TEMPERATURE AND OCEAN ACIDIFICATION EFFECTS ON THE IMMUNE SYSTEM OF *OCTOPUS RUBESCENS*

by

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ABSTRACT

As the climate changes, due primarily to considerable carbon dioxide emissions, oceans absorb the excess heat and carbon dioxide, resulting in a global ocean warming and acidification. Research has found that calcifying organisms are strongly impacted by these conditions, and many marine invertebrates may have impaired acid-base regulation. Studies on how climate change conditions will affect other physiological processes, such as immune response, have been insufficient, however. Additionally, with most of the studies focusing on calcifying organisms, many key taxa, including cephalopods, have largely gone overlooked. To therefore study the effects of climate change conditions on the immune response of a common octopus species, I measured four immunological parameters in *Octopus rubescens* following three weeks in treatments. These treatments involved a combination of control and increased pCO_2 and control and increased temperature, according to end-of-century predictions. I measured the difference in hemocyte count, phagocytosis, superoxide production, and lysozyme activity between the four treatments. Results indicate that increased pCO_2 may elicit a stress response in *O. rubescens*, evidenced by an increased number of circulating hemocytes, which are responsible for the cellular immune response. As a result, total phagocytosis is also increased. These results correspond with some similar studies on marine invertebrates, and differ from others, suggesting the need for more research into the immune response of various organisms to climate change conditions. Additionally, research indicates that cephalopods are among the most adaptable group of marine animals, suggesting that *O. rubescens* may adapt to these conditions and thrive in a future climate.

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INTRODUCTION

Introduction to Climate Change

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 (Fabry et al., 2008; Royal Society, 2005). The oceans have absorbed about one third of the anthropogenic CO_2 released since the Industrial Revolution (Sabine, 2004), resulting in a 26% increase in ocean acidity (IPCC, 2014).

As the oceans absorb carbon dioxide, the CO_2 reacts with water to form carbonic acid (H_2CO_3), which dissociates into a bicarbonate ion (HCO_3^-) and a proton (H^+), thus decreasing ocean pH. There has already been a drop in ocean pH by greater than 0.1 pH units (Orr et al., 2005; Pelejero, Calvo, & Hoegh-Guldberg, 2010; Royal Society, 2005), and we may see an additional doubling of current ocean acidity by 2100 (Dupont & Pörtner, 2013). This increase in acidity would result in a drop in pH of up to 0.32 pH units (IPCC, 2014), possibly reaching the lowest ocean pH in over 40 million years (Pelejero et al., 2010). If continued, the global average ocean pH is predicted to drop from 8.15 (pre-industrial) to 7.45 by 2200, a total decline of 0.7 pH units (Hofmann & Schellnhuber, 2009).

In addition to the ocean acting as a sink for carbon dioxide in the atmosphere, it also absorbs excess heat from the warming planet. Scientists have already recorded an average increase in land and ocean surface temperature of 0.85 °C (IPCC, 2014). It is predicted that if there are not additional emission mitigation efforts, the global average surface temperature will increase by 3.7-4.8 °C by 2100 (IPCC, 2014), with the coastal

sea surface temperature expected to increase 1-3 °C more than what has already been observed, increasing up to 4.8 °C above pre-industrial average ocean temperatures (IPCC, 2014; Parry et al., 2007).

Responses of Marine Organisms to Climate Change

There has been substantial research on ocean acidification and ocean warming over the last couple decades, most of which has focused on calcifying animals, which may be the most susceptible because decreased carbonate ion availability may impair skeletogenesis (Dupont & Pörtner, 2013; Fabry et al., 2008; Gibson et al., 2011; Hofmann & Schellnhuber, 2009). There has also been considerable research on the impacts of changing ocean conditions on the metabolic physiology of marine organisms, as another concern with ocean acidification is an organism's ability to maintain its acid-base regulation (Fabry et al., 2008). As environmental acidity increases, CO_2 diffuses into intra- and extracellular compartments of marine organisms, resulting in hemolymph acidosis. The natural buffering capabilities of the organism may be unable to compensate for these levels of pH reduction, changing their metabolic rate and negatively adjusting their energy budget (Gazeau et al., 2013).

Most studies have looked at either ocean acidification or warming, but more research studying the interactive effects of the two is needed (Gibson et al., 2011). Additionally, while research on changing ocean conditions has increased over the years, research on the effects of these conditions on other physiological processes, such as the immune response, should become more prominent.

Few studies have looked at marine invertebrates' immune response to potentially stressful climate change conditions, and those that have focused mostly on ocean

acidification effects on bivalves or echinoderms. Results of these few studies showed mixed results, with some indicating that ocean acidification and/or ocean warming may suppress immune function, such as through reduced hemocyte counts and phagocytic abilities (Bibby et al., 2008; Brothers et al., 2016; Hernroth et al., 2011, 2012; Mackenzie et al., 2014). Others conclude that ocean acidification or warming conditions elicit an immune response via an increase in phagocytic ability, ROS production, or hemocyte count (Dupont & Thorndyke, 2012; Li et al., 2015; Mackenzie et al., 2014; Truscott & White, 1990). These few studies represent a substantial amount of the published research investigating invertebrate immune responses to changing ocean conditions, revealing a lack of information on many important marine taxa, such as cephalopods.

Cephalopod Immune System

Cephalopods, similar to all invertebrates, have only an innate immune response and no immunological memory. Common pathogens in cephalopods, such as octopuses, include bacterial, protozoan, cestode, trematode, nematode, dicyemid, and crustacean infections (Castellanos-Martínez & Gestal, 2013). They respond to these pathogenic agents through cellular and humoral components of their innate immune response.

Cellular components of the octopus immune response include hemocytes, also called leukocytes, 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) (Castellanos-Martínez et al., 2014; Malham & Runham, 1998). The production of ROS includes superoxide anion as the initial metabolite of these oxidative chemicals (Castellanos-Martínez & Gestal,

2013). It has been reported that this intracellular superoxide may be produced in response to stress (Malham et al., 2002).

In addition to internal defense mechanisms, hemocytes also assist in tissue damage repair, nutrient transport, and digestion (Castellanos-Martínez et al., 2014; Castellanos-Martínez & Gestal, 2013). Being vital components of the immune system and general welfare of octopuses, hemocyte concentration may be used as an indicator of the immune response capability of an octopus, as well as a biomarker of the animal's general health, where reduced hemocyte count may increase disease susceptibility (Harvell et al., 2002), and both a decrease and an increase in hemocyte count may suggest a stress response (Castellanos-Martínez & Gestal, 2013; Gestal & Castellanos-Martínez, 2015). Furthermore, phagocytosis by circulating hemocytes is considered the main internal defense mechanism in octopuses, and phagocytic activity may also be used to measure health and welfare (Castellanos-Martínez et al., 2014).

The humoral components of the cephalopod immune system consist of opsonins, agglutinins, and lysozyme dissolved in the blood (Castellanos-Martínez & Gestal, 2013; Malham & Runham, 1998). Lysozyme is present both in the blood plasma and the hemocytes of octopuses, and they seem to react non-specifically to a variety of foreign microbial substances (Malham, Runham, & Secombes, 1998). Environmental factors, such as temperature and water quality, may either lower the immune response capability of cephalopods (Malham & Runham, 1998; Malham et al., 2002) or elicit an immune response (Locatello et al., 2013). Therefore, studying how changing environmental conditions will impact the immune response of octopuses is an important factor in assessing the risks that climate change will have on these animals.

Objectives and Hypotheses

Though there has been a sharp increase in studies on ocean acidification and sea temperature warming effects on marine organisms, research is lacking for octopuses. In 2013, a review article was published on the effects of ocean acidification on mollusks, and they discovered that only about 4% of ocean acidification research on mollusks involves cephalopods, and of those studies, all were conducted with squid and cuttlefish (Parker et al., 2013). Not only are there few studies on changing ocean conditions and cephalopods, but there are no studies addressing the effects on the immune response to these future conditions. Therefore, the goal of my thesis research was to determine the impacts of ocean acidification, warming ocean temperatures, and the combination of these two stressors on four immune responses in *Octopus rubescens* (Berry, 1953).

The four immunological parameters I measured include changes in hemocyte count, hemocyte phagocytosis activity, hemolymph lysozyme activity, and hemocyte superoxide anion production. Since stressful environmental conditions may elicit an immune response in octopuses (Locatello et al., 2013), I expected to see an increase in hemocyte count in the three experimental treatment groups, similar to how Stumpf & Gilbertson (1978) discovered a significant increase in hemocyte count with increasing temperatures in a species of freshwater snail and how Malham et al. (1998) measured a significant increase in octopus hemocyte count when exposed to pathogenic bacteria. Additionally, I predicted that phagocytosis activity would significantly increase in response to the stressors, as was found in the common octopus when exposed to air for five minutes to induce stress (Malham et al., 2002).

Similar to how two recent studies found a significant increase in lysozyme activity in the cell-free hemolymph of *O. vulgaris* in response to stress (Grimaldi et al., 2013; Locatello et al., 2013), I hypothesized a similar increase in the activity of hemolymph lysozymes in the experimental groups. Lastly, one study observed that when the lesser octopus was exposed to a stressful out-of-water situation, there was a significant increase in production of intracellular superoxide anion (Malham et al., 2002). Another study on squid hatchlings found that increased temperatures increased superoxide production (Rosa et al., 2012) and I expected to find similar results.

METHODS

Octopus collection

I collected 24 *Octopus rubescens* by SCUBA from Driftwood Park, Whidbey Island (Island County, WA). Octopuses were found in discarded glass bottles on the ocean floor, and the bottles were brought to the surface in sealable, plastic storage bags. To ensure adequate volumes of blood could be drawn for immune assays, I only kept octopuses of an estimated mass of 150 grams or more (Malham, Secombes, & Runham, 1995). I then transferred the octopuses estimated to be of adequate mass from the glass bottle to a red plastic bottle for transporting, and returned those that appeared to be under 150 grams. I transported the octopuses to Rosario Beach Marine Laboratory (Anacortes, WA) in individual bottles covered in mesh inside a large cooler of seawater with constant bubbling (Malham et al., 1998).

Upon arrival, I recorded the sex and mass of each octopus and put them in individual 113.5 L coolers in a closed system or 27.5 L individual enclosures in an open system for holding until ready to begin the experiments. Due to the mass requirements, only male octopuses were large enough to use, so no females were used in this study. Octopuses were fed purple shore crabs (*Hemigrapsis nudus*) *ad libitum* throughout the experiment.

Tank Design

Each tank was made using a 113.5 L insulated cooler. I removed the hinges and added a handle to the shorter end of the cooler lid. To allow light into the tank and easy viewing of the tank's inhabitant without needing to open the tank and disturb the animal,

I cut a 20.3 cm x 45.7 cm rectangular hole in the top of the lid and glued a 2.4 mm acrylic pane over the cutout to make a window and sealed the exposed foam with silicone.

To keep the water chilled and aerated, I put an inflow and an outflow on the tank. The outflow, which was located at the bottom at one end of the tank, was connected to a Pondmaster Magnetic Drive Model 5 (31.5 L/min) or Model 7 (44.2 L/min) water pump with 1.9 cm inner diameter (ID) vinyl tubing. The water was then pumped into a 75 watts Hydrofarm Active Aqua Chiller, through a venturi injector to introduce air into the water, and back into the tank through an inflow placed near the top on the opposite end of the tank (Fig 1). This design allowed for continuous water flow through the chiller and aerator, maintaining the tank at a constant temperature and oxygen partial pressure (pO_2).

