Immunological Assays of Hemocytes in the Northern Quahog *Mercenaria mercenaria*

Yangqing Zeng[1], Yuanzi Huo[1], and Huiping Yang[1]*

[1] *School of Forest, Fisheries, and Geomatics Sciences, Institute of Food and Agricultural Sciences, University of Florida, 7922 NW 71st Street, Gainesville, Florida, U. S. 32653*

Running title: Assays of Hemocytes in *Mercenaria mercenaria*

*Corresponding author
Huiping Yang
7922 NW 71st Street,
Gainesville, FL 32653
Email: huipingyang@ufl.edu
Phone: 352-294-0671

Abstract

The northern quahog *Mercenaria mercenaria* (commonly named hard clam) is an important aquaculture and fishery species along the Atlantic west coast. Environmental stresses, such as heat shock, fluctuating salinity, and harmful algal blooms are major challenges for clam aquaculture. In response to environmental stresses, hemocytes would change dynamically for defense and immunity. The goal of this study was to characterize basic immunological assays of hemocytes in the northern quahog by use of flow cytometry. The objectives were to: 1) develop a non-lethal method for hemolymph collection and dilution; 2) verify the capability of flow cytometry for hemocyte count and type identification through comparison with microscopic observation; 3) validate hemocyte viability assay based on plasma membrane integrity, and 4) develop hemocyte phagocytosis assay by use of fluorescein labeled microbeads. A non-lethal hemocyte collection method was developed using needle insertion through the ligament. Osmolality measurement of serum was the same as that of culture seawater. The pH measurement of serum (7.2) was significantly different from that of culture seawater (8.4). By microscopic observation, three types of hemocytes were identified with granulocytes, the dominant cell type (70 ± 16%), agranulocyte (14 ± 4%), and blast-like cell (16 ± 4%), and no differences were found from the measurements by flow cytometer on FSC/SSC plot (cell size/granularity). The viability of hemocytes based on plasma membrane integrity was 88 ± 6% ranging from 70 to 97% (n = 60, three populations), and viability protocol was further validated with the pre-set expected viability ($p \geq 0.424$). Phagocytosis assay of hemocytes with fluorescence beads showed a mean capacity of 10 ± 5% (n = 60, three populations). Incubation time (up to 6 h) or bead concentrations (2:1 or 5:1 to hemocytes) did not affect the phagocytosis measurement. Overall, this study reported the basic characteristics of hemolymph (serum and hemocytes) of northern quahogs. It is expected that the assay methodologies will be applied to evaluation of hemocyte responses to environmental stresses for clam aquaculture.

Keywords: Viability; Phagocytosis; Flow cytometry; Northern quahogs; Hemocyte; Non-Lethal sampling; *Mercenaria mercenaria;* Granulocyte; Agranulocyte; Blast-like cell.

Abbreviations: CB, Cytochalasin B; FCM, flow cytometry; MAS, modified Alsever's solution; ROS; reactive oxygen species.

1. Introduction

The northern quahog *Mercenaria mercenaria* (Family Veneridae, also commonly named hard clam) is an important aquaculture and fishery species along the Atlantic west coast. Aquaculture of *M. mercenaria* was initiated in the 1970s, and has developed into a $56 million (sales value) industry in 2017 [1] in the U. S. with Virginia and Florida as the major producers [2]. As an aquaculture species, *M. mercenaria* has been introduced into other states, such as California, Puerto Rico, and Florida west coast and other countries for aquaculture [3]. Additionally, as an endobenthic bivalve species, *M. mercenaria* plays a significant role in coastal ecosystem structure and functioning.

As poikilothermic osmo-conformers, molluscan bivalves generally use their hemolymph as the first defender against environmental changes [4]. The hemocytes could prevent loss of hemolymph following tissue injury, interact with and clear microorganisms introduced by trauma, and initiate the process of wound repair and tissue remodeling [5]. Further studies on hemocytes (and hemolymph) in molluscan bivalves indicated that hemocytes could influence homeostasis [6], nutrient digestion, transportation, and distribution [7], detoxification processes [8], and cellular immune defense. Additionally, bivalve hemocytes were found to have the ability to recognize, bind, and phagocytize bacteria microbes [9], and gene-environment relationships in hemocytes were identified [9] to provide a useful marker for bivalve health condition or cultured environment evaluation [5].

Since its first invention in 1953 [10], flow cytometry (FCM) has become one of the most powerful technologies for cell analysis, and is routinely used as a standard laboratory tool for fundamental and applied immunology studies and sorting of subpopulations [11]. Application of flow cytometer for molluscan immunology started in 1989 [12]. To date, FCM has been used for the analysis of hemocyte cell count, viability, cell types, phagocytosis, oxidative stress, apoptosis, DNA, and protein content [13]. However, compared to the research in mammals, immunological assays in mollusks by use of FCM is still limited. With rapid development of fluorescence probes [14] and multiple-color FCM system [15], more immunological assays of hemocytes in mollusks would be developed by use of FCM analysis.

For *M. mercenaria*, studies on hemocyte immunity were firstly on the cell cytochemical property and cytoplasmic structures [16], and eosinophil granular were identified in the cytoplasm with dehydrogenase activity and reaction with phospholipid. The first description of hemocyte morphological and behavioral characteristics revealed three types of hemocytes, granulocyte, fibrocyte, and hyalinocytes, at a concentration of $1.4\text{-}1.9 \times 10^6$ cells/ml, and no correlations between animal sizes and hemocyte size and number [17]. Furthermore, electron microscopic observation of hemocytes suggested that fibrocytes were actual granulocytes, which were at the terminal phase of their physiologic cycle relative to the degradation of phagocytized “non-self” materials, and lipid-like droplets were reported in hemocytes for the first time [18]. Since then, functions of hemocytes, such as cytochemical enzyme activities, phagocytosis, agglutinins, and shell repair, were studied by use of microscopic observations and spectrophotometry. Application of flow cytometry on hemocytes of *M. mercenaria* was firstly reported on cell type, cell count, and oxidant stress induced by zymosan [19]. Additionally, flow cytometry was used in studies of hemocytes in response to quahog parasite unknown（QPX) disease in *M. mercenaria* [20]. Overall, the hemocyte assays in *M. mercenaria* by use of flow cytometer included total cell count, reactive oxygen species (ROS) production, and phagocytosis on bacteria.

Environmental stresses, such as summer heat shock, fluctuating salinity, and harmful algal blooms are major challenges for the hard clam aquaculture industry in Florida, U.S.. In response to these environmental stresses, hemocytes would change dynamically for defense and immunity [21, 22]. With prolonged stresses, the immune system would show damage, and subsequently, the susceptibility to pathogens could increase and create disease outbreaks [5]. In oysters, immune gene expression and genomic evolutionary adaptations to extreme environmental changes were discovered [23] and up/down regulation of immune genes were identified after exposure to heat shock and/or hypoxia [24].

The goal of this study was to characterize basic immunological assays of hemocytes in the northern quahog by use of flow cytometry. The objectives were to: 1) develop a non-lethal method for hemolymph collection and dilution; 2) verify the capability of flow cytometry for hemocyte count and type identification through comparison with microscopic observation: 3) validate hemocyte viability assay based on plasma membrane integrity, and 4) develop hemocyte phagocytosis assay by use of fluorescein labeled microbeads. It is expected that these assay methodologies will be applied to estimate hemocyte responses to environmental changes and develop effective biomarkers for environmental stresses and for genetic breeding.

