Establishment of a Long-Term Primary Culture of Oyster Hemocytes and Novel Insights About Their Function, Metabolism, and Behavior

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Abstract

This study aims to develop a comprehensive and standardized protocol for the primary cell culture of oyster hemocytes and their structural and functional analyses. Hemocytes are crucial for immune defense and overall bivalve health. Current primary culture systems for bivalve hemocytes are limited in duration, making long-term studies challenging. Here, we adopted the approach of utilizing hemolymph plasma as our complete and natural culture medium maintaining live, active, and immunocompetent oyster hemocytes throughout 21 days of culture period. Distinct types of hemocytes are described based on their morphology and function in primary culture. Granulocytes and hyalinocytes displaying various shapes and spreading patterns, in addition to blast-like cells, were cultured for up to 7 days. Between 14 and 21 days, blast-like cells and hyalinocytes decreased in number with signs of apoptosis, revealing a subpopulation of hemocytes with a higher resistance for *in vitro* maintenance. Live-cell imaging revealed that the remaining cells were kept alive and active throughout the 21 days of culture. Two primary cell motility behaviors were observed: pseudopod-dependent and lamellipodium-dependent membrane extensions, which are reminiscent of the mesenchymal and amoeboid migration modes, respectively. The proportion of about 40 % of the hemocytes displaying phagocytic activity within fresh hemolymph was maintained throughout the 21-day culture period. However, the phagocytic capacity was higher within phagocytes present after 14 and 21 days of culture. Metabolic assays revealed alterations in reactive oxygen species production and neutral lipid storage, suggesting physiological alterations in response to the culture environment. Our research established a robust protocol for long-term primary cell culture and assessment of bivalve hemocyte health, function, and metabolism. This protocol, potentially applicable to hemocytes of other marine bivalve species, paves the way to enhance our understanding of bivalve immunology and cellular physiology and ecophysiology. This valuable tool can be applicable for future research in marine biology, biotechnology, environmental science, ecotoxicology, and comparative physiology.

Keywords: Invertebrates; *Crassostrea gigas*; immune cells; live-cell imaging.

1. Introduction

Hemocytes are specialized cells found in the circulatory system of invertebrates, such as marine bivalves, and play crucial roles in physiology and health. Hemocytes are immune cells acting as the first line of defense against pathogens, parasites, and foreign particles. The most important functions of hemocytes are the detection and engulfment of invading microorganisms through phagocytosis and the production of proteolytic and antimicrobial molecules, effectively neutralizing these threats and preventing infections. Hemocytes are also involved in encapsulation, a mechanism where they surround larger foreign bodies or parasites that cannot be readily phagocytosed. By encapsulating these threats, hemocytes prevent their spread and limit the damage caused to the tissues. Hemocytes also participate in wound healing and tissue repair when a bivalve sustains an injury: they migrate to the site of damage and assist in sealing the wound, facilitating the regeneration of damaged tissues. They also contribute to other physiological processes than immunity, including nutrient transport, maintaining osmotic balance, shell mineralization, and the elimination of metabolic wastes from the hemolymph [1–3].

The study of hemocyte biology has emerged as a fundamental aspect of ecotoxicology and ecophysiology research in understanding the resilience and adaptability of marine bivalves in their natural environments and the context of environmental changes. Measuring the functions of hemocytes (*e.g.,* phagocytosis and viability) and their abundance (*e.g.*, number of cells per volume of hemolymph) have long been used as key indicators of the overall health and physiological condition of bivalves [4]. Indeed, changes in hemocyte numbers, morphology, or function may reflect the exposure to pollutants, toxins, or environmental stressors, making them valuable biomarkers for assessing environmental quality and the impact of human activities on marine ecosystems [5–7]. Thus, research focusing on hemocyte biology is crucial for predicting and mitigating the impacts of a diversity of stressors like ocean acidification, pollution, and climate change on bivalve populations. This can support the development of strategies aiming at the conservation and management of bivalve populations and their coastal environments under current and future environmental conditions.

The Pacific oyster *Crassostrea gigas* (Thunberg, 1793) is a marine invertebrate that holds a major economic and ecological significance at the global scale. It is one of the most cultivated shellfish around the world and an abundant mollusk species in an increasing number of coastal areas. *C. gigas* is widely used as a sentinel bivalve species for biomonitoring studies in coastal marine environments [8] and to understand the impacts of environmental stressors [9]. In such studies, the effects on oyster health are often evaluated by assessing hemocyte parameters *ex vivo* [10–16]. However, typically, this approach requires large facilities, expensive experimental conditions, co-maintenance of microalgae cultures for animal feeding, and intensive hemolymph sampling on the days of experimentation.

In vitro studies provide numerous advantages for biological research. They are potent and valuable tools in marine bivalve research, offering crucial insights into the physiology, health, and responses of these organisms to various environmental stressors [17–21]. *In vitro* cell studies can provide the investigation of precise mechanisms (gene expression, enzyme activities, biomarkers) linked to changes in cell function, morphology, and metabolism, shedding light on how individuals adapt their cellular responses to environmental challenges. Another key advantage lies in the possibility of controlling the microenvironment around cells and providing standardized conditions for such studies. For example, temperature, pH, salinity, and pollutant concentrations can be controlled to study isolated or combined factors or to simulate real-world conditions. This is particularly important for bivalves, who are constantly exposed to a variety of environmental stressors such as temperature, food, and oxygen fluctuations within the intertidal zone, as well as pathogens and pollutants [22–25]. Additionally, *in vitro* studies reduce ethical concerns associated with *in vivo* experiments, as they minimize the number of animals needed for large-scale experimentation. *In vivo* studies not only require a greater number of animals to be sacrificed but also, in the case of waterborne pollutant exposures, they generate significantly larger amounts of chemical waste. Nonetheless, maintaining viable and functional bivalve hemocytes under *in vitro* conditions for periods longer than 2-4 days has been a major challenge [26]. In this study, we developed a standardized protocol to maintain primary cell cultures of *C. gigas* hemocytes viable and in an active immunological state for at least 21 days. Additionally, we provide a panel of structural, functional, and metabolic parameters that can be used as indicators of hemocyte physiological state. Therefore, this study provides a promising *in vitro* cellular platform paving the way for in-depth investigation of hemocyte functions *in vitro* that supplement *in vivo* research on cellular biology, immunology, ecophysiology, and ecotoxicology of marine bivalves.

2. Methods

2.1 Animals

Hatchery-produced adult *C. gigas* (triploid, size n°2 – 86-110 g of whole-body weight) were obtained from an oyster farm (Beg Ar Vil) located at the Aber Benoit (France), an area considered to have a low anthropogenic impact [27]. The main reasons why choosing triploid oysters over the naturally occurring diploid ones are their easy access through the aquaculture industry, the possibility of obtaining hemolymph without contamination by gametes all year long, and the absence of major metabolic changes induced by reproductive maturation. Oysters were conditioned from 48 h up to 4 weeks before cell sampling. They were maintained either in natural seawater with natural food source at Ifremer's experimental site (Ste Anne, Plouzané, France) or under controlled conditions with seawater temperature at 16±1.5 °C, constant aeration, water flow, and daily feeding by a commercial phytoplankton diet (0.5 mL per animal; SA Live Marine Phytoplankton®, Premium Blend) in the laboratory (Plouzané, France).

2.2 Hemolymph collection

Hemolymph was collected by performing a small notch in the shell immediately before sampling. A cold 1 mL syringe coupled with a 23G x 1" needle was injected into the adductor muscle, and hemolymph was gently withdrawn, avoiding bubbles and seawater contamination. A drop of each hemolymph sample collected was used to inspect hemolymph quality under a light microscope using both 4× and 10× objectives. Only samples containing zero visualization of contaminants (*e.g.*, tissue and cellular debris and visible microorganisms: bacteria, protozoa, and microalgae) and no excessive hemocyte aggregation were used. Hemolymph samples that passed this rigorous quality check were pooled in 15 mL centrifuge tubes maintained on ice. Hemolymph collection usually lasted about 1 h but depending on the volume needed and easiness of collection, collection time varied between 1-3 h. Both the rigorous quality check of each hemolymph extraction and the limited time on ice (up to 3 h) were critical factors for the success of the primary cultures. The hemolymph sampling for the optimization experiments was conducted between February 2022 and September 2022. Samplings for the three independent 21-day experiments were conducted in November 2022 (autumn), April 2023 (spring) and May 2023 (spring). In the first experiment, two independent pools of hemolymph were analyzed (N=2), while in the second and third experiments, five independent pools of hemolymph were analyzed (N=5 for each). Each hemolymph pool consisted of samples from 2-6 oysters.

