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Mytilus galloprovincialis releases immunologically functional haemocytes to the intervalvar space in response to tissue injury and infection

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ABSTRACT

Haemocytes of *Mytilus galloprovincialis* represent the main component of the internal self-defence system. Although haemocytes from haemolymph are usually studied to analyse these animals' immune response, the presence of haemocytes in the intervalvar liquid, which is essentially sea water, led us to characterize them. Several functional (ROS production, phagocytosis, gene expression, travel velocity and distance) and morphological (area, size and granularity) assays were performed by applying different stimuli to the mussels (waterborne infection, shell injury and their combination). Our results revealed that intervalvar liquid haemocytes share common characteristics with haemolymph haemocytes (for instance, the cell morphology and the cell population structure divided in three main groups) but also show significant differences in size (usually smaller in the intervalvar liquid), mobility (commonly faster in the intervalvar liquid), ROS production (higher in non-stimulated intervalvar liquid cells) and gene expression (IL17, Myd88 and CathL are over expressed in liquid intervalvar cells compared to haemolymph cells). Moreover, differences were observed when mussels were subjected to the mentioned treatments. These free intervalvar haemocytes could constitute the first line of defence as external sentinels extending the immunological alert system outside of the mussel body.

1. Introduction

The Mediterranean mussel *(Mytilus galloprovincialis)* is a bivalve mollusc distributed worldwide and cultured in several countries with high production levels (the main producers are China, Chile and Spain) [1]. From an ecological point of view, it is considered an invasive species [2], and its sessile condition as well as its filter-feeding way of life explain why these animals serve as pollution bioindicators [3,4]. They are characterized by being highly resistant to pathogens, as far as only punctual and scarce mass mortalities have been reported in natural environments contrary what happens in other bivalve species sharing similar ecosystems, such as clams (*Ruditapes decussatus* and *R. philippinarum*) and oysters (*Crassostrea gigas* and *Ostrea edulis*) [5–10].

As with all invertebrates, mussels rely only on their innate immune system, in which haemocytes play the main role. These macrophage-like cells cooperate in the repair of injured tissues and the fight against infections by different mechanisms, such as chemotaxis, encapsulation, phagocytic activity and release of oxygen and nitrogen radicals. Moreover, haemocytes activate intracellular signalling pathways to trigger the synthesis of antimicrobial effectors such as defensins [11], mytilins [12], mytimycins [13] and myticins [14]. These molecules show high genetic variability [15–17], and in addition to antiviral, antibacterial and antifungal activities, their involvement in tissue injury and regeneration processes has been described [18].

Most of the studies on the immune response of mussels have been conducted in the haemolymph that is usually collected in the posterior adductor muscle with a syringe [19], where bivalves have an easily accessible sinus [19,20]. Most likely due to the semiopen circulatory system, haemocytes can reach different tissues, and it is well known that they have an important capacity for migration. The presence of haemocytes associated with mucus covering the mantle, gills and body wall has been confirmed in *Crassostrea virginica,* where they can migrate in a bidirectional way between the mucosal layer and the circulatory system. Migration was also induced after infection with pathogens such as *Vibrio splendidus* in *C. gigas* [21] or after injury in *M. galloprovincialis* [22]. Moreover, haemocytes have been detected in the extrapallial fluid that can be found in the very small space (extrapallial space) enclosed between the shell and mantle of *C. virginica* [23]. These haemocytes could

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be used by pathogens such as *Perkinsus marinus* as a vehicle to enter the oyster [24,25]. However, as Eggermont et al. [19] stated, extrapallial fluid has a composition very similar to that of haemolymph and should not be confused with pallial fluid or intervalvar liquid, which is seawater. In our studies on the mussel immune response, the observation by chance of cells similar to haemocytes in the intervalvar space (delimited by the shells, surrounded by the mantle and where the gills are located), where supposedly there is only seawater, led us to further study these cells and compare them with the haemocytes that can be found inside the mussel body. The presence of haemocytes in the intervalvar liquid was previously demonstrated after conditions of high temperature stress [26]. These authors have already suggested that these cells could act as Trojan horses for spreading bacterial diseases among mussels. However, until now, there has not been a comparison between these cells from the intervalvar cavity and internal haemocytes. It is also unknown whether the presence of haemocytes in the intervalvar liquid is normal in bivalves and whether they could act as sentinels extending the immunological alert system beyond the body surface.

In this study, we analysed the characteristics of the haemocytes of the intervalvar liquid of mussels and their response against different stimuli (a tissue injury and a waterborne bacterial infection) in comparison with the haemocytes from haemolymph. Number, size, movement, reactive oxygen species production, phagocytosis, and the expression of genes related to migration and immune functions of haemocytes outside (intervalvar liquid) and inside the mussel body were analysed and compared.

2. Materials and methods

2.1. Mussels

Adult mussels (*Mytilus galloprovincialis)* were obtained from a commercial shellfish farm (Vigo, Galicia, Spain). The animals were 7.63 ± 0.93 cm in length. Their shells were externally cleaned of environmental residues, and the animals were maintained in open-circuit seawater tanks at 15 °C with aeration and fed daily with the algae *Phaeodactylum tricornutum* and *Isochrysis galbana* for a week before use. The experiments were conducted on February 2022 when the animals were in a ripe stage after spawning.

2.2. Collection of haemocytes and cell quantification

The animals were maintained without food for 24 h before sampling to avoid interference from algae. The fluids retained inside the animal (intervalvar liquid) were sampled using a disinfected spatula to separate the two shells. The spatula was slightly introduced through the region of the byssus where the valves are physically separated to avoid breaking the mantle and contaminating the sample with extrapallial fluids. The intervalvar liquid was collected in a sterile Petri dish by gravity. Haemolymph was extracted from the adductor muscle using a syringe fitted with a 25 gauge needle and immediately diluted in cold sterile filtered sea water (FSW) in a 1/50 ratio to avoid aggregation.

The number of cells present in the suspensions was determined by microscopy. One millilitre of sample was dispensed in a 24-well polystyrene plate. Haemolymph extracted from the muscle was directly distributed in the wells, and the intervalvar liquid was previously filtered using a 40 μm nylon cell strainer (Falcon). Haemocytes were stained with Hoechst 33,342 (NucBlue™ Live ReadyProbes™ reagent, ThermoFisher) to discard cell debris and other noncellular elements present in the intervalvar liquid. Images were taken at 10X magnification using a Leica DMi8 motorized microscope (Leica), and the number of cells was counted in a 3.33 mm^2 area. The total number of haemocytes per mL was calculated by extrapolating the values to the entire well (200 mm^2) and considering the dilution factor.

