1
2 Morphological characterization via light and electron microscopy of
3 Atlantic jackknife clam (*Ensis directus*) hemocytes
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12 red; morphology
13

14 **Abstract**
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16 The Atlantic jackknife clam, *Ensis directus*, is currently being researched as a potential
17 species for aquaculture operations in Maine. The goal of this study was to describe the
18 hemocytes of this species for the first time and provide a morphological classification scheme.
19 We viewed hemocytes under light microscopy (using Hemacolor, neutral red, and Pappenheim's
20 stains) as well as transmission electron microscopy (TEM). The 2 main types of hemocytes
21 found were granulocytes and hyalinocytes (agranular cells). The granulocytes were subdivided
22 into large and small granulocytes while the hyalinocytes were subdivided into large and small
23 hyalinocytes. The large hemocytes had both a larger diameter and smaller nucleus to cell
24 diameter ratio than their smaller counterparts. A rare cell type, the vesicular cell, was also
25 observed and it possessed many vesicles but few or no granules. Using TEM, granulocytes were
26 found to contain both electron-lucent and electron-dense granules of various sizes. These
27 numerous granules were the only structures that took up the neutral red stain. Hyalinocytes had
28 few of these granules relative to granulocytes. Large hyalinocytes had both various organelles
29 and large vesicles in their abundant cytoplasm while small hyalinocytes had little room for
30 organelles in their scant cytoplasm. Total hemocyte counts averaged 1.96×10^6 cells mL⁻¹ while
31 differential hemocyte counts averaged 11% for small hyalinocytes, 12% for large hyalinocytes,

59% for small granulocytes, and 18% for large granulocytes. The results of this study provide a starting point for future studies on *E. directus* immune function.

1. Introduction

The Atlantic jackknife clam, *Ensis directus*, is native to the East Coast of North America as well as invasive to the Wadden Sea of Europe. The species currently supports a small fishery in North Shore, MA (Leavitt, 2010) and is in the development phases for large scale aquaculture (Burt et al., 2005). There is a small live and processed market in place for *E. directus* in the Northeast yet an inconsistent supply of product prevents its expansion (Leavitt, 2010). Sizable markets for razor clams already exist in Netherlands, United Kingdom, Ireland, Spain and Portugal, which will provide an outlet for any product that exceeds the local market's demand (Freundendahl and Nielsen, 2005). Literature on handling methods, habitat preference, and brood stock are all available (Freundendahl and Nielsen, 2005), however, very little is known about what potential pathogens *E. directus* is vulnerable to (Leavitt, 2010). Information on the health of the *E. directus* will be needed to assess the health of stocks as the razor clam aquaculture industry develops in the Northeast.

The circulating cells in bivalves, hemocytes, have been shown to be indicative of an animal's immune status (Cheng, 1984). This is because hemocytes serve a variety of immune functions as well as wound healing, nutrient digestion, transport, and excretion (Cheng, 1984). Establishing the morphology of healthy *E. directus* hemocytes will make comparisons of hemocytes possible between healthy and diseased bivalves (Mateo et al., 2009). Bivalve hemocytes can also be used as bio indicators for abiotic stressors like pollutants (Cheng and

Sullivan, 1984; Gagnaire et al., 2006; Seiler and Morse, 1988; Winston et al., 1996), salinity shifts (Fisher et al., 1987), warming (Fisher et al., 1987; Li et al., 2015), and acidification (Li et al., 2015). Hemocyte morphology, total hemocyte counts, and differential counts of the hemocytes are some of the basic parameters that can be used to help assess the immune capacity of bivalves (Estrada et al., 2013; Le Foll et al., 2010; Wootton et al., 2003). Transmission electron microscopy (TEM) can be used to take a more detailed look at hemocyte morphology and can thus be a useful tool in assessing the immune status of bivalves (Cajaraville et al., 1995; Moore et al., 1992; Seiler and Morse, 1988). These studies have shown that details, such as number of granules, integrity of the cytoskeleton, presence of pseudopodia, and size of lysosomes can be used to differentiate between diseased and healthy hemocytes

