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Functional and metabolic characterization of hemocytes of the green mussel, *Perna viridis*: *in vitro* impacts of temperature

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ABSTRACT

The green mussel, *Perna viridis*, is a bivalve mollusk native to Asia and was recently introduced to Florida, USA. Since its first observation in 1999 in Tampa Bay, Florida, green mussel population has expanded considerably, to reach the Atlantic coast of Florida, Georgia and South Carolina. Most of currently available studies about the ecology and biology of green mussels were performed in the Indian and Pacific oceans. Very recently, it has been suggested that due to a weak low temperature resistance, green mussels might have already reached the Northern edge of their distribution in the USA. However, there is currently an obvious lack of data about the adaptation capacities of *Perna viridis* to environmental conditions in Florida, especially at the physiological and cellular levels. In the present work, we determined and characterized the populations of circulating hemocytes, and the cellular components of hemolymph involved in various physiological functions, including immunity. Two main populations were characterized, hyalinocytes and granulocytes. Granulocytes accounted for 60% of circulating cells, and displayed higher phagocytic capacities, lysosomal content and basal oxidative metabolism than hyalinocytes. Hemocyte parameters were not influenced by the size of green mussels. In addition, hemocytes were subjected to acute temperature challenges (10, 20 and 30 °C) and their immune-related functions and metabolism analyzed. Our results showed that 10 °C represent a stressful condition for the Floridian green mussels, as depicted by a low phagocytosis capacity and an increase of oxidative metabolism.

1. Introduction

The green mussel *Perna viridis* is a bivalve mollusk native from the Indian coast and throughout the Indo-Pacific and Asia-Pacific. In the early to mid-1990s, populations were discovered along the coasts of Venezuela, Trinidad, and Jamaica [1,2]. In 1999, green mussels genetically related to the ones previously described in the Caribbean were observed in Tampa Bay, Florida, USA [3]. This was the first reported occurrence of this Indo-Pacific marine bivalve in North America. Green mussel population has then expanded considerably, most likely through local reproduction and settlement, along the coastal regions of southwestern Florida. Recruits of *Perna viridis* can now also be found on the Atlantic coast of Florida, Georgia and South Carolina [1,2,4]. Green mussels are known biofoulers of boats, navigational aids but also of submerged power plants and other industries infrastructures, potentially clogging water intakes and outflow pipes [1]. Furthermore, this non-native species seems to have the potential to displace local native species such as the Eastern oyster, *Crassostrea virginica* [5]. The long-term ecological and economic impact of the green mussel introduction to Florida might then be considerable but cannot be predicted at this time.

Due to their origin, the population dynamics [6–8] as well as the reproduction and growth [9–11] of green mussels were, until now, mostly studied in Asia, where *P. viridis* are widely used for the biomonitoring of coastal environments. Indeed, many studies measured the tissue levels of contaminants such as heavy metals, PAHs, PCBs or pesticides in *P. viridis* [12–15]. Similarly, the toxicological impacts of such contaminations on the green mussels were investigated, once again mostly in Asia [16–21]. The populations of green mussels from Florida were, until now, almost exclusively studied for their geographic distribution patterns, in order to understand their origin and to predict the population dynamics [1–4]. Very recently, Urian et al. [22] investigated on the effects of seawater temperature on the survival of Floridian *P. viridis*, and concluded that due to a weak low temperature resistance, green mussels might have already reached the Northern edge of their distribution in USA. Indeed, temperature is a predominant factor determining the geographic distribution of marine invertebrates

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[23]. For instance, cold temperatures have been shown to increase mortality of the tropical species *Perna perna* [24]. But even non lethal temperatures can induce a stress that may reduce long-term survival, growth and/or reproductive success [22,23]. There is currently an obvious lack of data about the adaptation capacities of *P. viridis* in Floridian environmental conditions, at the physiological and cellular levels.

Hemocytes are cells circulating in hemolymphatic system and infiltrating in tissues of bivalve mollusks [25–28]. Bivalve homeostasis relies, at least partially, on hemocytes through their involvement in various physiological functions [29] including nutrient digestion, transportation, and distribution [30,31], shell and tissue repair [32,33], detoxification processes [34–36] and cellular immune defense [37–41]. Although various hemocyte sub-populations have been described in marine bivalves [28], two main types are generally accepted: granulocytes, containing many intra-cytoplasmic granules, and hyalinocytes, with no granules [25,27,28]. Environmental factors including temperature, salinity, nutrients and toxicants affect marine bivalves. Hemocytes were suggested to be sensitive to variations of these factors, reflecting the physiological status of bivalves and their adaptation to the environment [29,41]. The characterization of hemocytes under natural, ambient condition, as well as their response to environmental variations is then essential to further understand cell-mediated responses of bivalves to anthropogenic and pathological stresses, as well as their adaptation capacities compared with other species.

The present study aimed at better understanding the Floridian green mussels' adaptation capacities to changing environmental conditions, such as temperature, through (*i*) the characterization of the biology and types of circulating hemocytes and (*ii*) the study of the *in vitro* effects of temperature variations on the hemocytes' functional and metabolic activities.

