

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 μatm) 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 ≈ 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 μatm , ppmv) to over 40 Pa ($\approx 400 \mu atm$) today (Caldeira and Wickett, 2005) and may reach 100 Pa (1000 μatm) 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 μatm 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 μatm), 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 μatm) CO_2 (Rosa et al., 2014).

However, Hu et al. (2014) found no effect of 160 Pa (1600 μatm) 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 μatm) (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 μ 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 μ 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 μ 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 (≈ 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 CO₂ 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 O₂ 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’ O₂ 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 CO₂ 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 O₂ covered 1/10th the trial duration and the time bins at low O₂ 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 CO₂ produced to O₂ 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 CO₂ 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 O₂ delivery model construction

A physiological model was developed to estimate the magnitude of an effect seawater P_{CO_2} has on blood O₂ 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_{CO_2} and pH, non-bicarbonate buffering capacity (β_{NB}), Bohr and Hill coefficients, hemocyanin P_{50} , arterial P_{O_2} at normoxia, and P_{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_{CO_2} must change symmetrically with seawater P_{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 CO_2 production, the flux rate must remain constant if gradual respiratory acidosis is to be avoided. Thus, an increase in seawater P_{CO_2} elevates blood P_{CO_2} and reduces blood pH. 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 O_2 -binding curve to the right, decreasing arterial hemocyanin (Hc)– O_2 saturation according to the Hc– 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_{O_2} necessary to maintain the same Hc– O_2 saturation

increases as well in a nonlinear relationship. As long as O_2 demand is unchanged (e.g. no change in temperature or physiological activity), P_{crit} is reached when Hc– O_2 saturation falls below a set threshold (Redfield and Goodkind, 1929; Speers-Roesch et al., 2012) at which point the amount of O_2 carried in the blood is insufficient to support cellular metabolism.

The change in arterial P_{O_2} for a given change in environmental P_{O_2} can be calculated from the arterial P_{O_2} under environmental air saturation and under anoxia assuming a linear relationship between environmental and arterial P_{O_2} (Eddy, 1974; Johansen et al., 1982; Houlihan et al., 1982; Speers-Roesch et al., 2012). The increase in arterial P_{O_2} required to reach the Hc– O_2 saturation threshold under hypercapnia translates to an increase in P_{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°C^{-1} to match *in vivo* temperature dependence of blood pH (Howell and Gilbert, 1976). The Bohr coefficient, P_{50} and P_{crit} were temperature-adjusted using species-specific temperature relationships (Table 2). The temperature dependence of P_{crit} in *D. pealeii* has not been measured to date. Therefore, the Q_{10} value for hemocyanin P_{50} was applied to P_{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 μatm CO_2) on lethal P_{O_2} in *D. pealeii*. Blood acid–base compensation was not incorporated to the model due to the acute 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°C . Three levels of environmental hypercapnia were also considered. First was a rise in P_{CO_2} of 60 Pa ($\approx 600 \mu\text{atm}$) as expected for the mean sea surface. Given a ΔP_{CO_2} of 60 Pa for air-equilibrated seawater, CO_2 dissociation constants from Lueker et al. (2000), average ocean TA (Lee et al., 2006) and an RQ of 0.75, environmental P_{CO_2} may be expected to rise by ≈ 130 Pa ($\approx 1300 \mu\text{atm}$) in regions with 50% air saturation, and ≈ 200 Pa ($\approx 2000 \mu\text{atm}$) in regions with 10% air saturation. These ΔP_{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°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°C . Metabolic rate was

Table 2. Species-specific physiological parameters for the blood O_2 supply capacity model

Run	<i>Doryteuthis pealeii</i>	<i>Dosidicus gigas</i>
Normocapnic blood P_{CO_2} (Pa)	300 ^{a,b,c}	300 ^{a,b,c}
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 ^b	4.89 ^g
Bohr coefficient	Bohr = $0.01557T - 1.4175^{b,e}$	Bohr = $0.03387T - 1.554^f$
Hill coefficient	4 ^b	3.15 ^f
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_{O_2} (kPa)	16 ^a	16 ^a
Normocapnic P_{crit} (kPa)	$P_{\text{crit}} = 2.345e^{0.0347T}$, $Q_{10} = 1.4^j$	$P_{\text{crit}} = 1.207e^{0.0487T}$, $Q_{10} = 1.62^{h,i,j}$

^aRedfield and Goodkind (1929); ^bPörtner (1990); ^cHu et al. (2014); ^dHowell and Gilbert (1976); ^eRedfield and Ingalls (1933); ^fSeibel (2013); ^gBrix et al. (1989) from *Todarodes sagittatus*; ^hGilly et al. (2006); ⁱTrueblood and Seibel (2013); ^jPresent study.

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

Species	Temperature (°C)	Salinity	P_{CO_2} range (Pa)	pH _T 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}_{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_{CO_2} had no detectable effect on temperature-adjusted P_{crit} in either species (Fig. 1B). The P_{crit} for *D. pealeii* at 15°C was 3.9 ± 0.8 kPa. *Dosidicus gigas* mean P_{crit} was 3.8 ± 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_{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_{crit} was 1.4 kPa lower than *D. pealeii* P_{crit} ($t_{24}=3.84$, $P<0.001$).