The two general types of hemocytes (haemocytes) present in most bivalves are granulocytes and hyalinocytes (agranulocytes). Granulocytes are defined by the presence of granules regardless of their size or density. Most of these granules are considered to be a type of lysosome as they contain acid hydrolases used to break down invading pathogens and cellular debris (Hine, 1999). The granulocytes can be further classified as large and small granulocytes. Most bivalves have at least one type of granulocyte although the lions-paw scallop, *Nodipecten subnodosus*, has no granulocytes at all (Estrada et al., 2013). Hyalinocytes are distinguished from granulocytes by their lack of granules. Some bivalves also have small hyalinocytes with a large nucleus to cytoplasm ratio sometimes referred to as blast-like cells or hemoblasts. These cells have already been observed in many bivalve species including *Tapes philippinarum* (Cima et al., 2000), *Saccostrea glomerata* (Aladaileh et al., 2007), *Crassostrea ariakensis* (Donaghy et al., 2009), *Saccostrea kegaki*, *Ostrea circumpicta*, *Hyotissa hyotis* (Hong et al., 2013). Their presence in a variety of bivalve species, low immune function capacity (Donaghy et al., 2009),

positive reading to the mammalian stem cell molecular marker anti-CD34 (in the case of *T. philippinarum*) (Cima et al., 2000), and the ability to divide (as found in *T. philippinarum*) (Matozzo et al., 2008) all provide evidence to suggest blast-like cells may actually stem cells that mature into the other cell types.

In addition to the more common hemocytes types listed above, there are 2 rare cell types found in only some bivalves: the vesicular cell (vacuolated granulocyte) and the serous cell (pigmented cell, rhogocyte, pore-cell, brown cell, morula cell). The vesicular cell is characterized by an abundance of clear vesicles and a lack or scarcity of granules. The function of this cell type in bivalves is unknown although its morphology may be the result of a granulocyte going through degranulation. This cell type has been reported in the mussel *Anodonta cygnea* (Salimi et al., 2009) oysters *S. kegaki*, *O. circumpecta*, *H. hyotis*, *C. gigas* (Chang et al., 2005; Hong et al., 2013), the cockle *Cerastoderma edule* (Russell-Pinto et al., 1994), and the clam *Scapharca inaequivalvis* (Holden et al., 1994). The serous cell is rounded and has a light brown to almost black coloration to it when viewed unstained (Cheng, 1981; Haszprunar, 1996). It is believed to perform functions such as excretion (Hine, 1999), detoxification of heavy metals (Haszprunar, 1996), removal of moribund parasites (Mackin, 1951), and cell aggregation initiation (Nakayama et al., 1997). Bivalves possessing this cell type include the clam *T. philippinarum* (Cima et al., 2000), the giant clam *Tridacna crocea* (Nakayama et al., 1997), and the cockle *C. edule* (Russell-Pinto et al., 1994).

Classifying the morphology of individual species is necessary as not all bivalves have the same types and frequency of hemocytes (Hine, 1999). For this reason, the number of bivalve species covered in the hemocyte literature has continued to increase over the years. Bivalve species recently added to this literature base include *Callista chione* (Matozzo and Bailo, 2015),

Bathymodiolus japonicas, *B. platifrons*, *B. septemdirum* (Tame et al., 2015), *S. kegaki*, *O. circumpecta*, *H. hyotis* (Hong et al., 2013), and *A. cygnea* (Salimi et al., 2009). The current study contributes to this effort by classifying the hemocytes of *E. directus*, and providing a detailed morphology of them using both transmission electron microscopy (TEM) and light microscopy. There is currently no information available on the hemocytes of *E. directus* although work has been done on the hemocytes of a related razor clam species, *E. siliqua* (Wootton et al., 2003). Total hemocyte counts and differential cell counts will also be presented for wild *E. directus*. These counts can be obtained without lethally sampling the animal and can be a valuable indicator of health status. Classifying and describing *E. directus* hemocytes will lay the foundation for future immune function studies in the species.

2. Methods

2.1. Clam and Hemolymph collection

E. directus were collected from the shore of the Damariscotta Estuary in Walpole, ME (43°56'05.8"N 69°34'49.1"W) using a clam rake on December 4th 2014 and January 19th 2015. They were all individually banded and transported in a cooler filled with water from the Damariscotta Estuary. All clams used in the study were undamaged and retracted all soft parts immediately upon removal from water. Clams ranged between 108 and 145mm in length and 20 to 25mm in width. Clams were kept in a 150L recirculating tank at the University of Maine, Orono filled with water from the Damariscotta Estuary. Clams used for hemocyte counts and light microscopy were sampled within 48 hours from the collection time while those used for

124 TEM were sampled a week after collection time. The glass tank was kept at $6.5 \pm 1^\circ\text{C}$ and had a
125 polypropylene tub placed in it filled with paver's sand to a depth of 100mm for the clams to
126 burrow in. All sand had larger rocks removed from it by running it through an onion bag and was
127 then rinsed with artificial sea water (made to a salinity of 30ppt using Crystal Sea Marinemix)
128 before being place in the tub. A Koralia circulation and wave pump was used to provide constant
129 water movement over the sand tub. A 38 L water change was done twice a week using artificial
130 seawater to maintain water quality when clams were kept more than 48 hours. Hemolymph was
131 withdrawn from the anterior abductor muscle using a 1ml syringe fitted with a 23-gauge needle.
132 A slight but constant pressure was maintained until hemolymph started to flow in the syringe.
133 After removing the needle, hemolymph was expelled into a 1.5ml centrifuge tube. There was no
134 need to pool hemolymph from multiple clams as at least 1ml of hemolymph could be obtained
135 from each clam.

