PHYSIOLOGICAL RESPONSE OF THE GIANT ACORN BARNACLE, *BALANUS NUBILUS,* TO AIR EXPOSURE

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ABSTRACT

Physiological response of the giant acorn barnacle, *Balanus nubilus,* to air exposure

Emily Jane Resner

The giant acorn barnacle, *Balanus nubilus*, is a resident of the subtidal and low intertidal rocky shoreline on the Pacific Coast of North America (Alaska to Baja California). *B. nubilus* is notable for having the largest muscle fibers in the animal kingdom; fiber diameters that can exceed 3mm in adults! At such extreme sizes these muscle cells may be at risk for insufficient oxygen delivery to mitochondria owing to low SA:V ratios and long intracellular diffusion distances. Oxygen limitation to these muscles may be further exacerbated during low tide air exposure (emersion) or environmental hypoxia events, which are increasing in frequency and duration along the world's coastlines. We are interested in characterizing the internal oxygen conditions of *B. nubilus* during air emersion and anoxia so that we can ultimately investigate the physiologic mechanisms by which *B. nubilus* maintains function in their giant muscle fibers during environmental oxygen limitation. To this end, we examined the effects of air emersion and anoxia on 1) hemolymph gas, pH and ion levels, 2) oxygen consumption rates (MO_2; emersion only), and 3) respiratory behaviors (e.g., cirri beating). In the first experiment, we measured hemolymph pO_2, pCO_2, pH and ion ($[Na^+]$, $[Cl^-]$, $[K^+]$, $[Ca^{2+}]$) concentrations at 0, 3, 6 and 9h of exposure to air emersion, anoxic immersion and normoxic immersion (control). Next, we compared the average MO_2 of barnacles held in water and air for 6h at three common temperatures (10, 15, or 20°C) using intermittent (aquatic) and closed-system (air) respirometry. Lastly, we investigated the respiratory behaviors (% time operculum open, %time testing, % time pumping, % time cirri beating, cirri beat frequency, opercular pulse frequency) of *B. nubilus* during acute (6h) exposure to air emersion, anoxic immersion and normoxic immersion (control). Our data revealed that hemolymph pO_2 was significantly decreased in the anoxic barnacles by 3h and remained significantly depressed relative to the normoxic control for 9h. The air-exposed barnacles, however, maintained hemolymph oxygen levels that were intermediate to the control and anoxia barnacles for the entire experiment, achieving levels that were significantly lower than normoxic barnacles only by 9h. We also found that oxygen consumption rates for *B. nubilus* held at ecologically realistic temperatures were similar in air and water. From these data we conclude that *B. nubilus* is relatively adept at taking up oxygen from the environment while out of the water, which is common for certain barnacle species, and that air emersion represents a relatively mild environmental stress for this species (at least from a gas-exchange perspective). Efficient aerial gas-exchange by the giant acorn barnacle is likely facilitated by seawater pools stored in the mantle cavity, which can directly take up oxygen from the air and make it accessible to soft tissues and gill-like structures on the inside of the shell. This strategy, however, would require complementary behaviors aimed at oxygenating the mantle cavity fluid (e.g, aperture opening, cirri extensions to facilitate mixing), and this is exactly what we see. In our behavior experiment we found that air-exposed barnacles (and, more surprisingly, anoxic barnacles) spent significantly more time with their cirri extended than our control animals, who engaged more in an aperture pumping behavior with their cirri retracted. These behavioral preferences existed even though there were no significant differences in

the total time spent with their aperture open (regardless of the behavior occurring while open) between any of the treatments. There were also interesting findings in the ion data. While there were no significant treatment effects on $[Na^+]$, $[Cl^-]$, or $[Ca^{2+}]$, we did observe significantly higher $[K^+]$ by 6h in both the emersion and anoxic groups relative to the normoxic group. We predict that this change in $[K^+]$ is likely attributable to its role in acid-base buffering. There was a strong correlation between pCO_2 levels and pH across all treatments; however, decreases in pO_2 levels in the hemolymph, which corresponded with increases in pCO_2 levels, had only minimal effects on the hemolymph pH, indicating a well-buffered system. In conclusion, we found that air exposure does not inhibit aerobic metabolism in *B. nubilus*, owing largely to efficient aerial oxygen uptake and perhaps also effective acid base-buffering. We therefore predict that muscle function would be preserved during periods of low-tide emersion. Anoxia, on the other hand led to a significant decline in hemolymph oxygen content, which suggests that environmental hypoxia is likely to diminish functionality of their giant muscle fibers. In a parallel study, we intend to investigate the plastic response of *B. nubilus* muscle fibers to the same conditions (air emersion and anoxic immersion).

Keywords: *Balanus nubilus*, barnacle, emersion, oxygen, pH, respirometry, behavior, muscle

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INTRODUCTION

The rocky intertidal environment poses extreme physiological challenges to its resident organisms. Daily tidal fluctuations result in alternating periods of seawater immersion and air emersion, and this causes substantial variation in both abiotic (e.g., temperature, oxygen, pH, salinity, and wave action) and biotic (e.g., competition, predation) stressors. Sessile organisms that live halfway between the air and water in the rocky intertidal have to cope with the threat of wave action which impedes larval settlement, compete for space within a narrow suitable microhabitat, avoid predation from both the air and water, and, most significantly, prevent desiccation when in the air while experiencing potentially severe changes in temperature, salinity and oxygen availability. Oxygen limitation in the intertidal zone can be further exacerbated by bouts of environmental hypoxia associated with coastal eutrophication and upwelling, which are increasing in number on the Pacific West coast (Grantham et al., 2004; Vaquer-Sunyer and Duarte, 2008), internal hypoxia that occurs in response to decreased salinity amid rain events (Davenport and Irwin, 2003), phytoplankton blooms (Heisler et al., 2008), and even periods of nighttime isolation in warm tidal pools that are dominated by cellular respiration and no photosynthesis (Truchot and Duhamel-Jouve, 1980).

The opposing need to avoid desiccation and facilitate oxygen uptake in the dehydrating air environment is particularly troublesome for intertidal organisms, as gas-exchange surfaces (i.e., gills or skin) are highly permeable and so prone to evaporative water loss. Despite this paradox, some intertidal invertebrates are still able to take up oxygen effectively in both air and water (e.g., decapod crustaceans, stalked barnacles). Others, however, entirely avoid aerial gas-exchange and water loss, isolating themselves from the environment by burrowing into the sediment or closing their shells/valves when

faced with air exposure (e.g., molluscs, polychaetes) (for review see Burnett, 1988). Animals that avoid gas-exchange in the air tend to have a high capacity for anaerobic metabolism (De Zwaan and Putzer, 1985), effective mechanisms for buffering the blood/hemolymph during any accompanying metabolic acidosis (e.g., high concentrations of blood buffering proteins; Burnett, 1988), and strategies for preventing oxidative stress associated with the recovery from hypoxia (e.g., high concentration of antioxidant enzymes; Sui et al., 2017, David et al., 2005). For example, the polychaete lugworm, *Arenicola marina*, lives in sand burrows in the intertidal zone, where it rapidly depletes oxygen stores during low-tide emersion. During these periods *A. marina* relies heavily on anaerobic metabolism, accumulating acidic anaerobic end products like succinate, and experiences a consequent metabolic and respiratory acidosis (Schottler et al., 1983). This hypoxia-driven acidosis, however, is minimized in this species by the effective buffering role of hemoglobin (e.g., the Haldane Effect) (Toulmond, 1973).

Among the intertidal animals that *can* facilitate gas-exchange in the aerial environment, there is tremendous variation in their effectiveness at oxygen uptake. Some animals, such as chitons (McMahon et al., 1991), decapod crustaceans (Burnett, 1988), and balanomorph (acorn) barnacles (Innes, 1985) can take in oxygen across moistened gills while emersed, though they still typically experience decreased hemolymph pO_2 and reduced oxygen consumption rates.The length of emersive activity in these organisms can be extended if the animal has the ability to store seawater around their gills [e.g., branchial fluid in decapod crustaceans, or mantle cavity fluid in acorn barnacles (see below)]. This fluid pool is in direct contact with the air and so is easily reoxygenated to serve the gills, yet the animal manages to avoid desiccation. Desiccation is still likely to set in eventually if the branchial fluid is not restored via immersion.

Other marine intertidal organisms, however, are extremely adept at aerial oxygen uptake in the absence of any fluid pool directly surrounding the gills. For example, the pedunculate (stalked) barnacles have significant gas-exchange across the integument of their peduncle, and in at least one case (*Calantica spinosa*) have been shown to be better able to take up oxygen from the air than the water (Innes, 1985)! Pedunculate barnacles are typically able to increase hemolymph pO_2 while in the air (relative to the water) due to their integumentary gas-exchange abilities, and either maintain (as with *C. spinosa*; Innes, 1985), or even increase (as with *P. polymerus*; Petersen et al., 1974) their oxygen consumption rates in the air relative to the water. Additionally, some species of stalked barnacles (e.g., *P. polymerus*) appear to have a respiratory binding pigment (based on the red coloration of their hemolymph; Lamb and Hanby, 2005) which would enhance their oxygen carrying capacity.

Balanomorph acorn barnacles, the focus of this study, are typically able to take up oxygen across moistened gills while in the air. Compared to their stalked barnacle cousins, acorn barnacles are found across a wider range of the intertidal zone (Ricketts et al., 1985). This distributional difference results largely from an increased ability to avoid desiccation as a result of their thick shells. Additionally, by virtue of having a hard, enclosing shell, acorn barnacles are able to store an exosomatic fluid pool, known as the mantle cavity fluid, around their gas-exchange surfaces. Mantle tissues line the entire inside surface of the shell and create a cavity inside of which the barnacle resides surrounded by seawater (Fig. 1; Anderson, 1994). While emersed, acorn barnacles can access and hold air inside their mantle cavity in order to oxygenate this fluid pool, which they do by slightly opening and closing their mobile upper plates (Barnes and Barnes, 1957). This movement creates a small opening called a pneumostome through which

atmospheric gases can come into contact with the mantle cavity space (Barnes and Barnes, 1958; Barnes et al., 1963). Despite their ability to oxygenate the mantle cavity fluid, these fluid pools have still been shown to be hypoxic during emersion. This may not altogether surprising given that even while immersed their mantle cavity fluid is regularly observed to be hypoxic (Davenport and Irwin, 2003). Emersed acorn barnacles will continue to open and close their upper plates until most of the mantle cavity fluid has been replaced by air, at which time the plates close until the barnacle is reimmersed. Once the plates are closed, oxygen levels in the mantle cavity fall rapidly, resulting in more severe internal hypoxic stress (Davenport and Irwin, 2003). Unlike some stalked or parasitic barnacles, acorn barnacles also seem to lack any respiratory binding pigments (e.g., hemoglobin, hemocyanin, myoglobin) in their hemolymph or muscle (Terwilliger and Ryan, 2001; Waite and Walker, 1988), and so must have a relatively low oxygen-carrying capacity. For reference, Antarctic Nototheniod fishes, whose blood lacks hemoglobin, have an arterial oxygen content that is about 10% of that found in closely related Nototheniods whose blood contains hemoglobin (Egginton, 1994; Holeton, 1974). In combination, these findings suggest that the hemolymph pO_2 of acorn barnacles would be depressed during air emersion compared to seawater immersion.

The largest balanomorph barnacle species in the world is the giant acorn barnacle, *Balanus nubilus*. *B. nubilus* is found in the subtidal to low intertidal zones from Alaska to Baja California. This species is most well-known for possessing the largest muscle fibers in the animal kingdom, with diameters exceeding 3 mm in adults (Hoyle and Smyth, 1963). At these extreme sizes, barnacle muscle fibers may confer the benefits of increased force output (Connaughton et al., 1997) and reduced energetic costs associated with membrane potential maintenance (Johnston, 2006; Jimenez et al., 2011). However,

very large fiber diameters pose potential limitations to aerobic metabolism owing to the low surface area to volume ratios (SA:V) and long intracellular diffusion distances that will limit oxygen flux into and within these cells (Kinsey et al., 2005, 2007). We are ultimately interested in understanding how *B. nubilus* maintains function of their muscle fibers given the suite of oxygen-limiting conditions they are potentially dealing with in concert: extreme fiber dimensions, the lack of any respiratory binding pigments, and potential internal hypoxia owing to low-tide air emersion or episodic environmental hypoxia.

