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Effects of ocean acidification and ocean warming on the behavior and physiology of a subarctic, intertidal grazer

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ABSTRACT: The global ocean is expected to both acidify and warm concurrently; thus, multiple-stressor manipulative experimentation is an emergent area of study that ultimately aims to examine the individual and interactive effects of these factors on marine organisms. We characterized the physiological responses to acidification and warming of the intertidal grazer *Lottia scutum*, and examined how these ocean change variables influenced predator–prey dynamics with *Evasterias troschelii*, a key sea star predator. Specifically, we conducted a laboratory experiment where we exposed limpets to factorial combinations of temperature (11 and 15°C) and pH (7.6 and 8.0), and measured effects on thermal tolerance, metabolic rate, cortisol concentrations, and behavioral responses to the predator. We found that ocean warming (OW) decreased the critical thermal maxima (CT_{max}) and increased cortisol levels in *L. scutum*, whereas ocean acidification (OA) increased the mass-specific metabolic rate in this species. Additionally, we found that there was no significant effect of OA or OW on the anti-predator behavior of *L. scutum* when exposed to *E. troschelii*. These results highlight the need for future studies to integrate multidisciplinary experimental designs (i.e. behavior and physiology) that span multiple levels of biological organization to make ecologically relevant predictions for how marine organisms will respond to ocean change.

KEY WORDS: Multiple-stressors · Ocean acidification · Ocean warming · Gulf of Alaska · Nearshore ecology · *Lottia*

1. INTRODUCTION

Our oceans are changing at an unprecedented rate, accelerated by recent anthropogenic activity such as deforestation and the burning of fossil fuels. Consequently, the world's oceans have absorbed 30% of anthropogenic carbon emissions (Doney et al. 2009). Rising CO_2 concentrations drive changes in seawater carbonate chemistry and reduce pH (Gattuso & Buddemeier 2000). As carbon dioxide (CO_2) concentrations in our atmosphere continue to rise, the oceans are projected to further acidify by 0.3 to 0.4 pH units by the year 2100, a process referred to as ocean acidification (OA) (Stocker et al. 2013). In polar and sub-polar regions such as Alaska, where cooler and fresher seawater support increased solubility of CO_2, alterations to the carbonate system are even more pronounced relative to temperate and tropical regions (Fabry et al. 2009). In addition to acting as a carbon sink, the oceans also absorb heat energy: since 1961, they have absorbed >90% of the increase in heat content on earth (Johnson et al. 2018). This absorption of heat is projected to increase global sea surface temperature by 2.0 to 4.5°C by the year 2100 (Stocker et al. 2013). As a function of thermodynamics, changes in ambient temperature can affect organismal physiological processes including metabolic rate, energy demands, and often, foraging drive and

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intensity (Schmidt-Nielsen 1997, Sanford 1999, Hofmann & Todgham 2010, Somero 2010). These organismal changes are underscored by changes at the molecular level; increasing temperature alters the thermal stability of essential proteins, regulates cellular membrane transport properties, and increases the rate of cell signaling and synaptic transmission (Hofmann & Todgham 2010). When increasing sea surface temperatures force organisms outside of their known tolerance windows, altering rates of metabolic processes, or changing patterns of development or reproduction, there can be indirect effects on species interactions that reverberate throughout marine communities (Hofmann & Todgham 2010). In order to understand how communities and ecosystems might respond to a changing ocean, it is foremost necessary to characterize individual species' vulnerabilities to future, multiple-stressor conditions and how they may further impact biotic interactions.

Organismal stress has been defined as 'the exposure of an organism to an abiotic or biotic forcing factor (a stressor) that results in a shift of one or more biological processes from their respective homeostatic set points' (Sokolova 2021, p. 3). When homeostasis is disrupted due to the exposure of one or more stressors, there can be direct implications on the energy balance of an organism as it expends energy to correct such a disruption (Sokolova 2021). In response to stress, there are coordinated sets of changes we see at the cellular level, e.g. cortisol expression, and organismal level, e.g. metabolic rates and/or anti-predator behavior (Lannig et al. 2010, Lagos et al. 2015, Jellison et al. 2016). These responses allow an organism to return to homeostasis, protect it from stress-induced damage, and minimize a decrease in organismal performance and fitness (Sokolova 2021). Stressors such as elevated temperature and decreased pH can affect acid–base homeostasis and incur additional energy costs for ion transport processes (Nattie 1990, Melzner et al. 2020). The compensatory increases in ion and acid–base transport and concomitant increase in energetic cost are one of the proposed mechanisms utilized to maintain biomineralization in marine calcifiers under OA conditions, making biocalcifiers particularly vulnerable to ocean change (Wood et al. 2010, Stumpp et al. 2012, Pan et al. 2015, Frieder et al. 2017, Clark 2020).

Organisms living within the rocky intertidal zone can help shed light on how different species might cope with future changes to environmental conditions as this ecosystem is subjected to strong daily and seasonal variations in tidal cycles, temperature and wave activity, and is structured by well-defined vertical zonation (Helmuth & Hofmann 2001). Vertical zonation directly reflects the adaptation of marine species to varying degrees of environmental stress (Stillman & Somero 2000). The dynamic physical and biological structure of the rocky intertidal zone is therefore an ideal study system to investigate the effects of ocean change on marine organisms. While many intertidal organisms experience fluctuations in temperature and pH well beyond near-future changes projected for the ocean, several studies show that some intertidal invertebrates are already living near their physiological tolerance limits (Stillman & Somero 2000, Tomanek 2008, Somero 2010), and shifts in baseline environmental conditions may produce suboptimal or lethal conditions for resident marine fauna.

One measure of a species' sensitivity to ocean change is the extent to which it is intolerant of emerging environmental changes that will require plastic acclimatization or genetic adaptation to cope with future conditions. Studies have shown that some species acclimated to warmer habitats exhibit greater rather than lesser sensitivity to future warming when compared with congeners or other individuals of the same species from cooler environments (Somero 2010, Kelley et al. 2011). One explanation for this pattern is that species from warmer environments operate closer to acute, potentially immutable, physiological limits, than those originating from cooler environments (Stillman & Somero 2000, Somero 2010). While there have been fewer studies to determine if a similar trend holds true for pH exposure, a recent meta-analysis determined that for most surveyed taxa, there was indication that the impact of a given experimental partial pressure of carbon dioxide (pCO_2)/pH scenario depended on the deviation from the upper pCO_2 level experienced by local populations (Vargas et al. 2022). In other words, it is necessary to incorporate local environmental variability into the understanding of a species' sensitivity to future ocean change.

