PREDATORY STRATEGIES AND BEHAVIORS OF *OCTOPUS RUBESCENS* IN RESPONSE TO OCEAN WARMING AND ACIDIFICATION

by

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For Max,
thank you for all your love and support

ABSTRACT

Increasing ocean temperature and acidity due to anthropogenic climate change poses threats to marine organisms. Climate change-related research conducted on marine organisms has focused on physiological mechanisms, with few looking at the interactive effects of acidification and warming. There is limited research investigating the behavioral effects of climate change on complex invertebrates, such as cephalopods. It is imperative to look at the behavioral response of cephalopods because they are ecologically and economically important. Behavioral changes of cephalopods due to climate change may have far-reaching implications that could alter ecosystem structure and function because they are highly adaptable generalist predators. I investigated the predatory strategies and drilling behavior of *Octopus rubescens* following a two-week exposure to year 2100-projected pH and temperature treatments. The predatory strategies I measured include latency to attack, striking distance, type of attack, predator-prey orientation, and body pattern during attack. I analyzed drill hole cluster variability using multi-distance spatial cluster analysis. Results indicate that elevated warming and temperature does not elicit an effect on predatory and drilling behaviors. These results are in contrast with similar research suggesting more investigation is needed to look into the behavioral responses of cephalopods. However, a significant difference in cluster variability exists between the anterior and posterior end of the *Venerupis philippinarum* shell, indicating that multi-distance spatial analysis can be utilized to discern point patterns of octopus drill holes. The outcome of this study suggests *O. rubescens* from the Salish Sea may be a population that is resilient to future ocean conditions.

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INTRODUCTION

Climate Change

Atmospheric carbon dioxide (CO_2) concentrations have increased by more than 40%, from 280 ppm to over 400 ppm, since the start of the industrial revolution. (Collins et al. 2013; Pachauri et al. 2015; Royal Society 2005). These CO_2 emissions are primarily due to fossil fuel use and deforestation (Sabine 2004; Sabine and Feely 2007). If we maintain our current trajectory, it is projected that CO_2 will reach over 1000 ppm by the end of the century (Collins et al. 2013; Pachauri et al. 2015; Royal Society 2005).

With the partial pressure of CO_2 (pCO_2) of the ocean surface being in approximate gas equilibrium with atmospheric CO_2 (Doney et al. 2009, 2012), the pCO_2 of the ocean rises alongside atmospheric concentrations (Doney et al. 2009, 2012).When CO_2 is absorbed by the ocean, it reacts with water to form carbonic acid (H_2CO_3) which dissociates into a bicarbonate ion (HCO_3^-) and a proton (H^+).The increasing proton concentration consequently causes the ocean pH to drop (Dickson et al. 2007; Riebesell et al. 2011). This decrease in ocean pH due to the uptake in atmospheric CO_2 is known as ocean acidification (OA) (Doney et al. 2009).

In addition to OA, the ocean is warming. Increasing atmospheric CO_2, along with other greenhouse gas emissions (GHG), has caused average global temperature to rise, creating excess heat which is absorbed by the ocean (Doney et al. 2009, 2012; Feely et al 2009; Pachauri et al 2015). The Representative Concentration Pathways (RCPs) are four end-of-century predictions that forecast potential climate responses to greenhouse gas emissions and atmospheric concentrations, air pollutant emissions and land use (Pachauri et al. 2015). RCP 8.5 is the most extreme of these scenarios which predicts the average global temperature to increase 3.7 - 4.8°C and ocean pH to decrease another 0.3 – 0.5 pH units (Collins et al. 2013.; Pachauri et al. 2015;

Royal Society 2005). These changes to ocean chemistry and temperature may affect numerous marine species by altering individual physiological performance which changes their behavior, affecting their population and trophic dynamics (Sabine 2004; Fabry et al. 2008; Doney et al. 2009), leading to changes in marine ecosystem functioning and biodiversity (Sabine 2004; Fabry et al. 2008; Doney et al. 2009; Harvey et al. 2013).

Marine Animal Behavior and Climate Change

Extensive reviews looking into the effects of OA and warming have reported responses varying in direction and magnitude (Harvey et al. 2013; Kroeker et al. 2013; Clements and Hunt 2015; Nagelkerken and Munday 2016). Much of the research looking into the behavioral effects of ocean warming and acidification have been conducted on coral reef fishes (Watson et al. 2013). These studies observed numerous detrimental changes in predator recognition, avoidance, and detection, prey capture, attack, and feeding rates, and prey detection (Allan et al. 2013; Clements and Hunt 2015; Nagelkerken and Munday 2016). However, a recent study attempted to replicate the effects of OA on fish behavior and found the effects to be negligible and results not reproducible (Kwan et al. 2017; Clark et al. 2020). In some fish species, the interaction of the OA and warming created different patterns in fish predatory behavior compared to when the effects were studied in isolation (Ferrari et al. 2015; Clements and Hunt 2015; Nagelkerken and Munday 2016). Despite the diverse results of these studies, each indicate the adverse effects of climate change to ecologically important predator-prey interactions (Allan et al. 2013; Ferrari et al. 2015)

Invertebrate studies looking into the predator-prey behavioral effects of elevated CO_2 resulted in a range of responses in their feeding, predatory avoidance, and defense behavior (Clements and Hunt 2015). Negative effects include the hindered predator avoidance by the

conch snail (Watson et al. 2013), and the reduction in predation rates of the cone snail (Watson et al. 2017). A positive effect observed includes the increased self-right ability of juvenile muricid snails (Manríquez et al. 2013). Slight changes in ocean temperature altered the predation rates of purple sea star, a keystone species whose presence maintains the biodiversity of intertidal zone (Sanford 1999). Additionally, the interaction of the OA and warming created different patterns in adult dog whelks as elevated CO_2 decreased movement speed, increased foraging distance and prey handling time, but foraging time was unaffected. When warming was introduced, it negated the effects of OA on movement speed and foraging distance (Queirós et al. 2015). Again, the varied outcomes of these studies investigating OA and warming showcases their adverse effects on invertebrate behavior.

Cephalopod Studies and Climate Change

Studies investigating the potential effects of OA and warming on more complex mollusks, such as cephalopods, have primarily focused on physiological traits. The physiological effects of climate change on cephalopods have been studied in numerous species. Elevated CO_2 caused metabolic suppression in squid (Rosa and Seibel 2008; Hu et al. 2014), but increased metabolic rate in octopus (Onthank et al. 2021). However, no change in metabolic rate has been observed in other studies of squid (Birk et al. 2018) and in cuttlefish (Gutowska et al. 2008). The varied results are perhaps attributable to the difference in species, life stages, and methods used to conduct each respective study.

Other research has looked into the acid-based regulatory mechanisms of cephalopods. These mechanisms enable cephalopods to stabilize their blood pH in hypercapnic or hypoxic environments by increasing their bicarbonate concentrations (Gutowska et al. 2010; Hu et al. 2013, 2014). Researchers speculate that these strong acid-base regulatory mechanisms may give

cephalopods an advantage over other species in the face of climate change (Strobel et al. 2012; Birk et al. 2018)

Cephalopod Behavior and Climate Change

Behavior is driven by internal physiological processes and external factors (Clements and Hunt 2015; Nagelkerken and Munday 2016) and is one of the first measurable responses to environmental change. Since, animal behavior is influenced by environmental conditions, understanding animal behavior can indicate the welfare of a particular species or population (Clements and Hunt 2015; Nagelkerken and Munday 2016; Beever et al. 2017; Hofmeister et al. 2018). Human-induced environmental perturbations, such as climate change, drive physiological and behavioral changes in marine animals (Doney et al. 2012; Dupont and Pörtner 2013; Pörtner et al. 2004). These changes can affect species interactions, ultimately changing marine ecosystem and biodiversity. However, in the past, climate change studies have paid minimal attention to animal behavior (Clements and Hunt 2015; Nagelkerken and Munday 2016). Studying behavior has now gained considerable attention in recent years with the realization of its far-reaching implications in the marine ecosystem. (Clements and Hunt 2015; Nagelkerken and Munday 2016).

There have only been three studies that have researched the behavioral effects of climate change on cephalopods, all studied the effects of OA on squid (Spady et al. 2014, 2018; Zakroff et al. 2018). Elevated CO_2 altered activity and defense in *Idiosepius pygmaeus* (Spady et al. 2014), altered predatory behaviors and strategies in *I. pygmaeus* and *Sepioteuthis lessoniana* (Spady et al. 2018), but only slightly altered paralarval swimming behavior of juvenile *Doryteuthis pealeii* (Zakroff et al. 2018). No investigation has looked at the behavioral responses of octopuses to climate change stressors.

Cephalopod Predatory Behavior

Cephalopods are important organisms due to their dual role as both predator and prey (André et al. 2010; Boyle and Rodhouse 2006). This duality may create a structuring role in marine ecosystems, because cephalopods link different trophic levels and food webs from different habitats (de la Chesnais et al. 2019). Not only are cephalopods important ecologically, they have had a significant and growing presence in global fisheries (FAO 2013, 2018; André 2010). Shifting environmental factors can alter the predatory strategies of cephalopods, therefore understanding the predatory responses of cephalopods can be helpful in forecasting the impacts of ocean change and providing insight to better aid future management strategies (André et al. 2010).

Octopuses are cephalopods which are common predators in marine intertidal and subtidal communities (Ambrose and Nelson 1983; Mather 1993). They are versatile and opportunistic predators that utilize visual and chemotactile senses to hunt and forage for prey (Fiorito and Gherardi 1999; Hanlon and Messenger 2018). Their diet can include crustaceans, bivalves, fish, gastropods, and other cephalopods (Wodinsky 1969; Cortez et al. 1998; Steer and Semmens 2003). Prey abundance and distribution can potentially be influenced by the octopus, because of their ability to consume so many species (Ambrose and Nelson 1983).

Attack Sequence

Octopuses can visually discriminate size, shape and orientation, allowing them to detect prey (Hanlon and Messenger 1996; Mather and Alupay 2016). However, prey do not need to move to be recognized as a food source since octopuses also consume stationary bivalves and gastropods (Hanlon and Messenger 1996). In studies where octopuses were fed crabs, the attack sequence typically follows a series of events where the octopus reduces the distances between

itself and the prey and ends at prey capture or escape (Maldonado 1964; Warren et al. 1974). Maldonado et al. (1964) analyzed the movements of the attack by studying the changes of acceleration throughout the attack sequence and divided into three parts: first time delay, second time delay, and final pattern of acceleration. The final two parts combined made up "movement time" where the octopus moved towards the prey. Before the "final pattern of acceleration" is a constant near-zero movement, followed by maximum acceleration, maximum deceleration, then prey capture. Warrant et al. (1974) described the attack sequence behaviors as successive "phases" known as before, detect, attack, land, capture, withdraw, and after (Phase I, II, III, IV, V, VI, VII). "Attack" in the Warrant et. al (1974) study included all movement made by the octopus to capture the crab. Henceforth, for clarity, I define "attack" as the final pattern of acceleration, or final movements, the octopus makes before capturing its prey.

The two most common types of attacks observed in octopus are the pounce, where the animal jumps toward the prey with its interbranchial web open, or the side arm grab (Hanlon and Messenger 1996; Mather and Alupay 2016). When the attack sequences commence, changes in body pattern and coloration occur (Warren et al. 1974; Hanlon and Messenger 1996). It is unknown why these changes occur, but previous research concludes that they are nonsystematic and inevitable due to changes in locomotor activity during attacks (Warren et al. 1974).

Drilling Behavior

Octopus are unique among cephalopods because they prey on organisms with strong defense mechanisms, such as crustaceans, bivalves and gastropods (Cortez et al. 1998). When preying on a shelled organism, octopuses face the problem of having to extract the visceral mass from out of the shell (Pilson and Taylor 1961; Wodinsky 1969; Anderson et al. 2008). Bivalves have adductor muscles that keep their shells closed and gastropods have their columellar muscle

to pull themselves into their shell and some have an operculum to seal their aperture (Fiorito and Gherardi 1999). Depending on the species, an octopus will exhibit different shell penetration techniques (Pilson and Taylor 1961; Anderson et al. 2008). First, an octopus will attempt to pull apart the shelled organism which requires a lot of energy, but a short handling time (Ebisawa et al. 2011). If the pulling action fails, the octopus will switch strategies and begin drilling their prey with the use of their radula and/or salivary papilla (Nixon 1979a, b; Ebisawa et al. 2011). When the shell has been penetrated, the salivary papilla is inserted into the hole to inject venom that paralyze the prey (Pilson and Taylor 1961; Fiorito and Gherardi 1999). Once the prey is weakened, the octopus is able to extract the prey from its shell and consume it (Steer and Semmens 2003; Anderson et al. 2008).

Drill hole Localization

Octopuses drilling behavior is selective because the location of these drill holes is not random, but are localized to certain places depending on the prey species (Ambrose and Nelson 1983; Cortez et al. 1998; Steer and Semmens 2003; Anderson et al. 2008; Blustein and Anderson 2016). Gastropods are drilled on their apical spire, while bivalves are drilled at their adductor muscle attachment, with a preference for the anterior muscle, or pallial line (Ambrose and Nelson 1983; Nixon and Maconnachie 1988; Runham et al. 1997; Cortez et al. 1998; Anderson et al. 2008).

In the Superfamily Veneroidea, the shell beneath the areas of muscle attachment is termed the myostracum (Taylor et al. 1969). The shells are two-layered, outer and inner, and are completely made up of aragonite (Taylor et al. 1969; Ambrose et al. 1988). The outer layer can be broken down into two arrangements. Aragonite crystals arranged radially make up the outermost arrangement of the outer layer (Taylor et al. 1969; Ambrose et al. 1988). The next

arrangement is made up of complex crossed laminar structure, which abruptly ends at the pallial line (Taylor et al. 1969; Ambrose et al. 1988). The pallial line is where the mantle muscles attach to the shell and is joined by the adductor muscle scars. At the pallial and adductor myostracum, the structure of the shell does not have a complex crossed laminar structure. Instead, the shell structure is made up of irregular prisms known as "myostracal pillars." The shift in shell structure from a complex crossed laminar to mystrocal pillar could explain the tendency for drill holes to occur at the edges of myostracum in bivalves. Perhaps, the junction between the two structures represents a "weak spot" in the shell that the octopus can identify. At this time, this question remains unanswered. In addition, the causal mechanisms that induce drilling behavior, how drill hole localization occurs, along with why the anterior adductor muscle is preferred has yet to be determined. By understanding drill hole localization characteristics, we familiarize ourselves to typical predatory activity and can recognize when atypical behaviors or changes occur.

Objectives and Hypothesis

It is important to understand how changing environmental conditions may impact the predatory response of octopuses as this information will be essential in assessing the risks that climate change will have on these animals, their population, and community. Alterations in predatory behavior due to climate change may have far-reaching implications that we have yet to understand. Therefore, the goal of the proposed research project is to investigate the impacts of OA, warming, and their co-occurrence on the predatory and drilling behaviors of a common octopus species in the northeastern Pacific Ocean, *Octopus rubescens.*

The predator behavior parameters that I will measure include latency to attack, striking distance, predator to prey orientation, type of attack, and body pattern during the attack. I

hypothesize to see a difference in these parameters between treatment groups and predict that the interaction of climate stressors will exacerbate the effects. I also will compare drill hole clustering variability between CO_2 and temperature treatment groups using multi-distance spatial cluster analysis, a more robust method of discerning spatial patterns. I hypothesize that there will be a difference in drill hole clustering variability between treatment groups and predict that future ocean conditions will increase this variability.

METHODS

Octopus Collection

I collected thirty-two *Octopus rubescens* by SCUBA from Driftwood County Park, Whidbey Island, WA. The octopuses were on the ocean floor in discarded glass bottles which I placed in sealable plastic bags and then brought to the surface. I collected any octopuses that appeared, so sampling was haphazard. The octopuses that appeared too small, gravid, or senescent were not used leading to a biased sample. If the octopus looked less than 50 g, or smaller than a golf ball, I deemed too small because I assumed that smaller octopuses would not be able to handle the stress of treatments and I wanted to keep mortality rates low. I then transferred the octopuses out of the glass bottles into red plastic bottles which I transported to Rosario Beach Marine Laboratory (RBML) in Anacortes, WA.

I recorded the mass and sex of each octopus upon arrival to RBML and placed each octopus into a 27.5 L enclosure which were connected to the ambient flow-through seawater system. Octopuses acclimated to laboratory conditions for a minimum of one week; acclimation time ranged from 7 to 33 days.