In this study, we aim to explore the physiological and behavioral response of *B. nubilus* to air emersion. To do this we investigated the effects of air exposure (6-9h) on 1) hemolymph pO_2, pCO_2, pH, and ion levels, 2) oxygen consumption rates (MO_2), and 3) respiratory behaviors in adult giant acorn barnacles, and compare these data to values obtained from barnacles held in normoxic and anoxic seawater. The data we gather will help us to understand the internal oxygen environment of these barnacles when faced with air emersion and anoxia so that in future studies we can begin to unravel how they maintain function of their giant muscle fibers during oxygen limitation.

2. Methods

2.1 Animal care and maintenance

Adult giant acorn barnacles, *Balanus nubilus*, were collected on SCUBA from the pilings of the Cal Poly Research Pier in San Luis Obispo Bay, CA (approximate collection depth ~5-6m). Barnacles were maintained atop the same pier in a 200L surge tank ('holding tank') that was supplied with equal parts filtered seawater (FSW) and raw

seawater (RSW) via a continuous flow-through seawater system, which pumped water from directly below the pier (~7m depth) to tanks housed above. Conditions in the holding tank, therefore, very closely approximated conditions experienced by the barnacles *in situ* (temperature, ~11-14°C; salinity, 32-33 ppt; ambient photoperiod). Barnacles fed on planktonic organisms naturally available in the raw seawater, and were kept in these holding tanks for a minimum for 1wk prior to experimentation. Care was taken to collect barnacles of similar size, and those used in our experiments had the following morphological measurements represented as mean ± SEM: weight (g), 540.57 ± 34.53; volume (mL), 373.57 ± 21.76; aperture width (cm), 4.03 ± 0.12; aperture length (cm), 4.49 ± 0.13; height (cm), 8.29 ± 0.22; base width (cm), 10.08 ± 0.44; base length (cm), 11.42 ± 0.41; scutum width (cm), 4.42 ± 0.12; scutum length (cm), 4.34 ± 0.11.

2.2 Hemolymph pO_2, pCO_2, pH and ion levels

In preparation for the measurement of hemolymph gas and ion levels, a rotary tool (Dremel) was used to drill a small hemolymph sampling port (i.e., a hole) through the outer shell of each barnacle in a location immediately superficial to the rostral sinus (Fig. 2; Burnett, 1977; Anderson, 1994). The hole was sealed with a double layer of dental latex affixed to the shell with cyanoacrylate glue, and this created a leak-proof diaphragm through which repeated hemolymph samples could be withdrawn by a syringe (1mL syringe; 25G x 1.5” needle). Prior to experimentation, barnacles were held for a minimum of two weeks after the port hole was drilled. Pilot studies were conducted to ensure that samples collected from this port were indeed hemolymph and not mantle cavity seawater fluid. After formation of the sampling port, barnacles were subsequently returned to their holding tanks. No mortality resulted from this process.

Isolated experimental tanks (38L; N=9) were placed inside a larger, seawater-filled acrylic tank, which served as a temperature bath. The surrounding acrylic tank was supplied with continuously flowing seawater pumped from the ocean directly below the pier. The experimental tanks were therefore held at the same temperature in which the barnacles had been maintained in their holding tanks prior to experimentation, and the same conditions in which they were initially found *in situ* (~11-14°C). This experimental system was located outdoors, at the end of the Cal Poly Research Pier, and so experienced ambient photoperiod conditions. Experimental tanks were manipulated to create the following oxygen treatments**:** *normoxic immersion* [N; filtered seawater (20L; 32-33 ppt) bubbled with atmospheric air], *air emersion* [E; empty tank], and *anoxic immersion* [A; filtered seawater (20L; 32-33 ppt) bubbled with pure nitrogen gas]. We used a total of three experimental tanks per treatment, with each tank holding three barnacles (n=9 barnacles/treatment).

Barnacles were moved from their holding tank and allowed to acclimate in the experimental tanks for 24h prior to experimentation. During this time all tanks were filled with 20L of filtered seawater (FSW), bubbled with atmospheric air, and temperature was held constant at ambient ocean levels via the water bath. At no time during the experiment was food added to the experimental tanks. At the initiation of the experiment, tank conditions were modified to achieve N, E or A, and animals were held under these conditions for a period of 9h. PO_2 levels in the anoxic treatment tanks fell to <0.5 mg L^{-1} in approximately 15 min. Temperature and PO_2 levels in each tank were periodically monitored with a ProODO (YSI) oxygen meter over the course of the 9h (temperature range: immersion tanks, 13-14.5°C; emersion tanks, 13-15°C). At times 0 (baseline), 3, 6 and 9h, barnacles were briefly removed from their experimental tank and hemolymph

samples (200 μL) were drawn from the sampling porthole using a 1mL BD syringe (25G x 1.5" needle). Care was taken to insert the syringe only just beyond the thickness of the outer shell so as to not pierce the internal body tissues of the barnacle. Hemolymph samples (65 μL) were aspirated directly from the collection syringe into an ABL90 FLEX blood gas analyzer (Radiometer) for the immediate measurement of: PO_2 (torr), PCO_2 (torr), pH, as well as $[Na^+]$, $[K^+]$, $[Cl^-]$, $[Ca^{2+}]$ ion levels (mM). We were most interested in changes in the hemolymph pO_2, but the blood gas analyzer gave us the capacity to measure several other related variables, including pCO_2, pH and ion concentrations. These variables were still of interest, however, given that 1) CO_2 levels are related to oxygen consumption rates, 2) hypoxia often results in a blood acidosis (Burnett, 1988), and 3) ion concentration changes occur during both desiccation and acid-base challenges (Henry and Wheatly, 1992). All hemolymph samples were analyzed in duplicate and were delivered to the blood gas analyzer from the same syringe in rapid succession (measurement time per sample was ~2 min). The opening of the syringe was covered with paraflim following aspiration of the first sample so as to prevent the diffusion of gases into or out of the syringe during the 2 min wait period before the duplicate could be run.

2.3 Respirometry

We used an intermittent-flow respirometry system (Loligo Systems; AutoResp Software) to measure oxygen consumption rates (MO_2; mg O_2 kg^{-1} hr^{-1}) of individual barnacles immersed in FSW (aquatic respirometry) and emersed in air (aerial respirometry) at each of the following temperatures: 10, 15, and 20°C. Oxygen consumption rates were determined for every barnacle [N=8 (aquatic) or N=10

(emersion)] under every temperature condition (10, 15 or 20°C). MO_2 values were derived from changes in the concentration of oxygen (mg O_2 L^{-1}) in the respirometry chamber (liquid or gas filled), as determined by a fiber-optic sensor (Witrox 4) that measured fluorescence levels emitting from a small oxygen-sensitive sensor spot glued to the inside wall of each glass chamber. The fiber-optic O_2 sensor and sensor spots were calibrated at the beginning of each set of data collection (i.e., aquatic measurements or aerial measurements). For measures of both aquatic and aerial MO_2, calibrations were carried out by immersion in FSW bubbled with atmospheric air (100% O_2) and in FSW saturated with sodium sulfite (0% O_2). Several days prior to experimentation, barnacles were scrubbed clean of all epibiotic growth to ensure that the measured MO_2 was not falsely elevated. Barnacles were not fed for 24h prior to data collection.

To determine the MO_2, barnacles were removed from their holding tank and individually placed into a cylindrical, acrylic respirometry chamber (4.25L) filled with either FSW (33 ppt; aquatic measurement) or air (emersion measurement). Respirometry chambers (N=3; n=2 containing a single barnacle; n=1 empty control) were held within a larger glass aquarium filled with 75L (aquatic) or 60L (emersion) of FSW (Fig. 3, 4), which served as a water bath (and as the source of recirculating/flushing water for the chambers in the aquatic respirometry experiments). The surrounding FSW was cooled to the desired experimental temperature (10, 15 or 20°C) with an aquarium chiller and was continuously bubbled with atmospheric air. The positions of the two barnacle chambers and the single, empty control chamber were randomized within the larger aquarium.

In the *aquatic* respirometry experiment, barnacles were held in the FSW-filled chambers at the desired experimental temperature (i.e., 10, 15 or 20°C) for 6h prior to data collection. At the end of the 6h acclimation period we began the intermittent

respirometry protocol; this consisted of a 5 min *flush cycle* - during which time the chamber was opened and the oxygen-depleted seawater in the chamber was replaced with fully-oxygenated water from the surrounding FSW, a 5 min *wait period* - during which time the chamber was closed and the concentration of oxygen was given time to stabilize following the re-initiation of recirculation, and a 1h *measurement period* - during which time the chamber was closed and MO_2 values were calculated based on the decreasing concentration of oxygen due to barnacle respiration. This entire flush, wait, measure cycle was repeated 5 times between approximately 12:00 am and 6:00 am.

In the *aerial* respirometry experiments, barnacles were held in air-filled chambers at the desired experimental temperature (i.e., 10, 15 or 20°C) for 30 min prior to data collection. The respirometry chambers (same chambers as in the aquatic experiments) were filled with 1.125L of large glass beads that served as inert space fillers. Despite the large overall size of adult *B. nubilus*, the actual oxygen-consuming internal body of the organism is relatively small for the size of chamber required to fit the whole shell. Thus, inert glass beads were used to reduce the volume of air in the chambers and hence permit measurable rates of oxygen consumption in the remaining air. Preliminary tests of the aerial respirometry system included pumping of an attached air-tight glass syringe in an effort to evenly mix the air within the chamber and generate accurate oxygen readings. We found that mixing had no effect on the oxygen reading and so this pumping was deemed unnecessary. At the end of the acclimation period, we began the closed system respirometry protocol; this consisted of leaving the air-filled chamber closed within the temperature-controlled water bath for 5-6h while recording a measurement of oxygen concentration (mg O_2 L^{-1}) once per second. This entire closed system MO_2 protocol took place between approximately 5:00pm and 11:00pm.

Both aquatic and aerial MO_2 values were calculated from the change in oxygen concentration over time using the following equation:

$$MO_2 = ([O_2]_{t_0} - [O_2]_{t_1}) \cdot \frac{V}{t} \cdot \frac{1}{BW}$$

MO_2 = oxygen consumption rate (mg O_2 kg^{-1} hr^{-1})
$[O_2]t_0$ = oxygen concentration at time t_0 (mg O_2 L^{-1})
$[O_2]t_1$ = oxygen concentration at time t_1 (mg O_2 L^{-1})
V = respirometer volume minus volume of experimental animal (L)
$T = t_1 - t_0$ (hr)
BW = body weight (kg)

To determine the final MO_2 value for each barnacle we subtracted the average MO_2 of the empty control tank from the average MO_2 of each barnacle-containing treatment tank from the same temperature treatment tank. Our final aerial MO_2 value required one additional conversion. Although the sensor spots used in this respirometry system are capable of measuring pO_2 in both aquatic and gas conditions, the most current version of the AutoResp software (V. 2.2.2) incorrectly assumes - by default - that you are measuring in water (of a specifiable salinity, temperature and atmospheric pressure) before converting these values to $[O_2]$ (e.g., mg L^{-1}). Thus, we had to multiply our final MO_2 values by a conversion factor that reflected the roughly 30-fold difference in oxygen concentration between air and water at the same partial pressure. This conversion factor was determined as the exact fold-increase in $[O_2]$ between the air and freshwater (0 ppt) at the same pO_2 for each measured temperature (10, 15, 20°C). These values were for 25.36 (10°C), 27.90 (15°C), and 30.42 (20°C).

Relative humidity in the laboratory, and thus chamber humidity at the start of the aerial respiration data collection ranged from 36 - 49% (mean=41.5±1%; mode=40%). Additionally, throughout the course of the 5-6 hour collection, many of the barnacles squirted stored seawater from their mantle cavity into the closed chambers causing slight pooling of water at the bottom of the chamber, condensation of water on the sides of the chamber and a probable increase in relative chamber humidity.

