EFFECTS OF INTERTIDAL POSITION ON THE RESPONSE TO OXYGEN AND DESICCATION STRESS IN THE COMMON ACORN BARNACLE, 

Balanus glandula

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COMMITTEE MEMBERSHIP

TITLE: Effects of intertidal position on the response to oxygen and desiccation stress in the common acorn barnacle, *Balanus glandula*

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ABSTRACT

Effects of intertidal position on the response to oxygen and desiccation stress in the common acorn barnacle, *Balanus glandula*

Megan Michelle Dotterweich

Sessile invertebrates in the rocky intertidal experience intermittent periods of air exposure due to tidal flux, presenting risks of temperature extremes, hypoxia, nutrient limitation, and most dangerously, desiccation. Microscale variation in severity and frequency of these risks is widely dependent on vertical position within the intertidal zone. Common acorn barnacles (*Balanus glandula*) have a wide vertical distribution in the intertidal, creating large differences in microhabitat between the highest and lowest individuals in the population. This study set out to explore whether tidal position dependent differences exist in the response to oxygen and desiccation stress in *B. glandula*. We hypothesized that *B. glandula* from relatively high tidal heights, which are exposed to the air for a greater duration, will be better suited to tolerate anoxic and desiccation stress than conspecifics from lower tidal heights. To explore this, we compared responses of *B. glandula* collected from high and low intertidal positions to A) anoxia (0 mg O$_2$/L) and hypoxia (≤ 2 mg O$_2$/L) on survival, behavior (closed opercular plates, cirral beating, pneumostome formation), enzyme activity (lactate dehydrogenase (LDH), superoxide dismutase (SOD)), and tissue-lactate accumulation, in addition to B) the effects of humid (98% RH) and dry (32% RH) air emersion (at 17°C) on survival, opercular behavior (open/closed), evaporative water loss (EWL) rates, and tissue-lactate accumulation. Relative to barnacles from the low intertidal, we found that barnacles from the high intertidal survive longer during anoxia and air emersion stress, close their operculum sooner in dry air, lose more water during air exposure at any humidity level, and tend to accumulate less D-lactate. We suspect that high intertidal *B. glandula* can survive desiccation longer by ejecting stores of mantle cavity fluid, thereby creating a moist lung-like, air-filled internal environment, then remaining largely closed and metabolically inactive when in air to avoid drying out and becoming anoxic. These differences may reflect plasticity or selective pressure in response to environmental stress during development and highlight the potential importance of microscale stress heterogeneity in influencing species climate change tolerance and potential distribution patterns.

Keywords: *Balanus glandula*, acorn barnacle, intertidal zonation, plasticity, oxygen stress, behavior, survival, desiccation, lactate, lactate dehydrogenase, superoxide dismutase
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1. INTRODUCTION

Alternating periods of air exposure and submersion in the rocky intertidal zone lead to frequent, wide fluctuations in metabolically influential factors, such as temperature, oxygen, and moisture (Dahlhoff, 2004; Ober et al., 2019; Somero, 2002). The success of sessile species in the intertidal is dependent on their ability to cope with emersion-related (e.g., desiccation, thermal stress, oxygen-uptake limitations) and submersion-related stressors (e.g., competition, predation, hypoxia) (Harley & Helmuth, 2003; Shick et al., 1988). Disparities in the magnitude and variability of these stressors between tidal heights can lead to the emergence of distinct tidal height-dependent differences in metabolic properties not only between species of intertidal invertebrates (Augenfeld, 1967; Stillman & Somero, 1996), but within species (Harley & Helmuth, 2003; Desai & Prakash, 2009).

Shore height has been documented to affect baseline metabolic characteristics in several intertidal barnacles. Within the barnacle *Balanus amphitrite*, individuals from low intertidal zone have lower antioxidant enzyme activity - of both catalase and superoxide dismutase - than individuals from the high intertidal zone (Desai and Prakash, 2009). Similarly, Horn et al. (2021) found that common acorn barnacles (*Balanus glandula*) from the low intertidal zone typically have smaller body sizes, reduced cirral activity during submersion, and significantly higher baseline levels of D-lactate and lactate dehydrogenase (LDH) activity compared to conspecifics from the high intertidal zone.

Intertidal position influences metabolic characteristics in barnacles when they are experiencing non-stressful, ‘baseline’ conditions (Horn et al., 2021). We predict that these
Tidal height-driven differences will be more numerous and/or more pronounced during environmental stress. Some evidence for this hypothesis has already been gathered. During thermal stress, for example, activity of the antioxidant enzyme superoxide dismutase (SOD) was significantly higher and survival times were significantly longer in *B. glandula* from the high intertidal zone compared to those from the low intertidal zone (Anderson, 2022). Changes in the activity of antioxidant enzymes such as SOD, often occur as part of the cellular stress response, and upregulation of antioxidants during low oxygen stress has been broadly observed across phyla (e.g., arthropods, mollusks, chordates, etc.) (Moreira et al., 2016).

The common acorn barnacle (*B. glandula*) represents an ideal organism for studying the effects of intertidal position on the stress response within a single species. This is due to their wide (~1 m) vertical distribution in the rocky intertidal zone, which creates large differences in emersion time between the highest and lowest barnacles in a population. *B. glandula* at the highest position in their vertical range spend on average less than 1% of each day fully submerged, compared to barnacles at the lowest point in their range which spend on average around 50-60% each day fully submerged (water level data, Port San Luis, NOAA). Sessile intertidal organisms respond to prolonged air exposure in a number of ways. Differences in these responses then influence oxygen uptake dynamics and metabolic strategies exploited during emersion. Mussels, for instance, close their shells in air to avoid desiccation, which limits oxygen exchange and induces facultative anaerobic metabolism (Gäde, 1983; Wang & Widdows, 1993). Acorn barnacles, on the other hand, take advantage of abundant oxygen in air by forming a
minute opening between their opercular plates, called a pneumostome, which allows access to atmospheric oxygen, yet resistance to desiccation (Barnes et al., 1963; Foster, 1971a). By doing this, barnacles can avoid switching to anaerobic respiration in air, generating more ATP per gram of food and limiting production of anaerobic byproducts, like lactate (Barnes et al., 1963; Davenport & Irwin, 2003; López et al., 2003; Ober et al., 2019b; Rangaswami et al., 2020; Vial et al., 1999). The strategy used by barnacles for tolerating air exposure is remarkably consistent with the tidal-height dependent differences in [D-lactate] and LDH activity we previously described for _B. glandula_ (Horn et al., 2021).

In the present study, we set out to determine if there were differences in the stress response to oxygen limitation and desiccation between _B. glandula_ anchored at relatively low and high shore heights within a rocky intertidal habitat on the Central California coast. To achieve this goal, we looked for effects of intertidal position on survival, behavior, evaporative water loss rates, enzymatic indicators of anaerobic metabolism [lactate dehydrogenase (LDH) activity and D-lactate concentration] and oxidative stress response [superoxide dismutase activity (SOD)] during hypoxia and air emersion.
2. METHODS

2.1 Barnacle Collection

Common acorn barnacles (*Balanus glandula*) were collected during low tide [Mean Lower Low Water (MLLW) ≤ 0 m] from the rocky intertidal zone at the base of the California Polytechnic State University Research Pier (Cal Poly Pier; 35°10'41.2"N 120°44'34.8"W) in Avila Beach, CA. Live adult *B. glandula* (operculum >2 mm) were removed by hammer and chisel (with substrate still attached) from the lowest and highest intertidal positions characteristic of this species’ vertical range at this field site. The designations of low and high intertidal positions at this site were: +1 m and +2.1 m above MLLW, respectively. Barnacles were collected at each of the two tidal heights every 1 m along a 15 m transect oriented parallel to the shoreline. At each meter mark, barnacles (n=3-5/tidal height) were chosen for collection by circling out from the transect tape until size-appropriate individuals were located. Barnacles were either chiseled off the underlying sandstone conglomerate rock or collected along with the mussels onto which they had settled. Mussels with barnacles attached were pried off the rock, shucked, cleaned of any mussel tissue, and excess shell was trimmed around where the barnacle was attached.

Barnacles that were collected for baseline ‘field’ measurements of metabolite or enzyme assays were measured and tissues were excised immediately upon collection (referred to as *in situ*). Barnacles that were collected for use in lab-based experiments were immediately transported back to the Cal Poly Research Pier or the main campus. Holding conditions for these individuals are outlined in each section below. For both groups of barnacles, we measured the following morphometrics to the nearest 0.01 mm
using digital calipers (VINCA, model DCLA-0605, Clockwise Tools Inc., Valencia, CA): total shell height, aperture height, aperture width, base height, and base width (see Fig. 2 in Horn et al., 2021 for diagram). For barnacles in which *in situ* tissue excision occurred we removed the prosoma and thorax with cirri, but excluded the mantle and gonad tissues which adhere to the inside surface of the shell. Hereafter, this sample is referred to as a whole-animal tissue. These samples were flash-frozen in liquid nitrogen and stored at -80°C until subsequent analysis.

