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ORIGINAL PAPER

Elevated immune response in *Octopus rubescens* under ocean acidification and warming conditions

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Abstract

To study the effects of ocean warming and acidification on the immune response of octopuses, 24 *Octopus rubescens* were collected from Driftwood Park, Washington (48°09′48.9"N 122°38′14.1"W) April–July 2018. Following 3 weeks in elevated pCO_2, elevated temperature, or the combination thereof, immunological parameters were measured including total hemocyte count, phagocytosis activity, superoxide production, and lysozyme activity. Increased pCO_2 elicited an increase in the number of circulating hemocytes, which are responsible for the cellular immune response, indicating a stress response. As a result, total phagocytosis also increased. This is the first study examining the effects of climate change on the immune system of cephalopods.

Introduction

Atmospheric carbon dioxide levels have been increasing at a rate of approximately 0.5% per year, due primarily to human activity, which is the most rapid change in CO_2 concentrations in the last 650,000 years (Raven et al 2005; Fabry et al 2008). The oceans have absorbed about one-third of the anthropogenic CO_2 released since the Industrial Revolution (Sabine et al 2004), resulting in a 26% increase in ocean acidity, with a further 250% increase in acidity predicted by the end of this century (Pachauri et al 2014).

In addition to the ocean acting as a sink for carbon dioxide in the atmosphere, it also absorbs excess heat from the warming planet, with land and ocean surface temperatures already rising 0.85 °C (Pachauri et al 2014). It is predicted that if there are no additional CO_2 emission mitigation efforts, the coastal sea surface temperature is expected to increase 1–3 °C more than what has already been observed (Parry et al 2007; Pachauri et al 2014). Previous studies have primarily examined either ocean acidification or warming, but more research studying the interactive effects of the two is needed (Gibson et al 2011).

There has been substantial research on the impacts of ocean acidification and ocean warming on calcifying marine organisms (Fabry et al 2008; Hofmann and Schellnhuber 2009; Gibson et al 2011; Dupont and Pörtner 2013) and the metabolic physiology of marine organisms (Fabry et al 2008). While research on changing ocean conditions has increased over the years, research on the effects of these conditions on non-calcification related physiological processes, such as the immune response, are needed. The few studies investigating marine invertebrate's immune response to potentially stressful climate change conditions have focused mostly on ocean acidification effects on bivalves and echinoderms, demonstrating the lack of information on many important marine taxa, such as cephalopods. The studies that do exist suggest that marine invertebrates exhibit variable immune responses to climate change conditions.

This study examines a common octopus species, *Octopus rubescens,* to understand how climate change may impact an under-studied, yet pivotal member of marine ecosystems. Octopuses are a ubiquitous group of marine mollusks found in all depths and in most marine habitats (Mather et al 2013), and they play a key role in marine ecosystems. Octopuses are important prey for many species of seabirds, pinnipeds, cetaceans, and fishes (Boyle and Rodhouse 2005). Octopuses feed on a wide variety of taxa, including fish, bivalves, crustaceans, and gastropods, and use a generalist feeding strategy which optimizes caloric intake (Anderson et al 2008; Onthank and

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Cowles 2011). This prominent role as both predator and prey coupled with very high gross conversion efficiencies near 50% (Van Heukelem 1976; Rigby and Sakurai 2005) mean that octopuses are an indispensable link between trophic levels in many marine ecosystems.

Cephalopods have only an innate immune response with cellular and humoral components. Cellular components include hemocytes, which assist in wound repair, coagulation, phagocytosis, encapsulation, and the production of cytotoxic substances such as reactive oxygen species (ROS) and nitric oxide (NO), a common reactive nitrogen species (RNS) (Malham and Runham 1998; Castellanos-Martínez et al 2014). Phagocytosis by circulating hemocytes is considered the main immune defense mechanism in octopuses (Castellanos-Martínez et al 2014). Elevated hemocyte concentration may be used as an indicator of an immune response and reduced hemocyte count may indicate increased disease susceptibility (Harvell et al 2002), and either an increase or decrease may suggest a stress response (Castellanos-Martínez and Gestal 2013; Gestal and Castellanos-Martínez 2015). The humoral components of the cephalopod immune system consist of opsonins, agglutinins, and lysozyme dissolved in the blood (Malham and Runham 1998; Castellanos-Martínez and Gestal 2013). Environmental factors, such as temperature and water quality, have been shown to both lower the immune response capability of cephalopods (Malham and Runham 1998; Malham et al 2002) and elicit an increased immune response (Locatello et al 2013; Pascual et al 2019). Therefore, studying how changing environmental conditions will impact the immune response of octopuses is important to assess the impact that climate change will have on these animals.

