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RESEARCH ARTICLE

Hypoxia tolerance in elasmobranchs. I. Critical oxygen tension as a measure of blood oxygen transport during hypoxia exposure

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SUMMARY

The critical O_2 tension of whole-animal O_2 consumption rate ($\dot{M}_{O_2}$), or P_{crit}, is the water P_{O_2} (Pw_{O_2}) at which an animal transitions from an oxyregulator to an oxyconformer. Although P_{crit} is a popular measure of hypoxia tolerance in fishes because it reflects the capacity for O_2 uptake from the environment at low Pw_{O_2}, little is known about the interrelationships between P_{crit} and blood O_2 transport characteristics and increased use of anaerobic metabolism during hypoxia exposure in fishes, especially elasmobranchs. We addressed this knowledge gap using progressive hypoxia exposures of two elasmobranch species with differing hypoxia tolerance. The P_{crit} of the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*, 5.10±0.37 kPa) was significantly lower than that of the comparatively hypoxia-sensitive shovelnose ray (*Aptychotrema rostrata*, 7.23±0.40 kPa). Plasma [lactate] was elevated above normoxic values at around P_{crit} in epaulette sharks, but increased relative to normoxic values at Pw_{O_2} below P_{crit} in shovelnose rays, providing equivocal support for the hypothesis that P_{crit} is associated with increased anaerobic metabolism. The $\dot{M}_{O_2}$, arterial P_{O_2} and arterial blood O_2 content (Ca_{O_2}) were similar between the two species under normoxia and decreased in both species with progressive hypoxia, but as Pw_{O_2} declined, epaulette sharks had a consistently higher $\dot{M}_{O_2}$ and Ca_{O_2} than shovelnose rays, probably due to their significantly greater *in vivo* haemoglobin (Hb)–O_2 binding affinity (*in vivo* Hb–O_2 P_{50}=4.27±0.57 kPa for epaulette sharks *vs* 6.35±0.34 kPa for shovelnose rays). However, at Pw_{O_2} values representing the same percentage of each species' P_{crit} (up to ~175% of P_{crit}), Hb–O_2 saturation and Ca_{O_2} were similar between species. These data support the hypothesis that Hb–O_2 P_{50} is an important determinant of P_{crit} and suggest that P_{crit} can predict Hb–O_2 saturation and Ca_{O_2} during hypoxia exposure, with a lower P_{crit} being associated with greater O_2 supply at a given Pw_{O_2} and consequently better hypoxia tolerance. Thus, P_{crit} is a valuable predictor of environmental hypoxia tolerance and hypoxia exposures standardized at a given percentage of P_{crit} will yield comparable levels of arterial hypoxaemia, facilitating cross-species comparisons of responses to hypoxia.

Key words: respiration, metabolic rate, haemoglobin, P_{crit}, P_{50}, haematology, pH, CO_2, energy metabolism, fish.

INTRODUCTION

Environmental hypoxia is a common abiotic stressor affecting the survival and distribution of aquatic species, but its occurrence varies in magnitude and spatio-temporal scale depending on habitat (Diaz and Breitburg, 2009). Consequently, many species of fish have evolved the ability to survive periods of low O_2 exposure but the severity and duration of hypoxia that can be tolerated is highly species specific. Identification of the physiological responses contributing to hypoxia tolerance among fishes is an area of major research interest. The increasing occurrence worldwide of environmental hypoxia due to anthropogenic activities, in particular, has led to the desire for simple physiologic metrics of hypoxia tolerance in fishes and other aquatic organisms. Even so, relatively few comparative studies exist on this topic in fishes.

One simple metric that holds promise in this regard is the critical O_2 tension of whole-animal O_2 consumption rate ($\dot{M}_{O_2}$), or P_{crit}, which is the water P_{O_2} (Pw_{O_2}) at which the $\dot{M}_{O_2}$ of an organism transitions from oxyregulation to oxyconformation. P_{crit} is thought to reflect the ability of an organism to extract O_2 from the environment to maintain routine $\dot{M}_{O_2}$ as Pw_{O_2} decreases, with a low P_{crit} being associated with greater hypoxia tolerance presumably because of improved O_2 uptake and transport to tissues at low Pw_{O_2}. Consequently, P_{crit} has been employed routinely as an important measure of hypoxia tolerance in aquatic organisms including fishes (Chapman et al., 2002; Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008; Pörtner and Grieshaber, 1993; Routley et al., 2002). Physiological modifications at any step in the respiratory cascade may affect P_{crit}, including changes in ventilation, gill surface area, blood O_2 capacity (including blood haemoglobin concentration [Hb] and Hb–O_2 binding affinity), circulation of O_2 (e.g. cardiac output), diffusion into tissues and mitochondrial O_2 turnover (Dejours, 1981; Farrell and Richards, 2009). Whole-blood Hb–O_2 P_{50} (i.e. Hb–O_2 binding affinity), in particular, has received attention as a determinant of P_{crit} as well as hypoxia tolerance in fishes because

of its important role in controlling blood O_2 content and O_2 uptake (Brauner and Wang, 1997). A phylogenetically independent comparison of O_2 transport in sculpins showed that P_{crit} is strongly correlated with Hb–O_2 P_{50}; species possessing a low P_{crit} typically also have a low Hb–O_2 P_{50} (Mandic et al., 2009). Other fishes known to have a high Hb–O_2 binding affinity typically also have a low P_{crit} and are often hypoxia tolerant (Burggren, 1982; Jensen and Weber, 1982; Sollid et al., 2005). Overall, however, the relationship between P_{crit} and Hb–O_2 P_{50} has rarely been directly investigated and it remains incompletely defined. Also, it has not been unequivocally demonstrated that a low P_{crit} is associated with greater arterial blood O_2 transport during hypoxia exposure in fishes. In fact, little is known in fishes about the responses of blood gas transport at and around P_{crit}.

At Pw_{O_2} below P_{crit}, O_2-independent energy production (e.g. anaerobic glycolysis) is increasingly relied upon to meet energy demands at a time when aerobic metabolism is constrained. Measurements of P_{crit} and anaerobic end-product accumulation in certain animals provide evidence for the hypothesis that the increased activation of anaerobic metabolism coincides with P_{crit} (Pörtner and Grieshaber, 1993), although support from other studies is equivocal (McKenzie et al., 2000; Nonnotte et al., 1993). The link between P_{crit} and increased activation of anaerobic metabolism is thus uncertain, especially in fishes. Also, there are no direct comparisons of the onset of lactate accumulation in hypoxia-tolerant and -sensitive fishes that differ in P_{crit}.

In the present study, we investigated the relationship between P_{crit} and arterial O_2 transport properties, including *in vivo* Hb–O_2 P_{50} and arterial total O_2 content (Ca_{O_2}), during progressive hypoxia exposure in two tropical elasmobranch species with similar lifestyles and activity levels, the epaulette shark, *Hemiscyllium ocellatum* (Bonnaterre), and the eastern shovelnose ray, *Aptychotrema rostrata* (Shaw). Epaulette sharks inhabit shallow coral reef environments where nocturnal hypoxia occurs commonly and they can tolerate hours of severe hypoxia (<1.0 kPa) exposure and up to 45 min of anoxia exposure (Renshaw et al., 2002; Routley et al., 2002). In contrast, eastern shovelnose rays are found in generally well-oxygenated coastal sandy and muddy benthic habitats in eastern Australia, including our sampling site of Moreton Bay, where hypoxic events are rare and dissolved O_2 is usually close to air saturation at all depths (Dennison et al., 2004; Gabric et al., 1998; Kyne and Bennett, 2002). Indeed, preliminary observations from the present study showed that shovelnose rays are relatively hypoxia sensitive, succumbing rapidly if held at or below a Pw_{O_2} of 2.0 kPa for more than approximately 30 min. We predicted that the more hypoxia-tolerant epaulette shark would have a lower P_{crit} than the shovelnose ray and that the lower P_{crit} in the epaulette shark would be associated with a lower Hb–O_2 P_{50} and a correspondingly greater Ca_{O_2} at similar hypoxic Pw_{O_2}. Furthermore, we predicted that at each species' P_{crit} or at the same percentage of P_{crit}, Hb–O_2 saturation and Ca_{O_2} would be similar between the species despite being exposed to different Pw_{O_2} values. Finally, we measured arterial blood metabolic status including pH, [lactate] and CO_2 status in order to further characterize the physiological correlates of P_{crit} in fishes and test the hypothesis that P_{crit} is associated with increased activation of anaerobic metabolism. Overall, the present study provides a comprehensive picture of the physiological responses associated with P_{crit} in two fishes, elucidating for the first time the relationship between P_{crit} and arterial blood O_2 transport characteristics during hypoxia exposure, and providing comparative insight into the respiratory and metabolic attributes associated with hypoxia tolerance in fishes.