The seawater intake system at Rosario Beach Marine Laboratory (RBML) pumps water in from Rosario Bay, which has a lower pH than Driftwood Park where the octopus were collected. For this reason, I raised the pH of the seawater used in treatments by attaching an EcoPlus Eco Air 7 (225 L/min) air pump to a Bulk Reef Supply (BRS) CO_2 scrubber filled with soda lime beads (BRS Color Changing Medical Grade CO_2 Absorbent). This CO_2-scrubbed air was then pumped into a manifold, where 0.48 cm ID silicone tubing connected to the venturi injector on each tank allowed for CO_2-scrubbed air to be introduced into tank circulation.

Each tank also had a slow, constant water exchange system. Water flowed in at approximately 100 mL/min from the seawater system through 1.0 cm ID vinyl tubing and drained at the same rate from an overflow port, maintaining a constant inflow and outflow of water. This prevented buildup of ammonia or wastes in each tank without

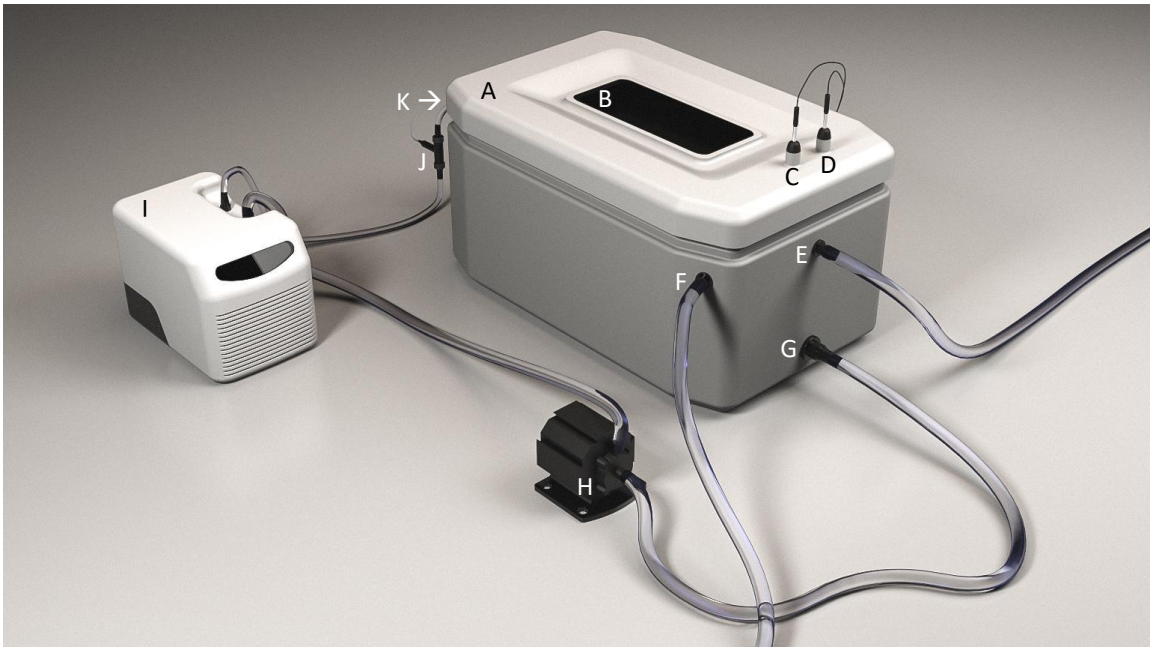


Figure 1. Model of the custom designed tank system used in this study. Modeled and rendered by Jon Spracklen. A) Holding tank for octopus; B) Animal viewing window; C) Single-junction pH probe connected to the custom control hardware; D) PT-100 temperature probe connected to the custom control hardware; E) Overflow water drained to ocean at ~100 mL/min; F) Inflow of seawater from Rosario Bay at ~100 mL/min; G) Outflow of water from tank in closed system; H) Water pump; I) Chiller; J) Venturi injector introduced CO_2-scrubbed air into circulation; K) Inflow of water into tank in closed system.

having to do large, full-tank water changes which would interrupt the controlled pH and temperature of the treatments.

Control System

Each tank was controlled with custom tank control hardware, which received input from a three-wire PT-100 temperature probe and a single junction glass pH electrode inserted into the water through holes drilled in the cooler lids. 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 CO_2 was slowly bubbled into the tank when signaled by the control system. To regulate the temperature, the chiller also received signals from the control system to turn on or off. I manually entered the setpoints for both the temperature and pH daily, and the control hardware adjusted the chiller and CO_2 injection as needed, respectively.

Carbonate Chemistry Measurements

The carbonate chemistry of each tank was measured and controlled using four independent measurements, including total pH (pH_T), total alkalinity (A_T), salinity, and temperature. To ensure these measurements were accurate, I compared each method of measurement to a reference material, as described below (Fig 2).

During the experiment, I measured the pH of each tank one to four times per week using a modified spectrophotometric pH method according to standard operating procedure (SOP) 3b (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

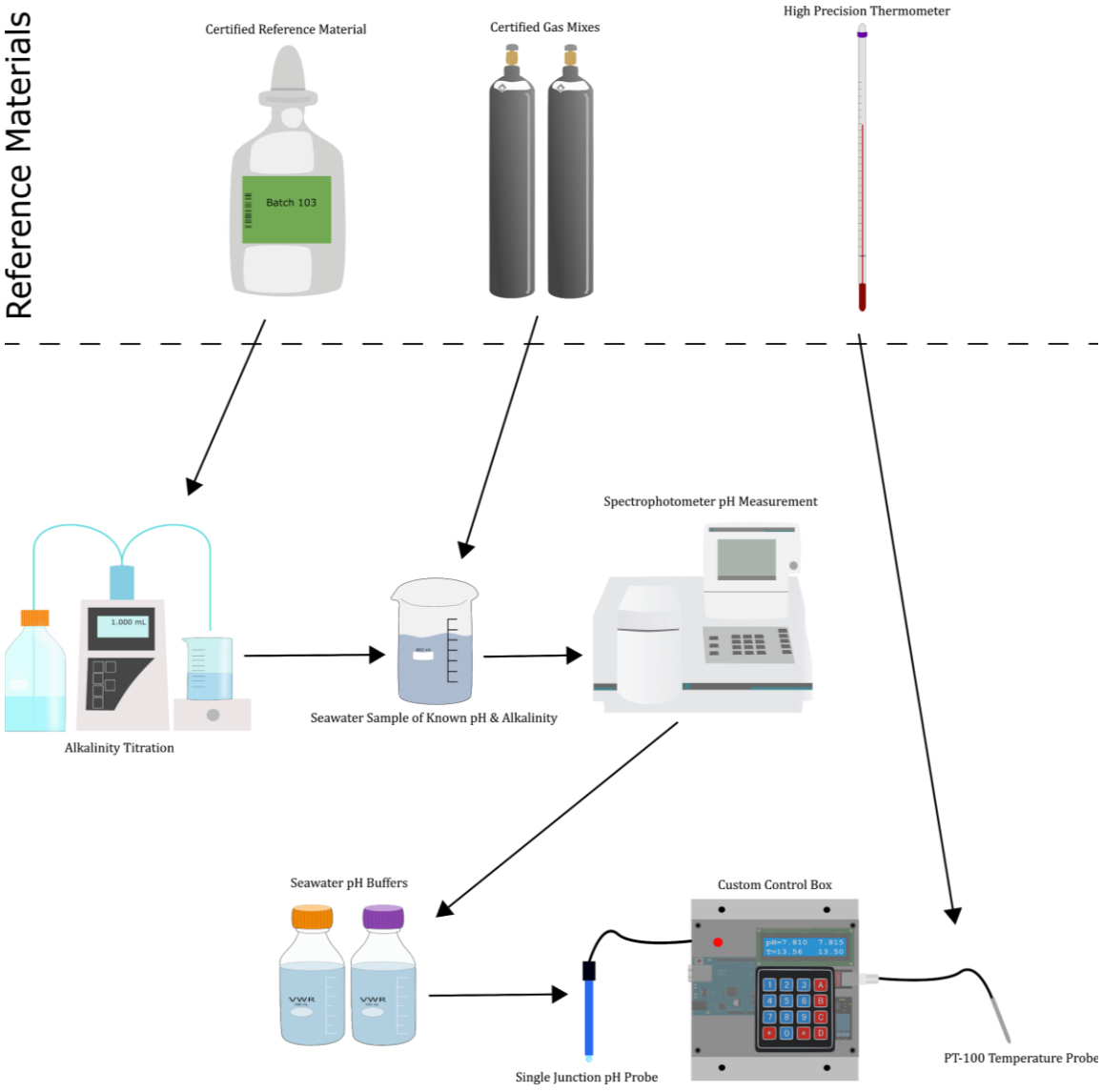


Figure 2. Flow chart of calibration protocol used in this study.

seconds, and using a water bath to warm the samples rather than a thermostated spectrophotometer. This method achieved measurements with 0.004 pH unit accuracy.

I measured pH on the total scale (pH_T), which is the standard pH scale used for ocean acidification research (Dickson, 2010). This scale accounts for the high ionic strength of seawater, which the NIST or NBS scales do not address. Additionally, it is used instead of the seawater (pH_{SWS}) or free (pH_F) scales because it considers the effects of both protons and sulfate ions, but not fluoride ions as they occur at a much smaller concentration in seawater.

The spectrophotometric method for measuring pH_T was calibrated using two samples of seawater of known alkalinity and pCO_2. If alkalinity, pCO_2, salinity, and temperature of a sample of seawater is known, pH_T can be exactly calculated. To make seawater samples of a known alkalinity and pCO_2, I vigorously bubbled a certified reference material (CRM) of known alkalinity obtained from Andrew Dickson's lab (UCSD, Scripps Institute of Oceanography, San Diego, CA) with two NorLab® certified gas mixtures of 199 ± 2 ppm CO_2 and 1490 ± 15 ppm CO_2 that 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., 2018), I calculated the pH_T of these two solutions using the A_T and pCO_2, which I then used to calibrate the spectrophotometric method for measuring pH_T in my study. The spectrophotometric method was used to verify the pH of the seawater buffers used to calibrate the pH probes.

In addition to pH measurements, I measured the total alkalinity (A_T) of each treatment tank approximately weekly. I measured alkalinity using an open-cell titration based off the Dickson et al. (2007) SOP 6b with 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 six minutes to 10 minutes 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. I used the measured A_T and target treatment pCO_2 to calculate pH setpoints, and therefore updated tank pH setpoints each time A_T was measured in that tank.

I measured the tank temperature using the PT-100 temperature probe, which was verified using a high precision thermometer. I measured salinity with a Vernier salinity probe and calibrated this measurement against a Vernier salinity standard. Lastly, I calculated the pCO_2 of each tank using the tank temperature, pH_T, salinity, and A_T using the Seacarb package in R.

Treatments

After a minimum one week acclimation period, I assigned each octopus to one of four 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 four treatments were interspersed throughout the room to avoid any difference between treatments in light or temperature due to location of the tanks. I determined the control temperature and pCO_2 by measuring the temperature and pCO_2 of the seawater at depth where I caught the octopuses. I used an alcohol thermometer to measure the temperature of the collection site at depth and collected water samples in triplicate at depth using air-tight plastic collection bottles.

These samples were kept on ice and transported back to RBML, where I determined the pCO_2 of each using the salinity, alkalinity, temperature, and pH of each sample within four hours of collection.

