Korean J. Malacol. 41(4): 221-228 2025
https://doi.org/10.9710/kjm.2025.41.4.221

Characterization of Hemocytes and Immune Parameters of the Variegated Tapes *Ruditapes aspera* (Quoy & Gaimard, 1835) from Jeju Island

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ABSTRACT

We examined the hemocyte types and immune function of *Ruditapes aspera* from the sandy-pebble intertidal zone in Wimi Harbor on Jeju Island, using microscopy and flow cytometry. Three types of hemocytes were identified under light microscopy: granulocytes, hyalinocytes, and blast-like hemocytes. The ratio of nuclear size to cell size (N/C) of blast-like cells (0.77 ± 0.09) was slightly larger than that of granulocytes (0.52 ± 0.08) and hyalinocytes (0.65 ± 0.09). The N/C ratios of *R. aspera* hemocytes were similar to those of *R. philippinarum* (granulocyte 0.55 ± 0.06, hyalinocyte 0.61 ± 0.07, and blast-like cell 0.75 ± 0.07) collected from Jeju Island. Flow cytometry revealed that *R. aspera* contained 7.8 ± 2.9 × 10^5 hemocytes per mL, which is similar to that of *R. philippinarum* (10.0 ± 3.4 × 10^5 cells/mL). Among the three types of hemocytes in *R. aspera*, hyalinocytes accounted for 69.2% of the total hemocytes, followed by granulocytes (18.2%) and blast-like cells (10.2%). The lysozyme content, a proxy for the innate immune capacity of invertebrates, was highest in granulocytes (11.5 ± 4.4 × 10^4 A.U.), significantly higher than that in hyalinocytes (2.1 ± 0.4 × 10^4 A.U.), indicating that, like other marine bivalves, granulocytes are the main cells involved in defense. Phagocytosis rates measured by flow cytometry also showed that granulocytes in *R. aspera* are the primary defense cells, with a rate (58.5 ± 20.5% after 90 min) significantly higher than that of hyalinocytes (10.3 ± 6.4%; ANOVA, $P < 0.05$). Similarly, reactive oxygen species (ROS) production in granulocytes (4.7 ± 0.6 × 10^6 A.U. after 120 min) was significantly higher than in hyalinocytes (3.0 ± 0.3 × 10^6 A.U. after 120 min), confirming granulocytes as the key hemocytes involved in defense.

Keywords: *Ruditapes aspera*, hemocytes, flow cytometry, phagocytosis, ROS production

INTRODUCTION

Described initially as *Venus aspera*, *Ruditapes aspera* (= *Ruditapes variegatus*, *R. variegata*, *Tapes variegata*, *Venerupis aspera*, *V. variegata*) is a warm-water venerid clam first collected from Port Dorey, Nouvelle-Guinée (= Manokwart Bay, West Papua, Indonesia) by Quoy and Gaimard (1835), who reported numerous new species of mollusks obtained from Southeast Asia during the 1826 and 1829 Voyage de l'Astrolabe. Known as "Variegated Tapes", *R. aspera* has a wide geographical distribution range, from tropical Australia, Indonesia, Vietnam, to subtropical Taiwan, Japan and southern China (Abbott and Dance, 1982; Hylleberg, 2000; Lee and Chao, 2003; Kurihara, 2003; Vargas *et al*., 2010; Bai *et al*., 2016; Tang *et al*., 2022). *R. aspera* also occurs on the intertidal pebble beach in Jeju Island and the south coast of Korea, and often they co-occur with Manila clam *R. philippinarum* (Noseworthy *et al*., 2007; Silina, 2014; Kang *et al*., 2016). Currently, eco-physiological studies of *R. aspera* are limited compared to those of

