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Ecophysiology of Oxygen Supply in Cephalopods

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Ecophysiology of Oxygen Supply in Cephalopods

by

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A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
College of Marine Science
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DEDICATION

I dedicate this work to Jen Goff and Pam Kimber, two of my high school teachers that saw potential in me and invested their time and energy in me in hopes to make a difference in my life. You have. I also dedicate this work to my parents, Greg and June Birk, for their consistent support of my ambitions and their many sacrifices to give me a better life.

To my bright and beautiful daughter, Natalie, and your siblings after you: I pray you discover your passion, and your calling, and that you love each step of the journey set before you. To my beautiful bride, Marianne: you know well that this work would not have been possible without you. You have supported me in so many ways through this journey. I thank you for your many sacrifices and your commitment to me through five years of moving, research cruises, loneliness, and busyness. My heart has trusted in you and I have had no lack of gain.

Finally, I dedicate this work to the Creator and Redeemer of all things. What an opportunity it has been to see first-hand the many beautiful, wonderful, and amazingly complex aspects of God's creation, and to contribute one tiny piece more to our knowledge of His work, that we may better know Him.

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ABSTRACT

Cephalopods are an important component of many marine ecosystems and support large fisheries. Their active lifestyles and complex behaviors are thought to be driven in large part by competition with fishes. Although cephalopods appear to compete successfully with fishes, a number of their important physiological traits are arguably inferior, such as an inefficient mode of locomotion via jet propulsion and a phylogenetically limited means of blood-borne gas transport. In active shallow-water cephalopods, these traits result in an interesting combination of very high oxygen demand and limited oxygen supply. The ability to maintain active lifestyles despite these metabolic constraints makes cephalopods a fascinating subject for metabolic physiology. This dissertation focuses on the physiological adaptations that allow coleoid cephalopods to maintain a balance of oxygen supply and demand in a variety of environmental conditions.

A critical component of understanding oxygen supply in any animal is knowing the means of oxygen delivery from the environment to the mitochondria. Squids are thought to obtain a fairly large portion of their oxygen via simple diffusion across the skin in addition to uptake at the gills. Although this hypothesis has support from indirect evidence and is widely accepted, no empirical examinations have been conducted to assess the validity of this hypothesis. In Chapter 2, I examined cutaneous respiration in two squid species, *Doryteuthis pealeii* and *Lolliguncula brevis*, by using a divided chamber to physically separate the mantle

cavity and gills from the outer mantle surface. I measured the oxygen consumption rate in the two compartments and found that, at rest, squids only obtain enough oxygen cutaneously to meet demand of the skin tissue locally (12% of total). The majority of oxygen is obtained via the traditional branchial pathway. In light of these findings, I re-examine and discuss the indirect evidence that has supported the cutaneous respiration hypothesis.

Ocean acidification is believed to limit the performance of squids due 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}), defined as the PO_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 Chapter 3, I assessed the effects of CO_2 (40 to 140 Pa) on the metabolic rate and P_{crit} of two squid species: *Dosidicus gigas* and *Doryteuthis pealeii.* Carbon dioxide had no effect on metabolic rate or hypoxia tolerance in either species. Furthermore, considering oxygen transport parameters (e.g. Bohr coefficient, blood P_{50}) and blood PCO_2 values from the literature, I estimated an increase in seawater PCO_2 to 100 Pa (≈1000 μatm/ppmv) would result in a maximum drop in hemocyanin-O_2 saturation by 6% at normoxia and a P_{crit} increase of ≈1 kPa (≈5% air saturation) in the absence of active extracellular pH compensation. Such changes are unlikely given the capacity for acid-base regulation in many cephalopods. Moreover, this estimated change is within the 95% confidence intervals of the P_{crit} measurements reported here. Squid blood-O_2 binding is more sensitive to pH than most other marine animals measured to date. Therefore, the lack of effect in squids suggests that ocean acidification is unlikely to have a limiting effect on blood-O_2 supply in most marine animals.

The pelagic octopod, *Japetella diaphana*, is known to inhabit meso- and bathypelagic depths worldwide. Across its range, individuals encounter oxygen levels ranging from nearly air-saturated to nearly anoxic. In Chapter 4, we assessed the physiological adaptations of individuals from the eastern tropical Pacific (ETP) where oxygen is extremely low. Ship-board measurements of metabolic rate and hypoxia tolerance were conducted and a metabolic index was constructed to model suitable habitat for aerobic metabolism. I found that animals from the ETP had a higher metabolic rate than animals from more oxygen-rich habitats. Despite their higher oxygen demand, they maintained better hypoxia tolerance than conspecifics from oxygen-rich Hawaiian waters. Furthermore, I found that hypoxia tolerance in *Japetella* has a reverse temperature dependence from most marine ectotherms, a characteristic that uniquely suits the physical characteristics of its oxygen-poor environment. Even with their high tolerance to hypoxia, the OMZ core likely has insufficient oxygen supply to meet the basal oxygen demand of *Japetella.* Despite the limited aerobic habitat in this region, species abundance was comparable to more oxygenated ocean regions, suggesting that physiological or behavioral plasticity such as altered hypoxia tolerance or hypoxic avoidance in this globally-distributed species is sufficient to maintain species fitness in this extreme environment.

These findings contribute towards our understanding of the impacts of climate change on cephalopod physiology and biogeography. The study of environmental physiology provides a mechanistic basis for the understanding and prediction of ecological responses to climate change.

CHAPTER ONE:

INTRODUCTION

Cephalopods are marine mollusks that inhabit ocean environments worldwide. They are a moderately diverse group with nearly 800 species that occupy environments from the intertidal to seafloor hydrothermal vents by employing diverse life histories, behaviors, and ecologies (Hanlon and Messenger 1996). The evolutionary success of this group is often attributed to competition with fishes. Coleoid cephalopods (octopuses, squids, cuttlefishes, and their relatives) share a number of traits with fishes such as active locomotion, complex behaviors, and visually-oriented predation (Packard 1972). Both groups occupy similar ecological and trophic niches, and compete with, and are predated upon by, each other.

Although cephalopods compete successfully with fishes, a number of their important physiological traits are apparent constraints (O'Dor and Webber 1986). Of primary importance is their means of locomotion. Unlike fishes, which use high efficiency undulatory fins, many cephalopods rely heavily on jet propulsion, a highly inefficient mode of locomotion, particularly in a viscous medium like seawater. For example, the Froude efficiency, or the ratio of power output to input, in the squid *Doryteuthis opalescens* is less than a third that of a similar sized trout (O'Dor and Webber 1986). Additionally, the squid *Illex illecebrosus* uses six times as much energy per unit distance swimming via jetting than a salmon of the same length undulating fins (Webber and O'Dor 1985).

This locomotory inefficiency, combined with high non-asymptotic growth rates (Moltschaniwskyj 2004), result in high energy demands for many coleoid cephalopods, and thus high metabolic rates. Indeed, when adjusted for temperature and size, active shallow-water squids have higher metabolic rates than many crustaceans, fishes, and mammals (Seibel 2007).

Such high O_2 demand requires an equally capable oxygen supply pathway from seawater to the mitochondria. In this respect, however, cephalopods are constrained by their molluscan ancestry. Unlike fishes, which transport their oxygen-binding protein hemoglobin within red blood cells, cephalopods possess very large (3.5-4.5 MDa) hemocyanin molecules freely-dissolved in their blood (Markl 2013). This arrangement limits the concentration of hemocyanin molecules due to viscous and osmotic constraints (Shadwick et al. 1990). As a result, cephalopod blood contains no more than about 2 mmol O_2 L^{-1} (Pörtner 1990; Redfield and Goodkind 1929), compared to 7 mmol O_2 L^{-1} attainable in fish blood (Root 1931).

Additionally, cephalopods do not possess an intracellular oxygen transport protein such as myoglobin in their tissues, nor are their muscle tissues as highly vascularized as fish muscle (Bone et al. 1981). To maintain high O_2 supply despite these constraints, cephalopods have evolved a number of physiological adaptations. These include a closed high-pressure cardiovascular systems (10-20 kPa; Gosline and Shadwick 1982) with very high cardiac outputs (100-250 mL / kg / min; Bourne 1987), higher than fishes and even comparable to mammals in some species (Wells 1992), and a highly pH-sensitive respiratory protein that ensures nearly complete use of all oxygen carried in the blood (Brix et al. 1989).

Focus statement

The ability to maintain active lifestyles despite the above metabolic constraints makes coleoid cephalopods a fascinating subject for metabolic physiology. This dissertation focuses on the physiological ability of coleoid cephalopods to maintain a balance of oxygen supply and demand in a variety of different environmental conditions.

Chapter 2: Do squids breathe through their skin?

One adaptation that has been proposed to alleviate the circulatory and metabolic constraints is that coleoid cephalopods utilize cutaneous respiration, obtaining oxygen via simple diffusion across the skin in addition to that delivered through the circulatory system. Previous research found that a small proportion of O_2 was taken up across the skin in *Octopus* (Wells and Wells 1983), but no *in vivo* measurements have ever been made in squids. In Chapter 2 of this dissertation, I present results of such experiments in two representative squid species that demonstrate that squids do not utilize cutaneous respiration, contrary to 30 years of conjecture in the cephalopod physiology literature.

Chapter 3: Is squid metabolism impaired by ocean acidification?

The highly pH-sensitive hemocyanin O_2-binding protein mentioned above is adaptive when tissues are producing large quantities of CO_2 as it facilitates O_2 loading at the relatively alkaline gill-seawater interface and unloading at the tissues where it is consumed (Pörtner 1990). When an animal is exposed to high CO_2 in the ambient seawater, however, hemocyanin function shifts towards O_2 release rather than binding at the gills, a potentially maladaptive response in light of recent anthropogenic ocean acidification.

The oceans take up roughly 3 Gt C / year from the atmosphere, or about a third of anthropogenic CO_2 emissions (Pilson 2013). Upon dissolution in seawater, much of the CO_2 undergoes hydration and dissociation to form protons and decrease the pH of the surface ocean (Dickson 2010). This increase in CO_2 and associated decrease of pH and $[CO_3^{2-}]$ have various, and so far unpredictable, impacts on marine organisms including effects on metabolism (Lefevre

2016), calcification (Hofmann et al. 2010), behavior (Nilsson et al. 2012), growth rate (Kroeker et al. 2013), and reproduction (Ross et al. 2011). These physiological responses lead to impacts on ecosystems (Hall-Spencer et al. 2008) and fisheries (Le Quesne and Pinnegar 2012).

While there are various physiological systems that are independently affected by hypercapnia (high CO_2) (Heuer and Grosell 2014), one of the most important may be the hypercapnic limitation of blood O_2 supply as this can directly limit the scope for locomotion, calcification, growth, and reproduction (Pörtner and Gutt 2016).

Amongst the broad animal diversity in the oceans, the sensitivity of blood O_2 supply to CO_2 (pH) can vary markedly. Therefore, the examination of those taxa thought to be most sensitive, such as the squids discussed above, should be most informative, as it should provide a reasonable estimate of an upper bound on the expected effect of CO_2 on blood O_2 supply in marine animals generally.

Active, muscular, shallow-water squids such as those examined in Chapter 3 of this dissertation are believed to possess blood with some of the greatest sensitivity to increases in environmental CO_2 known to date (Pörtner and Reipschläger 1996; Seibel 2016). In Chapter 3, I present results of both experimental and theoretical approaches to quantify the impact of CO_2 levels anticipated by the end of the century (1000 μatm) on blood O_2 supply in squids. In so doing, I propose that the blood O_2 supply pathways of even the most sensitive marine animals such as squids are quite robust to hypercapnic levels expected to occur by the year 2100.

Chapter 4: How do mesopelagic octopods breathe in the oxygen minimum zone?

Although cephalopod O_2 supply may be resilient to relatively high CO_2, low dissolved oxygen levels in the ocean are a common, and increasingly severe, environmental condition that can strongly affect all aerobic organisms, including cephalopods (Breitburg et al. 2018). Hypoxia naturally occurs on diel to seasonal timescales in coastal environments and on geologic timescales in intermediate waters underlying productive eastern boundary currents (Rabalais et al. 2010). In these environments, oxygen is utilized by heterotrophs faster than it can be replenished by air-sea gas exchange, primary production, or advection.

Animals living in these hypoxic environments may utilize a number of strategies to survive acute hypoxic events, such as migration, metabolic suppression, or increasing anaerobic capacity. However, for animals that live in chronically hypoxic environments, such as oxygen minimum zones (OMZs), survival depends on the ability of O_2 supply pathways to meet demand even under extreme hypoxia (Childress and Seibel 1998). Oceanic OMZs have strong impacts on the physiology, abundance, and distribution of marine organisms as well as the biogeochemical cycles they sustain (Levin 2003).

In Chapter 4 of this dissertation, I examine the metabolic adaptations of *Japetella diaphana*, a little-studied meso- and bathypelagic octopod, to OMZs in the eastern tropical Pacific. I examine metabolic rate, hypoxia tolerance, and abundance of this species in an OMZ to assess the metabolic capabilities these animals have evolved to inhabit this extreme environment. I also present a physiology-based habitat suitability model to demonstrate that although *Japetella* are very tolerant to hypoxia, a large span of the OMZ core has insufficient oxygen supply to meet the oxygen demand of this species.

Relevance and implications

The marine environment is changing at an accelerated rate due to anthropogenic climate change. This includes rises in seawater temperature and CO_2 content as well as declines in oxygen content (Breitburg et al. 2015). Each of these perturbations influences the balance of oxygen supply and demand in marine organisms both independently and synergistically (Pörtner et al. 2005). The research findings presented in this dissertation contribute towards our understanding of the physiological mechanisms of oxygen supply and demand and the responses of those mechanisms to changing environmental conditions. This mechanistic approach to organismal responses is likely to be a key to understanding and predicting responses of marine organisms to their changing environment (Lefevre et al. 2017).

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CHAPTER TWO:

DO SQUIDS BREATHE THROUGH THEIR SKIN?

Abstract

Squids are thought to obtain a large portion of their oxygen via simple diffusion across the skin in addition to uptake at the gills. Although this hypothesis has support from indirect evidence and is widely accepted, no empirical examinations have been conducted to assess the validity of this hypothesis. In this study, we examined cutaneous respiration in two squid species, *Doryteuthis pealeii* and *Lolliguncula brevis*, by using a divided chamber to physically separate the mantle cavity and gills from the outer mantle surface. We measured oxygen consumption and ammonia excretion rates in the two compartments and found that, at rest, squids only obtain enough oxygen cutaneously to meet demand of the skin tissue locally (12% of total) and excrete little ammonia across the skin. The majority of O_2 is obtained via the traditional branchial pathway. In light of these findings, we re-examine and discuss the indirect evidence that has supported the cutaneous respiration hypothesis.

Introduction

Cutaneous oxygen uptake, the acquisition of O_2 molecules from the environment into the skin, is likely to occur to some extent in nearly all animals by simple diffusion due to an oxygen gradient from the environment into skin tissue (Krogh 1941). However, the acquisition of oxygen not just into the skin, but across the skin and into the underlying blood or tissues is less universal but still contributes notably to oxygen supply in a diversity of animals including most amphibians, several fishes (especially air-breathing fishes), copepods, many small terrestrial arthropods, echinoderms, pycnogonids, bryozoans, cnidarians, a variety of worms, and occurs in a number of embryonic and larval stages before respiratory structures have developed (Krogh 1941; Feder and Burggren 1985; Lane et al. 2018).

In addition to oxygen uptake, metabolically produced ammonia may also be excreted cutaneously. This has been demonstrated in several seawater and freshwater fishes including Pacific hagfish (*Eptatretus stoutii*; Clifford et al. 2016, 2017), mangrove killifish (*Kryptolebias marmoratus*; Frick and Wright 2002; Cooper et al. 2013), and sea lamprey (*Petromyzon marinus*; Blair et al. 2017), as well as the fully aquatic African clawed frog (*Xenopus laevis*; Cruz et al. 2013), and freshwater leech (*Nephelopsis obscura*; Quijada-Rodriguez et al. 2015). However, the role of skin in the removal of nitrogenous waste has not been investigated in cephalopods to date.

Wells and Wells (1983) were the first to demonstrate cutaneous O_2 uptake in cephalopods. They found that *Octopus vulgaris* acquire roughly 13% of their resting O_2 consumption cutaneously (across the skin) on the web, arms, and suckers in addition to branchially (across the gills). Soon thereafter, Wells et al. (1988) proposed that since squids have a higher surface area-to-volume ratio than octopuses, cutaneous O_2 uptake is likely to occur to a greater extent in squids. They estimated, in the complete absence of evidence, that it may contribute to 20% of oxygen acquisition.

In the 30 years since, the idea that squids obtain a notable portion of their O_2 cutaneously rather than branchially has pervaded the literature, with proposed contributions of the skin ranging from 20 to 73% of total O_2 uptake (Wells et al. 1988; Pörtner 1994). The cutaneous respiration hypothesis has been incorporated into calculations of branchial oxygen extraction efficiency (Trübenbach et al. 2013a), used to explain calculations of surprisingly high cardiac power output (O'Dor et al. 1990; Shadwick et al. 1990; Wells 1992) and blood hemocyanin properties (Pörtner 1990, 1994, 2002), and has been incorporated into discussion of skin morphology (Madan and Wells 1997), muscle ultrastructure (Pörtner 2002; Seibel 2016), growth rates (O'Dor and Hoar 2000; Moltschaniwskyj 2004; Moltschaniwskyj and Carter 2010), metabolic scaling (Seibel 2007; Rosa et al. 2009; Trübenbach et al. 2013b), and hypoxia tolerance (Seibel 2013, 2016).

This hypothesis was based on several lines of indirect evidence in addition to the direct measurements made in octopus. When examining the O_2 binding properties of the squid respiratory protein, hemocyanin, Pörtner (1990) observed that the Bohr coefficient (a metric of pH sensitivity) was less than -1, meaning that deoxygenated hemocyanin in tissue capillaries removes more CO_2 from solution than is produced during metabolism from the O_2 delivered. This suggests that venous blood should be less acidic than arterial blood, which would increase hemocyanin O_2-affinity, thus inhibiting the delivery of oxygen to the tissues. In fact, such a venous alkalosis is not observed in squids (Redfield and Goodkind 1929). Instead, they exhibit the typical venous acidosis, which supports O_2 delivery to the tissues. To explain this paradox, Pörtner (1990) supposed that if cutaneous oxygen acquisition were substantial, the CO_2 produced from this cutaneously-derived oxygen could enter the blood and provide sufficient CO_2 into the blood to support a venous acidosis.