Ventilatory changes

Ventilation rate in *D. pealeii* had a breakpoint relationship with P_{O_2} , remaining stable at high P_{O_2} (0.77 Hz) but increasing by 0.04 Hz kPa^{-1} O_2 with progressive hypoxia below 9 kPa (Fig. 3A). Although O_2 strongly influenced ventilation, no effect of seawater P_{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 O_2 delivery model

Running the O_2 supply capacity model with physiological parameters from the literature for *D. pealeii* (Table 2) matched the empirical measurements of lethal P_{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– O_2 binding affinity (higher P_{50}), the 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_{crit} and fall in arterial hemocyanin saturation from the same CO_2 exposure (Fig. 5B–D). For both species, the expected ΔP_{crit} was largely insensitive to temperature. The expected rise in P_{crit} from a 60 Pa increase in 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_{O_2} relative to P_{50} .

DISCUSSION

Effects of CO_2 on metabolism

We found no effect of seawater P_{CO_2} up to 143 Pa (1410 μatm) on metabolic rate or hypoxia tolerance in either species (Fig. 1). Rosa and Seibel (2008) had previously found that a P_{CO_2} of ≈ 100 Pa (1000 μatm) had caused a 20% decrease in *D. gigas* RMR relative to 30 Pa (300 μatm) and had attributed this suppression to hypercapnia-induced limitation to blood– O_2 binding. A 20% decline in RMR (black diamond in Fig. 1) fell outside of the 95% confidence band of the 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 CO_2 that is unrelated to blood O_2 supply. Rosa and Seibel (2008) measured metabolic rate for up to 6 h during exposure to CO_2 , whereas the present study allowed the squid to acclimate to treatment CO_2 for at least 5 h and on average 10 h before beginning metabolic rate measurements.

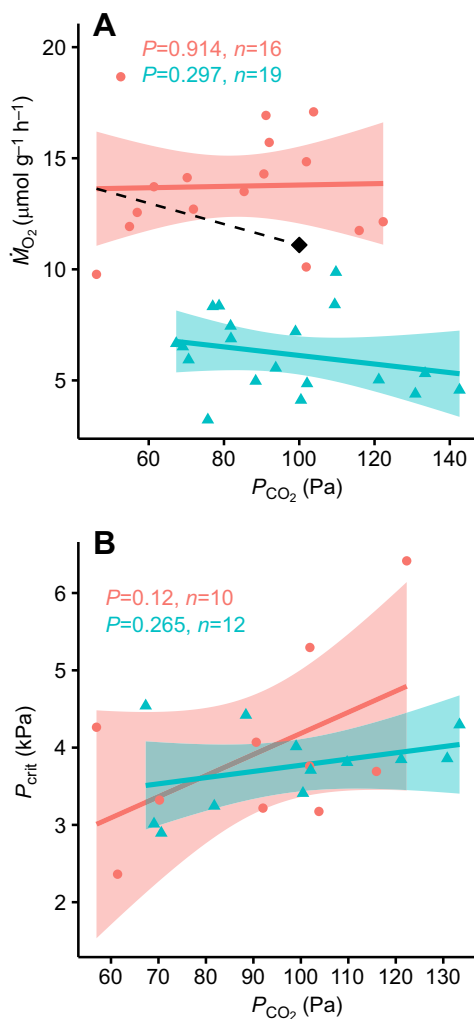


Fig. 1. Effect of seawater P_{CO_2} on squid metabolism. Effect of seawater P_{CO_2} on (A) routine metabolic rate (\dot{M}_{O_2}) and (B) critical P_{O_2} (P_{crit}) in *Dosidicus gigas* (red circles; 25°C) and *Doryteuthis pealeii* (blue triangles; 15°C). Shaded bands are 95% confidence intervals. 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}_{O_2}) and 1.8 (P_{crit}). When adjusted to a common temperature (15°C), *D. gigas* \dot{M}_{O_2} was 1.8 $\mu\text{mol O}_2 \text{ g}^{-1} \text{ h}^{-1}$ higher and P_{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 CO_2 .

