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Early host-pathogen interactions in marine bivalves: Evidence that the alveolate parasite *Perkinsus marinus* infects through the oyster mantle during rejection of pseudofeces

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ABSTRACT

Parasites have developed myriad strategies to reach and infect their specific hosts. One of the most common mechanisms for non-vector transmitted parasites to reach the internal host environment is by ingestion during feeding. In this study, we investigated the mechanisms of oyster host colonization by the alveolate *Perkinsus marinus* and focused on how oysters process infective waterborne *P. marinus* cells during feeding in order to determine the portal(s) of entry of this parasite to its host. We also compared the infectivity of freely-suspended cells of *P. marinus* with that of cells incorporated into marine aggregates to link changes in particle processing by the feeding organs with infection success and route. Finally, we evaluated the effect of oyster secretions (mucus) covering the feeding organs on *P. marinus* physiology because these host factors are involved in the processing of waterborne particles. The ensemble of results shows a unique mechanism for infection by which the parasite is mostly acquired during the feeding process, but not via ingestion. Rather, infection commonly occurs during the rejection of material as pseudofeces before reaching the mouth. The pseudofeces discharge area, a specialized area of the mantle where unwanted particles are accumulated for rejection as pseudofeces, showed significantly higher parasite loads than other host tissues including other parts of the mantle. Aggregated *P. marinus* cells caused significantly higher disease prevalence and infection intensities when compared to freely-suspended parasite cells. Mucus covering the mantle caused a quick and significant increase in parasite replication rates suggesting rapid impact on *P. marinus* physiology. A new model for *P. marinus* acquisition in oysters is proposed.

1. Introduction

Marine diseases are now recognized as a major factor shaping population dynamics and modulating the functions of marine ecosystems (Harvell et al., 1999, 2004; Powell et al., 2008; Ward and Lafferty, 2004). One group of ecologically important marine animals that has suffered severe alterations caused by diseases is the bivalve mollusks (Ford, 1996; Ward and Lafferty, 2004). Since the middle of the twentieth century, concern over introduction of disease has been heightened by epizootic mortalities associated with pathogens in several species of mollusks along the coasts of the United States and in Western Europe (Ford, 1992; Renault, 1996). These epizootics, initially favored by transplantations and introductions of mollusks, have been sustained in the environment by changes in temperature and salinity regimes (Ford, 1992; Ford and Chintala, 2006). One of the most studied diseases of mollusks is Perkinsosis or Dermo disease which involves the eastern Oyster *Crassostrea virginica* and the protistan parasite *Perkinsus marinus*. Initially placed in the phylum Apicomplexa (Levine, 1978), subsequent phylogenetic studies support the inclusion of *P. marinus* within the Dinozoa (which also includes dinoflagellates; (Adl et al., 2005; Reece et al., 1997; Siddall et al., 1997)). Dermo disease has caused severe epizootics in *C. virginica* throughout the east coast of the United States from the Gulf of Mexico to Maine (Ford and Chintala, 2006) and is believed to cause 50% yearly mortality of the market-size component of oyster populations in the Gulf of Mexico (Mackin, 1962; Powell et al., 1996). Because of the importance of environmental factors on disease development, Dermo disease has featured prominently as a case study in the emerging field of research concerned with disease dynamics in the oceans

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as related to climate change (Cook et al., 1998; Ford and Smolowitz, 2007; Kim and Powell, 1998; Lafferty et al., 2004).