Received: December 17, 2025; Revised: December 23, 2025; Accepted: December 30, 2025
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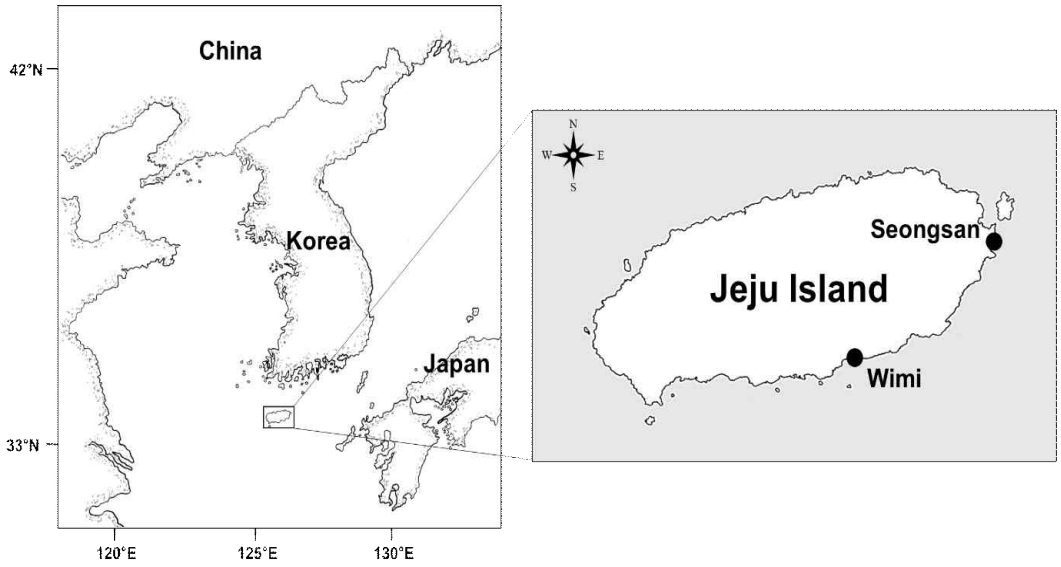

Fig. 1. Sampling locations of *Ruditapes philippinarum* and *R. aspera*. *R. philippinarum* was collected from sandy sediment, whereas *R. aspera* was collected from gravelly sediment.

R. philippinarum, one of the most crucial shellfish resources worldwide (Vargas *et al*., 2010; Tang *et al*., 2022; Humphreys *et al*., 2025), possibly because of its lower economic value.

Hine (1999) reviewed extensive information on the types and functions of hemocytes in various marine bivalve species, based on the appearance of stained cells under light microscopy or the ultrastructure of hemocytes. According to the review (Hine, 1999), most marine bivalves have "granula" and "agranula" hemocytes, although agranula hemocytes are further differentiated into blast-like cells, basophilic macrophage-like cells, and hyalinocytes. Agranula and granula blood cells are also identified in venerid clams, as Cima *et al*. (2000) and Donaghy *et al*. (2009a) reviewed studies on hemocyte types and their functions in Manila clam *R. philippinarum* and the grooved carpet shell *R. decussatus*. These hemocytes are involved in gas exchange, the transport of nutritional substances, tissue and shell repair, and cell-mediated defense activities, such as phagocytosis and the secretion of reactive oxygen species (ROS) to destroy ingested foreign cells or tissues (Donaghy *et al*., 2009a, 2015; Cima *et al*., 2000). For the characterization of marine bivalve hemocytes, flow cytometry has been adopted and applied since it allows rapid multiparametric analysis of large numbers of hemocytes, including measurement of ROS production, counting live and dead hemocytes in the total hemocyte population, and assessing the level of phagocytosis (Hong *et al*., 2013, 2016; Donaghy *et al*., 2016; Kim *et al*., 2020). Flow cytometry confirmed that granulocytes in Manila clams are the primary hemocytes involved in cell-mediated defense activities, including phagocytosis and ROS production (Donaghy *et al*., 2009b; Hong *et al*., 2014; Kim *et al*., 2020).

Like other marine bivalves, understanding the cellular defenses of the variegated clams is crucial for preventing disease-related mortality. Using light microscopy and flow cytometry, we first characterized the hemocyte types and their immune capacity in *R. aspera* collected from Jeju Island. The present study compares the types of hemocytes and their immune capacities with those of the Manila clam, also collected from Jeju Island.