Though there have been many studies on ocean acidification and sea temperature warming effects on marine organisms, very little work has addressed cephalopods (Parker et al 2013) and to date only one paper has investigated octopuses (Onthank et al 2021). Additionally, there have been no studies addressing these effects on the cephalopod immune response. We set out to determine the impacts of ocean acidification, warming ocean temperatures, and the combination of these two stressors on four immune system parameters in *O. rubescens*: hemocyte count, hemocyte phagocytosis activity, plasma lysozyme activity, and hemocyte superoxide anion production. Since stressful environmental conditions may elicit an immune response in octopuses (Locatello et al 2013), we hypothesized an increase in overall immune response due to these potentially stressful environmental conditions, and, therefore, predicted to see an increase in hemocyte count, phagocytosis rate, lysozyme activity, and superoxide production.

Materials and methods

Octopus collection

Octopus rubescens ($n = 24$) were collected by SCUBA from Driftwood Park, Whidbey Island (Island County, Washington 48°09′48.9"N 122°38′14.1"W) and were transported to Rosario Beach Marine Laboratory (Anacortes, Washington). Upon arrival, the sex and mass of each octopus was recorded, and each was put into individual 113.5 L insulated holding tanks in a closed system for holding until ready to begin the experiments. Only octopuses with a mass of 150 g or more were used so that enough blood could be drawn for assays without sacrificing the octopus (Malham et al 1995). By chance, no female octopuses were collected that met this mass requirement, so none were used in this study. Octopuses were fed purple shore crabs (*Hemigrapsis nudus*) ad libitum throughout the experiment.

Holding tank design

Each holding tank was made using an insulated cooler with an overhead window in the lid to allow light into the tank. The tank system included a chiller and venturi injector to maintain the proper temperature and oxygen partial pressure (pO_2). To decrease the pCO_2, each venturi injector was connected to a CO_2 scrubber to supply reduced CO_2 air. Lastly, each aquarium had a slow, constant water exchange system; fresh seawater flowed in at approximately 100 mL min^{-1} from the marine lab flow-through seawater system and drained from an overflow port. This prevented buildup of ammonia or wastes in each tank without having to perform large, full-tank water changes which would interrupt the controlled pH and temperature of the treatments.

Treatments

After a minimum 1 week acclimation period (Onthank et al 2021), each octopus was randomly assigned to one of 4 treatment groups: control temperature/control pCO_2, control temperature/high pCO_2, high temperature/control pCO_2, or high temperature/high pCO_2. The physical arrangement of the tanks within these 4 treatments were interspersed throughout the room to avoid any difference between treatments in light or temperature due to the location of the tanks. The control temperature and pCO_2 were determined by measuring the temperature and pCO_2 of the seawater at the depth the octopuses were caught. An alcohol thermometer was used to measure the water temperature at the collection site at depth, and triplicate water samples were collected at depth using air-tight plastic collection bottles. These samples were

kept on ice and transported back to Rosario Beach Marine Laboratory and within 4 h of collection the pCO_2 of each was determined using the salinity, alkalinity, temperature, and pH of each sample.

The Salish Sea, where the octopuses were collected, is a region of temporally and geographically persistently high seawater pCO_2, with levels commonly exceeding 700 µatm (Murray et al 2015; Onthank et al 2021). The pCO_2 measured at the octopus collection location was 740 µatm and the temperature was 10.1 °C, which in turn were our targeted control conditions. Target treatment pCO_2 was 530 µatm higher than control, and the target treatment temperature was 3.5 °C higher than the control temperature treatment, both based on the RCP8.5 prediction for conditions by 2100 (Riahi et al 2007; Pachauri et al 2014), which is a commonly used scenario which assumes high greenhouse gas emissions and no additional mitigation efforts. Based on observed pCO_2 and temperature at the collection site, these increases yielded treatment targets of 1270 µatm pCO_2 and 13.6 °C.