MATERIALS AND METHODS

Animals

Epaulette sharks and shovelnose rays of mixed sexes were supplied by Cairns Marine (Cairns, QLD, Australia) or Seafish Aquarium Life (Dunwich, QLD, Australia), respectively. The animals were collected under A1 level commercial harvest licences granted by the Department of Primary Industries, Australia. Epaulette sharks were caught on the Great Barrier Reef and transported in flow-through seawater tanks to holding tanks at Cairns Marine, where they were kept for 2 days without feeding before being transported by air and automobile to Moreton Bay Research Station, North Stradbroke Island, QLD, Australia. Shovelnose rays were caught in Moreton Bay, transferred to the mainland in flow-through seawater tanks, and transported to Moreton Bay Research Station by automobile. All fish were held in a recirculating seawater system (28°C) for at least 5 days before experimentation. Animals were fed every other day and fasted for at least 24 h before experimentation. All experiments were conducted according to guidelines set out by the Canadian Council for Animal Care and protocols approved by the University of British Columbia Animal Care Committee and the Griffith University Animal Ethics Committee.

Experimental and analytical protocols

Surgical protocol

Epaulette sharks (1.29±0.04 kg, N=7) and shovelnose rays (1.54±0.06 kg, N=8) were netted from the holding tanks and anaesthetized in water containing a final concentration of 0.1 g l^{-1} benzocaine (initially dissolved in 95% ethanol; 0.001% ethanol in anaesthetic bath). Fish were then moved to a surgery table where the gills were continuously irrigated with aerated seawater (28°C) containing 0.075 g l^{-1} benzocaine.

To permit periodic sampling of blood, a PE50 (Clay-Adams, Parsippany, NJ, USA) cannula was fitted in the caudal artery *via* a lateral incision in the caudal peduncle, as described previously (De Boeck et al., 2001). The cannula was filled with heparinized (50 U ml^{-1}) elasmobranch saline (in mmol l^{-1}: 257 NaCl, 7 Na_2SO_4, 6 $NaHCO_3$, 0.1 Na_2HPO_4, 4 KCl, 3 $MgSO_4 \cdot H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 300 urea and 100 trimethylamine oxide). The cannula was exteriorized through a PE160 grommet and sutured to the skin. The incision was closed with silk sutures. The fish were also fitted with ventral aorta flow probes as described in the accompanying paper (Speers-Roesch et al., 2012).

Experimental protocol

Following surgery, the instrumented fish was immediately moved to a cylindrical acrylic respirometer (18.2 l for epaulette sharks and 28.4 l for shovelnose rays) that was submersed inside an opaque aquarium that received seawater from the same recirculating system used for the fish holding tanks (28°C). A submersible pump inside the aquarium provided a continuous flow of water to the respirometer that ensured complete water mixing inside the respirometer and maintained Pw_{O_2}. The respirometer was covered with black plastic to prevent visual disturbance of the fish. The cannula and the flow probe lead from the fish were exteriorized through a hole in the respirometer fitted with a soft rubber stopper modified with a slit. The fish was allowed to recover from surgery and habituate to the respirometer for at least 12 h before any experimental procedures were performed.

Stable baseline conditions were confirmed by monitoring routine cardiovascular variables (see Speers-Roesch et al., 2012) for 1–2 h at a normoxic Pw_{O_2} of between 15 and 16 kPa (74–78% air saturation; 100% air saturation=20.4 kPa=153 Torr). An aortic blood

sample (1 ml) was taken at the end of this period for immediate measurement of normoxic resting levels of arterial whole-blood pH, P_{O_2}, [Hb], haematocrit (Hct) and total O_2 content as described below. Plasma was separated by centrifugation (5000 ***g***, 5 min), frozen in liquid nitrogen within 5 min of sampling, transported to Canada in a dry shipper and kept frozen at –80°C for several weeks until analyses of metabolites and [total CO_2] (see below). The red blood cell pellet was re-suspended in elasmobranch saline to a final volume of 1 ml and this was injected *via* the cannula to replace the blood removed. The respirometer was then closed by connecting the inflow and outflow tubes of the respirometer *via* a submersible pump that re-circulated the water inside the respirometer. The fish was allowed to consume O_2 in the respirometer and the rate of depletion of $P_{W_{O_2}}$ was used to calculate $\dot{M}_{O_2}$ as described below. The changes in water parameters (e.g. pH, P_{CO_2}) potentially associated with the use of closed respirometry have been shown previously to have no effect on P_{crit} in fish (Henriksson et al., 2008). Indeed, only modest increases in arterial P_{CO_2} ($P_{a_{CO_2}}$) were observed in the present study (see Results and Discussion). Arterial blood samples were taken and treated as described above at regular intervals including $P_{W_{O_2}}$ at approximately 11.8, 7.7, 5.8, 3.8 and 2.0 kPa in both species, as well as approximately 1.0 and 0.1 kPa in epaulette sharks (see Table 1). Fish were held in the closed respirometer until $P_{W_{O_2}}$ reached ~0.1 kPa for epaulette sharks and ~1.6 kPa for the shovelnose rays, which took 135±8 and 71±6 min, respectively, from the point at which the normoxic blood sample was taken and the respirometer was closed. These nadirs of $P_{W_{O_2}}$ were chosen based on previous studies on epaulette sharks (e.g. Renshaw et al., 2002) and preliminary observations of hypoxia-exposed animals, which clearly revealed that epaulette sharks tolerated prolonged bouts of severe hypoxia whereas shovelnose rays showed distress or loss of equilibrium if held at or below 2.0 kPa for more than 30 min. The rate of O_2 depletion was similar between species (see Table 1). Once the nadir in $P_{W_{O_2}}$ (~0.1 or ~1.6 kPa) was reached, normoxic water was reintroduced to the respirometer. A final blood sample was taken at 60 min of recovery in normoxic water and analysed as previously described. In some cases, cannulae were damaged by the movements of the fish during the overnight acclimation period or during the experimental exposure and therefore sample sizes of measured parameters vary slightly (see figure captions for final *N* values). At the end of the trials the fishes were terminally anaesthetized in seawater containing benzocaine.

Data acquisition and calculation of oxygen consumption rate and P_{crit}

$P_{W_{O_2}}$ in the respirometer was measured using an Oxyguard probe (Mark IV, Point Four Systems, Richmond, BC, Canada), modified to give a ±1 V output signal, that was placed in a custom-made Plexiglas® chamber connected in line with the circulation pump. The probe output was fed to a Power Lab unit (ADInstruments, Castle Hill, NSW, Australia) and subsequently analysed using LabChart Pro software (v. 6.0; ADInstruments).