OA and ocean warming (OW) have been known to affect common behaviors in marine organisms such as settlement, recruitment, habitat selection, feeding, reproduction, and anti-predator behavior (Wang & Wang 2020; see Clark et al. 2020 and Clements et al. 2022 for criticism of some of this work in vertebrates). In a recent review analyzing the ways in which marine organismal behavior is affected by OA, 53% of the relevant anti-predator behaviors of marine organisms showed negative responses to OA (Wang & Wang 2020). However, the underlying mechanisms by which behavior is altered are poorly understood. Recently, scientists have largely given their attention to neuro-

logical pathways in order to understand OA-related changes in behavior, particularly γ-aminobutyric acid type A ($GABA_A$) receptor theory. $GABA_A$ receptor theory suggests that a decrease in pH causes an imbalance in plasma chloride and bicarbonate ion concentrations as a result of acid–base regulation, which then causes the reversal of ionic fluxes through $GABA_A$ receptors in marine organisms, leading to altered neuronal function (Tresguerres & Hamilton 2017). OA-related changes in behavior can have widespread detrimental effects not only for individuals, but for ecosystems via direct and indirect effects (Jellison et al. 2016, Manríquez et al. 2021).

Few studies have measured changes in both behavior and physiological responses of marine fauna in response to multiple stressors. Common metrics for physiological responses are thermal tolerance and metabolic rate (Hofmann & Todgham 2010, Todgham & Stillman 2013, Peck et al. 2014). Both heart rate and metabolic rate can increase as temperature increases, subsequently increasing the energetic demand needed to maintain metabolism in ectotherms or to maintain thermoregulation in endotherms (Braby & Somero 2006, Peck et al. 2014). Furthermore, there can be an interactive effect of elevated temperature and decreased pH on metabolism, as shown in oysters (Lannig et al. 2010). With increased energetic demand, essential behavioral responses may be affected. Some studies relate altered behavioral responses to changes in metabolic pathways (Lannig et al. 2010), or to a disruption in neural pathways (e.g. $GABA_A$ receptors) (Hamilton et al. 2014). Therefore, designing experiments that investigate both biotic interactions mediated by behavioral changes and physiological responses to stressors is key to understanding how ocean change will not only affect individuals but communities in the future.

In Alaska's nearshore habitat, predation by the mottled star *Evasterias troschelii* on the Pacific plate limpet *Lottia scutum* represents an important predator–prey dynamic in the rocky intertidal zone (Branch et al. 1985). The Pacific plate limpet, found from the intertidal and shallow subtidal zone, is an important grazer along the Alaskan coastline. In response to predation pressure, the plate limpet has developed a series of anti-predator responses when they detect waterborne or tactile cues from their predators (Branch et al. 1985). Plate limpets are often preyed upon by seabirds and sea stars such as the mottled star *E. troschelii* (Branch et al. 1985). The mottled star has a geographical range that extends from the Aleutian Islands, Alaska, to Monterey Bay, California, USA (Rogers & Elliott 2013). They are often found within protected shorelines on rocks, cobbles, docks or pilings, from the low intertidal to subtidal zone. *L. scutum* and *E. troschelii* overlap in both geographical and vertical ranges, providing a well understood predator–prey relationship (Margolin 1964, Rogers & Elliott 2013) that can serve as a model for characterizing the impacts of future ocean change on the behavioral ecology of rocky intertidal species. The predator–prey dynamic demonstrated by these 2 species represents an appropriate model for characterizing the impacts of future ocean change on the behavioral ecology of species that inhabit the rocky intertidal zone. We examine both the individual and interactive effects of an acidifying and warming ocean on the behavior and physiology of *L. scutum.*

2. MATERIALS AND METHODS

2.1. Experimental overview

Adult individuals of both *Lottia scutum* and *Evasterias troschelii* were collected from the rocky intertidal habitat in Kasitsna Bay, Alaska, USA, located adjacent to the joint University of Alaska Fairbanks (UAF) and NOAA Kasitsna Bay Laboratory (59.4677° N, 151.5510° W), in July 2020. Following collection, specimens were immediately placed into large flow-through seawater aquaria (1 per species) at Kasitsna Bay Laboratory and were left to acclimate to the laboratory environment for 7 d. The holding tanks were supplied with seawater pumped directly from Kasitsna Bay, and individuals were fed *Ulva fenestrata* ad libitum throughout the acclimation period. Feeding was halted at the end of the acclimation period prior to individuals being placed in their respective experimental treatments. The total length of the experiment, beginning after the 7 d laboratory acclimation, was 14 d. During the experimental period, *L. scutum* underwent a thermal tolerance assay, closed-system respirometry, 2 behavioral assays, and cortisol measurements.

2.2. Seawater treatments

Following the 7 d laboratory acclimation period, *L. scutum* and *E. troschelii* were randomly divided among 4 treatments. Each treatment consisted of 5 (19 l) *L. scutum* culture vessels, and 1 (19 l) *E. troschelii* culture vessel (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/m711p031_supp.pdf). *L. scutum* culture vessels each contained 30

individuals, and *E. troschelii* culture vessels each contained 1 individual. Treatments consisted of an ambient temperature/ambient pH treatment, (11°C and pH 8.0, present-day conditions), an ambient temperature/decreased pH treatment (11°C and pH 7.6, OA conditions), an elevated temperature/ambient pH treatment (15°C and pH 8.0, OW conditions), and an elevated temperature/decreased pH treatment (15°C and pH 7.6, future predicted conditions). Ambient pH and temperature (pH 8.0 and 11°C) were determined based on incoming seawater measurements at Kasitsna Bay Laboratory. Decreased pH and elevated temperature (pH 7.6 and 15°C) were calculated based on the IPCC, RPC 8.5 projections for the year 2100, which predicts a decrease in pH by 0.4 units and an increase in sea surface temperature by 4°C (Stocker et al. 2013, RPC 8.5). Culture vessels were sealed with a lid to allow all individuals to be completely submerged within their treatments throughout the experiment, and to prevent gas exchange between the culture vessels and atmosphere.

The manipulation of carbonate chemistry (the addition of lab-grade CO_2 to a flow-through seawater culture vessel) to achieve the desired pH of each treatment was maintained via the flow-through Ocean Change Experimental System (OCES) following the 'Guide to best practices for ocean CO_2 measurements' (Dickson et al. 2007). In brief, OCES first scrubs air of all CO_2 using a CO_2 adsorber unit. The air is then continually mixed with a discrete continuous flow of pure CO_2 gas into 1 reservoir head-tank per treatment using a Venturi injector and mass flow controllers (MFC) until the desired pH is achieved (Fangue et al. 2010, Yu et al. 2013). Treatment water is then pumped from the head-tanks into each of the culture vessels using a positive flow dripper so that the seawater from other culture vessels (i.e. predator vessel) never mix with each other (Fig. S1). The flow rate for each vessel was maintained at 7.57 l h^{-1}. The temperature of each treatment was maintained by placing all culture vessels in a flow-through water bath, set at the desired temperature for that treatment (Fig. S1).

