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Characterization of hemocytes from different body fluids of the eastern oyster *Crassostrea virginica*

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ABSTRACT

Bivalve hemocytes are involved in a variety of physiological and immunological functions. Circulating hemocytes in the hemolymph represent the main component of the internal self-defense system while hemocytes present in the extrapallial space (between the mantle and the shell) are actively involved in biomineralization and shell formation. This study focused on the characterization of hemocytes from different body fluids of the eastern oyster *Crassostrea virginica*. Hemocytes present in the hemolymph were compared to those contained in the extrapallial fluid. Hemocytes associated with the mucus layer covering pallial organs (mantle, gills, body wall) were also investigated because of their potential role as sentinel cells. Hemocytes were characterized using flow cytometry in conjunction with fluorescent epitope markers (clusters of differentiation, lectins) as well as functional assays (i.e. phagocytosis and reactive oxygen species -ROS). Compared with the hemolymph, there was a significantly greater percentage of granulocytes and agranulocytes among extrapallial and pallial hemocytes, respectively. Accounting for the different percentages of hemocyte sub-populations, significant differences in surface carbohydrate and clusters of differentiation signatures were also revealed between the different fluids. Most informative epitope markers included concanavalin A, peanut agglutinin, soybean agglutinin, CD11b and CD14. Functional assays revealed significant differences in phagocytic activity and ROS production between hemocytes from the extrapallial fluid and hemolymph; however, less robust differences were observed between hemolymph cells and hemocytes associated with the pallial mucus. Findings from this study suggest that there are markedly different hemocyte populations in the three body fluids. The role of peripheral cells, particularly those associated with the pallial mucus, requires further investigations.

1. Introduction

Bivalves, like most mollusks, have an open circulatory system populated by hemocytes that circulate in hemolymph vessels and sinuses as well as throughout soft tissues. In these animals, hemocytes are involved in a variety of biological functions such as phagocytosis of invading microbes and encapsulation of larger or refractory invaders, wound healing, food digestion and transport of nutrients, reproduction, excretion, shell formation, and production and secretion of humoral factors [1–3]. Circulating hemocytes in the hemolymph have been the subject of many investigations and were shown to represent the main component of the internal self-defense system [3–7]. In contrast, hemocytes present in the extrapallial space (between the mantle and the shell) are involved in shell biomineralization in addition to immunological functions [8–13]. Virtually all previous investigations assessing bivalve cellular immunity focused on hemocytes present in the circulatory system, and much less information is available on hemocytes present in other body fluids (e.g. extrapallial fluid) or those resident in soft tissues. There is a rising need to address these shortcomings given the growing body of information on the importance of peripheral components of the immune system in the maintenance of microbial homeostasis and health. In particular, the role of immune components associated with mucosal tissues in health maintenance in mollusks remain largely unknown even though there is growing evidence suggesting these host-environment interfaces play an important role in the outcome of early host-pathogen interactions [1,14,15]. Mucosal tissues are often well-irrigated by the blood due to their primary role in exchanges with the surrounding environment for processes such as oxygen or nutrient extraction, and connective tissues of the gills and sub-epithelial tissues along the digestive tract are among the most hemocyte-rich tissues in mollusks. These hemocytes are able to cross the basement membrane and wander at the surface of the epithelial barrier in close association with the mucus layer covering the surface of these organs [1,8,16–18]. This transepithelial migration of immune

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cells is well described in vertebrates where respiratory and gastrointestinal mucus contains representatives of innate and adaptive immune cells such as neutrophils and dendritic cells that cross epithelial surfaces to interact with environmental microbes and mount an appropriate host response [19]. In bivalve mollusks, the pioneering works by Takatsuki [17] and Yonge [18] were the first to report the presence of hemocytes associated with mucosal secretions covering the pallial cavity and in the gut lumen, respectively. More recently, transepithelial migration of hemocytes was reported in the clam *Ruditapes philippinarum* [20] and the oyster *Crassostrea virginica* [16] where hemocytes were found associated with mucus covering pallial organs (gills, mantle). Hemocytes lining pallial epithelia are in direct contact with environmental microbes present in the shell cavity, therefore one hypothesis is that they may play a sentinel role providing a first alert system.

Several hemocyte types were previously reported in *C. virginica* based on cell size and the presence and abundance of cytoplasmic granules. These include granulocytes, agranulocytes (otherwise known as hyalinocytes), intermediate hemocytes and small granulocytes [6,21–23]. Granulocytes are highly mobile with macrophage-like characteristics and are generally more phagocytic than agranulocytes. They also produce reactive oxygen species (ROS) and a wide range of hydrolytic enzymes [6,21,22,24]. In contrast, agranulocytes are cells that have little-to-no granulation, but are important in the early inflammatory response due to their ability to infiltrate wounds, phagocytize and sequester cellular debris and foreign particles [6,7,25]. Morphological features of hemocytes do not offer sufficient resolution to unravel the functional differentiation of various types of blood cells. For this reason, previous studies in vertebrates and invertebrates have used a wide range of methods to "phenotype" various types of blood cells. In many instances, these methods include the use of specific carbohydrate-binding proteins (lectins) or antibodies (e.g. cluster of differentiation or CD) that recognize specific patterns associated with blood cells. While lectins are commonly used to distinguish carbohydrate surface epitopes that may differ between different cell types, CDs are functional molecules dispersed on the cell membrane and are used as targets for specific antibodies to immunotype and identify different leukocyte types in vertebrates. Interestingly, several CDs have been shown to be conserved throughout evolution and antibodies raised against vertebrate targets are reactive against homolog invertebrate proteins [26–30]. For these reasons, both lectins and CDs have been successfully used to characterize hemocytes in various invertebrate species [26–32].