2. Materials and methods

2.1. Experiment animals

Northern quahogs (12-14-month-old) were purchased from three farmed populations in Cedar Key, Florida, U.S. (hereafter named as P1, P2, and P3, n =138) with shell length of 47.33 ± 3.35 mm (38.54 - 54.15 mm), shell height of 39.13 ± 2.74 mm (31.38- 44.62 mm), shell width of 25.67 ± 2.03 mm (20.8 -31.2 mm), and body weight of 33.15 ± 6.97 g (17.44-54.24 g). The seed of these three clam populations were known to come from different hatcheries. After collection, clams were cleaned using freshwater to remove the mud and sand and transported to the laboratory in Gainesville, Florida (1-hr driving) in a cooler with ice bags. Upon arriving, clams were cultured in a recirculating system equipped with a UV light and a bead filter (Endurance 4000, Aquaculture Systems Technologies, Baton Rouge, LA) at 22-24°C, salinity of 25 ppt and pH of 8.35. These clams were fed twice a day with mixing microalgae, including *Isochrysis galbana*, *Thalassiosira weissflogii*, and *Tetraselmis suecica* at 50,000 cell/ml. Water quality was measured using a saltwater aquaculture kit (LaMotte seawater kit, Apopka, FL, USA) once a week to maintain ammonia lower than 0.01 ppm, nitrite lower than 0.01 ppm, and nitrate lower than 7 ppm.

2.2. Flow cytometer (FCM) used in this study

A flow cytometer (Accuri C6, BD Biosciences, San Jose, CA, USA) equipped with 488-nm and 640-nm lasers was used in this study. Before analyzing experimental samples, the performance of the flow cytometer was verified using fluorescent validation beads (Spherotech beads, BD Biosciences, San Jose, CA, USA), and the coefficient of variation values were controlled < 3.0% (based on full peak height). All samples were run at a medium flow rate (35 µL per min). Data collected from flow cytometry were analyzed using the manufacturer's software (CFlow® version 1.0.202.1, BD Biosciences, San Jose, CA, USA).

2.3. Experiment I: non-lethal hemolymph sampling and physio-chemical properties

To allow clams alive after hemolymph sampling, a non-lethal method was developed and was used for hemolymph collection in all experiments in this study. Additionally, osmolality and pH of

the serum were measured to provide the basis for handling hemolymph samples for further analysis.

2.3.1. Non-lethal hemolymph sampling

The length, height, and width of 50 individual clams were measured using a caliper with 0.01 mm accuracy (Mitutoyo, Aurora, IL) (**Figure 1A** and **Figure 1B**). The acute angle between the inserted needle through the hinge ligament was measured on empty shells using a protractor with 1-degree accuracy after dissection of clams.

To validate the non-lethal hemolymph sampling method, hemolymph was sampled from 60 clams (20 from each population) by use of a 21-gauge needle (25.5 or 51 cm depending on clam size) attached to a 3-ml syringe (BD safety-Lok). Following the measured angles, needle was pierced through the hinge ligament edge at posterior side to reach the posterior adduct muscle (Figure 1 C and D). Due to the resistance of adduct muscle, it was distinguished from other tissues. After hemolymph sampling, clams were put back into the recirculating system to monitor mortality on day 1, 3, and 7, and to compare with the mortality of controls without hemolymph sampling (20 from each population).

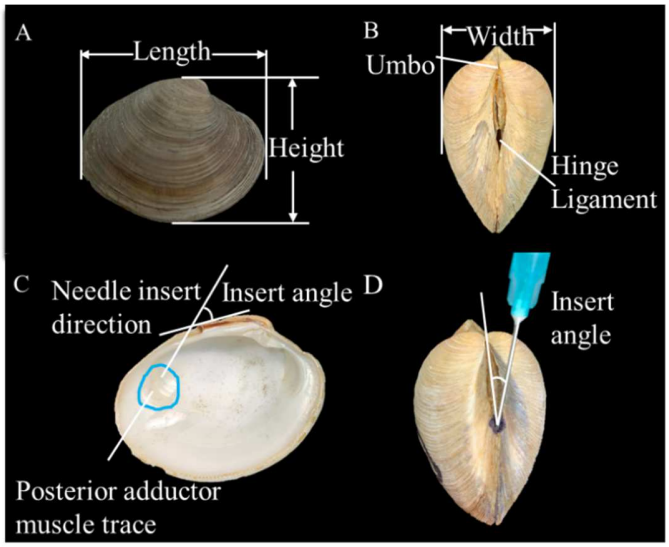

Figure 1. Measurements of the northern quahog *Mercenaria mercenaria* for non-lethal hemolymph collection. A. Measurement of shell length and height. B. Measurement of shell width and the location in ligament to pierce needle for hemolymph sampling from posterior adduct muscle. C. Inside view for the needle insertion to reach posterior adduct muscle for hemolymph sampling. D. Abridged general view for non-lethal hemolymph collection.

2.3.2. pH and osmolality of hemolymph and choice of anti-aggregate buffer

Hemolymph after non-lethal collection from each clam was transferred into a microcentrifuge tube on ice after filtering through a 20-µm mesh. Immediately, hemolymph (~1000 µL) was centrifuged at 4,400 rpm at 4°C for 5 mins (Eppendorf 5424R, Hamburg, Germany), and supernatant was removed into another tube for pH and osmolality measurements. The osmolality was measured using a freezing-point osmometer (model 3320, Advanced instruments, Inc. Horn, Netherland) at a 20-µL sample volume. The pH was measured with a fine pH paper with 0.2 accuracy (Fisher Brand, Catalogue #13-640-510, Ottawa, ON, Canada) due to the small sample

volume. Three measurements were made for samples from each clam. Meanwhile, the osmolality and pH of the seawater in the clam culture recirculating system were measured.

To prevent hemocytes from agglutination during bleeding, different types of anti-agglutination solutions have been reported, such as modified Alsever's solution (MAS) [9] from original Alsever's solution for human blood cells [25] and cold seawater [26]. In this study, MAS (MAS: 27 mM sodium citrate, 336 mM sodium chloride, 115 mM glucose, 9 mM EDTA in deionized water) was used to dilute the hemolymph samples immediately after collection at a ratio of 1:1, pH (7.2) and osmolality (860 mOsmol/kg) were adjusted to the same values as that of serum.

2.4. Experiment II: Hemocyte cell types and cell count

FCM has been used to measure cell concentration and cell types [27] simultaneously with other immunological assays. To test if the hemocyte type and count in hard clams can be recorded by use of FCM, microscopic observation was performed and compared to FCM record. Hemolymph samples (500 µL, n = 15 and 5 for each population) were collected by non-lethal method and immediately mixed with an equal volume of MAS buffer. One half of each sample (500 µL) was used for microscopic observation (section 2.4.1), and another half (500 µL) was used for FCM analysis (section 2.4.2).

2.4.1 Cell type and determined by use of microscopic observation.

Hemolymph samples were centrifuged at 4,000 rpm (~ 1500 g) at 4°C for 5 mins, the supernatant was discarded, and the hemocyte pellet was re-suspended gently by adding 20-µL MAS buffer. Hemocyte suspension (20 µL) was pipetted onto a pre-cleaned and labeled slide (~ 0.5 cm to the edge) (Fisher Scientific, Pittsburgh, PA). Spread of hemocytes on the whole slide was made by using another slide against the hemocyte suspension drop at a 30-degree angle and moving gently towards the opposite side of the slide. Hemocyte slides were dried at 4°C in a refrigerator by placing them on a shelf in a moist container with wet tissue paper on the bottom.

