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RESEARCH ARTICLE

Hypoxia tolerance in elasmobranchs. II. Cardiovascular function and tissue metabolic responses during progressive and relative hypoxia exposures

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SUMMARY

Cardiovascular function and metabolic responses of the heart and other tissues during hypoxia exposure were compared between the hypoxia-tolerant epaulette shark (*Hemiscyllium ocellatum*) and the hypoxia-sensitive shovelnose ray (*Aptychotrema rostrata*). In both species, progressive hypoxia exposure caused increases in stroke volume and decreases in heart rate, cardiac output, cardiac power output (CPO, an assessment of cardiac energy demand) and dorsal aortic blood pressure, all of which occurred at or below each species' critical P_{O_2} for whole-animal O_2 consumption rate, $\dot{M}_{O_2}$ (P_{crit}). In epaulette sharks, which have a lower P_{crit} than shovelnose rays, routine levels of cardiovascular function were maintained to lower water P_{O_2} levels and the changes from routine levels during hypoxia exposure were smaller compared with those for the shovelnose ray. The maintenance rather than depression of cardiovascular function during hypoxia exposure may contribute to the superior hypoxia tolerance of the epaulette shark, presumably by improving O_2 delivery and waste removal. Compared with shovelnose rays, epaulette sharks were also better able to maintain a stable cardiac high-energy phosphate pool and to minimize metabolic acidosis and lactate accumulation in the heart (despite higher CPO) and other tissues during a 4 h exposure to 40% of their respective P_{crit} (referred to as a relative hypoxia exposure), which results in similar hypoxaemia in the two species (~16% Hb–O_2 saturation). These different metabolic responses to relative hypoxia exposure suggest that variation in hypoxia tolerance among species is not solely dictated by differences in O_2 uptake and transport but also by tissue-specific metabolic responses. In particular, lower tissue [lactate] accumulation in epaulette sharks than in shovelnose rays during relative hypoxia exposure suggests that enhanced extra-cardiac metabolic depression occurs in the former species. This could facilitate strategic utilization of available O_2 for vital organs such as the heart, potentially explaining the greater hypoxic cardiovascular function of epaulette sharks.

Key words: heart, cardiorespiratory, energy metabolism, P_{crit}, pH, fish.

INTRODUCTION

The ability to tolerate environmental hypoxia varies greatly among fishes and the physiological attributes that underlie this variation in hypoxia tolerance remain incompletely understood. A growing body of evidence from comparative studies suggests that one important determinant of hypoxia tolerance in fishes is the ability to maintain O_2 uptake at low water P_{O_2} ($P_{W_{O_2}}$), which is reflected by the $P_{W_{O_2}}$ at which whole-animal O_2 consumption rate ($\dot{M}_{O_2}$) transitions from being independent to dependent on environmental O_2; this inflection point is termed P_{crit} (Mandic et al., 2009; Speers-Roesch et al., 2012). At $P_{W_{O_2}}$ below P_{crit}, hypoxic survival becomes dependent on maintenance of cellular energy balance in the face of decreases in aerobic energy supply (Richards, 2009). This may be achieved by an increased reliance on O_2-independent energy supply (e.g. anaerobic glycolysis) as well as a profound, reversible metabolic rate depression in which large decreases in cellular energy demand occur (Richards, 2009).

The cardiovascular system is an essential component of the respiratory cascade and is driven by the heart, a hypoxia-sensitive organ with high energy demands. In fishes exposed to hypoxia the cardiovascular system is vital for transport of available O_2 as well as in the distribution of fermentable fuel and the removal of waste. However, maintenance of cardiac function may be constrained by the limited energy supply during hypoxia exposure and in hypoxia-sensitive species catastrophic cardiac failure may occur as a result of a mismatch between energy supply and demand in the heart that leads to perturbed cardiac energy status (i.e. the levels of high-energy phosphate compounds) (Farrell and Stecyk, 2007). Hypoxia-tolerant fishes are able to avoid this fate, but the cardiovascular responses during hypoxia exposure (including $P_{W_{O_2}}$ at and below P_{crit}) that are associated with hypoxia tolerance remain unclear, in part because the responses reported are varied and because of the relatively few species studied. In most fishes exposed to hypoxia, bradycardia is observed and this generally occurs when $P_{W_{O_2}}$ reaches P_{crit}, suggesting that hypoxia-tolerant fishes with lower P_{crit} may be able to maintain heart rate (f_H) to lower $P_{W_{O_2}}$ (Farrell, 2007; Speers-Roesch et al., 2010). However, responses of other cardiovascular parameters at and below P_{crit} are more varied, confusing interpretation of their role in hypoxia tolerance. In some fishes, routine cardiac output ($\dot{Q}$) is defended to an extent by

increases in stroke volume (V_S), even as $P_{W_{O_2}}$ falls below P_{crit} (Butler and Taylor, 1975; Gamperl and Driedzic, 2009; Gamperl et al., 1994; Petersen and Gamperl, 2011). In other fishes, V_S is unchanged and $\dot{Q}$ falls because of the decrease in f_H below P_{crit} (Iversen et al., 2010; Speers-Roesch et al., 2010; Stecyk and Farrell, 2006). Studies on fish species that allow $\dot{Q}$ to fall during hypoxia exposure, including tilapia (*Oreochromis* hybrid) and common carp (*Cyprinus carpio*), suggest that the reductions in f_H and $\dot{Q}$ below P_{crit} enable a decrease in cardiac power output (CPO), which represents a lowering of cardiac energy demand (Speers-Roesch et al., 2010; Stecyk and Farrell, 2006). Depression of CPO may be an important component of hypoxia tolerance because it allows cardiac energy demand to be matched to reduced energy supply at $P_{W_{O_2}}$ below P_{crit} where aerobic metabolism is limited, thus facilitating the maintenance of stable cardiac energy status and function (Farrell and Stecyk, 2007). The crucian carp (*Carassius carassius*), in contrast, has a low routine CPO that apparently can be sustained anaerobically, probably explaining how its $\dot{Q}$ remains relatively unchanged during hypoxia exposure (Stecyk et al., 2004). Overall, there is uncertainty about the degree to which depression or maintenance of cardiovascular function including CPO is associated with hypoxia tolerance and P_{crit} in fishes.

Here, we carried out two series of experiments to assess cardiovascular and heart metabolic responses to progressive and relative hypoxia exposure in two species of elasmobranchs, the hypoxia-tolerant epaulette shark, *Hemiscyllium ocellatum* (Bonnaterre), and the comparatively hypoxia-sensitive Eastern shovelnose ray, *Aptychotrema rostrata* (Shaw). In one series of experiments, we report on cardiovascular function in epaulette sharks and shovelnose rays during progressive hypoxia exposure to examine how cardiovascular responses at and below P_{crit} compare between a hypoxia-tolerant and a hypoxia-sensitive elasmobranch, given the lack of previous direct comparisons of the hypoxic responses of CPO between hypoxia-tolerant and -sensitive fishes. We hypothesized that routine cardiovascular function is maintained to a lower $P_{W_{O_2}}$ in the epaulette shark because of its greater hypoxia tolerance and lower P_{crit} compared with the shovelnose ray (Speers-Roesch et al., 2012). At progressively lower $P_{W_{O_2}}$ below P_{crit}, we hypothesized that there would be a greater depression of cardiovascular function including CPO in the epaulette shark compared with the shovelnose ray. In a second series of experiments, we assessed metabolic status (i.e. pH and levels of metabolites of energy metabolism, e.g. high-energy phosphates and lactate) in cardiac and other tissues of hypoxic epaulette sharks and shovelnose rays held for ≤4 h at a $P_{W_{O_2}}$ representing the same percentage of each species' P_{crit}. Our accompanying study revealed that exposure to the same percentage of P_{crit} in these two species would yield a similar level of hypoxaemia (Speers-Roesch et al., 2012). Therefore, the present experiment allowed us to assess whether the greater hypoxia tolerance of epaulette sharks is associated with more stable hypoxic cardiac and extra-cardiac metabolic status compared with the shovelnose ray, even when variation in interspecific O_2 supply is controlled for. In turn, this allowed us to test the hypothesis that the ability to take up and transport O_2 at low $P_{W_{O_2}}$, as indicated by P_{crit}, dictates tissue-level hypoxia tolerance. In this case, we predicted that tissues of the two species during relative hypoxia exposure would have similar energy status, lactate accumulation and pH levels. Overall, these experiments and those in the accompanying study (Speers-Roesch et al., 2012) provide insight into the cardiorespiratory and metabolic responses that contribute to hypoxia tolerance in elasmobranchs and other fishes.

MATERIALS AND METHODS

Animals

Epaulette sharks and shovelnose rays of mixed sexes were collected and held in a recirculating seawater system (28°C) at Moreton Bay Research Station, North Stradbroke Island, QLD, Australia, as described in the accompanying paper (Speers-Roesch et al., 2012).

