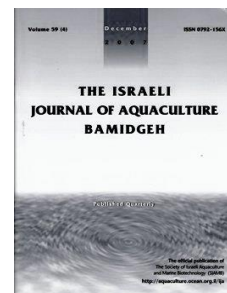




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Effects of Air Exposure and Re-Submersion on Oxidative Stress of Marine Gastropod, *Babylonia areolata*

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Keywords: air exposure; re-submersion; oxidative stress; *Babylonia areolata*

Abstract

The effects of air exposure on the antioxidant capacity of marine gastropod, *Babylonia areolata*, were evaluated. Superoxide dismutase (SOD) activity, malondialdehyde (MDA) content, and total antioxidant capacity (T-AOC) levels in the muscle and hepatopancreas in *B. areolata* were measured after air exposure and re-submersion. Results showed that SOD activity minimally increased in the hepatopancreas and muscle, after air exposure for 4 h. SOD activity in the hepatopancreas was lower than the normal level after 20 h of air exposure and air exposure followed by 4 h of re-submersion. T-AOC levels in the hepatopancreas and muscle of *B. areolata* decreased significantly ($P < 0.05$) following the period of air exposure. MDA content in the hepatopancreas of *B. areolata* subjected to air exposure for 24 and 28 h was significantly higher than the normal level. SOD activity in the hepatopancreas and T-AOC level in the hepatopancreas and muscle of *B. areolata* recovered to the normal level after 12 h of air exposure followed by 8 h of re-submersion. Air exposure can cause oxidative damage to *B. areolata*. The antioxidative system can be restored after air exposure for less than 12 h followed by re-submersion for 8 h.

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Introduction

Normal oxygen consumption by aerobic organisms produces potentially reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2) (Fridovich et al., 2004). ROS play a crucial role in various physiological processes. In the intertidal zone culture model, *Babylonia areolata* lives in the intertidal zone, which is a rigorous environment with extreme oxygen variations. To survive in this environment, *B. areolata* must endure periodic changes in oxygen, water availability, salinity, and temperature. The most serious situation is air exposure. Furthermore, juvenile *B. areolata* are often cultured in concrete ponds and transported to the intertidal zone. *B. areolata* suffer various forms of stress due to current handling practices, air exposure, re-immersion, and size selection. Air exposure is harmful to shellfish because it affects antioxidant defenses, immune responses, acid base status, respiration, energy-producing mechanisms, and survival (Chen et al., 2007; Dwyer and Burnett, 1996; Ellen et al., 2010). Excessive ROS production can increase oxidative stress (Kim et al., 2009). Environmental stresses, including air exposure, temperature, pH, algal toxin, and metals, induce a generation of ROS in shellfish (Almeida et al., 2004; Almeida and Bainy 2006; Qiu et al., 2013; Hu et al., 2015). Marine coastal ecosystems contain varied oxygen concentrations. Intertidal organisms must cope daily with large oxygen variations. These organisms are exposed to air twice a day during low tide and thus experience periodic hypoxia or anoxia. With incoming tides, tissues undergo rapid re-oxygenation, which potentially leads to hyperoxia (Sussarellu et al., 2012). ROS production increased significantly in scallops (*Chlamys farreri*) exposed to air at 17°C and 25°C (Chen et al., 2007).

The spotted babylon *B. areolata*, is widely distributed from Sri Lanka and the Nicobar Islands through the Gulf of Siam, along the Vietnamese and Chinese coast to Taiwan (Regteren and Gittenberger, 1981). *B. areolata* has been a commercially important aquaculture species in China and Thailand but in nature, its numbers are decreasing (Guilan et al., 2013; Chaitanawisuti et al., 2002). The annual output of *B. areolata* is more than 1,000,000 kg, which corresponds to more than \$15 million in China (Guilan et al., 2013). There are several models for culturing this species, including flow-through and static seawater systems in concrete/canvas ponds, earthen pond culture model, and the intertidal zone culture model (Kritsanapuntu al., 2009).