The target increase in pCO_2 for the high pCO_2 treatments compared to the control pCO_2 treatments was 530 µatm, which corresponds with the RCP8.5 predicted increase in pCO_2 by 2100 (Riahi, Grübler, & Nakicenovic, 2007). Since the Salish Sea, where this study took place, has naturally high pCO_2 (Murray et al., 2015), the target control pCO_2 was 740 µatm and the target increased pCO_2 treatment was 1270 µatm. The target increase in temperature for the high temperature treatment was 3.5 °C higher than the control temperature treatment, based on the RCP8.5 prediction that oceans will warm 2.6 °C to 4.8 °C by 2100 (IPCC, 2014). The target control temperature treatment was 10.1 °C, and the target increased temperature treatment was 13.6 °C. The Representative Concentration Pathways (RCPs) are four end-of-century predictions based on continued greenhouse gas (GHG) and pollutant emissions, atmospheric gas concentrations, and land use. RCP8.5 is a high GHG emission scenario with no additional efforts to limit emissions by 2100 (IPCC, 2014).

After 20-22 days in treatment, I took blood samples to perform the immunological assays described below. Following a minimum of a two-day recovery period, I returned the octopuses to Driftwood Park.

Blood Collection

Following approximately three weeks in the designated treatment, I anaesthetized each octopus by submerging them in 2.5% ethanol (EtOH) in seawater (SW) until the octopus was unresponsive (Malham, Secombes, & Runham, 1995). I recorded the amount

of time the octopus was in the EtOH-SW, which ranged from 8-19 minutes. I then drew blood from their branchial vein through the mantle opening with a 31 G x 0.8 cm hypodermic needle into a sterile 1.0 mL syringe. The target volume of blood was 0.5 mL, and actual amount drawn ranged from 0.1-0.6 mL. I then returned the octopus to well-oxygenated seawater for recovery, ensuring that the out-of-water portion of the procedure took less than ten minutes total.

Next, I removed a 20 µL aliquot of the blood and mixed it with 200 µL of marine anticoagulant (MA, Appendix A) to perform a hemocyte count. I centrifuged the remainder of blood at 800 g for 5 minutes at 4 °C (Locatello et al., 2013; Malham et al., 1998). Following centrifugation, I removed the supernatant (cell-free plasma) and stored it on ice for the lysozyme assay. Next, I washed the isolated hemocytes with Octopus Ringer's Solution (OR, Appendix A) and centrifuged at 800 g for 5 minutes at 4 °C, then repeated this process. Lastly, I removed the OR, resuspended the hemocytes in 1.0 mL Modified Hanks' Balanced Salt Solution (MHBSS, Appendix A) and stored them 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 1:10 in marine anticoagulant and kept on ice to prevent the rapid formation of clumps (morulae) for hemocyte count (Malham et al., 1995). I then used a hemocytometer to count the concentration of hemocytes/mL in triplicate within 30 minutes 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), I prepared the fluoresceinated bacteria solution by growing *Vibrio anguillarum* at room temperature (approx. 18-20 °C) in Zobell Marine Broth for 3 days. I then killed the bacteria in formalin by mixing 10% formalin 2:1 with the bacteria solution and incubating for approximately 3 hours. Next, I washed the killed bacteria with 0.85% saline three times, spinning at 10k rpm for 5 minutes between washes, before diluting it in saline so a 1:10 dilution had an optical density (OD) of 0.540 at 540 nm.

Next, I diluted this bacteria-saline slurry 1:5 in 0.5 M carbonate/bicarbonate buffer (Appendix A). I labeled the killed bacteria with fluorescein 5-isothiocyanate, Isomer I (FITC) as first detailed by Gelfand et al. (1976) by mixing this bacteria solution 2:1 in a 0.03% solution of FITC. The FITC solution was made by dissolving 2.0 mg FITC in 2 mL 100% acetone, then adding this solution to 4.67 mL carbonate/bicarbonate buffer.

Following a 2 hour and 20 minute incubation at room temperature, I centrifuged the fluoresceinated bacteria (FB) at 10k rpm for 10 minutes, removed the supernatant, and washed the FB 1:10 in gelatin veronal-buffered saline ($GVBS^{2+}$) three times. I then resuspended the FB in 5 mL phosphate buffered saline (PBS) and stored the solution at -20 °C.

To perform the phagocytosis assay, I placed 100 µL of hemocytes diluted in MHBSS on a glass slide and incubated for 10 minutes in a moist incubation chamber. Next, I rinsed the slide with 100 µL MHBSS before adding 100 µL of thawed FITC-labelled *V. anguillarum* to the slide and incubated for 30 minutes at room temperature. I

then rinsed the slide with 100 µL of MHBSS again and counterstained the unphagocytosed bacteria with 100 µL ethidium bromide (50 µg/mL in PBS) for two minutes. Lastly, I mounted the reaction with Vectashield® Mounting Medium for Fluorescence, sealed with a coverslip and clear nail polish, and stored the slide at 4 °C for later analysis.

I analyzed these slides using a Leica DMIRB fluorescent microscope at 400x magnification with a 488 nm fluorescent emission filter. This process involved taking a photo of the hemocytes in the phase contrast mode of the microscope, and then taking another photo in the same position with the fluorescence turned on to capture the fluorescent bacteria. I then overlaid the hemocyte photo over the fluorescent photo, which resulted in a single photo with black hemocytes and green fluorescence, indicating bacteria. I photographed a minimum of 200 cells/slide.

I used ImageJ to analyze the photos for each slide by uploading the batch of photos corresponding to one slide. I first segmented the photo to select hemocytes in each photo using the threshold function, and determined the location and area of each hemocyte in each photo. I then isolated the fluorescent bacteria using the color threshold function and again determined the location and area of each occurrence of fluorescence (Fig 3, Appendix B).

I counted the number of phagocytic vs. nonphagocytic cells in R (Appendix C). Phagocytic cells were those which consumed the fluoresceinated bacteria (FB). A hemocyte was considered phagocytic if it was co-located with fluorescence of a minimum area, and nonphagocytic if the hemocyte was not surrounded by fluorescence. Hemocytes in large areas of fluorescence were thrown out as these were generally

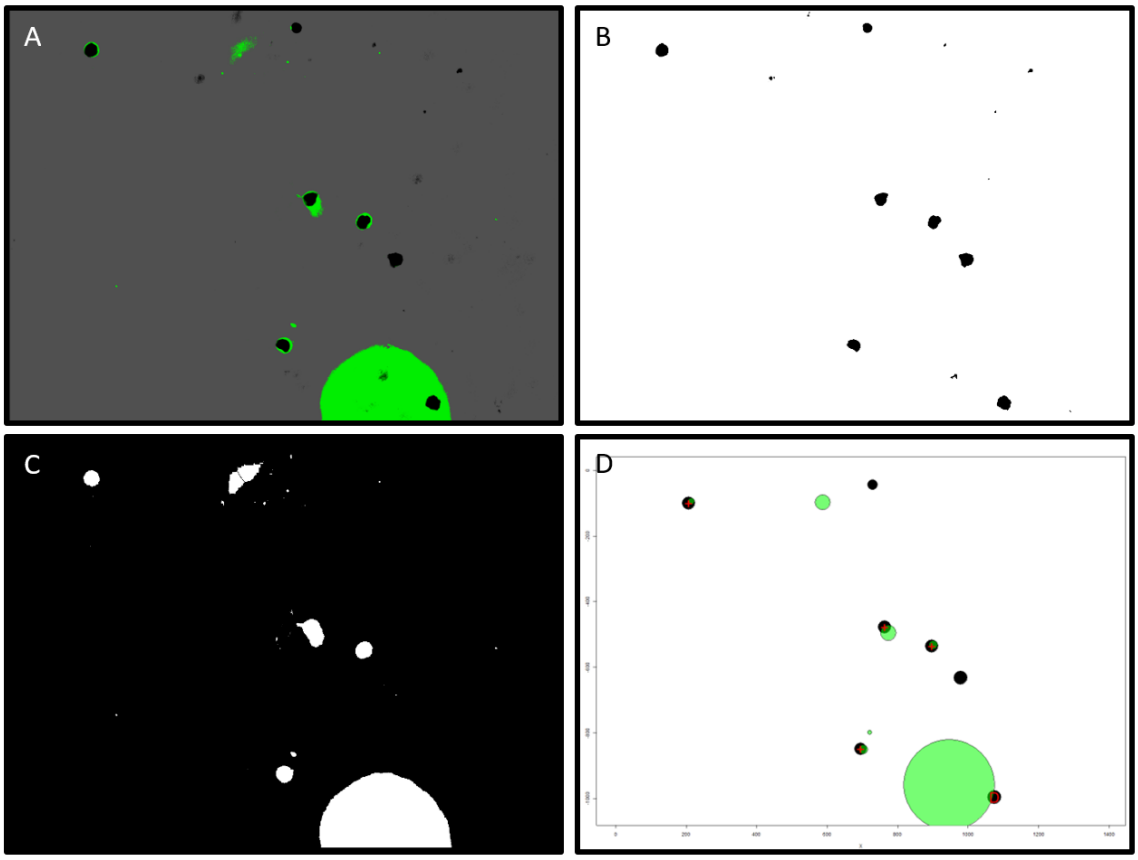


Figure 3. Sequence of photos depicting the steps for analysis of the phagocytosis assay. A) Overlap of original two photos taken using the Leica DMIRB fluorescent microscope at 400x magnification; B) Photo of the hemocytes, segmented using the threshold function in ImageJ; C) Photo of the fluorescence, segmented using the color threshold function in ImageJ; D) Image created by R analysis showing hemocytes which did not phagocytose the bacteria (depicted as black circles), hemocytes that did phagocytose the bacteria (depicted by the red "+" symbol), and a hemocyte that was dropped due to being within a large area of fluorescence (depicted by the "D").

hemocytes in large clumps of fluorescent bacteria and I could not determine if these hemocytes had phagocytosed bacteria. I calculated a proportion of phagocytic hemocytes compared to the total, as well as the total phagocytosis per volume blood.

Superoxide Production Assay

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

I incubated these tubes for one hour at room temperature before spinning for 10 minutes at 120,000 g, removing the supernatant, and washing the cells twice with 200 μL MHBSS. Next, I added 200 μL of 100% methanol (MeOH) to each tube, vortexed each tube gently, and let incubate for 10 minutes at room temperature. I then centrifuge the tubes at 300 g, removed the supernatant, and allowed the cells to air dry. Next, I rinsed the cells three times with 200 μL of 50% MeOH, and then added 240 μL of potassium hydroxide (KOH) and 280 μL dimethyl sulfoxide (DMSO). I heavily vortexed the tubes and transferred the supernatant into 1.5 mL cuvettes. Lastly, I measured the optical density (OD) of each sample at 620 nm and reported the results as (OD values $\times 10^6$) $cell^{-1}$ and (OD values $\times 10^6$) mL^{-1} blood.

Lysozyme Assay

I used a turbidimetric method for measuring lysozyme activity of the cell-free hemolymph isolated from the blood of each octopus (Grimaldi et al., 2013; Locatello et al., 2013; Malham et al., 1998). I began by dissolving lyophilized *Micrococcus lysodeikticus* 0.0075 g/10 mL citrate/phosphate buffer (Appendix A). I then added the *M. lysodeikticus* solution and hemolymph to a 1.5 mL cuvette 3:1 and immediately measured the absorbance at 450 nm. I measured absorbance over a 5-10 minute period, noting when the absorbance remained steady. I used the citrate/phosphate buffer to blank the spectrophotometer before measuring the change in turbidity due to lysozyme activity. Results are expressed as lysozyme units/mL, where one unit is equal to the amount of lysozyme that causes a 0.001 decrease in absorbance per minute (Grimaldi et al., 2013; Locatello et al., 2013).

Statistical Analysis

I tested for normality and homoscedasticity within each treatment for each data set using the Shapiro-Wilk test and Bartlett test, respectively. I then performed a 2-way Analysis of Variance (ANOVA) on each data set, and noted which data sets were not normal and/or homoscedastic. A Tukey test was then used when the ANOVA results were significant. I used the conservative Bonferroni correction to control for type I error resulting from multiple comparisons ($\alpha = 0.00833$). All analyses were performed using the statistical program R (Appendix D).

