# Identification of distinct haemocyte populations from freshwater bivalves Anodonta cygnea and Anodonta anatina using wheat-germ agglutinin (WGA)

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Identification of distinct haemocyte populations from freshwater bivalves *Anodonta cygnea* and *Anodonta anatina* using wheat-germ agglutinin (WGA)


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Abstract

Haemocytes play a major role in molluscs immunity. Functional studies are however impaired by limited available experimental tools to identify and sort distinct haemocyte populations. Therefore, using nonlethal methods, we aimed at evaluating whether lectin staining combined with flow cytometry could be used to distinguish circulating haemocyte populations from freshwater bivalves of Unionidae species, *Anodonta anatina* (Linnaeus, 1758) and *Anodonta cygnea* (Linnaeus, 1758). Based on classical classification, haemocytes were distinguished as granulocytes and hyalinocytes and cytological features were visualized using transmission microscopy and staining techniques. Size, granularity, viability and surface staining using lectins as specific probes were analysed by flow cytometry and fluorescence microscopy. The microscopic proportions of granulocytes and hyalinocytes significantly differed, being of 70% and 30% for *A. cygnea* and of 85% and 15% for *A. anatina*, respectively. Two haemocyte populations were sorted by flow cytometry based on size and granularity and confirmed as granulocytes and hyalinocytes. Interestingly, two different granulocyte populations could be further discriminated in *A. cygnea* according to their binding affinity to wheat-germ agglutinin (WGA) while granulocytes of *A. anatina* all stained similarly. Our results show that WGA-labelling combined with flow cytometry can be used to better discriminate *Anodonta* haemocyte populations and obtain purified populations for functional studies.

Keywords: freshwater bivalves; Unionidae; *Anodonta*; haemocytes; immune system; lectin affinity
1. Introduction

Anodonta cygnea Linnaeus 1758 and Anodonta anatina Linnaeus 1758 are two freshwater bivalve species native in Europe belonging to the Unionoida order. The unionoids have been suffering a substantial decrease in their populations mainly in the last 50 years (Bauer 1988; Bogan 1993; Neves et al. 1997; Graf and Cummings 2007). The reasons for this decline are mainly anthropogenic and are essentially due to habitat degradation including siltation, pollution and river system compartmentalization. In this context, research on freshwater bivalve immunology acquires particular relevance to better understand the causes for their decline or expansion, having in mind that the immune system of these animals may be differentially affected by several factors (Pipe and Coles 1995; Girón-Pérez 2010).

As most bivalves, these species have an open circulatory system, the haemolymph is pumped from the heart to the arteries and sinuses and from these spaces and tissues the haemolymph enters into the veins and eventually is carried back to the heart (Brand 1972; Cheng 1981). The main components of the bivalve immune system are the haemocytes (Danilova 2006; Glinski and Jarosz 1997). These cells can be mainly found in the circulating haemolymph and play several roles, not only those more closely associated with immune function, like wound and shell repair and phagocytosis, but also in coagulation, oxygen transport, nutrient digestion and biomineralization (Cajaraville and Pal 1995; Soares-da-Silva et al. 2002; Cheng 1981).

In the last years, few researchers have addressed the different roles played by haemocytes in the immune response. Haemocytes classification is still not consensual, but most researchers use the terminology proposed by Cheng in 1981. This
classification is based only on morphological parameters without the use of biochemical markers. Several other authors have distinguished two haemocyte types in bivalves, based on morphological, cytochemical and functional characteristics (Hine 1999; Chang et al. 2005). In these studies, hyalinocytes are characterized as cells with fewer granules in their cytoplasm and a higher nucleus to cell ratio, when compared with the granulocytes, which have abundant granules. In other studies, density gradient centrifugation (Carballal et al. 1997; Friebel and Renwrantz 1995; Bachère et al. 1988), flow cytometry (Ashton-Alcox and Ford 1998; Hégaret et al. 2003a; Hégaret et al. 2003b; Xue et al. 2001; Allam et al. 2002) and functional assays have been also used to separate and identify haemocyte populations, from which more haemocyte types have been proposed (Pipe et al. 1997; Nakayama et al. 1997; Carballal et al. 1997; Lopez et al. 1997). However, morphological characteristics are difficult to analyse since they often vary with taxa, life stage, with the different haemocytes sampled and culture techniques (Ittoop et al. 2001; Carballal et al. 1997). So, an ambiguity surrounding cell classification persists and a more functional classification is needed, being one of the missing links associated with the lack of knowledge on the origin of haemocytes in bivalves.