This study was designed to characterize and contrast hemocytes present in different body fluids of the oyster *C. virginica*. Hemocytes collected from the extrapallial fluid were characterized and compared to those present in the circulatory system and to hemocytes associated with the pallial mucus. Morphological, physiological and cell surface epitope profiles were determined in an aim to provide a better understanding of the function of hemocyte subpopulations in each locus.

2. Material and methods

2.1. Oysters

Adult *C. virginica* (90–100 mm in length) were obtained from Frank M. Flower and Sons (Oyster Bay, NY). Upon arrival, oysters were stripped of debris and fouling organisms. Oysters were acclimated in aerated seawater (salinity 28 ppt, 23 °C) for 7–10 days prior to the experiment and were fed daily with a commercial diet (DT's Live Marine Phytoplankton).

2.2. Collection of hemocytes

Hemocytes from the 3 different compartments (pallial surfaces, circulatory system and extrapallial space) were investigated. The extrapallial fluid, defined as the fluid found between the external epithelium of the mantle and the inner shell, was collected using a micropipette through a hole drilled into the center of the left valve and diluted immediately with ice-cold filtered seawater (FSW, salinity 28 ppt) [8]. Care was taken to avoid breaching the underlying mantle tissue. Hemocytes associated with the pallial mucus (i.e. pallial hemocytes) were collected via a notch created on the edge of the shell. Water in the pallial cavity was drained and the mucosal surfaces lining the cavity were washed gently with FSW twice to remove debris before refilling the cavity with FSW for a final wash and collection of the fluid. Oysters were gently agitated prior to being drained again for fluid collection. Wash fluid was compared to collected fluid via microscopy in preliminary assays to confirm low loss of hemocytes. The hemolymph, which contained circulatory hemocytes, was collected using a 26-gauge needle from the adductor muscle through the notch and diluted to a 2:1 ratio of FSW to hemolymph. Fluids from 16 individual oysters were utilized for hemocyte lectin analysis. Four out of these 16 individuals were used for CD analysis (see below). Fluids from 20 different individual oysters were analyzed for morphology. ROS production, viability, and phagocytosis were analyzed on another 20 individual oysters. All samples were placed on ice immediately after collection.

2.3. Flow cytometry

Morphological and functional characterization of hemocytes was completed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences). FSW (salinity 28 ppt) was used as the sheath fluid. Fluorescence, forward and side scatter data were collected for 10,000 cells for each sample.

2.4. Hemocyte morphology and viability

Aliquots of collected diluted hemocytes (300 μl) from each fluid were stained with ethidium homodimer (1 μM) and calcein AM (1 μM) to determine the proportion of dead and live cells, respectively. Samples and dye were combined and incubated on ice in the dark for 30 min and immediately processed through flow cytometry. Based on previous studies, four subpopulations of hemocytes were defined using the freeware WinMDI based on differences in forward scatter (size) and side scatter (granularity) [23,33]. Samples were gated such that debris (low forward and side scatters) and dead cells (negative for calcein AM and positive for ethidium homodimer) were excluded from the analysis [23,34]. The percent of these sub-populations compared to the entire gated sample was recorded. Statistical analysis was performed to compare differences in cell sub-populations between the extrapallial, pallial and hemolymph samples.

2.5. Functional assays: hemocyte phagocytic activity and ROS production

Functional assays were performed on hemocytes from the different fluids following previously described methods using flow cytometry. Phagocytic activity was measured using 2 μm fluorescent latex beads as described in Refs. [35] and [23] with a 100 bead to hemocyte ratio. ROS (H_2O_2) production was measured using dichlorofluorescein diacetate (DCFH-DA) (10 mM final concentration) as adapted from Perrigault et al. [36] and Goedken and De Guise [25]. Briefly, aliquots of hemocytes (300 μl) from each fluid were incubated with DCFH-DA on ice in the dark for 20 min and green fluorescence signals (indicative of ROS levels) were measured by flow cytometry.

2.6. Lectins and cluster of differentiation (CD)

Fluorescein isothiocyanate (FITC) labeled lectin epitope markers listed in Table 1 (Sigma and Invitrogen) were used to identify differences in cell surface carbohydrates between the different cell types

Table 1
Lectins and CD antibodies used in the study. Previous studies that employed these to characterize hemocytes in invertebrates are also listed.