2.3. Treatments

To determine if an external stimulation induced an increase in the number of haemocytes present in the intervalvar liquid or in the haemolymph, mussels were subjected to the following treatments: (1) physical damage, consisting of a small notch in the shell, injuring the border of the mantle. The notch was located in the dorsal back part of the shell, close to the adductor muscle. This notch was done using a grinding machine; (2) a waterborne infection with *Vibrio splendidus,* only by diluting the bacteria in the tank water and allowing the filtration of the bacteria; and (3) the double stimulus (physical damage and infection) was performed combining the two previously described stimuli (first the notch followed by the bath infection with *Vibrio splendidus*).

The intervalvar liquid of all animals was examined before the stimulation. The animals showing a poor condition or an abnormally high number of haemocytes in the intervalvar liquid at this moment were not used in the experiments (this poor condition is determined by an exceptionally high number of cells in intervalvar liquid that could be the result of a previous injury).

For bath infection, *V. splendidus* was grown on 1.5% NaCl tryptic soy agar (TSA) plates at 22 °C for 24 h. A suspension of *V. splendidus* was prepared on FSW and added to 5 L tanks to obtain a final bacterial concentration of 10^8 CFU/mL.

2.4. Changes in cellular counts induced by injury and bath infection

Six animals were used for each treatment, including a nonstimulated control group. All mussels were marked in the shell with polish nail to allow tracking of the individuals. Mussels were sampled 3 and 24 h after stimulation, and the number of haemocytes was evaluated as previously described. The experiment was conducted 4 times. Fold changes values represent the ratio between treatments at different times and the experimental t0.

2.5. Cell populations characterization

Samples were evaluated by flow cytometry to characterize the populations of haemocytes in the intervalvar liquid and compared to those from the haemolymph extracted from the same animal. Moreover, the effect of the treatments was also determined. A total of 6 animals per treatment were evaluated. The size and complexity (FSC/SSC) were measured by using a FACSCalibur cytometer (Becton and Dickinson). An FSC threshold value was set to 20 to exclude sample debris. Data were analysed using CellQuest software (Becton and Dickinson).

2.6. ROS production

Haemocytes from the intervalvar liquid and haemolymph were obtained 24 h after stimulation to analyse their functional activity. The production of reactive oxygen species (ROS) was analysed using the luminol-enhanced chemiluminescence (LC) method. Haemocytes from the intervalvar liquid and haemolymph were extracted from 8 animals. This experiment was conducted twice. Only mussels injured 24 h before the experiment were used to obtain enough cells in the intervalvar liquid to produce a detectable ROS signal. The cell concentration was measured in an automated cell counter (CellDrop, DeNovix) and adjusted to a final concentration of 10^5 cells/mL in FSW. One hundred microlitres of cell suspension was plated in duplicate in a white opaque 96-well flat microplate (Thermo Fisher) and incubated for 30 min at 15 °C. Zymosan A (Sigma Aldrich) and PMA (phorbol myristate acetate, Sigma Aldrich) were used to trigger ROS production, and luminol (5-amino-2,3-dihydro-1,4-phthalazindione) (Sigma Aldrich) was used as a light emitter. Cells of the same 8 animals were tested for the three triggering conditions. A 0.1 M luminol solution in dimethyl sulfoxide (DMSO, Sigma Aldrich) was diluted in FSW to obtain a final concentration of 10 mM. Zymosan A and PMA were diluted in the luminol

solution at final concentrations of 1 mg/mL and 1 μg/mL, respectively. One hundred microlitres of zymosan A or PMA was added to each well. The generation of relative luminescence units (RLU) was measured in a Glomax Discover Microplate device (Promega) nine times at intervals of 5 min.

2.7. Phagocytosis

The phagocytic activity of haemocytes was evaluated by fluorescence microscopy. Briefly, haemocytes extracted from the intervalvar liquid (maximum extracted volume) and haemolymph (2 mL) were distributed in 6-well polystyrene plates (Falcon) and allowed to settle for 30 min at room temperature (22 °C). Supernatants were removed, and the attached haemocytes were incubated for 1 h at 18 °C with 2 mL of carboxylate-modified fluosphere (Invitrogen) at a final concentration of 10^7 beads/mL. Noningested particles were removed by two consecutive washes with PBS. The percentage of phagocytosis was calculated by counting the total number of cells that ingested at least one fluorescent particle in 3 random regions of the well using a DMi8 fluorescence microscope (Leica). This experiment was conducted twice using haemocytes extracted from three individuals per treatment.

2.8. Cell area and migration

The size of haemocytes was analysed by microscopy. Briefly, haemocytes extracted from 3 individuals per treatment were distributed in 6-well plates were fixed in 2% paraformaldehyde (PFA) for 10 min, stained with 0.1 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) and visualized on a Leica DMi8 fluorescence microscope (Leica). Pictures were taken at 20X magnification, and the cell area was measured in a mean of 150 cells per treatment using ImageJ software.

The movement of the haemocytes was analysed by time-lapse images by measuring the velocity and distance travelled by the cells. Haemocytes extracted from five animals per treatment were placed in 24-well polystyrene plates (24h after the stimulation) and imaged using a motorized Leica DMi8 microscope (Leica). Multiposition time-lapse recordings were performed, and 15 images were captured at intervals of 2 min (30 min in total). The individual images of the time-lapse sequence were analysed using the Manual Tracking and the Chemotaxis and Migration Tool 2.0 plugins for ImageJ analysis software to calculate the velocity and distance travelled by the cells. This experiment was performed four times. In each experiment, at least 60 cells were analysed, and the mean velocity and accumulated distance were measured.

2.9. Gene expression analysis

Differences in the gene expression profile between haemocytes isolated from the intervalvar liquid and from haemolymph were evaluated by qPCR. Moreover, the effects of injury and bacterial infection on gene expression were also analysed. A total of 144 mussels were stimulated as previously described. Animals were sampled 24 h after stimulation to generate 6 pooled samples of haemocytes extracted from 6 animals in the four experimental conditions (control, injury, bacterial infection and double stimulation).