Hemocyte slides were stained using a Wright-Giemsa stain kit (Camco Quik Stain II, Fort Lauderdale, FL) by following the manufacturer's manual. Briefly, slides were dipped in Wright-Giemsa staining solution for 10s, washed in deionized water for 90s in a jar with water flowing, and air-dried at room temperature. Hemocytes were observed using a microscope at 400× and 1000× (Olympus, BX43, Tokyo, Japan) and photographs of hemocytes were taken using a microscope camera (Infinity HD, Lumenera Co, Ottawa, ON, Canada) at 1000× magnification. For each clam, hemocytes (at least 100 cells) were counted and sorted, and the sizes of cell and nucleus were measured using a microscopic ruler.

2.4.2. Total hemocyte count and hemocyte types determined using FCM

Hemolymph samples were analyzed by FCM with a total of 50,000 events recorded (could be performed simultaneously with cell membrane integrity analysis with staining of SYBR Green I and propidium iodide in section 2.5). The forward scatter (FSC) vs. side scatter (SSC) plot was used to identify the hemocyte types based on cell size (correlated to FSC) and intracellular complexity (correlated to SSC). Proper gates were used to exclude debris on FSC/SSC plots and include different types of hemocytes. The concentration of total hemocytes and different hemocyte types were recorded using the cell number and sample volume after proper gating on the FSC and SSC plot.

2.5. Experiment III: Hemolymph viability assay by FCM

Viability of hemocytes was analyzed based on the plasma membrane integrity. A pilot study was conducted to determine the staining concentrations. In 493-uL of hemocyte sample after mixing with MAS buffer at 1:1 ratio, a 5-µL of SYBR green I (10,000× concentration in DMSO, Invitrogen, Eugene, Oregon) and a 2-µL of propidium iodide (PI, 1mg/ml stocking solution in deionized water, Invitrogen, Eugene, Oregon) were needed. SYBR green I is a membrane permeable nucleic acid stain, while PI is a non-membrane permeable nucleic acid stain. After staining for 20 min in dark at room temperature (20°C), hemocyte samples were analyzed by use of FCM. Gated cells were analyzed on a scatter plot of FL1 (SYBR Green I) vs. FL2 (PI) with fluorescence compensation (FL1 was compensated by FL2 with 1.76%, and FL3 was compensated by FL1 with 11.16%). Hemocyte viability was expressed as the percentage of cells stained with SYBR green I (alive) in the total cells stained with SYBR green and PI. Hemolymph samples from 60 individuals (20 clams for each population) were analyzed for viability analysis.

To verify the effectiveness of the protocol for viability analysis, hemolymph samples (n = 9, each was pooled from two clams) were aliquoted and half of the sample was heated at 80 °C in water bath for 1 min. By mixing different ratios of fresh hemocyte sample and heat-treated hemolymph samples, five groups (500 µL for each) were generated with known viable hemocyte percentages: 1) 100% (100:0); 2) 75% (75:25); 3) 50% (50:50); 4) 25% (25:75), and 5) 0% (0:100). These samples were analyzed using the protocol set up in this experiment, and viability measurements were compared with the expected viability to verify the effectiveness of the protocol.

2.6. Experiment IV: Phagocytic capabilities of hemocytes by use of FCM

The phagocytic activity of hemocytes was determined by the uptake of dragon green fluorescence latex beads (2.07 µm, Bangs Laboratories Inc, IN, USA). Cytochalasin B (CB, EMD Millipore Corp, USA), a cell-permeable mycotoxin to block the formation of contractile microfilaments, was used to inhibit phagocytosis as negative controls at a final concentration of 10 µg/mL [12, 28]. Hemocyte samples (n = 5, each pooled from 4 clams) were aliquoted into four sub-samples for the following bead concentrations: 1) 2×10^6 /ml (2:1 ratio of bead: hemocyte); 2) 2×10^6 /ml plus CB; 3) 5×10^6/ml (5:1 ratio of bead: hemocyte), and 4) 5×10^6/ml plus CB. The samples were analyzed with FCM after incubation for 10 seconds, 0.5h, 1 h, 2 h, 3 h, 4 h, 5 h, and 6 h in dark at room temperature. The fluorescence of beads was detected at a 530 ± 30 nm band-pass filter at FL1 histogram. The measurement of hemocytes with CB indicated the beads attached to hemocyte only, and that without CB indicate beads were gulped. The difference in percentage between CB and no CB groups defined as the percentage of phagocytosis capacity of the hemocytes [29].

The methodology for determining phagocytic capabilities of hemocytes was established based on the optimal incubation time and bead concentration. To validate this method, hemocyte samples from 60 individual clams (20 for each population) were analyzed for their phagocytic capabilities.

2.7. Data analysis

All results in this study were presented as the mean ± standard deviation. The percentage data were transformed as the arcsine of the square root before ANOVA was conducted. Data analysis was performed by JMP pro (version 15.0.0, SAS Institute Inc, Cary, NC). One-way or multiple analysis of variance (ANOVA) and Tukey's post-hoc test was performed to compare the

differences. Correlation analysis was used to identify the relationship between parameters. The significance of difference was set as $p < 0.050$.

3. Results

3.1. Experiment I: Non-lethal collection of hemolymphs

The acute angle measured between the inserted needle for reaching the posterior adduct muscle and hinge ligament was 39 ± 2º ranging from 34º to 44º. Correlation analysis indicated that this acute angle did not correlated with clam shell length (50.9 ± 4.0 mm, $p = 0.460$), shell width (28.7 ± 2.2 mm, $p = 0.220$), or shell height (42.4 ± 3.6 mm, p = 0.490). The mortality of clams after non-lethal hemolymph sampling did not show significant difference with the control group at day 1 ($p = 1.000$), day 3 ($p = 0.120$), or day 7 ($p = 0.230$) (**Figure 2**).

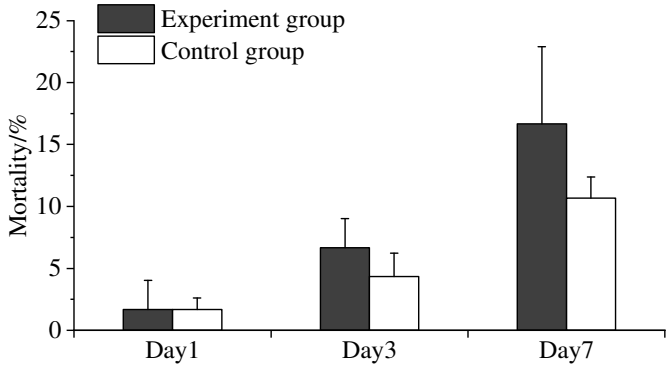

Figure 2. Mortality of *Mercenaria mercenaria* after non-lethal hemolymph collection (black column). No differences were found comparing to the mortality in control groups (white column) at different day.

The osmolality of serum was measured as 881 ± 10 mOsmol/kg, not significantly difference from osmolality of seawater in the culture system (878 ± 6 mOsmol/kg, $p = 0.884$). The pH of serum was measured as 7.2 ± 0.1, significantly lower than that of the seawater (8.35 ± 0.01, $p < 0.001$).

3.2 Experiment II: Hemocyte types observed by microscopic observation and FCM analysis

Microscopic observation of hemocytes identified four different cell-types based on morphology, cell size, nucleus size and proportion (nucleus: cytoplasm ratio) (**Figure 3A**).