Lectin[a]	Carbohydrate specificity	Usage in invertebrates
ConA	α-D-mannose and α-D-glucose	[31,39,40]
PNA	galactose- β(1,3)-N-acetylgalactosamine	[41]
SBA	N-acetyl-D-galactosamine	[31]
WGA	N-acetyl-β-D-glucosaminyl and N-acetyl-β-D-glucosamine	[31,39,42]
UEA	L-fucose	[40]
CD	**Vertebrate protein target description (and function)**	
CD14	Co-receptor for TLR (pattern -in particular LPS-recognition receptor)	[26,37]
CD11b	Integrin alpha-M.beta-2 (adhesive interactions)	[26,29,30,37]
CD11c	Integrin alpha-X/beta-2 (fibrinogen receptor, adhesive interactions)	[26,29]
CD16	Fc receptor (bind to Fc portion of immunoglobulins)	[29,30,37]
CD54	Intercellular Adhesion Molecule 1 (facilitates leukocyte transmigration)	[37,38]

[a] ConA: from *Canavalia ensiformis*, PNA: from *Arachis hypogaea*, SBA: from *Glycine max*, WGA: from *Triticum vulgare*, UEA: from *Ulex europeus*.

collected from the 3 fluids. Fluids from 20 oysters were collected as described above and aliquots containing at least 20,000 cells (200 µl circulatory, 200 µl extrapallial, 500 µl pallial) were incubated with each lectin on ice in the dark for 30 min. Flow cytometry data was collected, including forward and side scatters and green fluorescence. The proportion of fluorescently tagged cells was compared between cell subpopulations and between fluids. CDs are listed in Table 1 and were selected based on previous studies that successfully utilized target antibodies to label various invertebrate blood cells [26–30,37,38]. Antibodies against selected CDs labeled with FITC (CD14, CD54, CD16) or R-phycoerythrin (R-PE) (CD11b, CD11c) were also tested alongside the lectin marker experiment for 4 of the oysters. Sample processing followed the antibody manufacturer's recommendation (incubated 30 min, on ice), and data collection was performed as for the fluorescent lectins.

Fluorescent microscopy was used to validate flow cytometry measurements for each assay prior to running the full experiment. Random samples were selected from all experiments and used to microscopically validate and assure that cell surface was fluorescently stained and the background was kept at a minimum. After collection with the flow cytometer, all raw data were analyzed using WinMDI. Statistical analyses of data were performed using SPSS by implementing appropriate parametric and non-parametric tests (see below).