The literature on Dermo disease in oysters is extensive (reviewed by Villalba et al. (2004)) and *P. marinus* is routinely cultured in vitro using standard cell culture media greatly facilitating experimental studies. The life cycle of *P. marinus* is composed of three main stages including trophozoite, tomont and zoospore stages, all of which can be infective to *C. virginica*. The trophozoite stage occurs in host tissues where it reproduces using vegetative proliferation creating up to 32 daughter cells. These cells reside in live tissues or can be released through feces leading to the spread of the disease to nearby oysters (Bushek et al., 2002; Villalba et al., 2004). As a facultative intracellular parasite, *P. marinus* has adopted several strategies allowing its proliferation inside the tissues of *C. virginica*. For example, oyster immune cells (hemocytes) readily phagocytose *P. marinus* after recognizing cell surface epitopes (Tasumi and Vasta, 2007), however the parasite is able to neutralize host microbicidal processes and evade intracellular destruction (Ahmed et al., 2003; Fernandez-Robledo et al., 2008; Schott et al., 2003; Schott and Vasta, 2003; Volety and Chu, 1995; Wright et al., 2002). This ultimately allows *P. marinus* to divide and proliferate inside hemocytes inducing their rupture and release of daughter parasite cells, greatly facilitating its dissemination throughout host tissues (Villalba et al., 2004). The parasite also produces a battery of "universal virulence factors" thought to be involved in oyster invasion including various proteases (Brown et al., 2005; Brown and Reece, 2003; Faisal et al., 1999; Garreis et al., 1996; Joseph et al., 2010; La Peyre et al., 1996) and anti-oxidant enzymes (Ahmed et al., 2003; Fernandez-Robledo et al., 2008).

Despite the availability of abundant information on the cellular and molecular processes that allow *P. marinus* to survive and disseminate throughout host tissues, our understanding of initial steps of host invasion is limited. This situation is not limited to Dermo disease in oysters. One major problem limiting our understanding of resistance to infections in bivalves lies in our modest knowledge of the early host-pathogen interactions, including basic elements such as portal(s) and mechanism of entry of pathogens and of the factors that facilitate or inhibit entry. In the case of *P. marinus*, early histopathological investigations suggested that waterborne *P. marinus* cells were ingested and that the gut was the principal portal of entry. In this model, cells of the parasite are phagocytized by hemocytes present in the lumen, and carried inside tissues by hemocyte diapedesis across epithelia (Mackin, 1951; Mackin and Boswell, 1956). Based on his laboratory challenge experiments using *P. marinus* zoospores, Perkins (1988) proposed an alternative route by which the parasite can infect its host through the epithelia of the pallial organs. This was further supported in subsequent studies using more sensitive and quantitative techniques. For instance, based on their immunolabeling study, Dungan et al. (1996) suggested that *P. marinus* enters through pallial interfaces after finding parasite cells in the epithelia of the pallial organs (mantle and gill) but not in digestive tract epithelia. Using quantitative culture-based techniques, Bushek et al. (1997) demonstrated that exposing oysters to *P. marinus* via shell cavity injection had significantly higher infection levels than animals exposed by feeding. Similarly, Chintala et al. (2002) also suggested that pallial organs (mantle, gills and labial palps) were more important than the gut route. The discrepancy between more recent investigations and earlier histopathological studies likely comes from the fact that *P. marinus* cells are very small and that early infection stages might simply fall below the technique's detection limit (a 6-μm thick histological section represents a 1/15,000th subsample of a 9-cm oyster). Nevertheless, despite the fact that earlier histopathological studies reported prevalent infections in digestive epithelia, *P. marinus* cells were also occasionally detected in the mantle (Mackin, 1951; Ray, 1952) or gill (Ray, 1966) tissues when none had been seen in the digestive epithelium. These observations further support the concept that infection may be initiated in pallial organs.

Oysters, like other suspension-feeding bivalves, are capable of processing over 100 L of seawater per day, per individual, in their quest to capture nutritious particles from the water column. This mode of feeding exposes oysters to the multitude of microbes suspended in the water column or associated with flocculated organic matter (e.g. marine aggregates; see Lyons et al., 2007). Another characteristic of suspension-feeding bivalves is that they are able to sort captured material, ingesting nutritious particles and reject unwanted ones as pseudofeces (reviewed by Ward and Shumway (2004)). This mechanism is in part a result of specific biochemical interactions between suspended particles and mucus covering the feeding organs (Pales Espinosa et al., 2010a, 2009). The ability of suspension-feeding bivalves to capture and ingest (or reject) waterborne pathogens has never been investigated.

