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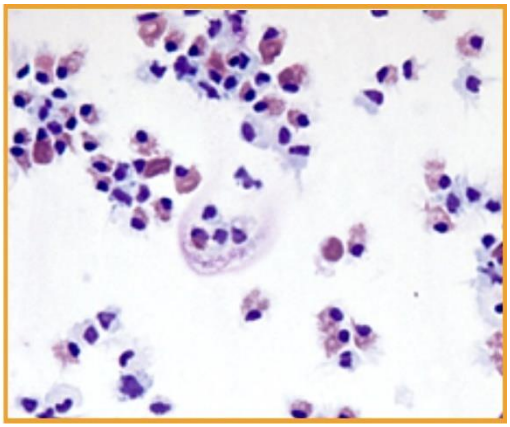

Lead Partner for Output	Centro de Investigacións Mariñas (CIMA)
Contributors	Silvia Lorenzo-Abalde, Asunción Cao, David Iglesias, María J. Carballal, Antonio Villalba
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1. Executive Summary

Considering the multiple stressing factors threatening cockle populations, special effort has been devoted to characterise the cockle immune system, one of the main defences of the cockles to face stress. The immune system of the cockles is scarcely known; in the case of the bivalve species in which the immune system has been more deeply studied, haemocytes represent the backbone of their immune system, playing a major role in phagocytosis of invading microbes and encapsulation of larger or refractive invaders as well as being involved in wound healing, food digestion and transport of nutrients, reproduction, excretion, shell formation, and production and secretion of humoral factors involved in the immune response. This is because the attention of this study has been focused on the main cell effectors of the immune system, the haemocytes. The specific objectives were to characterise the haemocyte types; to search for differences between haemocyte types in some immune abilities, namely phagocytosis, production of reactive oxygen intermediates (ROIs), lysosomal content and non-specific esterase content; and to evaluate the influence of environmental factors (namely temperature and salinity), internal physiological factors as the cockle gonad condition, as well as the most serious pathological conditions on the total haemocyte count (THC) and the relative abundance of each haemocyte type, that is to say the differential haemocyte count (DHC).

Presuming that the most serious disease of cockles in Galicia, marteiliosis, could significantly influence some of those haemocytic (immune) parameters, the shellfish bed of Noia (ria of Muros-Noia), where marteiliosis outbreaks have never been detected, was chosen as source of cockles for the study. Additionally, adult cockles were collected from the bed of Noia and transplanted into the bed of Lombos do Ulla (ria of Arousa) to evaluate the effect of marteiliosis on cockle THC and DHC. From 30 to 33 adult cockles were collected monthly from each bed and transported to the laboratory of CIMA to be processed for various analyses. Sampling in Noia was performed from April 2018 to March 2020, while in Lombos do Ulla from May to December 2018; the high mortality of the transplanted cockles in the latter bed, mostly due to marteiliosis, impeded farther sampling. Fresh haemolymph was withdrawn from the posterior adductor muscle of the cockles and then a tissue piece from each cockle was processed with standard histology for disease diagnosis and assessment of gonad condition. An aliquot of the haemolymph sample of each cockle was used to estimate the total haemocyte count (THC), expressed as the number of cells per ml of haemolymph, using a counting chamber Malassez and a light microscope. Additionally, 150 µl of haemolymph were cytocentrifuged to attach the haemocytes onto a slide and the cells were fixed, stained and examined with a light microscope to estimate the relative abundance of each recognizable haemocyte type in the haemolymph sample, that is to say the differential haemocyte count (DHC).

Six haemocyte types were distinguished in the cockle haemolymph according to morphological characters and staining properties, namely (from more to less abundant in average) eosinophilic granulocytes, large hyalinocytes, basophilic granulocytes, haemocytes

with a large vacuole (HLV), haemoblasts and mixed granulocytes. One of these types, the HLV, has not been identified in bivalve species other than *Cerastoderma* spp. The temporal variation of THC in the haemolymph of the cockles collected from Noia through the two year-study period showed a seasonal pattern. Regarding the temporal variation of DHC, the percentage of granulocytes (and that of eosinophilic granulocytes) showed maxima in mid-late autumn and early spring, while minima in summer. Two environmental variables with seasonal pattern of variation, seawater temperature and salinity, were significantly associated with the THC and the DHC of the haemolymph of cockles collected from Noia. Another factor with seasonal pattern of variation, the gonad condition, seemed to influence THC because significantly higher values of THC corresponded to cockles with reabsorbing or resting gonads compared to those in the remaining classes of gonad condition. Stating which factors with seasonal pattern of variation have a more direct influence on THC is difficult because the maximum values of THC occurred in late summer, when temperature and salinity reached maxima and most cockles showed either reabsorbing or resting gonads. Similarly, the minimum values of THC occurred in winter to early spring, when temperature and salinity minima were recorded and gonads showed gametogenesis progression. Regarding DHC, it seemed to be influenced by the gonad condition too; higher mean percentage of eosinophilic granulocytes were detected in cockles with reabsorbing and resting gonad than in cockles with ripe or spawned gonads. Conversely, the mean percentage of basophilic granulocytes was lower in cockles with reabsorbing and resting gonad than in cockles with ripe or spawned gonads. Consistently, the seasonality of DHC could be influenced by the gonad condition because the maximal mean percentages of eosinophilic granulocytes occurred in November coinciding with maximum percentages of cockles in resting gonad stage, whereas the minimal mean percentages of eosinophilic granulocytes occurred in the period May-July, coinciding with the spawning period, that is to say when cockles showed ripe or spawned gonads

Marteiliosis, significantly influenced the THC and the DHC of the haemolymph of cockles that had been transplanted into Lombos do Ulla. The THC of cockles heavily affected by marteiliosis was significantly lower than that of non-infected cockles. Concerning the DHC, heavy marteiliosis involved decrease of the relative abundance of eosinophilic granulocytes and increase of mixed granulocytes, large hyalinocytes and haemoblasts. The association of heavy marteiliosis with THC decrease was opposite to the general trend in bivalves of THC rise associated with heavy infections, according to literature, and would deserve further research. In this study, contrary to the case of marteiliosis, a THC drop was not observed in association with any of the other pathological conditions and contrasted with the THC rise associated with the infection with *M. tapetis* and the heavy haemocytic infiltration of tissues. The drop of the relative abundance of eosinophilic granulocytes associated with heavy marteiliosis was not observed in the other pathological conditions while heavy granulomatosis was associated with increase of the relative abundance of mixed granulocytes.

Flow cytometry allowed discrimination of cockle haemocytes in four fractions (R1-R4) depending on their size and cell complexity. The correspondence of those four fractions with

the six recognisable morphological types with light microscopy was not straight, although most granulocytes (particularly eosinophilic granulocytes) would be expected to be included in the R1 fraction of flow cytometry and most haemoblasts would be expected in the R4 fraction. Sorting the haemocytes from each region was possible with a sorter device coupled to the flow cytometer although each sorted fraction was contaminated to some extent with cells from the other fractions. Flow cytometry was used to estimate some immune abilities, namely the ability to phagocytose bacteria, the production of reactive oxygen intermediates (ROIs), and the lysosomal and non-specific esterase content. The phagocytic ability was evaluated by *in vitro* challenging cockle haemolymph with fluorescence-labelled bacteria. Granulocytes showed the highest ability to phagocytose bacteria, while large hyalinocytes and haemoblasts showed very limited ability to do it. Regarding production of ROIs, all the cockle haemocyte types showed similar ability. Finally, larger, more complex haemocytes (likely granulocytes) showed the highest lysosomal and non-specific esterase content, while the other cell types showed lower to null content.

2. Introduction

In the context of the multiple variety of stressing factors that cockles have to face and the cockle capabilities to address them, an objective of the project COCKLES was to get knowledge on the cockle immune system. General knowledge of the bivalve mollusc immune system derives from studies mainly focused on the most important species from a commercial point of view, such as oysters, mussels, some clams and, to a lesser extent, some scallops. However, very few studies have focused on the immune system of cockles, which is largely unknown. Bivalve molluscs are able to avoid and neutralise multiple pathogens because they possess a potent and efficient innate immune system, physical barriers, and behavioural avoidance (Gerdol *et al*. 2018). The shell is the first physical barrier; due to the filter feeding of the bivalves, surfaces of their mantle and gills are exposed to large volumes of water containing microbes and plankton but bivalves are able to distinguish non-nutritious or potentially harmful particles (including some micropathogens) on the basis of size, physical, and chemical cues, and reject (expel) these particles using mucociliary mechanisms (Ben Horin *et al*., 2015). They are also able to shut down feeding and keep the valves tightly closed under unfavourable environmental conditions (e.g., low oxygen or blooms of an undesirable phytoplankton species). Mucosae constitute the next barrier to micropathogens, not only physical but representing the first line of immune defence with a relevant role of mucous (Allam & Pales-Espinosa 2016). The innate immune system of the bivalves involves, as its major players, circulating haemocytes and a broad range of diverse molecular effectors dissolved in the plasma which work in a complementary fashion to neutralize invading organisms. Haemocytes represent the backbone of the bivalve immune system; they are multi-potent and contribute to several biological functions, playing a major role in phagocytosis of invading microbes and encapsulation of larger

or refractive invaders, but they are also involved in wound healing, food digestion and transport of nutrients, reproduction, excretion, shell formation, and production and secretion of humoral factors involved in the immune response (Allam & Raftos, 2015). Molluscan haemocytes are generally classified based on morphological appearance and ‘‘granularity’’ of their cytoplasm, thus distinguishing two main types, granular cells (granulocytes) and agranular cells (hyalinocytes) in most studied species, to which blast-like cells are added in some cases (Cheng, 1984; Hine, 1999; Allam & Raftos, 2015). Some functional differentiation between mollusc haemocyte types has been found using flow cytometry (Lambert *et al.*, 2007; Donaghy *et al.*, 2009a; Hong *et al.*, 2014a; Wikfors & Alix, 2014; Li *et al.*, 2018; Rolton & Rag, 2020; Rolton *et al.*, 2020) as well as transcriptomic (Mao *et al.*, 2020) and proteomic (de la Ballina *et al.*, 2020; de la Ballina *et al.*, submitted) approaches. The total haemocyte count (THC) in the haemolymph is influenced by environmental conditions, particularly temperature (Carballal *et al*., 1998; Flye-Sainte-Marie *et al*., 2009) and pollution (Mansour *et al*., 2017; Matozzo *et al*., 2019) among others, and by infection with some pathogens (Carballal *et al.*, 1998; da Silva *et al*., 2008; Flye-Sainte-Marie *et al*., 2009; Choi *et al*., 2011).

Regarding cockles *Cerastoderma edule*, Russell-Pinto *et al.* (1994) distinguished three haemocyte types: granulocytes, the largest type, with pseudopods, abundant cytoplasmic granules and eccentric nucleus, representing *ca.* 64% of the haemocytes; hyalinocytes, the smallest type, without pseudopods, with a central nucleus and scarce cytoplasm, representing *ca.* 22% of the haemocytes; and a particular third type (named type III), with intermediate size, rounded, without pseudopods, with a large vacuole occupying most of the cell and very eccentric nucleus, representing *ca.* 14% of the haemocytes. Wootton *et al*. (2003a) also recognised those three haemocyte types in cockles *C. edule* and stated two granulocyte subtypes, eosinophilic (eosinophilic) and basophilic; the average percentage of each cell type was 44.9%, 30.2%, 15.8% and 9.1% for eosinophilic granulocytes (named granular eosinophils), basophilic granulocytes (named granular basophils), hyalinocytes (named agranular basophils) and type III (named type III eosinophils), respectively. Matozzo *et al.* (2007) studied the haemocytes of the Mediterranean cockle *Cerastoderma glaucum* and also reported the occurrence of basophilic and eosinophilic granulocytes, basophilic hyalinocytes and haemocytes with a large vacuole with eosinophilic content (similar to the type III described by previous authors) that the authors called eosinophilic hyalinocytes. Both haemocytes and haemolymph plasma of cockles *C. edule* showed antibacterial activity, even higher than that of mussels *Mytilus galloprovincialis*, oysters *Ostrea edulis* and *Crassostrea gigas*, and clams *Ruditapes decussatus* and *Ruditapes philippinarum* (Casas *et al*., 2011). High antibacterial activity as well as antiviral activity in extracts from cockles *C. edule* and *C. glaucum*, even higher than those in other bivalve and gastropod molluscs, was also reported in other studies without allocation into organ, tissue or cell types (Defer *et al*., 2009; Ghorbanalizadeh *et al*., 2018; Mona *et al*., 2021). Concerning the phagocytic ability, the percentage (ca. 80%) of cockle *C. edule* haemocytes that phagocytosed zymosan (protein-carbohydrate complexes derived from yeast cell wall) particles (about 3 µm in diameter) was similar to that of mussel *Mytilus edulis*

haemocytes, while the percentages of cockle haemocytes phagocytosing heat-killed bacteria cells of 3 different species were significantly lower than those of mussel haemocytes; the phagocytosing haemocytes were identified as granulocytes (Wooton *et al*., 2003a). Around 80% of *C. edule* haemocytes phagocytosed inert beads (1 µm in diameter, polystyrene microspheres) (Diaz *et al*., 2011). Matozzo *et al*. (2007) reported that the ability of cockle *C. glaucum* haemocytes to phagocytose yeast cells was significantly higher if the yeast cells had been "opsonised" (incubated with cockle plasma); the authors found that granulocytes had more ability than hyalinocytes while the haemocytes with large vacuole did not phagocytose yeast cells. The haemocytes of *C. edule* (Wootton *et al*., 2003a,b; Díaz *et al*. 2011), as well as all the haemocyte types of *C. glaucum* (Matozzo *et al*., 2007), produce reactive oxygen intermediates (ROIs), which contribute to neutralise pathogens. Regarding lysosomal enzymes, also contributing to degrade pathogens, the occurrence of acid-phosphatase, non-specific esterase and aryl sulphatase was demonstrated in *C. edule* haemocytes, predominantly in eosinophilic granulocytes (Wootton *et al*., 2003a,b). Similarly, Díaz *et al*. (2011) detected higher lysosomal content and non-specific esterase levels in *C. edule* granulocytes than in hyalinocytes. In the case of *C. glaucum* haemocytes, the occurrence of acid-phosphatase, N-acetyl-β-glucosaminidase, non-specific esterase, acid esterase and N-acetyl-β-hesoxaminidase was detected, with higher levels in granulocytes than in hyalinocytes and no detection in haemocytes with large vacuole (Matozzo *et al*., 2007). Haemocytes of *C. edule* are able to bind to concanavaline A, and *Tetragonolobus purpurea*, *Helix pomatia* and wheat germ agglutinins (Wootton *et al*., 2003a). Lysozyme activity was found to be higher in haemocytes of *C. glaucum* than in plasma (Matozzo *et al*., 2007).

Knowledge of the immune cockle system should contribute to understand how the cockle interacts with pathogens and why some infections can progress causing severe damage, even host death. Furthermore, it could contribute to find ways to overcome cockle pathological problems. Multiple parasites and diseases have been identified in cockles through the Atlantic Area, both previously and in the context of the project COCKLES, with a wide range of pathogenicity and potential to threaten cockle populations (de Montaudouin *et al*., 2021). Particular attention deserves the infection caused by the protistan *Marteilia cochillia*, a highly pathogenic parasite that invades and disables the cockle digestive gland, resulting in energy deprivation and cockle death. This parasite is the agent of cockle marteiliosis, a disease that caused cockle fishery collapse in the southern rias of Galicia (NW Spain) (Villalba *et al*., 2014; Iglesias *et al*., 2015).

The specific objectives of our study of the *C. edule* immune system were to characterise the haemocyte types; to search for differences between haemocyte types in some immune abilities, namely phagocytosis, production of ROIs, lysosomal content and non-specific esterase content; and to evaluate the influence of environmental factors (namely temperature and salinity), internal physiological factors as the cockle gonad condition, as well as the most serious pathological conditions on the total haemocyte count (THC) and the relative abundance of each haemocyte type, that is to say the differential haemocyte count (DHC).

3. Materials and Methods

3.1. Sampling

Two shellfish beds of Galicia (NW Spain) were selected for this study (Fig. 1). Considering the high pathogenicity of marteiliosis (infection with the protistan *Marteilia cochillia*) for cockles (Villalba *et al*., 2014) and presuming that this disease could significantly influence THC and DHC, one shellfish bed located in Noia (inner side of the ria of Muros-Noia, 42°47'25''N – 8°55'22''W), an area with high cockle production where marteiliosis (infection with the protistan *Marteilia cochillia*) outbreaks have never been detected (Iglesias *et al.*, 2017), was chosen as source of cockles for the study to avoid the interference of this disease. Additionally, adult cockles were collected from the bed of Noia and transplanted into the bed of Lombos do Ulla (inner side of the ria of Arousa, 42°37´45.4'' N – 8°46'31.3'' W) in April 2018, which is thoroughly described in the section 3.2 of the deliverable 7.2 of this project COCKLES (Cao *et al*., 2021), to evaluate the effect of marteiliosis on some variables considered in the study. Briefly, 2160 cockles collected from Noia were distributed into 18 plastic boxes (120 cockles per box, around 600 cockles / m^2) partially filled with shellfish bed sediment; the boxes were covered with a plastic net (10 mm mesh), to avoid predation, and arranged in three frame structures (six boxes in each frame structure); those structures with the boxes were submerged to the bottom, on the shellfish bed of Lombos do Ulla, and kept connected with a rope to a buoy (Fig. 2).

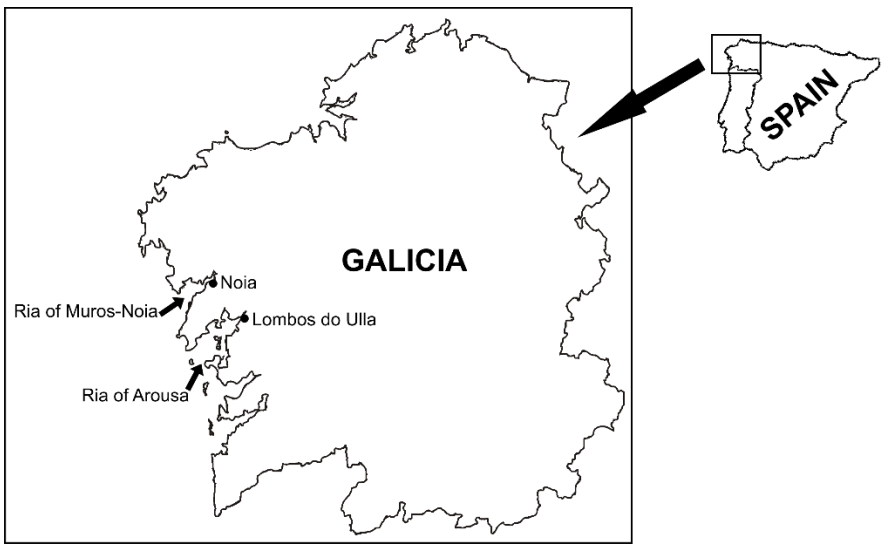

Fig. 1. Map showing the location of the two shellfish beds involved in the study, Noia and Lombos do Ulla.