2.3 Primary hemocyte cultures

The first step in preparing the primary hemocyte cultures was to determine the concentration of hemocytes in hemolymph pools. For this, 50 µL of well-homogenized hemolymph pool was mixed with 50 µL of filtered sterile seawater (FSSW; 0.22 µm polyethersulfone -PES- syringe filter) containing 3.9 % formaldehyde to avoid cell clumping and adherence to the counting chamber, which are detrimental factors for accurate counting). Then 10 µL of the fixed hemolymph was added to the counting slides adapted to the EVE™ Automatic Cell Counter. Once the cell concentration was determined, whole hemolymph pool samples containing 500.000 or 80.000 cells were seeded into each well of 24 or 96-well plates (~250 000 cells/cm^2), respectively. Plates were pre-coated with poly-D-lysine either from the manufacturer or at the laboratory following the manufacturer's protocol (Gibco™, ThermoFisher Scientific, A3890401; for microplate references, see Supp. Tab. 1). The microplates containing the freshly seeded cells were then incubated for at least 1 h (up to 3 h) at 16±1 °C for adherence using a standard cooling incubator (Memmert IPP55; which uses ambient air, *i.e.*, no O_2 or CO_2 control). The supernatant was then gently completely removed and replaced with the hemocyte culture media. As a first step, different media and other culture conditions were tested (Supp. Tab. 2). The quality of hemocyte primary cultures within the different test conditions was screened by microscopic examination daily, observing cell morphology and adherence, and possible signs of contamination (see Results section for a detailed description of our definition of cultures presenting good quality conditions). The cell culture medium that provided optimum cell culture conditions was composed of filtered-sterile oyster plasma (FSOP) containing penicillin (+P) 100 U/ml and streptomycin (+S) 100 µg/ml (Gibco™, ThermoFisher Scientific, 15140148). The FSOP medium predominantly consisted of two pools of whole hemolymph samples from about 100 oysters (each collected at two different periods, September 2022 and March 2023), which were filtered (0.22 µm PES syringe filter), split into aliquots, and stored without antibiotics at -80 °C until use. A minor part of fresh FSOP was eventually used at the start

of the cultures, whereas FSOP from the -80 °C bank was always used for complete medium renewal every 7 days. A final volume of 500 µL or 100 µL of the cell culture medium FSOP+P/S was used for the 24 or 96-well plates, respectively.

2.4 Characterization of cell morphotypes and behavior

We combined both May-Grünwald Giemsa staining with live-cell imaging to characterize the cells present in our oyster primary hemocyte cultures over time. All assays described in this section were optimized to be conducted in cells cultured in 96-well microplates.

For the May-Grünwald Giemsa (MGG; RAL Diagnostics, 320070) staining, microplates were centrifuged (1000 ×g, 5 min, 16 °C), the supernatant was carefully removed, replaced with 100 µL of May-Grünwald and incubated for 3 min at room temperature. Then, 200 µL of distilled H_2O was added and samples were incubated for an extra 1 min. The supernatant was then removed, cells were rinsed once with 200 µL of distilled H_2O, and incubated with 100 µL of Giemsa R solution for 10 min. Finally, the supernatant was removed, and cells were rinsed with 200 µL of distilled H_2O. Plates were left to dry, and just before the microscope visualization, a drop of microscopy immersion oil was added to each well to improve cell visualization. Images were recorded using an inverted microscope (Zeiss AXIO Observer Z1) coupled with a color camera system (Canon EOS 700D).

Live unstained cells were imaged through phase-contrast microscopy (EVOS XL Core Imaging System, ThermoFisher Scientific) or Confocal Laser Scanning Microscopy (Zeiss LSM780, Carl Zeiss) using the transmitted-light channel equipped with a photomultiplier (TPMT). Phase-contrast and transmitted-light TPMT images were taken every 10s or 3s, using ×10 or ×40 air objectives, respectively.

The phagocytic behavior of hemocytes in primary cultures was performed by time-lapse imaging of cells incubated with Fluoresbrite® YG Microspheres beads (2 µm, Polysciences Inc.). The beads were added to the primary cultures at a final concentration of 0.005 %, and cultures were incubated for 4 hours at 16±1 °C before imaging. Image acquisition was performed every 10 s for 3 min at room temperature (20±1 °C) using the transmitted-light channel of the Zeiss AXIO Observer Z1 coupled with the Canon EOS 700D color camera system.

Time-lapse videos were created with the MakeAVI software using 5 frames per second (each second of the video corresponds to 50 s in real-time), except for the videos using confocal transmitted-light TPMT images, which were created with the ZEN 2012 software (Carl Zeiss) using 10 frames per second (each second of the video corresponds to 30 s in real-time).

2.6 Cellular physiological parameters

Cellular parameters involving size, complexity, viability, immune function, and metabolism were analyzed on hemocytes maintained in primary cultures over time. Assays were performed through flow cytometry (cell viability, ROS production, phagocytosis, and mitochondrial and neutral lipid contents) or by multimode plate readers (ATP content and NADH production) as described below. For these assays, the start of each primary cell culture was staggered, meaning that cells were seeded 21, 14, and 7 days before the day of the analysis and compared to fresh hemolymph samples (Day 0). The assays were thus performed on the same day so that all cell culture time points could be more readily comparable. Therefore, it is important to highlight that each time-point of the cell-based assays corresponded to cultures obtained from different hemolymph pools (thus different oysters).

Flow cytometry analyses were performed from primary hemocyte cultures maintained in 24-well plates (Supp. Tab. 1). Cells were recovered from the wells by gentle repetitive pipetting of the cell culture medium within each well and transferred to centrifuge microtubes on ice. The fresh hemolymph (Day 0, used as control) was collected and maintained in centrifuge microtubes on ice without deposition into the microplate to avoid cell loss (as within the first hours of cell culture, most hemocyte types tend to be more adherent to the bottom of the well and cannot be easily detached). Flow cytometry assays were performed by transferring 50 µL of cells into flow cytometer tubes containing 150 µL of FSSW containing different fluorophores. Cell viability was measured by incubation with 10 µg/mL of propidium iodide (Sigma, France) and 1X SYBR Green I (10,000X stock; Sigma, France) for 30 min, at 18 °C, in the dark [28]. Cell size and complexity were quantified using the same samples stained for cell viability through

measurements obtained by the side scatter (SSC), representing the granularity or internal complexity of particles, and the forward scatter (FSC), estimating the size of particles. SYBR Green I-stained hemocytes could be distinguished from other hemolymph particles by gating and differentiating them using a density plot visualization of SSC vs. FL1, displayed on a log scale [28]. For the quantification of ROS production, incubation was performed with 10 µM dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma, France) for 60 min, at 18 °C, in the dark [29]. Hemocyte phagocytosis was measured using the final concentration of Fluoresbrite® YG Microspheres beads at 0.005 % (2 µm, Polysciences Inc., France) and incubation was performed for 120 min, at 18 °C, in the dark. The percentages of active phagocytes were defined as the percentage of cells that ingested three or more beads [30]. Mitochondrial content, mitochondrial ROS production, and neutral lipid content were measured according to the manufacturer's protocols. Mitochondrial content was assessed by incubating cells with a 1/1,000 dilution of MITO-ID® Green (Enzo Life Sciences) for 1 h at 18 °C, in the dark. Mitochondrial ROS production was quantified using 5 µM MitoSOX Red (Thermo Fisher Scientific) and a 30 min incubation period at 18 °C, in the dark. Neutral lipids content was measured by incubating cells with 10 µM of BODIPY™ 493/503 (Thermo Fisher Scientific) for 15 min at 18 °C, in the dark. Cell viability, ROS production, and phagocytosis analyses were performed on a BD FACSVerse™ flow cytometer (BD Biosciences, San Jose, CA, USA). Mitochondrial content and ROS production and neutral lipid content analyses were performed on a Guava® easyCyte HT System (Luminex, Merck Millipore).

ATP production was measured in primary hemocyte cultures maintained in 96-well plates (Supp. Tab. 1) using the kit CellTiter-Glo™ 3D (Promega, G9681), following the manufacturer's protocol with some minor modifications. On the day of the assay, plates were centrifuged (1,000 ×g, 5 min, 16 °C), and the supernatant was carefully removed and replaced with 50 µL of FSSW plus 50 µL of the ATP kit reagent. Plates were submitted to linear shaking for 2 min, then samples were transferred to opaque white 96-well plates (Supp. Tab. 1), incubated for 10 min at room temperature, in the dark, and luminescence was recorded using the EnSpire® Multimode Plate Reader (PerkinElmer©). ATP absolute values were calculated based on ATP standard curves (0, 1, 10, 100, 625, 1250, 2500 nM) performed on the same day and plate with the samples.

