

## RESEARCH ARTICLE

# Ocean acidification does not limit squid metabolism via blood oxygen supply

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## ABSTRACT

Ocean acidification is hypothesized to limit the performance of squid owing to their exceptional oxygen demand and pH sensitivity of blood–oxygen binding, which may reduce oxygen supply in acidified waters. The critical oxygen partial pressure ( $P_{crit}$ ), the  $P_{O_2}$  below which oxygen supply cannot match basal demand, is a commonly reported index of hypoxia tolerance. Any  $CO_2$ -induced reduction in oxygen supply should be apparent as an increase in  $P_{crit}$ . In this study, we assessed the effects of  $CO_2$  (46–143 Pa; 455–1410  $\mu$ atm) on the metabolic rate and  $P_{crit}$  of two squid species – *Dosidicus gigas* and *Doryteuthis pealeii* – through manipulative experiments. We also developed a model, with inputs for hemocyanin pH sensitivity, blood  $P_{CO_2}$  and buffering capacity, that simulates blood oxygen supply under varying seawater  $CO_2$  partial pressures. We compare model outputs with measured  $P_{crit}$  in squid. Using blood– $O_2$  parameters from the literature for model inputs, we estimated that, in the absence of blood acid–base regulation, an increase in seawater  $P_{CO_2}$  to 100 Pa ( $\approx$ 1000  $\mu$ atm) would result in a maximum drop in arterial hemocyanin– $O_2$  saturation by 1.6% at normoxia and a  $P_{crit}$  increase of  $\approx$ 0.5 kPa. Our live-animal experiments support this supposition, as  $CO_2$  had no effect on measured metabolic rate or  $P_{crit}$  in either squid species.

**KEY WORDS:** Acid–base balance, Blood– $O_2$  binding, Hypercapnia, Cephalopod, Hypoxia tolerance, *Dosidicus*

## INTRODUCTION

Atmospheric carbon dioxide ( $CO_2$ ) partial pressure ( $P_{CO_2}$ ) has increased from the pre-industrial mean of 28 Pa (280  $\mu$ atm, ppmv) to over 40 Pa ( $\approx$ 400  $\mu$ atm) today (Caldeira and Wickett, 2005) and may reach 100 Pa (1000  $\mu$ atm) by the year 2100 (IPCC, 2014). Elevated environmental  $P_{CO_2}$  will influence marine organisms indirectly via global warming. However, anthropogenic  $CO_2$  also diffuses into the ocean, where it reacts with water, resulting in reduced pH. This phenomenon, known as ocean acidification (OA), may affect animal performance in numerous ways (Fabry et al., 2008; Clements and Hunt, 2015). For example, it has been proposed that OA may impair the oxygen ( $O_2$ ) supply capacity of marine animals via its effect on pH-sensitive respiratory proteins (Widdicombe and Spicer, 2008; Fabry et al., 2008; Pörtner, 2012; Miller et al., 2016; Seibel, 2016). Even small losses in  $O_2$  supply capacity could hinder an animal's exercise performance or environmental hypoxia tolerance.

Shallow-water active squid, such as those in the families Loliginidae and Ommastrephidae, are ideal study organisms for examining impacts of OA on  $O_2$  supply because their respiratory proteins (hemocyanins) are among the most pH sensitive of any marine animal (Brix et al., 1989; Bridges, 1994; Pörtner and Reipschläger, 1996; Seibel, 2016). Sensitivity to pH, quantified as the Bohr coefficient (Bohr et al., 1904), is optimal for  $O_2$  delivery to the tissues at half the respiratory quotient (Lapennas, 1983), which would be between  $-0.35$  and  $-0.5$  in cephalopods. Squid hemocyanin, however, often has a Bohr coefficient of  $<-1$  (Bridges, 1994). The extreme sensitivity in cephalopods may result in large impairments in blood– $O_2$  binding affinity from relatively small changes in blood pH.

Furthermore, hemocyanins are not contained within red blood cells, but are freely dissolved in the blood, limiting their concentration owing to viscosity and osmotic constraints. Unlike fishes or invertebrates with red blood cells, which can increase hemoglobin concentration or hematocrit to increase  $O_2$  supply (Johansen and Weber, 1976), squid are constrained in their blood  $O_2$  carrying capacity, and are fully dependent on their cardiovascular system for  $O_2$  delivery (Birk et al., 2018). Squid are thought to utilize most of the  $O_2$  available in their blood with very little venous reserve even under resting conditions (Wells, 1992; Pörtner, 1994). Cephalopods, unlike fishes and crustaceans, are not known to rely on organic cofactors such as adenosine phosphates and lactate to modify hemocyanin– $O_2$  affinity (Johansen and Weber, 1976; Mangum, 1997).

Such physiological considerations have led to the concern that, in the absence of acclimation or adaptation, squid metabolism may be strongly affected by OA (Pecl and Jackson, 2007; Seibel, 2016). In fact, Pörtner (1990) estimated that a 0.1–0.15 unit decrease in arterial pH would be lethal for active squid. Redfield and Goodkind (1929) found that blood– $O_2$  transport in the loliginid squid *Doryteuthis pealeii* was impaired by acute exposures (10–15 min) to  $P_{CO_2}$  levels up to 3200 Pa (31,500  $\mu$ atm  $CO_2$ ), resulting in death. Rosa and Seibel (2008) reported reduced metabolic rate and activity at much more modest  $CO_2$  levels (100 Pa; 1000  $\mu$ atm), which they attributed to the high pH sensitivity of hemocyanin in *Dosidicus gigas*. Similar results have been found in embryonic squid exposed to 170 Pa (1650  $\mu$ atm)  $CO_2$  (Rosa et al., 2014).

However, Hu et al. (2014) found no effect of 160 Pa (1600  $\mu$ atm)  $CO_2$  on metabolism even after 1 week of exposure in the loliginid squid *Sepioteuthis lessoniana*. Cuttlefish, which have lower  $O_2$  demand but hemocyanin with similarly high pH sensitivity, also exhibited no effect on metabolism after 24 h or growth rate over 40 days at  $P_{CO_2}$  levels up to 615 Pa (6100  $\mu$ atm) (Gutowska et al., 2008). Such tolerances may be attributed to the high capacity for blood acid–base regulation in most cephalopods (Melzner et al., 2009; Hu and Tseng, 2017). The studies to date are not directly comparable, each having employed a different species,  $P_{CO_2}$  level, exposure duration and method. Thus, the variable results are

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**List of symbols and abbreviations**

DML	dorsal mantle length
$M_{O_2}$	metabolic rate
OA	ocean acidification
OMZ	oxygen minimum zone
$P_{50}$	partial pressure of oxygen required to achieve 50% hemocyanin saturation
$P_{CO_2}$	carbon dioxide partial pressure
$P_{crit}$	critical $P_{O_2}$
$P_{O_2}$	oxygen partial pressure
$Q_{10}$	temperature coefficient
RMR	routine metabolic rate
RQ	respiratory quotient
TA	total alkalinity

perhaps not surprising. A mechanistic physiology-based model can help elucidate these diverse whole-animal metabolic responses to  $CO_2$  reported in the literature.

Although all loliginid and ommastrephid squid have rather active lifestyles, individual species have evolved in very different environments that may select for quite different  $CO_2$  or hypoxia tolerances. For example, *Dosidicus gigas* is an ommastrephid squid that inhabits the eastern tropical Pacific, where a pronounced oxygen minimum zone (OMZ) exists. They encounter strong gradients in  $P_{O_2}$  ( $\Delta > 10$  kPa),  $P_{CO_2}$  ( $\Delta \geq 100$  Pa; 1000  $\mu$ atm) and temperature ( $\Delta > 10^\circ C$ ) during their daily migration into the OMZ (Gilly et al., 2006, 2012; Franco et al., 2014). The squid suppress total metabolism by 50% while in the core of the OMZ during daytime hours (Seibel et al., 2014). In contrast, the loliginid squid *Doryteuthis pealeii* inhabits coastal and shelf waters in the western Atlantic, and never encounters such extreme hypoxia or hypercapnia [though bays can reach  $P_{CO_2} > 50$  Pa (500  $\mu$ atm) in the summer months; Turner, 2015]. As such, *D. gigas* is adapted to more extreme environmental conditions than *D. pealeii*.