Madan and Wells (1996a) measured respiration rates of dissected skins from a variety of cephalopods including two squids, *Lolliguncula brevis* and *Sepioteuthis lessoniana,* and found that they were comparable to the dissected skin of octopuses. In a separate experiment, they also found that intact *Octopus vulgaris* skin from the dorsal mantle could support up to 82% of O_2

demand. These findings are suggestive that squid skin may be able to support comparable rates, but *in vivo* measurements have still only been conducted in *Octopus* to date (Wells and Wells 1983).

In addition to the evidence described above, an assortment of circumstantial evidence also supports the cutaneous respiration hypothesis in squids. The general body plan of a squid is a hollow tube, with inner and outer mantle surfaces exposed to seawater. The resultant high surface area to volume ratio could support a high contribution of cutaneous respiration. Unlike in the body plans of most animals, the SA:V ratio of loliginid squids has been reported to scale isometrically over several orders of magnitude in size due to allometric lengthening of the mantle and fins, such that large squids have just as much cutaneous area, relative to respiring mass, as small squids (O'Dor and Hoar 2000).

Additionally, the composition of circular muscle in the mantle is arranged in a "sandwich" where the outer layers of muscle tissue (both on the outside of the animal and the inner mantle cavity) are mitochondria-rich aerobic fibers and have high capillary density, while the central layer contains mitochondria-poor anaerobic fibers with lower capillary density (Bone et al. 1981; Mommsen et al. 1981). This arrangement may be conducive for cutaneous oxygen acquisition and the delivery of cutaneously derived CO_2 to the blood as proposed by Pörtner (1990). The active, water-column dwelling lifestyle of squids and their jet propulsion biomechanics also provide ample ventilation across the skin, minimizing large boundary layers, which may limit cutaneous respiration in octopuses (Madan and Wells 1996a). Finally, squid skin is quite thin; it is merely 300 μm in *Loligo vulgaris* and 150 μm in *Illex illecebrosus* compared to the $\approx 1300\ \mu m$ skin of *Octopus vulgaris* (Madan and Wells 1997). Moreover, octopus epidermal cells secrete a thin cuticle over the whole body while squids lack these secretions (Packard 1988). All else being the same, according to Fick's law (Fick 1855; see Discussion), respiratory gases diffuse faster across shorter distances.

Despite the indirect and circumstantial evidence compiled above, to date, there have been no direct empirical measurements quantifying the magnitude of cutaneous oxygen uptake in squids nor determining whether they "breathe" through their skin. In this study, we provide the first measurements of *in vivo* cutaneous oxygen consumption in two squid species, *Doryteuthis pealeii* and *Lolliguncula brevis*. In addition to oxygen exchange, we also examine the potential role of squid skin in metabolic ammonia excretion for the first time.

Methods

Animal capture and maintenance

Doryteuthis pealeii (Lesueur 1821; n = 17) were caught in the Vineyard Sound by benthic otter trawl by the R/V *Gemma* in October 2017 and held in a large aerated tank at the Marine Biological Laboratory at 19 °C until experiments were performed. Trials were conducted within 24 hours of capture for all but two animals that were fasted for up to three days. Only animals with skin in excellent condition were chosen for experiments. One individual was jigged aboard the R/V *Gemma* to determine whether capture by the trawl net had any effect on the results even in the absence of visible skin damage. *Lolliguncula brevis* (Blainville 1823; n = 4) were similarly caught by otter trawl in Tampa Bay, Florida, USA from April through June 2017 by the Florida FWCC FWRI Fisheries Independent Monitoring program and tested within 24 hours of capture. Mass and dorsal mantle length of both species are shown in Table 2.1. All *L. brevis* experiments were run at 20°C, while *D. pealeii* experiments were conducted at 11 and 19°C. Salinity was 30-34 in all trials.

Divided chamber setup

We developed a divided chamber setup to physically isolate cutaneous and branchial oxygen consumption in squids. This setup was conceptually similar to that used by Wells and Wells (1983) to measure cutaneous respiration in *Octopus*. Each squid was gently patted dry around the anterior edge of the mantle and a custom-sized rubber collar was attached to the anterior margin of the mantle with cyanoacrylate glue. The entire procedure typically lasted less than one minute. Collars were constructed with 0.4 mm thick, 70 duro nitrile (West American Rubber Company LLC, Orange, California, USA). The collars were truncated cones arranged so that they widened posteriorly and were attached to one end of a transparent acrylic tube (9x17 cm). The other end of the acrylic tube was covered with a flexible rubber oxygen-impermeable membrane so that ventilatory inspirations were not impeded by pressure inside the tube. This tube, the “mantle respirometer”, contained water in contact with the exterior mantle and fins (Figure 2.1). On average, 35% and 45% of the skin was contained inside the “mantle” respirometer for *D. pealeii* and *L. brevis* respectively. With the collar attached, animals were observed to ventilate at a normal rate (Chapter 3).

The mantle respirometer and squid were then placed into a larger respirometer, the “gill respirometer”, which contained water in contact with the gills, mantle cavity, head, arms, and tentacles. Magnetic stir beads were placed inside both respirometers to ensure uniform mixing (Figure 2.1). In one of the trials, food coloring was added to the mantle respirometer water to demonstrate that there was no water exchange between the mantle and gill respirometers. P_{O_2} was measured with oxygen-sensitive spots adhered to the inside of both respirometers (PreSens Fibox 3 (Regensburg, Germany) and Loligo Systems Witrox O_2 meters (Viborg, Denmark)). Oxygen meters were calibrated with air-saturated seawater and concentrated $NaSO_3$ solution

($P_{O_2} = 0$). Oxygen consumption rates (M_{O_2}) in both respirometers were calculated with the R package “respirometry” (Birk 2018). Only M_{O_2} values derived from P_{O_2}-time relationships with an $R^2 > 0.8$ were used.

Calculation of cutaneous oxygen uptake

Cutaneous surface area was measured for each squid by photographing the flattened mantle after the viscera and gladius were removed, fins, head, arms, and tentacles and quantifying surface area via ImageJ (Abràmoff et al. 2004). Total measured cutaneous oxygen uptake (M_{O_2}) was then calculated according to Equation 2.1. The amount of cutaneous oxygen consumption expected for exclusively localized use within the skin tissue was then calculated according to Equation 2.2 by multiplying the total surface area, skin thickness of 300 μm (Madan and Wells 1997), a tissue density of 1.05 $g \cdot cm^{-3}$ (Packard 1972) and the animal’s mass-specific metabolic rate as determined by the combined oxygen consumption in both the mantle and gill respirometers. The measured and local cutaneous rates were then compared with a paired t-test.

$$\text{Measured cutaneous } \mathrm{M_{O_2}} = \frac{\text{total surface area}}{\text{surface area inside mantle respirometer}} \times \mathrm{M_{O_2}} \text{ of mantle respirometer}, \quad (2.1)$$

$$\text{Local cutaneous } \mathrm{M_{O_2}} = \text{total surface area} \times 0.03\ \text{cm} \times 1.05\ \text{g} \cdot \text{cm}^{-3} \times \text{mass-specific } \mathrm{M_{O_2}}, \quad (2.2)$$

Ammonia excretion rates

To quantify ammonia excretion rates, water samples were collected from both respirometers at the end of the experiments and stored at -80°C until processed. The concentration of excreted ammonia was measured using a phenol method adapted from Ivančič and Degobbis (1984). Briefly, duplicate water samples (2 mL) were treated with phenol, nitroprusside, alkaline citrate, and dichloro-iso-cyanuric acid, and incubated for 12 h in the dark. Blue-colored indophenol was formed in the presence of ammonia in the sample. Ammonia concentration was measured with a spectrophotometer (Shimadzu UV-1700 (Kyoto, Japan) by observing absorbance at 635 nm. Ammonia excretion measurements were conducted for six and one *D. pealeii* at 11 and 19°C, respectively, and two *L. brevis.*

Results

Oxygen consumption

The proportion of whole animal M_{O_2} that was acquired from cutaneous uptake ranged from 5 to 19% (Table 2.2; Figure 2.2). The cutaneous uptake of the one jigged specimen (8%) fell within the range of those collected by trawl suggesting that trawl damage was not a factor. The contribution of cutaneous uptake was indistinguishable between species (p_{19} = 0.327) or temperature treatments (p_{15} = 0.086). *Doryteuthis pealeii* ranged in size from 51 to 150 g and 10.8 to 18.1 cm dorsal mantle length (Table 2.1). Across this size spectrum, the squids' surface area to volume ratio decreased with increasing size according to SA:V = 15.6 × mass$^{-0.28}$ (p = 0.004), and with it, the contribution of cutaneous M_{O_2} to total M_{O_2} (p_{15} = 0.003).

To determine whether the oxygen taken up by the skin is in excess of that used locally by the skin, the measured cutaneous M_{O_2} was compared to the expected cutaneous M_{O_2} from local (the skin tissue only) oxygen demand, which in turn, was derived from cutaneous surface area and mass-specific M_{O_2}. Total cutaneous surface area was 424 $\pm$ 78 cm^2 and 113 $\pm$ 13 cm^2 for *D. pealeii* and *L. brevis,* respectively (Figure 2.3A). The mass-specific metabolic rate (M_{O_2}) of *D. pealeii* was 11.3 $\pm$ 2.4 $\mu mol\ O_2 \cdot g^{-1} \cdot hr^{-1}$ at 11∞C and increased to 14.3 $\pm$ 4.1 $\mu mol\ O_2 \cdot g^{-1} \cdot hr^{-1}$ at 19°C (Figure 2.3B). *Lolliguncula brevis* mass-specific M_{O_2} at 20°C was 12.7 $\pm$ 2.6 $\mu mol\ O_2 \cdot g^{-1} \cdot hr^{-1}$, which was similar to *D. pealeii* at 19°C (p_{12} = 0.502; Figure 2.3B). The measured cutaneous M_{O_2} was significantly less than that expected from local tissue demand (p_{20} < 0.001; Figure 2.3C).

The area-specific cutaneous M_{O_2} ($\mu mol\, O_2 \cdot cm^{-2} \cdot hr^{-1}$) was similar between the two species at similar temperatures (p_{12} = 0.188) and increased with increasing temperature in *D. pealeii* (p_{15} = 0.015) with a Q_{10} of 1.6, which was similar to the Q_{10} for mass-specific M_{O_2} of 1.3. In fact, the area-specific cutaneous M_{O_2} was related to mass-specific M_{O_2} within both temperature treatments for *D. pealeii* (11°C: p_5 = 0.003; 19°C: p_8 = 0.025) and for all the animals from both species (p_{19} = 0.019).

Ammonia excretion

Doryteuthis pealeii skin excreted 7.5 ± 1.6% of the metabolically produced ammonia, with the remainder excreted via the gills and renal sac (Table 2.2). The atomic ratio of O_2 consumed to NH_3 excreted by the whole animal (O:N) varied widely, from 17 to 59 with a median of 27. The animal exhibiting the highest O:N was fasted for three days before the trial. The O:N ratio in the mantle respirometer was significantly higher than in the gill respirometer (p_8 = 0.033; Figure 2.4).

Discussion

Contrary to popular conjecture, we found that squids do not breathe through their skin. By this we mean the uptake of O_2 across the skin was likely consumed locally by the skin tissue rather than being incorporated into the blood for systemic utilization. The proportion of total O_2 uptake across the skin was less than any previous estimates. Since the measured cutaneous uptake was on average 23% less than the estimated amount required by the skin tissue locally, squid skin does not seem to be a net source of O_2 to the animal, but rather a sink, at least partially dependent on blood-borne oxygen.

We also found that O:N was higher across the skin than across the gills, suggesting that the capacity of ammonia excretion across the skin is lower than the gills. This is not surprising given the presence of ammonia transport proteins in the gills (Hu and Tseng 2017). The gill side of the respirometer was also in contact with the renal sac in the mantle cavity, which is also involved in ammonia excretion (Boucher-Rodoni and Mangold 1994) though to a lesser extent than the gills (Hu et al. 2017).

Madan and Wells (1996a) found that isolated skin from both *L. brevis* and *Sepioteuthis lessoniana* had an M_{O_2} of $0.12 \pm 0.04\ \mu mol\ O_2 \cdot cm^{-2} \cdot hr^{-1}$ at 20-24°C. These experiments, however, were in an unstirred chamber such that a hypoxic boundary layer was allowed to form. When performing similar experiments on *Octopus vulgaris* skin*,* they found that adding a magnetic stir bar more than doubled the *in vitro* rate of O_2 consumption. When the same factor is applied to the squid skin they examined, isolated squid skin should have an M_{O_2} of 0.26-0.27 $\pm$ 0.09 $\mu mol\ O_2 \cdot cm^{-2} \cdot hr^{-1}$. The *in vivo* results for *L. brevis* and *D. pealeii* at 19°C from our study are indistinguishable from these values ($p > 0.05$ for all species-wise comparisons), which suggests that the O_2 consumed in our experiments can be fully explained by localized consumption without any uptake by the blood and other body tissues.

The O:N ratios observed here were higher than is typical for squids (11-17), which have a well-known protein-rich diet (Ikeda 2016). One animal was fasted for three days and had a notably higher O:N than any other individual, suggesting a use of lipid reserves. This matches well the lipid utilization by *Sepia officinalis* after 3-5 days of starvation (Speers-Roesch et al. 2016).

Limitations to cutaneous uptake

Diffusion distance is likely the most important factor limiting cutaneous respiration in squids. Squid skin is thinner than *Octopus* skin, but unlike octopods, it does not seem to be vascularized (Madan and Wells 1997). Blood vessels are commonly found in the skin of cutaneously respiring animals (Krogh 1941; Feder and Burggren 1985). Thus, O_2 from seawater would have to diffuse across the entire squid skin thickness (150-300 μm) before reaching capillaries. Oxygen diffusion across animal tissue is slow, however, three times slower than in water and one million times slower than in air (Krogh 1941). Diffusion limitation of metabolism is possible even on the intracellular scale, influencing mitochondrial placement within myocytes (Kinsey et al. 2007).

A simple calculation of diffusion capacity reveals the situation. In another loliginid squid, *Alloteuthis subulata*, total branchial surface area and diffusion distance for a 100 g individual are 1063 cm^2 and 6.2 μm, respectively (Eno 1994) yielding a diffusion capacity of 519 $\mu mol\ O_2 \cdot kPa^{-1} \cdot hr^{-1}$ according to Equation 2.3. This mass-specific branchial surface area is comparable to many marine fishes (Gray 1954) and other cephalopods (Madan and Wells 1996b). With a skin thickness of 300 μm (Madan and Wells 1997), the *D. pealeii* in our study had a cutaneous diffusion capacity of only 4 $\mu mol\ O_2 \cdot kPa^{-1} \cdot hr^{-1}$. As a useful comparison, the inter-capillary distance in aerobic circular muscle of the squid mantle is no more than 80 μm (Bone et al. 1981; Kier and Thompson 2003). Thus, the furthest distance that oxygen must travel from capillaries to mitochondria is 40 μm, far less than the skin thickness.

$$\text{Diffusion capacity} = \text{K} \times \frac{\text{surface area}}{\text{diffusion distance}}, \quad (2.3)$$

At 20°C, K is ≈3.03 $\mu mol\ O_2 \cdot cm^{-2} \cdot \mu m \cdot kPa^{-1} \cdot hr^{-1}$ (Krogh 1919).

The estimates of total cutaneous M_{O_2} in this study were derived from cutaneous uptake across the outer mantle and fins and extrapolated to the inner mantle, head, and appendages. Cutaneous diffusion capacity is inversely proportional to the distance from the skin surface to the blood vessels or underlying tissue. Therefore, variations in skin thickness across the body could lead to differences in cutaneous O_2 uptake. However, Madan and Wells (1996a) found no difference between *in vitro* cutaneous M_{O_2} on the web and the dorsal mantle of *Octopus*, suggesting that variations in skin thickness across the body are unlikely to cause notable effects. In addition, Wells and Wells (1982) demonstrated through a series of manipulative experiments in *Octopus* that there is no measurable uptake of oxygen within the mantle cavity once water has passed the gills.

Why is octopus skin vascularized while squid skin is not? One plausible explanation is that octopus skin is more metabolically active than squid skin. Octopus skin contains both muscular papillae that can alter the skin's three-dimensional texture (Allen et al. 2014) and 1-2 orders of magnitude more chromatophores than squids (Messenger 2001). Such active muscular structures in the skin may require greater O_2 supply than can be supplied by diffusion from the skin surface.

Three species of loliginid squids have now been examined through a combination of *in vitro* (Madan and Wells 1996a) and *in vivo* (this study) measurements and all show similar area-specific cutaneous O_2 uptake rates. The ommastrephid squid, *Illex illecebrosus*, has thinner skin ($150\,\mu m$) than most loliginids but it also lacks cutaneous vascularization (Madan and Wells 1997) and thus likely also has a low cutaneous diffusion capacity. As part of this study, we conducted a short preliminary experiment on an ommastrephid, *Sthenoteuthis oualaniensis.* Its cutaneous respiration was 12.7% of total M_{O_2}, similar to our results for loliginid squids. Although further examination is needed, it seems likely, given our current knowledge, that ommastrephid squids do not breathe through their skin either.

The cutaneous diffusion capacity of deep-water squids also remains poorly studied. Morphological measurements of branchial diffusion capacity have been made in a number of taxa (Madan and Wells 1996b) and generally seem sufficient to support their lower metabolic rates (Seibel 2007). Additionally, the hypoxic waters that many deep-sea squids inhabit lowers the diffusion gradient from the environment into the skin compared to aerated surface waters, making notable cutaneous uptake unlikely.

Where did previous studies go wrong?