Whole-animal $\dot{M}_{O_2}$ during the respirometry trials was calculated from the rate of decline in $P_{W_{O_2}}$ over 10 min periods bracketing $P_{W_{O_2}}$ at regular intervals from approximately 14.8 to 0.67 kPa in epaulette sharks and approximately 13.7 to 1.9 kPa in shovelnose rays, following the methods of Henriksson et al. (Henriksson et al., 2008). Blanks without fish were run for both chambers and background $\dot{M}_{O_2}$ was subtracted from fish $\dot{M}_{O_2}$. The corrected $\dot{M}_{O_2}$ was plotted against $P_{W_{O_2}}$ and the inflection point at which $\dot{M}_{O_2}$ transitions from being independent to being dependent on $P_{W_{O_2}}$ (i.e. P_{crit}) was calculated using the BASIC program designed by Yeager and Ultsch (Yeager and Ultsch, 1989). At $P_{W_{O_2}}$ above P_{crit}, $\dot{M}_{O_2}$ values were constant and not significantly different from one another within a species, therefore confirming that $\dot{M}_{O_2}$ is independent of $P_{W_{O_2}}$ above P_{crit}. The $\dot{M}_{O_2}$ was not measured at the

Table 1. Arterial Hct, [Hb], MCHC, plasma [glucose], plasma [β-HB], [HCO_3^-], P_{CO_2} and [total CO_2] of epaulette sharks and shovelnose rays exposed to progressive decreases in $P_{W_{O_2}}$, and after a subsequent 60 min of recovery in normoxic water

	Sample	Time (min)	$P_{W_{O_2}}$ (kPa)	Hct (%)	[Hb] (mmol l^{-1})	MCHC ([Hb]/Hct)	[Glucose] (mmol l^{-1})	[β-HB] (mmol l^{-1})	[HCO_3^-] (mmol l^{-1})	$P_{a_{CO_2}}$ (kPa)	[Total CO_2] (mmol l^{-1})
Epaulette shark	1	0	16.01±0.43	13.4±0.7	0.45±0.02	3.39±0.15	1.51±0.18	0.69±0.21	4.04±0.58	0.18±0.02	4.09±0.59
	2	13.3±2.0	11.80±0.06	13.9±0.7	0.50±0.02	3.60±0.17	1.47±0.14	0.49±0.11	4.15±0.35	0.19±0.01	4.19±0.35
	3	27.7±2.7	7.65±0.10	13.4±1.0	0.49±0.04	3.64±0.11	1.43±0.12	0.50±0.13	4.45±0.24	0.21±0.01	4.51±0.24
	4	35.7±3.4	5.73±0.02	13.9±1.0	0.52±0.04	3.71±0.13	1.39±0.12	0.46±0.13	4.45±0.24	0.24±0.02*	4.51±0.25
	5	43.4±3.4	3.81±0.12	12.7±1.3	0.49±0.03	3.90±0.13	1.32±0.10	0.49±0.14	4.03±0.25	0.26±0.02*	4.10±0.25
	6	57.4±3.4	1.85±0.03	13.4±1.0	0.48±0.04	3.62±0.12	1.60±0.15	0.44±0.11	3.86±0.49	0.31±0.04*	3.94±0.50
	7	66.8±2.8	1.00±0.02	14.0±1.6	0.51±0.06	3.67±0.27	1.42±0.18	0.36±0.09	3.64±0.57	0.36±0.03*	3.74±0.58
	8	130±9	0.11±0.03	13.4±1.5	0.42±0.06	3.13±0.17	1.05±0.19	0.53±0.17	2.65±0.46	0.97±0.11*	2.91±0.48
	Recovery	195±7	14.07±1.08	16.2±0.9*	0.55±0.04	3.37±0.20	1.31±0.25	0.51±0.36	2.18±0.53*	0.42±0.11*	2.30±0.56*
Shovelnose ray	1	0	15.34±0.42	12.2±0.7	0.46±0.04	3.76±0.23	1.19±0.08	0.23±0.14	4.56±0.31	0.25±0.02†	4.64±0.32
	2	9.0±1.2	11.86±0.46	12.2±1.0	0.44±0.04	3.64±0.17	1.32±0.12	0.70±0.33	4.44±0.43	0.24±0.03	4.51±0.44
	3	24.3±2.3	7.88±0.07	11.9±0.5	0.44±0.03	3.75±0.21	1.26±0.10	0.41±0.25	4.29±0.26	0.23±0.02	4.21±0.26
	4	32.9±2.8	5.83±0.09	11.1±0.5†	0.42±0.04†	3.74±0.30	1.30±0.09	0.40±0.27	4.35±0.38	0.26±0.03	4.42±0.39
	5	44.2±3.6	3.82±0.07	10.5±0.4	0.39±0.03†	3.72±0.20	1.36±0.10	0.41±0.24	4.08±0.36	0.29±0.03	4.17±0.36
	6	63.4±5.8	2.07±0.03	10.2±0.5†	0.39±0.04†	3.82±0.33	1.38±0.17	0.29±0.17	3.52±0.41	0.39±0.05*	3.63±0.41
	Recovery	131±6	14.28±0.42	10.5±0.6†	0.41±0.05	3.87±0.21	1.52±0.09	0.43±0.17	3.84±0.60†	0.56±0.24*	4.12±0.47†

Data are means ± s.e.m. (*N*=4–6 for epaulette sharks except for recovery where *N*=3; *N*=5–6 for shovelnose rays). Total water P_{O_2} range of exposures: ~16.0 kPa and 15.3 kPa (normoxia) to ~0.1 kPa and 1.6 kPa, in epaulette sharks and shovelnose rays, respectively; total duration of exposure from initial blood sample 1 and closing of respirometer to $P_{W_{O_2}}$ nadir: 135±8 min and 71±6 min; respectively; see Materials and methods for further details.

$P_{W_{O_2}}$, water P_{O_2}; Hct, haematocrit; Hb, haemoglobin; MCHC, mean cellular haemoglobin content; β-HB, β-hydroxybutyrate; $P_{a_{CO_2}}$, arterial P_{CO_2}.

*Value is significantly different from the resting value (sample 1) within species (samples 2–6 for both species: two-way ANOVA with Holm–Sidak test; sample 7–8 for epaulette sharks: one-way ANOVA with Holm–Sidak test; recovery values for both species: two-way ANOVA with Holm–Sidak test; $P<0.05$);

†shovelnose ray value is significantly different from that of the epaulette shark at the same sample point, for samples 1–6 or recovery (two-way ANOVAs with Holm–Sidak tests, $P<0.05$). Time and $P_{W_{O_2}}$ were excluded from statistical analyses.

initial normoxic P_{wO_2} (~15.3–16.0 kPa) or the normoxic recovery period because of the flow-through conditions.

Analytical protocols

Blood [Hb] was measured spectrophotometrically (Blaxhall and Daisley, 1973). Hct was determined following centrifugation at 5000 ***g*** in a sealed capillary tube. Mean cellular Hb content (MCHC) was calculated as ([Hb]/Hct)100. Arterial blood P_{O_2} (P_{aO_2}) was measured with a Radiometer P_{O_2} (E-5046) electrode, thermostatically controlled in a D616 cell at 28°C, in conjunction with a PHM 71 acid–base analyser (Radiometer, Copenhagen, Denmark). Whole-blood C_{aO_2} was measured following the method of Tucker (Tucker, 1967). In order to calculate the percentage O_2 saturation of Hb in arterial blood, the quantity of O_2 bound to Hb was calculated by subtracting physically dissolved O_2 in the blood [calculated from measured P_{aO_2} and published blood O_2 solubility coefficients (Christiforides and Hedley-Whyte, 1969)] from the C_{aO_2} measured in whole blood. The quantity of O_2 bound to Hb was then expressed as a percentage of the theoretical maximum quantity of O_2 bound to Hb, which was calculated using measured [Hb] values and assuming four O_2 bound per Hb tetramer at 100% saturation. *In vivo* blood P_{50} and Hill coefficient values were then calculated from Hill plots based on data for arterial P_{O_2} and Hb–O_2 saturation.