In this study, we examined the effects of  $CO_2$  on hypoxia tolerance in two squid species with similar  $O_2$  demands but differing hypoxia tolerances, *D. gigas* and *D. pealeii*, to determine what impact OA may have on  $O_2$  supply in squid. We applied two independent approaches to this question. First, we conducted laboratory experiments to examine the effect of  $CO_2$  on hypoxia tolerance. Second, we constructed a model of blood acid–base balance and  $O_2$  delivery with variable inputs for blood– $O_2$  affinity,  $CO_2$  sensitivity of hemocyanin and buffering capacity to predict the physiological changes in  $O_2$  supply expected by end-of-the-century OA.

**MATERIALS AND METHODS****Animal capture and maintenance**

Adult and juvenile *Dosidicus gigas* (D'Orbigny, 1835;  $n=16$ ) were jigged at night in Guaymas Basin, Gulf of California, Mexico, from 16 May 2015 to 1 June 2015 aboard the R/V Oceanus. Juvenile and adult *Doryteuthis pealeii* (Lesueur, 1821;  $n=29$ ) were caught in southern Narragansett Bay, RI, USA, by either hand jigs or, less commonly, benthic otter trawl in April through November 2014–2016. Morphometrics of both species are shown in Table 1. *Dosidicus gigas* were placed immediately in a respirometer aboard ship for acclimation, whereas *D. pealeii* were transported in an aerated cooler to the Durbin Aquarium facility at the University of Rhode Island, where they were held in tanks of at least 540 liters with flow-through filtered seawater. *Doryteuthis pealeii* were fed grass shrimp (*Palaemonetes* sp.) or 1 cm wide herring (*Clupea harengus*)

**Table 1. Morphometrics of animals used in hypoxia tolerance experiments**

Species	$n$	Mass (g)	Dorsal mantle length (cm)	Gender
<i>Doryteuthis pealeii</i>	29	112 $\pm$ 58	18 $\pm$ 5	F:15, M:11
<i>Dosidicus gigas</i>	16	233 $\pm$ 64	21 $\pm$ 1	F:15, M:1

Values are mean $\pm$ s.d.

steaks *ad libitum* during the holding period before experiments were conducted. Prior to acclimation and experimental trials with *D. pealeii*, temperature was maintained at 15°C (within 5°C of capture temperature) and  $P_{CO_2}$  varied with ambient conditions in Narragansett Bay, where  $P_{CO_2}$  typically ranges from 10 to 70 Pa (100–700  $\mu$ atm; Turner, 2015).

**Hypoxia tolerance assessment**

Hypoxia tolerance was assessed by measuring  $O_2$  consumption rates of individual squid under progressively declining seawater  $P_{O_2}$  using intermittent respirometry. All experiments were conducted in a 90 liter swim tunnel respirometer (Loligo Systems, Viborg, Denmark) with a 70 $\times$ 20 $\times$ 20 cm working section in which the animal was confined. Trials were conducted at surface pressure, as hydrostatic pressure has little effect on metabolism in squid (Belman, 1978). Acclimation and trials were conducted at 15°C for *D. pealeii*. However, for *D. gigas*, temperature was maintained at ambient sea surface temperature, which varied from 23 to 27°C. Measurements were adjusted accordingly (see below).  $CO_2$  treatment began immediately upon placing the animal in the respirometer. Median acclimation duration was 10 and 13 h for *D. gigas* and *D. pealeii*, respectively, but varied from 8 to 13 h for *D. gigas* and from 5 to 59 h for *D. pealeii*. This allowed time for thermal acclimation (for *D. pealeii*), completion of digestion from any previous meal (Wells et al., 1983; Katsanevakis et al., 2005), acclimation to  $CO_2$  conditions (Gutowska et al., 2010a), and recovery from handling stress. Oxygen consumption rates from squid in swim-tunnel experiments extrapolated to zero activity are similar to rates measured after 6 h acclimation (Seibel, 2007). Previous measurements (Trueblood and Seibel, 2013) have shown that *D. gigas* stabilize metabolic rate by 6 h after enclosure in respirometers. Animals were free to move within the working section of the respirometer, which was 3–4 mantle lengths long. Thus, we refer to the metabolic rates measured here as routine metabolic rates (RMR) rather than standard metabolic rates (SMR), although the animals often rested on the bottom of the chamber.

After acclimation for each animal, the respirometer was closed and the  $P_{O_2}$  was drawn down by the animal. Every 4–5 kPa  $O_2$  (every 1–7 h depending on the rate of metabolism), the respirometer was flushed with seawater at matching  $P_{O_2}$  and experimental  $P_{CO_2}$  to minimize  $NH_3$  and  $CO_2$  accumulation. The average flush provided a 70% water exchange, based on estimated mixing efficiency from Steffensen (1989).  $P_{O_2}$  was measured every 10 s with an  $O_2$ -sensitive spot (Fibox 3 meter and PSt3 spots; PreSens Precision Sensing GmbH, Regensburg, Germany). The oxygen meter was calibrated with air-saturated seawater and concentrated  $NaSO_3$  solution ( $P_{O_2}=0$ ). Water velocity inside the respirometer was kept low ( $\approx 5$  cm  $s^{-1}$ ) to allow homogeneous mixing.

Flush water was UV-treated, then stored in a reservoir, brought to treatment temperature and bubbled with pure nitrogen gas to draw down dissolved  $P_{O_2}$ . To produce high  $CO_2$  conditions, pure  $CO_2$  gas (AirGas 'Bone Dry' grade, Radnor, PA, USA) was dispersed through a peristaltic pump and bubbled into the intake of

a submersible aquarium pump to enhance dissolution (Jokiel et al., 2014). The treated reservoir water was then flushed through the respirometer. The appropriate volume of CO<sub>2</sub> gas added for each flush was calculated using the R package ‘respirometry’ (<https://cran.r-project.org/web/packages/respirometry/index.html>). Water samples were collected from the respirometer output at the start of each flush for carbonate chemistry analyses. For the *D. pealeii* trials, water samples were also analyzed from the incoming flush water.

Metabolic rate ( $\dot{M}_{O_2}$ ) was monitored in real time and the trial was ended soon after the animal reached  $P_{crit}$ , the environmental  $P_{O_2}$  below which aerobic metabolism (indicated by O<sub>2</sub> consumption rate) decreases. The average duration of the trials after acclimation was 7 and 23 h for *D. gigas* and *D. pealeii*, respectively, owing to temperature and animal size. This resulted in an average total exposure duration to treatment conditions by the time the animal reached  $P_{crit}$  of 17 and 36 h, respectively. At the end of each trial, the animal was removed and the ‘background’ O<sub>2</sub> consumption rate of the microbial community in the respirometer was measured and deducted from calculated squid  $\dot{M}_{O_2}$ . Gill length relative to dorsal mantle length (DML) was measured in both species.

### Ventilation

During 17 of the *D. pealeii* trials, the animals were filmed for 1 min every 30 min to monitor ventilation rate. The camera was placed above the respirometer and a mirror was placed at a 45 deg angle to the camera, allowing simultaneous monitoring of the animal from a dorsal and lateral view.

As hypoxia progressed, three possible effects on ventilation rate were considered: (1) ventilation rate is unaffected by hypoxia, (2) ventilation rate increases linearly with progressive hypoxia and (3) ventilation rate is unaffected at moderate  $P_{O_2}$  levels but increases at more extreme hypoxia (i.e. breakpoint relationship). A model was fit for each of these relationships using maximum likelihood estimation with normal error distributions with the `mle2()` function from the R package ‘bbmle’ (<https://cran.r-project.org/web/packages/bbmle/index.html>). The best-fitting model was chosen using the Bayesian information criterion (BIC). Effects of  $P_{CO_2}$  on ventilation rate were assessed by linear regression based on the estimated CO<sub>2</sub> level of each ventilatory observation.