If squids do not breathe through their skin, how are we to interpret previously reported findings that have been used to support this hypothesis? The first indirect evidence was Pörtner (1990)'s paradox of blood pH rising at the tissue capillaries unless some additional source of CO_2 or other acid is provided independently of blood-delivered O_2. There are two theoretical issues with this prediction, however. Firstly, for the hypothesis to work, CO_2 produced in the tissue mitochondria would have to diffuse into the blood rather than diffuse out into seawater. Seawater P_{CO_2}, however, is $\approx$ 7x lower than blood (Redfield and Goodkind 1929; Pörtner 1990; Hu et al. 2014), which should result in a higher diffusion rate out into seawater rather than into the blood. In fact, it is common among skin-breathing animals for much larger quantities of CO_2 to be released than O_2 absorbed across the skin (Krogh 1919; Feder and Burggren 1985).

Secondly, Pörtner (1990)'s hypothesis that extra CO_2 from the skin enters the blood could only function for capillaries very near the skin. If CO_2 produced by the consumption of cutaneously-sourced O_2 were required for hemocyanin to function efficiently, how would blood-bourne oxygen be efficiently delivered across capillaries in internal organs, such as the brain, that are far removed from the skin? This leaves the very high Bohr coefficients found in many squids and octopuses unexplained.

Madan and Wells (1996a) found that *in vitro* squid skin has similar oxygen uptake rates as *in vitro* octopus skin. However, caution must be used before predicting *in vivo* estimates from their experiments. Blood convection is a highly important factor in facilitating cutaneous respiration (Feder and Burggren 1985). Intact *Octopus* skin tissue had an oxygen uptake rate over 5x higher than dissected skin tissue (Madan and Wells 1996a), likely because blood convection through the tissue capillaries in the intact animal could maintain a steep diffusion gradient in the skin tissue. Since squids lack cutaneous blood vessels, they lack this ability to draw oxygen deeper into the body. Also, as noted above, *Octopus* skin is likely more metabolically active than squid skin due to the higher density of chromatophores (Messenger 2001) and muscular papillae (Allen et al. 2014).

Finally, although squids do indeed have a higher surface area-to-volume ratio than most animals (O'Dor and Hoar 2000), we found that, at least for *D. pealeii* over the very limited size ranges examined here, the surface area-to-volume ratio declines with increasing size, scaling at a similar rate as expected for geometric solids (b = -0.33). Thus, the small contribution of cutaneous oxygen uptake found here should be even smaller in larger individuals. Finally, even though squid mantle circular musculature is arranged such that O_2-dependent aerobic muscle fibers are on the exterior margins (Bone et al. 1981; Mommsen et al. 1981), these are ultimately not relevant to cutaneous respiration because the diffusion limitation is on a much smaller scale within the skin than across the mantle. When Mommsen et al. (1981) originally described the "sandwich" pattern of muscle fibers in the mantle, they proposed an entirely biomechanical explanation for this arrangement. They proposed that when only a small portion of circular muscles are being utilized, as during breathing or routine swimming contractions (Bone et al. 1981), this arrangement allows the passive central mantle muscle to be pressurized as a hydrostatic working fluid.

This study demonstrates that, contrary to a commonly held but untested hypothesis, squids do not acquire large quantities of oxygen through their skin for systemic use. This finding has important implications for our understanding of branchial and cardiac performance in squids. The squid cardiovascular system is already believed to be delivering near-maximal quantities of oxygen (O'Dor et al. 1990; Shadwick et al. 1990; Wells 1992). Based on this research, cutaneous oxygen uptake does not alleviate the oxygen delivery demand on the cardiovascular system. This makes the physiological adaptations of active squids to provide sufficient oxygen supply to meet their high oxygen demand (Seibel 2007) all the more interesting.

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Tables

Table 2.1. Morphometrics of animals used in divided chamber experiments. Values are mean ± s.d.

Species	n	Mass (g)	Dorsal mantle length (cm)	Gender
Doryteuthis pealeii	17	94 ± 24	15 ± 2	F:3, M:14
Lolliguncula brevis	4	31 ± 5	8 ± 1	F:2, M:2

Table 2.2. Gas exchange rates and skin arrangements in divided chamber experiments. Values are mean ± s.d.

Species	*Doryteuthis pealeii* (11 °C)	*Doryteuthis pealeii* (19 °C)	*Lolliguncula brevis* (20 °C)
Total cutaneous surface area (cm^2)	435 ± 77	416 ± 82	113 ± 13
Cutaneous MO_2 (µmol O_2 / cm^2 / hr)	0.23 ± 0.04	0.32 ± 0.09	0.43 ± 0.2
Branchial MO_2 (µmol O_2 / hr)	1053 ± 347	1135 ± 455	343 ± 83
Mass-specific MO_2 (µmol O_2 / g / hr)	11.33 ± 2.41	14.28 ± 4.09	12.74 ± 2.59
MO_2 from cutaneous uptake (%)	9 ± 1	11 ± 4	12 ± 4
Cutaneous MNH_3 (nmol NH_3 / cm^2 / hr)	12.5 ± 4.52	11.07	87.36, 27.09
Branchial and renal MNH_3 (µmol NH_3 / hr)	69 ± 20	50	38, 20
MNH_3 from cutaneous uptake (%)	7 ± 2	7	23, 14

Figures

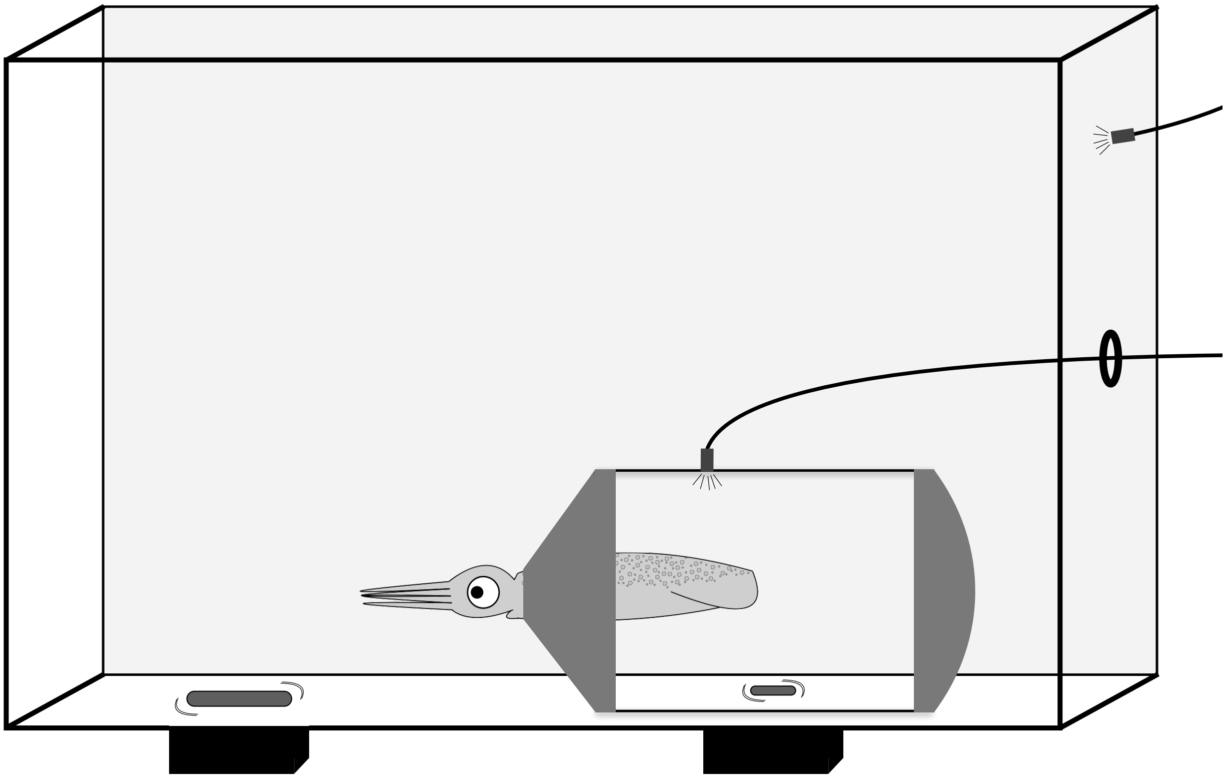

Figure 2.1. Divided chamber setup for cutaneous respirometry experiments. Squids were adhered to a rubber collar and placed inside a small cylindrical “mantle” respirometer to isolate cutaneous from branchial O_2 consumption. Both chambers were mixed with magnetic stir bars. P_{O_2} was measured with oxygen-sensitive spots via fiber optic cables to oxygen meters.

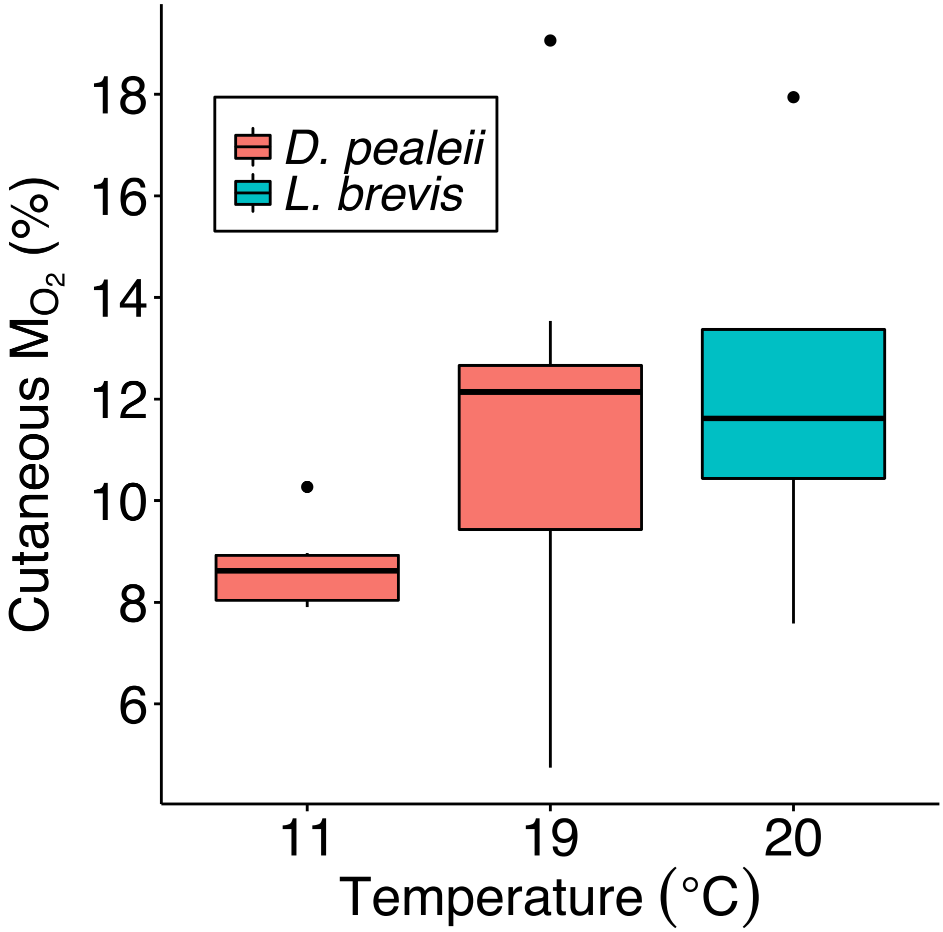

Figure 2.2. Contributions of total cutaneous O_2 uptake to whole-animal M_{O_2} in two species of squids.

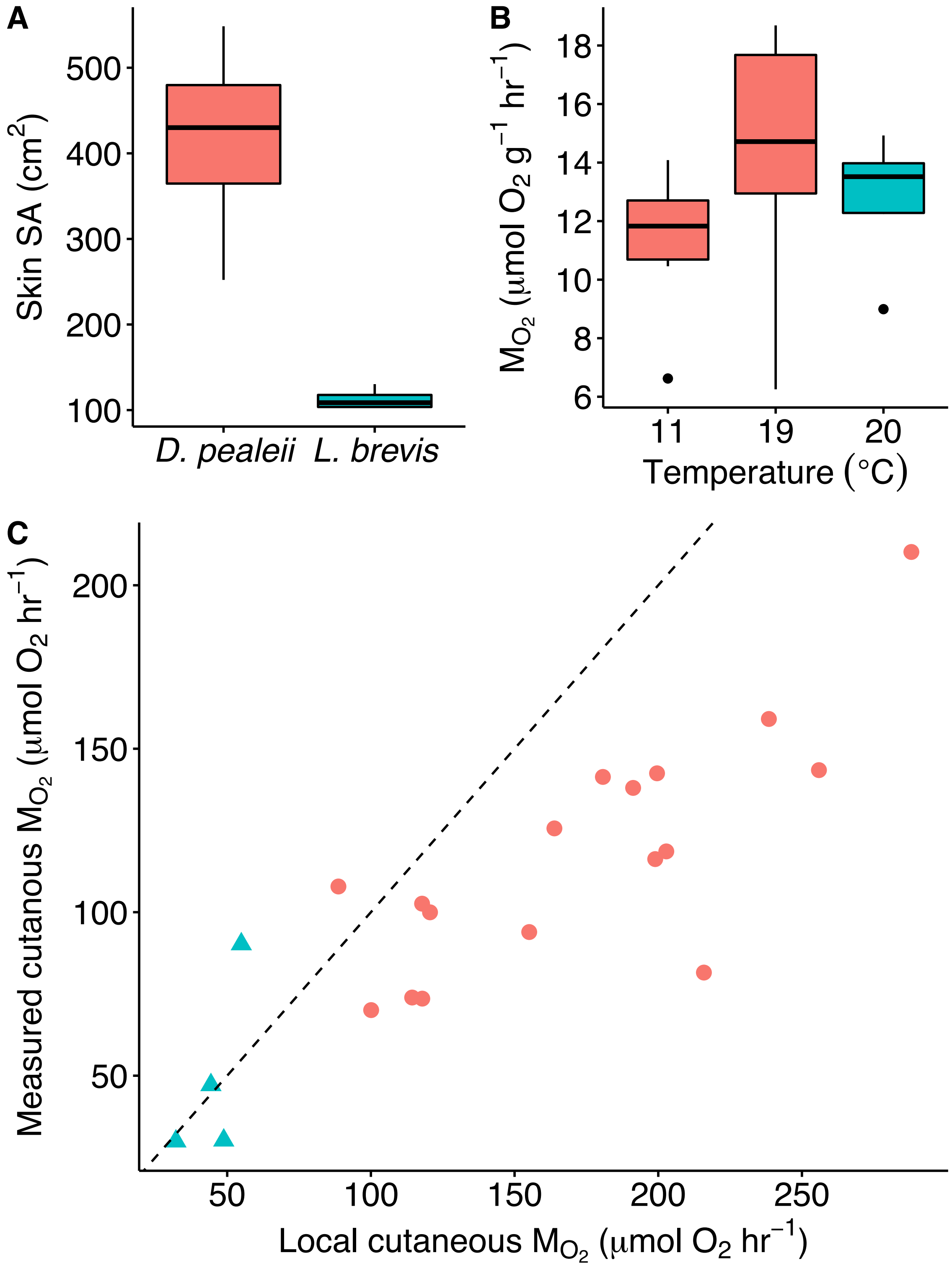

Figure 2.3. Comparison of measured and local cutaneous oxygen uptake. **A**. Total cutaneous surface area. **B**. Mass-specific metabolic rate. **C**. Measured versus predicted cutaneous M_{O_2} in squids. Red circles represent *Doryteuthis pealeii* and blue triangles represent *Lolliguncula brevis*. Grey dashed line is the unity line.

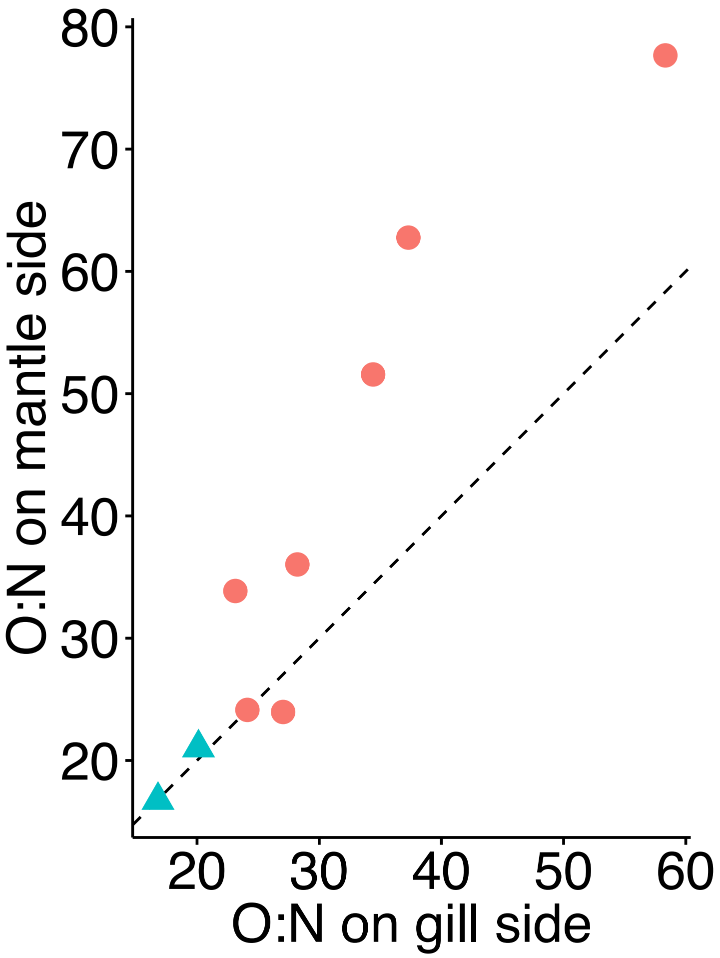

Figure 2.4. Metabolic quotient of O_2 consumed and NH_3 excreted. The gill side of the respirometer was in contact with the gills, viscera, and skin from the mantle cavity, head, arms, and tentacles. The mantle side was in contact with the outer surface of the mantle and fins. Red circles represent *Doryteuthis pealeii* and blue triangles represent *Lolliguncula brevis*. Grey dashed line is the unity line.