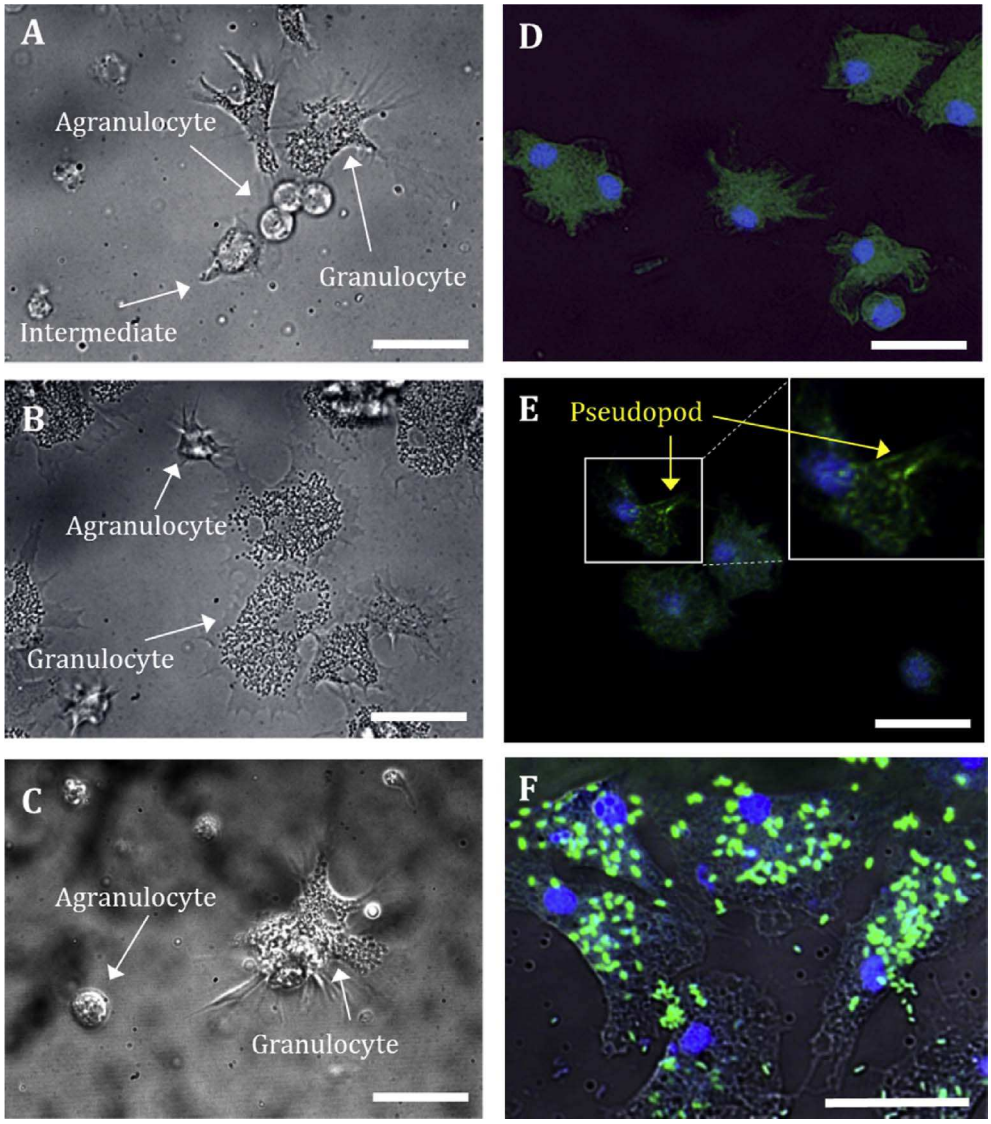

Fig. 1. Phase contrast (A–C) and fluorescence (D–F) microscope images of hemocytes from extrapallial fluid (A), circulatory hemolymph (B, D, E), and mucus associated with pallial surfaces (C and F). Hemocytes labeled with PNA and CD14 are shown in D and E, respectively (note brightly labeled pseudopod in E). F shows phagocytosis of FITC-labeled bacteria (*Vibrio alginolyticus*) by pallial hemocytes. Nuclei (blue) were stained with Hoechst 33342. Scale bar = 20 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.7. Statistics

Data was analyzed through the use of IBM Statistical Package for the Social Sciences (SPSS) software. Morphological data was analyzed by one-way ANOVA ($P < 0.05$). ROS and phagocytosis data was separately analyzed using an index calculated as the product of percentage positive cells and geometric mean (gmean) of fluorescence intensity of these cells (i.e. channel number generated by the flow cytometer). The resulting index was then analyzed using one-way ANOVA with Tukey HSD post-hoc test. To normalize percentage data, the percent of lectin labeled hemocytes was arcsine transformed. PCA factor analysis was used to analyze the results from the multiple lectin epitope markers and discriminant analysis of fluorescent intensity was used to evaluate the data. One-way ANOVA was subsequently used with Tukey HSD post-hoc to analyze individual lectin epitope marker results. CD data was arcsine transformed and one-way ANOVA was performed. Differences in percentage of labeled hemoyctes were considered significant at $P < 0.05$.

3. Results

3.1. Morphological characterization of hemocytes

Microscopic observations confirmed the presence of functional hemocytes in pallial mucus, circulatory- and extrapallial fluid samples (Fig. 1 and Supplementary Fig. S1). Dilution of fluids in cold FSW and storage on ice was sufficient to limit hemocyte aggregation. Flow cytometry profiling of viable hemocytes indicated the presence of 4 subpopulations (agranulocytes, intermediate hemocytes, granulocytes, and small granulocytes) in all three body fluids, in agreement with previous reports of hemocyte make-up in the circulatory system of *C. virginica* [23]. There were significantly different proportions of these subpopulations of hemocytes associated with the three body fluids, with higher proportion of agranulocytes (42%) among hemocytes associated with the pallial mucus as compared to the circulatory (19%) and extrapallial (10%) hemocytes (Fig. 2). In contrast, circulatory hemocytes had significantly higher proportion of intermediate hemocytes (49%) as compared to the extrapallial fluid and pallial mucus (39% each). The highest proportion of granulocytes was measured in the extrapallial fluid (49%) followed by hemolymph (31%) and pallial mucus (19%). Finally, the proportion of small granulocytes was highest (2%) in the extrapallial fluid and lowest (0.2%) in the pallial mucus. However, since small hemocytes made up such a small proportion of the overall population, they were not considered for the subsequent analyses.

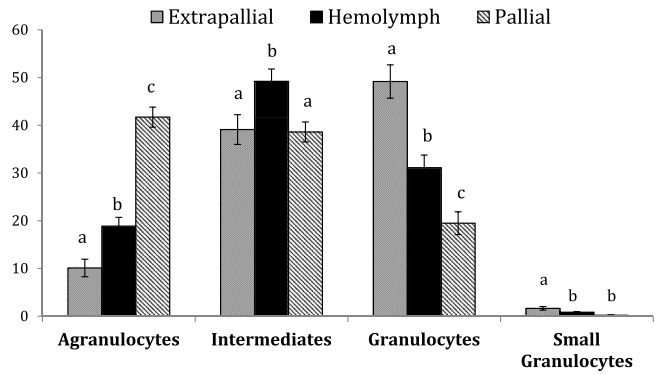

Fig. 2. Percentage of each cell type in each body fluid (mean ± SE). Different letters indicate significant difference in percent of each cell type between fluids (one-way ANOVA Tukey HSD post-hoc test, $P < 0.05$, $n = 20$).

