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Transepithelial migration of mucosal hemocytes in *Crassostrea virginica* and potential role in *Perkinsus marinus* pathogenesis

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ABSTRACT

We have recently described the presence of hemocytes associated with mucus covering the pallial organs (mantle, gills, and body wall) 3 of the eastern oyster *Crassostrea virginica*. These hemocytes, hereby designated "pallial hemocytes" share common general characteristics with circulating hemocytes but also display significant differences particularly in their cell surface epitopes. The specific location of pallial hemocytes as peripheral cells exposed directly to the marine environment confers them a putative sentinel role. The purpose of this study was to gain a better understanding of the source of these pallial hemocytes by evaluating possible exchanges between circulatory and pallial hemocyte populations and whether these exchanges are regulated by pathogen exposure. Bi-directional transepithelial migrations of hemocytes between pallial surfaces and the circulatory system were monitored using standard cell tracking approaches after staining with the vital fluorescent dye carboxyfluorescein diacetate succinimidyl ester (CFSE) in conjunction with fluorescent microscopy and flow cytometry. Results showed bi-directional migration of hemocytes between both compartments and suggest that hemocyte migration from the pallial mucus layer to the circulatory system may occur at a greater rate compared to migration from the circulatory system to the pallial mucus layer, further supporting the role of pallial hemocytes as sentinel cells. Subsequently, the effect of the obligate parasite *Perkinsus marinus* and the opportunistic pathogen *Vibrio alginolyticus* on transepithelial migration of oyster hemocytes was investigated. Results showed an increase in hemocyte migration in response to *P. marinus* exposure. Furthermore, *P. marinus* cells were acquired by pallial hemocytes before being visible in underlying tissues and the circulatory system suggesting that this parasite could use pallial hemocytes as a vehicle facilitating its access to oyster tissues. These results are discussed in light of new evidence highlighting the role of oyster pallial organs as a portal for the initiation of *P. marinus* infections in oysters.

1. Introduction

The eastern oyster *Crassostrea virginica* is one of the most economically- and ecologically-important marine species along the East coast of North America. However, due to overfishing and disease, the eastern oyster has been in decline (Kirkley, 1997). One major disease that affects *C. virginica* is Perkinsiosis, commonly known as dermo disease. This warm-water disease is caused by the protozoan parasite *Perkinsus marinus* and results in mass mortalities of *C. virginica* (Perkins, 1993). *P. marinus* was originally misclassified as a fungus (*Dermocystidium marinum*) but is currently reclassified as a member of the Alveolates in a clade closely related to dinoflagellates (Adl et al., 2005; Adl et al., 2012; Reece et al., 1997; Saldarriaga et al., 2003; Villalba et al., 2004). Originally discovered in *C. virginica* in the Gulf of Mexico, it is now detected in different oyster species throughout the western Atlantic ocean from Maine to Brazil (da Silva et al., 2013; Mackin et al., 1950; Pecher et al., 2008; Ray, 1952) as well as the Pacific coasts of Panama and Mexico (Lohan et al., 2016, 2017)

P. marinus can be transmitted directly through the water column from infected to uninfected oysters (Chu, 1996). The modes of transmission include direct infection via dead infected oysters and additionally, through shedding of *P. marinus* in feces and pseudofeces (material bound in mucus and rejected prior to ingestion) from live infected oysters (Bushek et al., 2002). Gut epithelium was presumed to be the primary portal of entry for *P. marinus*, however, more recent studies have found that pallial organs such as labial palps, gills, and especially, the mantle are likely to play important roles in the infection process (Allam et al., 2013; Chintala et al., 2002). The pseudofeces discharge area, defined by Allam et al. (2013) as the area of the mantle where non-ingested particles bound in mucus are accumulated prior to rejection as pseudofeces, was shown to harbor significantly higher loads of *P. marinus* cells than other tissues during early infection stages. The

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possibility of pallial surfaces, like the mantle, playing a role in the infection process is not surprising given that the pallial surfaces are the first host tissues encountered by waterborne microbes such as *P. marinus*.

Recent studies have reported the presence of hemocytes associated with the mucus lining pallial surfaces (epithelial tissues associated with the gills, mantle and palps) of *C. virginica* (Lau et al., 2017). These findings follow those reported as early as 1934 in the European oyster *Ostrea edulis* (Takatsuki, 1934) and later in the Manila clam *Ruditapes philippinarum* (Allam and Paillard, 1998) where hemocytes were detected in association with mucosal secretions covering pallial organs. These findings have major implications for the mechanisms of early host-pathogen interactions in bivalves. The pallial surfaces are bathed in the marine environment exposing them directly to the multitude of microbes present in the water column. Therefore, hemocytes associated with these pallial surfaces represent the first host cells that can potentially interact with waterborne microbes, unlike circulatory hemocytes that only interact with microbes that succeed in breaching physical barriers. This is particularly relevant to *P. marinus* since pallial organs (mantle in particular) have been identified as a potential portal of entry for the parasite into oyster tissues (Allam et al., 2013; Chintala et al., 2002).