RNA was automatically extracted in a Maxwell robot (Promega) using a simplyRNA Tissue Kit (Promega) according to the manufacturer's protocol. The concentration and purity of the RNA were measured using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA). The RNA concentration was adjusted to 50 ng/mL, and first-strand cDNAs were synthesized using the NZY first-strand cDNA synthesis kit (Nzytech). Specific primers for the analysis of *myticin C*, *IL-17-2, IL-17-3, Myd88, galectin-4, cathepsin-L, cathepsin-D* and *18S* genes were used. 18S was used as reference gene for normalization. The sequences of the primers and their amplification efficiencies are presented in Table 1.

Gene expression was assayed by qPCR using the StepOnePlus Real-Time PCR System (Applied Biosystems) by mixing 1 μL of cDNA template with 0.5 μL of each primer (10 μM) and 12.5 μL of SYBR Green PCR Master Mix (Thermo Fisher Scientific) in a final volume of 25 μL. Standard cycling conditions were 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 30 s. All reactions were performed in technical duplicates. Relative gene expression levels were calculated using ribosomal 18S as a reference gene following the method described by Pfaffl [27]. Fold change units were calculated by dividing the normalized expression values obtained in stimulated samples by the normalized expression values obtained in the control. The results were expressed as the mean ± standard error of the six biological samples.

Table 1
Primers used for qPCR analysis. The efficiency of amplification is indicated.

Name	Sequence (5′-3′)	Efficiency
Myticin C	For: ATTTGCTACTGCCTTCATTG Rev: TCCATCTCGTTGTTCTTGTC	−3.52
IL-17-2	For: TACGCTGTCGTTGTCAAGGT Rev: ACACCATTTTCGCAAACTCC	−3.29
IL-17-3	For: CGCTGTATGGCGTCTTCTCT Rev: TTTTTCACGGAAGGTTGGAC	−3.04
Myd88	For: AGATGTTGTGTTGGATAGCG Rev: TCGTACCTGTCACTTTCTGG	−3.65
Galectin-4	For: GTGTACCAAATCCAACAGCA Rev: CCAACTTCCGTTCTGACA	−3.90
Cathepsin-L	For: GGTTCTGTAGTCAGACCAGACTTG Rev: CTGGCTGATGTTGAGATACCAC	−3,14
Cathepsin-D	For: GAGTTACTCCTTGGAGGCAGTG Rev: ACAGGTCCAGCTAAGAGTGAGG	−3,82
18S	For: GTACAAAGGGCAGGGACGTA Rev: CTCCTTCGTGCTAGGGATTG	−3.30

2.10. Statistical analyses

Obtained data were statistically analysed as follows: i) changes in cellular counts induced by injury and bath infection were evaluated by the nonparametric Friedman test; ii) differences in the percentage of granulocytes and hyalinocytes to characterize cell populations were evaluated by ANOVA; iii) ROS production results were analysed by *t*-test; iv) phagocytosis was analysed by ANOVA with a post hoc Tukey test; v) cell area and migration results were tested by ANOVA; vi) gene expression results were evaluated by the Mann–Whitney and Kruskal–Wallis non parametric tests. All the statistical tests were run using GraphPad Prism software (San Diego; CA; USA), and the results were considered significant with a threshold p value < 0.05.

3. Results

3.1. Morphological characterization of intervalvar liquid haemocytes

Microscopic observations confirmed the presence of haemocytes in the intervalvar liquid of mussels. The morphology of these cells was similar to that of the haemocytes found in the haemolymph, including granulocytes and hyalinocytes without granules in the cytoplasm (Fig. 1a). The distribution of haemocytes into different cell populations according to their size and granularity was also analysed by flow cytometry (Fig. 1b). Cells were mainly distributed into three populations: R1 included cell debris and small haemocytes, R2 included smaller haemocytes with low complexity values (hyalinocytes) and R3 included large granular haemocytes (granulocytes). Intervalvar liquid haemocytes showed a cell distribution similar to that observed in the haemolymph, with a significantly higher percentage of cells in the R1 region (97.5% ± 0.9 in intervalvar liquid and 85.8% ± 10.1 in haemolymph, $p < 0.05$). Due to the small size of most haemocytes and the lack of resolution in the R1 region, we decided to measure the cell size by microscopy to be able to discriminate haemocytes from other materials or cell debris present in the intervalvar liquid. The mean area of the

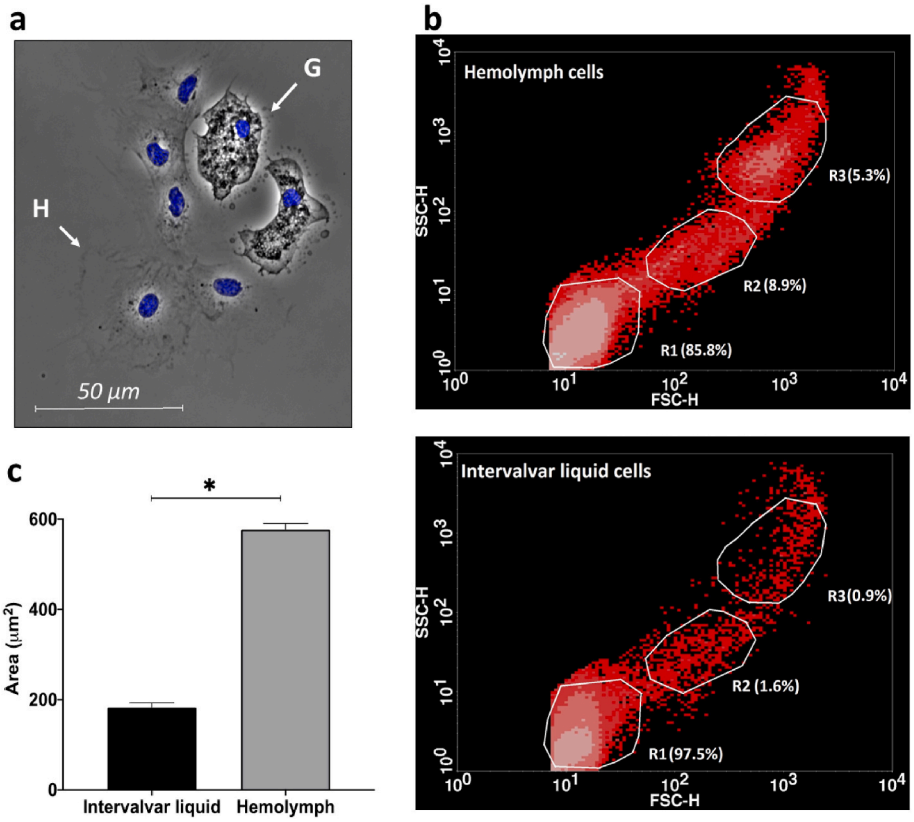

Fig. 1. (a) Contrast phase image showing the presence of haemocytes in the intervalvar liquid. (G) Granulocytes. (H) Hyalinocytes. Nuclei are stained blue with Hoechst. Scale bar at 50 µm. **(b)** Density plots comparing the distribution of the haemolymph haemocytes and the intervalvar liquid. The average percentage of cells included in each region (R1, R2 and R3) is indicated in brackets. **(c)** Average ± SEM area of the haemocytes from the intervalvar liquid (152 cells) and from the haemolymph (342 cells). Asterisk (*) indicates significant differences at $p \leq 0.0001$.

intervalvar liquid haemocytes was significantly smaller than that registered in the haemocytes isolated from the haemolymph (Fig. 1c).