Blood pH was measured using a Radiometer pH micro-electrode thermostatically held at 28°C and displayed on a Radiometer PHM 71 acid–base analyser. True plasma [total CO_2] was measured according to Cameron (Cameron, 1971), immediately following thawing of the plasma on ice. To confirm that freezing of plasma and storage at –80°C has no effect on [total CO_2], we compared [total CO_2] measured in freshly sampled plasma from normocarbic, normoxic tilapia (*Oreochromis* hybrid sp.) with an aliquot of the same plasma that was frozen in liquid nitrogen and stored at –80°C for 4 weeks. [Total CO_2] was 7.1±0.7 mmol l^{-1} in the fresh samples *vs* 7.0±0.8 mmol l^{-1} in the frozen samples (N=6; paired t-test, P>0.05; B.S.-R., unpublished); thus, freeze–thaw of plasma does not appear to affect [total CO_2]. We have seen similar results in hagfish (*Eptatretus stoutii*) (D. W. Baker and C.J.B., unpublished). P_{aCO_2} and [HCO_3^-] were calculated from measured values of pH and [total CO_2] through manipulation of the Henderson–Hasselbalch equation (Brauner et al., 2000) with appropriate constants for elasmobranchs (Boutilier et al., 1984). Plasma [lactate] and [glucose] were measured on deproteinized and untreated plasma, respectively, according to the protocols outlined by Bergmeyer (Bergmeyer, 1983). Plasma [β-hydroxybutyrate] ([β-HB]) was measured on deproteinized plasma following the protocol of McMurray et al. (McMurray et al., 1984).

Statistics

The effects of species and P_{wO_2} on blood gas, acid–base and metabolite parameters were tested for samples 1–6 (at overlapping P_{wO_2} between species; see Table 1) using a two-way ANOVA followed by Holm–Sidak *post hoc* (H–S) tests against species or the normoxic resting values measured at ≥15.3 kPa (P_{wO_2} values were not statistically different between species at each sample point, allowing this two-way design; Student's t-test, P>0.05). Samples 7 and 8 in epaulette sharks (see Table 1) were tested separately for significance against the normoxic resting sample 1 (16.0 kPa) using a one-way ANOVA with H–S tests. Species differences in the *in vivo* blood P_{50} and Hill coefficient (n_H) values were examined using a Student's t-test and the effect of O_2 on Hb–O_2 saturation was compared within species using a one-way ANOVA with H–S tests (comparisons between species were not made because arterial P_{O_2} at sample points were not always comparable). The relationship between Hb–O_2 saturation and P_{crit} was examined *via* linear regression analysis of Hb–O_2 saturation against P_{wO_2} expressed as the percentage of P_{crit} (using values from individual animals). This analysis was carried out up to a Hb–O_2 saturation of ~75% because at higher values Hb saturation curves are asymptotic. Recovery values were compared between species and with the normoxic resting values at ≥15.3 kPa within species using a two-way ANOVA with H–S test.

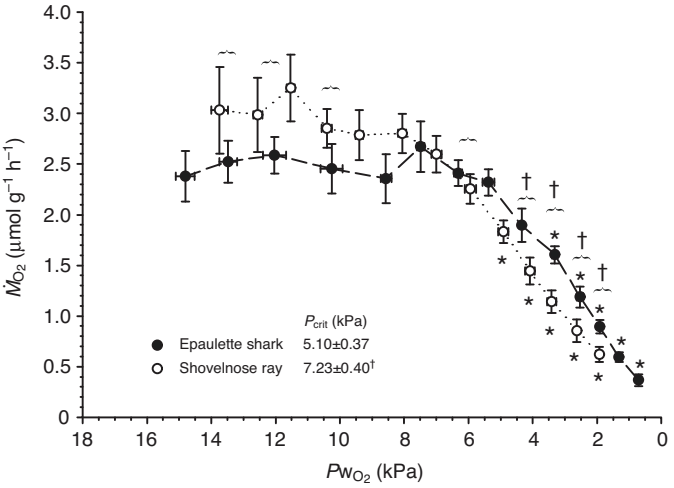

Fig. 1. Oxygen consumption rate ($\dot{M}_{O_2}$) and critical O_2 tension (P_{crit}) values of epaulette sharks and shovelnose rays exposed to progressive decreases in water P_{O_2} (P_{wO_2}). See Table 1 and Materials and methods for further information on time course and starting and ending P_{wO_2} of the exposures. Data are means ± s.e.m. (N=7 for epaulette sharks; N=8 for shovelnose rays). *Statistically significant difference from the first normoxic resting value within species. †Statistically significant difference between species for P_{crit}, and statistically significant difference between the epaulette shark value and the shovelnose ray value bracketed by a horizontal brace (where the two species values were taken at statistically similar P_{wO_2}); the absence of a dagger indicates that the two bracketed species values are not statistically different from each other. See Materials and methods for details on statistical methods.

The critical P_{wO_2} of $\dot{M}_{O_2}$ were compared between species using a Student's t-test. The effects of species and P_{wO_2} on $\dot{M}_{O_2}$ were tested using a two-way ANOVA with H–S tests using data from eight sampling points of overlapping P_{wO_2} at approximately 13.6, 12.3, 10.3, 6.1, 4.2, 3.4, 2.5 and 1.9 kPa and the points of statistical comparison are denoted by horizontal braces on the figures. Overlapping P_{wO_2} values were not statistically different between species (Student's t-test, P>0.05). Data from other sampling points were omitted from these analyses. However, in order to fully assess the effect of P_{wO_2} on $\dot{M}_{O_2}$ in each species, one-way ANOVA were run across all sampling points within each species, with H–S comparisons against the first normoxic resting value. The effect of P_{wO_2} on measured parameters was found to be similar for both two-way and one-way ANOVA designs.

Statistical significance was accepted when P<0.05 and analyses were carried out using SigmaStat 3.0 or GraphPad Prism 5.0. Data were log or square-root transformed prior to statistical analyses if assumptions of equal variance or normality were not met. Repeated measures ANOVA could not be carried out because experimental constraints negated the use of data from the same animal at every single sample period. In any case, the standard ANOVA procedures utilized here result in a conservative statistical assessment of our data.