The process of immune cells transiting across epithelial tissues is common across many taxa. In mammals, specialized representatives of the immune cells (neutrophils, dendritic cells) cross epithelial surfaces to interact with environmental microbes at mucosal interfaces (Rescigno et al., 2001). This migration is further enhanced during inflammatory responses allowing leukocytes to cross through epithelial tissues and migrate to the site of infection or injury (Liu et al., 2004). Similar to the inflammatory response in mammals, hemocytes of the tunicate *Ciona intestinalis* are able to migrate through the mantle epithelial tissues to the tunic in response to injury (Di Bella and De Leo, 2000). In bivalves, migration of cells across epithelial tissues can also be triggered by inflammation and pathogen exposure. In scallops, the harmful algal species *Prorocentrum minimum*, was shown to induce hemocytes to migrate into the alimentary tract (Li et al., 2012). Increased bivalve hemocyte concentrations have been shown in the extrapallial fluid in response to both shell repair and immune challenge (Allam et al., 2000a, 2000b, 2001, 2006; Mount et al., 2004).

Despite previous reports confirming the presence of hemocytes associated with mucosal tissues (both digestive and pallial) in bivalves (Allam, 1998; Allam and Paillard, 1998; Allam and Espinosa, 2016; Feng et al., 1977; Lau et al., 2017; Takatsuki, 1934; Yonge, 1926), it remains unclear whether these hemocytes originates from the circulatory system or are a specialized fraction of hemocytes resident in mucosal tissues but that venture at the surface of epithelial layers to "sample" environmental microbes or to accomplish other functions. Further, it remains unclear if these "mucosal hemocytes" do penetrate back into tissues to reach the circulatory system. If exchanges between mucosal hemocytes and internal milieu exist, this may represent a route for external microbes to penetrate host tissues and establish infection. For instance, penetrating external barriers such as epithelial layers represent one of the first challenges for internal pathogens when establishing infection. Previous studies have reported that *P. marinus* cells are phagocytosed by hemocytes but evade intracellular degradation (La Peyre et al., 1995; Volety and Chu, 1995). Given their peripheral location, hemocytes associated with the pallial surfaces may be the first host cells to interact with *P. marinus* and are likely to respond by internalizing the pathogen. However, given that *P. marinus* can persist within hemocytes, the pallial hemocytes may serve as a vector and a portal of entry for this parasite.

This study was designed to investigate whether exchange exists between hemocytes associated with the pallial surfaces and those present in hemolymph. Further, the study evaluated the effect of pathogen exposure (e.g. *P. marinus*) on hemocyte trafficking between both compartments. Results are discussed with an emphasis on the potential role of pallial hemocytes as vehicles facilitating the entry of specialized waterborne microbes.

2. Materials and methods

2.1. Oysters

Adult *C. virginica* were obtained from Frank M. Flower and Sons (Oyster Bay, New York, USA) for baseline trans-epithelial migration experiments and Pemaquid Oyster Company (Damariscotta, Maine, USA) for pathogen exposure experiments. Upon arrival, oysters were stripped of debris and fouling organisms. Oysters were acclimated in aerated filtered (1μm) and UV-sterilized seawater (28ppt, 23 °C) for 7–10 days prior to experiments and were fed daily with a commercial diet (DT's Live Marine Phytoplankton).

2.2. In vivo tracking of circulatory hemocytes

To track hemocytes transiting from the circulatory system to the pallial surfaces, carboxylfluorescein diacetate succinimidyl ester (CFSE) was injected into the circulatory system of 35 oysters. CFSE, a membrane permeable non-transferring dye, expresses becomes fluorescent and membrane impermeable when the acetate side chains are cleaved by intracellular esterases. The succinimidyl group is able to bind to intracellular amines leading to stable fluorescence that could last for several months in mammalian cells (Lyons, 1999; Parish, 1999). Preliminary experiments confirmed that CFSE staining does not affect hemocyte viability. Preliminary experiments confirmed that CFSE staining does not affect hemocyte viability and that hemocytes remain stained up to 14 days. CFSE was injected (50 μl at 40 μM in filtered –0.2 μm– sterile seawater –FSW– twice with 1 h between injections) through a small hole carefully drilled in the right valve directly above the adductor muscle. The hole was then plugged with softened wax and the oysters were placed back in tanks. At each collection time, hemocytes associated with the pallial surfaces and circulatory hemocytes were collected. A random subset of sampled oysters was retained and subsequently re-sampled at the next time point to evaluate whether shell notching impacts hemocyte trafficking. Oysters were sampled at 24 h (11 oysters), 3 days (10 new oysters, 4 from previous sampling), 7 days (10 new oysters, 6 from previous sampling) and 14 days (5 from previous sampling) post-injection. These time points were chosen based on preliminary trials. These time points were chosen based on preliminary trials completed to evaluate the optimal times to obtain signal. At each collection time point, the initial fluid in the pallial cavity was drained via a notch created on the edge of the shell and 1 mL isoosmotic ethylenediaminetetraacetic acid (EDTA) anti-aggregant solution (1 L distilled water, 14.4 g $Na_2HPO_4{\cdot}2H_2O$, 2.6 g $NaH_2PO_4{\cdot}H_2O$, 25 g NaCL, 10 g EDTA; pH 7.4) (Allam and Paillard, 1998) was pipetted into the cavity to help dissociate hemocytes from the pallial surfaces. Oysters were then covered with parafilm at the notch and gently agitated. Subsequently, the fluid was collected, allowed to settle in 24 well plates and viewed under a fluorescence microscope to record the number of fluorescent cells. Microscopy was used for pallial hemocytes instead of flow cytometry to ensure that only intact hemocytes are counted and eliminate any risk of false positive events that could occur with flow cytometry. For each sample, a minimum of 3 brightfield pictures were recorded and counted using the program Image J (Schneider et al., 2012). Results were averaged and used to extrapolate the total number of hemocytes for each well. The total number of fluorescent cells per well was counted for each sample using fluorescence microscopy and was divided by total number of hemocytes per well × 100 to obtain the percentage of stained hemocytes that migrated from the circulatory system to the pallial surfaces. Circulatory hemocytes were collected via a syringe through the hole drilled on top of the adductor muscle (Chaney and Gracey, 2011) and fluorescent and non-fluorescent cells were counted using a FACSCalibur flow cytometer (Becton Dickinson

Biosciences) by analyzing a minimum of 10,000 events. After the last collection, oysters were opened to confirm the accuracy of the hole placement and data from oysters with holes not centered on the adductor muscle was discarded (4 out of 35 oysters were discarded).