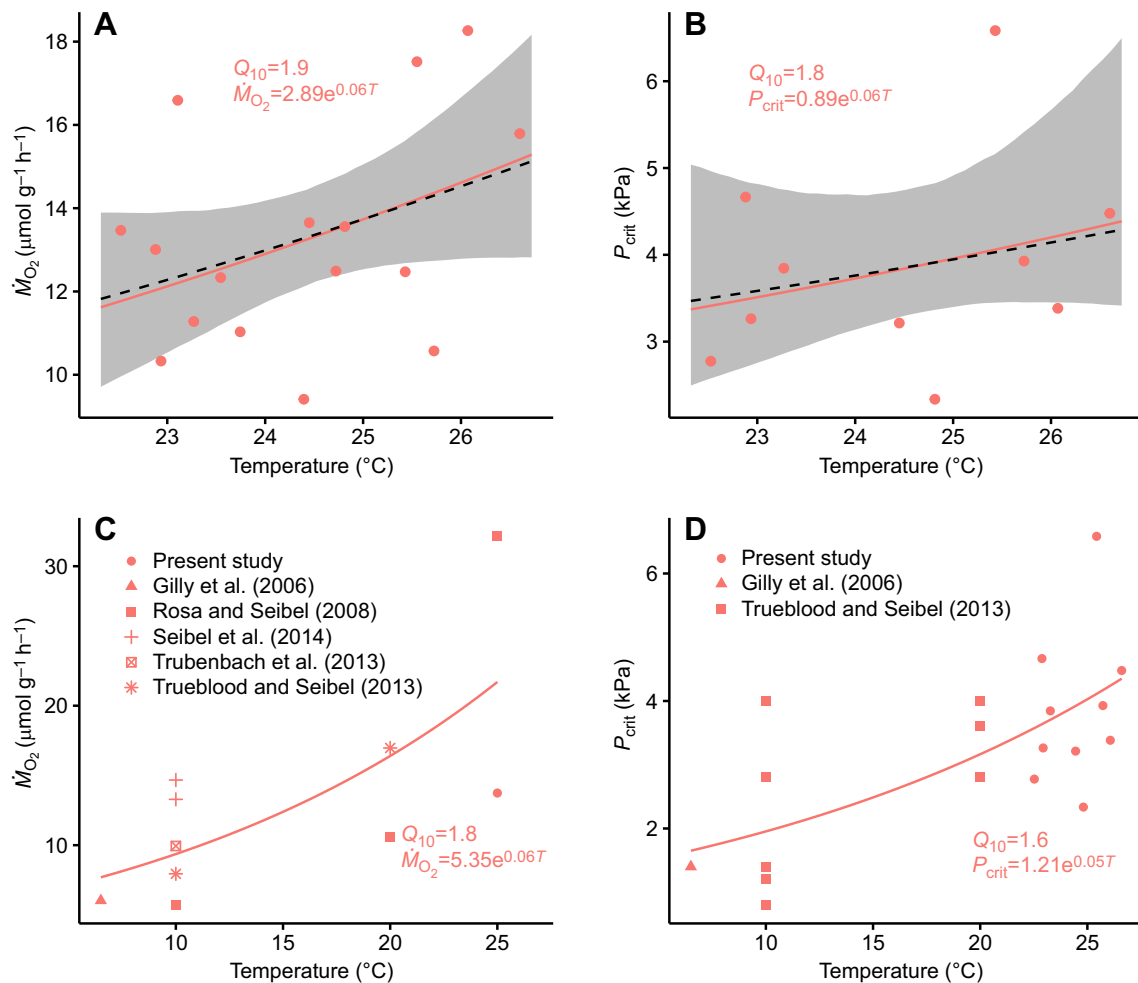


Fig. 2. Effect of temperature on squid metabolism. Effect of temperature on (A) routine metabolic rate (\dot{M}_{O_2}) and (B) critical P_{O_2} (P_{crit}) in *Dosidicus gigas*. The black dashed line represents a temperature effect corresponding to a Q_{10} of 2. Shaded bands are 95% confidence intervals. *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 μ 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 μ atm) CO_2 (Hu et al., 2014). Similarly, when exposed to 600 Pa (5920 μ 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).

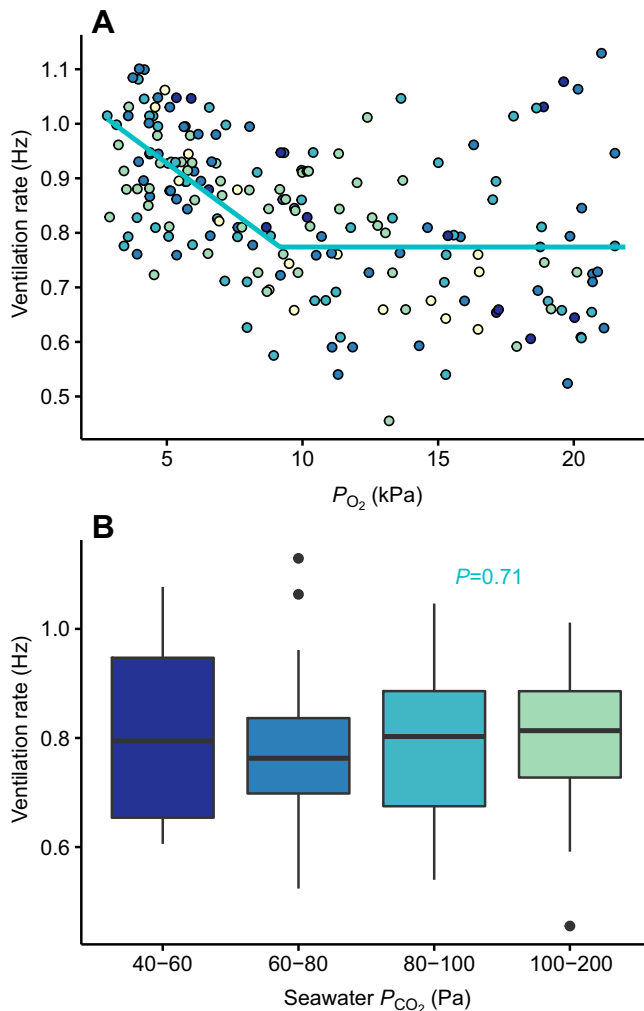


Fig. 3. Effect of dissolved gas levels on squid ventilation rate. Ventilation rate in *Doryteuthis pealeii* (15°C) as a function of (A) P_{O_2} at varying P_{CO_2} levels and (B) P_{CO_2} in normoxia ($P_{O_2} > 9$ kPa). Colors denote the same P_{CO_2} range in both panels. A best-fit model analysis (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 μatm 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 μatm). 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

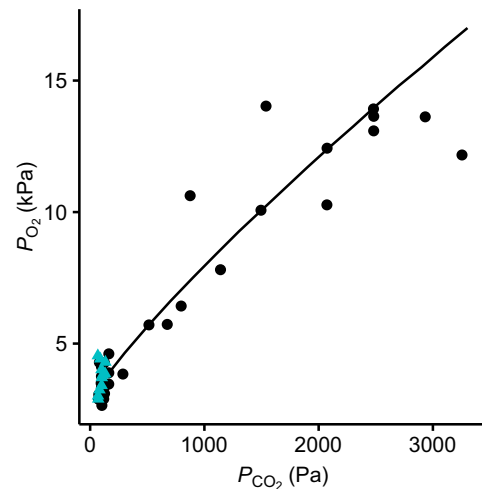


Fig. 4. Model predictions of hypoxia tolerance (critical P_{O_2} , kPa)

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 μatm) 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 ≈ 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 μatm ; 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

