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Sustaining immunity during starvation in bivalve mollusc: A costly affair

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ABSTRACT

Complete or partial depletion of resource in a freshwater habitat is a common phenomenon. As a consequence, aquatic fauna including bivalve molluscs may be exposed to dietary stress on a seasonal basis. Haemocyte based innate immune profile of the freshwater mollusc *Lamellidens marginalis* (Bivalvia: Eulamellibranchiata) was evaluated under starvation induced stress for a maximum period of 32 days in a controlled laboratory condition. During starvation, the bivalve haemocytes maintained a homeostasis in phagocytic efficacy and nitric oxide generation ability with respect to the control. The mollusc maintained a significantly high protein content in its haemolymph and tissues under the nutritional stress with respect to the control. The dietary stress had no significant impact on the activity of digestive tissue derived α-amylase till sixteenth day but by 32 days the enzyme activity went down significantly. The histopathological profile revealed that the bivalve was adapted to maintain a steady immune profile by incurring degeneration of its own tissue structure. The total haemocyte count surged significantly till 16 days but differed insignificantly with respect to the control at 32 days implying probable haematopoietic exhaustion. The study reflects the instinctive urge of the bivalve to maintain immune physiology at heavy metabolic cost under nutrient limited condition.

1. Introduction

Molluscs, including the bivalves, occupy an important position in the freshwater ecosystem of the tropics by stabilising it against natural as well as anthropogenic stresses (Dudgeon et al., 2006; Chakraborty et al., 2012; Fritts et al., 2015). The filter-feeding habits of molluscs, including bivalves, regulate the productivity and health of an aquatic environment (Officer et al., 1982; Newell, 2004). The sensitivity of the bivalves to the environmental stressors is manifested through the altered structural and functional attributes of their circulating haemocytes (Chakraborty et al., 2008; Calisi et al., 2008; Chakraborty and Ray, 2009; Martins and Costa, 2015; Matozzo, 2016) and tissues (Belcheva et al., 2011; Chakraborty et al., 2013; deOliveira et al., 2016). The circulating haemocytes of the molluscs constitute a vital component of its defence strategy and nutrient dynamics (Feng et al., 1977; Cheng, 1981). Phagocytosis of non-self particulates and generation of reactive nitrogen intermediates (RNIs) like nitric oxide are evolutionarily conserved strategies which are employed by the molluscan haemocytes to combat pathogenic insults (Araya et al., 2009; Chakraborty et al., 2009; Park et al., 2012). Studying the fluctuations of the molluscan haemocyte morphotypes with relation to diverse stressors seems important in order to perceive the environmental changes (Pamparinin et al., 2002). Bivalves thus have become a dependable model for environment biomonitoring (Sanders, 1993; Fernández-Tajes et al., 2011; Fritts et al., 2015) and their immunological profile represents the health of their resident environment (Sauvé et al., 2002; Girón-Pérez, 2010; Bhunia et al., 2016).

Introduction of bivalve to aquasystems deprived of unionid fauna is a popular environment management strategy to incorporate trophic stability in oligotrophic environment (Patterson et al., 1999; Vaughn and Hakenkamp, 2001; Xu et al., 2008). Their inclusion in an existing population may improve its genetic diversity (Cope and Waller, 1995; Szumowski et al., 2012). The relocated bivalve population may thus be temporarily subjected to a con-

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dition of complete food deprivation due to the founder effect (Hoftyzer et al., 2008; Gilg et al., 2013) or the reinforced population may suffer from partial food deprivation due to augmented competition (Wacker and von Elert, 2002; Guernic et al., 2015). Partial or complete starvation may alter the biochemical composition of bivalves (Whyte et al., 1990), decrease their metabolic activity (Rodhouse and Gaffney, 1984) and change the haemocyte defence capacity (Butt et al., 2007).

Lamellidens marginalis (Mollusca; Bivalvia) is a common bivalve mollusc of the freshwater aquasystems of eastern India including West Bengal (Fig. 1a). It is often used as a dietary supplement for poultry as well as human feed in rural West Bengal and adjoining states (Chakraborty et al., 2008; Madhyastha et al., 2010). It is a potential culturable aquacrop since pearls are naturally formed in the bivalve (Barik et al., 2004; Madhyastha et al., 2010). Its physiology has exhibited dose-responsiveness to diverse environmental pollutants like cadmium, chromium, arsenic (Das and Jana, 2004; Satyaparameshwar et al., 2006; Chakraborty et al., 2008, 2012, 2013) and stressors like oxygen and ammonia (Indira and Chetty, 1994; Das and Jana, 2003) which validates its prospective role in environment biomonitoring. However, the effect of starvation on the immunological profile of the haemocytes with relation to the morphological stability of tissue has not been investigated in details in molluscan model. The study was designed to understand the alterations in total count and selected immunological profiles of the haemocytes of *L. marginalis* subjected to dietary stress for a defined time span under controlled laboratory conditions. Histopathological study of selected organs of the starved molluscs would be helpful to characterise the effect of the stressor on its tissue structure. The data would be useful to analyse the impact of starvation induced stress on aquatic biota which would assist in formulation of a sustainable strategy of conservation of freshwater aquasystems.