2.7 Statistical Analyses

Statistical analyses of the cellular parameter results were performed using the one-way analysis of variance followed by Tuckey's post hoc test. Results were considered significant at $p<0.05$. All experiments were repeated three times, except for the neutral lipid content and mitochondrial ROS production, which were repeated twice.

3. Results

3. 1 Filtered sterile oyster plasma allows 21 days of robust maintenance of oyster hemocytes *in vitro*

Several cell culture conditions were tested to achieve an optimal condition for the *in vitro* maintenance of oyster hemocytes. The conditions included different cell culture media (Supp. Tab. 2), temperatures (20 and 16°C), and plate surfaces (with or without poly-D-lysine coating). The quality of the cultures was first monitored through visual microscopy inspection. Quality criteria included the presence or absence of bacterial and protozoa contamination, and overall cell morphology, diversity, and adherence. Culture contamination events were rare (about 1 in every 10 cultures) due to our strict quality control check during hemolymph collection (see Section 2.3 for details) and were mainly due to protozoa when occurred. The culture was considered healthy and not under significant stress when the overall morphology of cells resembled Day 0 primary cultures. Day 0 cultures were characterized by the presence of a mixed population (which will be described in further detail in Section 3.2), with a similar proportion of strongly adhered morphotypes and partially adhered and highly refringent cell morphotypes (Fig. 1). At first, all cell culture media tested contained Leibovitz's L-15 medium in different concentrations (Supp. Tab. 2). These media were tested with or without the

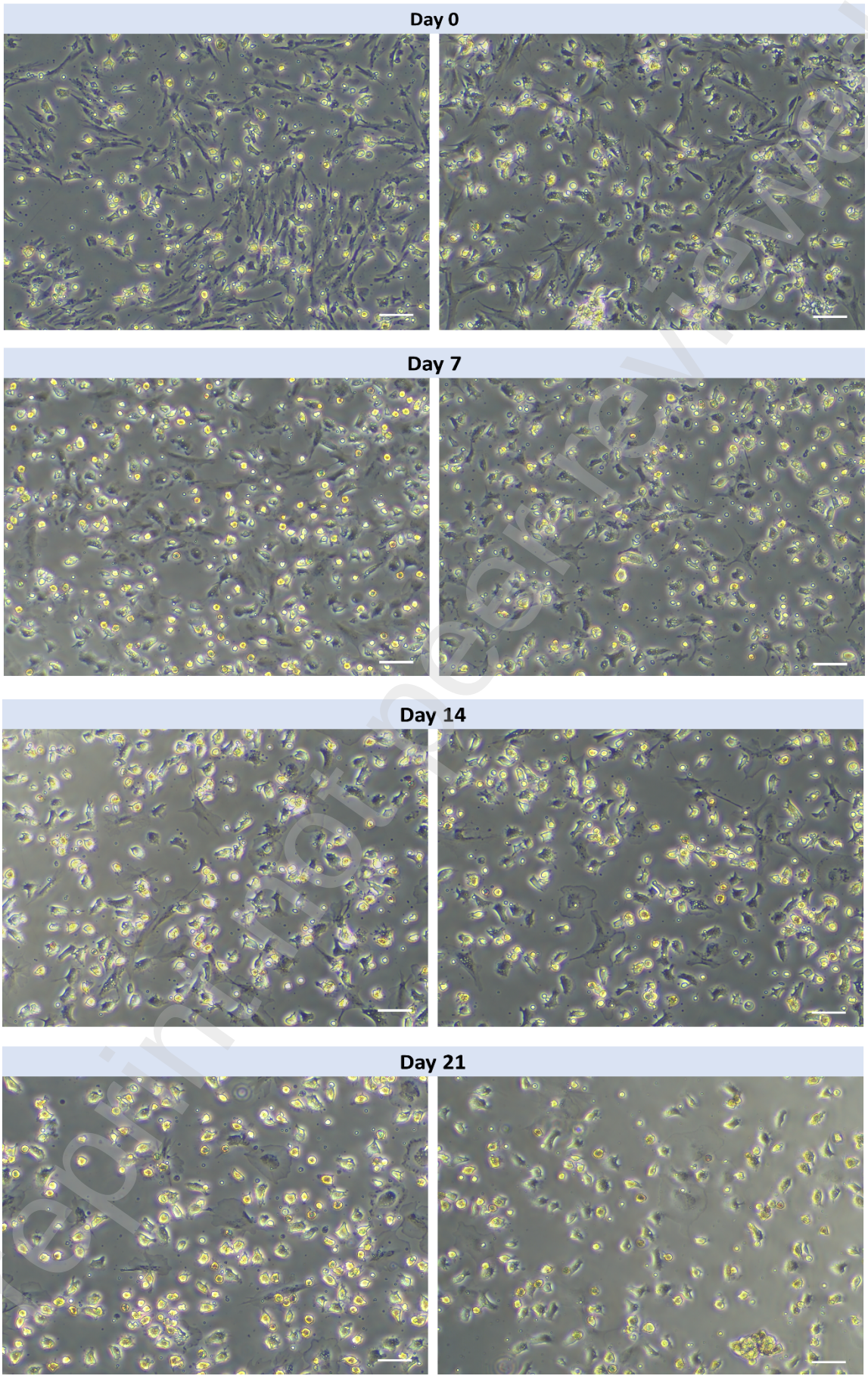

Figure 1. Primary cultures of oyster *Crassostrea gigas* hemocytes throughout 21 days. Cells (500,000/well) were cultivated with filtered-sterile oyster's blood plasma containing antibiotics (FSOP+PS) in 24-well plates coated with poly-D-lysine. The two images from each day correspond to independent cultures. Bar = 50 µm.

addition of fetal bovine serum and with or without the addition of oyster *C. gigas* cell-free hemolymph (filtered sterile oyster plasma or FSOP). All media formulations which were based predominantly on the synthetic Leibovitz's L-15 medium were not successful in maintaining healthy oyster hemocyte primary cultures for periods longer than 2 days, as indicated by a predominance of detached round cells, with several cells presenting loss of refringence, and cell aggregates (Supp. Fig. 1). Whereas the addition of 50 % of FSOP within the different media formulations always improved the quality of the cell culture condition by maintaining cell diversity and adhesion (Supp. Fig. 1). This led us to test 100 % FSOP with antibiotics (FSOP+PS) as a culture medium (see Section 2.3 for details). Indeed, cell culture quality improved significantly at this condition, which was further improved by culturing cells using poly-D-lysine as a surface coating.

Thus, by cultivating oyster hemocytes within a natural culture medium such as the FSOP combined with low temperatures (~16°C), surface coating (poly-D-lysine), and antibiotics to prevent bacterial contamination, we were able to maintain primary cultures for at least 21 days (Fig. 1). Some culture replicates were able to survive over this period (some lasted over 2 months), but the possibility of obtaining this longer durability was somewhat variable.

3.2 Overall hemocyte diversity and morphology are maintained during the first week of culture

The FSOP+PS medium allowed the maintenance of the same diversity of cell morphotypes as in Day 0 for about 7 days of culture (Figs. 1 and 2). During this first week, six major hemocyte morphotypes were identified derived from the three commonly described hemocyte subpopulations: granulocytes, hyalinocytes, and blast-like cells (Fig. 2). Three different cell morphotypes were identified among granulocytes: two presenting high and one presenting low spreading. Granulocytes with low spreading (Fig. 2A) usually displayed a round or dendritic-like shape (Fig. 2A). Granulocytes with high spreading presented either a semi-circular shape (half-moon) (Fig. 2B) or a macrophage-like shape (similar to a fried-egg shape) (Fig. 2C). Among hyalinocytes, we also identified three different cell morphotypes: again, two with high and one with low spreading. Similarly to the granulocytes, hyalinocytes with low spreading also presented a round or dendritic-like shape (Fig. 2D). Hyalinocytes with high cellular spreading were represented by small cells with irregular or half-moon shape (Fig. 2E) or very large cells with elongated (fibroblast-like) or irregular shape (Fig. 2F). Finally, blast-like cell morphotypes exhibiting the characteristic high nucleus/cytoplasm ratio and small size were also present (Fig. 2G).

3.3 Notable morphological and cell diversity changes are observed in cells maintained after two weeks of culture

Between 14 days and 21 days of culture, all three commonly described hemocyte subpopulations were represented; however, the blast-like cells and large highly spread hyalinocytes (fibroblast-like cells) were usually less present (Fig. 1). When present, the large highly-spread hyalinocytes seemed larger and mostly of irregular shape (Fig. 2K and 2P). Moreover, from 14 to 21 days, the larger granulocytes presented mostly round or oval shape (Figs. 2H and 2M). Also, larger hyalinocytes with round or oval shapes were identified on days 14 and 21 (Figs. 2J and 2O), which were not seen in cultures within the first week.