Immunity in freshwater mussels is not only assured by haemocytes as it is complemented by the humoral components of the haemolymph such as agglutinins (lectins), antibacterial peptides and enzymes, although these elements are more extensively characterized in marine species, where several antibacterial peptides have already been identified (Mydlarz et al. 2006).

Nowadays it is fundamental to avoid lethal methods to study the freshwater bivalve species. It is thus essential to assure the health status of the organisms by using appropriate sampling techniques to collect small samples of haemolymph avoiding
damaging their integrity (Gustafson et al. 2005), since these are endangered species. The modern technologies used to study vertebrate blood cells, such as flow cytometry, which allow the analysis of large numbers of cells and can be coupled with other techniques such as fluorescence labelling, contribute to haemocytes characterization and fill some of the gaps in the knowledge on cells differentiation, function and respective classification. In the present study, nonlethal methods and a multi-technique approach were used to characterize Anodonta anatina and Anodonta cygnea circulating haemocytes, and establish an easier and more accurate classification of haemocyte populations.
2. Materials and Methods

2.1 Animals collection

The two species of freshwater bivalves used in this study, were collected in northern Portugal. *Anodonta cygnea* was collected from the “Barrinha” lagoon (Mira-Portugal - 40° 27’ 22” N, 8° 48’ 7” W) and *A. anatina* from the Tâmega River near Mondim de Basto (41º 24’ 52” N, 7º 57’ 51” W) in two time periods, January and May, each from a different year. They were kept in aerated tanks with dechlorinated water and were acclimatised in these conditions for two weeks. The animals were fed daily with a microalgae diet. The organisms were considered healthy if the surface of the shell was smooth and shiny and if they closed the valves when disturbed. Only organisms considered healthy were included in the study.

2.2 Haemolymph collection

Haemolymph from twenty-four organisms of each species (*A. cygnea* and *A. anatina*), was carefully extracted, (ca. 1 mL) using a 21G needle (Braun) attached to a 2 mL sterile syringe (Braun), by insertion between the valves across the inner layer of the mantle into the intraepithelial space. Each haemolymph tube was maintained on ice, immediately after collection, to avoid aggregation (Gagnaire et al. 2004; Silva et al. 2000) and an anticoagulant solution of N-ethylmaleimide 0,05 M was added in a proportion of 1/10 of total volume (Hinzmann et al. 2013).

2.3 Light Microscopy
2.3.1 Haemocytes morphology and cell counts

Haemocytes were immediately analysed after collection. Briefly, fresh cell preparations of each of the previously collected samples were made and observed under a light microscope (Olympus BX 41) coupled with a digital camera (Olympus DP70). Morphological differentiation of cell types was done and respective measurements of cell and nuclei diameters were taken. For cell counting an improved Neubauer haemocytometer (Marienfeld) was used. The relative percentage of the different haemocyte populations was calculated based on the results obtained for 10 animals, and the cell measurements were made on a minimum of 100 cells.

2.3.2 Light microscopy coloration

2.3.2.1 Haematoxylin-eosin and May-Grünwald Giemsa

Haemolymph was collected as described above and an aliquot was set onto a glass slide for adhesion. The coloration technique was conducted as described in the procedure of Hemacolor® (Merck, Kenilworth, NJ, USA) and May-Grünwald’s eosin methylene blue for microscopy (Merck) for air dried smears fixed with methanol 100%. In the end, slides were washed and let to dry. Prior to observation in the light microscope, slides were mounted with DPX (Merck) for long term preservation.