I only used twenty-nine octopuses for the experiments out of the original thirty-two octopuses collected. The three octopuses I did not use stopped eating while acclimating to laboratory conditions. After acclimation, I randomly placed octopuses into 113.5 L recirculating slow flow-through coolers with inner dimensions of 34” L x 14” W x 13.25” H. A predetermined treatment was assigned to each 113.5 L cooler before housing the octopus.

Animal Husbandry

I fed the octopuses *Hemigrapsus nudus* crabs *ad libitum* while acclimating to laboratory conditions. Octopuses were also exposed to an approximate 12 hour light cycle. It should be

noted that octopuses captured during the summer months may have been exposed to more light compared to animals captured during the fall months due to ambient sunlight entering through the glass doors of the seawater hallway where the specimens were kept before treatment. The light schedule stayed the same when animals were transferred to the experimental treatment tanks, but the feeding regimen changed. I fed octopuses *Venerupis philippinarum* clams while they were in treatment. The octopuses always had at least two clams in the experimental tank with them. This was done to prevent starvation by ensuring there was enough food for sufficient lipid absorption (Onthank and Cowles 2011).

Octopuses were conditioned to light exposure during the acclimation period. Additionally, octopuses were exposed to direct overhead lighting during the daily experimental tank calibrations. This conditioning was done to prevent the specimens from being "alarmed" during the predatory behavior trials.

Tank Design & Control System

Tank design and control systems utilized for this experiment were based on the thesis work of Culler (2019) (Fig. 1). I modified the design by adding 201.34 W Hydor-in-line external heater (12.7 mm or 15.8 mm outer diameter) in between the chiller and venturi injector. The external heaters made it possible for the high-temperature treatment tanks to approach the target value of 15.8 °C. External heaters were added to only half of the twelve treatment tanks.

I made another alteration to the design by adding a 6.4 mm hose barb to 4.8 mm hose barb nylon reduction coupler connecting the tubing between the venturi injector and CO_2 scrubber. The addition of the coupler created a tighter seal between the venturi injector and CO_2 scrubber, with the hope that this change would decrease the waste of CO_2 scrubbed air. Finally, with the addition of the external heater, I updated the software controlling temperature to include

a heat or chill function that would control either the heater or chiller rather than just the chiller.

Carbonate Chemistry Measurements

I measured pH (pH_T), total alkalinity (A_T), salinity, and temperature to calculate and control the carbonate chemistry of each tank. I compared each method of measurement to a reference material to ensure accuracy (Fig. 2). Because A_T would vary in time, I used measured A_T along with target pCO_2, salinity and temperature of the tank to calculate the target pH.

I used pH and alkalinity measurements based on the methods developed by Culler (2019), which in turn were modifications of from Dickson et al. (2007). I measured the pH of each tank at least six times a week. This method of measuring pH achieved an accuracy of 0.002 pH units. Once the pH of the tank was calculated, I calibrated the pH probes to the calculated pH value while the probe was submerged in the seawater of the tank (Culler, 2019).

I used two seawater samples of known salinity, alkalinity, and pCO_2 to calibrate the spectrophotometric method for measuring pH_T. Certified reference material (CRM) of known A_T and salinity were obtained from the Dickson Lab at Scripps Institute of Oceanography in San Diego, CA. I bubbled the CRM with water saturated NorLab certified gas mixtures of either 199 ± 2ppm CO_2 or 1490 ± 15p ppm CO_2 to obtain a CRM sample of known pCO_2. I calculated the pH_T of the gas-saturated CRM using its known A_T, salinity, and pCO_2. I used these values to calibrate the spectrophotometric method of measuring pH_T.

I measured the alkalinity of each tank once a week. I conducted titrations on CRM of known alkalinity, achieving an accuracy of $\pm$ 8 μmol kg^{-1}. I used weekly alkalinity measurements and target pCO_2 values to determine the pH_T setpoints of each tank.

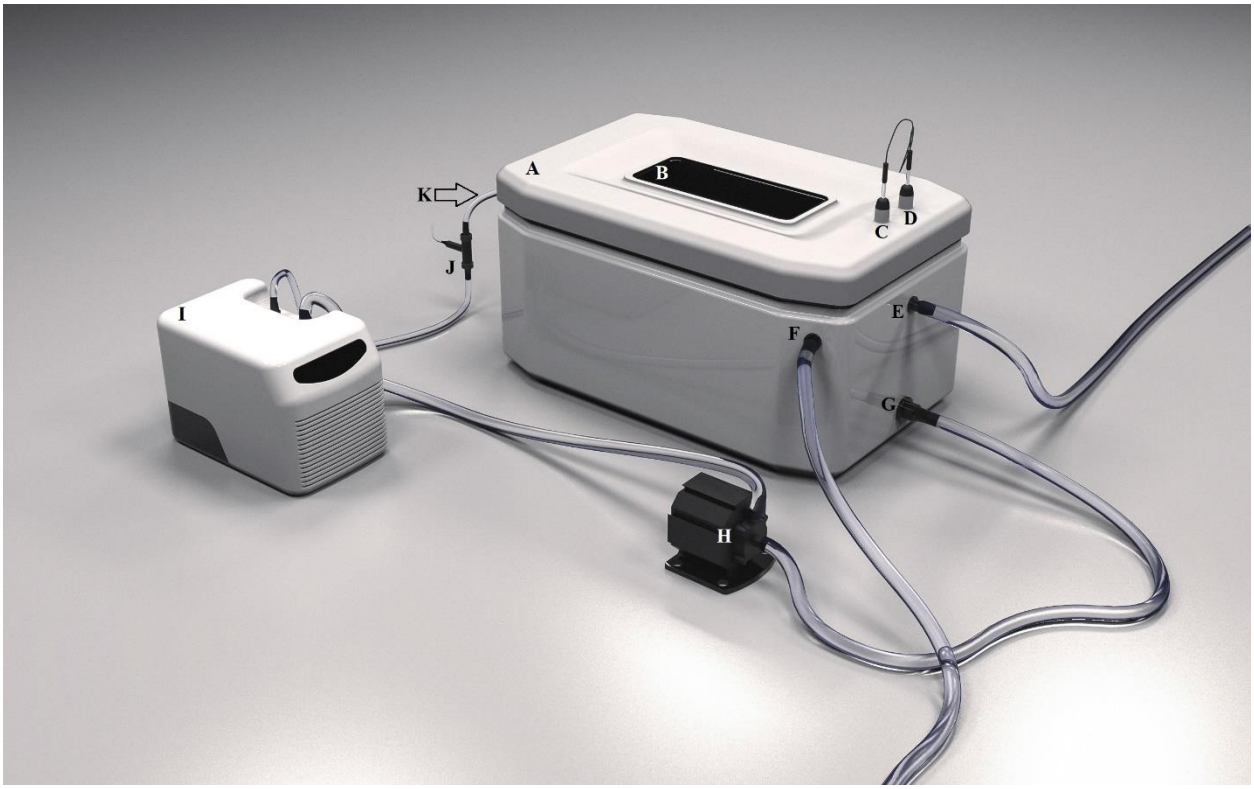

Figure 1. Model of custom tank system designed by Monica Culler. Modeled and rendered by Jon Spracklen. A) Holding tank for octopus; B) Animal viewing window; C) Single-junction pH probe connected to custom control hardware; D) PT-100 temperature probe connected to custom control hardware; E) Water overflow at ~100 mL/min; F) Water inflow at ~100 mL/min; G) Recirculating closed-system water outflow; H) Water pump; I) Chiller; J) Venturi injector introduced CO_2-scrubbed air into circulation; K) Recirculating closed-system water inflow. Modifications (not shown) include the installment of a 200-watt Hydor In-line External Heater (½" or ⅝" outer diameter) in between the (I) chiller and (J) venturi injector.

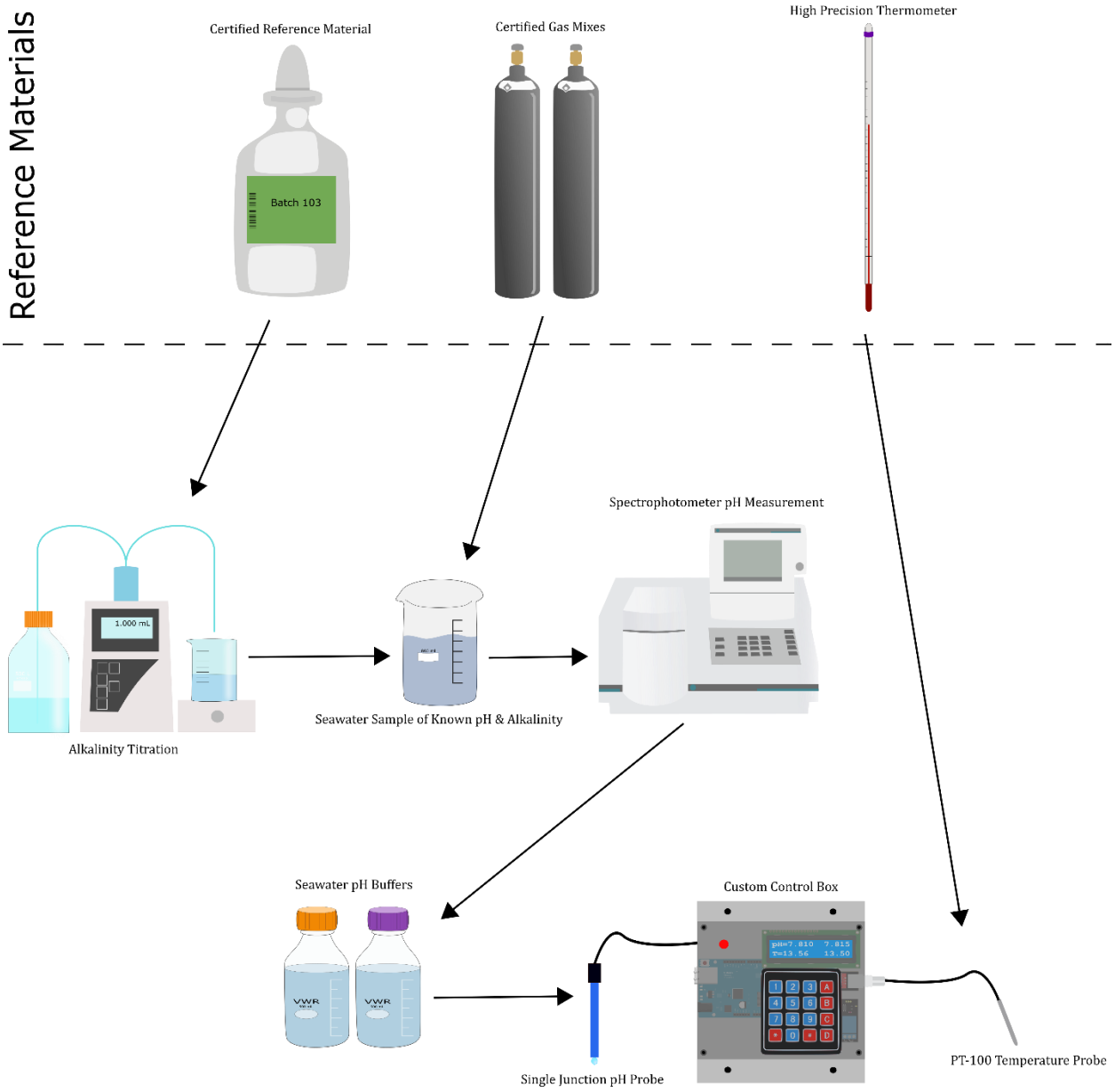

Figure 2. Flow chart of calibration protocol used by this study. Image rendered by Monica Culler.

I used PT-100 temperature probes to measure the temperature of the tanks. I calibrated the temperature probes daily against a high precision, NIST traceable thermometer. I used a Vernier Salinity Sensor calibrated against 35 ppt Vernier Salinity Standard to measure the salinity of seawater samples. Finally, I calculated the pCO_2 of each tank using the tank's respective values for temperature, salinity, pH_T, and A_T. I used the *seacarb* package in R (Gattuso et al. 2019) to for all carbonate chemistry calculations with the exception of the spectrophotometric pH calculations, which were made with OTools (Onthank 2019).

Treatments

Using a random number generator, I assigned octopuses to one of four experimental treatments after an acclimation period of at least one week. Experimental treatments were control pCO_2 /control temperature (CONTROL), control pCO_2 /projected temperature, projected pCO_2/control temperature, and projected pCO_2/projected temperature.

I acquired seawater at depth where octopuses were collected and used it to determine the control pCO_2. Seawater samples were transferred on ice to RMBL to minimize respiration. Once at RBML, I determined the salinity, temperature, alkalinity, and pH of the seawater sample to calculate the pCO_2.

To determine the control temperature, I obtained raw CTD data collected by the Hansville mooring located at 47° 54.44' N and 122° 37.62' W from Northwest Environmental Moorings. I chose this mooring because it was the closest buoy to the octopus collection site, at a distance of 28 km away. The raw data included the temperature profiles of the water column up to a depth of 100 meters between the dates of November 18, 2005 and May 28, 2019. I created a subset of the raw data to only include seawater temperatures during the months of June through

September, the months during which the experiment was conducted, and between the depths of 18 to 22 meters, where we typically find octopuses. I calculated the average temperature of the data to determine the control temperature. Next, I determined the range of temperatures at a depth between 18 to 22 meters (APPENDIX A). I established that the range of temperatures was 7.2 °C to 13.9 °C between the depths of 18 to 22 meters (APPENDIX A). I performed this step to verify that the projected treatment temperature was outside of the range of temperatures experienced by *O. rubescens* at depth. A linear regression analysis was used to determine if there was evidence supporting ocean temperature warming during the summers months between the years of 2005 and 2019. I observed that warming does occur over time, however, this increase was not significant ($r^2 = 0.308$, $F_{(1,8)} = 3.574$) (APPENDIX A).

I chose a projected temperature 3.7°C higher than the control temperature, and a projected pCO_2 treatment 550 µtm higher than the control based the RCP 8.5 predictions (Collins et al. 2013.; Pachauri et al. 2015). Control and projected temperatures were 12.1°C and 15.8°C, respectively. The projected temperature value was 1.9 °C warmer than the highest temperature measured at the Hansville mooring data. Control and treatment pCO_2 were 800 µatm and 1350 µatm, respectively.

I kept octopuses in treatment for 14 - 16 days. I recorded video of predatory behavior at the end of the treatment period. Afterwards, I removed the octopuses from treatment and placed them into enclosures connected to the flow-through ambient seawater system. Octopuses recovered in the enclosures before being released back to Driftwood County Park.

Predatory Behavior Trials

I recorded the predator-prey interactions of each octopus after their two-week exposure to treatment. The experiments were conducted in the treatment tanks to reduce the variability that arose from handling stress. I attached a GoPro Hero 7 digital camera to a custom-built mount

which I placed on top of the treatment tank to record the predator-prey interactions at 30-60 fps. Two 80-watt bulbs illuminated the tank to create light. I gave the octopuses ten minutes to acclimate to the mount and lights before recording. During this time, I measured the length of the carapace and mass of the crab. Predator to prey mass ratio was noted to control for these factors should it affect the dependent variables. After the acclimation time, I dropped a purple shore crab (*Hemigrapsus nudus*) into the tank on the opposite side of the octopus. I used a PVC chute pipe to drop the crab into the tank to avoid being seen by the octopus. Start time of the experiment began as soon as the crab touched the water and ended once the prey had been successfully captured, or ten minutes after the start of the trial. I extracted data from the videos with the use of either VLC 3.0.8 media player or ImageJ 1.52p. I repeated the predatory behavior trials per octopus up to three times, waiting 12 to 24 hours between each trial. If the octopus exhibited the same feeding behavior two trials in a row – captured crab or not captured crab – then, a third trial did not occur. I recognized afterwards that I should have conducted three trials for all octopuses, regardless of the feeding behavior pattern exhibited. Since I made this mistake, I only utilized one set of trial data per octopus. The trial selected was based on the fastest latency time.

Latency to Attack

Latency to attack is the amount of the time the octopus waited before attacking the crab. It encompasses the moment the crab touched the water until the moment the octopus tried to capture the crab either with a pounce or arm grab. I measured latency to attack in seconds using VLC 3.0.8 media player and the Time v3.2 add-on (VideoLan Organization). Time began as soon as the video started, and I noted the timestamp when the crab touched the water and when the octopus made its first attempt to capture the crab. The difference between the two timestamps (crab introduction and octopus' first attempt at capture) provided the latency to attack time in

seconds. If the octopus did not attempt to capture the crab within ten minutes, total latency time was 600 seconds.