CHAPTER THREE:

WILL OCEAN ACIDIFICATION LIMIT METABOLISM VIA BLOOD O_2 SUPPLY?: MAXIMAL EFFECTS ON A MODEL ORGANISM WITH EXTREME BLOOD PH-SENSITIVITY

Abstract

Ocean acidification is hypothesized to limit the performance of squids due 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 respiratory protein pH-sensitivity, blood P_{CO_2}, and buffering capacity, that simulates blood oxygen supply in any animal with respiratory proteins under varying seawater CO_2 partial pressures. We compare model outputs to measured P_{crit} in squids. Using blood-O_2 parameters from the literature for model inputs, including a Bohr coefficient of -1.5, we estimated that, in the absence of non-bicarbonate blood buffering or 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 6% at normoxia and a P_{crit} increase of

$\approx$1 kPa. Because squid blood is among the most sensitive to pH of any animal, and because most marine animals have at least some capacity for acid-base regulation, this small predicted change in P_{crit} represents the maximum change expected in any animal. In fact, more realistic assumptions of buffering capacity and acid-base regulation result in much smaller expected changes that are within the error of the measurement. Our live-animal experiments support this supposition as CO_2 had no effect on measured metabolic rate or P_{crit} in either squid species. We conclude that reports in the literature of CO_2 effects on metabolism or performance are unlikely to be due to limitation in blood oxygen supply.

Introduction

Atmospheric carbon dioxide (CO_2) partial pressure (P_{CO_2}) has increased from the pre-industrial mean of 28 Pa (280 μatm, ppmv; 1 Pa $\approx$ 10 μatm) 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 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 oxygen supply capacity could hinder an animal's exercise performance or environmental hypoxia tolerance.

Shallow-water active squids, such as those in the families Loliginidae and Ommastrephidae, are ideal study organisms for examining impacts of ocean acidification (OA) on oxygen supply because their respiratory proteins 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 < -1 (Bridges 1994). Bohr coefficients in many other animals are much smaller, zero, or (rarely) even have a reverse effect (Mangum 1997). The extreme sensitivity in cephalopods may result in large impairments in blood-O_2 binding affinity from relatively small changes in blood pH.

Their blood also has rather low non-bicarbonate buffering capacity (β_{NB}) when compared to vertebrates (Heisler 1986; Withers 1992) and some marine worms (Toulmond and Tchernigovtzeff 1989), and is similar to other invertebrates such as crabs (Spicer et al. 2007; Pane and Barry 2007), shrimps (Taylor and Spicer 1991), scallops (Duncan et al. 1994), and peanut worms (Portner et al. 1984). Squid β_{NB} is larger than some taxa such as mussels (Booth et al. 1984) and sea urchins (Spicer et al. 1988, 2011; Taylor et al. 2014). When β_{NB} is low, small changes in blood P_{CO_2} lead to relatively large changes in pH.

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). Furthermore, hemocyanins are not contained within red blood cells but are freely dissolved in the blood, which limits their concentration due to viscosity and osmotic constraints. Unlike fishes or invertebrates with red blood cells, which can increase hemoglobin concentration or hematocrit to increase oxygen supply (Johansen and Weber 1976), squids are constrained in their O_2 carrying capacity. Finally, unlike most fishes and mammals, squids 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).

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 squids, consistent with the findings of Redfield and Goodkind (1929). They found that blood-oxygen transport in the loliginid squid *Doryteuthis pealeii* was impaired by acute exposures to P_{CO_2} levels up to 3200 Pa resulting in death. Rosa and Seibel (2008) reported reduced metabolic rate and activity at much more modest CO_2 levels (100 Pa), which they attributed to the high pH-sensitivity of hemocyanin in *Dosidicus gigas*. Similar results have been found in embryonic squids (Rosa et al. 2014). However, Hu et al. (2014) found no effect of 160 Pa CO_2 on metabolism even after one week exposure in the loliginid squid *Sepioteuthis lessoniana*. Cuttlefish, which have lower oxygen demand but respiratory proteins with similarly high pH-sensitivity, also showed no effect on metabolism or growth rate at P_{CO_2} levels up to 615 Pa (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 perhaps not surprising.

Although all loliginid and ommastrephid squids 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} (Δ > 10 kPa), P_{CO_2} ($\Delta \geq 100$ Pa), and temperature (Δ > 10 °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 in the summer months; Turner 2015). As such, *D. gigas* is adapted to more extreme environmental conditions than *D. pealeii*.

Of the 28 known marine animal phyla, 11 utilize specialized oxygen binding proteins for oxygen transport to metabolizing tissues (Mangum 1997). Among the taxa examined thus far, blood oxygen binding affinity in squids is among the most sensitive to environmental hypercapnia (Pörtner and Reipschläger 1996). Yet the whole-animal metabolic responses to CO_2 reported in the literature are highly variable (for reviews, Lefevre 2016; Kelley and Lunden 2017; Hannan and Rummer 2018).

In this study, we examined the effects of CO_2 on hypoxia tolerance in two squid species with similar oxygen demands but differing hypoxia tolerances, *Dosidicus gigas* and *Doryteuthis pealeii*, to determine what impact ocean acidification may have on O_2 supply in squids. 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-oxygen affinity, CO_2 sensitivity of respiratory proteins and buffering capacity, to predict the physiological changes in O_2 supply expected by end-of-the-century ocean acidification for squids and marine animals generally.

Methods

Animal capture and maintenance

Dosidicus gigas (D'Orbigny 1835; n = 16) were jigged at night in Guaymas Basin, Gulf of California, Mexico from 16 May 2015 to 01 June 2015 aboard the *R/V Oceanus*. *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 of 2014-2016. Morphometrics of both species are shown in Table 3.1. *Dosidicus gigas* were placed immediately in a respirometer aboard ship for acclimation while *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 L with flow-through filtered seawater. *Doryteuthis pealeii* were fed grass shrimp (*Palaemonetes* sp.) or 1 cm wide herring steaks, *Clupea harengus, 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, RI, where P_{CO_2} typically ranges from 10 to 70 Pa (Turner 2015).

Hypoxia tolerance assessment

Hypoxia tolerance was assessed by measuring oxygen consumption rates of the squids under progressively declining seawater P_{O_2} using intermittent respirometry. All experiments were conducted in a 90 L swim tunnel respirometer (Loligo Systems, Viborg, Denmark) with a 70x20x20 cm working section in which the animal was confined. Trials were conducted at surface pressure since hydrostatic pressure has little effect on metabolism in squids (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 hours for *D. gigas* and *D. pealeii,* respectively. This allowed time for thermal acclimation (for *D. pealeii*), recovery from handling stress, completion of digestion from any previous meal (Wells et al. 1983; Katsanevakis et al. 2005), and acclimation to CO_2 conditions. 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 hours 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 (Steffensen 1989). P_{O_2} was measured every ten seconds with an oxygen-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 ($\approx 5\ cm \cdot sec^{-1}$) to allow homogenous 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, Pennsylvania, 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_2 gas added for each flush was calculated using the R package "respirometry" (Birk 2018). 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 (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 oxygen consumption rate) decreases. The average duration of the trials after acclimation was 7 and 23 hours for *D. gigas* and *D. pealeii*, respectively, due 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 hours, respectively. At the end of each trial, the animal was removed and the "background" oxygen consumption rate of the microbial community in the respirometer was measured and deducted from calculated squid 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 one minute every 30 minutes to monitor ventilation rate. The camera was placed above the respirometer and a mirror was placed at a 45° angle to the camera, allowing simultaneous monitoring of the animal from a dorsal and lateral view.

As hypoxia progressed, three 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" (Bolker 2017). The best fitting model was chosen by the Bayesian information criterion (BIC).

M_{O_2} *and* P_{crit} *analysis*

M_{O_2} was calculated from the slope of a linear regression of P_{O_2} over time. The number and quality of 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_2 covered 1/10th the trial duration and the time bins at low O_2 covered 1/100th the trial duration. This provided an optimal balance between precision and resolution throughout each trial. 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 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 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. This sub-PI method resulted in a lower variability in P_{crit} measurements than the traditional breakpoint method.

Only M_{O_2} measurements with mean $P_{O_2} > P_{crit}$ were considered when calculating routine metabolic rate (RMR). For trials where no P_{crit} could be reliably established, RMR was determined as the mean of the M_{O_2} values.

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

$$Q_{10}=(k_2/k_1)^{10/(T_2-T_1)}$$

where k_1 and k_2 are the calculated values (e.g. 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 "respirometry" R package (Birk 2018) 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 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 "respirometry" R package (Birk 2018). A respiratory quotient (RQ; ratio of CO_2 produced to O_2 consumed) of 0.85 was used since 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). 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" (Gattuso et al.

2015). 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. Due to the unavoidable CO_2 buildup 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 $\mathbf{O_2}$ delivery model

A physiological model was developed to estimate the magnitude of an effect seawater P_{CO_2} has on blood O_2 transport and P_{crit} in a marine animal. 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 squids at 25 °C. Importantly, the model inputs for those blood traits that make squids more sensitive to CO_2 than nearly any other marine animal (e.g. large Bohr coefficient) can be maximized, while the characteristics that make squids more resilient to CO_2 than some other animals (buffering capacity and acid-base regulation) could be minimized or neglected. This allowed us to demonstrate the maximum possible effect that future CO_2 levels are likely to have on blood-oxygen transport in any animal.

According to Fick's law of diffusion (Equation 3.1; 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. This has been observed in cephalopods (Gutowska et al. 2010a; Häfker 2012; Hu et al. 2014) as well as fishes exposed to hypercapnia (Janssen and Randall 1975; Esbaugh et al. 2012, 2016; Strobel et al. 2012a; Ern and Esbaugh 2016; for a review: 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 (Table 3.2). CO_2 solubility and dissociation constants for seawater (S = 35) from Lueker et al. (2000) are used. These constants are similar to values calculated in crab hemolymph (Truchot 1976), which has similar ionic properties to squid blood.

$$\text{Diffusion rate}=\text{K}\times\frac{\text{gill surface area}}{\text{gill membrane thickness}}\times\left(\text{P}_{\text{CO}_{2_{\text{seawater}}}}\text{-}\text{P}_{\text{CO}_{2_{\text{blood}}}}\right),\ (3.1)$$

A decrease in blood pH increases P_{50} according to Equation 3.2 (Table 3.2). This acidosis also decreases $Hc - O_2$ binding cooperativity (Hill coefficient, n; Seibel 2013; Table 3.2). The rise in P_{50} shifts the O_2-binding curve to the right, decreasing arterial hemocyanin O_2 saturation according to the hemocyanin-O_2 binding equation (Equation 3.3):

$$\text{Bohr coefficient}=\frac{\Delta\log(\text{P}_{50})}{\Delta\text{pH}},\ (3.2)$$

$$\%\,\text{Hc-O}_2\text{ saturation}=\frac{(\text{P}_{\text{O}_2})^{\text{n}}}{(\text{P}_{50})^{\text{n}}+(\text{P}_{\text{O}_2})^{\text{n}}}\times 100,\ (3.3)$$

According to Equation 3.3, 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 oxygen 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 (Table 3.2) and that under anoxia assuming a linear relationship between environmental and arterial P_{O_2} (Eddy 1974; Houlihan et al. 1982; Johansen 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 (Fig. S3F) translates to an increase in P_{crit}.

To assess the model's reliability, the model was run with published β_{NB}, Bohr and Hill coefficient, and P_{50} values for *D. pealeii* from Pörtner (1990; Table 3.2: "Realistic squid") and compared to empirical data from Redfield and Goodkind (1929). They examined the effects of acute (10-15 min) seawater hypercapnia (up to 3200 Pa 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 with one of the largest Bohr coefficients measured for squids (Brix et al. 1989) and no non-bicarbonate buffering capacity (Table 3.2: "Worst case scenario"). Finally, the model was run with buffering capacity and respiratory protein pH-sensitivity more typical of most marine animals (Mangum 1997; Table 3.2: "Typical marine animal"). For all parameter inputs, the model was run with environmental P_{CO_2} values ranging from 40 to 416 Pa to cover a broad range of P_{CO_2} conditions that marine animals may encounter (Melzner et al. 2013).

Results

Experimental seawater parameters from the trials are shown in Table 3.3. All results are expressed as mean ± standard deviation.

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; Figure 3.1A). Furthermore, seawater P_{CO_2} had no detectable effect on temperature-adjusted P_{crit} in either species (Figure 3.1B).

Dosidicus gigas had a routine metabolic rate (RMR) of 13.2 $\pm$ 2.6 $\mu mol\, O_2\, g^{-1}\, h^{-1}$ 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 (Figure 3.2A). *Doryteuthis pealeii* had an RMR of 6.5 $\pm$ 2.5 $\mu mol\, O_2\, g^{-1}\, h^{-1}$ at 15 °C. Once adjusted to a common temperature (15 °C) using a Q_{10} of 1.8 (derived from *D. gigas* M_{O_2} measurements from the literature spanning from 6.5 to 25 °C; (Gilly et al. 2006; Rosa and Seibel 2008; Trueblood and Seibel 2013; Trübenbach et al. 2013; Seibel et al. 2014); this study; Figure 3.2C), *D. gigas* metabolic rate was 1.8 $\mu mol\, O_2\, g^{-1}\, h^{-1}$ higher than in *D. pealeii* (t_{35} = 3.31, p = 0.002).

The P_{crit} for *D. pealeii* at 15 °C was 3.9 $\pm$ 0.8 kPa. *Dosidicus gigas* mean P_{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 (Figure 3.2B). 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; Figure 3.2D), 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 $Hz \cdot kPa^{-1}\, O_2$ with progressive hypoxia below 9 kPa (Figure 3.3A). While O_2 strongly influenced ventilation, no effect of seawater P_{CO_2} was found on normoxic ($P_{O_2} > 9$ kPa) ventilation rate (t_{14} = -0.38, p = 0.71; Figure 3.3B). *Dosidicus gigas* relative gill length (38% of DML) was longer than *D. pealeii* (29%) (t_{25} = 5.39, p < 0.001).

Blood O_2 delivery model

Running the model with the inclusion of non-bicarbonate buffering and a Bohr coefficient from the literature for *D. pealeii* (Table 3.2: “Realistic squid”) matched the empirical measurements of lethal P_{O_2} from Redfield and Goodkind (1929) very well even up to 3200 Pa CO_2 (Figure 3.4). Assuming no non-bicarbonate buffering capacity and a Bohr coefficient of -1.5 (Table 3.2: "Worst case scenario") resulted in a large overestimate of the observed rise in lethal P_{O_2} especially at high CO_2 levels (Figure 3.4).

When running the "Worst case scenario" model (Table 3.2), an increase in environmental P_{CO_2} from 40 to 100 Pa raises P_{crit} by only 1.02 kPa and lowers arterial hemocyanin-O_2 saturation in normoxia by only 6%. The ΔP_{crit} is approximately linear with increasing P_{CO_2} (Figure 3.5). The effects of CO_2 were even less pronounced in the "Typical marine animal" model (Figure 3.5). The ΔP_{crit} for a typical marine animal due to a rise in environmental P_{CO_2} from 40 to 100 Pa was 0.2 kPa and arterial O_2 saturation fell by only 1.7%. Even when environmental P_{CO_2} rose by 234 Pa, P_{crit} rose by only 0.66 kPa and arterial saturation fell by 7%.

Discussion

Effects of CO_2 on metabolism

We found no effect of seawater P_{CO_2} up to 143 Pa on metabolic rate or hypoxia tolerance in either species (Figure 3.1). Rosa and Seibel (2008) had previously found that a P_{CO_2} of 100 Pa had caused a 20% decrease in *D. gigas* RMR relative to 30 Pa and had attributed this suppression to hypercapnia-induced limitation to blood-O_2 binding. A 20% decline in RMR (black diamond in Figure 3.1) 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.

Given what is now known about blood-O_2 binding in *D. gigas* (Seibel 2013), impairment of oxygen 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 oxygen 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. It is possible that the reduced rate they observed was due to a short-term behavioral effect as has been documented in the marine worm, *Sipunculus nudus* (Reipschläger et al. 1997). In this animal, hypercapnia alters neuromodulator concentration in nervous tissue, which suppresses metabolism independently of O_2-supply constraints.

As has been demonstrated in other adult cephalopods (Gutowska et al. 2010a; Häfker 2012; Hu et al. 2014), 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 hours of exposure to 420 Pa CO_2 (Hu et al. 2014). Similarly, when exposed to 600 Pa 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 mM lowers free $[Mg^{2+}]$ by ≈1% due 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).

Impact of ocean acidification on O_2 supply in squids

To determine what effect P_{CO_2} might reasonably be expected to have on P_{crit}, we modeled the effect of P_{CO_2} across a range of physiological traits and conditions. The "Worst case scenario", including a modeled animal with extreme pH sensitivity of oxygen binding (Bohr coefficient = -1.5) and no capacity for non-bicarbonate buffering or acid-base regulation, results in only a 1.02 kPa increase in P_{crit} due to a rise in P_{CO_2} to 100 Pa. This increase in P_{crit} is within the range of existing intraspecific variability measured in this study and others (Redfield and Goodkind 1929; Trueblood and Seibel 2013). The "Realistic squid" model formed with physiological parameters from Redfield and Goodkind (1929) and Pörtner (1990) matches independent empirical data up to 3200 Pa CO_2 (Redfield and Goodkind 1929). This suggests that despite its simplicity, our model accurately captures whole-animal metabolic responses to CO_2 in squids at least in the short-term.

Many of the parameters in the model are temperature-dependent; the CO_2 dissociation constants, Bohr coefficient, normocapnic P_{50} value, and arterial P_{O_2} at P_{crit} are all influenced by temperature. Of these parameters, the P_{50} and normocapnic P_{crit} values are highly influential and both decrease with decreasing temperature (Brix et al. 1989; Seibel 2013). Therefore, CO_2 should have an even lesser effect on blood O_2 transport in these squids at colder temperatures than calculated here for 25 °C.