2.4 Transmission Electron Microscopy

A haemolymph pool from three organisms was assessed for each species, to reduce individual variation. The cells were fixed in 2.5% glutaraldehyde (Merck) in 0.1 M
cacodylate (Sigma, St Louis, MO, USA) buffer, pH 7.2 for 2 h at 4 ºC. The cells were then centrifuged at 3000 rpm for 10 min at 4 ºC and washed twice in cacodylate buffer. The pellet was fixed in 1% osmium tetroxide (TAAB, Sigma) in 0.1 M cacodylate (Sigma) buffer for 1 h and then washed in cacodylate buffer and dehydrated in crescent ethanol (Merck) solutions (30%, 50%, 70%, 90%, 95%, 100%), 15 min for each, at 4 ºC until the 95% ethanol solution and then two washes at room temperature for the final concentration). Then propylene oxide 100% (Sigma) was added to the cells prior to impregnation in epoxy resin (Fluka, St Louis, MO, USA). Semi-thin sections were stained with methylene blue and observed under a light microscope. Thin sections were stained with uranyl acetate (Sigma) and lead citrate (Sigma) and observed in a Jeol 100 CXII (JEOL, Inc, Peabody, MA, USA) transmission electron microscope.

2.5 Flow cytometry analysis

Flow cytometry analysis was performed using a EPICS XL flow cytometer equipped with a 488 nm laser and the standard filter setup using the EXPO32ADC software (Beckman Coulter, >Miami, FL, USA). PBS was used as sheath liquid. Data were analysed using the Flowjo 10.1 software (Tree Star, Ashland, OR, USA).

Haemolymph was collected as described above and filtered, through 100 µm pore filter to remove aggregates and detritus. Samples were incubated with propidium iodide (PI, Sigma), a marker of membrane integrity, at 5 µg/ ml for 5 min at room temperature prior to acquisition to determine cell viability. Fresh haemocyte samples from 24 individuals of each species were analysed based on forward Scatter (FSC), Side Scatter (SSC) parameters and, when suitable, on green channel fluorescence.
2.6 *Anodonta cygnea* Haemocyte Sorting

Haemolymph from two *Anodonta cygnea* individuals was collected as described above. Haemocytes were sorted by flow cytometry in a FACSria II cell sorter equipped with the FACSDiva software (Becton Dickinson, San Jose, CA, USA) based on FSC and SSC parameters. One thousand sorted cells of each population were spotted onto microscope slides, fixed with methanol 100%, stained with Haematoxylin-eosin, washed and let to dry. At least 10 spots were collected per population, per animal. Prior to observation in the light microscope, slides were mounted with DPX (Merck) for long term preservation.

2.7 Lectin labelling

To assess lectin binding to haemocytes, $2 \times 10^5$ freshly collected haemocytes were incubated for 15 min in the dark on ice with each of the following fluorescein conjugated-lectins: wheat germ agglutinin (WGA) at 5 µg/mL (Sigma), peanut agglutinin (PNA) at 2 µg/mL (Sigma) and phytohemagglutinin-L (PHA-L) at 2 µg/mL (Vector Laboratories, Burlingame, CA, USA). Fluorescein-conjugated streptavidin at 2 µg/mL (Vector) was as a negative control of fluorescence. PI was added at the end of the incubation period to exclude dead cells. Cells were analysed by flow cytometry based on FSC, SSC and green channel fluorescence parameters.

2.8 Fluorescence Microscopy of lectin-labbeled cells

Haemocytes were extracted as mentioned previously, aliquots were incubated for 15 min with fluorescein-conjugated WGA, PNA and PHA-L at the concentrations
mentioned above. After this incubation, cells were centrifuged for 5 min at 1100 rpm in a Cytospin 3 (Thermo Fisher Scientific Inc.; USA), cytospins were air dried for 10 min, fixed with paraformaldehyde 4% for 10 min, washed with PBS 0.5 mM, and mounted with 15 µl Vectorshield mounting medium with DAPI and phalloidin. Cytospins were kept at 4°C for 30 min prior observation under the fluorescence microscope Olympus BX 40 with the digital photocamera DP70, and DAPI, FITC and TRITC filters (Olympus). Combined images from DAPI, FITC, and TRITC were made to assess lectin labelling of the cells.