Predator to Prey Orientation

I defined predator to prey orientation as the angle of the octopus to the prey right before the attack. Using the time when the octopus attacked the crab, I captured an image right before the attack using VLC 3.0.8 media player (VideoLan Organization). I used ImageJ 1.52p to measure predator to prey orientation and striking distance between the octopus and crab. I drew a horizontal line between the centers of the eyes (eye line) and a vertical line down the middle of the mantle perpendicular to the previous line (mantle line) to define 0° (Fig. 3). I ensured the lines were perpendicular by setting the Rotate Tool to 90°. The two lines created a crosshair which I used as the foundation to measure the predator to prey orientation and striking distance. The three points needed to use the Angle Tool in ImageJ 1.52p were the center of the crab, the center of the crosshair, and anterior point on the mantle line. Once all three points were selected, the angle was measured by selecting Analyze → Measure. Angle measurements never exceeded 180°. If a crab was on the left side of the octopus, the value obtained by the Angle Tool would be subtracted from 0° to obtain its true angle orientation. Values less than 0° represented the left side and values greater than 0° right side of the octopus.

Striking Distance

I defined striking distance as the distance between the octopus and crab the moment right before the attack, as typically the octopus will pause before attacking (Maldonado 1964). I used the same image from the previous section to measure striking distance. I used the center of the crosshair to measure the distance between the octopus and crab.

Choice of Attack

I defined choice of attack as the movement the octopus used to capture the crab. Potential attacks include the pounce and arm grab. I defined these behaviors based on previous research (Warren et al. 1974; Hanlon and Messenger 1996). The pounce involves a 'forward-jet propulsion' during which the arms trail behind the octopus. Before the octopus lands to capture the prey, the interbranchial web opens up like a parachute. An arm grab involves using the arm closest to the prey to seize it and pull it under its web. I evaluated predator behavior videos in VLC media player to determine the type of attack used and compared them between treatment groups.

Body Pattern

I defined body pattern as the octopus' color I observed during the attack and based these definitions on previous research (Warren et al. 1974). Warren et al. (1974) described pattern displays as being uniform, spotted, mottled combined with the colors grey, blush, red, or transparent. During an attack, only uniform color displays in blush or grey were observed by Warren et al. (1974). I reviewed each attack video and observed the colors grey, blush, or red. As previously stated, the attack behavior is identified as the movement the octopus makes in an attempt to capture the crab. This can be the movement of just an arm or the jetting of the octopus into a pounce.

Drill-hole Data Collection

I fed the octopuses *Venerupis philippinarum* (manila clams) while in treatment. I checked daily for discarded shells and replaced consumed clams. I labeled shells with the date, treatment, and cohort. I captured images of shells with a drill holes using a Canon EOS Rebel SL1. I compared drill holes spatial patterns between pCO_2 and temperature treatments, respectively. I

also compared drill holes spatial patterns between the anterior and posterior end of clams.

I made a clam outline and used it to standardize rotation and scale of all other clam images in GNU Image Manipulation Program v2.10.12 (GIMP). I positioned the outline over a clam image and adjusted the image so that the margin of the clam in the image matched the outline. I accomplished this by using the Scale Tool with the Keep Aspect guide turned off. I highlighted drill hole locations for easier detection later. Then, I placed a one-pixel box to surround the perimeter of the clam (Fig. 5). Vertically, the box spanned from the umbo to the ventral margin of the clam. Horizontally, the box spanned from the anterior to posterior end of the clam. Once all clam shells were standardized, I measured drill hole locations using g3data 1.5.4. Using the one-pixel box, the height of the clam defined the y-axis and the length of the clam defined the x-axis. Location of the drill hole was defined as a proportion to the (x,y) plane based on the shell.

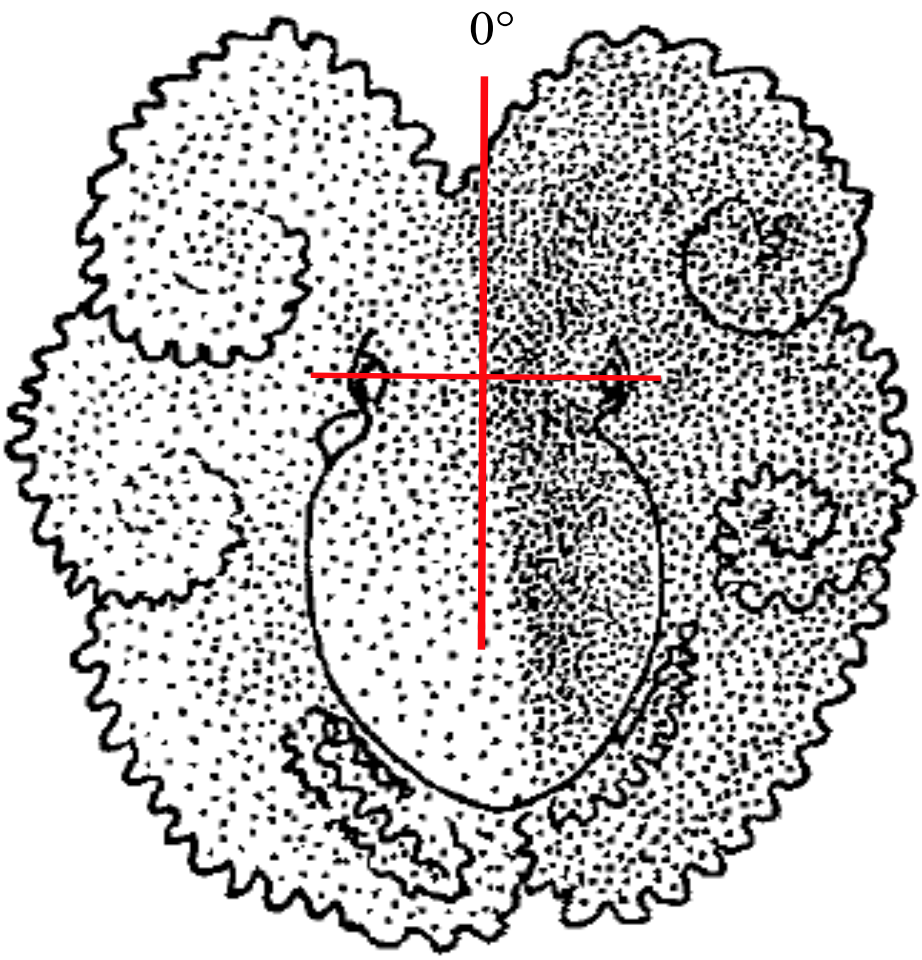

Figure 3. Top-down view of an octopus (Packard and Sanders 1971). A horizontal line between the eyes (eye line) and a vertical line down the middle of the mantle, perpendicular to the previous line (mantle line) defines 0°. Values less than and greater than 0° represented the left and right side of the octopus, respectively. Striking distance was measured from the center of the crosshair to the center of the crab (not shown).

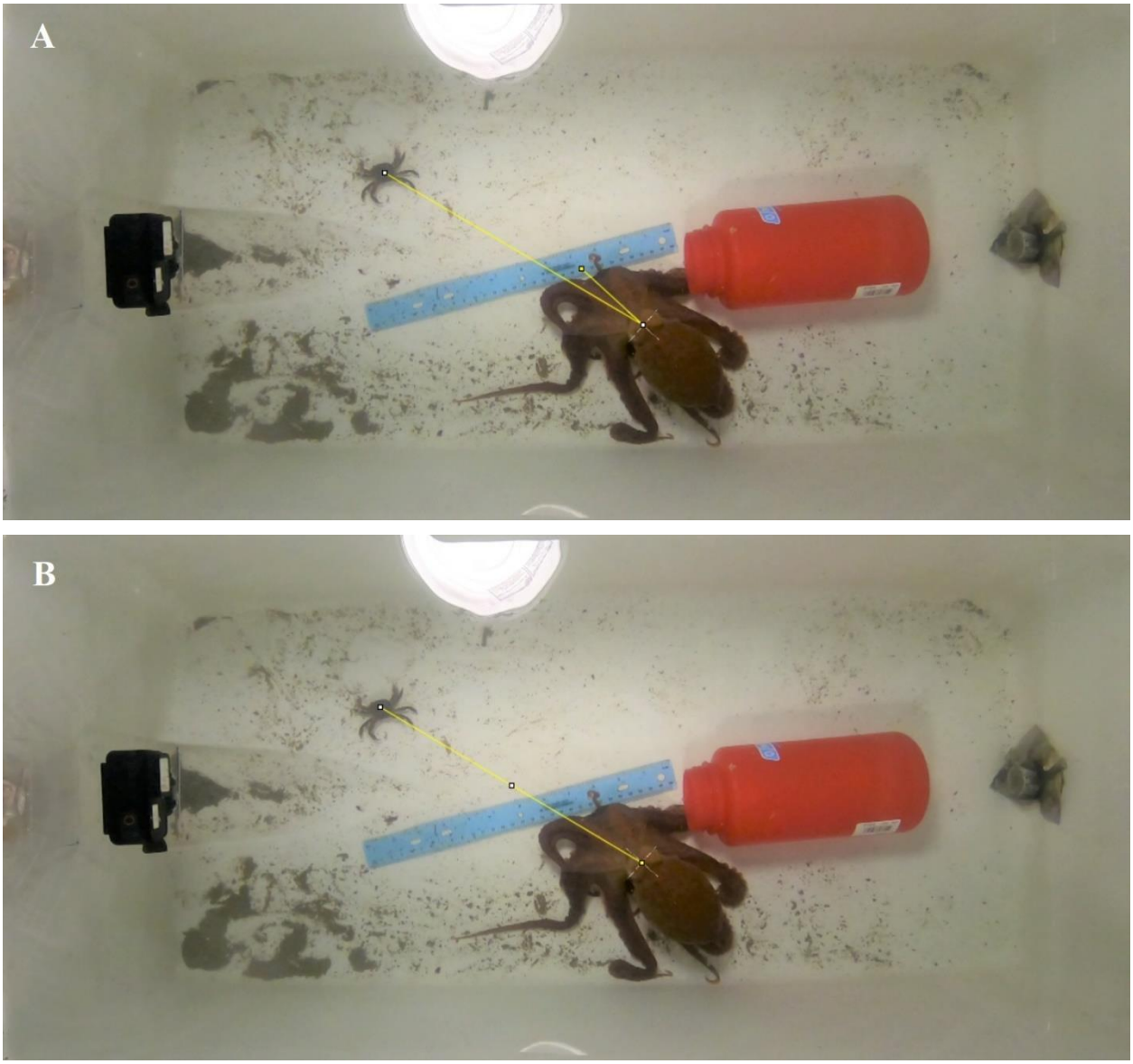

Figure 4. Two of the same image of *Octopus rubescens* right before attacking *Hemigrapsus nudus* showing the artificially drawn crosshair. The top images shows how to measure predator to prey orientation (A) and the bottom image shows how to measure striking distance (B) in ImageJ.

Figure 5. Artificial outline superimposed over an image of a discarded *Venerupis philippinarum* shell. The drill hole, located towards the left, has been highlighted for easier data extraction. A one-pixel box surrounds the perimeter of the clam. The horizontal plane (x) spans the from the anterior to posterior end of the clam. The vertical plane (y) spans from the umbo to the ventral margin of the clam.

Statistical Analysis

Predatory Behavior Trials

Out of the twenty-nine octopuses placed in treatment, twenty-one of them ate a crab during the trials. I used these twenty-one octopuses in the following analyses. I used both Multivariate Shapiro-Wilks tests and Bartlett Tests to test for the normality and homoscedasticity of the continuous variables: latency to attack, striking distance, and attack orientation. I cube root transformed latency to attack values and used a Box-Cox power transformation to determine the ideal power transformation to make striking distance values normally distributed. I determined the appropriate exponent to transform the striking distance data using the *boxcox* function in the *MASS* package in R (Venables & Ripley 2002). Once the assumptions were met, I ran a Two-way multivariate analysis of variance (MANOVA). I used separate Cochran-Mantel-Haenszel tests to compare the categorical variables: type of attack and body pattern.

Drill Hole Cluster Analysis

Ripley's K function is a spatial analysis method used to describe the spatial arrangement of point patterns. It allows us to discern whether patterns are clustered, random, or regularly dispersed over a range of distances. In brief, Ripley's K is calculated based off how many point pairs are within a given distance of each other. You can calculate Ripley's K for a series of distances with the following equation:

$$\widehat{K}(r) = \lambda^{-1} E_r$$

Where λ is the density of points, and E is the mean number of points within distance r of each point.

If the spatial distribution of points is random, the mean number of points that can be expected with distance r of any given point is equal to the point density (λ) times the area of the

circle with radius *r* (A). Therefore, we can replace *E* with λA to yield the expected K(*r*) for random data and simplify the above equation, as such:

$$\widehat{K}(r) = \frac{1}{\lambda}(\lambda A) = \frac{1}{\lambda}(\lambda \pi r^2) = \pi r^2$$

This demonstrates that the expected K(*r*) for spatially random points would be equal to the area of a circle with radius *r* (Fig. 6A). Clustered point patterns would yield more points within distance *r* of any given point than expected by a random distribution, and therefore would yield K(*r*) values greater than expected. Regular point patterns would yield K(*r*) values less than expected. Ripley's L is a transformation of Ripley's K for which expected values for spatially random points at all values of *r* are 0 (Fig. 6B).

$$\hat{L}(r) = \left(\frac{\widehat{K}(r)}{\pi}\right)^{\frac{1}{2}} - r$$

L(*r*) values above or below zero are considered clustered or regular, respectively. Randomization is used to determine if the K-function or L-function for an observed point pattern is significantly different from what is likely to be produced by a random point pattern (Fig. 6C). A random point pattern is produced with the same number of points, with the same density and in the same spatial window, and the K- or L-function is recalculated. This process is done many times and the 95% limits, or randomization envelope, of these functions produced from random point patterns is determined.

To determine the relative clustering of two separate point patterns, I took the difference between L(*r*) of each point pattern and refer to this as M(*r*) (M-function).

$$\widehat{M}(r) = \hat{L}(r)_{Group\ 1} - \hat{L}(r)_{Group\ 2}$$

The expected M(r) is set to zero, indicating that the relative clustering between two point patterns are equal. Since we are now determining the relative clustering between two point patterns, any M(r) values above zero indicates that $\hat{L}(r)_{Group\ 1}$ is larger than $\hat{L}(r)_{Group\ 2}$ and therefore group 1 is more tightly clustered than group 2, and any M(r) below zero indicates that $\hat{L}(r)_{Group\ 2}$ is larger than $\hat{L}(r)_{Group\ 1}$ and therefore group 2 is more tightly clustered than group 1.

But before using the M-function, I used Ripley's L function to determine if drill holes were clustered (Fig. 7). When I determined the pattern was significantly more clustered than random, I moved forward and used the M-function to compare relative degrees of clustering among sets of drill hole locations by treatment (Temperature or pCO_2). Next, I used the gap statistic method to determine the number of drill hole clusters, and k-mean clustering to assign drill holes to one of two clusters designated 'anterior' or 'posterior.' Finally, I compared degrees of clustering between the two clusters using the M function, as well. The process is similar when producing a randomization envelope for the M-function, except the observed points are randomized between the two groups, or treatments.