2. Material and methods

2.1. Animals

Adult green mussels were collected from Estero Bay, Florida, USA (26°22′N 81°51′W; depth from 1.5 to 2 m), where ambient salinity was 31.6 and seawater temperature 22 °C. For characterization of hemocytes, mussels were field collected and processed immediately. The size of mussels was determined as the length of the shell. The size of *P. viridis* individuals ranged from 86 to 138 mm, with a mean value of 109.75 ± 7.5 mm. Mussels used in temperature experiments (Section 2.4) were placed in tanks of fully aerated artificial seawater (salinity 30) and acclimated for at least 1 week prior to experiments. Mussels were fed daily with Shellfish Diet algae mix (Reed Mariculture Inc.).

2.2. Hemolymph collection

Using a 3 mL syringe fitted with a 25-G needle, approximately 1.5–2.0 mL of hemolymph was collected from each mussel. The needle was inserted between shell valves into the posterior adductor muscle. Collected hemolymph was microscopically examined for purity, filtered through 41 μm nylon filters (according flow cytometer manufacturer recommendations) and kept on ice to minimize cell clumping. All subsequent analyses were performed on individual samples.

2.3. Flow cytometry analyses

Flow cytometry analyses were performed on a Cytomics FC500 flow cytometer (Beckman Coulter), equipped with a 488 nm Argon laser.

2.3.1. Hemocyte concentration, cytomorphology and mortality

Determination of hemocyte concentration, cytomorphology and mortality was performed using a double staining procedure including SYBR Green I (1/1000 of stock solution) (Invitrogen) and Propidium iodide (PI; 10 μg mL^{-1}) (Sigma) [42]. Hemocyte morphology was based upon relative flow–cytometric parameters, Forward Scatter (FSC) and Side Scatter (SSC). FSC and SSC commonly measure particle size and internal complexity, respectively. Membranes of viable cells do not allow PI to penetrate; whereas, altered membranes are permeable to PI. Dead cells are characterized by loss of membrane integrity and are, therefore, double stained by SYBR Green I and PI. Concentration is reported as the number of cells mL^{-1} of hemolymph; morphological parameters are expressed in arbitrary units (A.U.), and hemocyte mortality is the percentage of double stained cells.

2.3.2. Lysosome quantification

The presence and relative amount of lysosomes in hemocytes was determined using LysoTracker Red (Molecular Probes, Invitrogen), a membrane permeable, fluorescent probe (emission maximum at ~ 590 nm) that accumulates within lysosomal compartments. Hemolymph was diluted in artificial filtered seawater (AFSW) containing LysoTracker Red (final concentration 1 μM). Mixed solutions were incubated 60 min in the dark, at room temperature. Relative intracellular lysosomal quantity is expressed as the level of red fluorescence (FL3 detector of the flow cytometer) in arbitrary units (A.U.).

2.3.3. Phagocytic capacities

Evaluation of phagocytic capacities was based on the ingestion of fluorescent latex microbeads (2.0 μm, Polysciences Inc.) by hemocytes. Incubation of hemolymph with microbeads was performed at 20 °C for 120 min. Conditions of incubation for *in vitro* experiment are described in Section 2.4. Phagocytic capacities were then defined as (*i*) the percentage of cells that had ingested three or more microbeads [42,43], and (*ii*) the average number of microbeads per phagocytic hemocytes [44].

2.3.4. Intracellular oxidative metabolism

Determination of intracellular oxidative metabolism was performed using 2′7′-dichlorofluorescein diacetate (final concentration 10 μM) (DCFH-DA; Molecular Probes, Invitrogen), a membrane permeable, non-fluorescent probe. Inside hemocytes, the -DA radical is first hydrolyzed by esterase enzymes. Intracellular hydrogen peroxide (H_2O_2), as well as superoxide ion ($O_2^{\bullet -}$ [45,46];), then oxidizes DCFH to the fluorescent DCF molecule. Oxidation of DCFH can also be mediated by nitrite radicals (NO_2 or N_2O_3) [47] and various oxidase and peroxidase enzymes [48]. DCF green fluorescence is proportional to the intracellular oxidative metabolism of hemocytes. Relative intracellular oxidative metabolism is therefore expressed as the level of green fluorescence (FL1 detector) in arbitrary units (A.U.). Incubation of hemolymph with DCFH-DA was performed at 20 °C for 30 min. Conditions of incubation for *in vitro* experiment are described in Section 2.4.

2.3.5. Mitochondrial membrane potential

Estimation of mitochondrial membrane potential (MMP) was performed using JC10 (final concentration 5 μM) (Enzo Life Sciences), a membrane permeable fluorescent probe. JC-10 accumulates and aggregates in mitochondria, selectively generating an orange emission profile (λ 590 nm). As membrane potential decreases, JC-10 monomers are generated, resulting in a shift to green fluorescence emission (λ 525 nm). Relative MMP intensity is expressed as the ratio between the levels of orange fluorescence (FL2 detector) and green fluorescence (FL1 detector).