Temperature and pCO_2 of each tank were controlled with a custom pH-stat system which received input from a three-wire PT-100 temperature probe and a single junction glass pH electrode inserted into the water. Each pH electrode was calibrated daily using TRIS and 2-aminopyridine seawater buffers (Dickson et al 2007), and each temperature probe was calibrated daily using an alcohol thermometer. To decrease the seawater pH, pure gaseous CO_2 was slowly bubbled into the tank when a solenoid valve was actuated by the pH-stat whereas temperature was controlled using a chiller operated in a similar manner.

Blood was collected 20–22 days after experiments commenced, particularly to perform the immunoassays. Previous research has found that routine metabolic rates of *O. rubescens* return to normal after a week in a 1500 µatm pCO_2 treatment, suggesting that acute impacts of elevated pCO_2 have largely abated by that time (Onthank et al 2021). A treatment period of approximately 3 weeks was used because it was considered safe to assume that after that period only chronic effects would be measured. Following a minimum 2 day recovery period (based on our maintenance experience) after the blood collection procedure, the octopuses were released at the original collection site.

Carbonate chemistry measurements

The carbonate chemistry of each tank was measured and controlled using four independent measurements: total pH (pH_T), total alkalinity (A_T), salinity, and temperature. Due to time and staffing constraints, these measurements were taken at irregular intervals throughout the experiment. The slow, constant water exchange built into the system prevented large fluctuations in salinity and alkalinity; consequently we believe that the inconsistency in the measurements did not have a negative effect on the treatment control.

During the experiment, the pH of each tank was independently verified 1–4 times per week using a modified spectrophotometric pH method according to standard operating procedure (SOP) 3b of Dickson et al (2007). Modifications included using a 1 cm pathlength acetate cuvette, drawing the seawater sample and m-cresol purple dye with a regular micropipette tip, not flushing the cuvettes out with the seawater sample for 15–20 s, and using a water bath to warm the samples rather than a thermostated spectrophotometer. This method achieved measurements with 0.004 pH unit accuracy.

The spectrophotometric method for measuring pH_T was calibrated using two samples of seawater of known alkalinity and pCO_2. To make seawater samples of known alkalinity and pCO_2, a certified reference material (CRM #143) (Dickson 2010) was vigorously bubbled with one of two NorLab® certified air mixtures, either 199 ± 2 ppm CO_2 or 1490 ± 15 ppm CO_2. Air mixtures were water-saturated by bubbling through seawater in a sealed Erlenmeyer flask before bubbling into the CRM to avoid evaporation. Using the seacarb package in R (Gattuso et al 2015), the pH_T of these two solutions was calculated using the A_T and pCO_2, which were then used to calibrate the spectrophotometric method for measuring pH_T in this study. The spectrophotometric pH measurement method was used to verify the pH of the seawater buffers used to calibrate the pH probes.

In addition to pH measurements, the total alkalinity (A_T) of each treatment tank was measured approximately weekly using an open-cell titration based on SOP 6b of Dickson et al (2007) with the following modifications. The temperature of the seawater was held at 30 °C (which was an increase from the 25 °C in the protocol) to increase the rate of CO_2 off-gassing. Additionally, the off-gassing period was increased from 6 to 10 min and accomplished by vigorous stirring, but no air was bubbled through the solution as is also done in the published protocol. Alkalinity titrations were verified using the CRM of known alkalinity, which resulted in a measurement accurate to within 60 µmol kg^{-1}. The measured A_T and target treatment pCO_2 were used to calculate pH setpoints, and tank pH setpoints were therefore updated each time A_T was measured in that tank.

The tank temperature was measured using the PT-100 temperature probe which was verified daily using a high precision thermometer. Salinity was measured with a Vernier salinity probe every time alkalinity was measured, and calibrated against a Vernier salinity standard. Lastly, the pCO_2 of each tank was calculated using the tank temperature, pH_T, salinity, and A_T using the seacarb package in R (Gattuso et al 2015).