136 An additional batch of 5 clams (with a size range of 105 to 170mm) was collected on
137 January 21st, 2016 off of Beals Island in Beals ME ($44^\circ 30' 24.6''\text{N}$ $67^\circ 36' 15.1''\text{W}$) and used
138 exclusively for the Pappenheim's stain preparations. They were banded and transported back to
139 the Bowden lab wrapped in paper towels saturated with natural sea water. These animals were
140 kept in the same tank set-up as described above with the exceptions of a larger aquaria (76 L)
141 and the use of sand from the collection site. A stainless steel sieve with 2.5mm mesh was used to
142 remove any large animals or rocks from the sand prior to placing the sand in the tubs. Salinity
143 was maintained between 32 and 33ppt while temperature was kept at $4.5^\circ\text{C} \pm 0.5^\circ\text{C}$. These clams
144 were fed ad libitum with instant algae (shellfish diet 1800, Reed Mariculture) on a daily basis
145 and kept at least a week in the lab before hemolymph was drawn. Hemolymph was added to a

tube containing an equal amount of 0.38% sodium citrate (Fisher Scientific) in 0.2µm-filtered artificial seawater (FASW) to reduce clotting (Matozzo and Bailo, 2015).

2.2. Total cell counts (THCs)

All cell counts were done immediately by loading freshly drawn hemolymph into a Neubauer improved hemocytometer and observing the cells at x250 magnification using a Leitz Wetzlar light microscope. Hemolymph was also checked for gamete contamination at this time. Small hemocyte aggregates were present but the cells within them were still distinguishable from each other and were thus included in the counts.

2.3. Cytological preparation for Hemacolor and Pappenheim's panoptical staining

Slides to be stained in Hemacolor were prepared by placing 50µl of hemolymph on glass slides within a moisture chamber placed in a refrigerator. Hemolymph was allowed to incubate for 30min at 6°C. Excess hemolymph was then removed by tilting the slide to one side and drying the droplet with a paper towel. Slides were then immediately stained using a Harleco Hemacolor® stain kit (65044) consisting of methanol, eosin, and methylene blue. Staining was done according to the manufacturer's directions. After air drying, the slide was fitted with a cover slip using Triangle Biomedical Sciences SHUR/Mount™ toluene-based liquid mounting media.

Slides to be stained in Pappenheim's mixture were prepared by placing 50µl of hemolymph on slides within a moisture chamber left at room temperature. Hemolymph was allowed to incubate for 30min before excess hemolymph was removed by pipette. The attached

hemocytes were then covered with 60µl of a 1% glutaraldehyde solution made in 32ppt FASW containing 1% sucrose. Slides were then allowed to fix for 30min at 6°C within their moisture chambers. Slides were then rinsed with filtered artificial seawater before being dipped in May-Grunwald stain (Electron Microscopy Sciences) for 3min. Slides were then dipped in distilled water before being dipped in a 5% Giesma (Sigma) bath for 5 minutes (Matozzo and Bailo, 2015). Slides were rinsed once more with distilled water before being mounted with a coverslip as described above.

2.5. Neutral red staining for lysosomes

Cells were stained using the Neutral Red staining procedure previously used by (Lowe et al., 1995) with a few modifications. The neutral red stock solution was made by dissolving 20 mg neutral red in 1 ml of DMSO. The stock solution was then frozen at -20°C until a working solution was needed. The working solution was made by mixing 10µl of stock solution with 5 ml of FASW. Once made, the working solution was again filtered through a 0.22 µm syringe-driven filter to remove any remaining clumps of neutral red dye. Slides were prepared for staining with neutral red using the same procedure as previously listed for Hemacolor staining preparations. After disposing of the excess hemolymph, 50µl of freshly prepared neutral red working solution was placed onto the attached hemocytes. A cover slip was placed on and hemocytes were allowed to incubate a further 15 min in the moisture chamber kept in a fridge at 6°C. The sides of the cover slip were then blotted with a tissue before being viewed in an Olympus BX60 light microscope at various magnifications. Immersion oil was used when viewing through the 100x objective lens.

2.6. Differential counts and hemocyte measurements

An Olympus BX60 light microscope was used to view stained slides at 40x and 100x objective lens. Immersion oil was used with the 100x objective lens. Pictures were taken with an Olympus DP71 microscope camera. Differential counts were taken under the 40x objective lens. For each animal, hemocytes were counted and categorized by cell type until 100 cells were counted. Extremely rare cell types were excluded from the counts. Hemocyte diameters, nucleus diameters, and nucleus to hemocyte ratios were obtained from the Hemacolor micrographs using Image J photo software.