2.4 Respiratory behaviors

Three 19L opaque, plastic buckets ('experimental treatment tanks') filled with FSW (15L; 33 ppt) were placed inside a single larger glass aquarium (150L) filled with freshwater, which served as a temperature bath. The water in the surrounding glass aquarium was cooled to an environmentally relevant temperature (12°C) with an aquarium chiller. We allowed 24h for the temperature of the FSW in the experimental tanks to equilibrate with the temperature of the surrounding water bath, as confirmed by a glass thermometer held in each bucket. At this time, barnacles were removed from their holding tanks at the pier and individually placed into each experimental tank. Here, they were held in FSW bubbled with atmospheric air for 1h prior to experimentation. Following this acclimation period, each of the three experimental tanks was rapidly manipulated to create one of the following oxygen treatments**:** *normoxic immersion* [N; FSW bubbled with atmospheric air], *air emersion* [E; empty bucket], and *anoxic immersion* [A; filtered seawater bubbled with pure nitrogen gas]. In the anoxia treatment we covered the surface of the water with a thin, transparent, floating layer of plastic cut to the size of the bucket opening to prevent the exchange of gases between the atmosphere and the seawater. PO_2 levels in the anoxic tanks fell to <0.5 mg L^{-1} in ~15 min. In both

immersion treatments we maintained the experimental tanks with no flow (other than that created by the bubblers), so as to try and limit behaviors that were aimed at feeding and instead observe behaviors that were intentionally respiratory in nature (i.e., aimed at oxygen uptake). Flow is known to encourage feeding behaviors (Crisp and Southward, 1961; Southward and Crisp, 1965; Trager et al., 1990) and the lack of flow may result in lower and less stable oxygen concentrations in the mantle cavity fluid (Davenport and Irwin, 2003). The position of the experimental tank assigned to each treatment (i.e., N, E, or A) within the larger temperature bath was randomized in each trial. Barnacles were exposed to the experimental oxygen treatments for 6h, during which time a single waterproof camera (GBB 4K Ultra HD WiFi Sport Action Camera; 720P 30fps) was positioned directly above the animal and recorded behavior for the entire 6h duration. At the end of the 6h measurement period, barnacles were returned to their holding tank.

Video recordings were later analyzed and scored for the following behaviors: 1) *operculum closed,* 2) *testing* (operculum open with no visible cirri protrusions or aperture pumping/pulsing, and no apparent water flow through the aperture), 3) *pumping beat* (operculum open with visible aperture pumping and apparent water flow through the aperture, though no visible cirri extensions), and 4) *cirral beating* (operculum open with cirri extended as indicated by any form of visible cirri protrusion through the operculum regardless of its rate of beating or degree of extension or fanning) (see Discussion for more detail on this classification scheme). Additionally, we measured the *pumping beat frequency* (PBF), which we defined as the rate of opercular opening and closing in the absence of cirri extensions, and the *cirral beat frequency* (CBF), which we defined as the rate at which the cirri appeared (in any form) and disappeared through the open operculum. In total, we analyzed the behavior of N=9 barnacles per treatment, whereby

every barnacle was exposed to each treatment once, in a randomized order (repeated measures design). Barnacles were given a minimum of four days of rest between treatments.

2.5 Statistical analyses

All statistical analyses were conducted using JMP Pro software (v. 12.1). Data were tested for normality using the Shapiro-Wilk test, and for homogeneity of variance using the Levene test. Two-way ANOVA models were used to determine if there were any significant oxygen treatment (N, E, A), time, and their interaction effects on pO_2, pCO_2, pH, and ion concentration (Na^+, Cl^-, K^+, and Ca^{2+}), as well as to determine if there were any significant fluid medium (air versus water), temperature, and their interaction effects on MO_2. When significant main effects were detected in the ANOVA models, *post-hoc* pairwise comparison tests were performed using either Tukey's HSD tests or Dunnett's tests. Repeated measures ANOVA models were used to determine if there were any significant effects of temperature (10, 15, 20°C) on oxygen consumption rates (MO_2) in air and in water. Further, we used a two-way ANOVA to determine if there were significant differences in MO_2 between air and water at each temperature. Repeated measures ANOVA models were used to reveal any significant treatment effects (N, E, A) on the suite of examined respiratory behaviors. When significant main effects were detected in the ANOVA models, *post-hoc* pairwise comparison tests were performed using a Tukey's HSD tests. All values are reported as mean±SEM. All results were considered to be significant at the α=0.05 level.

3. Results

3.1 Hemolymph pO_2, pCO_2, pH and ion levels

A two-way ANOVA on data collected during a 9h exposure period to normoxic immersion, air emersion, and anoxic immersion (i.e., 'oxygen treatment'), revealed a significant oxygen treatment*time interaction effect ($F_{6,93}$=2.5423, p=0.0253) on pO_2 in *B. nubilus*. *Post-hoc* Dunnett's test indicated that by 3h, barnacles exposed to anoxia had a significantly lower pO_2 than their time 0 control, and this reduction was maintained for the duration of the experiment (Fig. 5). Animals in the emersion group did not experience a significant reduction in pO_2 over time (when compared to its own time 0h value), although this value was significantly lower than the normoxic control group by 9h. Normoxic control animals did not show any significant change in pO_2 over the 9h experiment, although there was a notable upward trend in pO_2 by 9h; this trend was also observed in the emersion and anoxic animals. Thus, hemolymph oxygen levels in barnacles exposed to anoxia dropped significantly compared to barnacles held under normoxic conditions, whereas animals held in the air-maintained hemolymph pO_2 levels that were intermediate to them both.

A separate two-way ANOVA on data from the same 9h exposure period revealed a significant main effect of oxygen treatment ($F_{2,94}$=4.6617, p=0.0117) and time ($F_{3,94}$=17.1723, p<0.001) on pCO_2. *Post-hoc* Dunnett's test indicated that pCO_2 levels were significantly elevated by 6h in the emersion group, and by 9h in both the anoxic and emersion group, relative to their time 0 control (Fig. 6). There were no significant differences in the pCO_2 values over time in the normoxic control group. At 3h pCO_2 levels in the anoxic group were significantly lower than the normoxic control. Thus, with small exception (anoxia at 3h), hemolymph CO_2 levels were not significantly different

between treatments, though the pCO_2 did increase slightly in all three treatment groups over the 9h exposure period.

An additional two-way ANOVA revealed a significant main effect of oxygen treatment ($F_{2,95}$=3.7530, p=0.0270) and time ($F_{3,95}$=2.9753, p=0.0354) on pH. *Post-hoc* Dunnett's test indicated that, relative to their time 0 control, hemolymph pH was significantly elevated at 3h in the anoxic group (which corresponded to the significant decline in pCO_2 at the same time), but had returned to baseline levels by 6h and remained there for the duration of the experiment (Fig. 7). There were no significant changes in hemolymph pH over time in the emersion or normoxic control groups. The pH was not significantly different between treatment groups at any time point. We also compared the hemolymph pH to the pCO_2 within each treatment and found that the changes in pH exactly mirrored the changes in pCO_2 (Fig. 9). This trend held true when we regarded the data collected from barnacles in each treatment (N, E, and A) separately, or when we regarded the entire data set as a whole and ignored treatment (Fig. 8).

We also analyzed hemolymph ion (K^+, Cl^-, Ca^{2+}, and Na^+) data following the 9h exposure periods, and a two-way ANOVA revealed a significant main effect of oxygen treatment ($F_{2,9}$7=7.0377, p=0.0014) and time ($F_{3,95}$=6.7764, p=0.003) on [K^+] (Fig. 10). Hemolymph [K^+] levels were found to be significantly elevated from their 0h baseline by 6h in the anoxic and emersion groups, and remained elevated by 9h in anoxic group (*Post-hoc* Dunett's test). Furthermore, by 6hr in the emersion treated animals and by 9h in both the anoxic and emersion groups, [K^+] levels were significantly higher than in the normoxic group. There were no significant differences in the [K^+] levels over time in the normoxic control group. K^+ was the only ion to show any significant change in concentration in response to air emersion or anoxia exposure (i.e., treatment effect).

We also found a significant main effect of time on hemolymph [Cl^-] ($F_{3,95}$=3.0665, p=0.0317; two-way ANOVA); however, the *post-hoc* Dunnett's test indicated that [Cl^-] levels were not significantly different from the time 0 control *within* any specific treatment group (Table 1). We did not see any significant main effect of oxygen treatment (p=0.4589) nor a treatment*time interaction effect (p=0.8728) on [Cl^-]. Thus, hemolymph [Cl^-] levels did not vary between treatment groups at any time (Table 1).

Lastly, a two-way ANOVA revealed a significant main effect of time on hemolymph [Ca^{2+}] ($F_{3,95}$=6.2821, p=0.0006) and on hemolymph [Na^+] ($F_{3,95}$=5.1051, p=0.0026), with *post-hoc* Dunnett's tests indicating that [Ca^{2+}] and [Na^+] levels at 6h were significantly higher from the time 0 control in the emersion group. In the normoxic and anoxic groups, there were no significant differences in either [Ca^{2+}] or [Na^+] across time. We did not see any significant main effect of oxygen treatment with either [Ca^{2+}] (p=0.1091) or [Na^+] (p=0.1579), nor a treatment*time interaction effect for [Ca^{2+}] (p=0.7326) or [Na^+] (p=0.8456). Thus, hemolymph [Ca^{2+}] and [Na^+] levels did not vary between treatment groups at any time (Table 1).

3.2 Respirometry

Increasing temperature resulted in an increase in oxygen consumption rates in barnacles exposed to both seawater immersion and air emersion (Fig. 11). These relationships were statistically confirmed by repeated-measures ANOVAs, which revealed a significant main effect of temperature on MO_2 in seawater ($F_{2,14}$=8.7076, p=0.0035) and in air ($F_{2,18}$=6.4241, p=0.0078). *Post-hoc* Tukey's HSD tests found that

MO_2 values were significantly elevated at 20°C compared to 10°C in both water and air (Fig. 11). Further, *B. nubilus* appear to have extremely similar MO_2 values whether in the water or in the air.

In a separate two-way ANOVA, we found a significant main effect of temperature on MO_2 ($F_{2,48}$=6.5753, p=0.0030), whereby MO_2 values increased significantly as temperature increased (Fig. 11). There was no significant main effect of fluid medium (air versus water) ($F_{1,48}$=0.0684, p=0.7949) or the fluid medium X temperature interaction effect ($F_{2,48}$=0.7458, p=0.4798) on MO_2.

3.3 Respiratory behaviors

We determined that air emersion and anoxia immersion altered respiratory behaviors in *B. nubilus*, relative to animals held in normally oxygenated seawater. Barnacles exposed to emersion and anoxia spent significantly greater time with cirri extended from the operculum and significantly less time performing opercular pumping compared to barnacles held in normoxia (Fig. 12). A repeated measures ANOVA revealed a significant main effect of oxygen treatment on % time cirral beating ($F_{2,16}$=4.5087, p=0.0280) and % time performing a pumping beat ($F_{2,16}$=5.023, p=0.0203), though there were no significant differences between treatment groups in time spent testing (i.e., open with cirri retracted; p=0.1439) or overall % total time spent open for any behavior (p=0.1697; Fig. 12, inset graph). Additionally, barnacles held in the air and in anoxic seawater had a significantly lower cirri beat frequency, though not opercular pulse frequency, than barnacles held in normoxic seawater (Fig. 13). A repeated measures ANOVA revealed a significant effect of oxygen treatment on cirri beat

frequency ($F_{2,16}$ = 17.7244, p<0.0001), though not on opercular pulse frequency (p=0.1364).

It should be noted that there was no significant effect of time on any behavior (CBF, p=0.9535; OPF, p=0.7766; testing, p=0.9749; pumping beat p=0.9993; cirral beating, p=0.9992).

4. Discussion

Given the innate oxygen limitations faced by the giant acorn barnacle – (extremely large muscle fibers, the lack of a respiratory binding pigment, and potential environmental oxygen limitation during low-tide air exposure or coastal eutrophication/upwelling events) – *Balanus nubilus* represents an ideal model for studying the maintenance of whole organism function under hypoxia. Here, we provide a baseline characterization of the physiological and behavioral response of *B. nubilus* to air exposure and anoxic immersion, and present evidence for mild to no oxygen limitation during air exposure, with more predictably severe oxygen depletion during anoxia. These data will enable us to understand more fully how *B. nubilus* maintains function of their giant muscle fibers during environmental oxygen limitation (e.g., low tide air exposure, environmental hypoxia).

4.1 Hemolymph pO_2, pCO_2, pH and ion levels

4.1.1 Hemolymph pO_2 and pCO_2

The first step in understanding the internal oxygen environment of *B. nubilus* during oxygen limitation was to measure the partial pressure of oxygen and carbon

dioxide in the hemolymph in response to air emersion and anoxic immersion, and compare these to control barnacles held in normally oxygenated seawater. *B. nubilus* showed a significant drop in hemolymph pO_2 following 9h exposure to anoxic immersion (Fig 4). Without the addition of new oxygen, existing stores would, predictably, be used to support any ongoing aerobic metabolism, causing the observed drop in pO_2. Barnacles that were emersed in air, however, showed no significant pO_2 decrease over time, nor a significant difference of hemolymph pO_2 from the normoxic control group before 9h. This indicates that *B. nubilus* is effectively bringing oxygen into their tissues while in the air.