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 three weeks in either elevated CO_2, temperature, or both, I measured blood total hemocyte count (THC), phagocytosis activity, hemocyte superoxide production, and blood plasma lysozyme activity of each octopus. I found an effect of CO_2 on the hemocyte concentration ($p = 0.00452$, Table 2, Fig 4) and total phagocytosis per volume blood ($p = 0.00222$, Table 2, Fig 5), 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 6), production of superoxide anion per cell (Table 2, Fig 7), production of superoxide anion per volume blood (Table 2, Fig 8), or lysozyme activity (Table 2, Fig 9).

It should be noted that the lysozyme concentrations present in *O. rubescens* blood plasma appear below the detectable limit of 3.2 ng/mL using the turbidimetric method, determined by generating a standard curve using egg white lysozyme. Therefore, the blood plasma lysozyme results may not accurately display the effects of these treatments.

Table 1. Seawater carbonate chemistry values for individual tanks and overall treatments. Treatment averages represent mean ± sd (n = 6 per treatment). Measured values: temperature (T), salinity (S), pH_T, alkalinity (A_T), and pCO_2.

Treatment	T (˚C)	S (ppt)	pH_T	A_T (ppm)	pCO_2 (µatm)
Control	10.1 ± 0.2	30.0 ± 0.4	7.717 ± 0.020	2052 ± 21	844 ± 48
	10.1 ± 0.0	29.8 ± 0.4	7.679 ± 0.011	2065 ± 3	929 ± 24
	10.1 ± 0.0	29.8 ± 0.3	7.715 ± 0.060	2079 ± 15	861 ± 120
	10.9 ± 0.9	29.8 ± 0.2	7.728 ± 0.020	2068 ± 10	833 ± 42
	11.0 ± 0.4	30.3 ± 0.7	7.722 ± 0.022	2066 ± 13	844 ± 43
	10.8 ± 0.4	30.7 ± 0.9	7.691 ± 0.037	2092 ± 19	919 ± 88
Avg. Control	**10.5 ± 0.4**	**30.1 ± 0.4**	**7.709 ± 0.019**	**2070 ± 14**	**872 ± 42**
Acidified	10.2 ± 0.1	30.0 ± 0.4	7.543 ± 0.048	2055 ± 4	1296 ± 155
	10.1 ± 0.0	29.8 ± 0.1	7.494 ± 0.026	2064 ± 0	1455 ± 97
	10.1 ± 0.0	29.8 ± 0.3	7.544 ± 0.048	2131 ± 104	1315 ± 129
	11.6 ± 0.7	30.3 ± 0.7	7.521 ± 0.066	2073 ± 1	1395 ± 226
	11.0 ± 0.2	30.4 ± 0.8	7.511 ± 0.063	2076 ± 1	1476 ± 225
	10.5 ± 0.3	30.7 ± 0.9	7.533 ± 0.071	2085 ± 25	1343 ± 215
Avg. Acidified	**10.6 ± 0.6**	**30.2 ± 0.4**	**7.524 ± 0.020**	**2081 ± 27**	**1380 ± 74**
Warming	12.9 ± 0.7	30.1 ± 0.4	7.698 ± 0.022	2060 ± 14	892 ± 52
	13.4 ± 0.3	30.0 ± 0.3	7.743 ± 0.009	2058 ± 15	804 ± 20
	13.0 ± 0.8	29.9 ± 0.3	7.677 ± 0.049	2066 ± 14	951 ± 106
	13.4 ± 0.4	30.4 ± 0.9	7.735 ± 0.012	2077 ± 15	825 ± 25
	13.1 ± 0.4	30.4 ± 0.8	7.712 ± 0.032	2088 ± 10	883 ± 75
	13.4 ± 0.8	31.1 ± 0.7	7.684 ± 0.062	2113 ± 17	916 ± 140
Avg. Warming	**13.2 ± 0.2**	**30.3 ± 0.4**	**7.708 ± 0.027**	**2077 ± 21**	**885 ± 63**
Acidified + Warming	13.4 ± 0.3	29.9 ± 0.2	7.566 ± 0.029	2054 ± 1	1244 ± 88
	13.2 ± 0.6	29.9 ± 0.1	7.512 ± 0.065	2064 ± 0	1427 ± 223
	13.0 ± 0.7	30.0 ± 0.3	7.511 ± 0.032	2075 ± 9	1431 ± 109
	13.5 ± 0.1	30.4 ± 0.7	7.599 ± 0.015	2071 ± 7	1154 ± 39
	13.1 ± 0.4	30.7 ± 0.8	7.585 ± 0.075	2098 ± 19	1220 ± 210
	13.4 ± 0.3	30.6 ± 0.8	7.531 ± 0.067	2104 ± 26	1382 ± 216
Avg. Acidified + Warming	**13.3 ± 0.2**	**30.3 ± 0.4**	**7.551 ± 0.038**	**2078 ± 20**	**1310 ± 119**

Table 2. Two-way ANOVA results of the effects of pCO_2 and temperature on immunological parameters measured in blood drawn from *Octopus rubescens* after three weeks in treatment (n = 5-6 per treatment). Significant values shown in bold ($p < 0.00833$).

LT: Ambient temperature treatment; HT: High temperature treatment; LC: Ambient pCO_2 treatment; HC: High pCO_2 treatment.

Measurement	Factor	df	Sum Sq.	Mean Sq	*F*-value	*p*-value	Tukey's HSD
Hemocyte Concentration	pCO_2	1	1.385×10^{14}	1.385×10^{14}	10.223	**0.00452**	LTHC > LTLC
	Temp	1	6.407×10^{12}	6.407×10^{12}	0.473	0.49959	
	pCO_2*Temp	1	9.754×10^{12}	9.754×10^{12}	0.720	0.40624	
Total Phagocytosis per mL Blood	pCO_2	1	1.027×10^{14}	1.027×10^{14}	12.296	**0.00222**	LTHC > LTLC & HTLC
	Temp	1	5.675×10^{12}	5.675×10^{12}	0.679	0.41954	
	pCO_2*Temp	1	1.063×10^{13}	1.063×10^{13}	1.272	0.32727	
Proportion of Phagocytic Cells	pCO_2	1	0.005262	0.005262	0.935	0.345	
	Temp	1	0.000152	0.000152	0.027	0.871	
	pCO_2*Temp	1	0.006489	0.006489	1.153	0.296	
Superoxide Production per Cell	pCO_2	1	0	0	0	0.998	
	Temp	1	0	0	0.001	0.979	
	pCO_2*Temp	1	0.0975	0.0975	1.784	0.197	
Superoxide Production per mL Blood	pCO_2	1	2.887×10^{13}	2.887×10^{13}	2.990	0.100	
	Temp	1	1.016×10^{12}	1.016×10^{12}	0.105	0.749	
	pCO_2*Temp	1	4.155×10^{12}	4.155×10^{12}	0.430	0.520	
Lysozyme Activity	pCO_2	1	0.002	0.002	0.001	0.982	
	Temp	1	4.208	4.208	1.275	0.274	
	pCO_2*Temp	1	3.653	3.653	1.107	0.307	

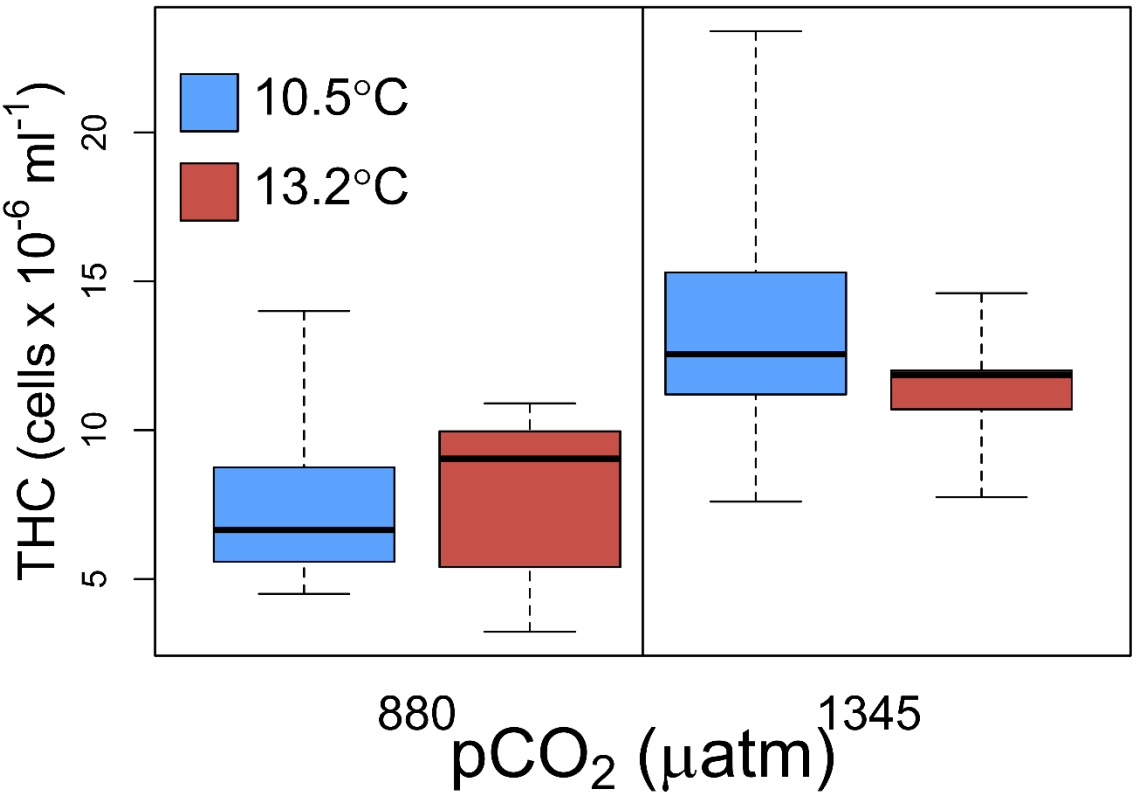


Figure 4. Total hemocyte concentration (THC) of *Octopus rubescens* blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (CO_2 p-value = 0.0042; *n* = 6 per treatment).