3.2. Effect of stimulation on the concentration and morphology of intervalvar liquid haemocytes

The intervalvar liquid extracted from unstimulated mussels showed about 60 haemocytes/ml (measures performed in 24 animals per treatment). This concentration was not significantly altered when the intervalvar liquid was extracted again from the same animal at 3 and 24 h after the first extraction. This result confirms that the technique used to obtain the intervalvar liquid had a very low impact on welfare and did not affect the normal physiology of the animal (Fig. 2a). The concentration of haemocytes in the intervalvar liquid increased when animals were injured and/or infected (Fig. 2a and b). The bacterial infection induced a small but significant increase in cells at 3 h poststimulation. The physical injury to the shell induced an important increase in the cell concentration at 3 and 24 h poststimulation with a more than 100-fold increase compared with the control mussels. The combination of both stimuli (injury and infection) in the same animal also significantly increased the concentration of haemocytes at all sampling points, but no significant differences were obtained compared to animals that were only injured to the shell (Fig. 2a and b). For comparative purposes, the concentration of haemocytes in the haemolymph (extracted from the muscle) was also analysed in stimulated animals. In this case, none stimuli modified the number of haemocytes after 24 h (Fig. 2c).

The stimulation of mussels modified the proportion of granulocytes and hyalinocytes in a similar way in the haemolymph and in the intervalvar liquid (Fig. 3). In both samples, the percentage of granulocytes increased in mussels 24 h after bacterial challenge, changing from 30% in controls to 69% in infected animals. However, a significant reduction in the number of hyalinocytes was also registered in both compartments when individuals were subjected to a double stimulation (Fig. 3).

Changes in haemocytes induced by the different treatments were also evaluated by measuring the cell area by microscopy. Several changes in the distribution of cells were observed in both haemocytes from the intervalvar liquid and haemolymph after treatment (Fig. 4). A generalized increase in the cell area of haemocytes in the intervalvar liquid was observed after all stimulations. The percentage of small cells (0–200 μm^2) observed in the intervalvar liquid from control animals (46%) decreased to 4% and 14% after infection and injury, respectively. At the same time, the percentage of haemocytes included in larger groups (601–800, 801–1000 μm^2) increased with all types of stimulation compared to controls (Fig. 4). The size distribution of cells from the haemolymph was also modified after stimulation. The bacterial infection increased the number of cells included in the largest sizes (801–1000 and > 1000 μm^2) to 19% and 15%, respectively. In contrast, injured animals had the highest percentage of cells belonging to the 201–400 μm^2 range (38%) and less than 2% of cells in the larger size. The double stimulation induced a homogeneous distribution of cells in all size groups (Fig. 4).

When comparing haemocytes from intervalvar liquid and from haemolymph, a higher proportion of larger cells was observed in the haemolymph than in the intervalvar liquid after infection. An opposite response was observed after injury, where the haemolymph had a higher percentage of cells with small areas. Finally, a similar cell distribution was observed in both types of haemocytes when animals were subjected to a double stimulus (Fig. 4).

3.3. Effect of stimulation on cell activity

The effect of the different stimuli on the activity of haemocytes was assayed by measuring the velocity and distance travelled by the cells in time-lapse images. A total of 60 haemocytes per treatment were tracked for 30 min (Fig. 5c shows an example of how the measures were performed). In the absence of stimulation, the haemocytes from the intervalvar liquid moved significantly faster and travelled longer distances than the haemocytes from the haemolymph. The mean velocity of