To the best of our knowledge, there are few papers on the effects of air exposure and re-submersion on oxidative stress of shellfish species namely on the bivalve *Perna perna* (Almeida et al., 2005), *C. farreri* (Chen et al., 2007), *Crassostrea virginica* (Willson and Burnett, 2000), and freshwater gastropods *Nacella concinna* (Ellen et al., 2010), and *Cipangopaludina chinensis malleata* (Havel, 2011).

B. areolata is a marine gastropod that lives in intertidal zones where there are extreme oxygen variations. Since we found no information on the response of antioxidant enzyme activities to air exposure followed by re-submersion, we studied the effects of air exposure and re-submersion on the behavior and oxidant levels of *B. areolata*. Superoxide dismutase (SOD) activity, total anti-oxidative capacity (T-AOC) level, and malondialdehyde (MDA) content in the

muscle and hepatopancreas were determined after exposure to air and re-submersion. This study aims to provide a base for selecting marine zones for culture, and for designing a suitable strategy to decrease oxidative stress and mortality of *B. areolata* during transfer and other aquaculture activities.

Materials and Methods

Experiments were conducted in Zhanjiang Tengfei Industry Co., Ltd. (Zhanjiang, Guangdong, P. R. China). A batch of apparently healthy *B. areolata* (mean body weight 13.5 ± 0.5 g) was transferred to the laboratory from the culture pond. Only healthy and undamaged individuals were selected and maintained in an air-conditioned room at 28°C. They were fasted for at least 12 h prior to the experiment.

The air exposure experiment was performed by placing them in individual opaque foam tanks (120 L capacity) without water but covered with wet gauze to maintain air humidity. Individuals were subjected to air exposure stress for 0, 4, 8, 12, 16, 20, 24, and 28 h. After air exposure at 28°C, individuals at different points in time were re-submersed in aerated seawater (28°C) for 4 h. In another experiment, individuals exposed to air for 12 h were re-submersed in aerated seawater (28°C) for 4, 8, 12, 16, 20, and 24 h. Various forms of behavior were observed after air exposure and re-submersion.

Samples of hepatopancreas (digestive gland) and foot muscle were collected after air exposure and/or re-submersion for analysis of antioxidant parameters. For each condition and sampling time (air exposure and re-submersion), 3 individuals were sampled and analyzed respectively. All *B. areolata* individuals were placed on ice prior to anesthetization and dissection. The excised hepatopancreas and muscle tissues were homogenized in Tris-HCl buffer (pH 7.4) at 4 °C. The homogenates were centrifuged at 4000 g for 10 min at 4°C, and the clear supernatant was directly used for antioxidant parameter analysis (Liu et al., 2015). SOD activity, T-AOC, and MDA content were evaluated using the corresponding commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to manufacturers' instructions.

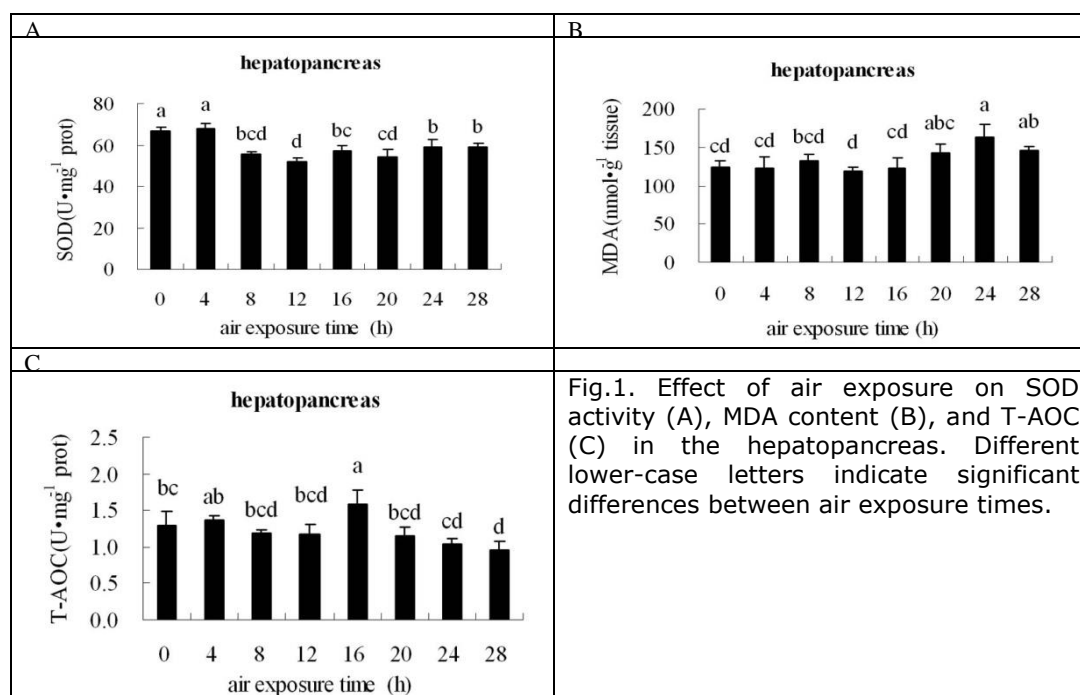
Results were analyzed with one-way analysis of variance and Duncan's multiple comparisons of the means were used to determine statistical differences. Statistical analyses were performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, IL, USA).