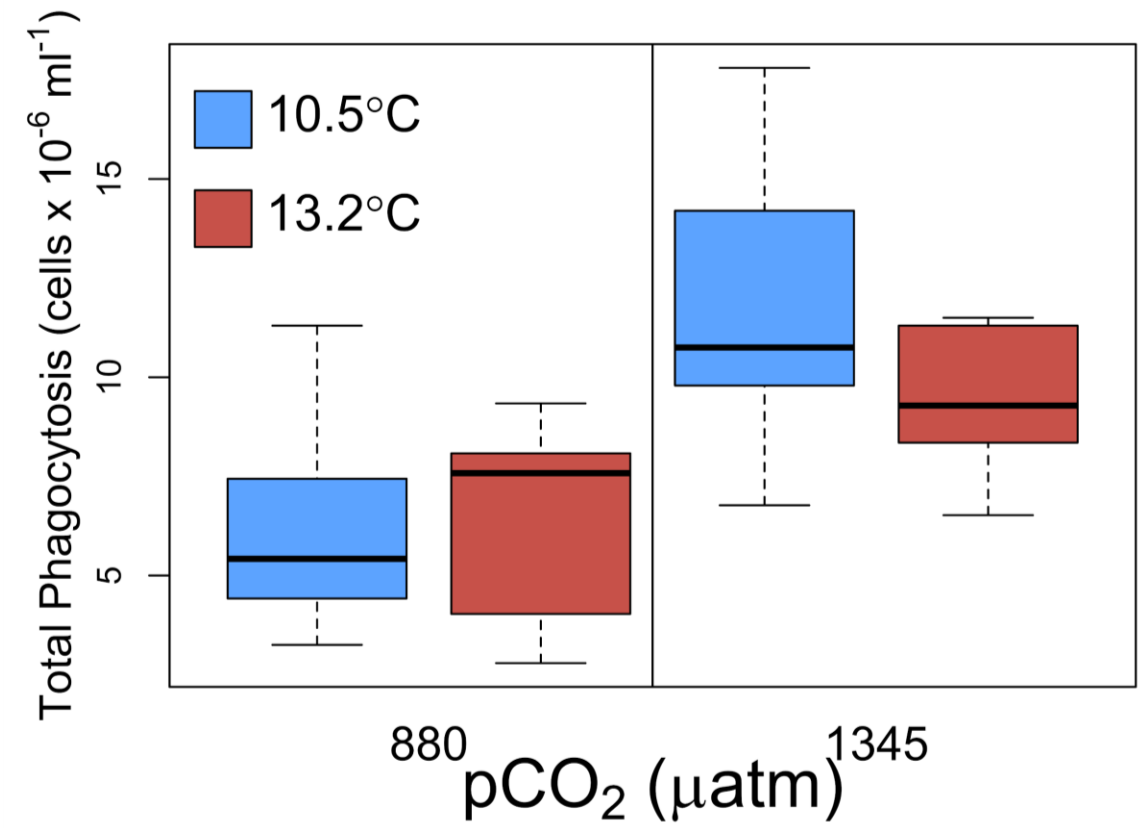


Figure 5. Total phagocytosis in *Octopus rubescens* blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (CO_2 p-value = 0.00222; *n* = 6 per treatment).

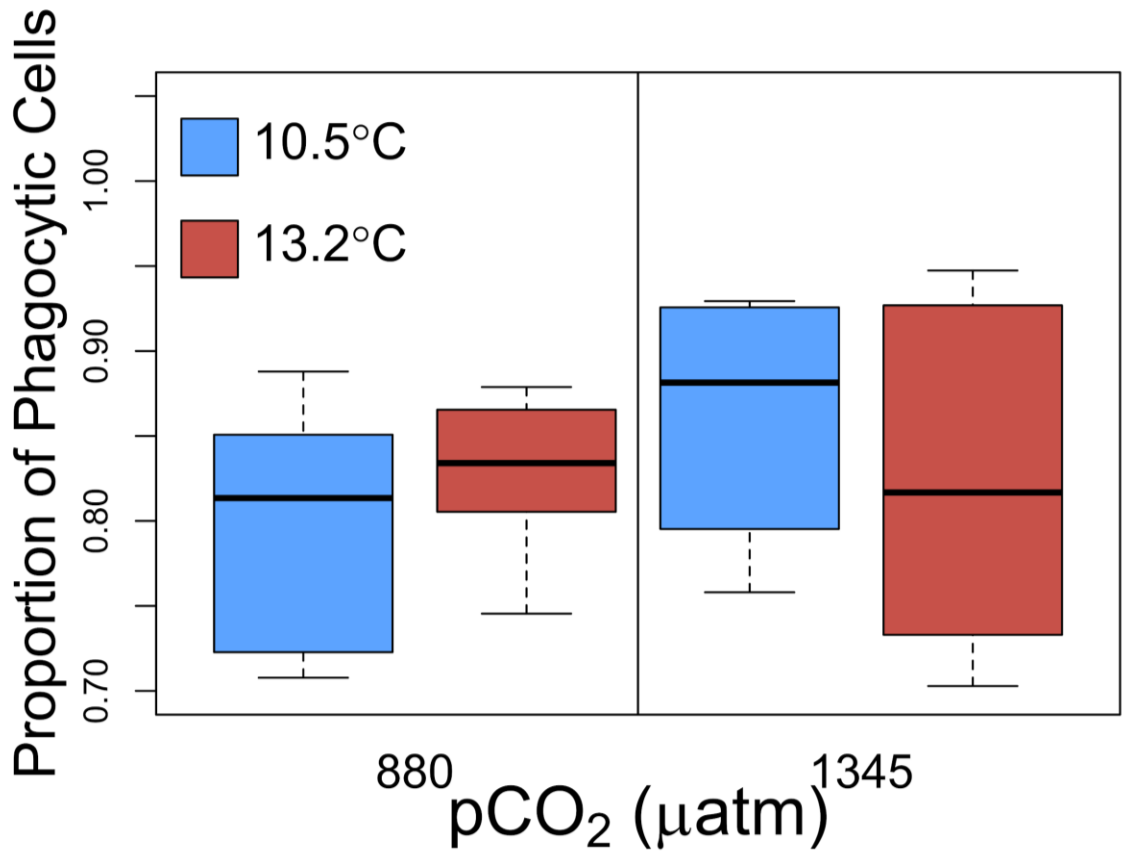


Figure 6. Proportion of phagocytic cells in *Octopus rubescens* blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (n = 6 per treatment).

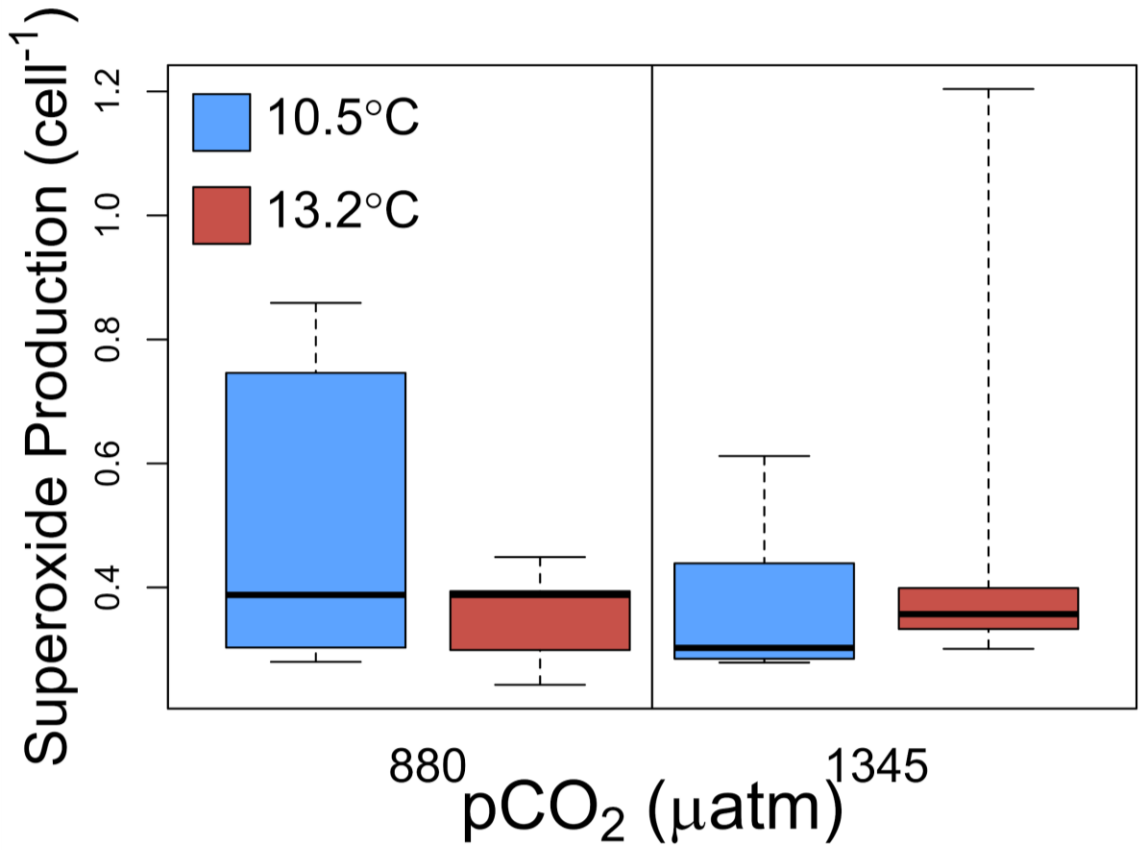


Figure 7. Production of superoxide anion per cell in *Octopus rubescens* blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (*n* = 5-6 per treatment).

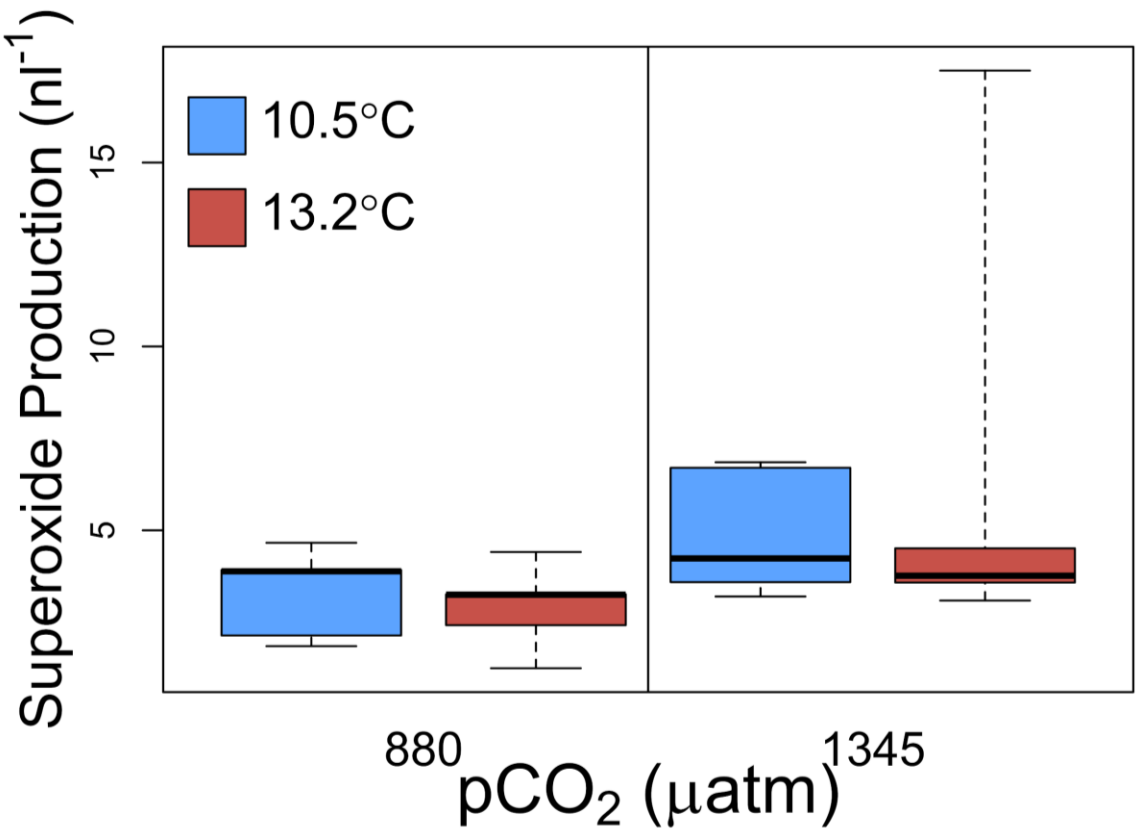


Figure 8. Production of superoxide anion per volume in *Octopus rubescens* blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (*n* = 5-6 per treatment).