MATERIALS AND METHODS

1. Sampling Effort

Fig. 1 shows the sampling locations of *R. aspera* and *R. philippinarum* analyzed in this study. The

variegated clams occurred on the intertidal pebble substrate at a density of 20–50/m^2 in a harbor on the south coast of Jeju Island. As a control, adult Manila clams were collected from a lagoon on the east coast of Jeju Island. For flow cytometric analysis, clams were acclimated to 20 °C in the laboratory for 48 hrs.

2. Hemocyte collection

Hemolymphs of the variegated clams and Manila clams were withdrawn from the posterior adductor muscle using a 1 mL syringe equipped with a 26G × 1/2″ needle, according to Hong *et al*. (2014, 2016). Approximately 1 mL of hemolymph was harvested from each Manila clam, while only 200 to 400 µL of hemolymph could be withdrawn from the variegated clam, possibly due to its size. The harvested hemolymph was then stored in a 1 mL plastic tube placed on ice to prevent hemocyte aggregation.

3. Microscopy of hemocytes

For hemocyte staining and subsequent microscopic observation, forty µL of hemolymph from each clam was placed on glass slides coated with poly-L-Lysine (MAS-11; Matsunami Glass Ind., Ltd., Japan). The hemolymph-coated slides were kept in a humidified chamber and incubated at room temperature for 30 minutes. The attached hemocytes were then stained with Hemacolor (Merck, Germany) prior to microscopic examination. The different types of hemocytes observed under the microscope were digitized using a digital camera. To identify hemocyte types, cell and nuclear diameters of hemocytes were measured using image analysis software (Zen 2.3 Lite, ZEISS, Germany).

4. Total hemocyte count and type proportion

A SYBR Green I (Invitrogen, USA) staining assay was conducted to determine the total hemocyte count (THC) and the proportion of each hemocyte type within THC, following Kim *et al*. (2020). For the assay, 50 µL of clam hemolymph was diluted with an equal volume of antiaggregant solution (AASH, 2.5% NaCl, and 1.5% EDTA in 0.1 M phosphate buffer, pH 7.4) and incubated with 1,000× SYBR Green I (final concentration = 10×) for 30 min in the dark at room temperature. The hemocyte type of *R. aspera* was then identified from a flow cytometry diagram displaying internal complexity (granularity, measured by side scatter, SSC) and cell size (forward scatter, FSC).

5. Oxidative activity

In flow cytometry, the oxidative activity in clam hemocytes using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen, USA), a membrane-permeable, non-fluorescent probe (Kim *et al*. 2020). Inside hemocytes, reactive oxygen species (ROS) and reactive nitrogen species (RNS) oxidize DCFH-DA into the highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF). Additionally, the oxidative capacity was boosted by adding phorbol 1,3-acetate (PMA; Sigma-Aldrich, USA) to the hemolymph. To measure the level, 150 µL of hemolymph diluted with an equal volume of filtered seawater (FSW) was mixed with DCFH-DA (final concentration = 10 µM) and PMA (final concentration = 10 µg/mL), then incubated in the dark at room temperature. The oxidative activities were recorded at 10, 30, 60, 90, and 120 min after the reaction. The level of oxidative activity was finally expressed in arbitrary fluorescence units (A.U.).

6. Phagocytosis capacity

Clam hemocyte phagocytosis (PHG) was stimulated by adding Fluoresbrite® Yellow Green Microspheres (2.0 µm in diameter, Polysciences Inc., USA) into the hemolymph. For the assay, 150 µL of hemolymph was mixed with an equal volume of FSW, then 2% fluorescence-labeled beads were added to the diluted hemolymph (final concentration = 0.2%). The hemocyte-fluorescence bead mixture was incubated in the dark at room temperature. The phagocytic capacity of hemocytes was determined at 10, 30, 60, and 90 min after incubation using a green fluorescence detector in flow cytometry. Finally, the level of hemocyte phagocytosis was expressed as the

percentage of hemocytes that engulfed more than three beads.