Blood collection

Following approximately 3 weeks in the designated treatment, each octopus was anesthetized by submersion in 2.5% ethanol in seawater until the octopus was unresponsive (Malham et al 1995). Blood was drawn from the branchial vein through the mantle opening with a 31 G × 0.79 cm hypodermic needle into a sterile 1.0 mL syringe. The target volume of blood was 0.5 mL with the actual amount drawn ranging from 0.1 to 0.6 mL. The octopus was promptly returned to well-oxygenated seawater for recovery with the procedure taking less than 10 min total after sedation.

Following blood extraction, a 20 µL aliquot of the blood was used to perform a hemocyte count (Malham et al 1998b). The remainder of blood was centrifuged at 800 g for 5 min at 4 °C (Malham et al 1998a; Locatello et al 2013). Following centrifugation, the supernatant (cell-free plasma) was removed and stored on ice for the lysozyme assay. The isolated hemocytes in the pellet were washed with octopus Ringer's solution (Malham et al 1998b) and centrifuged at 800 g for 5 min at 4 °C 2 times. Lastly, the octopus Ringer's solution was removed and the hemocytes were resuspended in 1.0 mL Hank's balanced salt solution modified with 3 µg 100 mL^{-1} EGTA (MHBSS) and stored on ice for the phagocytosis and superoxide production assays.

Hemocyte count

Immediately following blood collection, a 20 µL aliquot of the collected blood was diluted with 200 µL of marine anticoagulant (1:10 v:v) and kept on ice to prevent the rapid formation of clumps (morulae) for hemocyte count (Malham et al 1995). A hemocytometer was used to count the concentration of hemocytes mL^{-1} in triplicate within 30 min of extraction. This count was also used to determine the concentration of hemocytes used for the phagocytosis and superoxide production assays.

Phagocytosis assay

Following the phagocytosis assay methods described by Lacoste et al (2002) and Malham et al (2002), a fluoresceinated bacteria solution was prepared by growing *Vibrio anguillarum* at room temperature (approx. 18–20 °C) in Zobell Marine Broth for 3 days. The bacteria were then killed in formalin by mixing 10% formalin 2:1 (v:v) with the bacteria solution and incubating for approximately 3 h. Subsequently, the bacteria were washed with 0.85% saline 3 times, spun at 10 k rpm for 5 min between washes, and diluted in saline so a 1:10 (v:v) dilution had an optical density (OD) of 0.540 at 540 nm. This bacteria-saline slurry was further diluted 1:5 (v:v) in a buffer of 0.375 M sodium bicarbonate and 0.125 M sodium carbonate (carbonate/bicarbonate buffer). The bacteria were labeled with fluorescein 5-isothiocyanate, Isomer I (FITC) as first detailed by Gelfand et al (1976) by mixing this bacteria solution 2:1 (v:v) in a 0.03% solution of FITC. The FITC solution was made by dissolving 2.0 mg of FITC in 2 mL 100% acetone and this solution was added to 4.67 mL of carbonate/bicarbonate buffer.

Following a 2 h and 20 min incubation at room temperature, the fluoresceinated bacteria (FB) were centrifuged at 10 k rpm for 10 min, the supernatant removed, and the FB washed 1:10 (v:v) in gelatin veronal-buffered saline ($GVBS^{2+}$) 3 times. The FB was resuspended in 5 mL of phosphate buffered saline (PBS) and stored at −20 °C.

To perform the phagocytosis assay, 100 µL of hemocytes diluted in MHBSS was placed on a glass slide and incubated for 10 min in a moist incubation chamber. Next, the slide was rinsed with 100 µL MHBSS before 100 µL of thawed FITC-labelled *V. anguillarum* was added to the slide and incubated for 30 min at room temperature. The slide was again rinsed with 100 µL of MHBSS and the unphagocytosed bacteria counterstained with 100 µL of ethidium bromide (50 µg mL^{-1} in PBS) for 2 min. Lastly, the reaction was mounted with Vectashield® Mounting Medium for Fluorescence, sealed with a coverslip and clear nail polish, and stored at 4 °C for later analysis.