### 2.2 Effects of intertidal position on survival and behavior during anoxia

Following collection events, barnacles \([n=8/\text{tidal height (low, high)}]\) were brought to the Cal Poly campus where they were evenly divided between two 40L glass tanks filled with oxygenated filtered seawater (FSW; 30L; 15°C, 34ppt and bubbled with atmospheric air). After a 24 h holding period, one tank was deoxygenated via bubbling with nitrogen gas (>0.5mg O\(_2\)/L), while the other tank continued to be maintained at oxygen-saturating conditions (=normoxic control; 8.3 mgO\(_2\)/L). Individual barnacles were then checked once daily for mortality until all barnacles in the anoxic treatment were dead (no mortality was observed in the control). Barnacle survival was determined by probing the opercular plates with a blunt dissecting needle. If plates depressed inward or wobbled when probed, the barnacle was considered dead and removed from the tank. Animals were not fed during the experiment. This experiment was carried out in this way three separate times between September 2021 and January 2022.

In addition to monitoring survival daily, we observed behavior in accordance with the behavior scheme used in Horn et al (2021). Barnacle behavior was classified as
either: 1) cirri beating (cirri extended from the operculum and rhythmically beating), 2) forming pneumostome (opercular plates open with cirri retracted), or 3) opercular plates closed. Behavior was observed and recorded prior to probing the barnacles to determine if alive or dead. Behavioral observations were then quantified as the proportion of barnacles from each tidal height and treatment group that were engaged in each behavior, and these proportions were averaged between the three runs of the experiment (n=3). Only observation from 5 min, 6 h, 24 h, 48 h, 72 h, and 96 h time points were included in behavior analysis to reflect the behavior of the treatment barnacles prior to when mass mortality began. Aside from the observation done at t=6 h which was offset from the rest by 6 h, all behavioral observations were made at the same time each day.

Barnacle pneumostome behavior was compared to overall survival time and proximity to death (‘days until death’) for each barnacle within the anoxic treatment. To compare barnacle behavior with proximity to death, we subtracted the day of each behavior observation from the time that each barnacle was observed dead. This variable (days until death) was grouped into the following time bins for analysis: greater than 10 days, 8-10 days, 6-8 days, 4-6 days, 2-4 days, and less than 2 days until death. Proportions of barnacles engaged in pneumostome formation for each treatment and tidal position in each time bin were calculated for each round of the experiment, and an average value of the three trials was determined.

2.3 Effects of intertidal position on lactate accumulation during hypoxia

Following collection (March 2020), barnacles [n=30/tidal height (low and high)] were transported to the Cal Poly Pier where we affixed latex labels with superglue to
allow subsequent identification. Clumped barnacles were separated where it was possible to break individuals apart without damaging their shells. If separation was not possible, a tag was placed on each individual in the clump. Barnacles from each tidal height were randomly assigned to one of three replicate 40 L exposure tanks - such that every replicate tank had individuals from every intertidal height – and tanks were each filled with FSW that was allowed to continuously overflow onto a water table. Barnacles were held in these tanks for 24 h at 15˚C, 34 ppt and ambient light conditions. After the 24 h adjustment period, tissues were collected (as described in section 3.1) from a subset of control (time=0 h) barnacles (n=15 barnacles per tidal height), then flash-frozen in liquid N\textsubscript{2} and stored at -80˚C. After the time 0h barnacles were removed, free-flowing FSW was stopped, and we began bubbling N\textsubscript{2} gas into each of the three replicate tanks to deoxygenate the seawater. Plastic sheets were floated on top of the water in each tank to prevent the inward diffusion of atmospheric O\textsubscript{2}. Dissolved oxygen fell to <2 mg O\textsubscript{2}/L in ~30 min. We used a PRO ODO YSI to intermittently monitor oxygen levels in each of the three replicate tanks to ensure DO levels did not rise above 2 mg O\textsubscript{2}/L over the course of the experiment. Barnacles were held under these conditions for 48 h. After this time, whole body tissues from the remaining barnacles in each tank (n=15/tidal height) were excised, flash-frozen in liquid N\textsubscript{2}, and stored at -80˚C until subsequent lactate analysis.

2.4 Lactate Measurements

Whole-animal D-lactate concentrations were quantified using spectrophotometric assays as outlined in Horn et al. (2021). Tissue samples were homogenized in a 5-fold dilution of 10% trichloroacetic acid, centrifuged for 15 minutes at 10,000 x g at 4˚C, and
supernatants were stored at -80°C until subsequent processing analysis. Samples were later thawed on ice and 16 μL of undiluted supernatant was added to UV-transparent 96-well plate in triplicate. For each triplicate sample, two wells received 210 μL of the standard assay buffer [combined at a ratio of 1mL glycine buffer (Sigma-Aldrich #G5418), 2mL distilled water, 18 μL D-LDH, and 5mg NAD⁺], while one well received an equal volume of the assay buffer minus D-LDH (to serve as the negative control). Reactions were allowed to incubate for 45 min at room temperature before endpoint absorbance values were measured at 340nm (Victor X4 Multimode Plate Reader, Perkin Elmer). Unknown sample lactate concentrations (mM) were then determined by comparison to a standard curve generated from duplicate D-lactate samples of known concentration (0-5 mM) on each plate. Final values for all samples and standards were corrected for background absorbance from a sample of lactate-free assay buffer.

2.5 Effects of intertidal position on LDH and SOD activity during hypoxic immersion and reoxygenation

Following collection (July 2021), barnacles from high and low tidal positions were transported to the main campus where they were randomly assigned to one of two 40 L tanks filled with 20 L of oxygen-saturated, FSW (15°C and 34 ppt). After a 24 h adjustment period, tanks were either changed to hypoxic condition (2 mg O₂/L) or maintained at normoxic control conditions (8.3 mg O₂/L). The hypoxic tank was regulated at 2 mg O₂/L using an Arduino-based oxygen control system, which was programed to bubble nitrogen gas into the tank when oxygen levels exceeded 2 mg O₂/L. In the control treatment, normoxic conditions were maintained by continued bubbling
with atmospheric air. Surface gas-exchange was minimized by floating a plastic sheet on
the surface of the water in all tanks. Oxygen concentrations in both treatment conditions
were monitored with the Arduino oxygen control system and secondary oxygen sensors
(Loligo Systems). After 48 h, the hypoxic tank was reoxygenated with atmospheric air to
initiate a 24 h recovery period. Barnacles from normoxic control and hypoxic treatment
tanks were sacrificed and tissues were excised (as described in Section 3.1) at each of the
following time points: immediately at the end of the 24 h adjustment period (t=0 h), after
48h in hypoxic or normoxic conditions (t=48 h), and again at the end of the 24h recovery
period (t=72 h). Barnacles were not fed for the entire duration of the experiment. This
experiment was repeated as described here two separate times, with two weeks between
each trial. Over the two trials, we sampled n=12 barnacles from each tidal height (low
and high) at each timepoint (0, 48 and 72 h) across both treatments (normoxia or
hypoxia). Dissected tissues were stored at -80°C until subsequent LDH and SOD
analysis.

2.6 LDH and SOD measurements

Frozen tissues were thawed on ice and homogenized (PowerGen homogenizer) at
high power for 30 s in a 1:10 (w/v) dilution of 50 mM potassium phosphate (KPO) buffer
(pH 7.5). Homogenates were centrifuged at 14,000 RCF for 10 min at 4°C. After
centrifugation, supernatants were further diluted in a 1:10 of 50 mM KPO buffer (LDH
assay) or 1:20 in 100 mM sodium phosphate buffer (NaPO; pH 8.0) (SOD assay). Diluted
supernatants were frozen at -80°C until subsequent processing.
LDH activity (U/mg protein) was quantified using a standard 96-well spectrophotometric assay as outlined in Horn et al. (2021). In brief, this assay measures the facilitated reduction of pyruvate to lactate by endogenous LDH activity present in the diluted supernatants. Activity is measured via the corresponding rate of disappearance of NADH that occurs as lactate is produced, and which is spectrophotometrically absorbent at 340 nm (Victor X4 Multimode Plate Reader, Perkin Elmer). All samples were run in triplicate, and assays were carried out at 24°C.