2.9 Statistics

Statistical analysis was performed using GraphPad software (Version 6.0, GraphPad Software Inc, La Jolla, CA, USA). Unless otherwise indicated, statistical analysis between populations was performed using unpaired two-way ANOVA followed by Sidak’s multiple comparisons test. Column graphs are represented showing means plus one SD. P-values lower than 0.05 were considered statistically significant.
3 Results

3.1 Haemocyte analysis

3.1.1 Microscopy - Haemocyte morphology and relative abundance

Live cells were observed under light microscopy for measurement and counting, so that their characteristics were kept as intact as possible. Two main cell types were distinguished, in both studied species (Table 1, Fig. 1A, 1B1 and 1B2, 2A and 2B1 and 2B2). After adhesion haemocytes comprising the larger and more abundant cell type, tended to form thin extensions (pseudopods) and to aggregate (Fig.1A and Fig. 2A). These cells also presented an eccentric nucleus, few to several refringent vacuoles in the cytoplasm and granules that were not always visible, and were designated here as granulocytes. The other visible cell type was composed by smaller and less abundant cells, with a rounder shape, a centric nucleus, low cytoplasm content, less refringent vacuoles and absence of granules. These cells were designated as hyalinocytes.

In *A. cygnea* the population of granulocytes comprised a mean of 70% of total cells found in the haemolymph. The remaining cells were hyalinocytes (30.3±17.93 cells/ml). In *A. anatina* the predominance of granulocytes was more marked, around 86%, but these values varied between organisms. A high individual variability among biochemical and cellular parameters is common when working with wild mussels, and was more evident in *A. cygnea*, which presents a larger deviation from the mean values. In terms of abundance variability also occurred. The number of cells varied substantially among organisms, but in average, the haemolymph of *A. cygnea* had more cells, around $8 \times 10^5$ cells/ ml, while in *A. anatina* the number was around $5.5 \times 10^5$ cells/ ml.
The granulocytes in both species varied considerably in size. These cells presented a diameter in the range 10-20 µm, low nucleus/cytoplasm ratio and a more constant nucleus diameter; some cells also presented extensions that varied in size and shape, from small and thin to long and thicker projections, designated as pseudopods. Among the hyalinocytes, the dimensions were more constant, the cells were smaller, rounder and with a higher nucleus/cytoplasm ratio. Most of the cytoplasm was occupied by the nucleus. Granulocytes were slightly smaller in *A. anatina* when compared with *A. cygnea* and an inverse pattern occurred with hyalinocytes (Table 1).

The morphometric measures and abundances data supported the classification adopted to characterize the haemolymph haemocytes from both *A. cygnea* and *A. anatina*. The established groups of cells were significantly different for both species (Table 1).

### 3.1.2 Light microscopy observation of stained cells

Staining with May-Grünwald blue methylene or Hemacolor® on spontaneously adhering cells also revealed the two main types of haemocytes: granulocytes and hyalinocytes. The cytoplasmatic granules were not always visible by light microscopy, probably because the staining procedure favours degranulation, being only possible to infer about the acidophilic or basophilic properties of the cells, given by the coloration of the cytoplasm. In *A. cygnea* most cells from the haemolymph presented a violet coloration, indicating that most of the granulocytes and hyalinocytes have a basophilic content (Fig. 1D1 and 1D2). In this species, few acidophilic granulocytes and hyalinocytes were detected. Similar results were obtained for *A. anatina*. However, using the May Grunwald’s eosin methylene coloration technique the granules of *A. anatina* were in some cases slightly more evident (data not shown). In fact, with light
microscopy the granules of *A. cygnea* were only visible in the thin sections stained with methylene blue (Fig.1F); this coloration technique was also suitable to visualize the granules of *A. anatina* granulocytes (Fig. 2F).

### 3.1.3 Transmission electron microscopy

Under transmission electron microscopy, the same cell types were found for both species and their organelles could be analysed in more detail (Fig. 1A, 1B, 2A and 2B). Granulocytes (Fig. 1B and 2B) display eccentric nuclei with large clumps of chromatin, a few thin cytoplasmic projections, and a low nucleus/cytoplasm ratio. Variable numbers of mitochondria, Golgi complexes, vesicles and cytoplasmic granules were simultaneously contained in the cytoplasm. The granules were electron-dense and electron-lucent and the shape and size varied. Granulocytes had more-prominent pseudopods, and residual bodies were occasionally detected in the cytoplasm. The hyalinocytes of both species examined (Fig. 1A and 2A), contrarily to granulocytes, had few to no cytoplasmic granules. The nuclei were larger than in granulocytes, with stippled chromatin, surrounded by long profiles of rough endoplasmic reticula.