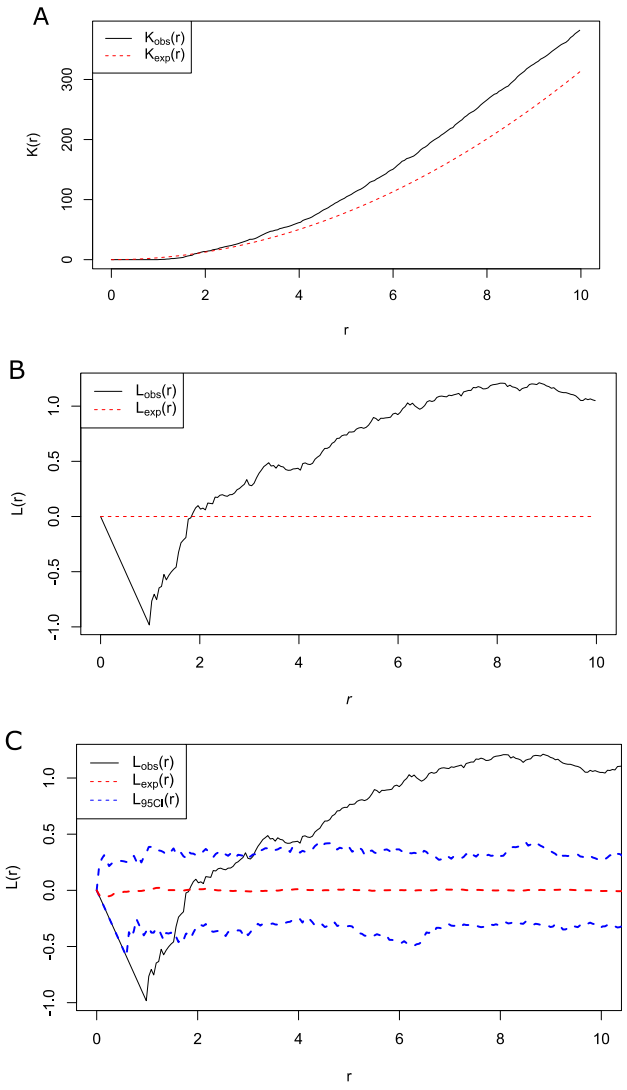

Figure 6. (A) Ripley's K-function. The red dashed and black solid lines are the expected K(r) and observed K(r), respectively, at multiple distances of *r*. (B) Ripley's L transformation of the previous graph. A regular spatial pattern is observed (L_{obs} is below zero) at lower values of *r* and a clustered pattern is observed (L_{obs} is above zero) at higher values of *r*. (C) Randomization creates a 95% confidence interval envelope (blue dashed lines) which surrounds the expected L(r). L(r) values outside the bounds of the confidence envelope indicates significance at distance *r*.

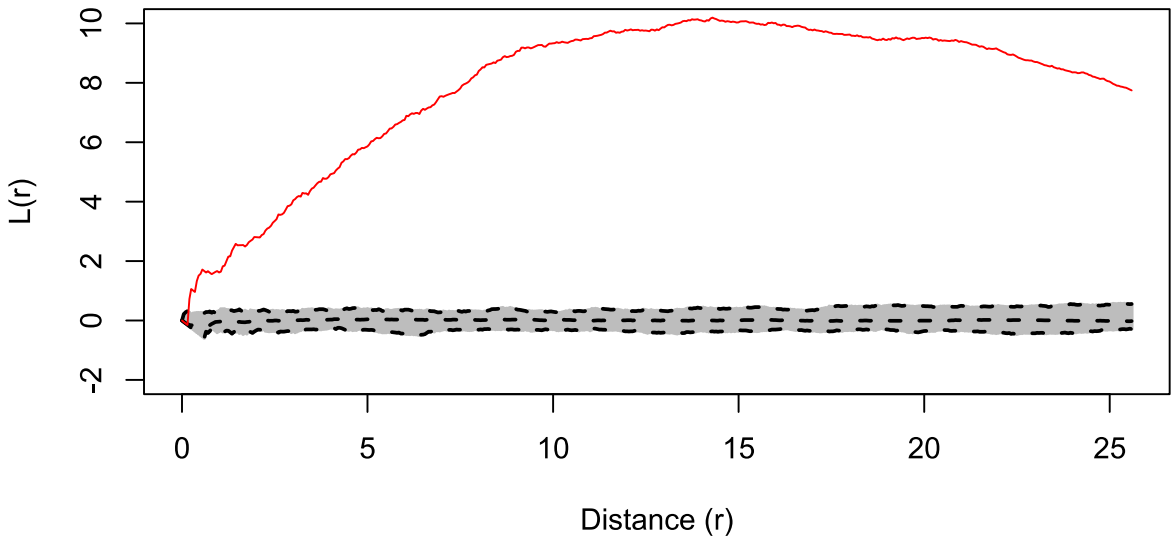

Figure 7. Clustered spatial pattern determination of drill hole on *Venerupis philippinarum* shells. The red line represents the observed L(r) above the significance envelope (black dashed lines) at all values of *r*.

RESULTS

Carbonate Chemistry

A custom pH-stat system which bubbled pure CO_2 into insulated aquaria controlled the carbonate chemistry of each treatment. The target control pCO_2 and temperature were 800 μatm and 12.1 °C, and the control pCO_2 and temperature achieved were 869.4 ± 172.1 μatm and 12.1 ± 0.2 °C, respectively (Table 1). The target experimental pCO_2 and temperature were 1350 μatm and 15.8°C, and the experimental pCO_2 and temperature achieved were 1347.0 ± 290.4 μatm and 15.4 ± 0.8 °C, respectively (Table 1). In addition, I determined the carbonate chemistry parameters experienced by every octopus used in the study (Table 2).

Predatory Behavior Trials

Predator-prey interactions between *Octopus rubescens* and *Hemigrapsus nudus* were recorded after a two-week exposure to treatment. After all videos were collected, latency to attack, striking distance, and predator-prey attack orientation were measured, while body pattern during attack and type of attack were categorized. CO_2 did not significantly affect latency to attack, striking distance, or predator-prey orientation (2-Way MANOVA; $p = 0.65, 0.07, 0.69$) (Table 3). Temperature did not significantly affect latency to attack, striking distance, or predatory-prey orientation (2-Way MANOVA; $p = 0.91, 0.3, 0.38$) (Table 3). The interaction of CO_2 and temperature did not significantly affect latency to attack, striking distance, or predatory-prey interaction (2-Way MANOVA; $p = 0.15, 0.75, 0.89$) (Table 3). Moreover, the effect of CO_2 on striking distance was nearly significant ($p = 0.07$). At 800 μatm of pCO_2, the average striking distances of octopuses in 12.1°C and 15.8°C were 31.57 cm and 21.07 cm, respectively. At 1350 μatm of pCO_2, the average striking distances of octopuses in 12.1°C and 15.8°C were 16.42 cm and 9.96 cm, respectively. I observed a decrease in striking distance from 800 μatm of pCO_2 to 1350 μatm of pCO_2. But this difference is not significant.

Drill Hole Analysis

Octopus rubescens were fed *Venerupis philippinarum* (manila clams) throughout treatment. I collected any discarded shells to determine if any drill holes were present. A total of 140 clams were consumed, where 80 of these clams had incomplete or completed drill holes. In eight instances, more than one drill hole was found on the same valve or separate valves of the clam. However, all drill holes were considered independent.

Once drill hole locations were determined, I used Ripley's L function and discerned a clustered point pattern on the *V. philippinarum* shells (Figure 12). Next, I used M(*r*) to determine if different factors affected relative cluster variability between two point patterns. I determined the 95% confidence interval, average M(*t*) value, and p-value for each factor (Table 4). The isolated effects of CO_2 and temperature did not create a difference in the relative degree of clustering between point patterns ($p = 0.82$ & 0.47, Table 4). However, I determined that there was a significant difference in the relative degree of clustering between the point patterns of the anterior and posterior end of the *V. philippinarum* shells ($p < 0.01$, Table 4).

Table 1: Seawater carbonate chemistry - pH_T, temperature (T), salinity (S), alkalinity (A_T), and pCO_2 for each treatment level. Values are the means and standard deviations.

	pH_T	St. Dev	T(°C)	St. Dev	S (ppt)	St. Dev	A_T(μmol/kg)	pCO_2(μatm)	St. Dev
Control	7.73	0.08	12.11	0.31	32.98	1.20	2103.29	859.84	168.61
Warming	7.72	0.08	15.51	0.80	33.23	0.95	2096.05	881.26	177.26
Acidified	7.55	0.08	12.09	0.08	33.10	1.22	2084.19	1328.57	262.73
Warming+Acidified	7.55	0.07	15.50	0.79	32.88	3.52	2091.59	1353.22	259.83

Table 2: Seawater carbonate chemistry - pH_T, temperature (T), salinity (S), alkalinity (A_T), and pCO_2 for each octopus. Values are the means and standard deviations

Octopus	pH_T	St. Dev	T(°C)	St. Dev	S (ppt)	St. Dev	A_T(μmol/kg)	pCO_2(μatm)	St. Dev
Control									
Arthur	7.71	0.07	12.13	0.05	31.68	0.92	2102.52	882.46	147.01
Drake	7.68	0.12	11.98	0.17	33.20	0.42	2108.50	985.01	289.71
Ernesto	7.76	0.08	12.08	0.07	31.71	0.38	2119.46	805.23	161.96
Ezra	7.73	0.05	12.03	0.13	33.18	0.40	2073.95	824.38	103.36
Kitty	7.73	0.05	12.53	0.85	32.21	1.25	2087.06	833.92	105.81
Mario	7.75	0.09	12.08	0.04	34.40	0.82	2086.50	797.49	160.60
Quill	7.72	0.07	12.09	0.04	34.19	0.37	2141.88	883.99	144.98
Uma	7.74	0.05	12.09	0.04	34.21	0.39	2106.39	817.48	89.81
Warming									
Bob	7.73	0.05	15.67	0.28	32.24	0.77	2113.69	862.76	105.81
Cory	7.74	0.12	14.91	1.30	33.31	0.48	2068.94	839.62	251.08
Crystal	7.68	0.08	15.44	0.48	32.47	0.44	2105.34	985.00	212.06
Hank	7.73	0.08	15.77	0.91	32.25	0.68	2084.09	862.16	145.17
Pedro	7.74	0.04	15.72	0.26	34.08	0.66	2116.74	831.38	92.44
Yarrow	7.68	0.06	15.83	0.14	34.00	0.53	2105.66	974.03	123.88
Zeke	7.75	0.07	15.81	0.09	34.07	0.73	2100.97	826.48	134.80
Acidified									
Alphonse	7.53	0.07	12.04	0.11	33.36	0.50	1995.94	1300.05	196.13
Dan	7.58	0.09	12.11	0.03	31.99	0.71	2111.86	1234.36	257.53
Fred	7.50	0.07	12.10	0.00	31.60	0.18	2114.94	1508.24	249.04
Isabelle	7.56	0.07	12.06	0.11	32.80	1.14	2114.19	1281.01	209.53
Oscar	7.54	0.06	12.20	0.12	34.62	0.48	2126.14	1356.77	196.62
Tako	7.54	0.11	12.10	0.00	34.25	0.65	2085.33	1377.60	354.51
Valentino	7.55	0.13	12.10	0.00	34.38	0.48	2107.92	1353.51	405.53
Warming + Acidified									
Benjie	7.57	0.09	15.12	1.37	33.25	0.45	2078.30	1288.51	313.55
Guadalupe	7.52	0.08	15.81	0.08	32.74	0.66	2088.92	1439.02	292.41
Jomar	7.57	0.04	15.24	0.46	32.01	0.97	2084.25	1259.64	124.85
Nestor	7.55	0.07	15.40	0.33	32.65	1.35	2095.76	1332.10	189.25
Rusty	7.53	0.10	15.68	0.68	34.08	0.74	2103.82	1422.88	352.78
Sevro	7.53	0.08	15.83	0.10	34.06	0.73	2097.96	1431.34	305.83
Xena	7.55	0.05	15.53	0.84	31.39	8.78	2100.06	1325.55	155.55

Table 3: Two-way MANOVA results of the effects of pCO_2 and temperature on predatory behavior parameters of *Octopus rubescens* after two weeks in treatment (n = 5-6)

Factor	df	Sum Sq	Mean Sq	f-value	p-value
Latency to Attack					
pCO_2	1	0.36	0.36	0.22	0.65
Temp	1	0.02	0.02	0.01	0.91
pCO_2:Temp	1	3.76	3.76	2.25	0.15
Residuals	17	28.45	1.67		
Striking Distance					
pCO_2	1	0.20	0.20	3.83	0.07
Temp	1	0.06	0.06	1.15	0.3
pCO_2:Temp	1	0.01	0.01	0.1	0.75
Residuals	17	0.90	0.05		
Predator-Prey Orientation					
pCO_2	1	948.90	948.90	0.17	0.69
Temp	1	4650.80	4650.80	0.83	0.38
pCO_2:Temp	1	112.70	112.70	0.02	0.89
Residuals	17	95487.49	5616.91		

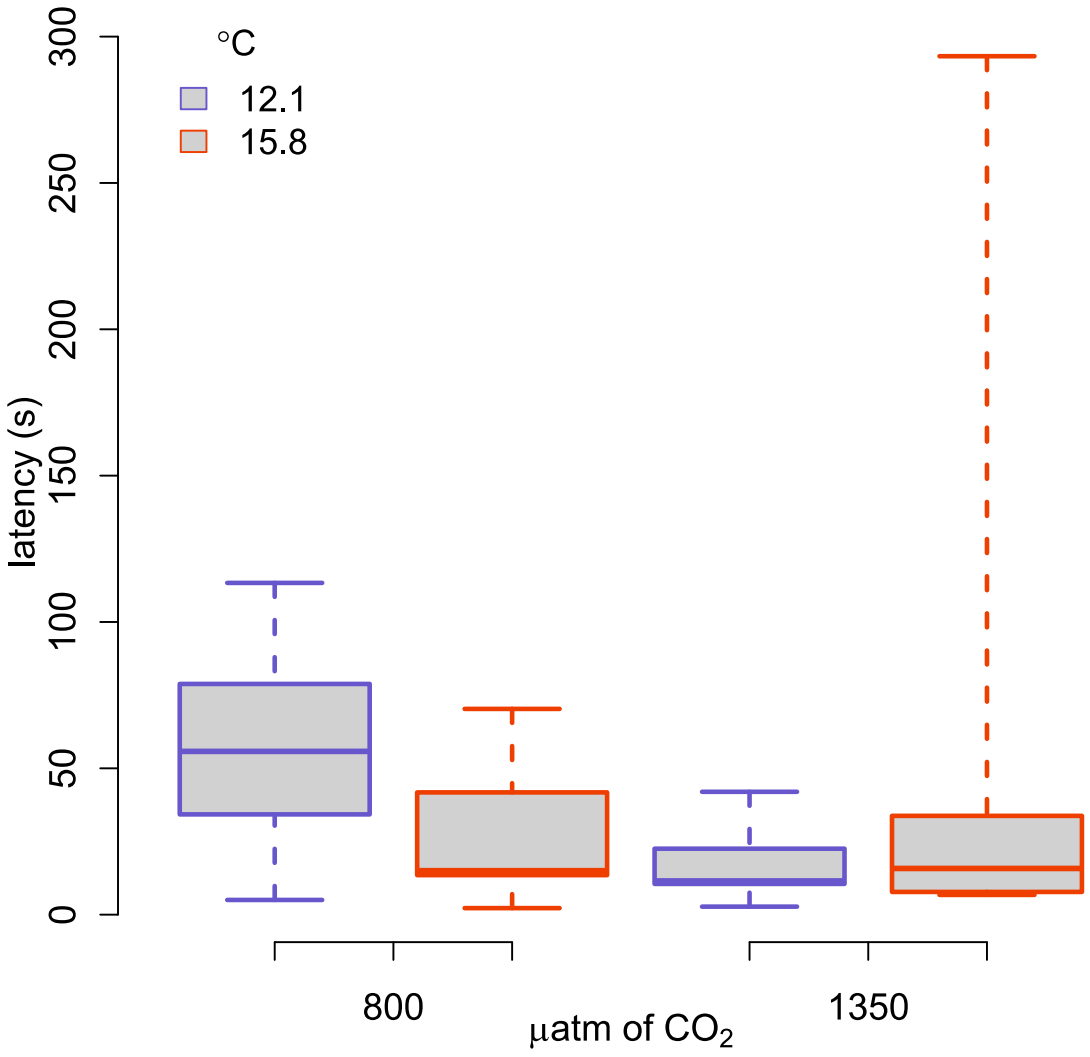

Figure 8. Latency to attack (s) of *Octopus rubescens* after the introduction of the prey. Response is after a two-week exposure to different pCO_2 and temperature treatments for approximately two weeks. Box-and-whisker plots represent the minimum, 1st quartile, median, 3rd quartile, and maximum value. Results were not significant (2-Way MANOVA; df = 1, $f = 2.25$, $p = 0.15$).