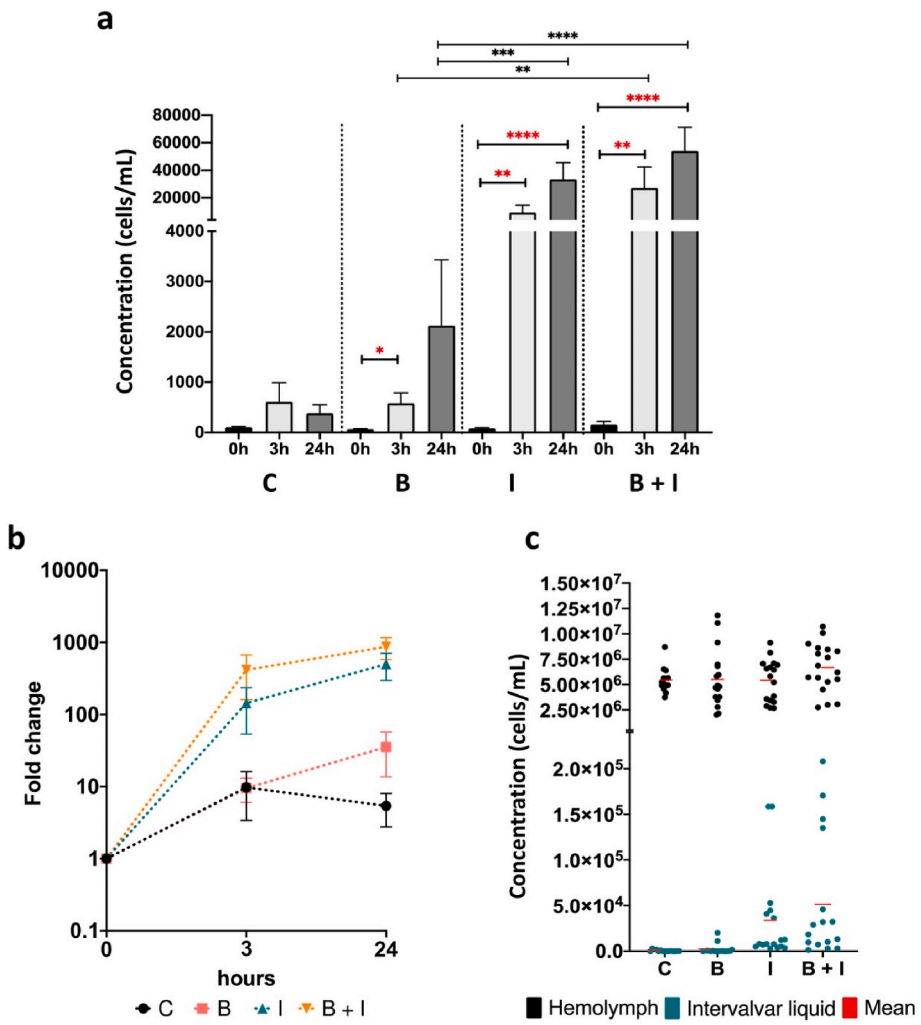

Fig. 2. (a) Concentration ± SEM of haemocytes in the intervalvar liquid of stimulated mussels at different time points (0 h, 3 h and 24 h). Asterisks indicate significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Red asterisks represent significant differences compared to each control. Black asterisks represent significant differences between treatments. **(b)** The evolution of the cell concentration in the intervalvar liquid is shown as the fold change. Fold change units were calculated by dividing the cell concentration obtained at 3 h and 8 h by the concentration registered at 0 h in each stimuli. **(c)** Comparison of the cell concentration in the intervalvar liquid and haemolymph 24 h after treatment. In the entire figure: C, control animals; B, animals infected with bacteria; I, injured animals; B + I, injured animals also infected with the bacteria.

intervalvar liquid haemocytes was 3.47 μm/min, while haemocytes in the haemolymph moved at 2.14 μm/min (Fig. 5a). Moreover, the intervalvar liquid haemocytes travelled a longer distance (100 μm) than that covered by the haemocytes extracted from the haemolymph (50 μm) (Fig. 5b). Haemocytes from the intervalvar liquid showed a significant increase in velocity and accumulated distance only after injury stimulation. No effects were observed when mussels were exposed to a bacterial infection or a double stimulation. In contrast, haemocytes extracted from the haemolymph significantly increased their velocity and distance only when mussels were subjected to double stimulation (Fig. 5a and b).

3.4. Effect of stimulation on ROS production and phagocytosis

ROS production was evaluated in order to stablish the differences between haemocytes from intervalvar liquid and from haemolymph. To compare the results, the higher triggering point was selected (35 min after trigger the ROS production). At the basal level (only detected using luminol), the production of reactive oxygen species was significantly higher in the intervalvar liquid haemocytes than in haemocytes from the haemolymph (Fig. 6a). A similar response was obtained when ROS production was triggered by PMA stimulation, although in this case, the increment in the luminescence levels registered after PMA stimulation was smaller than at the basal level (Fig. 6a). However, an opposite response was obtained when ROS production was triggered by zymosan stimulation: the production of ROS by haemocytes from the haemolymph was significantly higher than that induced in haemocytes from the intervalvar liquid (Fig. 6a).

In the absence of stimulation, the phagocytic activity was similar in haemocytes from the intervalvar liquid and haemolymph (Fig. 6b). However, a significant increase in phagocytic activity was observed in the intervalvar liquid haemocytes when animals were waterbornely infected. In contrast, the phagocytic activity of haemolymph haemocytes was not modified by any of the stimuli used.

3.5. Effect of stimulation on gene expression

We first compared the constitutive expression of seven genes involved in immune-related processes in the intervalvar liquid haemocytes with that of the haemocytes from the haemolymph (Fig. 7a). Significantly higher expression of the *IL17-2, IL17*-3 and *CathL* genes was found in haemocytes present in the intervalvar liquid cells than in those from haemolymph. The *Myd88* gene was also highly expressed in the intervalvar liquid, although it was not statistically significant compared to the expression in haemolymph haemocytes. No statistically significant differences were observed in the expression of *Galectin-4, Cathepsin D* and *Myticin C* genes in the intervalvar liquid haemocytes compared to the haemolymph haemocytes (Fig. 7a).

The gene expression profile of haemocytes present in the intervalvar liquid was not modulated by bacterial infection. None of the seven analysed genes showed significant changes compared to controls. Intervalvar haemocytes extracted from injured animals showed a reduction in the expression of both *IL17* isoforms, although only the expression of isoform 2 was significantly reduced. Additionally, the

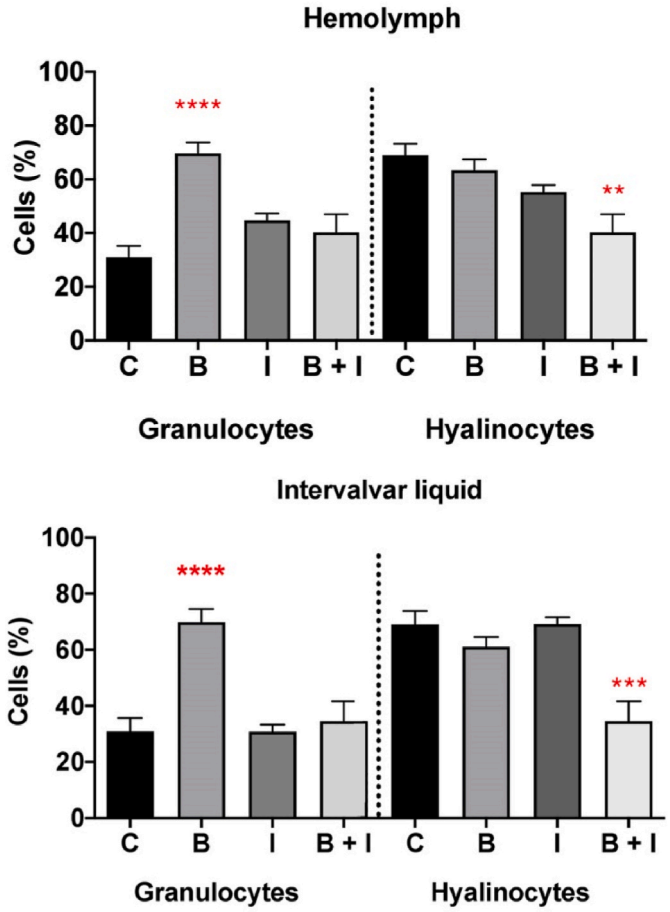

Fig. 3. Percentage of granulocytes and hyalinocytes ±SEM in haemolymph and intervalvar liquid and from mussels at 24 h poststimulation. Asterisks indicate significant differences at $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). C, control animals; B, animals infected with bacteria; I, injured animals; B + I, injured animals also infected with the bacteria.

expression of *CathD* and *Galectin-4* was respectively down and up modulated in injured animals. Haemocytes present in the intervalvar liquid extracted from animals with double stimulation showed significantly reduced expression of only the *IL17-2* and *CathD* genes (Fig. 7b).