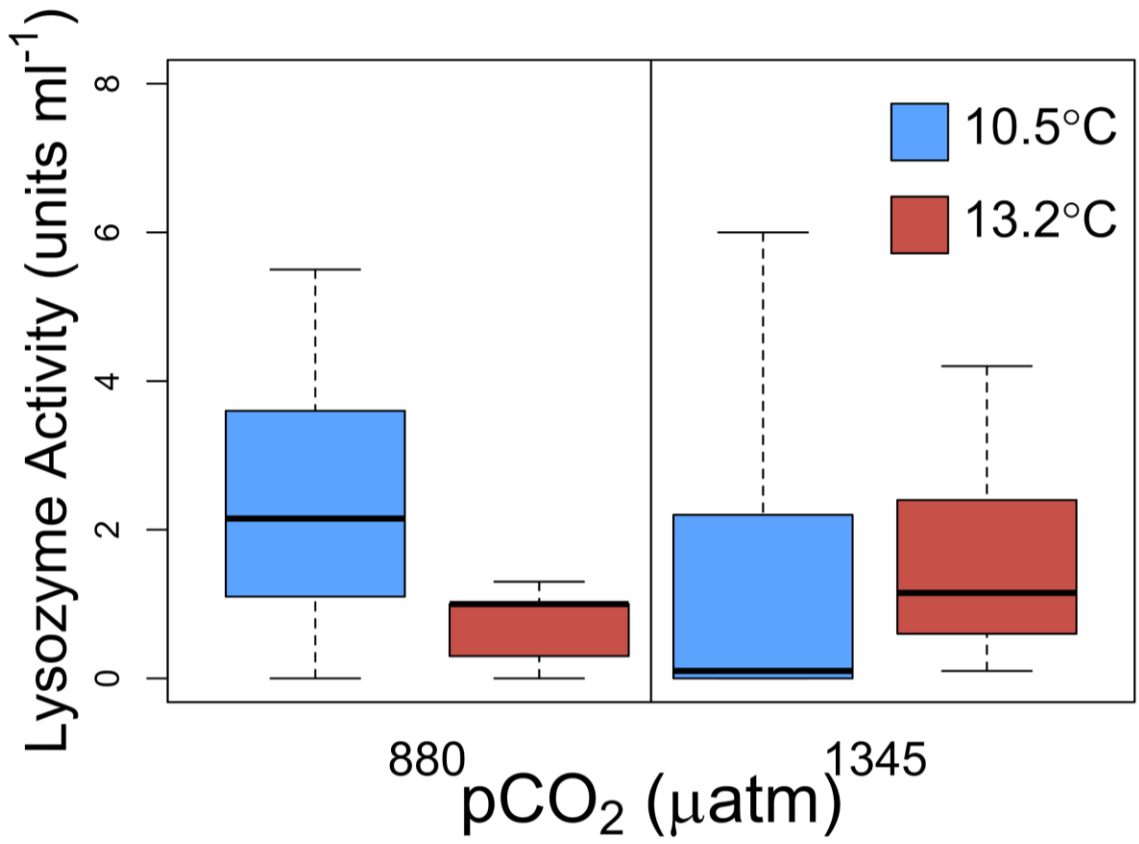


Figure 9. Lysozyme activity in *Octopus rubescens* cell-free blood after exposure to different pCO_2 and temperature treatments for approximately three weeks (n = 5-6 per treatment).

DISCUSSION

Immune Response of Octopus rubescens to Climate Change

Results from this study indicate that elevated CO_2 may elicit an increased immune response in *Octopus rubescens* as evidenced by higher hemocyte counts and increased total phagocytosis. No effect of temperature was measured; however this may be because the control temperature was measured in the spring, and an increase in 3.5 ˚C from that temperature may be close to the natural annual fluctuations in temperature the octopuses already experience in the wild.

An octopus' homeostasis can be disrupted by unusual occurrences, both natural or anthropogenic in origin, resulting in a stress response (Malham et al., 2002, 2003). Environmental changes may alter the immune response of invertebrates (Mydlarz, Jones, & Harvell, 2006), and there is a strong link between an organism's stress response and immunity (Malham et al., 2003). In fact, ecologists use immune cell counts as a way to assess stress in many animals (Davis et al., 2008). Therefore, an increase in relative immune response is indicative of a stress response in octopuses (Locatello et al., 2013). Increased temperature and hypercapnia are two of the environmental stressors that cause the most concern in our current changing climate, and these results indicate that *O. rubescens* may be among those which may respond negatively to future climate conditions.

Comparing Immune Responses between Taxa

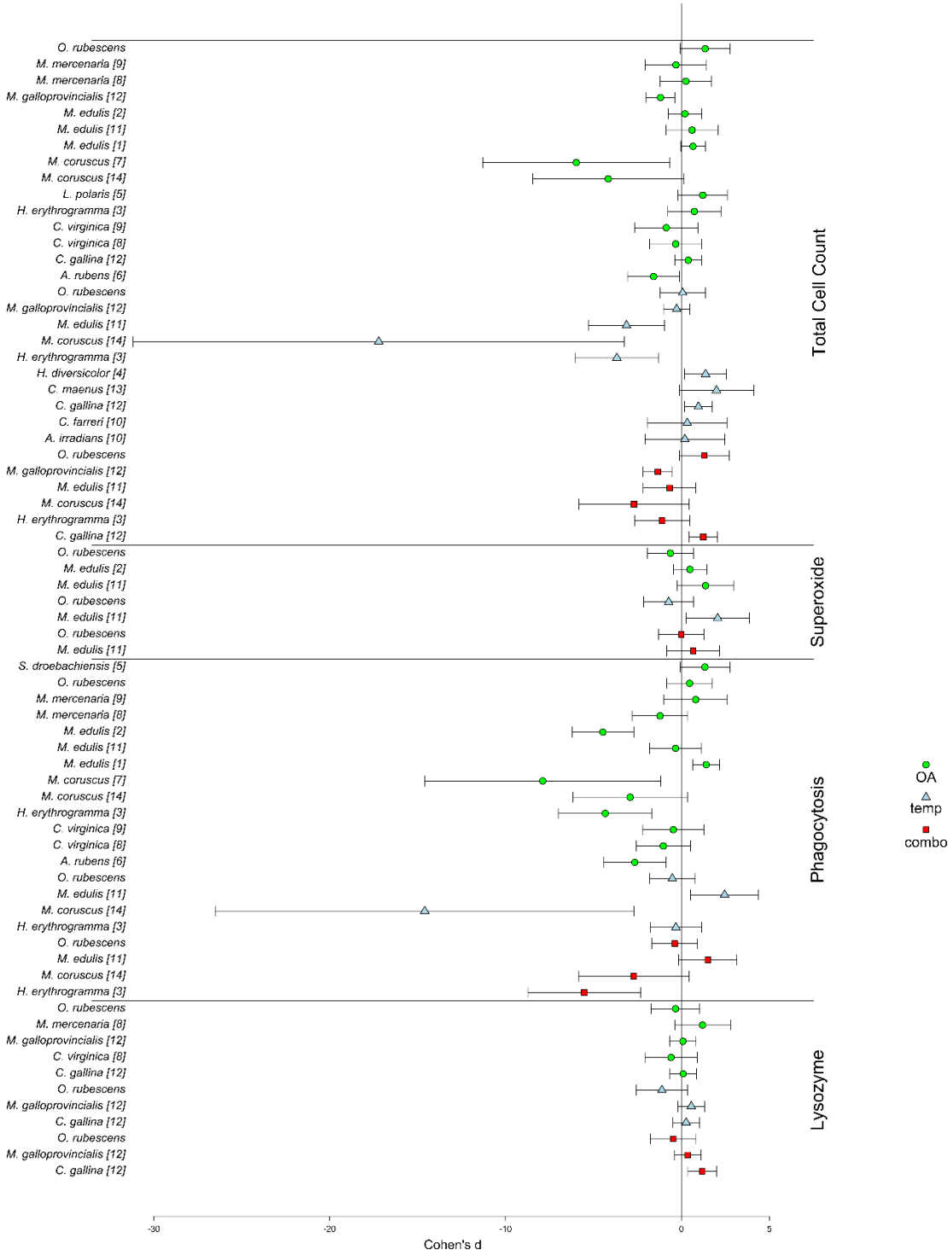
This research is the first known study on ocean acidification effects on the cephalopod immune response. A number of similar studies on other marine invertebrates indicate that stressful environmental conditions have a variety of effects on a variety of

marine animal taxa, and these results on *O. rubescens* are an important addition to this growing body of knowledge about how future climate conditions may impact marine invertebrate immune responses.

To put the results of this study into context, I determined Cohen's d effect sizes of immune responses of marine invertebrates to climate change conditions from the literature (Fig 10). Cohen's d effect size measures the magnitude of a study's results, taking the sample size into account. The criteria to be included in this comparison included providing a minimum of one of the following: p-value, F-value, or mean ± SD. If none of these values were provided, I was unable to include the study in this analysis. The following studies were among those which were initially analyzed but were not included in the effect size comparison due to lack of the minimum criteria: Cheng, Wang, & Chen, 2005; Hernroth et al., 2012; Leite Figueiredo et al., 2016; Li et al., 2015; Liu et al., 2016; Monari et al., 2007; Pascual et al., 2003; Stumpf & Gilbertson, 1978; Sui et al., 2016; Q. Wang et al., 2016; X. Wang et al., 2012; Yu et al., 2009.

Response to Ocean Warming

In an extensive review on the immune response of marine and freshwater invertebrates in response to various stressors, such as environmental conditions, Mydlarz et al. (2006) describe how temperature stress often results in negative effects on the physiological and immune function in marine invertebrates. That review explored several studies on the matter, and many concluded that temperature stress suppresses immune function, while others recorded an increase in immune function. There is no known mechanistic link between temperature and immunity to date, and it is therefore important


O. rubescens
M. mercenaria [9]
M. mercenaria [8]
M. galloprovincialis [12]
M. edulis [2]
M. edulis [11]
M. edulis [1]
M. coruscus [7]
M. coruscus [14]
L. polaris [5]
H. erythrogramma [3]
C. virginica [9]
C. virginica [8]
C. gallina [12]
A. rubens [6]
O. rubescens
M. galloprovincialis [12]
M. edulis [11]
M. coruscus [14]
H. erythrogramma [3]
H. diversicolor [4]
C. maenus [13]
C. gallina [12]
C. farreri [10]
A. irradians [10]
O. rubescens
M. galloprovincialis [12]
M. edulis [11]
M. coruscus [14]
H. erythrogramma [3]
C. gallina [12]
O. rubescens
M. edulis [2]
M. edulis [11]
O. rubescens
M. edulis [11]
O. rubescens
M. edulis [11]
S. droebachiensis [5]
O. rubescens
M. mercenaria [9]
M. mercenaria [8]
M. edulis [2]
M. edulis [11]
M. edulis [1]
M. coruscus [7]
M. coruscus [14]
H. erythrogramma [3]
C. virginica [9]
C. virginica [8]
A. rubens [6]
O. rubescens
M. edulis [11]
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O. rubescens
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O. rubescens
M. mercenaria [8]
M. galloprovincialis [12]
C. virginica [8]
C. gallina [12]
O. rubescens
M. galloprovincialis [12]
C. gallina [12]
O. rubescens
M. galloprovincialis [12]
C. gallina [12]
Total Cell Count
Superoxide
Phagocytosis
Lysozyme
OA
temp
combo
-30
-20
-10
0
5
Cohen's d

Figure 10. Effect sizes (Cohen's d) of immune responses of various marine invertebrates to climate change conditions ([1]Asplund et al., 2014; [2]Bibby et al., 2008; [3]Brothers et al., 2016; [4]Cheng et al., 2004; [5]Dupont & Thorndyke, 2012; [6]Hernroth et al., 2011; [7]Huang et al., 2016; [8,9]Ivanina, Hawkins, & Sokolova, 2014, 2016; [10]Liu et al., 2004; [11]Mackenzie et al., 2014; [12]Matozzo et al., 2012; [13]Truscott & White, 1990; [14]Wu et al., 2016).

to study a wide variety of organisms to understand the potential impacts a warming ocean may have on marine ecosystems.

