

Impact of Short- and Long-Term Exposure to Elevated Seawater PCO_2 on Metabolic Rate and Hypoxia Tolerance in *Octopus rubescens*

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ABSTRACT

Much of the CO_2 released by human activity into the atmosphere is dissolving into the oceans, making them more acidic. In this study we provide the first data on the short- and long-term impacts of ocean acidification on octopuses. We measured routine metabolic rate (RMR) of *Octopus rubescens* at elevated CO_2 pressure (PCO_2) with no prior acclimation and 1 or 5 wk of acclimation and critical oxygen pressure (P_{crit}) after 5 wk of acclimation. Our results show that with no prior acclimation, octopuses had significantly higher RMRs in 1,500- μatm PCO_2 environments than octopuses in 700- or 360- μatm environments. However, after both 1 and 5 wk of acclimation there was no significant difference in RMRs between octopuses at differing PCO_2 , indicating that octopuses acclimated rapidly to elevated PCO_2 . In octopuses acclimated for 5 wk at 1,500 μatm PCO_2 , we observed impaired hypoxia tolerance, as demonstrated by a significantly higher P_{crit} than those acclimated to 700 μatm PCO_2 . Our findings suggest that *O. rubescens* experiences short-term stress in elevated PCO_2 but is able to acclimate over time. However, while this species may be able to acclimate to near-term ocean acidification, compounding environmental effects of acidification and hypoxia may present a physiological challenge for this species.

Keywords: ocean acidification, *Octopus rubescens*, hypercapnia, critical oxygen pressure, metabolic rate.

Introduction

Atmospheric CO_2 has risen from preindustrial levels of approximately 275 ppm (MacFarling Meure et al. 2006) to over 400 ppm presently (Tans and Keeling 2015). As much as one-third of all anthropogenic CO_2 is absorbed by the oceans (Doney et al. 2009), resulting in an increased PCO_2 of oceanic waters and decreased average pH from 8.2 to a current average of 8.1, a process known as ocean acidification (OA; Caldeira and Wickett 2003).

Early work on the biological impacts of OA has largely focused on negative consequences for calcifying organisms. As oceanic pH drops, the ability of calcifying organisms to precipitate calcite and aragonite for use in calcareous structures is decreased, and dissolution of existing calcareous structures can occur (Fabry et al. 2008).

Biological consequences of OA are not limited to calcifying processes because many physiological processes are pH sensitive. Challenges to an organism's physiology are often reflected in changes in energy use and therefore can be observed as changes in aerobic metabolic rate. For example, respiratory physiology is particularly sensitive to pH disturbances due to Bohr and Root effects exhibited by respiratory pigments (Miller 1985; Bridges 1995; Widdicombe and Spicer 2008; Seibel 2016). This impacts the ability to obtain environmental oxygen for use in metabolic processes. Hypercapnic environments have been demonstrated to cause a reduction in oxygen pressure in hemolymph of crabs (Metzger et al. 2007; Walther et al. 2009). The aerobic scope of cardinal fish has been found to decrease as much as 47% when environmental PCO_2 was raised from ambient to 1,000 ppm (Munday et al. 2009). Seawater hypercapnia has been shown to depress oxygen consumption both in whole organisms, such as *Sipunculus nudus* (Linnaeus 1766) (Pörtner et al. 1998), and in isolated tissues, such as isolated hepatocytes of fish *Pachycara brachycephalum* (Pappenheim 1912) and *Lepidotothen kempfi* (Norman 1937) (Langenbuch and Pörtner 2003) and body wall muscle of *S. nudus* (Langenbuch and Pörtner 2002). Larval stages may be particularly sensitive because elevated CO_2 has been shown to impair their growth and lower survival of some invertebrates (Byrne et al. 2013) and fish (Baumann et al. 2011).

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Research on how OA may affect cephalopods has been sparse and generated conflicting results. Across a range of P_{CO_2} , responses in regard to calcification or aerobic metabolism are not consistent, with squid and cuttlefish responding differently. In studies with short-term exposure to more environmentally relevant ranges of 700–1,700 $\mu\text{atm } P_{CO_2}$, squid have been shown to have a reduction in aerobic metabolism (Rosa and Seibel 2008; Rosa et al. 2014), whereas cuttlefish show no significant change (Gutowska et al. 2008; Hu et al. 2014). Calcification rates have been shown to increase, with at least one species of squid, *Doryteuthis opalescens* (Berry 1911), developing larger statoliths when exposed to elevated P_{CO_2} (Navarro et al. 2016).

However, when P_{CO_2} is pushed to more extreme levels of 2,000–4,500 μatm , there continues to be variation in response. In cuttlefish, juvenile *Sepia officinalis* (Linnaeus 1758) experienced an increase in cuttlebone calcification (Gutowska et al. 2008, 2010b), whereas in the squid *Doryteuthis pealii* (Lesueur 1821) hatchlings are found to have malformed statoliths with increased porosity (Kaplan et al. 2013). Aerobic metabolism in squid still shows a negative response, with *Sepioteuthis lessoniana* (d'Orbigny 1826) showing a reduction in aerobic metabolism at P_{CO_2} above 4,000 μatm (Hu et al. 2014).