Results

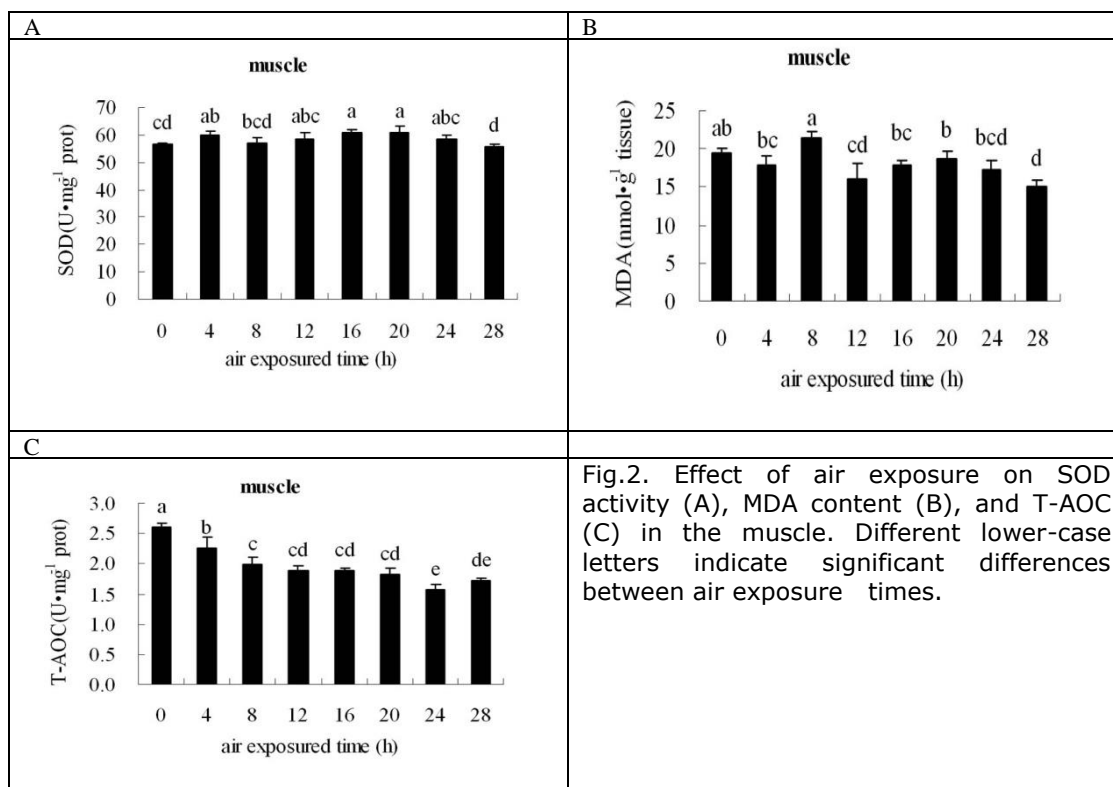
The state of each gastropod's foot was observed. After exposure to air, individuals opened their operculum and extended their feet. The foot was spread out in the air and retracted quickly at a slight touch. All re-submersed individuals were able to crawl. After 16 h of air exposure, some (40%) did not retract their foot into the shell completely when touched slightly but re-submersed individuals were able to crawl after 4 h of re-submersion. After 24 h of air exposure, individuals retracted their foot slowly when touched but could not retract fully into

their shell. 40% could crawl after 4 h of re-submersion. After 28 h of air exposure, 20% of the individuals died. Five individuals were re-submersed in the water for 4 h but only one could crawl a short distance. The remaining four individuals could not crawl.