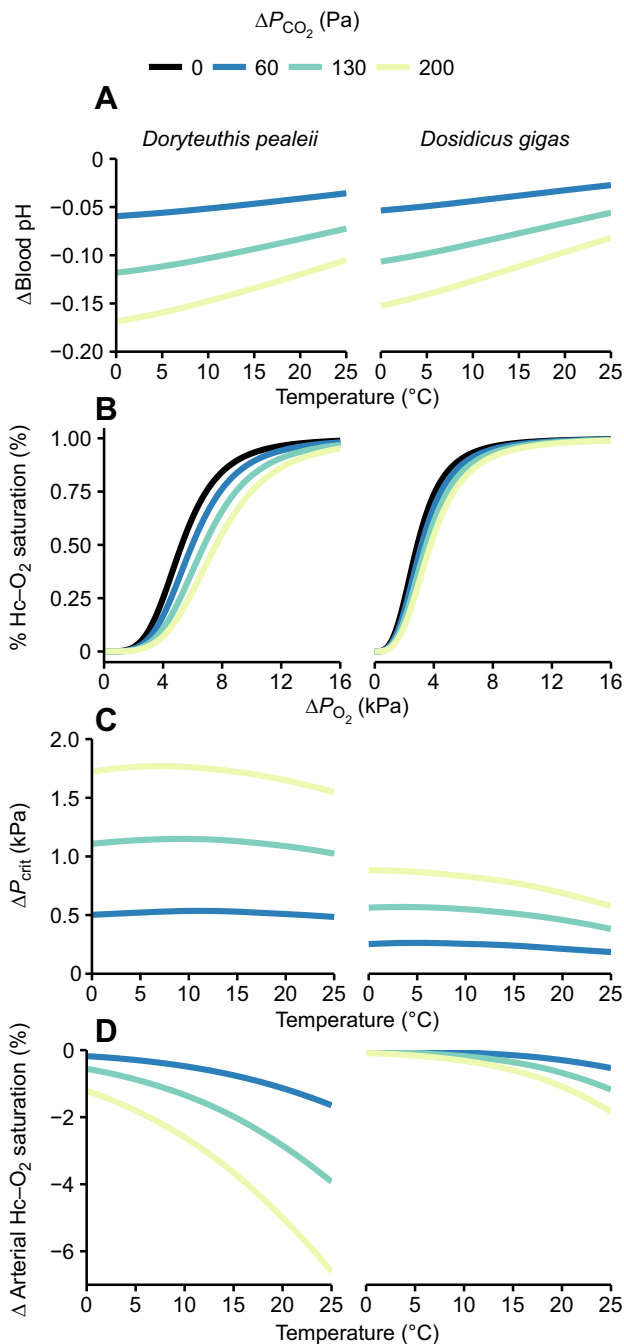


Fig. 5. Expected effect of environmental P_{CO_2} on physiological parameters for two squid species at various temperatures without any blood pH compensation. (A) Blood pH, (B) hemocyanin– O_2 affinity at 15°C , (C) P_{crit} and (D) arterial hemocyanin saturation. Rises in environmental P_{CO_2} reflect expected changes in various marine environments (see Materials and Methods, Model application).

higher P_{50} , these species have been held up as models of 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_{crit}), maximal metabolic rate and aerobic scope are also unlikely to be impacted by OA in active squid.

Although it is unlikely that the 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 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 O_2 past the gills as the 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 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 O_2). At this extreme hypoxic condition (below P_{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_{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).

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 CO_2 , then no increase in ventilation rate is necessary to maintain 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 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 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 μatm), 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>