3.2. Lectins

Lectin epitope markers indicated differences in hemocyte surface carbohydrates among the various body fluids. Results of discriminant analysis of lectin binding intensity showed that the hemocytes associated with the three different body fluids are distinctively grouped based on cell surface carbohydrate profiles (Fig. 3). However, given the differences in the percentage of each hemocyte subpopulation among the different body fluids, further analysis by one-way ANOVA for the results of each lectin epitope marker was conducted to contrast the percentage of positive cells within each hemocyte subpopulation. One-way ANOVA (Tukey HSD post-hoc test, $p < 0.05$) analysis indicated that the percentage of cells labeled with concanavalin A (Con A) was significantly greater among agranulocytes from the extrapallial fluid (68%) as compared to those from the circulatory system (26%) and pallial mucus (16%; Fig. 4a). Similarly, the percentage of cells positive for soybean agglutinin SBA was significantly greater in agranulocytes collected from the extrapallial fluid (66%) as compared to those from the circulatory system (29%) and pallial mucus (37%; Fig. 4b). Finally, the percentage of cells positive for peanut agglutinin (PNA) was significantly greater among agranulocytes collected from the extrapallial fluid (80%) as compared to those associated with the pallial mucus (45%, Fig. 3c). No differences were noted from labeling hemocytes with WGA (agranulocytes 66%–90%, intermediate cells 87%–97%, granulocytes 94–99%), or UEA (agranulocytes 24–50%, intermediate cells 54–63%, granulocytes 72–95%).

3.3. CDs

The percentage of cells positive for CD11b was significantly greater among granulocytes collected from the extrapallial fluid (52%) as compared to those from the circulatory system (10%) (Fig. 5a). In parallel, the percent of granulocytes that were positive for CD14 was significantly higher among hemocytes associated with the pallial mucus (70%) as compared to circulatory hemocytes (26%) (Fig. 5b). No significant difference was noted between the three different body fluids for CD11c-(agranulocytes 8%–33%, intermediate cells 17%–39%, granulocytes 28%–45%), CD16-(agranulocytes 16%–55%, intermediate cells 20%–24%, granulocytes 33%–43%) or CD54-positive hemocytes (agranulocytes 36–62%, intermediate cells 46%–68%, granulocytes 56%–78%).

3.4. Functional assays

Phagocytic index was systematically higher in all three hemocyte subpopulations from the pallial mucus as compared to hemocytes derived from the circulatory system or the extrapallial fluid, and differences were significant for agranulocytes and intermediate hemocytes (Tukey HSD post-hoc test, $P < 0.05$; Fig. 6a). ROS production index was significantly higher in granulocytes from the pallial mucus and circulatory system as compared to those from the extrapallial fluid (Fig. 6b).

4. Discussion

This study indicates that hemocytes collected from different anatomical compartments of oysters have different morphological, functional, and cell surface characteristics supporting the idea that they may serve different roles. While the major hemocyte types recognized in eastern oyster (agranulocytes, intermediate hemocytes, granulocytes and small granulocytes) were found in all three investigated compartments (pallial mucus, hemolymph and extrapallial fluid), significant differences in the proportion of each cell type were noted. In particular, hemocytes associated with the pallial mucus consisted of significantly greater proportion of agranulocytes as compared to the hemocytes associated with the hemolymph and the extrapallial fluid. Agranulocytes

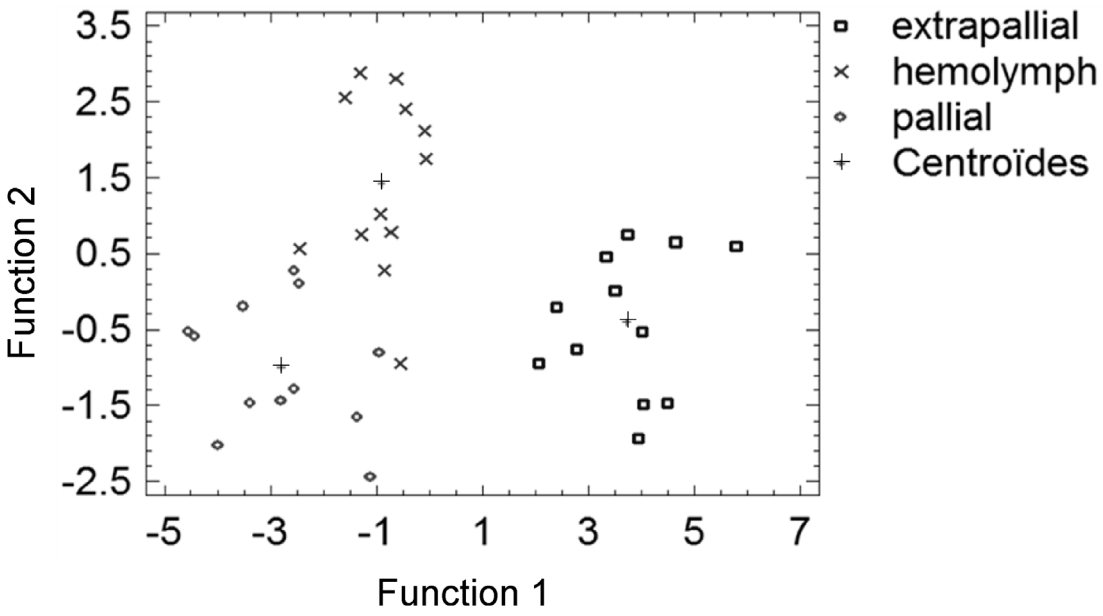

Fig. 3. Discriminant analysis of lectin binding intensity data combining information from the 6 tested lectins.