Experimental series I: cardiovascular responses to progressive hypoxia

Surgical protocol

Experimental series I is the same experiment as that described in our accompanying paper (Speers-Roesch et al., 2012) and the preparation of animals for surgery is fully described therein. In brief, cardiovascular responses to progressive hypoxia were monitored in epaulette sharks (1.29±0.04 kg, N=7) and shovelnose rays (1.54±0.06 kg, N=8) simultaneously with the measurements of $\dot{M}_{O_2}$ and blood O_2 transport properties. Measurement of dorsal aortic blood pressure (P_{DA}) was *via* the caudal artery cannula that also allowed periodic blood sampling. To measure ventral aortic blood flow (i.e. cardiac output or $\dot{Q}$), an ultrasonic flow probe was fitted around the ventral aorta *via* a midline ventral incision made through the skin and overlying muscle anterior from the fifth gill slit. The connective tissue surrounding the ventral aorta was inspected for superficial vessels before being cut to expose the ventral aorta. Where present, vessels were ligatured with 4-0 silk to prevent bleeding. A 2.5 mm ultrasonic SB-type flow probe (Transonic Systems, Ithaca, NY, USA) filled with acoustic gel (Transonic Systems) was then fitted around the exposed ventral aorta distal to the third, fourth, and fifth afferent branchial arteries. This flow probe placement allows measurement of ~37% of $\dot{Q}$ in elasmobranchs and this value does not change during hypoxia exposure [see Taylor et al. (Taylor et al., 1977) and Lai et al. (Lai et al., 1989), and references therein]. *In vivo* measurement of total $\dot{Q}$ is not possible in elasmobranchs because the afferent branchial arteries for the posterior gill arches arise as the conus arteriosus exits the pericardium and therefore no portion of the ventral aorta outside of the pericardium carries the entire cardiac output. Entering the pericardium is not an option in elasmobranchs because of its importance for cardiovascular function (Franklin and Davie, 1993; Stensløkken et al., 2004). After placement, the flow probe was secured with two 4-0 silk sutures tied to the surrounding muscle. The muscle incision was closed with interrupted 4-0 silk sutures and then the skin incision was closed with interrupted 1-0 silk sutures. The lead of the flow probe was secured to the skin and tied to the arterial cannula to prevent entanglement.

Experimental protocol

The experimental protocol and other details for the progressive hypoxia exposure are described in the 'Experimental protocol' section of the accompanying paper (Speers-Roesch et al., 2012). Routine cardiovascular variables (f_H, $\dot{Q}$, P_{DA}) were continuously recorded at a normoxic $P_{W_{O_2}}$ of approximately 16.0 kPa or 15.3 kPa for epaulette sharks and shovelnose rays, respectively (75–78% air saturation; 100% air saturation=20.4 kPa=153 Torr) for 1–2 h to ensure stable baseline conditions. After initial blood sampling and closing of the respirometer in which animals were exposed to progressive hypoxia, cardiovascular parameters were continuously monitored as $P_{W_{O_2}}$ was depleted as a consequence of fish respiration. The effects on cardiovascular function of changes in water parameters (e.g. pH, P_{CO_2}) potentially associated with utilization of closed respirometry are considered to be negligible (Speers-Roesch et al., 2010). In fact, in both species increases in arterial P_{CO_2} were

minor and apparently had no effect on other measured parameters during progressive hypoxia exposure, as described in the accompanying study (Speers-Roesch et al., 2012). The P_{wO_2} end points, rate of O_2 depletion, duration of the progressive hypoxia exposures, and recovery in normoxic water for 60 min are described in the accompanying paper (Speers-Roesch et al., 2012). In some cases, flow probes became damaged by water exposure or by fish movements (typically during the overnight acclimation period) and therefore samples sizes of measured parameters vary slightly (see figure captions for final N values). At the end of the trials, fishes were terminally anaesthetized in seawater containing benzocaine and the ventricle was excised, emptied of blood, blotted dry and weighed.

Data acquisition and calculation of cardiovascular variables

The dorsal aortic cannula was connected to a pressure transducer (Capto SP844 model MLT844, MEMSCAP AS, Skoppum, Norway) calibrated against a static water column with the water surface in the experimental tank serving as the zero pressure reference. The transducer signal was amplified with a ML221 bridge amplifier (ADInstruments, Castle Hill, NSW, Australia). Dorsal aortic blood pressure recordings made in the respirometer were compensated for the small pressure change (~0.5 kPa) that occurred depending on whether the respirometer was open or closed. The cannula was temporarily disconnected to allow for the periodic blood sampling described in the companion paper (Speers-Roesch et al., 2012). Cardiac output was recorded with a Transonic blood flow meter (Model T206, Transonic Systems, Ithaca, NY, USA). Flow probes were calibrated according to manufacturer guidelines at 28°C following the experiment to compensate for the effect of calibration temperature on flow readings. Water P_{O_2} in the respirometer was monitored as described previously (Speers-Roesch et al., 2012). Signal integration and analysis were carried out using a Power Lab unit (ADInstruments) and LabChart Pro software (v. 6.0; ADInstruments), respectively.

Cardiovascular parameters were analysed over 5–10 min sampling periods bracketing P_{wO_2} values at regular intervals that were similar to those used for $\dot{M}_{O_2}$ calculation, from approximately 16.0 to 0.1 kPa in epaulette sharks and approximately 16.0 to 1.9 kPa in shovelnose rays. Because of periodic blood sampling and routine variability in individual traces, it was not possible to analyse data at exactly the same P_{wO_2} values in each fish, so P_{wO_2} values are provided with standard errors. Cardiovascular function during 60 min of recovery in normoxic water was analysed over 5–10 min intervals bracketing each time point (5, 15, 30, 45 and 60 min).

Cardiac output was calculated directly from the flow trace in LabChart Pro and corrected for the estimated loss of flow (63%) due to the location of the flow probes on the ventral aorta, as discussed previously (Lai et al., 1989; Taylor et al., 1977). P_{DA} was calculated using the blood pressure analysis module in LabChart Pro. Because of limited animal numbers, we were unable to directly measure ventral aortic blood pressure (P_{VA}). Therefore, we estimated P_{VA} from our P_{DA} measurements, using a percentage correction (P_{VA}=1.3×P_{DA}) based on previous simultaneous measurements of P_{VA} and P_{DA} in normoxia and hypoxia in epaulette sharks and spotted catsharks (*Scyliorhinus canicula*) (Short et al., 1979; Stensløkken et al., 2004; Taylor et al., 1977). CPO (mW g^{-1} wet ventricular mass) was calculated as the product of P_{VA} (kPa) and $\dot{Q}$ (ml s^{-1}) divided by the wet ventricular mass (g), where 1 J=1 kPa l. Heart rate (f_H) was calculated from the pulsatile pressure or flow trace. Cardiac stroke volume (V_S) was calculated as $\dot{Q}/f_H$ and systemic peripheral resistance (R_{SYS}) was calculated as $P_{DA}/\dot{Q}$, with the assumption that central venous blood pressure is zero. Cardiovascular parameters were plotted against P_{wO_2} to identify the inflection points where each parameter ceased to be independent of P_{wO_2} (i.e. the critical P_{wO_2} of each cardiovascular parameter), as previously described for calculation of P_{crit} of whole-animal $\dot{M}_{O_2}$ (Speers-Roesch et al., 2012).

Experimental series II: tissue metabolic status during relative hypoxia exposure

Experimental protocol

Epaulette sharks (0.388±0.048 kg) and shovelnose rays (1.07±0.110 kg) were transferred from holding tanks to aquaria (~300 l) supplied with aerated recirculating filtered seawater (28°C). Six to 10 epaulette sharks were distributed equally between two aquaria and the same was done for shovelnose rays in two separate aquaria. The fishes were allowed to acclimate for 12 h under well-aerated conditions. Then, one or two fish from the normoxic control group were gently removed from each aquarium and quickly immersed in a bucket of aerated seawater containing benzocaine (0.2 g l^{-1} benzocaine, initially dissolved in 95% ethanol). Animals struggled minimally during this procedure. Following anaesthesia (<1 min), mixed arterial–venous blood was sampled *via* caudal puncture and placed on ice until measurement of whole-blood pH as described before (Speers-Roesch et al., 2012). Following blood sampling, the fish was killed by severing the spinal cord posterior to the head. The heart was quickly removed, emptied of blood, blotted dry and frozen in liquid N_2. White muscle from the caudal peduncle, liver and plasma obtained by centrifuging whole blood were also sampled. Frozen samples were transported to Canada in a dry shipper and kept at –80°C until analysis.

Following sampling of the normoxic fishes, hypoxia was induced by bubbling N_2 into each aquarium, which was covered with plastic bubble wrap to prevent O_2 ingress. Epaulette sharks were exposed to a P_{wO_2} of 2.0 kPa (10% air saturation) and shovelnose rays were exposed to 3.1 kPa (15% air saturation), both of which represented 40% of each species' P_{crit} and thus resulted in a similar level of physiological hypoxia (~16% Hb–O_2 saturation; ~0.6 vol. % arterial O_2 content) (Speers-Roesch et al., 2012). These exposures are referred to henceforth as relative hypoxia. The hypoxic levels were reached after ~30 min of N_2 bubbling. Levels of O_2 were monitored using hand-held O_2 meters and manually adjusted as needed by N_2 bubbling. At 2 and 4 h of hypoxia exposure, fishes were sampled as previously described. This entire protocol was then repeated on subsequent days to achieve a total sample size of 4–7 fish per species and time point.

In a separate trial, the same protocol was repeated except epaulette sharks were exposed to a P_{wO_2} of 1.0 kPa (5% air saturation) and shovelnose rays to 2.0 kPa (10% air saturation) in order to investigate the effects of deeper hypoxia in both species and to compare the effect of a similar level of environmental hypoxia (2.0 kPa) between species. At 2.0 kPa, however, shovelnose rays succumbed to hypoxia in <30 min, negating sampling, but epaulette sharks tolerated 1.0 kPa, allowing sampling at 2 and 4 h, as described previously.