The exact behavioral strategy by which *B. nubilus* facilitates aerial gas-exchange had not been established prior to this study. In general, not a tremendous amount is known about the details of external respiration (oxygen uptake) in the air in acorn barnacles. It is understood that when submerged, movement of the cirri facilitates respiration via circulation of oxygenated seawater into and out of the mantle cavity where gas-exchange surfaces are located (i.e., gill-like branchiae structures, mantle tissues, and the prosoma), and that this movement is controlled by a combination of mantle muscles and hydraulic lift of the prosoma (Anderson, 1994). In the air, however, intertidal and subtidal barnacles appear to have different behaviors. Intertidal acorn barnacles are known to actually expel the fluid pooled in their mantle cavity and subsequently create a small opening with the soft-tissues of their opercular valves (known as a 'micropylar pneumostome') through which they bring air into this cavity. In this way they can directly oxygenate the moist gas- exchange surfaces inside the shell while largely avoiding desiccation (Barnes and Barnes, 1957; Barnes et al., 1963; Grainger and Newell, 1965; Davenport and Irwin, 2003). Subtidal barnacles, however, have a more varied

response to air emersion (Barnes et al., 1963; Lopez et al., 2003). *Balanus crenatus*, for example, repeatedly extrudes their collapsed cirri while in the air, but without intentional expulsion of mantle cavity fluid stores or the formation of a pneumostome (Barnes et al., 1963). *Austromegabalanus psittacus*, another giant subtidal to low intertidal species like *B. nubilus,* has been observed to form a pneumostome during emersion, though in many cases they kept this aperture closed and avoided aerial respiration and gas-exchange altogether (Lopez et al., 2003). In the current work, we observed that *B. nubilus* too forms a pneumstome aperture during emersion, which it often repeatedly opens and closes (i.e., testing and pumping behaviors) in a likely effort to bring air into the mantle cavity. Though this species is capable of rapid, forceful expulsion of seawater upon air exposure, it does not appear to do this consistently or upon immediate exposure to air (like most small intertidal barnacles). Rather, *B. nubilus* will shoot out a stream of seawater from its mantle cavity when agitated or probed, as if deterring a predator. Thus, *B. nubilus* seems to keep its mantle cavity largely filled with seawater while emersed, but cycles fresh air into this space to keep the fluid oxygenated.

The pCO_2 data seem to suggest that the production of CO_2 does not vary much between control and emersion barnacles, despite the slight differences in hemolymph oxygen content we observed. We found that the control barnacles had no significant change in the hemolymph pCO_2 over the 9h exposure period, and the pCO_2 levels in the air emersion barnacles were not significantly different from the control at any time point (Fig. 6). These data are further supported by our oxygen consumption findings, in which the MO_2 values for barnacles held at a common temperature were very similar between the air and the water (Fig. 11). Resting oxygen consumption rates in most animals are held constant over some environmental oxygen range, and only begin to decline once the

pO_2 has fallen to a particular, species-specific P_{crit} value (i.e., the pO_2 at which an organism can longer hold MO_2 constant and it begins to decline proportionally with the oxygen content.) We suspect that the oxygen content of the mantle cavity fluid does not drop below the P_{crit} value for *B. nubilus* during emersion and so CO_2 production by aerobic metabolism is not affected.

Hemolymph pCO_2 in anoxic barnacles, however, took a dip at 3h, during which time it was significantly lower than the pCO_2 in the control animals (Fig. 6). And though it recovered by 6h, it remained slightly lower than that of the control and air-exposed animals for the remainder of the experiment. In a separate study, we have also seen that pCO_2 levels in anoxic *B. nubilus* barnacles were, in fact, significantly lower than those in air and normoxic water after 6h (Grady et al., in prep). Taken together, these findings may indicate that barnacles in anoxic conditions experience a whole-animal metabolic depression as a way to conserve energy during oxygen limitation. This is a common strategy in intertidal invertebrates experiencing hypoxia or hypercapnia (Grieshaber et al., 1994; Guppy, 2004; Reipschläger and Pörtner, 1996). For example, the mud-burrowing, xanthid crab, *Eurytium albidigitum*, does not compensate for the effects of aerial exposure when exposed at low tide; rather, they undergo a metabolic depression until reimmersed (Burnett and McMahon, 1987). Crustaceans are the group with the highest LC_{50} (median lethal oxygen concentrations) and the shortest LT_{50} (median lethal time) in a review of 872 published experiments reporting oxygen thresholds and/or lethal times for a total of 206 species spanning the full taxonomic range of benthic metazoans, so such activity is not surprising given that crustaceans as a whole show the lowest ability to deal with hypoxia (Vaquer-Sunyer and Duarte, 2008). Though they do not fare well in oxygen limited environments, it is likely that most species of crustaceans would not be forced to

deal with hypoxia for any extended period of time given their mobile nature. As sessile crustaceans, barnacles are unable to move to avoid hypoxia and must rely on alternative forms of avoidance, such as a metabolic depression.

Another interesting pattern emerged from our hemolymph-gas data. We observed a slight trend toward increasing pO_2 and pCO_2 values over the 9h for all three treatments. Hemolymph oxygen levels went up slightly, though not significantly, between 6h and 9h (Fig. 5), particularly in normoxia, and hemolymph CO_2 levels rose significantly in the emersion and anoxia barnacles by 6-9 h, though the increase is not significant for the control barnacles (Fig. 6). Our first inclination was to attribute this upward trend to a change in temperature, but we confirmed that there was no temperature variation in the experimental tanks over the 9h exposure period via regular, repeated temperature readings. We further hypothesized that this trend may be due to changes in the hemolymph volume that resulted from successive sampling of each individual barnacle in this experimental design. With a smaller total hemolymph volume, the concentration of oxygen in the hemolymph (and hence the oxygen partial pressure) would increase more quickly during gas-exchange, such that reoxygenation rates would increase relative to the rate of oxygen use in the tissues. This is supported by the relative lack of increase in hemolymph oxygen in the anoxic group (where there is no new oxygen in the seawater) compared to the control and air exposed barnacles between 6 and 9h (Fig. 5). Likewise, if the same quantitiy of CO_2 is diffusing from the tissues into the hemolymph at a now smaller volume, the concentration would artificially rise.

It is also possible that the slight increase in pCO_2 over time is not artificial, but is due to an activity-induced increase in aerobic metabolism. Support for this hypothesis can be found in our behavior data. We found that *B. nubilus* exposed to air and anoxia

both have an increase in cirri extension activity relative to the normoxic control animals (Fig. 12), which could explain the rise in pCO_2 in these groups. This activity requires that the barnacles still have oxygen in their hemolymph (and potentially still in the mantle cavity fluid), which is supported by our pO_2 data (Fig 4). Additionally, we know *B. nubilus* do not accumulate lactate in their muscles during air exposure and anoxia (at least for periods up to 6hr), so it appears they are not using anaerobic metabolism to power these cirri extensions in any of these conditions (Grady et al., in prep).

4.1.2 Hemolymph pH

Exposure to the air or anoxic seawater did not significantly alter the hemolymph pH over time (Fig. 9). There was a slight elevation of pH in the anoxia treatment at 3h, which is likely due to the concomitant drop in the anoxic pCO_2 at that same time point (Fig. 9C). In general, we saw strong correlations within each treatment between pH and pCO_2 (Fig. 9), and predict that the slight changes in pH observed during this experiment are being driven by changes in pCO_2 as opposed to lactate accumulation (Melzner et al., 2009). It is important to note that while the pH changes closely mirror those of pCO_2, they are not significant changes. We suspect pH changes are minimized due to effective buffering occurring within the hemolymph of *B. nubilus*. Intertidal invertebrates that are exposed to the air end up experiencing acidosis whether they tend to avoid air exposure or tend to facilitate gas-exchange from the air directly. In the former case, a metabolic acidosis results from anaerobic metabolism; in the latter case, a respiratory acidosis results from retention of carbon dioxide due to hypoventilation as a result of the much higher oxygen capacity of air than of water (Burnett, 1988). This intermittent acidosis necessitates that intertidal organisms, including *B. nubilus,* be able to buffer against large

changes in pH effectively. This regulation can be achieved through myriad acid-base balancing mechanisms. These could include increasing plasma buffering proteins (e.g., hemocyanin, phosphates; Henry and Wheatly, 1992), altering ventilation rates to increase CO_2 clearance (McMahon, 2001), and/or preferentially excreting or accumulating H^+ or HCO_3^- (Burnett, 1988; McMahon et al., 1991). In prawns and lobsters, for example, hypoxia- and hypercapnia-induced metabolic acidosis is often counteracted by increasing circulating bicarbonate buffers taken from calcium carbonate shell (Dissanayake et al., 2010; Taylor and Whiteley, 1989). Innes (1986) found that changes in hemolymph pH in response to changes in hemolymph pCO_2 in *Calantica spinosa,* a species of stalked barnacle, were similar in magnitude to that of other decapod crustaceans, despite their lack of a respiratory binding pigment. They suggest that other non-hemocyanin buffering proteins must be responsible for acid-base balance in barnacles. At this time, we do not know the exact mechanisms of acid-base buffering in *B. nubilus*, but it is reasonable to assume that they too are exploiting bicarbonate stored in their shell, as well as other plasma buffering proteins.

4.1.3 Hemolymph ion levels

Increasing (or decreasing) the concentration of plasma buffering proteins or HCO_3^- to counteract pH changes in the hemolymph will also require compensatory measures to offset osmotic changes or facilitate transporter exchange. This compensation is sometimes accomplished by changes in the concentration of certain inorganic ions (Taylor et al., 1987; Taylor and Whiteley, 1989; Henry and Wheatly, 1992; Randall et al., 2002). Taylor and Whiteley (1989) found that circulating Ca^{2+} and Cl^- levels in the lobster *Homarus gammarus* rose during a metabolic acidosis associated with air

emersion, which they speculate to be related to modification of the [HCO_3^-]. In the current study we measured the hemolymph concentration of Na^+, Cl^-, K^+ and Ca^{2+} during air emersion and anoxic immersion. Hemolymph K^+ was the only ion that was significantly different in the emersion and anoxic groups relative to the normoxic group, with levels significantly higher in the air exposed barnacles by 6h (and 9h) and in the anoxia exposed barnacles by 9h (Table 1; Fig. 10). Barnacles held in normoxic conditions did not show an increase in hemolymph K^+ over time.

We hypothesize that the rise in hemolymph [K^+] observed in barnacles exposed to anoxia and emersion could be occurring as part of a strategy to prevent a respiratory acidosis due to the significant increase in pCO_2 that occurred under the same conditions. That is to say, this increase in [K^+] is part of an acid-base regulation strategy that likely involves simultaneous changes in non-hemocyanin buffering proteins. This suggestion is supported by several previous studies. Hemolymph K^+ levels have been shown to increase in *Procambarus clarkii* over the first 24h of exposure to an acidic environment (Wheatly et al., 1996) and in a species of freshwater fish (*Cyprinus carpio)* during long-term low pH exposure (Mathan et al., 2010). Also, metabolic acidosis has been shown to result in the movement of K^+ from the cells to the extracellular space in canids (Pitts, 1954). Ultsch et al. (1981) has also observed an increase in plasma K^+ levels in carp when exposed to low pH, and proposed that the K^+ is released into the tissues from the cells in exchange for H^+ when there is a surplus of H^+ that require buffering.

In addition to a rise in K^+ during anoxia and air emersion, we also saw an elevation in Na^+, Ca^{2+} and K^+ over time in *all* treatments, whereby ion levels at 6h were significantly higher than the 0h control, but in all cases, levels had returned to baseline by 9h (Table 1). It is possible that such an ion-wide increase is the consequence of a reduced

blood volume owing to our successive hemolymph sampling regime. Perhaps *B. nubilus* is increasing its plasma osmolarity by increasing its plasma ion concentrations to facilitate the osmotic influx of water across the gills, skin or antennal glands and increase the hemolymph volume. Such a link between ion transport and blood volume has been observed previously (Lockwood, 1976).