are important in the early stages of inflammation and can infiltrate wounds and interact with foreign particles. For example, in the Pacific oyster *Crassostrea gigas*, agranulocytes were found to be important in wound plugging [43] and previous reports highlighted the prominent role of agranulocytes in aggregation in the Sydney rock oyster [44]. In *C. virginica*, several studies also reported the role of agranulocytes in encapsulation [45,46]. Since the physical barrier of the pallial surfaces and the overlying mucus are the first line of immune defense against potential pathogens, a greater presence of agranulocytes could assist in the early immune response and quickly plug wounds that may occur on these epithelial tissues. Interestingly, the highest proportion of granulocytes was measured in the extrapallial fluid. Granulocytes are generally more phagocytic than agranulocytes and function similarly to mammalian macrophages [6,7,25]. Previous studies highlighted the role of hemocytes associated with the extrapallial fluid in immunity in the clam *Ruditapes philippinarum* where the resistance against a bacterial disease (brown ring disease) was linked to the abundance of granulocytes in that compartment [47]. In addition to immune functions, granulocytes in the extrapallial fluid were also shown to be involved in calcium carbonate crystal formation and shell mineralization [10]. Therefore, it is not surprising to find high levels of granulocytes in the compartment where shell maintenance and growth take place.

In addition to difference in the proportion of each hemocyte type in the three investigated fluids, it is noteworthy that hemocytes belonging to each of these categories displayed different cell surface epitopes (carbohydrates profiles, CD11b-like and CD14-like; Figs. 3 and 5) based on their sampling location. Different cell surface carbohydrate profiles could significantly impact how hemocytes interact with microbes. For example, the protozoan parasite *Perkinsus marinus* was suggested to mimic the cell surface carbohydrates of *C. virginica* hemocytes to gain entry via phagocytosis into these cells [48]. Similarly, differences in CD labeling between hemocytes derived from the different oyster fluids could indicate functional specialization of hemocytes. Antibodies produced against mammalian CDs have been successfully used in invertebrates to identify functionally-different cell types [26–30,37,38]. This is particularly the case for CD11b and CD14 that appear to be molecularly (similar antibody specificity and molecular weight) and functionally conserved in invertebrates (e.g. the leech *Glossiphonia complanata*) as compared to their mammalian counterparts [26,29,37]. In vertebrates, CD14 interacts with a lipopolysaccharide (LPS)-binding protein to initiate a Toll-like Receptor (TLR)-induced cascade [49], which in turn can enhance phagocytosis [50]. The same function was also proposed in the sipunculan worm *Themiste petricola* where the majority of phagocytic coelomocytes were CD14-positive [26]. Therefore, if a CD14-like protein is functionally conserved in *C. virginica*, more CD14-positive hemocytes may confer an immunological advantage. It should be noted that the highest CD14 signals were measured in granulocytes associated with the pallial mucus, which also displayed the highest phagocytic activity. Hemocytes in the pallial mucus are likely the first hemocytes to interact with microbes present in seawater and a high reactivity towards microbes (e.g. LPS detection, high phagocytic activity) would be expected if they were to serve as sentinel cells. Interestingly, most intense CD14-like signals were noted in pseudopods (Fig. 1E) further suggesting a link between this epitope and phagocytic activity. Further investigations into possible differences in reactivity to LPS by CD14-positive pallial granulocytes may provide insight into any associated functional differences. Finally, the fact that CD14 is a common constituent of mucosal secretions of vertebrates [51] could suggest this may represent a conserved mechanism of mucosal innate immunity in metazoans. In parallel, CD11b has been suggested to be well conserved between invertebrates and vertebrates [52] and genomic data indicates that there is a CD11b homolog in oysters. CD11b is part of the integrin family and regulates leukocyte adhesion and migration [53], mediates inflammatory response [54], chemotaxis and phagocytosis [55]. Interestingly, CD11b also contributes to the regulation of bone formation in vertebrates where CD11b-deficient mice displayed low bone formation and bone mass [56]. In this context, if CD11b-like proteins in *C. virginica* hemocytes are functionally conserved, higher levels of CD11b-positive granulocytes in the extrapallial fluid may be a reflection of the role of these cells in shell mineralization [10]. Nevertheless, inferring specific biological functions based on CD labeling results remains speculative without further investigations into the role of target proteins in oyster immunity.