SOD activity was analyzed using a protocol adapted from Peskin and Winterbourn (2017). This reaction is based on the principle that xanthine oxidase oxidizes hypoxanthine at a standardized rate, forming superoxide radicals that reduce WST-1 to form formazan dye visible at 450 nm. The extent to which SOD activity inhibits superoxide generation, and therefore inhibits formazan dye formation, is used to quantify activity of SOD in a sample. It should be noted that this assay quantifies total SOD activity and does not distinguish between different isoforms of this enzyme. To perform this assay, a reaction mixture was prepared by adding 100 µL of 10 mM WST-1 solution (Dojindo Molecular Technologies #W201-10), 100 µL of 2 mg/mL catalase solution (Sigma Aldrich #C40), and 400 µL of xanthine oxidase (in an ammonium sulfate suspension diluted 1:100 in deionized water; Sigma Aldrich #X1875) to a 19.3 mL assay buffer (100 mM NaPO pH=8, 0.1 mM diethylenetriamine-pentaacetic acid; Sigma-Aldrich #D6518), and 0.1 mM hypoxanthine (Sigma-Aldrich #H9377)). SOD standards from Bovine Cu, Zn SOD (Sigma-Aldrich, #S9697) were diluted in phosphate buffer to 0.6, 1.2, 2.4, 4.8, 6, 12, and 18 µg/mL. We added 10 µL of a blank (NaPO buffer), diluted supernatant or SOD standard to each well of a 96-well plate and followed this with the
addition of 190 µL of reaction mix. The plate was then immediately placed into the plate reader, shaken for 30 seconds, and absorbance was measured at 450 nm over 5 minutes (Victor X4 Multimode Plate Reader, Perkin Elmer). The rate of change in absorbance per min was calculated for each well and averaged between triplicates. Percent inhibition of each sample and standard was calculated by comparison to a SOD-free blank, which would exhibit maximal formazan production without any inhibition. SOD activity (U/mg protein) was then calculated from a standard curve of percent inhibition by SOD concentration, where the U of activity per mg of SOD standards was known. All samples were run in triplicate, and assays were carried out at 24°C. Total protein content for both LDH and SOD assays was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific).

2.7 Effects of intertidal position on survival, behavior, and evaporative water loss during air exposure

Following collection events (November 2021 and twice in January 2022), barnacles [n=15/tidal height (low, high)] were transported to the main campus where they were held in FSW at 15°C and 34ppt for 24h. After this adjustment period, barnacles were placed in 3L acrylic chambers manipulated to have either dry air or humid air conditions (n=5 high and n=5 low per treatment tank; repeated three times). Inside each chamber, barnacles were placed on a plastic mesh false bottom that held them above either deionized water (humid air treatment) or silica desiccant crystals (dry air treatment). The sealed, air-tight chambers were then submerged in a water bath chilled to 15°C, which maintained the chamber temperature at 17°C. A hygrometer (iButton humidity and temperature logger; DS1923-F5#, iButtonLink, LLC, Whitewater, WI,
USA) was placed inside each chamber to track humidity and temperature. The humid treatment was maintained at an average of 98.0% relative humidity (RH) and the dry treatment was maintained at an average of 31.8% RH.

Barnacle mortality was monitored three times a day (10:00, 14:00, 22:00) until all barnacles in the dry chamber died, then once a day (14:00) until all the barnacles in the humid chamber died. Barnacles were considered dead when they could no longer hold their opercular plates closed, and we were therefore able to easily pry open their plates with a small dissecting probe. Once dead, barnacles were removed from the chamber, weighed and body morphometrics measured.

While monitoring survival, we also quantified barnacle behavior over the first two days of air exposure using the same behavioral classification scheme described in Section 2.2 above. Although barnacle behavior was observed beyond 52h, observations beyond this time were not included in the analysis due to significant mortality in the dry condition. Included in analysis are observations taken at 5 min, 4 h, 8 h, 20 h, 28 h, 32 h, 44 h, and 52 h. We quantified the proportion of barnacles that were engaged in each of the three behaviors (i.e., cirral beating, pneumostome formation or closed) at each observation time from each treatment group (dry or humid air) and tidal position (low or high) and then analyzed these values across the three trial rounds of this experiment.

Barnacles were also weighed daily (at 14:00) on an analytical scale to estimate the amount of water lost via evaporative water loss from low and high intertidal B. glandula exposed to air emersion stress. Forceps were used for handling all barnacles in this experiment to minimize heat transfer from hands, and barnacles remained out of the chambers for no more than one minute during weighing.
2.8 Effects of intertidal position on lactate accumulation during air exposure

Barnacles [n=35/tidal height] were collected from the low and high intertidal positions at the base of the Cal Poly Pier in April of 2022. Of these, n=7 barnacles from the high and low intertidal positions, respectively, were sacrificed *in situ* and tissues were flash-frozen for ‘field baseline’ lactate measurements. The remaining barnacles were transported to the main campus where they were held in FSW at 15°C and 34ppt for 24 h. After this adjustment period, an additional seven barnacles per tidal height were sacrificed to obtain ‘t=0h’ pre-treatment lactate levels. An additional seven barnacles per tidal height were then transferred to a sealed acrylic chamber (same one as used in section 3.5), where they were exposed to either humid air (control), dry air, or anoxic humid air. The temperature of all air treatments was maintained by water bath and treatment conditions of humid and dry air were maintained as described above for previous desiccation experiment, with the addition of an anoxic humid air treatment. The anoxic humid treatment was set up identically to the normoxic humid air chamber, except oxygen was purged from the chamber using nitrogen gas and then the chamber was sealed. Oxygen concentration and relative humidity levels were monitored in all three chambers using Loligo systems oxygen sensors, and iButton hygrometers, respectively. iButton hygrometers also monitored temperature within each chamber. After 24h in their assigned treatment conditions, all barnacles were dissected and tissues flash-frozen and stored at -80°C until subsequent analysis. Whole-animal tissues were homogenized in a 1:6 dilution in a 10% w/v trichloroacetic (TCA) acid solution. D-lactate concentration was analyzed, as described in Section 2.4 above.
2.9 Statistical analysis

All statistical analyses were conducted using JMP software (v. 16.2.0). Data were tested for normality of distribution using the Shapiro-Wilk/Anderson-Darling test, and for homogeneity of variance using the Levene’s test. Data that did not meet the assumptions for normality were transformed (log10 [x+1] or square root[x]), and assumptions of normality and homoscedasticity were re-checked before use with parametric statistics. Effects of intertidal position on survival were analyzed with Cox proportional hazards models and parametric survival models. When assumptions of proportionality were not met for the Cox model, parametric survival models were used. The distribution used for analysis in a parametric survival model was chosen using the distribution with the lowest AICc value. Effects of intertidal position on behavior were analyzed using general linear mixed models (GLMM) with random effects of individual barnacle identification number, trial round, and/or tank. Rao-Scott-corrected likelihood ratio chi-square tests were also used to analyze behavior differences when appropriate. To test the effects of intertidal position on lactate, LDH activity, SOD activity and EWL rates during stress, we used two-way ANOVA and GLMM models where appropriate. Table 1 identifies the statistical test used to analyze each data set.

In each experiment, a principal component analysis of the five measured body size metrics was also conducted to generate a single composite variable (principal component) that could be used to represent ‘body size’ in subsequent analyses. Body mass cannot be used as an indicator of size in barnacles, unlike most animals, due to their having rocks or mussel shell still attached to their body following collection. This problem is an unavoidable consequence of removing *B. glandula* barnacles from the
rocks such that they remain alive for future lab experiments. From each PCA analysis, we obtained the principal component with the highest eigenvalue and with a % variation > 56%, which was always the single Principal Component 1 (PC1), and this variable was used in any statistical analyses where an index of body size needed to be included. Linear regression analyses were used to determine if there was a significant relationship between barnacle body size (as PC1) on each response variable. If PC1 was determined to have a significant effect on that variable, it was included in the analysis (see Table 1).

All values throughout are reported as mean ± SEM, unless otherwise noted. Statistical significance was set at α= 0.05.
3. RESULTS

We looked for effects of intertidal position on various aspects of stress tolerance (survival, behavior, evaporative water loss rates), anaerobic metabolism ([D-lactate], LDH activity) and the cellular stress response (SOD activity) in *B. glandula* exposed to various forms of oxygen-limitation and desiccation stress.

3.1 Survival and behavior during anoxia

*B. glandula* from higher intertidal positions were found to survive for longer in anoxic seawater than conspecifics from lower shore heights (Cox proportional hazards model; Hazards Ratio = 0.31; 95% CI = 0.15-0.62; p = 0.001) (Fig. 1). LT$_{50}$ (=median survival time) values in anoxia were 12.2 d and 9.4 d in high and low shore height barnacles, respectively. There was no significant effect of body size (as PC1) on survival ($R^2 = 0.0416$; $F_{1,43} = 1.87; p = 0.1789$), in this experiment.

Despite a biologically significant increase in survival times in the highest shore height animals during anoxic immersion, we did not find any statistical evidence that this enhanced survivorship was the result of behavioral differences from the lower shore height animals. *B. glandula* from both shore heights were observed to have their opercular plates closed approximately the same amount of time relative to one another whether they were in anoxic [71 ± 5% (low position) and 70 ± 5% (high position) closed] or normoxic treatments [65 ± 5% (low position) and 71± 5% (high position)] over the first four days of exposure (Proportion Closed GLMM; Treatment*Tidal Position, $F_{1,206.5} = 0.67; p = 0.4137$) (Fig. 2C). Further, there were no significant effects of tidal height on cirral beating behavior or pneumostome formation behavior between barnacles from
different tidal heights (Cirral Beating GLMM; Treatment*Tidal Position, F\textsubscript{1,154.2}=1.93; p = 0.1671; Pneumostome Formation GLMM; Treatment*Tidal Position, F\textsubscript{1,288.1}=0.14; p = 0.7038) (Fig. 2 A, B), whereas the oxygen treatment did strongly affect their behavioral strategy [Likelihood ratio chi-square test; Cirral Beating, \(\chi^2(2) = 75.29; p < 0.0001\); Pneumostome Formation, \(\chi^2(2) = 47.26; p < 0.0001\)] (Fig. 2D).