2.3. In vivo tracking of pallial hemocytes

To track hemocyte migration from pallial surfaces into the circulatory system, hemocytes associated with the pallial surfaces were collected and stained. Oysters were notched on the shell edge carefully without disturbing the mantle. The pallial fluid was then collected as described above excluding the use of sterile seawater instead of an anti-aggregant solution. Oysters were then placed in 50 µm dopamine baths for one hour to reduce filtration rates (Anador et al., 2011). Concurrently, the collected pallial fluid was centrifuged (200*g*, 10 min), and the supernatant was collected and retained on ice. The pelleted hemocytes were resuspended and stained with 5 µM CFSE in 500 µl FSW for 10 min in the dark. Hemocytes were washed, centrifuged (200*g*, 7 min) and supernatant was discarded. Washed cells were recombined with previously saved cell-free pallial liquid and introduced back into the pallial cavity of each original oyster using a pipette. Oysters were sealed with parafilm and incubated on a cell culture shaker (50 RPM) for 30 min on ice followed by 30 min at room temperature. After incubation with stained cells, circulatory and pallial hemocytes were collected (one hour post-incubation with CFSE-stained hemocytes). Oysters were then placed back in tanks containing sterile seawater and hemocytes associated with the pallial surfaces and circulatory hemolymph were subsequently collected at 3, 24, 30 and 48 h. Fluorescent and non-fluorescent hemocytes in the hemolymph and pallial samples were counted as described above via flow cytometry for hemolymph and fluorescence microscopy for pallial samples.

2.4. Effects of pathogen exposure on hemocyte trafficking

To assess the effect of pathogen exposure on hemocyte trans-epithelial trafficking, oysters were exposed to FSW (negative control), *P. marinus*, or *V. alginolyticus* before the migration of fluorescently-labeled hemocytes was assessed. *P. marinus*-naïve oysters were obtained from Maine (see Section 2.1). *P. marinus* status was confirmed on a subset of 16 oysters using the standard fluid thioglycollate assay; (Burreson et al., 2005) to ensure results were not skewed by pre-exposure to *P. marinus*. For challenge experiments, exponentially-growing *P. marinus* cells (ATCC 50439) grown in DME/F12-3 media (Burreson et al., 2005) at 23 °C were collected via centrifugation, washed with FSW and resuspended in FSW. *Vibrio alginolyticus* was grown on a shaker at room temperature in marine broth and cultures were collected via centrifugation, washed with FSW and resuspended in FSW.

Effect of pathogen exposure on bi-directional trafficking of both circulatory and pallial hemocytes was assessed. Circulatory hemocytes were labeled with CFSE as previously described 2 days prior to pathogen challenge to track the change in motility of hemocytes from circulatory system to pallial surfaces after pathogen exposure. Oysters were incubated in dopamine for 1 h to reduce filtration (see above) before exposed to *P. marinus* (10^6 cells in 1 mL FSW, introduced into the pallial cavity with a pipette), *V. alginolyticus* (10^8 cfu in 1 mL FSW) or FSW (control). The notch that served to introduce the inoculum was covered with parafilm and oysters were incubated for 1 h on a shaker (50 RPM) at room temperature before being placed back into separate seawater tanks.

To evaluate changes in pallial hemocyte trafficking following pathogen exposure, pallial hemocytes were collected and stained ex-vivo as described above. Concomitantly, oysters were incubated in seawater containing dopamine before pallial hemocytes were reintroduced into the pallial cavity with a pipette (see Section 2.3). Immediately after addition of stained pallial hemocytes, 1 mL *P. marinus* (10^6 cells in FSW), *V. alginolyticus* (10^8 cfu FSW) or FSW (control) was introduced into the pallial cavity of individual oysters with a pipette. Oysters were sealed with parafilm and incubated for 1 h on a shaker at room temperature before being placed back into separated tanks.

Hemocyte trafficking was then evaluated by analyzing change in the proportion of fluorescent hemocytes in pallial mucus and hemolymph at different time points. To track circulatory hemocytes, hemocytes associated with the pallial surfaces and circulatory hemocytes were collected at 3, 6, and 24 h post-pathogen exposure. To track the movement of pallial hemocytes, hemocytes associated with the pallial surfaces and hemolymph were collected at 3, 24, 30, and 48 h post-pathogen injection. The number of fluorescent and non-fluorescent hemocytes in each sample was determined as described in Section 2.2.