Overall, variations in cell surface epitopes between hemocytes derived from pallial mucus, extrapallial fluid and hemolymph suggest hemocytes from the three fluids are distinctly different. These differences may be linked to the probable diversity in the make-up of the microbial flora that interacts with hemocytes present in each of these compartments. As discussed above, hemocytes associated with the pallial surfaces have the potential to interact with the multitude of microbes present in seawater and that attach to pallial mucus. In contrast, the extrapallial fluid is a more confined environment and only

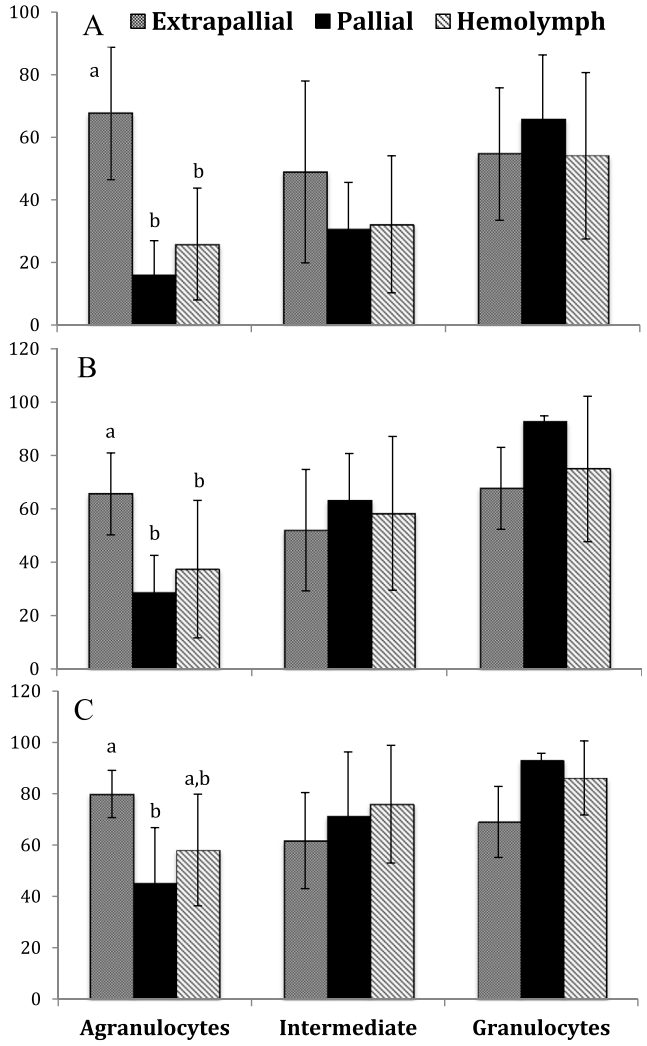

Fig. 4. Percentage of hemocytes that were positive for (A) ConA, (B) SBA, (C) PNA among each cell type from each compartment (mean ± SD). Different letters (a and b) denote significant differences between different fluids within agranulocytes (one-way ANOVA Tukey HSD post-hoc test on arcsine-transformed values, $P < 0.05$, $n = 14$, $n = 13$, $n = 16$ respectively). No significant differences were noted for intermediate cells and granulocytes.

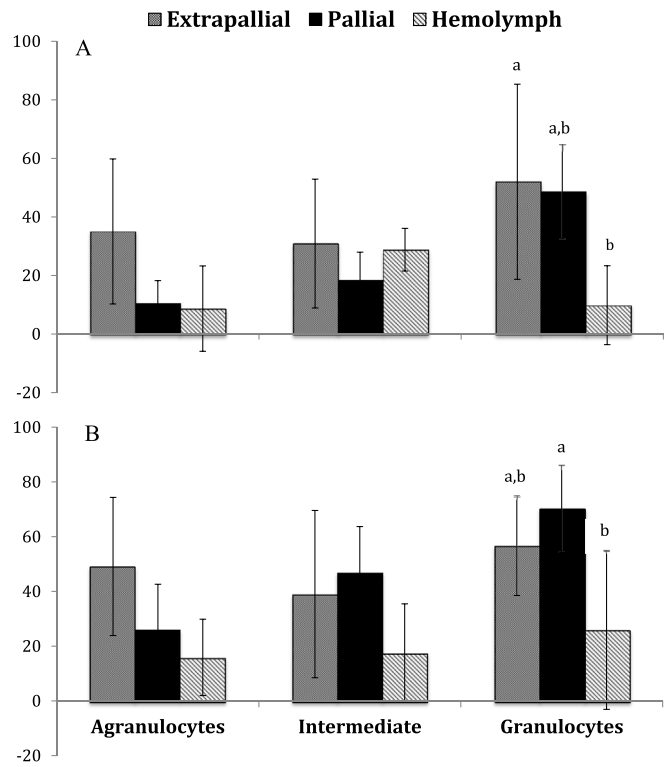

Fig. 5. Percentage of hemocytes that were positive for (A) CD11b and (B) CD14 within each cell type (mean ± SD) (one-way ANOVA Tukey HSD post-hoc test on arcsine transformed values, $P < 0.05$). Different letters (a and b) denote significant differences between different fluids within granulocytes (one-way ANOVA Tukey HSD post-hoc test on arcsine-transformed values, $P < 0.05$). No significant differences were noted for agranulocytes and intermediate cells.