The present study focused on how oysters process infective waterborne *P. marinus* cells in order to better characterize the portal(s) of entry of this parasite to its host tissues. We also manipulated the method of *P. marinus* delivery to link changes in particle processing by the feeding organs of oysters with infection success and route. Finally, we evaluated the effect of mucus secreted by the feeding organs of oysters on *P. marinus* physiology. This mucus contains biochemical factors that are involved in the processing of waterborne particles (Pales Espinosa et al., 2010a, 2009, 2010b; Ward and Shumway, 2004), and represent the first host material encountered by infective *P. marinus* cells.

2. Materials and methods

2.1. Processing of freely suspended P. marinus by oysters

In this experiment, we determined whether cells of the parasite, *P. marinus*, were preferentially ingested or rejected in pseudofeces by oysters compared to a nutritious microalga (Prymnesiophyceae, *Isochrysis galbana*). Oysters (*C. virginica*, 43.8 ± 4.7 mm in height, mean ± standard deviation) were obtained from a commercial source (Frank M. Flower and Sons Oyster Company, Oyster Bay, New York, USA), and cleaned of sediment and epibionts. Oysters were acclimated in the laboratory for a minimum of 1 week (salinity of 28, 23 °C), during which time they were fed daily using DT's Live Marine Phytoplankton (15% of dry meat weight) (Pales Espinosa and Allam, 2006). One day prior to being used in the exposure experiments, oysters were placed in filtered seawater (0.45 μm) to allow them to purge their gut. The microalgae *I. galbana* (CCMP 1323) was grown in F/2 medium (Guillard, 1975) at 15 °C under a 14-h light/10-h dark cycle. Cells of *P. marinus* (ATCC 50439) were inoculated at 10^5 cells mL^{-1} into 4 × 300-mL culture flasks containing sterile DME/F12-3 culture medium (Burreson et al., 2005). Cultures were grown at 23 °C for 15 days and then subjected to centrifugation (400 g, 15 min, room temperature) to concentrate the cells. Pellets from each replicate flask were resuspended in 300 mL of sterile seawater (filtrated at 0.22 μm) and kept overnight at 23 °C. On the following morning, parasite cultures were pooled and equal concentrations of each cell type (i.e., *I. galbana* and *P. marinus*) were mixed together and suspended in filtered seawater (5×10^5 cells mL^{-1} final concentration) and delivered to oysters maintained in individual 1-L tanks ($n = 12$). Cells were kept in suspension by means of gentle stirring every 5 min. Water samples from each tank were taken periodically and sedimentation of cells compared to that from a control tank containing an empty shell. Pseudofeces were collected from each tank 2 h after onset of production. Pseudofeces were homogenized to disperse aggregates, fixed with 10% buffered formalin and their composition determined using flow cytometry as described previously (Pales Espinosa et al., 2010a, 2009). Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson Biosciences, CA, USA),

and a minimum of 10^4 events were analyzed. The 488 nm argon laser was used for excitation, and cells identified and characterized based on their forward (FSC) and side (SSC) light scatters, and chlorophyll auto-fluorescence (FL3, 675 nm).