References

- Bartol, I. K., Mann, R. and Vecchione, M. (2002). Distribution of the euryhaline squid *Lolliguncula brevis* in Chesapeake Bay: effects of selected abiotic factors. *Mar. Ecol. Prog. Ser.* **226**, 235–247.
- Belman, B. W. (1978). Respiration and the effects of pressure on the mesopelagic vertically migrating squid *Histioteuthis heteropsis*. *Limnol. Oceanogr.* **23**, 735–739.
- Birk, M. A. (2018). Ecophysiology of oxygen supply in cephalopods. PhD thesis, University of South Florida, Saint Petersburg, FL, USA.
- Birk, M. A., Dymowska, A. K. and Seibel, B. A. (2018). Do squid breathe through their skin? *J. Exp. Biol.* **221**, jeb185553.
- Bohr, C., Hasselbach, K. and Krogh, A. (1904). Ueber einen in biologischer Beziehung wichtigen Einfluss, den die Kohlensäurespannung des Blutes auf dessen Sauerstoffbindung übt. *Skand. Arch. Physiol.* **16**, 402–412.
- Bridges, C. R. (1994). Bohr and Root effects in cephalopod haemocyanins – paradox or pressure in *Sepia officinalis*? *Mar. Freshw. Behav. Physiol.* **25**, 121–130.
- Brix, O., Bårdgard, A., Cau, A., Colosimo, A., Condò, S. G. and Giardina, B. (1989). Oxygen-binding properties of cephalopod blood with special reference to environmental temperatures and ecological distribution. *J. Exp. Zool.* **252**, 34–42.
- Burnett, L. E. and Stickle, W. B. (2001). Physiological responses to hypoxia. In *Coastal Hypoxia: Consequences for Living Resources and Ecosystems* (ed. N. N. Rabalais and R. E. Turner), pp. 101–114. American Geophysical Union.
- Caldeira, K. and Wickett, M. E. (2005). Ocean model predictions of chemistry changes from carbon dioxide emissions to the atmosphere and ocean. *J. Geophys. Res.* **110**, C09S04.
- Childress, J. J. (1971). Respiratory adaptations to the oxygen minimum layer in the bathypelagic mysid *Gnathophausia ingens*. *Biol. Bull.* **141**, 109–121.
- Childress, J. J. (1975). The respiratory rates of midwater crustaceans as a function of depth of occurrence and relation to the oxygen minimum layer off Southern California. *Comp. Biochem. Physiol. A Physiol.* **50**, 787–799.
- Clayton, T. D. and Byrne, R. H. (1993). Spectrophotometric seawater pH measurements: total hydrogen ion concentration scale calibration of m-cresol purple and at-sea results. *Deep Sea Res. J. Oceanogr. Res. Papers* **40**, 2115–2129.
- Clements, J. C. and Hunt, H. L. (2015). Marine animal behaviour in a high CO_2 ocean. *Mar. Ecol. Prog. Ser.* **536**, 259–279.
- Deutsch, C., Ferrel, A., Seibel, B. A., Pörtner, H.-O. and Huey, R. B. (2015). Climate change tightens a metabolic constraint on marine habitats. *Science* **348**, 1132–1135.
- Dickson, A. G., Sabine, C. L. and Christian, J. R. (2007). Guide to best practices for ocean CO_2 measurements. *PICES Special Publ.* **3**, 191.
- D'Orbigny, A. D. (1835). Voyage dans l'Amérique Méridionale Paris, pp. 50–54. Chez Pitois Levrault.
- Eddy, F. B. (1974). Blood gases of the tench (*Tinca tinca*) in well aerated and oxygen-deficient waters. *J. Exp. Biol.* **60**, 71–83.
- Eddy, F. B. and Morgan, R. I. G. (1969). Some effects of carbon dioxide on the blood of rainbow trout *Salmo gairdneri* Richardson. *J. Fish Biol.* **1**, 361–372.
- Ern, R. and Esbaugh, A. J. (2016). Hyperventilation and blood acid-base balance in hypercapnia exposed red drum (*Sciaenops ocellatus*). *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **186**, 447–460.
- Esbaugh, A. J., Heuer, R. and Grosell, M. (2012). Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **182**, 921–934.
- Esbaugh, A. J., Ern, R., Nordi, W. M. and Johnson, A. S. (2016). Respiratory plasticity is insufficient to alleviate blood acid-base disturbances after acclimation to ocean acidification in the estuarine red drum, *Sciaenops ocellatus*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **186**, 97–109.
- Fabry, V. J., Seibel, B. A., Feely, R. A. and Orr, J. C. (2008). Impacts of ocean acidification on marine fauna and ecosystem processes. *ICES J. Mar. Sci.* **65**, 414–432.
- Feely, R. A., Alin, S. R., Carter, B., Bednaršek, N., Hales, B., Chan, F., Hill, T. M., Gaylord, B., Sanford, E., Byrne, R. H. et al. (2016). Chemical and biological impacts of ocean acidification along the west coast of North America. *Estuarine Coastal Shelf Sci.* **183**, 260–270.
- Franco, A. C., Hernández-Ayón, J. M., Beier, E., Garçon, V., Maske, H., Paulmier, A., Färber-Lorda, J., Castro, R. Sosa-Ávalos, R. (2014). Air–sea CO_2 fluxes above the stratified oxygen minimum zone in the coastal region off Mexico. *J. Geophys. Res. Oceans* **119**, 2923–2937.
- Friedman, J. R., Condon, N. E. and Drazen, J. C. (2012). Gill surface area and metabolic enzyme activities of demersal fishes associated with the oxygen minimum zone off California. *Limnol. Oceanogr.* **57**, 1701–1710.
- Gilly, W. F., Markaida, U., Baxter, C. H., Block, B. A., Boustany, A., Zeidberg, L., Reisenbichler, K., Robison, B., Bazzino, G. and Salinas, C. (2006). Vertical and horizontal migrations by the jumbo squid *Dosidicus gigas* revealed by electronic tagging. *Mar. Ecol. Prog. Ser.* **324**, 1–17.
- Gilly, W. F., Zeidberg, L. D., Booth, J. A. T., Stewart, J. S., Marshall, G., Abernathy, K. and Bell, L. E. (2012). Locomotion and behavior of Humboldt squid, *Dosidicus gigas*, in relation to natural hypoxia in the Gulf of California, Mexico. *J. Exp. Biol.* **215**, 3175–3190.
- Gilmour, K. M. (2001). The CO_2 /pH ventilatory drive in fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **130**, 219–240.
- Gräns, A., Jutfelt, F., Sandblom, E., Jonsson, E., Wiklander, K., Seth, H., Olsson, C., Dupont, S., Ortega-Martinez, O., Einarsdottir, I. et al. (2014). Aerobic scope fails to explain the detrimental effects on growth resulting from warming and elevated CO_2 in Atlantic halibut. *J. Exp. Biol.* **217**, 711–717.