The decreased aerobic metabolism observed in squid has often been associated with limitations of their respiratory pigments (Seibel 2013). Cephalopods appear to be strong pH regulators (Gutowska et al. 2010a); however, their respiratory physiology may be adversely affected by environmental hypercapnia, as evidenced by metabolic suppression (Rosa and Seibel 2010) and decreased blood oxygen binding (Seibel 2013) in *Dosidicus gigas* as well as impaired hypoxia tolerance in *S. officinalis* (Rosa et al. 2013). This has been hypothesized to be due in part to constraints of cephalopod hemocyanins, which exhibit a low oxygen carrying capacity (O'Dor and Webber 1991) and pronounced Bohr and Root effects (Bridges 1995). However, recent work by Birk and Seibel (Birk et al. 2018) suggests that epipelagic squid blood oxygen carrying capacity is minimally impacted by changes in environmental pH. Their proposed model suggests a Bohr coefficient of -1.5 in squid as being a worst-case scenario for considering impacts of OA on cephalopods. This value is reasonable in light of the range of Bohr coefficients recorded for epipelagic squid of -1.56 to -0.38 (Rosa and Seibel 2008). However, studies of octopus hemocyanin show that changes in pH have a greater effect on their blood oxygen binding with Bohr coefficients of -1.7 in *Octopus dofleini* (Miller 1985) and -1.99 in *Octopus macropus* (Lykkeboe and Johansen 1982). This suggests that hypercapnia may have a greater effect on octopus blood oxygen binding comparatively and, as a result, aerobic metabolism.

Research into the impacts of OA on cephalopods has largely focused on cuttlefish and squid, and to date no investigation has explored the impacts of OA on octopuses. Also, examinations of the impacts of hypercapnia on the physiology of cephalopods have been undertaken using either short-term exposure <24 h (Rosa and Seibel 2010) or long-term exposure ~ 6 wk (Gutowska et al. 2010a; Hu et al. 2010); however, there has been no examination explicitly comparing responses to

both short-term and long-term exposures to hypercapnia in the same species. Short-term exposure to hypercapnia will likely induce stress responses that would not be representative of the responses of acclimated organisms. Nevertheless, a great deal of investigations using only short-term hypercapnic exposure exist in the literature, and studies comparing responses to acute and chronic hypercapnia could help clarify how short-term responses can inform organismal responses to long-term OA.

Here, we examine *Octopus rubescens* (Berry 1953), a small octopus common to the intertidal and nearshore subtidal habitats of the west coast of North America (Hochberg 1998). This octopus is relatively easily collected and maintained in aquaria, making it an ideal octopus for laboratory physiological investigations (Onthank and Cowles 2011). The Salish Sea, where this research was conducted, presents a unique opportunity for OA research, because the shallow basin receives water from the CO_2 -rich California Undercurrent and allows for persistent hypercapnic conditions to exist, often near 700 $\mu\text{atm } CO_2$ (Murray et al. 2015). Such coastal habitats will also experience accentuated acidification due to local hypoxia and eutrophication (Cai et al. 2011; Melzner et al. 2013). The goal of this investigation was to determine not only the impacts of hypercapnia on the aerobic metabolic rates of octopuses but also to compare the relative effects of short-term and long-term exposure to elevated CO_2 . We hypothesized that the extreme Bohr effects observed in octopuses would lead to aerobic metabolic rate suppression and elevation of critical oxygen pressure (P_{crit}) in the face of elevated environmental P_{CO_2} .

Methods

Seawater pH Measurement

This research was carried out at the Rosario Beach Marine Laboratory (RBML) in Anacortes, Washington. To characterize spatial patterns in seawater pH and P_{CO_2} in the region from which octopuses were collected, water samples were taken along transects extending from RBML to Friday Harbor, San Juan Island, Washington, and from RBML to Driftwood Park, Whidbey Island, Washington. Surface water samples were taken by submerging a 200-mL high-density polyethylene (HDPE) sample container approximately 10 cm below the water surface and capping the container with a HDPE screw lid to exclude air. At a subset of sampling sites, water samples from a depth of 15 m were collected using a Van Dorn water sampler and transferred to a 200-mL HDPE container. All water samples were immediately placed on ice and transported to RBML, where pH on the total scale (pH_T) was measured using the *m*-cresol purple spectrophotometric method (Dickson et al. 2007), within 3 h. To explore potential spatial autocorrelation of seawater pH_T , the nugget (the semivariance when lag distance is equal to 0), sill (the semivariance reached when semivariance and distance are no longer correlated), and range (the distance at which the sill is reached) of the pH_T semivariogram were estimated using a Gaussian covariance model as implemented in the geoR package in R (Ribeiro and Diggle 2001). Seawater P_{CO_2} was estimated based on pH_T at each

location and assuming a seawater alkalinity of $2,080 \mu\text{mol kg}^{-1}$, an average alkalinity found in the area (Murray et al. 2015).

Octopus Collection

Thirty-two male *Octopus rubescens* (mass = 20–310 g) were collected in 2014, and 10 males were collected in 2015 (mass = 46–270 g) from Driftwood County Park, Whidbey Island, Washington, by scuba (see appendix). Octopuses were located by examining discarded glass bottles, which *O. rubescens* is known to occupy (Anderson et al. 1999). Bottles containing octopuses and without eggs were placed into plastic resealable bags for transport to shore. On shore, octopuses were removed from bottle dens by draining water from the bottle and waiting for the octopus to voluntarily leave the bottle. Octopuses were immediately placed in 1-L red plastic bottles to minimize stress; these bottles were then stored in a large cooler of seawater aerated by battery-powered bubblers and transported to RBML.

