasmobranchs following capture, cannulation and exercise (Soivio *et al.*, 1973; Wood *et al.*, 1977; Turner *et al.*, 1983; Wells *et al.*, 1986), upon acclimation to elevated temperature (adult horn sharks, *Heterodontus francisci*; Neale *et al.*, 1977) and in response to anoxia (grey carpet shark, *Chiloscyllium punctatum*, and epaulette shark; Chapman and Renshaw, 2009). In teleosts, acute changes can be associated with adrenergic red blood cell (RBC) swelling (Caldwell *et al.*, 2006), a mechanism in place to protect RBC pH and oxygen transport during stress,
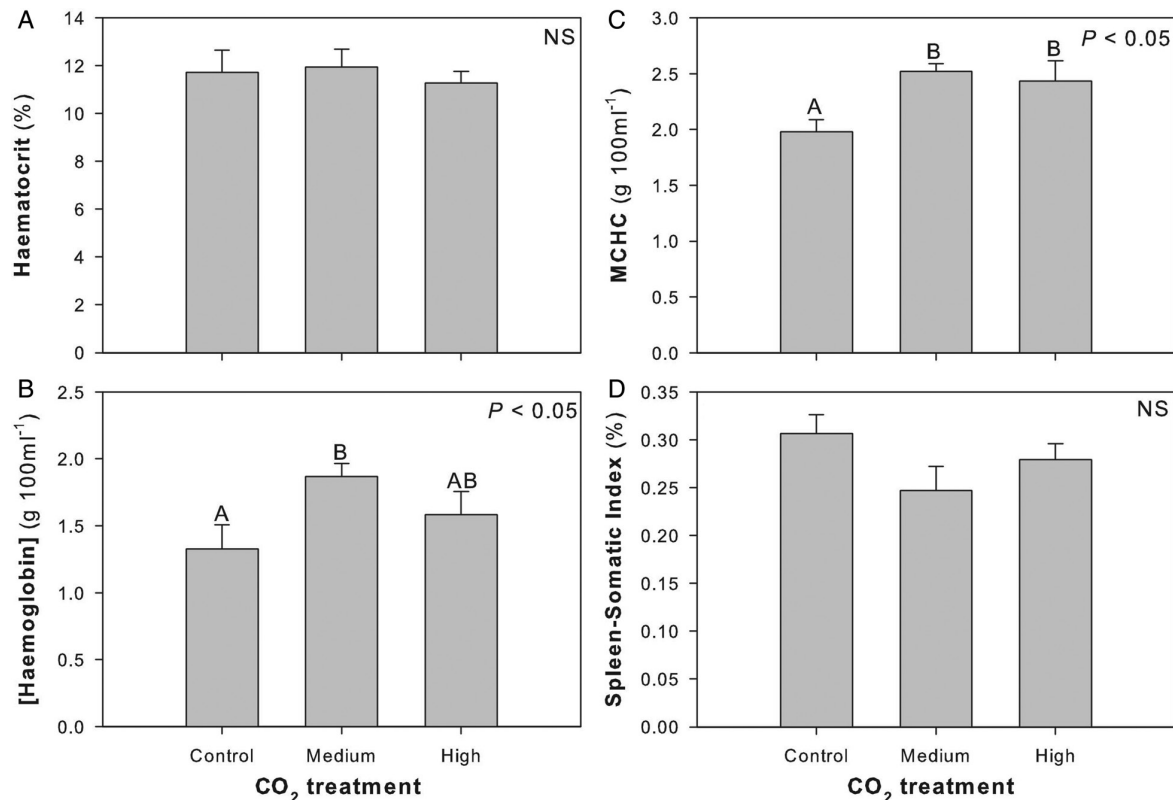
Figure 3: Changes in haematocrit (**A**), haemoglobin concentration (**B**), mean cell haemoglobin concentration (MCHC; **C**), and spleen–somatic index (**D**) after sharks were exposed to control, medium or high CO_2 for ~90 days. Different letters within a panel demarcate significant differences between treatment groups, and statistical significance is noted in the top right corner of each panel. Abbreviation: NS, not significant.