3.4 Granulocytes are the most prevalent subpopulation at later stages of culture

Overall, it was noticeable by eye a decrease in cell density throughout the culture and a predominance of granulocytes at day 21 (Fig. 1). Such cell loss was correlated with signs of apoptosis, which could be identified by the MGG staining (Fig. 2; bottom panel). Cells presenting the typical signs of apoptosis such as nuclear condensation, loss of cell volume, and nuclear fragmentation were mostly observed on day 7 (Fig. 2Q). Such signs were also seen on days 14 and 21 (Figs. 2T and 2V), however with a lower frequency. Other nuclear abnormalities were also mostly observed at day 7, such as micronuclei and blebbing or amorphous nuclei, which were mainly seen among the large and highly spread hyalinocytes (Fig. 2R). In all days of culture (except on day 0), highly pigmented granules were observed within cells presenting normal nucleus morphology, suggesting that these cells had performed the phagocytosis of apoptotic corpses (Figs. 2S, 2U, 2X, and 2Y). Interestingly, cells presenting highly pigmented granules were predominantly hyalinocytes and more frequently seen in cultures from day 7 (Figs. 2S,

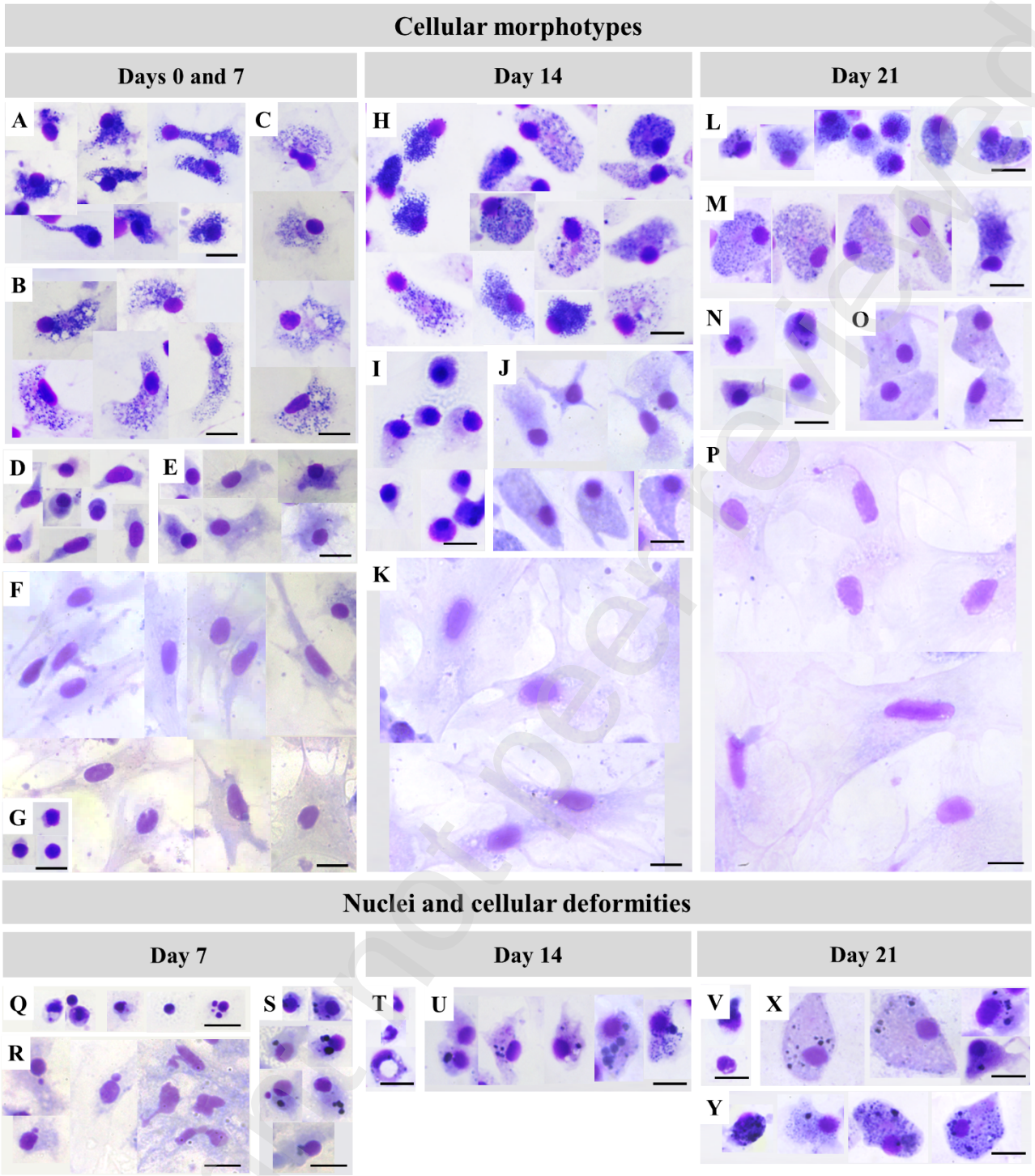

Figure 2. Morphotypes (top panel) and deformities (bottom panel) of *Crassostrea gigas* hemocytes in primary culture over time. Cells were cultivated with filtered-sterile oyster's blood plasma with antibiotics (FSOP+PS) in 96-well plates (µClear bottom) coated with poly-D-lysine and colored with May-Grünwald Giemsa. **Morphotypes at days 0 and 7**: (A) small granulocytes with round or dendritic-like shape; (B) large granulocytes with a semi-circular or half-moon shape; (C) large granulocytes with a macrophage-like or fried-egg shape; (D) small hyalinocytes with round shape; (E) small hyalinocytes with irregular or half-moon shape; (F) large hyalinocytes with fibroblast-like or irregular shape; (G) blast-like cells with high nucleus/cytoplasm ratio and small size. **Morphotypes at day 14**: (H) granulocytes with mostly round or oval shape; (I) small hyalinocytes with mostly round shape; (J) hyalinocytes with oval or dendritic-like shape; (K) large hyalinocytes with fibroblast-like or irregular shape. **Morphotypes at day 21**: granulocytes with mostly round or oval shape presenting two different size categories overall: small (L) and large (M); (N) small hyalinocytes with mostly round shape; (O) larger hyalinocytes with oval shape; (P) large hyalinocytes with mostly irregular shape. **Nuclei and cellular deformities at days 7, 14, and 21**: (Q, T, and V) cells presenting the typical signs of apoptosis such as nuclear condensation, loss of cell volume, and/or nuclear fragmentation; (R) nuclear abnormalities on day 7 cultures such as micronuclei and blebbing or amorphous nuclei; (S, U, X, and Y) highly pigmented granules within cells presenting normal nucleus morphology, suggesting phagocytosis of apoptotic corpses. Bar = 10 µm for all images.

2U, and 2X). Nonetheless, these highly pigmented granules were also detected within granulocytes at day 21 (Fig. 5Y). The enrichment of granulocytes was further corroborated by the flow cytometry analyses which are described below in section 3.6.

3.5 Resistant hemocyte subpopulations are still active and immunocompetent

Despite the signs described above suggesting cell death, live-cell imaging revealed that most cells from all days of culture were alive as demonstrated by their active motility (see supplementary video files 1-12). We distinguish two main cell movement strategies employed by the hemocytes in culture: movement through pseudopod formation, also known as "mesenchymal migration mode" (Fig. 3A; supplementary video file 13), and movement through lamellipodium formation, also known as "amoeboid migration mode" (Fig. 3B; supplementary video file 14). By comparing the morphotypes seen through the MGG staining images and the ones observed through the time-lapse videos, we suggest that cells displaying a half-moon shape can represent a movement state of the round or dendritic-like morphotypes (Fig. 3B) and of the fried-egg shape morphotype (Fig. 3C). The large highly spread hyalinocytes (fibroblast-like; Fig. 2F) and granulocytes (fried-egg shape) displayed cell membrane expansion and retraction (Fig. 3C), however, the cells did not display mobility.