### 3.2 Flow cytometry

Flow cytometric analysis of fresh haemocytes also revealed two viable populations differing in size and granulosity/internal complexity according to FCS and SSC parameters, respectively (Fig. 3A and 4A). Since these parameters correlated to morphological characteristics of these two distinct cell populations previously observed by light microscopy techniques, the two populations of haemocytes collected from *A.*
cygnea were sorted by flow cytometry and observed under light microscopy upon hemacolor staining. This allowed identifying the cell population with the smallest size and lowest granulosity gated in R1 as hyalinocytes and the other cell population with higher size and granulosity, gated in R2, as granulocytes (Figure 3B).

The identification of the two populations detected by flow cytometry also allowed the evaluation of the relative abundance of viable hyalinocytes and granulocytes. Although wide inter- and intra-mussel variability could be observed, a significantly higher percentage of hyalinocytes was found in A. cygnea comparatively to A. anatina (Fig. 4A and 4B). These relative abundances were consistent with the ones found by light microscopy analysis. The relative percentage of hyalinocytes in A. anatina was nevertheless slightly lower when evaluated by flow cytometry.

3.4 Lectin binding analysis

The extent to which WGA, PHA-L and PNA bound A. anatina and A. cygnea haemocytes was evaluated by flow cytometry and confirmed by fluorescence microscopy. Granulocytes from live A. cygnea exhibited two distinct WGA binding levels, while granulocytes from live A. anatina had roughly the same fluorescence intensity due to WGA labelling. Contrastingly, WGA bound live hyalinocytes from both species to a much lower extent (Fig. 5A). WGA total staining intensity was significantly higher in A. cygnea than in A. anatina (Figure 5C). The disparate binding of WGA to hyalinocytes and granulocytes was confirmed by fluorescence microscopy (Fig. 5B). PNA and most notably PHA-L bound haemocytes to a lower extent than WGA (Fig. 5C). No differences were observed between the fluorescence intensity detected in granulocytes and hyalinocytes due to PNA or PHA-L binding. Also, no notorious
differences were found in the staining due to PNA binding between *Anodonta* species, although the mean fluorescence intensity (MFI) of PHA-L was slightly higher in *A. cygnea* than in *A. anatina* (Figure 5C).
4 Discussion

Invertebrate animals, which lack an adaptive immune system, have developed a defence system that responds to common antigens on the surface of potential pathogens (Mitta et al. 1999). One of the main components of their immune system is composed by the cells circulating in their body fluids, the haemocytes, although their origin in bivalves is still unclear when compared with other molluscs (Pila et al. 2016). These are involved in several immune functions such as phagocytosis, detoxification processes or even in the formation of microspheres (Hinzmann et al. 2014). In the present work, cells from the haemolymph were analysed in detail and their main morphologic features were described.

Morphological criteria are the basis of most studies addressing the characterization of haemocytes. However, the nomenclature adopted is ambiguous and inconsistent among researchers, varying according to the researcher and the used technique (Cheng 1981; Salimi et al. 2009). Based on morphological data, Cheng (1981) already proposed the classification of invertebrate haemocytes dividing these cells in two main types: granulocytes and hyalinocytes. However, Hine (1999) pointed out that classifying haemocytes into granular and agranular forms was too simplistic, posing great constraints in our understanding of haemocytes functions.