While not explicitly explored in this study, it should also be noted that warming temperatures are accompanied by more frequent cases of hypoxia (Mydlarz et al., 2006). Hypoxia is considered more of a danger to benthic, sessile organisms (Boyd & Burnett, 1999), however octopus may also be susceptible to increased hypoxic events. Octopuses are historically a difficult group of organisms to track in the field (Mather, Resler, & Cosgrove, 1985), and therefore little is known about their geographic range of movement. However, *O. rubescens* have been found from the intertidal down to depths of 200 m (Laidig & Adams, 1995), and they likely experience periods of hypoxia within this wide range. Increased hypoxic events may thus limit the available range for *O. rubescens*. Therefore, although I recorded no significant effect of warming sea temperatures on the immune response of *O. rubescens*, there may be a variety of other negative effects global ocean warming will have on their physiology.

Response to Ocean Acidification

The content of carbon dioxide in the water, which directly affects the pH and carbonate ion availability, is also considered to be one of the most important environmental factors impacting invertebrate physiology (Byrne, 2011). As previously mentioned, shelled invertebrates are considered more at risk to ocean acidification due to the reduction in available carbonate ion. However, more research is now being done on other physiological effects of hypercapnia on marine invertebrates, such as metabolic and immune responses. Ocean acidification has an overall negative effect on the various taxa explored, especially during larval stages (Byrne, 2011). However, similar to warming

effects, the immune response of marine organisms to ocean acidification is unclear, with some organisms having a reduced immune capability, some having no change, and some, including *O. rubescens*, seeing an increased immune response (Fig 10). Therefore, it is pertinent that all marine taxa and life stages be examined for the impacts that future climate change conditions may have on various physiological responses, including immunity.

Potential Adaptation to Future Climate Conditions

While these results suggest that *O. rubescens* elicits a stress response to the ocean acidification conditions after three weeks, it is also important to understand the long-term effects. Ottaviani & Franceschi (1996) studied stress in a variety of invertebrates and vertebrates. They concluded that the stress response is widely conserved between taxa, and may significantly contribute to adaptation. So while a stress response is generally considered a negative response to environmental conditions, the potentially positive long-term effects should also be taken into consideration.

As the climate continues to change, there will without a doubt be large shifts in the global ocean distribution of marine organisms. These shifts are already being observed in association with rising temperatures, especially in algal and plankton populations in high-latitude oceans (Adger et al., 2007). Therefore, it is only those able to adapt to these shifting environmental conditions that will thrive in the future. Cephalopods are known to adapt quickly due to their relatively short life spans and high plasticity throughout their life history (Rodhouse et al., 2014). In fact, Doubleday et al. (2016) found that cephalopod populations, including octopus, have grown worldwide over the last 60 years, as evidenced by an increase in cephalopod fishery catch.

Scientists estimate that near-future climate conditions may result in the extinction of 20-30% of all species worldwide (Adger et al., 2007; Lovejoy, 2006). Some at-risk species may include those which prey on *O. rubescens* or compete with them for food sources. So while my results suggest that *O. rubescens* may be susceptible to ocean acidification, as evidenced by an increase in their immune response suggesting a stress response, the recent proliferation of cephalopods worldwide indicate that they may be adapting to changing climate conditions. Therefore, although an increased immune response may reduce some metabolic function in *Octopus rubescens*, such as through the redistribution of energy to the immune system, the overall effects of a changing climate may be in their favor if they have less predation and competition, as well as the ability to adapt quickly to environmental conditions. 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 the future ocean.

CONCLUSION

Results from this study suggest that future environmental conditions may have a negative effect on the overall health and immune response of *Octopus rubescens*. As evidenced by an increase in hemocyte concentration and total phagocytosis after three weeks in high pCO_2 treatments, I conclude that *O. rubescens* may respond negatively to future ocean acidification conditions by eliciting a stress response. With extra energy dedicated towards an enhanced immune response, there is less overall energy available for other physiological functions such as growth, reproduction, movement, and venom production. However, evidence from other studies indicates that octopus are able to adapt to environmental conditions relatively quickly, perhaps within one lifetime rather than over several generations. Therefore, a similar study conducted for a longer period of time would provide better insight into the effects of future climate conditions on *Octopus rubescens*.

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APPENDIX A

Octopus Ringer Solution (OR): Recipe according to Malham, Runham, & Secombes (1998)

- Sodium chloride (NaCl; 2.433 g/100 mL)
- Glucose ($C_6H_{12}O_6$; 1.4 g/100 mL)
- Ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA; 0.015 g/100 mL)
- Potassium chloride (KCl; 0.082 g/100 mL)
- Monopotassium phosphate (KH_2PO_4; 0.004 g/100 mL)
- Store at 4 ℃

Marine Anticoagulant (MA): Recipe according to Malham, Runham, & Secombes (1998)

- Sodium chloride (NaCl; 2.63 g/100 mL)
- Glucose ($C_6H_{12}O_6$; 1.8 g/100 mL)
- Tri-sodium citrate ($Na_3C_6H_5O_7$; 0.088 g/100 mL)
- Citric acid ($C_6H_8O_7$; 0.055 g/100 mL)
- Ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA; 0.029 g/100 mL)
- Store at 4 ℃

Carbonate/Bicarbonate Buffer:

- 3 volumes Sodium bicarbonate ($HNaCO_3$; 0.5 M)
- 1 volume Sodium carbonate (Na_2CO_3; 0.5 M)
- Store at room temperature

Modified Hank's Balanced Salt Solution (MHBSS):

- Ethylene glycol-bis(β-aminoethylether) N, N, N', N', -tetraacetic acid (EGTA; 3 µg/100 mL)
- Hank's Balanced Salt Solution
- Store at room temperature

Nitroblue Tetrazolium Chloride (NBT) Solution: Recipe derived from Malham et al. (2002)

- Nitroblue tetrazolium chloride (NBT; 2 mg/mL)
- Tris(hydroxymethyl)aminomethane (Tris; 12.1 g/100 mL)
- Sodium chloride (NaCl; 2 g/100 mL)
- Reduce pH to 7.6 using concentrated hydrochloric acid (HCl)
- Store at room temperature, avoid light

Phosphate/Citrate Buffer:

- Sodium phosphate dibasic dihydrate ($NaHPO_4 \cdot 2H_2O$; 25 mM)
- Citric acid ($C_6H_8O_7$; 14 mM)
- Distilled water
- Store at room temperature

APPENDIX B

Using ImageJ to Analyze Hemocytes in Phagocytosis Slide Photos:

1. Import sequence of photos from one slide as 8-bit files
2. Apply the "Threshold" function
    a. Move sliders to 5 and 255
    b. Threshold Dropdown: Default
    c. Background Dropdown: B&W
    d. Check the "Dark background" box
    e. "Apply"
    f. In the next window:
        i. Method: Default
        ii. Background: Dark
        iii. Check the "Calculate threshold for each image" and "Black background (of binary masks)" boxes
3. Apply the "Invert" function
4. Open the "Set Measurements…" box
    a. Check the "Area" and "Centroid" boxes
5. Apply the "Analyze particles" function
    a. Size: 350-infinity
    b. Circularity: 0.60-1.00
    c. Show: Outlines
    d. Check the "Display results", "Clear results", and "Summarize" boxes
6. Save the "Summary" and "Results" files for further analysis in R

Using ImageJ to Analyze Fluorescence in Phagocytosis Slide Photos:

7. Import sequence of photos from one slide
    a. Do not convert to 8-bit
8. Apply the "Color Threshold"
    a. Hue: Sliders at 0 and 255
    b. Saturation: Sliders at 0 and 255
    c. Brightness: Sliders at 125 and 255
    d. Thresholding method: Default
    e. Threshold color: B&W
    f. Color space: HSB
    g. Check the "Dark background" box
    h. "Stack"
9. Apply the "Analyze particles" function
    a. Size: 100-infinity
    b. Circularity: 0.00-1.00
    c. Show: Outlines
    d. Check the "Display results", "Clear results", and "Summarize" boxes
10. Save the "Summary" and "Results" files for further analysis in R

Appendix C

Phagocytosis Data Analysis

```
knitr::opts_chunk$set(warning=FALSE)
```

Preparing Data Files

```
setwd(paste("C:/Users/mcull/Google Drive/",
            "M.S. in Biology/Research/",
            "Phagocytosis Slides/1",sep=""))
hemo=read.csv('hemo.results1b.csv')
hemo_sum=read.csv('hemo.summ1b.csv')
fluor=read.csv('fluor.results1b.csv')
fluor_sum=read.csv('fluor.summ1b.csv')
```

```
hemo_slice=rep(hemo_sum$Slice[1],hemo_sum$Count[1])
for (i in 2:length(hemo_sum$Slice)){
  hemo_slice=c(hemo_slice,rep(hemo_sum$Slice[i],hemo_sum$Count[i]))
}
hemo$slice=hemo_slice
```

```
fluor_slice=rep(fluor_sum$Slice[1],fluor_sum$Count[1])
for (i in 2:length(fluor_sum$Slice)){
 fluor_slice=c(fluor_slice,rep(fluor_sum$Slice[i],fluor_sum$Count[i]))
}
fluor$slice=fluor_slice
```

Analyzing Hemocytes and Fluorescence

```
hemo$consume="n"
for (i in 1:nrow(hemo)){
  last.count.loop=i
  far=as.matrix(dist(rbind(hemo[i,3:4],fluor[fluor$slice==hemo$
                                                slice[i],3:4])))[,1]
  if (length(far)>1) {
    shadow=sum(far<sqrt(hemo$Area[i]/pi)*2)
    shadow.area=fluor$Area[as.numeric(names(which.min(far
                                                [2:length(far)])))]
    big.things=fluor[fluor$Area>=5000&fluor$slice==hemo$slice[i],]
    howmanybig=nrow(big.things)
```

```
    in.big.things=1
    for (j in 1:howmanybig){
      in.big.things[j]=as.numeric(dist(rbind(hemo[i,3:4],big.things
          [j,3:4]))-sqrt(hemo$Area[i]/pi)-sqrt(big.things$Area[j]/pi))
    }
    if (is.na(in.big.things)){in.big.things=0}
    if (length(in.big.things)==0){in.big.things=0}
    if (shadow>1){hemo$consume[i]="y"}
    if (shadow.area<180){hemo$consume[i]="n"}
    if (min(in.big.things)<0){hemo$consume[i]="drop"}
  }
}
hemo$consume=as.factor(hemo$consume)
```

Generating Figures

```
for (i in unique(hemo$slice)){
  png(filename=paste("slice",i,".png",sep=""),width=1392,height=1040)
  plot(-1*Y~X,data=hemo[hemo$slice==i,],ylim=c(-1040,0),xlim=c(0,1392)
       ,cex=sqrt(hemo$Area[hemo$slice==i]/pi)/3,pch=21,bg="black")
  points(-1*Y~X,data=fluor[fluor$slice==i,],pch=21,bg="#06fb008b",
         cex=sqrt(fluor$Area[fluor$slice==i]/pi)/3)
  points(-1*Y~X,data=hemo[hemo$slice==i&hemo$consume=="y",],pch="+",
         col="red",cex=3)
  points(-1*Y~X,data=hemo[hemo$slice==i&hemo$consume=="drop",],pch="D"
         ,col="red",cex=3)
  dev.off()
}
```

Calculating Proportion of Phagocytic Cells

```
yes=sum(hemo$consume=="y")
no=sum(hemo$consume=="n")

proportion=yes/(yes+no)
```