Fig. 2. A: six plastic boxes filled with shellfish-bed sediment, arranged within a frame structure; the cockles on the sediment had just been set and they would burrow within the sediment once boxes were submerged. B: the six boxes covered with a net to avoid predation. C and D: a frame structure with six boxes in the process of submersion in Lombos do Ulla.

From 30 to 33 adult cockles were collected monthly from each bed and transported to the laboratory of CIMA to be processed for various analyses. Sampling in Noia was performed from April 2018 to March 2020, while in Lombos do Ulla from May to December 2018; the high mortality of the transplanted cockles in the latter bed, mostly due to marteiliosis, impeded farther sampling. Once in the laboratory, the cockles from Noia were kept in closed circuit with filtered, UV-treated, aerated seawater until the next day to purge them; the cockles from Lombos do Ulla were kept in open circuit with seawater pumped from the ria because of the close location of the laboratory and the shellfish bed.

3.2. Haemolymph collection and histological processing

Fresh haemolymph was withdrawn from the posterior adductor muscle of the cockles using a cold syringe 1ml with a 30G needle, through a small notch made in the shell margin with a carpenter file (Fig. 3). About 700-900 µl of fresh haemolymph were transferred into microtubes and kept on ice, to avoid haemocyte aggregation, until further use. Anti-aggregating solutions were not used to prevent interferences in the subsequent functional immunoassays. A drop of each haemolymph sample was examined with light microscopy to assess quality; the samples contaminated with debris, gametes or any other indication of contamination with

water from pallial cavity or tissues other than haemolymph were discarded. Haemolymph samples with neoplastic cells were also discarded due to the interference of those cells on haemocyte counts. After collecting haemolymph, each cockle was shucked and a piece of tissues (5 mm thick) containing visceral mass, foot, mantle lobes, and gills was taken, fixed in Davidson's solution for 24 h at 4 °C, dehydrated in ethanol series and embedded in paraffin; 5 µm thick sections were stained with Harris' haematoxylin and eosin (Howard *et al*., 2004) and examined with light microscopy for disease diagnosis and assessment of gonad condition.

Fig. 3. Photograph showing haemolymph collection from the posterior adductor muscle of a cockle.

3.3. Total and differential haemocyte counts

An aliquot of the haemolymph sample of each cockle was used to estimate the total haemocyte count (THC), expressed as the number of cells per ml of haemolymph, using a counting chamber Malassez and a light microscope. Additionally, 150 µl of haemolymph were centrifuged (92 *g*, 5 min, 4 °C) to attach the haemocytes onto a slide, using a Megafuge 1.0R (Heraeus) cytocentrifuge. To do this, the haemolymph was transferred into a microtube with an opening in the bottom, which was set above a slide and hold in a cyto-container (Fig. 4); two microtubes (two haemolymph samples) were hold in each cyto-container, thus resulting two areas of a single slide covered with attached haemocytes (cytospins), each area corresponding to a different cockle. After centrifugation, the slides with the haemocytes were allowed to dry, and then the cells were fixed and stained with the kit Hemacolor® (Merck). The stained cytospins were examined with a light microscope to estimate the differential haemocyte count (DHC), that is to say the relative abundance of each recognizable haemocyte type in the haemolymph sample. To do this, from 100 to 500 cells occurring in each cytospin were assigned to one of the recognised haemocyte types; then, the percentage of the total cells corresponding to each haemocyte type was calculated.

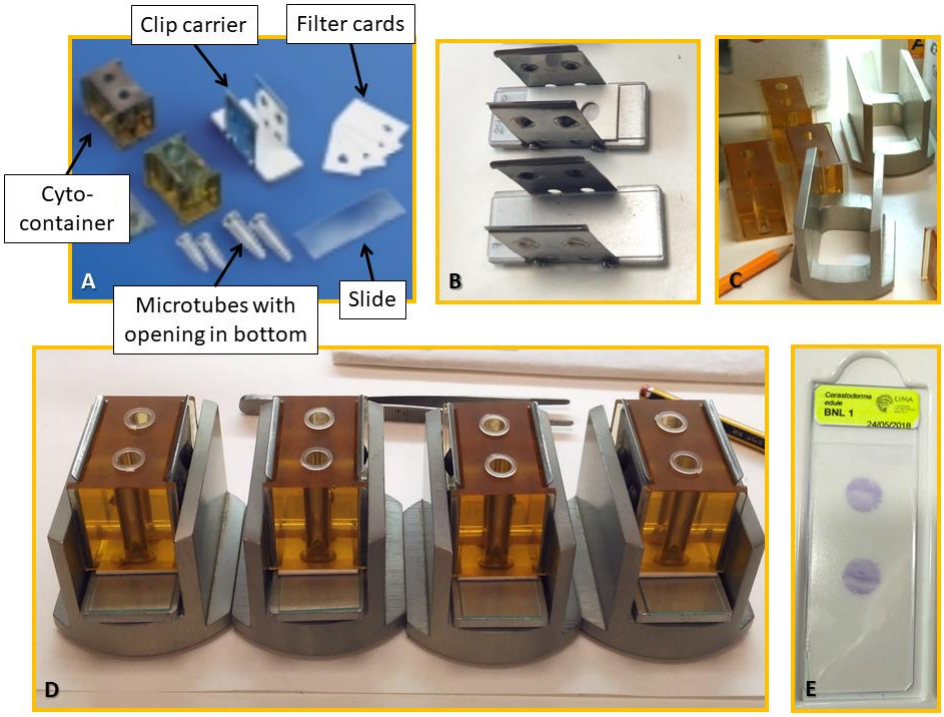

Fig. 4. Accessories used in the cytocentrifugation process to attach the haemocytes of haemolymph samples onto slides. A: Image showing the accessories. B: Two clip carriers with each slide; the clip carrier of the top has a filter card, with two circular holes (only one is visible), on the slide. C: Cyto containers and support inserts. D: Assembled accessories; each cyto-container, holding two microtubes, is mounted on a slide, with a filter card between the slide and the cyto-container. E: Slide with two circles covered with attached haemocytes (cytospins) after cytocentrifuging, fixation, staining and covering.

3.4. Evaluation of health and gonad condition

The histological sections of the cockles were examined with light microscopy to detect parasites and pathological conditions. Special attention was devoted to those conditions considered the most pathogenic for cockles (de Mountaudouin *et al*. 2021), namely infection with *M. cochillia*, infection with *Minchinia tapetis*, infestation with trematode sporocysts, granulomatosis and inflammatory reaction. As explained above, cockles affected by neoplasia were discarded of this study. Each cockle was assigned to a severity category as follows: 0 (null), 1 (light severity), 2 (moderate severity), and 3 (heavy severity). In the case of the infection with *M. cochillia*, cockles were assigned to one of the following infection stages: 0 (null), 1 (early stage), 2 (intermediate stage), 3 (advanced stage), 4 (post-spore release) (Iglesias *et al.*, in prep.). Additionally, each cockle was assigned to a gonad condition stage as follows: 0 (resting gonad), 1 (early gametogenesis), 2 (advanced gametogenesis), 3 (ripe gonad), 4 (spawned gonad), 5 (reabsorbing gonad) (Díaz *et al*., 2016; Maia *et al.*, 2021).

3.5. Information on environmental conditions

A data-logger (conductivity-temperature recorder Compact-CT, JFE ALEC Co.) was set above the sediment in one of the boxes with transplanted cockles from Noia that were deployed in the shellfish bed of Lombos do Ulla; it recorded salinity and temperature values every 10 min. In the case of the shellfish bed of Noia, the information on seawater temperature and salinity derived from the weekly records obtained with a CTD device in the sampling station M7 (the closest to the shellfish bed) of the oceanographic sampling net of INTECMAR (http://www.intecmar.gal/Informacion/fito/Estacions/Default.aspx?sm=b5), which is located around 2 Km far from the shellfish bed (42° 47' 45.6'' – N 08° 56' 32.4'' W).

3.6. Statistical analyses on factors influencing THC and DHC

The influence of pathological conditions, gonad condition, temperature and salinity on the THC was analysed using multiple linear regression, considering four pathological conditions (infection with *M. tapetis*, infestation with trematode sporocysts, granulomatosis, and haemocytic infiltration of tissues) in the case of cockles collected from Noia, while five pathological conditions (the same four as in Noia plus infection with *M. cochillia*) were considered in the case of the cockles that had been transplanted into Lombos do Ulla. THC values were $\log_{10}$ transformed to meet statistical requirements. Regarding DHC, the sum of the percentages of each haemocyte type is constrained to a constant, thus they constitute a compositional variable (Aitchison, 1982, 1986); because of this, the isometric log-ratio transformation was used (Egozcue *et al*., 2003). Multiple linear regression for compositional data was used to evaluate the influence of the pathological conditions, temperature and salinity, again considering separately the cockles collected from Noia and those that had been transplanted into Lombos do Ulla.

3.7. Flow cytometry assays

Various assays were carried out by flow cytometry to assess functional abilities of the cockle haemocytes, namely, phagocytosis ability, production of ROIs, and lysosomal and non-specific esterase contents. Multiple assays were performed to optimise the experimental conditions and procedures. All the assays were performed with haemolymph from cockles that had been collected from Noia the previous day and kept in a tank with filtered, mild-aerated seawater until haemolymph withdrawal. First of all, the cockle haemocytes were analysed in a forward scatter (FSC: particle size) vs side scatter (SSC: internal complexity) in logarithmic scale dot plot, using a BD FACSARIA III cell Sorter flow cytometer and the DRAQ5 dye (Biostatus), which stain nucleic acids to discriminate the nucleated cells. Propidium iodide (SIGMA) was used to identify dead cells. At least 10000 events were recorded in each flow-cytometer assay. The Flowlogic software (Inivai) was used for analysis. Discrimination of cell groups based on their relative size and complexity was performed by delimiting regions with more complex and

larger cells and regions with less complex and smaller cells in the FSC vs SSC dot plots. A trial was performed to evaluate the correspondence between the cell discrimination provided by this flow cytometry procedure and the previously haemocyte types established by morphological and staining features, based on light microscopy examination. Cockle haemolymph from 20 cockles was pooled; a subsample of the haemolymph was passed through the flow cytometer to assess the distribution of the cells in different size-complexity regions; another subsample of the haemolymph pool was centrifugated in a Percoll® (Sigma) discontinuous density gradient composed of 60, 50, 40, 30 and 20% (v/v) Percoll® in filtered seawater; after centrifugation (680 x g, 30 min, 4 °C) the cells present at each interphase were washed with filtered seawater, passed throw the flow cytometer and the distribution of the cells from each interphase in regions according to size and complexity was assessed. Furthermore, a cytospin was performed with a subsample of the cells accumulated in the interphase 50/40, that with higher number of cells, to assess the relative abundance of each recognisable haemocyte type.

Additionally, the efficacy of using a **FACSARIA III Sorter** module to separate the cells of the different size-complexity regions delimited with the flow cytometer was evaluated. A cockle haemolymph pool was passed through the flow cytometer; the cells within each size-complexity region were gated and sorted; then, the cells sorted from each region were passed again through the flow cytometer to reassess their distribution in the different size-complexity regions.

3.7.1. Phagocytosis assays

Phagocytic ability was evaluated by *in vitro* challenging haemocytes with commercial pHrodo™ Green *Eschericia coli* BioParticles™ (Invitrogen). These bacterial conjugates are non-fluorescent outside the cell at neutral pH, but fluoresce brightly green at acidic pH, such as inside phagosomes. Thus, non-phagocytosed bacteria sticking to the outer cell membrane of the haemocytes are not detected (do not fluoresce), whereas those internalised in haemocytic lysosomes are highly fluorescent (Lindner *et al*., 2020). The lack of fluorescence of the bacteria outside the haemocytes eliminates the need for wash steps and quencher dyes. The haemolymph collected from 20 cockles was pooled; an aliquot was used to estimate the total haemocyte count, as explained above, and the concentration was adjusted to $1x10^6$ haemocytes/ml with filtered seawater (FSW). The adjusted haemolymph pool was dispensed into 6-wells plates. Four wells were filled with 3 ml of haemolymph ($3x10^6$ haemocytes per well); pHrodo™ Green *E. coli* BioParticles were added at a ratio 1:20 (haemocyte:bioparticles) and incubated at room temperature for 2 hours. Other haemocyte:bacteria ratios were tested (1:40 and 1:80) for tuning up the procedure and, although 1:40 ratio showed better resolution in flow cytometer, subsequent haemocyte type identification became very difficult due to the presence of the bacteria in the samples, so 1:20 seemed to be the optimum. Controls with haemocytes but without bioparticles and wells with haemocytes to add the bioparticles at the same ratio just before passing through the flow cytometry (t=0) were included. After incubation, the cells

were collected from the wells with light scraping with the plunger of a 1 ml syringe (because the cells were attached to the well bottom) and were filtered by a 40 µm mesh cell strainer (Corning) to obtain a homogeneous cell suspension, without clumps. The cells were analysed by flow cytometry and gates were selected based on their fluorescent signal. Negative, intermediate and fluorescein isotiocyanate (FITC) positive gates were drawn. Cells from each fraction were sorted using the **FACSARIA III Sorter** module. Sorted cells were kept at 4 °C until use. Once the cells were sorted, a cytospin of each fraction was performed and stained as described above. Highly diluted cells were previously centrifuged at 300 g, 5 min, 4 °C in order to get enough cells in the cytospin. Cytospins produced after sorting cells as wells as those produced before the haemolymph was incubated with bioparticles were observed under standard light microscopy and confocal fluorescence microscopy (Leica SP5 confocal module).

3.7.2. ROI production assays

One of the weapons contributing to neutralise pathogenic microorganism is the production of reactive oxygen intermediates (ROIs). The production of ROIs was measured by flow cytometry using the chloromethyl derivative of the dichlorofluorescein-diacetate probe (CM-H2DCFDA, Invitrogen). This probe is a cell permeant indicator for ROIs that is nonfluorescent until removal of the acetate groups by intracellular esterases. Subsequent oxidation yields a green fluorescent adduct that is trapped inside the cell. Green fluorescence, detected on the FL1 detector of the flow cytometer, is proportional to the total oxidative activity of haemocytes. Individual haemolymph samples and pooled haemolymph from the same cockles were used. Haemolymph was withdrawn as explained before, an aliquot was used to estimate the THC, and the concentration was adjusted to $1x10^6$ haemocytes/ml with FSW. The assay was performed in the presence and in absence of zymosan; zymosan was used as a supposed stimulus of ROI production. The zymosan was used at a final concentration of 0.1 mg/ml, i.e. a ratio 1:20 (haemocyte:zymosan particles). For the assay, 400 µl of haemolymph (200,000 haemocytes) were incubated in wells with 400 µl of zymosan (or FSW in the non-stimulated samples). After 40 min, CM-H2DCFDA was added at 8.6 or 4.3 µM final concentration, and incubated for 30 min in the dark. After incubation, the cells were collected from the wells with light scraping with the plunger of a 1 ml syringe and were filtered by a 40 µm mesh cell strainer (Corning) to obtain a homogeneous cell suspension, without clumps. Part of the sample was incubated 30 more minutes and, before the flow cytometry analysis, 3 µl of propidium iodide (PI, Sigma) was added to differentiate the dead cells; PI does not penetrate in viable cells, whereas altered membranes of dead cells are permeable to this dye. The flow cytometry assay was performed three times, using each time haemolymph from three individual cockles plus pooled haemolymph from those three cockles. Similar assays were performed with mussel *Mytilus galloprovincialis* haemocytes used as reference from a bivalve model species, with the same experimental conditions as those used for cockle haemocytes.

3.7.3. Lysosomal and non-specific esterase content assays

Lysosomes and esterases are related with immune function because of their involvement in bacterial destruction (Bang *et al.*, 2014). The lysosome content in the cockle haemocytes was determined using LysoTracker Deep Red (Invitrogen). This deep red-fluorescent dye accumulates in intra-cytoplasmic acid compartments in live cells, which are mainly lysosomes. Additionally, the esterase content was determined using fluorescein diacetate (FDA, Invitrogen), a non-fluorescent cell-permeant esterase substrate, which produces fluorescein upon hydrolysis by intracellular esterases. Determination of both lysosomal and non-specific esterase contents was performed simultaneously, using haemolymph individual samples from five cockles plus pooled haemolymph from those five cockles. Haemolymph was withdrawn as explained before, an aliquot was used to estimate the THC, and the concentration was adjusted to $1x10^6$ haemocytes/ml with FSW. For the flow cytometry assay, 400 µl of haemolymph (400,000 haemocytes) were incubated with 15 µl of LysoTracker® (75 nM final concentration), at room temperature for 1h in the dark. Then, 2 µl FDA (3.2 uM) was added and samples were incubated for other 30 min., at room temperature in the dark. A well with 400 µl of haemolymph without probes was included as control. After incubation, the cells were collected from the wells with light scraping with the plunger of a 1 ml syringe and were filtered by a 40 µm mesh cell strainer (Corning) to obtain a homogeneous cell suspension, without clumps. The haemocytes were passed through the flow cytometer and FSC, SSC, FITC (FDA signal, indicative of the esterases level) and APC (lysotracker signal, indicative of the lysosomal content) data were recorded.