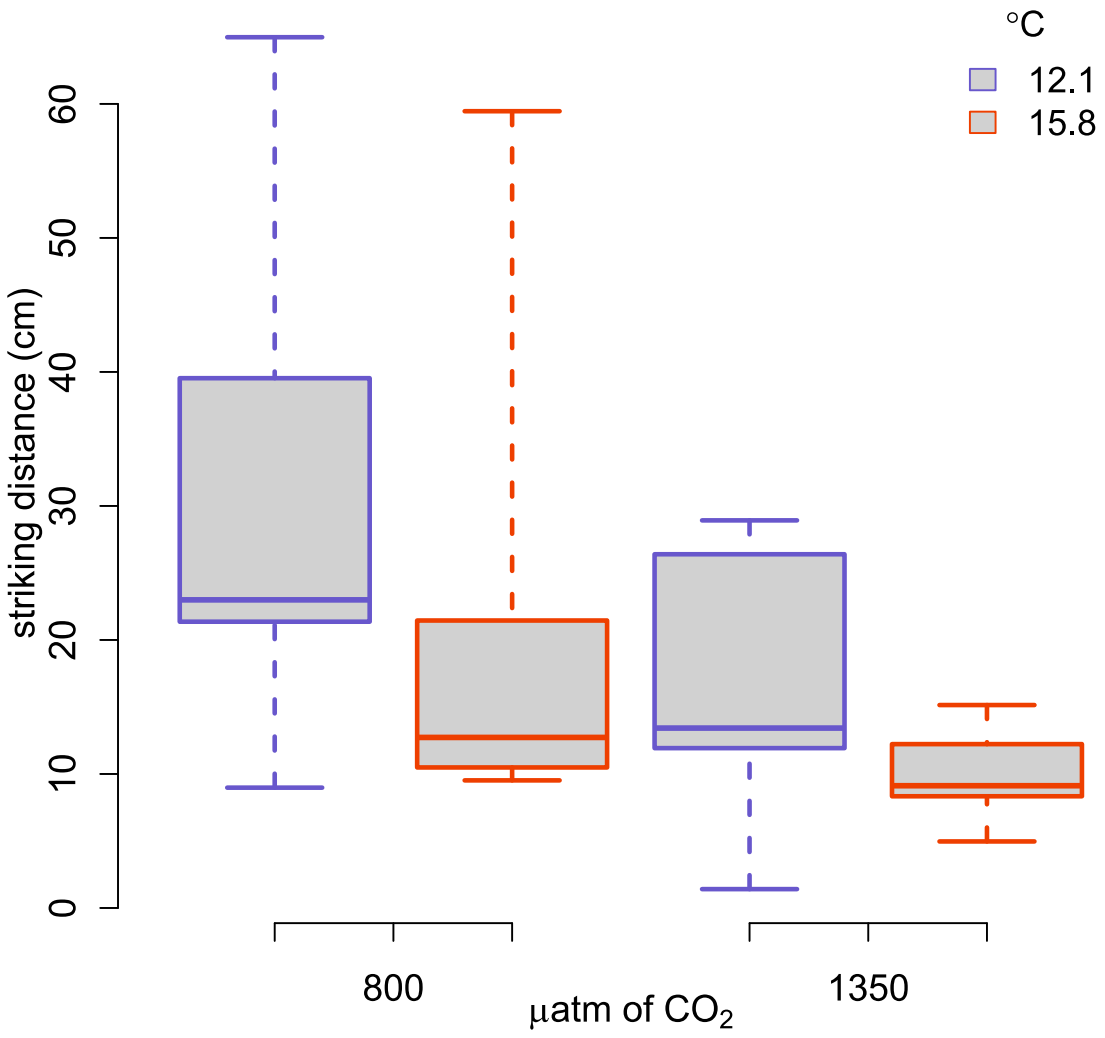

Figure 9. Distance (cm) between *Octopus rubescens* and *Hemigrapsus nudus* when attack sequence was initiated, also known as strike distance. Response is after a two-week exposure to different pCO_2 and temperature treatments for approximately two weeks. Box-and-whisker plots represent the minimum, 1st quartile, median, 3rd quartile, and maximum value. Results were not significant (2-Way MANOVA; df = 1, $f = 0.1$, $p = 0.75$)

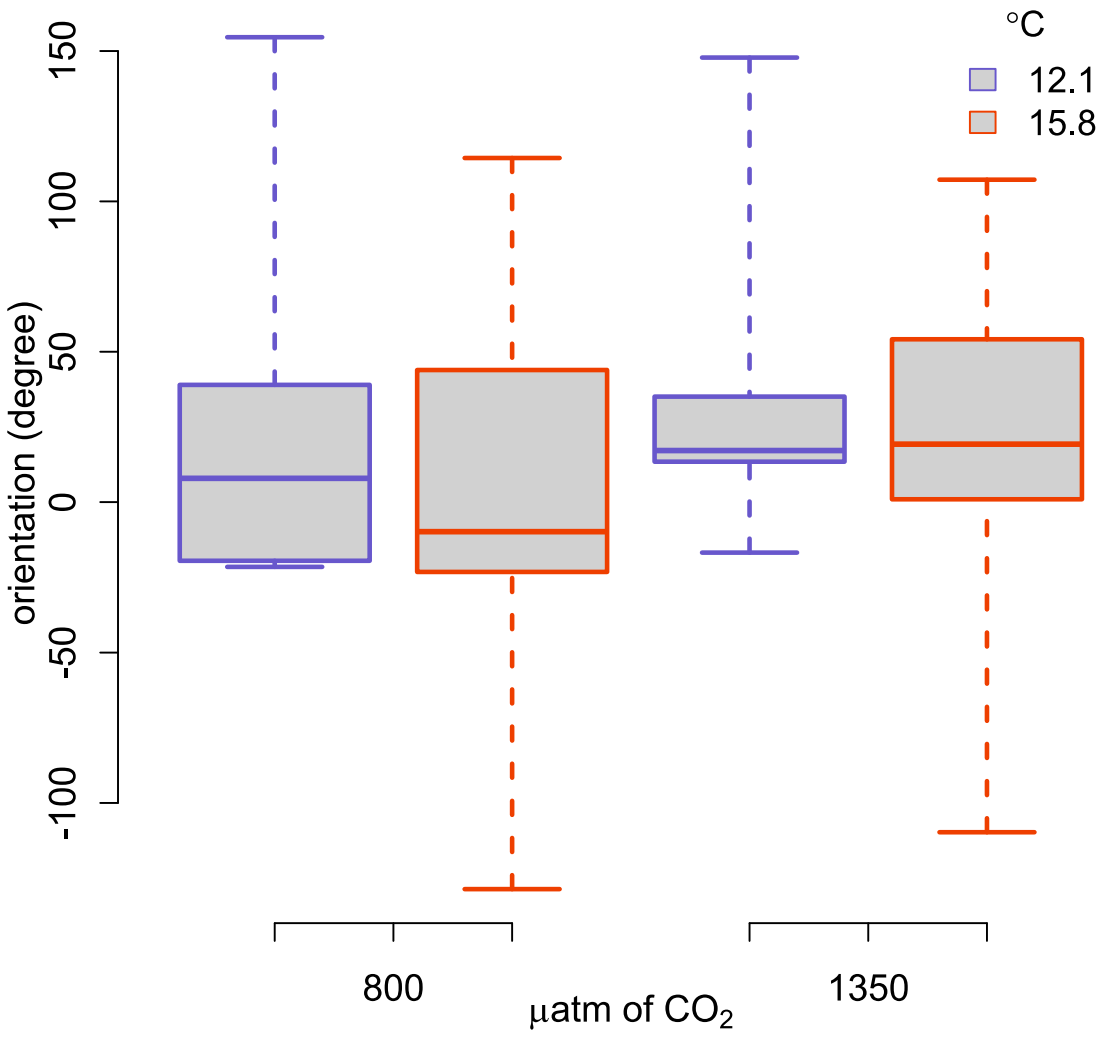

Figure 10. Orientation (degrees) of *Octopus rubescens* to *Hemigrapsus nudus* right before initiating attack sequence, also know predatory to prey orientation . Response is after a two-week exposure to different pCO_2 and temperature treatments for approximately two weeks. Box-and-whisker plots represent the minimum, 1st quartile, median, 3rd quartile, and maximum value. Results were not significant (2-Way MANOVA; df =1, $f = 0.02$, $p = 0.89$)

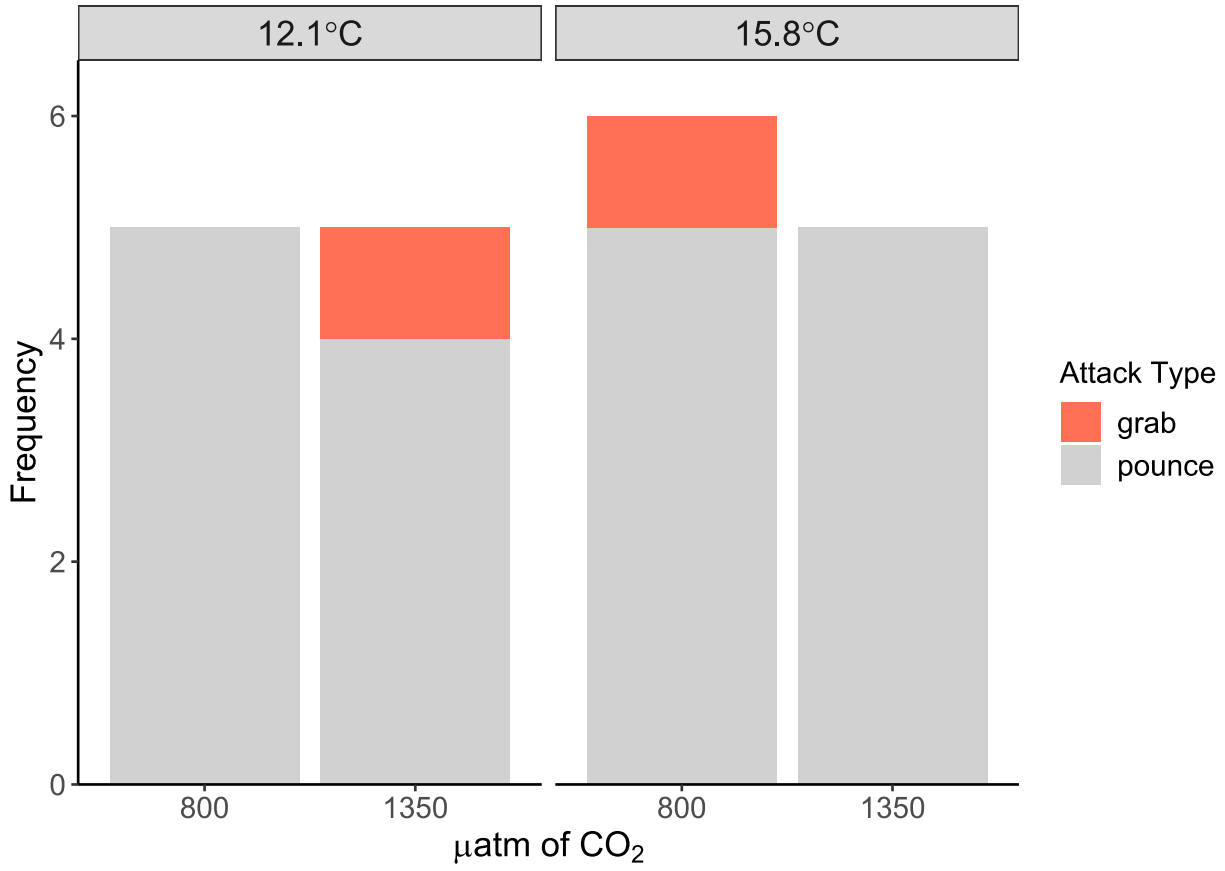

Figure 11. Type of attack chosen by *Octopus rubescens* to capture prey after two-week exposure to different pCO_2 and temperature treatments. Grey bars represent the animal choosing to pounce capture the prey. The orange bars represent the animal choosing an arm grab to capture the prey. Results were not significant (Cochran-Mantel-Haenszel; df = 1, $\chi^2 = 0.004$, $p = 0.95$).

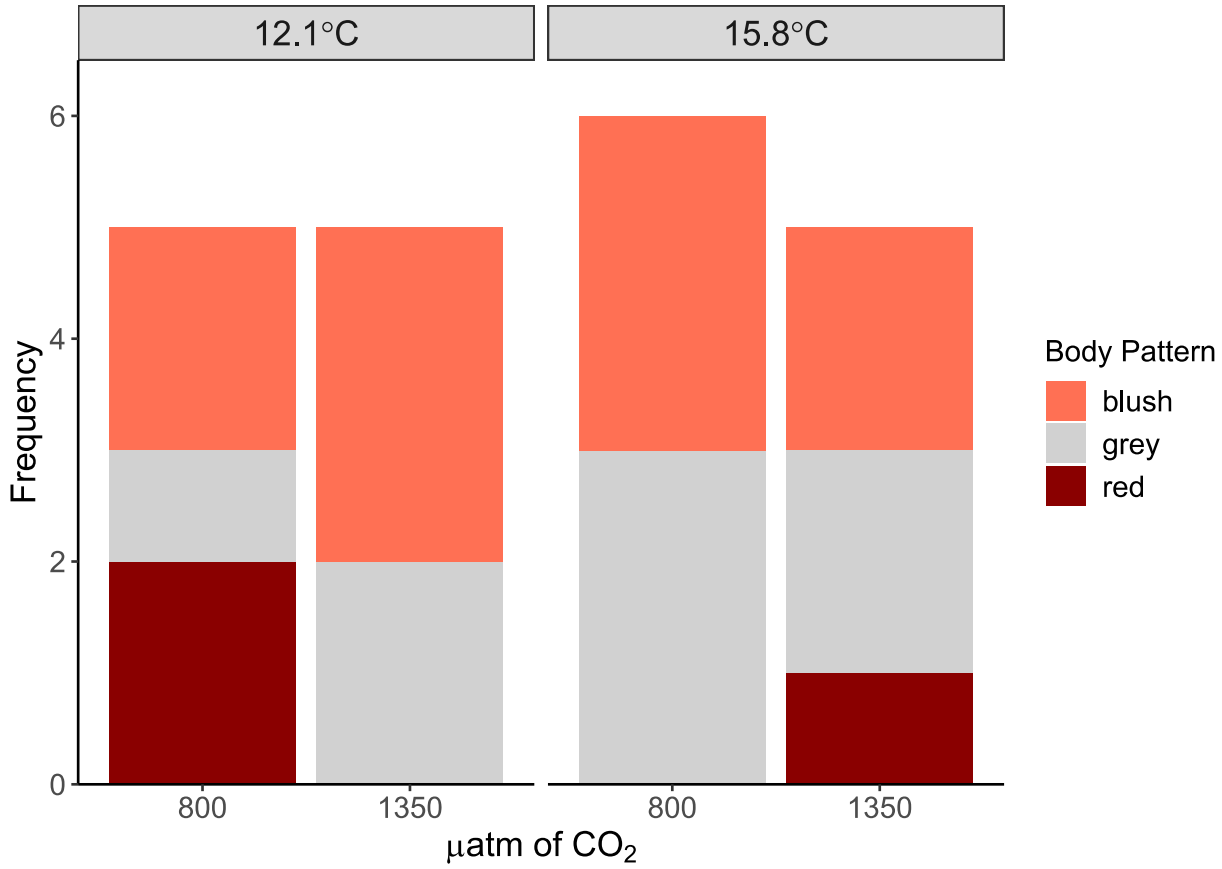

Figure 12. Body pattern chosen by *Octopus rubescens* while attacking prey after two-week exposure to different pCO_2 and temperature treatments. Grey, red, and blush bars represent the body patterns use by animal during the attack. Results were not significant (Cochran-Mantel-Haenszel; df = 2, $\chi^2 = 0.75$, $p = 0.69$).

Table 4: Multi-distance spatial cluster analysis results (Ripley's m-function) - Groups, sample size(n), average M_{est} value, 95% confidence interval, and p-value for each factor.