Here a detailed microscopic and flow cytometric analysis of haemolymph haemocytes from *A. anatina* was presented, which complements previous reported data on *A. cygnea* haemolymph haemocytes (Soares-da-Silva 2002; Salimi et al. 2009). Both species had similar haemocyte types – granulocytes and hyalinocytes – easily differentiated by microscopy, although the relative abundance varied among specimens, likely influenced by natural, anthropogenic or stress non-controllable factors (Wootton et al 2003; Pipe and Coles 1995). Soares-da-Silva et al. (2002) had already reported the presence of
these same types of haemocytes in *A. cygnea*. Under light microscopy the granules were not always observed, this effect might be attributed to the anticoagulant solution used that, as most anticoagulants, favour degranulation (Burkhard et al. 2009). Nevertheless, its use was fundamental to disaggregate the cells. Smith and Soderhall (1983) found that the haemocytes of the freshwater crayfish *Astacus astacus* (Linnaeus, 1758) easily showed profound degranulation and lysis under stress conditions. Therefore, a similar process may occur in the haemocytes of *Anodonta* species. Consistent with this hypothesis, granules were easily observed by electron microscopy in these species. Most of the cells of *A. cygnea* showed basophilic properties, although some acidophilic cells were also found. The characteristic granules of the granulocytes were not always visible in this species what we assume to be caused by some of the components of the coloration technique that may favour the degranulation process. Nonetheless, their presence was proved by using blue methylene in semi-thin sections. *A. anatina* haemocytes showed basophilic properties, and the granules stained well with May-Grünwald blue methylene and blue methylene coloration. Cheng (1981) suggested that acidophilic granulocytes are the more mature cells and are probably originated from basophilic granulocytes which are partially in line with the results from Lin et al. (2013) showing that eosinophilic granulocytes are more active phagocytes than basophilic granulocytes. Similarly, two distinct cell populations were identified in other bivalve species, although some authors also include an intermediate group of granulocytes, usually more heterogeneous, comprised of medium-sized cells with few granules. The reports cover species as different as the clams *Mya arenaria* (Linnaeus, 1758) ((Huffman et al. 1982), *Mercenaria mercenaria* (Linnaeus, 1758) (Pipe et al. 1997; Allam et al. 2002), *Ruditapes philippinarum* (Adams and Reeve, 1850) (Allam et al. 2002), the mussels *Mytilus edulis* (Linnaeus, 1758) (Pipe et al.; 1997) and *Mytilus*
galloprovincialis (Lamarck, 1819) (Cajaraville and Pal 1995), the oysters Ostrea edulis (Linnaeus, 1758) (Bigas et al. 2006; Cochenne-Elalou et al. 2003), Crassostrea virginica (Gmelin, 1791) (Allam et al. 2002; Goedken and Guise 2004) and Crassostrea rhizophorae (Guilding, 1828) (Rebelo et al. 2013), and the deep sea mussels of the Bathymodiolus genus (Bettencourt et al. 2010; Tame et al. 2015).

A constraint of microscopy is that it is very time consuming and limits the study to a small number of cells (Goedken and Guise 2004) when compared with flow cytometry analysis. By using flow cytometry, more populations of cells could be analysed and sub-populations could be discriminated, namely profiting from distinct lectin binding profiles. Moreover, the granularity of the cells is more accurately assessed by using this technique, since different levels of granularity can be discriminated (Ashton-Alcox and Ford 1998). In the present study, the two cell types identified for both Anodonta species by using light microscopy were also detected by flow cytometry. Flow cytometric assisted cell sorting of the two distinct haemocyte populations further confirmed the identification of hyalinocytes and granulocytes in A. cygnea. This technique has been widely used to confirm the identification of the haemocyte populations distinguished by FSC and SSC parameters (Ashton-Alcox and Ford 1998; Allam et al. 2002; Goedken and Guise 2004; Rebelo et al. 2013). The correlation between microscope and flow cytometry estimates for cell population’s relative percentages was high, which is in accordance with previous reports (Ashton-Alcox and Ford 1998; Allam et al. 2002). Thus, characterization of the haemocytes by flow cytometry will enable a quicker, simpler and less subjective quantification of these cells. We observed distinct relative hyalinocyte/granulocyte proportions between the studied species. Putative effects of the different habitats from which the organisms were collected, as well as intrinsic factors, on the abundance of these cells cannot be ruled out. Indeed, it has already been reported
that the haemocyte subpopulations undergo changes in their proportions upon infection (Mateo et al. 2009). As the organisms were collected in different periods, each of a distinct year nevertheless displaying similar cell profiles between collection periods, it rather seems that the observed differences represent a characteristic feature of the respective species. This is further suggested by a hyalinocyte/granulocyte profile reported for *A. cygnea* in a previous study (Soares-da-Silva et al. 2002).