4.2 Respirometry

Oxygen consumption rates for sessile intertidal organisms in the air vary widely, but it has been consistently observed that rates are highest in those organisms which are able to facilitate oxygen uptake from the atmosphere (Lopez et al., 2003; Barnes & Barnes, 1957; Barnes et al., 1963; Littlewood, 1989). Blue mussels (*Mytlius spp.*), which tend to close their valves during emersion to prevent desiccation (and hence, naturally, oxygen exchange), have relatively low oxygen consumption rates in the air. For example, *Perumytilus purpuratus* and *Mytilus chilensis* have oxygen consumption rates in air that are only 30-50% and 5-15% of the rates during immersion, respectively (Simpfendorfer et al., 1995). Acorn barnacles, which keep their moist (or fluid-filled) mantle cavity in contact with the atmosphere - either through slight openings in the aperture (a.k.a, micropylar aperture or pneumostome; Barnes and Barnes, 1957, 1958; Barnes et al., 1963) or through repeated cirral extensions (pers. observation) - can take up oxygen moderately well in air as compared to water (Grainger and Newell, 1965). The giant Chilean acorn barnacle, *Austromegabalanus psittacus*, is capable of aerial respiration rates that are 60% of those in water (Lopez et al., 2003). On the other end of the spectrum, some intertidal organisms are nearly equal in their ability to consume oxygen in the water and the air. The intertidal chiton, *Chiton stokesii*, which pulls air into its

pallial cavity and facilitates direct oxygen uptake from the air by the gills, has aerial MO_2 values that are only about 75% of those in the water, though this difference was not significant (McMahon et al., 1991). The small, intertidal acorn barnacle *Jehlius cirratus* is capable of aerial oxygen consumption rates that are 80-100% of oxygen uptake in water (Simpfendorfer et al., 1995), and the stalked gooseneck barnacle, *Pollicipes polymerus,* has oxygen consumption rates that are actually higher in air than in water (Peterson et al., 1974)! This results from substantial oxygen uptake occurring across the integument of their peduncle.

It is generally accepted that species living higher in the intertidal are better adapted to air exposure and would therefore possess an increased capacity for aerial respiration. Such a pattern has been observed in several other studies of intertidal invertebrates, including snails, mussels, barnacles, and crabs (e.g., McMahon and Russell-Hunter, 1977; Houlihan, 1979; Houlihan and Innes, 1982; Tagliarolo et al., 2012; Simpfendorfer et al., 1995; Stillman and Somero, 1996), though others have found there to be no difference in aerial respiration rates between several species of marine snails (Sandison, 1966) or chitons (Murdoch and Shumway, 1980) from different positions in the intertidal. We found that *B. nubilus* had relatively similar MO_2 (mg O_2 kg^{-1} hr^{-1}) values in the water and in the air (between 10°C and 20°C), despite the fact that this species primarily occupies the subtidal to low intertidal zone (Fig. 11). Adult *B. nubilus* hold several milliliters of seawater in their mantle cavity (pers. obs.), which allows them to keep their respiratory exchange surfaces in direct contact with oxygenated fluid. Further, we found that *B. nubilus* hold their opercular plates open ~65% of the time (for up 6h) when held in the air (Fig. 12; inset), often repeatedly extending and retracting the cirri, and this will reoxygenate the seawater in the cavity as it is depleted of oxygen.

Given this strategy, it may be no surprise that *B. nubilus* has the same average MO_2 values in air and water. Houlihan et al. (1981) observed that aerial oxygen consumption rates of three species of intertidal gastropods were lowered by the loss of mantle cavity fluid bathing their gills, and suggested a general correlation between a substantial mantle cavity fluid pool, the presence of a gill in the mantle cavity, and a large reduction in oxygen consumption upon the loss of that fluid.

We also saw a significant and equal increase in average MO_2 as temperature increased from 10°C to 20°C in both aquatic and air respirometry (Fig. 11), which is expected of a marine ectotherm like *B. nubilus*. However, the average MO_2 in air was constant from 10°C to 15°C with a steep increase between 15°C and 20°C, while in water, average MO_2 at 15°C was almost perfectly intermediate to the average MO_2 values at 10°C and 20°C. This finding does not fit the expected prediction of a proportional increase in respiration rates with temperature, though others have observed the same phenomenon. Lopez et al., (2003) found that temperature did not affect aerial respiration rates in *A. psittacus* between 10°C and 20°C, though immersion respiration rates did increase directly with temperature over this same range. A possible explanation for this lack of influence of temperature on air respiration may be that the mantle shell insulates the mantle cavity fluid to some degree and the fluid is therefore protected from rapid changes in air temperature.

4.3 Respiratory behaviors

In their highly descriptive account, Crisp and Southward (1961) proposed a classification scheme for typical acorn barnacle behaviors that has since been adopted

into general use (see also Anderson, 1981 and Anderson and Southward, 1987). The following five modes of behaviors are recognized in this scheme: (1) *testing*, in which the scutal and tergal valves are held slightly open, the cirri are not protruded and there is presumably no water flow occurring through the aperture; (2) *pumping beat*, in which strong rhythmic opening and closing movements of the operculum occur and the curled cirri may be slightly protruded, though not fully extended; (3) *normal beat*, a development of pumping, but with the cirri fully extended into a fanned out (uncurled) position then withdrawn in rhythm with the opercular movements; (4) *fast beat*, with less opercular movement, but strong and fast rhythmic cirral movements that occur exclusively outside of the mantle cavity; and (5) *extension*, in which the cirri are held extended and uncurled outside the shell for varying periods without any rhythmic movements. For the purposes of our own experiment, we had to slightly modify this behavioral classification scheme in a way that allowed for the same behaviors to be observed under water and in the air. Certain behaviors (e.g., normal beat, fast beat, extension) are not equally possible in water and air, as cirri clump together when exposed to air and are therefore unable to fan despite the barnacle's intention to fan or not. Thus, we classified the barnacle behaviors as follows: 1) *testing,* operculum open with no visible cirri protrusions or aperture pumping/pulsing, and presumably little to no water flow through the aperture, 2) *pumping beat*, operculum open with visible aperture pumping and apparent water flow through the aperture, though no visible cirri extensions, and 3) *cirral beating,* operculum open with cirri extended as indicated by any form of visible cirri protrusion through the operculum regardless of its rate of beating or degree of extension or fanning.

Barnacles in general are known to be extremely tolerant of air exposure and anoxia (e.g., Castro and Vial, 2001). *A. psittacus*, a subtidal to low intertidal giant acorn barnacle, has been shown to survive in the air for >7 days (Lopez et. al., 2003), and the high intertidal adapted *Cthamalus spp.* has been shown to survive out of water for 119 day (Foster, 1971)! Intertidal acorn barnacles are well established to expel seawater from their mantle cavity, which then functions like a lung to take up oxygen directly from the air (Barnes et al., 1963; Grainger and Newell, 1965). But desiccation is still a real problem, especially for smaller barnacles with a low SA:V. As we discussed above, subtidal barnacles have a more varied response to air emersion, with some species more likely to retain their mantle cavity fluid and remain largely closed, and others more sporadic in their activity patterns and with clear penumostome formation (Barnes et al., 1963; Lopez et al., 2003). We predicted that *Balanus nubilus* exposed to air and anoxia would keep their operculum closed more or less the entire duration of the experiment, whereas barnacles in normoxic seawater would engage in frequent bouts of aperture opening and cirral extensions. Air-exposed barnacles would be expected to remain closed to prevent desiccation (and perhaps also avoid predation or conserve energy), particularly given that *B. nubilus* lives mostly in the subtidal to lowest intertidal and is much less adapted to air exposure. For anoxia-exposed barnacles, remaining closed would prevent the oxygen content in the mantle cavity fluid from declining due to mixing with the surrounding anoxic seawater. This closure would allow them to maintain continued aerobic metabolism, albeit at a likely decreased rate (Guppy and Withers, 1999; Livingstone, 1991; English and Storey, 2003; Hill et. al., 1991), using the available oxygen trapped inside their shell. In both cases we would predict that as the oxygen content of the fluid pool is depleted during valve closure, (which it does in <1h in other

smaller barnacle species during a salinity challenge; Davenport and Irwin, 2003), they would switch to a reliance on anaerobic metabolism and perhaps also enter a state of metabolic depression. These are both common strategies for tolerating hypoxia and air emersion in intertidal molluscs and crustaceans (e.g., Burnett, 1988; Guppy and Withers, 1999; Vial et al., 1999).

Surprisingly, we found that barnacles in all three treatments (normoxic immersion, anoxic immersion and air exposure) spent an approximately equal amount of time (43%, 57% and 64% of the time, respectively) with their operculum open in some form (i.e., testing, pumping or cirral beating; Fig. 12, inset). Interestingly, though, they differed in the relative time allocated to each of these specific behaviors while open (Fig. 12). When active (i.e., operculum open), barnacles in oxygenated water spent the majority of their time engaged in pumping [24% of the total time, compared to 7% (air) and 1.7% (anoxia)], whereas barnacles in the air or in anoxic seawater spent the majority of their time open engaged in cirral beating (42% and 43% of the total time, respectively, compared to only 14% in normoxic barnacles) and substantially less time carrying out a typical pumping beat (Fig. 12). While initially surprising, these findings proved to be consistent with several other behavior studies of acorn barnacles in the air. Vial et al. (1999) found that high intertidal barnacles (*Chthamalus jossus* and *Balanus glandula*) kept very still and tended to close up their operculum when in air, whereas subtidal and low intertidal acorn barnacles (*Tetraclita squamosa* and *Balanus tintinnabulum*) remained vigorously active while in the air (see also Barnes and Barnes, 1957). Presumably, these behavioral differences reflect the dissimilarities in their body size and the resultant tendencies to desiccate in the air (which are much higher in small barnacles characteristic of the high intertidal zone). Thus, the large size of *B. nubilus* and relative

'unfamiliarity' of this species with air exposure may have led them to more dynamic aerial behaviors.

Of similar interest was our finding that air- and anoxia-exposed barnacles engaged in different behaviors than the control barnacles when their valves were open. Specifically, these barnacles engaged more frequently in cirral beating behaviors while open, compared to barnacles in normoxia, which engaged largely in pumping behaviors while open. For barnacles in anoxia, we did expect to see small amounts of testing or pumping over the 6h period as the barnacles monitored the oxygen levels in the surrounding seawater to see if they had returned to normal, but it was quite a surprise to discover that they spent so much time extending their cirri into the completely deoxygenated water. Perhaps after *many* hours (>>6h) you would expect to see them begin to open their operculum again and fully extend their cirri after they have completely depleted their internal oxygen stores and continued reliance on anaerobiosis becomes untenable, but certainly not within such a short time period. So why do they do they engage in such extensive cirral beating, and remain open for more than half of the time while in anoxia? We hypothesize that anoxic barnacles engaged in cirral beating are still merely sampling their environment to see if oxygen conditions have improved, but in a more aggressive fashion than simple testing or pumping. It is possible that testing and pumping are less comprehensive ways of sampling the chemical content (including oxygen, food and/or other chemical cues) of the seawater, whereas cirral extension (full or partial) gives a more complete picture of the seawater composition. Balanomorph barnacles have chemoreceptors and photoreceptors on their cirri and setae (Anderson, 1994; Chan et al., 2008), and that the chemoreceptors on some barnacle species allow them to engage in food selectivity (i.e., choosing which food particles are brought to the

mouth, rather than eating everything their setae catch). Based on the presence of these receptors, there is the potential that oxygen receptors could also be found on the cirri, though no one has yet confirmed this. There is evidence that isopods (Lockwood, 1968) and mud shrimp (Farley and Case, 1968) have external oxygen receptors located on their pleopods. Such sensors might require the barnacle to more *fully* extend their cirri into the surrounding seawater to test the oxygen level, and as such, testing and pumping would not be sufficient for the oxygen sensors to interact with the surrounding environment. Southward and Crisp (1965), similarly observed an increase in the duration of cirri extensions as seawater oxygen tensions declined across several species of acorn barnacles.

In the air these increased cirri beating behaviors are a bit easier to explain. We have already described the relative ease with which *B. nubilus* keeps their hemolymph oxygenated and consumes oxygen while in the air environment, but these abilities require concomitant measures aimed at continuously re-oxygenating the fluid enclosed in the mantle cavity. As discussed above, we found that *B. nubilus* retains the seawater in the mantle cavity during emersion and forms a pneumostome aperture that it repeatedly opens and closes likely to bring air into the mantle cavity. Thus, *B. nubilus* seems to keep its mantle cavity largely filled with seawater while emersed, and cycles fresh air into this space to keep the fluid oxygenated. Full extensions and retractions of the cirri are much more likely to increase the amount of oxygen brought into this fluid pool rather than simple pumping behaviors, and this will consequently increase the oxygen available to the branchiae or other soft-tissues inside the shell. Thus, cirral extensions in the air may help facilitate an increased reliance on aerobic metabolism during low tide air emersion in larger barnacles, given that desiccation is unlikely for a species with such a large

mantle cavity fluid pool. Barnacles in normally oxygenated seawater are less likely to engage in full cirral extensions, as we observed, as they are not experiencing oxygen limitation, and with a lack of flow and food in our experimental treatment tanks are also less likely to extend their cirri for the purposes of feeding.