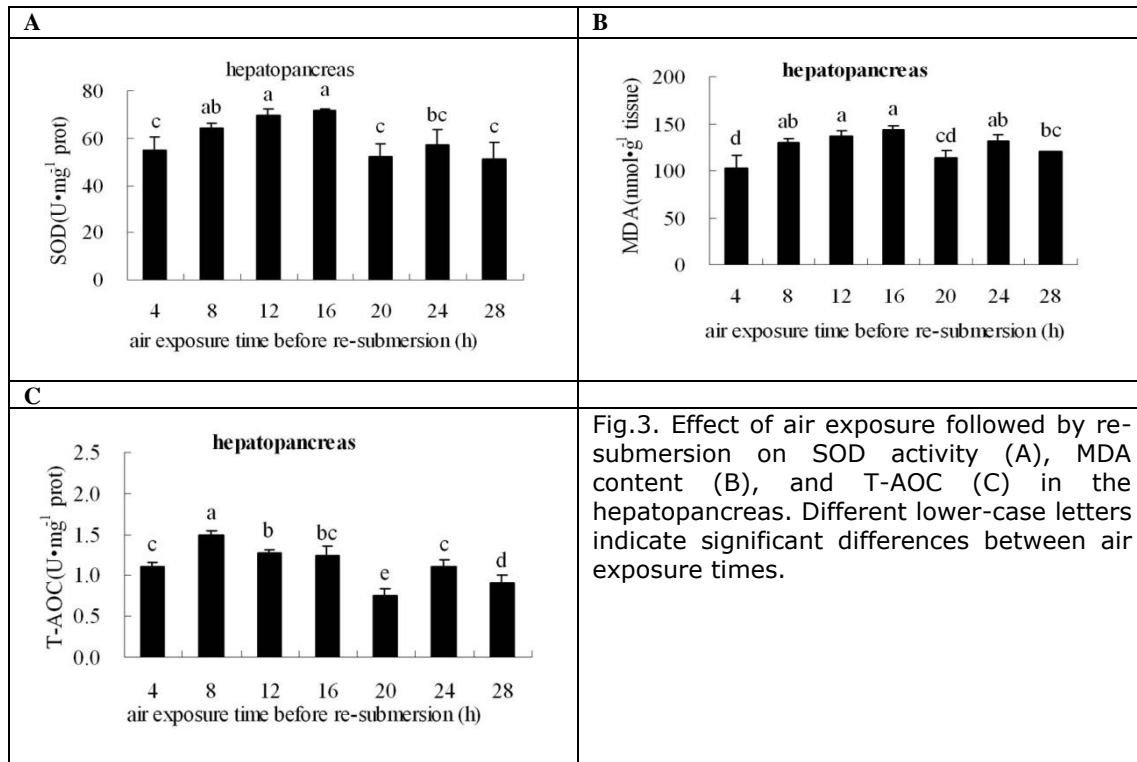
SOD activity in the hepatopancreas decreased significantly after 8 h of air exposure ($P < 0.05$) and showed a fluctuating trend (Fig.1.A). MDA content in the hepatopancreas was affected significantly ($P < 0.05$) by air exposure. MDA content reached the maximum value after 24 h of air exposure (Fig.1.B). T-AOC level in the hepatopancreas was also affected significantly ($P < 0.05$) by air exposure. T-AOC level reached the maximum value after 16 h of air exposure, and then decreased gradually after 20–28 h of air exposure (Fig.1C).