### $\dot{M}_{O_2}$ and $P_{crit}$ analysis

$\dot{M}_{O_2}$  was calculated from the slope of a linear regression of  $P_{O_2}$  over time. The number and quality of  $\dot{M}_{O_2}$  measurements obtained from this technique are dependent on the width of the time bins used. The time bin width scaled with  $P_{O_2}$  such that the time bins at high O<sub>2</sub> covered 1/10th the trial duration and the time bins at low O<sub>2</sub> covered 1/100th the trial duration. This provided an optimal balance between precision and resolution throughout each trial.  $\dot{M}_{O_2}$  measurements derived from regressions with an  $R^2 < 0.7$  were discarded.

To calculate  $P_{crit}$ , a traditional breakpoint relationship was fit using the `segmented()` function from the ‘segmented’ R package (Muggeo, 2008), which fits a broken-stick regression to the relationship between  $\dot{M}_{O_2}$  and  $P_{O_2}$ . Then, a 95% prediction interval was added around the oxyregulating line to encapsulate a space in which all observed  $\dot{M}_{O_2}$  values can reasonably be considered within the oxyregulating space. The ‘sub-PI’  $P_{crit}$  is defined as the  $P_{O_2}$  at which the oxyconforming line intersects the lower limit of the 95% prediction interval (see Figs S1 and S2). This sub-PI method resulted in a lower variability in  $P_{crit}$  measurements than the traditional breakpoint method.

Trials in which the animal did not exhibit a clear breakpoint response to  $P_{O_2}$  were removed from  $P_{crit}$  analyses. Only  $\dot{M}_{O_2}$  measurements with mean  $P_{O_2} > P_{crit}$  were considered when calculating mean RMR. Median RMR and the lowest 10% of  $\dot{M}_{O_2}$  observations for each individual were also estimated and gave similar results. For trials where no  $P_{crit}$  could be reliably established, RMR was determined as the mean of the  $\dot{M}_{O_2}$  values.

To compare measurements made at different temperatures, we calculated a temperature coefficient,  $Q_{10}$ , according to:

$$Q_{10} = (k_2/k_1)^{10/(T_2-T_1)}, \quad (1)$$

where  $k_1$  and  $k_2$  are the calculated values (e.g.  $\dot{M}_{O_2}$  or  $P_{crit}$ ) measured at temperatures  $T_1$  and  $T_2$ , respectively. Typical  $Q_{10}$  values for metabolic rate in ectotherms range from 2 to 3 (Hochachka and Somero, 2002), meaning that metabolic rate doubles or triples with a 10°C increase in temperature. The  $Q_{10}$  was calculated using the `Q10()` function from the R package ‘respirometry’, and bootstrap bias-corrected and accelerated confidence intervals were fit to form confidence bands.

### Carbonate chemistry

Seawater carbonate chemistry was assessed by measuring pH (total scale) and total alkalinity (TA) from water entering and expelled from the respirometer during acclimation and flushes. pH was measured spectrophotometrically at 25°C with m-Cresol Purple, a pH-sensitive dye (Clayton and Byrne, 1993), using standard operating procedure (SOP) 6b from Dickson et al. (2007), modified for use with a 1 cm path length cuvette. Based on pH measurements from flush water samples, seawater pH inside the respirometer during the inter-flush periods of the trials was calculated using the `predict_pH()` function from the R package ‘respirometry’. A respiratory quotient (RQ; ratio of CO<sub>2</sub> produced to O<sub>2</sub> consumed) of 0.85 was used because cephalopods mainly utilize protein catabolism (Hoeger et al., 1987). For the *D. pealeii* trials, where water samples were analyzed from both respirometer input and output at every flush, pH was calculated from both the start and end of each inter-flush measurement period. The values from these two methods of calculation differed by only 0.06 pH units on average, which corroborates this RQ value, and were averaged.

Alkalinity was measured either by SOP 3b from Dickson et al. (2007; potentiometric titration) or by Liu et al. (2015; spectrophotometric titration). Alkalinity measurements were calibrated with certified reference materials provided by Andrew Dickson (Scripps Institution of Oceanography, La Jolla, CA, USA).  $P_{CO_2}$  was calculated from inter-flush pH and trial-averaged TA using carbonate dissociation constants from Lueker et al. (2000) via the R package ‘seacarb’ (<https://cran.r-project.org/web/packages/seacarb/index.html>). Mean  $P_{CO_2}$  values for each trial were chosen as the environmental metric for analysis. The  $P_{CO_2}$  at  $P_{crit}$  was also estimated and gave similar results. Because of the unavoidable CO<sub>2</sub> build-up when the respirometer was closed, seawater pH during each trial varied within the respirometer over a median range of 0.21 and 0.28 pH units in the *D. gigas* and *D. pealeii* trials, respectively.

### Blood O<sub>2</sub> delivery model construction

A physiological model was developed to estimate the magnitude of an effect seawater  $P_{CO_2}$  has on blood O<sub>2</sub> transport and  $P_{crit}$  in squid. This model intentionally does not include any ability for blood acid–base compensation, a well-developed trait in squid that makes it highly unlikely that OA would result in long-term blood acidosis (Melzner et al., 2009; Hu and Tseng, 2017), for two reasons: (1) we have no specific knowledge on the rate or extent of

acid–base compensation in either species studied, and (2) by removing the ability to compensate for blood acidosis in this model, a reasonable upper bound on expected hypercapnic impact can be assessed. Physiological parameters [normocapnic blood  $P_{\text{CO}_2}$  and pH, non-bicarbonate buffering capacity ( $\beta_{\text{NB}}$ ), Bohr and Hill coefficients, hemocyanin  $P_{50}$ , arterial  $P_{\text{O}_2}$  at normoxia, and  $P_{\text{crit}}$ ] were collected from the literature for *D. pealeii* and *D. gigas* (Table 2).

According to Fick's law of diffusion (Eqn 2; where  $K$  is Krogh's diffusion coefficient, a gas- and tissue-specific constant) and without any change in ventilatory dynamics, blood  $P_{\text{CO}_2}$  must change symmetrically with seawater  $P_{\text{CO}_2}$  in order to maintain the same diffusive flux from the body:

$$\text{Diffusion rate} = K \times \frac{\text{gill surface area}}{\text{gill membrane thickness}} \times (P_{\text{CO}_2, \text{seawater}} - P_{\text{CO}_2, \text{blood}}). \quad (2)$$

This has been observed in cephalopods (Gutowska et al., 2010a; Hu et al., 2014; Häfker, 2012) as well as fishes exposed to hypercapnia (Janssen and Randall, 1975; Strobel et al., 2012a; Esbaugh et al., 2012, 2016; Ern and Esbaugh, 2016; for a review, see Heuer and Grosell, 2014). In the absence of any change in metabolic rate or  $\text{CO}_2$  production, the flux rate must remain constant if gradual respiratory acidosis is to be avoided. Thus, an increase in seawater  $P_{\text{CO}_2}$  elevates blood  $P_{\text{CO}_2}$  and reduces blood pH.  $\text{CO}_2$  solubility and dissociation constants for seawater ( $S=35$ ) from Lueker et al. (2000) were used. These constants are similar to values calculated in crab hemolymph (Truchot, 1976), which has ionic properties similar to those of squid blood.

A decrease in blood pH increases  $P_{50}$  according to Eqn 3:

$$\text{Bohr coefficient} = \frac{\Delta \log(P_{50})}{\Delta \text{pH}}. \quad (3)$$

This rise in  $P_{50}$  shifts the  $\text{O}_2$ -binding curve to the right, decreasing arterial hemocyanin (Hc)– $\text{O}_2$  saturation according to the Hc– $\text{O}_2$  binding equation (Eqn 4):

$$\% \text{ Hc–O}_2 \text{ saturation} = \frac{(P_{\text{O}_2})^n}{(P_{50})^n + (P_{\text{O}_2})^n} \times 100. \quad (4)$$

According to Eqn 4, as blood  $P_{50}$  increases due to acidosis, the arterial  $P_{\text{O}_2}$  necessary to maintain the same Hc– $\text{O}_2$  saturation

increases as well in a nonlinear relationship. As long as  $\text{O}_2$  demand is unchanged (e.g. no change in temperature or physiological activity),  $P_{\text{crit}}$  is reached when Hc– $\text{O}_2$  saturation falls below a set threshold (Redfield and Goodkind, 1929; Speers-Roesch et al., 2012) at which point the amount of  $\text{O}_2$  carried in the blood is insufficient to support cellular metabolism.