Analytical protocols

Frozen tissue was broken into small pieces under liquid N_2 using an insulated mortar and pestle. For extraction of metabolites, 1.0 ml of ice-cold 1 mol l^{-1} $HClO_4$ was added to a microcentrifuge tube containing 50–100 mg of tissue and the mixture was immediately sonicated on ice for three bursts of 10 s using a Kontes sonicator on its highest setting. An aliquot was frozen at –80°C for

measurement of [glycogen] (Bergmeyer, 1983). The remaining homogenate was centrifuged (10,000 ***g***, 10 min, 4°C) and the supernatant neutralized with 3 mol l^{-1} K_2CO_3. Neutralized extracts were assayed spectrophotometrically for [adenosine triphosphate] ([ATP]), [CrP], [creatine] (heart only), [lactate] and [glucose] following methods described elsewhere (Bergmeyer, 1983). [Glycogen] was corrected for measured endogenous glucose levels. Intracellular pH (pH_i) was measured in frozen heart tissue using previously published methods [(Pörtner et al., 1991), as validated by Baker et al. (Baker et al., 2009)] and a thermostatically controlled (28°C) BMS3 Mk2 capillary microelectrode with PHM84 pH meter (Radiometer, Copenhagen, Denmark). Levels of cardiac free adenosine diphosphate and adenosine monophosphate (ADP_{free} and AMP_{free}) were calculated as described previously (Speers-Roesch et al., 2010). Blood pH and plasma [lactate], [glucose] and [β-HB] were measured as described before (Speers-Roesch et al., 2012).

Statistics

The effects of species and O_2 on cardiovascular measurements (experimental series I) were tested *via* a two-way ANOVA with Holm–Sidak (H–S) *post hoc* tests using data from 11 sampling points of overlapping $P_{W_{O_2}}$ at approximately 16.0, 13.3, 11.7, 10.4, 8.3, 6.1, 5.1, 4.3, 3.1, 2.4 and 1.9 kPa and the points of statistical comparison are denoted by horizontal braces on the figures. Overlapping $P_{W_{O_2}}$ values were not statistically different between species (Student's *t*-test, $P>0.05$). Data from other sampling points were omitted from these analyses. In order to fully assess the effect of O_2 on cardiovascular parameters 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 O_2 on measured parameters was found to be similar for both two-way and one-way ANOVA designs. The critical $P_{W_{O_2}}$ values of cardiovascular variables were compared with each other and between species using a two-way ANOVA with H–S tests. The critical $P_{W_{O_2}}$ of $\dot{M}_{O_2}$ (P_{crit}) measured in the accompanying study (Speers-Roesch et al., 2012) was included in this analysis to determine whether the critical $P_{W_{O_2}}$ of cardiovascular variables coincided with P_{crit}. Cardiovascular recovery values were compared between species and with the normoxic resting values measured at ~16.0 kPa using a two-way ANOVA with H–S tests.

The effects of species and hypoxia exposure on physiological parameters in fishes exposed to relative hypoxia (experimental series II) were tested using a two-way ANOVA with H–S tests. The data for epaulette sharks exposed to a $P_{W_{O_2}}$ of 1.0 kPa for 2 h were omitted from these analyses, but these were compared with the normoxic control using a Student's *t*-test.

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

RESULTS

Experimental series I: cardiovascular responses to progressive hypoxia

A hypoxia-induced bradycardia was observed in both species, with the onset (i.e. the critical $P_{W_{O_2}}$ of heart rate) occurring at a significantly lower $P_{W_{O_2}}$ in the epaulette sharks than in the shovelnose rays (Fig. 1A; Table 1). At $P_{W_{O_2}}$ higher than ~7.3 kPa, f_H was similar in the two species, but at lower $P_{W_{O_2}}$, f_H was greater in epaulette sharks, even after bradycardia commenced in both species (Fig. 1A). f_H showed a plateau below ~3.3 kPa in shovelnose rays and both species showed a maximal f_H depression of ~65% compared with normoxic resting values (Fig. 1A).

The responses of $\dot{Q}$ and CPO to progressive hypoxia exposure paralleled that of f_H, with the decreases commencing at a lower $P_{W_{O_2}}$ in the epaulette sharks than in the shovelnose rays (Table 1; Fig. 1B,C). Above ~6.0 kPa, $\dot{Q}$ was similar in the two species, but it was significantly greater in epaulette sharks at lower $P_{W_{O_2}}$ (Fig. 1B). A maximal depression of $\dot{Q}$ of approximately 50% and 60% was observed in shovelnose rays and epaulette sharks, respectively (Fig. 1B). CPO was significantly higher in epaulette sharks at all $P_{W_{O_2}}$, and at similar $P_{W_{O_2}}$ below the epaulette shark critical point for CPO, the percentage depression from the normoxic resting value was always greater in the shovelnose rays (e.g. at ~3.1 kPa, CPO in epaulette sharks is 30% lower than the resting level compared with 55% lower in shovelnose rays; at ~1.9 kPa, CPO is decreased 40% in epaulette sharks and 75% in shovelnose rays) (Fig. 1C).

In the two species, V_S was similar under normoxic resting conditions and increased by 30–40% in response to progressive hypoxia exposure, with a plateau seen in shovelnose rays below ~3.3 kPa (Fig. 2A). The increase occurred at a significantly lower $P_{W_{O_2}}$ in epaulette sharks than in shovelnose rays (Table 1). A decrease in P_{DA} (and calculated P_{VA}, data not shown) of up to 45% was observed in both species during progressive hypoxia exposure (Fig. 2B). This decrease commenced at a significantly lower $P_{W_{O_2}}$ in epaulette sharks than in shovelnose rays (Table 1). At $P_{W_{O_2}}$ below ~4.0 kPa, P_{DA} was higher in epaulette sharks, whereas at higher $P_{W_{O_2}}$, P_{DA} was similar in the two species (Fig. 2B). Systemic peripheral resistance was similar for the two species and increased modestly during progressive hypoxia exposure in both species but reached statistical significance only for epaulette sharks (Fig. 2C). Critical $P_{W_{O_2}}$ values for R_{SYS} were not calculated because of the absence of major changes with decreasing $P_{W_{O_2}}$.

The relationship between the critical $P_{W_{O_2}}$ values of cardiovascular parameters and the critical $P_{W_{O_2}}$ of $\dot{M}_{O_2}$ (P_{crit}) measured in the accompanying study (Speers-Roesch et al., 2012) were relatively

Table 1. Critical $P_{W_{O_2}}$ (kPa) of cardiovascular parameters and whole-animal oxygen consumption rate in epaulette sharks and shovelnose rays exposed to progressive decreases in $P_{W_{O_2}}$

	Epaulette shark	Shovelnose ray
$\dot{Q}$	5.12±0.95^{a}	8.86±1.13^{†,y}
$\dot{M}_{O_2}$	5.10±0.37^{a}	7.23±0.40^{†,z,y}
CPO	4.80±0.48^{a,b}	7.03±0.59^{†,z,y}
f_H	3.94±0.61^{a,b,c}	7.66±0.59^{†,z,y}
V_S	3.22±0.58^{b,c}	6.99±0.59^{†,z}
P_{DA}	2.61±0.51^{c}	4.78±0.68^{†,x}

See Materials and methods for information on time course and starting and ending $P_{W_{O_2}}$ of the exposures. For comparison, the critical $P_{W_{O_2}}$ of whole-animal oxygen consumption rate (P_{crit}) is included from our accompanying paper (Speers-Roesch et al., 2012). Parameters are listed in descending order of the values for epaulette sharks. Values are means ± s.e.m. (N=5).

$P_{W_{O_2}}$, water P_{O_2}; $\dot{Q}$, cardiac output; $\dot{M}_{O_2}$, whole-animal oxygen consumption rate; CPO, cardiac power output; f_H, heart rate; V_S, stroke volume; P_{DA}, dorsal aortic blood pressure.

†Significant difference between shovelnose and epaulette values. Values with different letters within species are significantly different (two-way ANOVA with Holm–Sidak tests, $P<0.05$). The critical $P_{W_{O_2}}$ for ventral aortic blood pressure (P_{VA}) is the same as for P_{DA} and the critical $P_{W_{O_2}}$ for systemic resistance (R_{SYS}) was not calculated (see text).