7. Intracellular lysosome levels

LysoTracker Red (Invitrogen, USA), a red-fluorescent dye, was used to stain lysosomes in hemocytes and assess lysosomal levels by flow cytometry. For the assay, 150 µL of hemolymph from clams was first mixed with an equal volume of FSW. The diluted hemolymph in FSW was then mixed and incubated with LysoTracker Red (final concentration = 10 µM) for 60 min at laboratory conditions. The lysosomal components in the hemocytes, measured by flow cytometry, was expressed as the intensity of red fluorescence in arbitrary units (A.U.).

RESULTS

Three hemocyte populations were identified in *R. aspera* and *R. philippinarum* using FSC-A/SSC-A parameters in flow cytometry (Fig. 2). Consistently, light microscopic observation following Hemacolor staining identified three types of hemocytes: granulocytes containing eosinophilic granules, hyalinocytes, and blast-like cells (Fig. 2). The cell size, nucleus size, and N/C ratio of each hemocyte type in *R. aspera* and *R. philippinarum* are presented in Table 2. The average cell sizes of granulocytes and hyalinocytes in *R. aspera* were 8.93 ± 1.44 µm and 7.30 ± 1.05 µm, respectively, slightly

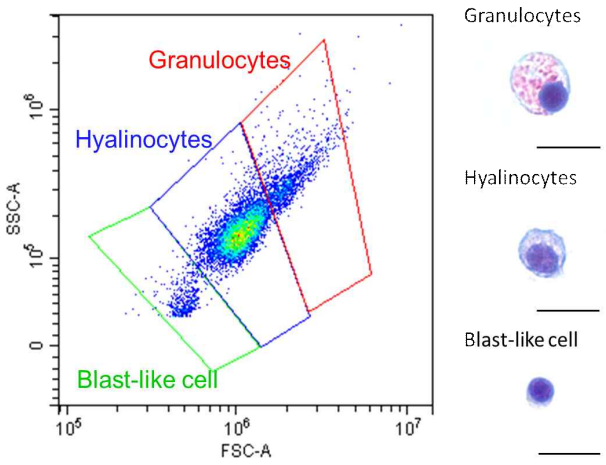

Fig. 2. Flow Cytometric Gating Strategy for Hemocyte Subtypes in *R. aspera* and Representative Hemacolor-Stained Granulocytes, Hyalinocytes, and Blast-like Cells. Scale bar = 10 µm.

larger than those in *R. philippinarum*, while the sizes of blast-like cells were similar in both species. No significant differences in N/C ratios were observed between species (Table 2). The relative proportions of hemocyte types in *R. aspera* were 18.2% for granulocytes, 69.2% for hyalinocytes, and 10.2% for blast-like cells, indicating hyalinocytes are the dominant population, followed by granulocytes and blast-like cells (Fig. 3). Similarly, hyalinocytes were the most common hemocyte type in *R. philippinarum* (79.3%), but the proportion of blast-like cells (11.3%) was higher than that of granulocytes (9.1%). The THC of *R. aspera* and *R.*

Table 1. Sampling information for *Ruditapes philippinarum* and *R. aspera*

Species	Sampling date	Site	N	SL
R. philippinarum	2025-07-06	Seongsan	5	36.00 ± 2.96
R. aspera	2025-07-15	Wimi	10	24.83 ± 1.62

Table 2. Cell Size, Nuclear Diameter, and Nucleus-to-Cell (N/C) Ratio of Hemocyte Types in *Ruditapes philippinarum* and *R. aspera* Stained with Hemacolor. Values are presented as mean ± standard error. N: number of analyzed cells

Species	Cell type	Cell size (µm)	Nucleus size (µm)	N/C
R. philippinarum	Granulocytes	7.58 ± 1.01	4.15 ± 0.48	0.55 ± 0.06
	Hyalinocytes	5.95 ± 0.80	3.59 ± 0.51	0.61 ± 0.07
	Blast-like cell	4.45 ± 0.46	3.33 ± 0.36	0.75 ± 0.07
R. aspera	Granulocytes	8.93 ± 1.44	4.56 ± 0.50	0.52 ± 0.08
	Hyalinocytes	7.30 ± 1.05	4.67 ± 0.46	0.65 ± 0.09
	Blast-like cell	4.78 ± 0.38	3.68 ± 0.51	0.77 ± 0.09

philippinarum, measured by flow cytometry, was similar: 7.8 ± 2.9 × 10^5 cells/mL in *R. aspera* and 10.0 ± 3.4 × 10^5 cells/mL in *R. philippinarum* (Fig. 4).

