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# Haemocyte morphology and function in the Akoya Pearl Oyster, *Pinctada imbricata*

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ABSTRACT

The morphology and cytochemistry of *Pinctada imbricata* haemocytes were studied *in vitro*. Three distinct blood cell types were identified; hyalinocytes, granulocytes, and serous cells. Haemocytes were classified based on the presence/absence of granules, and nucleus to cytoplasm ratio. Granulocytes were the most common cell type (62 ± 2.81%), followed by hyalinocytes (36 ± 2.35%), and serous cells (2 ± 0.90%). Granulocytes, and hyalinocytes were found to be immunologically active, with the ability to phagocytose Congo red stained yeast. Of the cells involved in phagocytosis, granulocytes were the most active with 88.8 ± 3.9% of these haemocytes engulfing yeast. Cytochemical stains (phenoloxidase, peroxidase, superoxide, melanin, neutral red) showed that enzymes associated with phagocytic activity were localised in granules within granulocytes. Based on their affinities for Giemsa/May–Grünwald stain, haemocytes were also defined as either acidic, basic or neutral. Hyalinocytes and serous cells were found to be eosinophilic, whilst granulocytes were either basophilic (large granulocytes), eosinophilic (small granulocytes) or a combination of the two (combination granulocytes). Light, differential interference contrast and epifluorescence microscopy identified three sub-populations of granulocytes based on size and granularity; small (4.00–5.00 μm in diameter, with small granules (0.05–0.5 μm in diameter), large (5.00–9.00 μm in diameter, with large granules (0.50–2.50 μm in diameter) and combination (5.00–9.00 μm in diameter, with both large and small granules). These observations demonstrate that *P. imbricata* have a variety of morphologically and functionally specialized haemocytes, many of which maybe associated with immunological functions.



## 1. Introduction

The increasing prevalence of infectious diseases in aquaculture has become a significant problem for many previously lucrative industries (Huchette and Clavier, 2004; Travers et al., 2008). Between 1993 and 1996 the Japanese Akoya pearl industry experienced a significant slump in production of both shell and pearls, representing a decline in productivity of almost 50% (O'Connor et al., 2003). This disruption in the market was attributed to coastal degradation and the proliferation of disease (O'Connor et al., 2003). A thorough understanding of immunological responses by aquatic species may help to lessen the impact of disease, especially within monocultures. The characterisation of haemocytes within invertebrate aquatic species is a useful first step in defining immunological processes because these cells are thought to be primary mediators of host defence.

In bivalve molluscs, haemocyte-mediated immune responses include nodule formation, encapsulation, phagocytosis, melanisation and the production of reactive oxygen species (ROS; Bayne, 1990; Pipe, 1992; Hégaret et al., 2003; Aladaileh et al., 2007; Butt and Raftos, 2008), whilst humoral responses involve the synthesis of effector proteins and antimicrobial enzymes (Bayne, 1990; Boulanger et al., 2006; Aladaileh et al., 2007). Aside from their immunological functions, mollusc haemocytes are also thought to be involved in shell mineralization, excretion, metabolite transport, digestion and wound repair (Cheng, 1981; Mount et al., 2004).

A number of studies have investigated the structure and function of bivalve mollusc haemocytes (Cheng, 1984; Auffret, 1988; McCormick-Ray and Howard, 1991; Cajaraville and Pal, 1995; Ballarin and Cima, 2005; Chang et al., 2005; Aladaileh et al., 2007). They have been based on a variety of techniques including density gradient centrifugation (Cheng et al., 1980; Friebel and Renwrantz, 1995), flow cytometry (Xue et al., 2001; Hégaret et al., 2003), and transmission, light and scanning electron microscopy (Morona and Mingyi, 1989; Zhang et al., 2006; Travers et al., 2008). Early characterisations of bivalve haemocytes were often contradictory because a variety of techniques were used for their classification; as a result numerous terminologies were adopted. It was not until Cheng (1981, 1984) and Hine (1999) synthesised the literature that the existence of three major circulating haemocyte classes, un-differentiated cells, hyalinocytes and granulocytes, were hypothesised.

Both hyalinocytes and granulocytes are phagocytic and play a central role in host defence (Auffret, 1988). In a recent study by Aladaileh et al. (2007), granulocytes from the Sydney rock oyster (*Saccostrea glomerata*) were found to be more phagocytically active

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than hyalinocytes. Similar granulocytes have also been identified in many invertebrate species other than molluscs, and have been classified as either eosinophilic, basophilic or neutrophilic (McCormick-Ray and Howard, 1991; López et al., 1997). Cytochemical assays have demonstrated that granules within granulocytes contain a number of intracellular antimicrobial compounds, including superoxide anion and melanin (Aladaileh et al., 2007), as well as defensive enzymes, such as phenoloxidase (PO), acid phosphatase, and peroxidase.

In contrast to granulocytes, bivalve haemocytes containing few or no cytoplasmic granules are defined as hyalinocytes. In 1999, Hine described hyalinocytes as having a hyaline cytoplasm of a "silky" appearance (Hine, 1999). Superficially, hyalinocytes can be divided into two classes; small hyalinocytes with large nuclei and scanty cytoplasm lacking organelles, and large hyalinocytes with reniform or irregular nuclei, large cytoplasms, and often a variety of organelles.