Appendix D

Statistical Analysis

Preparing Data Files

```
setwd("C:/Users/mcull/Google Drive/M.S. in Biology/Research/R/Data Analysis")
immuno=read.csv("Immunoassay_Results.csv",header = T)
immuno$temp="hi"
immuno$temp[grep("LT",immuno$trmt)]="lo"
immuno$co2="hi"
immuno$co2[grep("LC",immuno$trmt)]="lo"
immuno$temp=as.factor(immuno$temp)
immuno$temp=factor(immuno$temp,levels=levels(immuno$temp)[c(2,1)])
immuno$co2=as.factor(immuno$co2)
immuno$co2=factor(immuno$co2,levels=levels(immuno$co2)[c(2,1)])
```

Statistical Analysis

Hemocyte Concentration

```
shapiro.test(immuno$hemo[immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$hemo[immuno$trmt == "LTLC"]
## W = 0.86052, p-value = 0.1909
```

```
shapiro.test(immuno$hemo[immuno$trmt=="LTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$hemo[immuno$trmt == "LTHC"]
## W = 0.90446, p-value = 0.401
```

```
shapiro.test(immuno$hemo[immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$hemo[immuno$trmt == "HTLC"]
## W = 0.89348, p-value = 0.3368
```

```
shapiro.test(immuno$hemo[immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$hemo[immuno$trmt == "HTHC"]
## W = 0.9205, p-value = 0.509
```

```r
bartlett.test(immuno$hemo,immuno$trmt)
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$hemo and immuno$trmt
## Bartlett's K-squared = 3.7755, df = 3, p-value = 0.2868
```

```r
hemo.aov=aov(hemo~co2*temp,data=immuno)
summary(hemo.aov)
```

```
##             Df    Sum Sq   Mean Sq F value  Pr(>F)
## co2          1 1.385e+14 1.385e+14  10.223 0.00452 **
## temp         1 6.407e+12 6.407e+12   0.473 0.49959
## co2:temp     1 9.754e+12 9.754e+12   0.720 0.40624
## Residuals   20 2.710e+14 1.355e+13
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```r
TukeyHSD(hemo.aov)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = hemo ~ co2 * temp, data = immuno)
##
## $co2
##          diff     lwr     upr     p adj
## hi-lo 4805000 1670263 7939737 0.0045228
##
## $temp
##           diff      lwr     upr    p adj
## hi-lo -1033333 -4168070 2101403 0.499592
##
## $`co2:temp`
##                    diff       lwr        upr     p adj
## hi:lo-lo:lo  6080000.0    131570 12028430.0 0.0440294
## lo:hi-lo:lo   241666.7  -5706763  6190096.7 0.9994554
## hi:hi-lo:lo  3771666.7  -2176763  9720096.7 0.3139916
## lo:hi-hi:lo -5838333.3 -11786763   110096.7 0.0555557
## hi:hi-hi:lo -2308333.3  -8256763  3640096.7 0.7017357
## hi:hi-lo:hi  3530000.0  -2418430  9478430.0 0.3692612
```

Proportion of Phagocytic Cells

```r
shapiro.test(immuno$phago[immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago[immuno$trmt == "LTLC"]
## W = 0.92822, p-value = 0.5664
```

```r
shapiro.test(immuno$phago[immuno$trmt=="LTHC"])
```

```
##
```

```
##  Shapiro-Wilk normality test
##
## data:  immuno$phago[immuno$trmt == "LTHC"]
## W = 0.88873, p-value = 0.3116
```

```
shapiro.test(immuno$phago[immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago[immuno$trmt == "HTLC"]
## W = 0.91651, p-value = 0.4806
```

```
shapiro.test(immuno$phago[immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago[immuno$trmt == "HTHC"]
## W = 0.93146, p-value = 0.5914
```

```
bartlett.test(immuno$phago,immuno$trmt)
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$phago and immuno$trmt
## Bartlett's K-squared = 2.2053, df = 3, p-value = 0.5309
```

```
phago.aov=aov(phago~co2*temp,data=immuno)
summary(phago.aov)
```

```
##             Df  Sum Sq  Mean Sq F value Pr(>F)
## co2          1 0.00526 0.005262   0.935  0.345
## temp         1 0.00015 0.000153   0.027  0.871
## co2:temp     1 0.00649 0.006489   1.153  0.296
## Residuals   20 0.11255 0.005628
```

Total Phagocytosis (cells/mL blood)

```
shapiro.test(immuno$phago_total[immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago_total[immuno$trmt == "LTLC"]
## W = 0.90598, p-value = 0.4105
```

```
shapiro.test(immuno$phago_total[immuno$trmt=="LTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago_total[immuno$trmt == "LTHC"]
## W = 0.96199, p-value = 0.835
```

```
shapiro.test(immuno$phago_total[immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago_total[immuno$trmt == "HTLC"]
## W = 0.88711, p-value = 0.3033
```

```
shapiro.test(immuno$phago_total[immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$phago_total[immuno$trmt == "HTHC"]
## W = 0.94038, p-value = 0.6622
```

```
bartlett.test(immuno$phago_total,immuno$trmt)
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$phago_total and immuno$trmt
## Bartlett's K-squared = 2.2567, df = 3, p-value = 0.5209
```

```
phago_total.aov=aov(phago_total~co2*temp,data=immuno)
summary(phago_total.aov)
```

```
##             Df    Sum Sq   Mean Sq F value  Pr(>F)
## co2          1 1.027e+14 1.027e+14  12.296 0.00222 **
## temp         1 5.675e+12 5.675e+12   0.679 0.41954
## co2:temp     1 1.063e+13 1.063e+13   1.272 0.27271
## Residuals   20 1.671e+14 8.353e+12
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
TukeyHSD(phago_total.aov)
```

```
##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = phago_total ~ co2 * temp, data = immuno)
##
## $co2
##          diff     lwr     upr     p adj
## hi-lo 4137500 1676253 6598747 0.0022208
##
## $temp
##          diff      lwr     upr     p adj
## hi-lo -972500 -3433747 1488747 0.4195382
##
## $`co2:temp`
##                    diff        lwr        upr     p adj
## hi:lo-lo:lo  5468333.3   797906.5 10138760.2 0.0181755
## lo:hi-lo:lo   358333.3 -4312093.5  5028760.2 0.9963840
## hi:hi-lo:lo  3165000.0 -1505426.8  7835426.8 0.2608910
## lo:hi-hi:lo -5110000.0 -9780426.8  -439573.2 0.0288583
## hi:hi-hi:lo -2303333.3 -6973760.2  2367093.5 0.5254072
## hi:hi-lo:hi  2806666.7 -1863760.2  7477093.5 0.3586414
```

Superoxide Anion Production (per cell)

```
shapiro.test(immuno$SO[immuno$SO>=0&immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO[immuno$SO >= 0 & immuno$trmt == "LTLC"]
## W = 0.83364, p-value = 0.1154
```

```
shapiro.test(immuno$SO[immuno$SO>=0&immuno$trmt=="LTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO[immuno$SO >= 0 & immuno$trmt == "LTHC"]
## W = 0.762, p-value = 0.02604
```

```
shapiro.test(immuno$SO[immuno$SO>=0&immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO[immuno$SO >= 0 & immuno$trmt == "HTLC"]
## W = 0.94194, p-value = 0.6797
```

```
shapiro.test(immuno$SO[immuno$SO>=0&immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO[immuno$SO >= 0 & immuno$trmt == "HTHC"]
## W = 0.59174, p-value = 0.0003642
```

```
bartlett.test(immuno$SO[immuno$SO>=0],immuno$trmt[immuno$SO>=0])
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$SO[immuno$SO >= 0] and immuno$trmt[immuno$SO >= 0]
## Bartlett's K-squared = 8.5852, df = 3, p-value = 0.03535
```

```
SO.aov=aov(SO~co2*temp,data=immuno[immuno$SO>=0,])
summary(SO.aov)
```

```
##             Df Sum Sq Mean Sq F value Pr(>F)
## co2          1 0.0000 0.00000   0.000  0.998
## temp         1 0.0000 0.00004   0.001  0.979
## co2:temp     1 0.0975 0.09749   1.784  0.197
## Residuals   19 1.0381 0.05464
```

Superoxide Anion Production (per mL blood)

```
shapiro.test(immuno$SO_total[immuno$SO_total>=0&immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
```

```
## data:  immuno$SO_total[immuno$SO_total >= 0 & immuno$trmt == "LTLC"]
## W = 0.84385, p-value = 0.1403
```

```
shapiro.test(immuno$SO_total[immuno$SO_total>=0&immuno$trmt=="LTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO_total[immuno$SO_total >= 0 & immuno$trmt == "LTHC"]
## W = 0.84594, p-value = 0.1459
```

```
shapiro.test(immuno$SO_total[immuno$SO_total>=0&immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO_total[immuno$SO_total >= 0 & immuno$trmt == "HTLC"]
## W = 0.96724, p-value = 0.8572
```

```
shapiro.test(immuno$SO_total[immuno$SO_total>=0&immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$SO_total[immuno$SO_total >= 0 & immuno$trmt == "HTHC"]
## W = 0.57486, p-value = 0.0002254
```

```
bartlett.test(immuno$SO_total[immuno$SO_total>=0],immuno$trmt[immuno$
                                                    SO_total>=0])
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$SO_total[immuno$SO_total >= 0] and immuno$trmt[immuno$SO_total >= 0]
## Bartlett's K-squared = 17.133, df = 3, p-value = 0.0006635
```

```
SO_total.aov=aov(SO_total~co2*temp,data=immuno[immuno$SO_total>=0,])
summary(SO_total.aov)
```

```
##             Df    Sum Sq   Mean Sq F value Pr(>F)
## co2          1 2.887e+13 2.887e+13   2.990  0.100 .
## temp         1 1.016e+12 1.016e+12   0.105  0.749
## co2:temp     1 4.155e+12 4.155e+12   0.430  0.520
## Residuals   19 1.835e+14 9.655e+12
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

Lysozyme Activity

```
shapiro.test(immuno$lyso[immuno$lyso>=0&immuno$trmt=="LTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$lyso[immuno$lyso >= 0 & immuno$trmt == "LTLC"]
## W = 0.95952, p-value = 0.816
```

```r
shapiro.test(immuno$lyso[immuno$lyso>=0&immuno$trmt=="LTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$lyso[immuno$lyso >= 0 & immuno$trmt == "LTHC"]
## W = 0.75428, p-value = 0.03259
```

```r
shapiro.test(immuno$lyso[immuno$lyso>=0&immuno$trmt=="HTLC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$lyso[immuno$lyso >= 0 & immuno$trmt == "HTLC"]
## W = 0.89819, p-value = 0.4
```

```r
shapiro.test(immuno$lyso[immuno$lyso>=0&immuno$trmt=="HTHC"])
```

```
##
##  Shapiro-Wilk normality test
##
## data:  immuno$lyso[immuno$lyso >= 0 & immuno$trmt == "HTHC"]
## W = 0.89732, p-value = 0.3583
```

```r
bartlett.test(immuno$lyso[immuno$lyso>=0],immuno$trmt[immuno$lyso>=0])
```

```
##
##  Bartlett test of homogeneity of variances
##
## data:  immuno$lyso[immuno$lyso >= 0] and immuno$trmt[immuno$lyso >= 0]
## Bartlett's K-squared = 7.1093, df = 3, p-value = 0.06849
```

```r
lyso.aov=aov(lyso~co2*temp,data=immuno[immuno$lyso>=0,])
summary(lyso.aov)
```

```
##             Df Sum Sq Mean Sq F value Pr(>F)
## co2          1   0.00   0.002   0.001  0.982
## temp         1   4.21   4.208   1.275  0.274
## co2:temp     1   3.65   3.653   1.107  0.307
## Residuals   18  59.39   3.299
```