Dosidicus gigas hemocyanin has among the lowest P_{50} (highest O_2 affinity) measured in cephalopods (Seibel 2013) and, thus, may retain full blood oxygen saturation under hypercapnia where other squids may not. Another ommastrephid squid, *Illex illecebrosus,* has one of the highest P_{50} values measured in squids ($P_{50} = 8.1$ kPa at 15 °C; Pörtner 1990), a Bohr coefficient of -1.07 (Pörtner 1990), and a normocapnic P_{crit} of 4.9 kPa at 20 °C (Birk, unpublished). For comparison, if P_{50} and P_{crit} are both adjusted to 25 °C using a Q_{10} of 1.7 (Seibel 2016), *Illex illecebrosus'* arterial hemocyanin O_2 saturation would decrease by 14%, while P_{crit} would increase by 1.03 kPa under 100 Pa CO_2 assuming no non-bicarbonate buffering and no blood pH compensation. According to the model, hemocyanin at this high temperature is only 61% saturated at the gills in air-saturated water at current P_{CO_2} levels, which is not surprising given how rarely *I. illecebrosus* is found at such high temperatures (Whitaker 1980). This does suggest, however, that such a high P_{50} (13.8 kPa) can strongly constrain available habitat in squids (Bridges 1994). Assuming even modest non-bicarbonate buffering capacity greatly reduces the possible effect of OA on blood-oxygen transport while the strong acid-base regulation found in active squids will likely render OA irrelevant for oxygen transport despite the extreme pH sensitivity of their blood.

While it is unlikely that the oxygen supply pathway of active squids will be affected by ocean acidification, there remain other mechanisms of concern for hypercapnia to impact squid fitness. Blood acid-base disturbance from environmental hypercapnia has been shown to increase cuttlebone calcification in cuttlefish (Gutowska et al. 2010b). Ocean acidification has also been demonstrated to alter behavior in marine animals (Clements and Hunt 2015), including squids (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).

Implications for other marine animals

We propose that the CO_2 sensitivities shown here (Figure 3.5) represent the maximal anticipated effect of ocean acidification on oxygen supply of marine animals generally. There may be some isolated cases where the Bohr coefficient is extreme or the P_{50} and arterial P_{O_2} at P_{crit} are very high, which could render a given species susceptible. However, moderate blood buffering capacity or acid-base regulatory abilities would seem sufficient to protect blood from the very modest declines in pH one might otherwise expect. Squids were predicted to be among the most sensitive (Pörtner and Reipschläger 1996), and our analysis suggests that OA in most marine habitats could have, at most, a minor effect on blood pH and oxygen transport.

We have been quite conservative in this conclusion by intentionally constructing a model that does not incorporate a number of physiological phenomena that would further minimize OA's effect on blood O_2 supply. There has been no incorporation of pH compensation via branchial ion transport or non-bicarbonate buffering in the blood. In fact, such compensation and buffering have been demonstrated in squids (Pörtner 1991; Hu et al. 2014) and would alleviate impacts of increased CO_2 (Melzner et al. 2009). 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 (Gutowska et al. 2010a; Ern and Esbaugh 2016). There are many additional known mechanisms to modulate blood-O_2 binding in other animals (e.g. lactate or phosphate cofactors, proton pumps in red blood cells), but all of them to our knowledge would enhance oxygen delivery and lessen any anticipated hypercapnic effect.

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; Eddy and Morgan 1969). In addition, a minor fall in arterial saturation may be compensated 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}. Fishes, crustaceans, and cephalopods 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; Chapter 4).

In addition to the Bohr effect, teleost hemoglobin is also sensitive to CO_2 via the Root effect (Root 1931), but this occurs with changes in blood pH that are an order of magnitude greater than those due to ocean acidification (Pelster and Weber 1991). Additionally, some fishes have a hypercapnia-induced catecholamine release response that may facilitate oxygen uptake when animals are exposed to hypercapnia (Rummer et al. 2013a). In a recent meta-analysis, Hannan and Rummer (2018) demonstrated that oxygen uptake in fishes is generally insensitive to environmentally relevant P_{CO_2} levels. Of the relatively few studies conducted to date that have examined the effect of CO_2 near OA levels on hypoxia tolerance, most have found no effect in fishes (Cochran and Burnett 1996; Burnett and Stickle 2001; Couturier et al. 2013; Heinrich et al. 2014; Dixon et al. 2017; Ern et al. 2017), crustaceans (Mickel and Childress 1978; Cochran and Burnett 1996; Kiko et al. 2016), and gastropods (Lefevre et al. 2015). Many taxa (echinoderms, bivalves, etc.) have much lower non-bicarbonate buffering capacity and ion regulatory capacity than squids, but the pH-sensitivities of the respiratory proteins in these taxa are typically much less than squids (Mangum 1997).

Although some studies to date have found effects of CO_2 at OA levels on metabolism in marine animals (for reviews, see Lefevre 2016; Kelley and Lunden 2017; Hannan and Rummer 2018), it seems unlikely that hypercapnia-induced O_2 supply limitation is the physiological mechanism for these effects. Hu et al. (2014) found that after one week exposure to 420 Pa CO_2 *Sepioteuthis* squid were significantly metabolically suppressed even though their blood remained fully pH-compensated. Rummer et al. (2013b) have also found that fish exposed to high CO_2 can have suppressed resting M_{O_2} yet have enhanced maximal M_{O_2}, which would be impossible if O_2 supply is insufficient to even sustain basal 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 oxygen delivery. We find it highly unlikely that any documented case of a decline in standard metabolic rate from OA-relevant CO_2 exposures is attributable to limitations in blood oxygen supply.

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 (Hughes 1973; Wells and Wells 1995; 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. 2017).

CO_2 had no effect on ventilation rate (Figure 3.3B). If blood acid-base balance can be fully compensated 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 squids had similar metabolic rates. Although the two species are phylogenetically rather distant (different orders), they are both active squids 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. Aquatic animals' hypoxia tolerance can closely define their distribution and suitable habitat on spatial scales from meters (Mandic et al. 2009) to 100s of kilometers (Deutsch et al. 2015). It is therefore unsurprising that *D. gigas* has better hypoxia tolerance than *D. pealeii* because the former species are closely associated with the strong oxygen minimum zone of the eastern tropical Pacific (Nigmatullin et al. 2001) whereas the latter are not known to frequently encounter such extreme hypoxia. While 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*. *Dosidicus gigas* relative gill length is 30% longer than *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. D. gigas* store 2-4x higher concentration of phosphoarginine in its mantle muscle than *D. pealeii* (Storey and Storey 1978; Seibel et al. 2014), which should be more advantageous for surviving subcritical oxygen levels. Glycogen reserves have not been quantified in *D. pealeii* mantle muscle, but glycogen concentration in *D. gigas* mantle is $\approx 300\,\mu mol\, glucosyl\, units\, g^{-1}$ (Seibel et al. 2014), which is much higher than in most fishes (Nilsson and Östlund-Nilsson 2008; Richards 2009) and even bivalves that can survive months in anoxia (Oeschger 1990).

In this experiment, we found that P_{CO_2} levels up to 122 Pa, near the P_{CO_2} in the oxygen minimum zone (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 squids have high oxygen demand and constrained oxygen supply, their blood-oxygen carrying capacity, hypoxia tolerance, and oxygen demand seem to be unaffected by near-future CO_2 levels. Because squid blood O_2 delivery is expected to be more sensitive to hypercapnia than in nearly any other marine animal, we suggest that OA is unlikely to strongly influence blood oxygen transport in most marine species.

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Tables

Table 3.1. Morphometrics of animals used in hypoxia tolerance experiments. Values are mean ± s.d.

Species	n	Mass (g)	Dorsal mantle length (cm)	Gender
Doryteuthis pealeii	29	112 ± 58	18 ± 5	F:15, M:11
Dosidicus gigas	16	233 ± 64	21 ± 1	F:15, M:1

Table 3.2. Physiological parameters for various runs of the blood oxygen supply capacity model.

Run	"Realistic squid"	"Worst case scenario"	"Typical marine animal"
Normocapnic blood PCO_2 (Pa)	300[1,2,3]	300	300
Normocapnic blood pH	7.4[4,5]	7.4	7.4
Non-bicarbonate buffering capacity (mmol / L / pH unit)	5.8[2]	0	1
Bohr coefficient	-1.15[2]	-1.5[6]	-0.5
Hill coefficient	4[2]	3[2,7,8]	3
Normocapnic P_{50} (kPa)	7[2]	6[7]	6
Arterial PO_2 (kPa)	16[1]	16	16
Normocapnic P_{crit} (kPa)	3.8	3.8	3.8

[1]Redfield and Goodkind 1929; [2]Pörtner 1990; [3]Hu et al. 2014; [4]Howell and Gilbert 1976; [5]Pörtner et al. 1991; [6]Brix et al. 1989; [7]Seibel 2013; [8]Lykkeboe and Johansen 1982

Table 3.3. Seawater parameters experienced by squids during hypoxia tolerance tests. Values are mean ± s.d.

Species	Temperature (°C)	Salinity (psu)	PCO_2 range (Pa)	pH_T range	Total alkalinity (µmol / kg)
Doryteuthis pealeii	14.8 ± 0.3	31.2 ± 0.5	67 – 143	7.51 – 7.82	2073 ± 31
Dosidicus gigas	22.5 – 26.6	35.3 ± 0.1	46 – 122	7.62 – 7.99	2399 ± 97

Figures

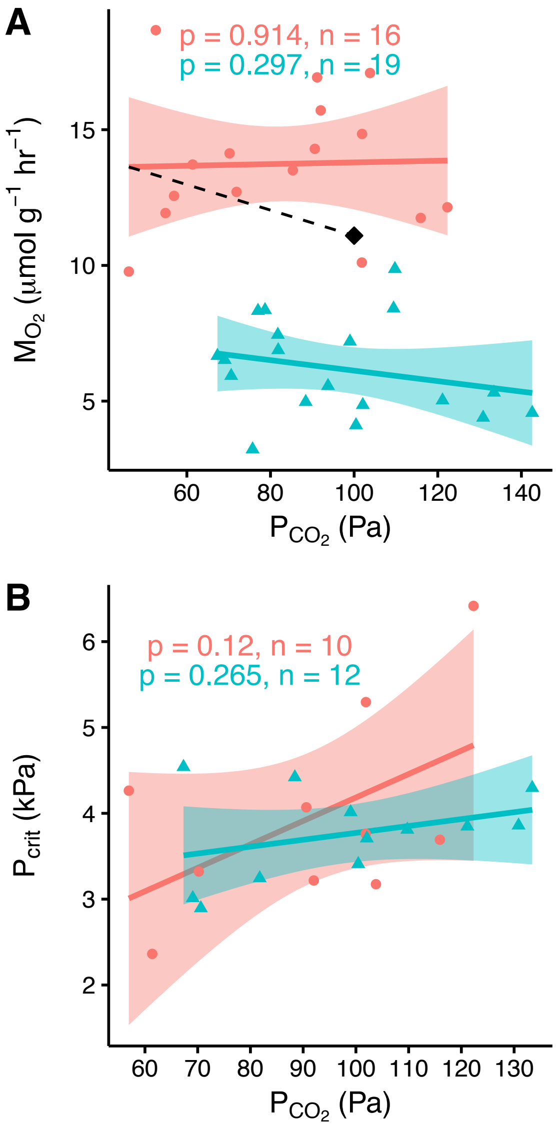

Figure 3.1. Effect of seawater P_{CO_2} on ***A***) routine metabolic rate (RMR) and ***B***) critical P_{O_2} (P_{crit}) in *Dosidicus gigas* (red circles; 25 °C) and *Doryteuthis pealeii* (blue triangles; 15 °C). Shaded bands are 95% confidence intervals. Because *D. gigas* trials covered a temperature range, all data were temperature-adjusted to 25 °C using a Q_{10} of 1.9 (M_{O_2}) and 1.8 (P_{crit}). When adjusted to a common temperature (15 °C), *D. gigas* M_{O_2} was 1.8 $\mu mol\ O_2\ g^{-1}\ h^{-1}$ 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.

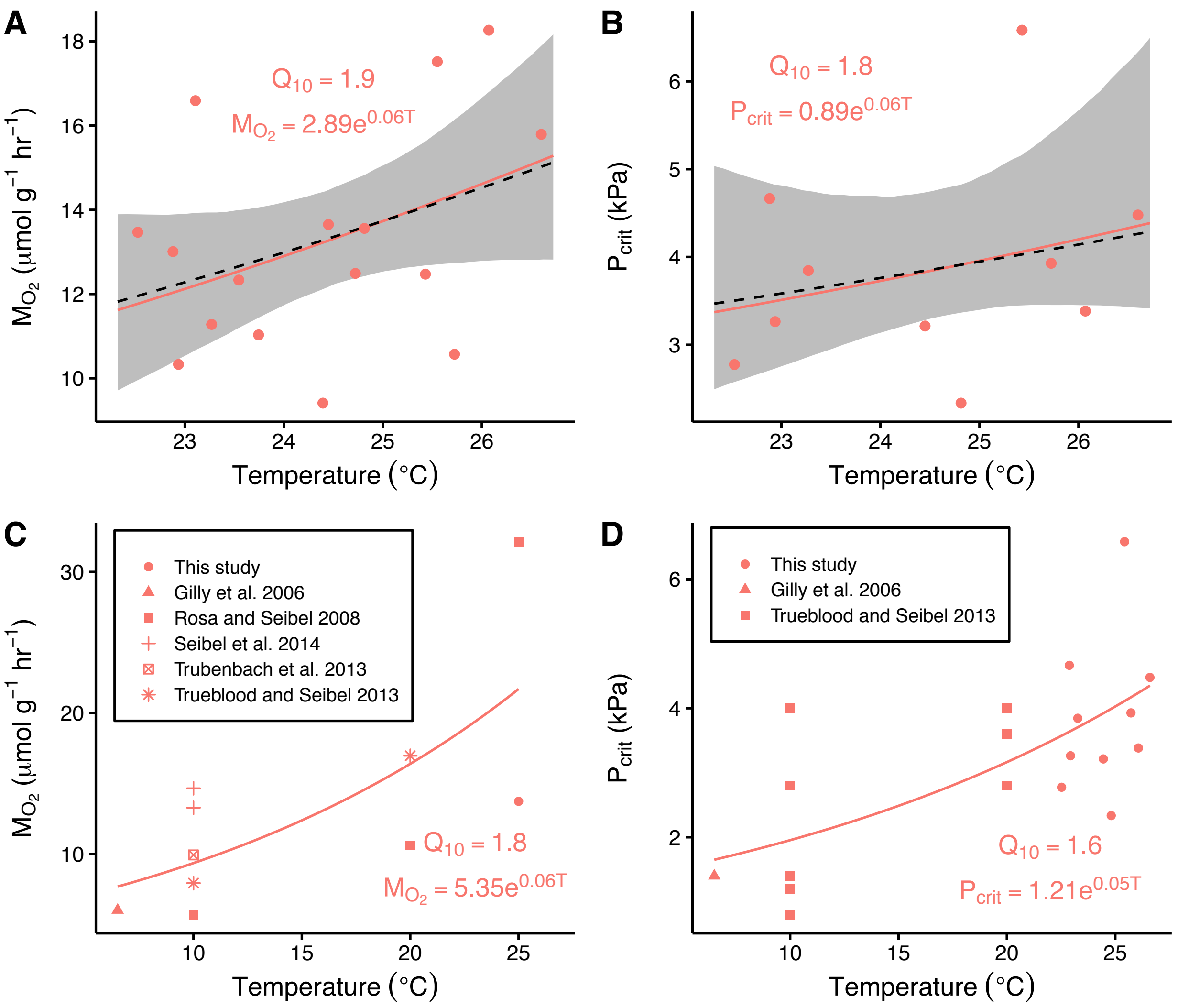

Figure 3.2. Effect of temperature on $\boldsymbol{A}$) routine metabolic rate and $\boldsymbol{B}$) critical P_{O_2} (P_{crit}) in *Dosidicus gigas*. The black dashed line represents a temperature effect corresponding to a Q_{10} of 2. Shaded bands are 95% confidence intervals. *Dosidicus gigas* mean metabolic rate ($\boldsymbol{C}$) and P_{crit} ($\boldsymbol{D}$) compared to 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.

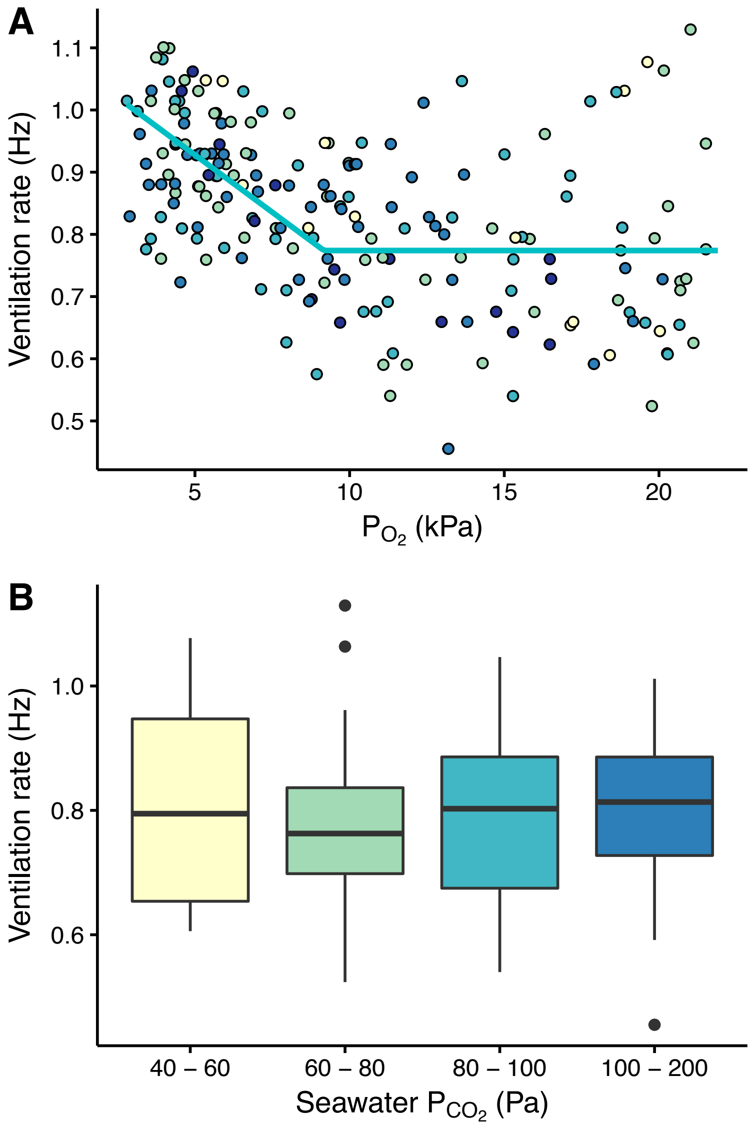

Figure 3.3. Ventilation rate in *Doryteuthis pealeii* as a function of ***A***) P_{O_2} at varying P_{CO_2} levels and ***B***) P_{CO_2} in normoxia (P_{O_2} > 9 kPa). Colors denote the same P_{CO_2} range in both panels. A best-fit model analysis (see text) reveals a hypoxic threshold of 9 kPa below which ventilation increases (n = 17). Colors represent seawater P_{CO_2} at the time of observation. There was no effect of CO_2 on ventilation rate at any oxygen level.