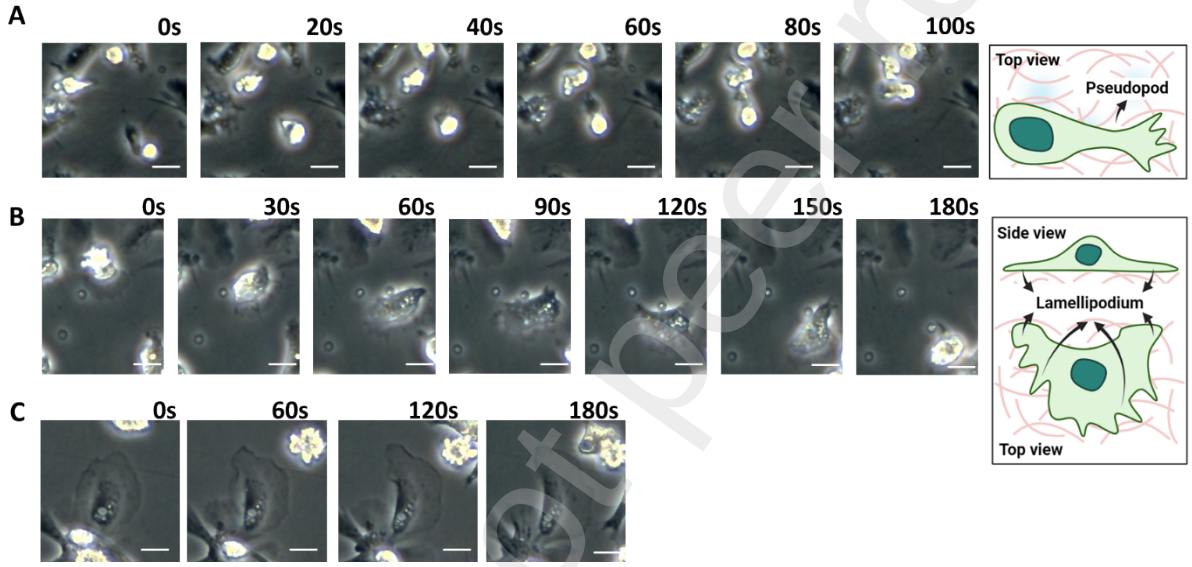

Figure 3. Cell movement behavior of *Crassostrea gigas* hemocytes in primary cultures. Cells were cultivated with filtered-sterile oyster's blood plasma with antibiotics (FSOP+PS) in 96-well plates (µClear bottom) coated with poly-D-lysine. Live-cell imaging revealed two main cell movement strategies employed by the hemocytes in culture: movement through pseudopod or lobopodian formation (A), and movement through lamellipodium formation (B). (C) Cell transitioning from a fried egg shape to a half-moon shape through cell membrane expansion and retraction, but that do not display mobility.

To assess if the cell mobility observed in the primary cultures over time could also be associated with immune function, we performed live-cell imaging of the hemocytes in culture in the presence of polystyrene microbeads to observe their phagocytic activity. Cells capable of engulfing the microbeads were observed in all days of culture (Fig. 4). Observation of the time-lapse images revealed distinct phagocytic behaviors for the different cell morphotypes. Overall, three main observations were established: (i) highly spread fibroblast-like cells do not display phagocytic behavior (Fig. 4B, a); (ii) not all highly mobile cells present phagocytosis behavior as some highly mobile cells with (Fig. 4B, c) or without (Fig. 4B, d) granules did not display phagocytic behavior (supplementary video file 15 and 16, respectively); and, finally, (iii) phagocytosis behavior can also be performed by cells presenting none or low motility in culture, such as the highly spread macrophage-like (fried-egg like) cells (Fig. 4B, b; supplementary video 17). We also observed that day 21 cultures seemed to present cells capable of engulfing more microbeads (Fig. 4B-e*) than most phagocytes observed in the other days of culture (Fig. 4B-e).

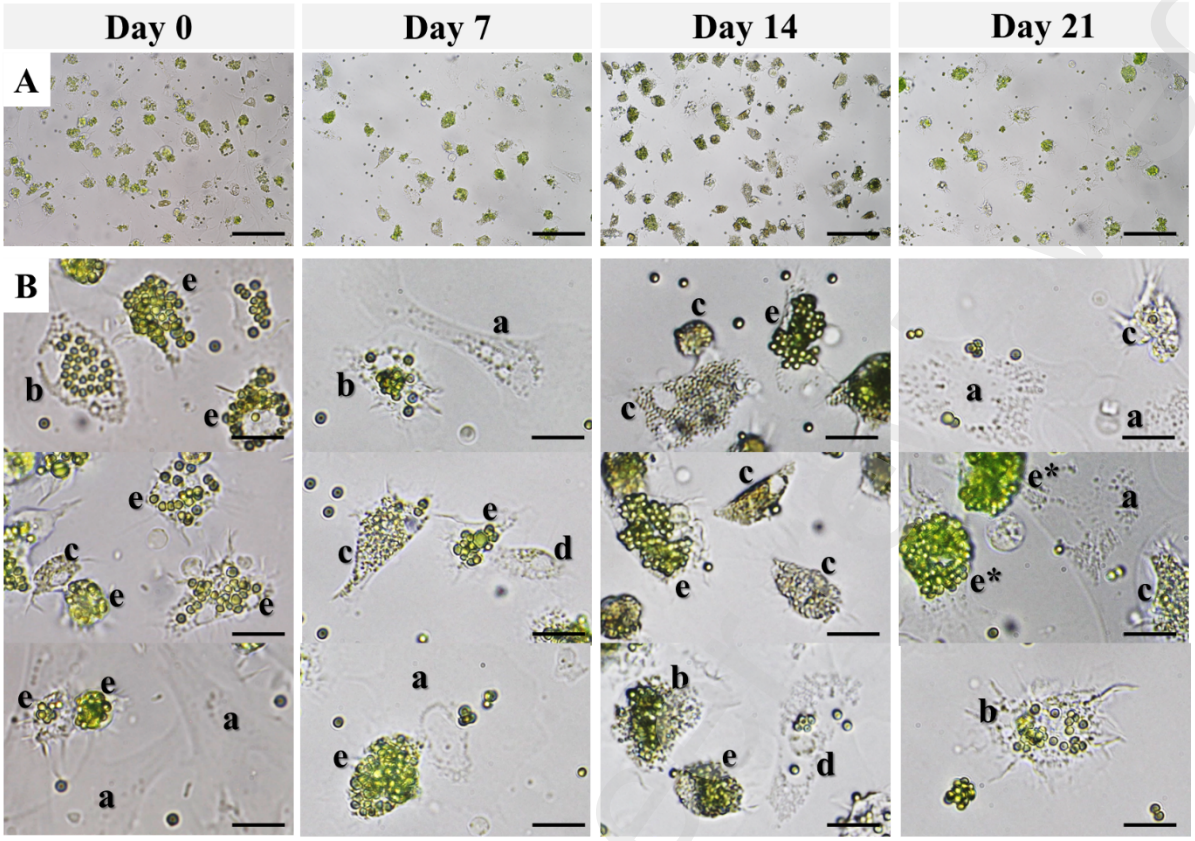

Figure 4. Phagocytic behavior of *Crassostrea gigas* hemocytes in primary culture over time. Cells were cultivated with filtered-sterile oyster's blood plasma with antibiotics (FSOP+PS) in 96-well plates (µClear bottom) coated with poly-D-lysine and incubated with microsphere beads (2 µm) for 4 hours at 16±1 °C before imaging. Cells capable of engulfing the microbeads were observed in all days of culture (A). The detailed (B) observation of images combined with the time-lapse videos revealed distinct phagocytic behaviors of the different cell morphotypes: highly spread fibroblast-like cells without phagocytic behavior (a); (ii) highly spread macrophage-like (fried-egg) cells which did not display mobility with phagocytosis behavior (b); highly mobile cells with (c) or without (d) granules without phagocytosis behavior; highly mobile cells capable of concentrating a great number of beads (e), and possibly an even greater amount within the day 21 cultures (e*). Bar = 10 µm.

In summary, the different morphological and behavioral analyses performed through microscopy imaging revealed the maintenance of live, active, and immunocompetent cells throughout the 21 days of culture. The cultures evolved to display fewer hemoblasts and fibroblast-like cells, which possibly died through apoptosis (Fig. 2). Older cultures seemed to display larger cells overall and present phagocytes with a higher phagocytic capacity.

3.6 Maintenance of live and immunocompetent hemocytes with higher complexity

We proceeded to flow cytometry analyses to further confirm the live-cell imaging findings and, more precisely, quantify changes in cell viability, size, and phagocytic capacity of the hemocytes in primary cultures over time.