During four days of normoxic immersion, barnacles that were open (~29-37% of individuals) were primarily engaged in cirral beating, and this proportion was remarkably consistent between tidal groups and over time (Fig. 2B,D). During four days of anoxic exposure, however, \textit{B. glandula} exhibited significantly less cirral beating (Cirral Beating GLMM; Treatment * Time, F\textsubscript{1,500.3} = 3.95; p = 0.0475). The barnacles in anoxia that were open - which again existed in similar proportions between barnacles from both tidal positions (~27-30%) - were all primarily engaged in pneumostome formation, independently of tidal height origin (Fig. 2A, D). Five minutes after anoxic conditions were induced, \textit{B. glandula} in anoxia exhibited similar pneumostome behavior to the normoxic group; but, after 4 hours in anoxia and onward, barnacles from both tidal positions exhibited increases in pneumostome behavior in the anoxic exposure relative to the normoxic control (Pneumostome GLMM; Treatment*Time, F\textsubscript{1,499.9} = 9.39; p = 0.0023) (Fig. 2A).

Within the anoxic treatment, \textit{B. glandula} formed pneumostomes significantly more often as they got closer to death (two-way ANOVA; Days until death, F\textsubscript{5,750.7} = 30.71; p < 0.0001) (Fig. 3), and this effect was independent of tidal position. When barnacles were between 5 and 10 days from their death day, their behavior did not differ significantly from when they were >10 d away from death; whereas when barnacles were
4 days or less from dying, there was a significant uptick in pneumostome formation compared to the initial pneumostome behavior in anoxic barnacles that were >10 d away from death (Fig. 3) (Dunnet’s test post hoc; p<0.05). There was no difference in the relationship between pneumostome behavior and time to death between barnacles from different tidal positions (Tidal Position*Days until death, F_{5,751.1} = 1.1966, p = 0.3091) (Fig. 4). Additionally, the total duration of survival time was not significantly affected by the proportion of time an individual spent with an open pneumostome over the course of the experiment (i.e., barnacles that pneumostome more often in general are not predicted to die sooner or later than those that did not) (Regression: y = 0.0095x+10.4843; R^2 = 0.0047; F_{1,46} = 0.2160; p = 0.6443).

**3.2 D-lactate accumulation during hypoxia**

A two-way ANOVA revealed a significant effect of oxygen treatment (F_{1,47} = 20.94; p < 0.0001) and tidal position (F_{1,47} = 5.67; p = 0.0214) on (log-transformed) D-lactate levels in *B. glandula* (Fig. 4). The interaction effect, however, was not significant (F_{1,47} = 1.52; p = 0.2238). In barnacles from high intertidal positions, D-lactate concentration was significantly higher after 48h exposure to hypoxic submersion compared to their control values (Tukey HSD post hoc; p = 0.0006). In barnacles from low intertidal positions the increase in D-lactate following hypoxia exposure was not significantly higher than their control values (Tukey HSD post hoc; p = 0.1128), which may reflect the high variability that seems to characterize physiological parameters in the lower intertidal barnacles.
3.3 LDH and SOD activity during hypoxia

A GLMM found that there was no effect of 48h of hypoxia on LDH activity in *B. glandula* compared to normoxic control animals at the same time point (Tukey HSD post hoc; p = 0.8350), and following suit, there were no differences in the LDH activity response of barnacles to 48h of hypoxia (or 24h recovery from hypoxia) based on tidal position (Tidal Position, $F_{1,74.22} = 0.03$, $p = 0.8611$; Treatment*Tidal Position, $F_{1,74.0} = 0.08$, $p = 0.7806$; Tidal Position*Treatment*Time, $F_{1,74.64} = 0.24$, $p = 0.7873$) (Fig. 5).

A GLMM found no significant effect of tidal position, oxygen treatment, sampling time or their interactions on SOD activity from *B. glandula* ($p > 0.3704$) (Fig. 6).

3.4 Survival, behavior and evaporative water loss rates during air exposure

In both humid and dry air, survival analyses revealed that high intertidal zone barnacles lived longer than those from the low intertidal zone (Humid: Tidal Position*PC1*Round, $\chi^2 (2) = 6.36$; $p = 0.0416$; Dry: Tidal Position*Round, $\chi^2 (2) = 11.78$; $p=0.0028$) (Fig. 7). One barnacle (from the high position), lived 40 d in humid air, after which the experimental observation period was stopped and the barnacle was returned to seawater where it immediately exhibited cirral beating behavior. This barnacle was censored in the analysis. LT$_{50}$ (median survival time) values in humid conditions were 8.1 d (low) and 15.2 d (high), and in dry conditions were 3.8 d (low) and 3.9 d (high) (Fig. 7). Across all treatments, body size was a strong predictor of survival, with
larger barnacles (analyzed as PC1) surviving significantly longer than smaller barnacles (Linear regression; Humid: \( y = 2.91x + 13.86; R^2 = 0.37; F_{1,27} = 16.02; p = 0.0004; \) Dry: \( y = 1.01x + 4.997; R^2 = 0.23, F_{1,30}=10.66, p=0.0027 \) (Fig. 8). For this reason, PC1 was included in the survival model, despite the fact that the mean composite body size variable was not significantly different between tidal heights (t-test; t ratio = -0.05831, p = 0.9537).

In the first 52 h in dry air, barnacles from high positions exhibited more pneumostome formation than those from low positions (Dry: \( \chi^2 (1) = 14.38; p = 0.0001 \)), but this difference was not present in humid air (Humid: \( \chi^2 (1) = 1.29; p = 0.2560 \)).

In high humidity air (98% RH), about 20% of \( B. \) glandula were consistently observed to have their opercular plates open at any time, and this proportion was not significantly affected by tidal position (Time, \( F_{7,336} = 1.4796; p = 0.1735; \) Tidal Position, \( F_{1,46.1} = 0.81; p = 0.3725; \) Time*Tidal Position, \( F_{7, 336} = 0.63; p = 0.7351 \) (Fig. 9A).

In dry air (32% RH), however, \( B. \) glandula exhibited a decline in the proportion of individuals with open opercular plates over time until, eventually, all barnacles were closed and remained closed (Fig. 9B). Barnacles from high positions closed their operculum more quickly during dry emersion than barnacles from the low (Time*Tidal Position, \( F_{7,322} = 2.10; p = 0.0429 \) (Fig. 9B). Barnacles from high positions were never observed to open their opercular plates after 20 h in dry air, whereas barnacles from low positions were not all closed until 44 h in dry air (Fig. 9B).

A repeated measures ANOVA revealed a significant interaction effect between time and tidal position (\( F_{2,88} = 11.9073; p < 0.0001 \), and time and humidity condition
(F\textsubscript{2,88} = 9.2022; p = 0.0002) on EWL rate in \textit{B. glandula} exposed to air (Fig. 10). Over 48 h in air, all barnacles lost water due to evaporation, with the highest rates of EWL occurring in the first 4 h (Fig. 10B), and significantly more water was lost by barnacles in dry air than in humid air (Fig. 10A). In both humidity conditions, barnacles from the high intertidal zone lost more water than barnacles from the low intertidal zone, with the difference in EWL rates between tidal populations being much more pronounced during dry air exposure.