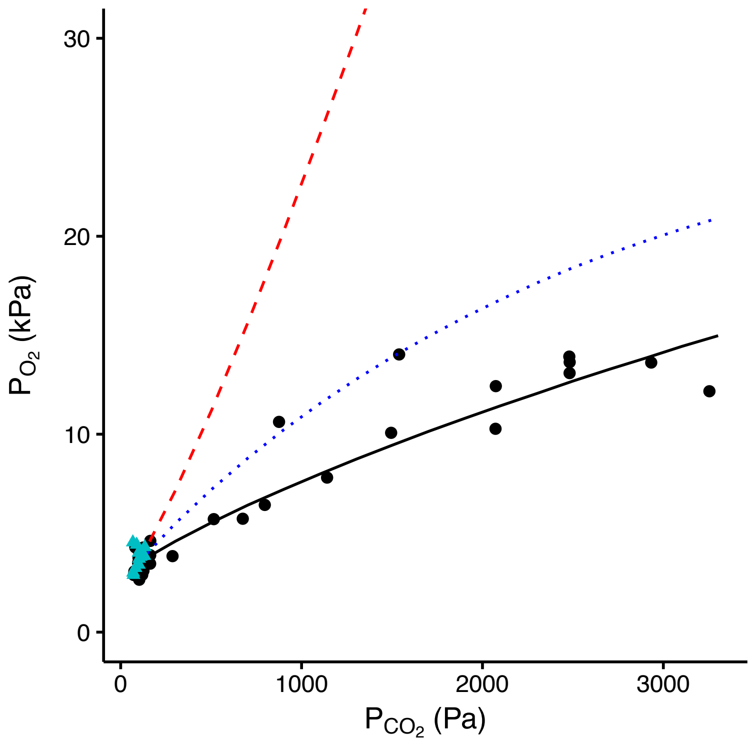

Figure 3.4. Model predictions of hypoxia tolerance (critical P_{O_2}, kPa) compared with independently-derived empirical data. Black points denote lethal combinations of O_2 and CO_2 for *Doryteuthis pealeii* from Redfield and Goodkind (1929). Blue triangles represent P_{crit} for *D. pealeii* from this study. The black line uses realistic physiological parameters for buffering capacity (Pörtner 1990; see text), while the dashed red line and dotted blue line use no non-bicarbonate buffering capacity and a Bohr coefficients of -1.5 and -1, respectively.

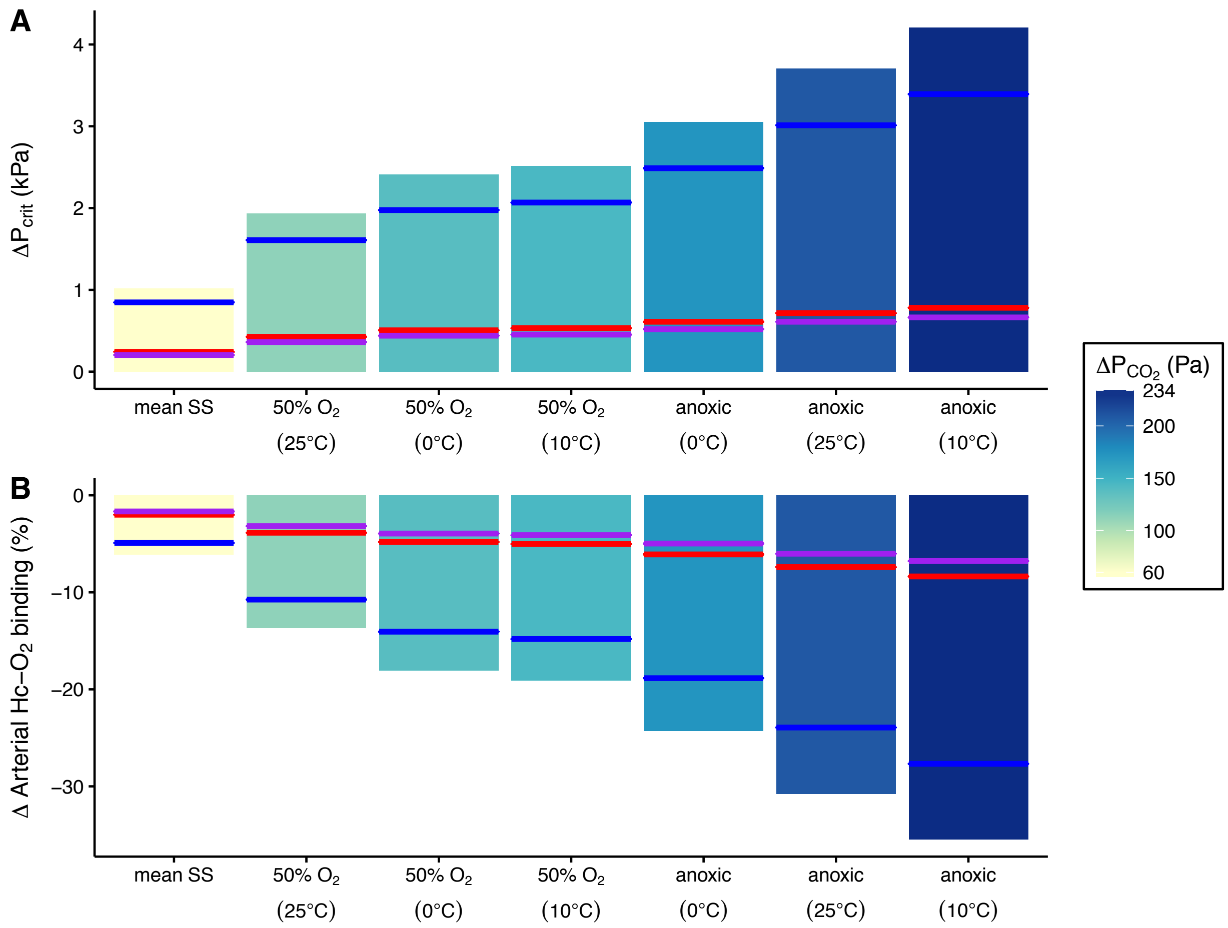

Figure 3.5. Maximal expected effect of environmental P_{CO_2} on P_{crit} and arterial hemocyanin saturation for a hypothetical animal at CO$_2$ levels predicted to occur in various environments. Changes in environmental P_{CO_2} were calculated as an air-equilibrated water mass from 40 to 100 Pa CO_2 and assuming a respiratory quotient of 0.75. Column color indicates the rise in P_{CO_2} from current to future conditions. Columns show the maximal effect given no non-bicarbonate buffering capacity ($\beta_{NB} = 0\ mmol \cdot L^{-1} \cdot pH\ unit^{-1}$) and a Bohr coefficient of -1.5. Blue lines depict the same Bohr coefficient and $\beta_{NB} = 1\ mmol \cdot L^{-1} \cdot pH\ unit^{-1}$. Red lines depict a Bohr coefficient of -0.5 and $\beta_{NB} = 0\ mmol \cdot L^{-1} \cdot pH\ unit^{-1}$. Purple lines depict the combination of Bohr coefficient = -0.5 and $\beta_{NB} = 1\ mmol \cdot L^{-1} \cdot pH\ unit^{-1}$.

CHAPTER FOUR:

METABOLIC ADAPTATIONS OF THE PELAGIC OCTOPOD, *JAPETELLA DIAPHANA*, TO OXYGEN MINIMUM ZONES

Abstract

The pelagic octopod, *Japetella diaphana*, is known to inhabit meso- and bathypelagic depths worldwide. Across its range, individuals encounter oxygen levels ranging from nearly air-saturated to nearly anoxic. In this study, we assessed the physiological adaptations of individuals from the Eastern Tropical Pacific (ETP) oxygen minimum zone (OMZ). Ship-board measurements of metabolic rate and hypoxia tolerance were conducted and a metabolic index was constructed to model suitable habitat for aerobic metabolism. We found that animals from the ETP had a higher metabolic rate than animals from more oxygen-rich habitats, yet maintained better hypoxia tolerance than conspecifics from oxygen-rich Hawaiian waters. Furthermore, hypoxia tolerance in *J. diaphana* has a reverse temperature dependence from most marine ectotherms, a characteristic that uniquely suits the physical characteristics of the lower oxycline, where oxygen decreases and temperature increases in shallower water towards the OMZ core. Even with their high tolerance to hypoxia, the OMZ core likely has insufficient oxygen supply to meet the basal oxygen demand of *J. diaphana.* Despite the limited aerobic habitat in this region, species abundance was comparable to more oxygenated ocean regions, suggesting that physiological or behavioral plasticity such as altered hypoxia tolerance or

hypoxic avoidance in this globally-distributed species is sufficient to maintain species fitness in this extreme environment.

Introduction

The octopod *Japetella diaphana* (Octopoda: Bolitaeninae; Hoyle 1885) inhabits tropical and subtropical meso- and bathypelagic waters worldwide (Norman and Finn 2013). Bolitaenins are among the most abundant pelagic octopods (Judkins et al. 2017). Throughout its range, *J. diaphana* encounter a wide range of oxygen conditions ranging from the well-oxygenated north Atlantic to the near-anoxic Eastern Tropical Pacific (ETP), including all of the major oceanic oxygen minimum zones (Norman and Finn 2013). They are gelatinous and can rapidly change between transparency and pigmentation to hide from predators (Zylinski and Johnsen 2011). Their gelatinous musculature allows them to have lower metabolic costs, and better tolerance to hypoxia than most shallow-water benthic octopods from which they likely evolved (Seibel and Childress 2000). Despite these adaptations, their distributions may still be impacted by hypoxia in strong oxygen minimum zones (OMZs) such as in the Eastern Tropical Pacific.

In addition to their wide horizontal distribution, *J. diaphana* undergo an ontogenetic vertical descent. Off the coast of California, young individuals (< 30 mm mantle length (ML)) primarily stay at 600-800 m depths, while older larger individuals (> 30 mm ML) are evenly distributed from 600 m down to at least 1200 m (Roper and Young 1975). Similarly, in the north Atlantic, individuals < 20 mm ML were always found between 100 and 1000 m, while individuals > 20 mm ML were only found deeper than 1000 m (Clarke and Lu 1975). Furthermore, starting at 300 m, there was a strong relationship between ML and depth. Off Hawaii, animals larger than 20 mm ML were always found deeper than 725 m while smaller individuals were abundant between 170 and 800 m (Young 1978).

Sexual maturation and mating seems to occur near the lower end of their depth range. Gravid and brooding females have only been found deeper than 700 m (Young 1978), and are often deeper than 1000 m. These females possess a large yellow circumoral photophore, one of the few known bioluminescent structures in any octopod, presumably to attract males (Robison and Young 1981; Herring et al. 1987). Unlike benthic octopods, female pelagic octopods brood their eggs and possibly paralarvae as well (Young 1972). There is some suggestion that brooding females return to shallower depths (≈800 m) after mating occurs near the deepest part of their range (Young 1978; Voight 1995).

In the ETP, the core of the OMZ occurs near the top of *J. diaphana's* range where juveniles begin their ontogenetic vertical migration. In the OMZ core, P_{O_2} can fall as low as 0.14 kPa (1.8 $\mu mol \cdot kg^{-1}$, 10 °C; Wishner et al. 2013), lower than any cephalopod is currently known to be able to maintain basal metabolism (Seibel et al. 1997). Accordingly, *J. diaphana* population density in the Gulf of Panama is anomalously low around the OMZ (Thore 1949).

Animals require oxygen to fuel metabolic production of ATP for maintenance, prey capture and consumption, locomotion, and growth (Wells and Clarke 1996). Populations are sustained when there is sufficient oxygen to support an "aerobic scope" for such activities. Deutsch et al. (2015) developed a "metabolic index" to quantify the suitability of a habitat for aerobic metabolism. They found that populations of a variety of marine organisms typically require ambient O_2 levels 2-5 times larger than that required by an individual at rest.

The *J. diaphana* population that inhabits the ETP should be sufficiently adapted to tolerate, or sufficiently mobile to move through the OMZ core. Construction of a metabolic index for *J. diaphana* in the ETP OMZ should increase our understanding of how this poorly understood species is adapted for this extreme environment. In this study, we investigate the effects of environmental P_{O_2} on hypoxia tolerance and oxygen consumption rate in the ontogenetically descending mesopelagic octopod, *J. diaphana,* in order to construct a metabolic index of its habitat and determine what adaptations allow it to survive in this region.

Methods

Animal collection

Japetella diaphana were collected for physiological measurements with a 3 m^2 Tucker trawl with a 30 L insulated cod-end to protect animals from temperature shock (Childress et al. 1978). Trawls were conducted between 22 January and 11 February 2017 ≈600 km off the Pacific coast of Mexico near 21.5 °N and 117.5 °W aboard the R/V *Sikuliaq* (Figure 4.1A). Upon capture, animals were transferred to fresh chilled seawater for 9 to 55 hours (mean = 27 hrs) prior to measurement to acclimate them to measurement temperature and to reduce the contribution of digestion to metabolic rate measurements.

Environmental parameter profiles of temperature, salinity, and O_2 were obtained by CTD casts where animals were collected. O_2 profiles were measured with a Seabird SBE 43 dissolved O_2 sensor. One additional animal was also collected from a 1 m^2 Multiple Opening/Closing Net and Environmental Sensing System (MOCNESS) but was not used in physiological experiments. In total, 2.37×10^5 and 1.13×10^5 m^3 of water were filtered by the Tucker trawl and MOCNESS, respectively.

Respirometry

Animals were placed in darkened sealed chambers filled with 0.2 μm filtered seawater that had been treated with antibiotics to minimize bacterial respiration. Trials were conducted at surface pressure since hydrostatic pressure has little effect on metabolism in mesopelagic cephalopods (Belman 1978). Ambient P_{O_2} was measured optically with either a Loligo Systems

Witrox 4 meter or a PyroScience FireStingO2 meter. Upon placement in the chamber, animals were allowed to breathe down the ambient oxygen until their O_2 consumption rate could no longer be sustained. The trials lasted 12 hours on average and ranged from 5 to 25 hours. To assess the temperature-dependence of metabolism, 8 and 4 individuals were tested at 5 and 10 °C, respectively. Temperature was maintained with Lauda E100 and ThermoFisher NESLAB RTE-7 water baths. Oxygen meters were calibrated with air-saturated seawater and concentrated $NaSO_3$ solution ($P_{O_2} = 0$). Chamber water was mixed with magnetic stirrers (Cole-Parmer Immersible Stirrer EW-04636-50).

After experiments were completed, animals were preserved in 10% formalin before being weighed and measured. Dorsal mantle length (DML) was measured as the linear distance from the midpoint between the eyes to the posterior end of the mantle.

$\boldsymbol{P_{crit}}$ *analysis*

The P_{O_2} level below which an animal was unable to sustain its routine oxygen consumption rate was defined as the critical partial pressure of oxygen, or the P_{crit}. The P_{crit} for each animal was calculated using two methods: first, we used the traditional "breakpoint" method, which fits a linear breakpoint function to the relationship between M_{O_2} and P_{O_2}. This calculation was performed with the "segmented" package in R (Muggeo 2008). The "breakpoint" P_{crit} was defined as the P_{O_2} at the intersection between the relatively flat oxyregulating line and the steeply sloping oxyconforming line (Figure 4.2, blue line).

The P_{crit} determined using the traditional breakpoint method is biased, typically toward higher values, due to variability in M_{O_2} values above and below the breakpoint caused by spontaneous changes in behavior and ventilation. In an attempt to calculate a more accurate P_{crit}, the "sub-PI" method of P_{crit} calculation was developed. Using this method, a traditional breakpoint relationship was fit, as before, but a 95% prediction interval was added around the oxyregulating line to encapsulate a space in which all observed M_{O_2} values can reasonably be considered within the oxyregulating space (Figure 4.2, dashed lines). 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 (Figure 4.2, red line).

The sub-PI method results in a systematically lower P_{crit} estimation than the traditional breakpoint method. In this dataset, the sub-PI method returned a 36% lower P_{crit}, on average. The temperature-dependences of P_{crit} metrics were fit using an exponential model from the "Q10" function of the R package "respirometry" (Birk 2018).

Metabolic rate and hypoxia tolerance data were also compared with Seibel et al. (1997) who used similar methods of collection and experimentation with *J. diaphana* collected off the coasts of California and Hawaii (Figure 4.1; note: the *J. heathi* species reported by Seibel et al. (1997) has been merged into the *J. diaphana* taxon). Metabolic rates were normalized for each trial and the mean normalized M_{O_2} at multiple P_{O_2} levels was computed for each population. Due to the large size range of specimens captured, differences in M_{O_2} between populations were assessed by an ANCOVA with size as a covariate.

Metabolic index

The metabolic index, or the ratio of oxygen supply to demand, is a temperature-dependent metric to quantify suitable habitat for aerobic metabolism (Equation 4.1; Deutsch et al. 2015). E_0 and A_0 are physiological parameters defined as the slope and intercept, respectively, of the linear relationship between the natural logarithm of P_{crit} and $1/k_B$T, where k_B is the Boltzmann constant and T is temperature in Kelvin. It can also be parameterized as the ratio of ambient P_{O_2} to temperature-dependent critical P_{O_2} (P_{crit_T}).

$$\mathrm{MI}=\mathrm{A}_0\frac{\mathrm{P}_{\mathrm{O}_2}}{\exp(\text{-}\mathrm{E}_0/\mathrm{k}_\mathrm{B}\mathrm{T})}=\frac{\mathrm{P}_{\mathrm{O}_2}}{\mathrm{P}_{\mathrm{crit}_\mathrm{T}}}, (4.1)$$

An MI < 1 represents a region where demand exceeds supply (ambient P_{O_2} is below P_{crit}) and should not be able to support steady-state basal aerobic metabolism. An MI > 2-5 is typically required to sustain populations of marine animals (Deutsch et al. 2015). Multiple MI depth profiles were constructed using the environmental data from CTD casts and the measured relationship between P_{crit} and temperature. Distinct MI profiles were constructed based on 1) breakpoint P_{crit}, 2) sub-PI P_{crit}, 3) the 95% confidence interval of the sub-PI P_{crit}, and 4) the lowest measured sub-PI P_{crit} from each temperature.