2.7. TEM

Freshly drawn hemolymph was used to fill iced 1.5ml centrifuge tubes halfway. Up to 3ml of hemolymph could be extracted from a single clam. A 4% glutaraldehyde solution with a salinity of 30ppt was made by mixing a 0.22 μ m filtered 60ppt salt solution (Crystal Sea Marinemix) with a stock 8% glutaraldehyde solution (made in DI water) in a 1:1 ratio. This 4% glutaraldehyde fixative solution was then added to the 1.5ml centrifuge tube in a 1:1 ratio. The hemolymph was then gently mixed by inversion and kept in the fridge at 6°C for 14 hours before being centrifuged at 700xg for 10min. Supernatant was removed and cell pellets were re-suspended in 30ppt salt solution in order to remove the glutaraldehyde. This rinse step was repeated 2 more times before the pellets were postfixed with 1% osmium tetroxide solution (salinity 30ppt) for 1h at 6°C. The 1% osmium solution was then switched out for chilled 30ppt

salt solution and the tube was allowed to sit in the fridge overnight. The 30ppt salt solution was removed by pipette and pellets were dehydrated in an acetone series. After drying for 48h, ultrathin sections were cut using a diamond knife on a microtome. Sections were dried onto copper grids and stained with uranyl acetate and lead citrate (Chang et al., 2005). Sections were then viewed in a Phillips CM10 transmission electron microscope at 100KV and pictures were taken with an Orius SC200 digital CCD camera, model 830.

3. Results

3.1. Light Microscopy

Observation of live hemocytes stained with neutral red and fixed hemocytes stained with Hemacolor or Pappenheim's mixture allowed for identification of both hyalinocytes (agranular hemocytes) and granular hemocytes based on the presence or scarcity of granules. Both of these hemocytes types were then divided into large and small categories based on their nucleus to cytoplasm ratio. Neutral red was used only as a vital stain to check for the presence of lysosomes since it is difficult to discern granulocytes from hyalinocytes when using this stain (Fig. 2).

3.1.1 Hyalinocytes

Hyalinocytes were classified as 2 sub-types: large hyalinocytes and small hyalinocytes. Large hyalinocyte nuclei were oval in shape and stained a dark shade of purple in Hemacolor (Fig. 1 A). These cells were usually spread out onto the glass slide and thus had a high cell diameter. Their average nucleus to cell ratio was mid-range even though their average nucleus

size was greater than that found in any of the other hemocyte types (Table 1). Their cytoplasm took up very little stain and had either scattered granules or none at all. Extended pseudopodia were always absent in the hyalinocytes that had no granules (Fig 1B). An abundance of small round vesicles was present in the cytoplasm (Fig 1B) although many of them were difficult to discern due to the lightly-stained cytoplasm. Both round and elongated cell morphologies were observed (Fig. 1A and B).

Small hyalinocytes were also present which were characterized by their rounded, dark blue nucleus. They had both a very high nucleus to cell diameter ratio and small cell diameter. (Table 1; Fig. 1A and E). This made it impossible to discern if granules were present in their scant cytoplasm using light microscopy. Few of these cells had extended pseudopodia (Fig. 1E).

3.1.2 Granulocytes

Granulocytes could be identified by the presence of granules in the cytoplasm and were further classified as large and small granulocytes. All the granulocytes had granules that were not well defined in Hemacolor and often blurred together to form one mass (Fig. 1A). In Pappenheim's stain preparations however some of the individual granules could be distinguished (Fig. 1D). Scattered vesicles were also apparent in some the large granulocytes stained in Pappenheim's mixture (Fig. 1C). Neutral red preparations showed an abundance of tightly packed red granules filling most of the cytoplasm however the nucleus remained unstained (Fig. 2). Large granulocytes were almost always spread out onto the glass slide and thus had a high average cell diameter (Table 1). They appeared in oval, elongated, and spindle morphologies with extended pseudopodia (Fig. 1A, C, D, and E). The nucleus to cell diameter ratio was the

smallest of the 5 cell types (Table 1). The granules stained either dark pink (Fig 1C) or purple (Fig 1D) and cytoplasm took up very little stain around the edges of the cells (Fig 1C and D). Their nucleus was either round or oval in shape and always stained a dark blue (Fig. 1C, and D).