2.3. Water chemistry

Incoming seawater (100 ml) was sampled to measure total alkalinity (A_T) once weekly (Table 1). A_T samples were preserved using 10 µl of super-saturated mercuric chloride ($HgCl_2$) to reduce any background respiration that could possibly alter the carbonate chemistry before analysis. A_T was measured using open-cell titration with a Metrohm Titrino 848 according to SOP 3b at the end of the experiment (Dickson et al. 2007). A 2-point calibration was used to determine acid calibration using methodology outlined in standard operating procedure SOP 3a, Chapter 4, in Dickson et al. (2007). Certified Reference Materials (CRMs) of seawater were used to determine the A_T accuracy. Seawater pH was measured at 25°C every other day using a Shimadzu 1800 spectrophotometer (SOP 6b in Dickson et al. 2007); using meta-cresol purple from Acros (batch # 30AXM-QN). A dye impurity correction factor was calculated for batch # 30AXM-QN (Douglas & Byrne 2017) and applied to the final calculation of pH_T (total hydrogen scale). On the days that we did not measure seawater pH using the spectrophotometer, we used an Orion™ ROSS™ Sure-Flow™ pH Electrode to make sure the pH of each culture vessel was at the target pH. Seawater pH reported in Table 1 were measurements taken from the Shimadzu 1800 spectrophotometer. *In situ* pH of each culture vessel was calculated using A_T, $pH_{25°C}$, temperature, and salinity, using CO2Calc with CO_2 constants from Mehrbach et al. (1973) refit by Dickson & Millero (1987) (Table 1). A YSI 3100 conductivity meter was used to measure the salinity of incoming lab seawater every other day (Table 1). The temperature of each seawater culture vessel was recorded every other day (Table 1).

Table 1. Mean (±SD) pH_T (total hydrogen scale), pCO_2, and temperature for each treatment over the experimental period. Mean (±SD) total alkalinity (A_T) and salinity recorded from a separate incoming seawater line over the course of the experiment

Treatment	pH_T *in situ*	pCO_2 (µatm)	Temperature (°C)	A_T (µmol kg^{-1})	Salinity (ppt)
Ambient	7.95 ± 0.07	486.72 ± 88.63	11.34 ± 0.31	–	–
Decreased pH	7.58 ± 0.06	1276.40 ± 218.32	11.33 ± 0.30	–	–
Increased temperature	7.90 ± 0.10	584.60 ± 155.68	15.73 ± 1.78	–	–
Increased temperature/ decreased pH	7.58 ± 0.07	1287.22 ± 252.96	15.82 ± 1.79	–	–
Incoming	–	–	–	2105.01 ± 8.97	31.16 ± 0.12

2.4. Thermal tolerance

On Days 7 and 8, *L. scutum* individuals from each treatment (n = 2 from each culture vessel, N = 10 total) were randomly selected to undergo a thermal tolerance assay. A thermal tolerance assay was conducted on the ambient pH/ambient temperature (pH 8.0, 11°C) and decreased pH/ambient temperature (pH 7.6, 11°C) treatments on Day 7, while on Day 8 a thermal tolerance assay was conducted on the decreased pH/elevated temperature (pH 7.6, 15°C) and ambient pH/elevated temperature (pH 8.0, 15°C) treatments. Assays were not conducted on the same day due to the length of each assay and the equipment available at the field station, but were done sequentially 2 days in a row. Each individual was placed in a separate plastic vessel filled with water originating from their respective culture vessel, which was then subsequently placed within a temperature-controlled water bath. Each vessel was aerated using an air stone throughout the assay. Temperature was increased at a rate of roughly 2°C h^{-1} (Miller & Kelley 2021), and mortality temperature was recorded for each individual. Mortality was confirmed by applying pressure to each individual's muscular foot and observing if its withdrawal reflex was still intact. The critical thermal maxima (CT_{max}) of each individual was established and the mean CT_{max} for each culture vessel was calculated (see Kelley et al. 2011).

2.5. Respirometry

On the day of specimen collection, 5 *L. scutum* were gathered from the rocky intertidal habitat directly adjacent to Kasitsna Bay Laboratory and underwent closed-system respirometry to measure the metabolic rate (MO_2) in their natural environment. After laboratory acclimation and before the experimental period (time zero of the experiment), we measured MO_2 on 5 individuals randomly sampled from the large acclimation tank in which all experimental *L. scutum* were being kept. Finally, on Days 7 and 14, one individual from each culture vessel (n = 5 per treatment) was sampled and underwent 2 final closed-system respirometry measurements. Individuals were placed in separate respirometry chambers, each fitted with a Presens fiber-optic spot. Fiber-optic spots were factory calibrated, which we verified with 100% air-saturated seawater (Jones et al. 2021). Chambers were filled with fully oxygenated water originating from each individual's specific treatment conditions. The respirometry chambers were then placed in a temperature-controlled water bath throughout the assay (11 or 15°C, depending on treatment). The size of the respirometry chamber (300 ml) was chosen such that the estimated body volume of each organism did not exceed ~10% of the chamber volume. Due to the size of the respirometry chambers, we did not use magnetic stirrers to recirculate chamber seawater; however, the chambers were picked up every 30 min to take measurements and were gently mixed during this period. Organisms were not fed during the experimental period; therefore, we were able to avoid postprandial effects while calculating metabolic rate, i.e. these metabolism measurements represent the lower bound of carbon consumption, as metabolic rates typically increase following feeding (Chapelle et al. 1994). During each assay, 3 control chambers were filled with fully oxygenated seawater originating from the respective treatment condition, but did not include an *L. scutum* individual. These control chambers were measured to account for possible background respiration from bacteria and other organisms suspended in the water column. Oxygen concentration in each chamber was measured every 30 min over a 3 h period to allow for handling-stress recovery, and for the capture of an oxygen consumption rate without surpassing an 80% O_2 saturation stress threshold. Oxygen concentration was measured using a Fibox4. At the completion of the assay, all individuals were wrapped in tinfoil, then kept at −18°C until the end of the experiment. Frozen individuals were transported to the University of Alaska Fairbanks, where the wet mass of each thawed individual was recorded, followed by the desiccation of each individual over 72 h at 150°C. After desiccation, the samples were removed from the drying oven and the dry masses were recorded. The samples were then ignited at 650°C for 6 h, and the ash-free dry mass of each individual was recorded by calculating the difference in pre- and post-ignition masses.