These slides were analyzed using a Leica DMIRB fluorescent microscope at 400 × magnification with a 488 nm fluorescent emission filter. A minimum of 200 cells $slide^{-1}$ were photographed and ImageJ was used to determine locations of hemocytes and fluorescence in the photos for each slide. Hemocytes were determined to be phagocytic based on co-localization with fluorescence using R (see code provided in Dryad data repository).

Superoxide production assay

Hemocyte superoxide anion production was measured using a nitroblue tetrazolium (NBT) reduction assay (Malham et al 2002). A 200 µL aliquot of hemocyte solution in MHBSS was added to a 1.5 mL microcentrifuge tube in duplicate or triplicate and 200 µL of NBT solution (Malham et al 2002) was added. In addition to these 2 or 3 reaction tubes, a blank with 200 µL of NBT solution in 200 µL of MHBSS, a negative control with 200 µL of hemocyte solution in 200 µL of MHBSS, and a positive control of 300 units of superoxide dismutase (SOD) with 200 µL of NBT solution diluted in 200 µL of MHBSS were also made.

These tubes were incubated for 1 h at room temperature before centrifuged at 120,000 g for 10 min, the supernatant removed, and the cells washed twice with 200 µL of MHBSS. To each tube 200 µL of 100% methanol (MeOH) was added and vortexed gently before incubating for 10 min at room temperature. The tubes were centrifuged at 300 g,

the supernatant removed, and the cells allowed to air dry. The cells were rinsed 3 times with 200 µL of 50% MeOH, and 240 µL of potassium hydroxide (KOH) after which 280 µL of dimethyl sulfoxide (DMSO) was added and the tubes were heavily vortexed. The supernatant was transferred into 1.5 mL cuvettes and the OD of each sample was measured at 620 nm; and the results reported as OD values $\times 10^6$ cell^{-1} and OD values $\times 10^6$ mL^{-1} blood.

Lysozyme assay

A turbidimetric method was used for measuring lysozyme activity of the cell-free hemolymph isolated from the blood of each octopus (Malham et al 1998a; Locatello et al 2013; Grimaldi et al 2013). Initially, 0.0075 g of lyophilized *Micrococcus lysodeikticus* was dissolved in 10 mL of citrate/phosphate buffer with 25 mM sodium phosphate dibasic dihydrate and 14 mM citric acid. The *M. lysodeikticus* solution and hemolymph were then added to a 1.5 mL cuvette in a 3:1 v:v ratio and the absorbance immediately measured at 450 nm. The absorbance was measured over a 5–10 min period, and it was noted when the absorbance stabilized. The citrate/phosphate buffer was used to blank the spectrophotometer before measuring the change in turbidity due to lysozyme activity. Results are expressed as lysozyme unit's mL^{-1}, where one unit is equal to the amount of lysozyme that causes a 0.001 decrease in absorbance per minute (Locatello et al 2013; Grimaldi et al 2013).

Statistical analysis

All datasets were tested for normality and homoscedasticity within each treatment for each data set using the Shapiro–Wilk test and Bartlett test, respectively. A two-way analysis of variance (ANOVA) was then performed on each data set and a Tukey post hoc test was used when the ANOVA results were significant. To control for type I error resulting from multiple comparisons, *p* values were corrected using the Benjamini–Hochberg FDR correction as implemented in the p.adjust function in R (Benjamini and Hochberg 1995; R Core Team 2008). All analyses were performed using the statistical program R.

All data and analyses used in this study can be found on the Dryad data repository at https://datadryad.org/stash/share/4Hhumbv2fnMUkWh7vDgmcZXmVLRDuduqKHEWFcX1ox0.

Results

Carbonate chemistry

The carbonate chemistry of each treatment was controlled using a custom pH-stat system which bubbled pure CO_2 into insulated aquaria. The target ambient pCO_2 and temperature were 740 µatm and 10.1 °C, respectively, and the achieved ambient pCO_2 and temperature were 880 ± 50 µatm and 10.5 ± 0.5 °C (Table 1). The target experimental pCO_2 and temperature were 1270 µatm and 13.6 °C, respectively, and the achieved experimental pCO_2 and temperature were 1345 ± 100 µatm and 13.2 ± 0.2 °C (Table 1).