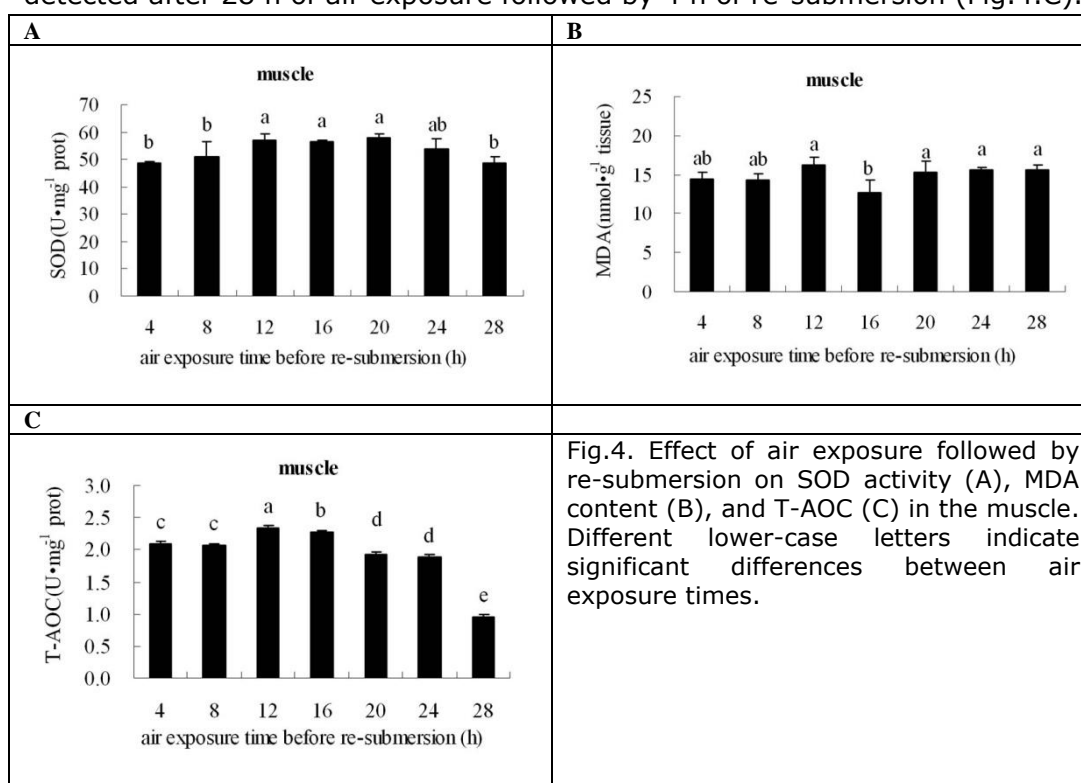
There was a significant difference ($P < 0.05$) in SOD activity in the muscle between groups. SOD activity reached a maximum value after 16 and 20 h of air exposure, and then decreased to a minimum value after 16 h of air exposure (Fig.2.A). Unexpectedly, MDA content in the muscle showed a decreasing trend, but 28 h was the only time point at which the MDA content was significantly lower ($P < 0.05$) than at other time points (Fig.2.B). T-AOC level in the muscle was affected significantly by air exposure ($P < 0.05$). T-AOC level decreased gradually with prolonged air exposure.