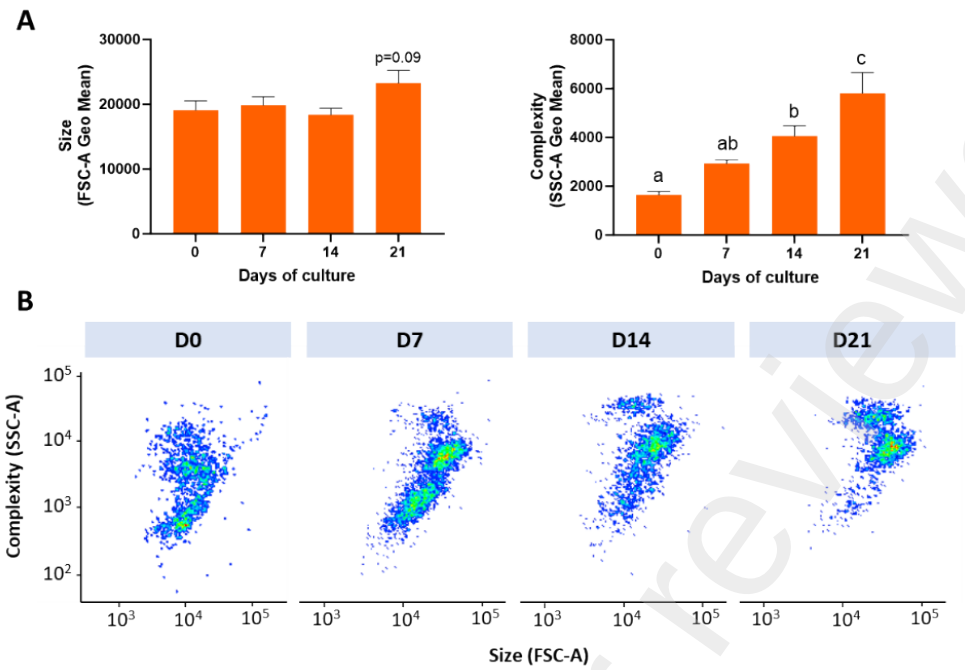

Figure 5. Flow cytometry analyses of the size and complexity of oyster *Crassostrea gigas* hemocytes in primary culture over time. Cells (500,000/well) were cultivated with filtered-sterile oyster's blood plasma containing antibiotics (FSOP+PS) in 24-well plates coated with poly-D-lysine. (A) Geometric means of cell size and complexity; and (B) representative forward scatter (FSC-A; cell size) vs side scatter (SSC-A; cell complexity) dot plots, showing the cell distribution within the primary cultures throughout time as compared to control (Day 0, cells freshly collected). Bars represent means ± standard deviation. Different letters mean significant differences between groups. Results were considered significantly different when $p<0.05$.

Our results reveal that although the mean size of all cells present in the cultures from each day did not significantly vary, there was a marginally significant increase in size at day 21 and a time-dependent increase in the mean cellular complexity (Fig. 5A). Such differences were evident through the visualization of forward scatter (cell size) vs side scatter (cell complexity) dot plots, showing the cell distribution within culture from each culture period (Fig. 5B and Supp. Figs. 2-5). Classic gating of oyster hemocyte subpopulations, as generally performed in studies using fresh hemolymph, does not seem the most appropriate to be applied in this study because of the obvious evolution of cell morphology over time. This makes it difficult to determine which events correspond to hyalinocytes or granulocytes, for example. Nonetheless, we applied such gating analyses, which revealed a tendency to a decrease in the proportion of blast-like cells on days 14 and 21 (Supp. Fig. 2). However, no significant changes were detected in the proportion of "hyalinocytes" and "granulocytes" (Supp. Fig. 2).

It is important to note that highly adherent hyalinocytes could not be recovered from wells for the flow cytometry analyses, as we did not apply any trypsin treatment. This was a choice to avoid cell damage and dysfunction, as we used such samples for further immunocompetence and metabolic analyses. Thus, even though this approach might have introduced a bias toward the real proportion of granulocytes in the culture over time, it is evident through whole primary culture images (Fig. 1) and videos (supplementary video files 1-12) that such highly adherent hyalinocytes were much less present in cultures after 14 days. This is also evident through images taken after cells were harvested for the flow cytometry analyses (Supp. Fig. 6).

Further flow cytometry analyses were performed to gain quantitative information on hemocyte viability and immune function. This was done in primary cell cultures from day 0 to 21 days, using propidium iodide staining to measure cellular membrane integrity and fluorescent microbeads to measure phagocytosis. Despite the decrease in cell number over time, as discussed above, the remaining cells presented a constant percentage of viable cells from day 0 (fresh, non-plated hemolymph samples) until day 21 (Fig. 6A). Most importantly, these cells kept their immunocompetence, as demonstrated by the phagocytosis assay (Fig. 6B and C). Interestingly, while the percentage of phagocytic cells did not change over time (Fig. 6B), the number of microbeads each cell was able to engulf

(phagocytic capacity) was higher after 14 and 21 days of culture (Fig. 6C), corroborating the results from the microscopy images (Fig. 4).

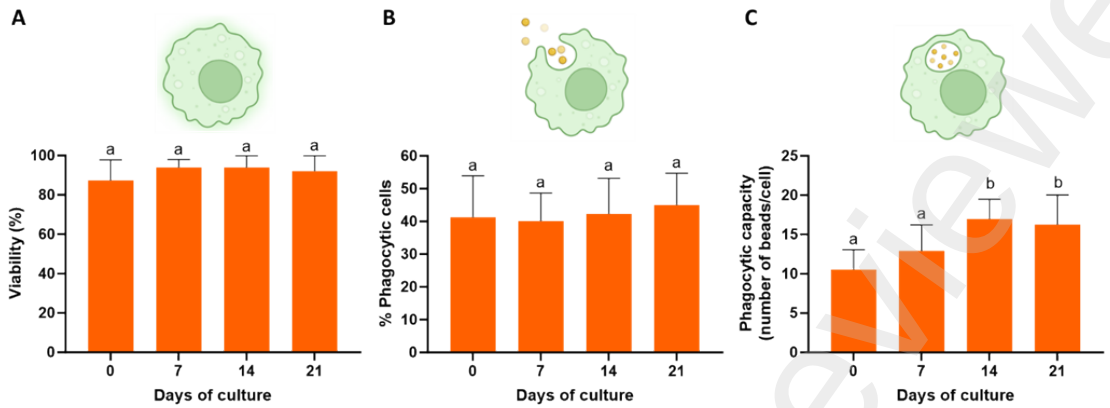

Figure 6. Viability and immune function of *Crassostrea* gigas hemocytes in primary cultures over time. Cells were cultivated with filtered-sterile oyster's blood plasma with antibiotics (FSOP+PS) in 24-well plates. Day 0 corresponds to cells freshly collected from oysters and not placed in culture. (A) cell viability, (B) the percentage of cells capable of engulfing polystyrene beads, and (C) the average number of polystyrene beads engulfed per cell. Bars represent means ± standard deviation. Different letters mean significant differences between groups. Results were considered significantly different when $p<0.05$.

3.7 Hemocytes present in the long-term primary cultures display some metabolic changes to the *in vitro* environment

Additional assays were conducted over time to determine the overall physiological state of the hemocytes in primary cultures. Quantification of basal ROS production, measured through DCFH-DA staining, revealed that cells in culture under all timepoints presented lower intracellular ROS levels than fresh hemolymph samples (Fig. 7A). On the other hand, analysis of mitochondrial superoxide production using the MitoSOX Red staining did not indicate changes on mitochondrial ROS production overtime (Fig. 7B). No changes were also detected in mitochondrial content, measured by the MitoID Green staining (Fig 7C). It is important to highlight that these three parameters were analyzed by flow cytometry and were calculated as average signal per cell. Additional analyses of mitochondrial parameters by luminescence and fluorescence spectroscopy indicated lower ATP levels after 14 and 21 days (Fig. 7D) as well as lower NADH production rates after 7 and 21 days (Fig. 7E). Contrary to the assays with flow cytometry, these parameters were measured as the average luminescence or fluorescence signal per well. Finally, flow cytometry analysis of the neutral lipid reserve content within each cell revealed a time-dependent higher accumulation, reaching statistically significant values within cells from the day 21 cultures (Fig. 7F).