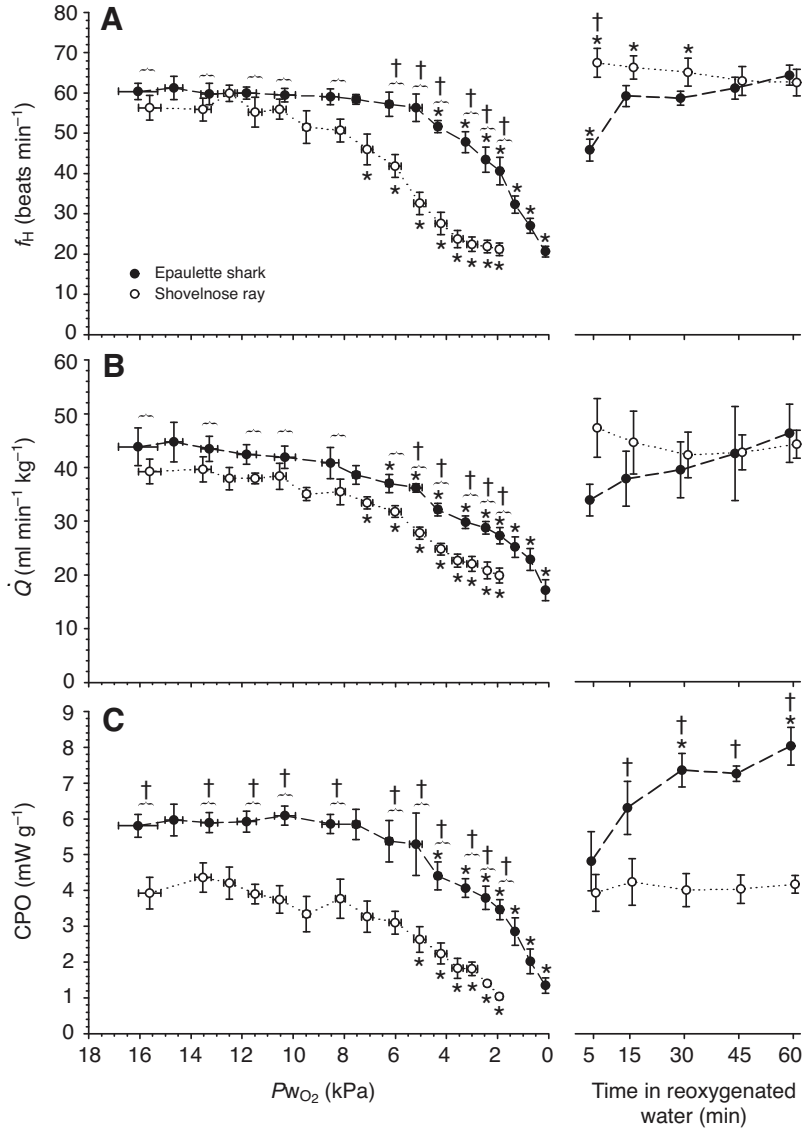

Fig. 1. Heart rate (f_H, A), cardiac output ($\dot{Q}$, B), and cardiac power output (CPO, C) of epaulette sharks and shovelnose rays exposed to progressive decreases in water P_{O_2} (P_{wO_2}) over 135±8 and 71±6 min, respectively, followed by up to 60 min of recovery in normoxic water. Data are means ± s.e.m. (N=5 for both species except epaulette sharks in recovery, where N=4 for f_H and $\dot{Q}$ and N=3 for CPO). Recovery values are offset at each time point for clarity. *Significantly different from the first, normoxic resting value at ≥15.3 kPa within species. †The two species values bracketed by horizontal braces (at statistically similar P_{wO_2}) are significantly different between species; the absence of a dagger indicates the two bracketed species values are not statistically different from each other. For recovery, a dagger denotes a significant difference between epaulette sharks and shovelnose rays at each time point.

similar between species. In each species the critical P_{wO_2} values of $\dot{Q}$, CPO and f_H were statistically similar to one another and to P_{crit} (Table 1). In each species the critical P_{wO_2} of V_S was significantly lower than that of $\dot{Q}$ only, except in epaulette sharks where it was also lower than the P_{crit} (Table 1). In shovelnose rays the critical P_{wO_2} of P_{DA} was significantly lower than that of all other cardiovascular parameters as well as P_{crit} whereas in epaulette sharks the same was true except that the critical P_{wO_2} values for V_S and f_H were not significantly different from P_{DA} (Table 1).

During the first 30 min of normoxic recovery from progressive hypoxia exposure, f_H in shovelnose rays was significantly elevated above the normoxic resting value. In contrast, f_H in epaulette sharks was significantly lower than the resting value after 5 min of recovery, returning to resting values by 15 min of recovery (Fig. 1A). $\dot{Q}$ returned to resting values within 5 min of recovery in both species and remained unchanged for the full 60 min recovery period (Fig. 1B). CPO returned to resting values after 5 min of recovery in both species, but as recovery progressed CPO continued to increase to above resting values in epaulette sharks but not in shovelnose rays (Fig. 1C). The same response was seen for P_{DA} (and P_{VA}, data not shown) during recovery resulting in significantly greater P_{DA} during recovery in epaulette sharks than in shovelnose rays (Fig. 2B). During recovery in both species, V_S and R_{SYS} returned to resting values within 5 min and remained unchanged throughout the 60 min recovery period (Fig. 2A,C).

Experimental series II: tissue metabolic status during relative hypoxia exposure

Cardiac [ATP] was similar in the two species and was unaffected by 4 h of relative hypoxia exposure (Fig. 3A). The two species had similar normoxic levels of cardiac [CrP]. During the relative hypoxia exposure, heart [CrP] was unchanged in epaulette sharks but decreased significantly in the shovelnose rays (Fig. 3B). Cardiac [lactate] was similar in the two species under normoxic conditions and increased significantly during relative hypoxia exposure in shovelnose rays but not in epaulette sharks (Fig. 4A). Cardiac [glucose] was similar in the two species under normoxic conditions and was unaffected by relative hypoxia exposure (Table 2). Cardiac [glycogen] was significantly higher in epaulette sharks than in shovelnose rays in normoxia and relative hypoxia exposure had no effect on cardiac [glycogen] in either species (Table 2). Cardiac [ADP_{free}] and [AMP_{free}] were unchanged by relative hypoxia exposure in epaulette sharks whereas levels of both increased significantly in shovelnose rays (Table 2).

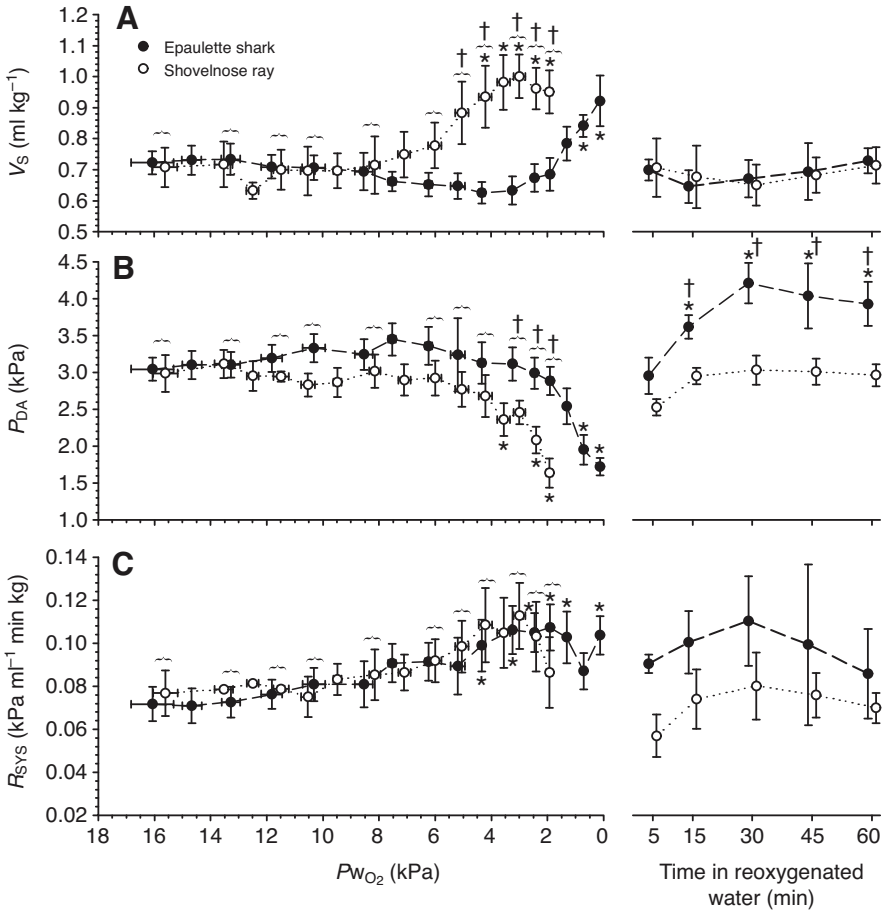

Fig. 2. Stroke volume (V_S, A), dorsal aortic blood pressure (P_{DA}, B), and systemic resistance (R_{SYS}, C) of epaulette sharks and shovelnose rays exposed to progressive decreases in Pw_{O_2} over 135±8 and 71±6 min, respectively, followed by up to 60 min of recovery in normoxic water. Data are means ± s.e.m. (N=5 for both species except epaulette sharks in recovery, where N=4 for V_S and N=3 for P_{DA} and R_{SYS}). Recovery values are offset at each time point for clarity. See Fig. 1 legend for details on symbols denoting statistical comparisons.

Cardiac pH_i was similar between species in normoxia (Fig. 5A). Shovelnose rays showed a significant decrease in cardiac pH_i throughout the relative hypoxia exposure, whereas a significant decrease was not observed until 4 h in epaulette sharks. At 2 h of exposure, epaulette sharks had significantly higher cardiac pH_i compared with shovelnose rays, but not at 4 h (Fig. 5A). Blood pH remained unchanged during relative hypoxia exposure in epaulette sharks, but it decreased significantly in shovelnose rays (Fig. 5B).

White muscle [ATP] was unchanged in both species during relative hypoxia exposure but liver [ATP] decreased (Table 2), and did so more rapidly (within 2 h) in shovelnose rays than in epaulette sharks (within 4 h). White muscle [CrP] was similar in the two species in normoxia but was depleted more rapidly and by a greater amount in shovelnose rays during relative hypoxia exposure. Liver [CrP] was highly variable and unchanged in both species during relative hypoxia exposure (Table 2). Epaulette sharks had higher normoxic resting levels of liver [glycogen] compared with shovelnose rays (Table 2). Relative hypoxia exposure caused no change in white muscle [glycogen] whereas a significant decrease of liver [glycogen] occurred in shovelnose rays but not epaulette sharks (Table 2). White muscle [glucose] decreased after 4 h of relative hypoxia exposure in shovelnose rays but was unchanged in epaulette sharks. In liver, [glucose] was unaffected by relative hypoxia exposure (Table 2). [Lactate] in white muscle increased more rapidly and accumulated to a greater amount in shovelnose rays than in epaulette sharks exposed to relative hypoxia (Fig. 4B). Lactate accumulation in liver, however, was similar for the two species (Fig. 4C).