P_{CO₂} Level Selection

At the time of this experiment, summer of 2014 and 2015, open-ocean P_{CO_2} levels were at approximately $360 \mu\text{atm } P_{\text{CO}_2}$, as recorded at station Aloha (Dore et al. 2009). As discussed above, the Salish Sea has persistent hypercapnic conditions often at or near $700 \mu\text{atm } \text{CO}_2$ (Murray et al. 2015) and in the future may reach as high as $1,500 \mu\text{atm } \text{CO}_2$ (Barry et al. 2010; Cai et al. 2011; Melzner et al. 2013; Bianucci et al. 2018). *Octopus rubescens* is common to the intertidal and nearshore subtidal habitats of the west coast of North America (Hochberg 1998). Individuals in most of these areas experience $360 \mu\text{atm } P_{\text{CO}_2}$ as normcapnic (Dore et al. 2009). The selected range of P_{CO_2} allowed us to examine whether Salish Sea populations are impacted by comparatively hypocapnic (normcapnic for open-ocean conspecifics) and predicted hypercapnic levels.

Long-Term Hypercapnia Exposure

Octopuses, all of which were collected in 2014, were individually placed with their 1-L red bottles into 27.5-L enclosures. Octopuses in long-term treatments were fed purple shore crabs (*Hemigrapsus nudus*) ad lib. during acclimation and throughout the experimental period (Onthank 2008; AZA AITAG 2014).

The $1,500\text{-}\mu\text{atm } P_{\text{CO}_2}$ ($n = 5$) and $700\text{-}\mu\text{atm } P_{\text{CO}_2}$ ($n = 5$) treatment systems were supplied with seawater from 415-L temperature-controlled mixing aquaria $11.0^\circ \pm 0.1^\circ\text{C}$. Another 10 octopus enclosures were supplied with unmodified seawater directly from the lab seawater system seawater, which uses seawater pumped from Rosario Bay. Octopuses were acclimated to the enclosures at their native P_{CO_2} , which was approximately $700 \mu\text{atm}$ for 1 wk before the start of treatments. Octopuses that did not begin feeding readily during the acclimation period, defined as eating at least one crab per day, were excluded from the study. This left a total of 10 octopuses in the long-term hypercapnia exposure: four in the $1,500\text{-}\mu\text{atm } P_{\text{CO}_2}$ treatment, two in the $700\text{-}\mu\text{atm } P_{\text{CO}_2}$ treatment, and four in the unmodified flow-

through system (see “Results” for unmodified flow-through system P_{CO_2}). After acclimation, systems were brought to experimental pH stepwise over the course of 24 h. After 5 wk in treatments, all octopuses included in the trials remained in apparent good health, as evidenced by continued healthy appetite and lack of skin lesions that often accompany poor health or senescence.

Mixing aquaria were equipped with a pH-stat system to maintain aquaria at a controlled pH by the addition of CO_2 . The pH-stat system consisted of a Vernier pH glass electrode connected to a laptop through a National Instruments/Vernier SensorDAQ. Custom software opened a solenoid valve on a CO_2 regulator connected to a standard aquarium bubbler airline and bubble stone in the aquarium if the measured pH rose above a specified threshold. Glass pH electrodes were calibrated daily against National Institute of Standards and Technology buffers. While this system allowed us to elevate P_{CO_2} for prolonged periods of time, it was not capable of decreasing P_{CO_2} below local environmental conditions. As a result we were not able to do long-term data collection at $360 \mu\text{atm } P_{\text{CO}_2}$.

Seawater alkalinity of the mixing aquaria and unmodified seawater system outflow was determined daily by open-cell titration (Dickson et al. 2007), and alkalinity values were calculated from titration data using the seacarb package in R (Gattuso et al. 2015). Measured alkalinity was used to calculate target pH to maintain desired P_{CO_2} . The pH of seawater samples was independently measured immediately after collection from aquaria for alkalinity measurements using a SI Analytics glass electrode with a Schott Instruments ProLab 1000 meter. This measurement was used to verify the measurements of the pH-stat system and was also used to calculate P_{CO_2} achieved in the aquaria.

Measurement of Routine Metabolic Rate

Routine metabolic rates (RMRs) were determined for octopuses in long-term treatments after 1 wk and 5 wk in the treatment. After fasting for 24 h, octopuses were placed into 6-L flow-through, water-jacketed respirometers. The same type of pH-stat system used in the experimental aquaria was used to adjust the pH of seawater in a 114-L cooler, which was then pumped into the incurrent port of these respirometers. PyroScience Firesting O_2 flow-through cells (OXFTC2) and robust temperature probes (TSUB36) were placed on the incurrent and excurrent stream of each respirometer. Flow rates through each respirometer were measured at the beginning and end of each respirometry session by timing how long it took the respirometer outflow to fill a 100-mL graduated cylinder. Octopuses were placed into respirometers for 23 h, and aerobic metabolic rates were measured throughout; however, the first 3 h were discarded to remove handling effects on oxygen consumption and washout. After 23 h of respirometry, octopuses were removed, and oxygen consumption was measured in the empty respirometer to determine background oxygen consumption, which was typically about 5% of octopus respiration. After background respiration was measured, inflow and outflow optodes were connected immediately in a series to evaluate drift. No drift was detectable after each of the first seven 23-h respirometry measurements, and measurements were

discontinued thereafter. RMR was calculated from raw oxygen data using the `resp.pyro` function in the `OTools` package in R (<https://github.com/KirtOnthank/OTools>).