Oxygen consumption rate (MO_2, µmol O_2 l^{-1} min^{-1}) for each individual was then calculated from the linear regression of oxygen concentration over time (Table S1). The average MO_2 of the 3 control chambers from each assay was subtracted from the measured organismal rates to account for background respiration or production. Rates were converted to µmol O_2 h^{-1} based on the net volume of water contained in the incubation chamber (net volume = volume of respirometry chamber − volume of organism). These rates were then converted to mass specific MO_2 (µmol O_2 g^{-1} h^{-1}) by dividing by the ash-free dry

mass of each individual. Lastly, we calculated the linear regression of ash-free dry mass versus wet mass to determine if the 2 metrics have a 1:1 linear relationship.

2.6. Behavioral assay

A behavioral assay was conducted on Day 14. Five *L. scutum* individuals from each culture vessel (n = 25 from each treatment) were randomly selected and relocated to 8 l behavioral arenas (n = 5 per 8 l arena). Each individual was placed at the center of their corresponding behavioral arena, which was filled with 2 l of seawater collected from their source culture vessel. Individuals were acclimated to the behavioral arenas for 10 min before the start of the experiment to decrease the possibility of handling stress effects. After the 10 min acclimation period, 1 l of predator-conditioned water was added to each arena and responses were video recorded over a 30 min period. Predator-conditioned water was achieved by ceasing water flow in the sea star culture vessels (19 l) for 2 h before the start of the experiment, allowing predator cues to accumulate. Behavioral response was then measured by analyzing the video recording for each arena over the 30 min period. All video recordings were additionally deposited in an open-access online data repository (www.datadryad.org). As *L. scutum* are known to rotate around their visceral mass and actively flee from their predators when they encounter waterborne or tactile predator cues, we discretely observed whether our individuals exhibited an initial rotation around their visceral mass when introduced to predator-conditioned water, and whether they actively fled throughout the behavioral assay (Espoz & Castilla 2000, Escobar & Navarrete 2011, Aguilera et al. 2019).

To test $GABA_A$ receptor theory, we treated *L. scutum* with gabazine on Day 21 and conducted a second behavioral assay using the same methodology described above. Gabazine (SR 95531) is a $GABA_A$ neurotransmitter receptor antagonist known to inhibit GABA binding to $GABA_A$ receptors in vertebrates (Heaulme et al. 1986), and has also been found to inhibit GABA-induced ion currents or GABA binding to receptors in some invertebrates (Watson et al. 2014). Gabazine has been shown to restore normal anti-predator behavior in the marine mollusk *Gibberulus gibbosus*, which displayed OA-induced behavioral disruptions (Watson et al. 2014). Following the same methodology as Watson et al. (2014), we placed *L. scutum* in 100 ml of seawater (originating from each respective culture vessel) containing 4 mg l^{-1} of gabazine for 30 min before the start of the second behavioral assay.

2.7. Cortisol extraction

Five *L. scutum* individuals were collected from the rocky intertidal habitat adjacent to the lab before the start of the experiment. They were placed in cryovials and flash frozen with liquid nitrogen. These individuals were used to establish the baseline cortisol levels of *L. scutum in situ*. Additionally, 5 individuals were collected after lab acclimation, and 1 individual was collected from each culture vessel (n = 5 from each treatment) on Days 7 and 14. On Day 14, one additional individual from each culture vessel (n = 5 from each treatment) was sampled and flash frozen with liquid nitrogen after being exposed to predator-conditioned seawater and undergoing a behavioral assay. This allowed us to characterize cortisol levels in *L. scutum* both before and after exposure to their predator. All sampled individuals were placed in separate cryovials and kept in liquid nitrogen until the end of the experimental period, where samples were then transported to the University of Alaska Fairbanks and immediately stored at −80°C. We then used a commercial enzyme-linked immunosorbent assay (ELISA) kit (EA65; Oxford Biomedical Research) to measure cortisol levels in our samples, per manufacturer's recommendations (Lagos et al. 2015). In brief, 0.05 g of muscular foot tissue from each sample was homogenized in 1× extraction buffer and centrifuged for 15 min at 5000 × *g*. The supernatant of each sample was collected and immediately stored at −80°C. Once the supernatant of each sample was collected, a 96-well microplate (coated with a rabbit anti-cortisol antibody) was loaded with 50 µl of known cortisol standard solutions and 50 µl of sample supernatants. The microplate was then left to incubate at room temperature for 1 h, after which it was washed 3 times with 300 µl of wash buffer per well. We then added 150 µl of tetramethylbenzidine (TMB Substrate, ThermoFisher) to each well for color development and left the microplate to incubate at room temperature for 30 min. Lastly, the microplate was read at 650 nm using a standard UV-vis 96-well plate reader for spectrophotometric measurements. Cortisol concentration was then determined for each sample by comparing the results to the standard curve (n = 8, $R^2 = 0.99$), which was calculated using the absorbance values of the known cortisol standard solutions:

$$y = 9.43e^{-12.1x} \quad (1)$$

2.8. Statistical analysis

The data from this experiment were analyzed using R Software (version 3.5.1; R Core Team 2016) with the RStudio Workbench. A 2-way ANOVA was used to determine if pH and/or temperature had a significant effect on the CT_{max} of *L. scutum*. Separate 3-way ANOVAs were then used to determine if MO_2 or cortisol concentration in *L. scutum* varied with pH, temperature, and/or time during the experiment. All assumptions for ANOVA were verified and met in R Software. There were no extreme outliers in our dataset (R function: 'identify_outliers()'). We used a Shapiro-Wilk test of normality to confirm a normal distribution of our data. Finally, we applied a Levene's test to confirm the homogeneity of variances among the different treatments. Statistically significant results ($\alpha = 0.05$) from each ANOVA were then followed by a Tukey's post-hoc test to determine which treatment groups differed from one another. Lastly, we applied chi-squared tests to determine whether pH and/or temperature had a significant effect ($\alpha = 0.05$) on the anti-predator behavior of *L. scutum.*

3. RESULTS

3.1. Thermal tolerance

Acclimation treatment temperature had a significant effect on the CT_{max} of *Lottia scutum* ($p = 0.03$). Interestingly, acclimation to a higher temperature (15°C) decreased the overall CT_{max} in *L. scutum* (Fig. 1). The mean (±SD) upper CT_{max} for the ambient temperature treatments was 32.68 ± 2.25°C (OA conditions) and 31.88 ± 1.99°C (present-day conditions), whereas the mean upper CT_{max} for the high temperature treatments were 30.63 ± 0.69°C (future predicted conditions) and 31.44 ± 1.45°C (OW conditions) (Fig. 1). Additionally, individuals that were acclimated to predicted future ocean conditions (pH 7.6, temperature 15°C) exhibited the lowest thermal tolerance across all the treatments with a mean upper CT_{max} of 30.63 ± 0.69°C (Fig. 1). pH did not have a significant effect on the CT_{max} of *L. scutum* nor were there interactive effects of pH and temperature ($p = 0.956$ and 0.156, respectively) (Fig. 1).