Haemocytes from the haemolymph showed a different gene expression profile after stimulation. In this case, bacterial infection induced a significant reduction in *CathD* gene expression. Injured mussels showed a significant increase in *Gal4* and *Myd88* gene expression. In contrast, the expression of *CathD* was significantly inhibited. Finally, the double stimulation of mussels significantly reduced the expression of only the *CathD* gene.

4. Discussion

Mussel haemocytes, the cells responsible for immune defence, are able to move freely in a semiopen circulatory system, reaching the surface of the body and the extrapallial cavity [24–26]. In our work, we have confirmed the presence of these specialized cells in the intervalvar liquid, which is essentially sea water [26]. The morphology, functional activity and gene expression of these cells were similar to those of haemocytes from the haemolymph, which are the immune cells usually studied in bivalve immunology. In normal circumstances, there is a limited number of haemocytes in the intervalvar liquid; however, after a strong thermal stress [26], or, as we described, after a tissue injury or a bacterial infection, there is an important increase in the number of these cells. We hypothesize that the cells found in the intervalvar liquid come from the haemolymph migrating after a stimulus. However, interestingly, the number of haemocytes in the haemolymph did not decrease after stimulation. If the haemocytes of the intervalvar liquid have migrated from the haemolymph, proliferation may occur, which deserves further investigation.

Another issue that remains to be studied is the effect that the life cycle could have in all this cellular immune system. There is no information linking the reproductive stage of mussels (or other bivalves) and the presence of haemocytes in the intervalvar liquid. However, this kind of studies have been performed in relation to haemocytes present in the haemolymph. For instance, in *R. philippinarum*, it could be determined that the total number of haemocytes as well as the ROS production did not change in any reproductive stage [28]. However, in *C. gigas*, it was evident a decrease in haemocytes density at the same time as spawning

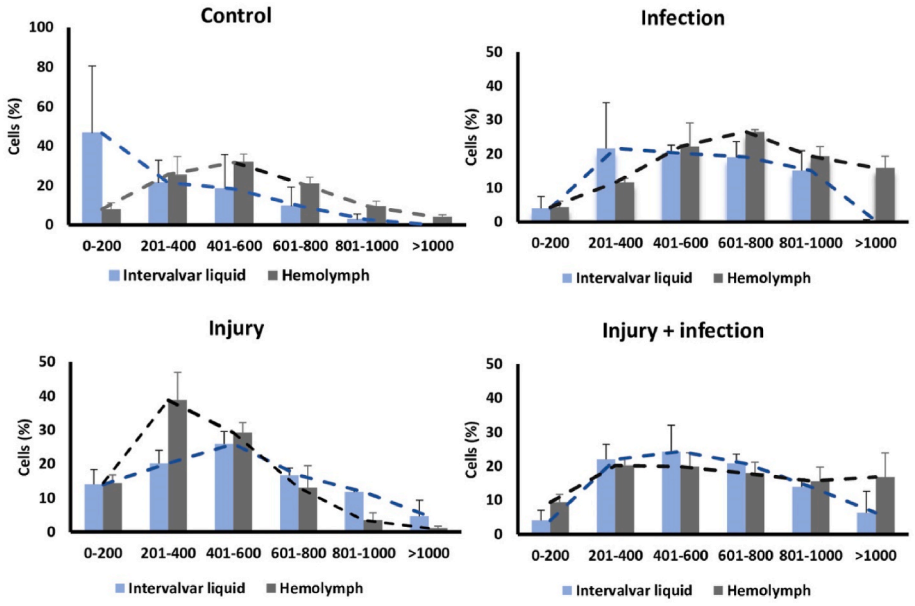

Fig. 4. Percentage of cells ± SEM included in different ranges of cell area (in μm^2) in the intervalvar liquid and in haemolymph after the different treatments. The cell areas were measured in a mean of 150 cells per treatment.

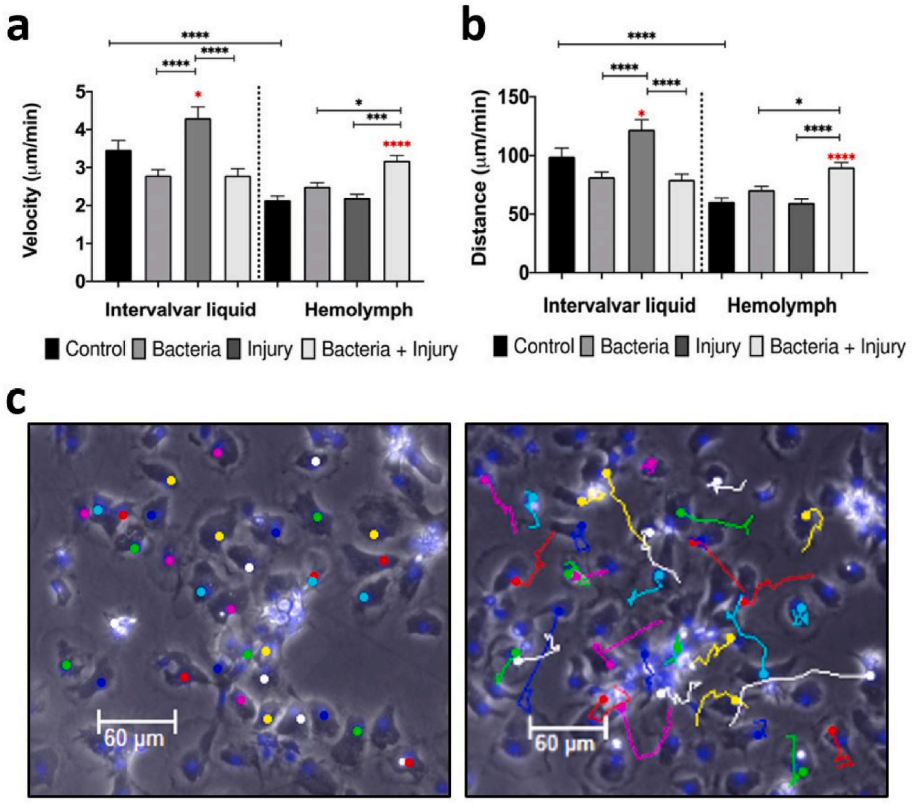

Fig. 5. Effect of treatments on cell motility. **(a)** The mean ± SEM velocity (µm/min) of haemocytes is shown 24 h poststimulation. **(b)** The value of the cumulative distance (µm) travelled by cells for 30 min was registered in haemocytes after 24 h of stimulation and is represented with the SEM values. Asterisks indicate significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Red asterisks represent significant differences compared to each control. Black asterisks represent significant differences between treatments. **(c)** Image showing the haemocytes position at T0 and T30 min showing the strategy to do the measures.