Data were analyzed using goodness-of-fit tests (G test). Two series of tests were performed comparing the proportion of each cell type in samples of the diet (taken from each of the 1-L tanks) and pseudofeces collected from the oysters (Pales Espinosa et al., 2010a). The first series of tests ensured that replicates within diet or pseudofeces samples were homogeneous. The second series tested the null hypothesis that the proportion of each cell type in the diet and the pseudofeces was not different. In addition to the comparison of raw counts, a sorting efficiency (SE) index was calculated in order to examine particle selection (Iglesias et al., 1992). This index was defined as: SE = 1- (P/D), where P (pseudofeces) and D (diet) represent the proportion of the cell of interest in the pseudofeces and diet, respectively. A positive SE for a given cell type indicates that it is preferentially ingested (cell type is depleted in the pseudofeces, compared to diet), a negative SE indicates rejection (cell type is enriched in the pseudofeces compared to diet), and zero indicates the absence of selection by the oyster. After confirming their normal distributions, calculated SE values obtained for each of the two cell types were compared to zero using a one-sample *t*-test (two-tailed). The null hypothesis was that the selection efficiencies were equal to zero (i.e., no selection). Two-sample *t*-tests were then used to examine differences in SE between the two particle types.

2.2. Infectivity of freely suspended and aggregated P. marinus

In this series of experiments, the infectivity of freely suspended cells of *P. marinus* to oysters was compared to that of cells incorporated into marine aggregates. The experiments tested whether the method of delivery of *P. marinus* cells affects disease prevalence and intensity in different organs of the oyster. Naïve oysters (68.7 ± 9.2 mm in height, mean ± SD) were obtained from Dr. Chris Davis (Pemaquid Oyster Co., Waldoboro, Maine, USA). They were cleaned of epibionts, attached to wooden craft sticks using Velcro© and marine epoxy (see Ward and Kach (2009) for details), and maintained in an aerated, recirculating tank at 22 °C and salinity of 32 for a minimum of 3 days. Water for the tank was collected from Long Island Sound (LIS, Groton, Connecticut, USA), and passed through a series of filters ending with a 1-μm nominal pore size. Oysters were fed a maintenance ration of Instant Algae Shellfish Diet 1800 (Reed Mariculture).

Aggregate production and feeding trials followed the general protocol of Kach and Ward (2008) and Ward and Kach (2009), and are briefly described below. Exponentially-growing *P. marinus* cells propagated in DME/F12-3 medium (Burreson et al., 2005) supplemented with oyster tissue homogenates (Earnhart et al., 2004) were suspended at a concentration of ca. 8.3×10^4 *P. marinus* cells mL^{-1} in natural seawater (from LIS) that had been passed through a 210-μm sieve. The cell suspension was then distributed in two series of 1-L polycarbonate jars (Nalgene). The first series of jars was placed on a rolling table for 24 h at 15 rpm to produce *P. marinus*-laden marine aggregates (Shanks and Edmondson, 1989; as modified by Ward and Kach (2009)). A second series of jars was placed next to the rolling table for the same period of time. For an experiment, rolled and un-rolled jars were inverted to re-suspend parasite cells. Samples of water and aggregates from two representative jars were then taken to count parasite cells and determine the percentage of *P. marinus* incorporated into aggregates which typically ranged between 50% and 75%. All other jars were then immediately used for the challenge experiment.

Experiments were conducted in an environmental chamber held at ca. 20 °C. Given the complex nature of these experiments, four separate trials were performed following the same protocol. Each trial included oysters exposed to one of three treatments: (1) *P. marinus* cells incorporated into aggregates (4–5 oysters/trial); (2) cells freely suspended (3–4 oysters/trial); and (3) seawater from LIS (sieved to 210 μm) without added *P. marinus* cells (controls, 3–4 oysters/trial). One oyster was placed (hinge side down) into a jar containing one of the aforementioned treatments and its craft stick secured to the lip of the container with a wooden clip. A stir bar was added and the bottle placed on an electromagnetic stir plate. To ensure that aggregates and cells remained suspended, jars were supplied with gentle aeration and stirred for 10 s every 15 min (160 rpm). Latex beads (10-μm diameter) were added to each bottle (ca. 2×10^3 beads mL^{-1}) and monitored over time to assess the feeding activity of each oyster (Ward and Kach, 2009). Oysters were allowed to feed for 2 h, after which time they were transferred to glass isolation tanks (57 L, one tank for oysters in each treatment) supplied with recirculating, filtered seawater (1 μm, LIS). Tanks were cleaned and water replaced at least twice per week. Oysters were fed a daily ration (see Urban et al., 1983) of Instant Algae Shellfish Diet (1800, Reed Mariculture), and were held for 3–4 weeks prior to being screened for infection.