Short-Term Hypercapnia Experiment

RMR was measured in octopuses used in short-term-exposure experiments, maintained in the flow-through systems that matched the P_{CO_2} and temperature of the collection location, using a system similar to that described for RMR measurements for long-term-exposure octopuses, with the exception that incurrent water was gas equilibrated in an equilibration column into which O_2 , N_2 , and CO_2 were bubbled using three mass flow controllers. This allowed for the rapid adjustment and precise control of gas parameters of the respirometer. For this study 22 octopuses were held at ambient P_{CO_2} levels of $700 \mu\text{atm}$, and metabolic rate was recorded. Immediately afterward, without removing octopuses from the respirometer, they were then exposed to either 360 (in 2014) or 1,500 (in 2015) μatm P_{CO_2} while oxygen consumption was again measured. These values were selected to match current open-ocean, current, and potential future Salish Sea pH levels (Dore et al. 2009; Barry et al. 2010; Bianucci et al. 2018). RMRs were measured in the same manner as in long-term trials described above.

Measurement of P_{crit}

After 5 wk in long-term-exposure treatments, P_{crit} was determined for octopuses. This included six octopuses that were exposed to the 700–360- μatm short-term treatment and subsequently held in 700- μatm P_{CO_2} for 5 wk along with other long-term octopuses, but RMR was not measured at 1 wk. Following a 23-h RMR measurement, the respirometer was closed by connecting the outflow of the respirometer to the inflow of the pump supplying the respirometer. Oxygen concentration in the respirometer was allowed to fall to at least 3% atmospheric saturation. P_{crit} was determined from aerobic metabolic rate (R) as a function of oxygen pressure (P_{O_2}) by using nonlinear least squares regression as implemented in the `nls` function in R (R Development Core Team 2008) to fit the following modified Weibull function to the measured data (Marshall et al. 2013):

$$R = M_R(1 - e^{-(P_{O_2}/0.59P_{crit})}).$$

Statistical Analysis

The effects of short-term P_{CO_2} exposure on RMR were examined using a repeated-measures linear mixed effect model with mass and P_{CO_2} included as fixed factors and octopus ID as a random factor (R Development Core Team 2008). The effect of long-term P_{CO_2} exposure on RMR was examined using a linear mixed effects model using mass, P_{CO_2} , and weeks in treatment as fixed effects and octopus ID as random effects. Estimated marginal means (covariate-corrected means, in this case, mass) were determined for each P_{CO_2} category using the `emmeans` package in R (Lenth 2018). Differences in P_{crit} by treatment P_{CO_2} were tested using a one-tailed- permutation t -test.

Data Availability

All data underlying figures in this article have been submitted to the Dryad data repository and are publicly available (<https://datadryad.org/stash/share/lmVWP-sb-daJT3yLnLWQBfthAVZDODXXSGNTJyRYDHE>).

Results

Sea surface pH_T was consistently lower than 7.8 throughout the geographic region studied, ranging from 7.658 to 7.773, and water samples from 15-m depth were nearly always lower than corresponding surface measurements, ranging from 7.579 to 7.741 (fig. 1). Estimated P_{CO_2} ranged from 792 to 1,048 μatm , with a mean of 927 μatm in surface water, and from 857 to 1,257 μatm , with a mean of 10,22 μatm at 15-m depth. Surface seawater pH demonstrated spatial autocorrelation to a range of 24.9 km. The pH of seawater sampled at 15 m also showed spatial autocorrelation but a sill was not reached by the maximal pairwise distance (50.9 km).

High- CO_2 long-term treatments had a measured P_{CO_2} of $1,422 \pm 116 \mu\text{atm}$; low- CO_2 long-term treatments had a measured P_{CO_2} of $687 \pm 84 \mu\text{atm}$; long-term flow-through treatments fed directly from the marine lab seawater system had a measured P_{CO_2} of $714 \pm 40 \mu\text{atm}$ (table 1). The P_{CO_2} of the two low- CO_2 treatments were not significantly different (permutation t -test, $B = 10,000$, $P = 0.095$); therefore, the results for these two treatments were pooled for all subsequent analyses.

Short-term exposure to elevated- P_{CO_2} environments impacted RMR of octopuses, with RMRs being significantly higher at 1,500 μatm (estimated marginal mean [EMM] = $3.10 \mu\text{mol } O_2 \text{ g}^{-1} \text{ h}^{-1}$) than at 700 μatm (EMM = $2.03 \mu\text{mol } O_2 \text{ g}^{-1} \text{ h}^{-1}$) or 360 μatm (EMM = $1.64 \mu\text{mol } O_2 \text{ g}^{-1} \text{ h}^{-1}$; fig. 2; analysis of deviance, $\chi^2 = 13.27$, $P = 0.0013$, Tukey post hoc). However, RMR was not significantly different between 700 and 1,500 μatm P_{CO_2} in long-term exposure (fig. 2; analysis of deviance, $\chi^2 = 0.0017$, $P = 0.97$). RMR tended to decrease with acclimation time at both P_{CO_2} levels (fig. 2). P_{crit} was significantly higher in long-term high- CO_2 treatments than in control CO_2 treatments (fig. 3).