	Groups	n	M_{est}	95% CI	p-value
CO_2	800 vs 1350	52, 68	-227.7	-2129.7, 1555.5	0.82
Temperature	12.1 vs 15.8	66, 44	-716.1	-2110.3, 2245/1	0.47
Side	anterior vs posterior	48, 62	4106.3	-1627.9, 1168.6	0.01*

Note:
* = siginficant result

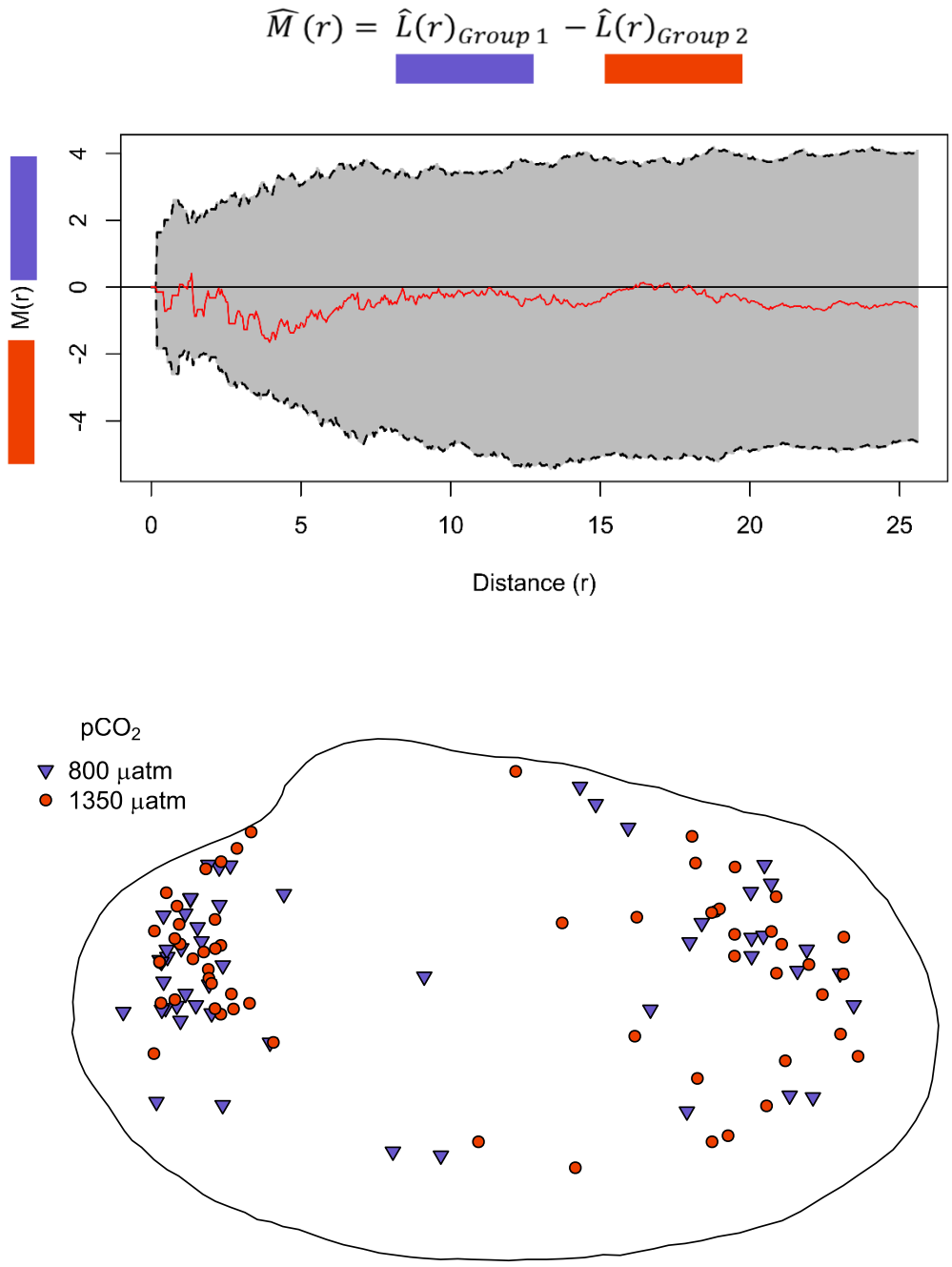

Figure 13. Top: Relative degrees of clustering among sets of drill hole locations by CO_2 treatment. The gray area bordered by black dashed lines represent 95% CI at distance *r*. The red line represent the observed values of M(*r*). Bottom: Drill hole point patterns on *Venerupis philippinarum* shell outline by CO_2 treatment. (M-function; $p = 0.82$).

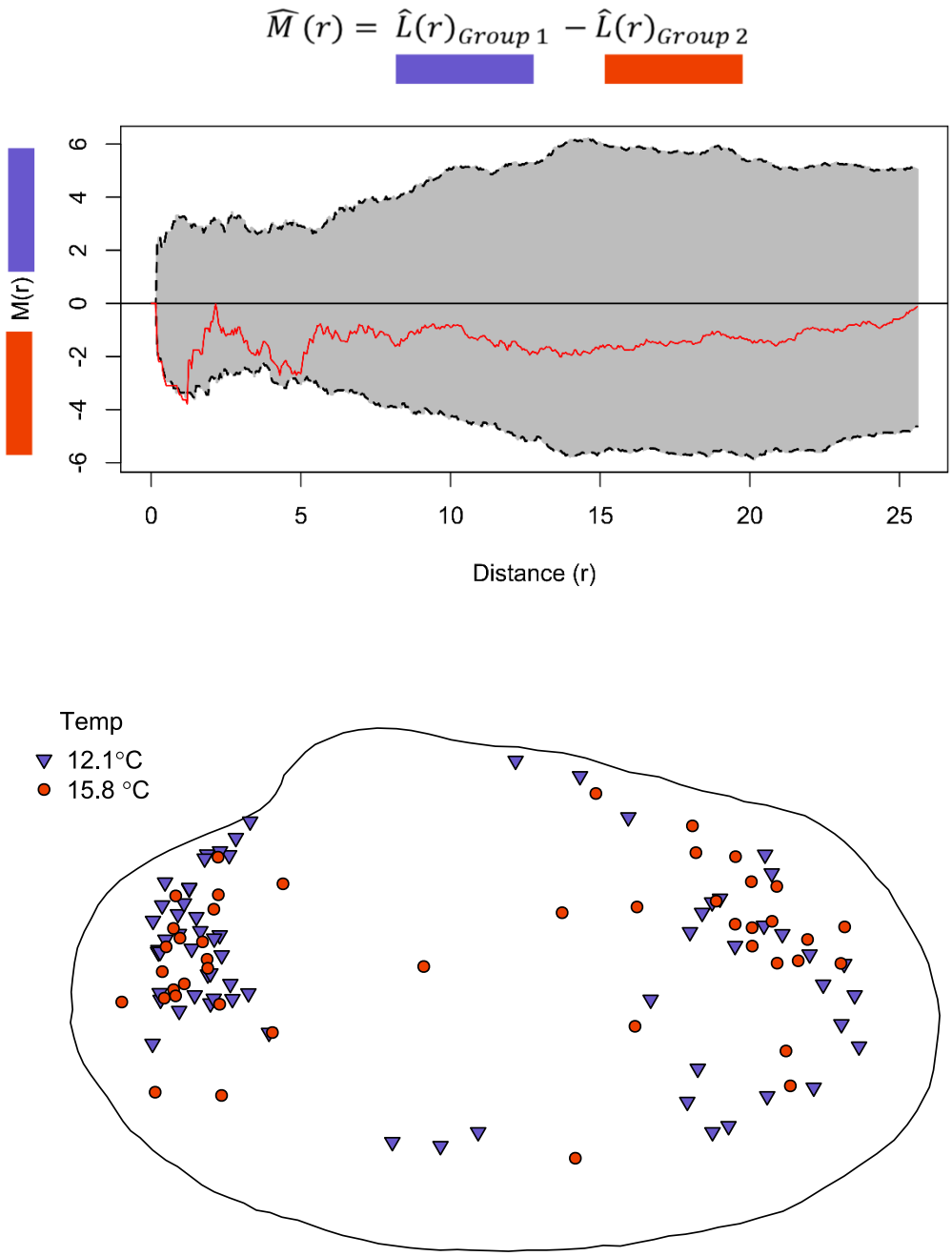

Figure 14. Top: Relative degrees of clustering among sets of drill hole locations by temperature treatment. The gray area bordered by black dashed lines represent 95% CI at distance *r*. The red line represent the observed values of M(*r*). Bottom: Drill hole point patterns on *Venerupis philippinarum* shell outline by temperature treatment. (M-function; $p = 0.47$).

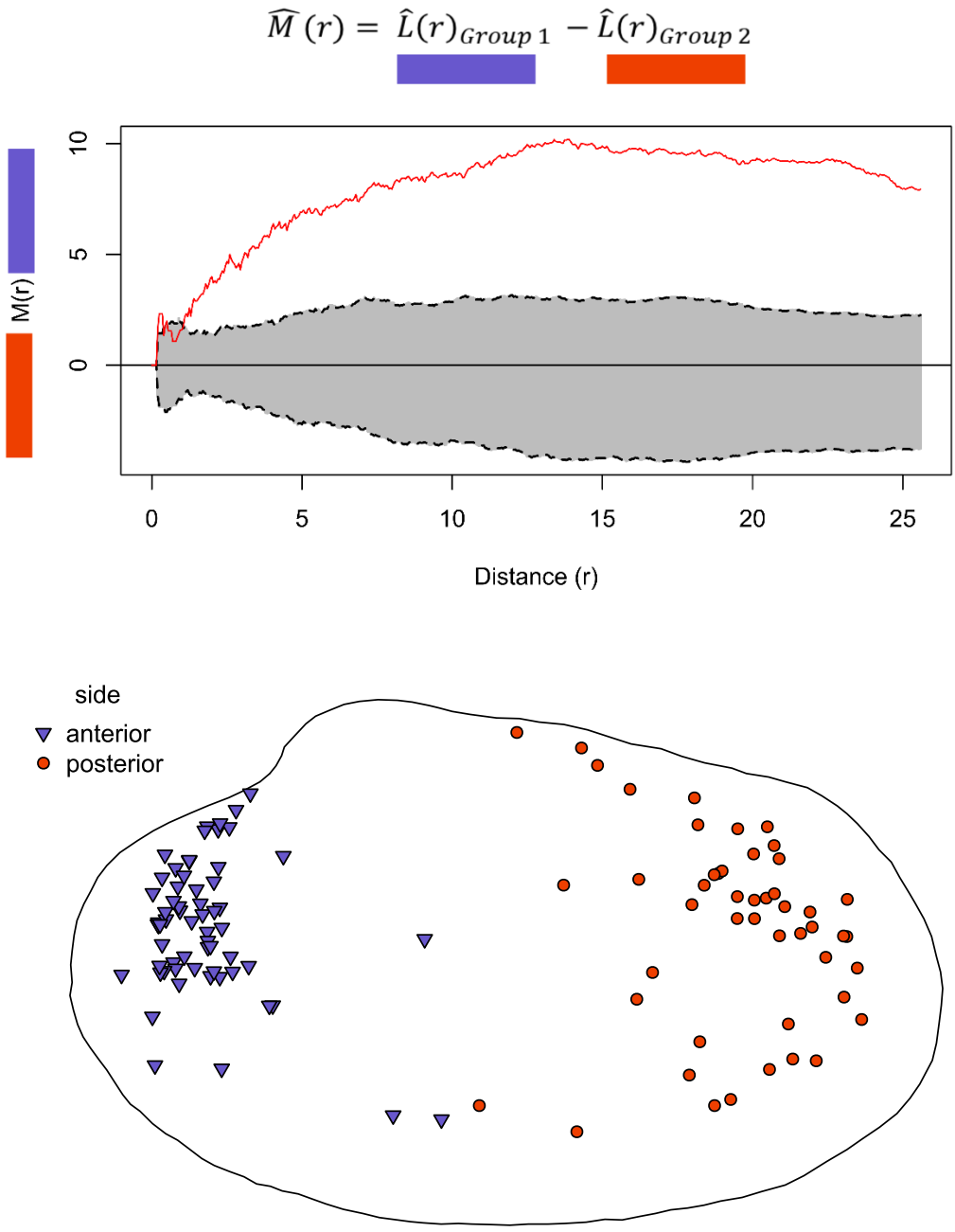

Figure 15. Top: Relative degrees of clustering among sets of drill hole locations by side treatment. The gray area bordered by black dashed lines represent 95% CI at distance *r*. The red line represent the observed values of M(*r*). Bottom: Drill hole point patterns on *Venerupis philippinarum* shell outline by side treatment. (M-function; $p < 0.001$).

DISCUSSION

Predatory Responses of Octopus rubescens to Climate Change Conditions

The results of this study indicated that elevated CO_2 and temperature had no measurable effect on the predatory behavior strategies: latency to attack, striking distance, predatory-prey orientation, type of attack, and body pattern during attack of *Octopus rubescens*. However, striking distance in response to elevated CO_2 was shown to be approaching significance ($p = 0.067$). In similar research, striking distance significantly increased in *Idiosepius pygmaeus* amongst three CO_2 treatments (Spady et al. 2018). *Idiosepius pygmaeus* in the moderate and high CO_2 treatment had a striking distance of 0.31 and 0.29 cm, respectively. This is a 50% increase in the mean striking distance compared to the control treatment, which averaged of 0.20 cm. In comparison, *O. rubescens* in control CO_2 and temperature had a mean striking distance of 57.46 cm. A 50% increase would be 86.19 cm (about 33 in.). *O. rubescens* in this experiment were limited in the maximum distance they could strike because they were contained in a 113.5 L cooler with inner dimensions of 86.4cm L x 35.6cm W x 33.7cm H. The longest possible distance within the cooler was about 94 cm which measures one corner to another. The size difference between *O. rubescens* and *I. pygmaeus* is great. Adult *O. rubescens* can weigh between 100-400 g, but the adult pygmy squid weighs less than one gram. Also, *O. rubescens* and *I. pygmaeus* are two animals exhibiting different lifestyles and hunting behaviors. The attack behavior of *I. pygmaeus* includes stalking prey, holding an attack pose, followed by the quick splaying of its arms to release the striking tentacles to capture prey. All the while, taking place in the water column (Hanlon and Messenger 1996; Spady et al. 2014, 2018). Depending on the situation or individual, *O. rubescens* will launch into attack with a ballistic pounce or arm grab from a surface (Warren et al. 1974; Hanlon and Messenger 1996; Mather and Alupay 2016).

Finally, a limitation of this study was that I only investigated predatory response to OA and warming and did not explore the potential impacts to the behavior of prey. Reviews have addressed that prey can respond negatively, positively, or neutrally to future ocean conditions (Clements and Hunt 2015; Nagelkerken and Munday 2016; Draper and Weissburg 2019). The larger size of *O. rubescens*, difference in hunting style, and size limitations of the experimental arena may have contributed to the non-significant results observed in striking distance.

Drill Hole Analysis

The analysis of drill hole patterns produced varied results. The isolated stressors of elevated temperature or CO_2 did not affect the drill hole degree of clustering between point patterns. When temperature and CO_2 treatment were disregarded, a significant difference in drill hole clustering variability between the anterior and posterior end of the *V. philippinarum* shells was found. Previous studies showed that octopuses prefer drilling on the anterior end of clams (Nixon 1979b; Nixon and Maconnachie 1988; Cortez et al. 1998) and this study was able to discern that the point pattern is more tightly clustered, as well (Fig. 15). These results show that *O. rubescens* drill hole patterns are more precise on the anterior end compared to the posterior end. Whatever mechanisms that allow *O. rubescens* drill hole patterns to be more precise on the anterior end of the clam do not seem to be affected by elevated temperature or acidification. In spite of the mixed results, we were able to gather a new understanding about octopus drilling behavior on clam prey.

In this regard, this research is the first of its kind in two ways. There are numerous studies that look into octopus drilling behaviors but none that studied the effects of ocean acidification or warming on octopus drill holes. Also, this study was the first to utilize multi-

distance spatial cluster analysis to discern the pattern on octopus drill holes.

Test Power Analysis

Using a power analysis, I calculated the minimum difference between the means of control and experimental groups that would be necessary to maintain a test power of 0.8 for latency to attack, strike distance, and predatory-prey orientation (Table 5). I determined the smallest mean difference to maintain a test power of 0.8 for each test by calculating the Cohen's d effect size by standard power analysis and dividing it by the pooled standard deviation. This allowed me to set up an upper likely bound on the possible true mean difference between control and experimental treatment despite non-significant hypothesis test results (Table 5). For example, the latency to attack control mean was 57.46 s, and the minimum mean difference calculated 63.21 s. I can therefore claim that, even though my statistical test was not significant, latency to attack likely does not more than double when octopuses are exposed to an increase of 550 μatm pCO_2. If it did, I would have had a ≥80% chance of detecting the difference with this experiment and statistical test. These calculations showcase the importance of having a large sample size to extrapolate findings between treatment groups, as the small sample of each treatment led to a large effect size which created large mean differences with implausible results. For instance, if pCO_2 caused a substantial increase in striking distance, I would not have been able to detect because of the size limitation of the holding tank

Comparing Cephalopod Behavior Studies

This research is the first known study on cephalopod predatory behavioral effects to OA and warming. Where all three studies detected behavioral effects due to OA, this one did not. It is possible that the methods that I used to measure data limited my abilities to detect an effect, but I believe the largest issue was the small sample size in each treatment group.