Similar to heart and white muscle, plasma [lactate] increased significantly during relative hypoxia exposure in both species, but the magnitude of accumulation was greater in shovelnose rays than in epaulette sharks (Fig. 4D). Plasma [glucose] was comparable between species and was unaffected by relative hypoxia exposure (Table 2). Plasma [β-HB] was higher in normoxia in epaulette sharks compared with shovelnose rays but levels were similar in the two species during relative hypoxia exposure because plasma [β-HB] in epaulette sharks decreased significantly whereas levels in shovelnose rays were unchanged (Table 2).

Compared with the normoxic group, the responses of metabolite levels in tissues of epaulette sharks exposed to 2 h of hypoxia at 1.0 kPa generally were similar to those exposed to 2.0 kPa (Table 2). However, the 2 h exposure at 1.0 kPa caused significant decreases in liver [ATP], white muscle [CrP] and cardiac pH_i as well as significant increases in cardiac and white muscle [lactate], which at 2.0 kPa were only apparent after 4 h of exposure (Table 2; Fig. 4A,B; Fig. 5A). Qualitatively, a greater accumulation of plasma and liver [lactate] also was apparent in the epaulette sharks exposed to 1.0 kPa for 2 h compared with 2.0 kPa for 2 h (Fig. 4C,D). Finally, at 1.0 kPa, but not at 2.0 kPa, 2 h of hypoxia exposure caused a significant decrease in cardiac [glycogen] and a trend (P=0.06) of decreased liver [glycogen] (Table 2).

DISCUSSION

The impressive hypoxia tolerance of the epaulette shark is associated with enhanced cardiovascular function and more stable cardiac energy status during hypoxia exposure compared with the less hypoxia-tolerant shovelnose ray. Similar routine levels of cardiovascular function were maintained above P_{crit} in the two species (5.10±0.37 kPa, epaulette shark; 7.23±0.40 kPa, shovelnose ray) (Speers-Roesch et al., 2012). Hypoxic cardiovascular responses occurred at or below P_{crit} and always at a lower Pw_{O_2} in epaulette

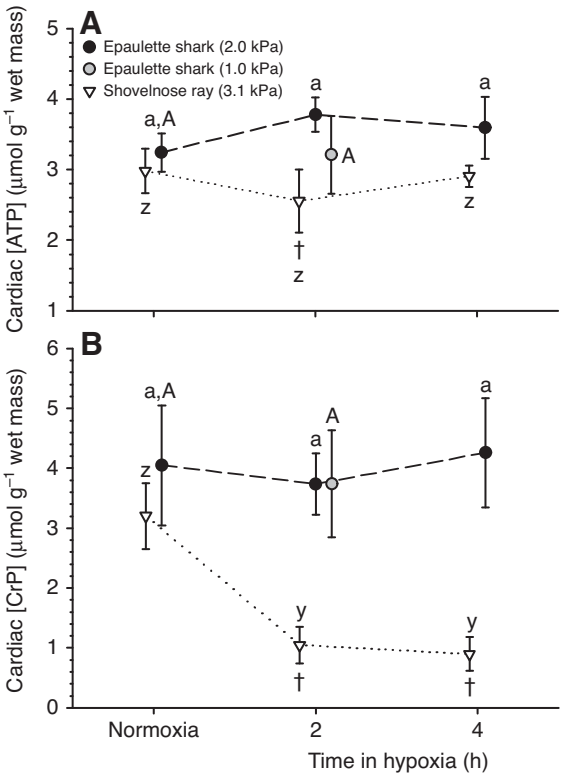

Fig. 3. Cardiac [ATP] (A) and [creatine phosphate] ([CrP], B) in epaulette sharks and shovelnose rays exposed either to normoxia or to 2 and 4 h of hypoxia representing 40% of each species' respective critical O_2 tension of whole-animal O_2 consumption rate, P_{crit} (Pw_{O_2}=2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P_{crit} (Pw_{O_2}=1.0 kPa). Data are means ± s.e.m. (N=5–7 for epaulette sharks; N=4–7 for shovelnose rays). Values are offset at each time point for clarity. Values with different lowercase letters are significantly different from each other within species; between species differences at each time point are denoted by daggers. For epaulette sharks, a significant difference between the 1.0 kPa group and the normoxic group is denoted by different uppercase letters.

sharks. Thus, routine cardiovascular function was maintained to lower Pw_{O_2} in epaulette sharks compared with shovelnose rays. Also, epaulette sharks maintained greater levels of cardiovascular function, including higher CPO, during hypoxia exposure. Depression of cardiac energy demand may be of secondary importance for hypoxia tolerance compared with the ability to maintain greater cardiovascular function during hypoxia exposure.

The ability of epaulette sharks to maintain cardiac energy status and to minimize lactate accumulation and metabolic acidosis during hypoxia exposure is also superior to that of shovelnose rays. This is especially significant because the experimental exposure was carried out at a relative percentage of P_{crit}, therefore equalizing the between-species variation in Ca_{O_2} (Speers-Roesch et al., 2012). Thus, while P_{crit} accurately reflects O_2 transport during hypoxia exposure (Speers-Roesch et al., 2012), it does not necessarily reflect tissue-level hypoxia tolerance, which may be affected by other factors such as tissue-specific metabolic rate depression. The more stable cardiac energy status of the epaulette shark is not apparently related to greater depression of cardiac energy demand, but perhaps is due to greater O_2 delivery to the heart as a result of enhanced $\dot{Q}$ as well as extra-cardiac metabolic depression. Strategic O_2 delivery to the heart, as well as greater blood O_2 capacity (Speers-Roesch et al., 2012), may help explain the enhanced hypoxic cardiovascular function in the epaulette shark compared with the shovelnose ray.

Cardiovascular responses to progressive hypoxia and recovery

With the exception of CPO, which was higher in shovelnose rays, normoxic levels of cardiovascular function were similar between epaulette sharks and shovelnose rays (Figs 1 and 2). The resting f_H and P_{DA} of epaulette sharks match those measured previously in this species (Stensløkken et al., 2004). In epaulette sharks and shovelnose rays, resting f_H, V_S, $\dot{Q}$, P_{DA} and R_{SYS} were similar to those in other elasmobranchs with similar activity levels and accounting for differences in experimental temperature (Butler and Taylor, 1975; Lai et al., 1989; Lai et al., 1990; Sandblom et al., 2006; Satchell et al., 1970). To our knowledge, the only other *in vivo* measurement of elasmobranch CPO is a mass-independent value for spotted catshark (Short et al., 1979), which is, in general, similar to our mass-specific values for epaulette sharks and shovelnose rays. Elasmobranchs have higher routine CPO values than typically seen in teleosts (0.5–3.0 mW g^{-1}) (Speers-Roesch et al., 2010; Stecyk and Farrell, 2006).

Progressive hypoxia exposure elicited a similar cardiovascular response in epaulette sharks and shovelnose rays, including bradycardia, increased V_S and decreased $\dot{Q}$, CPO and P_{DA} (Fig. 1; Fig. 2A,B). Bradycardia induced by hypoxia exposure is common in fishes and has been observed previously in elasmobranchs such as the epaulette shark, spiny dogfish (*Squalus acanthias*) and spotted catshark (Butler and Taylor, 1975; Sandblom et al., 2009; Stensløkken et al., 2004). Whereas spotted catsharks and spiny dogfish appear to achieve hypoxia-induced bradycardia *via* vagally mediated cholinergic inhibition, this is not the case in epaulette sharks where the mechanism is unclear (Stensløkken et al., 2004); nothing is known about mechanisms of hypoxic bradycardia in shovelnose rays. Acidosis has a negative chronotropic effect in fishes but does not appear to be a primary cause of bradycardia in epaulette sharks or shovelnose rays because, as shown in the accompanying study (Speers-Roesch et al., 2012), blood pH decreased at a lower Pw_{O_2} compared with the commencement of bradycardia [cf. fig. 5B in the accompanying study (Speers-Roesch et al., 2012) and Fig. 1A in the present study]. Bradycardia was initiated immediately below the Pw_{O_2} corresponding to a Pa_{O_2} just below the Hb–O_2 P_{50} in each species (Speers-Roesch et al., 2012) and direct hypoxaemic effects on f_H probably contributed to the bradycardia**.** Further studies are needed to elucidate the mechanisms of hypoxic bradycardia in elasmobranchs and especially the epaulette shark, including the suggested involvement of α-adrenoceptors (Stensløkken et al., 2004).