The last finding from our behavior assessment was that cirri beat frequency was significantly higher in our control barnacle than in the anoxia or emersion barnacles (Fig. 13). Under anoxic conditions, a decrease in the cirri beat frequency seems a natural consequence of the reduction in oxygen available to power muscular contractions. The drastic differences in the physical dynamics of air versus water, as well as the slightly reduced hemolymph pO_2 levels in air, also make it unsurprising that cirri beat frequencies would be reduced in air. We found no differences in the opercular pulse frequency between treatments, which is not surprising given the low mechanical work (and hence energetic requirement) required of this movement, and the relative similarity of this movement in air versus water.

5. Conclusion

To understand how *Balanus nubilus* maintains function of its extreme muscle fibers during environmental oxygen limitation, we characterized the hemolymph gas content, oxygen consumption rates and respiratory behaviors of barnacles exposed to air, anoxic immersion, and normoxic immersion. We found that aerobic metabolism in *B. nubilus* is not inhibited by air exposure, presumably owing to efficient aerial oxygen uptake and perhaps also effective acid base-buffering. We therefore predict that muscle function would be preserved during periods of low-tide emersion. Anoxia, on the other

hand leads to a significant decline in hemolymph oxygen content, which suggests that environmental hypoxia is likely to diminish functionality of their giant muscle fibers. In a parallel study, we have been investigating the plastic response (e.g., [lactate], lactate dehydrogenase and citrate synthase activity, fiber size, mitochondrial density, etc.) of *B. nubilus* muscle fibers to the same conditions (air emersion and anoxic immersion). Our upcoming interpretation of these findings will be more complete now that we understand the internal oxygen conditions experienced by these fibers during environmental oxygen limitation.

6. Tables

Table 1. Concentration (mM) of Na^+, Cl^-, Ca^{2+}, and K^+ in *B. nubilus* hemolymph after acute (9h) exposure to normoxic immersion, air emersion, and anoxic immersion.

Electrolytes (mM)	Time (h)	Treatment		
		Normoxic (Control)	Air Emersion	Anoxic Immersion
Na^+	0	484.00 ± 5.91	485.83 ± 3.66	490.15 ± 4.21
	3	487.00 ± 4.08	498.00 ± 3.39	489.80 ± 3.55
	6	491.70 ± 5.16	**505.00 ± 6.51 ***	501.11 ± 6.46
	9	487.70 ± 2.77	488.00 ± 1.97	494.73 ± 2.34
Cl^-	0	423.31 ± 15.73	445.75 ± 13.57	452.15 ± 11.72
	3	422.90 ± 11.93	438.11 ± 14.16	451.20 ± 12.88
	6	453.70 ± 8.96	452.20 ± 6.94	446.67 ± 10.41
	9	465.00 ± 2.55	462.17 ± 2.28	460.55 ± 4.03
Ca^{2+}	0	6.51 ± 0.55	6.93 ± 0.52	7.61 ± 0.40
	3	6.11 ± 0.4	7.35 ± 0.49	7.21 ± 0.47
	6	7.50 ± 0.38	**8.37 ± 0.26 ***	8.17 ± 0.39
	9	8.12 ± 0.16	7.74 ± 0.16	8.24 ± 0.11
K^+	0	9.02 ± 0.13	9.02 ± 0.10	9.16 ± 0.10
	3	9.07 ± 0.09	9.31 ± 0.09	9.27 ± 0.08
	6	9.16 ± 0.12	**9.83 ± 0.21 + ***	**9.58 ± 0.12 ***
	9	8.99 ± 0.05	**9.54 ± 0.16 +**	**9.62 ± 0.11 + ***

Note: Means with different letters are significantly different (*post-hoc* Tukey's HSD, α=0.05). Values are means ± SEM, n=9 barnacles per treatment group. Asterisks (*) indicate means that are significantly different from time 0h value in the same oxygen treatment group (Dunnett's test). Plus signs (+) indicate means that are significantly different from the normoxic control group at each time point (Dunnett's test).

7. Figures

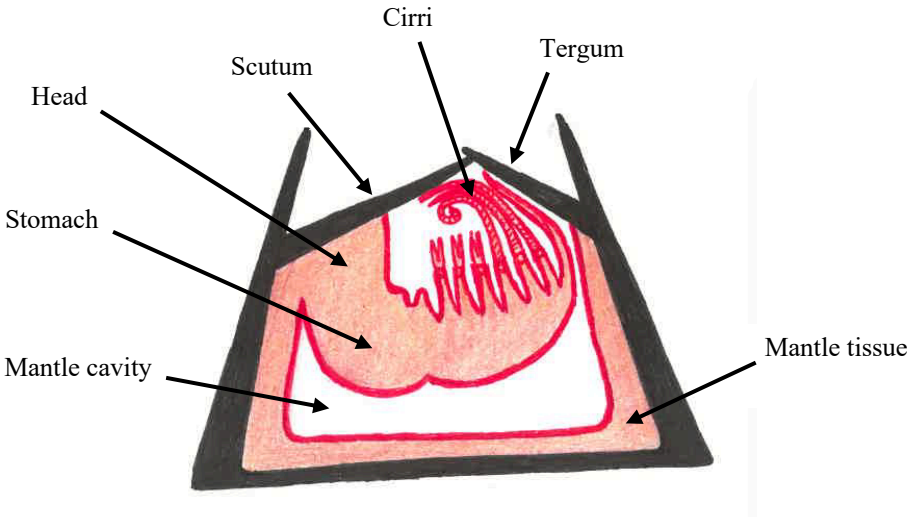

Fig. 1. Simplified internal anatomy of the giant acorn barnacle, *Balanus nubilus*. White space represents the mantle cavity and colored regions represent body tissues all contained within the black shell. There are two scutal and two tergal plates, only one of each can be seen in this diagram, which together form the operculum.

Fig. 2. Individual giant acorn barnacle, *Balanus nubilus,* with a smaller individual affixed to its lateral surface. Larger individual is shown with a latex-sealed hemolymph sampling port created with a rotating Dremel drill. The hole is positioned just superficial to the rostral sinus, which can be found immediately deep to the junction of the rostral and lateral shell plates. This sampling port provides direct access to hemolymph from the rostral sinus when punctured with a 1ml syringe (25G x 1.5” needle).

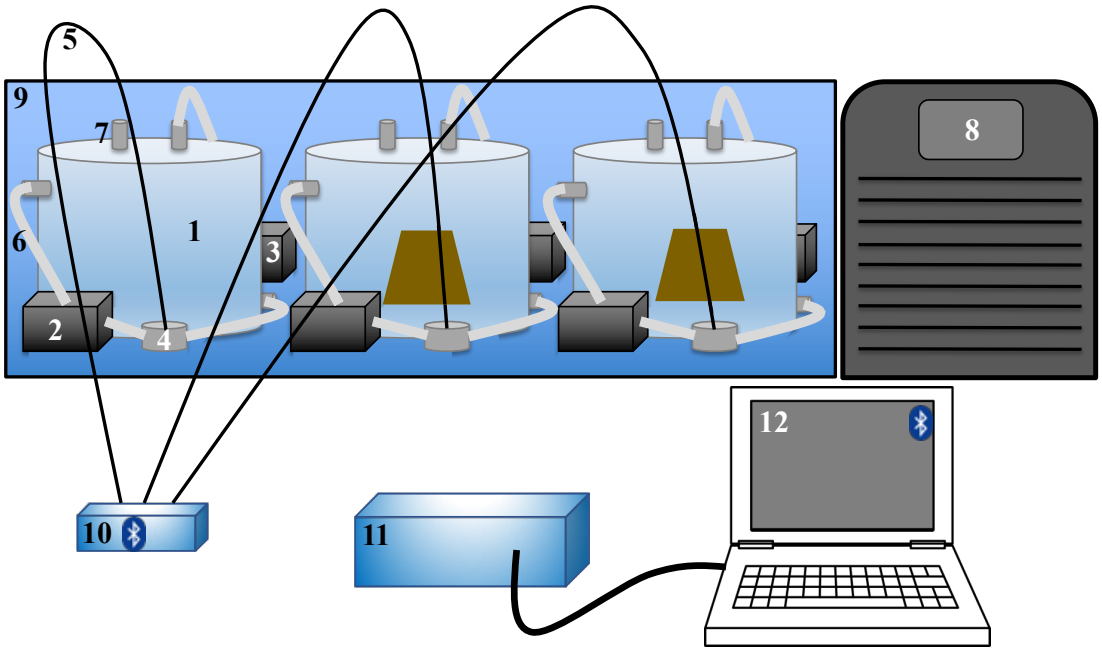

Fig. 3. Aquatic respirometry experimental system. This system consists of the following components: 1) experimental chamber, 2) recirculation pump, 3) flush pump, 4) oxygen sensor spot contained in a flow through chamber, 5) fiber optic cable anchored above the sensor spot connecting back to Witrox 4, 6) silicone tubing, 7) tubing connector, 8) aquarium chiller, 9) tank filled with filtered sea water, 10) Witrox 4 communicating fiber optic information to computer via Bluetooth, 11) DAQ-M controlling whether recirculation or flush pumps are on or off (plugs connected to pumps removed for simplicity), 12) computer running AutoResp software.

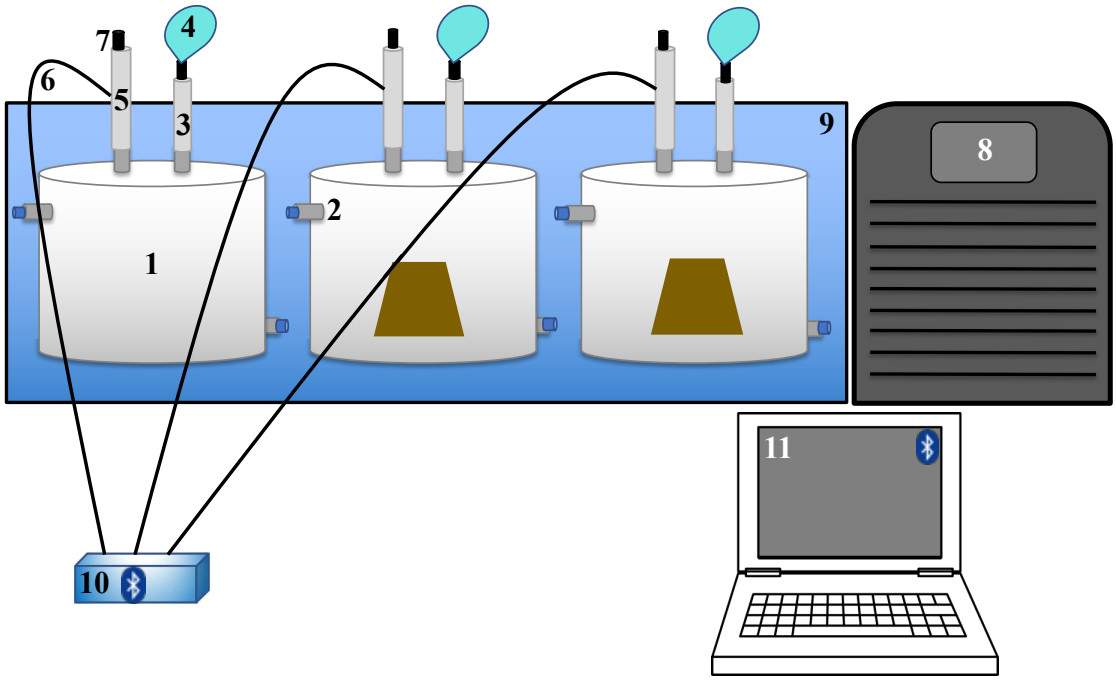

Fig. 4. Aerial respirometry experimental system. This system consists of the following components: 1) experimental chamber, 2) tubing connector plugged with a rubber stopper, 3) silicone tubing, 4) Mylar balloon to allow for pressure differences due to syringe pumping to ensure homogenous oxygen distribution in the tanks, 5) oxygen sensor spot contained in a flow through chamber connected on either side by silicone tubing, 6) fiber optic cable anchored above the sensor spot connecting back to Witrox 4, 7) Luer Lock stopcock to control air flow past the sensor spot when a Luer Lock glass syringe is connected and pumped, 8) aquarium chiller, 9) tank filled with filtered sea water, 10) Witrox 4 communicating fiber optic information to computer via Bluetooth, 11) computer running AutoResp software.

