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(Cirripedia: Thoracica)

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Running Title: Ecology of *Balanus amphitrite* Darwin.....

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ABSTRACT

The barnacle, *Balanus amphitrite* is a dominant intertidal and a major fouling organism. As its life cycle includes both sessile and pelagic phases, adaptation is a necessity to tolerate harsh conditions that are faced in the intertidal and pelagic environment. Recently a decreasing trend in the dissolved oxygen levels in coastal areas has been observed, effect of which would be more prominent on the sessile organisms inhabiting the intertidal habitats. *B. amphitrite* being an important intertidal inhabitant the impact of reduced dissolved oxygen (hypoxia) and no oxygen (anoxia) on the adult and nauplii was assessed. Their response to hypoxia and anoxia was evaluated by estimating the levels of antioxidant enzymes, catalase and superoxide dismutase. Impact of desiccation on antioxidant enzymes in adult *B. amphitrite* collected from different tidal heights was also evaluated. Under hypoxia, 100% mortality of nauplii was observed after 64h and under anoxia before 24h when nauplii were starving. However, when nauplii were exposed to hypoxia and anoxia along with food their tolerance level increased. A decrease in naupliar feeding rate and oxygen consumption was observed when exposed to hypoxia and anoxia. A significant difference in the antioxidant enzymes in adult *B. amphitrite* collected at different tidal levels was observed, and it increased with increase in the exposure duration, suggesting their capability to adapt to stress under different niches. Nauplii showed antioxidant defense by increased enzyme activity under hypoxia and anoxia. The magnitude of changes in enzymes suggests that these enzymes could be potential biomarkers of stress.

KEY WORDS: *Balanus amphitrite*; Desiccation; Hypoxia; Anoxia; Catalase; Superoxide dismutase

INTRODUCTION

On the edge of any static body of water there is a gradation of environmental conditions from terrestrial to aquatic, and this is paralleled by abrupt change in the nature of the organisms. The situation is more complex in coastal environments by the semidiurnal rise and fall of the tide which results in periodic exposure and emersion of the intertidal zone and these zones also have spatially and temporally varying physico-chemical factors (Newell 1979).

Barnacles are the characteristic intertidal sessile rocky shore organisms (Stephenson and Stephenson 1972) and when the environmental factors become adverse they have to adapt for extreme conditions which must include increased tolerance or mechanisms to protect the tissues under all likely conditions both physically and physiologically. The barnacle *Balanus amphitrite* (= *Amphibalanus amphitrite*) (Clare & Hoeg 2008) inhabits the intertidal region in the coastal marine environment and is also a dominant fouling organism (Fernando 1990, Anil 1991, Desai & Anil 2005). The life cycle of *B. amphitrite* includes both sessile (juvenile and adult) and pelagic (nauplii and cypris) phases. The adult release instar I nauplii which molts into instar II in short duration (2-3 hours). From instar II to instar VI they undergo a planktotrophic naupliar development. The instar VI nauplii undergo metamorphosis into a cypris larva which is a non-feeding stage. The role of the cypris larva is to explore a suitable substratum for settlement. Thus, they should adapt to tolerate any harsh conditions occurring at the air-water interface in the intertidal region and also in pelagic environment to successfully complete planktonic phase. In recent years a decreasing trend in the levels of dissolved oxygen has been observed in the coastal areas world wide (Diaz 2001). Concerns over the health of estuarine habitats has prompted researches to shift their attention to the responses of organisms to low oxygen conditions i.e. hypoxia. The seasonal shifts in the spatial distribution of oxygen can result in a dramatic shift in the composition of benthic and pelagic communities and the availability of the habitat to estuarine species. Oxygen depletion

occurs more frequently in coastal areas with longer water residence time, higher nutrient loads and stratified water columns (Harned & Davenport 1990, Breitburg 1992, Howell & Simpson 1994, Paerl et al. 1998; Diaz 2001). No other environmental variable of such ecological importance to estuarine and coastal marine ecosystems around the world has changed so drastically in a short period of time as dissolved oxygen, and is a issue of growing global concern.

Release of larvae in the hypoxic or anoxic waters could have an impact on their survival and development during planktonic phase of their life cycle. Investigations have been carried out with barnacles on their capacity to withstand desiccation, habitat selection, lifecycle stages, planktotrophic larval dispersal, biological factors such as abundance of phytoplankton on naupliar development and importance of barnacles in marine fouling (Barnes & Barnes 1968, Crisp 1954, Hurley 1973, Lewis 1975, Desai & Anil 2004, Khandeparker et al. 2003, Richardson & Brown 1990).