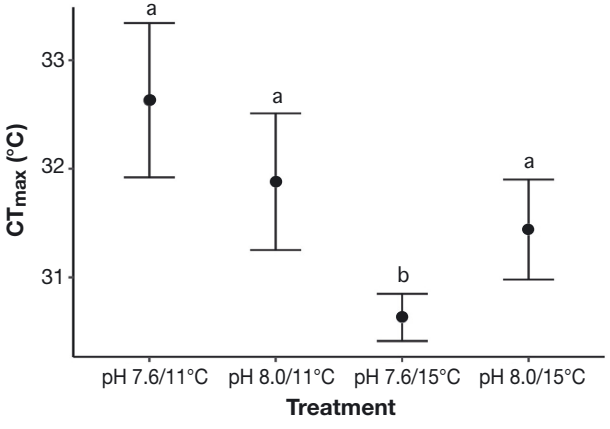

Fig. 1. Variation among treatments (pH/temperature) in the thermal tolerance (mean CT_{max}) of *Lottia scutum*. Error bars denote ±SD for each treatment. Means with different lowercase letters are significantly different ($p < 0.05$, Tukey's post-hoc test)

3.2. Metabolism

After lab acclimation, all individuals expressed a significant increase ($p < 0.001$) in MO_2 after they were placed into their specified treatment conditions (lab acclimation to Day 7) (Fig. 2). The MO_2 of individuals that were acclimated to pH 7.6 (pH for the year 2100) significantly increased ($p < 0.001$) over the course of the experiment (lab acclimation to Day 14) (Fig. 2). The mean (±SD) MO_2 of individuals during lab acclimation was 6.47 ± 6.67 µmol O_2 g^{-1} h^{-1}, whereas the mean MO_2 of individuals in the low pH treatments on Day 7 increased to 9.88 ± 3.56 µmol O_2 g^{-1} h^{-1} (future predicted conditions) and 12.69 ± 4.60 µmol O_2 g^{-1} h^{-1} (OA conditions). On Day 14, the mean MO_2 of individuals in low pH treatments increased to 22.78 ± 2.34 and 20.16 ± 4.89 µmol O_2 g^{-1} h^{-1}, respectively. However, after an initial increase in MO_2 from lab acclimation to Day 7 of the experiment, MO_2 in individuals that were acclimated to pH 8.0 did not increase from Day 7 to Day 14 (Fig. 2). Additionally, MO_2 was the highest in the future predicted oceanographic conditions treatment (pH 7.6, temperature 15°C) by Day 14 (Fig. 2). When compared to the MO_2 of 'field-caught' individuals, the MO_2 of *L. scutum* was significantly lower ($p < 0.001$) after being acclimated to the laboratory environment for 7 d (Fig. 2). The mean MO_2 of field-caught individuals was 13.62 ± 5.52 µmol O_2 g^{-1} h^{-1}, while the mean MO_2 of lab-acclimated individuals was 6.47 ± 6.79 µmol O_2 g^{-1} h^{-1}. MO_2 of experimental *L. scutum* on Day 7 increased and were then comparable to MO_2 of field-caught individuals (Fig. 2). However, by Day 14, individuals that were acclimated to a lower pH exhibited a significantly higher ($p = 0.004$) MO_2 compared to the field-caught *L. scutum* (Fig. 2). We found that there was no significant effect ($p = 0.727$)

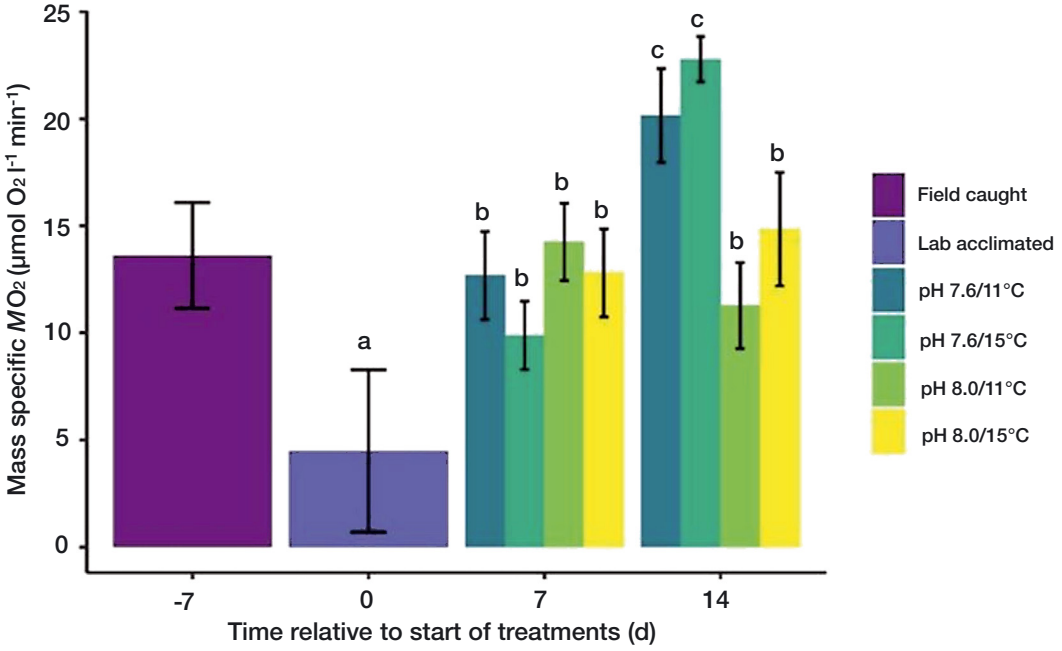

Fig. 2. Variation among experimental treatments and through time in the metabolic rate (MO_2) of *Lottia scutum*. Colored bars represent the mean MO_2 of each treatment. Mean MO_2 of field-caught individuals were measured 1 wk before the start of the treatments, whereas mean MO_2 of individuals acclimated to the laboratory environment were measured right before the start of the treatments. Error bars denote ±SD. Means with different lowercase letters are significantly different ($p < 0.05$, Tukey's post-hoc test)