Results

Abundance

In total, 15 animals were collected in the ETP between 170 and 1011 m depths, with a total abundance of 5.32 individuals per $10^5\ m^3$. Thirteen were juveniles (11-37 mm DML). A mature male (52 mm DML) and female (72 mm DML) were both caught in the same trawl between 779-1011 meters. No trawls were conducted deeper than 1282 meters.

Metabolism and metabolic index

Mass-specific M_{O_2} of individuals captured in the ETP was over twice as high as those found off California and Hawaii, even when tested at the same temperature (5 °C) over similar trial durations (p = 0.003; Figure 4.3A). Mass-specific M_{O_2} decreased with increasing size, with a scaling coefficient (b) of -0.33 at 5 °C (n = 7). Despite their higher oxygen demand, animals from the ETP had similar hypoxia tolerance to those from California and were more tolerant than animals from Hawaii (Figure 4.3B).

Among animals in the ETP, mass-specific M_{O_2} increased significantly with increasing temperature, from $0.41 \pm 0.115\ \mu mol\ O_2 \cdot g^{-1} \cdot hr^{-1}$ at 5 °C to $0.68 \pm 0.205\ \mu mol\ O_2 \cdot g^{-1} \cdot$

hr^{-1} at 10 °C (p = 0.019; Q_{10} = 2.75; Figure 4.4A). Mean breakpoint P_{crit} decreased significantly over the same temperature range from 1.8 $\pm$ 0.73 kPa at 5 °C to 0.88 $\pm$ 0.34 kPa at 10 °C (p = 0.043; Q_{10} = 0.24; Figure 4.4B). The lowest sub-PI P_{crit} recorded at each temperature was lower than that obtained using the breakpoint method: 0.63 and 0.31 kPa at 5 and 10 °C, respectively.

We found that the OMZ in the ETP had a minimum P_{O_2} of 0.143 kPa (5.6 °C) and that a wide depth range (at least 543 m) had an MI < 1, even with the most liberal metabolic index, constructed from the lowest sub-PI P_{crit} observations from each temperature treatment (Table 4.1; Figure 4.5B). The depths at which many animals were captured had an MI < 1 (Figure 4.5B).

Discussion

Aerobic habitat and distribution

By all methods of calculating the metabolic index, MI < 1 for a large depth range of the OMZ, suggesting that oxygen supply in this region is insufficient to meet the basal oxygen demand of *J. diaphana*. In other marine animals, an MI of 2-5 has been found to be the lower limit for populations to be sustained (Deutsch et al. 2015); this is higher than the MI levels where many of the octopods were found in this study. *Japetella diaphana* does not seem to undergo diel vertical migration (Young 1978), making it unlikely that the animals we captured were temporarily metabolically suppressed in the OMZ core as part of their life history strategy, a behavior exhibited by more muscular cephalopods in the eastern Pacific OMZ (Hunt and Seibel 2000; Seibel et al. 2014).

Although none of the animals we examined were able to sustain aerobic metabolism below the most extreme hypoxia encountered in the OMZ core, 0.14 kPa (2 $\mu mol \cdot kg^{-1}$), the

P_{O_2} within the OMZ core was within the 95% prediction interval of the P_{crit} values we measured (Figure 4.5A), suggesting that some individuals may be able to maintain aerobic metabolism at this low oxygen level. It is important to consider that net-caught and confined mesopelagic specimens may be stressed or injured in ways that, while not visible or detectable, may nonetheless impair tolerance to hypoxia compared to an individual *in situ.* Thus, it is likely that the mean P_{crit} values reported here are overestimates, thus underestimating the "true" metabolic index of this region for *J. diaphana*.

It seems likely that the ambient oxygen levels in the core of the OMZ would, at the very least, constrain aerobic performance in these animals relative to the more oxygenated waters above and below. It is not surprising, therefore, that many of the animals were captured in the upper and lower oxyclines but few were captured where the hypoxia was most extreme; though, admittedly, the sampling effort was limited. Thore (1949) similarly reports that in the Gulf of Panama, where a strong OMZ also exists, *J. diaphana* density has a minimum between 200 and 1000 m, unlike more oxygenated stations. It is not uncommon to find aggregations of zooplankton and micronekton in oxyclines around the OMZ (Wishner et al. 2013). For predatory *J. diaphana*, this may be a feeding ground rich in euphausiids, fishes, calanoid copepods, decapod crustaceans, and other mollusks upon which they feed (Passarella and Hopkins 1991). As such, the foraging opportunities may outweigh the limited oxygen available.

In the ETP, the ontogenetic vertical migration of *J. diaphana* passes through the core of the OMZ. Based on the ontogenetic vertical distributions described previously, oxygen supply is insufficient to meet basal demand in much of the juvenile habitat but only in the upper range of adult habitat. It would not be surprising for juveniles to descend rapidly through the OMZ core to avoid the limited aerobic scope, thus producing a "punctuated" ontogenetic vertical migration.

Off the California coast, the OMZ is not as strong (Helly and Levin 2004), and many *J. diaphana* are found near the lowest O_2 depths (Roper and Young 1975). Relative gill size declines with increasing body size in *J. diaphana* (Thore 1949). This trend is not an adaptation to oxygen profiles as it occurs across individuals from many ocean basins due to lower mass-specific oxygen demand in larger individuals (Figure 4.3A), but it should nevertheless be beneficial for young juveniles in the ETP that encounter the strongest hypoxia, as they will have relatively larger gills than larger animals from more oxygenated depths.

Bolitaenins are one of the most abundant pelagic cephalopod groups in the Gulf of Mexico (Judkins et al. 2017). We found that *J. diaphana* abundance in the ETP was comparable or even exceeded abundance estimates from the North Atlantic off the coast of Africa (Clarke and Lu 1975) and from the Gulf of Mexico (H. Judkins, pers. comm). This is surprising given the apparent paucity of aerobic habitat for juveniles, but this may be counteracted by the high productivity and prey biomass in this region (Fernández-Álamo and Färber-Lorda 2006). Thore (1949) reported *J. diaphana* density in the Gulf of Panama to be comparable or larger than most other stations of the circumglobal *Dana* expeditions, and found that *J. diaphana* density often correlated with primary productivity. Admittedly, our sampling effort was small and comparisons between studies are complicated by differences in sampling gear, but it seems unlikely that the extreme hypoxia in this region has strong negative effects on the local abundance of *J. diaphana.*

Metabolic adaptations to hypoxia

Individual *J. diaphana* from the ETP had a higher M_{O_2} than individuals from California and Hawaii. The physiological mechanism or adaptive significance of such differences is unclear. Despite their higher oxygen demand, individuals from the ETP maintained a lower P_{crit}

(improved hypoxia tolerance) compared to individuals from Hawaii (Seibel et al. 1997). Similarly, in some mesopelagic crustacean species, P_{crit} is higher in individuals from the relatively well-oxygenated Hawaiian waters than from conspecifics in the OMZ off California (Cowles et al. 1991; Childress and Seibel 1998). It has been demonstrated that crustacean and fish species also alter their hypoxia tolerance to match ambient conditions on much smaller spatial scales such as within the Monterey Canyon or Southern California basins (Childress 1975; Friedman et al. 2012). In pelagic animals generally, P_{crit} correlates with the minimum encountered ambient P_{O_2} (Seibel 2011).

The physiological mechanisms underlying differences in P_{crit} between populations of *J. diaphana* are currently unknown, but are likely to involve differences in blood O_2 binding affinity and gill surface area. The vampire squid, *Vampyroteuthis infernalis,* a mesopelagic octopodiform that inhabits the OMZ off California, has very high blood O_2 binding affinity and relatively large gills for its low metabolic rate (Seibel et al. 1999). In addition, specimens of the squid *Bathyteuthis abyssicola* caught in the ETP have larger gills than conspecifics from the Atlantic or Southern Oceans (Roper 1969). The congeneric *B. bacidifera* is endemic to hypoxic equatorial waters in the eastern Pacific and Indian Oceans, and has even larger gills than *B. abyssicola* (Roper 1969). Midwater crustaceans in the relatively hypoxic waters off California have higher hemocyanin-O_2 binding affinity than species off Hawaii where P_{O_2} is higher (Childress 1995). Similar physiological responses have been observed in a diversity of animals from a variety of hypoxic environments (Snyder 1985; Mandic et al. 2009; Friedman et al. 2012). Although oxygen demand increased under higher temperature, as is typical of ectotherms, the P_{crit} decreased by 0.92 kPa (i.e. hypoxia tolerance improved) from 5 to 10 °C. In ectotherms, hypoxia tolerance typically decreases (i.e. P_{crit} rises) with rising temperature (Rogers et al.

2016) due to a combination of higher oxygen demand and possibly reduced oxygen binding affinity of the respiratory proteins. However, this is not always the case. Freshwater darter fishes have been observed to have lower P_{crit} despite higher M_{O_2} with rising temperature (Ultsch et al. 1978). The physiological mechanism behind such effects may include a larger increase in ventilation or cardiac output with temperature than is typical or an inverse temperature-dependence of blood-O_2 binding affinity. Whatever the mechanism, the functional benefit of this inverse temperature-dependence is clear for a meso- and bathypelagic animal living in a strong OMZ. Within the depth range where *J. diaphana* are typically found (400-1200 m), warmer waters have lower P_{O_2} and thus require a lower P_{crit} to maintain aerobic metabolism. Accordingly, a similar inverse temperature-dependence of P_{crit} has recently been documented in lower oxycline krill and shrimp species (Wishner et al., in review).

The *J. diaphana* examined here from the OMZ are more tolerant of hypoxia than more active cephalopods examined to date in the eastern Pacific. The muscular active squids *Dosidicus gigas* and *Gonatus onyx* undergo diel migration to mesopelagic depths, but have mean P_{crit} of 1.9 and 4 kPa, respectively (Seibel et al. 1997; Hunt and Seibel 2000; Trueblood and Seibel 2013). Unlike *J. diaphana,* these squids suppress metabolism during the daytime and return to oxygenated surface waters at night, which allows them to pay back any oxygen debt incurred at oxygen levels below their P_{crit} (Hunt and Seibel 2000; Seibel et al. 2014).

Japetella diaphana exhibit similar hypoxia tolerance to the mesopelagic squid *Histioteuthis heteropsis* in the California Current (0.9-1.5 kPa; Belman 1978; Seibel et al. 1997) and *Octopus californicus* residing in the Santa Barbara Basin just above the anoxic sill (Seibel and Childress 2000). Hawaiian *J. diaphana* have similar hypoxia tolerance with the Hawaiian bathypelagic squid *Bathyteuthis abyssicola* (2.4 kPa; Seibel et al. 1997). *Japetella diaphana*s

P_{crit} is also not statistically distinguishable from that of *Vampyroteuthis infernalis,* which lives permanently in the core of the OMZ off California. The latter is reportedly one of the most hypoxia tolerant cephalopods measured to date (0.96 kPa; Seibel et al. 1997).

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Tables

Table 4.1. Hypoxia tolerance and metabolic index of *Japetella diaphana* in the Eastern Tropical Pacific. Values are shown as mean ± standard deviation.

Method	Temperature (°C)	P_{crit} (kPa)	Minimum MI	Depth range of MI < 1 (m)	Breadth of MI < 1 (m)
Breakpoint	5	1.8 ± 0.73	0.09	346 – 1455	1109
	10	0.88 ± 0.34			
sub-PI	5	1.17 ± 0.37	0.13	373 – 1207	834
	10	0.68 ± 0.35			
95% CI of sub-PI	5		0.18	422 – 1021	599
	10				
Minimum sub-PI	5	0.63	0.25	427 – 970	543
	10	0.31			

Figures

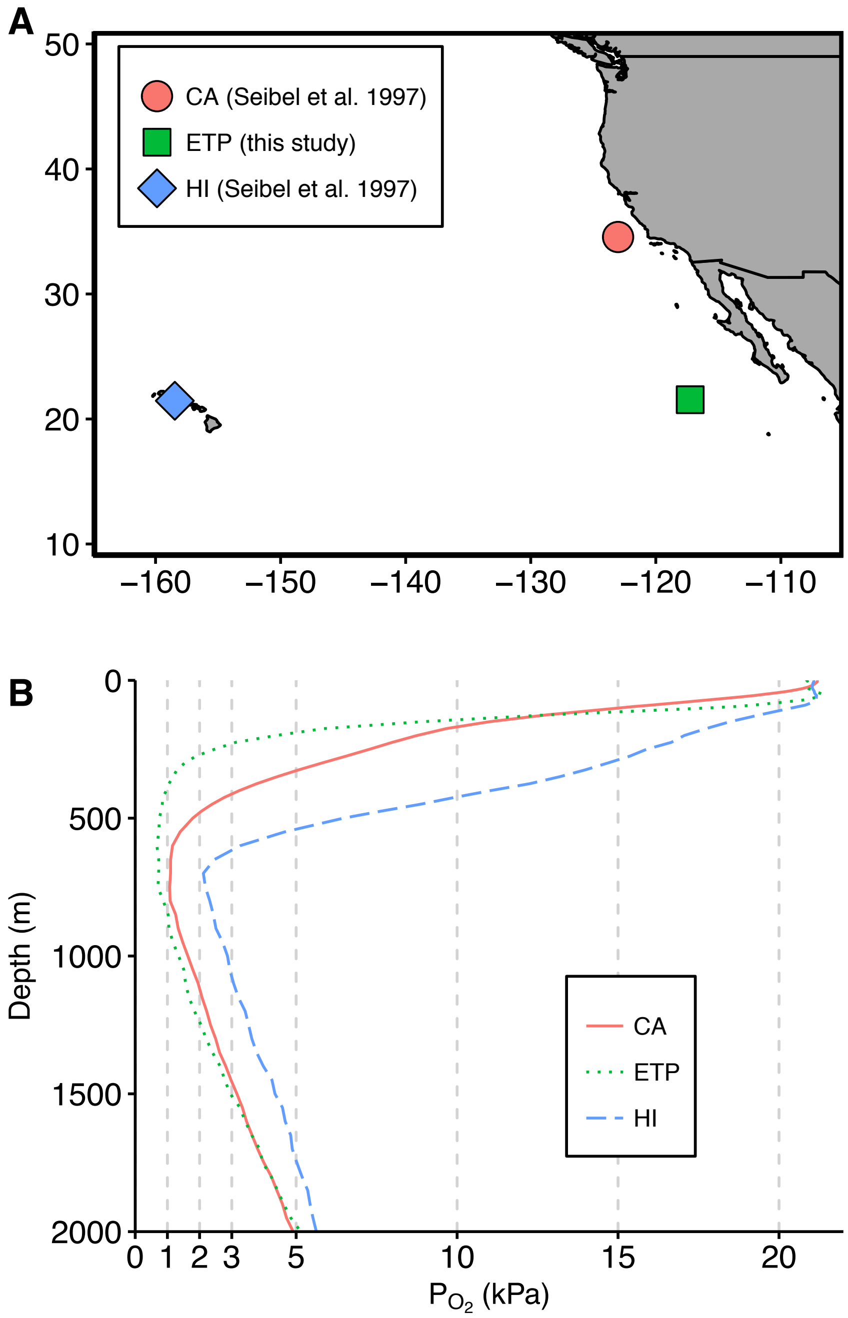

Figure 4.1. Sampling sites of *Japetella diaphana* and depth profiles. ***A***) Location of animal collection sites. ***B***) P_{O_2} depth profiles at each collection site from the World Ocean Atlas 2013.

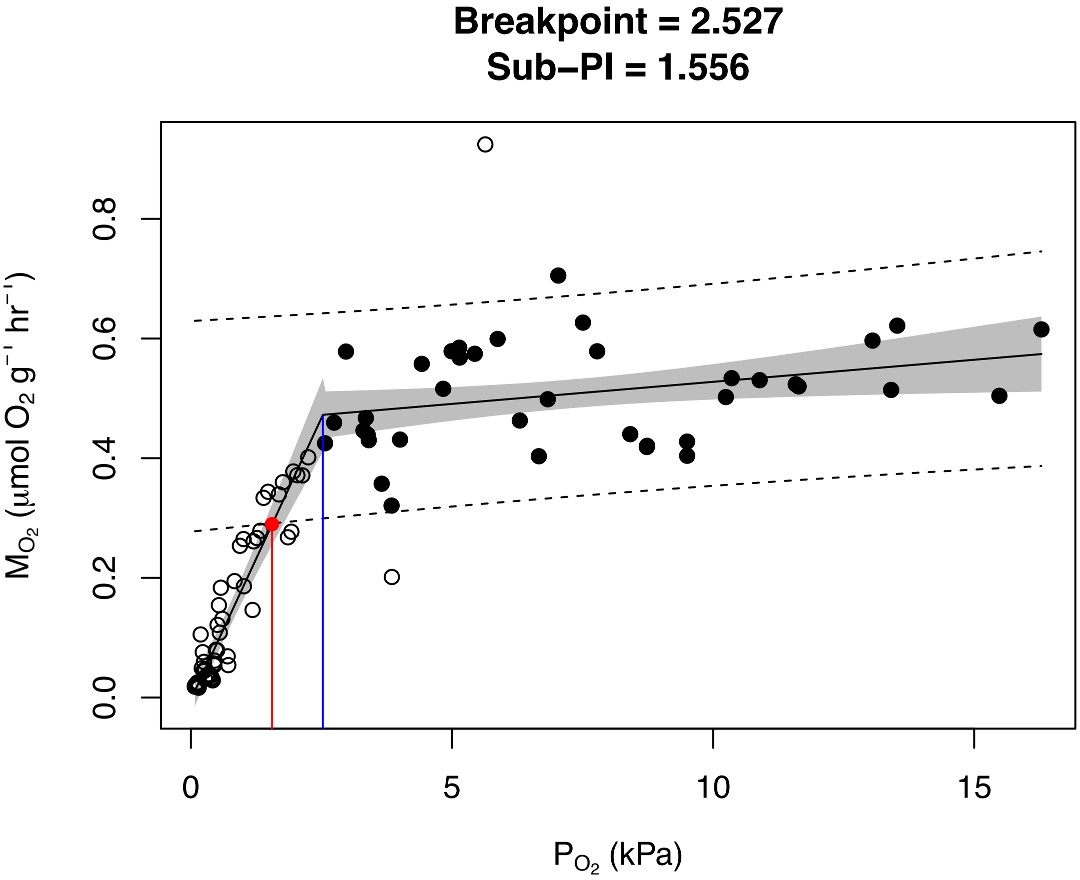

Figure 4.2. A representative respirometry experiment showing the routine metabolic rate ($\mu mol\ O_2 \cdot g^{-1} \cdot hr^{-1}$) of *Japetella diaphana* as a function of oxygen partial pressure (kPa). The P_{crit} calculation methods are indicated by vertical colored lines. The blue line indicates the P_{crit} calculated using the breakpoint method. The red line indicates the P_{crit} calculated using the sub-PI method. The shaded region represents the 95% confidence interval and the dashed lines represent the 95% prediction interval for the regulated oxygen consumption rate.