Lectin binding to surface or intracellular polysaccharides has been previously used to characterize bivalve haemocytes, since different types of haemocytes frequently display distinct lectin binding patterns or levels. This technique also allows the characterization of the glycoconjugates composing the surface membranes of haemocytes by using fluorescence-labelled lectins with defined sugar-binding specificities. Here we show that different lectins bind the surface of *Anodonta* haemocytes at different extents allowing discriminating cell types. While WGA only stained granulocytes, PNA indistinctly labelled the two cell types with lower staining intensity. In contrast, PHA-L bound weakly to both cell types. The disparate ability of different lectins to bind bivalve haemocytes has already been noticed in previous reports. WGA was shown to bind the plasma membrane of granulocytes from the clam *Tapes semidecussata* (Reeve, 1864) (Montes et al. 1995), the mussel *Mytilus edulis* and species of the genus *Bathymodiolus* (Bettencourt et al. 2010; Tame et al. 2015) but not that of hyalinocytes. Although we have shown that PNA bound *Anodonta* haemocytes, such labelling was not found in *Mytilus edulis* (Pipe 1990 Wootton et al. 2003), *Cerastoderma edule* (Linnaeus, 1758) and *Ensis siliquae* (Linnaeus, 1758) (Wootton et al. 2003). Most studies also indicate that PHA-L had no affinity with the membrane of bivalve haemocytes (Tame et al. 2015). Consistent with these results, Kannaley and Ford reported in 1990 that WGA agglutinated oyster haemocytes while PHA did not. Curiously, here we observed that
WGA labelled *A. cygnea* granulocytes with two distinct fluorescence intensities, which contrasts the uniform staining of WGA on *A. anatina* and also reported for *Mytilus edulis* granulocytes (Pipe 1990). Tame et al. (2015) in a study with *Bathymodiolus* species reported that WGA bound to two distinct types of granulocytes but made no reference to differences in intensity staining between them. It could be hypothesized that lectin binding strength could reflect physiological states of haemocytes, since binding affinities of *Drosophila* lamellocytes to WGA were correlated with intracellular Ca$^{2+}$ concentrations (Tirouvanzian et al. 2004). Whether the relative abundance of N-acetylglucosamine and/or N-acetylneuraminic acid, the ligands of WGA, on the surface of *A. cygnea* granulocytes has a physiological role remains unknown and would be worth exploring.

In conclusion, we characterized *A. anatina* and *A. cygnea* haemocytes in detail and have shown that two major cell populations, hyalinocytes and granulocytes, could be identified by microscopy and by flow cytometry. The last technique proved reliable and much easier and faster than microscopy for the characterization of haemolymph cells’ relative abundance. We have also shown that haemolymph cell populations exhibited distinct interspecific lectin binding, as well as differences in binding among cells of a single individual. Further studies exploring the glycoconjugates composing the membranes of the haemocytes and their potential physiological consequences could extend our knowledge on these important cells of the innate defence of bivalves.

5. Conflict of interest

The author declares no conflict of interest.
Acknowledgments

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References


Figure Legends

Table 1 – Comparison of haemocytes from *Anodonta anatina* and *Anodonta cygnea*, by microscopy.

**Fig. 1.** Microscopy analysis of *Anodonta cygnea* haemocytes. Images A, B, D and E were obtained using light microscopy. A. Aggregate of live granulocytes, B. Live haemocytes: B1 hyalinocytes and B2 granulocyte (arrow marking the presence of pseudopods). D. Semi-thin section stained with methylene blue, D1 granulocytes and D2 hyalinocyte; E. Cells stained with hemacolor, E1 granulocyte and E2 hyalinocytes; In light microscopy images scale bars correspond to 10 µm. Transmission electron microscopy images: C. hyalinocyte, scale bar corresponds to 5µm and F. granulocyte, scale bar correspond to 10 µm; n – nucleus and gr – granules.

**Fig. 2.** Microscopy analysis of *Anodonta anatina* haemocytes. Images A, B, D and E were obtained using light microscopy. A. Aggregate of live granulocytes, B. Live haemocytes: B1 granulocyte and B2 hyalinocyte. D. Semi-thin section stained with methylene blue, D1 granulocytes and D2 hyalinocyte; in E. Cells stained with hemacolor, E1 – granulocyte and E2 – hyalinocyte. Transmission electron microscopy images: C. hyalinocyte and F. granulocyte, n – nucleus and gr – granules. Scale bars correspond to 10 µm.

**Fig. 3.** Flow cytometry sorting of haemolymph cells from *A. cygnea*. A. Representative dot plots showing the gating strategy used to sort the two distinct cell populations, as assessed by flow cytometry in Region 1 (R1) and Region 2 (R2), respectively. B.
Representative pictures of unsorted and sorted cells, as indicated, stained with hemacolor and observed in a bright-field microscope. Bar = 10 µm.

