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Comparisons of the metabolic responses of two subtidal nassariid gastropods to hypoxia and re-oxygenation

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ABSTRACT

Changes in the levels of carbohydrate, lipid, protein and anaerobic metabolites (succinate, lactate, acetate, fumarate and propionate), upon exposure to hypoxia (1.5 mg O_2 l^{-1}) and after reoxygenation in subtidal gastropods *Nassarius siquijorensis* and *N. conoidalis*, were compared. A significant decrease of the glycogen content was observed under hypoxia in *N. conoidalis* but not in *N. siquijorensis*. A greater increase in the concentrations of anaerobic metabolites was observed in *N. conoidalis* under hypoxia, and their levels did not return to baseline after returning to normoxia for 24 h. In contrast, a lower rate of accumulation of the metabolites was observed in *N. siquijorensis*, and complete recovery from anaerobic metabolism was observed after reoxygenation. The results lend further support to the role of hypoxia in governing the different distributional patterns between the two subtidal gastropods in Hong Kong waters.

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1. Introduction

Hypoxia, defined as dissolved oxygen (DO) level in water < 2.0 mg O_2 l^{-1}, is now a global problem occurring in coastal bottom waters and affects hundreds of thousands of square kilometers worldwide (Diaz and Rosenberg, 1995; Zhang et al., 2010). Although hypoxia can be a natural phenomenon, coastal eutrophication due to organic pollution is considered the most important causative factor of this phenomenon (Rabalais and Turner, 2001). Hypoxia may cause changes in species abundance and distribution, and it may alter community composition by eliminating sensitive species, resulting in the proliferation of a few tolerant species (Dauer, 1993; Weisberg et al., 2008).

Under aerobic conditions, substrates used for energy production in organisms include carbohydrates, lipids and amino acids. The types of substrate used, however, vary considerably among species and organs within the same species (Larade and Storey, 2002). When the ambient oxygen level is reduced below the critical level (environmental hypoxia) or the internal oxygen pressure fall because of intensive muscular activity (functional hypoxia) (Gäde et al., 1984), full or partial anaerobic metabolism begins to occur (Gnaiger, 1991). Under this condition, carbohydrates become the primary substrate (Larade and Storey, 2002). Four anaerobic pathways have been identified in invertebrates: (1) glucose–lactate pathway (end product: lactate); (2) glucose–opine pathway (end product: opines); (3) glucose–succinate pathway (end products: succinate) and (4) aspartate–succinate pathway (end products: succinate and alanine) (Hochachka and Somero, 1984; Livingstone, 1983). Although energy can be produced at a faster rate in the first and second pathways, energy production is more efficient in the third and fourth pathways, as they can yield almost twice as much energy (ATP) as the first two pathways. All of these pathways have been demonstrated in various marine bivalves under both long-term and short-term hypoxia (de Zwaan, 1983), and the end products (including lactate, succinate and alanine) are the most important compounds accumulated under anaerobic stress (de Zwaan and Wijsman, 1976; Brodey and Bishop, 1992).

Information about anaerobic metabolism of gastropods is scarce and fragmentary (Santini et al., 2001), although a number of anaerobic end products have been found, including lactate, octopine, alanine, succinate, acetate, propionate, butyrate, strombine and alanopine (Livingstone and de Zwaan, 1983). The metabolic pathways vary with taxonomic groups (Larade and Storey, 2002), environmental conditions (Wieser, 1980; Kooijman et al., 1982; Prabhakara Rao and Prasada Rao, 1982) and type of hypoxia (functional and environmental hypoxia) (Gäde, 1975; Gäde et al., 1984). Under hypoxic stress, lactate is an important end product in terrestrial and freshwater gastropods (Livingstone, 1982), but they are replaced by alanine, succinate, acetate and propionate in marine species where carbohydrates are similarly catabolized by

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glycolysis, but the flux is diverted towards the end of the glycolytic sequence by the carboxylation of phosphoenolpyruvate (PEP) (reaction catalyzed by PEP-carboxykinase). The product oxaloacetate may be further metabolized to succinate and then to propionate and other volatile fatty acids (VFA). The duration of exposure to anoxia or hypoxia also determines the types of end product accumulated (de Zwaan and van Marrewijk, 1973; Kluytmans et al., 1975). In the soft-shell clam *Mya arenaria*, lactate accumulation under hypoxia is accelerated after some time at the expense of further accumulation of volatile acids (Rosen, 1966). In the blue mussel *Mytilus edulis*, lactate is the initial end product, but alanine and succinate are formed at a ratio of about 2:1 after 24 h of anaerobiosis (de Zwaan and van Marrewijk, 1973). No further production of alanine, however, has been found if anaerobiosis persists, resulting in an alanine:succinate ratio of 2:3 after 72 h of anaerobiosis (Kluytmans et al., 1975). Additionally, rapid recovery of the energy reserves following reoxygenation can be an adaptive response in burrowing species for survival in hypoxic or anoxic conditions (Hervant et al., 1997).