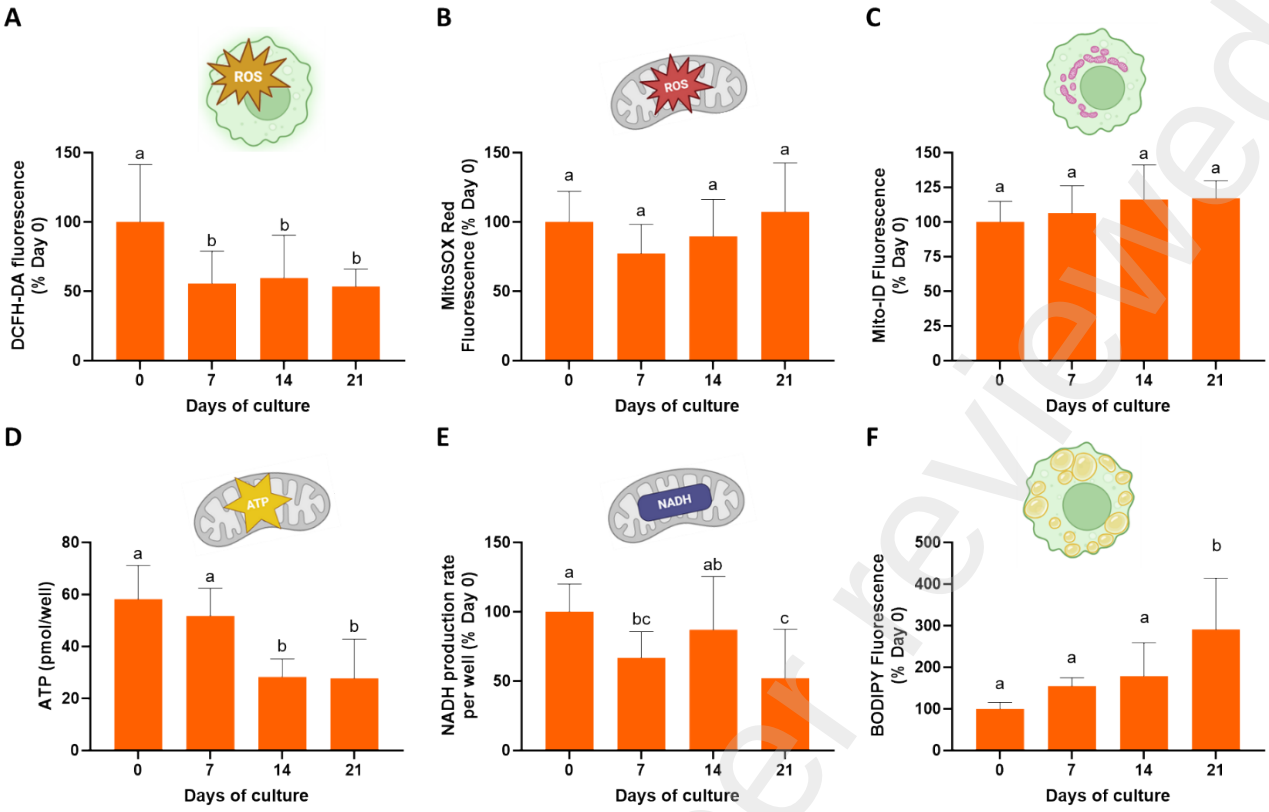

Figure 7. Metabolism-related parameters of *Crassostrea gigas* hemocytes in primary culture over time. Cells were cultivated with filtered-sterile oyster's blood plasma with antibiotics (FSOP+PS) in 24-well or 96-well plates and harvested at the specified days of culture. Day 0 corresponds to cells freshly collected from oysters and not placed in culture. Flow cytometry analyses were made to quantify (A) basal ROS production, (B) mitochondrial ROS production (C) mitochondrial content, and (F) lipid reserves per cell. Plate reader assays were conducted to quantify (D) ATP and (E) NADH production per well. Bars represent means ± standard deviation. Different letters mean significant differences between groups. Results were considered significantly different when $p<0.05$.

4. Discussion

Despite attempts dating back to the 1960s, at present, primary cultures are the only approach for culturing mollusk cells as not a single marine mollusk cell line is available [31]. Researchers have been developing primary culture systems for bivalves since the mid-1900s, which led to the initial establishment of oyster heart and mantle tissue cell cultures [32]. Even though such cell types can be cultured for several weeks, hemocytes are still bivalves most used primary cells, since they are key indicators of organism physiological and immune status [33,34]. Bivalve hemocytes have been the subject of great interest in biology and environmental sciences due to their crucial role in immune defense and maintenance of bivalve health. The diverse functions of bivalve hemocytes encompass a wide range of biological processes, including pathogen recognition, phagocytosis, immune signaling, encapsulation, tissue repair, detoxification, nutrient transport, wound healing, ion regulation, and waste clearance [2,35,36]. Indeed, the study of bivalve hemocyte biology is an area of active research that holds great promise for advancing our understanding of animal immune function, metabolic regulation, stress responses, and ecotoxicology. Given the significance of marine bivalve aquaculture to the global economy and prevailing disease threats, the need for a marine bivalve immune cell culture tool is critical. However, until now, researchers have relied mostly on short-term cultures, limiting their applicability in research.

Moreover, the literature related a huge diversity in the culture conditions of hemocytes. A recent study reviewed the conditions for primary cell culture of hemocytes from the mussels *Mytilus edulis* and *Mytilus galloprovincialis* [26]. The protocols mentioned by the review were developed to be applied in 24 or 96-well plates at cellular densities similar to the ones proposed in this study (2 – 10 x 10^5 cells/ml). The main difference was the use of anti-aggregation solution

(containing EDTA), commercial culture media (Eagle's Basal Medium or Leibovitz L-15) adjusted to an osmolarity of 990 – 1050 mOSM/kg, and supplementation with fetal bovine serum or glucose. Studies with *C. gigas* hemocytes also commonly use L-15 medium [33,37] or 0.22 µm filtered seawater [19,38]. In such circumstances, hemocytes can typically survive for 2-4 days when cultured [26]. It is important to note that Eagle's Basal Medium and L-15 medium do not contain certain amino acids like proline and taurine found in high concentrations in oyster hemolymph [39]. In addition, traditional cell culture media are developed to saturate cell culture with the necessary nutrients to maintain stable and maximal proliferation of cancer cells [40], which is not the case for hemocyte primary cultures. Such balance between antibiotic supplementation and culture medium components is critical to ensure hemocyte health and immunological function during primary culture conditions. Our hemocyte culture approach differs from other studies as we use hemolymph plasma as a natural cellular culture medium. This approach mimics the hemocyte's natural microenvironment, thus providing the cells with a context that is closer to their *in vivo* conditions. This method allows the maintenance of viable and functional *C. gigas* hemocytes for 21 days and even beyond in some cases, while many studies with bivalves detect a loss of viability before 2-7 days of culture [26,34]. Indeed, the use of a plasma-like cell culture medium has also recently been adopted for mammalian cell culture, based on increasing evidence that artificial cell media can largely interfere with cellular processes and metabolism [40]. Hemolymph plasma also contains immune system components such as neurotransmitters, opsonins, hormones, and LPS-binding proteins [41,42], making it an ideal cellular medium for studies on immune responses, inflammation, and host-microbe interactions. Nonetheless, one major disadvantage of this approach is the lack of a standardized cell culture medium. Indeed, oyster plasma composition can vary among individuals and environmental factors [43,44], thus possibly introducing differences in cellular responses to abiotic and biotic factors. For example, different plasma compositions could impact the characterization of the cytotoxicity risk of pollutants. To minimize this drawback, we adopted the strategy of creating a big *C. gigas* plasma bank to be used throughout a same set of experiments. Our success in culturing *C. gigas* hemocytes using plasma can now foster future studies focusing on a thorough characterization of oyster plasma organic and inorganic composition to support the creation of a plasma-like standardized culture medium for oyster cells.

Live-cell imaging has become an invaluable technique in cell biology, allowing researchers to observe dynamic processes in living cells in real-time. In the study of bivalve hemocytes, live-cell imaging offers crucial insights into the cellular and molecular mechanisms underlying immune responses, environmental stress adaptations, and disease resistance. In this study, we adopted live-cell imaging techniques to monitor hemocyte primary cultures over time. This non-invasive, label-free approach allowed us to easily screen between the different cell culture conditions during the primary culture optimization phase to determine if hemocytes were alive based on their motility behavior. Moreover, by combining such a technique with MGG staining, we were able to determine that some morphotypes corresponded to different movement states of the cells. From such observations, we suggest that fresh hemolymph samples are composed of at least 5 distinct hemocyte types: blast-like cells, two small highly mobile cell types with and without granules (dendritic-like cells), and two less mobile cell types without granules (fibroblast-like cells) and with granules (macrophage-like cells). Such findings differ from what most publications describe regarding the diversity of *C. gigas* hemocyte subpopulations, which report mainly three hemocyte types: blast-like cells, hyalinocytes, and granulocytes [2]. We believe this distinction is because most studies usually analyze fixed hemocyte samples, while in our study hemocytes are alive and, therefore free to acquire different shapes. Such an approach has the advantage of offering additional support in understanding the role of each morphotype. For example, large hyalinocytes that appeared highly spread and adhered to the cell culture plate (fibroblast-like cells) might have a function of aggregation and wound healing, as seen for other bivalve species [2]. Our live-cell imaging of hemocytes together with microbeads strengthens this hypothesis, as these fibroblast-like cells did not display any phagocytic capacity. Our study, therefore, underlines the need to further investigate the function of the different types of hemocytes to help understand their respective role *in vivo* and the need to get a more comprehensive and unified characterization of these different types of hemocytes. Our primary cultures maintained the above-mentioned hemocyte variety of at least 5 different hemocyte types for 7 days. Cell diversity loss became more evident after 14 and 21 days, with a noticeable decrease in the proportion of blast-like cells and fibroblast-like cells, and an enrichment of more complex cells. Thus, we hypothesize granulocytes represent a more resistant hemocyte subpopulation to our *in vitro* conditions. Interestingly, the surviving subpopulations present at later culture periods, rather than exhibiting symptoms of aging and functional loss, exhibited stable immunological function as measured by phagocytic competence.