1) **Granulocytes 1**. Granulocytes 1 showed varied shapes between round and ovoid. The cytoplasm was filled with eosinophilic (Giemsa stain) granules, stained either dark pink or purple. Nucleus was stained dark blue. This type accounted for 54 ± 7% of total hemocytes.
2) **Granulocytes 2**. Granulocytes 2 possessed an ameboid shape with small eccentric nuclei, stained a dark blue. Their cytoplasm often showed pseudopodia and contained basophilic granules, stained a blue color in Giemsa-Wright stain. This type accounted for 16 ± 4% of total hemocytes.
3) **Hyalinocytes or agranulocytes.** Agranulocytes possessed a round shape, large basophilic nucleus with rough structure and narrow cytoplasm surrounding it. This type accounted for 14 ± 4% of total hemocytes.

4) **Blast-like cells**. Blast-like cells were the smallest hemocytes with a spherical shape, high nucleus: cytoplasm ratio and no cytoplasmic granules in the cytoplasm. This type accounted for 16 ± 4% of total hemocytes.

Among the three clam populations, no differences were found in hemocyte size ($p \geq 0.170$) or nuclei size ($p \geq 0.080$) for each type of hemocyte (**Table 1**). However, the percentage of hemocyte types showed differences among the three populations. Granulocytes 1 ($p \geq 0.100$) and granulocytes 2 ($p \geq 0.220$) did not show differences among P1, P2, and P3; Agranulocyte in P2 was significantly lower than that in P1 or P3 ($p \leq 0.006$); Blast-like cells in P3 was significantly higher than that in P1 and P2 ($p \leq 0.020$).

Table 1. Hemocyte cell type, size, nucleus size, and nucleus/cell ratio (N/C) in the northern quahog *Mercenaria mercenaria* by use of light microscopic observation (n = 15, 5 quahogs from each population).

Cell type		Population 1	Population 2	Population 3
Granulocyte 1	Cell (µm)	17.48 ± 0.51	17.70 ± 0.23	17.72 ± 0.93
	Nucleus (µm)	9.86 ± 0.90	9.97 ± 0.67	8.70 ± 0.62
	N/C	0.56 ± 0.04	0.56 ± 0.04	0.49 ± 0.04
Granulocyte 2	Cell (µm)	17.77 ± 0.48	17.50 ± 1.34	18.14 ± 1.20
	Nucleus (µm)	11.99 ± 1.03	12.30 ± 0.97	12.24 ± 1.14
	N/C	0.67 ± 0.22	0.70 ± 0.01	0.67 ± 0.02
Agranulocyte	Cell (µm)	15.99 ± 0.42	17.88 ± 1.75	16.44 ± 1.53
	Nucleus (µm)	12.05 ± 0.89	12.55 ± 0.40	11.95 ± 0.89
	N/C	0.75 ± 0.06	0.70 ± 0.09	0.73 ± 0.06
Blast-like cell	Cell (µm)	12.68 ± 0.24	12.90 ± 0.71	13.48 ± 0.76
	Nucleus (µm)	10.96 ± 0.13	10.97 ± 0.79	10.75 ± 0.76
	N/C	0.87 ± 0.02	0.85 ± 0.04	0.80 ± 0.03

Flow cytometric analysis of hemocytes showed three distinctive cell populations on FSC/SSC plot (**Figure 3B**). Based on their position of FSC and SSC, the granulocytes, which was corresponding to granular hemocytes 1 and 2 from microscopic observation, were recognized as one population on FCM analysis and accounted for the most abundant population for 72 ± 16% of the total hemocytes, which was significantly higher than agranulocytes and blast-like cells with 14 ± 10% and 14 ± 11% in the total hemocytes ($p < 0.0001$, **Figure 4**). The total hemocyte concentration recorded by FCM was $2.02 \times 10^6 \pm 1.02 \times 10^6$ cell/ml (n = 15), ranging from 1.11×10^6 cell/ml to 5.31×10^6 cell/ml.

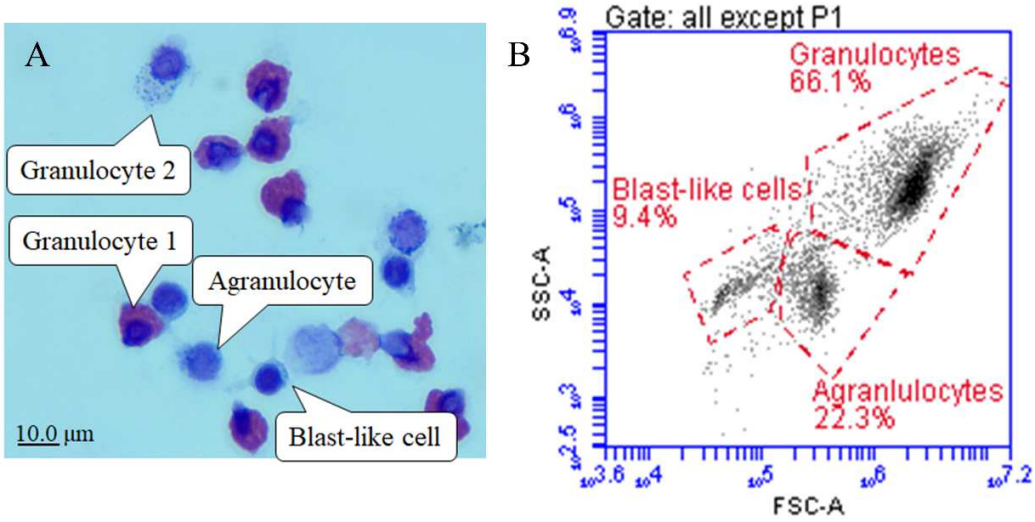

Figure 3. Comparison of hemocyte types of *Mercenaria mercenaria* by use of microscopic morphology observation and flow cytometry assay. **A.** Microscopic observation showing three types of hemocytes: Granulocytes 1 and 2, Agranulocytes, and Blast-like cell. **B.** Forward scatter (FSC) vs. side scatter (SSC) density plot of flow cytometry assay showed three hemocyte subpopulations of hemocytes in corresponding to the three types of hemocytes from microscopic observation.

Comparing the results of microscope observation and FCM analysis, no significant differences were found in the percentages of granulocytes ($p = 0.470$), agranulocytes ($p = 0.940$), and blast-like cells ($p = 0.110$) (**Figure 4**).

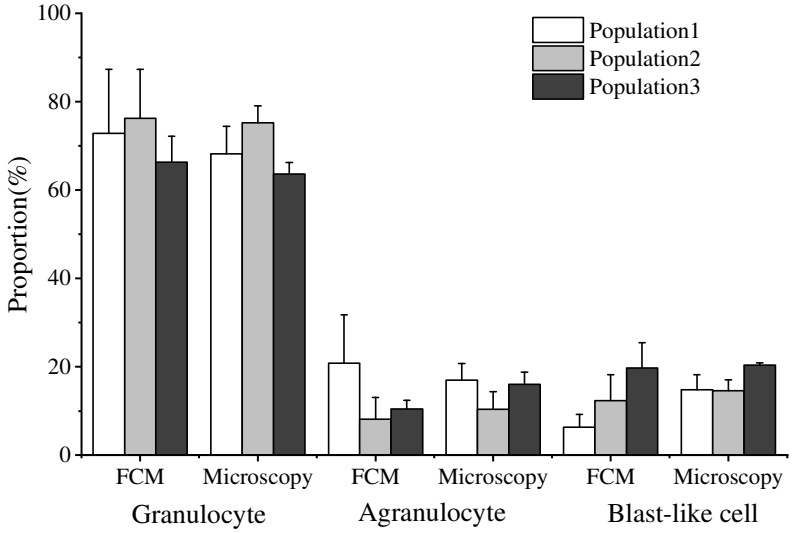

Figure 4. Compositions of different types of hemocytes of *Mercenaria mercenaria* by use of microscopic observation and flow cytometry assay in three different populations (population 1: white bar; population 2: light grey bar, and population 3: black bar).