Discussion

The Salish Sea is a persistently high- CO_2 environment, both temporally (Murray et al. 2015) and spatially (this study). The geographic pervasiveness of elevated P_{CO_2} we found in the Salish Sea, coupled with previous data on the lasting low pH in the region, provides strong evidence that the low pH experienced by octopuses collected for study was not anomalous. We observed P_{CO_2} over 1,200 μatm at 15 m, demonstrating that experimental P_{CO_2} values of 1,500 μatm are not unreasonable. Long-term monitoring at Friday Harbor Laboratory on San Juan Island between 2011 and 2013 found a pH average near 7.8 and a P_{CO_2} consistently greater than 650 μatm (Murray et al. 2015). We found that mean sea surface pH was 7.71. This creates an ideal natural laboratory to explore the effects of long-term acidification on marine organisms and ecosystems. Our estimated

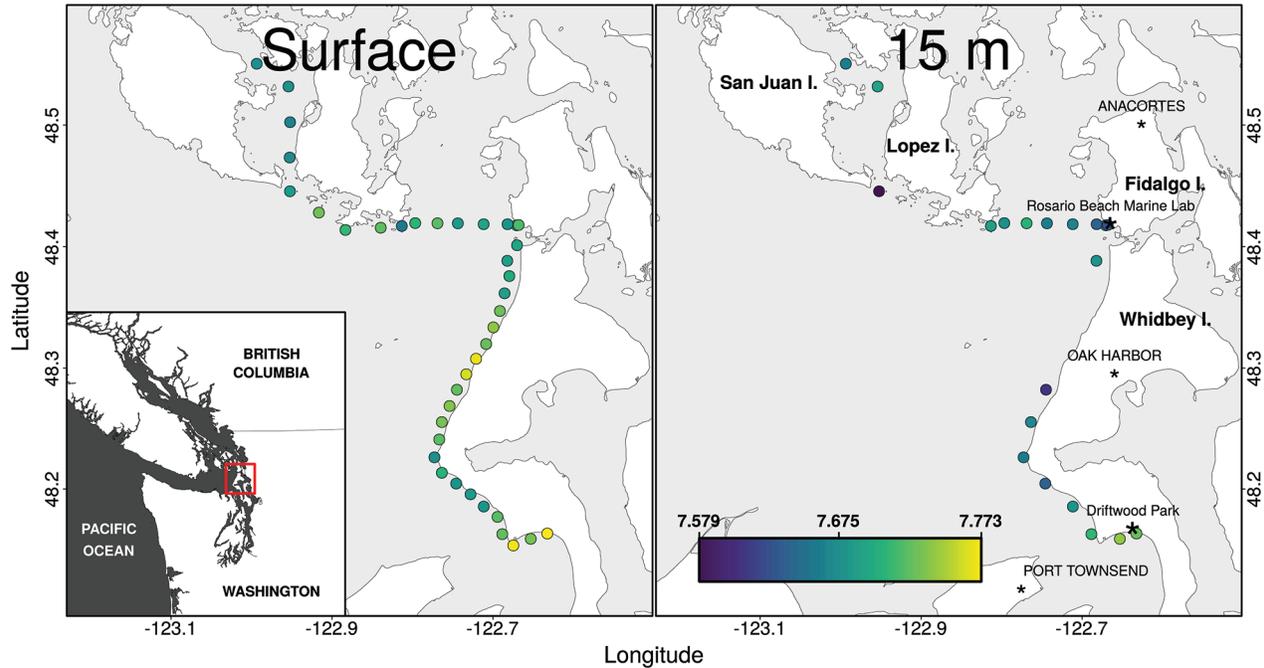


Figure 1. Geographic variation in surface seawater pH near Rosario Beach Marine Lab and Driftwood Park, where octopuses were collected, at the surface (left) and at 15-m depth (right).

mean surface seawater P_{CO_2} of $878 \mu\text{atm}$ exceeds all but the most extreme atmospheric P_{CO_2} projections for the end of this century (IPCC 2013). Mesocosm studies of organismal responses to elevated CO_2 will always lack the acclimation time necessary to replicate that of projected real-world CO_2 changes. This makes persistently elevated CO_2 regions, such as the Salish Sea, a valuable resource to explore the impact that OA may have on organisms after many generations.

Previous work on the impact that hypercapnia has on cephalopods has shown a decrease in RMR and elevation of P_{crit} during short-term exposure at or above $1,000 \mu\text{atm}$ CO_2 (Rosa and Seibel 2010; Hu et al. 2014; Seibel 2016). However, as cephalopods are inherently difficult to work with during long-term studies, currently we are unaware of any studies that examine the effect that both short-term exposure and long-term exposure have on metabolic and respiratory physiology in the same species. Thus, our data set provides a unique view of the effect of long-term exposure to OA on the metabolic physiology of cephalopods. During this study we were able to maintain individuals for 5 wk at 700 and $1,500 \mu\text{atm}$. This duration provided the unique opportunity to see whether the decreased RMR that seems common in short-term exposures (Rosa and Seibel 2008; Rosa et al. 2014) is present or whether there are other

responses and potential for acclimation and restoring RMR to nonhypercapnic rates.

Our data show that hypercapnia has an effect on the RMR of *Octopus rubescens*, but it is dependent on duration of exposure. Unlike a decreased RMR in response to hypercapnia observed in squid (Rosa and Seibel 2008; Rosa et al. 2014), we observed a significant increase in RMR as P_{CO_2} levels increased during short-term exposure. However, following long-term exposure to elevated CO_2 , RMR returned to levels observed in nonhypercapnic and hypocapnic treatments, but P_{crit} was significantly higher. This response in RMR suggests that *O. rubescens* is able to acclimate to elevated CO_2 over time.