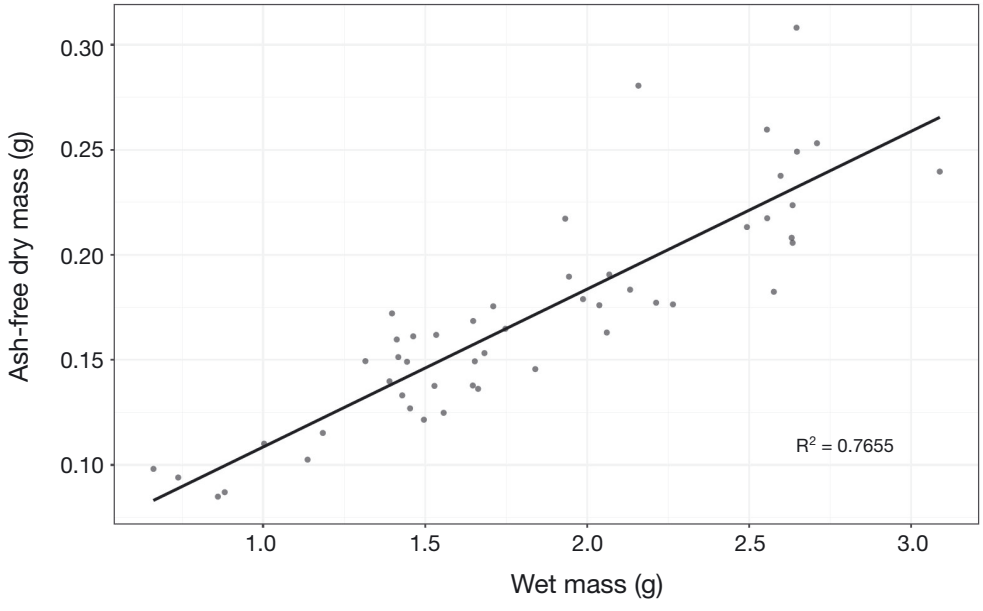

Fig. 3. Linear regression of wet mass versus ash-free dry mass of *Lottia scutum* individuals ($p < 0.001$)

of acclimation temperature on MO_2 of *L. scutum*. Lastly, the wet mass versus ash-free dry mass linear regression of *L. scutum* individuals showed a moderately high level of correlation, with $R^2 = 0.7655$ and $p < 0.001$ (Fig. 3).

3.3. Behavior

In our control treatment, representing present-day conditions (pH 8.0, temperature 11°C), 44% of individuals actively fled from cues of their predator, and

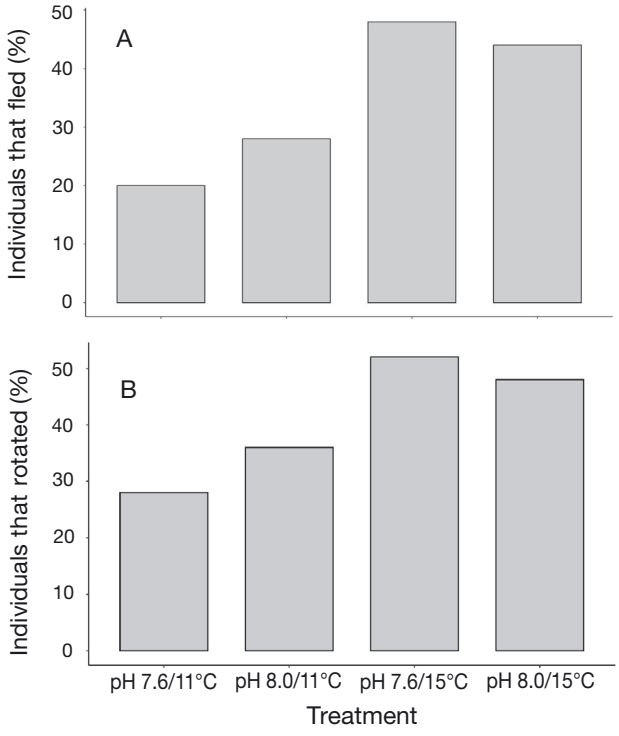

Fig. 4. Percentage of *Lottia scutum* from each treatment (pH/temperature) that exhibited anti-predator behavior when introduced to cues of their sea star predator: (A) individuals that fled from predator cues; (B) individuals that rotated from predator cues

52% of individuals initially rotated around their visceral mass (Fig. 4). However, in our future predicted oceanographic conditions treatment (pH 7.6, temperature 15°C), we found that only 28% of individuals actively fled from predator cues, and 36% of individuals rotated around their visceral mass (Fig. 4). A chi-squared test determined that neither pH nor temperature significantly affected ($p = 0.124$, $p = 0.288$) the antipredator behavior of *L. scutum.* Additionally, *L. scutum* showed no significant change ($p = 0.745$, $p = 0.1701$) in behavior after being dosed with gabazine on Day 21. In our control treatment, 20% of individuals actively fled from cues of their predator, and 24% of individuals initially rotated around their visceral mass. Comparatively, in our future predicted oceanographic conditions treatment, we found that only 12% of individuals actively fled from predator cues, and 56% of individuals rotated around their visceral mass.

3.4. Cortisol

Our results demonstrated a significant increase ($p < 0.001$) in cortisol concentration in *L. scutum* through time, with the highest cortisol concentration originating from the high temperature treatments (temperature 15°C) (Fig. 5). On Day 7, the cortisol concentration among treatments were not significantly different ($p = 0.631$), and the mean cortisol concentration averaged across all treatments was 62.12 pg g^{-1}. However, on Day 14, cortisol concentrations in the high temperature treatments increased to 376.76 pg g^{-1} (future predicted conditions) and 389.76 pg g^{-1} (OW conditions). There was no significant difference ($p = 0.457$) in cortisol concentration between field-caught *L. scutum* and lab-acclimated *L. scutum* (Fig. 5). We saw no significant effect ($p = 0.328$) of pH on cortisol levels throughout the experiment. Additionally, we found no significant difference ($p = 0.469$) in cortisol concentration before or after the individuals were exposed to predator cues (Fig. 6).

4. DISCUSSION

Our results suggest that both temperature and pH, separately and differentially affected the physiology of *Lottia scutum.* Temperature is often thought of as one of the most important factors influencing the physiology and ecology of ectotherms (Castañeda et al. 2004, Mora & Maya 2006, Peck et al. 2014). Additionally, temperature plays a significant role in determining the biogeographical distributions of ectotherms in thermally heterogeneous environments (Somero 2002). In our thermal tolerance study, we found that acclimation to a higher temperature decreased the overall CT_{max} of *L. scutum,* suggesting that an increase in acclimation temperature (as projected for the year 2100) is enough to induce a high physiological toll on this species (Fig. 1). This is somewhat surprising in that many marine species that have been acclimated to higher temperatures in the laboratory environment generally exhibit an overall increase in CT_{max} (Podrabsky & Somero 2006, Bilyk & DeVries 2011, Kelley 2014). However, a few studies report similar results to our thermal tolerance study in other marine invertebrates (especially those originating from high latitude environments) (Peck et al. 2009, 2010, 2014).