- Gutowska, M. A., Pörtner, H.-O. and Melzner, F. (2008). Growth and calcification in the cephalopod *Sepia officinalis* under elevated seawater pCO₂. *Mar. Ecol. Prog. Ser.* **373**, 303-309.
- Gutowska, M. A., Melzner, F., Langenbuch, M., Bock, C., Claireaux, G. and Pörtner, H.-O. (2010a). Acid-base regulatory ability of the cephalopod (*Sepia officinalis*) in response to environmental hypercapnia. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 323-335.
- Gutowska, M. A., Melzner, F., Pörtner, H.-O. and Meier, S. (2010b). Cuttlebone calcification increases during exposure to elevated seawater pCO₂ in the cephalopod *Sepia officinalis*. *Mar. Biol.* **157**, 1653-1663.
- Häfker, N. S. (2012). Effects of hypoxia and hypercapnia on blood and tissue physiology of the common cuttlefish *Sepia officinalis*. MSc thesis, University of Bremen, Bremen, Germany.
- Heuer, R. M. and Grosell, M. (2014). Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R1061-R1084.
- Hochachka, P. W. and Somero, G. N. (2002). *Biochemical Adaptation: Mechanism and Process in Physiological Evolution*. Oxford University Press.
- Hoeger, U., Mommsen, T. P., O'Dor, R. and Webber, D. (1987). Oxygen uptake and nitrogen excretion in two cephalopods, octopus and squid. *Comp. Biochem. Physiol. A Physiol.* **87**, 63-67.
- Houlihan, D. F., Innes, A. J., Wells, M. J. and Wells, J. (1982). Oxygen consumption and blood gases of *Octopus vulgaris* in hypoxic conditions. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **148**, 35-40.
- Howell, B. J. and Gilbert, D. L. (1976). pH-temperature dependence of the hemolymph of the squid, *Loligo pealei*. *Comp. Biochem. Physiol.* **55A**, 287-289.
- Hu, M. and Tseng, Y.-C. (2017). Acid-base regulation and ammonia excretion in cephalopods: an ontogenetic overview. In *Acid-Base Balance and Nitrogen Excretion in Invertebrates* (ed. D. Weihrauch and M. O'Donnell), pp. 275-298. Springer International.
- Hu, M. Y., Guh, Y.-J., Stump, M., Lee, J.-R., Chen, R.-D., Sung, P.-H., Chen, Y.-C., Hwang, P.-P. and Tseng, Y.-C. (2014). Branchial NH₄⁺-dependent acid-base transport mechanisms and energy metabolism of squid (*Sepioteuthis lessoniana*) affected by seawater acidification. *Front. Zool.* **11**, 55.
- Hughes, G. M. (1973). Respiratory responses to hypoxia in fish. *Am. Zool.* **13**, 475-489.
- IPCC (2014). Climate Change 2014: synthesis report. In *Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change Team* (ed. The Core Writing Team, R. K. Pachauri and L. Meyer). Geneva, Switzerland: IPCC.
- Janssen, R. G. and Randall, D. J. (1975). The effects of changes in pH and PCO₂, in blood and water on breathing in rainbow trout, *Salmo gairdneri*. *Respir. Physiol.* **25**, 235-245.
- Johansen, K. and Weber, R. E. (1976). On the adaptability of haemoglobin function to environmental conditions. *Perspect. Exp. Biol.* **1**, 219-234.
- Johansen, K., Brix, O. and Lykkeboe, G. (1982). Blood gas transport in the cephalopod, *Sepia officinalis*. *J. Exp. Biol.* **99**, 331-338.
- Jokiel, P. L., Bahr, K. D. and Rodgers, K. S. (2014). Low-cost, high-flow mesocosm system for simulating ocean acidification with CO₂ gas. *Limnol. Oceanogr.* **12**, 313-322.
- Kaniewska, P., Campbell, P. R., Kline, D. I., Rodriguez-Lanetty, M., Miller, D. J., Dove, S. and Hoegh-Guldberg, O. (2012). Major cellular and physiological impacts of ocean acidification on a reef building coral. *PLoS ONE* **7**, e34659.
- Kaplan, M. B., Mooney, T. A., McCorkle, D. C. and Cohen, A. L. (2013). Adverse effects of ocean acidification on early development of squid (*Doryteuthis pealeii*). *PLoS ONE* **8**, e63714.
- Katsanevakis, S., Protopapas, N., Miliou, H. and Verriopoulos, G. (2005). Effect of temperature on specific dynamic action in the common octopus, *Octopus vulgaris* (Cephalopoda). *Mar. Biol.* **146**, 733-738.
- Lapennas, G. N. (1983). The magnitude of the Bohr coefficient: optimal for oxygen delivery. *Respir. Physiol.* **54**, 161-172.
- Lee, K., Tong, L. T., Millero, F. J., Sabine, C. L., Dickson, A. G., Goyet, C., Park, G.-H., Wanninkhof, R., Feely, R. A. and Key, R. M. (2006). Global relationships of total alkalinity with salinity and temperature in surface waters of the world's oceans. *Geophys. Res. Lett.* **33**, L19605.
- Lesueur, C. A. (1821). Descriptions of several new species of cuttle-fish. *J. Acad. Nat. Sci. Philadelphia* **2**, 86-101.
- Liu, X., Byrne, R. H., Lindemuth, M., Easley, R. and Mathis, J. T. (2015). An automated procedure for laboratory and shipboard spectrophotometric measurements of seawater alkalinity: continuously monitored single-step acid additions. *Mar. Chem.* **174**, 141-146.
- Lueker, T. J., Dickson, A. G. and Keeling, C. D. (2000). Ocean pCO₂ calculated from dissolved inorganic carbon, alkalinity, and equations for K₁ and K₂: validation based on laboratory measurements of CO₂ in gas and seawater at equilibrium. *Mar. Chem.* **70**, 105-119.
- Mandic, M., Todgham, A. E. and Richards, J. G. (2009). Mechanisms and evolution of hypoxia tolerance in fish. *Proc. R. Soc. B Biol. Sci.* **276**, 735-744.
- Mangum, C. P. (1997). Invertebrate blood oxygen carriers. In *Handbook of Physiology* (ed. W. H. Dantzler), pp. 1097-1135. Oxford University Press.
- Melrose, D. C., Oviatt, C. A. and Berman, M. S. (2007). Hypoxic events in Narragansett Bay, Rhode Island, during the summer of 2001. *Estuaries Coasts* **30**, 47-53.