Immunoassays

After 3 weeks in either elevated CO_2, temperature, or both, the total hemocyte count (THC), phagocytosis activity, hemocyte superoxide production, and blood plasma lysozyme activity of each octopus was measured. There was an effect of CO_2 on the hemocyte concentration (two-way ANOVA, $F_{(1,20)} = 10.223$, $p = 0.041$, Table 2, Fig. 1A) and total phagocytosis per volume blood (two-way ANOVA, $F_{(1,20)} = 12.296$, $p = 0.040$, Table 2, Fig. 1B), with no effect of temperature or significant interaction. There was no effect of CO_2, temperature, or the combined effects on the proportion of phagocytic cells (Table 2, Fig. 1C), production of superoxide anion per cell (Table 2, Fig. 1D), production of superoxide anion per volume blood (Table 2, Fig. 1E), or lysozyme activity (Table 2).

It should be noted that the lysozyme concentrations present in *O. rubescens* blood plasma appear below the detectable limit of 3.2 ng mL^{-1} using the turbidimetric method, determined by generating a standard curve using egg white lysozyme. Therefore, the blood plasma lysozyme values are indistinguishable from zero and are not included in any further analyses. These results were unexpected since similar studies detected measurable amounts of lysozyme activity in octopus blood plasma (Locatello et al 2013; Barragán-Méndez et al 2019). However, Malham et al (1998b) suggests

Table 1 Seawater carbonate chemistry values for the four treatments, displayed as mean ± sd ($n = 6$ per treatment)

Treatment	T (℃)	S	pH_T	A_T (ppm)	pCO_2 (µatm)
Control	10.5 ± 0.4	30.1 ± 0.4	7.709 ± 0.019	2070 ± 14	872 ± 42
Acidified	10.6 ± 0.6	30.2 ± 0.4	7.524 ± 0.020	2081 ± 27	1380 ± 74
Warming	13.2 ± 0.2	30.3 ± 0.4	7.708 ± 0.027	2077 ± 21	885 ± 63
Acidified + warming	13.3 ± 0.2	30.3 ± 0.4	7.551 ± 0.038	2078 ± 20	1310 ± 119

Measured values: temperature (T), salinity (S), pH_T, alkalinity (A_T), and pCO_2

Table 2 Two-way ANOVA results of the effects of pCO_2 and temperature on immunological parameters measured in blood drawn from *O. rubescens* after 3 weeks in treatment ($n = 5$–6 per treatment). Significant values shown in bold (Adj. $p < 0.05$)

Measurement	Factor	*F* value	*P* value	Adj. *p* value	Tukey's HSD
Hemocyte concentration	pCO_2	10.223	0.004	**0.041**	HCLT > LCLT
	Temp	0.473	0.500	0.720	
	pCO_2*Temp	0.720	0.406	0.687	
Total phagocytosis per vol. blood	pCO_2	12.296	0.002	**0.040**	HCLT > LCLT & LCHT
	Temp	0.679	0.420	0.687	
	pCO_2*Temp	1.272	0.273	0.687	
Proportion of phagocytic cells	pCO_2	0.935	0.345	0.687	
	Temp	0.027	0.871	0.998	
	pCO_2*Temp	1.153	0.296	0.687	
Superoxide production per cell	pCO_2	0.000	0.998	0.998	
	Temp	0.001	0.979	0.998	
	pCO_2*Temp	1.784	0.197	0.687	
Superoxide production per Vol. blood	pCO_2	2.990	0.100	0.600	
	Temp	0.105	0.749	0.963	
	pCO_2*Temp	0.430	0.520	0.720	
Lysozyme activity	pCO_2	0.001	0.982	0.998	
	Temp	1.275	0.274	0.687	
	pCO_2*Temp	1.107	0.307	0.687	

LC ambient pCO_2 treatment, *HC* high pCO_2 treatment, *LT* ambient temperature treatment; *HT* high-temperature treatment

that lysozyme may be present in the blood plasma but only when released by the hemocytes, where it originates. Therefore, it is conceivable that there may have been no circulating lysozyme in the blood plasma when the hemocytes were separated in this study.

