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Inducible variation in anaerobic energy metabolism reflects hypoxia tolerance across the intertidal and subtidal distribution of the Pacific oyster (*Crassostrea gigas*)

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ABSTRACT

Pacific oyster (*Crassostrea gigas*) distribute a steep gradient of environmental stress between intertidal and subtidal habits and provide insight into population-scale patterns and underlying processes of variation in physiological tolerance. In this study, 1-year-old-F_1 oysters, collected from subtidal and intertidal habitats, were obtained after common garden experiment. Genetic differentiation and physiological responses under air exposure were examined to determine whether they had evolved into local adapted subpopulations. Mortality rate, anaerobic glycolysis metabolism, and energy status indicated that oyster had initiated metabolism depression and anaerobic glycolysis metabolism in both intertidal and subtidal oysters under air exposure. However, the subtidal oysters displayed the larger energy metabolism depressions and the earlier anaerobic glycolysis responses. This may indicate that subtidal oysters were more sensitives to hypoxia stress, which may lead the higher mortality rate under long term of air exposure. Based on a common garden experimental design, we propose that this diversification may have a genetic background. Overall, the clear differences between intertidal and subtidal oysters under air exposure have provided an important reference for their aquaculture and transportation used in commercial production.

1. Introduction

Oysters comprise the largest molluscan group cultured in China, accounting for 33% of total marine molluscan yield (China Fishery Statistical Yearbook, 2015). The Pacific oyster (*Crassostrea gigas*) is widely distributed along the coastal areas of northern China and can inhabit both the very variable and stressful intertidal zone and the more stable sub-littoral zone (Guo et al., 1999). These long-term environmental differences may accelerate specific adaptation in oysters and cause physiological diversification under stress conditions, eventually leading to genetic differentiation of the subpopulations (Li et al., 2006). Elucidation of the genetic and phenotypic differentiation between intertidal and subtidal oysters could provide a reference for understanding the influence of environmental change on population differentiation at a fine-scale.

Many studies have revealed that marine invertebrates acclimatized to intertidal conditions have acquired a higher tolerance to aerial exposure, temperature, and other stress factors than those from the subtidal area. In this regard, marked phenotypic variations have been observed, including those in body size, size at sexual maturity, habitat preference, resistance to desiccation and thermal stress, and morphological defense against predators (Altieri, 2006; Botta et al., 2014; Vermeij, 1972; Weihe and Abele, 2008). However, little studies have been conducted to observed the adaptive differentiations between subtidal and intertidal oyster populations of *C. gigas*. In the intertidal environment, oysters experience hours of exposure to air, during which time they have no access to water. Then oysters will close their shells and switch to anaerobic metabolism. In contrast, in subtidal oysters, the hypoxic stress induced by air exposure is never experienced (Dunphy et al., 2006). These strong environmental gradients may accelerate the population differentiations. However, the different metabolic responses between intertidal and subtidal oysters under air exposure remains unknown.

Marine bivalves are champions of hypoxia tolerance and they have

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evolved the special hypoxia adaptive strategies: (1) strong metabolic rate depression that greatly lowers the rate of ATP utilization that can be sustained over the long term by the ATP output of fermentation reactions alone; (2) special anaerobic glycolysis process: coupling of glycolysis to additional substrate-level phosphorylation reactions catalyzed with PEPCK to increase the ATP output relative to lactate producing pathways in mammals; (3) production of alternative end products, including succinate, propionate and malate et al., with volatile or less acidic to maintain cellular acid homeostasis during long term anoxia; (4) large tissue stores of fermentable fuels (mainly glycogen but also selected amino acids and lipids). Among these adaptations, regulation of anaerobic metabolism plays a key role in hypoxia survival and tolerance in marine mollusks (Larade and Storey, 2009; Russell and Storey, 1995). Therefore, hypoxia affecting energy metabolism, including ATP and organic acids production, gene expression and enzyme activities participating in anaerobic glycolysis, can be expected to evaluate hypoxia tolerance.

Cellular responses to hypoxia in animals are mainly controlled by hypoxia-inducible factor (HIF)- prolyl hydroxylase (PHD) pathway, which facilitates both O_2 delivery and adaptation to O_2 deprivation (Zhang et al., 2014). Under hypoxia condition, HIF-α will rapidly be accumulated due to inactivation of the prolyl hydroxylase-2 (PHD2) gene. Then the accumulated HIF-1α initiates the expression of genes that control cellular processes including a switch from oxidative to anaerobic metabolism. High transcriptional expression of *HIF-1α* and *PHD* during hypoxic stress has been observed in mammals, insects, nematodes, fish, and mollusks, and their expression reflects differences in the sensitivity to hypoxic stress (West and Blader, 2015). HIF-1α regulates the anaerobic production of lactate as an energy source via pyruvate kinase (PK) in the terminal step of glycolysis (Semenza, 2007). However, marine invertebrates may have an alternative phosphoenolpyruvate carboxykinase (PEPCK) pathway during sustained severe hypoxia (Spicer, 2014). The related transcriptional regulation mechanisms are, however, unknown.