4. Results and Discussion

4.1. Haemocyte types

Examination of cytospins showed different morphological characters in cockle haemocytes, namely with or without cytoplasmic granules, among which eosinophilic and basophilic granules were observed; with or without a large vacuole; with or without pseudopods; and higher or lower nucleus:cytoplasm (N:C) ratio. Six morphotypes were recognised among cockle haemocytes. The granulocytes were large cells with abundant cytoplasmic granules, pseudopods, and low N:C ratio; depending on the staining of the cytoplasmic granules, three types of granulocytes were distinguished: eosinophilic granulocytes (Fig. 5A); basophilic granulocytes, usually with fine granules (Fig. 5B) but occasionally with coarser ones (Fig. 6A); and mixed granulocytes, with both eosinophilic and basophilic granules in their cytoplasm (Fig. 5C). Large hyalinocytes were agranular cells, with pseudopods and low N:C ratio (Fig. 5D). Another particular haemocyte type was designated as haemocyte with large vacuole (HLV),

because of the occurrence of a striking large vacuole occupying most of the cytoplasm, with the nucleus in peripheral location; the vacuole usually was filled with eosinophilic material (Fig. 5E) but occasionally showed irregular, fragmented, eosinophilic content (Fig. 6B). Finally, the smallest type corresponded to agranular cells with scarce cytoplasm, thus showing a high N:C ratio, resembling blast cells, that were designated as haemoblasts; their cytoplasms used to be more or less basophilic (Figs. 5F and 6C) although occasionally showed eosinophilic areas (Fig. 6D).

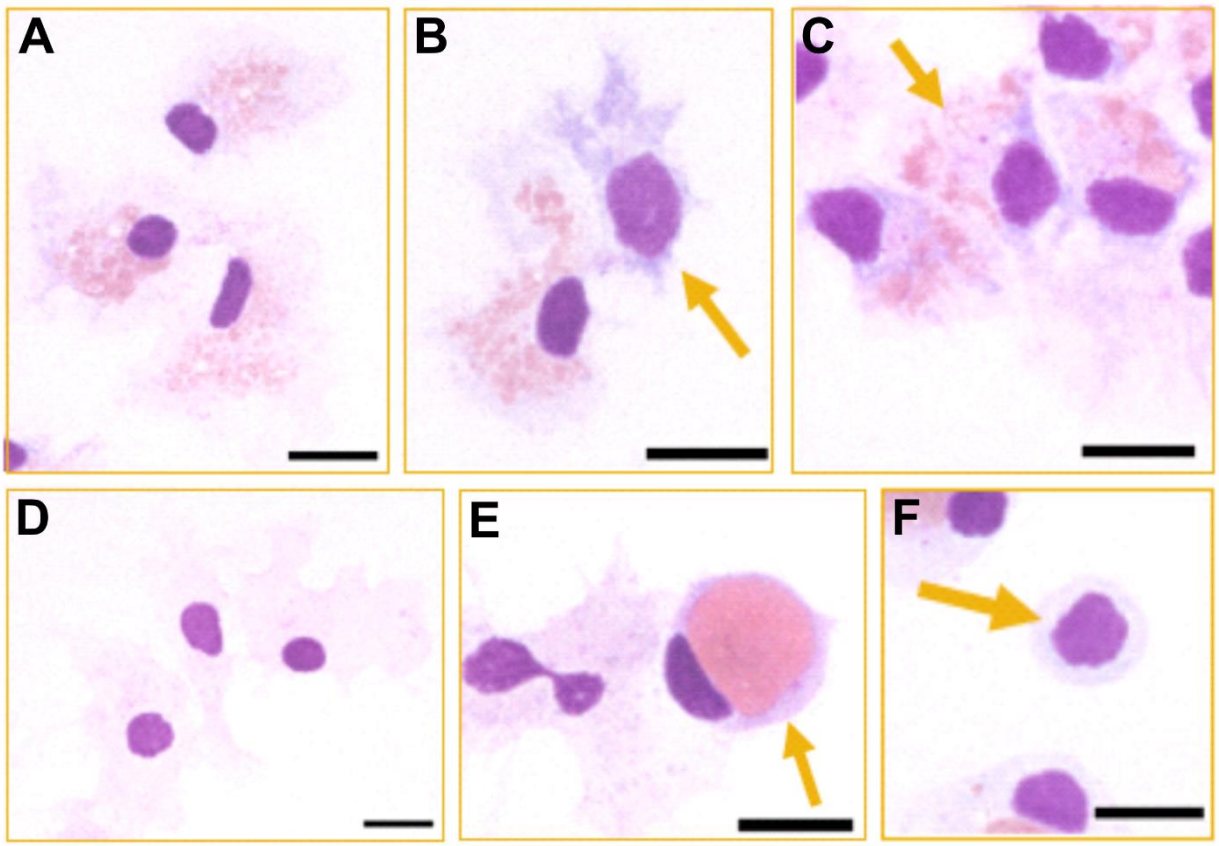

Fig. 5. Cockle haemocyte morphotypes recognised in cytospins. A: eosinophilic granulocytes; B: basophilic granulocyte (arrow); C: mixed granulocyte (arrow); D: large hyalinocytes; E: haemocyte with large vacuole (HLV, arrow); F: haemoblast (arrow). Scale bar: 10 µm.

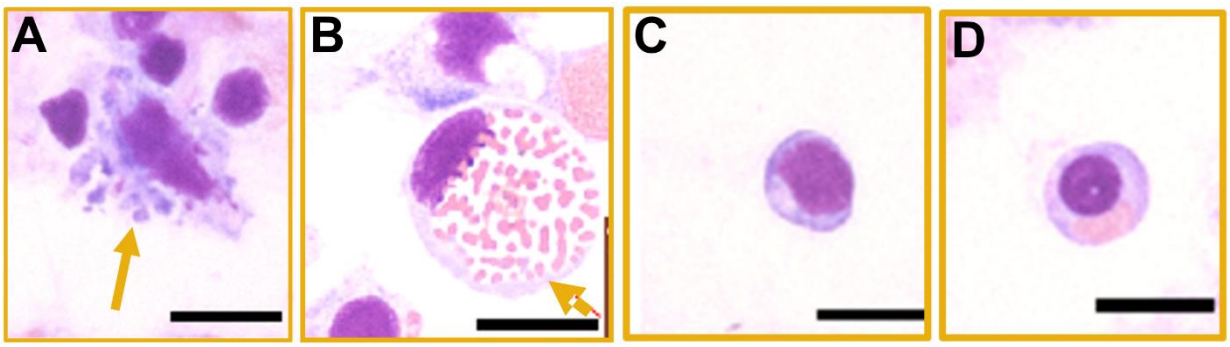

Fig. 6. Particular appearance of some haemocyte types. A: Basophilic granulocyte (arrow) with granules coarser than usual. B: Haemocyte with large vacuole (arrow) showing fragmented, irregular vacuolar content. C: Haemoblast with basophilic cytoplasm. D: Haemoblast with eosinophilic area in the cytoplasm. Scale bar: 10 µm.

4.2. Variability of haemocyte counts, THC and DHC

High variability was detected in both THC and DHC in the haemolymph from cockles collected from Noia in the two-year study period. Table 1 summarises statistical information of both THC and DHC. The distribution of cockles in classes of THC is shown in Fig. 7. The mean THC value recorded for the whole two-year study, 1.861 x 10^6/ml (SD = 1.138; SE = 0.048), was lower than values provided in other studies on *C. edule*, such as 3.3 x 10^6/ml (SE = 0.4, Casas *et al.*, 2011), and 4.54 x 10^6/ml (SE = 0.70; Wootton *et al.*, 2003a), and on *C. glaucum*, 5.5 x 10^6/ml (SD = 2.15; Matozzo *et al.*, 2007). Nevertheless, the values provided in those previous studies corresponded to cockles collected in a single sampling event or in a short period and, as explained below (section 4.2.1), the cockle THC has marked seasonal variation. Furthermore, multiple factors (see below, section 4.2.2) significantly influence cockle THC, which has to be considered when comparing values derived from different places or under different conditions. The DHC also showed high variability although the general trend was that granulocytes, particularly eosinophilic granulocytes, were the most abundant haemocytes, while haemoblasts were much less abundant and large hyalinocytes and HLVs showed intermediate abundance; the least mean value corresponded to mixed granulocytes (Table 1, Fig. 8).

Table 1. Summary of statistical data showing the variability of the total haemocyte count and the relative abundance of each haemocyte type in the haemolymph samples from the cockles (N=570) collected from Noia in the study period.

Cockle haemocytes	Range	Mean	SD	SE	95% Interval confidence	99% Interval confidence
Total haemocyte count (x 10^6/ml)	0.276 – 9.689	1.861	1.138	0.048	1.767 – 1.954	1.737 – 1.984
Eosinophilic granulocytes (%)	2.9 – 85.4	43.52	16.049	0.672	42.20 – 44.84	41.78 – 45.26
Basophilic granulocytes (%)	0.0 – 60.6	13.54	10.560	0.442	12.68 – 14.41	12.40 – 14.69
Mixed granulocytes (%)	0.0 – 44.4	2.02	4.318	0.181	1.67 – 2.38	1.56 – 2.49
Large hyalinocytes (%)	1.8 – 79.9	28.66	13.479	0.565	27.55 – 29.77	27.20 – 30.12
HLV (%)	0.0 – 44.0	10.04	6.622	0.277	9.50 – 10.59	9.33 – 10.76
Haemoblasts (%)	0.0 – 25.7	2.21	2.578	0.108	1.99 – 2.42	1.93 – 2.48

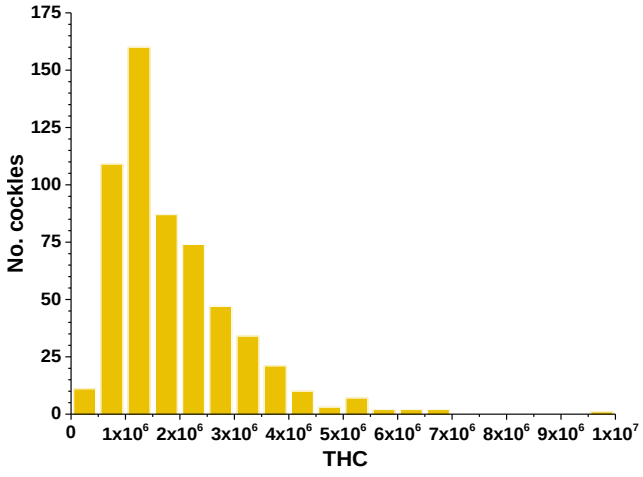

Fig. 7. Histogram of the total haemocyte count (THC, expressed as no. of haemocytes/ml of haemolymph) in the haemolymph of the cockles collected from Noia in the study period.

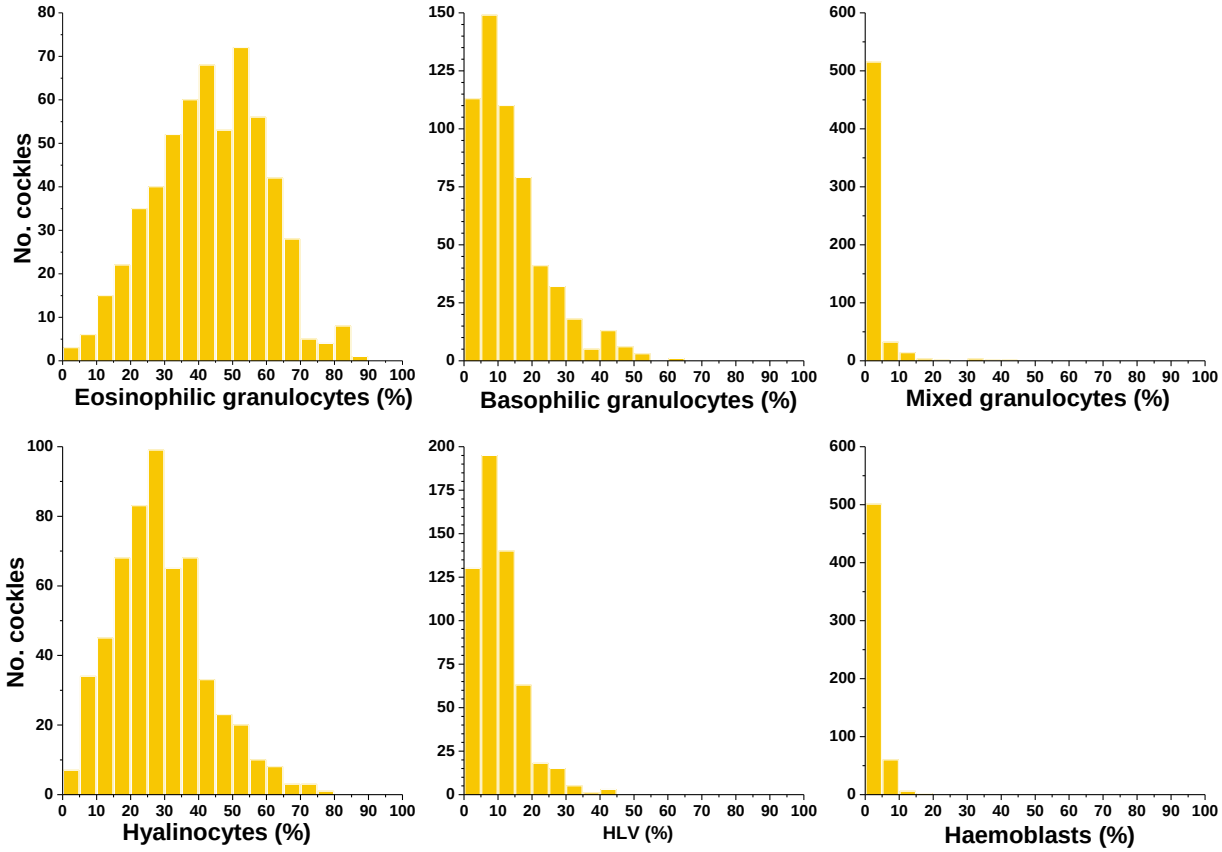

Fig. 8. Histogram of the relative abundance of each haemocyte type in the haemolymph of the cockles collected from Noia in the study period.

Russell-Pinto *et al*. (1994) described four types of cockle *C. edule* haemocytes: granulocytes, among which two types were further distinguished (called types Ia and Ib) depending on the cell size and the length of their pseudopods, hyalinocytes (called type II), and a third type (called type III) similar to the haemocytes with large vacuole identified in our study. Those authors did not discriminate the granulocytes depending on the staining properties of the cytoplasmic granules and, considering the relative abundance of their types Ia and Ib, 26.7 % and 37.4%, respectively, their joint value of 64.1% would not be far from the joint mean relative abundance of all the granulocytes in our study, 59.08 %. The authors remarked the high N:C of the type II haemocytes, which could lead to match up them with the haemoblasts of our study, but the relative abundance assigned to type II haemocytes (21.6%) would suggest that those authors joined all the agranular cockle haemocytes (including both the large hyalinocytes and the haemoblasts considered in our study) within the type II. Finally, the relative abundance provided for the type III, 13.9%, reasonably matches with that of the HLVs recorded in our study. Wootton *et al*. (2003a) did consider the staining properties and distinguished four types of cockle *C. edule* haemocytes, granular eosinophils, granular basophils, agranular basophils and type III eosinophils (with a very large vacuole). The relative abundance of their granular eosinophils (44.87%) and their type III eosinophils (9.13%) was similar to that of the eosinophilic

granulocytes and HLVs, respectively, in our study; the relative abundance of their granular basophils (30.25%) was higher than that of the basophilic granulocytes in our study, while that of their agranular basophils (15.75%) was lower than the joined relative abundance of large hyalinocytes and haemoblasts in our study. Matozzo *et al*. (2007) distinguished the same four types as Wootton *et al*. (2003a) among haemocytes of *C. glaucum*, calling them acidophil granulocytes (60%), basophil granulocytes (25%), acidophil hyalinocytes (with a large vacuole, 5%) and basophil hyalinocytes (10%). A common finding of the previous studies focused on the haemocytes of the cockles *C. edule* and *C. glaucum*, confirmed in our study, was the occurrence of a particular haemocyte type that has not been identified in other bivalve genera, the HLV. Knowing that this cell type is one of the haemocyte types occurring in the cockle is important because, frequently, it has been misidentified as a protistan parasite. Further study focused on HLV function would be advisable. The consideration of haemoblast (or blast-like cell) as a cell type within the agranular haemocytes has been frequently assumed when describing haemocytes of other bivalve species (Hine, 1999). The other haemocyte type identified in our study with very low abundance, the mixed granulocyte, was not mentioned in previous studies on cockle haemocytes and it was likely included within eosinophilic or basophilic granulocytes. Granulocytes with both eosinophilic and acidophilic cytoplasmic granules were also found in mussels *M. galloprovincialis* (Carballal *et al*., 1997).

The influence of various environmental and biological factors on the variability of both THC and DHC is analysed in the following two sections.

4.2.1. Temporal variability of THC and DHC

The temporal variation of THC in the haemolymph of the cockles collected from Noia through the two year-study period showed a seasonal pattern, with maxima (mean values above 3 x 10^6/ml) in late summer and minima (mean values ca. 1 x 10^6/ml) in early spring, and seemed to be associated with that of seawater temperature (Fig. 9), which ranged from 12.3 °C to 16.7 °C. The temporal variation of THC in the cockles transplanted from Noia to Lombos do Ulla in the shorter eight-month study period showed a similar pattern to that recorded in the cockles from Noia (Fig. 9) and also appeared associated with seawater temperature variation; temperature ranged from 13.1 °C to 19.7 °C in Lombos do Ulla in that period. The variation of the seawater salinity, which ranged from 20.1 psu to 35.5 psu and from 20.4 psu to 30.3 psu in Noia and Lombos do Ulla in their respective study periods, showed poorer association with the variation of THC in both places (Fig. 10). Regarding the temporal variation of DHC in the haemolymph of cockles collected from Noia, the percentage of granulocytes (and that of eosinophilic granulocytes) showed maxima in mid-late autumn and early spring, while minima from May to July in 2018 and from June to August in 2019 (Fig. 11). The temporal variation of DHC in the haemolymph of cockles transplanted from Noia into Lombos do Ulla was quite different from that observed in cockles collected from Noia (Fig. 12) but, as explained below, the DHC in of cockles in Lombos do Ulla was markedly affected by marteiliosis, mainly in the

period August-December, when the prevalence of marteiliosis was higher and the advanced infection stages tended to be more prevalent.