The most evident differences I found when comparing the studies were the species' natural habitat and ocean acidification systems. First, all other studies were conducted on squid who inhabit either tropical or neritic zone environments that tend to be more stable than coastal temperate upwelling zones, where *O. rubescens* can be found (Byrne 2010). Because of the high variability of temperature and pH changes, the Salish Sea could have made the *O. rubescens* living there more resilient to climate stressors.

Second, all three previous studies used ocean acidification systems involved bubbling CO_2 into larger holding tanks to create their respective treatments. The acidified water was then distributed into the treatment tanks containing two or more specimens. This is considered sacrificial pseudoreplication since the specimens were not independent of one another but were counted as such, thereby artificially inflating their sample size. In this study, each experimental tank was controlled by its own pH control system, making each specimen independent of one another. Albeit this type of ocean acidification system can lead to small sample sizes (n = 5, 6 per treatment group), as in this experiment.

Octopus rubescens in Future Climate Conditions

The results of this study suggest that the predatory strategies and drill hole behavior of *O. rubescens* are not affected by OA and warming. One interpretation of these results could be that *O. rubescens* may be a species that is somewhat resilient to climate change perturbations. As a highly adaptable generalist predator with advanced acid-base regulatory capabilities, this potential resiliency may alter marine ecosystems and decrease biodiversity. Recent studies have shown that species that are behaviorally plastic (Beever et al. 2017) or generalist species (Colossi Brustolin et al. 2019) will likely adapt and proliferate in a changing ocean. This concept is supported by the increased abundance (Doubleday et al. 2016) and range expansion (Hiemstra

2015) of cephalopod species worldwide (Doubleday et al. 2016). Since no behavioral effects due to OA and warming were not detected in my study, it is possible that the Salish Seas *O. rubescens* may be a population that "succeeds" in future ocean conditions.

Table 5: Minimum difference between the means of the control and experimental groups to maintain at least a power of 0.8 in null-hypothesis significance tests (Mean Difference) of latency to attack (s), striking distance (cm), and predatory-prey orientation (degrees) with our given sample size (n). Minimum Cohen's d effect size (Effect Size) to maintain at least a power of 0.8, and mean of control are also included. Control pCO_2 was 800 (μatm) and experimental pCO_2 was 1350 (μatm).

Temperature (°C)	n	Effect Size	Mean of Control	Mean Difference
Latency to attack(s)				
12.1	5,5	2.02	57.46	63.21
15.8	6,5	1.91	26.32	171.27
Striking distance (cm)				
12.1	5,5	2.02	31.57	34.90
15.8	6,5	1.91	21.07	26.53
Predatory-prey orientation (degrees)				
12.1	5,5	2.02	32.10	138.16
15.8	6,5	1.91	-2.17	153.39

CONCLUSION

This study set out to examine the potential impacts of ocean acidification and warming on the predatory strategies and drilling behavior of *Octopus rubescens*. The parameters in which the predatory behaviors of *Octopus rubescens* were measured in this study did not indicate a response to ocean acidification or warming, as demonstrated by the non-significant results. It is possible that *O. rubescens*' strong acid-base regulatory mechanisms, behavioral plasticity, and adaptability make it resilient to climate change perturbations, leading to measurable change in behavior. It is also likely that the power to see the effects caused by ocean warming or temperature were reduced by high inter-individual variability and small sample size of this study. More research is required to further investigate the behavioral effects of climate change on cephalopods to determine to what extent they are resilient and adaptable to future ocean conditions, along with what this may mean to marine ecosystem functioning and biodiversity. Regardless of the absence of evidence within the predatory behavior of *O. rubescens*, this study inspired the development of a novel multi-distance spatial cluster analysis function, M(r). This study is the first to utilize such methods to discern the point patterns in octopus drill holes. It is my hope that this study will be the first of many to utilize more robust computational statistical methods to analyze octopus drill hole point patterns.

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"I want to stand as close to the edge as I can without going over. Out on the edge you see all kinds of things you can't see from the center." Kurt Vonnegut

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APPENDIX A: Hansville Mooring Temperature Data

Determining the temperate range between the depths of 18-22 meter at all months of the year and during the months of the experiment.

Libraries

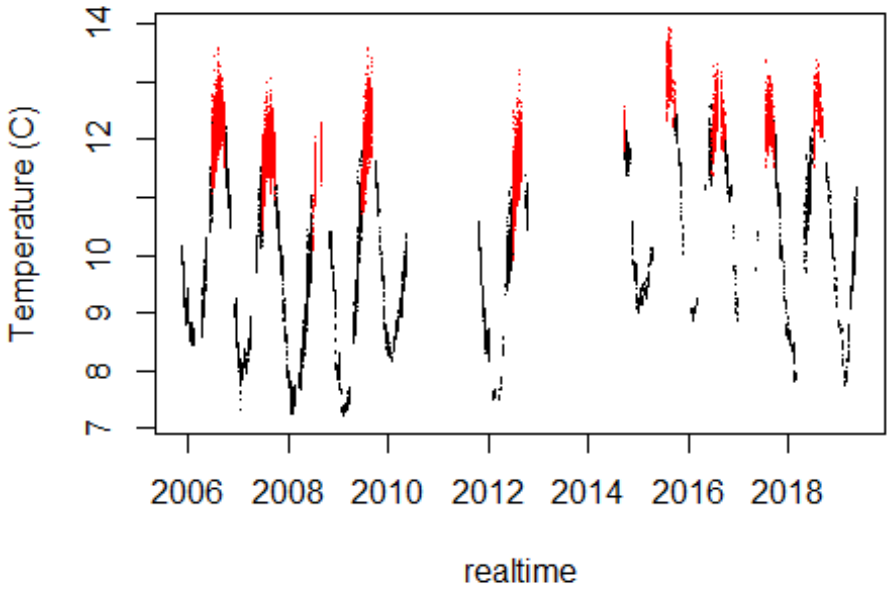
```
library(xlsx)
library(openxlsx)
library(rmatio)
```

Reading in and formating data

```
origin=strptime("00/1/1","%D")
mat=read.mat("HC_NB_CTD_data_bin_web.mat")
mat.new=data.frame(cbind(as.vector(mat$Btime),as.vector(mat$Bdepth),as.vector
(mat$Btemp)))
colnames(mat.new)=c("time","depth","temp")
mat.new$realtime=origin+(60*60*24)*mat.new$time
hans=mat.new[complete.cases(mat.new),]
hans$month=as.numeric(format(hans$realtime,"%m"))
hans$year=as.numeric(format(hans$realtime,"%Y"))
```

Plotting add data for 18-22 meters

```
plot(temp~realtime,data=hans[hans$depth>18&hans$depth<22,],pch=".",ylab="Temp
erature (C)")
points(temp~realtime,data=hans[hans$depth>18&hans$depth<22&hans$month>=7&hans
$month<=9,],
       col="red",pch=".")
```



Temp mean for July-September

Because there is not the same number of data points per year, I am taking the average of each year, and then taking the average for all year averages.

```
yearly=aggregate(temp~year,data=hans[hans$depth>18&hans$depth<22&hans$month>=
7&hans$month<=9,],FUN="mean")
mean(yearly$temp)
```

```
## [1] 12.08402
```

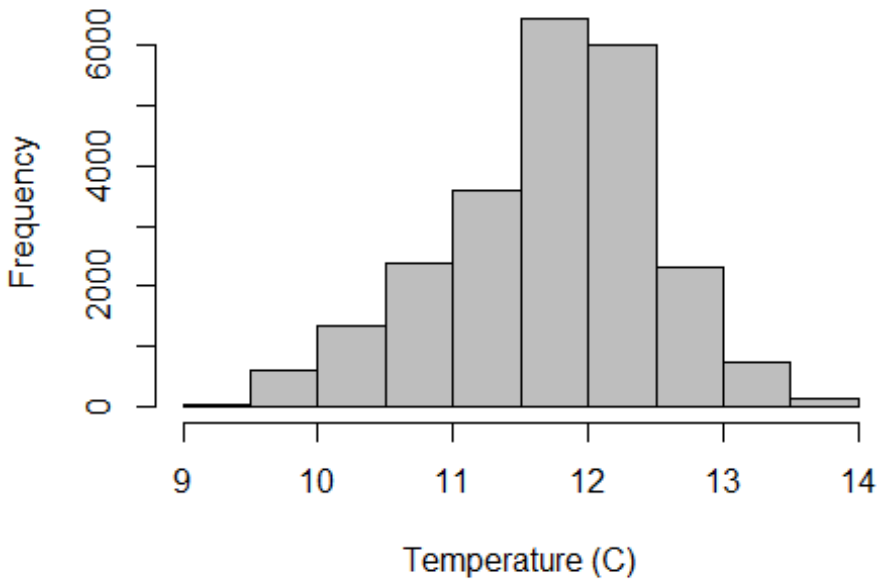
Range of temperatures found for June-September for all years

```
range(hans$temp[hans$depth>18&hans$depth<22&hans$month>=6&hans$month<=9])
```

```
## [1]  9.256645 13.911058
```

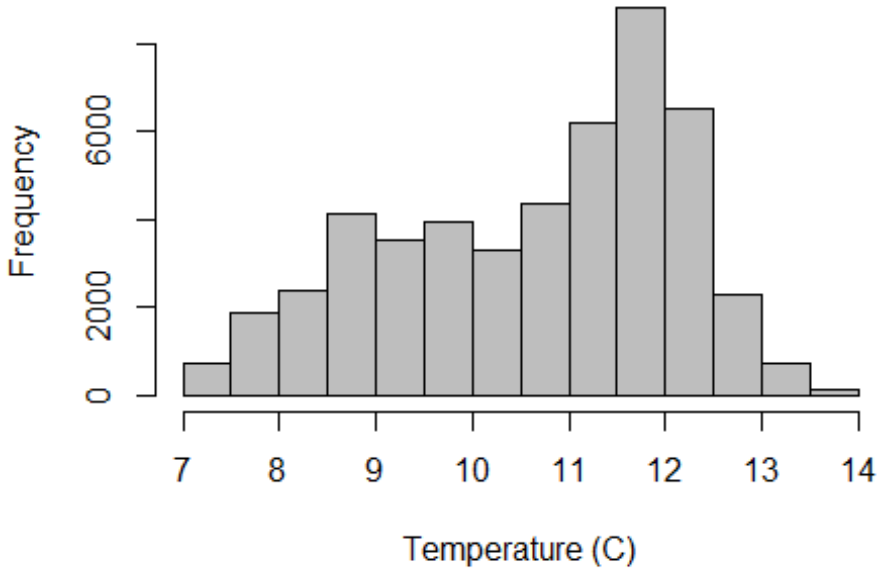
Histogram of temperatures for June-September for all years

```
hist(hans$temp[hans$depth>18&hans$depth<22&hans$month>=6&hans$month<=9],col="
grey",
     main="",xlab="Temperature (C)")
```



Historgram of all temperatures at all months for depths 18-22 meters

```
hist(hans$temp[hans$depth>18&hans$depth<22],col="grey",main="",xlab="Temperat
ure (C)")
```



Range of temperatures for all months at 18-22 meters

```
range(hans$temp[hans$depth>18&hans$depth<22])
```

```
## [1]  7.18498 13.91106
```

APPENDIX B: Predatory Behavior Statistical Analysis

Load Packages Required

```
library(mvnormtest)
library(MASS)
library(plotrix)
library(ggplot2)
library(heplots)
```

Load data and create new column

```
data = read.csv(file = "data.csv", header = T)
data$ate = TRUE
data$ate[data$ate_crab == "no"] = FALSE
```

Checking if the mass of the octopus and predator-prey ratio infuenced octopuses ability to eat crab

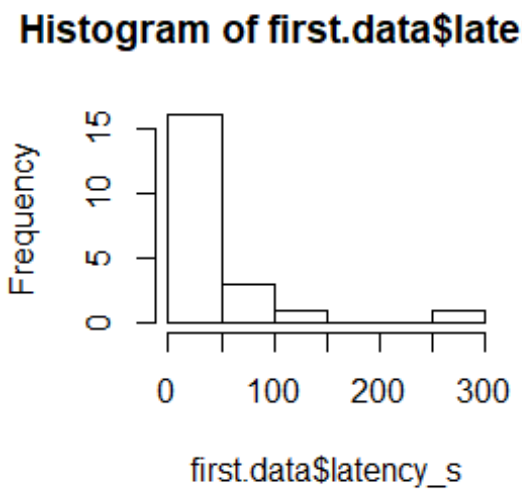
```
summary(aov(data$mass~data$ate))

##             Df Sum Sq Mean Sq F value Pr(>F)
## data$ate     1  12469   12469   1.645  0.204
## Residuals   62 469971    7580

summary(aov(data$pp_ratio~data$ate))

##             Df Sum Sq Mean Sq F value Pr(>F)
## data$ate     1   1668    1668   1.025  0.315
## Residuals   62 100838    1626

# no, they did not. We move on.
```

Create new data frame, remove octopuses who did not eat crab. Remove duplicates, keeping the data points with the fastest latency time. Clean up data and make pretty.

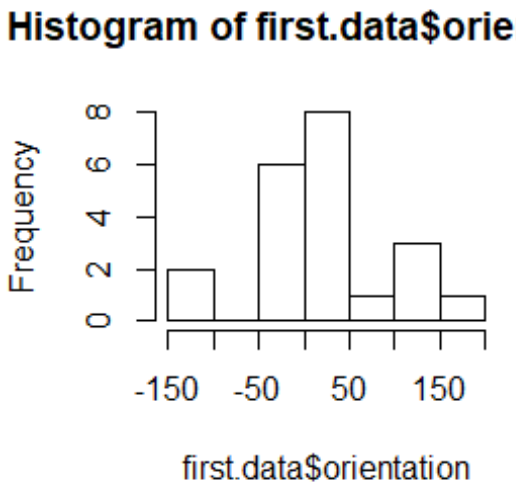
```
first.data = data[(data$ate=="TRUE"),]
first.data = first.data[order(first.data$octo_ID, abs(first.data$attempt)),]
first.data = first.data[ !duplicated(first.data$octo_ID),]
row.names(first.data) = NULL
first.data$orientation = as.numeric(as.character(first.data$orientation))
first.data$strike_dist = as.numeric(as.character(first.data$strike_dist))
first.data$orientation[first.data$orientation >= 180] = -(360 - (first.data$o
rientation[first.data$orientation >= 180]))
first.data = first.data[order(first.data$octo_ID),]
first.data$bp_attack = gsub("blush\n", "blush", first.data$bp_attack)
first.data$bp_attack = gsub("grey\n", "grey", first.data$bp_attack)
first.data$attack_type = gsub("grab\n", "grab", first.data$attack_type)
first.data$attack_type = gsub("pounce ", "pounce", first.data$attack_type)
first.data$attack_type = as.character(first.data$attack_type)
orientation = first.data$orientation
```

Checking distribution & Multivariate Normality Test

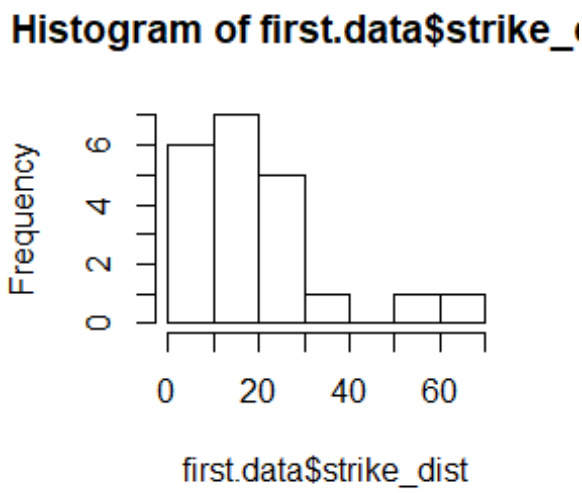
```
hist(first.data$latency_s)
```




```
hist(first.data$orientation)
```