3.3. Experiment III: Viability assays by use of FCM

Based on the FCM FL1/FL2 plot, the membrane integrated hemocyte population and membrane disrupted hemocyte population were distinctive (**Figure 5A**). The viabilities of hemocytes in clams from P1, P2, and P3 were 87 ± 8% (70 to 97%, n = 20), 89 ± 4%, (78 to 96%, n=20), and 87 ± 5% (79% to 96%, n=20). No differences in the viabilities measured by FCM among the clam populations ($p \geq 0.106$). Overall, the viability of hemocytes from combining of three clam populations was 88 ± 6% ranging from 70 to 97%.

For protocol effectiveness test, viability measurements in the five pre-set groups (100%, 75%, 50%, 25%, and 0% fresh hemocytes) were 77 ± 11%, 59 ± 8%, 40 ± 9%, 22 ± 8% and 3 ± 3%, respectively. After normalizing the data by consider the viability (77 ± 11%) in the 100% fresh hemocyte as 100% viability, the viabilities after transformation in 75%, 50%, and 25% fresh hemocyte groups were 77%, 52%, and 29%, which matched with the pre-set expected viability ($p \geq 0.424$) (**Figure 5B**).

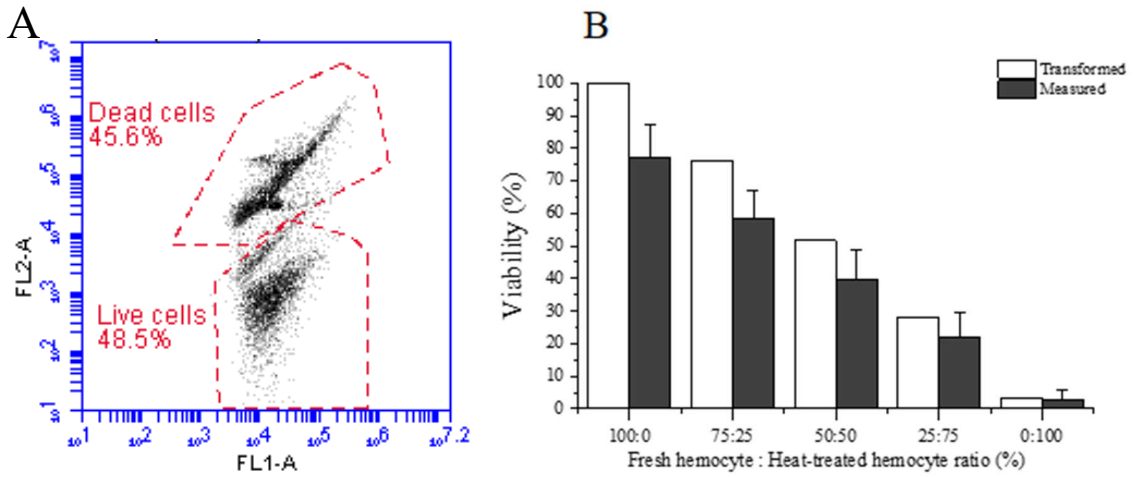

Figure 5. Viability assay of hemocyte in *Mercenaria mercenaria* based on plasm membrane integrity by double staining of SYBR green I (100× final concentration) and propidium iodide (4 µg/ml final concentration). **A.** FL1 vs. FL2 density plot of flow cytometry assay showed membrane integrated cells stained by SYBR green I and membrane disrupted cells stained by propidium iodide and SYBR green I. **B.** Hemolymph sample was heated at 80°C in water bath for 1 min to produce dead hemocytes. By mixing different proportions of the heat treated and fresh hemolymph samples, five groups were generated with known heat-treated proportions: 1) 0%; 2) 25%; 3) 50%; 4) 75%, and 5) 100%. The actual viability measurements by use of flow cytometry (black column) from these samples were transformed by considering the 0% heat-treated (100% fresh hemocytes) as 100%.

3.4. Experiment IV: Phagocytosis analysis by use of FCM

Between the two bead concentrations, the phagocytosis rate did not show significant differences at each incubation time point ($p \geq 0.110$) (**Figure 6**). Among different incubation times, no significant differences were found within the concentrations of 2×10^6 beads/ml group ($p \geq 0.600$) or 5×10^6 beads/ml group ($p \geq 0.490$). The highest phagocytosis rate in the 2×10^6 beads/ml group was 12 ± 8% at 2 h of incubation; the highest phagocytosis rate in the 5×10^6 beads/ml

group was 10 ± 8 % at 3 h of incubation. The lowest phagocytosis rates were 4% (2×10^6 beads/ml group) and 3% (5×10^6 beads/ml group) after 6h of incubation.

Among the three clam populations, no significant differences ($p \geq 0.380$) were found in the mean phagocytosis capacity of hemocytes: 10 ± 4% ranging from 5-21% in P1; 10 ± 4% ranging from 5-18% in P2, and 9 ± 6% ranging from 1-21% in P3.

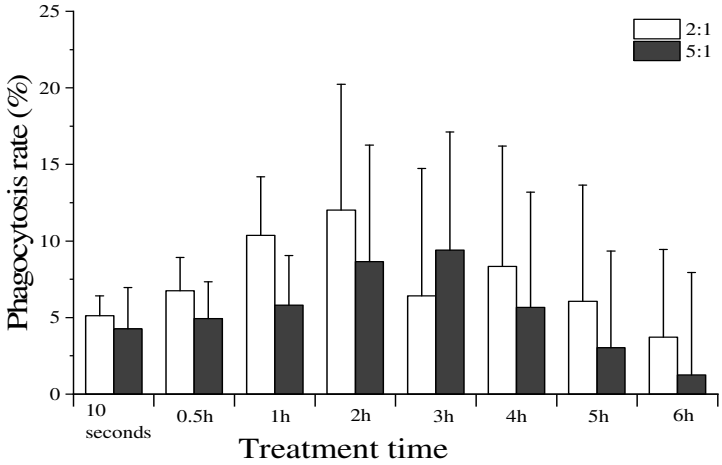

Figure 6. Phagocytosis rate of hemocyte in *Mercenaria mercenaria* (1×10^6 cells/ml) after incubated with florescence bead at concentrations of 2×10^6 or 5×10^6 beads/ml for different time periods at room temperature (22°C) in dark.

4. Discussion

4.1. Development of non-lethal hemocyte collection method

Non-lethal hemolymph collection can allow the animals alive for repeated bleeding or other research use. For molluscan bivalves, non-lethal hemolymph sampling for disease diagnosis or immunological assays is commonly performed by mechanically drilling or notching of shells, such as oysters and clams [30], prising and wedging two shells, such as freshwater mussels [31] or pearl oysters, or chemical anesthesia [32]. Overall, hemolymph withdrawal has been reported from adduct muscle (posterior or anterior), ventricle, pedicard, or pallial space depending the anatomy structure [33]. To elucidate the hemolymph perfusing locations, a 3-D visualization described the vascular-related anatomy in *Mytilus edulis* for hemolymph sampling, and adduct muscle was showed to be a suitable location for hemolymph sampling.