One way to compare the response of different species and populations to thermal acclimation in the laboratory is to calculate the acclimation response ratio (ARR) (Claussen 1977, Kelley 2014). A higher ARR value indicates that an organism is able to provide a greater increase in their thermal tolerance threshold after acclimation to a higher temperature

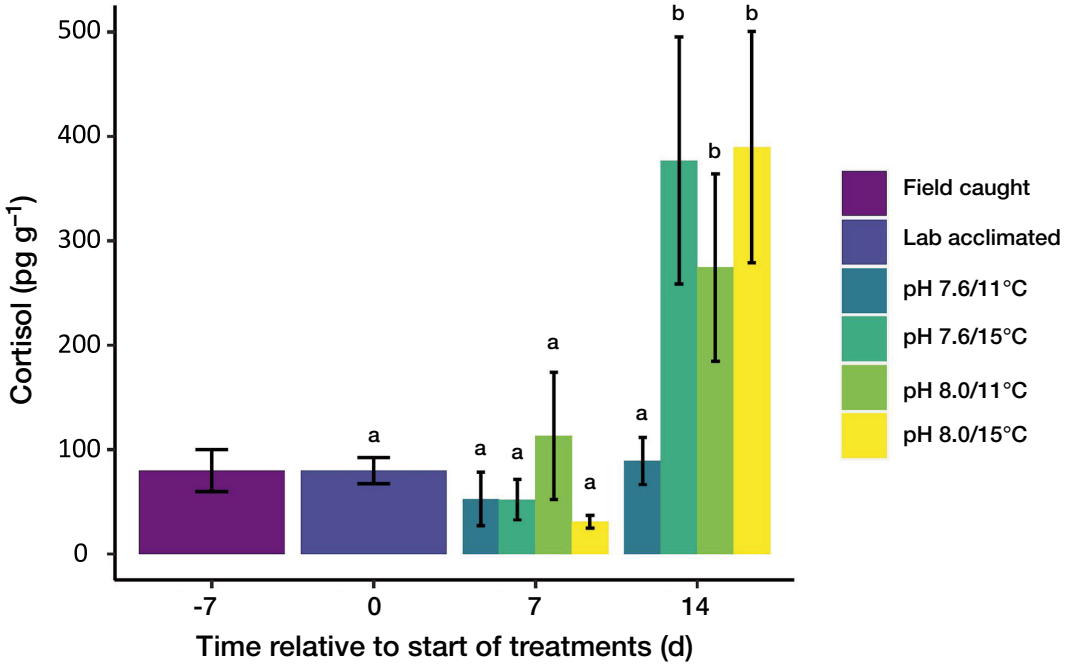

Fig. 5. Variation among experimental treatments and through time in the cortisol concentration of *Lottia scutum*. Colored bars represent the mean cortisol concentration of each treatment. Mean cortisol concentration of field-caught individuals were measured 1 wk before the start of the treatments, whereas mean cortisol concentration of individuals acclimated to the laboratory environment were measured right before the start of the treatments. Error bars denote ±SD. Means with different lowercase letters are significantly different ($p < 0.05$, Tukey's post-hoc test)

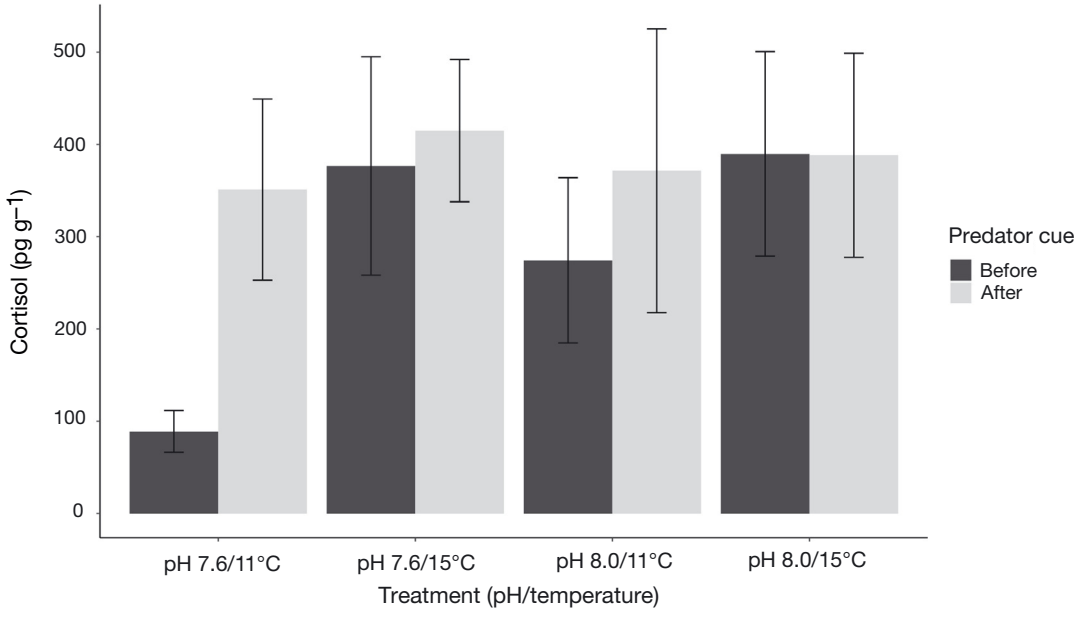

Fig. 6. Variation among treatments (pH/temperature) in mean cortisol concentrations of *Lottia scutum* before and after being introduced to cues of their sea star predator. Error bars denote ±SD for each treatment

than an organism whose upper thermal tolerance threshold increases only marginally after acclimation (Kelley 2014). A recent meta-analysis found that every invasive species surveyed demonstrated a positive ARR value (Kelley 2014). However, 6 out of 19 native species exhibited a negative ARR value, where acclimation to a higher temperature decreased the overall upper thermal tolerance value in

subpolar terrestrial arthropods and several freshwater mussel species, with ARR values of −0.1 and −1.1, respectively (Slabber et al. 2007, Pandolfo et al. 2010, Kelley 2014). When comparing the CT_{max} of *L. scutum* from the present-day and future predicted conditions treatment, we calculated an ARR value of −0.31.

Understanding how ocean change will affect marine organisms requires a detailed knowledge of how closely individual species are currently living to their natural thermal tolerance limits, and what capacity they have to adapt to future oceanographic conditions (Orr et al. 2020). Similar to previous studies, our findings suggest that *L. scutum* are already living near the edge of their thermal tolerance window, and could be at risk in a warming ocean (Stillman & Somero 2000, Peck et al. 2014). While our results do not demonstrate any statistically significant interactive effects of both OW and OA on the upper thermal tolerance of *L. scutum,* other studies report a narrowing of the thermal tolerance window of marine ectotherms due to the synergistic effects of elevated temperature and decreased pH (Pörtner & Farrell 2008). Nonetheless, the lowest recorded CT_{max} value, 30.63 ± 0.69°C, was reported in the future treatment condition, 15°C, pH 7.6 for *L. scutum*, suggesting the effects of OA and OW were interactive in nature (Fig. 1).

