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Pinna nobilis: A big bivalve with big haemocytes?

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ABSTRACT

The fan mussel *Pinna nobilis* (Linnaeus, 1758) is one of the biggest bivalves worldwide. Currently, no updated information is available in the literature concerning the morpho-functional aspects of haemocytes from this bivalve species. Consequently, in this study, we characterised *P. nobilis* haemocytes from both a morphological and functional point of view. The mean number of haemocytes was about 5 ($\times 10^5$) cells mL haemolymph^{-1}, and the cell viability was about 92–100%. Two haemocyte types were distinguished under the light microscope: granulocytes (51.6%), with evident cytoplasmic granules, and hyalinocytes (48.4%), with a few granules. The granules of the granulocytes were mainly lysosomes, as indicated by the *in vivo* staining with Neutral Red. Haemocytes were further distinguished in basophils (83.75%), acidophils (14.75%) and neutrophils (1.5%). After adhesion to slides and fixation, the cell diameter was approximately 10 μm for granulocytes and 7 μm for hyalinocytes. The granulocytes and hyalinocytes were both positive to the Periodic Acid-Schiff reaction for carbohydrates. Only granulocytes were able to phagocytise yeast cells. The phagocytic index (6%) increased significantly up to twofold after preincubation of yeast in cell-free haemolymph, suggesting that haemolymph has opsonising properties. In addition, haemocytes produce superoxide anion and acid and alkaline phosphatases. Summarising, this preliminary study indicates that both the granulocytes and hyalinocytes circulate in the haemolymph of *P. nobilis* and that they are active immunocytes.

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1. Introduction

The fan mussel *Pinna nobilis* (Linnaeus, 1758) is the largest bivalve species in the Mediterranean Sea and one of the biggest bivalves worldwide [1]. It is present in coastal and estuarine zones between 0.5 and 60 m depth [2], on sand and muddy-sand bottoms, preferentially associated with *Posidonia oceanica* and *Cymodocea nodosa* meadows [3]. *P. nobilis* is a long-lived species, with a maximum reported age of 27 years [4]. Populations of *P. nobilis* have greatly reduced in the last 20–30 years as a result of reckless fishing, as well as incidental killing by trawling and anchoring. Consequently, the fan mussel has been listed as an endangered species in the Mediterranean Sea [5], the preservation of which is promoted mainly through the Barcelona Convention (protocol ASPIM Annex 2), and the Habitats Directive (Annex IV) [6]. All forms of deliberate capture or killing of fan mussel specimens are prohibited (EEC 1992). In order to effectively protect this endangered species there is an urgent need to updated information on the biology and ecology of *P. nobilis* populations.

Beyond physical (shell) and biological barriers (mucus and epithelia), the internal immune defence is fundamental to protect animal against pathogen assault. As well-known, the circulatory system of bivalves is open, and haemocytes circulate freely in haemolymph. Haemocytes play a crucial role in the immune response, mainly through the phagocytosis of foreign particles, and the production of hydrolytic enzymes, reactive oxygen intermediates and antimicrobial peptides [7–11].

Currently, no information is available in the literature on the haemocytes of *P. nobilis*. Consequently, the aim of this work was to study the morpho-functional features of the haemocytes from this bivalve species, in order to increase the knowledge on the role of mollusc haemocytes in immune responses. The morphological and physiological study of the bivalve haemocytes is very important because they are target species in aquaculture and can be vectors of agents of diseases that affect other organisms, as vertebrates.

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2. Materials and methods

2.1. Bivalves

This study was performed according to all national and international guidelines for animal welfare. Ten specimens of *P. nobilis* (with a shell length ranging from 23 cm to 53 cm and shell width from 9 to 18.3 cm) were collected from the Strait of Messina, Sicily, central Mediterranean (38°15′35.04″N, 15°37′44.74″E). Bivalves were collected at 1.5–4.5 m depth and transferred as soon as possible (max 15 min) to the laboratory where they were maintained for 7days in a tank containing synthetic sea water (SNSW, Nutri-SeaWater®Aquarium Saltwater, pH = 8 ± 0.1; salinity = 37 ± 1 ppt; T = 17 ± 1 °C; (π = 1100 mOsm/kg) with continuous aeration. Mussels were fed once a day with algal slurry (Liquifry Marine, Interpet, Dorking, UK) and SNSW was changed every two days.

2.2. Haemolymph collection

After two weeks of acclimation, haemolymph was collected via a 23 gauge needle from the posterior adductor muscle of mussels The syringe used for haemolymph sampling was previously filled with approximately 300 μL of 2% EDTA in physiological solution (550 mM NaCl, 12.5 mM KCl, 8 mM $MgSO_4$, 20 mM 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid (HEPES), 10 mM D-glucose, 4 mM $CaCl_2$, 40 mM $MgCl_2$; pH 7.3; 1100 mOsm). For each animal, a volume of about 1.5 mL of haemolymph was collected. Haemolymph samples were placed in tubes and immediately centrifuged at 1000g for 10 min at 4 °C. The supernatant was discarded and the pellet was suspended in physiological solution and part fixed with glutaraldehyde (1% and 2.5%) in physiological solution (see above).