The aim of the present study was to investigate the biochemical and molecular responses of intertidal and subtidal oyster populations to air exposure. Physiological and gene expression differences in these responses would indicate an adaptation to the stressful environment. We mainly focused on the capacity of ATP production, oxygen sensing, anaerobic glycolysis genes expression, and the production of organic acids in response to air exposure. We accordingly observed clear differences between the intertidal and subtidal oysters in response air exposure. To the best of our knowledge, this study represents the first comparative analysis of energy metabolism in intertidal and subtidal oysters collected in China. It will provide an important reference for intertidal and subtidal oyster aquaculture, as well as the handling and transport conditions used in the commercial production of Pacific oysters.

2. Materials and methods

2.1. Oyster collection and common garden experiments

Intertidal oysters were collected at Bayuquan (40°18′N), North China in April 2013. During spring (April) tides, oysters in the intertidal zone regularly experience emersion times of up to 4 h (personal observation). Then the subtidal individuals were collected in 10–13 m water depth in Bayuquan at about 0.5 km from the intertidal sampling location. The temperature was maintained around 16–18 °C during sampling process. All samples (9–15 cm in shell length) were then immediately transferred to Qingdao for a 1-month acclimation period and held in aerated, 16–18 °C seawater prior to breeding.

Common garden experiments for intertidal and subtidal oyster groups are described briefly as follows in Qingdao, in May 2013. Eggs from 30 mature female oysters were mixed and divided into 30 replicates. Each sample of mixed eggs was fertilized individually with sperm from 30 mature male oysters in 70-L plastic containers. At the D-shaped stage, the contents of six containers were combined to form one group with three culture replicates of each. The 1-year old oysters were collected in May 2014 and the sea water temperature was around 20 °C.

The air exposure experiment was conducted as followings. One-year old of F_1 generation of intertidal and subtidal oysters (6–8 cm in shell length) were acclimated to laboratory conditions for 2 weeks in filtered seawater at 20 °C (pH 8.1 ± 0.1, salinity 30 ± 1) that was changed daily during this period. Then they were treated with air exposure and the room temperature was maintained at 20 °C overall process. The oysters were collected at different time points.

2.2. DNA extraction and population structure analysis

Genomic DNA was extracted from frozen adductor muscles. About 100 mg of the tissue was digested overnight at 37 °C in 0.7 ml of lysis buffer (6 M urea, 10 mM Tris–HCl, 125 mM NaCl, 1% SDS, 10 mM EDTA, pH 7.5) and 35 μL of proteinase K (20 mg/ml). The reaction mixture was extracted with phenol: chloroform (1:1), precipitated with isopropanol, and dissolved in1 × TE buffer. A partial of *COI* (Cytochrome Oxidase C subunit I) fragments were amplified with universal primer pairs: LCO 1490 (5′-GGTCAACAAATCATAAAGATAT TGG-3′), and HCO 2198 (5′- TAAACTTCAGGGTGACCAAAAAATCA-3′) (Folmer et al., 1994). The Cycling conditions entailed initial denaturation at 94 °C for 3 min, followed by 40 cycles of 94 °C for 45 s, 50 °C for 30 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The 16s (16SrDNA: the large subunit rRNA-coding gene) fragment was amplified with L16s (5′-CGCCTGTTTATCAAAAACAT-3′) and H16s (5′-CCGGTCTGAACTCAGATCACGT-3′) (Banks et al., 1993). The cycling condition entailed initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, 48 °C for 30 s and 72 °C for 45 s, followed by a final extension at 72 °C for 10 min. The PCR products were insert to pEasy-T1 vectors and used for sequencing.

To analyze the genetic data, we calculated the number of polymorphic sites and number of haplotypes for *COI* and *16s* with DNASP software (Librado and Rozas, 2009). Population subdivision was evaluated by computing global FST using haplotype frequencies with ARLEQUIN software (Excoffier et al., 2005). To explore the partitioning of genetic variation among the samples, we conducted a hierarchical analysis of molecular variance (AMOVA) in ARLEQUIN by grouping populations either by site or by intertidal zone. The significance of both the FST and AMOVA tests was assessed by 10,000 permutations of the data.

2.3. Air exposure and mortality rate

To investigate the responses of intertidal and subtidal oysters to air exposure, 100 individuals from each group were placed on small plastic buckets for up to 210 h, with the temperature being maintained at 20 °C throughout the experiment. Of the 100 individuals, 50 were used for mortality rate analysis and the other 50 were used for analyses of physiological indices and gene expression. If the valves of an oyster did not close and the mantle did not react after stimulation, it was dead. The numbers of dead oysters in each tank were recorded until 210 h post injection. Then the adductor muscles were collected and immediately frozen in liquid nitrogen using freeze clamps for further analysis.

2.4. Physiological experiments

Frozen adductor muscle was used for physiological experiments. A total of 10 mg freeze drying samples was diluted with 200 μL deionized water and used to conduct the ultrasounds for 1 h. After centrifugation, the sample was filtered with 0.45 m filtering membrane. Then they were used for organic acids detection, including acetate, lactate, succinate, propionate, and fumarate, by HPLC. The 0.1 g frozen tissues