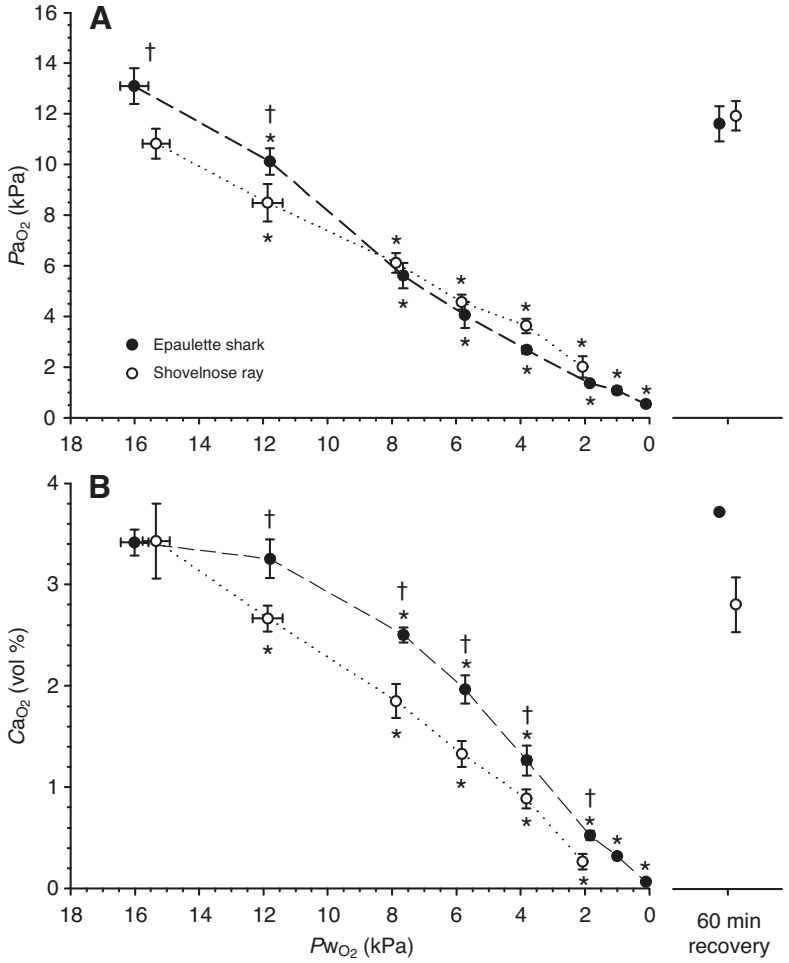

Fig. 2. Arterial P_{O_2} (Pa_{O_2}) (A) and arterial O_2 content (Ca_{O_2}) (B) of epaulette sharks and shovelnose rays exposed to progressive decreases in Pw_{O_2} and after a subsequent 60 min of recovery in normoxic water. See Table 1 and Materials and methods for further information on time course and starting and ending Pw_{O_2} of the exposures. Data are means ± s.e.m. (N=4–6 for epaulette sharks except for recovery where N=3; N=5–6 for shovelnose rays). Recovery values are offset for clarity. *Statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. †Shovelnose ray value is significantly different from that of the epaulette shark at the same sample point. See Materials and methods for details on statistical methods. Recovery values were not significantly different between species or when compared with resting values in both species.

RESULTS

Under normoxic conditions, individuals of both species were generally quiescent. As Pw_{O_2} decreased, there was a modest and temporary increase in activity level associated with exploratory behaviour in some individuals of each species. As Pw_{O_2} decreased further, the fishes again became quiescent. In a couple instances in each species, severe agitation occurred temporarily (<10 s) at low Pw_{O_2}, causing the cannula to be dislodged and negating further blood sampling.

A typical relationship between $\dot{M}_{O_2}$ and Pw_{O_2} was observed in both species, each with a zone of O_2-independent $\dot{M}_{O_2}$ occurring at higher Pw_{O_2} followed by a zone of O_2 dependence below P_{crit}, where $\dot{M}_{O_2}$ decreased with decreasing Pw_{O_2} (Fig. 1). The P_{crit} was significantly lower in epaulette sharks than in shovelnose rays (Fig. 1). When Pw_{O_2} was above ~5 kPa, $\dot{M}_{O_2}$ was similar between species. However, when Pw_{O_2} was below ~5 kPa, $\dot{M}_{O_2}$ was consistently greater for epaulette sharks than for shovelnose rays at any similar Pw_{O_2} (Fig. 1).

Pa_{O_2} decreased linearly with decreasing Pw_{O_2} and was not different between species, except at the two highest Pw_{O_2} where epaulette sharks had higher Pa_{O_2} compared with shovelnose rays (Fig. 2A). Total Ca_{O_2} decreased with decreasing Pw_{O_2} with the exception of between 16.01±0.43 and 11.80±0.06 kPa in epaulette sharks. Total Ca_{O_2} was the same in both species at the highest Pw_{O_2}, but at all lower Pw_{O_2} it was greater in epaulette sharks than in shovelnose rays (Fig. 2B). Recovery in normoxic water returned Pa_{O_2} and Ca_{O_2} to values similar to those measured at the beginning of the progressive hypoxia trial (Fig. 2). Hb–O_2 saturation decreased as Pa_{O_2} fell below approximately 9.0 and 6.0 kPa in shovelnose rays and epaulette sharks, respectively (Fig. 3). The *in vivo* Hb–O_2 binding affinity was significantly higher (lower P_{50}) in epaulette sharks than in shovelnose rays (Fig. 3). The Hill coefficient was similar between species (Fig. 3). At each species' P_{50}, arterial pH was ~7.82 in epaulette sharks and ~7.81 in shovelnose rays, and Pa_{CO_2} was ~0.23 kPa in both species (extrapolated from pH and Pa_{CO_2} data at the extrapolated Pw_{O_2} at P_{50}).

A significant linear relationship was found for each species when Hb–O_2 saturation was regressed against Pw_{O_2} expressed as a percentage of P_{crit}, and because the slopes and y-intercepts of these regression lines were not significantly different between species, one line of best fit was applied to the pooled data for the two species (Fig. 4). Above ~175% of P_{crit}, the Hb–O_2 saturation became asymptotic so these data were excluded from the linear regression. Thus, Hb–O_2 saturation was similar between species at Pw_{O_2} values representing the same percentage of each species' P_{crit} (up to ~175% of P_{crit}). Because there were no major differences in [Hb] between species (see below), the same relationship existed for Ca_{O_2} (data not shown). Overall, these data show that P_{crit} is predictive of Hb–O_2 saturation and Ca_{O_2} at Pw_{O_2} lower than about 175% of P_{crit}.

In both species progressive hypoxia had no effect on Hct, [Hb] or MCHC (Table 1). Hct and [Hb] were significantly higher in epaulette sharks at sampling points 4 (Pw_{O_2}=5.73±0.02 kPa in epaulette sharks and 5.83±0.09 kPa in shovelnose rays) and 6 (Pw_{O_2}=1.85±0.03 kPa in epaulette sharks and 2.07±0.03 kPa in shovelnose rays) and [Hb] was also higher at sampling point 5 (Pw_{O_2}=3.81±0.12 kPa in epaulette sharks and 3.82±0.07 kPa in

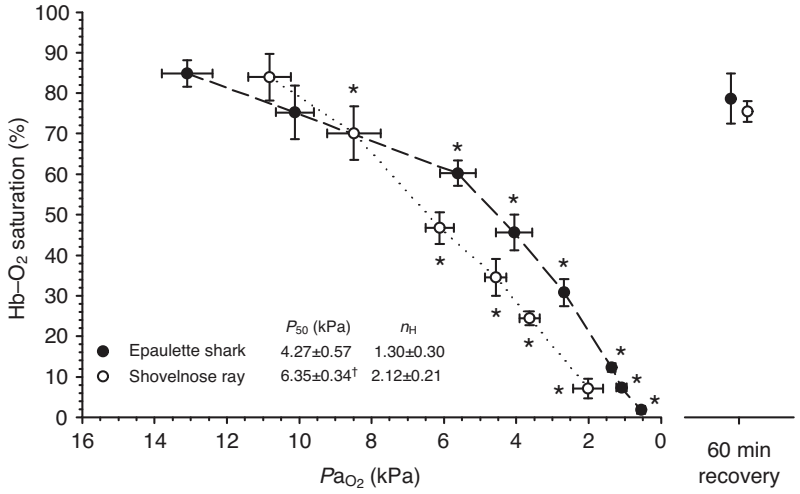

Fig. 3. Arterial haemoglobin–O_2 (Hb–O_2) saturation as a function of arterial P_{O_2} (Pa_{O_2}) of epaulette sharks and shovelnose rays exposed to progressive decreases in Pw_{O_2} and after a subsequent 60 min of recovery in normoxic water. *In vivo* Hb–O_2 P_{50} values and Hill coefficients (n_H) were calculated from Hill plots (see Materials and methods) and are presented in the figure. At each species' P_{50}, arterial pH was ~7.82 in epaulette sharks and ~7.81 in shovelnose rays, and Pa_{CO_2} was ~0.23 kPa in both species (extrapolated from pH and Pa_{CO_2} data at the extrapolated Pw_{O_2} at P_{50}). See Materials and methods for details on calculation of Hb–O_2 saturation. See Table 1 and Materials and methods for further information on time course and starting and ending Pw_{O_2} of the exposures. Data are means ± s.e.m. (N=4–6 for epaulette sharks except for recovery where N=3; N=5–6 for shovelnose rays). Recovery values are offset for clarity. *Statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. †Shovelnose ray value for Hb–O_2 P_{50} is significantly different from that of the epaulette shark. The Hill coefficients were not significantly different between species. See Materials and methods for details on statistical methods. Recovery values were not significantly different between species or when compared with resting values in both species.

shovelnose rays) (Table 1). Recovery [Hb] and MCHC were similar to normoxic resting values but Hct was higher in epaulette sharks during recovery (Fig. 2; Table 1).