2.3. Cell count

Immediately after sampling, a haemolymph aliquot was put on a haemocytometer (Burker chamber) to determine total cell number (x10^5/mL) and cell viability by the Trypan blue exclusion assay [12–16].

2.4. Stability of lysosomal membranes

The test is based on the use of a cationic probe neutral red which is taken up into cells by membrane diffusion where it becomes ion trapped within the lysosomal compartment. Having obtained the haemolymph samples, the stability of lysosomal membrane by the neutral red retention (NRR) assay was evaluated. The neutral red stock solution was prepared by diluting 4 mg Neutral Red dye in 1.0 mL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy). After preincubation of cell for 15 min with neutral red working solution (1%), a drop of this suspension was deposited on clean slides. Poly-L-lysine (Sigma-Aldrich, Milan, Italy), at a concentration of 0.1%, was used as a coating agent to promote cell adhesion on slides (10 min). Haemocytes were observed under a Leitz Diaplan microscope. If haemocytes were vital the lysosomes appeared red.

2.5. Cytochemical assays

Haemocyte monolayers were fixed for 30 min at 4 °C in a solution of 1% glutaraldehyde (Fluka, Milan, Italy) in physiological solution, washed in phosphate buffered saline, pH 7.2 (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4) and stained according to various cytochemical methods:

i) *Giemsa's dye.* Haemocytes were stained for 10 min in a 10% Giemsa (Fluka, Milan, Italy) aqueous solution and washed in distilled water. Slides were then mounted in Acquovitrex (Carlo Erba, Milan, Italy) and observed under a light microscope. Nuclei appeared blue and cytoplasm pale blue or violet.
ii) *Pappenheim's panoptical staining.* Haemocytes were stained for 3 min in May-Grünwald's dye (Fluka). After washing in distilled water, they were stained for 5 min in 5% Giemsa, washed in distilled water, and mounted. Basophil granules appeared blue and acidophils dark pink.
iii) *Ehrlich's triacid mixture.* The mixture is composed of 12 parts of saturated Orange G aqueous solution, 8 parts of saturated acid fuchsine aqueous solution, 10 parts of saturated methyl green aqueous solution, 30 parts of distilled water, 18 parts of absolute ethanol, and 5 parts of glycerine. Haemocytes were stained for 15 min, washed in distilled water, and then mounted. Basophilic granules appeared pale green, neutrophilic violet, and acidophilic coppery red.
iv) *Carbohydrate detection (Periodic Acid-Schiff reaction).* Fixed haemocytes were incubated in 1% periodic acid for 10 min, rinsed in tap water and stained with Schiff's reagent for 30 min at 37 °C. Haemocytes were then incubated in a solution of 0.6% sodium metabisulphite in 0.02 M HCl for 6 min, washed in tap water for 10 min, and then in distilled water. Slides were mounted as described above. Positive sites appeared primary red.

Light microscopic images were acquired by a system consisting of a colour video camera (JVC 3-CCD), a Leica DM-LB microscope (1000× magnification), and a PC provided with ImageNT and Microimage software developed by CASTI Imaging (Italy). Each assay was repeated in triplicate.

2.6. Electron microscopy

A haemocyte suspension was fixed in physiological solution (see above) containing 2.5% glutaraldehyde for 30 min. Cells were postfixed in 1% osmium tetroxide for 60 min and 0.25% uranyl acetate overnight. Subsequently, samples were dehydrated through a graded ethanol series and embedded in EPON resin overnight at 37 °C, 1 day at 45 °C and 1 day at 60 °C. Ultrathin sections (80 nm) were cut parallel to the substrate and observed with a transmission electron microscope (TEM, FEI Tecnai G12) operating at 100 kV. Digital images were acquired with an Olympus VELETA camera.

2.7. Short-term haemocyte cultures, phagocytosis and opsonisation assays

Pooled haemolymph was centrifuged at 1000g for 10 min at room temperature, and haemocytes were resuspended in an equal volume of a physiological solution. Short-term haemocyte cultures (30 min at room temperature to allow adhesion of the haemocytes to coverslips) were prepared according to Ballarin et al. (1994).

After the adhesion of haemocytes to the coverslips, the physiological solution was discharged from the culture chambers and replaced with an equal volume of a yeast (*Saccharomyces cerevisiae*) suspension in physiological solution (yeast: haemocyte ratio = 10:1). Monolayers were incubated for 60 min at 25 °C, washed several times in physiological solution to eliminate uningested yeast cells and fixed in a solution of 1% glutaraldehyde (Fluka Milan, Italy) in physiological solution at 4 °C for 30 min. Monolayers were then stained with 10% Giemsa (Fluka, Milan, Italy) for 5 min, mounted on glass slides, and observed under the light microscope. With the aim of determining the percentage of

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