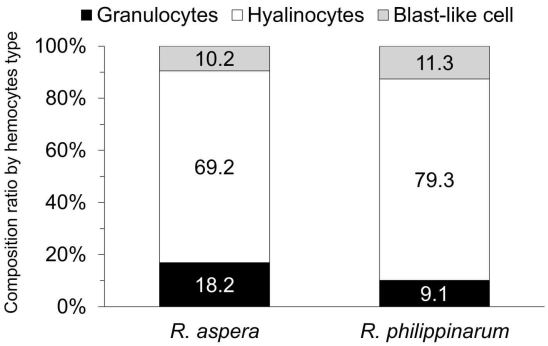

Fig. 3. Proportion of hemocyte types in *Ruditapes philipinarum* and *R. aspera*.

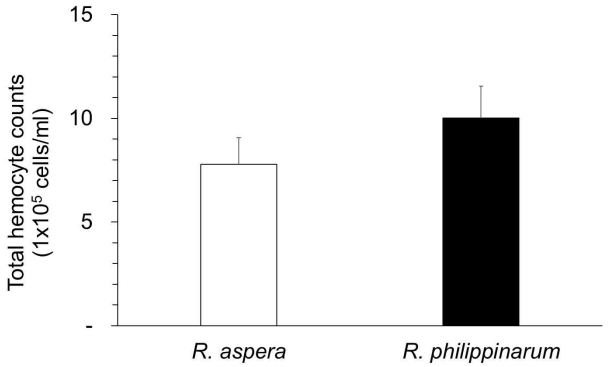

Fig. 4. Total hemocyte count in *Ruditapes philippinarum* and *R. aspera*

Immune function assays were performed separately for granulocytes and hyalinocytes to assess ROS capacity, PHG, and lysosomal content. In granulocytes of both species, ROS capacity peaked at 30 min after stimulation. It then either remained stable or declined, with a more pronounced decrease in *R. aspera* than in *R. philippinarum* (Fig. 5). In *R. aspera*, granulocyte ROS capacity ranged from 0.4 × 10^6 to 11.1 × 10^6 A.U. throughout the reaction period. In contrast, hyalinocytes exhibited lower capacity, ranging from 0.1 × 10^6 to 4.7 × 10^6 A.U., indicating that granulocytes produced more ROS. A similar trend was observed in *R. philippinarum*, with

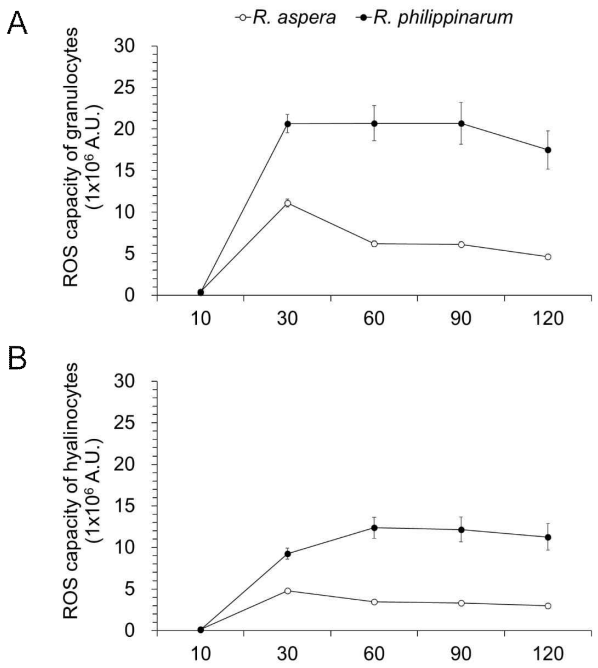

Fig. 5. ROS production capacity of granulocytes (**A**) and hyalinocytes (**B**) in *Ruditapes philippinarum* and *R. aspera*.

granulocyte ROS capacities ranging from 0.3 × 10^6 to 20.7 × 10^6 A.U., and hyalinocyte capacities ranging from 0.1 × 10^6 to 12.4 × 10^6 A.U. Although the average ROS capacity was slightly higher in *R. philippinarum* than in *R. aspera*, no statistically significant differences were detected at any reaction time point.