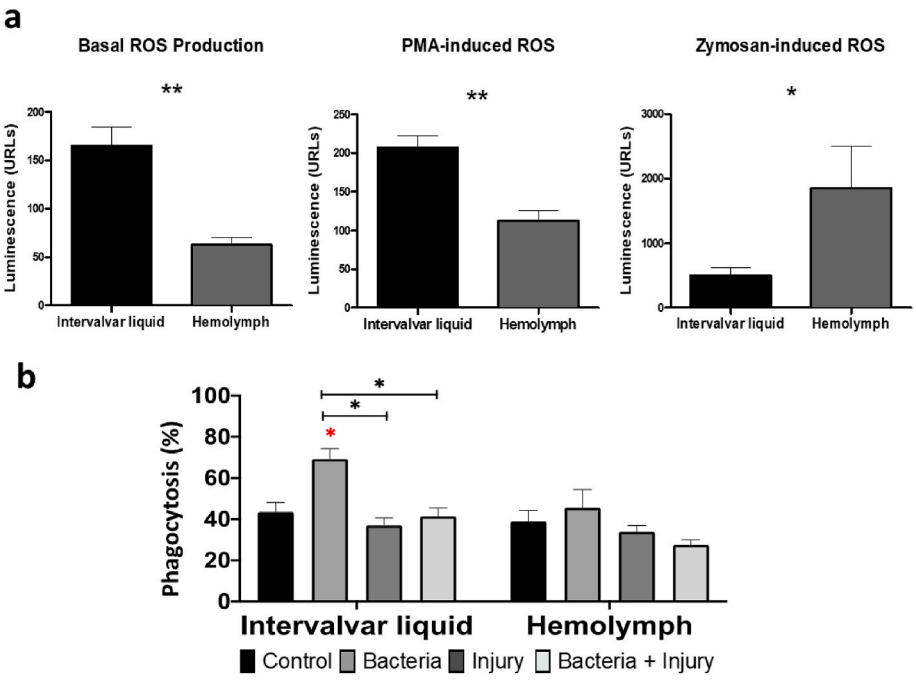

Fig. 6. Production of ROS and phagocytic activity ± SEM of haemocytes from the intervalvar liquid and haemolymph. **(a)** The production of ROS was evaluated at the basal level and after PMA and zymosan stimulation. Haemocytes from the intervalvar liquid and haemolymph were extracted from injured mussels (N = 8). **(b)** Phagocytic activity of haemocytes extracted from the intervalvar liquid and haemolymph 24 h after stimulation. Asterisks indicate significant differences at $p \leq 0.05$ (*) and $p \leq 0.01$ (**). Red asterisks represent significant differences compared to each control. Black asterisks represent significant differences between treatments.

occurs [29], but the number of cells is fully recovered two days after. Some variations may also occur in terms of phagocytosis, being the ripe stage the more active phase and the spawning stage a point of phagocytosis drop. This phagocytosis inhibition during the spawning phase seems to be widespread as it has been seen in *M. edulis, C. gigas* and *R. phillipinarum* [28–30]. Further research is needed to determine if this is also applicable to the cells found in intervalvar liquid.

Although intervalvar liquid and haemolymph haemocyte populations have the same morphology and cell structure, there were differences between them: intervalvar liquid haemocytes showed smaller size, higher migration ability with faster and longer movements and higher constitutive expression of some immune genes such as *IL17*. Additionally, the basal level of ROS, even after triggering with PMA, was higher. We could therefore consider that these cells are in a more highly