The change in arterial  $P_{\text{O}_2}$  for a given change in environmental  $P_{\text{O}_2}$  can be calculated from the arterial  $P_{\text{O}_2}$  under environmental air saturation and under anoxia assuming a linear relationship between environmental and arterial  $P_{\text{O}_2}$  (Eddy, 1974; Johansen et al., 1982; Houlihan et al., 1982; Speers-Roesch et al., 2012). The increase in arterial  $P_{\text{O}_2}$  required to reach the Hc– $\text{O}_2$  saturation threshold under hypercapnia translates to an increase in  $P_{\text{crit}}$ . Blood acid–base compensation was intentionally left out of the model because acid–base regulatory ability can minimize sensitivity to OA (Melzner et al., 2009). In so doing, a reasonable upper bound on expected hypercapnic impact can be assessed.

### Model application

Models were run for both species at a range of temperatures using physiological parameters from the literature from these or closely related species (Table 2). Blood pH was temperature-adjusted by  $-0.02^\circ\text{C}^{-1}$  to match *in vivo* temperature dependence of blood pH (Howell and Gilbert, 1976). The Bohr coefficient,  $P_{50}$  and  $P_{\text{crit}}$  were temperature-adjusted using species-specific temperature relationships (Table 2). The temperature dependence of  $P_{\text{crit}}$  in *D. pealeii* has not been measured to date. Therefore, the  $Q_{10}$  value for hemocyanin  $P_{50}$  was applied to  $P_{\text{crit}}$  in *D. pealeii* because of the close correlation between these parameters (Mandic et al., 2009).

To assess the model's reliability, the model was run with published physiological parameters for *D. pealeii* (Table 2) and compared with empirical data from Redfield and Goodkind (1929). They examined the effects of acute (10–15 min) seawater hypercapnia (up to 3200 Pa or 31,500  $\mu\text{atm}$   $\text{CO}_2$ ) on lethal  $P_{\text{O}_2}$  in *D. pealeii*. Blood acid–base compensation was not incorporated to the model due to the acute  $\text{CO}_2$  exposures (10–15 min) by Redfield and Goodkind (1929).

The model was also run for both species at temperatures ranging from 0 to  $25^\circ\text{C}$ . Three levels of environmental hypercapnia were also considered. First was a rise in  $P_{\text{CO}_2}$  of 60 Pa ( $\approx 600 \mu\text{atm}$ ) as expected for the mean sea surface. Given a  $\Delta P_{\text{CO}_2}$  of 60 Pa for air-equilibrated seawater,  $\text{CO}_2$  dissociation constants from Lueker et al. (2000), average ocean TA (Lee et al., 2006) and an RQ of 0.75, environmental  $P_{\text{CO}_2}$  may be expected to rise by  $\approx 130$  Pa ( $\approx 1300 \mu\text{atm}$ ) in regions with 50% air saturation, and  $\approx 200$  Pa ( $\approx 2000 \mu\text{atm}$ ) in regions with 10% air saturation. These  $\Delta P_{\text{CO}_2}$  levels were examined to cover a broad range of conditions that loliginid and ommastrephid squid may encounter in future oceans (Melzner et al., 2013).

### RESULTS

Experimental seawater parameters from the trials are shown in Table 3. All results are expressed as means  $\pm$  s.d.

#### Metabolic rate and hypoxia tolerance

There was no effect of hypercapnia on metabolic rate for either species (*D. pealeii*:  $t_{17} = -1.08$ ,  $P = 0.297$ ; temperature-adjusted *D. gigas*:  $t_{14} = 0.11$ ,  $P = 0.914$ ; Fig. 1A). *Dosidicus gigas* had an RMR of  $13.2 \pm 2.6 \mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$  at  $22.5$ – $26.6^\circ\text{C}$ . Its rate increased significantly with temperature ( $t_{14} = 1.6$ ,  $P = 0.123$ ), with a  $Q_{10}$  of 1.9 (Fig. 2A). *Doryteuthis pealeii* had an RMR of  $6.5 \pm 2.5 \mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$  at  $15^\circ\text{C}$ . Metabolic rate was

**Table 2. Species-specific physiological parameters for the blood  $\text{O}_2$  supply capacity model**

Run	<i>Doryteuthis pealeii</i>	<i>Dosidicus gigas</i>
Normocapnic blood $P_{\text{CO}_2}$ (Pa)	300 <sup>a,b,c</sup>	300 <sup>a,b,c</sup>
Normocapnic blood pH	pH = $-0.027T + 7.84^d$	pH = $-0.027T + 7.56^f$
Non-bicarbonate buffering capacity ( $\text{mmol l}^{-1} \text{ pH unit}^{-1}$ )	5.8 <sup>b</sup>	4.89 <sup>g</sup>
Bohr coefficient	Bohr = $0.01557T - 1.4175^{b,e}$	Bohr = $0.03387T - 1.554^f$
Hill coefficient	4 <sup>b</sup>	3.15 <sup>f</sup>
Hemocyanin $P_{50}$ (kPa)	$P_{50} = 3.162e^{0.0347T}$ , $Q_{10} = 1.4^{b,e}$	$P_{50} = 1.043e^{0.0687T}$ , $Q_{10} = 1.97^f$
Arterial $P_{\text{O}_2}$ (kPa)	16 <sup>a</sup>	16 <sup>a</sup>
Normocapnic $P_{\text{crit}}$ (kPa)	$P_{\text{crit}} = 2.345e^{0.0347T}$ , $Q_{10} = 1.4^j$	$P_{\text{crit}} = 1.207e^{0.0487T}$ , $Q_{10} = 1.62^{h,i,j}$

<sup>a</sup>Redfield and Goodkind (1929); <sup>b</sup>Pörtner (1990); <sup>c</sup>Hu et al. (2014); <sup>d</sup>Howell and Gilbert (1976); <sup>e</sup>Redfield and Ingalls (1933); <sup>f</sup>Seibel (2013); <sup>g</sup>Brix et al. (1989) from *Todarodes sagittatus*; <sup>h</sup>Gilly et al. (2006); <sup>i</sup>Trueblood and Seibel (2013); <sup>j</sup>Present study.

**Table 3. Seawater parameters experienced by squid during hypoxia tolerance tests**

Species	Temperature (°C)	Salinity	$P_{\text{CO}_2}$ range (Pa)	pH <sub>T</sub> range	Total alkalinity ( $\mu\text{mol kg}^{-1}$ )
<i>Doryteuthis pealeii</i>	14.8±0.3	31.2±0.5	67–143	7.51–7.82	2073±31
<i>Dosidicus gigas</i>	22.5–26.6	35.3±0.1	46–122	7.62–7.99	2399±97

Means are presented  $\pm$ s.d.

1.8  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$  higher in *Dosidicus gigas* than in *D. pealeii* ( $t_{35}=3.31$ ,  $P=0.002$ ) once adjusted to a common temperature (15°C) using a  $Q_{10}$  of 1.8 (derived from *D. gigas*  $\dot{M}_{\text{O}_2}$  measurements from the literature spanning from 6.5 to 25°C; Gilly et al., 2006; Rosa and Seibel, 2008; Trübenbach et al., 2013; Trueblood and Seibel, 2013; Seibel et al., 2014; present study; Fig. 2C).