Stroke volume increased in epaulette sharks and shovelnose rays during progressive hypoxia exposure but $\dot{Q}$ still decreased because of a large depression of f_H (Fig. 1B; Fig. 2A). The large fall in $\dot{Q}$ with only a modest increase in R_{SYS} resulted in the observed reduction in P_{DA} in epaulette sharks and shovelnose rays, although the critical Pw_{O_2} for P_{DA} was lower than that for $\dot{Q}$ (Table 1). Epaulette sharks show no change in gill resistance during hypoxia exposure so this is not likely to contribute to reduced P_{DA} in this species (Stensløkken et al., 2004). The absence of a barostatic reflex to maintain arterial blood pressure is consistent with previous studies showing that the reflex is weak in response to hypotension in fishes and especially elasmobranchs (Olson and Farrell, 2006). Additionally, the barostatic reflex may be reset in the face of hypoxia-induced bradycardia in fishes (Stecyk and Farrell, 2006). Like the two species in the present study, an increase in V_S and a

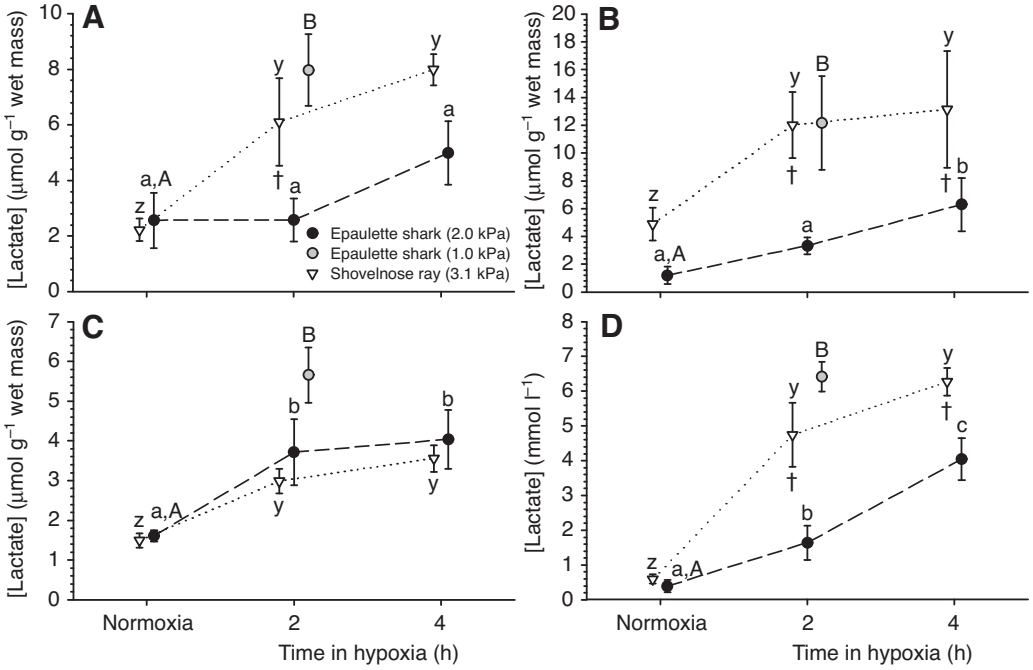

Fig. 4. [Lactate] in heart (A), white muscle (B), liver (C) and mixed arterial–venous plasma (D) in epaulette sharks and shovelnose rays exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species' respective P_{crit} (Pw_{O_2}=2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P_{crit} (Pw_{O_2}=1.0 kPa). Data are means ± s.e.m. (N=5–7 for epaulette sharks; N=4–7 for shovelnose rays). Values are offset at each time point for clarity. See Fig. 3 legend for details on symbols denoting statistical comparisons.

decrease in P_{DA} were seen in spotted catsharks exposed to progressive hypoxia, but no decrease in $\dot{Q}$ was observed, possibly because only moderate hypoxia was achieved (Butler and Taylor, 1975). In constrast, in spiny dogfish exposed to severe hypoxia, Sandblom and colleagues (Sandblom et al., 2009) observed decreases in $\dot{Q}$ and V_S but P_{DA} was unchanged. These data suggest that the pattern of cardiovascular responses to hypoxia, in particular V_S and P_{DA}, vary among elasmobranchs, as they do in teleosts.

Epaulette sharks and shovelnose rays showed a similar pattern of cardiovascular responses to progressive hypoxia and it remains unclear whether a specific suite of hypoxic cardiovascular responses correlates with hypoxia tolerance in fishes. There were, however, two key differences between the species: (1) all hypoxic cardiovascular responses in epaulette sharks occurred at a significantly lower Pw_{O_2} compared with shovelnose rays (Table 1); and (2) despite the presence of similar routine levels of cardiovascular function (i.e. above the critical Pw_{O_2} of each parameter; Table 1), the level of function of most parameters was greater at the same hypoxic Pw_{O_2} (below the critical Pw_{O_2}) in the epaulette shark compared with the shovelnose ray (Fig. 1; Fig. 2A,B). In other words, compared with the shovelnose ray, the more hypoxia-tolerant epaulette shark can maintain cardiovascular function at routine levels to lower Pw_{O_2} and once hypoxic responses commence the change from routine levels is smaller. This may benefit hypoxia tolerance by improving O_2 delivery and management of wastes.

The decreases in f_H, $\dot{Q}$ and CPO in epaulette sharks and shovelnose rays coincided with the decreases in whole-animal $\dot{M}_{O_2}$ seen during progressive hypoxia (Speers-Roesch et al., 2012) and the critical Pw_{O_2} of these parameters were statistically similar within species (Table 1). A correlation between P_{crit} and the critical Pw_{O_2} value of heart rate appears to be a common phenomenon among fishes (Fig. 6), suggesting that P_{crit} is a good predictor of the point at which hypoxic bradycardia is initiated. Likewise, co-occurrence of P_{crit} and the critical Pw_{O_2} of $\dot{Q}$ and CPO also has been observed in fishes (Iversen et al., 2010; Speers-Roesch et al., 2010). These relationships are perhaps unsurprising considering the link between $\dot{M}_{O_2}$ and convective O_2 delivery (Webber et al., 1998). Other hypoxic cardiovascular responses (e.g. V_S, P_{DA}) were not necessarily correlated with P_{crit}, although they always occurred at or below P_{crit} (Table 1). Overall, these data suggest that in fishes exposed to hypoxia, P_{crit} indicates the lowest Pw_{O_2} at which routine cardiovascular function is maintained and this may explain in part why epaulette sharks maintain routine cardiovascular function to lower Pw_{O_2}. However, P_{crit} may not fully predict the capacity for hypoxic cardiovascular function at Pw_{O_2} below P_{crit}, because at the same relative percentages below P_{crit} cardiovascular function remained closer to routine levels in epaulette sharks compared with shovelnose rays (e.g. Fig. 1; Fig. 2A,B). Because at relative percentages of P_{crit} the $\dot{M}_{O_2}$ and Ca_{O_2} are similar in the two species, this observation also suggests that the improved cardiovascular performance at Pw_{O_2} below P_{crit} in epaulette sharks cannot be explained completely by its lower P_{crit} and greater Ca_{O_2} (Speers-Roesch et al., 2012).

Cardiac energy demand as measured by CPO decreased as whole-animal $\dot{M}_{O_2}$ fell in both epaulette sharks and shovelnose rays [cf. Fig. 1C in present study and fig. 1 in the accompanying paper (Speers-Roesch et al., 2012)], probably due to the depression of $\dot{Q}$ associated with bradycardia. A similar result has been observed in tilapia (Speers-Roesch et al., 2010). Unlike tilapia, however, reductions in P_{DA} and P_{VA} probably also contribute to depressed CPO during hypoxia exposure in elasmobranchs, including epaulette sharks (Fig. 2B) (Stensløkken et al., 2004). Our results support previous findings showing that the depression of CPO is an integral component of hypoxia-induced whole-animal metabolic rate depression in many fishes that may contribute to hypoxia tolerance

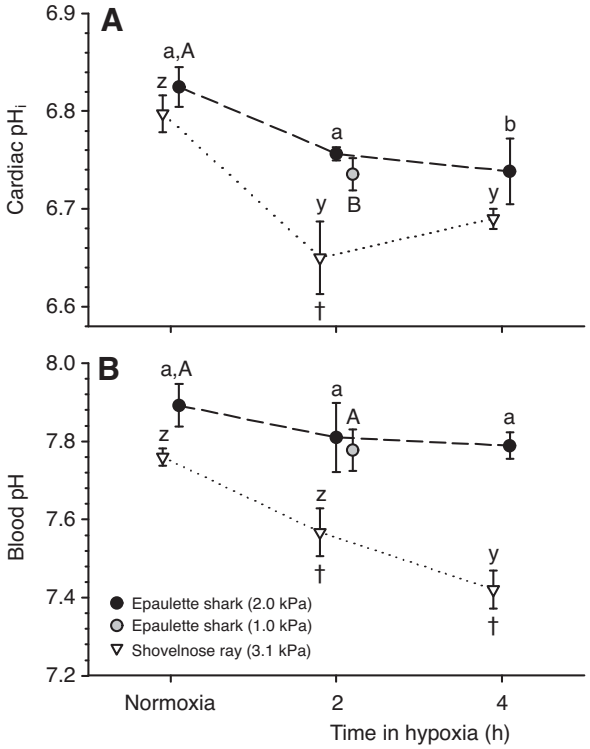

Fig. 5. Cardiac intracellular pH (pH$_i$, A) and blood (mixed arterial–venous) pH (B) in epaulette sharks and shovelnose rays exposed either to normoxia or to 2 h and 4 h of hypoxia representing 40% of each species' respective P_{crit} (Pw_{O_2}=2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P_{crit} (Pw_{O_2}=1.0 kPa). Data are means ± s.e.m. (pH$_i$, N=4 for epaulette sharks and N=4–7 for shovelnose rays; blood pH, N=4–6 for epaulette sharks and N=3–6 for shovelnose rays). Values are offset at each time point for clarity. See Fig. 3 legend for details on symbols denoting statistical comparisons.