In barnacles, respiratory exchange is regulated during altered pH and, to a lesser oxygen tension, by modification of the duration of activity bursts and not altering the frequency of the cirral beat (Southward & Crisp 1965). Barnacles resist the entry of water into the mantle cavity by controlled use of the opercular valves (Foster 1970). However, anoxic metabolism in barnacles has not been well documented. Lopez et al (1995) reported that the presence of predators can also lead to prolonged closure of valves in barnacles and this may provoke an anoxic condition, even during immersion. Oxygen is essential for most life forms, but it is also inherently toxic due to its biotransformation into reactive oxygen species. Direct consequence of anaerobic life is the production of deleterious reactive oxygen species (ROS) generated by the reduction of molecular oxygen to water by the addition of four electrons (Di Giulio et al. 1989). Organisms are able to adapt to some chronic situations of high exposure to ROS by increasing the antioxidant enzymes and many other forms of defense/response and

repair of oxidative damage (Demple 1999, Halliwell & Gutteridge 1986). Key enzymatic players in such defense mechanism against ROS include catalase, superoxide dismutases, glutathione reductases, glutathione peroxidases, glutathione S-transferases etc. Variations in the levels or activity of antioxidant defenses have often been used to indicate ROS-mediated toxicity (Halliwell 1978, Halliwell & Gutteridge 1986, Pryor & Godber 1991, Winston & Di Giulio 1991, Ahmad 1995). *B. amphitrite* is regularly exposed in the intertidal area of the study region to tidal fluctuations from 0.25 to 2.5m. Being an intertidal habitant it has to undergo lot of immersion and emersion conditions. This study on *B. amphitrite* was conducted to assess (1) impact of desiccation on the antioxidant defense of adult barnacles collected from different tidal levels from field (2) influence of hypoxia and anoxia on adult and nauplii of *B. amphitrite* in the laboratory (3) influence of hypoxia and anoxia on larval survival, food ingestion rate and oxygen consumption in the laboratory in presence and absence of food.

MATERIALS AND METHODS

The intertidal area of Dona Paula Bay (15°27.5'N 73°48'E) situated at the mouth of the Zuari estuary along the central west coast of India was selected as the study site. The local tidal amplitude ranges from 0.25m during neap tides to 2.5m during spring tides. Perennial connections of this estuary with the Arabian Sea result in a rhythmic exchange of marine and estuarine plankton populations (Devassy & Goes, 1989). The study site is influenced by the monsoons from the south-west. Adult *Balanus amphitrite* were collected from the intertidal area of this bay to carry out different experiments. These adults were brought to the laboratory and exposed to air for short duration (~30 min) and then placed in sea water which triggered the release of instar I nauplii. Instar I nauplii is non-feeding and molt into instar II and these nauplii were used to carryout different experiments. The main aim of using instar II nauplii to carryout the experiments, was being the first feeding stage of barnacle naupliar

development and thus would be the first one to get affected by the stress induced by both physical and biological parameters.

A reservoir containing filtered seawater of 35‰ was deoxygenated by continuous bubbling of dry nitrogen gas for 2 to 3 hours prior to incubation (Widdows et al. 1989). Syringes (100ml) were used for removing the hypoxic seawater from the reservoir without exposing the water to the surrounding atmosphere. This sea water was immediately fixed with Winkler's reagents to quantify dissolved oxygen. Anoxic sea water (no oxygen) was obtained by bubbling filtered sea water with dry nitrogen gas for a longer duration.

Larval development of *B. amphitrite* in hypoxia and anoxia

Hypoxic sea water was taken out from the reservoir using syringes and transferred to 30 ml DO bottles without exposing to the surroundings. II instar nauplii were incubated in hypoxic seawater of 0.5 and 1 ml l⁻¹ dissolved oxygen in 30ml DO bottles. Thirty individuals were taken for the developmental studies (ind⁻¹ ml⁻¹). Control bottles were maintained separately with oxygen level of 5 ml l⁻¹. Observations on naupliar mortality and molting were taken after 24 hours from each DO bottle. Surviving nauplii were transferred to fresh DO bottles with hypoxic sea water with food. Similar experiments were carried out with anoxic sea water. All the experiments were repeated three times.

Oxygen consumption by adult and nauplii of *B. amphitrite*

Oxygen consumption of adult barnacles and nauplii in control (5 ml l⁻¹ of DO) and hypoxic and anoxic conditions were observed in the laboratory at 25±1°C temperature and 35‰ salinity. Adult barnacles collected from Dona Paula bay were brought to the laboratory, cleaned for the epibiotic growth by using a nylon brush and acclimatized in the laboratory for 24h. After 24h of acclimatization five adults of 12-14mm RCD (RCD; rostro carinal diameter) were chosen for evaluating oxygen consumption. These adults were incubated in 60ml DO bottles in 0.22µm filtered seawater in dark under normal oxygen conditions without

food. The observation on oxygen consumption was monitored after 24 hours (Strickland & Parsons 1968). These experiments were repeated three times with different batches of adults. Oxygen consumption was also monitored for II instar nauplii after incubating for 24h in filtered (0.22 μ m) sea water in 60 ml DO bottles without food in dark. The experiment was repeated three times with different batches of larvae.

Oxygen consumption of adult and nauplii under hypoxia was also observed. II instar nauplii were incubated in hypoxic seawater without food in 60 ml DO bottles in dark. Set of 8 bottles (60ml) containing 60 nauplii (a larva ml⁻¹) were maintained for prolonged exposure to hypoxic sea water as well as control (5 ml l⁻¹ DO). Each bottle with nauplii was sacrificed every 8 hours for detecting mortality and dissolved oxygen levels.