Seawater  $P_{\text{CO}_2}$  had no detectable effect on temperature-adjusted  $P_{\text{crit}}$  in either species (Fig. 1B). The  $P_{\text{crit}}$  for *D. pealeii* at 15°C was  $3.9\pm 0.8$  kPa. *Dosidicus gigas* mean  $P_{\text{crit}}$  was  $3.8\pm 1.2$  kPa, but it increased with temperature from 23°C to 27°C with a  $Q_{10}$  of 1.8

(Fig. 2B). When adjusted to a common temperature (15°C) using a  $Q_{10}$  of 1.8 (derived from *D. gigas*  $P_{\text{crit}}$  values from the literature ranging from 6.5 to 27°C; Gilly et al., 2006; Trueblood and Seibel, 2013; Fig. 2D), mean *D. gigas*  $P_{\text{crit}}$  was 1.4 kPa lower than *D. pealeii*  $P_{\text{crit}}$  ( $t_{24}=3.84$ ,  $P<0.001$ ).

### Ventilatory changes

Ventilation rate in *D. pealeii* had a breakpoint relationship with  $P_{\text{O}_2}$ , remaining stable at high  $P_{\text{O}_2}$  (0.77 Hz) but increasing by 0.04 Hz  $\text{kPa}^{-1}$   $\text{O}_2$  with progressive hypoxia below 9 kPa (Fig. 3A). Although  $\text{O}_2$  strongly influenced ventilation, no effect of seawater  $P_{\text{CO}_2}$  was found on normoxic ( $P_{\text{O}_2}>9$  kPa) ventilation rate ( $t_{14}=-0.38$ ,  $P=0.71$ ; Fig. 3B). *Dosidicus gigas* relative gill length (38% of DML) was longer than that of *D. pealeii* (29% of DML;  $t_{25}=5.39$ ,  $P<0.001$ ).

### Blood $\text{O}_2$ delivery model

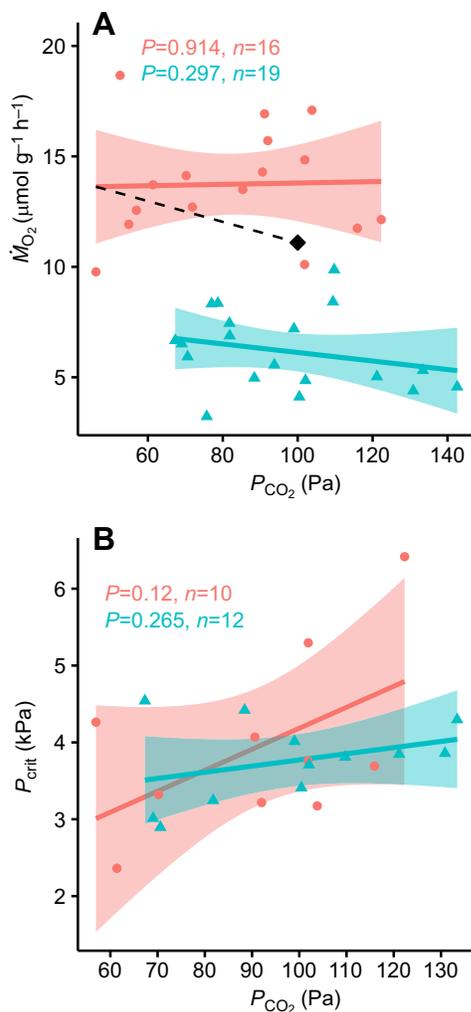
Running the  $\text{O}_2$  supply capacity model with physiological parameters from the literature for *D. pealeii* (Table 2) matched the empirical measurements of lethal  $P_{\text{O}_2}$  from Redfield and Goodkind (1929) very well even up to 3200 Pa or 31,500  $\mu\text{atm CO}_2$  (Fig. 4).

Both species experienced similar levels of arterial blood acidosis (Fig. 5A), with blood pH declining by 0.169 units at most under  $\Delta P_{\text{CO}_2}=200$  Pa. Under more modest hypercapnia ( $\Delta P_{\text{CO}_2}=60$  Pa), blood pH declined by 0.045 units, on average. Because of its lower hemocyanin– $\text{O}_2$  binding affinity (higher  $P_{50}$ ), the  $\text{O}_2$  binding curve for *D. pealeii* was more strongly impacted by blood acidosis than the curve for *D. gigas*, leading to a larger rise in  $P_{\text{crit}}$  and fall in arterial hemocyanin saturation from the same  $\text{CO}_2$  exposure (Fig. 5B–D). For both species, the expected  $\Delta P_{\text{crit}}$  was largely insensitive to temperature. The expected rise in  $P_{\text{crit}}$  from a 60 Pa increase in  $\text{CO}_2$  was 0.52 and 0.24 kPa for *D. pealeii* and *D. gigas*, respectively. The arterial hemocyanin saturation was minimally affected in both species even at the highest hypercapnia exposure owing to the high arterial  $P_{\text{O}_2}$  relative to  $P_{50}$ .