3.5 D-lactate accumulation during air exposure

A two-way ANOVA model was used to determine if there was any significant effect of intertidal position on [D-lactate] during different forms of emersion (when compared to a t = 0 h baseline) (Fig. 11). This analysis revealed a significant effect of emersion treatment (F\textsubscript{4,58} = 5.23; p = 0.0011) and intertidal position (F\textsubscript{1,58} = 7.75; p = 0.0072) on [D-lactate] in \textit{B. glandula}, but there was no significant interaction effect (p = 0.1801) (Fig. 11). Low sample sizes (due to the harmful algal bloom die-off event) reduced our statistical power and prevented us from detecting significant differences in our post-hoc comparisons, despite significant main effects in the model. Our interpretations of these results are therefore made in this context. Among the barnacles whose tissues were collected in situ (i.e., excised and flash-frozen immediately in the rocky intertidal zone), D-lactate levels were found to be higher in individuals collected from the low intertidal zone compared to those from the high intertidal zone. This difference in [D-lactate] disappeared, however, when barnacles were brought back to the lab and held in ‘common garden’ conditions (34 ppt, 15°C) for 24 h before tissue
collection (Fig. 11; t = 0 h). In general, barnacles from the low intertidal tend to accumulate more D-lactate than barnacles from the high intertidal. Emersion in any form led to an increase in D-lactate in barnacles from the low intertidal, and this increase was largest under anoxic conditions. Barnacles from the high intertidal did not accumulate any lactate in humid air, but barnacles in both dry and anoxic air exhibited a large – and comparably sized - increase from the t = 0 h baseline.
DISCUSSION

The rocky intertidal zone provides an ideal habitat in which to assess the effects of environmental stress on a species’ phenotype, stress tolerance limits and distribution patterns over conveniently small spatial scales. We have previously observed that conspecific sessile, acorn barnacles (*Balanus glandula*) from high versus low intertidal shore heights exhibit differences in certain baseline metabolic characteristics (e.g., [lactate], LDH activity, cirral behavior and body size) (Horn et al., 2021). Anderson et al. (2022) went on to reveal that *B. glandula* from different tidal heights also exhibit differences in their survivability during stress – specifically thermal stress. In the present study, we found that *B. glandula* from different shore heights similarly exhibit differences in their response to low oxygen and emersion stress. Compared to barnacles from the low intertidal zone, we specifically observed that *B. glandula* from the high intertidal zone: survive longer during anoxia and air emersion stress (Fig. 1, 7), tend to accumulate less D-lactate in air (Fig. 11), close their operculum sooner in dry air (Fig. 9B), and lose more water during air exposure at any humidity level (Fig. 10). These differences may reflect plasticity or selective pressure in response to environmental stress during development and highlight the potential importance of microscale stress heterogeneity in influencing species climate change tolerance and potential distribution patterns.

4.1 Effects of intertidal position on survival during stress

Barnacles exhibit an impressive tolerance to anoxia both in their larval stages (Eerkes-Medrano et al., 2013) and extending into adulthood (Castro et al., 2001; López et al., 2003). For example, the upper intertidal barnacle *Jehlius cirratus* can survive for up
to 80 days in anoxic immersion (Castro et al., 2001). We wanted to explore whether anoxia tolerance limits – like thermal tolerance limits (Anderson, 2022) - were influenced by position in the rocky intertidal zone within a single barnacle species. As we predicted, our investigations revealed that B. glandula that settled and grew to adulthood in the high intertidal zone had greater survivability during exposure to anoxic seawater in the lab compared to conspecific barnacles which had settled in the low intertidal zone (Fig. 1). These findings are consistent with past studies of inter-species comparisons, which found that high intertidal zone barnacle species (e.g., J. cirratus, B. balanoides, C. stellatus) can survive longer in anoxia compared to lower intertidal or subtidal zone barnacle species (e.g., A. psittacus, B. crenatus) (Augenfeld, 1973; Barnes et al., 1963; Castro et al., 2001; Foster, 1971a; López et al., 2003). This recurring pattern led us to question why barnacles at higher shore heights would have greater survival during anoxia.

The driving force that explains anoxia tolerance generally in B. glandula as a species, may also reflect how intertidal position will affect their tolerance to anoxia stress. We have put forth many hypotheses to explain why intertidal barnacles possess such extreme hypoxia tolerance. This trait may be an adaptive response to 1) frequent coastal hypoxia events experienced during periods of immersion, 2) prolonged shell closure occurring during air emersion to prevent desiccation, 3) prolonged shell closure during submersion to avoid predation (refuted by Anderson et al., 2023), or 4) simply the consequence of a body plan in which all tissues are housed inside an impermeable shell that contains poorly mixed and typically hypoxic seawater (see Davenport and Irwin, 2003 for support). Regarding the first hypothesis, the location of our barnacle collection site in San Luis Obispo Bay is known to experience frequent seasonal bouts of coastal
hypoxya due to upwelling, eutrophic events, or trapping of warm water within the bay (Walter et al., 2022), so adaptations (or plasticity) to tolerate these events would not be surprising in this population. If episodes of coastal hypoxya were driving anoxia tolerance in barnacles, however, we would expect *B. glandula* from the low intertidal zone to exhibit greater survivability during anoxic immersion, rather than the high intertidal barnacles, owing to the increased time they spend submerged. This trend is the opposite of what we observed, however (see Fig. 1). High wave-action characteristic of the intertidal zone likely prevents oxygen limitation in surface waters even during hypoxya events, and so precludes coastal hypoxya as an environmental pressure that varies across the intertidal zone influencing phenotype. Prolonged time in air may therefore be a stronger driver of anoxia tolerance in *B. glandula* than time submerged. If this is the case, then high intertidal barnacles should have greater survivability in anoxia than lower shore height individuals, which is exactly what observed in this study (Fig. 1).

Because high intertidal barnacles experience more time in air than barnacles from the low intertidal zone, we predicted that they would demonstrate longer survival times during emersion, in addition to during anoxia. We found that *B. glandula* did indeed survive for longer in air of high relative humidity (98% RH) when they were collected from the high intertidal zone compared to those from the low intertidal zone (Fig. 7; darker lines). The average median survival time (LT$_{50}$) from three survival trials was 15.2 and 8.1 d for high and low intertidal barnacles, respectively (Fig. 7). These data are consistent with past studies that have shown that species of barnacle dominant in the high intertidal zone lived for longer in air than species characteristic of the lower intertidal zone (Barnes et al., 1963; Foster, 1971; López et al., 2003, Castro et al., 2001). Further,
our observed survival times for *B. glandula* in humid air are of a similar duration to other intertidal barnacles [e.g., *Austromegabalanus psitticus* and *Jehlius cirratus*, whose LT$_{50}$ values in air were 7.6 d and 25 d, respectively (López et al., 2003, Castro et al., 2001)].

To our knowledge, only one other study has looked at the effects of intertidal position on survival during desiccation in a single barnacle species (*S. balanoides*; Ware and Hartnoll, 1996), and they likewise found that individuals from higher shore heights survived longer in air. When we modified the emersion treatment to create a more extreme form of dry air stress (32% RH), however, there was no longer any survival advantage based on intertidal position (Fig. 7; lighter lines). We suspect that this form of stress is so severe that any plastic or adaptive coping measures that barnacles from the high intertidal may possess, are simply not substantial enough to prevent death due to dehydration in these conditions. This explains why there is so little variability in the survival time data in barnacles from either shore height during air exposure (Fig. 7).

Regardless of relative humidity, a strong predictor of survival in these emersion trials was body size, with larger barnacles surviving longer (Fig. 8). This trend was more prominent in humid air compared to dry air (Fig. 8). Larger barnacles may be able to live longer due to a larger initial water and/or energy store in their body at the start of the experiment (Barnes et al., 1963). Mendt and Gosselin (2022) saw that starvation prior to desiccation stress significantly decreased survival time in newly settled *B. glandula*. The cause of death for barnacles in air is presumably some combination of osmotic stress due to water loss, as well as starvation since barnacles do not eat while in air (Foster, 1971a, 1971b). Having a larger body size may serve as a buffer for the lack of food during emersion, allowing for longer survival. Notably, this relationship between body size and
survival was not present in the anoxia immersion experiment, which occurred in seawater where food would typically be present and osmotic stress would be much less severe.

4.2 Effects of intertidal position on behavior during stress

The first line of defense for most organisms facing environmental stress is to engage in some form of behavioral avoidance, which typically occurs before any physiological response measures (West-Eberhard, 1989). Invertebrates in the intertidal zone will behaviorally avoid desiccation, for example, by engaging in shell closure (e.g., sessile barnacles, mussels) or hiding in moist microhabitats (e.g., limpets) during low tide (Barnes et al., 1963, Shick et al., 1988; Moisez et al., 2020). We hypothesized that *B. glandula* from the high intertidal zone would engage in more severe behavioral avoidance measures during anoxic immersion and air emersion compared to their lower intertidal counterparts. Further, we speculated that if this was occurring, these differences in behavior during stress might underlie the differences in survivability we observed between high and low intertidal barnacles during anoxia and emersion stress?

We did not, however, find any evidence for differences in cirral beating behavior or pneumostome activity in barnacles from higher intertidal heights compared to lower intertidal heights during anoxia stress (Fig. 2). Instead, we only found evidence that anoxia itself had a significant effect on behavior (in those barnacles that remained open.) Only about 30% of barnacles remained open at any time in seawater, whether the water was oxygenated or not, but of the individuals that were open, there was a clear difference in behavioral strategy depending on the oxygen content. In well-oxygenated seawater, *B. glandula* from both intertidal positions carried out mainly cirral beating activity with
sparse pneumostome formation (~5% of the observations). Presumably, this strategy accomplishes feeding and respiration simultaneously. Conversely, in anoxic seawater, cirral beating is rare and pneumostome formation is more common (Fig. 2B,D), indicating a clear de-prioritization of filter-feeding and energy consumptive physical activity during periods when oxygen is not available. The repeated formation of a small pneumostome opening between the opercular plates (when in air or water) has been well documented in the barnacle behavior literature (Anderson & Southward, 1987; Barnes et al., 1963; Barnes & Barnes, 1956; Castro et al., 2001; Davenport & Irwin, 2003; Foster, 1971a,b; Grainger & Newell, 1965; Horn et al., 2021; Ware & Hartnoll, 1996), and serves to ‘test’ the water for food and oxygen and/or promote oxygenation via mixing inside the mantle cavity without requiring cirral extensions (Davenport and Irwin, 2003). In this way, pneumostome behavior in anoxia may function as an energetically inexpensive strategy for reoxygenation. While this effort to reoxygenate is futile in a lab-engineered anoxic condition in which there is no oxygen to be gained by gaping, opening the opercular plates may be advantageous in a natural environment where some oxygen is likely to be present even during hypoxic episodes or it would be beneficial to intermittently test the surroundings to determine if oxygen had returned.