Naupliar ingestion rate

0.22 μ m filtered sea water with diatom, *Chaetoceros calcitrans* (1x10⁵ cells ml⁻¹) was deoxygenated by bubbling with dry nitrogen gas until 0.5 and 1 ml l⁻¹ of DO level was obtained. The II instar nauplii (30 nos.) were then transferred to this hypoxic seawater with food in DO bottles (30ml) and incubated in dark. Observations were made on feeding rate at the end of 24h by counting the number of *C. calcitrans* cells and corrected this for the control (without larvae). Similar experiments were carried out under anoxic conditions. The experiments were repeated three times with different batches of larvae.

Antioxidant enzyme activities of adult and nauplii under hypoxia and anoxia

Adult barnacles were collected from the field at three tidal levels (low, mid and high tide) from the intertidal area during low tide. These barnacles were immediately transferred to ice and brought to the laboratory in cold condition and stored at -20°C for the enzyme assays. The adult *B. amphitrite* having RCD of 10-14 mm were selected for the experiments. They were maintained in filtered seawater with aeration and acclimatized to the laboratory conditions for 24h after which they were incubated in hypoxic and anoxic seawater. Five

individuals (in three sets) were placed in each DO bottle and enzyme activity was analyzed after 24 hours of incubation in hypoxic and anoxic sea water. Organisms maintained in normal seawater with aeration were used as control. The experiments were repeated three times with different batches of adults.

Sample preparation and assay

Adults stored at -20°C were taken out and the soft body was dissected out by breaking the shell plates. The tissue was then rinsed with cold distilled water and extracted using glass homogenizer. Extraction was done by using 1mM EDTA, Sodium phosphate buffer (20mM at pH – 7.4) and Triton X-100 (0.1%) (Mourente et al. 1999). For this, 9 volumes of each solution were added one by one with the soft tissue before homogenization. The homogenized mixture was centrifuged at 4°C , at 600g for ten minutes. Supernatant was collected and used for enzyme assays. Similar procedure was used to extract enzymes from the nauplii exposed to hypoxic and anoxic conditions and also those maintained in control conditions. The total protein concentration was determined by the method described by Lowry et al (1951), using bovine serum albumin as the standard.

Catalase: Catalase activity was determined spectrophotometrically by measuring the rate of enzymatic decomposition of hydrogen peroxide, determined at the absorbance of 570nm (Sinha 1972). The activity of catalase was expressed as one unit of catalase that is the amount of enzyme that utilizes $1\mu\text{M}$ of hydrogen peroxide. Standardization was carried out using different concentrations of enzyme that were used to measure the decomposition of different micromoles of hydrogen peroxide.

Superoxide Dismutase: Superoxide dismutase activity was determined spectrophotometrically following a simple and rapid method (Marklund & Marklund 1974). The enzyme activity was measured as the degree of inhibition of autoxidation of pyrogallol,

at an alkaline pH, by superoxide dismutase. It is expressed as one unit of enzyme that corresponds to the amount of enzyme that inhibits 50% autoxidation of pyrogallol.

Standards were prepared using pyrogallol at different concentrations (Marklund & Marklund 1974). Autoxidation of pyrogallol was tested using 1mM EDTA and Tris- Cacodylic acid buffer at pH 8.2. Increasing concentration of pyrogallol was prepared in buffer from 0.1 to 0.5 mM. These standards were prepared and analyzed as per the procedure and the rate of autoxidation of increased concentration of pyrogallol was detected at 420nm. The observations were made from 1-3 minutes to detect the rate of autoxidation min^{-1} . Enzyme standards were prepared with the increasing concentration and presented in units ml^{-1} .

RESULTS

Effects of hypoxia and anoxia on *B. amphitrite* larvae under starvation

Instar II nauplii were subjected to starvation and exposed to anoxia and hypoxia. After 8h of exposure the initial mortality was 8% in the seawater with dissolved oxygen level of 0.5 ml l^{-1} , 3% in 1 ml l^{-1} dissolved oxygen and 1% in control (5 ml l^{-1} DO) (Fig. 1). Mortality increased with the exposure duration. 50% mortality was observed between 40-48h in seawater with 1 ml l^{-1} of DO and between 32-40h in case of 0.5 ml l^{-1} DO (Fig. 1). Under anoxic conditions 100% mortality was observed between 24–32h of exposure (Fig. 1). Two-way ANOVA results indicated significant ($p \leq 0.04$) influence of dissolved oxygen concentration with the duration of exposure on larval mortality.