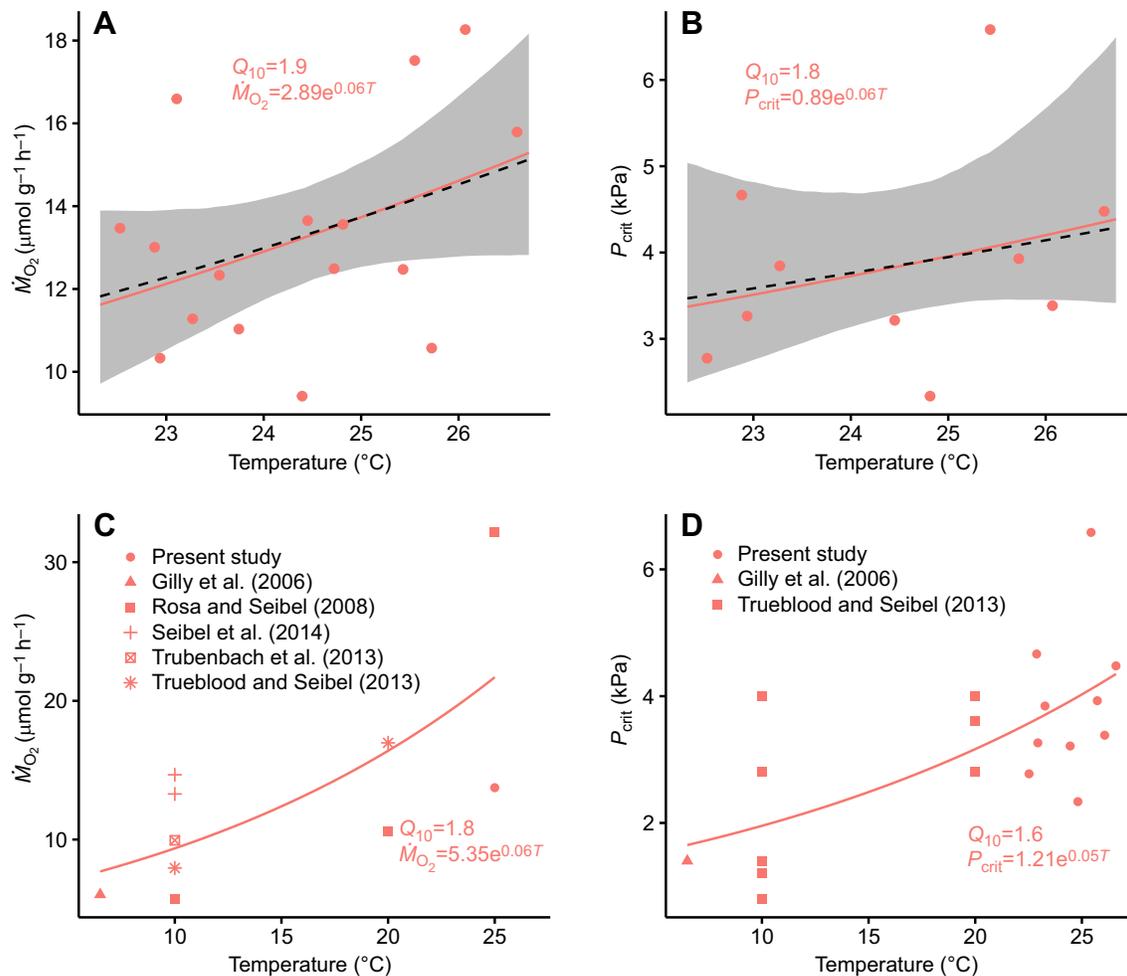
## DISCUSSION

### Effects of $\text{CO}_2$ on metabolism

We found no effect of seawater  $P_{\text{CO}_2}$  up to 143 Pa (1410  $\mu\text{atm}$ ) on metabolic rate or hypoxia tolerance in either species (Fig. 1). Rosa and Seibel (2008) had previously found that a  $P_{\text{CO}_2}$  of  $\approx 100$  Pa (1000  $\mu\text{atm}$ ) had caused a 20% decrease in *D. gigas* RMR relative to 30 Pa (300  $\mu\text{atm}$ ) and had attributed this suppression to hypercapnia-induced limitation to blood– $\text{O}_2$  binding. A 20% decline in RMR (black diamond in Fig. 1) fell outside of the 95% confidence band of the  $\text{CO}_2$  effect on RMR observed here. There were a number of differences between the studies that may have produced the disparate effects, such as animal size, acclimation duration and measurement technique. It is possible that the previous study documented a short-term response to high  $\text{CO}_2$  that is unrelated to blood  $\text{O}_2$  supply. Rosa and Seibel (2008) measured metabolic rate for up to 6 h during exposure to  $\text{CO}_2$ , whereas the present study allowed the squid to acclimate to treatment  $\text{CO}_2$  for at least 5 h and on average 10 h before beginning metabolic rate measurements.



**Fig. 1. Effect of seawater  $P_{\text{CO}_2}$  on squid metabolism.** Effect of seawater  $P_{\text{CO}_2}$  on (A) routine metabolic rate ( $\dot{M}_{\text{O}_2}$ ) and (B) critical  $P_{\text{O}_2}$  ( $P_{\text{crit}}$ ) in *Dosidicus gigas* (red circles; 25°C) and *Doryteuthis pealeii* (blue triangles; 15°C). Shaded bands are 95% confidence intervals. Because *D. gigas* trials covered a temperature range, all data were temperature-adjusted to 25°C using a  $Q_{10}$  of 1.9 ( $\dot{M}_{\text{O}_2}$ ) and 1.8 ( $P_{\text{crit}}$ ). When adjusted to a common temperature (15°C), *D. gigas*  $\dot{M}_{\text{O}_2}$  was 1.8  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$  higher and  $P_{\text{crit}}$  was 1.4 kPa lower than in *D. pealeii*. The dashed line and black diamond indicate a 20% reduction as observed by Rosa and Seibel (2008) when acutely exposing *D. gigas* to  $\text{CO}_2$ .



**Fig. 2. Effect of temperature on squid metabolism.** Effect of temperature on (A) routine metabolic rate ( $\dot{M}_{O_2}$ ) and (B) critical  $P_{O_2}$  ( $P_{crit}$ ) in *Dosidicus gigas*. The black dashed line represents a temperature effect corresponding to a  $Q_{10}$  of 2. Shaded bands are 95% confidence intervals. *Dosidicus gigas* mean metabolic rate (C) and  $P_{crit}$  (D) compared with literature values. All measurements are size-adjusted to 233 g (the mean mass in this study) using a scaling coefficient of  $-0.1$  (Seibel, 2007). The measurements in this study (A) were temperature-corrected to 25°C using a  $Q_{10}$  of 1.9 and averaged.

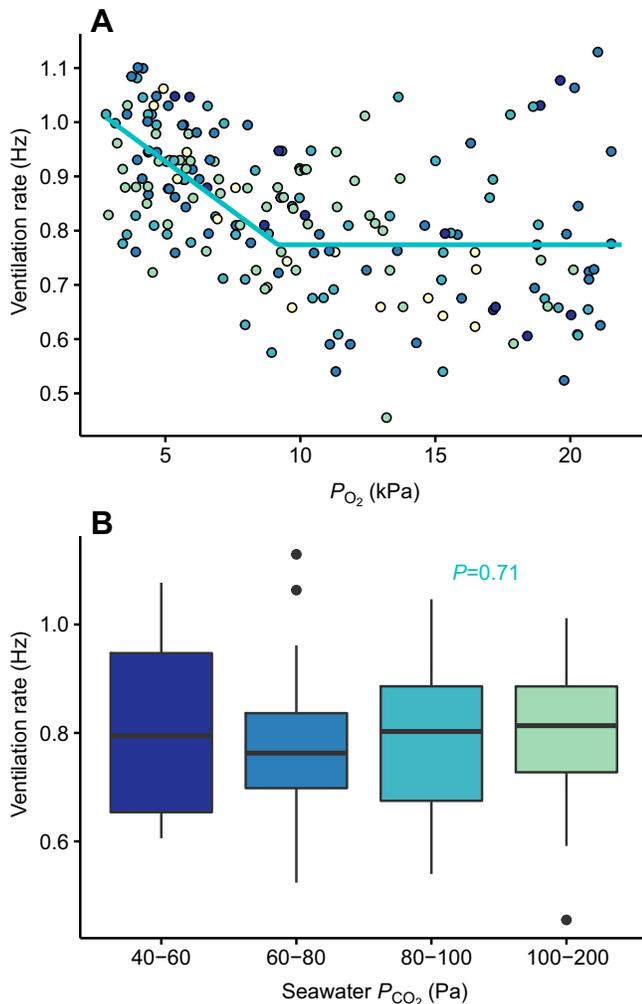
Given what is now known about blood- $O_2$  binding in *D. gigas* (Seibel, 2013), impairment of  $O_2$  supply could not have been the cause of the decline in metabolic rate and activity observed by Rosa and Seibel (2008). Based on the properties of *D. gigas* blood determined by Seibel (2013), the squid in the study by Rosa and Seibel (2008) should have had nearly completely  $O_2$ -saturated blood at all  $CO_2$  levels encountered. Furthermore, if  $O_2$  supply limitation were causing the decline in inactive and routine rates of metabolism they observed, then the much higher maximum metabolic rates documented should not have been attainable.

Other cases of lowered metabolic rate independent of  $O_2$  supply limitation have been documented in marine animals exposed to hypercapnia. Hu et al. (2014) found that after 1 week of exposure to 420 Pa (4130  $\mu$ atm)  $CO_2$ , *Sepioteuthis* squid were significantly metabolically suppressed even though their blood remained fully pH-compensated. Rummer et al. (2013) have also found that fish exposed to high  $CO_2$  can have suppressed resting  $\dot{M}_{O_2}$ , yet have enhanced maximal  $\dot{M}_{O_2}$ , which would be impossible if  $O_2$  supply is insufficient to even sustain resting needs. In the marine worm *Sipunculus nudus*, hypercapnia can cause metabolic suppression through alteration in neuromodulator concentration independently of  $O_2$  supply constraints (Reipschläger et al., 1997). Additionally, hypercapnia-induced metabolic suppression in corals has been

associated with differential gene expression of metabolic pathways at the tissue level (Kaniewska et al., 2012) rather than limited  $O_2$  delivery.

As has been demonstrated in other adult cephalopods (Gutowska et al., 2010a; Hu et al., 2014; Häfker, 2012), it is likely that the squid species examined here in high seawater  $P_{CO_2}$  compensated their blood pH by actively increasing  $[HCO_3^-]$ . *Sepioteuthis lessoniana* have been found to fully compensate for respiratory acidosis within 20 h of exposure to 420 Pa (4130  $\mu$ atm)  $CO_2$  (Hu et al., 2014). Similarly, when exposed to 600 Pa (5920  $\mu$ atm)  $CO_2$ , cuttlefish blood pH is nearly fully compensated and hemocyanin- $O_2$  saturation is not compromised (Gutowska et al., 2010a). In addition to raising blood pH, increasing  $[HCO_3^-]$  from 3 to 10 mmol  $l^{-1}$  lowers free  $[Mg^{2+}]$  by  $\approx 1\%$  owing to ion pairing. Although free  $Mg^{2+}$  is essential for proper hemocyanin function, such a small change has a negligible effect on hemocyanin  $P_{50}$  (Miller, 1985; Miller and Mangum, 1988).