Small granulocytes had a larger nucleus to cell ratio and a smaller cell diameter than large granulocytes (Table 1). In both Hemacolor and Pappenheim's stain preparations the cytoplasm looked much like that of the large granulocytes (Fig. 1A, D, and F). In neutral red preparations the tightly packed masses of granules in the cytoplasm of the small granulocytes stained a dark red, (Fig. 2D). The granules tended to blur together in both Hemacolor and neutral red preparations although individual granules were sometimes visible at the edges of these masses. The nucleus stained a dark blue/black color in Pappenheim's stain preparations and was round or oval in shape (Fig. 1D, and F).

3.1.3 Vesicular Cells

Vesicular cells were present in only some of the samples and were characterized by an abundance of vesicles surrounding the nucleus. Both round and elongate nucleus morphologies were observed. The cytoplasm stained a light blue color and thus made the large vesicles prominent in this cell type. Numerous extended pseudopodia were observed and the cell type possessed an elongate cell morphology. (Fig 1F).

Fig. 1 Light micrographs of fixed and stained *E. directus* hemocytes that were allowed 30 min to adhere to a glass slide. (A) Hemocytes collected without an anti-clotting solution fixed in methanol and stained with Hemacolor. (B-F) Hemocytes collected in a 0.38% sodium citrate solution and fixed in glutaraldehyde before being stained in Papenheim's mixture. LH- large hyalinocyte, LG- large granulocyte, SG- small granulocyte, SH- small hemocyte, VC- vesicular cell

Fig. 2 Light micrographs of live *E. directus* hemocytes stained with neutral red for 15min. The stained portions are granules and the clear circle within each cell is the nucleus. (A) Large hemocyte with many extended pseudopodia and some enlarged granules. (B) Large hemocyte filled with enlarged granules. (C) A large hemocyte where the granules have not yet become enlarged. (D) A small rounded hemocyte packed tightly with granules. (E) A small rounded hemocyte with enlarged granules.

Type	Nucleus (μm)			Cell (μm)			N/C ratio		
	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Min	Max	Mean $\pm$ SD	Min	Max
SH	3.25 $\pm$ 0.56	2.05	4.12	5.41 $\pm$ 1.04	3.68	8.07	0.61 $\pm$ 0.11	0.40	0.89
SG	3.65 $\pm$ 1.83	2.66	5.26	9.43 $\pm$ 1.83	5.57	13.76	0.39 $\pm$ 0.07	0.28	0.58
LH	4.79 $\pm$ 1.01	2.98	6.65	12.51 $\pm$ 2.16	8.68	16.71	0.36 $\pm$ 0.10	0.21	0.59
LG	3.88 $\pm$ 0.79	2.84	5.75	15.32 $\pm$ 3.19	10.53	22.03	0.26 $\pm$ 0.06	0.16	0.38

Table 1 The nucleus, cell diameter, and nucleus/cell ratio of the major hemocyte types found in *E. directus*. Vesicular cells are excluded from this table because they were far too scarce. Measurements were taken from methanol-fixed hemocytes that were stained using Hemacolor. SH; small hyalinocyte; SG; small granulocyte; LH: large hyalinocyte; LG: large granulocyte; Min: minimum; Max: maximum. n=30 for each hemocyte type.

3.1.3 Aggregates

Hemocytic aggregates of various sizes were observed in each of the Hemacolor preparations. Most aggregates formed a round or oval shape however there were the occasional odd shapes such as elongated chains and rings. These aggregates could get quite large when no anti-clotting solution was used (Fig 3A). The use of the sodium citrate anticoagulant solution prevented the hemocytes from forming these large aggregates, but still allowed for the binding of the hemocytes to the glass slides and the formation of small aggregates (Fig 3B and C). The core of the aggregates was much darker in the large aggregates than it was in the small ones. The hemocytes blended in with the dark blue/black color of the core once they joined the aggregate and thus became impossible to identify. Both hyalinocytes and granulocytes showed the capacity to form aggregates as they can be seen extending pseudopodia towards existing aggregates (Fig 3B and C). In neutral red preparations the stain could not penetrate past the outer perimeter of the aggregates so most of the aggregate appeared clear.

Fig. 3 Light micrographs of *E. directus* hemocyte aggregates stained after being allowed to adhere to a glass slide for 30 min. (A) Low magnification view showing the various possible shapes and sizes of methanol-fixed hemocyte aggregates stained in Hemacolor without anti-clotting solution. (B) Glutaraldehyde-fixed hyalinocytes collected in a 0.38% sodium citrate solution and stained using Pappenheim's mixture. (C) Glutaraldehyde-fixed granulocytes collected in a 0.38% sodium citrate solution and stained using Pappenheim's mixture.