**Fig. 4.** A. Representative example showing the gating strategy used to discriminate by flow cytometry live hyalinocytes and granulocytes from the haemolymph of *A. anatina* and *A. cygnea*. B. Percentage of hyalinocytes and granulocytes assessed by flow cytometry in the haemolymph of *A. anatina* and *A. cygnea*, as indicated. Bars represent means ± SD. **** *P* < 0.0001, Two-way ANOVA followed by Sidak’s multiple comparisons test. (n=24/group, pooled from the two collection periods).

**Fig. 5.** A. Representative example of flow cytometry analysis showing WGA staining of live cells from the haemolymph of *A. anatina* and *A. cygnea*. Gate 1 corresponds to cells with FSC and SSC parameters characteristic of hyalinocytes while gates 2 and 3 consist of cells displaying granulocyte-type parameters. B. Fluorescence microscopy image representative of haemocytes collected from *A. anatina* and *A. cygnea* after staining with FITC-conjugated WGA. DAPI was used to stain nuclei and phallolidin-TRITC was used to stain F-actin filaments. Bar = 10 µm; h-hyalinocyte and g-granulocyte. C. Histogram overlays showing the fluorescence resulting from the staining of *A. anatina* and *A. cygnea* haemocytes, as indicated, with fluorescein-conjugated WGA, PNA and PHA-L and analysed by flow cytometry. Negative controls correspond to cells incubated with FITC-conjugated streptavidin. Numbers below histograms correspond to means of the mean fluorescence intensities due to lectin staining ± SD for each group. **** *P* < 0.0001, Two-way ANOVA followed by Sidak’s multiple comparisons test between *A. anatina* and *A. cygnea* samples. (n=16/group for
WGA staining and n=4/group for PNA and PHA-L staining, pooled from the two collection periods).
Table 1 – Comparison of haemocytes from *Anodonta anatina* and *A. cygnea*, by microscopy.

<table>
<thead>
<tr>
<th></th>
<th><em>A. anatina</em></th>
<th><em>A. cygnea</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemocyte type</td>
<td></td>
<td></td>
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<tr>
<td>Granulocytes</td>
<td>4.72 ± 1.18</td>
<td>5.88 ± 1.74</td>
</tr>
<tr>
<td>Hyalinocytes</td>
<td>0.74 ± 0.19</td>
<td>2.78 ± 1.82</td>
</tr>
<tr>
<td>Cell diameter (µm)</td>
<td>13.61 ± 1.55***</td>
<td>15.24 ± 2.46***</td>
</tr>
<tr>
<td>Nucleus shape</td>
<td>oval to spherical</td>
<td>oval to spherical</td>
</tr>
<tr>
<td>Nucleus position</td>
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<td>eccentric</td>
</tr>
<tr>
<td>Cytoplasm pseudopod</td>
<td>prominent</td>
<td>long and thin</td>
</tr>
<tr>
<td>N/C ratio</td>
<td>0.41 ± 0.05***</td>
<td>0.39 ± 0.07***</td>
</tr>
<tr>
<td>n</td>
<td>164</td>
<td>100</td>
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<tr>
<td>Other</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staining</td>
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</tr>
<tr>
<td>Hemacolor</td>
<td>basophilic and acidophilic</td>
<td>basophilic</td>
</tr>
<tr>
<td>May_Grunwald's eosin methylene blue methylene in semi-thin sections</td>
<td>basophilic with purple granules</td>
<td>basophilic with pink granules</td>
</tr>
<tr>
<td>Granules size</td>
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<tr>
<td>Granules shape</td>
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<td>variable</td>
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<tr>
<td>Nucleus Chromatin</td>
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<td>dense</td>
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<tr>
<td>Statistics</td>
<td>Granulocytes vs Hyalinocytes where * p&lt;0.05, ** p&lt;0.01 and *** p&lt;0.001</td>
<td></td>
</tr>
</tbody>
</table>

*Proportion abundances (cells/ml x 10^5)*

**Statistics**

*Proportion abundances (cells/ml x 10^5)*
Figure 1

238x190mm (300 x 300 DPI)
Figure 3

208x185mm (300 x 300 DPI)
Figure 4

198x182mm (300 x 300 DPI)
Figure 5

207x280mm (300 x 300 DPI)