2.5. Uptake of P. Marinus at pallial surfaces

Uptake of *P. marinus* through pallial epithelia was investigated to evaluate the contribution of these surfaces as portals of entry for the parasite. Exponentially-growing *P. marinus* cells were injected (10^6 cells in 1 mL FSW) into the pallial cavity of naive Maine oysters in the presence (30 oysters) or absence (30) of dopamine (1 h bath exposure to dopamine prior to pathogen challenge, see above). Control oysters (30 oysters) were injected with 1 mL FSW. All oysters were incubated on a shaker (50 RPM) for 1 h at room temperature and replaced back into separate tanks. Oysters were collected at 6, 24, 72 and 168 h post-inoculation for dissection and tissue preservation. The following tissues were separately processed to identify their respective contribution as portal of entry: mantle, pseudofeces discharge area (PDA), gills, palps, and visceral mass. Different wash techniques such as the use of EDTA buffer (see above), DNA lysis buffer, bleach, and physical agitation (Kemp and Smith, 2005) were tested in preliminary trials for removal of *P. marinus* adhered externally to tissue surface, if any. The optimal technique for *P. marinus* cell removal from tissue surfaces was via capillary action by patting with disposable dry wipes. Following removal of surface *P. marinus,* tissues were weighed and homogenized in phosphate-buffered saline (PBS) (10 µl/1 µg tissue) for DNA extraction using the tissue nucleospin DNA extraction kit (Macherey-Nagel). DNA samples were tested via quantitative PCR (qPCR) for presence and concentration of *P. marinus*. Quantitative PCR primers and procedures were adapted from Audemard et al. (2004). Amplification reactions (5 µl 2× SYBR Green, 1 µl sample DNA, 1 µl of each of the 2 primer solutions, 0.15 µl reference dye, 1.85 µl dH2O) were run as follows: 95 °C for 10 min, 40 cycles of 95 °C for 30 s, annealing and extension at 60 °C for 1 min (fluorescent signal collected), and melting curve from 95 °C to 60 °C with temperature decreasing by 0.5 °C every 10 s. A standard curve made with DNA from known amounts of *P. marinus* cells was used to calculate the number of *P. marinus* cells in each sample.

To visually confirm the uptake of *P. marinus* by pallial hemocytes and subsequent translocation to tissues, exponentially-growing *P. marinus* cells were stained with the vital fluorescent dye PKH-67, before being introduced into the pallial cavity of adult oysters (10^8 cells/oyster) in the presence of dopamine as described above. Oysters were then incubated on a shaker for 2 h before placed back in aquaria. Pallial hemocytes, mantle tissues and hemolymph were subsequently collected at different time intervals (2, 6 and 24 h) and analyzed for the presence of fluorescent *P. marinus* cells using fluorescence microscopy. Fluid tissues (pallial mucus, hemolymph) were collected as described above, distributed in 12 well plates and microscopically screened for the presence of fluorescent parasite cells. Mantle tissues were processed for cryotomy and resulting sections (~20 µm in thickness) mounted on a glass slide and viewed under a fluorescence microscope.

2.6. Statistics

Data were analyzed using Microsoft Excel and IBM Statistical Package for the Social Sciences (SPSS) software. Percent of fluorescent hemocytes in the pallial mucus and the hemolymph was arcsin-

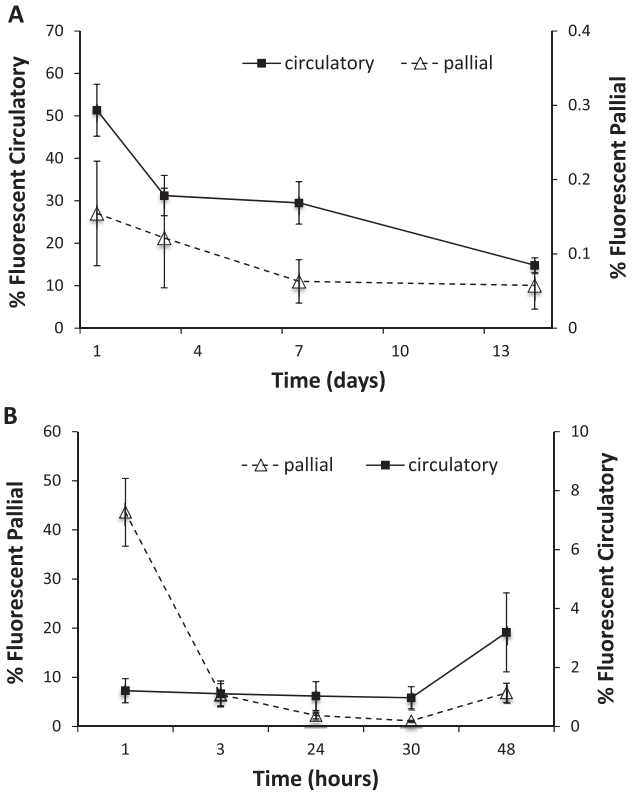

Fig. 1. Migration of hemocytes between hemolymph and pallial mucus. (A) Circulatory hemocytes were stained with CFSE and the presence of fluorescent cells was monitored in hemolymph and pallial mucus (Mean ± SE, N = 11–16 oysters/data point). (B) Pallial hemocytes were stained and the presence of fluorescent cells was monitored in both fluids (Mean ± SE, N = 10/data point).

transformed and ANOVA was performed to assess statistical significance. Quantitative PCR results (cells/mg oyster tissue) were log10 transformed and ANOVA was performed. When data normality and homoscedasticity could not be confirmed (e.g. effect of pathogen exposure of trans-epithelial migration), non-parametric tests (ANOVA on ranks and Mann-Whitney Rank Sum) tests were used. All differences were considered significant at $p < 0.05$.