3.2. TEM

Confirmation of the 5 morphologically distinct cell types that were identified using light microscopy was made using TEM. This also revealed many details in the hemocytes not visible in the light micrographs. At least 2 distinct granule types were observed, which were characterized as electron lucent and electron dense (Fig 5A-F). The term electron dense was reserved for the darkest colored granules while the medium to light gray granules were deemed electron lucent granules.

3.2.1 Hyalinocytes

Large hyalinocytes were amoeboid in shape and had a low nucleus to cell diameter ratio. They lacked dense granule concentrations but had some scattered granules and mitochondria. The nucleus was round and located near the center of the cell. A few large clumps of chromatin could be seen within the nucleus. All large hyalinocytes observed had relatively few granules compared to granulocytes. Granule size was variable (0.16-0.63 μm) and most granules were electron lucent. All granules were either round or oval in shape. Rough endoplasmic reticulum and multiple mitochondria were always present as well as large vesicles. Pseudopodia of various lengths were observed extending from the hyalinocyte's perimeter (Fig. 4A and B).

Small hyalinocytes were round or oval in shape and had a very large nucleus to cytoplasm ratio. The nucleus was always centric and oval in shape. Clumps of chromatin of various shapes were visible in the nucleus. The scarce cytoplasm usually contained at least a few mitochondria, but granules were either relatively scarce compared to granulocytes or completely

absent (Fig. 4C and D). When granules were present both electron lucent and electron dense granules were of variable size (1.00-0.18 μm). Both round and oval granule shapes were observed. Long pseudopodia were also observed which extended as much as 4um out from the edge of the cell (Fig. 4D).

Fig. 4 Transmission electron micrographs showing the hyalinocyte and the vesicular cell in *E. directus* hemolymph. (A) Large hyalinocyte with an abundance of empty space in the cytoplasm. (B) Close up of the large hyalinocyte shown in micrograph A. (C) Small hyalinocyte with both extended pseudopodia and some granules. (D) Small hyalinocyte with a large particularly long pseudopodium extended. (E) Vesicular cell with many vesicles and no granules. EDG- electron dense granule, ELG- electron lucent granule, N-nucleus, PS- pseudopodium, M- mitochondria, V-vesicle, rER- rough endoplasmic reticulum

3.2.2 Granulocytes

Large granulocytes had a low nucleus to cytoplasm ratio and the nucleus contained large clumps chromatin. The cell possessed an elongate morphology with the nucleus at one of the far ends. Only a few vesicles were present as most space was packed densely with various organelles including mitochondria, Golgi apparatus and rough endoplasmic reticulum. The rough endoplasmic reticulum was usually found near the nucleus. The most prominent feature however was the abundance of granules. Like those found in the hyalinocytes, these granules occurred in various sizes (0.20- 0.81 μm) and came in both round and oval morphologies. There were both electron lucent and electron dense granules present. Multiple short pseudopodia were also observed extending off the perimeter of each large granulocyte (Fig. 6D).

Small granulocytes had a higher nucleus to cytoplasm ratio than large granulocytes and possessed round or oval morphologies (Fig. 6A-C). The nucleus had large clumps of chromatin and was either centric (in round cell morphologies) or eccentric (in oval cell morphologies) (Fig. 6B; Fig. 5A and D). Both electron dense and electron lucent granules were found in them although electron dense granules were always more abundant than electron lucent granules within any given small granulocyte. These granules came in various sizes (0.20- 0.87 μm) and were observed to be packed particularly tight in small granulocyte sections that lacked a nucleus (Fig. 6A and C). Upon closer inspection of the granules we noticed some of the electron lucent granules had spots of more intense granular material within them (Fig. 5E and F). This could not be discerned in the darkest of the electron dense granules, which had a homogenous black coloration (Fig. 5B). The same variety of organelles found in large granulocytes was present and all cells possessed multiple pseudopodia.

Fig. 5 Transmission electron micrographs of granulocytes in *E. directus* hemolymph with accompanying close-up micrographs of the granule types found within them. (A) Small granulocyte located just outside a hemocyte pellet. (B) Large electron dense granule with homogeneous coloration within (C) A trio of granules, each with a different electron density (D) Small granulocyte with numerous large granules (E) Large electron lucent granule with only a few small patches of darker areas within. (F) Electron lucent granules with many patches of much darker granular material within. EDG- electron dense granule, ELG- electron lucent granule, N-nucleus, PS- pseudopodium, M- mitochondria, V-vesicle

Fig. 6. Transmission electron micrographs of granulocytes in *E. directus* hemolymph. (A) Small granulocyte packed tightly with electron dense granules. (B) Small granulocyte with electron dense granules extending multiple pseudopodia (C) Small granulocyte with a very prominent rER as well as a mixture of electron light and electron dense granules. (D) Large granulocyte with electron dense and electron lucent granules as well as an elongate morphology. EDG- electron dense granule, ELG- electron lucent granule, N-nucleus, PS- pseudopodium, M- mitochondria, V-vesicle, rER- rough endoplasmic reticulum, GA- golgi apparatus

3.2.3 Vesicular cells

A single vesicular cell was found using TEM (Fig. 4E). This hemocyte is characterized by numerous vesicles and a lack of organelles (with the exception of the nucleus). The vesicular cell has many rounded electron lucid vesicles. The nucleus is rounded and one of the few electron dense areas of the cell. The rest of the cell is entirely electron lucent with the exception a thin network of membranes that form the vesicles and cell perimeter.