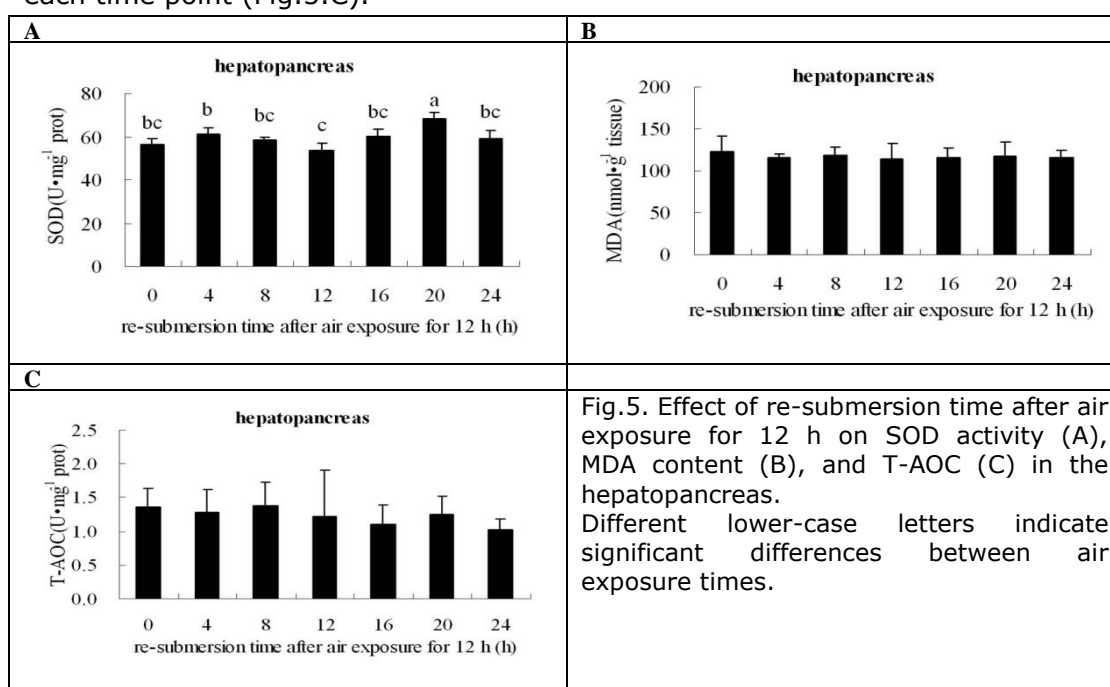
At different time points of air exposure at 28°C, *B. areolata* was re-submersed in aerated seawater for 4 h. SOD activity in the hepatopancreas after 12 and 16 h of air exposure followed by 4 h of re-submersion was significantly higher ($P < 0.05$) than at the other time points. SOD activity decreased after 16 h of air exposure followed by 4 h of re-submersion (Fig.3.A). MDA content in the hepatopancreas indicated a fluctuant change trend and reached a maximum value after 16 h of air exposure followed by 4 h of re-submersion. MDA content increased after 8, 12, and 16 h of air exposure followed by 4 h of re-submersion (Fig.3.B) compared with air exposure data (Fig.1.B). T-AOC levels increased significantly ($P < 0.05$) after 8 h of air exposure followed by 4 h of re-submersion, then decreased gradually (Fig.3.C).



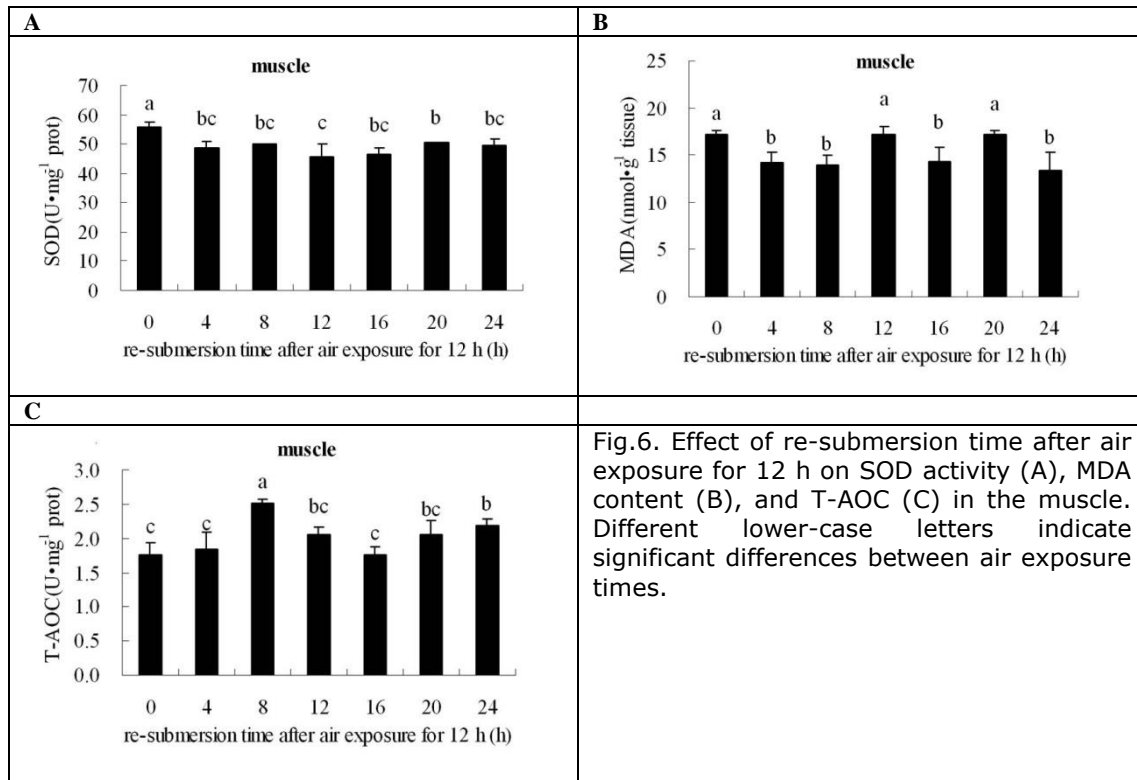
After air exposure which was followed by 4 h of re-submersion, the changing trend of SOD activity in the muscle was similar to that in the hepatopancreas. SOD activity increased significantly at several time points and then decreased (Fig.4.A). MDA content minimally changed and reached the maximum value after 12 h of air exposure followed by 4 h of re-submersion (Fig.4.B). The different changing trend of T-AOC levels was compared with air exposure data (Fig.2.C). T-AOC levels increased significantly after 12 and 16 h of air exposure followed by 4 h of re-submersion ($P < 0.05$), then decreased. The lowest level of T-AOC was detected after 28 h of air exposure followed by 4 h of re-submersion (Fig.4.C).