3. Results

3.1. Transmigration of hemocytes from the circulatory system to pallial surfaces

Fluorescent hemocytes associated with the pallial surfaces were detected within 24 h of CFSE injection into the adductor muscle (Fig. 1A). No significant differences were noted between oysters sampled once or twice; therefore the data was combined for all oysters sampled at each time point. The percentage of fluorescent circulatory hemocytes decreased (51.3% to 14.8%) over 14 days. Similarly, the percentage of fluorescent pallial hemocytes decreased (0.15%, corresponding to an average of 58 fluorescent hemocytes per culture well to 0.06%, corresponding to an average of 22 fluorescent hemocytes per well) over the course of 14 days. The percentage of fluorescent hemocytes in the circulatory fluid experienced a sharp decline initially but maintained similar levels by day 3–7 prior to experiencing another decline between day 7 and day 14. Fluorescent hemocytes associated with the pallial mucus slowly decreased from day 1–7 and then plateaued by day 7–14 (Fig. 1A).

3.2. Transmigration of hemocytes from pallial surfaces to the circulatory system

The migration of hemocytes associated with the pallial surfaces towards the circulatory fluid was observed one-hour post CFSE staining (Fig. 1B). Fluorescent hemocytes associated with the pallial surfaces had a sharp decrease (43.6–6.5%) within the first 3 h. This decrease continued for the next 2 sampling periods (2.2% at 24 and 1.1% at 30 h) before a mild increase was measured at the end of the experiment (6.8% at 48 h). Fluorescent hemocytes associated with the circulatory system maintained approximately the same percentage from 1 h (1.2%, 121 fluorescent hemocytes in a total of 1×10^4 counted hemocytes) to 30 h (1.0%) with a mild increase at 48 h (3.2%) similar to the trends found with hemocytes associated with the pallial surfaces (Fig. 1B).

3.3. Effects of pathogen exposure on hemocyte trafficking: in vivo

Circulatory hemocytes were stained with CFSE and tracked after individuals were injected with FSW (control), *P. marinus*, or *V. alginolyticus* in the pallial cavity. The percentage of fluorescent hemocytes in the circulatory system was similar among control and challenged oysters and ranged from 25.7% to 28.2% after 3 h and from 9.9% to 13.3% after 24 h (Fig. 2A). Fluorescent hemocytes appeared in the pallial mucus and their percentage was significantly higher at the 24-h sampling point in oysters injected with *P. marinus* (3.4%, average 29 fluorescent hemocytes per culture well; Fig. 2A) as compared to control oysters (1.4%, average 12 fluorescent hemocytes/culture well) and oysters exposed to *V. alginolyticus* (1.6%, average 17 fluorescent hemocytes/culture well). No significant changes in transmigration were

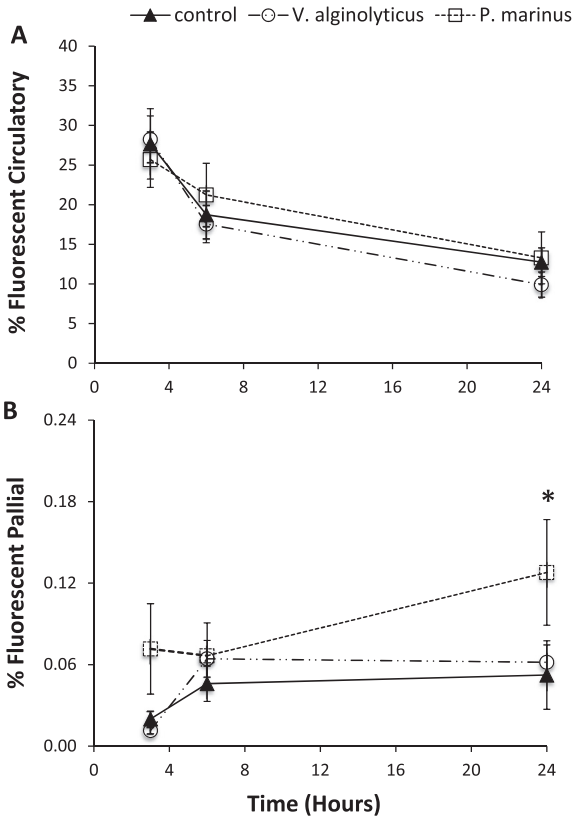

Fig. 2. Migration of hemocytes between hemolymph and pallial mucus in oysters injected with seawater (control), *V. alginolyticus* and *P. marinus*. Circulatory hemocytes were stained with CFSE and fluorescent cells were monitored in hemolymph (A) and pallial mucus (B) (Mean ± SE, n = 15–22 oysters/data point). * indicates significant difference between treatments (ANOVA on ranks followed by Mann-Whitney rank sum test, $P < 0.05$).