3.3 Total and differential hemocyte counts

Total cell counts were taken from 10 different razor clams, with 6 sampled in December and 4 sampled in January. We pooled results from both sample points since they were only sampled about a month apart from the same site and animals were within the same size and temperature range. The average total hemocyte count was $1.96 \times 10^6 \pm 6.78 \times 10^5$ cells mL⁻¹.

A total of 1,000 Hemacolor-stained cells (100 per sample) were counted under light microscopy for differential hemocyte counts and were comprised of 11% small hyalinocytes, 12% large hyalinocytes, 59% small granulocytes, and 18% large granulocytes (Fig. 7). At least 72% of the hemocytes counted were granulocytes in each of the samples used. The 27 cells that were counted from the electron micrographs were comprised of 26% small hyalinocytes, 15% large hyalinocytes, 48% small granulocytes, and 11% large granulocytes. Vesicular cells were not included in these counts as they were only discernable in Pappenheim's stain and TEM preparations. Vesicular cells were only present within 3 of these 5 Pappenheim's stain preparations and 1 of the TEM samples. None of these preparations contained more than 2 vesicular cells.

515 **Fig. 7** Population distribution of hemocyte types in *E. directus* hemolymph. Each bar stands for
 516 the mean of the given cell type counted for a total of 1,000 cells across 10 different animals.
 517 Error bars represent ± 1 standard error of the mean.
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520 4. Discussion

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522 Hemocyte classification in bivalves often varies from author to author as there are both
 523 multiple terms used to indicate the same hemocyte type and many different characteristics to
 524 classify by. In this work we have made an effort to keep the classification scheme simple by
 525 using only granularity and cell sizes as classification criteria. This means affinity for dyes was
 526 not used to classify cell types although observed colors are given for each cell type. In both light
 527 and electron microscopy we observed small hyalinocytes (agranular hemocytes), large

hyalinocytes, small granulocytes, large granulocytes, and vesicular cells. Serous cells (as described in the introduction) seem to be absent from *E. directus* hemolymph. Hyalinocytes and granulocytes are present in most bivalves (except some scallops) and their terminology is consistent throughout the literature (Hine, 1999). The classification scheme for the subtypes of these 2 general cell types however often vary between authors.

The small hyalinocytes identified in the current work resembled the blast-like cells (hemoblasts) found in many other bivalve species. They had the characteristic high nucleus to cell diameter ratio (Hine, 1999) and stained a dark blue in Pappenheim's stain (Aladaileh et al., 2007; Cima et al., 2000). Some the small hyalinocytes in the current study possessed a few granules as well as extended pseudopodia (Fig. 4C), both features not usually found in blast-like cells. It is possible then that the small hyalinocytes with a few granules are granublasts (Fig. 4C) while the small hyalinocytes without granules are hemoblasts (Fig. 4D). Granublasts have been hypothesized to be the stem cell for granulocytes and hemoblasts the stem cells for hyalinocytes (Cheng, 1981). The observations made on the hemocytes of the freshwater gastropod *Planorbarius corneus* support this theory (Franceschi et al., 1991). We chose to label these cells with a more general term (small hyalinocytes) since functional studies will be needed to determine whether they have the relatively low immune function capability that is characteristic of blast-like cells (Hong et al., 2013; Kuchel et al., 2010).

A rare type of hemocyte, the vesicular cell, was observed under TEM. This cell is unique because it has many vesicles and appeared to lack all organelles except the nucleus. It was only identifiable in TEM and Pappenheim's stain preparations and was very rare in all samples it was observed in. Their origin are unknown although it could possibly be a recently degranulated granulocyte. Granulocytes have granules comparable in abundance to the vesicles in the

vesicular cell so the vesicles could be phagosomes or degranulated granules. If this is the case then a low frequency of vesicular cells would be expected in healthy *E. directus* specimens. The authors would expect the frequency of this cell type to increase when the animals are exposed to a stressor. Such a frequency increase has been observed in tunicates exposed to tributyltin (Radford et al., 2000). Vesicular cell function is largely unknown although they have been observed to be involved with phagocytosis in the sea squirt *Phallusia mammillata* (Scippa et al., 1987) and the inflammatory response in the sea squirt *Ciona intestinalis* (Franceschi et al., 1991; Parrinello et al., 1990; Parrinello et al., 1984a; Parrinello et al., 1984b).