In this study, we observed the shell structure of the northern quahog and noticed that there is a narrow opening in the ligament area. This characteristic of shell morphology offers an opportunity to piercing the needle through the ligament to reach the posterior adduct muscle for hemolymph sampling. Therefore, without physical notching and drilling or chemical anesthesia, we developed a method for non-lethal hemolymph collection, and measurements of the angle to position needles through ligament were found no correlations with clam sizes, indicating that this method can work well regardless of clam sizes. The survival testing verified that this non-lethal hemolymph collection method did not cause significant mortality of clams. A similar technique has been used for hemolymph collection "from anterior adduct muscle through the hinge" in *Macrocallista nimbosa* [21], but no detailed quantified protocol was developed. Possibly, the non-lethal method for hemolymph collection developed in this study could be applied to other Veneridae species as

long as there is an opening in the ligament area. Based on our experience in using this method, cautions should be made on 1) sterilization of the needles; 2) adjustment of needle length and gauge size when working with different sizes of clams, and 3) no more than three times of piercing needles in for sampling.

4.2. Serum pH and osmolality and hemocyte agglutination

To prevent from agglutination of hemocytes, an anti-agglutination solution is required to dilute the hemolymph immediately after collection. Hemocyte activation and agglutination were found to involve in two intracellular signal transduction pathways, the CAMP pathway and the phospholipid/Ca^{2+} pathway [34]. Therefore, EDTA at concentrations of more than 0.6 mM were found to inhibit hemocyte from aggregation in *Mytilus californianus* [35] and *Crassostrea gigas* [4]. Based on one early study on anti-agglutination of human blood cells by the Alsever's solution [25], modification Alsever's solution (MAS) was made for marine organisms and has been used for the prevention of hemocyte agglutination in *Crassostrea gigas* [4], pearl oyster *Pteria hirundo* [36], and *Crassostrea rhizophorae* [9]. Temperature was another factor to prevent hemocyte agglutination [4]. Therefore, cold seawater (or artificial seawater) have been used for dilution of hemolymph samples to inhibit hemocyte agglutination [4]. In the current study, MAS at 1:1 ratio was chosen for sample dilution to prevent hemocyte agglutination. This decision was made based on our pilot studies, in which cold artificial seawater (with and without EDTA), cold 0.1 M phosphate buffer, Hanks' balanced salt solution without Ca^{2+}, and the MAS solution were used to dilute hemolymph samples at different dilution ratios for making hemocyte slides.

To assure the anti-agglutination solutions not to affect the physicochemical condition of hemolymph, the pH and osmolality of serum were measured seawater in this study and compared with that of the culture seawater. The pH measurement indicated that hard clams have the capability to adjust and maintain the pH value, which agreed with one early study [37] that the homogenized heart of norther quahogs (formerly called *Venus mercenaria*) could resistance and recover pH changes. The pH value can change hemocyte parameters and low pH increased hemocyte size significantly [38]. Osmolality measurement indicated that hemolymph of hard clams had the same osmolality as the culture seawater. Similar results were obtained in other molluscan bivalves, such as *Crassostrea virginica* and *Perna viridis*, and the acclimating capability was highly correlated to the salinity tolerance [39], and could be different depending on species. Based on the measurements of hemolymph pH and osmolality, the MAS buffer was formulated accordingly with the same pH and osmolality to assure no changes after anti-agglutination dilution in this study. It is worth noting that if the sweater salinity changes in the culture system, the osmolality of MAS buffer for hemolymph dilution may be required to change accordingly. Further investigations are warranted for physiochemical changes of hemocytes with environmental salinity changes.

4.3. Immunological assays by use of FCM

Hemocyte morphology in hard clams was first observed microscopically as amoebocytes in one cytochemistry study [16]. Later, light microscopic observation revealed three types of hemocytes, namely granulocytes, fibrocytes, and hyalinocytes [17]. These three types of hemocytes were further defined with electron microscopic observation, and fibrocytes were suggested as granulocytes at the terminal phase of their physiologic cycle due to the degradation of phagocytized materials [18]. In the current study, microscopic observation and FCM analysis were employed and compared for hemocyte type and cell count. Microscopic observation revealed three

types of hemocytes: granulocytes (1 and 2), agranulocytes, and blast-like cells, which matched the three distinctive sub-populations on the FCM FSC/SSC plots with no differences in hemocyte composition. This result indicated that FCM could be used to replace microscopy observation to identify hemocyte types in a fast and accurate way, and accordingly hemocyte cell types could be quantified and linked to their specific immune functions, such as phagocytosis, by use of FCM. Similar results were found in other molluscan bivalve species, such as *Crassostrea gigas* [40]. Classification of hemocytes has been studied in many molluscan bivalve species with varied results, and several comprehensive reviews have been published with summaries [41]. Readers interested in this aspect are referred to the review publications and hemocyte classification in other bivalves [9].

Viability based on membrane integrity by double staining of SYBR Green I and PI is a commonly used FCM assay [42] and has been applied in hemocyte viability test in molluscan bivalves such as *Macrocallista nimbosa* in response to salinity changes [21] and Manila Clam *Ruditapes philippinarum* in response to air exposure [43]. The mechanism for this assay is straightforward based on the membrane penetration of SYBR Green I and PI, and based on manufacturer's recommendation and published reports, the suitable staining concentration and staining time were determined in our pilot study. Specifically, the reliability of this assay was confirmed by comparing expected known viability and the actual measurement using FCM measurement. As the frontline of immune system, hemocyte viability was the basic assay to evaluate the response to environmental stresses.

Phagocytosis has been considered as an essential defense mechanism of immune response to pathogens among all eukaryote organisms, especially for invertebrates. Phagocytosis is a diverse physiological process, including recognition of endogenous foreign particles or pathogens by phagocyte surface receptor, uptake and engulfing into a plasma-membrane phagosome, signaling cascade to generate phagolysosome, fusion of phagosome with lysosomes, and degrading and cleared by the hydrolytic enzymes [44]. Phagocytosis is one of the most important immune activities of hemocytes in almost all organisms, including mammals. In the current study, phagocytosis capacity was defined using fluorescence plastic beads and cytochalasin B as an inhibitor of cell phagocytosis. Similar method has been used on hemocyte phagocytosis analysis in *Crassostrea virginica* [29] and *Mytilus californianus* [35]. The concentrations of fluorescence beads used in this study were at ratios of 2:1 and 5:1 to the hemocyte concentrations. Theoretically, bead concentration could affect phagocytosis, but in this study, no differences were found between the two tested bead concentrations. Considering the expensive cost of fluorescence beads, concentration of beads at a ratio of 2:1 to hemocytes would be suitable for the phagocytosis analysis. In other molluscan species, the concentration of beads for phagocytosis assays were 5 $\times 10^6$/ml in *Crassostrea virginica* [12] and 10 $\times 10^6$/ml in *Chamelea gallina* [45]. However, based on our observation, bead solution at 10 $\times 10^6$/ml was becoming viscous for pipetting.

Cytochalasin B is a cell-permeable mycotoxin and can block the formation of contractile microfilaments to inhibit cytoplasmic division. Therefore, cytochalasin B has been widely used to inhibit polar body release in fertilized eggs for polyploid production in mollusks at a concentration of 0.25-1 μg/mL [46]. As a phagocytosis inhibitor, a concentration of 10 μg/mL cytochalasin B (over 10 times more) has been used to inhibit actin polymerization to measure the phagocytosis of hemocytes in *Crassostrea virginica* [12, 28, 29]. In the current study, we used the same concentration of cytochalasin B as a negative control without further estimation.