The total duration of survival (in days) by B. glandula in anoxic conditions was also not associated with any particular behavioral strategy in our study (see Section 3.1). Interestingly however, when we explored temporal patterns in behavior during anoxia, we found that as barnacles neared their death day, pneumostome formation began to occur more often (Fig. 3). This pattern held regardless of how soon death occurred relative to the start of anoxia exposure and was independent of tidal height. Pneumostome formation
before death could be a ‘last-ditch’ effort to oxygenate the mantle cavity or an avenue for lactate excretion from the body following prolonged accumulation due to anaerobic glycolysis (Southward, 1955; Barnes et al., 1963). Alternatively, it may be indicative of severe physiological distress near death, occurring because the barnacle is no longer able to energetically sustain contraction of the adductor muscle that would keep its shell closed. Because pneumostome formation was the same between both tidal positions during anoxic stress, this behavior is likely neutral in relation to survival time in anoxia between tidal heights.

Unlike in anoxic immersion, we did find evidence that intertidal height influenced barnacle behavior when they were held continuously in air, though this effect only occurred in conditions of desiccating (dry) air exposure. When barnacles experience emersion stress under high humidity (98% RH, 17°C), ~20% of individuals are open and engaged in pneumostome formation at any time, and this behavior persists at these proportions in both tidal height groups for ~24h (Fig. 11A, C). The remainder of barnacles are closed during emersion. These results align closely with the findings of Ober et al. (2019), who found that ~25% of *B. glandula* were observed to be active (i.e., have open opercular plates) after 4 h in 15°C humid air (Ober et al., 2019). When we exposed barnacles to a more extreme ‘dry’ emersion condition (32% RH, 17°C), behaviors started to diverge significantly between barnacles from different tidal heights (Fig. 9B, C). In dry air, the frequency of high intertidal zone barnacles forming an open pneumostome decreased rapidly over time, falling to zero in the first 20 h, whereas it took twice as long (44 h) for the proportion of low intertidal barnacles forming a pneumostome to fall to zero (Fig. 9B). This decrease in pneumostome formation by high
intertidal barnacles in dry air agrees with the findings of Grainger and Newell (1965) and is quite certainly an effort to minimize dehydration of internal tissues. Given that high intertidal barnacles experience more time in air than barnacles from the low intertidal zone, it is not surprising that they would close their operculum sooner when emersed. We cannot determine from these data whether this is a learned behavior based on past stress experience, or if it represents a selective trait which enabled these individuals to persist at such high intertidal heights to begin with. Nevertheless, it is possible that this behavioral strategy is a partial driver of the increase in survival we observed in individuals from higher shore heights during dry air exposure (Fig. 7).

4.3 Effects of intertidal position on anaerobic metabolism during stress

Does greater anoxia tolerance in high intertidal animals result from an enhanced capacity for anaerobic metabolism? To investigate this question, we measured D-lactate levels and LDH activity in barnacles from both tidal heights during hypoxia and emersion exposure (Fig. 4, 5, 11). We have already seen in a previous study that sessile barnacles anchored lower in the intertidal zone maintain higher baseline LDH activity and D-lactate levels (Horn et al., 2021).

4.3.1 D-lactate accumulation

In the current study, we likewise found that low shore barnacles from our collection site tend to have higher control (Fig. 4) or field in situ (Fig. 11) D-lactate levels (Fig. 4, 11), although these differences can disappear following prolonged holding in common lab-acclimated conditions (Fig. 11; t = 0 h).
During hypoxia exposure, lactate levels in barnacles from both tidal heights increased, and to higher concentrations in lower shore height barnacles, although they did not reach levels that were significantly different (Fig. 4). The concentration of lactate achieved by *B. glandula* during hypoxia – up to 3.7 mM in the lows and 2.7 mM in the highs – is also not a particularly substantial amount of accumulation from a biological perspective. Other marine crustaceans will accumulate lactate at levels up to ~10-60 mM during anaerobioses (Henry et al., 1994; Rangaswami et al., 2020). Lactate levels during hypoxia may be relatively low in *B. glandula* because they are 1) producing lactate, but then quickly excreting it into the seawater (Barnes et al., 1963; Castro et al., 2001; López et al., 2003; Vial et al., 1999), 2) producing an alternate anaerobic end-product than D-lactate (e.g., succinate, alanine) (though very little support for this in barnacles exists; Achituv et al., 1980; see Vial et al., 1999 for review) or 3) not engaging in substantial amounts of anaerobic metabolism (perhaps opting instead to initiate a metabolic depression). A reduction in metabolic activity is a common response to stress in hypoxia- and emersion-tolerant marine invertebrates (Stickle et al., 1989; Grieshaber et al., 1994; Sokolova et al., 2012). Although direct measurement of metabolic rate as a function of oxygen tension has not yet been measured in *B. glandula*, we expect that metabolic reduction occurs *B. glandula* held in hypoxic conditions, since this pattern has been documented in other intertidal barnacle species (*B. tintinnabulum*, *B. amphitrite*; Prasada Rao & Ganapati, 1968).

During air exposure, low intertidal barnacles accumulated some amount of D-lactate whether the air was humid, dry or anoxic (Fig. 1), with levels highest during anoxia as expected. This accumulation confirms that anaerobic production of lactate is
occurring in *B. glandula*, though again not at levels as substantial as you might predict for an organism known to be so hypoxia tolerant. More importantly, however, we see that in all three conditions of air exposure (humid, dry and anoxia), barnacles from the low intertidal produce more lactate, or a statistically equivalent amount of lactate, compared to barnacles from the high intertidal. In humid air, which is most similar to the natural low tide aerial environment for this species, far more pronounced accumulation of lactate in low intertidal barnacles indicates anaerobic respiration is occurring for them in air, whereas the lack of lactate accumulation in high intertidal barnacles indicates that they are using aerobic metabolism to generate ATP in air (Fig. 11). This is once again, consistent with our own and other previous findings in barnacles that low intertidal barnacles are more likely to engage in anaerobic metabolism when emersed than high intertidal barnacles, which tend to preferentially exploit aerobic metabolism in air (Augenfeld, 1967, Simpfendörfer et al., 1995, Vial et al., 1999; Horn et al., 2021). *B. glandula* from low shore positions appear to be more similar to subtidal species such as *A. psittacus* that accumulate large amounts of D-lactate during both humid emersion and anoxic submersion (López et al., 2003).

On the other hand, barnacles in the dry air condition did not show any significant difference in the level of lactate they accumulated (Fig. 13). The relative humidity level we manipulated for this treatment (32%) is lower than realistic humidity levels for the intertidal zone at our field site [60-90% RH in Avila beach (CeNCOOS, Cal Poly Pier), and so represents a very extreme desiccation event. We had hoped this would better help us tease apart differences in the stress response between *B. glandula* from different tidal heights, but as with the survival analysis data under the same conditions, this stress
seemed to be so severe that any enhanced coping mechanisms that the high intertidal barnacles may possess were simply not able to overcome such severe desiccation.

Emersion in the intertidal zone often comes with a compounded stress of increased temperature (much higher than the non-stressful 17°C of the present study). Higher temperatures increase metabolic rates in barnacles (Ober et al., 2019) while at the same time increasing evaporative water loss rates. This will force sessile invertebrates into increasing anaerobic metabolic rates or entering into a metabolic depression. Rangaswami et al. (2020) found that lactate accumulation increased with higher emersion temperatures in *B. glandula* from a Washington population, but not in those from a Southern California population. Southern California population of *B. glandula* continued aerobic respiration during 5 h emersion, even at high temperatures up to 38°C (Rangaswami et al., 2020). Our Central California barnacle population may be more similar to the southern population in the high positions and more similar to more northern populations in the low intertidal. Meng et al. (2018) performed a similar investigation into the effects of intertidal distribution of the Pacific oyster, *C. gigas*, and found that intertidal oysters are less sensitive to hypoxic stress than subtidal oysters. Subtidal oysters initiated anaerobic glycolysis sooner and facilitated a more severe metabolic depression than intertidal oysters (Meng et al., 2018).