The observed increase in RMR may be the result of multiple acute responses to hypercapnia, possibly including both behavioral and physiological strategies. For example, the most adaptive response of octopuses that find themselves in a low-pH environment likely would be to move away from the area of low pH. The increased movement would result in the increased metabolic rate. Alternatively, the apparent increase in short-term metabolic rate at $1,500 \mu\text{atm}$ could, at least in part, be the result of increased activity in energy-dependent ion and acid-base regulation pathways. As with many aquatic metazoans, the primary site of ion regulation in cephalopods resides in the gills

Table 1: Carbonate system parameters measured in the three long-term treatments

Parameter	High CO_2	Low CO_2	Flow-through
pH	$7.51 \pm .04$	$7.81 \pm .05$	$7.77 \pm .02$
Total alkalinity ($\mu\text{mol kg}^{-1}$)	$2,091 \pm 38$	$2,096 \pm 42$	$2,005 \pm 11$
P_{CO_2} (μatm)	$1,422 \pm 116$	687 ± 84	714 ± 40

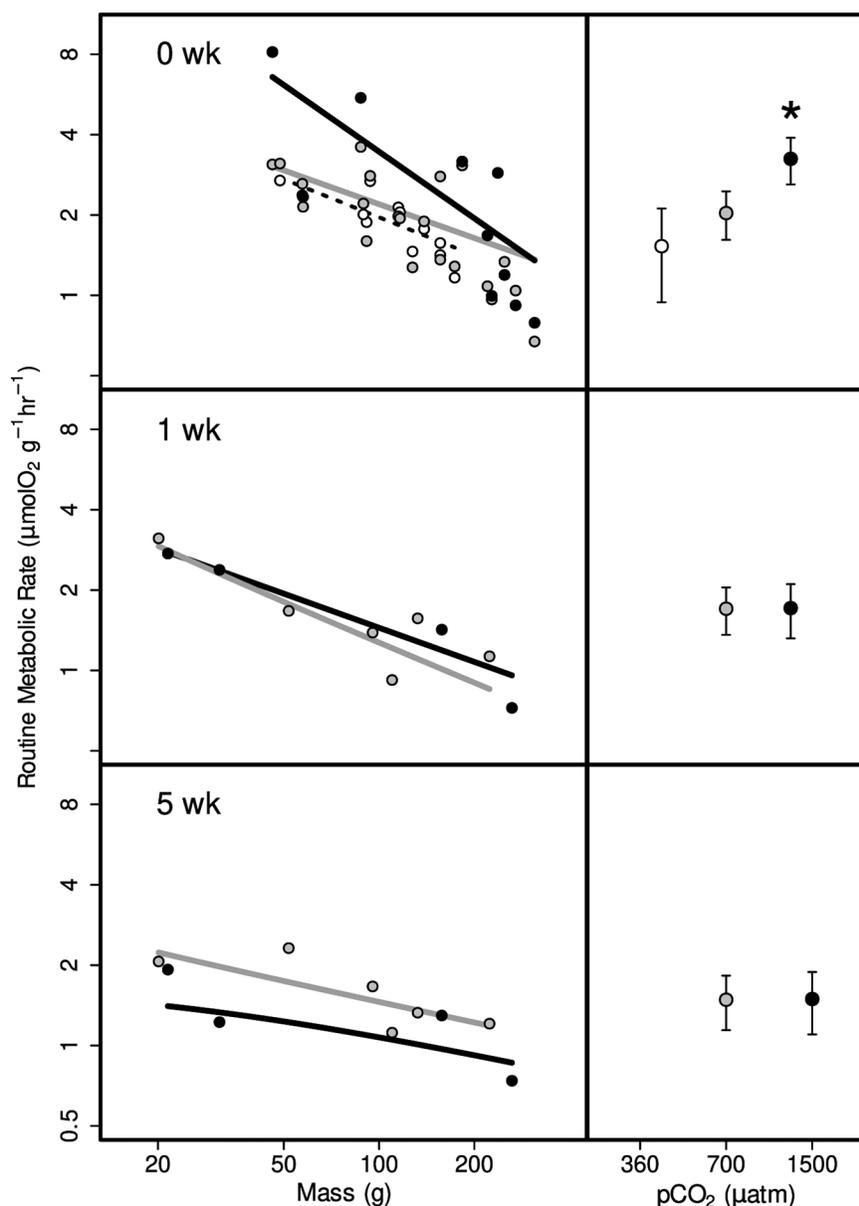


Figure 2. Routine metabolic rates (RMRs) of *Octopus rubescens* at three CO_2 partial pressures (P_{CO_2}): 360 μatm (white circles, $n = 12$), 700 μatm (gray circles, short-term $n = 22$, long-term $n = 6$), and 1,500 μatm (black circles, short-term $n = 10$, long-term $n = 4$), with no prior acclimation (0 wk), 1 wk of acclimation (1 wk), or 5 wk of acclimation (5 wk). Left panels display individual octopus RMR measurements, with nonlinear least squares power regression lines shown (dashed line = 360 μatm ; gray line = 700 μatm ; black line = 1,500 μatm). Right panels display mass-corrected means (ANCOVA estimated marginal means \pm 95% confidence intervals) at each P_{CO_2} . RMRs measured in octopuses with no acclimation are significantly different with respect to P_{CO_2} (ANCOVA, $F = 7.90$, $\text{df} = 2$, $P = 0.0013$), with RMRs at 1,500 μatm greater than at 700 μatm (Tukey post hoc, $t = 3.185$, $P = 0.0076$) and 360 μatm (Tukey post hoc, $t = 3.861$, $P = 0.0013$).