Effect of hypoxia and anoxia on the larvae of *B. amphitrite* in presence of food

After 1d exposure 20% mortality was observed in the case of 1 ml l^{-1} of DO and 26% in case of 0.5 ml l^{-1} of DO (Fig. 2a) in presence of food. 100% mortality was observed after 4d and 6d exposure in conditions with 0.5 and 1 ml l^{-1} DO levels respectively (Fig. 2a). Only 3% mortality was observed in control throughout the experiment (Fig. 2a). Two-way ANOVA

results indicated a significant variation ($p \leq 0.006$) in the percentage mortality of the nauplii when treated different concentration of DO. Under anoxic condition, 100% mortality of nauplii was observed after 30h of exposure (Fig. 2b). When compared the mortality of nauplii under anoxic conditions in presence and absence of food, 100% mortality was observed after about 30h in both the cases. However, this was not the case under hypoxic conditions as 100% mortality was observed after 64h (at 0.5 and 1 ml l⁻¹ DO) under starving conditions and after 4 and 6days at 0.5 and 1 ml l⁻¹ DO respectively under feeding conditions.

Oxygen consumption

Oxygen consumption of *B. amphitrite* larvae was observed at ambient oxygen concentrations. A significant difference (One-way ANOVA; $p \leq 0.006$) in the consumption of dissolved oxygen was observed in normal DO concentration (7.6×10^{-5} ml l⁻¹ lar⁻¹ h⁻¹) and reduction in utilization of DO by larvae was observed with a decrease in DO levels (3.15×10^{-6} ml l⁻¹ lar⁻¹ h⁻¹ at 0.5 ml.l⁻¹ and 9.5×10^{-6} ml l⁻¹ lar⁻¹ h⁻¹ at 1ml l⁻¹ DO respectively) (Fig. 3a). Similar trend was also observed in adult barnacles when they were exposed to different DO concentrations. Oxygen consumption of 1.35×10^{-3} ml l⁻¹ h⁻¹ mg⁻¹ wet weight was observed in normal DO condition and 2.25×10^{-4} and 6.35×10^{-5} ml l⁻¹ h⁻¹ mg⁻¹ wet weight was observed at 0.5 and 1 ml l⁻¹ of DO concentration respectively (Fig. 3b). The One-way ANOVA results indicated a significant variation ($p \leq 0.0001$) in the oxygen consumption levels with different concentration of DO.

Ingestion rate

Ingestion of *C. calcitrans* cells by *B. amphitrite* nauplii was observed at reduced DO levels. The ingestion rate varied with the dissolved oxygen concentrations (One-way ANOVA; $p \leq 0.0008$). Consumption of 5500 cells lar⁻¹ h⁻¹ was observed at normal conditions which were reduced to 4000 cells h⁻¹ lar⁻¹ at DO level of 1ml l⁻¹ and 2700 cells h⁻¹ lar⁻¹ at DO level

of 0.5 ml l⁻¹ (Fig. 4). Food consumption was negligible when nauplii were exposed to anoxia (Fig. 4).

Enzyme activity in *B. amphitrite* collected from field

A significant difference in the catalase activity was observed in the adults collected at three tidal exposure levels (One-way ANOVA; $p \leq 0.0002$). Adults at the low tidal level showed a low catalase activity when compared to adults from the mid tide and high tide regions (Fig. 5a). The amount of catalase was 6.83, 7.8 and 8.42 U mg⁻¹ Protein in the case of adults collected from low, mid and high tides respectively (Fig. 5a). Superoxide dismutase activity in these adults also showed an increase in the enzyme activity with the exposure duration. The amount of superoxide dismutase in the adult *B. amphitrite* increased from low tide (4.06 U mg⁻¹ Protein) to high tide (6.14 U mg⁻¹ Protein) level. At mid tide the superoxide dismutase activity was 5.2 U mg⁻¹ Protein (Fig. 5b).

Enzyme activity in *B. amphitrite* under hypoxia and anoxia

Catalase activity was analyzed from the adult barnacles exposed to different concentrations of DO in the laboratory. Adult *B. amphitrite* in control (5 ml l⁻¹ DO) showed very low catalase activity (Fig. 6a). However, when they were exposed to DO levels of 1.0 and 0.5 ml l⁻¹, the catalase activity was 4.15 U mg⁻¹ Protein and 5.57 U mg⁻¹ Protein respectively (One-way ANOVA; $p \leq 0.000007$). The maximum catalase activity of 7.08 U mg⁻¹ Protein was observed when the barnacles were exposed to anoxic condition (Fig. 6a). Superoxide dismutase activity also showed a similar trend as that of catalase. Significant increase in the superoxide dismutase activity of *B. amphitrite* was observed with a decreasing concentration of DO levels (One-way ANOVA; $p \leq 0.001$). Control showed very low enzyme activity. Enzyme activity was 5.82 U mg⁻¹ Protein in case of 1 ml l⁻¹ DO and 7.42 U mg⁻¹ Protein in case of 0.5 ml l⁻¹ DO. Under anoxia, the superoxide dismutase activity was 10.71 U mg⁻¹ Protein (Fig. 6b). It was observed that the enzyme activity and the oxygen consumption had an inverse

relation. In the control, oxygen consumption was high and the activity of catalase and superoxide dismutase was very low and this increased with decrease in the dissolved oxygen concentration when the oxygen consumption was low (Fig. 3b). Similar is the case with nauplii exposed to different levels of oxygen (Fig. 3b).