Despite these data on haemocyte morphology and function in other bivalve species, there is no comparable information for the Akoya pearl oyster (*Pinctada imbricata*). *P. imbricata* is currently being assessed by The Department of Industry and Investment New South Wales (NSW DII) as the basis of a local pearl oyster industry (O'Connor, 2003). The Akoya pearl oyster is native to the east coast of Australia, from NSW to the northern tip of Queensland. It is typically fished for pearls in both Japan and China (O'Connor et al., 2003).

This study characterises *P. imbricata* haemocytes on the basis of their morphology and function, particularly potential immunological activities including the production of ROS, the presence of PO, and phagocytosis.

## 2. Materials and methods

### 2.1. Haemolymph collection

*P. imbricata* were supplied by The Department of Industry and Investment New South Wales (NSW DII), who obtained them from Broken Bay Pearls Pty Ltd. (Tuncurry, NSW). Oysters (15 per tank) were housed at the Sydney Institute of Marine Science (SIMS) in 45 L flow-through sea water system maintained at 22 °C. Oysters were removed from aquaria 5 min prior to haemolymph extraction to drain excess water from their mantle cavities. Oysters were opened by severing the adductor muscle. Haemolymph was withdrawn from the area surrounding pericardial cavity and adductor muscle using 27-gauge needles fitted to 1 mL syringes.

### 2.2. Live cell analysis

Thirty microlitre aliquots of whole haemolymph were placed on acid-alcohol washed slides and allowed to adhere for 20 min. The haemocytes were then covered with glass coverslips and viewed at high magnification (60× objective, oil immersion) with an Olympus BH-2 microscope equipped with both epi-fluorescence and differential interference contrast (DIC) optics. The haemocytes were assessed on their ability to adhere to a glass slide, and the motility of adherent haemocytes was observed. Differential haemocyte counts were made to calculate the relative percentage of different haemocyte sub-populations; twenty random fields of view from five separate haemolymph samples were analysed.

### 2.3. Cytological analyses

Cytological analyses were performed by adding 30 μL of whole haemolymph onto acid-alcohol cleaned slides. Cells were allowed to adhere for 25 min in a humid chamber before being stained by the following protocols. After staining, haemocytes were observed with an Olympus BH-2 microscope equipped with both epi-fluorescence and differential interference contrast (DIC) optics. All of the reagents and buffers for cytological analysis were from Sigma–Aldrich (Castle Hill, NSW), unless indicated otherwise. For each treatment, haemolymph from seven oysters were analysed separately.

### 2.4. Giemsa/May–Grünwald stain

Haemocytes were stained with Romanowsky's Giemsa/May–Grünwald stain, to characterise basic cellular morphology (Aladaileh et al., 2007). Adherent haemocytes were fixed for 20 min with formaldehyde (4% w/v in filtered sea water, FSW). Slides were then immersed in May–Grünwald stain for 6 min, before being counterstained with Giemsa for a further 30 min and washed in phosphate buffered saline (PBS; 136 mM NaCl, 2.68 mM KCl, 10.14 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$, adjusted to pH 7.5). Slides were air-dried, mounted with Ultramount No. 7 (Fronine Laboratory Supplies, Taren Point, NSW). Under these conditions basic granules stained blue whilst acidic granules stained pink (Chang et al., 2005).

### 2.5. Neutral red staining for lysosomes

Neutral red was used to further differentiate between acidic and basic vesicles in *P. imbricata* haemocytes. A stock solution was prepared by dissolving 20 mg of neutral red in 1 mL dimethyl sulfoxide (DMSO). The stock solution was then filtered through Whatman No. 2 filter paper and diluted 1:5 in PBS (Lowe and Pipe, 1994). 20 μL of the diluted neutral red was then overlaid onto unfixed adherent cells for 5 min. Neutral red stained vacuoles from red (acidic) to yellow (basic; Lowe and Pipe, 1994).

### 2.6. Peroxidase staining

Adherent cells were fixed with formaldehyde (4% w/v in FSW) for 10 min before being washed in PBS and transferred to a Coplin jar containing 5 $mg/mL^{-1}$ 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 60 mM $H_2O_2$ (7.2 pH). Haemocytes were incubated in this solution at 37 °C for 2 h before being rinsed with PBS (adapted from Graham and Karnovsky (1966)). Peroxidase staining appeared as a yellow/brown precipitate in cytoplasmic granules using bright field optics (Cima et al., 2001; Aladaileh et al., 2007).

### 2.7. Intracellular phenoloxidase activity

Thirty microlitres of 20 μg $mL^{-1}$ lipopolysaccharide (LPS), 5 mM hydroquinine monomethyl ether (4HA; Fluka, Buchs, Switzerland) and 5 mM 3-methyl-2-benzothiazolinone hydrazone (MBTH) in FSW were overlaid onto live adherent haemocytes. The cells were stained for 25 min at room temperature before being mounted and inspected by light microscopy (Aladaileh et al., 2007). Quinones, the product of PO activity on polyphenol substrates, were detected under bright field illumination as a pink/red colouration within haemocytes (Cima et al., 2001; Aladaileh et al., 2007).

### 2.8. Staining for intracellular lipids

Adherent haemocytes were fixed in 4% paraformaldehyde for 30 min, before being bathed in 70% ethanol for 2 min. Sudan black B stain was prepared by adding 25 mg of Sudan black B to 50 mL of 70% ethanol. The fixed cells were then stained with Sudan black B for 15 min, rinsed with 70% ethanol and finally washed in distilled water. The slides were mounted and examined under DIC for purple/black deposits associated with Sudan black deposition.