In addition, hypoxia may even have an antagonistic effect of hypercapnia on blood pH. Seibel et al. (2014) reported that *D. gigas* blood pH increased under hypoxia, presumably to increase  $O_2$  affinity although at the expense of intracellular pH. Similar blood pH increases in response to hypoxia have been measured in *Octopus* and *Sepia* (Houlihan et al., 1982; Johansen et al., 1982).

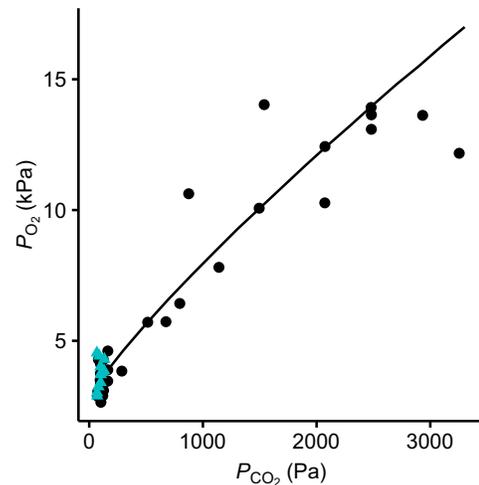


**Fig. 3. Effect of dissolved gas levels on squid ventilation rate.** Ventilation rate in *Doryteuthis pealeii* (15°C) as a function of (A)  $P_{O_2}$  at varying  $P_{CO_2}$  levels and (B)  $P_{CO_2}$  in normoxia ( $P_{O_2} > 9$  kPa). Colors denote the same  $P_{CO_2}$  range in both panels. A best-fit model analysis (see Materials and Methods, Ventilation) reveals a hypoxic threshold of 9 kPa below which ventilation increases ( $n=17$  individuals). Colors represent seawater  $P_{CO_2}$  at the time of observation. There was no effect of  $CO_2$  on ventilation rate.

### Impact of ocean acidification on $O_2$ supply in squid

To determine what effect  $P_{CO_2}$  might reasonably be expected to have on  $O_2$  supply capacity, we modeled the effect of  $P_{CO_2}$  across a range of physiological and temperature conditions. The model matches independent empirical data up to 3200 Pa or 31,500  $\mu\text{atm}$   $CO_2$  (Redfield and Goodkind, 1929), which suggests that despite its simplicity, our model accurately captures whole-animal metabolic responses to  $CO_2$  in squid when no blood acid–base compensation occurs. The model for *D. pealeii* (the more  $CO_2$  sensitive of the two species) results in only a 0.52 kPa increase in  $P_{crit}$  owing to a rise in  $P_{CO_2}$  to 100 Pa (1000  $\mu\text{atm}$ ). This increase in  $P_{crit}$  is well within the range of existing intraspecific  $P_{crit}$  variability measured in this study and others (Redfield and Goodkind, 1929; Trueblood and Seibel, 2013).

We have been quite conservative in this analysis by intentionally constructing a model that does not incorporate a number of physiological phenomena that would further minimize the effect of OA on blood  $O_2$  supply. There has been no incorporation of pH compensation via branchial ion transport. In fact, such compensation has been demonstrated in squid, and would greatly



**Fig. 4. Model predictions of hypoxia tolerance (critical  $P_{O_2}$ , kPa)**

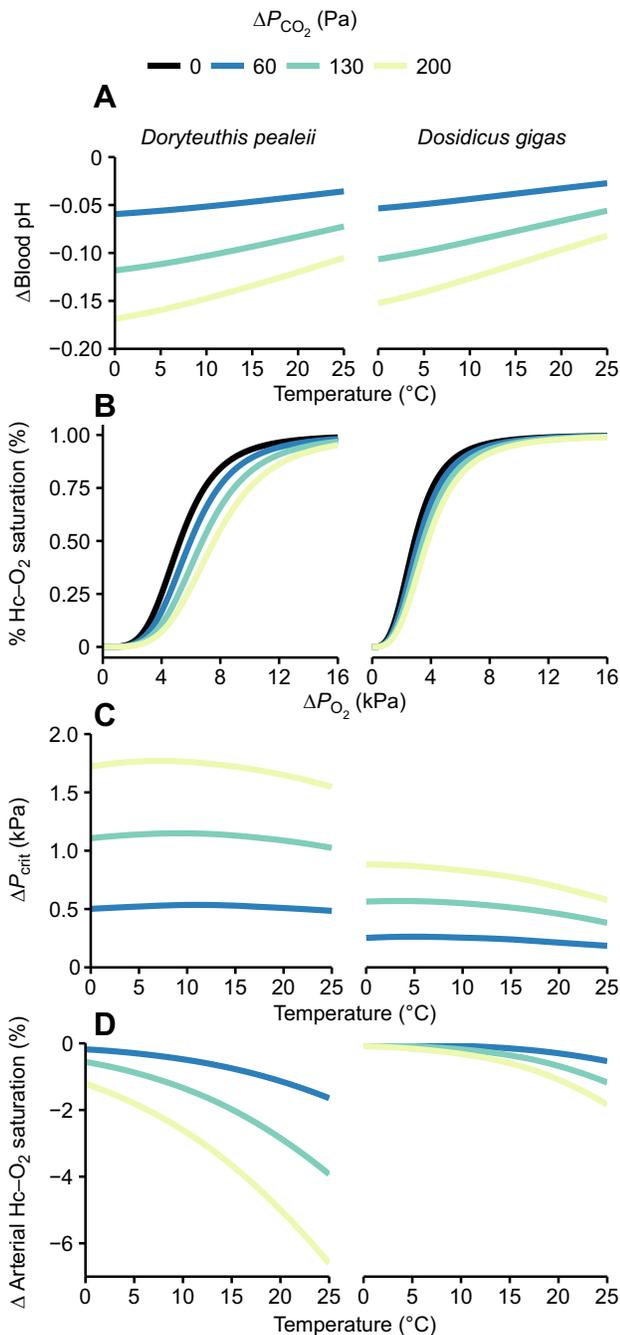
compared with independently derived empirical data. Black points denote lethal combinations of  $O_2$  and  $CO_2$  for *Doryteuthis pealeii* from Redfield and Goodkind (1929). Blue triangles represent  $P_{crit}$  for *D. pealeii* from this study. The black line models physiological parameters for *D. pealeii* (see Table 2).

alleviate impacts of increased  $CO_2$  (Melzner et al., 2009). Hu et al. (2014) found that the loliginid squid *Sepioteuthis lessoniana* exposed to 400 Pa (4000  $\mu\text{atm}$ )  $CO_2$  could raise blood pH by at least 0.13 units to completely return blood pH to normocapnic levels. Similarly, Gutowska et al. (2010a) found that *Sepia officinalis* could raise blood pH by  $\approx 0.2$  units in response to hypercapnia. Such compensation would completely relieve the effects of  $CO_2$  on  $O_2$  supply demonstrated here (Fig. 5A).

Some species of fishes, crustaceans and mollusks (including cephalopods) are known to produce multiple isoforms of respiratory protein subunits with different pH sensitivities (Johansen and Weber, 1976; Mangum, 1997; Strobel et al., 2012b). This could allow an animal to utilize a pH-insensitive isoform to further minimize impairment of  $O_2$  supply. Such a response seems to occur in rainbow trout exposed to very high  $CO_2$  ( $>1300$  Pa, 13,000  $\mu\text{atm}$ ; Eddy and Morgan, 1969). In addition, a minor fall in arterial saturation may be compensated for by a slight increase in cardiac output, which would be particularly advantageous at high blood  $P_{O_2}$ . Increases in cardiac output capacity have been observed in hypercapnia-exposed fishes (Gräns et al., 2014).

Water-breathers are also able to increase branchial surface area or decrease diffusion distance under projected OA-level hypercapnia (Esbaugh et al., 2016), which could lessen the increase in blood  $P_{CO_2}$ . Increased ventilation of gas-exchange structures (e.g. gills) would also lower arterial  $P_{CO_2}$  and raise blood pH, and has been documented in cephalopods and fishes exposed to hypercapnia (Gutowska et al., 2010a; Ern and Esbaugh, 2016). Cephalopods, fishes and crustaceans have all been shown to modulate their hypoxia sensitivity between populations that inhabit different  $O_2$  conditions, demonstrating that plasticity can further ameliorate environmental stressors to  $O_2$  supply (Childress, 1975; Friedman et al., 2012; Birk, 2018).