Prevalences and intensities of *P. marinus* infections were determined using a modified tissue burden assay based on the methods described by Bushek et al. (1994). Oysters were shucked and the following tissues isolated by dissection: (1) gills; (2) mantle; (3) sub-samples of the mantle tissue lying adjacent to the intersection of the labial palps and the gills (i.e., the principal pseudofeces discharge area [PDA]; Galtsoff, 1964; Ward et al., 1994); and (4) the visceral mass. Tissue samples were drained on clean paper towel and transferred to separate, sterile, pre-tared containers to determine the mass (g) of each. A volume of sterile seawater (salinity of 20) was added to each container so as to yield a ten-fold dilution of tissues after homogenization with a Polytron homogenizer (Brinkman Instruments). Aliquots (1 mL) of these tissue homogenates were used to inoculate 9 mL of Ray's fluid thioglycollate medium (RFTM, Bushek et al., 1994), supplemented with Chloramphenicol and Nystatin to limit the proliferation of microbial contaminants and lipid to promote enlargement of *P. marinus* hypnospores (Nickens et al., 2002). Following a one-week incubation in the dark at room temperature (21 ± 1 °C), cultures were centrifuged at 1500 rcf for 10 min (room temperature). Pelleted material was digested in 2 N NaOH (4 h at 60 °C) and processed for the detection and enumeration of hypnospores following the methodology of Nickens et al. (2002). Hypnospore counts in individual tissues were used in combination with tissue weights to calculate *P. marinus* counts per gram of whole oysters. Frozen (−20 °C) aliquots from samples that generated positive ($n = 10$) or negative ($n = 10$) results were submitted to PCR for confirmation of culture data using the internal transcribed-spacer-specific primers and methods described by Audemard et al. (2004). Means of tissue weight-normalized *P. marinus* cell counts were compared between different treatments and between different tissues within each treatment using analysis of variance (ANOVA) on Log10 transformed data.

2.3. Effect of pallial-organ mucus on P. marinus in vitro

This experiment studied the effect of the mucus secreted by the pallial organs of oysters on *P. marinus* physiological processes. These secretions are involved in the processing of waterborne microbes and represent the first host factors encountered by *P. marinus*. Mucus from the pallial organs was compared to extracts from the digestive gland because the digestive tract was traditionally considered as an important portal of entry for *P. marinus*. The posterior valve margins of adult *C. virginica* ($n = 12$, 91.1 ± 7.2 mm in height obtained from Frank M. Flowers Oyster Co., Oyster Bay,

NY) were carefully notched and hemolymph was withdrawn from the adjacent adductor muscle with a sterile hypodermic needle and syringe. Hemolymph samples were centrifuged at 900 rcf for 5 min (4 °C). The plasma was collected, filtered through 0.22-μm syringe filters, and held on ice until used as a supplement to culture media (typically within 2 h). Oysters were then shucked, the right valve carefully removed, and exposed tissues rinsed with seawater (salinity of 28). Mucus was collected from gills and mantle using cotton swabs (Pales Espinosa et al., 2009). The tips of each swab were removed and placed into 15 mL Falcon tubes containing 7 mL ice-cold seawater (salinity of 28). Tubes were gently shaken at 4 °C for 1 h after which time the liquid was centrifuged at 900 rcf for 15 min (4 °C). The supernatant was then filtered through 0.22-μm syringe filters and stored on ice. Following mucus collection, the digestive gland of each oyster was dissected and finely minced using a razor blade. The minced tissues were placed into 15 mL Falcon tubes containing 5 mL seawater. Tubes were gently shaken at 4 °C for 30 min after which time the liquid was centrifuged at 1000 rcf for 15 min (4 °C). To ensure removal of tissue debris, the supernatant was decanted and centrifuged again at 1000 rcf for 30 min (4 °C). Following centrifugation the extract was filtered through 1-μm and then 0.22-μm syringe filters. Aliquots of plasma, mantle and gill mucus, and digestive-gland extracts were used to determine protein concentration in each sample using a bicinchoninic acid assay (BCA; Pierce, Rockford, Illinois) and remaining samples were immediately used as experimental supplements to *P. marinus* cultures.