Enzyme activity in *B. amphitrite* nauplii under hypoxia and anoxia

Catalase activity was analyzed from the *B. amphitrite* nauplii which were exposed to reduced oxygen levels. Catalase activity of the nauplii in controlled condition was low (0.47 U mg^{-1} Protein). However, when the nauplii were exposed to 1 and 0.5 ml l^{-1} of DO, the catalase activity was 1.66 and 2.75 U mg^{-1} Protein respectively (One-way ANOVA; $p \leq 0.01$) (Fig. 7a). Under anoxic conditions the catalase activity was 6.38 U mg^{-1} Protein (Fig. 7a). Superoxide dismutase also showed a similar trend as that of catalase. In the control, the activity was low, 5.86 U mg^{-1} Protein (Fig. 7b). The activity of superoxide dismutase increased with the decrease in the DO levels (One-way ANOVA; $p \leq 0.02$) (Fig. 7b). Under anoxia the superoxide dismutase activity was 34.23 U mg^{-1} Protein (Fig. 7b).

DISCUSSION

Intertidal habitats are considered to be most dynamic habitats as they are at the interface of air and water and *B. amphitrite* is an important inhabitant of this region. In *B. amphitrite*, the juvenile and adult forms are sessile and the larval forms are planktonic indicating that they require both pelagic and sessile phases to complete their life cycle. Thus the tolerance to changes in the dissolved oxygen level by the nauplii and adult is different.

The median mortality time (for 50%) of starving and feeding larvae related to oxygen concentrations varied with the concentration of the dissolved oxygen, wherein feeding larvae tolerated longer duration of hypoxic and anoxic conditions. However, in none of the hypoxic/anoxic conditions irrespective of nauplii feeding or starving, cyprids were not

obtained indicating incomplete naupliar development. This indicates that the requirement of optimum amount of dissolved oxygen is an important factor in the larval development of *B. amphitrite*. It has been reported that mortality due to exposure to hypoxia and tolerances to hypoxia differ among species and their life stages (Dorfman & Westman 1970, Burton et al. 1980, Coutant 1985). Present study also reveals that starvation is an important factor in the naupliar development of this species as the limits of tolerating lower dissolved oxygen concentrations vary with presence or absence of food. Several studies have indicated a decrease in the feeding rate with a decrease in the DO levels. Cheung et al. (2008) made a similar observation with *Nassarius festivus*, a gastropod that the amount food consumed was reduced with reduction in DO level and also observed a decrease in the oxygen consumption at reduced oxygen levels and these observations were similar to those made in the present study. Thus the varying tolerance levels to hypoxia and anoxia by *B. amphitrite* in presence of food can be attributed to insufficient supply of oxygen that is required to meet all the metabolic needs.

Earlier studies on crustacean larvae have indicated the importance of starvation on larval development and mortality, and reported that the susceptibility to starvation depends upon the species, age of the larvae and on environmental factors (Anger & Dawirs 1981, Fenaux et al. 1988, Desai & Anil 2004). Thus the impact of hypoxia and anoxia on the larval development can be viewed as an interactive effect which depends on starvation or availability of food. The studies carried out on the larvae of mussel and oyster indicated that the tolerance of anoxic condition is poor in molluscan larvae as they showed 50% mortality in shorter duration of exposure to anoxic conditions than compared to *B. amphitrite* in the present study (de Zwaan et al. 2001, 2002). This indicates that *B. amphitrite* larvae can tolerate hypoxia and anoxia, suggesting that the larval stages have anaerobic metabolic pathways capable of sustaining life for a limited period of time. Thus, increase in the duration of tolerance to

hypoxia and anoxia could be an important factor in reproductive loss path way of this species. This suggests that the larvae could tolerate short term hypoxia and anoxia (hours) associated with low dissolved oxygen by their reduced feeding activity and oxygen consumption, however this may not be same for adults as the oxygen consumption is more in adults, indicating the effect to vary with different stages of the life cycle of organism.

Ingestion rate by *B. amphitrite* nauplii under normoxia showed a difference between the nauplii which were maintained at low DO concentrations. Feeding was consistent at the normal level and it was depressed in the hypoxic conditions and very low under anoxic condition, indicating the adverse effect of low dissolved oxygen concentrations in the food rich environments. Gallager (1988) while studying the effect of hypoxia on pediveliger larvae also observed reduced feeding and ingestion rates when exposed to moderate hypoxia and also showed the reduction in heat dissipation compared to normal conditions. Similar results were also reported for some bivalve larvae (Widdows et al. 1989, Wang & Widdows 1991).

It was observed that the oxygen consumption in *B. amphitrite* (adults and nauplii) under normal DO levels was more when compared to reduced oxygen levels. These observations suggest that *B. amphitrite* reduce their metabolic rate during oxidative stress. A critical oxygen tension level was observed in the two barnacle species *Balanus amphitrite* and *Balanus tintinnabulum tintinnabulum* and suggested that *Balanus amphitrite* which inhabits the oxygen deficient areas was able to regulate itself to much lower concentrations than *Balanus tintinnabulum tintinnabulum* (Prasada & Ganapati 1968).