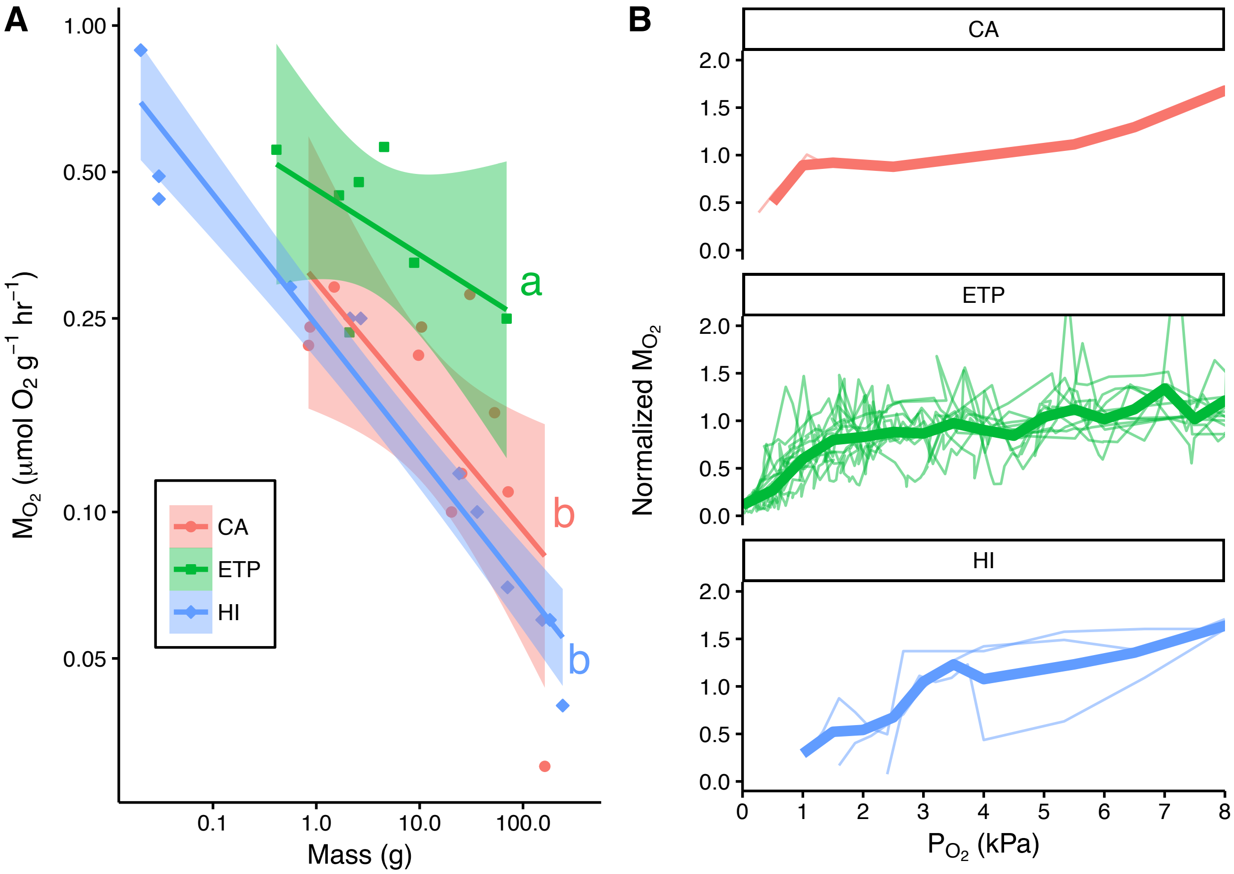

Figure 4.3. Differences in ***A***) oxygen consumption rates and ***B***) hypoxia tolerance between *Japetella diaphana* from California (red), the Eastern Tropical Pacific (green), and Hawaii (blue). Letters indicate statistically significant differences between populations. All measurements were conducted at 5 °C. Bold lines are trial averages.

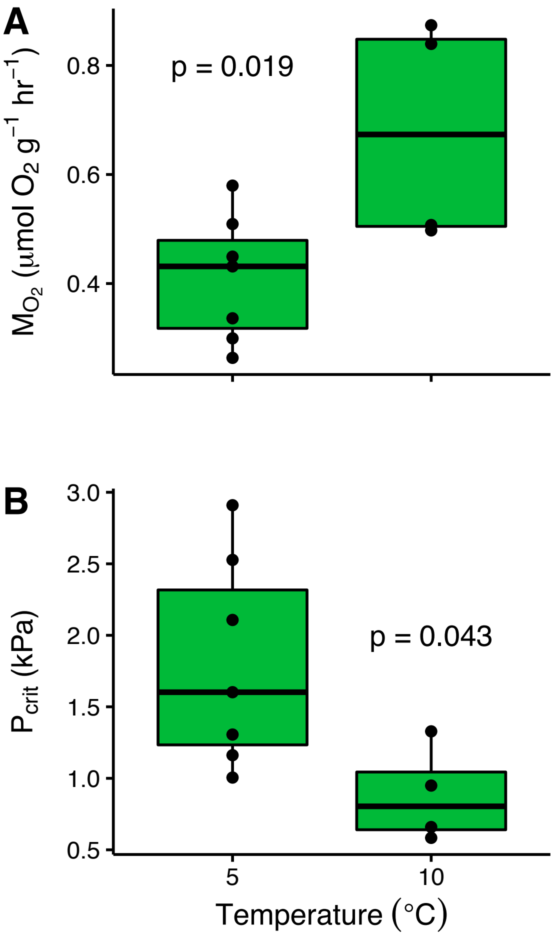

Figure 4.4. Effect of temperature on ***A***) oxygen consumption rate and ***B***) P_{crit} (breakpoint method) at 5 and 10 °C in *Japetella diaphana* from the Eastern Tropical Pacific.

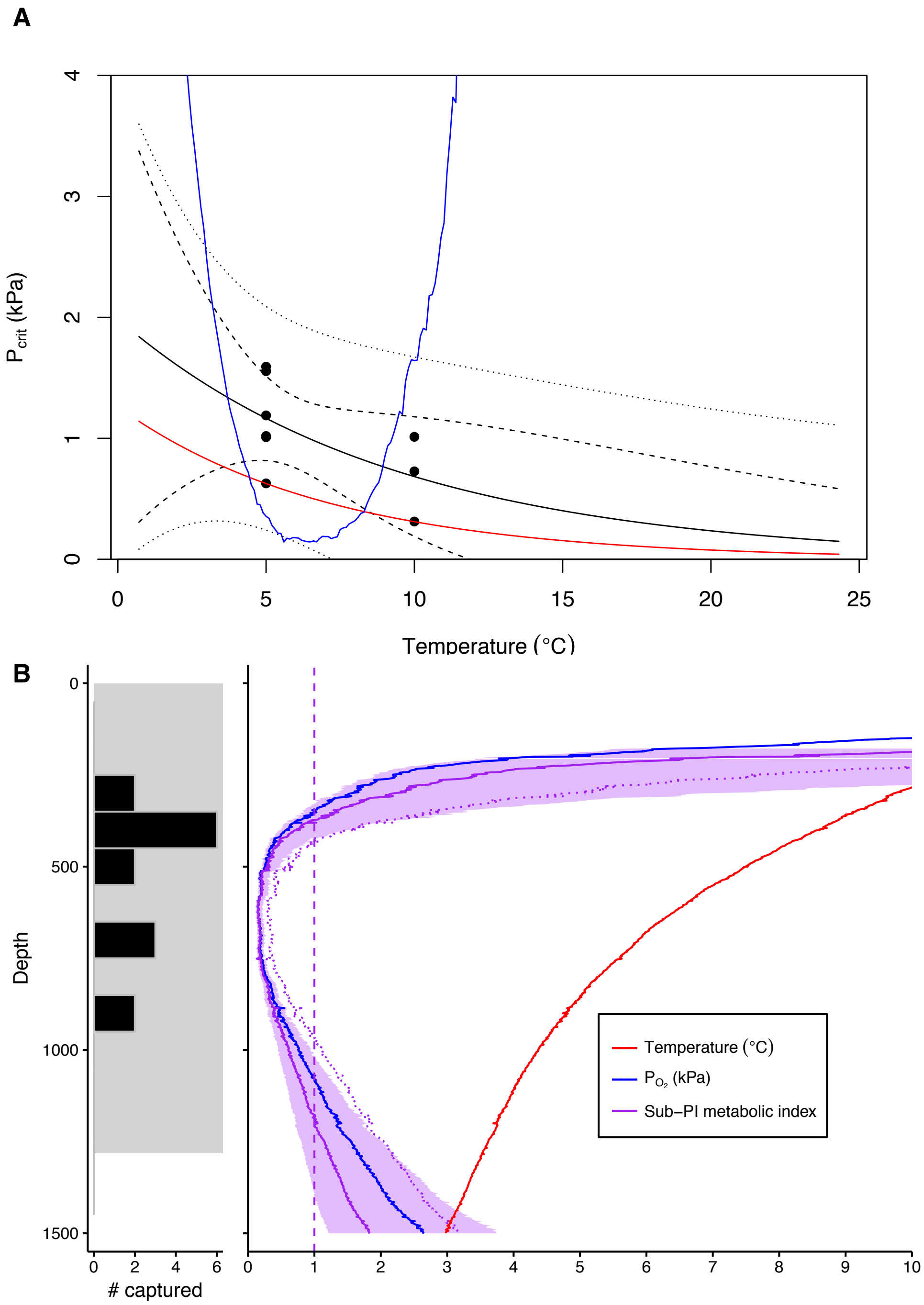

Figure 4.5. Metabolic index of *Japetella diaphana* in the Eastern Tropical Pacific. ***A***) Model of sub-PI P_{crit} vs. temperature. The dashed and dotted curves are the 95% confidence and prediction intervals, respectively. The red line is the best fit relationship using only the lowest values from each temperature. The blue curve is the lowest P_{O_2} encountered at each temperature. ***B***) Metabolic index of *J. diaphana* in the ETP. The solid purple line represents the MI from the mean sub-PI P_{crit} (solid curve in A). The shaded purple region represents the MI 95% confidence interval (dashed curve in A). The dotted purple line represents the MI from the minimum sub-PI P_{crit} (red curve in A). The number of animals captured is shown on the left. The shaded area represents the sampled depths.

CHAPTER FIVE:

RELEVANCE AND IMPLICATIONS

The objective of this dissertation is to advance our understanding of the physiological adaptations that allow coleoid cephalopods to maintain a balance of oxygen supply and demand amongst various environmental challenges. In this dissertation, I have provided novel evidence that confronts an established assumption about O_2 supply in the cephalopod physiology literature, provided experimental and theoretical evidence to further our understanding of tolerance to CO_2 in squid and, by extension, marine animals generally, and produced new data that show how the metabolic physiology of a little-known species may be affected by oxygen minimum zones.

Chapter 2 of this dissertation demonstrates that, contrary to a commonly-held but untested hypothesis, squids do not acquire large quantities of oxygen through their skin for systemic use. This finding has important implications for our understanding of branchial and cardiac performance in squids. The squid cardiovascular system is already believed to be delivering near-maximal quantities of oxygen (O'Dor et al. 1990; Shadwick et al. 1990; Wells 1992). Based on this research, cutaneous oxygen uptake does not alleviate the oxygen delivery demand on the cardiovascular system. This finding makes the physiological adaptations to balance oxygen supply and demand in cephalopods all the more interesting.

The advancements in our understanding of metabolic physiology presented in this dissertation come at an important time. A number of anthropogenic stresses, such as emissions of greenhouse gases and nutrient-enrichment of watersheds, are altering marine environments and influencing both the oxygen supply and demand pathways of marine animals. Chapter 3 of this dissertation addressed the effects of ocean acidification on metabolic physiology, while warming and ocean deoxygenation remain key stressors of concern (Breitburg et al. 2015).

Warming

Ocean warming has direct and indirect effects on oxygen balance in ectothermic marine animals. Rises in ambient temperature directly raise organismal oxygen demand (Hochachka and Somero 2002). Additionally, warming increases rates of oxygen diffusion in seawater, providing increased oxygen supply at the seawater-gill interface (Verberk et al. 2011). However, decreased O_2-binding affinity of respiratory proteins at high temperature in most animals decreases the delivery of oxygen to tissues (Mangum 1997). The net impact of increased temperature in most ectothermic animals is a rise in O_2 demand relative to supply, resulting in impaired hypoxia tolerance (but see Chapter 4 for an exception).

According to a popular but controversial hypothesis proposed by Pörtner (2001), limitation in oxygen supply is an important factor in the thermal limits of ectothermic marine animals. If true, then falling oxygen levels in a given environment will also limit the sustainable thermal range of animals in that environment having potential impacts on biogeography and phenology. Equatorward biogeographic limits of a number of marine species can be well explained by the combined effects of oxygen and temperature on metabolism (Deutsch et al. 2015). Thus, concomitant ocean warming and deoxygenation in the surface ocean have interconnected and possibly synergistic effects on oxygen balance in marine animals (Gobler and Baumann 2016).

Deoxygenation

Ocean deoxygenation is a global phenomenon caused by multiple drivers (Breitburg et al. 2018). In coastal environments, agricultural runoff causes nutrient-enrichment in rivers, leading to increased input of nitrogen and phosphorus into marine systems. These nutrients can result in blooms, and the subsequent microbial-induced decay produced by heterotrophic bacteria can lead to regions of oxygen poor bottom-water (Rabalais et al. 2014). These coastal “dead zones” often kill sessile taxa and encourage mobile taxa to migrate to more oxygenated waters (Diaz and Rosenberg 2008).

Oxygen minimum zones (OMZs) worldwide have also been growing in intensity and volume both horizontally and vertically. On average, OMZ cores are becoming more hypoxic by ~0.2 μmol O_2 / kg / year (Stramma et al. 2008). This is believed to be due to a combination of drivers such as lowered oxygen solubility in warmer surface waters (Weiss 1970), decreased mixing of O_2-rich surface water with the ocean interior due to increased stratification (Gruber 2011), a temperature-induced increase in microbial respiration (Devol 1978), and increased deep water residence time due to slowed meridional overturning circulation (Stocker and Schmittner 1997).

For cephalopods and other marine animals that inhabit ocean regions with OMZs, oxycline shoaling can result in vertical habitat compression, limiting the depth and duration at which an individual can inhabit a given depth. Compression has been well documented in zooplankton (Wishner et al. 2013), a number of pelagic fishes (Carlisle et al. 2017; Stramma et al. 2011), and jumbo squid (Seibel 2015). The consequences of such compression may include increased predation risk, altered trophic interactions in shallow well-lit surface waters, and alterations in biogeochemical cycles such as increased nutrient recycling in surface waters and weakening of the biological pump.

Implications of this research on climate change physiology

The behavioral and population-level responses of cephalopods and other marine ectotherms to climate change stressors are driven in part by responses at the physiological level (Helmuth 2009). Therefore, gains in understanding of animal physiology can help us better understand the responses of marine ecosystems and fisheries to changing climate (Cooke et al. 2012). The findings of this dissertation directly contribute to an improved understanding of the cephalopod response to climate change.

The discovery (Chapter 2) that squids do not obtain a large proportion of oxygen across the skin (contrary to what had been believed for the past 30 years) changes the predicted effect of temperature on oxygen supply. Under rising temperature, squids cannot depend upon increased oxygen supply provided by faster diffusion across the skin. Rather, nearly all oxygen is supplied through the cardiorespiratory system, a multi-step pathway with multiple temperature-dependent processes.

In Chapter 3, I argue that squid oxygen supply is insensitive to CO_2 increases anticipated in the surface ocean by the year 2100. Furthermore, since oxygen levels in squid blood are thought to be more sensitive to high CO_2 than the blood of nearly any other marine animal, this study produces upper bounds for the effects of ocean acidification on oxygen supply for marine animals in general.

Finally, in Chapter 4, I find that the eastern tropical Pacific OMZ has insufficient oxygen supply to meet the demands of *Japetella* octopods, which suggests that OMZ growth in future decades may impact the ontogenetic migration of this species in hypoxic ocean regions.

Physiology-based habitat suitability models such as the metabolic index (Deutsch et al. 2015) used in Chapter 4 are one means of connecting physiological knowledge to ecosystem-level changes. Such models can be valuable tools for understanding and predicting ecosystem responses to changing oceans (Teal et al. 2018).

Cephalopods play significant roles in many aspects of biological oceanography. They hold important niches in many marine ecosystems (Coll et al. 2013), play key roles in biogeochemical cycles (Arkhipkin 2013), and support substantial fisheries worldwide (Arkhipkin et al. 2015). Interestingly, cephalopods from various environments, life histories, and ocean regions seem to have been increasing in abundance over the past 60 years (Doubleday et al. 2016). Whether this is due to climate change, depletion of fish stocks, or some other driver is currently unknown. Despite the importance and increasing abundance of cephalopods and their intriguing physiological, behavioral, and evolutionary characteristics, much remains to be learned about these fascinating marine organisms.

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