Plasma [lactate] was low at Pw_{O_2} above 8 kPa, then significantly increased above normoxic resting levels by ~3.7 kPa in both species and increased further with decreasing Pw_{O_2}. Plasma [lactate] remained elevated after 60 min recovery in normoxic water (Fig. 5A). Plasma [lactate] was generally similar between species at all Pw_{O_2} during progressive hypoxia but was greater in epaulette sharks during recovery (Fig. 5A). A significant decrease in blood pH first occurred at ~3.8 kPa in both species, decreasing further with declining Pw_{O_2} and remaining low after 60 min recovery in normoxic water (Fig. 5B). Blood pH was similar between species but was significantly lower in the shovelnose rays at the final sampling point for this species at ~2.0 kPa (Fig. 5B). Arterial [HCO_3^-] and [total CO_2] were unchanged during progressive hypoxia exposure in both species (Table 1). An increase in Pa_{CO_2} occurred in both species and it remained after 60 min recovery in normoxic water (Table 1). In epaulette sharks only, normoxic recovery was associated with significantly lower [HCO_3^-] and [total CO_2] compared with normoxic resting levels as well as compared with the same parameters in shovelnose rays in recovery (Table 1). Plasma [glucose] or [β-HB] were similar between species and were unaffected by either progressive hypoxia or recovery in normoxic water (Table 1).

DISCUSSION

The present study demonstrates a link between P_{crit} and arterial blood O_2 transport characteristics during hypoxia exposure in two elasmobranch species, adding significantly to a growing body of evidence showing that P_{crit} is an important indicator of hypoxia tolerance in fish (Chapman et al., 2002; Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008). We provide the first comparative evidence that a lower P_{crit} is associated with maintenance of greater Ca_{O_2} during hypoxia exposure, which benefits hypoxia tolerance. Similarly, we show that P_{crit} is predictive of Hb–O_2 saturation and Ca_{O_2} (in the observed absence of changes in [Hb]) during hypoxia exposure (Fig. 4), supporting the notion that differences in Hb–O_2 binding affinity determine differences in P_{crit}. Indeed, the *in vivo* Hb–O_2 P_{50} of the epaulette shark was lower than that of the shovelnose ray and this is the likely explanation for the greater Ca_{O_2} observed in the former species at low Pw_{O_2}. The impressive hypoxia tolerance of the epaulette shark is probably attributable, in part, to its enhanced O_2 transport characteristics compared with those of the less tolerant shovelnose ray.

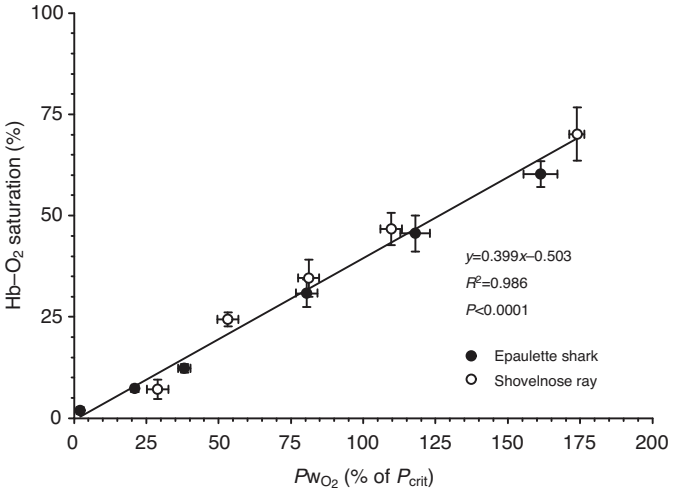

Fig. 4. Hb–O_2 saturation as a function of Pw_{O_2} represented as percentage of P_{crit} in epaulette sharks and shovelnose rays exposed to progressive decreases in Pw_{O_2} (i.e. P_{crit} of each species occurs at 100%). Data are means ± s.e.m. (N=4–6 for epaulette sharks; N=5–6 for shovelnose rays). The linear regression is statistically significant (P<0.0001, linear regression ANOVA).

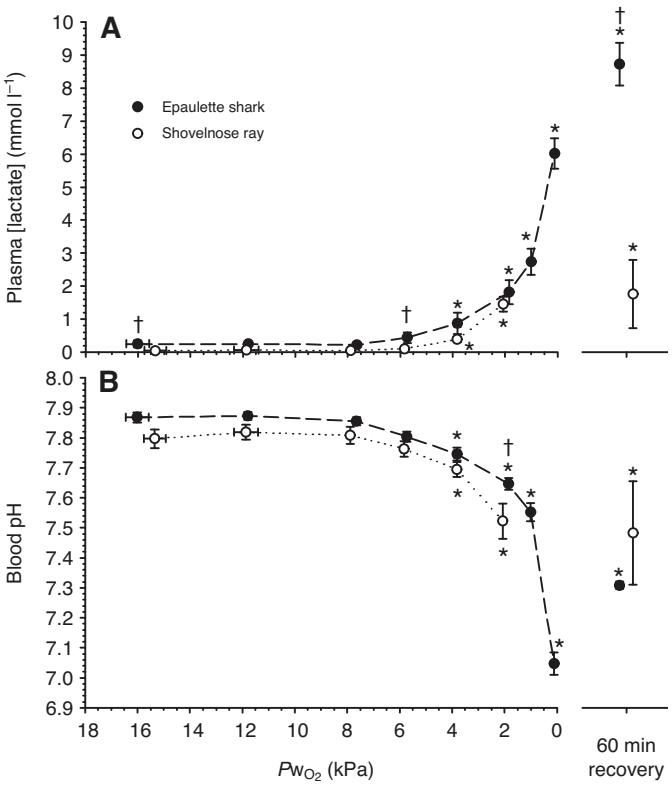

Fig. 5. Arterial plasma lactate (A) and arterial blood pH (B) of epaulette sharks and shovelnose rays exposed to progressive decreases in P_{wO_2} and after a subsequent 60 min of recovery in normoxic water. See Table 1 and Materials and methods for further information on time course and starting and ending P_{wO_2} of the exposures. Data are means ± s.e.m. (N=4–6 for epaulette sharks except for recovery where N=3; N=5–6 for shovelnose rays). Recovery values are offset for clarity. *Statistically significant difference from the first, normoxic resting value at ≥15.3 kPa within species. †Shovelnose ray value is significantly different from that of the epaulette shark at the same sample point.