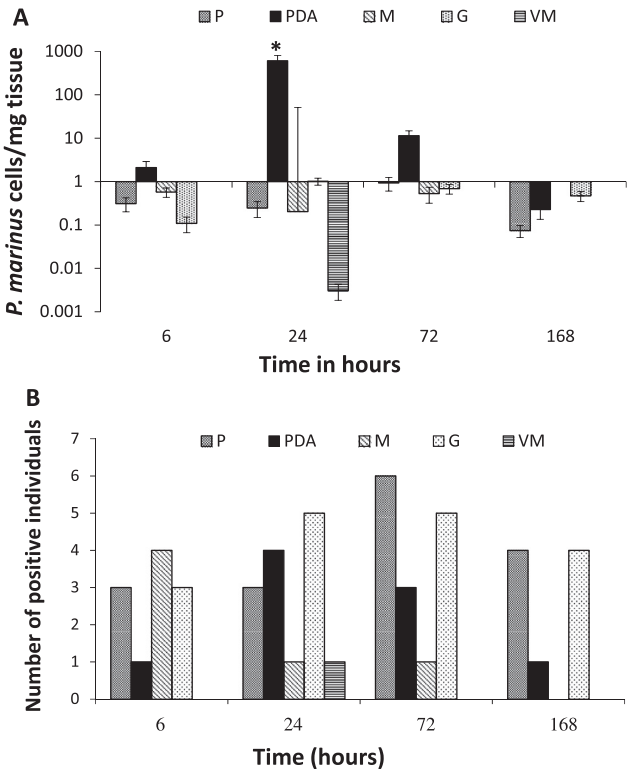

Fig. 3. (A) Average number of *P. marinus* cells/mg of oyster tissue and (B) number of positive samples following *P. marinus* exposure. P: labial palps, PDA: pseudofeces discharge area, M: mantle, G: gills, VM: visceral mass. * indicates significant difference as compared to all other tissues (ANOVA followed by Fisher LSD test on log10 transformed data, $P < 0.05$, $N = 12$ oysters/data point).

observed in oysters exposed to *V. alginolyticus* as compared to the controls.

Pallial hemocytes were also stained with CFSE and tracked after individuals were challenged with *P. marinus* or *V. alginolyticus,* or injected with FSW (control). The percentage of fluorescent hemocytes in the pallial mucus ranged between 5.5% and 7.0% after 3 h and continuously decreased to a range of 0.5–1.5% after 48 h (Suppl. Fig. S1A). Fluorescent hemocytes were detected in the circulatory system, representing 1.6–2.6% of the overall hemocyte population at the 3-h sampling time, and continuously decreased to reach 0.5% at the 48-h sampling (Suppl. Fig. S1B). Overall, no significant difference was noted at any sampling point between challenged and control oysters.

3.4. In vivo uptake of P. Marinus at pallial surfaces

Quantitative PCR results indicated differential uptake of *P. marinus* cells by pallial organs (Fig. 3A). In particular, the pseudofeces discharge area (PDA) had a higher load of *P. marinus* cells as compared to all other tissues throughout the first 3 days of the experiment with significant differences noted 24 h post exposure (over 600 cells/mg oyster tissue). *P. marinus* loads decreased afterward. In contrast, the overall number of positive samples was variable across sampling times and no clear trends between tissues was noted (Fig. 3B).

Two hours after *P. marinus*-challenged oysters were returned to seawater, fluorescent parasite cells were seen attached to mantle tissues and inside pallial hemocytes (Fig. 4A and B). Six hours following challenge, parasite cells were seen in the underlying connective tissues of the mantle (Fig. 4C), before being visible in the circulatory system after 24 h (Fig. 4D).

4. Discussion

A main finding from this study is that a bidirectional movement of hemocytes across the pallial epithelium exist in the oyster *C. virginica*. Although less well characterized in invertebrates than vertebrates (Friedl and Weigelin, 2008; Muller, 2003), a number of studies have observed the movement of hemocytes across epithelial tissues (Allam et al., 2000a, 2000b, 2001; Di Bella and De Leo, 2000; Galimany et al., 2008; Li et al., 2012; Mount et al., 2004). Therefore, it is not surprising that hemocytes associated with the pallial mucus in *C. virginica* have the ability to transit across the mantle into the circulatory system and vice versa.

A greater percentage of fluorescent hemocytes was measured in the circulatory system after staining pallial hemocytes as compared to the percentage of fluorescent hemocytes detected in pallial mucus after staining circulatory hemocytes. While these findings suggest different rates of transmembrane movement in both directions, the interpretation of these differences is challenging for several reasons. For instance, there are no methods to precisely determine the overall number, or the efficiency of recovery, of hemocytes from each compartment (for this reason data are presented as percentages). Therefore, the relative importance of the proportion of cells stained at the beginning of each experiment or recovered at each sampling point is difficult to establish. Furthermore, there is no way to determine if circulatory hemocytes present in peripheral tissues (e.g. mantle connective tissue and blood sinuses, which might be the closest source for pallial hemocytes) at the beginning of the experiment are as well stained as hemocytes present in the adductor muscle where the dye was injected. One can also argue that the change in the percentage of fluorescent hemocytes may indicate differential renewal kinetics of the cell population in each compartment (relative number of non-stained cells) more so than a change in the number of fluorescent hemocytes themselves. In addition, differences measured in the percentage of fluorescent cells in both compartments could reflect differences in the technical approach used for each set of experiments. Pallial hemocytes were stained *ex vivo* while circulatory hemocytes were stained by injecting CFSE directly into the circulatory system. These methods represented the best technical approaches to evaluate the transit given different constraints provided by both compartments. For instance, the circulatory system is relatively confined and injection of cell tracking dyes can be easily done without contamination of external surfaces. In contrast, injecting dyes directly to the pallial cavity can still lead to ingestion and absorption of CFSE that could ultimately stain circulatory hemocytes without the involvement of pallial hemocytes, yielding “false positive” signals. However, *ex vivo* labeling of pallial hemocytes means that only pallial hemocytes present at the time of sampling were labeled (instantaneous labeling) whereas circulatory hemocytes had sustained CFSE staining since the dye was directly injected into the hemolymph. This difference in staining may have contributed to the overall trends and variability in time scale leading to labeled circulatory hemocytes being traceable on a much longer timeframe of up to 14 days. In this context, the greater percentage of hemocytes observed to have migrated from the pallial surfaces to the circulatory system may be due to the higher percentage of hemocytes that were labeled at the beginning of the experiment; however, since pallial hemocytes were only instantaneously stained, migration to the circulatory system may have also been underestimated. In addition, the total residence time of the hemocytes on the pallial surfaces compared to the internal circulatory system is unknown. Considering the structure of the circulatory system of oysters, hemolymph bathing the adductor muscle first passes through the kidney before being split between the gills and accessory hearts, and finally directed to irrigate the mantle (Eble, 1996). Mantle hemocytes, however, move directly to the systemic heart and are subsequently directed to the adductor muscle. These differences could mean that circulatory hemocytes stained by injecting the dye in the adductor muscle may remain longer in internal tissues (e.g. kidney) before being directed to