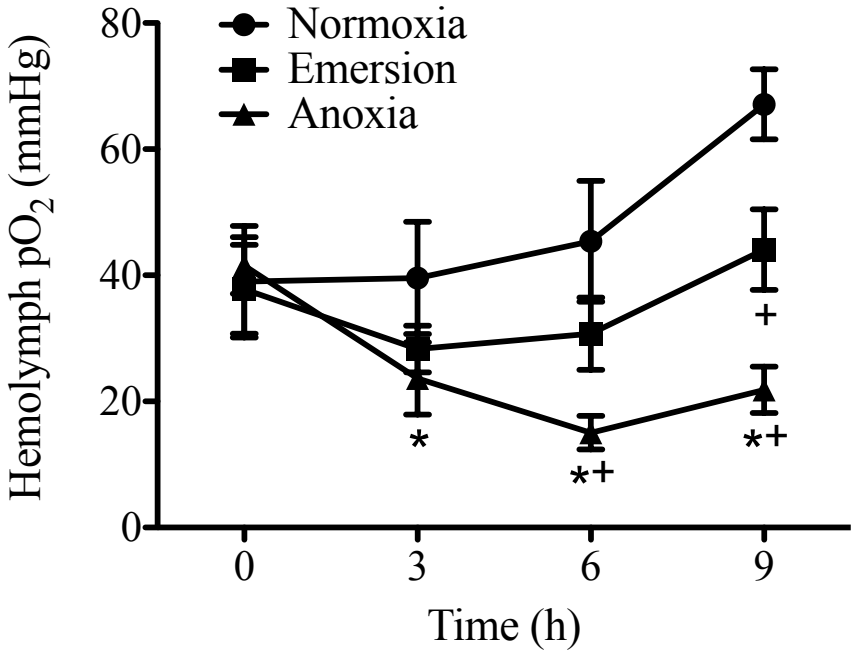

Fig. 5. Time course of hemolymph pO_2 (mmHg) in *B. nubilus* exposed to normoxic immersion, air emersion and anoxic immersion. Asterisks (*) indicate means that are significantly different from time 0h group in that oxygen treatment group (Dunnett's test). Plus signs (+) indicate means that are significantly different from the normoxic control group at each time point (Dunnett's test). Values are means±SEM; n=8-9 barnacles per time point.

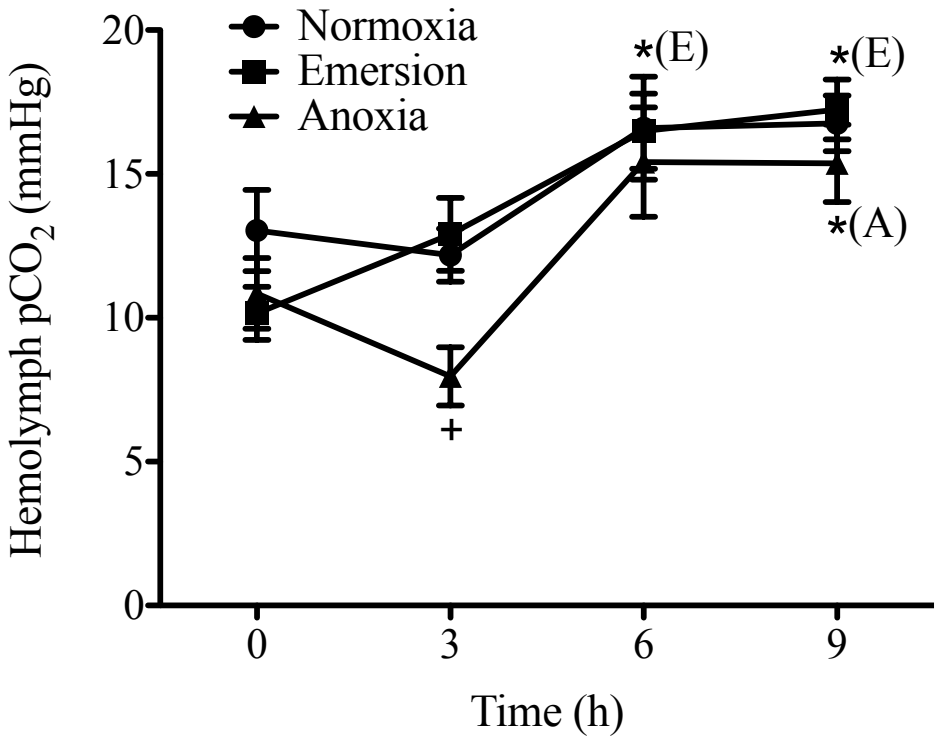

Fig. 6. Time course of hemolymph pCO_2 (mmHg) in *B. nubilus* exposed to normoxic immersion, aerial emersion and anoxic immersion. Asterisks (*) indicate means that are significantly different from time 0h group in that oxygen treatment group (Dunnett's test). Plus signs (+) indicate means that are significantly different from the normoxic control group at each time point (Dunnett's test). Values are means±SEM; n=8-9 barnacles per time point.

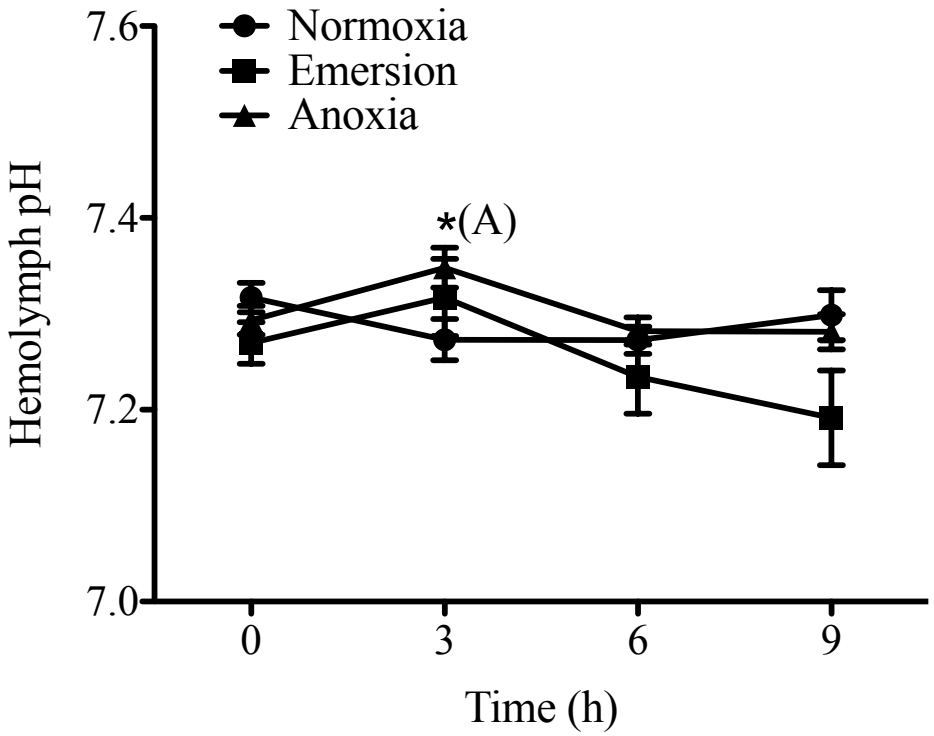

Fig. 7. Time course of hemolymph pH in *B. nubilus* exposed to normoxic immersion, aerial emersion and anoxic immersion. Asterisks (*) indicate means that are significantly different from time 0h group in that oxygen treatment group (Dunnett's test). Plus signs (+) indicate means that are significantly different from the normoxic control group at each time point (Dunnett's test). Values are means±SEM; n=8-9 barnacles per time point.

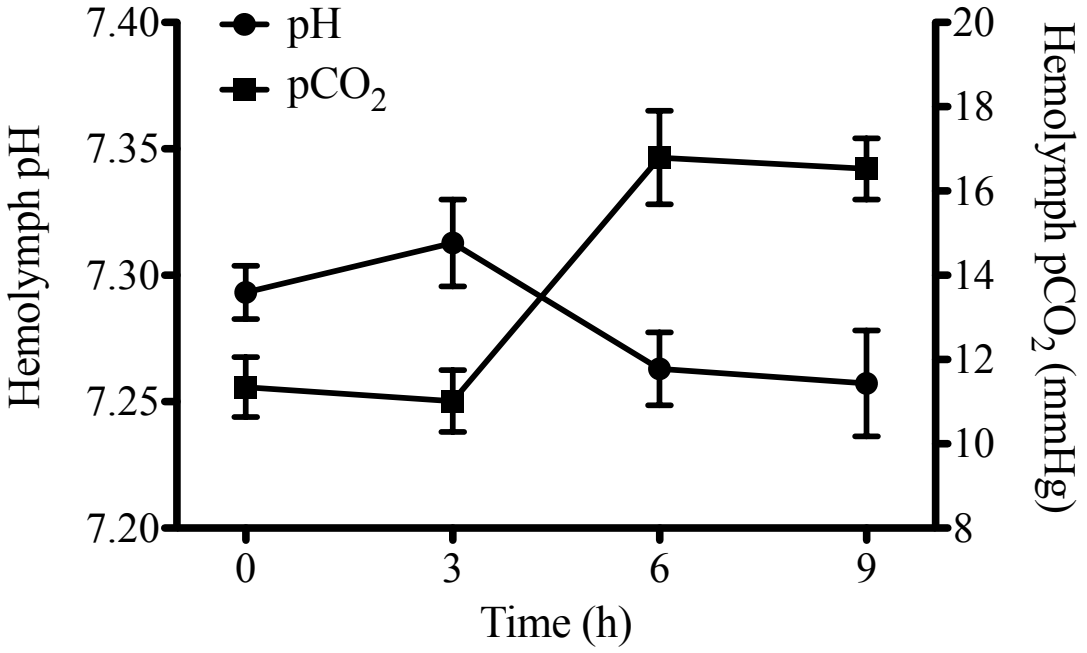

Fig. 8. Relationship between pH and pCO_2 in hemolymph from *B. nubilus* across the 9h exposure period. Values at each time point represent an average of all barnacles from all treatments (N, E, and A). Values are means±SEM; n=27 barnacles per time point.

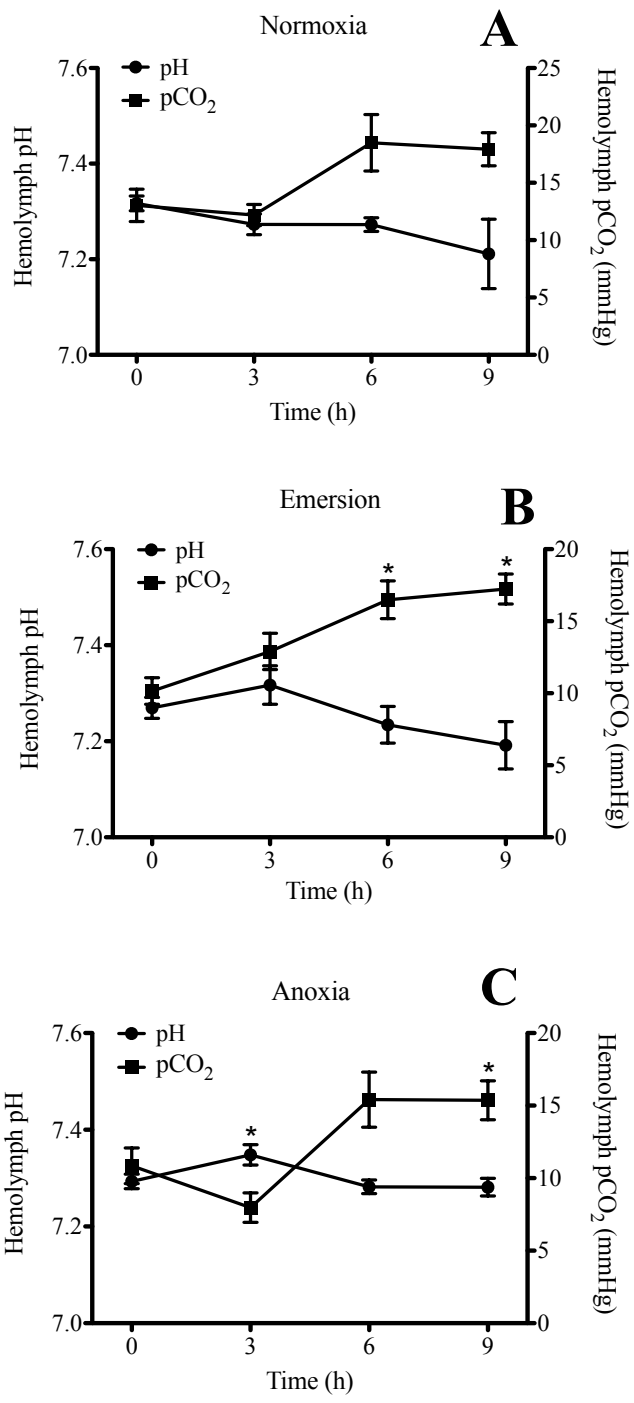

Fig. 9. Relationship between pH and pCO_2 in hemolymph from *B. nubilus* during the 9h exposure to A) normoxic immersion, B) air emersion or C) anoxic immersion. Values for pH and pCO_2 at each time point are from the same set of individuals. Asterisks (*) indicate means that are significantly different from time 0h data point (Dunnett's test). Values are means±SEM; n=8-9 barnacles per time point.