The findings in the present study can be compared directly to morphology studies done on other bivalve species. From a broad morphological standpoint, *E. directus* seems to have a hemocyte population similar to other most other bivalves as they possess both hyalinocytes and granulocytes of various sizes. This includes the closest relative to *E. directus* with hemocyte data available, *E. silique* (Wootton et al., 2003). Differential hemocyte counts revealed that *E. directus* has hemolymph dominated largely by granulocytes (77%) (Fig. 7), which is not unusual among bivalves. Other bivalves with hemocyte populations comprised of over 75% granulocytes include *M. edulis* (Wootton et al., 2003) *E. silique* (Wootton et al., 2003), *C. chione* (Matozzo and Bailo, 2015) and *Mya arenaria* (Huffman and Tripp, 1982).

The average total hemocyte count of *E. directus* is 1.96×10^6 cells mL^{-1} , which seems to fall into the low range of bivalve average THCs. The bivalves *Argopecten irradians* (37.5×10^6 cells mL^{-1}) (Zhang et al., 2006), *E. silique* (6.48×10^6 cells mL^{-1}) (Wootton et al., 2003) *M. edulis* (5.68×10^6 cells mL^{-1}) (Wootton et al., 2003), *T. philippinarum* (5.02×10^6 cells mL^{-1}) (Matozzo et al., 2008) and *Pinctada fucata* (4.9×10^6 cells mL^{-1}) (Li et al., 2015) all have higher THC than *E. directus* while *C. gigas* (1.6×10^6 cells mL^{-1}) (Duchemin et al., 2007) and *C. chione*

(1.2×10^6 cells mL⁻¹) (Matozzo and Bailo, 2015) both had lower THCs. It should be kept in mind that comparing total and differential hemocyte counts across many studies often does not account for possible seasonal variation, as has been observed in *C. gigas* (Duchemin et al., 2007). This includes the current work for which counts should only be considered representative of the winter season. Still, these results indicate substantial differences are present in the total hemocyte counts among various bivalve species and therefore must be examined on a species by species basis.

The TEM work revealed at least 2 morphologically distinct types of granules are present in *E. directus* granulocytes. Multiple granule types have also been identified in a variety of other bivalves such as *P. imbricata* (Kuchel et al., 2010), *C. gigas* (Chang et al., 2005), and *S. glomerata* (Aladaileh et al., 2007). The function of each granule type is unclear at the moment however the presence of dense patches of red granules in neutral red preparations indicates at least one of the granule types has low pH contents. Neutral red staining has been used to confirm the presence of lysosomes in *P. imbricata* (Kuchel et al., 2010) so we conclude here that at least one of the granule types found in *E. directus* is a lysosome. The small-medium sized electron dense granules are likely to be lysosomes as these were found in high enough densities to be comparable to the densities found in neutral red-stained granulocytes. Release of electron dense granules in response to phagocytosis of latex beads has been recorded in *C. virginica* (Hinsch and Hunte, 2012), which suggests that some of the electron dense granules in *E. directus* may have hydrolytic enzymes. The differences in electron density of the granules could thus be in part due to variations of hydrolytic enzyme contents. Various hydrolytic enzyme contents have already been detected in other bivalves such as *M. arenaria* (Huffman and Tripp, 1982) and *M. edulis* (Wootton et al., 2003).

Evidence from work on the oyster *C. rhizophorae* (de Freitas Rebelo et al., 2013) suggests that all hemocyte types in *C. rhizophorae* are actually different developmental stages of a single cell line. This could be the case for *E. directus* as well however it also possible there are 2 different stem cells as discussed above. Whichever the case, it is likely that granulocytes would degranulate if pathogens or environmental stressors are present (de Freitas Rebelo et al., 2013). After degranulation the granulocyte would likely have an abundance of empty vesicles and become a vesicular cell. The information presented in the present study neither confirms nor denies that all the *E. directus* hemocyte types described here are part of one or more cell lines as functional studies are still needed. The morphology of each hemocyte type established in the current work provides the foundation for these functional studies.

This study represents not only the first published record of *E. directus* hemocytes, but also the first TEM work on any razor clam species. The data presented here is both the start of the investigation into *E. directus* hemocytes and the foundation for future functional characterization studies. These studies should focus on assessing the capacity of *E. directus* hemocytes to perform immune functions known to occur in most other bivalve species such as phagocytosis, production of reactive oxygen species, and melanization (Allam and Raftos, 2015).

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