The phagocytosis rate, defined as the proportion of cells ingesting three or more beads, increased steadily over time in both species. In *R. aspera*, phagocytic rates of granulocytes and hyalinocytes ranged from 12.6% (10 min) to 58.5% (90 min) and from 1.0% (10 min) to 10.3% (90 min), respectively, showing significantly higher phagocytic activity in granulocytes (Fig. 6). Similarly, granulocytes of *R. philippinarum* displayed higher phagocytic rates (9.7-48.1%) than hyalinocytes (0.7-6.1%), with no notable differences between species. Lysosomal content, measured by LysoTracker Red staining, was highest in granulocytes, followed by hyalinocytes and blast-like cells in both species. In *R. aspera*, lysosomal content values were 11.5 × 10^4 A.U. for granulocytes, 2.1 × 10^4 A.U. for hyalinocytes, and 0.9

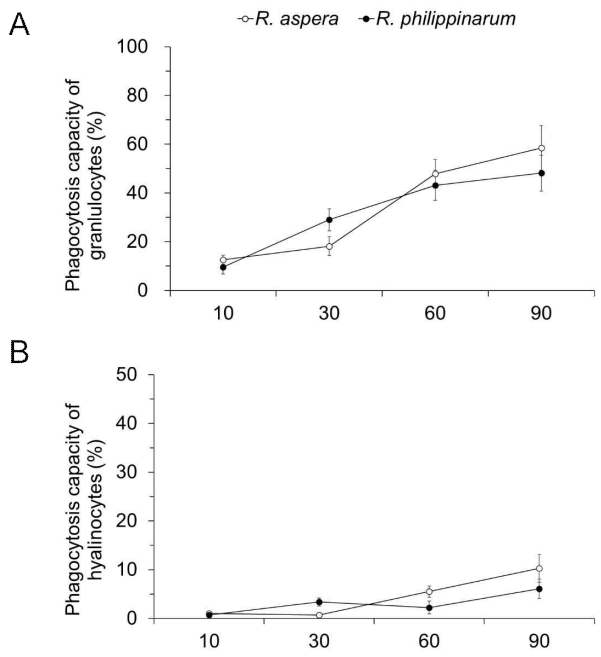

Fig. 6. Phagocytic capacity of granulocytes (**A**) and hyalinocytes (**B**) in *Ruditapes philippinarum* and *R. aspera*.

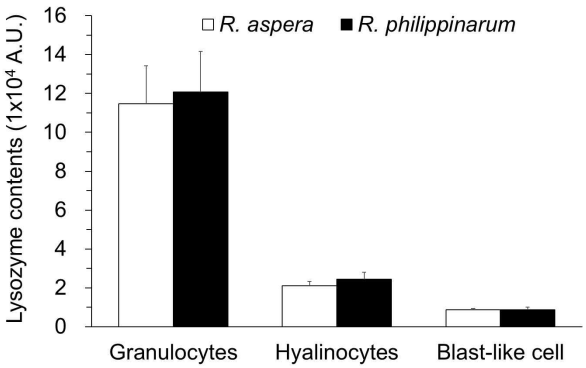

Fig. 7. Lysozyme contents by hemocyte type in *Ruditapes philippinarum* and *R. aspera*.

× 10^4 A.U. for blast-like cells (Fig. 7). Similar lysosomal content was observed in *R. philippinarum*, with lysosomal content of 12.1 × 10^4 A.U. in granulocytes, 2.4 × 10^4 A.U. in hyalinocytes, and 0.9 × 10^4 A.U. in blast-like cells.