(Schipf et al. 1979; Hu et al. 2015b). Previous work has shown that V-type H^+ -ATPase activity and Na^+/K^+ -ATPase activity increased after a 6-h \sim 1,600- μatm exposure in gill homogenates of squid *Sepioteuthis lessoniana*, along with mRNA transcript abundance of these enzymes (Hu et al. 2014). A small portion of the increased RMR may be the result of upregulation of antioxidant enzymes stimulated by the presence of radical oxygen species resulting from elevated CO_2 , as has been observed in

other species of mollusks (Beniash et al. 2010; Tomanek et al. 2011; Matozzo et al. 2012; Timmins-Schiffman and Roberts 2012; Matoo et al. 2013; Hu et al. 2015a). Additionally, some portion of the increased RMR could be the result of energy expended to produce isozymes of respiratory pigments that are better suited for the environmental pH being experienced, as observed in barnacles (Wong et al. 2011). Further studies would help to clarify which, if any, of these factors are driving the short-term

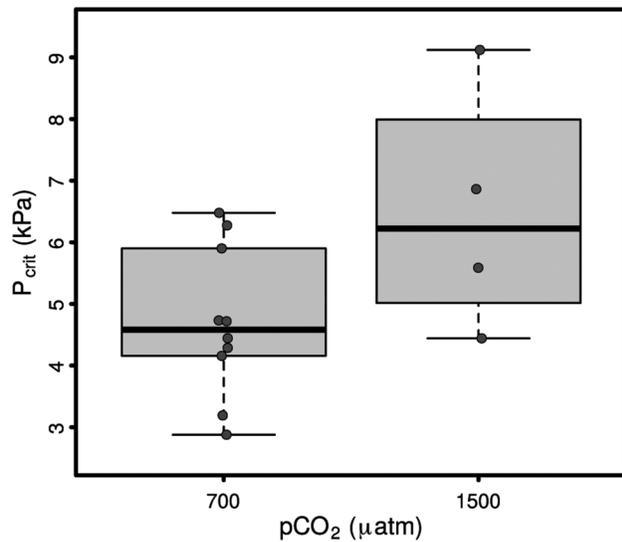


Figure 3. Critical oxygen pressure (P_{crit}) of *Octopus rubescens* after 5 wk of exposure to carbon dioxide partial pressures (P_{CO_2}) of 700 μatm ($n = 12$) and 1,500 μatm ($n = 4$). Critical oxygen pressures are significantly different between P_{CO_2} treatments after long-term exposure (one-tailed permutation t -test, $B = 10,000$, $P = 0.039$).

spike in RMR we observed, and any of these explanations would need to be consistent with the observed increase in P_{crit} after 5 wk of elevated P_{CO_2} exposure.

Octopus rubescens experienced some respiratory impairment at elevated CO_2 , manifesting as increased P_{crit} , which was 2.3 kPa higher for octopuses in 1,500- μatm long-term treatments than 700- μatm treatments. This is similar to the increase of P_{crit} observed in pre-hatchling *Sepia officinalis*, which were 1.1 kPa higher in eggs exposed to a pH 7.5 environment than those exposed to pH 8.0 (Rosa et al. 2013). This increase in P_{crit} suggests some form of oxygen delivery limitation resulting from elevated P_{CO_2} . It is possible that this oxygen delivery impairment is a result of lower hemocyanin oxygen affinity, owing to the exceptionally large Bohr effect of octopus hemocyanins, which exceeds even that of epipelagic squid (Lykkeboe and Johansen 1982; Miller 1985).

Many octopus species, including *Octopus rubescens*, depend on dens as a refuge from predators (Katsanevakis and Verriopoulos 2004). Octopuses will often choose dens with low

water permeability, as evidenced by their frequent use of discarded bottles (Anderson et al. 1999). These dens likely experience decreased oxygen levels while octopuses are in residence. Impaired hypoxia tolerance could reduce the possible dens usable by octopuses to only those well-ventilated dens or force octopuses out of dens entirely. Furthermore, estuaries such as the Salish Sea can frequently experience transient periods of hypoxia (Newton et al. 2007). Impaired hypoxia tolerance in octopuses could reduce octopus survival during such events.

Conclusions

Our data show that the RMR of *O. rubescens* is impacted by elevated CO_2 . The pattern we observe is different from those reported for epipelagic squid and cuttlefish. However, the marked increase during short-term exposure followed by a return to control RMR demonstrates that *O. rubescens* is able to acclimate to hypercapnic environments, even as high as 1,500 μatm . Further research is needed to clarify the mechanism driving the change in RMR. The observed decrease in hypoxia tolerance in long-term hypercapnic conditions suggests that *O. rubescens* is experiencing respiratory limitations.

Our data are the first to examine the response to acidification in octopuses and the first to compare short-term and long-term exposure to hypercapnia. Despite resilience to acidification, in-shore octopuses face both greater CO_2 concentrations (Cai et al. 2011; Melzner et al. 2013) and increasing episodic, acute environmental hypoxia in both open coastal environments (Grantham 2004) and semi-enclosed basins such as the Salish Sea (Newton et al. 2007) compared to open-ocean cephalopods. This makes octopuses from the Salish Sea an excellent model system for further study in how OA may impact cephalopods.