Phagocytosis is a crucial marker of immune function in hemocytes [45]. Our protocol shows no loss of hemocyte phagocytic activity and capacity over 21 days of culture. Moreover, we observed that the phagocytosis capacity (number of beads phagocyted per cell) increased at 21 days of culture. This increase correlates with the significant changes in hemocyte morphotypes present in culture at this time point. Indeed, we found a decrease in the abundance of non-phagocytic cell types such as the fibroblast-like hyalinocytes, and an enrichment of larger and more complex hemocytes. It is worth noting that more complex cells, such as granulocytes, are bivalves' most active phagocytic immune cells [46]. Thus, even if we note a noticeable loss of diversity within our 21-day cultures, it is likely that they were enriched with phagocyte cells, opening interesting avenues for bivalve immune system research and longer-term effects of environmental factors on immune capacity.

To further understand the physiology of our hemocyte primary cultures we analyzed key metabolic parameters to investigate possible metabolic alterations and reprogramming in response to the altered microenvironmental settings. ROS production is closely related to cellular metabolic activity. Indeed, mitochondrial ROS is the major contributor to ROS levels in *C. gigas* hemocytes [47]. In this study, we have shown that total basal ROS production is higher in fresh hemocytes on day 0, then becomes lower and stable until day 21 cultures. Recently, we have found that, in *C. gigas*, the average physiological oxygen level within oyster hemolymph is ~5 kPa, but it can vary between 0 kPa and 12 kPa depending on valve opening behavior and filtering activities [22]. However, ambient air is composed of 21 kPa O_2. Thus, when hemocytes are transferred from the *in vivo* to *in vitro* conditions, they are exposed to much higher levels of oxygen than they would naturally experience (hyperoxia). Indeed, ROS production in cells is known to increase at hyperoxia conditions [48,49], which could explain the high ROS levels in the fresh hemocytes (day 0). The subsequent decrease and stabilization of ROS production at the following periods of culture (Days 7, 14, and 21) can be due to hemocyte acclimation mechanisms. We hypothesize that such mechanisms may be mediated by the activation of the nuclear factor erythroid 2–related factor 2 (Nrf2) transcription factor which is a master regulator of antioxidant cellular responses [22]. This hypothesis is supported by the fact that the conserved role of Nrf2 in mediating redox changes was already confirmed in *C. gigas* [22,50,51], and that Nrf2 activation in cells submitted to hyperoxia condition (ambient air) is a well-described phenomenon within mammalian cell culture models [52]. Future studies performing gene expression analyses of Nrf2-target genes over the course of the hemocyte primary cultures should be performed to confirm this hypothesis.

However, throughout the 21 days of culture, we found stable levels of mitochondrial ROS, which can be a proxy for mitochondrial activity [53]. At first glance, this result seems to contradict our first hypothesis related to the Nrf2-mediated adaptive mechanisms of hemocytes in culture. Nonetheless, the differences reported between the total basal ROS and mitochondrial ROS can be related to differences in probe specificities. DCF is a broader marker of ROS, reacting with several different ROS types, whereas MitoSOX is a marker specific for superoxide anion related to mitochondrial activity, membrane potential, and size [54]. Moreover, the analysis of hemocyte mitochondrial content remained constant throughout the cell culture period. Cellular stress and mitochondrial dysfunction usually alter mitochondrial dynamics, often leading to changes in mitochondrial content through mitophagy or biogenesis mechanisms, for example [55]. Since this was not the case in our study, our primary hemocyte culture model offers stable conditions to study mitochondrial dynamics under different stress and disease scenarios.

Meanwhile, the total ATP levels detected in each well decreased at 7 days of culture and then stabilized at lower levels throughout the culture until 21 days. Additionally, the rate of total NADH production per well, which is also indicative of mitochondrial activity, was lower at days 7 and 21 of culture. We hypothesize that such lower levels are not necessarily a slowdown in the cellular metabolism, but rather indicative of cell death and/or loss over the 21-day culture period, leading to a decrease in the total cellular content per well. To better elucidate this hypothesis, future studies can be performed using high-content imaging and automated analysis of cellular content for a better normalization of the metabolic activity per cell, as soon as such tools become more available and adaptable to marine cell cultures.

Lipid droplets act as dynamic organelles for storing neutral lipids and play a crucial role in energy homeostasis and lipid metabolism. They are involved in various cellular processes, including the regulation of lipid synthesis and degradation, signaling pathways, and the maintenance of cellular lipid balance [56]. We have identified changes in intracellular dynamics of neutral lipid storage in hemocytes, leading to higher lipid accumulation at 21 days of cell culture. Lipid accumulation in cultured cells can be a sign of changes in lipid metabolism, or the ability to take up

lipids from the media, or as a result of autophagic processes [57]. Indeed, to interpret our result, there are at least three critical points to consider. Firstly, in bivalves, hemocytes are known to transport nutrients, including lipids, by absorbing them from the alimentary tract and transferring them to other tissues or developing oocytes [58,59]. Secondly, granulocytes, which were enriched at the 21-day cultures, have a higher neutral lipid content than other cell types in marine bivalves [60]. Thirdly, reserves of neutral lipids are related to the balance between synthesis and degradation in response to metabolic needs and shifts. Thus, it is likely that the absence of interaction between hemocytes and other tissues in the cultures blocked their ability to transport lipids to other cells, promoting a higher accumulation of lipids absorbed through the cell culture medium (FSOP). This higher neutral lipid accumulation could be related to the larger cell size observed after 21 days of culture. Taken together, the higher levels of neutral lipids in hemocytes in the second and third week of cell culture are possibly related to cellular size, composition, and energy allocation changes.

As discussed above, our results suggest potential changes in cellular metabolism over 21 days of culture. This presents a limitation when studying the biology of hemocytes in extended culture periods. However, it adds important information on the implications of cell microenvironment conditions to the success and function of bivalve primary hemocyte cultures. The established protocol presented here opens new avenues to explore biotic and abiotic conditions that can further improve hemocyte primary cultures and potentially even develop a cell line if conditions favoring proliferation are found. Moreover, it also provides the possibility to investigate the behavior of cell cultures under different stress conditions. For example, it is possible now to explore whether pathogens, contaminants, temperature, or plasma composition alterations will affect hemocyte viability and function over extended periods. Thus, this standardized long-term hemocyte primary culture, allied with the cell-based assays optimized in this study, allows the investigation of factors that can induce stress responses affecting cellular morphology, behavior, viability, immunocompetence, mitochondrial metabolism, and lipid storage. This could lead to new insights and discoveries in hemocyte biology and ecotoxicology research, thus expanding our understanding of bivalves' immunological and metabolic responses to the current and future challenges of our changing ocean.

5. Concluding remarks

In conclusion, our study of bivalve hemocytes in primary culture has provided valuable insights into their immunological competence, metabolic responses, and cellular behavior over an extended 21-day period. Bivalve hemocytes, known for their critical roles in immune defense and overall health, present a versatile model for studying various biological processes. Utilizing hemolymph plasma as a natural cellular culture medium, our approach offers a context approaching *in vivo* conditions. This method demonstrated sustained hemocyte phagocytic activity and capacity over the 21-day culture, highlighting the robustness of bivalve hemocytes in this unique culture medium. Notably, the increase in phagocytic capacity after 21 days was associated with shifts in hemocyte populations, indicating a dynamic response to prolonged culture conditions. We also revealed significant insights into the metabolic adaptation of hemocytes to *in vitro* conditions. Lastly, this proposed primary hemocyte culture protocol employs diverse health, function, and metabolic markers, presenting a valuable resource for *in vitro* research with hemocytes. The application of our protocols to different bivalve species holds promise for expanding our understanding of their immunological and metabolic responses in the face of ongoing and future challenges in our changing oceans. This can significantly support the ever-growing need for further hemocyte, immunology, and ecotoxicology research investigations.

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