Supplemented cultures were separately prepared in 12-well plates by combining 1 mL of DME/F12-3 culture medium, 165 μL of exponentially growing *P. marinus* culture maintained overnight in sterile seawater at 1.2×10^7 cells mL^{-1}, experimental supplements (0.15 mg protein mL^{-1} final concentration) and were adjusted to 2.5 mL with sterile artificial seawater (SAS, salinity of 28). A series of control cultures was prepared by replacing the experimental supplements with artificial seawater. Culture plates lids were sealed with paraffin tape to avoid evaporation and incubated at 23 °C. Cultures were microscopically monitored on a daily basis and subsamples of 200 μL were taken at Days 1, 4 and 8 and fixed with ethanol (33% final ethanol concentration). Cell counts of *P. marinus* were made on a BD FACSCalibur™ Flow Cytometer. Fixed samples were labeled with SYBR Green I (S-7563, Invitrogen, stock solution at 10,000×) at a final concentration of 10× and were incubated in the dark for 1 h. Flow cytometry data were analyzed using BD Cell Quest™ Pro software and *P. marinus* cells were identified according to forward scatter (FSC) and yellow-green fluorescence (FL1). Results are displayed as percent *P. marinus* growth (e.g. cell counts) as compared to unsupplemented control cultures although all statistical comparisons were made on raw cell counts in individual cultures using 2-way ANOVA with culture supplement type and incubation time as factors. Holm-Sidak post hoc test was used to compare individual data points when needed. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Processing of freely suspended P. marinus by oysters

Although the targeted proportion of each microorganism was 50%, an a posteriori count revealed the following proportions: 58.9 ± 3.5% (mean ± standard deviation) for *I. galbana* and 41.1 ± 3.5% for *P. marinus* (Fig. 1) yielding a total particle concentration of $4.6 \pm 0.1 \times 10^5$ cells mL^{-1}. The proportions of *I. galbana* and *P. marinus* in the experimental tanks remained stable over the course of the experiment indicating that differential settling of microorganisms had not occurred. When oysters were fed the

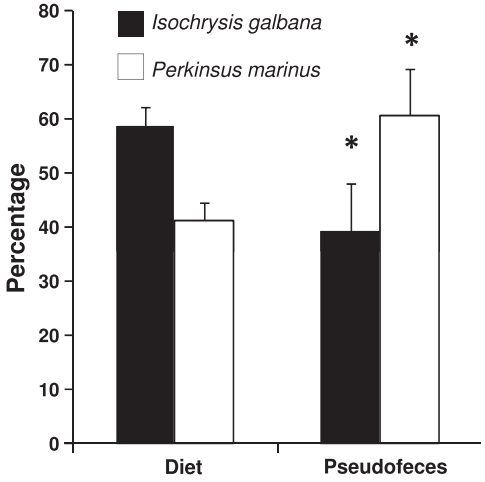

Fig. 1. Percentage (mean ± SD) of the two cell types in the water column (diet) and in pseudofeces of oysters exposed to a mixture of *Isochrysis galbana* and *Perkinsus marinus* cells. *indicates a significant difference for each particle type between the diet and the pseudofeces (G test, $p < 0.01$, $n = 12$).