- Melzner, F., Gutowska, M. A., Langenbuch, M., Dupont, S., Lucassen, M., Thorndyke, M. C., Bleich, M. and Pörtner, H.-O. (2009). Physiological basis for high CO₂ tolerance in marine ectothermic animals: pre-adaptation through lifestyle and ontogeny? *Biogeosciences* **6**, 2313-2331.
- Melzner, F., Thomsen, J., Koeve, W., Oschlies, A., Gutowska, M. A., Bange, H. W., Hansen, H. P. and Körtzinger, A. (2013). Future ocean acidification will be amplified by hypoxia in coastal habitats. *Mar. Biol.* **160**, 1875-1888.
- Miller, K. I. (1985). Oxygen equilibria of *Octopus dofleini* hemocyanin. *Biochemistry* **24**, 4582-4586.
- Miller, K. I. and Mangum, C. P. (1988). An investigation of the nature of Bohr, Root, and Haldane effects in *Octopus dofleini* hemocyanin. *J. Comp. Physiol. B* **158**, 547-552.
- Miller, S. H., Breitburg, D. L., Burrell, R. B. and Keppel, A. G. (2016). Acidification increases sensitivity to hypoxia in important forage fishes. *Mar. Ecol. Prog. Ser.* **549**, 1-8.
- Muggeo, V. M. R. (2008). segmented: an R package to fit regression models with broken-line relationships. *R News* **8**, 20-25.
- Nigmatullin, C. M., Nesis, K. N. and Arkhipkin, A. I. (2001). A review of the biology of the jumbo squid *Dosidicus gigas* (Cephalopoda: Ommastrephidae). *Fish. Res.* **54**, 9-19.
- Nilsson, G. E. and Östlund-Nilsson, S. (2008). Does size matter for hypoxia tolerance in fish? *Biol. Rev.* **83**, 173-189.
- Oeschger, R. (1990). Long-term anaerobiosis in sublittoral marine invertebrates from the Western Baltic Sea: *Halicyrtus spinulosus* (Priapulida), *Astarte borealis* and *Arctica islandica* (Bivalvia). *Mar. Ecol. Prog. Ser.* **59**, 133-143.
- Pan, T.-C. F., Applebaum, S. L. and Manahan, D. T. (2015). Experimental ocean acidification alters the allocation of metabolic energy. *Proc. Natl. Acad. Sci. USA* **112**, 4696-4701.
- Paulmier, A., Ruiz-Pino, D. and Garçon, V. (2011). CO₂ maximum in the oxygen minimum zone (OMZ). *Biogeosciences* **8**, 239-252.
- Pech, G. T. and Jackson, G. D. (2007). The potential impacts of climate change on inshore squid: biology, ecology and fisheries. *Rev. Fish Biol. Fish.* **18**, 373-385.
- Pörtner, H.-O. (1990). An analysis of the effects of pH on oxygen binding by squid (*Illex illecebrosus*, *Loligo pealei*) haemocyanin. *J. Exp. Biol.* **150**, 407-424.
- Pörtner, H.-O. (1994). Coordination of metabolism, acid-base regulation and haemocyanin function in cephalopods. *Mar. Freshw. Behav. Physiol.* **25**, 131-148.
- Pörtner, H.-O. (2012). Integrating climate-related stressor effects on marine organisms: unifying principles linking molecule to ecosystem-level changes. *Mar. Ecol. Prog. Ser.* **470**, 273-290.
- Pörtner, H.-O. and Reipschläger, A. (1996). Ocean disposal of anthropogenic CO₂: physiological effects on tolerant and intolerant animals. In *Ocean Storage of Carbon Dioxide Workshop 2 – Environmental Impact* (ed. W. G. Ormerod and M. Angel), pp. 57-81. Cheltenham: International Energy Agency Greenhouse Gas R&D Program.
- Pörtner, H.-O., Gutowska, M., Ishimatsu, A., Lucassen, M., Melzner, F. and Seibel, B. A. (2011). Effects of ocean acidification on nektonic organisms. In *Ocean Acidification* (ed. J.-P. Gattuso and L. Hansson), pp. 154-175. Oxford University Press.
- Redfield, A. C. and Goodkind, R. (1929). The significance of the Bohr effect in the respiration and asphyxiation of the squid, *Loligo pealei*. *J. Exp. Biol.* **6**, 340-349.
- Redfield, A. C. and Ingalls, E. N. (1933). The oxygen dissociation curves of some bloods containing hemocyanin. *J. Cell. Comp. Physiol.* **3**, 169-202.
- Reipschläger, A., Nilsson, G. E. and Pörtner, H.-O. (1997). A role for adenosine in metabolic depression in the marine invertebrate *Sipunculus nudus*. *Am. J. Physiol.* **272**, R350-R356.
- Richards, J. G. (2009). Chapter 10: metabolic and molecular responses of fish to hypoxia. *Fish Physiol.* **27**, 443-485.
- Rosa, R. and Seibel, B. A. (2008). Synergistic effects of climate-related variables suggest future physiological impairment in a top oceanic predator. *Proc. Natl. Acad. Sci. USA* **105**, 20776-20780.
- Rosa, R., Trübenbach, K., Pimentel, M. S., Boavida-Portugal, J., Faleiro, F., Baptista, M., Dionísio, G., Calado, R., Pörtner, H.-O. and Repolho, T. (2014). Differential impacts of ocean acidification and warming on winter and summer progeny of a coastal squid (*Loligo vulgaris*). *J. Exp. Biol.* **217**, 518-525.
- Rummer, J. L., Stecyk, J. A. W., Couturier, C. S., Watson, S.-A., Nilsson, G. E. and Munday, P. L. (2013). Elevated CO₂ enhances aerobic scope of a coral reef fish. *Conserv. Physiol.* **1**, cot023.
- Seibel, B. A. (2007). On the depth and scale of metabolic rate variation: scaling of oxygen consumption rates and enzymatic activity in the Class Cephalopoda (Mollusca). *J. Exp. Biol.* **210**, 1-11.
- Seibel, B. A. (2013). The jumbo squid, *Dosidicus gigas* (Ommastrephidae), living in oxygen minimum zones II: blood-oxygen binding. *Deep Sea Res. II Top. Stud. Oceanogr.* **95**, 139-144.
- Seibel, B. A. (2016). Cephalopod susceptibility to asphyxiation via ocean incalcescence, deoxygenation, and acidification. *Physiology* **31**, 418-429.
- Seibel, B. A., Häfker, N. S., Trübenbach, K., Zhang, J., Tessier, S. N., Pörtner, H.-O., Rosa, R. and Storey, K. B. (2014). Metabolic suppression