Individual and interactive stressors can strongly affect the energy metabolism of marine ectotherms and modify the energy fluxes within an organism and ecosystem (Kelley & Lunden 2017, Sokolova 2021). In general, 3 metabolic responses that aim to return an organism to homeostasis can be captured using respirometry techniques: an increase in metabolism, a decrease in metabolism, or no change in metabolism (Kelley & Lunden 2017). An increase in metabolism usually indicates the need for processes that require more cellular energy to attempt to return to or maintain homeostasis (Randall et al. 2002, Alberts et al. 2008). In contrast, metabolic suppression is generally considered a short-term response for dealing with stress, and involves down-regulating metabolic processes which can result in a decrease in protein synthesis, somatic tissue growth or maintenance (Comeau et al. 2010, Thomsen & Melzner 2010, Kültz 2020). Our results show that the metabolism of *L. scutum* increased throughout the experimental period, and that individuals that were acclimated to OA conditions expressed the greatest significant increase in metabolism. Additionally, we found that compared to the metabolism of field-caught *L. scutum*, experimental *L. scutum* demonstrated an initial short-term decrease in metabolism before increasing throughout the experimental period. However, it is important to note that our MO_2 measurements of field-caught *L. scutum* captured this species *in situ* metabolism, meaning that it is impossible to determine the feeding status of those individuals before we conducted closed-system respirometry. As the consumption of food can increase the MO_2 of individuals, it is possible that the differences in metabolism we measured between field-caught and lab-acclimated *L. scutum* were influenced by postprandial effects.

A similar study, which investigated the impact of OA on the metabolism of the oyster *Crassostrea gigas* found that with the addition of OW, standard metabolic rate (SMR) rose significantly in individuals acclimated to both ambient and decreased pH environments (Lannig et al. 2010). Moreover, there was a stronger increase in SMR of those individuals acclimated to decreased pH conditions compared to those acclimated to ambient pH conditions (Lannig et al. 2010). With an increase in metabolism, there is also a subsequent increase in the energetic demand needed to maintain this new metabolic rate. Several studies have demonstrated that the additional energy needed to maintain an increase in metabolism is at times reallocated away from other important processes such as growth, calcification, and reproduction (Kelley & Lunden 2017). Talmage & Gobler (2010) found that the shell size, diameter, and hinge thickness of the hard clam *Mercenaria mercenaria* decreased in individuals raised under OA conditions. For other species, it has been shown that individuals can maintain calcification but at the expense of growth and body size (Kroeker et al. 2014). Given these previous findings, it is possible that we might observe changes in calcification, growth, and/or body size in *L. scutum* individuals under future predicted oceanographic conditions. These trade-offs in animal life histories highlight the importance of considering bioenergetics in understanding organismal performance, plasticity, and adaptation to the challenges posed by future ocean change.

OA and OW have been shown to affect the behavior of a variety of marine species (Wang & Wang 2020); however, the mechanisms behind these changes in behavior have been far less studied. Sensory impairments, such as the disruption in chemoreception or the chemical alteration of cues under decreased pH conditions have been observed in various invertebrates (Briffa et al. 2012, Clements & Hunt 2014, Ashur et al. 2017, but see Clark et al. 2020 and Clements et al. 2022 for criticism of some of this work in vertebrates). It has also been hypothesized

that physiological effects of environmental stress can alter behavior via bioenergetics (Romero et al. 2009). While our *a priori* hypothesis was that OA and OW would affect the anti-predator behavior of *L. scutum* and that treating *L. scutum* with gabazine would restore their ability to detect their sea star predator, we concluded that pH and temperature had no significant effect on the behavior of *L. scutum* when exposed to *Evasterias troschelii*, and therefore treating *L. scutum* with gabazine was inconsequential. By designing experiments that integrate behavior, physiology, and molecular methodological approaches, we can begin to tease apart the various ways in which multiple stressors might affect marine organisms both directly and indirectly.

Cortisol is a corticosteroid frequently used as a stress biomarker in vertebrates (Hellhammer et al. 2009, Yeh et al. 2013, Gong et al. 2015). However, cortisol concentrations in marine invertebrates have been far less studied, and only 1 study, to date, has measured cortisol concentrations in a marine mollusk (Lagos et al. 2015). Our study found that there was a significant increase in cortisol over time, and that individuals acclimated to OW conditions expressed the highest levels of cortisol by the end of the experimental period (Fig. 5). It is important to note that organisms can release cortisol during conditions of starvation (Park et al. 2012, Dar et al. 2019). While it is unlikely for the duration of this experiment that *L. scutum* entered into a mode of starvation, we cannot rule out the possibility. We did not find any significant difference in cortisol concentrations before or after acutely exposing *L. scutum* to cues of their sea star predator. While this study is one of the first of its kind to characterize cortisol concentrations in a marine mollusk when exposed to multiple environmental stressors, caution is needed when attempting to compare our results to future studies. Exposure time, environmental stressors, and the type of tissue collected from individuals can lead to vastly different results (Binder et al. 2019). Future studies measuring cortisol concentrations in marine invertebrates should take these considerations into account for better comparisons between studies.

5. CONCLUSIONS

The oceans are both warming and acidifying concurrently. As the interactive effects of OA and OW can often vary from the individual effects of each stressor (Folt et al. 1999, Piggott et al. 2015), multiple-stressor research is a rapidly expanding field of science that aims to understand and ultimately predict the interactions between stressors. By characterizing these interactions, researchers can provide more ecologically relevant results for both the scientific community and environmental stakeholders. Additionally, multiple-stressor research needs to largely shift its focus towards higher levels of biological organization, as ecosystem managers and stakeholders are often more interested in the effect of stressors on communities and ecosystems, rather than individuals (De Laender 2018, Thompson et al. 2018). This study spanned multiple biological levels of organization from the molecular level to the level of interacting species. While we examined the relationship between an important intertidal grazer and its predator, future studies should begin to integrate additional trophic levels, such as primary producers, into their experimental designs (Gaylord et al. 2015, Jellison & Gaylord 2019). This is especially important as environmental stressors can often alter the trophic relationships between species (Arnold et al. 2012, Bruder et al. 2017). Lastly, future studies should take a multidisciplinary approach in designing experiments, in order to holistically understand the effects of multiple stressors on marine organisms. This is vital for the sustainable management of resources, for the conservation of biodiversity, and for the maintenance of ecosystem services.

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