Discussion

This study represents the first examination of cephalopod immune response, or immune response of any non-calcifying mollusk, to ocean acidification or warming. After a 3 week exposure to elevated pCO_2 and/or temperature we found a significant increase in blood hemocyte count and total phagocytosis in response to elevated CO_2, but no difference in those parameters in response to temperature, and no difference in superoxide production or proportion of phagocytic cells for either treatment or the combination of treatments. These results indicate that elevated CO_2 may elicit a limited but important increase in immune response in *O. rubescens.*

Environmental changes may alter the immune response of invertebrates (Mydlarz et al 2006), and there is a strong link between an organism's stress response and immunity (Malham et al 2003). In octopuses, hemocytes are known to increase in response to infection (Bullock et al 1987), as they do with many other mollusks (Da Silva et al 2008). Increased circulating hemocyte counts in octopuses have also been associated with acute stresses such as 5 min removal from water (Malham et al 2002) or intramuscular injection of titanium dioxide nanoparticles (Grimaldi et al 2013), and also chronic stresses such as once daily blood draws for 10 days (Malham et al 1998a). Hemocytes appear to be significantly involved in wound healing in cephalopods as well (Féral 1988; Imperadore et al 2017).

Previous work has observed an elevated routine metabolic rate (RMR) in *O. rubescens* after short-term exposure to elevated pCO_2, but RMR returned to control levels after 1 week of continuous exposure (Onthank et al 2021), indicating that acute stress responses do not persist for the 3 weeks these trials lasted. This same study, however, also found that hypoxia tolerance was impaired after 5 weeks of exposure, suggesting that subtler chronic stress effects are still present. Elevated hemocyte count in high pCO_2 treatment individuals after 3 weeks suggests that while these elevated CO_2 levels do not result in a detectable increase in RMR, there is nevertheless a sufficient physiological challenge to elicit an elevated immune response. The presence of a chronic, low-level stress response hint at long-term physiological consequences for cephalopods of persistently elevated pCO_2 that have not yet been measured.