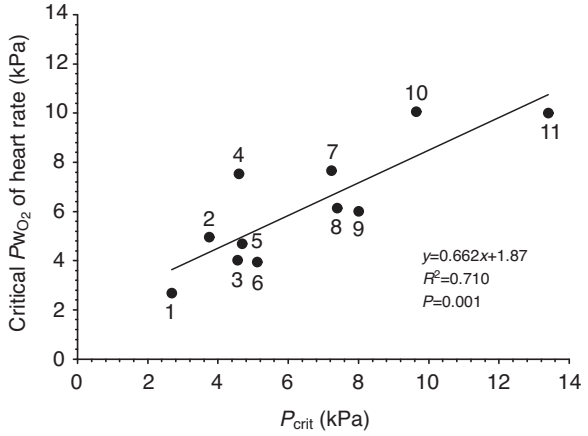

Fig. 6. The relationship between P_{crit} and critical Pw_{O_2} of heart rate (i.e. Pw_{O_2} at initiation of hypoxia-induced bradycardia) in fishes. (1) *Hoplias malabaricus*, 25°C (Rantin et al., 1993); (2) *Oreochromis* hybrid, 22°C (Speers-Roesch et al., 2010); (3) *Piaractus mesopotamicus*, 25°C (Rantin et al., 1998); (4) *Leiopotherapon unicolor*, 25°C (Gehrke and Fielder, 1988); (5) *Hoplias lacerdae*, 25°C (Rantin et al., 1993); (6) *Hemiscyllium ocellatum*, 28°C, present study and accompanying paper (Speers-Roesch et al., 2012); (7) *Aptychotrema rostrata*, 28°C, present study and accompanying paper (Speers-Roesch et al., 2012); (8) *Gadus morhua*, 10°C (McKenzie et al., 2009); (9) *Gadus morhua*, 10°C (Petersen and Gamperl, 2011); (10) *Scyliorhinus canicula*, 12°C (Butler and Taylor, 1975) (values estimated from graphical data); (11) *Anguilla anguilla*, 20°C (Iversen et al., 2010). The linear regression is significant (P=0.001, linear regression ANOVA).

by matching cardiac energy demand to lowered energy supply (Farrell and Stecyk, 2007; Speers-Roesch et al., 2010). There was no evidence, however, that depression of CPO was associated with the greater hypoxia tolerance of the epaulette shark, because epaulette sharks maintained a higher CPO at all similar Pw_{O_2}, even when expressed as a percentage of the normoxic resting level (Fig. 1C). Thus, CPO in epaulette sharks was maintained to lower Pw_{O_2} and at higher levels during hypoxia compared with shovelnose rays, probably because of the similar maintenance of f_H, $\dot{Q}$ and P_{DA}. Only at ~0.1 kPa did epaulette sharks reach the level of CPO depression seen at the lowest Pw_{O_2} (~2.0 kPa) tolerated by shovelnose rays, and at or below 0.1 kPa epaulette sharks (and presumably their hearts) remain responsive for at least 45 min (Renshaw et al., 2002). While flow traces did not show marked cardiac arrhythmia in shovelnose rays or epaulette sharks, the plateau of f_H and $\dot{Q}$ coupled with the continual decrease in CPO and P_{DA} below a Pw_{O_2} of ~2.7 kPa in shovelnose rays (Fig. 1; Fig. 2B) could be symptomatic of a failing heart, whereas no such pattern was evident for epaulette sharks.

Cardiovascular function returned to routine levels within 60 min of normoxic recovery following progressive hypoxia exposure in both epaulette sharks and shovelnose rays, with the exception of CPO in epaulette sharks, which steadily increased over time due to an increase in P_{DA} (Fig. 1C; Fig. 2B). Shovelnose rays had elevated f_H during the initial stage of recovery whereas epaulette sharks showed a non-elevated, gradual return to routine f_H (Fig. 1A). Cardiac output showed qualitatively the same trends as f_H (Fig. 1B). Further studies are needed to ascertain the role of these different cardiovascular responses for recovery from hypoxia exposure in epaulette sharks and shovelnose rays.

Metabolic status during relative hypoxia exposure

To investigate the effects of hypoxia on tissue metabolic status and to test the hypothesis that P_{crit} dictates tissue-level hypoxia tolerance, we exposed epaulette sharks and shovelnose rays for up to 4 h to a Pw_{O_2} representing 40% of the respective P_{crit}, which resulted in a similar level of arterial hypoxaemia in the two species [see fig. 2B in the companion paper (Speers-Roesch et al., 2012); interpolated Ca_{O_2} at 40% of respective P_{crit} is ~0.6 vol.% in both species]. Contrary to our prediction of similar interspecific metabolic status under these conditions, we found greater decreases in pH (Fig. 5), accumulation of lactate (Fig. 4A,B,D), and perturbation of tissue energy status (Fig. 3; Table 2) in shovelnose rays than in epaulette sharks. Perturbations of pH and energy status as well as high lactate load are thought to contribute to hypoxic death and hypoxia-tolerant animals are able to avoid or at least postpone such perturbation (Nilsson and Östlund-Nilsson, 2008). The more stable metabolic state of the epaulette shark compared with the shovelnose ray at a similar level of arterial hypoxaemia suggests that other factors work in concert with enhanced O_2 supply to explain the epaulette shark's superior hypoxia tolerance, possibly including effective acid–base regulation, metabolic rate depression and strategic utilization of O_2 (see below).

In the heart, [ATP] was stable in both species but whereas [CrP] was stable in epaulette sharks, it fell in shovelnose rays, leading to increases in [ADP$_{free}$] and [AMP$_{free}$] (Fig. 3; Table 2). The maintenance of cardiac energy status in epaulette sharks *vs* perturbation in shovelnose rays is consistent with previous results

Table 2. Levels of selected metabolites of energy metabolism in heart, liver, white muscle and plasma of epaulette sharks and shovelnose rays exposed either to normoxia or to 2 and 4 h of hypoxia representing 40% of each species' respective P_{crit} (Pw_{O_2}=2.0 and 3.1 kPa, respectively) and in epaulette sharks exposed to 20% of P_{crit} (Pw_{O_2}=1.0 kPa)

	Epaulette shark				Shovelnose ray		
	Normoxia	2 h at 2.0 kPa	4 h at 2.0 kPa	2 h at 1.0 kPa	Normoxia	2 h at 3.1 kPa	4 h at 3.1 kPa
Heart							
[ADP_{free}]	19.3±3.8^{a}	25.2±4.5^{a}	15.4±6.0^{a}	22.1±6.3	17.2±5.9^{z}	39.1±6.3^{y}	84.1±25.2^{y,†}
[AMP_{free}]	0.22±0.07^{a}	0.25±0.08^{a}	0.12±0.07^{a}	0.29±0.16	0.23±0.14^{z}	1.03±0.32^{y}	4.20±1.92^{y,†}
[Glycogen]	80.4±4.4^{a}	77.6±14.4^{a}	67.7±10.4^{a}	43.4±10.6*	54.7±8.0^{z,†}	53.0±10.8^{z}	52.8±8.8^{z}
[Glucose]	1.09±0.19^{a}	1.27±0.46^{a}	1.42±0.56^{a}	1.13±0.48	1.01±0.14^{z}	0.63±0.28^{z,†}	0.55±0.14^{z}
Liver							
[ATP]	1.41±0.14^{a}	1.10±0.25^{a}	0.44±0.07^{b}	0.37±0.06*	0.79±0.09^{z,†}	0.22±0.07^{y,†}	0.24±0.06^{y}
[CrP]	0.94±0.75^{a}	1.00±0.93^{a}	0.78±0.43^{a}	2.25±1.28	0.11±0.04^{z}	1.10±0.41^{z}	0.82±0.63^{z}
[Glycogen]	75.0±26.5^{a}	66.4±23.3^{a}	24.4±16.3^{a}	15.7±7.2	21.3±4.6^{z,†}	2.7±1.3^{y,†}	7.8±5.4^{y}
[Glucose]	2.92±0.24^{a}	3.33±0.75^{a}	2.81±0.86^{a}	4.21±1.37	3.20±0.22^{z}	1.12±0.42^{z}	2.36±0.46^{z}
White muscle							
[ATP]	5.91±0.84^{a}	6.59±0.82^{a}	5.54±1.13^{a}	7.40±0.41	7.88±0.37^{z}	8.33±0.85^{z}	7.95±0.70^{z}
[CrP]	41.7±2.7^{a}	38.1±1.5^{a,b}	32.7±3.1^{b}	25.7±4.1*	34.2±1.4^{z}	23.0±3.5^{y,†}	21.0±5.9^{y,†}
[Glycogen]	22.8±3.2^{a}	23.4±5.1^{a}	16.1±3.5^{a}	22.0±4.9	16.8±3.4^{z}	18.6±4.8^{z}	17.9±7.0^{z}
[Glucose]	0.20±0.08^{a}	0.19±0.07^{a}	0.30±0.13^{a}	0.37±0.07	0.39±0.09^{z}	0.14±0.10^{z}	0.03±0.03^{y,†}
Plasma							
[Glucose]	1.87±0.12^{a}	1.86±0.64^{a}	2.23±0.67^{a}	1.47±0.49	1.51±0.13^{z}	0.86±0.29^{z}	0.72±0.13^{z,†}
[β-HB]	0.97±0.13^{a}	0.52±0.05^{b}	0.34±0.07^{b}	0.57±0.10*	0.12±0.04^{z,†}	0.29±0.10^{z}	0.26±0.08^{z}

Values are means ± s.e.m. (N=5–8 for epaulette sharks; N=4–7 for shovelnose rays).

All data are expressed as μmol g^{-1} wet mass except glycogen (μmol glucosyl units g^{-1} wet mass), ADP_{free} and AMP_{free} (nmol g^{-1} wet mass), and plasma glucose and β-HB (mmol l^{-1}).