Based on the small to modest effects of  $CO_2$  on  $O_2$  supply demonstrated here without blood acid–base compensation and the well-established abilities of squid to regulate blood pH, we propose that  $O_2$  supply capacity in loliginid and ommastrephid squid is highly unlikely to be impaired to any great extent by OA predicted for the near future, even in hypoxic–hypercapnic environments. Although some squid may have slightly larger Bohr coefficients or



**Fig. 5. Expected effect of environmental  $P_{\text{CO}_2}$  on physiological parameters for two squid species at various temperatures without any blood pH compensation.** (A) Blood pH, (B) hemocyanin– $\text{O}_2$  affinity at  $15^\circ\text{C}$ , (C)  $P_{\text{crit}}$  and (D) arterial hemocyanin saturation. Rises in environmental  $P_{\text{CO}_2}$  reflect expected changes in various marine environments (see Materials and Methods, Model application).

higher  $P_{50}$ , these species have been held up as models of  $\text{CO}_2$ -intolerant species. We show that they are highly tolerant of likely OA scenarios even without inclusion of acid–base regulation capacity. This suggests that whole-animal metrics such as hypoxia tolerance ( $P_{\text{crit}}$ ), maximal metabolic rate and aerobic scope are also unlikely to be impacted by OA in active squid.

Although it is unlikely that the  $\text{O}_2$  supply pathway of active squid will be affected by OA, there remain other mechanisms of concern for hypercapnia to impact squid fitness. Although OA does not raise

overall  $\text{O}_2$  demand, the possibility remains that OA may alter the allocation of metabolic energy as a result of changes in the relative demands of the metabolic budget components (Strobel et al., 2012a; Pan et al., 2015). Blood acid–base disturbance from environmental hypercapnia has been shown to increase cuttlebone calcification in cuttlefish (Gutowska et al., 2010b). OA has also been demonstrated to alter behavior in marine animals (Clements and Hunt, 2015), including squid (Spady et al., 2014, 2018). Additionally, OA has been found to have negative impacts on embryonic growth rates and hatching success (Zakroff, 2013; Kaplan et al., 2013).

### Ventilation

We found that ventilation rate increases under hypoxia in the loliginid squid *D. pealeii*, which increases the flux of  $\text{O}_2$  past the gills as the  $\text{O}_2$  content of the seawater declines. Similar responses have been observed in other cephalopods and aquatic animals generally (Wells and Wells, 1995; Hughes, 1973; Burnett and Stickle, 2001). However, this finding contrasts with results obtained in studies of another loliginid squid, *Lolliguncula brevis*, in which progressive hypoxia had no effect on ventilation rate but, instead, animals increased  $\text{O}_2$  extraction efficiency (Wells et al., 1988). Extraction efficiency was not measured in the present study, so it is unclear whether *D. pealeii* exhibit a similar response in addition to their increased ventilation rate. Although ventilation was not measured in *D. gigas* here, Trübenbach et al. (2013) examined ventilation rate and stroke volume under normoxia and severe hypoxia (1 kPa  $\text{O}_2$ ). At this extreme hypoxic condition (below  $P_{\text{crit}}$ ), animals suppress metabolism. Thus, both ventilation rate and stroke volume are lower under extreme hypoxia than normoxia. It is still unclear, however, what effect intermediate  $P_{\text{O}_2}$  levels have on ventilation in this species before it begins to suppress metabolism. Pelagic crustaceans that also migrate daily into the OMZ have been found to increase ventilation with progressive hypoxia (Childress, 1971; Seibel et al., 2018).

$\text{CO}_2$  had no effect on ventilation rate (Fig. 3B). If blood acid–base balance can be fully compensated for by branchial ion-transport under elevated  $\text{CO}_2$ , then no increase in ventilation rate is necessary to maintain  $\text{O}_2$  supply. This is common in water-breathing animals, which rely much less on respiratory adjustments for acid–base balance than air breathers (Pörtner et al., 2011). However, cuttlefish and some fishes have been found to increase ventilatory dynamics under hypercapnia (Gutowska et al., 2010a; Ern and Esbaugh, 2016). In fishes, this is driven not only by blood acidosis but also by  $\text{CO}_2$ -sensitive chemoreceptors in the gills (Gilmour, 2001). It is not currently known whether cephalopods also have such branchial chemoreceptors.

### Species comparison

After adjusting for temperature, both species of squid had similar metabolic rates. Although the two species are phylogenetically rather distant (different orders), they are both active squid that inhabit shallow water and thus they both have strong selection for high metabolic rates (Seibel, 2007).

In this study, we found that the ommastrephid *D. gigas* has a better tolerance to hypoxia than the loliginid *D. pealeii* when compared at the same temperature. The hypoxia tolerance of aquatic animals can closely define their distribution and suitable habitat on spatial scales from meters (Mandic et al., 2009) to hundreds of kilometers (Deutsch et al., 2015). It is therefore unsurprising that *D. gigas* has better hypoxia tolerance than *D. pealeii* because the former is closely associated with the strong OMZ of the eastern tropical Pacific (Nigmatullin et al., 2001) whereas the latter is not

known to frequently encounter such extreme hypoxia. Although the bays that *D. pealeii* inhabit in the northern part of their range may occasionally become hypoxic (Melrose et al., 2007), it is likely that this species can easily find suitable habitat outside these small spatiotemporal regions (Bartol et al., 2002) and thus minimize the selective pressure to improve hypoxia tolerance.

*Dosidicus gigas* are demonstrably better equipped to handle hypoxia than *D. pealeii*. The relative gill length of *Dosidicus gigas* is 30% longer than that of *D. pealeii*, which suggests greater gill surface area. *Dosidicus gigas* hemocyanin requires less than half the blood  $P_{O_2}$  to saturate its hemocyanin as *D. pealeii* (Pörtner, 1990; Seibel, 2013). Finally, *D. gigas* maintain far higher anaerobic capacity than *D. pealeii*. *Dosidicus gigas* store 2–4× higher concentration of phosphoarginine in its mantle muscle than *D. pealeii* (Seibel et al., 2014; Storey and Storey, 1978), which should be more advantageous for surviving subcritical  $O_2$  levels. Glycogen reserves have not been quantified in *D. pealeii* mantle muscle, but glycogen concentration in *D. gigas* mantle is  $\approx 300 \mu\text{mol}$  glucosyl units  $\text{g}^{-1}$  (Seibel et al., 2014), which is much higher than in most fishes (Nilsson and Östlund-Nilsson, 2008; Richards, 2009) and even in bivalves that can survive months in anoxia (Oeschger, 1990).

In this experiment, we found that  $P_{CO_2}$  levels up to 122 Pa (1200  $\mu\text{atm}$ ), near the  $P_{CO_2}$  in the OMZ (Paulmier et al., 2011; Feely et al., 2016), had no measurable effect on *D. gigas*  $P_{crit}$ . Therefore, we do not expect that hypercapnia encountered in the OMZ has any additive or synergistic effect with hypoxia on *D. gigas* during its daily vertical migrations into this region.

In conclusion, although shallow-water squid species have high  $O_2$  demand and constrained  $O_2$  supply, their blood- $O_2$  carrying capacity, hypoxia tolerance and  $O_2$  demand seem to be unaffected by near-future  $CO_2$  levels.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: M.A.B., B.A.S.; Methodology: M.A.B., E.L.M., B.A.S.; Software: M.A.B.; Validation: M.A.B.; Formal analysis: M.A.B.; Investigation: M.A.B., E.L.M.; Resources: B.A.S.; Data curation: M.A.B.; Writing - original draft: M.A.B.; Writing - review & editing: M.A.B., E.L.M., B.A.S.; Visualization: M.A.B.; Supervision: B.A.S.; Project administration: B.A.S.; Funding acquisition: M.A.B., B.A.S.

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#### Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.187443.supplemental>

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