There are environmental variables with seasonal variation, such as temperature, salinity and food availability, that influence biological processes, such as growth, gonad cycle, and infection dynamics of multiple pathogens. Therefore, considering that haemocytes are involved in multiple functions, among them nutrient transport, gonad reabsorption and immune response, the temporal variation of both THC and DHC could be the consequence of multiple causes. A clear seasonal pattern was also observed for THC in cultured mussels *M. galloprovincialis* from Galicia, with maxima in late summer and minima in winter, while DHC did not show seasonal variation pattern (Carballal *et al*., 1998). Seasonal pattern of variation was also found for the total granulocyte count in the haemolymph of the Manila clam *R. philippinarum* from Brittany (France), with maxima in summer and minima in late winter (Flye-Sainte-Marie *et al*., 2009). The highest values of THC for the mangrove oyster *Crassostrea rhizophorae* from Santa Catarina (Brazil) were recorded in summer while the highest percentage of granulocytes in winter/spring (Barth *et al*., 2005). Seasonality was also found in the case of the THC of the giant honeycomb oyster *Hyotissa hyotis* from Korea (Hong *et al*., 2020).

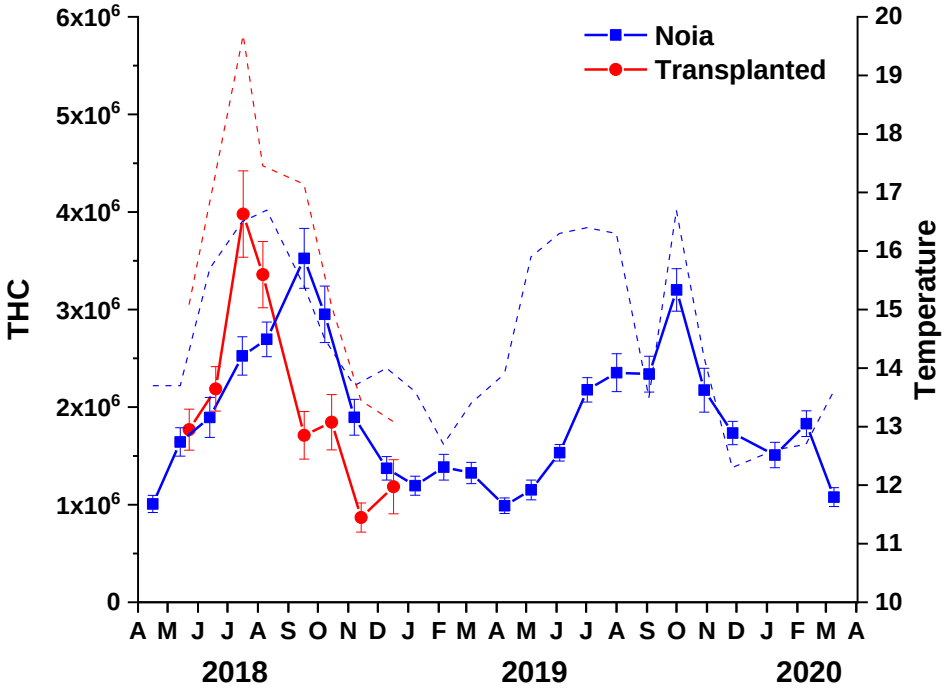

Fig. 9. Temporal variation of the mean value (±SE) of THC (blue, solid-line) in cockles collected from Noia through the two-year study period and the corresponding seawater temperature (blue, dashed-line) at sampling. The values corresponding to cockles transplanted from Noia to Lombos do Ulla are shown in red, the solid-line for THC and the dashed line for temperature.

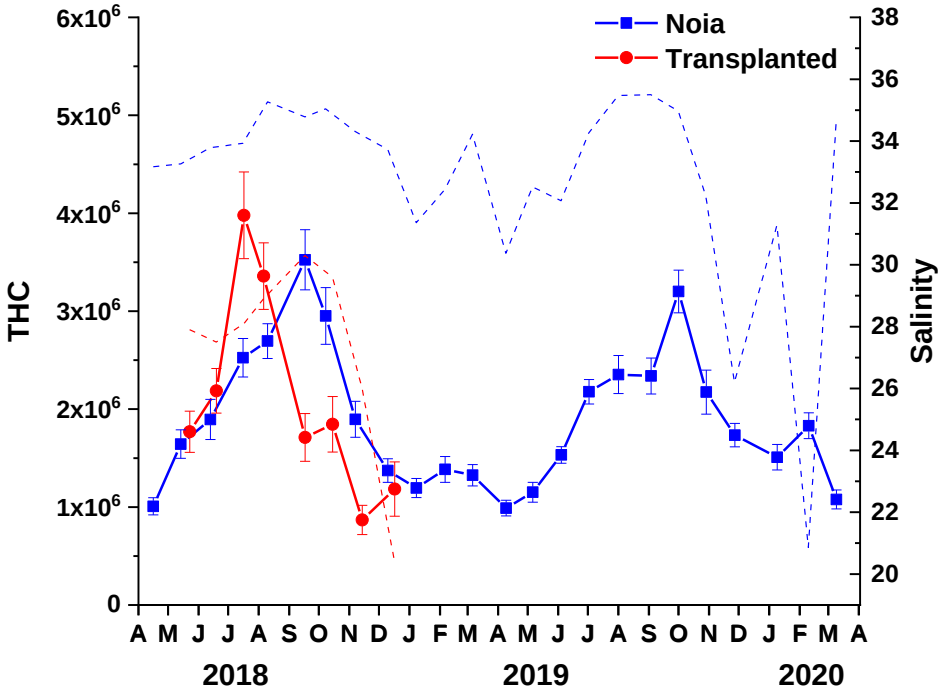

Fig. 10. Temporal variation of the mean value (±SE) of THC (blue, solid-line) in cockles collected from Noia through the two-year study period and the corresponding seawater salinity (blue, dashed-line) at sampling. The values corresponding to cockles transplanted from Noia to Lombos do Ulla are shown in red, the solid-line for THC and the dashed line for salinity.

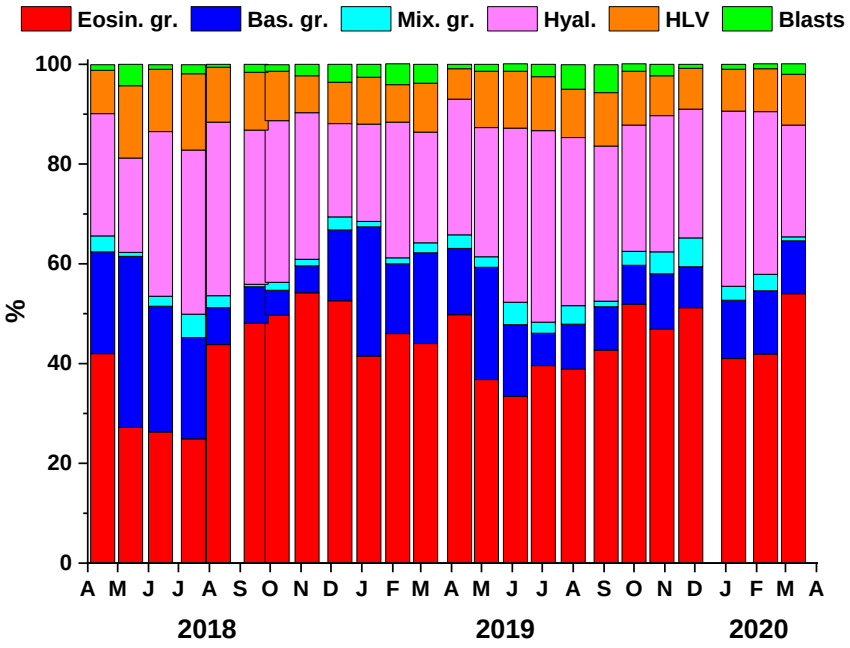

Fig. 11. Temporal variation of the relative abundance (expressed as the mean percentage) of each haemocyte type in the haemolymph of the cockles collected from Noia through the two-year study period.

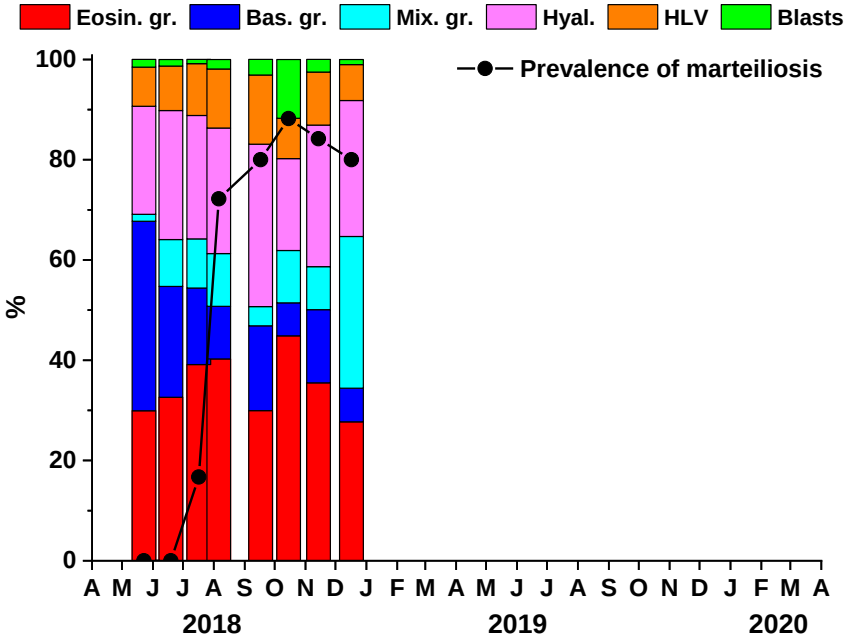

Fig. 12. Temporal variation of the relative abundance (expressed as the mean percentage) of each haemocyte type in the haemolymph of the cockles transplanted from Noia into Lombos do Ulla through the eight-month study period. The variation of the prevalence of marteiliosis is shown with a black solid line with black solid circles.

4.2.2. Influence of environmental and biological factors on the variability of THC and DHC

All the data deriving from sampling in Noia corresponded to a two-year period while data from Lombos do Ulla corresponded to an eight-month period, without completing one annual cycle. Because of this, we considered much more relevant the information deriving from sampling at Noia. Regarding the information deriving from sampling at Lombos do Ulla, most attention was focused on marteiliosis, which did not occur in Noia. Significant effects of seawater temperature and salinity on the THC of the haemolymph of cockles collected from Noia, while in the case of cockles transplanted into Lombos do Ulla, significant effects of temperature were detected but were not for salinity. Significant effects of both temperature and salinity on DHC were detected in both shellfish beds. A positive significant correlation between the THC in mussels *M. galloprovincialis* and the seawater temperature was reported (Carballal *et al*., 1998). Significant positive correlation of temperature with THC and with the total count of granulocytes but not with the total count of hyalinocytes of the Manila clam *R. philippinarum* was reported (Flye Sainte Marie *et al*., 2009). These authors did not find significant correlation of salinity with THC and with the total hyalinocyte count of this species but they found significant positive correlation of salinity with the total granulocyte count.

Another factor with seasonal pattern of variation, the gonad condition, seemed to be associated with THC because significantly higher values of THC corresponded to cockles with reabsorbing or resting gonads compared to those in the remaining classes of gonad condition (Fig. 13). Nevertheless, it is difficult to state which factors with seasonal pattern of variation have a more direct influence of THC because the maximum values of THC occurred in late summer, when temperature and salinity reached maxima (Figs. 9 and 10) and most cockles showed either reabsorbing or resting gonads (Fig. 14). Similarly, the minimum values of THC occurred in winter to early spring, when temperature and salinity minima were recorded and gonads showed gametogenesis progression. Regarding DHC, it seemed to be associated with the gonad condition too, particularly the relative abundance of eosinophilic and basophilic granulocytes; higher mean percentage of eosinophilic granulocytes were detected in cockles with reabsorbing and resting gonad than in cockles with ripe or spawned gonads (Fig. 13). Conversely, the mean percentage of basophilic granulocytes was lower in cockles with reabsorbing and resting gonad than in cockles with ripe or spawned gonads (Fig. 13). Consistently, the temporal variation of DHC appeared associated with that of the gonad condition because the maximal mean percentages of eosinophilic granulocytes occurred in November (2018 and 2019, Fig. 11) coinciding with maximum percentages of cockles in resting gonad stage (Fig. 14), whereas the minimal mean percentages of eosinophilic granulocytes occurred in the period May-July (2018 and 2019, Fig. 11), coinciding with the spawning period, that is to say when cockles showed ripe and spawned gonads (Fig. 14).

As mentioned above, the effects of gonad condition in THC and DHC are more difficult to be deduced from cockles collected from Lombos do Ulla because sampling there did not cover one annual cycle and the period dominated by reabsorbing and resting gonads (condition classes 5 and 0, respectively) was overrepresented regarding the period with gametogenesis progression (condition classes 1 and 2) (Fig. 15). Furthermore, cockles were heavily affected by marteiliosis there, which also could much interfere. Nevertheless, THC was also higher in cockles from Lombos do Ulla with reabsorbing gonads than in those with the other gonad conditions (Fig. 13). Regarding DHC, similarly to cockles from Noia, the minimum mean percentage of eosinophilic granulocytes and the maximum of basophilic granulocytes corresponded to cockles from Lombos do Ulla with ripe or spawned gonads (Fig. 13). The streaking higher percentage of mixed granulocytes in the cockles with reabsorbing (5), resting (0) and developing (1+2) gonad compared with those with ripe (3) and spawned (4) gonad is very likely due to marteiliosis because cockles with ripe and spawned gonad occurred in months (May-July 2018) with null or low marteiliosis prevalence whereas cockles with reabsorbing, resting or developing gonads occurred in months (August-December 2018) with high marteiliosis prevalence (Fig. 15). The lower THC of cockles with resting gonad from Lombos do Ulla compared to the THC of cockles from Noia with the same gonad condition could also be due to marteiliosis, because most cockles with resting gonad occurred in the period (August-December 2018) with high marteiliosis prevalence (Fig. 15). As explained below, the percentage of mixed granulocytes increased in cockles with moderate and advanced marteiliosis stages

while the THC decreased in cockles with advanced marteiliosis stages. The increase of THC associated with reabsorbing gonad could be due to the involvement of haemocytes in the process of gonad reabsorption; in fact, the gonad area, outside and inside gonad follicles, is massively invaded by haemocytes that destroy the gametes remaining in the follicles. Influence of the gonad condition on THC was also reported in the scallop *Chlamys farreri* from China although, contrarily to our study, THC was maximal in the ripe gonad period and minimal in the reabsorbing and resting gonad period (Lin *et al*., 2012); the percentage of granulocytes was higher in the ripe gonad period and lower in the reabsorbing and resting gonad period but the authors did not discriminate granulocyte subtypes. However, in the oyster *Saccostrea kegaki* from Korea, the THC minimum was recorded in the spawning period and it increased in the postspawning period (Hong and Choi, 2020), as in the case of the cockles in Galicia. No significant effects of gonad condition in *Mytilus galloprovincialis* (Carballal *et al*., 1998) and *Ruditapes philippinarum* THC (Hong *et al*., 2014b) were detected.

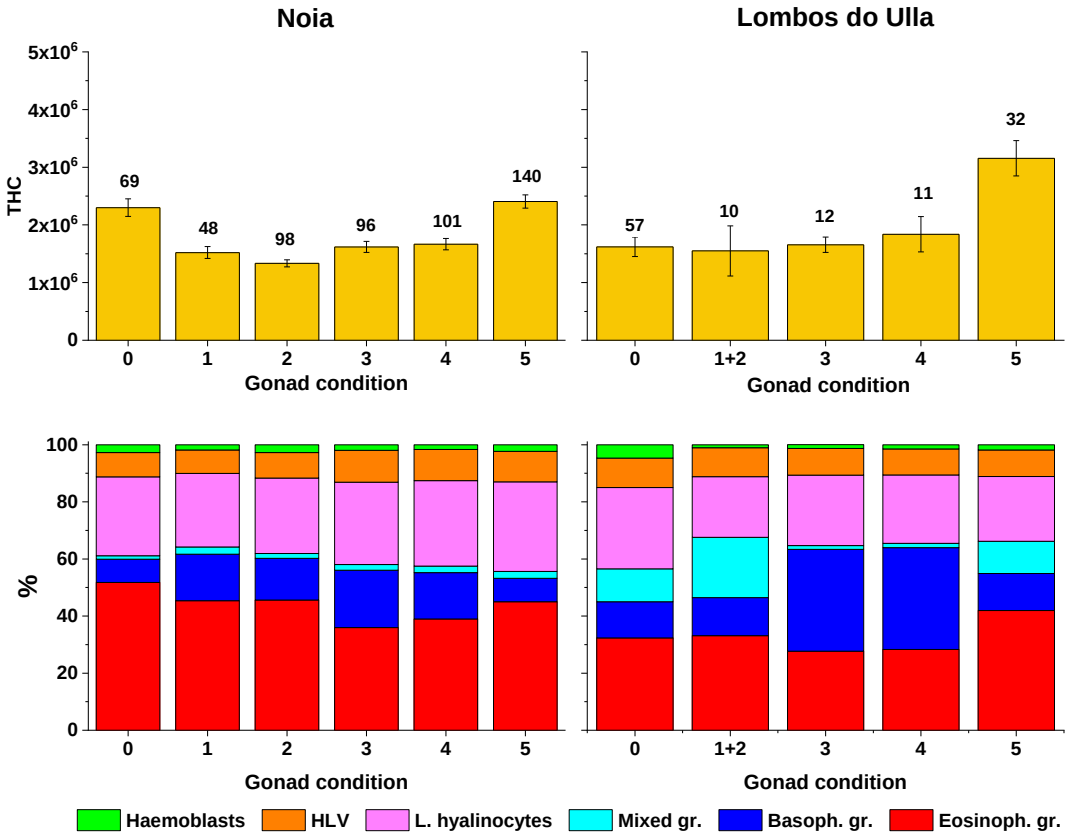

Fig. 13. Mean values (±SE) of total haemocyte count (THC, top graphs) and relative abundance (expressed as mean percentage) of each haemocyte type (bottom graphs) in the haemolymph of cockles classified according to gonad condition. The classes of gonad condition (0-5) are described in the section 3.4. Left graphs correspond to cockles collected from Noia through a two-year period; right graphs correspond to cockles transplanted into Lombos do Ulla and collected through an eight-month period. The classes of gonad condition 1 and 2 in cockles from Lombos do Ulla were combined because of the low number of cockles in those classes. The numbers of cockles included in each class are shown above bars in the top graphs.