but not known to occur in elasmobranchs (Berenbrink *et al.*, 2005). Both teleosts and elasmobranchs do, however, use their spleen to produce and store RBCs (Turner *et al.*, 1983; Fänge and Nilsson, 1985; Lai *et al.*, 2006) and can contract it to increase the proportion of RBCs in the circulation (Ken-Ichi, 1988; Lai *et al.*, 2006), presumably to aid in oxygen transport (Jensen *et al.*, 1992). We observed a decrease, although non-significant, in the SSI in sharks exposed to both medium and high CO_2, suggesting that splenic contraction(s) may have occurred at some point during the CO_2 exposure period. Periodic splenic contractions could also increase the proportion of immature RBCs in circulation, which could explain the slight increase in MCHC without significant changes in Hct. The temporal scale of splenic RBC release and subsequent increases in erythropoietin, the glycoprotein responsible for regulating RBC numbers, is well understood for teleosts (Lai *et al.*, 2006) and could be similar in elasmobranchs exposed to elevated CO_2 over extended periods of time, which is worth further investigation.

Plasma $[HCO_3^-]$ was elevated in sharks upon 90 days of exposure to elevated CO_2, which indicates some level of long-term acid–base compensation. This finding is supported by studies by Deigweiher *et al.* (2008), in which acclimation to elevated CO_2 over 6 weeks in a marine teleost resulted in upregulation of Na^+/HCO_3^- cotransporters (NBC1) and Na^+–K^+-ATPase at higher densities. Given the relationship between bicarbonate availability and synthesis of urea (the predominant osmolyte used by most elasmobranchs), acid–base compensatory mechanisms could have affected [urea] and therefore the efficiency of osmoregulatory pathways (Wood *et al.*, 1995). As [urea] did not change with CO_2 exposure, this may not be problematic at the CO_2 levels used here and/or over the 90 day duration. The activity of citrate synthase, the first enzyme of the Krebs cycle located within the mitochondria, can be a good indicator of aerobic capacity. Unchanged citrate synthase activity after prolonged CO_2 exposure further suggests that there is no limitation at the level of aerobic energy production in any of the tested tissues (McClelland *et al.*, 2005). Although there may have been no changes to aerobic capacity, changes may have been occurring in anaerobic pathways (e.g. activity of lactate dehydrogenase, the last enzyme of anaerobic glycolysis) to maintain energy production. This would be worthy of further investigation.
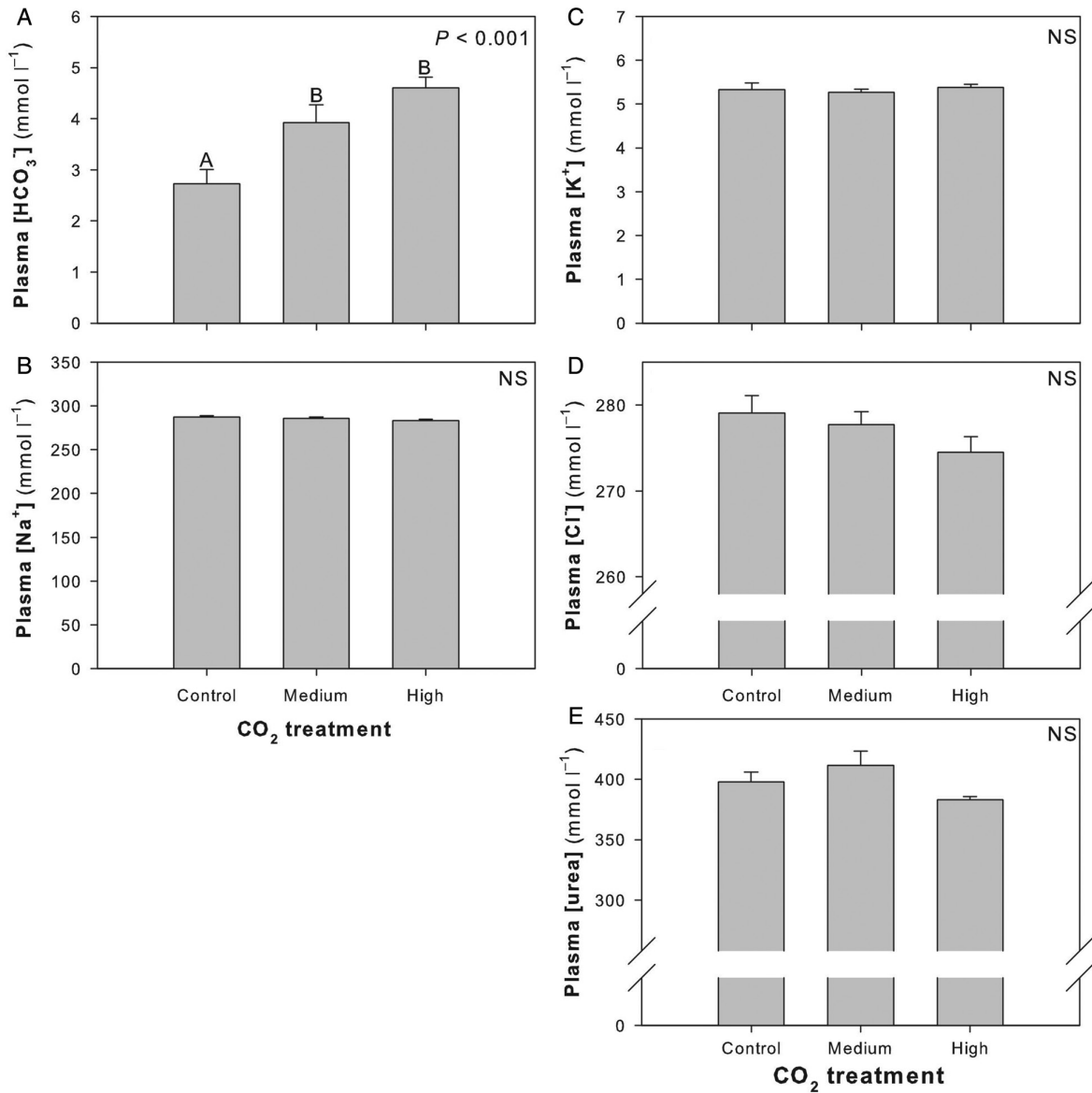
Figure 4: Changes in plasma parameters after sharks were exposed to control, medium or high CO_2 for ~90 days. Different letters within a panel demarcate significant differences between treatment groups, and statistical significance is noted in the top right corner of each panel. Abbreviation: NS, not significant.