DISCUSSION

Hemocytes form the core of the innate immune system in marine bivalves, which rely entirely on cellular defenses to fight against microbial pathogens, parasites, and environmental stressors. In this study, we characterized hemocyte cell types in *R. aspera* for the first time using the nuclear-to-cell ratio, a method previously employed to differentiate hemocyte types in molluscan bivalves (Cima *et al*. 2000; Donaghy *et al*., 2009b; Hong *et al*., 2013; Yang *et al*., 2015; de la Ballina *et al*. 2022). As with other marine bivalves, three types of hemocytes were identified in *R. aspera*: hyalinocyte, the most abundant and largest cell as the N/C ratio; granulocyte, most actively involved in defense; and blast-like cell, which is considered to be an undifferentiated hemocyte. Flow cytometry revealed that *R. aspera* hyalinocytes accounted for approximately 70% of the total hemocytes, whereas the phagocytosis rate measured 90 min after the reaction was only one-sixth of that of the granulocytes. Analysis of *R. aspera* hemocytes by flow cytometry also revealed that granulocytes have lysosomal content approximately 5 times greater than hyalinocytes and produce ROS twice as much. The phagocytic activity, lysosome abundance, and ROS production of blast-like hemocytes are negligible compared to granulocytes and hyalinocytes, suggesting that these cells are undifferentiated hemocytes, consistent with other studies. Therefore, granulocytes are the primary cells involved in cellular defense in *R. aspera*, as reported in various marine bivalves (Donaghy *et al*., 2009a; Hong *et al*., 2013, 2014, 2021; Yang *et al*., 2015; Kim *et al*., 2020).

Flow cytometric analysis of marine bivalve hemocytes is widely used to understand their ecophysiology and the impacts of both natural and human-made environmental stresses, such as oil pollution and heatwaves. By using flow cytometry to evaluate hemocyte immune functions, Donaghy *et al*. (2010, 2016) and Hong *et al*. (2016) studied the sublethal effects of the Hebei Spirit oil spill on oysters, mussels, and clams along the beaches of Taean on the west coast two years after the spill. Although environmental hydrocarbons from the spill have significantly decreased over two years, the immune functions of the Pacific oyster *Crassostrea*

gigas, the mussel *Mytilus galloprovincialis*, and Manila clam *R. philippinarum* still exhibit notable abnormalities in immune parameters, such as ROS production and phagocytosis rate. These studies also confirmed that flow cytometric analysis of immune responses can serve as a proxy for monitoring the sublethal effects of human-made stresses on marine organisms.

Natural stressors such as heatwaves and high air exposure, which caused significant desiccation, also affected the immune functions of clams and mussels. To understand the effects of air exposure and desiccation, Park *et al*. (2012) analyzed the immune response of Manila clams exposed to the atmosphere for several days. Flow cytometry showed that, compared to clams from their natural habitat, those subjected to long-term air exposure had a notable increase in total hemocyte count, DNA damage, and blood cell death, along with significant decreases in phagocytosis activity and spontaneous ROS production. These immune abnormalities were believed to result from prolonged exposure to air. Hong *et al*. (2021) also studied the effects of extended heatwaves, which led to high thermal conditions, on the mussel *Mytilisepta virgata* by analyzing hemocyte functions using flow cytometry. Under 5 days of heatwave conditions (40 °C) and 12 hrs of air exposure, *M. virgata* showed significantly higher ROS levels within 2 days of exposure compared to controls. After 3 days, hemocyte DNA damage increased markedly, and the phagocytosis rate dropped considerably 4 days after the start of the experiment, likely linked to the high ROS production initiated 2 days after exposure, which might have elevated oxidative stress in the mussel. As observed from these studies, flow cytometric analysis of marine bivalve hemocytes can serve as a proxy for assessing naturally occurring environmental stresses.

In summary, the different hemocyte types and their immune functions in the variegated clam *R. aspera* occurring on Jeju Island were first characterized using light microscopy and flow cytometry. As observed in the Manila clam, a sister species of the variegated clam, hyalinocytes are the most abundant hemocytes overall, while granulocytes are the primary cells involved in defense, although they are less numerous in the total cell count. It is believed that flow cytometric data from the variegated clam could provide insights into the immune systems of marine bivalves and be used to manage the *R. aspera* population on Jeju Island effectively.

ACKNOWLEDGEMENTS

This work was supported by the 2025 National University Development Project grant of Jeju National University, funded by the Ministry of Education.

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