I. galbana/*P. marinus* diet, the proportion of *P. marinus* increased significantly in the pseudofeces compared to the diet (60.6 ± 8.8%, Fig. 1, $n = 12$, G test, $p < 0.01$). Sorting efficiencies (SE) confirmed the above results and showed that oysters preferentially ingested *I. galbana* (SE = 0.33 ± 0.1) while they preferentially rejected *P. marinus* (SE = -0.48 ± 0.2, $n = 12$, *t*-test $p < 0.01$, Fig. 2).

3.2. Infectivity of freely suspended and aggregated P. marinus

Results from the infection studies showed significant effects of the method of delivery of *P. marinus* on infection prevalence and intensity in different organs of the oyster. Oysters that did not open and feed during the exposure trials (typically <10%) were excluded from the results. Infection prevalence was 56% (10 out of 18), 31%

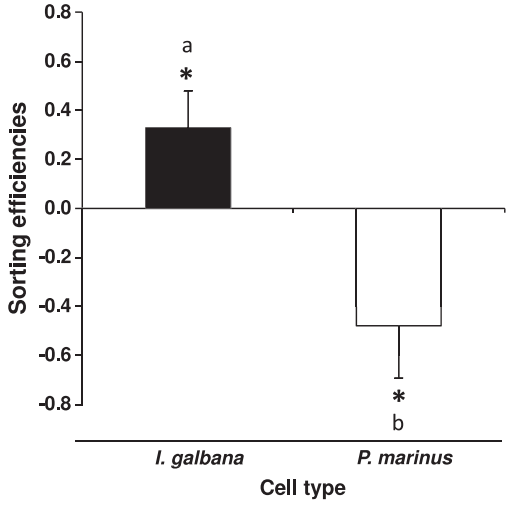

Fig. 2. Sorting efficiencies (mean ± SD) of oysters exposed to a mixture of *I. galbana* and *P. marinus* cells. A positive SE for a given particle type indicates that it is preferentially ingested (i.e., lower proportion in the pseudofeces, compared to diet), a negative SE indicates rejection (i.e., higher proportion in the pseudofeces compared to diet). (see Fig. 5) *indicates a sorting efficiency that is significantly different from 0 (one-sample *t*-test, $p < 0.001$). Letters denote significant differences in sorting efficiencies between the two cell types (two-sample *t*-test, $p < 0.01$, $n = 12$).

(4/13) and 18% (2/11) in oysters exposed to *P. marinus*-laden aggregates, suspended *P. marinus* cells and to control seawater, respectively. Mean total parasite cell counts was two orders of magnitude higher in the aggregate treatment (6515 hypnospores g oyster tissue^{-1}) as compared to the freely suspended (45 hypnospores g oyster tissue^{-1}) or control treatments (0.2 hypnospore g oyster tissue^{-1}; Fig. 3, ANOVA $p < 0.01$). The delivery method of *P. marinus* also caused a significant shift in parasite loads in various oyster tissues. In the aggregate treatment, hypnospore counts were 14 to 47 times higher in the principal pseudofeces discharge area (PDA; 74,956 hypnospores g^{-1}) of the mantle as compared to the remainder part of the mantle (5459 hypnospores g^{-1}), the visceral mass (5751 hypnospores g^{-1}) or the gill (1,598 hypnospores g^{-1}, Fig. 4A, ANOVA $p < 0.01$). The highest parasite loads were also detected in the PDA of oysters in the suspended and control treatments, although no significant differences were detected between tissues in these treatments because of the overall low parasite loads (Fig. 4B and C). Infection prevalence in different tissues from oysters exposed to *P. marinus* cells incorporated into aggregates followed the same trends with 9 out of 10 positive oysters displaying infections in the PDA, 6/10 in the gills, and 5/10 in both mantle and visceral mass tissues.