The above observations indicate that the organisms tolerate the changes in DO levels by controlling internal physiology. The cells are equipped with an impressive repertoire of antioxidant enzymes, as well as small antioxidant molecules (Yu 1994). These include enzymatic scavengers such as superoxide dismutase which hastens the dismutation of $O_2^{\cdot-}$ and H_2O_2 and catalase which converts H_2O_2 to water.

Adult *B. amphitrite* collected from different tidal heights showed different responses to levels of desiccation. The amount of catalase and superoxide dismutase in these adults increased from low tide (less exposure duration) to high tide level (more exposure duration). It is a well known fact that multiple demands are placed on the metabolism of marine organisms both from the stresses imposed by the external environment (e.g. variation in temperature, salinity, oxygen availability, etc.) and from internal physiological drives including reproduction and growth. Responses to such demands typically involve changes in flux through selected metabolic pathways and this generally translates into changes in the activities of pathway enzymes as they are the machines that run metabolism. The information obtained through this study suggests the existence of adaptation mechanisms in the barnacles against stress under different niches.

Enhancement of antioxidant defense and increase in the baseline activity of key antioxidant enzymes as well as secondary enzymatic defenses, were described in hypoxia and anoxia tolerant animals when subjected to environmental and metabolic stress which leads to oxygen limitation (Lima & Tania 2002). Higher activities of superoxide dismutase and catalase were also observed in marine gastropod *Littorina littorea* when exposed to anoxia (Pannunzio & Storey 1998). Higher catalase activity was observed in an estuarine polychaete *Laeonereis acuta* when exposed to hydrogen peroxide (da Rosa et al. 2007). Seasonal variation in antioxidant enzymes were also reported in adult barnacles of *Balanus balanoides* in relation to the abundance of polyaromatic hydrocarbons in tissues (Niyogi et al. 2001).

Catalase unlike superoxide dismutase was more active in the *B. amphitrite* collected from high tide than the low tide. This enzyme catalyzes the transformation of ROS, i.e. H_2O_2 to water. Adult barnacles which were maintained in the laboratory also showed marked difference when incubated in the hypoxic and anoxic seawater for 24 hrs. Controls which were maintained in aerated seawater for 24 hours did not show higher enzyme activity. The

magnitude of changes of catalase and superoxide dismutase observed from this study suggests that these two antioxidant enzymes could be used as the potential biomarkers to classify different ecosystems with respect to stress. Elucidation of genes expressed under such stressed environments needs further validation.

The present study demonstrated the response of two environments (laboratory and natural) and suggests the importance of environment as a modifying factor in determining the organism's response to hypoxia and anoxia. The response of nauplii also demonstrated a similar trend in enzyme response when maintained at ambient oxygen concentrations. Overall, the present data demonstrates that the enzymatic make-up of *B. amphitrite* intermediary metabolism is influenced by both long-term seasonal changes and by short-term environmental stress (hypoxia and anoxia). Further studies relating environmental parameters and seasonal variation in the antioxidant enzymes in the adult and nauplii collected from the field will provide insights into their physiological means of tolerance under stress.

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CAPTIONS FOR FIGURES

Figure 1. Effect of normoxia (control), hypoxia and anoxia on the mortality of *B. amphitrite* nauplii under starvation. In case of anoxia treatment all the nauplii were dead after 32h of exposure so no data were presented beyond 32h. (The error bars indicate the standard deviation from the mean). In

Figure 2. Effect of (a) hypoxia and normoxia (b) anoxia on the mortality of *B. amphitrite* nauplii when fed with *Chaetoceros calcitrans*. In control condition the mortality was observed only on day 1. (The error bars indicate the standard deviation from the mean).

Figure 3. Oxygen consumption (ml l^{-1}) in normoxia and hypoxia (a) Nauplii (consumption: $\text{lar}^{-1} \text{h}^{-1}$) (b) Adult (consumption: $\text{h}^{-1} \text{mg}^{-1}$ wet weight). (The error bars indicate the standard deviation from the mean).

Figure 4. Ingestion rates ($\text{cells lar}^{-1} \text{hr}^{-1}$) in *B. amphitrite* nauplii under hypoxia and normoxia. (The error bars indicate the standard deviation from the mean).

Figure 5. Activity of (a) Catalase (U mg^{-1} Protein) and (b) Superoxide dismutase (U mg^{-1} Protein) in *B. amphitrite* adults collected at different tidal height. LT – Low tide, MT- Mid tide and HT – High tide. (The error bars indicate the standard deviation from the mean).

Figure 6. Activity of (a) Catalase (U mg^{-1} Protein) and (b) Superoxide dismutase (U mg^{-1} Protein) in adult *B. amphitrite* exposed to hypoxia and anoxia in the laboratory. (The error bars indicate the standard deviation from the mean).

Figure 7. Activity of (a) Catalase (U mg^{-1} Protein) and (b) Superoxide dismutase (U mg^{-1} Protein) in *B. amphitrite* nauplii exposed to hypoxia and anoxia. (The error bars indicate the standard deviation from the mean).