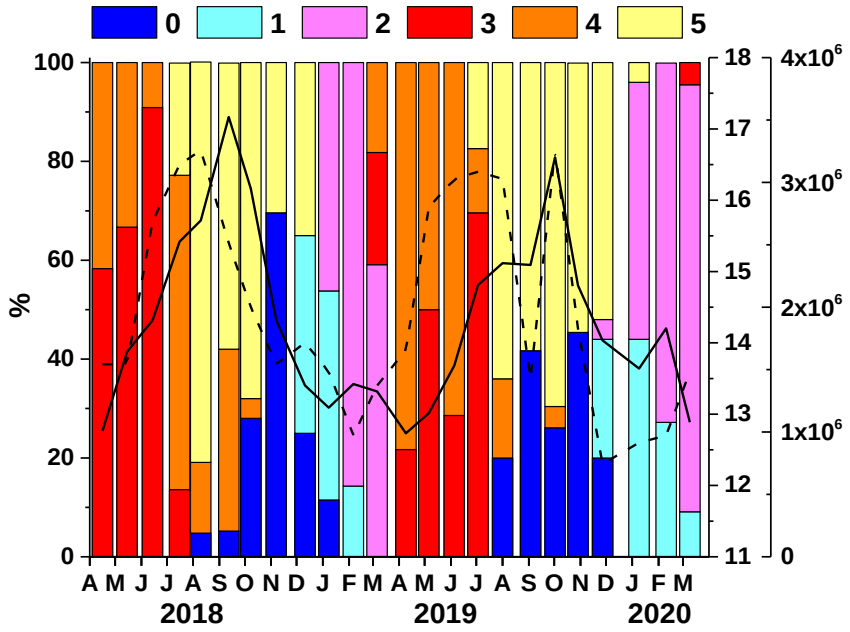

Fig. 14. Temporal variation of the distribution of cockles from Noia in classes of gonad condition through the 2-year study period. The classes of gonad condition (0-5) are described in the section 3.4. The variation of the temperature (dashed line, inner right axis) at sampling and that of the mean THC of the cockle haemolymph (solid line, outer right axis) are also shown.

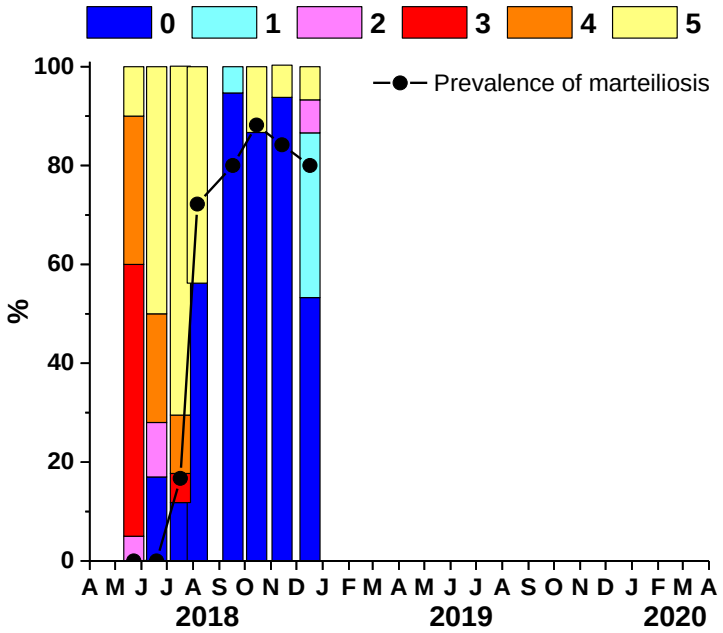

Fig. 15. Temporal variation of the distribution of cockles from Lombos do Ulla in classes of gonad condition through the 8-month sampling period. The classes of gonad condition (0-5) are described in the section 3.4. The variation of the prevalence of the infection with *Marteilia cochillia* (solid line) is also shown.

The severity of marteiliosis, the most serious pathological condition according to the mortality that it causes in Galician cockle populations, was significantly associated with the THC and the DHC of the haemolymph of cockles that had been transplanted into Lombos do Ulla. The THC of cockles in marteiliosis stages 3 (advanced sporulation) and 4 (after spore release) was significantly lower than that of non-infected cockles (stage 0) (Fig. 16). Regarding the DHC, the relative abundance of eosinophilic granulocytes was significantly lower in stages 3 and 4 of marteiliosis, while the relative abundance of mixed granulocytes was higher in marteiliosis stages 2-4, that of large hyalinocytes higher in marteiliosis stage 4 and that of haemoblasts higher in marteiliosis stages 2 and 4 (Fig. 16). Significant effects of marteiliosis (likely due to *Marteilia pararefringens* although the pathogen was reported as *Marteilia refringens*) on the THC and DHC of cultured mussels *M. galloprovincialis* from Galicia were also found (Carballal *et al*., 1998), although in that case, mussels with advanced marteiliosis showed higher THC than non-infected mussels. Decrease of the percentage of granulocytes in mussels with advanced marteiliosis could be deduced from the report of significantly higher total count of hyalinocytes but not significantly different total count of granulocytes in heavily infected mussels compared with non-infected ones (Carballal *et al*., 1998).

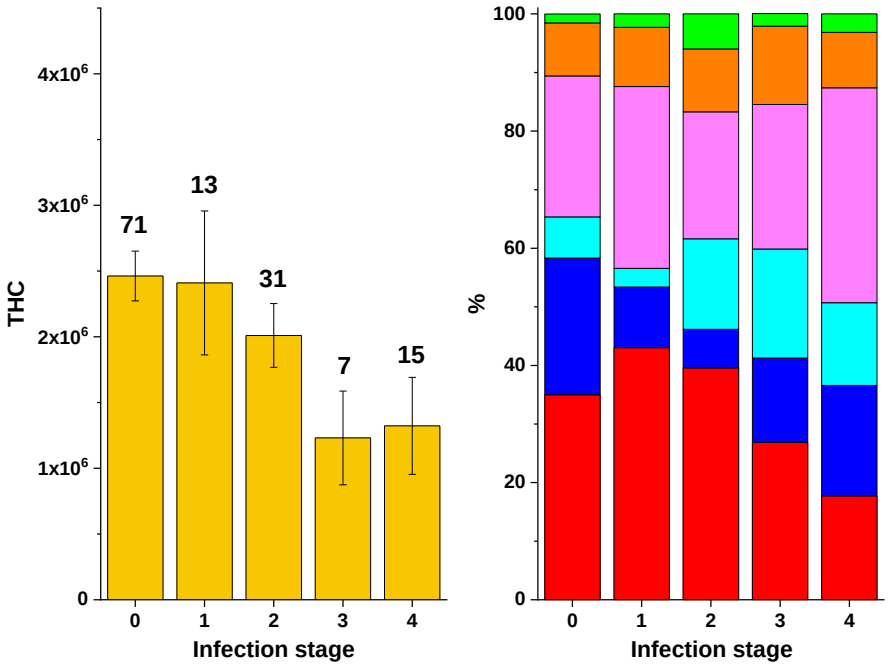

Fig. 16. Mean values (±SE) of total haemocyte count (THC, left graph) and relative abundance (expressed as mean percentage) of each haemocyte type (right graph) in the haemolymph of cockles classified according to the stage of the infection with *Marteilia cochillia*. The infection stages (0-4) are described in the section 3.4. The cockles had been transplanted from Noia into Lombos do Ulla and collected monthly through an eight-month period. The numbers of cockles included in each infection stage are shown above bars in the left graph.

Concerning the other pathological conditions considered in this study, significant increase of THC was detected in individuals with moderate infection with *M. tapetis* and those affected by heavy haemocytic infiltration among the cockles collected from Noia (Fig. 17). Regarding DHC in cockles from Noia, the percentage of mixed granulocytes was higher in cockles with moderate and heavy granulomatosis compared to that of non-affected cockles and slight decrease of the percentage of large hyalinocytes was detected in cockles heavily infested with trematode sporocysts (Fig. 18). As mentioned above, the samples from Lombos do Ulla are less relevant than those from Noia to analyse the effects of different factors on THC and DHC; nevertheless, a common trend of increase of the mean values of THC with increase of the severity of infection with *M. tapetis*, granulomatosis and haemocytic infiltration in cockles from Noia and those from Lombos do Ulla was recorded (Fig. 17). Cockles from Lombos do Ulla with moderate and heavy granulomatosis showed increased percentage of mixed granulocytes, as observed in cockles from Noia.

Effects of pathological conditions on THC and DHC of bivalve molluscs have been reported. Concerning THC, most frequently the effect consisted of increase of that variable, if any. That was the case of infestation of *Anadara trapezia* with trematode sporocysts (Dang *et al*., 2013), the infection of *O. edulis* with the protistan *Bonamia ostreae* (da Silva *et al*., 2008; Comesaña *et al*., 2012), the infection of *C. gigas* with the protistan *Marteilioides chungmuensis* (Choi *et al*., 2011), and among infections with perkinsid protistans, the infection of *Crassostrea virginica* with *Perkinsus marinus* (Anderson *et al*. 1992, 1995), the heavy infection of *Crassostrea gasar* with *Perkinsus* sp. (Queiroga *et al*., 2013) and the heavy infection of *R. philippinarum* with *Perkinsus* sp. (Flye-Sainte-Marie *et al*., 2009). In the case of the cultured mussel *M. galloprovincialis* from Galicia, two pathological conditions were found significantly associated with increase of THC, namely marteiliosis and heavy occurrence of granulocytomas, while other conditions as infestation with the copepod *Mytilicola intestinalis*, with trematode sporocysts of *Proctoeces maculatus*, the turbellarian *Urastoma cyprinae* or haemocytic infiltration of tissues did not affect the mussel THC (Carballal *et al*., 1998). The increase of THC in the haemolymph in those pathological conditions is generally explained as the mobilisation/production of haemocytes to fight the pathogen (Flye-Sainte-Marie *et al*., 2009; Comesaña *et al*., 2012). Therefore, the decrease of THC in the cockle haemolymph associated with the heavy infection with *M. cochillia*, appears as unusual, and deserves further study. There is scarce information on the effects of pathogens on mollusc DHC. The increase of the percentage of blast-like cells in the haemolymph of oysters *C. gasar* heavily infected with *Perkinsus* sp., together with the increase of THC, reinforce the hypothesis of haemopoietic proliferation to counteract the pathogen (Queiroga *et al*., 2013). The heavy infection of *R. philippinarum* with *Perkinsus* sp. is associated with increase of the relative abundance of granulocytes (Flye-Sainte-Marie *et al*., 2009). In our study, the increase of the percentage of haemoblasts observed in cockles with marteiliosis in stages 2 and 4 could denote attempts to compensate the THC decline; the implications of other cockle DHC changes associated with marteiliosis, such as decrease of the

relative abundance of eosinophilic granulocytes and the increase of that of mixed granulocytes, remain unknown.

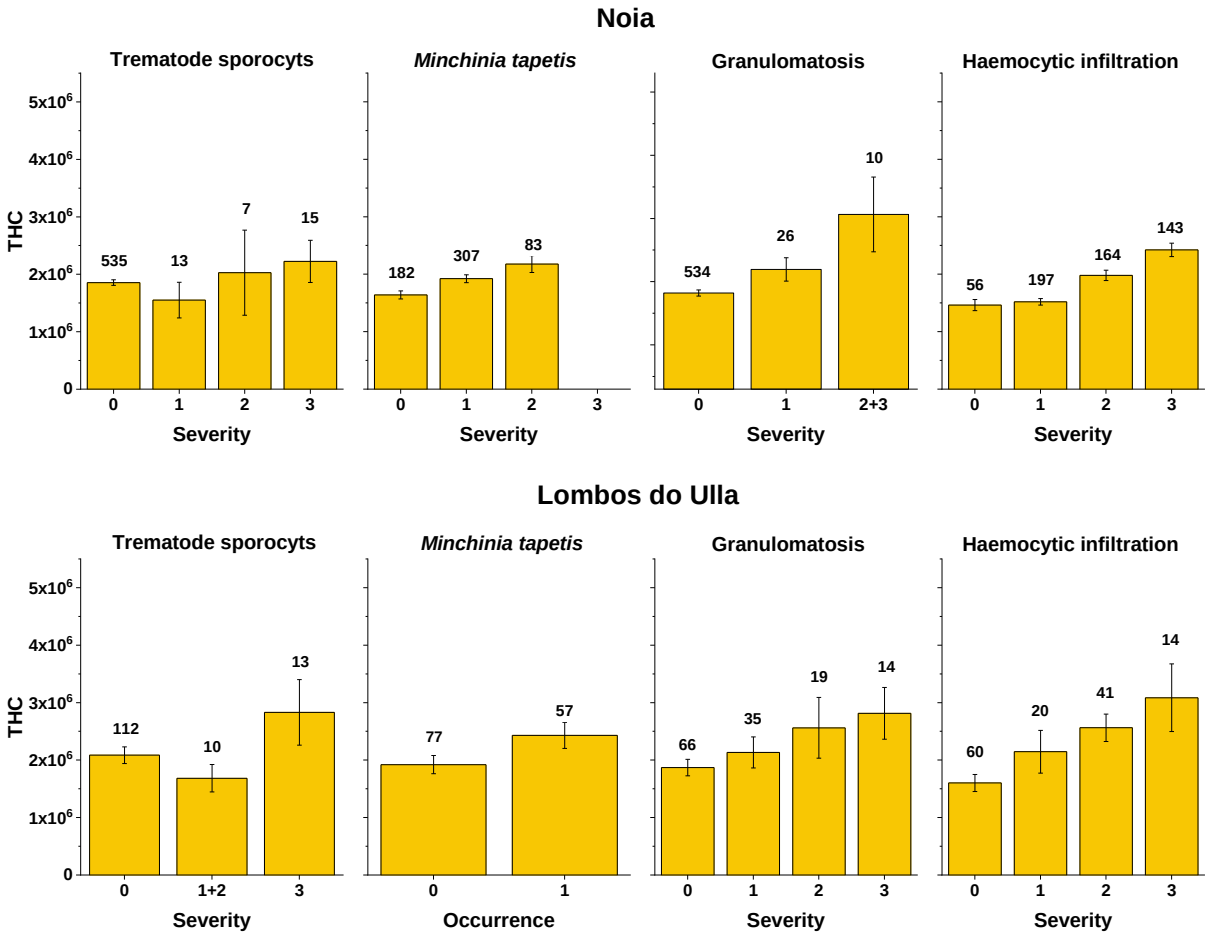

Fig. 17. Mean values (±SE) of total haemocyte count (THC) in the haemolymph of cockles classified according to severity classes of various pathological conditions (infestation with trematode sporocysts, infection with *Minchinia tapetis*, granulomatosis and haemocytic infiltration). The severity classes are described in the section 3.4. Top graphs correspond to cockles collected from Noia through a two-year period; bottom graphs correspond to cockles that had been transplanted from Noia into Lombos do Ulla and were collected through an eight-month period. No cockle with a heavy infection with *M. tapetis* was found in Noia; the classes of granulomatosis severity 2 and 3 in cockles from Noia and those of infestation with trematode sporocysts 1 and 2 in cockles from Lombos do Ulla were combined because of the low number of cockles in those classes. In the case of the infection with *M. tapetis* in cockles from Lombos do Ulla only occurrence (1) and non-occurrence (0) were considered. The numbers of cockles included in each class are shown above bars.

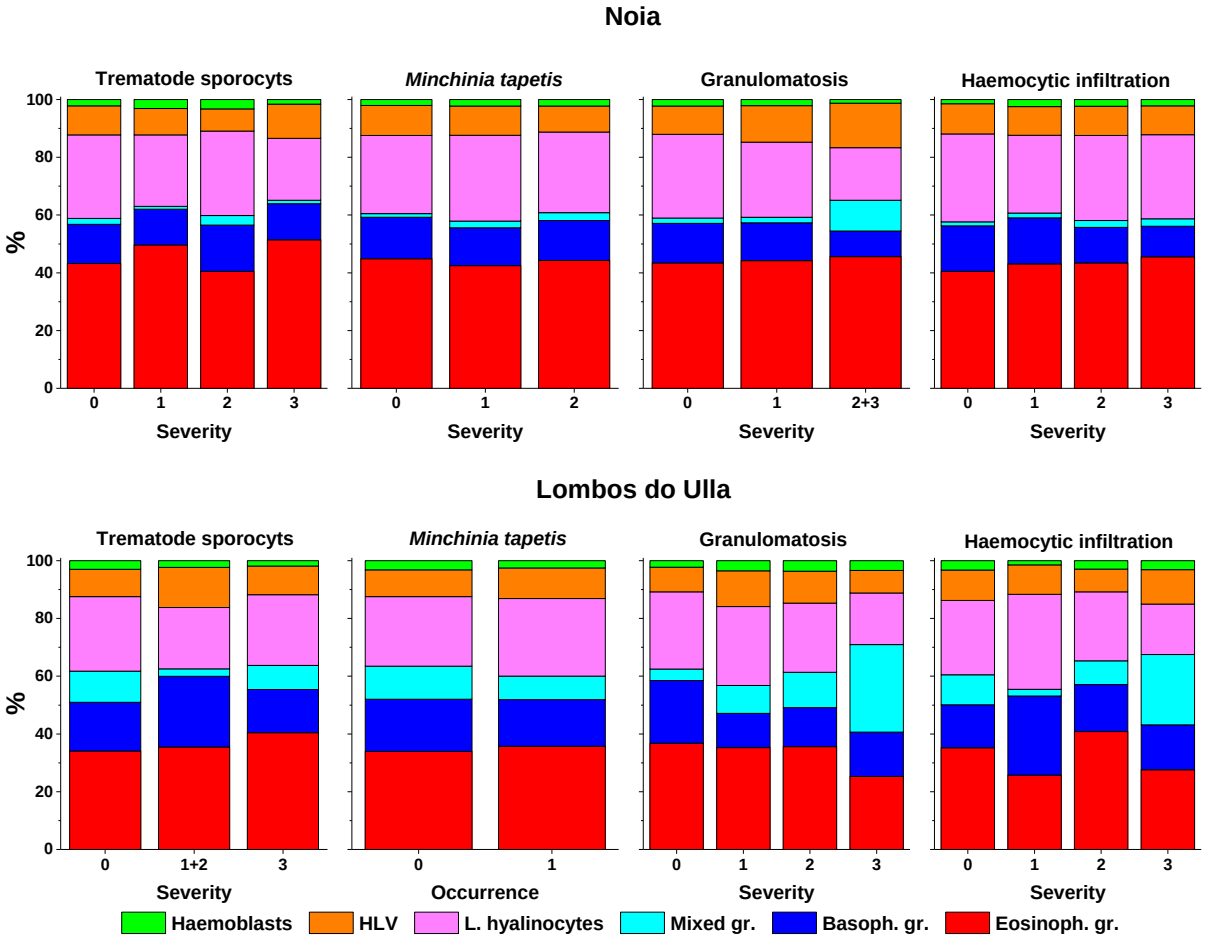

Fig. 18. Relative abundance (expressed as mean percentage) of each haemocyte type in the haemolymph of cockles classified according to severity classes of various pathological conditions (infestation with trematode sporocysts, infection with *Minchinia tapetis*, granulomatosis and haemocytic infiltration). The severity classes are described in the section 3.4. Top graphs correspond to cockles collected from Noia through a two-year period; bottom graphs correspond to cockles that had been transplanted from Noia into Lombos do Ulla and were collected through an eight-month period. No cockle with heavy infection with *M. tapetis* was found in Noia; the classes of granulomatosis severity 2 and 3 in cockles from Noia and those of infestation with trematode sporocysts 1 and 2 in cockles from Lombos do Ulla were combined because of the low number of cockles in those classes. In the case of the infection with *M. tapetis* in cockles from Lombos do Ulla only occurrence (1) and non-occurrence (0) were considered.