Incubation time at 10 seconds, 0.5h, 1h, 2h, 3h, 4h, 5h, and 6h did not show significant differences in phagocytosis at either tested bead concentrations in this study. This result was

somehow beyond our expectation because the first measurement was performed immediately (in 10 seconds) after mixing hemocytes and beads, indicating that the hemocytes were responded immediately to phagocyte the beads. Although not statistically significant, the highest phagocytosis capacity showed at 2 h after incubation. Therefore, an incubation time of 2 h is recommended for phagocytosis analysis considering phagocytosis research in other molluscan species such as *Crassostrea ariakensis* [27]. This recommended incubation time will also provide a suitable time window for researcher to do other FCM assays, such as viability assays, to make multiple assays from one single hemolymph sample.

In the present study, phagocytosis capacity of 60 clam hemocytes was 10 ± 4%, similar to the measurement in *Mya arenaria* [28]. However, previous research of *M. mercenaria* showed a mean phagocytosis capacity of 35 ± 1% [47]. The possible reason might be the different resources of working animals. As the leading mechanism of internal defense in bivalves, phagocytosis rate can be affected greatly by environmental conditions. Among the three clam populations, no significant differences were found, indicating that the assay methodology was effective for evaluation of phagocytosis capacity of hemocytes [28].

5. Conclusions

Overall, this study reported the basic characterization of hemolymph of the northern quahog. One non-lethal hemolymph collection method was developed and verified. Same as the previous reports, three types of hemocytes, granulocytes, fibrocytes, and hyalinocytes, were identified by microscopic observation and FCM analysis. It was verified that hemocyte concentration and cell types could be recorded by FCM simultaneously with other assays. Viability assay based on membrane integrity by use of double stains of SYBR green I and PI was developed and verified. Phagocytosis assay, which was considered as the major immune function of granular hemocytes in bivalves [41], was developed by use of fluorescence beads.

The current study provided the basis for immunological assays of hemocytes in the northern quahog by use of FCM including sample collection/dilution, cell count, cell type, viability, and phagocytosis. With the fast development of FCM technology and fluorescent probes, more hemocyte immune assays would be developed rapidly for aquaculture molluscan bivalves, such as reactive oxygen species production, lysozyme activity, cell apoptosis, cell cycle, protein expression, and gene expression. It is expected that FCM assays could serve as useful biomarkers to evaluate environmental stresses for aquaculture breeding and operation management.

ACKNOWLEDGEMENTS

This study was supported by funds from the National Sea Grant Aquaculture Initiative Award (NA18OAR4170344) and the National Institute of Food and Agriculture, United States Department of Agriculture (Hatch project FLA-FOR-005385). This study was partly supported by the Gulf States Marine Fisheries Commission (No. ACQ-210-039-2019-USM - Gulf of Mexico Oyster Genetics and Breeding Research Consortium Project).

REFERENCES

[1] USDA, 2017 Census of Agriculture: Census of Aquaculture, Special Study Part 2, 2018, p. 96.

[2] H. Yang, L.N. Sturmer, S. Baker, Molluscan shellfish aquaculture and production, http://edis.ifas.ufl.edu/fa191, http://edis.ifas.ufl.edu/fa191, Gainesville: University of Florida, Institute of Food and Agricultural Sciences, 2016, p. 8.

[3] K.K. Chew, Introduction of the hard clam (*Mercenaria mercenaria*) to the Pacific coast of North America with notes on its introduction to Puerto Rico, England, and France, in: J.N. Kreaeuter, M. Castagna (Eds.), Biology of the Hard Clam, Elsevier Science B. V., Amsterdam, The Netherlands, 2001, pp. 701-709.

[4] M. Auffret, R. Oubella, Hemocyte aggregation in the oyster *Crassostrea gigas*: In vitro measurement and experimental modulation by xenobiotics, Comp Biochem Physiol Part A Mol Integr Physiol 118(3) (1997) 705-712.

[5] L. Song, L. Wang, L. Qiu, H. Zhang, Bivalve Immunity, in: K. Söderhäll (Ed.), Invertebrate Immunity, Springer US, Boston, MA, 2010, pp. 44-65.

[6] L. Donaghy, C. Lambert, K.S. Choi, P. Soudant, Hemocytes of the carpet shell clam (*Ruditapes decussatus*) and the Manila clam (*Ruditapes philippinarum*): Current knowledge and future prospects, Aquaculture 297(1-4) (2009) 10-24.

[7] P.G. Beninger, G. Le Pennec, M. Le Pennec, Demonstration of nutrient pathway from the digestive system to oocytes in the gonad intestinal loop of the scallop *Pecten maximus* L., Biol Bull 205(1) (2003) 83-92.

[8] V. Matozzo, L. Ballarin, D.M. Pampanin, M.G. Marin, Effects of copper and cadmium exposure on functional responses of hemocytes in the clam, *Tapes philippinarum*, Arch Environ Contam Toxicol 41(2) (2001) 163-170.

[9] M.D. Rebelo, E.D. Figueiredo, R.M. Mariante, A. Nobrega, C.M. de Barros, S. Allodi, New insights from the oyster *Crassostrea rhizophorae* on bivalve circulating hemocytes, Plos One 8(2) (2013) e57384.

[10] W.H. Coulter, High speed automatic blood cell counter and cell size analyzer, Proceedings of the National Electronics Conference, 1956, p. 1034.

[11] G. Boeck, Current status of flow cytometry in cell and molecular biology, Int Rev Cytol 204 (2001) 239-298.

[12] M.R. Alvarez, F.E. Friedl, J.S. Johnson, G.W. Hinsch, Factors affecting invitro phagocytosis by oyster hemocytes, J Invertebr Pathol 54(2) (1989) 233-241.

[13] T.V. Nguyen, A.C. Alfaro, Applications of flow cytometry in molluscan immunology: Current status and trends, Fish Shellfish Immun 94 (2019) 239-248.

574 [14] C.W. Cunningham, A. Mukhopadhyay, G.H. Lushington, B.S.J. Blagg, T.E. Prisinzano, J.P.
575 Krise, Uptake, distribution, and diffusivity of reactive fluorophores in cells: Implications toward
576 target identification, Mol Pharmaceut 7(4) (2010) 1301-1310.

577 [15] S.P. Perfetto, P.K. Chattopadhyay, M. Roederer, Seventeen-colour flow cytometry:
578 Unravelling the immune system, Nature Reviews Immunology 4(8) (2004) 648-655.

579 [16] S.I. Zacks, The cytochemistry of the amoebocytes and intestinal epithelium of *Venus*
580 *mercenaria* (lamellibranchiata),with remarks on a pigment resembling ceroid, Q J Microsc Sci
581 96(33) (1955) 57-71.

582 [17] D.A. Foley, T.C. Cheng, Morphology, hematologic parameters, and behavior of hemolymph
583 cells of the quahog clam, *Mercenaria mercenaria*, Biol Bull 146(3) (1974) 343-356.

584 [18] T. Cheng, D.A. Foley, Hemolymph cells of the bivalve mollusc *Mercenaria mercenaria:* An
585 electron microscopical study, J Invertebr Pathol 26(3) (1975) 341-351.

586 [19] D.A. Bugge, H. Hegaret, G.H. Wikfors, B. Allam, Oxidative burst in hard clam (*Mercenaria*
587 *mercenaria*) haemocytes, Fish Shellfish Immun 23(1) (2007) 188-196.

588 [20] H. Hégaret, R.M. Smolowitz, I. Sunila, S.E. Shumway, J. Alix, M. Dixon, G.H. Wikfors,
589 Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum minimum* on northern
590 quahogs, *Mercenaria mercenaria*, Mar Environ Res 69(5) (2010) 337-344.