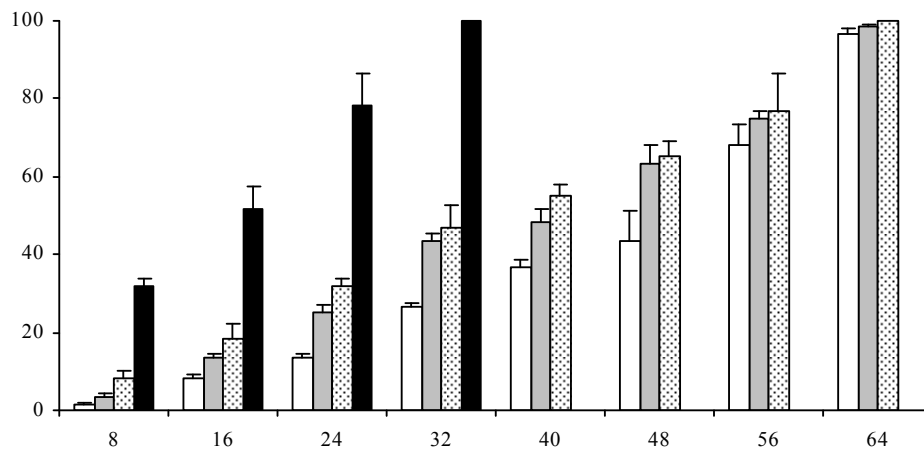


Figure 1

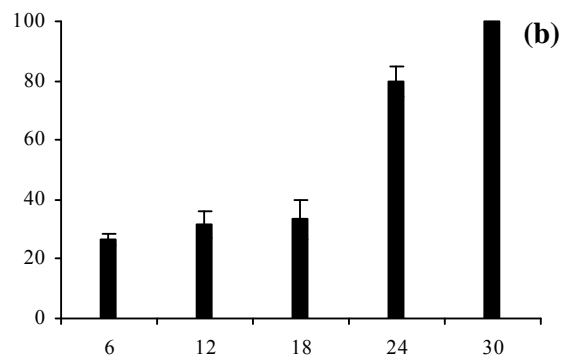
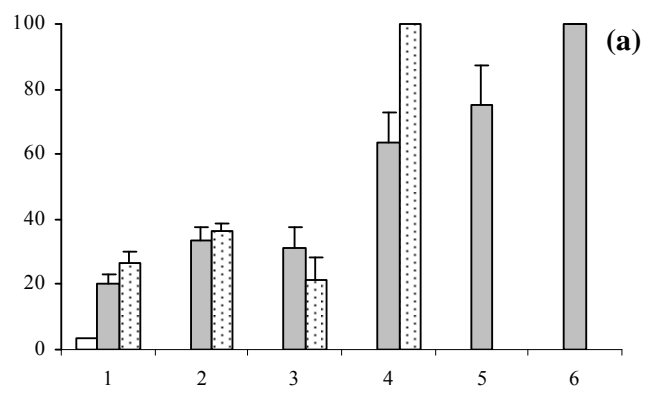