In an attempt to summarise the pathological and gonad conditions involving marked deviations from the mean values of THC and DHC of the cockle haemolymph, Table 2 shows the mean 95% confidence intervals of the cockles collected from Noia and the pathological and gonad conditions for which the mean THC and DHC values were markedly higher or lower than those intervals. Table 2 also includes the mean 95% confidence intervals of the cockles collected

from Lombos do Ulla, which had been transplanted there from Noia, as a reference for the marked deviations associated with marteiliosis.

Table 2. Conditions of cockles in which mean values of THC and DHC were markedly deviated (either low or high counts) compared with the mean 95% interval confidence of the cockles collected from Noia in the study period. In the cases of marteiliosis, the comparisons are referred to the mean 95% interval confidence of the cockles that had been transplanted into Lombos do Ulla.

Cockle haemocytes	Cockles from Noia Mean 95% confidence interval (N=570)	Cockles transplanted into Lombos do Ulla Mean 95% confidence interval (N=145)	High count	Low count
Total haemocyte count (x 10^6/ml)	1.767 – 1.954	1.874 – 2.381	Heavy haemocytic reaction, heavy granulomatosis, reabsorbing and resting gonad	Advanced stages (3 and 4) of marteiliosis
Eosinophilic granulocytes (%)	42.20 – 44.84	31.61 – 38.18	Resting gonad, heavy infestation with trematode sporocysts	Advanced stages (3 and 4) of marteiliosis
Basophilic granulocytes (%)	12.68 – 14.41	14.72 – 19.44		Reabsorbing and resting gonad, heavy haemocytic reaction, heavy granulomatosis
Mixed granulocytes (%)	1.67 – 2.38	6.90 – 13.13	Moderate and advanced stages (2-4) of marteiliosis, heavy granulomatosis	
Large hyalinocytes (%)	27.55 – 29.77	22.69 – 27.96	Marteiliosis stage 4	Heavy granulomatosis
HLV (%)	9.50 – 10.59	8.57 – 11.06	Heavy granulomatosis	
Haemoblasts (%)	1.99 – 2.42	1.99 – 3.74	Marteiliosis stages 2 and 4	

4.3. Flow cytometry assays

Flow cytometry assays were performed first to evaluate the feasibility of discriminating haemocytes based on size and complexity of the cells, using the DRAQ5 dye, which stain nucleic acids, to discriminate the nucleate cells. Four regions were tentatively delimited in the flow cytometer density plots, from R1 (more complex and larger cells) to R4 (less complex and smaller cells) (Fig. 19A, C). In addition to what we considered true nucleate cells, that is to say particles stained with DRAQ5, numerous non-stained particles were also detected, which were less complex (Fig. 19B) and could correspond to cell debris, subcellular particles such as released microvesicles or bacteria. Around 80% of the events detected by the flow cytometer corresponded to isolated cells (singlets, Table 3), while the remaining events corresponded to aggregated particles. More than 50% of the singlets corresponded to non-nucleate particles; among the delimited R1-R4 regions, R1 was the most numerous (Table 3).

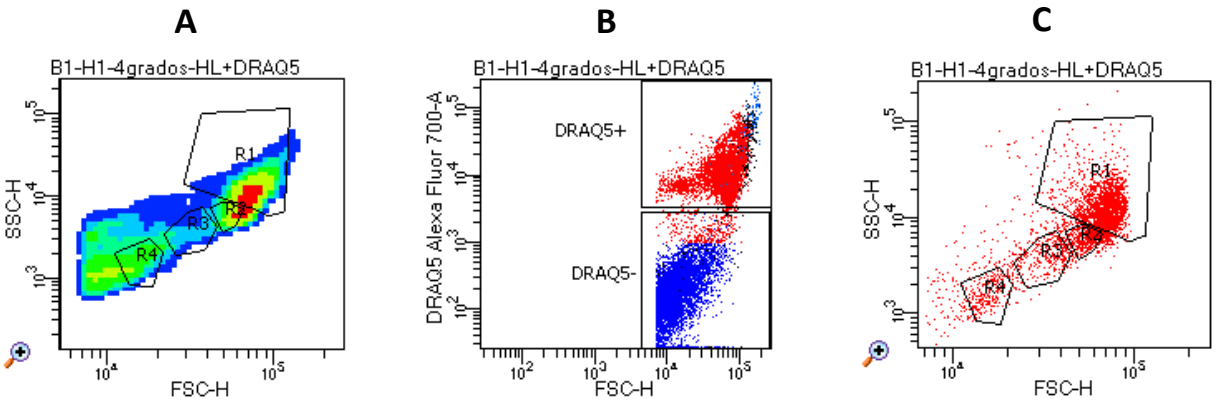

Fig. 19. Flow cytometer density plots [forward scatter (FSC) vs. side scatter (SSC)] of cockle fresh haemolymph cells plus DRAQ5 dye. A: density plot including all the events (stained and non-stained particles; four regions (R1-R4, from more complex and larger cells to less complex and smaller cells) appear delimited on the plot. B: density plot with areas discriminating stained (DRAQ5+, nucleate cells) and non-stained (DRAQ5-, non-nucleate) particles. C: density plot including stained particles (nucleate cells) only; again, the four regions (R1-R4) appear delimited on the plot.

Regarding the trial to evaluate the correspondence of the size-complexity regions with the previously established morphological haemocyte types, the separation of haemocytes by centrifugation in a Percoll® discontinuous density gradient, most cells were accumulated in the interphase 40/50 at glance, followed by 30/40, while cell accumulation in the other two interphases was not visible. A cytospin was produced with haemocytes accumulated in the interphase 40/50 but no attempt was performed with those from the other interphases to guarantee availability of cells for flow cytometry analysis of cells in each interphase. Examination of the cytospin showed that the group of haemocytes accumulated in the interphase 40/50 had higher percentage of eosinophilic granulocytes and lower of haemoblasts compared with the original haemolymph pool (before centrifugation in discontinuous density gradient) (Table 4), which is consistent with considering the eosinophilic granulocytes as denser

cells and the haemoblasts as less dense cells. Table 5 shows the distribution of the cells from each interphase in size-complexity regions after passing through the flow cytometer. The haemocyte groups accumulated at each interphase included particles corresponding to every size-complexity region although with different percentages. The groups of haemocytes accumulated in the denser interphases (50/60 and 40/50) had higher percentages of cells in the region R1 (larger and more complex cells) compared to the original haemolymph pool (before centrifugation); the group of haemocytes accumulated in the interphase 30/40 had higher percentage of cells in the region R2 compared to the original haemolymph pool, and the group of haemocytes accumulated in the interphase 20/30 had lower percentage of cells in the region R1 compared to the original haemolymph pool. Therefore, denser cells (particularly eosinophilic granulocytes) were more represented in R1 while less dense cells were more represented in R2-R4.

Table 3. Number of events (particles detected by flow cytometry) corresponding to singlets (isolated particles, in opposition to aggregated particles) and, within singlets, number of stained with DRAQ5 and non-stained particles, and number of particles in each of the regions (R1-R4) delimited in the FSC vs. SSC density plot. The percentages of particles in each size-complexity region (R1-R4) and DRAQ5 staining class (positive or negative) with regard to the total events and to the singlets are also shown.

	No. events	% of singlets	% of total events
Whole population	10,000	-	100
Singlets	8,238	100	82.4
DRAQ5+	3,510	42.6	35.1
R1	2,201	26.7	22.0
R2	532	6.5	5.3
R3	602	7.3	6.0
R4	914	11.1	9.1
DRAQ5-	4,698	57.0	47.0

Table 4. Relative abundance (percentage) of each recognisable haemocyte type in cytospins produced with haemocytes accumulated in the interphase 40/50, after centrifugation of cockle haemolymph in a Percoll® discontinuous density gradient, as well as with haemocytes from the original haemolymph pool (before centrifugation).

	Eosinophilic granuloc.	Basophilic granuloc.	Mixed granuloc.	Large hyalinoc.	HLV	Blasts
Original haemolymph pool	27	6	2	27	10	28
Interphase 40/50	47	6	0	26	12	10

Table 5. Distribution (percentage) of the haemocytes accumulated in each interphase, after centrifugation of cockle haemolymph in a Percoll® discontinuous density gradient, in four (R1-R4) size-complexity regions delimited in the FSC vs. SSC density plots by flow cytometry. The distribution of the haemocytes of the original haemolymph pool (before centrifugation) is also shown.

Size-complexity region	Original haemolymph pool	Interphase 20/30	Interphase 30/40	Interphase 40/50	Interphase 50/60
R1	26.7	14.8	28.4	44.0	30.8
R2	6.5	8.7	17.3	12.4	4.7
R3	7.3	5.9	7.7	6.1	3.5
R4	11.1	6.4	9.1	7.3	3.0

The flow cytometry assays performed to evaluate the efficacy of the sorting procedure showed substantial but imperfect cell separation. The evaluation procedure involved passing the cells of a haemolymph pool through the flow cytometer and then gating and sorting separately the cells delimited in each of the four (R1-R4) size-complexity regions. Each of the four fractions was passed again through the flow cytometer and most of the sorted cells were placed on the same region but some cells appeared in other regions (Table 6). Therefore, each sorted fraction was contaminated to some extent with cells from the other fractions.

Table 6. Distribution (percentage) of the haemocytes from each of the four (R1-R4) sorted fractions in the four (R1-R4) size-complexity regions after passing again (after sorting) through the flow cytometer. The distribution of the haemocytes of the original haemolymph pool (before sorting) is also shown.

Size-complexity region	Original haemolymph pool	Sorted cells from R1	Sorted cells from R2	Sorted cells from R3	Sorted cells from R4
R1	20.5	**62.4**	10.7	0.2	0.1
R2	4.6	6.8	**44.5**	3.2	0.0
R3	8.6	2.4	13.9	**57.2**	1.3
R4	12.6	1.8	1.7	10.2	**56.2**

Flow cytometry has been applied frequently to discriminate haemocyte types in the haemolymph of bivalve molluscs according to the cell size and inner cell complexity (granularity); variability among species ranges from one unique type to 5 types (Table 7).

Table 7. Literature summary on the use of flow cytometry to discriminate haemocyte types in the haemolymph of bivalve mollusc species and to evaluate some of their immune capabilities, namely phagocytic ability, production of ROIs, and lysosomal and non-specific esterase content. Differences in the number of "+" signs involve differences between haemocyte types in the same table row but not among rows. ND: not discriminated among haemocyte types. NF: Information not found. NS: not studied with flow cytometry

Taxon	Haemocyte types	Phagocytosis	ROIs	Lysosomes	Esterases	Citation
PTERIOMORPHIA						
Order Arcida, fam. Arcidae						
Anadara broughtonii	Erythrocytes-I	-	+	+	NS	Kim *et al.*, 2020
	Erythrocytes-II	-	++	+		
	Granulocytes	++	++	++		
	Hyalinocytes	+	+	+		
	Blast-like cells	-	-	+		
Anadara kagoshimensis	Erythrocytes-I	-	+	+	NS	Kim *et al.*, 2020
	Erythrocytes-II	-	++	+		
	Granulocytes	++	++	++		
	Hyalinocytes	+	+	+		
	Blast-like cells	-	-	+		
Anadara kagoshimensis	Subpopulation 1 (amebocytes)	NS	+	NS	NS	Kladchenko et al., 2020
	Subpopulation 2 (intermediate cells and erythrocytes)		+			
Anadara trapezia	Amebocytes	+	+	NS	NS	Dang *et al.*, 2013
	Erythrocytes	-	+			
Tegillarca granosa	Erythrocytes-I	-	+	+	NS	Kim *et al.*, 2020
	Erythrocytes-II	-	++	+		
	Granulocytes	++	++	++		
	Hyalinocytes	+	+	+		
	Blast-like cells	-	-	+		
Order Mytilida, fam. Mytilidae						
Aulacomya ater	Granulocytes	ND	NS	NS	NS	Caza *et al.*, 2015
	Hyalinocytes					

Mytilus coruscus	Granulocytes Hyalinocytes Blast-like cells	++ + -	++ + -	++ + -	NS	Yang *et al.*, 2015
Mytilus edulis	Hyalinocytes Small granulocytes Large granulocytes	NS	NS	NS	NS	Parisi *et al.*, 2008
Mytilus edulis	R1 (likely eosinophilic granulocytes R2 (likely hyalinocytes) R3 (likely basophils and hyalinocytes)	NS	NS	NS	NS	Le Foll *et al.*, 2010
Mytilus edulis desolationis	Granulocytes Hyalinocytes	ND	NS	NS	NS	Caza *et al.*, 2015
Mytilus galloprovincialis	R1 (large, complex, granular cells) R2 (large, intermediate complex, semigranular cells) R3 (smaller, intermediate complex, semigranular cells) R4 (small, less complex agranular cells)	++ + + -	+ + + -	NS	NS	García-García *et al.*, 2008
Mytilus galloprovincialis	Granulocytes Agranulocytes	NS	++ +	NS	NS	Andreyeva *et al.*, 2019
Perna canaliculus	Granulocytes Hyalinocytes Blast-like cells	+++ ++ +	+++ ++ +	NS	NS	Rolton and Ragg, 2020
Perna viridis	Subpopulation 1 (granulocytes) Subpopulation 2 (hyalinocytes)	++ +	++ +	++ +	++ +	Wang *et al.*, 2012
Modiolus kurilensis	R1 (haemoblasts and agranulocytes) R2 (hemigranulocytes) R3 (granulocytes)	NS	NS	NS	NS	Anisimova *et al.*, 2012
Modiolus kurilensis	Granulocytes Agranulocytes Blast like cells	NS	NS	NS	NS	Kumeiko *et al.*, 2018

Order Ostreida, fam. Margaritidae						
Pinctada fucata	R1 (small hyalinocytes) R2 (large hyalinocytes) R3 (granulocytes)	NS	NS	NS	NS	Li *et al.*, 2015
Order Ostreida, Fam. Ostreidae						
Ostrea chilensis	Granulocytes Hyalinocytes	++ +	++ +	++ +	ND	Rolton *et al.*, 2020
Ostrea edulis	R1 (small haylinocytes) R2 (large hyalinocytes) R3 (granulocytes)	ND	NS	NS	NS	Xue *et al.*, 2001
Crassostrea ariakensis	Granulocytes Hyalinocytes Blast-like cells	++ + -	+++ ++ +	NS	NS	Donaghy *et al.*, 2009
Crassostrea gasar	Granulocytes Hyalinocytes Blast-like cells	ND	ND	NS	NS	Queiroga *et al.*, 2013
Crassostrea gigas	R1 (small agranular cells) R2 (hyalinocytes) R3 (granulocytes)	NS	++ ++ +	NS	NS	Lambert *et al.*, 2003
Crassostrea gigas	Small agranular cells Hyalinocytes Granulocytes	NS	++ + NF	NS	NS	Lambert *et al.*, 2007
Crassostrea gigas	Granulocytes Hyalinocytes Blast-like cells	ND	ND	NS	NS	Donaghy *et al.*, 2010
Crassostrea gigas	Granulocytes Hyalinocytes Blast-like cells	++ + -	NS	NS	NS	Hong *et al.*, 2012
Crassostrea gigas	Subpopulation 1 (small non granulated cells) Subpopulation 2 (large cells with moderate granularity) Subpopulation 3 (largest cells with largest granularity)	NS	+ ++ +++	NS	NS	Andreyeva *et al.* 2020

Crassostrea hongkongensis	Granulocytes Hyalinocytes	++ +	++ +	NS	NS	Li *et al.*, 2018
Crassostrea niponna	Granulocytes Hyalinocytes Blast-like cells	++ + -	++ + +			Hong *et al.*, 2014a
Crassostrea rhizophorae	R1 (small to medium size cells with low to moderate granularity) R2 (medium to large size cells with high granularity)	NS	NS	NS	NS	Rebelo *et al.*, 2013
Crassostrea virginica	Group A (large size and granularity, further subdivided in A1, A2 and A3) Group B (small size and granularity, further subdivided in B1 and B2) Group C (intermediate between A and B) Group D (small, highly granular cells)	NS	NS	NS	NS	Allam *et al.*, 2002
Crassostrea virginica	Granulocytes Small granulocytes Large hyalinocytes Small hyalinocytes	ND	++ + + +	NS	NS	Hégaret *et al.* 2003a, 2003b
Crassostrea virginica	Granulocytes Intermediate cells Hyalinocytes	+++ ++ +	+ + -	NS	NS	Goedken and De Guise, 2004
Saccostrea glomerata	R1 (large size highly granular cells) R2 (medium size moderately granular cells) R3 (large agranular cells) R4 (small size highly granular cells)	NS	NS	NS	NS	Aladaileh *et al.*, 2007
Saccostrea glomerata	Granulocytes Hyalinocytes Small agranulocytes	+ + -	NS	NS	NS	Dang *et al.*, 2011
Saccostrea kegaki	Granulocytes Hyalinocytes Blast-like cells	+ + NF	NS	NS	NS	Hong and Choi, 2020