Oxygen uptake and blood oxygen transport properties

Under normoxic resting conditions (i.e. $P_{wO_2}>P_{crit}$) the $\dot{M}_{O_2}$ of epaulette sharks matches closely with that measured for this species by Routley et al. (Routley et al., 2002). Epaulette sharks and shovelnose rays had similar resting $\dot{M}_{O_2}$, which fell within the range of temperature-corrected $\dot{M}_{O_2}$ for elasmobranchs of a similar activity level (0.9–3.5 μmol g^{-1} h^{-1}) (Butler and Metcalfe, 1988). In both species, exposure to progressive hypoxia caused a pronounced reduction of $\dot{M}_{O_2}$ below P_{crit} (Fig. 1), similar to many teleosts (Mandic et al., 2009; Speers-Roesch et al., 2010) as well as at least one other elasmobranch, the spotted catshark (*Scyliorhinus canicula*) (Butler and Taylor, 1975). The P_{crit} for epaulette sharks (Fig. 1) matches the lower range of P_{crit} measured for this species by Routley et al. (Routley et al., 2002). The shovelnose ray had a significantly higher P_{crit} compared with the epaulette shark (Fig. 1), but it was lower than that of other previously studied elasmobranchs (see Routley et al., 2002). Although the level of O_2 demand can affect P_{crit} (Thuy et al., 2010), this probably does not explain the observed difference in P_{crit} because $\dot{M}_{O_2}$ under normoxic conditions was the same in each species (Fig. 1). The difference in P_{crit} between the hypoxia-tolerant epaulette shark and the comparatively hypoxia-sensitive shovelnose ray is consistent with the notion that a lower P_{crit} is associated with greater hypoxia tolerance in fish (Mandic et al., 2009; Nilsson and Östlund-Nilsson, 2008). The P_{crit} of the epaulette shark is generally similar to those of hypoxia-tolerant teleosts and it is the lowest known P_{crit} among elasmobranchs (Routley et al., 2002; Speers-Roesch et al., 2010). The lower P_{crit} of epaulette sharks was associated not only with maintenance of routine $\dot{M}_{O_2}$ to a lower P_{wO_2} compared with shovelnose rays but also with maintenance of greater $\dot{M}_{O_2}$ at all comparable hypoxic P_{wO_2} (i.e. below P_{crit}), where depression of $\dot{M}_{O_2}$ occurred in both species (Fig. 1). Thus, a low P_{crit} allows a fish to maintain $\dot{M}_{O_2}$ as high as possible during hypoxia exposure, minimizing reliance on inefficient anaerobic metabolism.

Haematological (i.e. Hct, [Hb], MCHC) and O_2 transport properties (i.e. P_{aO_2}, C_{aO_2} and Hb–O_2 saturation) in arterial blood of epaulette sharks and shovelnose rays under normoxic resting conditions (Figs 2 and 3, values at highest P_{wO_2} or P_{aO_2}; Table 1, sample 1) were generally similar between species and the values were typical for elasmobranchs (e.g. Hct, 10–20%; [Hb], 0.46–0.62 mmol l^{-1}; MCHC, ~3.3; P_{aO_2}, 8–15 kPa; C_{aO_2}, 3–5 vol. %; Hb–O_2 saturation, 75–100%) (Butler and Metcalfe, 1988; Butler and Taylor, 1975; De Boeck et al., 2001; Lai et al., 1990; Perry and Gilmour, 1996; Routley et al., 2002). The lack of 100% Hb–O_2 saturation in normoxia is consistent with other studies on elasmobranchs (e.g. Lai et al., 1990) and the presence of up to 27% methaemoglobin in fishes has been put forward as an explanation as to why some fish haemoglobins are not saturated to their theoretical maximum *in vivo* (Graham and Fletcher, 1986). The *in vivo* P_{50} values for epaulette sharks and shovelnose rays are higher than previously measured in other elasmobranchs (1.9–2.7 kPa) (Butler and Taylor, 1975; Cooper and Morris, 2004; Lai et al., 1990), but in the previous studies these were measured at lower environmental temperatures compared with the present study and environmental temperature appears to be positively correlated with P_{50} in marine fishes (Wells, 2005). The Hill coefficients were similar between species and relatively low, which is typical of elasmobranchs (Butler and Metcalfe, 1988).

The changes in P_{aO_2} and C_{aO_2} during progressive hypoxia (Fig. 2) were similar to those seen in other elasmobranchs (Butler and Taylor, 1975; Perry and Gilmour, 1996; Routley et al., 2002). The roughly linear decrease in P_{aO_2} was of similar magnitude in epaulette sharks and shovelnose rays, suggesting ventilatory responses to hypoxia are comparable between species (Fig. 2A). However, at all matched P_{wO_2} below the normoxic resting level, C_{aO_2} was greater in epaulette sharks than in shovelnose rays, which appears to be due to greater Hb–O_2 saturation resulting from the epaulette shark's higher *in vivo* Hb–O_2 binding affinity (i.e. lower P_{50}) (Fig. 2B; Fig. 3). This result is consistent with findings suggesting that Hb–O_2 P_{50} is an important component of hypoxia tolerance in fishes, with the most tolerant species possessing the lowest P_{50}, which result in greater blood O_2 loading at low P_{wO_2} (Jensen and Weber, 1982; Mandic et al., 2009; Perry and Reid, 1992).

Interestingly, the species differences in Hb–O_2 saturation and C_{aO_2} disappeared when these parameters were plotted against P_{wO_2} expressed as a percentage of P_{crit} (i.e. the relationships overlapped between species) (Fig. 4). Thus, when measured at a P_{wO_2} of the same percentage of each species' P_{crit}, values of Hb–O_2 saturation or C_{aO_2} (because changes in [Hb] were not apparent) were the same in epaulette sharks and shovelnose rays. These results suggest that P_{crit} is predictive of Hb–O_2 saturation and C_{aO_2} during hypoxia exposure, with species with lower P_{crit} having a greater capacity for arterial blood O_2 transport at similar hypoxic P_{wO_2}. These results also suggest that the differences in arterial Hb–O_2 saturation between epaulette sharks and shovelnose rays may represent the basis of species differences in P_{crit}. This observation, as well as the

fact that at P_{crit} the Pa_{O_2} in both species was similar to their respective *in vivo* Hb–O_2 P_{50} (Fig. 2A; Fig. 3), agrees with Mandic and colleagues' (Mandic et al., 2009) discovery of a close relationship between P_{crit} and Hb–O_2 P_{50} in sculpins and supports the idea that P_{50} is an important determinant of P_{crit} in fishes.

The lack of a change in Hct or [Hb] during progressive hypoxia (Table 1) is consistent with previous studies on epaulette sharks and other elasmobranchs (Perry and Gilmour, 1996; Routley et al., 2002; Short et al., 1979) and thus adjustments of these parameters may have a minimal role in improving O_2 transport in elasmobranchs exposed to hypoxia. Nonetheless, Hct and [Hb] were approximately 25% higher at some hypoxic Pw_{O_2} values in epaulette sharks compared with shovelnose rays (Table 1), probably contributing to the higher Ca_{O_2} seen in the former species. However, the difference of Ca_{O_2} between species was greater (40–100% higher in epaulette sharks) at these hypoxic Pw_{O_2}, affirming an important role for Hb–O_2 P_{50} in enhancing O_2 supply in the epaulette shark.