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APPENDIX

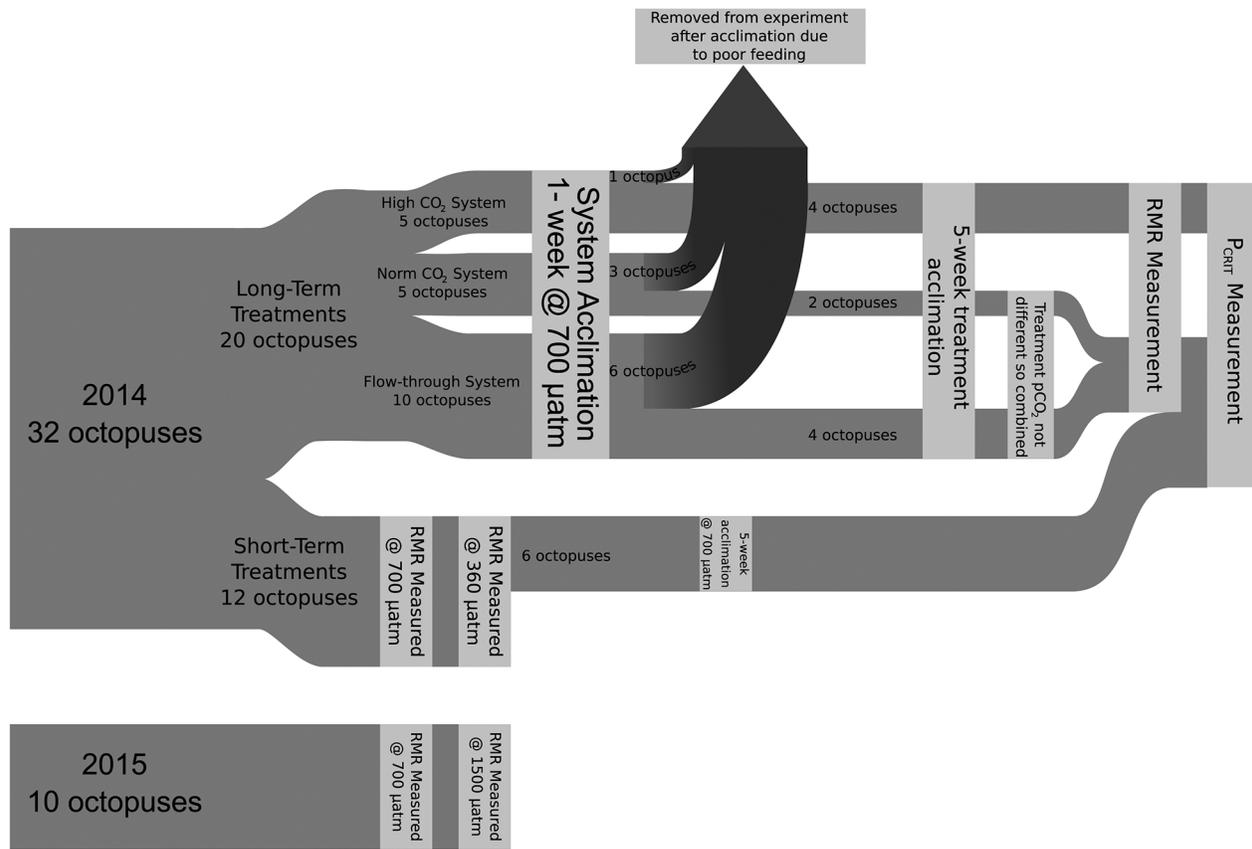


Figure A1. Flow diagram of the number of octopuses used (dark gray branches) and experimental events (light gray vertical bars) in this study. Width of branches is directly proportional to the number of octopuses in each division. A color version of this figure is available online.

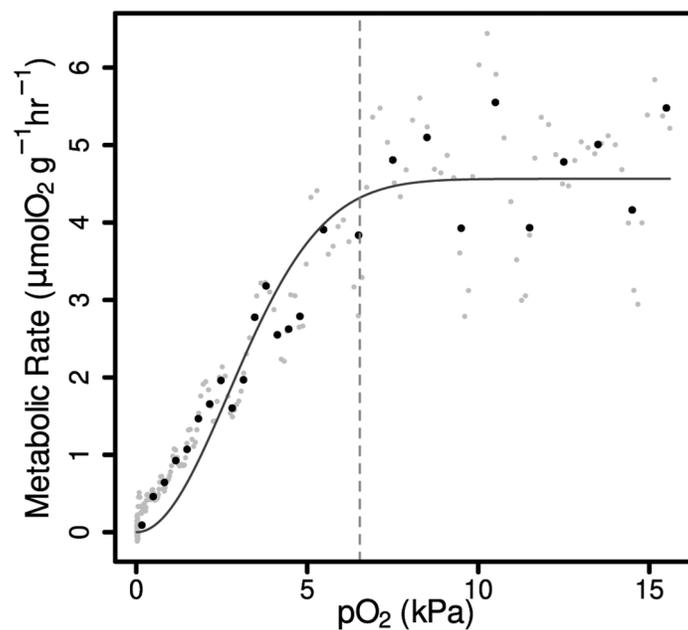


Figure A2. Example metabolic rate data plotted against environmental oxygen pressure (P_{O₂}) for one octopus (octopus 8), demonstrating critical oxygen pressure (P_{O₂}) determination. Small gray dots are raw metabolic rate measurements, while larger black dots are mean metabolic rates by 1-kPa bins. A fitted Weibull function is shown by the solid line, and a critical oxygen pressure estimation is shown by the dashed vertical line. A color version of this figure is available online.

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