Order Venerida, Fam. Cyrenidae						
Corbicula japonica	R1-A (small hyalinocytes) R1-B (large hyalinocytes) R2-A (basophilic granulocytes) R2-B (eosinophilic granulocytes)	NS	NS	NS	NS	Anisimova *et al.*, 2017
Order Venerida, Fam. Veneridae						
Chamelea gallina	R1 (agranular cells) R2 (granular cells)	NS	NS	NS	NS	Mosca et al., 2011
Macrocallista nimbosa	Unique cell population	+	+	+	NS	Jauzein *et al.*, 2013
Mercenaria mercenaria	Group A (large size and granularity, further subdivided in A1, A2 and A3) Group B (small size and granularity, further subdivided in B1 and B2)	NS	NS	NS	NS	Allam *et al.*, 2002
Meretrix lusoria	R1 (hyalinocytes) R2 (small granulocytes) R3 (large granulocytes)	+ + ++	NS	NS	NS	Tu *et al.*, 2007
Paphia malabarica	Granulocytes Agranulocytes	++ +	++ +	++ +	++ +	Gajbhiye and Khandeparker *et al.*, 2019
Ruditapes decussatus	R1 (granulocytes) R2 (hyalinocytes) R3 (size and granularity intermediate between R1 and R2)	++ + +	+++ + ++	NS	NS	Prado-Álvarez *et al.*, 2012
Ruditapes philippinarum	Group A (large size and granularity, further subdivided in A1, A2 and A3) Group B (small size and granularity, further subdivided in B1 and B2)	NS	NS	NS	NS	Allam *et al.*, 2002
Ruditapes philippinarum	Granulocytes Hyalinocytes Blast-like cells	ND	ND	NS	NS	Donaghy *et al.*, 2009b

Order Ostreida, fam. Pteriidae						
Pteria hirundo	Granulocytes Large hyalinocytes Small hyalinocytes Blast-like cells	ND	ND	NS	NS	Vieira *et al.*, 2017
HETEROCONCHIA — PALAEOHETERODONTA						
Order Unionida, Fam Unionidae						
Anodonta anatina	R1 (hyalinocytes) R2 (granulocytes)	NS	NS	NS	NS	Hinzmann *et al.*, 2017
Anodonta cygnea	R1 (hyalinocytes) R2 (granulocytes)	NS	NS	NS	NS	Hinzmann *et al.*, 2017
Anodonta cygnea	Granular cells Hyaline cells	NS	NS	NS	NS	Soares da Silva *et al.*, 2017
HETEROCONCHIA — EUHETERODONTA						
Order Adapedonta, Fam. Hiatellidae						
Panopea globosa	A (large and small hyalinocytes) B (granulocytes)	NS	NS	NS	NS	Hernández-Méndez *et al.*, 2020
Order Adapedonta, Fam. Pharidae						
Sininovacula constricta	P1 (granulocytes) P2 (hyalinocytes) P3 (semigranuocytes)	++ + ++	NS	++ + ++	NS	Nguyen *et al.*, in press
Order Myida, Fam. Dreissenidae						
Dreissena polymorpha	P1 (hyalinocytes) P2 (eosinophilic granulocytes) P3 (blast like cells)	ND	+++ ++ +	+++ ++ +	NS	Evariste *et al.*, 2016
Order Myida, Fam. Myidae						
Mya arenaria	A (granulocytes) B (hyalinocytes)	ND	NS	NS	NS	Brousseau *et al.*, 2000
Mya arenaria	A (granulocytes) B (hyalinocytes)	ND	NS	NS	NS	Fournier *et al.*, 2001
Mya arenaria	Subpopulation 1 (granulocytes) Subpopulation 2 (hyalinocytes)	++ +	NS	NS	NS	Mateo *et al.*, 2009

4.3.1. Phagocytic ability

Two experiments (A and B) were performed with the same experimental conditions, using different cockles each time, to assess reproducibility. Figure 20 shows flow cytograms at the incubation start (time 0, T_0) and that after incubation for 2 h (T_{2h}). At T_0, most (97% in A and 94% in B) haemolymph cells appeared concentrated in the area with no FITC fluorescence signal (negative area, no phagocytosis), while very few cells appeared in the background area (1% in A and 3% in B, no phagocytosis) and in the fully positive FITC signal area (0.01% in A and 0.02% in B, cells with phagocytosed bacteria). At T_{2h}, haemolymph cells appeared distributed in the

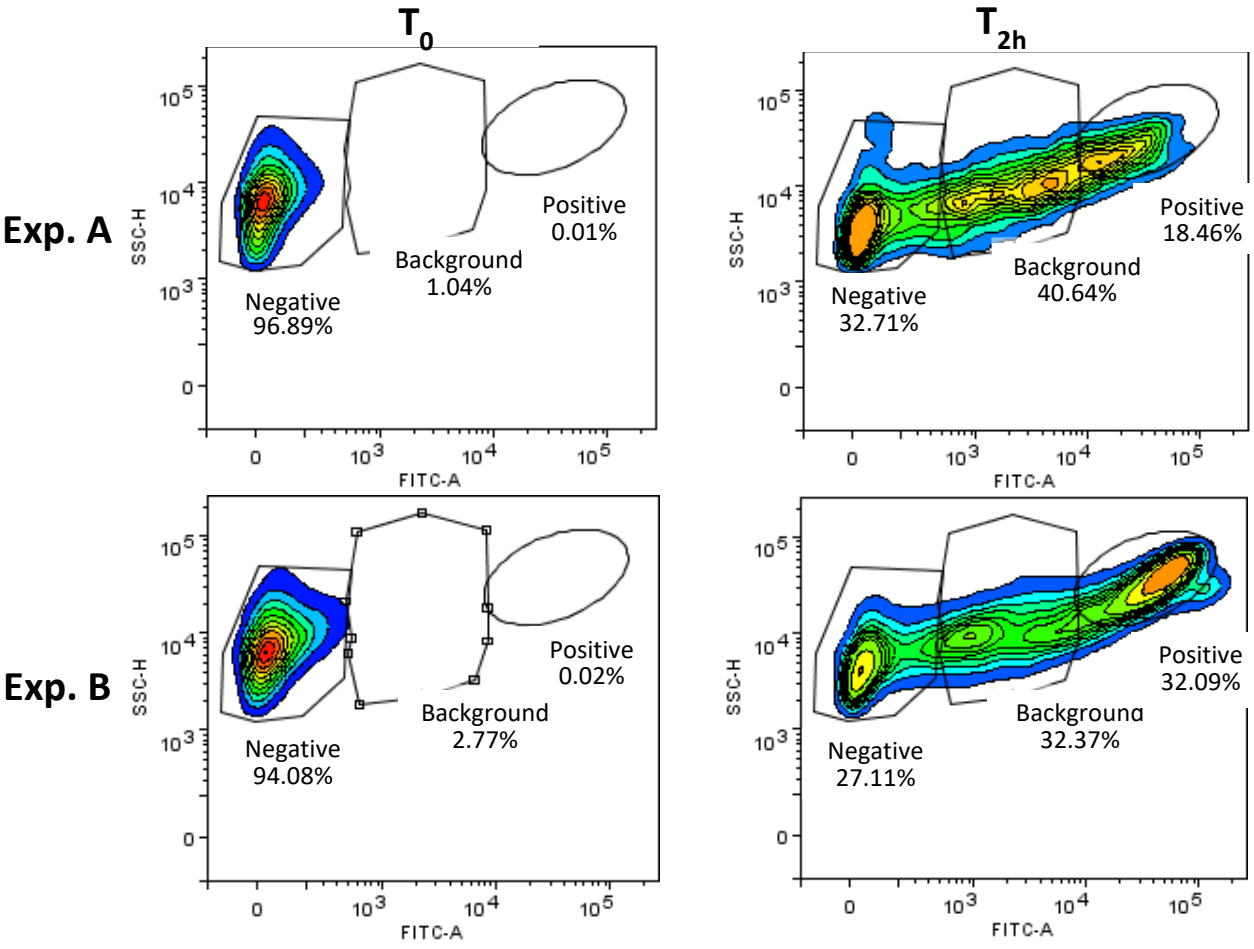

Fig. 20. Flow cytograms of *Cerastoderma edule* haemolymph cells incubated with commercial pHrodo™ Green *Escherichia coli* BioParticles™ (Invitrogen), at T_0 (left) and T_{2h} (right), corresponding to experiments A (top) and B (bottom). Three areas are depicted in the cytograms depending on the detected fluorescence signal (FITC-A), a negative area with no fluorescence (haemocytes without phagocytosed bacteria), an intermediate area corresponding to background fluorescence (haemocytes without phagocytosed bacteria), and a positive area corresponding to fully positive fluorescence signal (haemocytes with phagocytosed bacteria). The percentages of cells occurring in each area are shown in the cytograms.

negative (33% cells in A and 27% in B), background (41% in A and 32% in B) and fully positive (18% in A and 32% in B) FITC signal areas.

Cells occurring in each area at T_{2h} were sorted and cytocentrifuged to estimate the relative abundance of each haemocyte morphological type in each sorted fraction (Fig. 21). In both experiments (A and B), the fractions containing cells that had not phagocytosed bacteria (negative and background areas) appeared enriched with large hyalinocytes, and haemoblasts, while the relative abundance of granulocytes was reduced compared to the relative abundance of haemocyte types in both the original haemolymph pool used for the experiment and in the haemolymph pool incubated with bacteria at T_{2h} just before passing it through the flow cytometer (Table 8). Conversely, the fraction containing the cells that had phagocytosed bacteria (positive area) showed increased percentages of eosinophilic (experiment A) and basophilic (experiments A and B) granulocytes and reduced percentages of large hyalinocytes (A and B), HLV (A and B) and haemoblasts (B) compared to the relative abundance of haemocyte types in both the original haemolymph pool used for the experiment and in the haemolymph pool incubated with bacteria at T_{2h} just before passing it through the flow cytometer (Table 8).

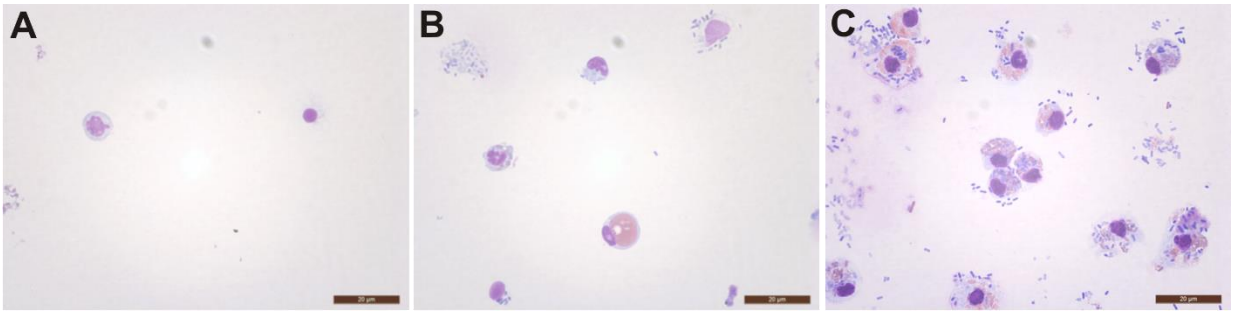

Fig. 21. Light micrographs of cytospins produced from each of the three sorted cell fractions after passing through the flow cytometer the cockle haemocytes that had been incubated with bacteria for 2 h. A: area with negative fluorescence; B: area with background fluorescence, C: area with positive fluorescence. Scale bars: 20 μm.

Additionally, examination of a sample of the fully positive fluorescence area with confocal microscopy showed the occurrence of fluorescent bacilli within haemocyte cytoplasm, thus confirming that bacteria had been phagocytosed, as well as non-fluorescent bacilli out of the haemocytes (Fig. 22). Most haemocytes with fluorescent bacilli in their cytoplasm corresponded to the granulocyte types (Fig. 22A) although some of them corresponded to the HLV type (Fig. 22B); some large hyalinocytes and haemoblasts without phagocytosed bacteria were also observed in the sorted positive fluorescent cell fraction (Fig. 22A).

Table 8. Relative abundance (%) of each haemocyte type estimated in cytospins produced from samples of the original cockle haemolymph pool used for the phagocytosis assay, the haemolymph incubated with bacteria for 2 h just before passing it through the flow cytometer, and each of the three sorted fractions after passing haemolymph cells through the flow cytometer, namely the fraction corresponding to the negative area (haemocytes without phagocytosed bacteria), that to the background area (haemocytes without phagocytosed bacteria) and that to the positive area (haemocytes with phagocytosed bacteria).

EXPERIMENT A	Eosinophilic granulocytes	Basophilic granulocytes	Mixed granulocytes	Large hyalinocytes	HLV	Haemo blasts
Original haemolymph pool	70.0	1.2	4.2	14.6	8.2	1.7
Haemolymph incubated with bacteria (T_{2h}) before passing through FC	64.8	6.5	2.6	15.8	8.0	2.4
Negative area*	0.0	0.0	0.0	25.0	0.0	75.0
Background area	9.8	9.8	0.0	29.4	11.8	39.2
Positive area	68.8	17.2	4.1	5.9	1.8	2.3

*Data based on 4 intact haemocytes, the remaining particles in the cytospin from this fraction corresponded to nuclei and cellular debris.

EXPERIMENT B	Eosinophilic granulocytes	Basophilic granulocytes	Mixed granulocytes	Large hyalinocytes	HLV	Haemo blasts
Haemolymph incubated with bacteria (T_{2h}) before passing through FC	63.7	8.8	0.0	5.9	10.8	10.8
Negative area*	6.3	4.2	0.0	29.5	6.3	53.7
Background area	6.0	4.0	0.0	21.0	8.0	61.0
Positive area	76.8	13.2	0.0	2.2	4.7	3.1

*Around 25% of the particles corresponded to haemocytes, the remaining particles in the cytospin from this fraction corresponded to nuclei and cellular debris.

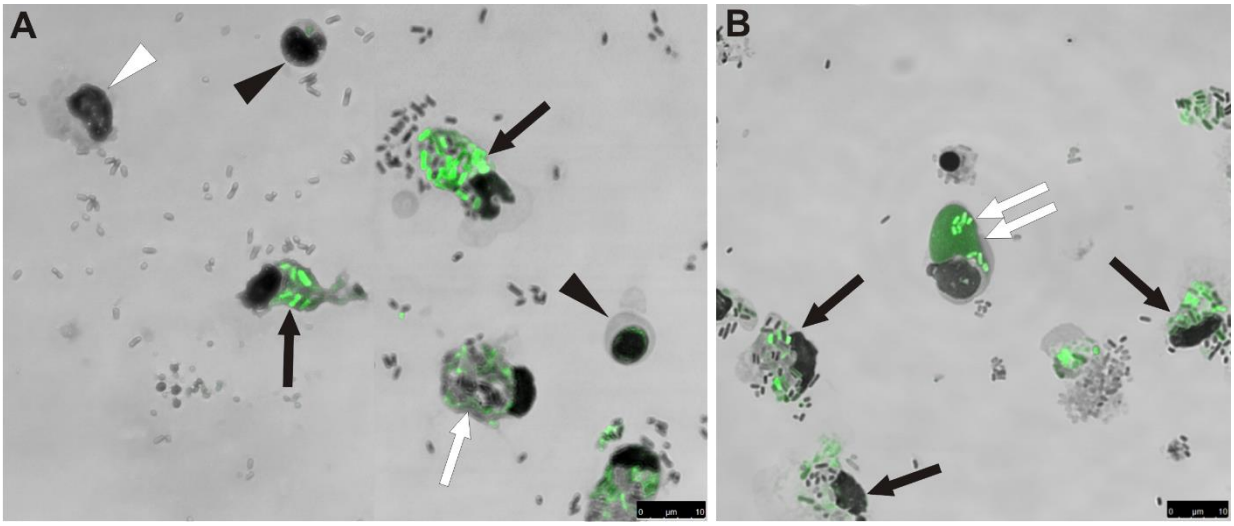

Fig. 22. Confocal micrographs showing sorted cells corresponding to the area of positive fluorescence after passing through the flow cytometer the cockle haemocytes that had been incubated with bacteria for 2 h. Green (fluorescent) bacilli correspond to bacteria occurring within haemocyte cytoplasm, while grey bacilli correspond to bacteria outside haemocytes. Black arrows: eosinophilic granulocytes; white arrow: basophilic granulocyte; white arrowhead: large hyalinocyte; black arrowheads: haemoblasts; double white arrow: haemocyte with large vacuole. Scale bars: 10 µm.

Results demonstrated that most cockle haemocytes phagocytosing bacteria were granulocytes, although the other haemocyte types were also represented in the sorted cell fraction of positive fluorescence. Considering that the sorting procedure was imperfect (as discussed above, section 4.3), some large hyalinocytes, HLVs and haemoblasts that were sorted within the positive fluorescent cell fraction corresponded to non-phagocytosing cells contaminating the sorted positive fluorescent cell fraction.

There were previous reports on phagocytosis ability of cockle haemolymph cells. Wootton *et al*. (2003a) reported intermediate ability to phagocytose various bacteria species of *C. edule* haemocytes compared to that of mussel *M. edulis* haemocytes (higher) and razor clam *Ensis siliqua* haemocytes (lower) but these authors did not discriminate between haemocyte types. Díaz *et al*. (2011) showed much higher ability of normal haemocytes to phagocytose fluorescent beads than haemolymph neoplastic cells, using flow cytometry, although no farther discrimination between normal haemocyte types was addressed. In the case of the lagoon cockle *C. glaucum*, Matozzo *et al*. (2007) found higher ability to phagocytose yeasts in granulocytes than in hyalinocytes. Our results were consistent with the general trend observed with flow cytometry in bivalve species, in which granulocytes are the haemocyte type with the highest phagocytic ability, while blast-like cells do not have that ability (Table 7).

4.3.2. ROI production

Flow cytometry assays to assess ROI production, using propidium iodide to discriminate live and dead cells, showed that most haemocytes were tightly grouped as live cells with positive fluorescent signal for ROI production, a minor percentage corresponded to dead positive cells, while the percentages of negative cells (either live or dead) were negligible (Fig. 23). Similar results were obtained stimulating cockle haemocytes either with zymosan or without stimulation, using CM-H2DCFDA at concentrations 4.3 µM or 8.6 µM, incubating the haemocytes with CM-H2DCFDA for 30 min or 60 min, and using haemolymph from individual cockles (3 different cockles) or pooled haemolymph. These results supported that all the cockle haemocyte types have similar ability to produce ROIs and therefore, no farther sorting was needed to discriminate between ROI-producing and non-producing haemocyte types.