After 12 h of air exposure, *B. areolata* was re-submersed in aerated seawater (28°C) for 4, 8, 12, 16, 20, and 24 h. Samples diagnosed showed that SOD activity in the hepatopancreas increased after 4 h of re-submersion, and reached normal levels after 20 h of re-submersion (Fig.5.A). No significant difference ($P > 0.05$) was observed in MDA content at different times after re-submersion (Fig.5.B). During re-submersion, T-AOC levels did not change significantly in the hepatopancreas ($P > 0.05$). Standard deviations of T-AOC levels were high at each time point (Fig.5.C).



B. areolata was re-submersed in water after air exposure for 12h. SOD activity in the muscle decreased significantly after re-submersion with a minimum value after 12h of re-submersion (Fig.6.A). MDA content reached the maximum value after 12 h of re-submersion (Fig.6.B). T-AOC levels increased after 8 h of re-submersion, which was approximately equal to the normal level (Fig.6.C).



Discussion

Many mollusks exhibit high tolerance to air exposure. These organisms experience air exposure and variations in oxygen levels during the tidal cycle, and thus develop several mechanisms to survive and recover under air exposure at low tides. A strategy commonly used by intertidal animals during tidal exposure is reduction in oxygen consumption. *P. canaliculus* decreased their oxygen uptake by 87% under these conditions (Marsden and Weatherhead, 1998). Only 0.1% of normal oxygen uptake was retained by the oyster *C. virginica* exposed to air (Willson and Burnett, 2000). Shell gapping also enhances the survival of mollusks exposed to air. The gills may approach the aerial environment, and water around the gill surface contains high oxygen levels. Relative humidity also influenced the tolerance of *Corbicula fluminea* to air exposure at 15°C (Byrne and Dietz, 1988). The tolerance of *C. chinensis* juveniles to air exposure was also influenced by humidity (Havel, 2011). These findings indicate that high humidity can maintain the moisture of gills and increase oxygen. In the present study, the operculum of *B. areolata* opened during air exposure, and the feet were spread out in air to allow the gills to obtain oxygen. Under relatively high humidity conditions (mean RH of 69%), mollusks showed high tolerance to air exposure (Byrne and Dietz, 1988). Strategies used by intertidal mollusks depend on their position in the intertidal zone. Low- and mid-littoral bivalve species generally close their valves and primarily rely on anaerobic pathways coupled with large reduction in metabolic rate (Mcmahon, 1988). Conversely, high littoral bivalves, such as *Modiolus demissus*, open their shells and obtain oxygen from the air (Lent, 1969).