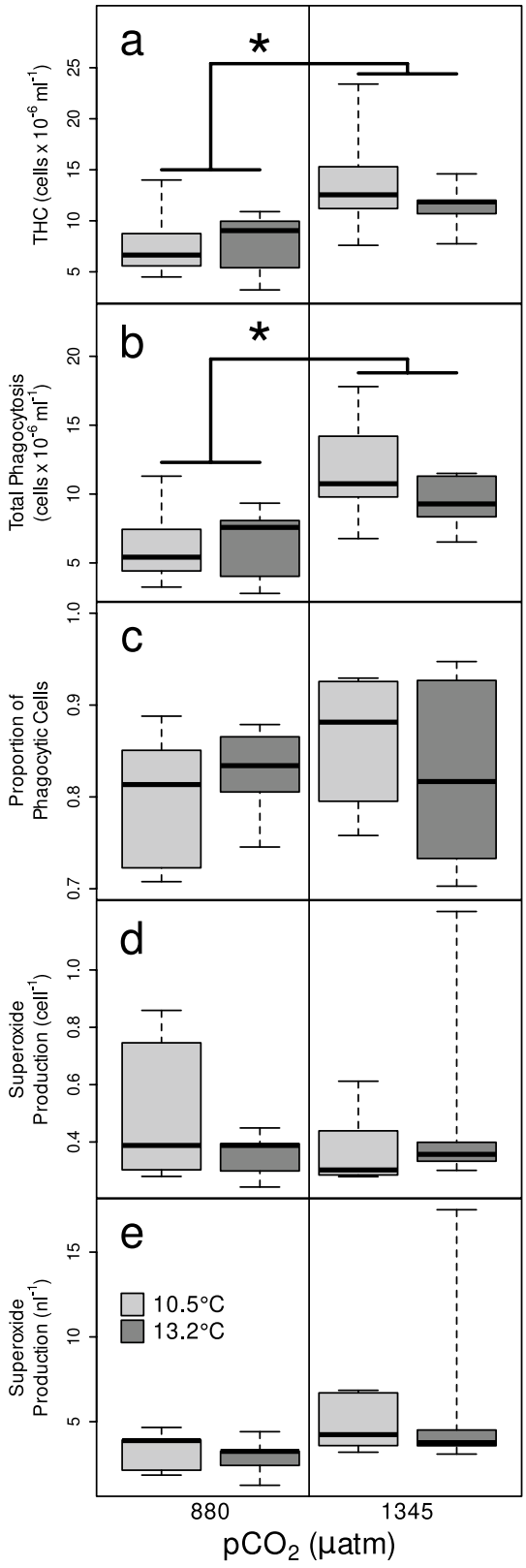

Fig. 1 Immune system parameters measured in *O. rubescens* following exposure to different pCO_2 and temperature treatments for approximately 3 weeks ($n = 5$–6 per treatment). **a** Total hemocyte concentration (THC) in blood. **b** Total phagocytic hemocyte cells per volume in blood. **c** Proportion of phagocytic hemocyte cells in blood. **d** Production of superoxide anion per cell in blood. **e** Production of superoxide anion per volume in blood. *P* values adjusted using the Benjamini–Hochberg correction. Significant differences between treatments denoted with a star (*)

The stress response is widely conserved between taxa and may significantly contribute to adaptation (Ottaviani and Franceschi 1996). It is estimated that near-future climate conditions may result in the extinction of 20–30% of all species worldwide (Lovejoy 2006; Parry et al 2007). While our results suggest that *O. rubescens* may be susceptible to ocean acidification, the recent proliferation of cephalopods worldwide indicates that they may, in fact, be net beneficiaries of changing climate conditions thus far (Doubleday et al 2016). However, more studies on octopus physiology, especially longer term and throughout different life stages, are necessary to better understand how this important ecological group will respond to future ocean conditions.

This research is the first study on the effects of global climate change on the cephalopod immune response. The literature on the impacts of acidification and warming on the immune responses of marine mollusks shows no clear, consistent trends. Our results indicate that *O. rubescens* exhibits an increase in THC and phagocytosis, but with no measurable change in proportion of phagocytic cells or superoxide production when exposed to increased pCO_2. In contrast, some studies found decreases in THC and phagocytosis when exposed to decreased pH (Liu et al 2016; Wu et al 2016; Huang et al 2016), while others measured no change in these parameters (Bibby et al 2008; Asplund et al 2014; Ivanina et al 2014, 2016; Mackenzie et al 2014).

In addition, we found no significant effect of temperature on the immune parameters measured in *O. rubescens*. Several other studies have found significant effects of temperature on marine mollusk immune response, with some measuring a decreased immune response (Wu et al 2016; Pascual et al 2019), one finding an increased immune response (Liu et al 2004), and some finding both increases and decreases in various immunological parameters (Monari et al 2007; Matozzo et al 2012; Mackenzie et al 2014). This diversity in immune responses of marine invertebrates to rising sea temperatures and ocean acidification demonstrates the importance of new data, such as ours on *O. rubescens*, to this growing body of knowledge.

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Author's contributions Conceptualization: MECJ and KLO; methodology: MECJ; formal analysis and investigation: MECJ and KLO; writing—original draft preparation: MECJ; writing—review and editing: MECJ and KLO; funding acquisition: KLO; resources: KLO; supervision: KLO.

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Data availability All datasets analyzed by the authors are available on Dryad. https://datadryad.org/stash/share/4Hhumbv2fnMUkWh7vDgmcZXmVLRDuduqKHEWFcX1ox0

Code availability All codes used for analyses are also available on Dryad. https://datadryad.org/stash/share/4Hhumbv2fnMUkWh7vDgmcZXmVLRDuduqKHEWFcX1ox0

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Organism collections for this project were permitted under a Washington Department of Fish & Wildlife collection permit (ONTHANK 18-150). All applicable laws concerning the ethical use of animals for scientific research were followed during the course of this study.

Informed consent No human subjects were used in this study and informed consent was, therefore, not applicable.No human subjects were used in this study and informed consent was, therefore, not applicable.

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