2. Materials and methods

2.1. Collection and maintenance of animals

Fresh specimens of *L. marginalis* measuring 5 cm (±0.342) × 3.5 cm (±0.164) were sampled from the selected freshwater ponds of the district of 24-Parganas (South) of West Bengal in India. The collected animals (90 bivalves for each experiment) were transported to the laboratory and acclimated for 7 days in well-aerated tanks (90 cm × 90 cm × 60 cm) in batches of 15 per tank. They were provided with a feed of chopped lettuce leaves. The water of the tanks was replaced every 12 h to remove unutilized food and metabolic wastes. The average dissolved oxygen content and hardness of the water in the storage tanks were maintained at 14.1 mg/l and 457 mg/l respectively after Raut (1991). The water, soil sediments from the site of animal collection and bivalves from the sampling sites were tested periodically to trace heavy metal and metalloid contamination which yielded negative results.

2.2. Exposure to stress

Six separate batches comprising 10 bivalves per batch were subjected to starvation for a time span of 1, 2, 4, 8, 16 and 32 days (D) respectively. Each batch of the bivalves was maintained in well-aerated glass water tanks of 20 l capacity. One similar batch of bivalves was maintained with standard feed as control. The water of the tanks was replaced every 12 h to remove unutilized food and metabolic wastes. The experimental bivalves were monitored for possible visible health abnormalities and death. The experiments were repeated for 5 times ($n = 5$).

2.3. Enumeration of total haemocyte count (THC)

Haemolymph was collected aseptically from the heart and posterior adductor muscle of the bivalves (Brousseau et al., 1999; Ahmad et al., 2011) in chilled glass vials. The THC was recorded separately from the haemolymph collected from the heart and posterior adductor muscle using Neubauer's haemocytometer (Chakraborty et al., 2008). The enumeration of THC was carried out on haemolymph sampled from each of the 10 bivalves per experimental batch and the experiment was repeated for 5 times.

2.4. Enumeration of phagocytic index

Cultured baker's yeast (*Saccharomyces cerevisiae*) was killed by boiling for 1 h and the resulting cell suspension was washed three times in pH 7.4 TBS/Ca^{2+} (20 mM Trizma base, 77 mM NaCl, 10 mM $CaCl_2$) by centrifugation at 650 × *g* for 10 min. The washed cells were then suspended at a concentration of 10^7 cells/ml in Grace's Insect Medium (Himedia). The phagocytic efficiency of the haemocytes was examined by challenging them with yeast suspension in a ratio of about 1:10 *in vitro* over slide. To the adherent monolayer of haemocytes, 1 μl of yeast (1×10^7 cells/ml) was added and incubated at 26 °C in a humid chamber for 2 h. After incubation, the monolayer was washed with sterile snail saline (5 mM HEPES, 3.7 mM NaOH, 36 mM NaCl, 2 mM KCl, 2 mM $MgCl_2 \cdot 2H_2O$, 4 mM $CaCl_2 \cdot 2H_2O$; pH 7.8) (Adema et al., 1991), stained with Giemsa's stain and observed under microscope (Axiostar Plus, Zeiss). A negative control for the assay was set using a known phagocytic inhibitor sodium azide (2%). More than 200 fields were examined for each slide and data was recorded to enumerate phagocytic index (PI), where PI = [(engulfed particles/phagocytic cells) × (phagocytic cells/total cells) × 100] (Elssner et al., 2004). The enumeration of PI was carried out on haemolymph sampled from each of the 10 bivalves per experimental batch and the experiment was repeated for 5 times.

2.5. Estimation of nitrite generation in haemocytes

The estimation of the generation of NO was carried out on haemolymph pooled separately from the heart and posterior adductor muscle of the 10 bivalves per experimental batch. The generation of NO in the haemocyte was measured with Griess reagent modified after Green et al. (1982). The density of haemocytes was adjusted to 10^6 cells/ml and 1 ml of the haemocyte suspension was incubated with equal volume of Griess reagent (1% sulphanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 5% orthophosphoric acid) at 26 °C for 30 min in a humid chamber. The absorbance was recorded in a spectrophotometer (Shimadzu 1800) at 550 nm against a standard blank. The generation of nitrite was determined using a standard curve of sodium nitrite. The generation of NO was expressed in terms of formation of nitrite as μM/min/10^6 cells and the experiment was repeated for 5 times.

2.6. Preparation of tissue lysates

The tissue samples were dissected out of the heart, gill and digestive organ of the 10 bivalves per experimental batch and pooled separately before washing in chilled SSS. The tissue samples were weighed, homogenized in SSS and washed twice by centrifugation at 3000 rpm for 20 min at 4 °C. The final pellet was resuspended with 1 ml of 0.1%. Triton X-100 and incubated under ice for 30 min. The suspensions were then centrifuged at 8000 rpm for 30 min at 4 °C and the supernatants were collected and stored

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