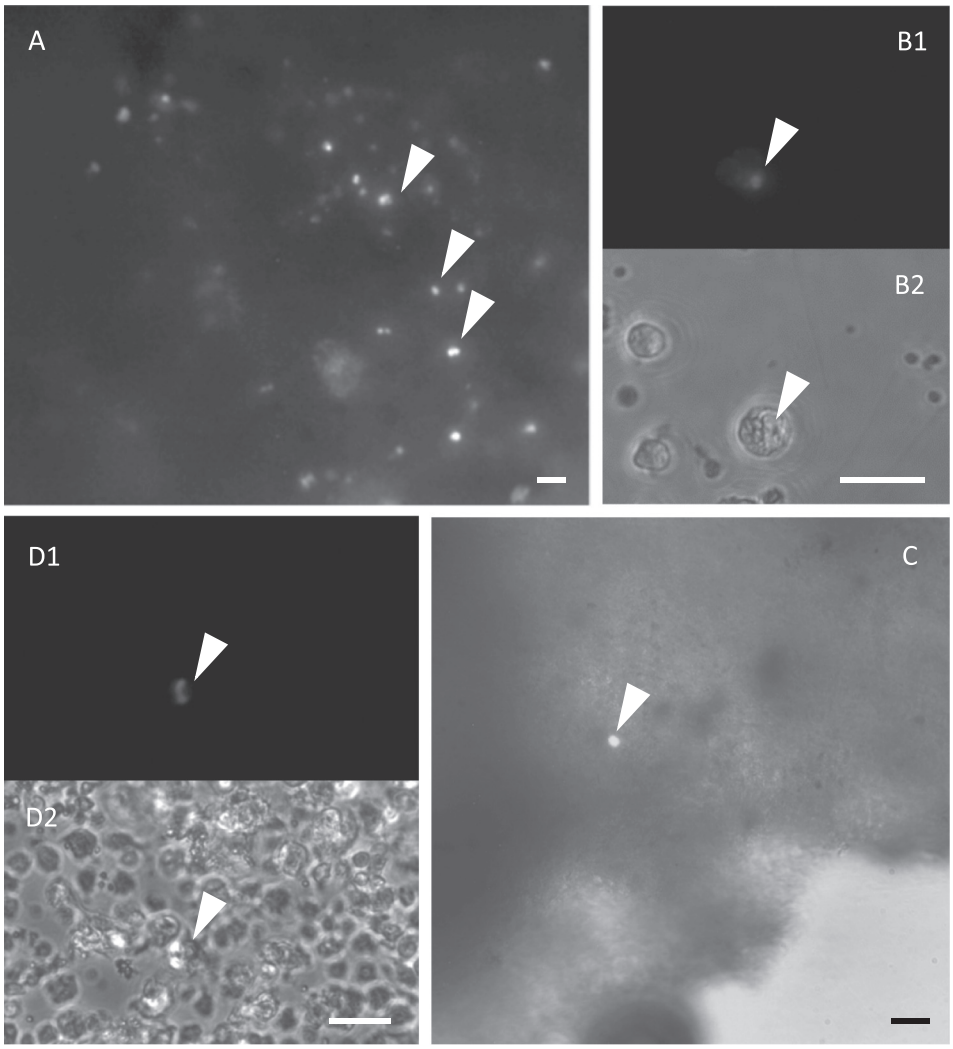

Fig. 4. Fluorescent *P. marinus* cells (arrowheads) are seen attached to mantle tissues (A, 2 h post-exposure), and inside pallial hemocytes (B1 and B2, 2 h) and mantle connective tissues (C, 6 h), before reaching the circulatory system (D1 and D2, 24 h). B1 and B2, and D1 and D2 represent the same microscopic fields viewed under fluorescence microscopy and phase contrast, respectively. Scale bars = 20 μm.

the pallial organs where they could still reach mucosal surfaces but at a much later time. In contrast, stained pallial hemocytes are quickly directed to the adductor muscle and could be rapidly detected in the hemolymph. Overall, a decrease of stained cells was observed in both the circulatory and pallial surfaces over 14 days, which could also be linked to loss in overall fluorescence and hemocyte turnover. CFSE binds to amine-containing molecules and a portion of these molecules are transient and/or can migrate out of cells within the first 24 h (Weston and Parish, 1990). Therefore, hemocyte fluorescence may slightly decrease in intensity as shown in non-dividing vertebrate cells where there is a drop of fluorescence intensity in the first 24–48 h after staining (Lyons, 1999). Despite these technical limitations that could lead to an underestimation of transmigration frequency, bidirectional migration of hemocytes was confirmed given the presence of fluorescent hemocytes across the pallial epithelial barrier. Additional studies are warranted to elucidate the circulatory path of hemocytes and the hematopoietic process to better understand the rate of transit of hemocytes across the pallial epithelium and between different body fluids.