4.3.2 Lactate dehydrogenase activity

Despite our finding of typically higher lactate levels in low intertidal barnacles during hypoxia (Fig. 4), LDH activity did not differ between tidal positions during hypoxia or subsequent reoxygenation (Fig. 5). The shorter survival time of the low
intertidal *B. glandula* during anoxia may indicate that higher anaerobic capacity does not translate to increased survival under long-term anoxia (a period in which barnacles are presumed to survive under some combination of anaerobic respiration and metabolic depression) (Fig. 1). However, because LDH activity can be seasonally variable (Anderson, 2022; Barnes & Blackstock, 1975), it is possible LDH activity was not higher in our barnacles from the low intertidal zone at the time of collection for the hypoxia experiment. Long-term and seasonal data on LDH activity in this population of *B. glandula* is needed to understand how anaerobic capacity in this species may fluctuate depending on environmental variability.

Changes in LDH activity after oxygen challenges have been seen in previous studies, however. Vial et al. (1999) found LDH specific activity increased in intertidal barnacles after both anoxic submergence and emersion, and that LDH activity (and therefore anaerobic capacity) was higher in lower intertidal barnacle species compared to higher species. In the current study, *B. glandula* does not appear to increase LDH activity in the time span of 48 h (Fig. 5), although other organisms exhibit changes in LDH activity over even shorter exposure periods in anoxia or hypoxia. For example, after 4h in anoxic conditions, *B. balanoides* nauplii have an increase in lactate and succinate, as well as an increase in LDH and MDH activity (Achituv et al., 1980). It is possible that adult *B. glandula* are less sensitive to hypoxia than their larval nauplii stages, and so lack a pronounced LDH regulatory response to hypoxia over this time frame.

### 4.4 Effects of intertidal position on SOD activity during stress
In a review by Moreira et al. (2016), superoxide dismutase (SOD) was found to be one of the most common enzymes to change in preparation for oxidative stress. Increases in antioxidant enzymes, like SOD, during hypoxia are hypothesized to occur in preparation for an influx of reactive oxygen species (ROS) during the aerobic re-payment of oxygen debt (Di Guilio et al., 1989). In shrimp, for example, activity of antioxidant enzymes increases during recovery from hypoxia and anoxia (Zenteno-Savín et al., 2006). We found that SOD activity in *B. glandula* did not change in response to hypoxia or recovery from hypoxia, nor were there any significant differences in SOD activity between individuals from different tidal positions (Fig. 6). At least in regard to this particular antioxidant stress response pathway, we do not see any evidence for tidal height-dependent plasticity or selection in *B. glandula*.

Though we did not see a change in *B. glandula*, SOD activity has been shown to increase after hypoxia in other barnacle species (Niyogi et al., 2001; Desai & Prakash, 2009). Further, in their study on the intertidal barnacle *Balanus amphitrite*, Desai and Prakash (2009) observed that individuals from low intertidal zone had lower baseline antioxidant enzyme activity (catalase and superoxide dismutase; SOD) than individuals from the high intertidal zone. Anderson (2022) reported similar tidal position-dependent differences in SOD activity within *B. glandula* conspecifics, whereby baseline SOD levels were higher in high intertidal barnacles. In a different study on *B. balanoides*, Niyogi et al. (2001) measured SOD activity levels within a similar range to our data (5-10 U/mg protein), and also demonstrated seasonal differences and increases in SOD due to pollution. It should be noted, however, that both Desai and Prakesh (2009) and Niyogi et al. (2001) studied intertidal barnacles in India, where surface temperatures are 25-30°C.
and are much warmer than the temperatures on the central California coast (avg. ~15°C). Patterns that may be seen in more tropical species may not translate to the temperate species we have locally. In temperate species, a more prolonged or chronic oxygen challenge may be needed to induce changes in activity in SOD.

It is also worth discussing that the molecular mechanism for oxygen response pathways in cirripede barnacles is not known. In most organisms, SOD expression is regulated by the highly conserved hypoxia inducible factor (HIF) pathway, an oxygen monitoring system ubiquitous throughout the animal kingdom (Freire et al., 2012; Hermes-Lima & Zenteno-Savín, 2002; Moreira et al., 2016). However, cirripede barnacles are one of the few organisms to have lost the HIF pathway (Graham et al., 2020). While barnacles do not utilize HIF pathways, they do still have enzymes typically regulated by HIF, such as the antioxidant SOD. It is possible that a mechanism for the oxygen-dependent regulation of SOD is not present or is not very sensitive in *B. glandula*.

### 4.5 Effects of intertidal position on evaporative water loss during stress

Barnacles held in dry air lost more water than those in humid air, as expected in more desiccating conditions (Fig. 12). More surprisingly though, was the finding that water loss rates were significantly greater in barnacles from high intertidal positions compared to those from the low intertidal positions. We had hypothesized that high intertidal barnacles would have more robust behavioral (e.g., shell closure) and/or physiological measures (e.g., cutaneous lipid insertion) in place to minimize evaporative water loss in air (e.g., Sokolova & Pörtner, 2001). Why do barnacles that have greater
survivorship during high temperatures (Anderson, 2022), emersion, and anoxia show greater water loss rates in air? Especially given our behavioral finding that a greater proportion of high intertidal barnacles have closed opercular plates when in dry air (Fig. 11).

We noticed that most of the water loss in barnacles occurred during the first 4h of air exposure, after which point water loss rates were much more minimal (Fig. 10). (The one exception to this was the low intertidal barnacles in humid air, which gained water in the first 4h of exposure presumably due to condensation on the shell). While this rapid initial water loss could be due simply to evaporative water loss, we propose an alternative explanation rooted in behavior. Several studies have observed that barnacles held in the air will purposefully expel the water from their mantle cavity (Barnes et al. 1963; Grainger and Newell, 1965; Vial et al., 1999; Resner et al., 2020). Given that air has a much greater oxygen concentration (~30X greater) than water at the same temperature and partial pressure, establishing a humid air-filled cavity would promote even more oxygen uptake into their tissues than a seawater filled chamber. Given the lack of a true filamentous gill structure in B. glandula which would collapse in air (Anderson, 1994), this hypothesis is plausible. It is possible that the rapid decrease in water in the first 4 h of air exposure is due to barnacles intentionally expelling water from inside their shell early during emersion to create a moist, air-filled internal cavity surrounding their gas-exchange surfaces that will promote prolonged oxygen availability (Vial et al., 1999), and thereafter closing their pneumostome to avoid dehydration.

We found that water loss rates were much more pronounced in barnacles from high positions than those from the low, which is consistent with their increased resilience
in air. If barnacles from high positions are engaging in this water expulsion behavior more often than low intertidal barnacles, we would expect to find lactate levels in high-position barnacles to remain low when in air as they exploit aerobic metabolism using atmospheric oxygen, rather than anaerobic glycolysis using stored glycogen. This is precisely what we observed: barnacles that were collected from higher shore heights continue to have lower lactate than low shore height barnacles for 24h in humid air (Fig. 11). Perhaps also, high intertidal barnacles keep their opercular plates closed more than lower barnacles in dry air because the abundance of humid oxygenated air in their mantle cavity allows them to remain closed for longer between intermittent opening of the opercular plates compared to a repository of oxygenated seawater in the mantle cavity (see also Grainger and Newell, 1965).

Low position *B. glandula* may then be better adapted for short bursts of anaerobic capacity during air emersion, rather than for prolonged aerial survival due to the relatively short periods they are exposed to air throughout the tidal cycle. The lower regions of the intertidal spend a much greater proportion of time in water, and even during emersion wave splashing keeps the shore relatively wet. Higher positions on the shore experience frequent and longer periods of emersion which are more challenging to survive. These environmental stress differences may lead to a selection preference in the high intertidal zone towards barnacles that prioritize sustained aerobic metabolism combined with a reduction in metabolic rate (metabolic depression) rather than anaerobic compensation during longer, hotter, or drier conditions. This approach would also be beneficial given that food is less available to higher shore filter-feeders.
5. CONCLUSIONS

We have found tidal height-dependent differences in the response of *Balanus glandula* to oxygen and desiccation stress. This study furthers our understanding of how barnacles cope with stress in the highly dynamic intertidal zone and emphasizes the importance of taking into account microscale population differences in physiological tolerance limits. This adds to the body of work examining the dynamic mosaic of intertidal microhabitats (Dahlhoff, 2004; Flight et al., 2010; Gilman & Rognstad, 2018; Harley & Helmuth, 2003; Helmuth & Hoffman, 2001; Helmuth et al. 2006a; Kroeker et al., 2016; Schmidt & Rand, 2001).