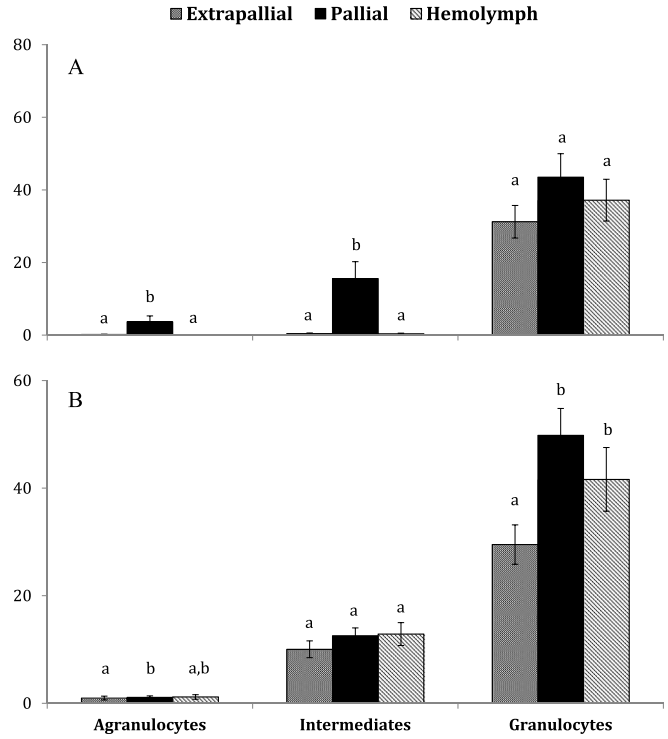

Fig. 6. Phagocytic (A) and reactive oxygen species production (B) index values of the different hemocyte subpopulations within the three body fluids (arbitrary index value scale, mean ± SD). Different letters (a and b) indicate significant difference between fluids within each cell type (Tukey HSD post-hoc test, $P < 0.05$, $n = 20$).

contains microbes that are capable of breaching outer physical barriers (periostracum, mantle edge) [8]. Finally, microbes that interact with circulatory hemocytes, whether invaders or more stable symbionts, likely represent a small fraction of the microflora present in the surrounding seawater and include microbes that were able to breach mucosal barriers lining soft tissues.

While the findings of higher phagocytic activities in granulocytes as compared to the other cell types is in agreement with previous findings in eastern oysters [6], it is noteworthy that higher phagocytic activities were measured in agranulocytes and intermediate cells associated with the pallial mucus as compared to their counterparts from the extrapallial fluid or hemolymph. These findings may again reflect a specialization of pallial hemocytes that continuously interact and "sample" environmental microbes. In particular, the markedly higher phagocytosis of agranulocytes (36 times higher phagocytic index in pallial hemocytes than in their circulatory counterparts) and intermediate cells (39 times higher phagocytic index in pallial hemocytes as in circulatory hemocytes) supports such specialization. Although agranulocytes are generally less phagocytic than granulocytes in the eastern oyster, it has been suggested that difference in phagocytic activity is also dependent on the nature of the particles that are being phagocytosed [6,7,57]. For

example, *C. virginica* agranulocytes are involved in phagocytosis of *Haplosporidium nelsoni* (MSX) and damaged host hemocytes in infected oysters while granulocytes are not [57]. Interestingly, ROS production and phagocytic activity were generally lower in granulocytes associated with extrapallial fluid compared to circulatory fluid and pallial mucus with significant differences for ROS. These differences further support that the primary role of extrapallial granulocytes may be shell biomineralization and not immune response.

5. Conclusion

Extrapallial hemocytes have been previously studied for their role in immunity [8,9,11,47,58] as well as mineralization [10,47]. This study provided an initial characterization of hemocytes associated with pallial mucus and showed that these share common morphological features but display different cell membrane (cell surface epitopes) and functional profiles (ROS, phagocytic activity) as hemocytes from the extrapallial fluid or hemolymph. The unique functional features of pallial hemocytes, as well as their physical location in close contact with environmental microbes, support a specific role for these hemocytes as sentinel cells occupying a physical niche similar to that of dendritic cells in the vertebrates. Further studies are needed to determine whether these functional differences derive from the migration of a specialized subset of circulatory hemocytes to the mucosal layer or if they result from a maturation of normal hemocytes in the mucosal tissues themselves. Similarly, additional studies are needed to unravel the potential role of pallial hemocytes in signaling and transmission of information to the circulatory system. Overall, pallial hemocytes represent an excellent and tractable model for the study of the cellular components of mucosal immunity in the invertebrates.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.10.025.

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