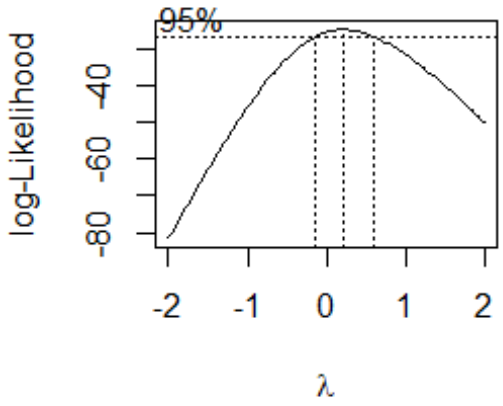

```
hist(first.data$strike_dist)
```




```
b = t(first.data[,13:15])
mshapiro.test(b)

##
##  Shapiro-Wilk normality test
##
## data:  Z
## W = 0.60665, p-value = 2.317e-06
```

Transform Data, Multivariate Normality, and Multivariate Homoscedatiscity Test

```
cube.lat = sign(first.data$latency_s) * abs((first.data$latency_s)^(1/3)) fir
st.data$cube.lat = cube.lat
boxcox(strike_dist ~ co2_atm + temp, data = first.data)
```



```
bc.strike = (first.data$strike_dist)^(.18)
first.data$bc.strike = bc.strike
c = t(cbind(cube.lat,bc.strike,orientation))
mshapiro.test(c)

##
##  Shapiro-Wilk normality test
##
## data:  Z
## W = 0.9098, p-value = 0.05441

magic = cbind(first.data$temp, first.data$co2_atm, first.data$latency_s, firs
t.data$strike_dist, first.data$bc.strike, first.data$cube.lat, first.data$ori
entation)
colnames(magic) = c("temp", "pco2", "latency", "strike", "bc.strike", "cube.l
at", "orient")
magic = as.data.frame(magic)
bartlettTests(magic[,5:7], magic$temp*magic$pco2)

## Bartlett's Tests for Homogeneity of Variance
##
##            Chisq df Pr(>Chisq)
## bc.strike 3.2207  3     0.3588
## cube.lat  3.7855  3     0.2856
## orient    0.2740  3     0.9648
```

Checking the influence of the mass of octopus and predator-prey ratio influence dependent variables, followed by Two-Way MANOVA

```
fatties = with(first.data, manova(cbind(cube.lat, bc.strike, orientation) ~ m
ass))
summary(fatties)

##           Df Pillai approx F num Df den Df Pr(>F)
## mass       1 0.1789   1.2347      3     17 0.3279
## Residuals 19

pp = with(first.data, manova(cbind(cube.lat, bc.strike, orientation) ~ pp_rat
io))
summary(pp)

##           Df  Pillai approx F num Df den Df Pr(>F)
## pp_ratio   1 0.15085   1.0066      3     17  0.414
## Residuals 19


please = with(first.data, manova(cbind(cube.lat, bc.strike, orientation) ~ co
2_atm*temp))
summary(please)
```

```
##              Df  Pillai approx F num Df den Df Pr(>F)
## co2_atm       1 0.29326  2.07474      3     15 0.1466
## temp          1 0.13726  0.79549      3     15 0.5153
## co2_atm:temp  1 0.19412  1.20442      3     15 0.3421
## Residuals    17
```

```
summary.aov(please)
```

```
##  Response cube.lat :
##              Df  Sum Sq Mean Sq F value Pr(>F)
## co2_atm       1  0.3609  0.3609  0.2156 0.6483
## temp          1  0.0223  0.0223  0.0133 0.9095
## co2_atm:temp  1  3.7633  3.7633  2.2487 0.1521
## Residuals    17 28.4507  1.6736
##
##  Response bc.strike :
##              Df  Sum Sq  Mean Sq F value Pr(>F)
## co2_atm       1 0.20290 0.202898  3.8291 0.0670 .
## temp          1 0.06068 0.060678  1.1451 0.2995
## co2_atm:temp  1 0.00553 0.005531  0.1044 0.7506
## Residuals    17 0.90082 0.052989
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
##
##  Response orientation :
##              Df Sum Sq Mean Sq F value Pr(>F)
## co2_atm       1    949   948.9  0.1689 0.6862
## temp          1   4651  4650.8  0.8280 0.3756
## co2_atm:temp  1    113   112.7  0.0201 0.8890
## Residuals    17  95487  5616.9
```

Mantelhaen Chi square test & Fisher's Exact (just in case!)

```
attack.freq = table(first.data$attack_type, first.data$temp, first.data$co2_a
tm)
mantelhaen.test(attack.freq)
```

```
##
##  Mantel-Haenszel chi-squared test without continuity correction
##
## data:  attack.freq
## Mantel-Haenszel X-squared = 0.0041494, df = 1, p-value = 0.9486
## alternative hypothesis: true common odds ratio is not equal to 1
## 95 percent confidence interval:
##   0.06448927 18.76281126
## sample estimates:
## common odds ratio
##               1.1
```

```
body.freq = table(first.data$bp_attack, first.data$temp, first.data$co2_atm)
mantelhaen.test(body.freq)
```

```
##
##  Cochran-Mantel-Haenszel test
##
## data:  body.freq
## Cochran-Mantel-Haenszel M^2 = 0.75378, df = 2, p-value = 0.686
```

APPENDIX C: Drill-hole M-function Analysis

Installing the MTest package from GitHub

```
library(devtools)
install_github("KirtOnthank/MTest")
library(MTest)
```

Now loading the rest of the libraries

```
library(spatstat)
library(readODS)
library(NbClust)
library(factoextra)
```

Reading in Data

```
holes = read.csv(file="drill_holes.csv")
hole.info=read_ods("Shell_info.ods",sheet=1)

## Parsed with column specification:
## cols(
##   Image_ID = col_double(),
##   Cohort = col_double(),
##   Tank = col_double(),
##   co2_uatm = col_double(),
##   temp = col_double(),
##   Date = col_character(),
##   Valve = col_character(),
##   no_dh = col_double(),
##   Same_animal = col_double()
## )

hole.info=hole.info[complete.cases(hole.info$Image_ID),]
outline=read.table("outline_2006.JPG.dat",header = F)
```

Formatting data

```
col.new=colnames(hole.info)[2:8]
holes[col.new]=NA
for (i in 1:nrow(holes)){
  holes[i,4:10]=hole.info[hole.info$Image_ID==holes$photo[i],2:8]
}
```

Clustering of holes into Anterior and Posterior

```
holes.k=kmeans(holes[2:3],centers=2)
holes$cluster=holes.k$cluster
holes$side="posterior"
holes$side[holes$cluster==1]="anterior"
```

M-Test of clustering by CO_2 data

First, set up two point patterns, each one with a group of points you want to compare

```
hi.pp = ppp(holes$X[holes$co2_uatm==1350], holes$Y[holes$co2_uatm==1350],
            poly=list(x=outline$V1,y=outline$V2))
lo.pp = ppp(holes$X[holes$co2_uatm==800], holes$Y[holes$co2_uatm==800],
            poly=list(x=outline$V1,y=outline$V2))
```

Next, Run the M-test with the function m.test()

```
co2.m=m.test(hi.pp,lo.pp)
co2.m$p

## [1] 0.82

co2.m$M.sum

## [1] -227.7309

co2.m$ConfInt

##      2.5%     97.5%
## -1763.100  1575.684
```

M-test of clustering by temperature

```
hot.pp = ppp(holes$X[holes$temp==15.8], holes$Y[holes$temp==15.8],
             poly=list(x=outline$V1,y=outline$V2))
cold.pp = ppp(holes$X[holes$temp==12.1], holes$Y[holes$temp==12.1],
              poly=list(x=outline$V1,y=outline$V2))

temp.m=m.test(hot.pp,cold.pp)
temp.m$p

## [1] 0.46

temp.m$M.sum

## [1] -716.1356

temp.m$ConfInt

##      2.5%     97.5%
## -2087.688  2219.166
```

M-test for relative clustering by side

```
ant.ppp = ppp(holes$X[holes$side=="anterior"], holes$Y[holes$side=="anterior"
],
              poly=list(x=outline$V1,y=outline$V2))

pos.ppp = ppp(holes$X[holes$side=="posterior"], holes$Y[holes$side=="posterio
r"],
              poly=list(x=outline$V1,y=outline$V2))

side.m=m.test(ant.ppp,pos.ppp)
side.m$p
```

```
## [1] "<0.01"

side.m$M.sum

## [1] -4106.285

side.m$ConfInt

##      2.5%     97.5%
## -1416.556  1375.821
```

APPENDIX D: Latency to Attack - Crab Survival

Using 600s as the latency period when octopuses do not attack the crab before the trial stops is not a very satifying way to deal with that situation. Fortunately, the problem of "When I am measuring the timing to an event and that even does happen before I stop looking" in statistical analysis has been pretty well worked out for some particularly kinds of analysis, and in particular in survival analysis. Here, I re-analyze the latency data using a "Crab Survival" analysis approach that better accounts for some crabs not being attacked before the trials were up. Spoiler Alert: Still not significant.

Load required packages

```
library(survival)
library(survminer)
library(dplyr)
```

Loading dataset

```
behav=read.csv("Behavior_data.csv")
head(behav)
```

```
##   Cohort   date octo_ID sex  mass co2_atm temp treatment tank attempt ate_
crab
## 1      1 29-Jul  Arthur   m 297.0     800 12.1      blue    5       1
no
## 2      1 29-Jul Crystal   f  43.0     800 15.8    yellow    6       1
yes
## 3      1 29-Jul    Fred   m 241.3    1350 12.1     green    7       1
yes
## 4      1 29-Jul Ernesto   m 250.0     800 12.1      blue    9       1
yes
## 5      1 29-Jul     Bob   m 312.0     800 15.8   yellow    10       1
yes
## 6      1 29-Jul     Dan   m  61.0    1350 12.1     green   11       1
no
##   nos_attack latency_s strike_dist orientation L_R bp_attack notes. attack
_type
## 1          -   600.000           -           -   -         -
-
## 2          1    13.515      21.456     358.403   L     blush             p
ounce
## 3          1    10.508      13.431      35.083   R      grey             p
ounce
## 4          1     5.007      64.973     154.566   R       red             p
ounce
## 5          1    14.264       9.529     341.974   L     blush             p
ounce
## 6          -   600.000           -           -   -
-
##   approach_type crab_mass  pp_ratio
## 1             -       2.9 102.41379
```

```
## 2           jet       2.9  14.82759
## 3           jet       3.4  70.97059
## 4           jet       3.5  71.42857
## 5         crawl       2.9 107.58621
## 6             -       4.6  13.26087
```

Making CO2 and Temperature into factor

```
behav$co2_atm=as.factor(behav$co2_atm)
behav$temp=as.factor(behav$temp)
```

Adding censoring information

```
behav$censor=1
behav$censor[behav$latency_s==600]=0
```

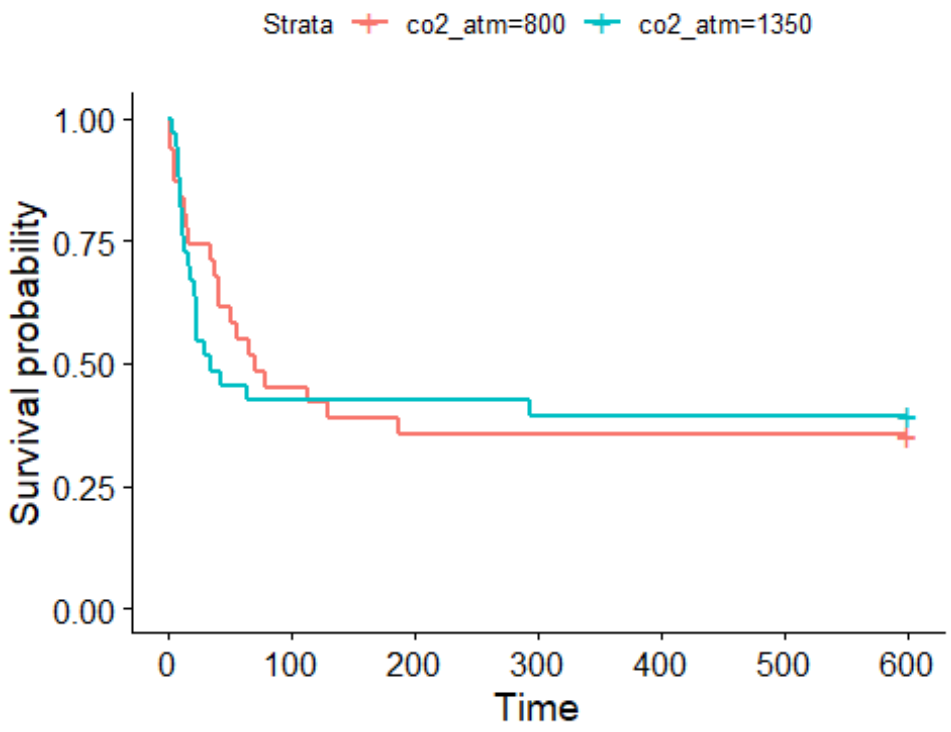
Building the Survival model

```
crab_surv=Surv(time=behav$latency_s,event=behav$censor)
crab_surv
```

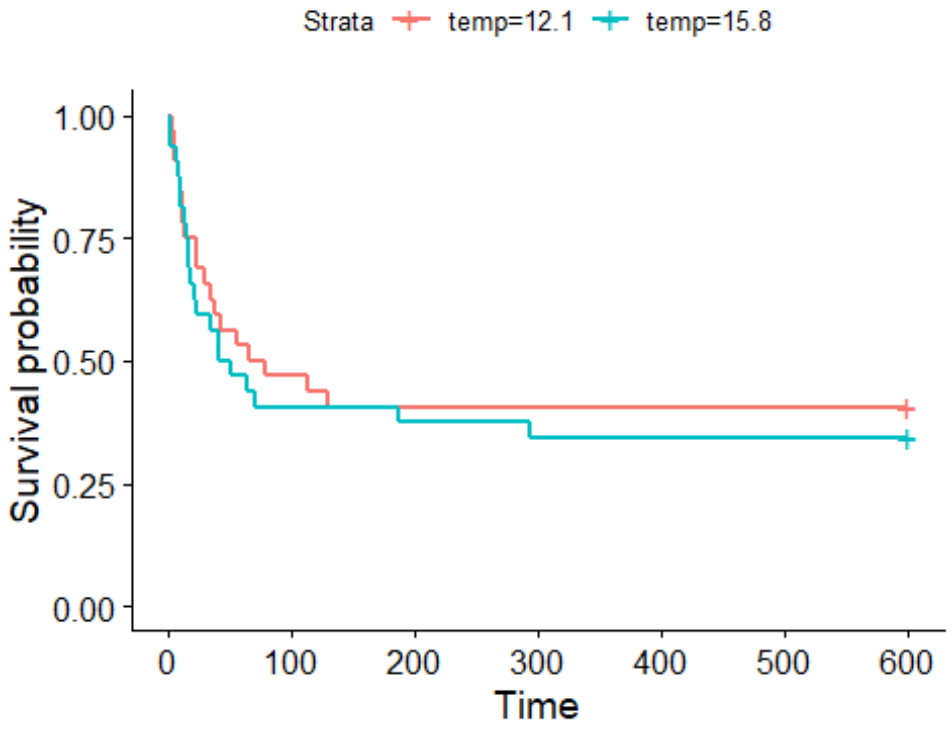
```
##  [1] 600.000+  13.515   10.508    5.007   14.264  600.000+ 600.000+ 186.93
9
##  [9]   9.006   65.814   50.300  600.000+ 600.000+  15.766  600.000+ 293.28
9
## [17]  33.783  600.000+ 600.000+ 600.000+ 600.000+ 600.000+  18.017    7.75
8
## [25] 600.000+  10.511   22.019   70.318   42.002   34.285   41.795   22.52
3
## [33]  41.794   10.758  129.126    9.255   13.517  113.365  600.000+ 600.00
0+
## [41]   6.757  600.000+ 600.000+  37.160  600.000+   2.752   63.814  600.00
0+
## [49] 600.000+  23.024  600.000+  15.769   11.515  600.000+   2.255   78.82
7
## [57] 600.000+ 600.000+  55.805    2.552    4.755   22.774   29.526  600.00
0+
```

Plotting survival curves

```
co2.fit=survfit(crab_surv~co2_atm,data=behav)
ggsurvplot(co2.fit,data=behav)
```




```
temp.fit=survfit(crab_surv~temp,data=behav)
ggsurvplot(temp.fit,data=behav)
```



Fitting a Cox proportional hazards model

This is a two-way test. The p-values for each factor are on the right had side of the grey boxes. The global p-value at at the bottom.

```
fit.coxph=coxph(crab_surv~co2_atm*temp,data=behav)
ggforest(fit.coxph,data=behav)

## Warning: Removed 2 rows containing missing values (geom_errorbar).
```