Figure 2

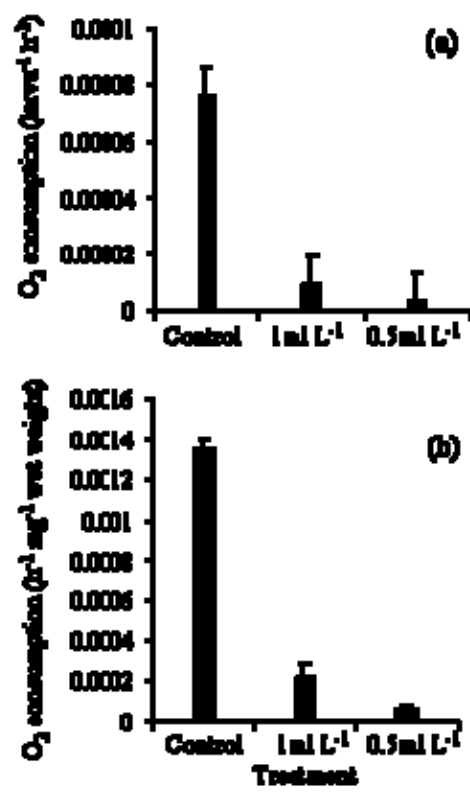


Figure 3

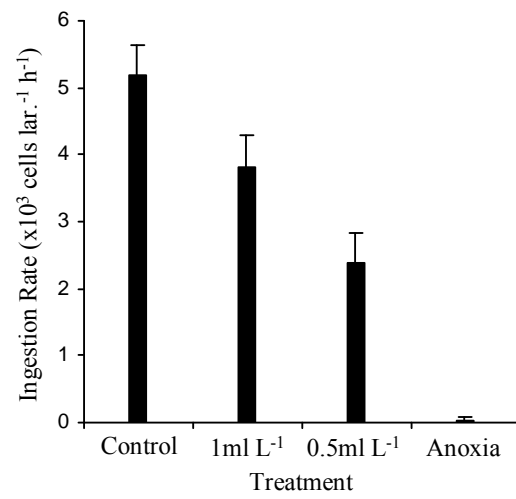


Figure 4

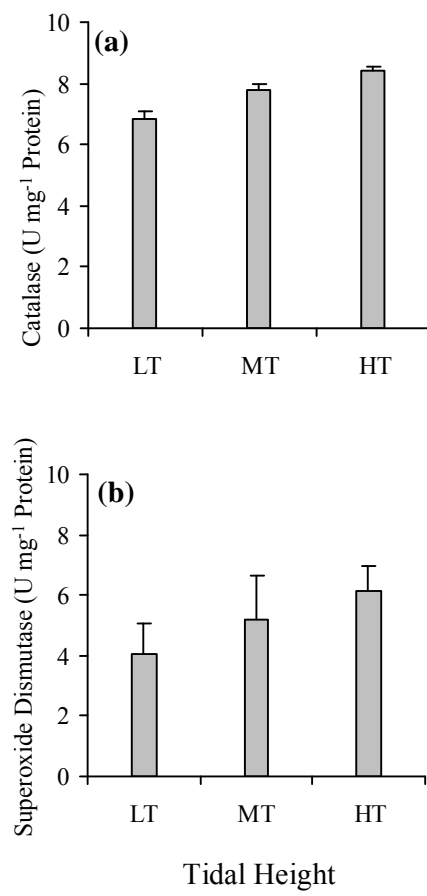


Figure 5

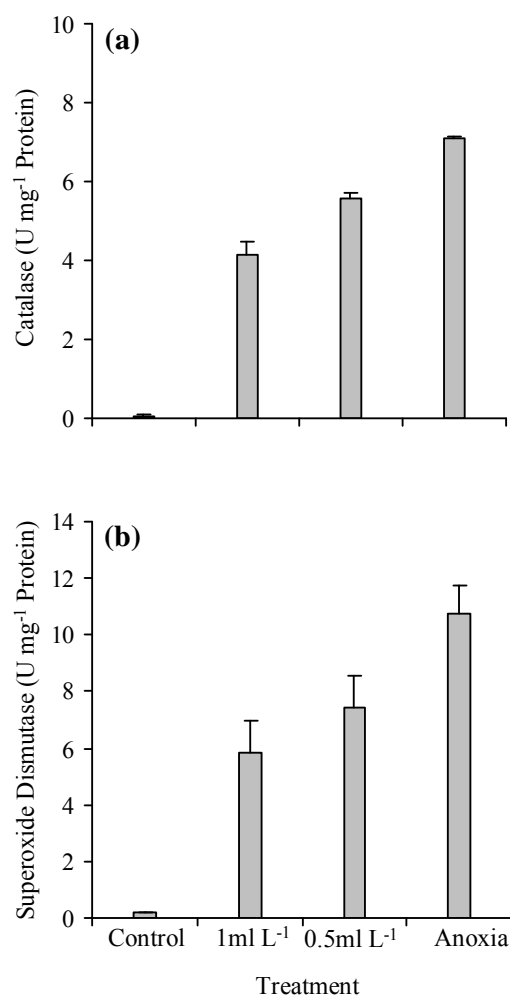


Figure 6

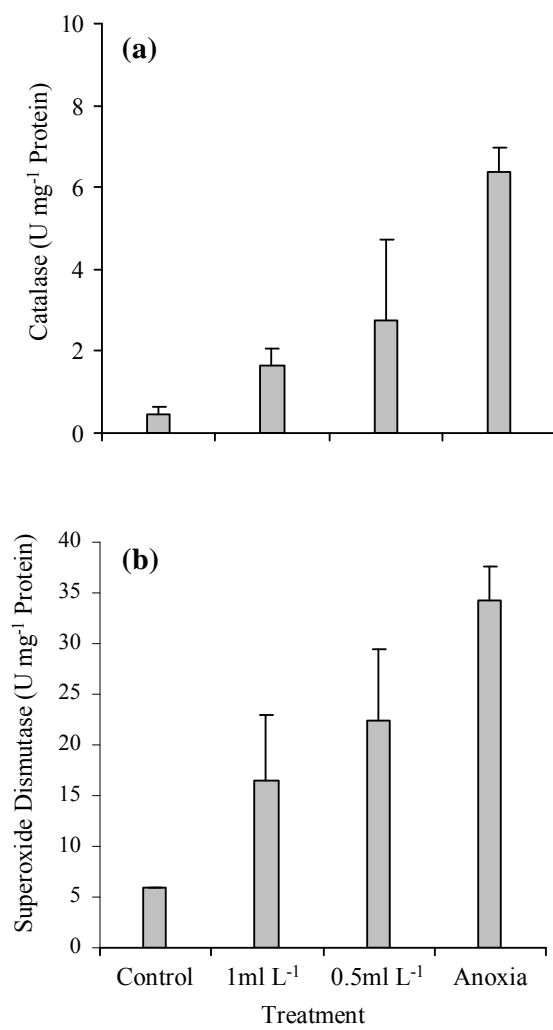


Figure 7