In this study, SOD activity in the hepatopancreas of *B. areolata* exposed to air for 4 h was not significantly different ($P > 0.05$) from the normal level (0 h of air exposure) but SOD activity both in the hepatopancreas and in the muscle slightly increased after air exposure for 4 h. *P. perna* exposed to the air for 4 h exhibited

high SOD activity in the digestive gland (Almeida and Bainy, 2006). This pattern of increase of some antioxidant enzyme activities was also found in other animals, such as *Paralomis granulosa* (Romero et al., 2011), *C. farreri* (Chen et al., 2007), and *Litopenaeus vannamei* (Liu et al., 2015). This response could be a preparative mechanism against oxidative stress during re-submersion, which could explain the low SOD activity after 4 and 8 h of air exposure followed by 4 h of re-submersion. However, SOD activity in the hepatopancreas was low after 8 and 12 h of air exposure. This trend indicated that animals switch from normal metabolism to low-oxygen consumption metabolism. This time-course response of SOD activity was also observed in *P. Perna* (Almeida et al., 2005).

During re-submersion, SOD activity in *B. areolata* showed a time-course response, which was affected significantly by duration of air exposure. SOD activity in the hepatopancreas was significantly higher ($P < 0.05$) after 8, 12, and 16 h of air exposure followed by 4 h of re-submersion than at the other time points. SOD activity both after 20 h of air exposure and air exposure followed by 4 h of re-submersion was lower than the normal level. This finding indicated that 16 h was the maximum time required by *B. areolata* to sustain normal antioxidant function after air exposure followed by re-submersion.

T-AOC comprises enzymatic and non-enzymatic antioxidants, the non-enzymatic antioxidants include glutathione (GSH), ascorbic acid, carotenoids, and their derivatives, etc. (Mahfouz et al., 2009). T-AOC levels in the hepatopancreas and muscle of *B. areolata* decreased significantly ($P < 0.05$) after air exposure. During air exposure, non-enzymatic antioxidants were mainly absorbed in these tissues. These results suggested that non-enzymatic antioxidants fulfill an important role against ROS or oxidative damage during air exposure. The importance of non-enzymatic antioxidants in protecting *Scrobicularia plana* from mercury pro-oxidant action was highlighted by investigating changes in ascorbic acid and GSH (Ahmad et al., 2012). During air exposure and re-submersion, the changing pattern of T-AOC levels in the hepatopancreas and muscle of *B. areolata* is similar to that of *L. vannamei* (Liu et al., 2015).

Lipid peroxidation leads to the formation of secondary products, such as MDA. This process has been evaluated as an indicator of environmental stresses in different tissues of the mussel *P. Perna* (Almeida et al., 2003; Almeida et al., 2004; Filho et al., 2001). When exposed to air for 24 h, the levels of lipid peroxidation in gills and digestive glands of *P. perna* increased significantly (Almeida et al., 2005). After air exposure, lipid oxidation increased in the tissues of Antarctic limpet *N. cocinna* and *P. granulose* (Ellen et al., 2010; Romero et al., 2007). In the present study, *B. areolata* exposed to air for 24 and 28 h showed significantly higher MDA content in the hepatopancreas compared with the normal level, however, MDA content in the muscle, did not increase significantly after air exposure. The results appear to be associated with the different tissues; hepatopancreas was more prone to oxidative damage than muscle. The underlying reason remains unclear but maybe be due to structural and functional

differences between the two tissue types. After 12 h of air exposure followed by re-submersion for 8 h, SOD activity in the hepatopancreas and T-AOC levels in the hepatopancreas and muscle of *B. areolata* recovered to normal levels. MDA content in the hepatopancreas and muscle of *B. areolata* after air exposure for 12 h and re-submersion for 12 h approached normal levels.

In summary, air exposure can cause oxidative damage to *B. areolata*. Oxidative damage can be restored when individuals are returned to their normal habitat, but the oxidative damage is irreversible and eventually causes death in animals after long periods of air exposure. The results of this study indicated that the critical time-period of air exposure is probably at 12 h for *B. areolata*. This information is useful to minimize oxidative stress in commercial aquaculture, and the capture process.

Acknowledgements

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