In the past 40 years, owing to intensive human disturbances such as trawling and pollution, the benthic community in Hong Kong once dominated by specialists has been gradually replaced by generalists (Taylor, 1993). Among gastropods (since the 1980s), predatory species with a more specialized diet were replaced by scavenging species from the family Nassariidae. Among seven nassariid species that found in Hong Kong waters, *Nassarius siquijorensis* and *N. conoidalis* were the most dominant (Chan and Morton, 1997). Nevertheless, the former species has a territory-wide distribution and is most dominant in sheltered harbors, such as Tolo Harbour, whereas the latter is restricted to southern waters with strong current flow. Such distributional differences were postulated, at least partially, to be a result of their differences in tolerance and adaptations to hypoxia, which occurs almost every year in the bottom waters of Tolo Harbour (EPD Hong Kong, 2007). Comparisons of the physiological responses of *N. siquijorensis* and *N. conoidalis* have shown that the former species is more tolerant to hypoxia. During exposure to 1.5 mg O_2 l^{-1} for two weeks, a larger reduction in the respiration rate was observed in *N. siquijorensis*, allowing them to maintain a positive energy balance, as shown in their scope for growth. In contrast, high metabolic rate under hypoxia resulted in negative scope for growth in *N. conoidalis* (Liu et al., 2011). Nevertheless, upon return to normoxia, the respiration rate and scope of growth of both species returned to levels comparable to the control, which was maintained under normoxia throughout the experiment.

The purpose of this study was to investigate the metabolic strategy of *N. siquijorensis* and *N. conoidalis* under short-term hypoxia exposure and post-hypoxic reoxygenation. Body composition, including total lipids and proteins, were analyzed, and glucose and glycogen were measured as indicators of energy flow and storage. The anaerobic metabolites, including succinate, lactate, acetate, fumarate and propionate, were also determined. We hypothesized that being more active under hypoxia, energy reserve would be depleted more quickly, and the accumulation of anaerobic metabolites would occur much faster in *N. conoidalis* than in *N. siquijorensis*, which should have a faster recovery rate from anaerobic metabolism.

2. Materials and methods

2.1. Experimental animals

N. siquijorensis and *N. conoidalis* adults were collected by trawling at depths between 10 and 30 m in southern Hong Kong waters (22°10′N, 114°10′E) in April 2009. Upon transportation to the laboratory, the gastropods were maintained in flowing seawater (30 psu, 24 °C) and fed with a superfluous supply of either shrimp (*Metapenaeus ensis*) or clam (*Ruditapes philippinarum*) meat (Liu and Morton, 1994), twice a week for at least two weeks before proceeding with the experiment.

2.2. Experimental design

Sixteen gastropods were equally divided into four groups, as replicates, and put into four experimental chambers, each with 500 ml filtered seawater (25 °C, 30 psu). The gastropods were allowed to acclimate for three days in the chambers, with an air supply. When the experiment started (Day 0), the gastropods were maintained under normoxia (6.0 mg O_2 l^{-1}). Dissolved oxygen level was then reduced to 1.5 mg O_2 l^{-1} from Days 1–3 by bubbling nitrogen and air into the seawater, at sufficient rates. Reoxygenation (6.0 mg O_2 l^{-1}) was carried out on Day 4 by bubbling air only. The gas flow rates were regulated using digital flow meters (Voegtlin GCR-B3SA-BA20), while the DO level was monitored continuously using an optical DO meter (TauTheta SOO-100). Another group of 16 individuals (in four separate chambers, as replicates) were exposed to normoxia throughout the experiment, as the control, by bubbling air only. None of the gastropods were fed during the experiment, and hypoxic exposure was limited to three days to minimize the effect of starvation on the metabolism of the tested gastropods (Borges et al., 2004).

On Days 0, 1, 3 and 4, an individual gastropod was removed from each experimental chamber. The shell of gastropod was broken carefully and body tissue was washed in freshwater and wiped with a paper towel. The tissue's wet-weight was measured to the nearest 0.1 mg using an electronic balance, and the body tissue was then immediately frozen in liquid nitrogen and preserved at −80 °C for further use. Before preparing the tissue extracts, the frozen samples were first lyophilized for 48 h with the tissue's dry weight measured to the nearest 0.1 mg, and the tissue dry weight to wet weight ratio of each individual was calculated. The dry tissue of individual specimens was powdered using an electronic homogenizer for further treatments.

2.3. Measurement of biochemical parameters

Total lipids were extracted according to Folch et al. (1957) and determined by the sulfophosphovanillin method (Frings and Dunn, 1970). Fish oil (47116, Sigma–Aldrich) dissolved in chloroform–methanol solvent was used as a standard. Results were expressed in mg g^{-1} wet weight of tissue. Total protein was measured using a CHNS/O analyzer (PerkinElmer 2400 Series II; PerkinElmer, Waltham, MA, USA), and the protein content was calculated from the N content by multiplying by 6.25 (Mariotti et al., 2008). Results were expressed in mg g^{-1} wet weight of tissue.

Samples in 30% KOH were saponified by keeping them at 50 °C for two hours for glycogen extraction. They were neutralized with 5 N HC1, and trichloroacetic acid was added to a final concentration of 3% for protein precipitation and then centrifuged at 20,000*g* for 20 min. The glycogen was precipitated by adding the same volume of 96% ethanol to the supernatant (van Handel, 1965). Glucose (G8270, Sigma–Aldrich) was used as a standard. The results were divided by 1.11, which is the factor determined by Morris (1948) for the conversion of glucose to glycogen and expressed in mg g^{-1} wet weight of tissue. The extract obtained from the organic acid analysis was also used for the determination of free glucose. Anthrone reagent was used to determine the glucose concentration in the supernatant. Glucose (G8270, Sigma–Aldrich) was used as the standard. The results were expressed in mg g^{-1} wet weight of tissue.

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