As Esbaugh *et al.* (2012) suggest, species that are already adapted to low levels of CO_2 may no longer rely on traditional short-term acid–base compensation strategies but instead use morphological changes (e.g. gill permeability, diffusion distances) or alter chemical equilibrium constraints in the blood over longer periods to maintain oxygen transport.

While there were no changes in metabolic performance in the sharks upon long-term CO_2 exposure, there was an

unexpected pattern of mass-specific metabolic rates, with larger sharks exhibiting higher mass-specific metabolic rates than smaller sharks. This contradicts the usual pattern exhibited by ectotherms, but may be related to their feeding patterns. For example, we examined sharks ranging in size from ~20 to 50 cm. However, we used a set 48 h fasting period prior to determining oxygen consumption rates and prior to blood and tissue sampling because of their small size and benthic lifestyle and previous feeding patterns while in
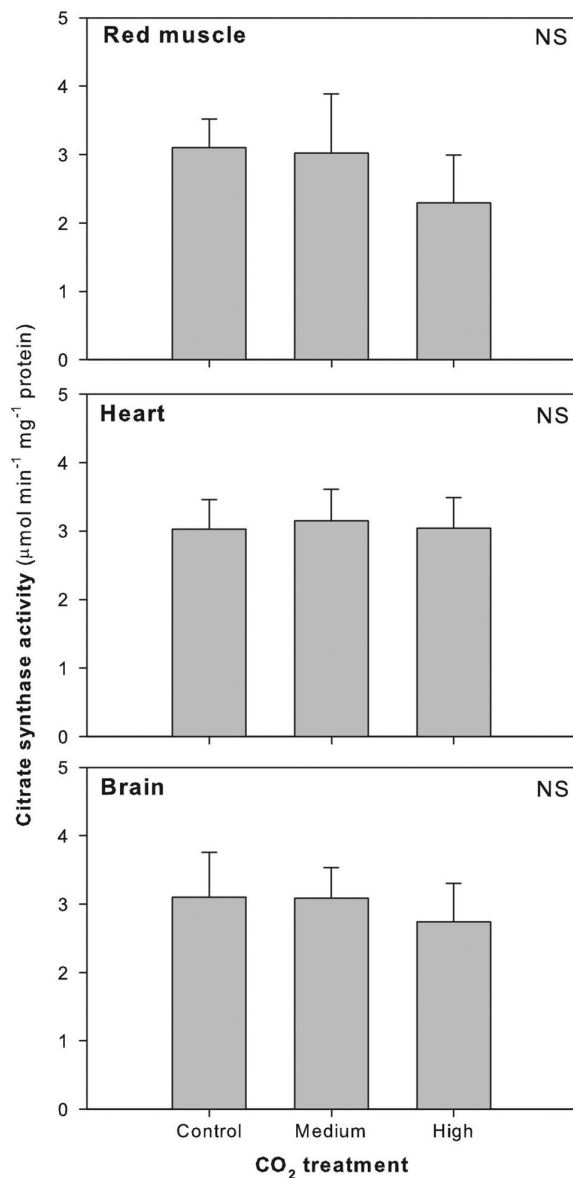
Figure 5: Changes in red muscle, heart and brain citrate synthase enzyme activity after sharks were exposed to control, medium or high CO_2 for ~90 days. Statistical significance is noted in the top right corner of each panel. Abbreviation: NS, not significant.