- during protracted exposure to hypoxia in the jumbo squid, *Dosidicus gigas*, living in an oxygen minimum zone. *J. Exp. Biol.* **217**, 2555-2568.
- Seibel, B. A., Luu, B. E., Tessier, S. N., Towanda, T. and Storey, K. B.** (2018). Metabolic suppression in the pelagic crab, *Pleuroncodes planipes*, in oxygen minimum zones. *Comp. Biochem. Physiol. B* **224**, 88-97.
- Spady, B. L., Watson, S.-A., Chase, T. J. and Munday, P. L.** (2014). Projected near-future CO₂ levels increase activity and alter defensive behaviours in the tropical squid *Idiosepius pygmaeus*. *Biol. Open* **3**, 1063-1070.
- Spady, B. L., Munday, P. L. and Watson, S.-A.** (2018). Predatory strategies and behaviours in cephalopods are altered by elevated CO₂. *Glob. Change Biol.* **24**, 2586-2596.
- Speers-Roesch, B., Richards, J. G., Brauner, C. J., Connolly, S. R., Hickey, A. J. R., Wang, Y. S. and Renshaw, G. M. C.** (2012). Hypoxia tolerance in elasmobranchs. I. Critical oxygen tension as a measure of blood oxygen transport during hypoxia exposure. *J. Exp. Biol.* **215**, 93-102.
- Steffensen, J. F.** (1989). Some errors in respirometry of aquatic breathers: how to avoid and correct for them. *Fish Physiol. Biochem.* **6**, 49-59.
- Storey, K. B. and Storey, J. M.** (1978). Energy metabolism in the mantle muscle of the squid, *Loligo pealeii*. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **123**, 169-175.
- Strobel, A., Bennecke, S., Leo, E., Mintenbeck, K., Pörtner, H.-O. and Mark, F. C.** (2012a). Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO₂. *Front. Zool.* **9**, 28.
- Strobel, A., Hu, M. Y. A., Gutowska, M. A., Lieb, B., Lucassen, M., Melzner, F., Pörtner, H.-O. and Mark, F. C.** (2012b). Influence of temperature, hypercapnia, and development on the relative expression of different hemocyanin isoforms in the common cuttlefish *Sepia officinalis*. *J. Exp. Zool. A Ecol. Genet. Physiol.* **317**, 511-523.
- Trübenbach, K., Pegado, M. R., Seibel, B. A. and Rosa, R.** (2013). Ventilation rates and activity levels of juvenile jumbo squid under metabolic suppression in the oxygen minimum zone. *J. Exp. Biol.* **216**, 359-368.
- Truchot, J.-P.** (1976). Carbon dioxide combining properties of the blood of the shore crab *Carcinus maenas* (L.): carbon dioxide solubility coefficient and carbonic acid dissociation constants. *J. Exp. Biol.* **64**, 45-57.
- Trueblood, L. A. and Seibel, B. A.** (2013). The jumbo squid, *Dosidicus gigas* (Ommastrephidae), living in oxygen minimum zones I: oxygen consumption rates and critical oxygen partial pressures. *Deep Sea Res. II Top. Stud. Oceanogr.* **95**, 218-224.
- Turner, C.** (2015). Verification of pH fluctuations in Narragansett Bay. Master's thesis, University of Rhode Island, Narragansett, RI, USA.
- Wells, M. J.** (1992). The cephalopod heart: the evolution of a high-performance invertebrate pump. *Experientia* **48**, 800-808.
- Wells, M. J. and Wells, J.** (1995). The control of ventilatory and cardiac responses to changes in ambient oxygen tension and oxygen demand in *Octopus*. *J. Exp. Biol.* **198**, 1717-1727.
- Wells, M. J., O'Dor, R. K., Mangold, K. and Wells, J.** (1983). Feeding and metabolic rate in *Octopus*. *Mar. Behav. Physiol.* **9**, 305-317.
- Wells, M. J., Hanlon, R. T., Lee, P. G. and Dimarco, F. P.** (1988). Respiratory and cardiac performance in *Lolliguncula brevis* (Cephalopoda, Myopsida): the effects of activity, temperature and hypoxia. *J. Exp. Biol.* **138**, 17-36.
- Widdicombe, S. and Spicer, J. I.** (2008). Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us? *J. Exp. Mar. Biol. Ecol.* **366**, 187-197.
- Zakroff, C.** (2013). CO₂-level dependent effects of ocean acidification on squid, *Doryteuthis pealeii*, early life history. Master's thesis, King Abdullah University of Science and Technology, Thuwal, Saudi Arabia.