To better understand the ability of pathogens in affecting the motility of hemocytes, circulatory and pallial hemocytes were both tracked for transmembrane movement after exposure to *V. alginolyticus* and *P. marinus*. Results showed that the opportunistic bacteria *V. alginolyticus* did not cause any change in hemocyte migration. Conversely, the obligate parasite *P. marinus* elicited a significant increase in hemocyte migration from the circulatory system to the pallial surfaces 24 h after challenge indicating the response is pathogen specific. Recruitment of mucosal sentinel cells (e.g. dendritic cells) to infection foci is well described in vertebrates (McWilliam et al., 1996) and oyster pallial hemocytes may play a similar sentinel role during the early interactions with *P. marinus*.

Our previous studies have suggested that oyster mantle, and the pseudofeces discharge area (PDA) of the mantle in particular, represents an important portal of entry for *P. marinus* (Allam et al., 2013). In the current study, significantly greater *P. marinus* loads were found in the PDA as compared to other tissues indicating that parasite acquisition may be concentrated in this area. Furthermore, microscopic observations support direct uptake of parasite cells at the mantle interface suggesting that pallial hemocytes may be involved in the uptake and dispersion of *P. marinus* as they could engulf parasite cells before migrating back into pallial tissues, thus directly transferring the pathogen into the body. Coupled with evidence that *P. marinus* can resist degradation in hemocytes post phagocytosis (La Peyre et al., 1995; Volety and Chu, 1995), an increase in hemocyte migration to the pallial surfaces may paradoxically facilitate *P. marinus* entry into the host via trans-epithelial migration of infected hemocytes. Although transmigration of hemocytes from the pallial surfaces to the circulatory system after exposure to *P. marinus* was maintained at basal levels, cells were only stained once at the beginning of the experiment. Therefore, only

hemocytes that were present on the pallial surfaces at the beginning of the experiment were tracked over the duration of the experiment. Further studies tracking hemocyte migration from the circulatory system to the pallial surfaces and back into the circulatory system are warranted. Nevertheless, hemocytes containing parasite cells were observed in the circulatory system 24 h after exposure to *P. marinus*. These observations strongly suggest that *P. marinus* may use pallial hemocytes as a vehicle to penetrate into their hosts through pallial epithelia.

Although the role of hemocytes associated with the pallial mucus has not been fully elucidated, a previous study conducted in our laboratory indicated that pallial hemocytes are morphologically and functionally distinct from circulatory hemocytes (Lau et al., 2017). Given their ability for transmembrane migration, pallial hemocytes may potentially act as sentinel cells relaying important information about the surrounding environment and subsequently eliciting a physiological response. In higher vertebrates, sentinel cells (e.g. dendritic cells) can be found on epithelial surfaces such as the skin or digestive linings and are able to capture and process antigens, migrate to lymphoid organs, produce cytokines, and subsequently stimulate other immune responses such as activating antigen-specific T-cells (Banchereau and Steinman, 1998). Dendritic cells have recently been found to play a similar role in the rainbow trout *Oncorhynchus mykiss* (Bassity and Clark, 2012). These findings suggest that the presence of sentinel peripheral immune cells capable of regulating immune functions is an evolutionarily conserved trait, at least across vertebrates. Similar processes may still occur in invertebrates, and hemocytes associated with oyster pallial mucus may serve an analogous sentinel role.

5. Conclusions

To our knowledge, this is the first study to quantitatively assess hemocyte exchanges between mucosal surfaces and the circulatory system in invertebrates. Findings from this study suggest there is bidirectional movement of hemocytes across the pallial epithelium and that *P. marinus* cells are able to increase hemocyte transepithelial motility. Given technical limitations, our results may represent an underestimation of the true levels of exchanges between pallial surfaces and the circulatory system. Combined with preliminary microscopic observations, our results support the idea that *P. marinus* is able to utilize hemocytes as a vector to gain entry to *C. virginica*. The possibility of pallial surfaces, like the mantle, playing an important role in the infection process is not surprising given that the pallial surfaces are directly exposed to seawater, which carries a wide range of pathogens including *P. marinus*. In this context, hemocytes associated with the pallial surfaces are the first host cells to interact with *P. marinus* and are shown to respond by phagocytosing the pathogen. Interestingly, hemocyte transmigration from the circulatory system to the pallial surfaces increased following *P. marinus* challenge. Since *P. marinus* survives inside hemocytes (La Peyre et al., 1995; Volety and Chu, 1995), increased hemocyte trafficking across pallial epithelia could potentially facilitate *P. marinus* entry into host tissues using a Trojan horse strategy. These conclusions, although supported by our preliminary microscopic observations, remain speculative and need validation. Our findings remain nevertheless important not only for a better understanding of entry mechanisms of waterborne pathogens, but also for unraveling a fundamental biological process of perception and interaction between bivalves (and most likely other aquatic invertebrates) and their surrounding environment. Fully unraveling the role of pallial hemocytes as sentinel cells and potential pathogen acquisition portals requires further investigations.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jip.2018.03.004.

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