captivity. It could have been that 48 h was sufficient fasting time for the smaller animals but not for the larger animals of that size range (Wood *et al.*, 2007). Therefore, the larger animals could have been exhibiting slightly increased metabolic rates due to specific dynamic action, which could also mask any acid–base processes occurring due to CO_2 exposure. The relationship between acid–base disturbances originating from feeding and those due to elevated water

CO_2 has ecological relevance and should be investigated in future studies.

Environment and lifestyle play an important role in physiological tolerance to changing environmental conditions (Pörtner and Farrell, 2008), and this study confirms that *H. ocellatum* is no exception. It is already known that *H. ocellatum* exhibits the lowest value of P_{crit} shown for any elasmobranch tested to date, suggesting an exceptional tolerance to short-term hypoxia, which is unique among chondrichthyans (Wise *et al.*, 1998; Routley *et al.*, 2002). *Hemiscyllium ocellatum* occupies shallow reef platforms that are subject to dramatic diurnal fluctuations in environmental O_2 and CO_2 conditions (Routley *et al.*, 2002; Diaz and Breitburg, 2009; Last and Stevens, 2009). During calm nights, the low O_2 tension encountered on coral reefs can drop below 10% air saturation (Routley *et al.*, 2002), usually as a result of respiration by reef organisms and especially during nocturnal low tides. This can also result in elevations in PCO_2, which have been reported to exceed 1000 µatm on shallow reef flats at night (Ohde and van Woesik, 1999; Shaw *et al.*, 2013). The CO_2 levels may even be higher in caves, reef crevices and restricted-flow habitats, which are used by *H. ocellatum* for shelter (Compagno, 2002; Last and Stevens, 2009). Indeed, diurnal or acute fluctuations in O_2 and CO_2 may play a role in signalling metabolism in species using such habitats. However, acute responses often differ dramatically from responses to prolonged exposure, and it is important to make this distinction. The increased uptake of CO_2 by the ocean will affect both the average CO_2 level and the magnitude of extreme CO_2 fluctuations (Ohde and van Woesik, 1999; Shaw *et al.*, 2013). This makes our finding that *H. ocellatum* exhibited no change in metabolic performance, including sensitivity to hypoxia, after prolonged exposure to projected future CO_2 levels even more important.

Adaptation to life on shallow reef platforms and lagoons may be the key to species like *H. ocellatum* for maintaining performance in projected future CO_2 concentrations (Melzner *et al.*, 2009b). While noteworthy, what was previously known about the physiological tolerance of the epaulette shark to challenging environmental conditions was related to acute exposure of minutes to hours. This is extremely relevant to a shelter-seeking, benthic, reef-dwelling species like the epaulette shark that would experience such conditions burrowing into coral caves to avoid predation or to exploit food sources, activities vital to biological fitness. Pelagic shark species, many of which function as apex predators in their respective environments (Last and Stevens, 2009), however, do not typically exhibit shelter-seeking behaviours in areas that would experience the routine fluctuations in water chemistry experienced by *H. ocellatum* and therefore may not tolerate prolonged periods of elevated CO_2. Given that increased uptake of CO_2 by the ocean may mean that the high CO_2 levels that the epaulette shark may already routinely experience could be the new average ocean CO_2 levels, some species may be able to tolerate future conditions better. Future studies should investigate the importance of fluctuating

environmental conditions in shaping an organism's tolerance. Differential effects on functional groups could impact predator–prey dynamics, affect the population structure of elasmobranchs and other aquatic organisms inhabiting coral reefs and, ultimately, impact ecosystem health. Investigating both sensitive and tolerant species from an array of habitat types would help to tease apart the role of the environment from other factors, including evolutionary history and behaviour, all of which is important when considering conservation measures under future climate change scenarios.

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