While barnacles are proven to be resilient under singular stressors, their tolerance limits can be challenged by multiple stressor events. In the spring of 2022, a mass barnacle die-off occurred at our collection site in San Luis Obispo Bay, CA. This occurred in response to a heat wave that coincided with a receding daytime low tide and resulted in the death of most of the high positioned barnacles from our sample population on the Avila Beach rocky shoreline. Additionally, during the summer of 2022, San Luis Obispo Bay experienced two extreme harmful algal bloom events (Cal Poly Pier, CalHABMAP), which killed most of the mussels and barnacles in the intertidal zone. Mussels appeared to be extremely susceptible to death during this bloom, however, *B. glandula* commonly settles on blue mussels and so many perished when their mussel substrates were swept away following death. Global climate change is predicted to make heat wave and HAB events like this more frequent, putting our intertidal communities at elevated risk. Sessile intertidal invertebrate populations alter the microhabitat structure of the coastline as they settle and grow on substrates, providing coarse settlement structures
for biofouling organisms and shelter for entire communities of other organisms in the rocky intertidal (Crisp & Barnes, 1954; Köhler et al., 1999). When these organisms are absent, the biodiversity of the site is likely to change rapidly. As of spring 2023, our collection site is still recovering from both these mortality events, with small barnacles finally beginning to settle in the newly available rocky intertidal real estate (personal observation).

Intertidal organisms are dually impacted by environmental change from both land and sea. Harsher storms predicted by climate change estimates will bring about increased wave action, influx of freshwater and rain, as well as pollution runoff, and increased erosion, in addition to rising air temperatures (IPCC, 2022). Rising sea levels may change the vertical ranges of intertidal organisms and alter species competition patterns, while rising sea surface temperatures may increase thermal stress, reduce oxygen availability, and increase the frequency of harmful algal blooms (IPCC, 2022; Helmuth et al., 2006b). As seen in this study, physiological tolerance to abiotic stress tolerance can vary in organisms settled mere meters apart. Because responses can differ greatly within a single population, this highlights the importance of considering microscale environmental variation in predicting species’ responses to different environments in the face of global climate change.
6. FIGURES & TABLES

Figure 1. Survival of B. glandula in anoxic and normoxic seawater. A) Survival of B. glandula collected from low (teal; n=24) or high (orange; n=24) intertidal positions during exposure to anoxic seawater (<0.5 mg O₂ L⁻¹, 33 ppt; 15°C), and exposure to a normoxic seawater control (gray; n=24/tidal position). B) Individual survival times for low and high intertidal B. glandula in anoxic seawater. Median survival time (LT₅₀) for low (9.4 d) and high (12.2 d) intertidal positions are denoted as dotted lines (in A) and solid black lines (in B) for the anoxic treatment.
Figure 2. Behavior of *B. glandula* in anoxic and normoxic seawater. Behavior of *B. glandula* collected from low (teal) and high (orange) intertidal positions, during the first four days (5 m, 4 h, and 1, 2, 3, 4 d) of exposure to either anoxia (solid lines) or normoxia (dashed lines) in seawater (33 ppt, 15°C). Data are reported as the percent of barnacles exhibiting each behavior [pneumostome formation (A), cirral beating (B), closed operculum (C)] in each treatment and tidal position for each time point, averaged between 3 runs of the experiment. Values are shown as means ±SEM. (D) Sum of the first four days of behavioral observations showing percent of barnacles in each group exhibiting pneumostome formation (pink), cirral beating (blue), or closed opercular plates (black). In total, n=23-24 barnacles per tidal position were monitored in each treatment.
Figure 3. Pneumostome behavior in relation to proximity to death in anoxic seawater. Percent of *B. glandula* from low (teal) and high (orange) intertidal positions forming a pneumostome at distinct time bins for ‘days until death’ in anoxic seawater. Each point is the mean ± SEM for the percent of barnacles with a pneumostome at the time bin for each of the three trial runs of the experiment. * Indicates values that differ significantly from the >10 days time bin (Dunnet’s test, α=0.05). Note: data are only shown for anoxic treatment group due to 100% survival in all control barnacles held in normoxia.
Figure 4. D-Lactate accumulation before and after hypoxia. Whole-animal [D-lactate] (mM) in B. glandula (n=9-16 per time point) collected from high (orange) and low (teal) intertidal positions before (normoxic control) and after 48 h of hypoxia exposure in FSW (≤2 mg O₂ L⁻¹; 33ppt, 15°C). Points shown are individual lactate values. Mean (solid black line) ± SEM (error bars) for each group is overlaid. Groups with different letters are significantly different (Tukey HSD; α=0.05).
Figure 5. Lactate dehydrogenase activity (LDH) activity following hypoxic exposure and reoxygenation. LDH activity in whole-animal tissues of *B. glandula* collected from high and low intertidal positions following 48h exposure to hypoxic seawater (darker shaded region) and subsequent reoxygenation (lighter shaded region) (left). A subset of barnacles was simultaneously exposed to normoxic seawater (right side) to serve as a control against which to compare the hypoxic treated barnacles. Tissues were collected for LDH activity prior to the start of hypoxia (time 0 h), following 48 h of hypoxia (time 48 h), and again following 24 h of recovery in fully oxygenated seawater (time 24 h). There were no significant effects of either time or oxygen-treatment on LDH activity in these barnacles. Values represent means ± SEM; n=4-11 per time point.
Figure 6. Superoxide dismutase (SOD) activity following hypoxic exposure and reoxygenation. SOD activity in whole-animal tissues of *B. glandula* collected from low and high intertidal positions following 48h exposure to hypoxic seawater (darker shaded region) and subsequent reoxygenation (lighter shaded region) (left). A subset of barnacles was simultaneously exposed to normoxic seawater (right) to serve as a control against which to compare to the hypoxic treated barnacles. Tissues were collected for SOD activity prior to the start of hypoxia (time 0h), following 48 h of hypoxia (time 48), and again following 24 h of recovery in fully oxygenated seawater (time 72h). There were no significant effects of either time, oxygen-treatment, or tidal position on SOD activity in these barnacles. Values represent means ± SEM; n=10-15 barnacles per tidal height.
Figure 7. Survival of *B. glandula* in humid and dry air. A) Survival of *B. glandula* collected from low (teal) or high (orange) intertidal positions and exposed to humid air (darker lines; 98% RH) or dry air (lighter lines; 32% RH) at 17 °C. In each treatment group, n=14-15 barnacles per tidal height were monitored. Note: observations ended at 40d. B) Individual survival times for low and high intertidal barnacles in humid (blue) and dry (tan) air. Median survival times (LT<sub>50</sub>) are denoted as dotted lines (in A) and solid black lines (in B) (Dry: 3.8, 3.9 d; Humid: 8.1, 15.2 d, for low and high respectively). Barnacles survived longer under humid conditions, with a slightly greater tolerance to air exposure in barnacles from higher shore heights.
Figure 8. Relationship between survival times and a composite body size variable (PC1). PC1 represented 68.6% of the variation across all measured body size indices (aperture length & width, base length & width, shell height) for barnacles exposed to humid air (blue) and dry air (tan) conditions. There is a significant positive relationship between body size and survival time in both the humid air ($y=2.91x + 13.86; R^2=0.37; F_{1,27}=16.02, p=0.0004$) and dry air ($y= 1.007x + 4.997; R^2=0.23; F_{1,30}=10.66, p=0.0027$) treatments when data from both tidal heights are pooled.
Figure 9. Behavior of *B. glandula* in humid and dry air. Percent of *B. glandula* collected from low (teal) and high (orange) intertidal positions engaged in pneumostome formation during the first 52 h (5 min, 4 h, 8 h, 20 h, 28 h, and 52 h) of exposure to humid air (98%, 17 °C; A) and dry air (32%, 17 °C; B). In each treatment group, n=23-25 barnacles per tidal height were monitored. (C) Sum of the first 52 h of behavioral observations for both treatment groups showing percent of barnacles in each group exhibiting pneumostome formation (pink) or closed opercular plates (black).
**Figure 10. Evaporative water loss of B. glandula in humid and dry air.** Total evaporative water loss (TEWL; A) and evaporative water loss (EWL) rates (B) in *B. glandula* collected from low (teal) and high (orange) intertidal positions during exposure to humid air (darker solid lines; 98% RH) or dry air (lighter dashed lines; 32% RH) at 17°C. TEWL and EWL rates were greater in dry air, and barnacles from the high tidal position lost significantly more water than barnacles from the low tidal position in dry air (p<0.0001). Values represent means ± SEM; n=20-25 barnacles per tidal height.
Figure 11. D-Lactate accumulation of *B. glandula* from different intertidal heights following air emersion. D-Lactate concentration (mM) in whole-animal tissues of *B. glandula* from low (teal) and high (orange) intertidal positions following 24h exposure to different emersion treatment [humid air (98%RH), dry air (32%RH) or anoxic humid air (98%RH)]. These values are compared against a time 0h ‘lab-acclimated’ baseline, which was measured in the lab after a 24h holding period in control conditions (aerated FSW at 34ppt and 15˚C) and an *in-situ* D-lactate concentration from the immediate time of field collection. Values represent means ± SEM; n=6-7 barnacles per tidal height.
Table 1. Summary of statistical tests.

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<th>Experiment</th>
<th>Response Variables</th>
<th>Explanatory Variables</th>
<th>Statistical Model</th>
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<td>Cox proportional hazards</td>
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<td>c) closed</td>
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