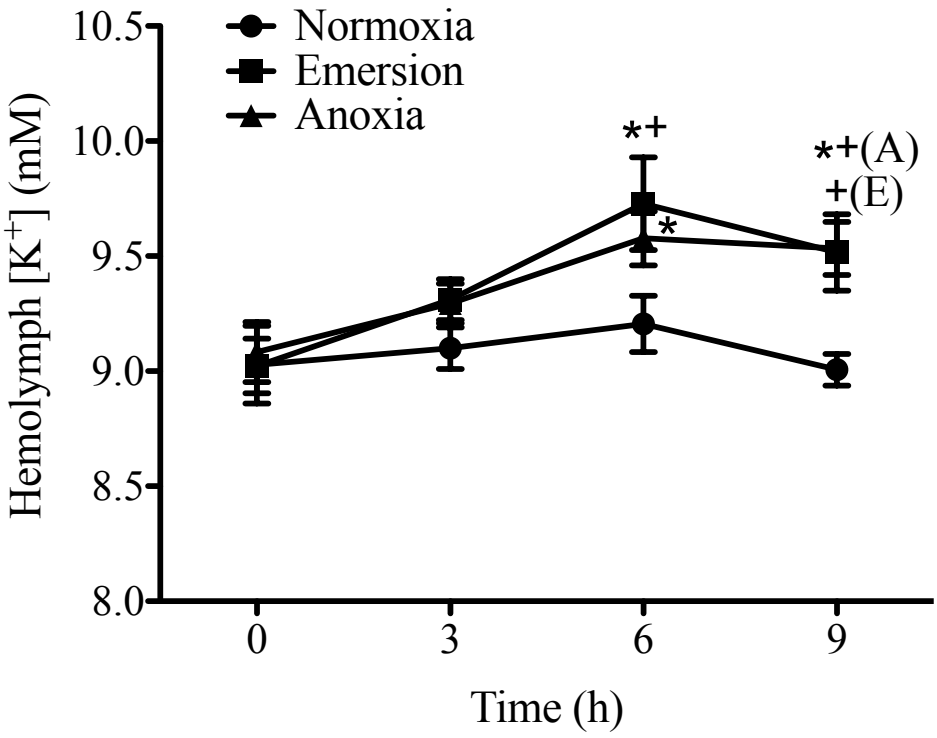

Fig. 10. Time course of hemolymph [K^+] (mM) in *B. nubilus* exposed to normoxic immersion, aerial emersion and anoxic immersion. Asterisks (*) indicate means that are significantly different from time 0h group in that oxygen treatment group (Dunnett's test). Plus signs (+) indicate means that are significantly different from the normoxic control group at each time point (Dunnett's test). Values are means±SEM; n=8-9 barnacles per time point.

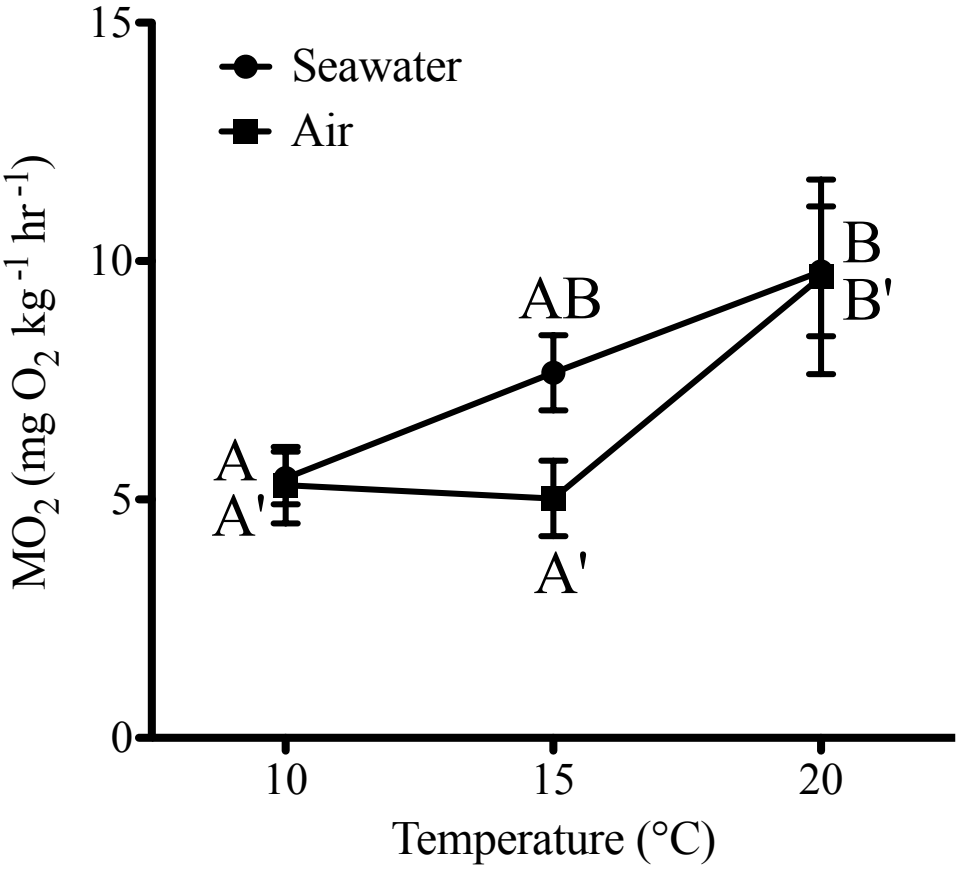

Fig. 11. Effect of temperature (°C) on oxygen consumption rates (MO_2; mg O_2 kg^{-1} hr^{-1}) in air emersed and seawater immersed *B. nubilus,* as determined by closed system (air) and intermittent (seawater) respirometry. Values with different letters are significantly different; the prime symbol, ' , indicates a separate analysis (*post-hoc* Tukey's HSD test). Values are means±SEM; N=8 (seawater) or N=10 (air) repeated across each temperature.

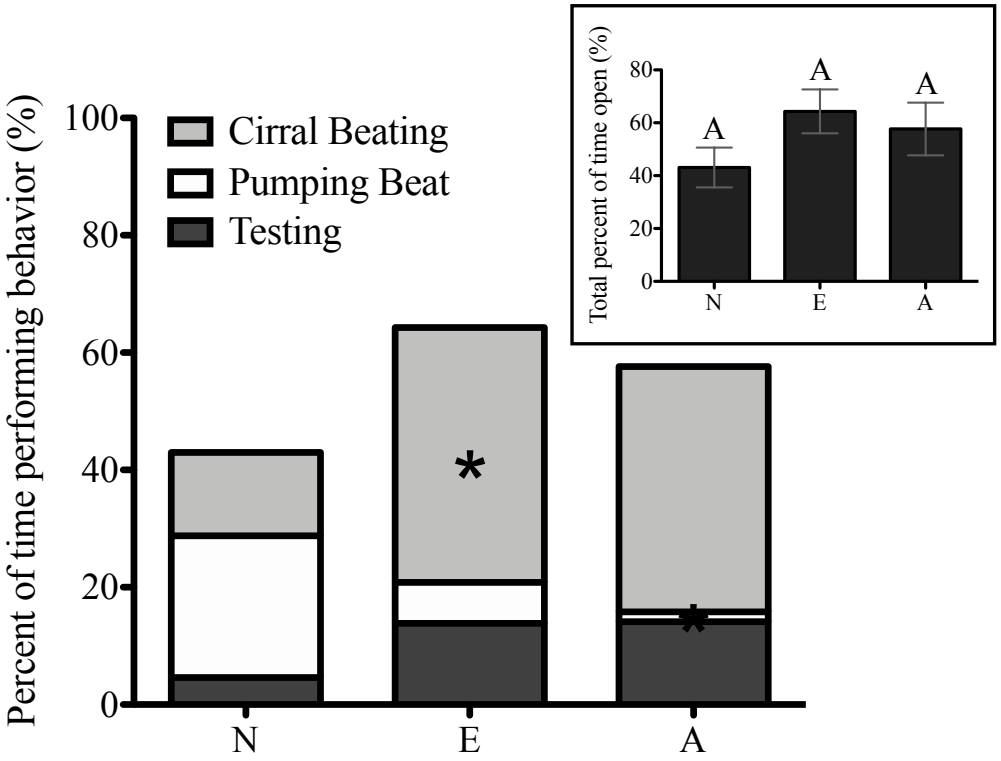

Fig. 12. Percentage of time *B. nubilus* spent performing various respiratory behaviors (testing, pumping beat, cirral beating) during a 6h exposure to normoxic immersion (N), air emersion (E), and anoxic immersion (A). Asterisks (*) indicate means for a specific behavior that are significantly different from the normoxic control group for that same behavior (*Post-hoc* Tukey's HSD test; α=0.05). Inset: Total percentage of time the operculum was open - regardless of the specific behavior occurring - during the 6h exposure to each oxygen treatment (N, E, A). Columns with different letters are significantly different (Repeated measures ANOVA; p=0.1697). Values are means±SEM; N=9 barnacles, repeated across treatments.

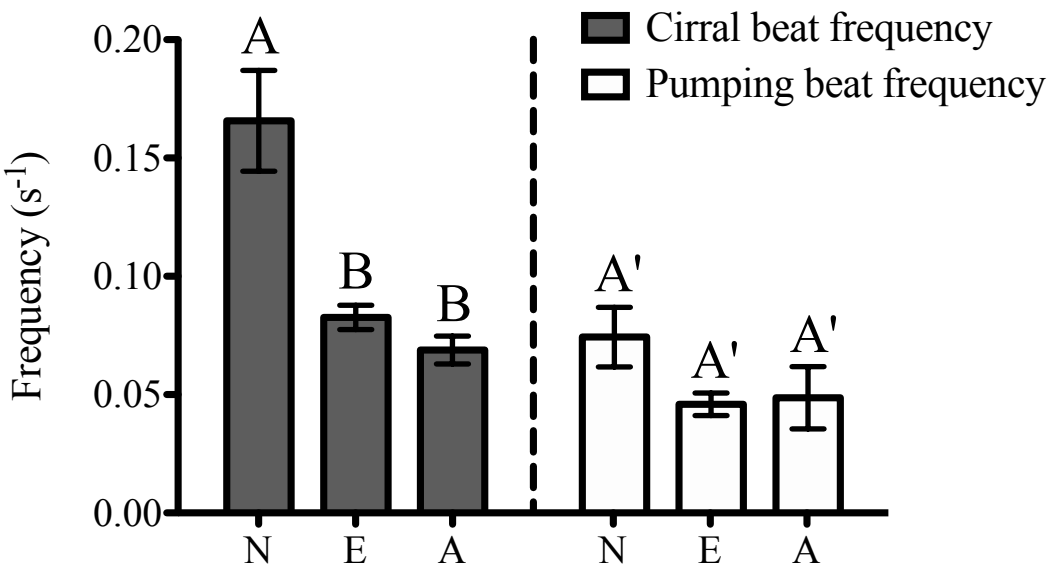

Fig. 13. Average cirri beat frequency (gray) and pumping beat frequency (white) during a 6h observation of *B. nubilus* exposed to normoxic immersion (N), air emersion (E), and anoxic immersion (A). Columns with different letters are significantly different within each behavior (Tukey HSD post-hoc test). Values are means±SEM; N=9 barnacles, repeated across each treatment.

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