β-HB, β-hydroxybutyrate; CrP, creatine phosphate.

Within each species, values with the same letter are not significantly different from one another (two-way ANOVA with Holm–Sidak *post hoc* test, P<0.05).

†Value for shovelnose ray is significantly different from the value for epaulette shark in normoxia or at the same time point at 2.0 kPa (two-way ANOVA with Holm–Sidak *post hoc* test, P<0.05). *Epaulette shark value for 2 h at 1.0 kPa is significantly different from the epaulette shark normoxia value (Student's *t*-test, P<0.05)

for hypoxia-tolerant *vs* -sensitive fishes exposed to hypoxia (Dunn and Hochachka, 1986; Jorgensen and Mustafa, 1980; Speers-Roesch et al., 2010). Although [CrP] stabilized at a lower level in hypoxia-exposed shovelnose rays, this may not represent a stable functional state considering that the increase of inorganic phosphate associated with CrP depletion, rather than ATP depletion, is thought to be a major contributor to hypoxic heart failure in mammals and fishes (Arthur et al., 1992; Neubauer, 2007). Notably, the Pw_{O_2} of the relative hypoxia exposure of shovelnose rays was only marginally higher than the Pw_{O_2} where possible signs of cardiac failure were seen during the progressive hypoxia exposure (see above). The energy status measurements suggest that matching of cardiac energy supply and demand during hypoxia exposure is much less perturbed in epaulette sharks than in shovelnose rays, even when hypoxaemia is similar. Under the more severe hypoxia exposure (Pw_{O_2}=1.0 kPa), epaulette shark hearts still maintained stable energy status (Fig. 3), suggesting that energy supply and demand remain well matched even under severe hypoxic conditions where shovelnose rays cannot survive.

The ability of epaulette sharks to better match cardiac energy supply and demand is not due to a greater depression of energy demand (i.e. CPO) compared with shovelnose rays because epaulette sharks actually maintain higher levels of CPO during progressive hypoxia exposure, including when levels are compared at the species-specific Pw_{O_2} values used in the relative hypoxia exposure (Fig. 1C). Although CPO in epaulette sharks may have decreased more over the duration of the relative hypoxia exposure compared with the shorter progressive hypoxia exposure, we consider this unlikely because the first sample time point (2 h) of the relative hypoxia exposure is comparable in duration to the progressive hypoxia exposure. Also, in other fish, levels of CPO and other heart parameters measured at each Pw_{O_2} during progressive hypoxia exposure appear to be a good indicator of the levels that are seen during prolonged exposure at the same, stable Pw_{O_2} (Speers-Roesch et al., 2010).

Strategic delivery of available O_2 to the heart during relative hypoxia exposure could explain, in part, how a stable cardiac energy status was maintained alongside greater cardiac function in epaulette sharks but not shovelnose rays, despite similar arterial hypoxaemia. Consistent with this hypothesis, there was minimal lactate accumulation in the heart of epaulette sharks and greater lactate accumulation in the heart of shovelnose rays during relative hypoxia exposure (Fig. 4A), even though cardiac energy demand was higher in the epaulette sharks (see above). Although not investigated in the present study, coronary perfusion of the myocardium could be more extensive in epaulette sharks, improving myocardial O_2 supply compared with shovelnose rays. In epaulette sharks, hypoxia exposure causes changes in gill blood flow that may deliver blood directly to the heart (Stensløkken et al., 2004). Also, whole-animal metabolic rate depression, especially in non-essential tissues such as white muscle, could be greater in the epaulette sharks than in shovelnose rays, thus sparing O_2 for the heart. The lesser accumulation of lactate in white muscle and plasma in epaulette sharks (Fig. 4B,D) supports this hypothesis because, at similar arterial hypoxaemia, lactate accumulation can be used as a rough proxy to compare energy demand between species. Liver [lactate] was similar between species (Fig. 4C), but in this case interpretation

is complicated by the potential accumulation of lactate in liver for glycogen or glucose synthesis. A caveat is that the relative hypoxia exposure did not control for the higher $\dot{Q}$ seen during hypoxia exposure in epaulette sharks (Fig. 1B), which may facilitate tissue O_2 delivery in this species despite Ca_{O_2} being the same in the two species. This may explain some of the species differences in lactate accumulation in non-cardiac tissues. Unfortunately, logistical constraints in the present study negated measurement of O_2 content in blood returning to the heart and use of the Fick method produced unreliable estimates of venous O_2 content, consistent with previous findings showing the inaccuracy of the Fick method during hypoxia exposure in elasmobranchs (Metcalfe and Butler, 1982). Further studies are needed to directly assess the idea that strategic cardiac utilization of O_2 contributes to the superior hypoxia tolerance of epaulette sharks.

There was no increase in plasma [glucose] during relative hypoxia exposure in either species, similar to the results of the progressive hypoxia exposure (Speers-Roesch et al., 2012) and similar to previous studies on epaulette sharks and other elasmobranchs exposed to hypoxia (Routley et al., 2002; Speers-Roesch and Treberg, 2010). However, glycogen was mobilized in the liver (Table 2), suggesting that glucose flux increases. Plasma [β-HB] decreased during relative hypoxia exposure in the epaulette sharks (Table 2). The role of ketone bodies during hypoxia exposure in epaulette sharks warrants attention considering the protective effects of ketone bodies seen in mammalian ischaemia and because of the parallels with the hypoxic decreases of plasma free fatty acids seen in some hypoxia-tolerant teleosts, which could be related to metabolic rate depression (Speers-Roesch and Treberg, 2010; Speers-Roesch et al., 2010).

CONCLUSIONS

The results of the present study and the accompanying study (Speers-Roesch et al., 2012) show that in comparison with the relatively hypoxia-sensitive shovelnose ray, the hypoxia-tolerant epaulette shark possesses greater blood O_2 transport, greater cardiovascular function and superior maintenance of pH and cardiac energy status during hypoxia exposure. These attributes probably contribute to the exceptional hypoxia tolerance of the epaulette shark and generally may be hallmarks of hypoxia tolerance in fishes. The enhanced hypoxic cardiorespiratory performance of the epaulette shark also highlights the importance for hypoxia tolerance of maintenance of a superior O_2 supply including cardiovascular function in order to optimize aerobic energy production during hypoxia exposure.

A regulated depression of CPO in fishes is thought to be an important component of cardiac and consequently whole-animal hypoxia tolerance (Farrell and Stecyk, 2007; Speers-Roesch et al., 2010). We hypothesized that the hypoxia-tolerant epaulette shark would show greater hypoxia-induced depression of CPO compared with the hypoxia-sensitive shovelnose ray. In fact, the epaulette shark's greater hypoxic cardiovascular function was associated with a smaller reduction of CPO compared with the shovelnose ray (Fig. 1C). Although this finding does not refute the importance of depression of CPO for hypoxia tolerance, it does suggest that rather than outright cardiac depression, the maintenance of higher levels of cardiac function and therefore energy demand may be an equally important strategy for hypoxia tolerance in fishes because of the benefits for O_2 supply and management of fuel and waste. Interestingly, unlike shovelnose rays, epaulette sharks appear to be able to avoid perturbation of cardiac energy status during hypoxia exposure (Fig. 3) despite maintaining a higher cardiac energy demand. Considering that this relative hypoxia exposure equalized Ca_{O_2} between species, these results and those for tissue [lactate] imply that during hypoxia exposure, O_2 delivery to the heart in epaulette sharks is superior to that of shovelnose rays, possibly in part due to O_2 sparing related to metabolic depression in non-essential tissues. Further studies are needed to test this hypothesis directly. Overall, epaulette sharks, unlike shovelnose rays, appear to be able to coordinate depression of cardiac energy demand (i.e. decreases in CPO) with improvements in cardiac energy supply (e.g. O_2 supply) in order to achieve stable cardiac energy status, enhanced cardiac function and, consequently, improved hypoxia tolerance.

Finally, the present study provides insight into the use of P_{crit} as a measure of hypoxia tolerance in fishes. Previous work has shown that P_{crit} is an excellent indicator of the ability of a fish to take up and transport O_2 at low Pw_{O_2} (Mandic et al., 2009; Speers-Roesch et al., 2012). Here, we also provide support for the idea that P_{crit} provides an indication of the ability of a fish to maintain routine cardiovascular function to low Pw_{O_2}. However, data from our relative hypoxia exposure show that P_{crit} does not necessarily determine the metabolic status and hypoxia tolerance of tissues, where metabolic depression may also play a major role. Overall, our results suggest that P_{crit} is an important measure of respiratory hypoxia tolerance and that improved hypoxic O_2 supply associated with a low P_{crit} is only one, albeit important, component of hypoxia tolerance in fishes.

LIST OF SYMBOLS AND ABBREVIATIONS

$\dot{Q}$	cardiac output
β-HB	β-hydroxybutyrate
ADP_{free}	free adenosine diphosphate
AMP_{free}	free adenosine monophosphate
ATP	adenosine triphosphate
Ca_{O_2}	arterial blood O_2 content
CPO	cardiac power output
CrP	creatine phosphate
f_H	heart rate
Hb	haemoglobin
Hb–O_2 P_{50}	haemoglobin–O_2 binding affinity
$\dot{M}_{O_2}$	whole-animal O_2 consumption rate
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_{DA}	dorsal aortic blood pressure
pH_i	intracellular pH
P_{O_2}	partial pressure of O_2
P_{VA}	ventral aortic blood pressure
Pw_{O_2}	water P_{O_2}
R_{SYS}	systemic peripheral resistance
V_S	stroke volume

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