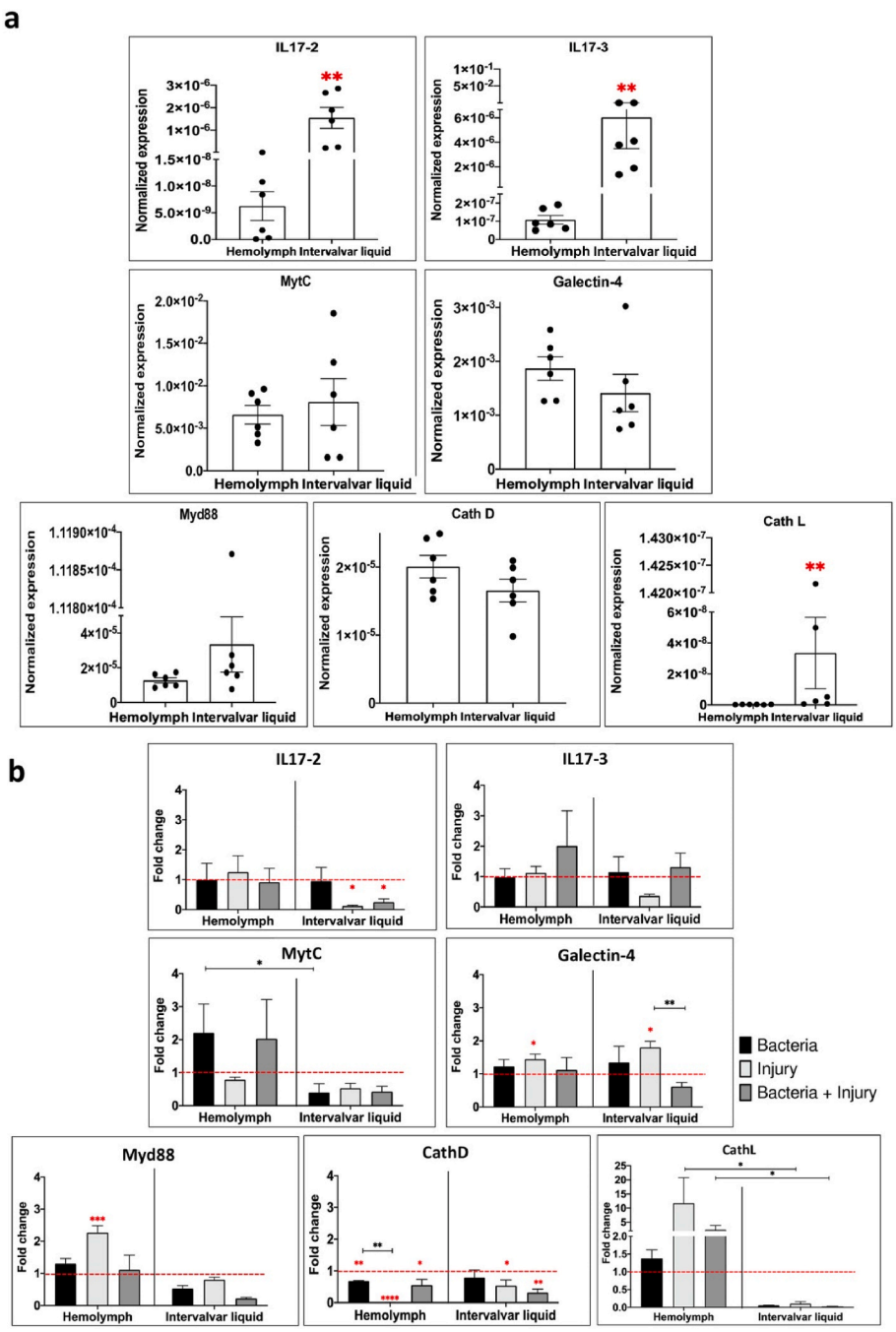

Fig. 7. Gene expression of haemocytes from the intervalvar liquid and haemolymph. **(a)** Normalized expression of selected genes in haemocytes extracted from the haemolymph and from the intervalvar liquid. The results represent the 6 individual data points (black dots) and the mean value ± SEM. **(b)** Fold change in gene expression calculated by comparing each treatment with the control value and represented as the mean value ± SEM. The red discontinuous line highlights the fold change value 1. Asterisks indicate significant differences at $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) and $p \leq 0.0001$ (****). Red asterisks represent significant differences compared to each control. Black asterisks represent significant differences between treatments.

activated stage than haemolymph haemocytes.

After stimulation, there was a different response between the two haemocyte populations. A bacterial infection was enough to increase the phagocytic activity in intervalvar liquid haemocytes, but this did not happen in the haemolymph haemocytes. Additionally, the mobility of haemocytes from intervalvar liquid was increased after injury; however, haemocytes from haemolymph need a double stimulus (bacteria and injury) to induce a significant increase in velocity and distance travelled. The stimulation induced by shell injury in haemocytes was previously reported [22], suggesting that it constitutes a stronger danger signal for mussels than the presence of bacteria or a pathogen-associated molecular pattern (PAMP) from bacteria, probably due to permanent contact by filtering feeding with microorganisms in the marine environment.

The higher constitutive expression of *IL17-2*, *IL17-3* and *Cath* L in intervalvar liquid haemocytes suggests that intervalvar haemocytes are in a higher activation stage. *Interleukin-17* (*IL-17*) constitutes one of the few known proinflammatory cytokines in mussels that presents a large expansion of isoforms in *M. galloprovincialis* [31]. Certain *IL-17* isoforms, including the two analysed in this work, were specifically involved in the response to a bath infection with *Vibrio splendidus* in mussel gills [32]. Gills along with intervalvar liquid cells are the first barriers interacting with microorganisms, and *IL17* may be determinant in haemocyte migration to the intervalvar cavity. Cathepsins are enzymes that participate in several processes, including the immune response of bivalves. Recently, the repertoire of cathepsins coded in the mussel genome was described [33], as well as a wide range of stimuli that modulate their expression, such as bacterial infections or PAMPs simulating pathogens [33–35]. Myticin C, a mussel antimicrobial peptide [15], was not differentially expressed in either haemocyte population, probably because it is usually stored in haemocyte granules ready to act

when needed [18,36].

Our results suggest the existence of cells in a putative more activated state that would cope with different physical and environmental challenges, playing crucial roles in the immune response. Intervalvar haemocytes could be sentinel cells moving around the mussel body ready to act as the first alarm against external threats. Previous studies have even hypothesized that "herd immunity" [26] could be built benefiting clusters of mussels bound together by the byssus [37]. Whether these cells outside the mussel body constitute a permanent or a transient population deserves further study. However, it can be hypothesized that clusters of mussels sharing sentinel haemocytes warning and defending the whole community to be able to respond faster and better against putative pathogens or injuries. This idea is consistent with the transmission of disseminated neoplasia, a leukaemia-like disease of multiple bivalve species, which is one of the few transmissible cancers reported [38]. García-Souto et al. [39] and Giersch et al. [40] suggested that the neoplasia of haemocytes was likely transmitted through marine water, inducing cancer in several susceptible bivalve species. The pool of extracorporeal haemocytes in the intervalvar liquid would be key for this transmission. Additionally, because of their location and the ability to internalize pathogens and particles, haemocytes from the intervalvar liquid could be used by pathogens to enter the bivalve body and produce infection, as previously suggested [24,26].

In conclusion, although most of the studies in mussels are conducted with haemolymph extracted from the posterior adductor muscle, which seems to proceed from small spaces and fissures between the muscle fibres connected to arteries [19], the population of haemocytes from the intervalvar liquid should be further studied, especially when the mussels are in stressful situations.

The main differences among the two cell populations could be summarized as follows: i) interalvar cells are significant smaller than haemolymph cells (about 200 μm^2 vs. about 600 μm^2); ii) interlvalvar cells are more active (they can cover distances about 40 μm/min more than the haemolymph haemocytes); iii) basal ROS production is a 37% higher in intervalvar liquid cells; and finally, iv) some immune marker genes such as IL17 and CathL are constitutively more expressed in intervalvar liquid cells.

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CRediT authorship contribution statement

A. Panebianco: Formal analysis, Investigation, Writing – original draft. **M. Rey-Campos:** Formal analysis, Investigation, Writing – original draft. **A. Romero:** Formal analysis, Investigation, Writing – original draft.

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

Data will be made available on request.

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