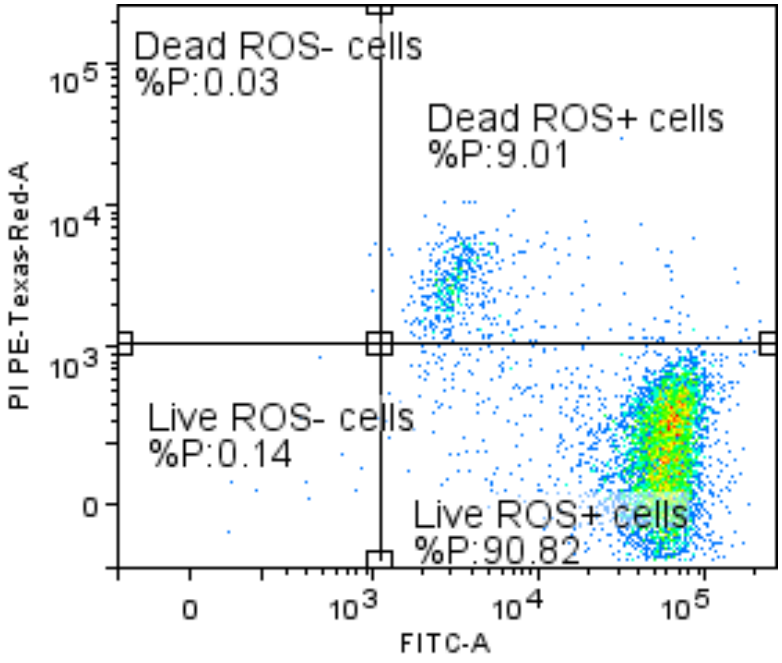

Fig. 23. Flow cytogram corresponding to an assay to assess ROI production of cockle haemocytes, performed with 200,000 haemocytes from a haemolymph pool deriving from three cockles, without zymosan stimulation and incubating them with CM-H2DCFDA 8.6 µM for 60 min. The particles are distributed according to their fluorescence for ROI (synonym of ROS) production (X axis) and their staining with propidium iodide (PI, Y axis). Four areas are depicted in the cytogram: PI positive (dead) – ROS negative cells, PI positive (dead) – ROS positive cells, PI negative (live) – ROS negative cells, and PI negative (live) – ROS positive cells. The percentages of cells occurring in each area are shown in the cytogram.

Considering that the production of ROIs in all the cockle haemocyte types deviates from the common trend described in other bivalve species of differences in ROI production ability between haemocyte types, new flow cytometry assays were performed using haemocytes of the mussel *M. galloprovincialis*, a bivalve model species, as a way to validate our flow cytometry

procedure. As expected, according to literature, and contrary to the cockle, differences in ROI production were found between mussel haemocyte types. Flow cytometry assays showed the occurrence of a group of cells (the lowest percentage of cells) that did not produce ROIs, another group with moderate production (the highest percentage of cells) and a third group of haemocytes with high ROI production (intermediate percentage of cells, Fig. 24). Smaller, less complex cells (likely hyalinocytes) were dominant in the groups of cells with negative and moderate ROI production, whereas larger, more complex cells (likely granulocytes) were dominant in the group with high ROI production (Fig. 24). Contrarily to the cockle, high interindividual variability was observed in the mussel; similarly to the cockle, analogous results were obtained stimulating mussel haemocytes either with zymosan or without stimulation, using CM-H2DCFDA at concentrations 4.3 µM or 8.6 µM, and incubating the haemocytes with CM-H2DCFDA for 30 min or 60 min. Therefore, our procedure yielded results consistent with literature regarding ROI production ability of *M. galloprovincialis* haemocytes (Table 7), which contributes to validate our results on ROI production ability of cockle haemocytes.

The ability of *C. edule* haemocytes to produce ROIs without phagocytic stimulation had been reported although without any specific mention to haemocyte types (Wootton *et al.*, 2003a). Higher production of ROIs was detected in the cockle haemolymph neoplastic cells than in the normal haemocytes, without phagocytic stimulation, using flow cytometry (Díaz *et al.*, 2011). In the case of the lagoon cockle *C. glaucum*, the ability to produce ROIs by every haemocyte type, with and without phagocytic stimulation, was reported (Matozzo *et al.*, 2007). Our results did not agree with most reports on ROI production by haemocytes from other bivalve species analysed with flow cytometry; in most cases, differences between haemocyte types were found, being higher in granulocytes. However, no differences were found between haemocyte types in the case of the ark-shells *Anadara kagoshimensis* and *Anadara trapezia*, while hyalinocytes were the haemocyte type with the highest ROI production in the zebra mussel *Dreissena polymorpha* (Table 7).

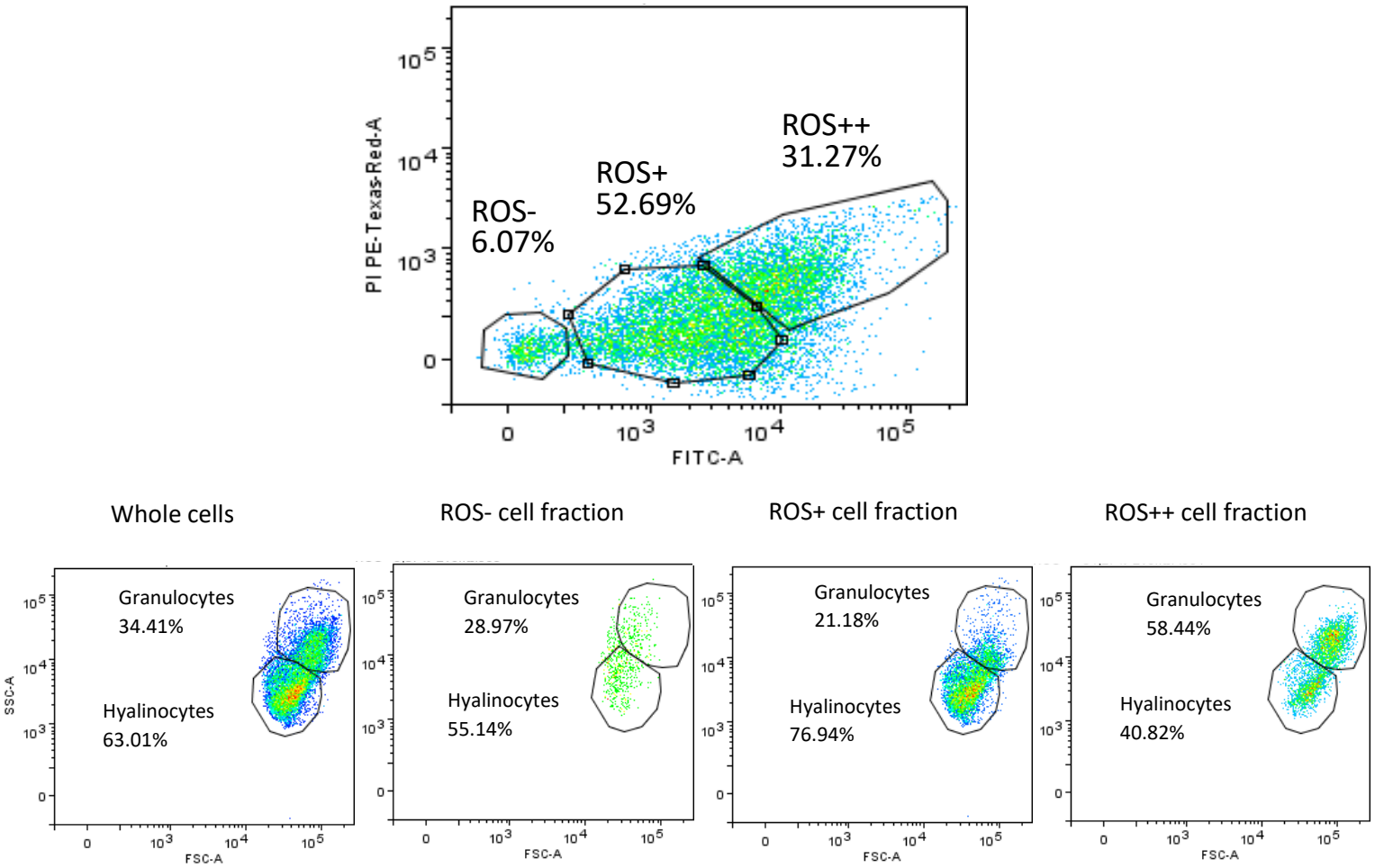

Fig. 24. Flow cytograms corresponding to an assay to assess ROI production of *Mytilus galloprovincialis* haemocytes, performed with 200,000 haemocytes from a haemolymph pool deriving from three mussels, without zymosan stimulation and incubating them with CM-H2DCFDA 8.6 µM for 60 min. Top graph: the particles are distributed according to their fluorescence for ROI (synonym of ROS) production (X axis) and their staining with propidium iodide (PI, Y axis); three areas are depicted in this cytogram, a negative area (ROS-), a moderately positive area (ROS+) and a highly positive area (ROS++). The percentages of cells occurring in each area are shown in the cytogram. Bottom graphs: flow cytometer density plots [forward scatter (FSC) vs. side scatter (SSC)] corresponding to the whole cells passed through the flow cytometer, the ROS- cell fraction, the ROS+ cell fraction, and the ROS++ cell fraction. Two areas are depicted in the bottom graphs, one corresponding to larger, more complex cells (likely granulocytes) and another area corresponding to smaller, less complex cells (likely hyalinocytes); the percentages of cells occurring in each area are shown in the cytograms.

4.3.3. Lysosomal and non-specific esterase content

Flow cytometry assay for lysosomal and non-specific esterase content showed that most (close to 90%) cockle haemocytes were positive for both characters (Fig. 25). According to FSC (cell size) and SSC (cell complexity) parameters, the cells with high fluorescence levels for both lysosomal and non-specific esterase content corresponded to larger, more complex cells (likely granulocytes), the cells with low fluorescence levels corresponded to smaller, less complex cells, while most cells negative for both lysosomal and non-specific esterase content corresponded to small, much less complex cells (likely haemoblasts). Unfortunately, the cells were much damaged after incubation, passing through the flow cytometer and sorting, thus haemocyte types could not be recognised in the cytospins performed with sorted cells after gating cytogram areas with different characters.

Wootton *et al*. (2003a) reported that the percentage of haemocytes positive for non-specific esterases was higher in *C. edule* than in *M. edulis* and *E. siliqua*, without discriminating between haemocyte types, using a cytochemical method. Díaz *et al*. (2011) applied flow cytometry and found differences in lysosomal and non-specific esterase content between *C. edule* haemolymph cell types, with low content in haemocytes of small size and lower inner complexity (likely hyalinocytes), moderate content in larger and more haemocytes (likely granulocytes), and high content in haemolymph cells of size and inner complexity corresponding to neoplastic cells. Regarding the lagoon cockle *C. glaucum*, Matozzo *et al*. (2007) found both granulocytes and hyalinocytes positive for non-specific esterases using a cytochemical procedure. The lysosomal and non-specific esterase content has been analysed in few bivalve species with flow cytometry (Table 7); in most cases, the content of lysosomes and non-specific esterases was higher in granulocytes than in the other haemocyte types, although in the case of the zebra mussel *D. polymorpha*, the lysosomal content was higher in hyalinocytes than in the other haemocyte types (eosinophilic granulocytes and blast like cells) (Table 7).

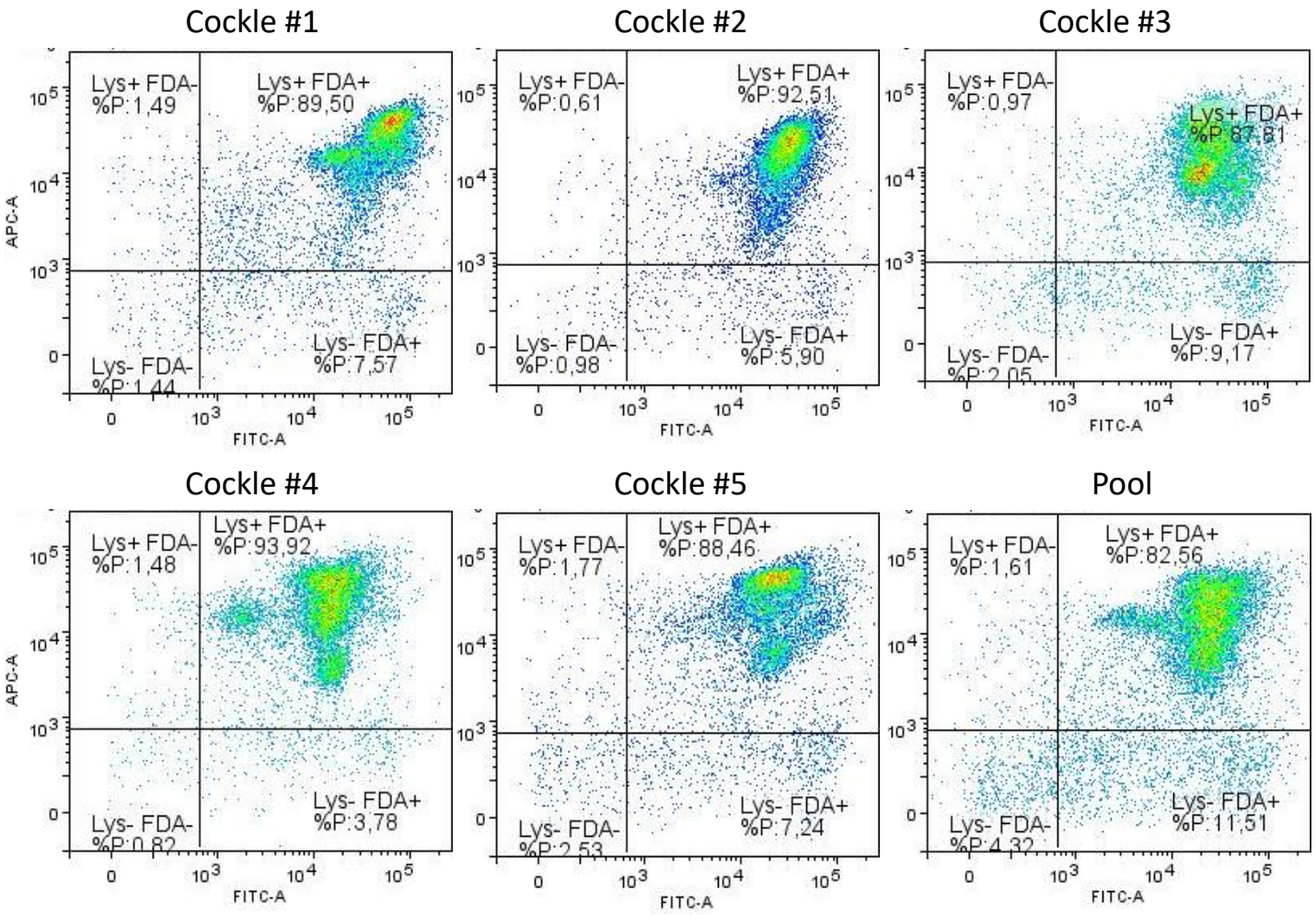

Fig. 25. Flow cytograms corresponding to assays performed to assess lysosome and non-specific esterase content of cockle haemocytes using separately haemolymph from each of five cockles (1-5) and pooled haemolymph from those five cockles. The particles appear distributed according to their fluorescence due to accumulation of Lysotracker-Deep-Red in their lysosomes (lysosomal content, APC-A) and their fluorescence due to hydrolysis of fluorescein diacetate (FDA) by intracellular esterases (FITC-A). Four areas are depicted in the cytograms: Lysotracker (Lys) positive (with lysosomes) – FDA negative (without esterases) cells, Lys positive – FDA positive (with esterases) cells, Lys negative (without lysosomes) – FDA negative cells, and Lys negative – FDA positive cells. The percentages of cells occurring in each area are shown in the cytograms.

5. Conclusions

Six haemocyte types were distinguished in the cockle haemolymph according to morphological characters and staining properties, namely (from more to less abundant in average) eosinophilic granulocytes, large hyalinocytes, basophilic granulocytes, haemocytes with a large vacuole (HLV), haemoblasts and mixed granulocytes. One of these types, the HLV, has not been identified in bivalve species other than *Cerastoderma* spp. Both the THC and the DHC showed high variability; some factors influencing them were identified. A pattern of seasonal variation was detected, with THC maxima in late summer and minima in early spring, while the relative abundance of eosinophilic granulocytes showed maxima in early autumn and early spring and minima in summer. Seawater temperature, salinity and gonad condition, all of them with seasonal variation, influenced THC and DHC. Marteiliosis, the most serious pathological condition affecting Galician cockles, according to the mortality that it causes, significantly influenced the THC and the DHC; heavy marteiliosis was associated with marked decrease of the THC and the relative abundance of eosinophilic granulocytes and increase of the relative abundance of mixed granulocytes, large hyalinocytes and haemoblasts. The association of heavy marteiliosis with THC decrease was opposite to the general trend in bivalves of THC rise associated with heavy infections, according to literature, and would deserve further research. In this study, contrary to the case of marteiliosis, a THC drop was not observed in association with any of the other pathological conditions and contrasted with the THC rise associated with the infection with *M. tapetis* and the heavy haemocytic infiltration of tissues. The drop of the relative abundance of eosinophilic granulocytes associated with heavy marteiliosis was not observed in the other pathological conditions while heavy granulomatosis was associated with increase of the relative abundance of mixed granulocytes.

Flow cytometry allowed discrimination of cockle haemocytes in four fractions (R1-R4) depending on their size and cell complexity. The correspondence of those four fractions with the six recognisable morphological types with light microscopy was not straight, although most granulocytes (particularly eosinophilic granulocytes) would be expected to be included in the R1 fraction of flow cytometry and most haemoblasts would be expected in the R4 fraction. Sorting the haemocytes from each region was possible with the sorter device although each sorted fraction was contaminated to some extent with cells from the other fractions. Granulocytes showed the highest ability to phagocytose bacteria, while large hyalinocytes and haemoblasts showed very limited ability to do it. All the cockle haemocyte types showed similar ability to produce ROIs; it is rather exceptional among studied bivalve molluscs, which mostly showed differences in the ability to produce ROIs between haemocyte types. Larger, more complex haemocytes (likely granulocytes) showed the highest lysosomal and non-specific esterase content, while the other cell types showed lower to null content.

The new knowledge on the cockle immune system should contribute to understand the cockle ability to face pathogens, which could be particularly useful in case of emerging diseases.

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