591 [21] C. Jauzein, L. Donaghy, A.K. Volety, Flow cytometric characterization of hemocytes of the
592 sunray venus clam *Macrocallista nimbosa* and influence of salinity variation, Fish Shellfish
593 Immun 35(3) (2013) 716-724.

594 [22] M. Perrigault, S.F. Dahl, E.P. Espinosa, L. Gambino, B. Allam, Effects of temperature on
595 hard clam (*Mercenaria mercenaria*) immunity and QPX (Quahog Parasite Unknown) disease
596 development: II. Defense parameters, J Invertebr Pathol 106(2) (2011) 322-332.

597 [23] X. Guo, Y. He, L. Zhang, C. Lelong, A. Jouaux, Immune and stress responses in oysters
598 with insights on adaptation, Fish Shellfish Immun 46(1) (2015) 107-119.

599 [24] X. Zhang, J. Shi, Y. Sun, Y.J. Habib, H. Yang, Z. Zhang, Y. Wang, Integrative transcriptome
600 analysis and discovery of genes involving in immune response of hypoxia/thermal challenges in
601 the small abalone *Haliotis diversicolor*, Fish Shellfish Immun 84 (2019) 609-626.

602 [25] J.B. Alsever, R.B. Ainslie, A new method for the preparation of dilute blood plasma and the
603 operation of a complete transfusion service., N. Y. State J Med 41 (1941) 126-131.

604 [26] F. Cima, V. Matozzo, M.G. Marin, L. Ballarin, Haemocytes of the clam *Tapes philippinarum*
605 (Adams & Reeve, 1850): Morphofunctional characterisation, Fish Shellfish Immun 10(8) (2000)
606 677-693.

607 [27] L. Donaghy, B.K. Kim, H.K. Hong, H.S. Park, K.S. Choi, Flow cytometry studies on the
608 populations and immune parameters of the hemocytes of the Suminoe oyster, *Crassostrea*

609 *ariakensis*, Fish Shellfish Immun 27(2) (2009) 296-301.

610 [28] M.T. Araya, A. Siah, D.R. Mateo, F. Markham, P. McKenna, G.R. Johnson, F.C.J. Berthe,
611 Morphological and molecular effects of *Vibrio splendidus* on hemocytes of softshell clams, *Mya*
612 *arenaria*, J Shellfish Res 28(4) (2009) 751-758.

613 [29] H. Hégaret, G.H. Wikfors, P. Soudant, Flow cytometric analysis of haemocytes from eastern
614 oysters, *Crassostrea virginica*, subjected to a sudden temperature elevation II. Haemocyte
615 functions: aggregation, viability, phagocytosis, and respiratory burst, J Exp Mar Biol Ecol 293(2)
616 (2003) 249-265.

617 [30] D.W. Howard, C.S. Smith, Histological Techniques For Marine Bivalve Mollusks, NOAA
618 Technical Memorandum NOS NCCOS 51983.

619 [31] L.L. Gustafson, M.K. Stoskopf, A.E. Bogan, W. Showers, T.J. Kwak, S. Hanlon, J.F. Levine,
620 Evaluation of a nonlethal technique for hemolymph collection in *Elliptio complanata*, a
621 freshwater bivalve (Mollusca : Unionidae), Dis Aquat Organ 65(2) (2005) 159-165.

622 [32] H. Yang, J. Supan, X. Guo, T.R. Tiersch, Nonlethal sperm collection and cryopreservation in
623 the Eastern oyster *Crassostrea virginica*, J Shellfish Res 32(2) (2013) 429-437.

624 [33] M. Eggermont, P. Cornillie, M. Dierick, D. Adriaens, N. Nevejan, P. Bossier, W. Van den
625 Broeck, P. Sorgeloos, T. Defoirdt, A.M. Declercq, The blue mussel inside: 3D visualization and
626 description of the vascular-related anatomy of *Mytilus edulis* to unravel hemolymph extraction,
627 Sci Rep 10(1) (2020) 16.

628 [34] J. Chen, Cell activation model of hemocyte aggregation and adhesion in the California
629 mussel, *Mytilus californianus*, Oregon State University, 1992, p. 160.

630 [35] J. Chen, C.J. Bayne, Bivalve mollusc hemocyte behaviors: Characterization of hemocyte
631 aggregation and adhesion and their inhibition in the california mussel (*Mytilus californianus*),
632 Biol Bull 188(3) (1995) 255-266.

633 [36] G.C. Vieira, P.M. da Silva, M.A. Barracco, A.F. Hering, M.C.P. de Albuquerque, J.D.
634 Coelho, E.C. Schmidt, Z.L. Bouzon, R.D. Rosa, L.M. Perazzolo, Morphological and functional
635 characterization of the hemocytes from the pearl oyster *Pteria hirundo* and their immune
636 responses against *Vibrio* infections, Fish Shellfish Immun 70 (2017) 750-758.

637 [37] S.I. Zacks, J.H. Welsh, Cholinesterase and lipase in the amoebocytes, intestinal epithelium
638 and heart muscle of the quahog, *Venus mercenaria*, Biol Bull 105(1) (1953) 200-211.

639 [38] Q. Xue, T. Renault, S. Chilmonczyk, Flow cytometric assessment of haemocyte
640 sub-populations in the European flat oyster, *Ostrea edulis*, haemolymph, Fish Shellfish Immun
641 11(7) (2001) 557-567.

642 [39] K. McFarland, L. Donaghy, A.K. Volety, Effect of acute salinity changes on hemolymph
643 osmolality and clearance rate of the non-native mussel, *Perna viridis*, and the native oyster,
644 *Crassostrea virginica,* in Southwest Florida, Aquat Invasions 8(3) (2013) 299-310.

[40] A.Y. Andreyeva, E.S. Kladchenko, O.Y. Vyalova, T.A. Kukhareva, Functional characterization of the Pacific oyster, *Crassostrea gigas* (bivalvia: ostreidae), hemocytes under normoxia and short-term hypoxia, Turk J Fish Aquat Sc 21(3) (2021) 125-133.

[41] P.M. Hine, The inter-relationships of bivalve haemocytes, Fish Shellfish Immun 9(5) (1999) 367-385.

[42] A. Nescerecka, F. Hammes, T. Juhna, A pipeline for developing and testing staining protocols for flow cytometry, demonstrated with SYBR Green I and propidium iodide viability staining, J Microbiol Methods 131 (2016) 172-180.

[43] K.I. Park, L. Donaghy, H.S. Kang, H.K. Hong, Y.O. Kim, K.S. Choi, Assessment of immune parameters of Manila clam *Ruditapes philippinarum* in different physiological conditions using flow cytometry, Ocean Sci J 47(1) (2012) 19-26.

[44] R.S. Flannagan, V. Jaumouille, S. Grinstein, The Cell Biology of Phagocytosis, Annu Rev Pathol-Mech 7 (2012) 61-98.

[45] V. Matozzo, M. Monari, J. Foschi, G.P. Serrazanetti, O. Cattani, M.G. Marin, Effects of salinity on the clam *Chamelea gallina*. Part I: Alterations in immune responses, Mar Biol 151(3) (2007) 1051-1058.

[46] H. Yang, X. Guo, J. Scarpa, Tetraploid induction and establishment of breeding stocks for all-triploid seed production, https://edis.ifas.ufl.edu/publication/FA215 2019(3) (2019) 8.

[47] H. Hégaret, P.M. da Silva, G.H. Wikfors, H. Haberkorn, S.E. Shumway, P. Soudant, In vitro interactions between several species of harmful algae and haemocytes of bivalve molluscs, Cell Biol Toxicol 27(4) (2011) 249-266.