Metabolites and acid–base status

An increased reliance on anaerobic glycolysis and a consequent metabolic acidosis during hypoxia exposure was indicated by a large increase in plasma [lactate] and a decrease in blood pH at or below P_{crit} in both species (Fig. 5), similar to results observed in other hypoxia-exposed fishes (Routley et al., 2002; Scott et al., 2008). At the lowest Pw_{O_2} exposure of shovelnose rays, blood pH was significantly lower compared with epaulette sharks (Fig. 5B), suggesting that the latter species may possess more effective acid–base regulation or less reliance on anaerobic energy production, potentially due to a superior O_2 supply. Nevertheless, at lower Pw_{O_2} epaulette shark blood pH dropped precipitously and like other hypoxia-tolerant vertebrates this species must be able to tolerate severe metabolic acidosis during hypoxia exposure (Driedzic and Gesser, 1994). The Pw_{O_2} at which epaulette sharks first showed a significant increase in plasma [lactate] was approximately the same as found previously for this species (Routley et al., 2002) and coincided with its P_{crit} (Fig. 5A). In shovelnose rays, however, the increase in plasma [lactate] did not coincide with its P_{crit} but rather occurred at a similar Pw_{O_2} to that of the epaulette sharks and accumulated at a similar rate (Fig. 5A). Although our data do not provide solid evidence for the hypothesis that P_{crit} correlates with increased activation of anaerobic metabolism in fishes (Pörtner and Grieshaber, 1993), they are consistent with this activation not occurring above P_{crit}. It is also possible that a non-equilibrium state during early progressive hypoxia exposure including interspecific differences in blood volume or lactate handling may have obscured our ability to detect the onset of an increase of anaerobic metabolism at P_{crit} in shovelnose rays. This may also explain why the rate of plasma lactate accumulation was similar between species despite lower blood O_2 content in shovelnose rays. We consider the effect of changes in fish activity (see Results) on plasma [lactate] to be negligible because of the low measurement variability and the consistently low values at Pw_{O_2} above P_{crit} (Fig. 1). Also, most fish activity was moderate and in the few instances of excessive activity, cannula dislodgement meant that plasma [lactate] was no longer measured.

The metabolic acidosis seen in epaulette sharks and shovelnose rays during progressive hypoxia resulted in a downward trend in $[HCO_3^-]$ and [total CO_2] (Table 1). In both species Pa_{CO_2} increased, possibly due to a build-up of CO_2 in the closed respirometer. This elevation in CO_2 probably had no major effect on other measured parameters, because the magnitude of the Pa_{CO_2} increase was not large until the final sample point and no major differences were observed between the final sample point and the previous sample point for any measured parameters. In any case, hypercarbia commonly accompanies environmental hypoxia so in this regard the present exposures are more ecologically relevant. The responses of blood CO_2 parameters to progressive hypoxia in the present study differ from the respiratory alkaloses seen in hypoxic spiny dogfish (*Squalus acanthias*) (Perry and Gilmour, 1996) or spotted catshark (Butler et al., 1979), but these other studies utilized moderate rather than severe hypoxia that probably mitigated metabolic acidosis – Butler et al. (Butler et al., 1979) found no increase in plasma [lactate]. The relatively low normoxic resting levels of Pa_{CO_2}, $[HCO_3^-]$ and [total CO_2] in epaulette sharks and shovelnose rays are typical of elasmobranchs (Butler and Metcalfe, 1988; De Boeck et al., 2001; Lai et al., 1990).

Plasma [glucose] was unaffected by progressive hypoxia (Table 1), which is unlike most teleosts but mirrors previous findings in hypoxia-exposed epaulette sharks and spiny dogfish (Routley et al., 2002; Speers-Roesch and Treberg, 2010). In some hypoxia-tolerant teleosts, large decreases in plasma [non-esterified fatty acid], an important aerobic fuel, occur during hypoxia exposure (Speers-Roesch et al., 2010). There was no similar response in epaulette sharks or shovelnose rays for plasma concentration of β-HB, which in elasmobranchs serves an important role as an alternative lipid-derived aerobic fuel (Speers-Roesch and Treberg, 2010).

Recovery from progressive hypoxia exposure

Epaulette sharks and shovelnose rays showed comparable recovery of respiratory parameters after progressive hypoxia and followed a trajectory similar to that of other fishes (Hughes and Johnston, 1978; Van Raaij et al., 1996). Following 60 min of recovery in normoxic water, normoxic arterial O_2 parameters were restored in both species (Fig. 2B; Fig. 3); however, recovery from metabolic acidosis and high [lactate] was incomplete, particularly in epaulette sharks, which had experienced more severe hypoxia and hypoxia-induced acidosis and lactate accumulation (Fig. 5B). Arterial $[HCO_3^-]$ in recovery remained lower than normoxic levels in epaulette sharks, probably as a consequence of the persistence of acidosis, whereas shovelnose rays had recovered (Table 1).

CONCLUSIONS

The present study on the hypoxia-tolerant epaulette shark and the hypoxia-sensitive shovelnose ray provides the first evidence that P_{crit} is predictive of arterial Hb–O_2 saturation and Ca_{O_2} during hypoxia exposure in fishes. At the same level of hypoxia, fishes with a low P_{crit} can maintain higher Ca_{O_2} than fishes with a higher P_{crit} and this appears to be due to the presence of a lower *in vivo* Hb–O_2 P_{50} that allows greater Hb–O_2 saturation (Fig. 2B; Fig. 3). Additionally, at Pw_{O_2} of the same percentage of P_{crit}, Hb–O_2 saturation (and Ca_{O_2}) is the same between species (Fig. 4). These results suggest that the interspecific differences in Hb–O_2 saturation may represent the basis of species differences in P_{crit}, providing strong support for the notion that Hb–O_2 P_{50} is a major determinant of P_{crit} as well as hypoxia tolerance in fishes (Mandic et al., 2009).

Our finding of a link between P_{crit}, Hb–O_2 saturation and Ca_{O_2} provides a mechanistic explanation for the argument that P_{crit} is a good indicator of hypoxia tolerance in fishes. A low P_{crit} is associated with greater Ca_{O_2} and therefore improved O_2 delivery to tissues during hypoxia exposure, which presumably enhances energy supply and reduces the accumulation of deleterious anaerobic end products, thus improving hypoxia tolerance. Importantly, our findings also show that hypoxia exposures that are standardized to

P_{crit} will yield comparable levels of arterial hypoxaemia, facilitating cross-species comparative analyses. However, the results of the accompanying study (Speers-Roesch et al., 2012) warn that even when hypoxia exposures are scaled to P_{crit}, tissue-specific differences may occur in the metabolic response to the same amount of circulating O_2.

Although our measurements of plasma [lactate] showed no difference in the onset Pw_{O_2} or the rate of accumulation of lactate between species despite differing P_{crit}, our ability to detect such a difference may have been obscured by non-steady-state lactate dynamics during initial hypoxia exposure. Accumulation of lactate in each species did occur at or below P_{crit}, providing equivocal support for the hypothesis that P_{crit} is associated with increased activation of anaerobic metabolism (Pörtner and Grieshaber, 1993). Investigations of lactate turnover in hypoxia-exposed fish, including consideration of the potential effect of metabolic depression (which could blunt lactate accumulation), are needed to further test this hypothesis.

The superior O_2 uptake and blood O_2 capacity during hypoxia exposure in epaulette sharks probably explains in part the renowned hypoxia tolerance of this elasmobranch (Nilsson and Renshaw, 2004). Epaulette sharks also show enhanced hypoxic cardiovascular function compared with shovelnose rays (Speers-Roesch et al., 2012), which further improves O_2 delivery to tissues. Maintenance rather than depression of O_2 supply and aerobic metabolism at low levels of Pw_{O_2} may be an important component of hypoxia tolerance in fishes.

LIST OF SYMBOLS AND ABBREVIATIONS

β-HB	β-hydroxybutyrate
Ca_{O_2}	arterial blood O_2 content
Hb	haemoglobin
Hb–O_2 P_{50}	haemoglobin–O_2 binding affinity
Hct	haematocrit
MCHC	mean cellular haemoglobin content
$\dot{M}_{O_2}$	whole-animal O_2 consumption rate
n_H	Hill coefficient
Pa_{CO_2}	arterial blood P_{CO_2}
Pa_{O_2}	arterial blood P_{O_2}
P_{CO_2}	partial pressure of CO_2
P_{crit}	critical O_2 tension of whole-animal O_2 consumption rate
P_{O_2}	partial pressure of O_2
Pw_{O_2}	water P_{O_2}

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