3.3. Effect of mucus covering pallial organs on P. marinus in vitro

Results from the in vitro experiments showed differential impacts of various extracts from the oyster on *P. marinus* proliferation in vitro. Parasite cell numbers increased gradually in unsupplemented controls from 0.9×10^6 at Day 1 to 2.9×10^6 and 6.3×10^6 at Day 4 and 8, respectively. Mantle mucus induced a rapid and significant increase (45% increase after 1 day, $p < 0.01$, Holm-Sidak post hoc test) in growth of *P. marinus* compared to unsupplemented control cultures (Fig. 5). In contrast, plasma and digestive-gland extracts were inhibitory and caused a significant reduction (47% and 60% decrease, respectively) in growth of the parasite compared to controls at Day 1 ($p < 0.01$). Interestingly, maximal inhibition was detected at Day 1 in cultures supplemented with digestive-gland extracts, causing a significantly different growth response than that found in the other three treatments. The inductive effect of mantle mucus on *P. marinus* growth leveled off at Day 4, with cell counts being similar to those found in cultures supplemented with gill mucus and in unsupplemented controls. Cell counts in cultures supplemented

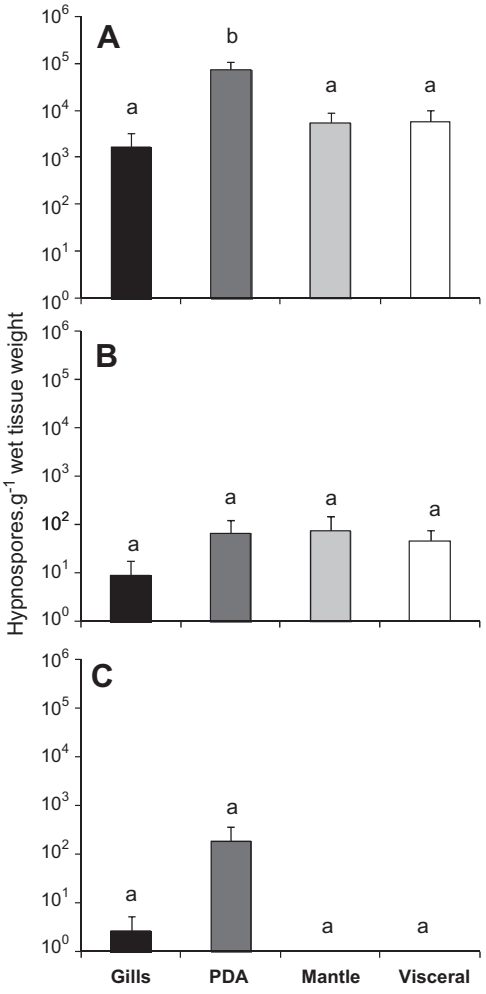

Fig. 4. Loads of *P. marinus* (mean ± SD) in different tissues of oysters exposed to parasite cells incorporated into aggregates (A), cells freely suspended (B) or seawater without added parasite cells (C). PDA designates the principal pseudofeces discharge area. Different letters (a, and b) indicate statistically significant differences between different tissues within each treatment (Holm-Sidak post hoc test, $p < 0.05$).

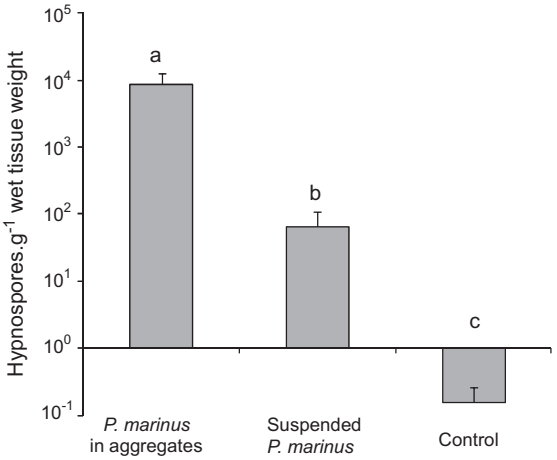

Fig. 3. Loads of *P. marinus* (mean ± SD) in tissues of oysters exposed to parasite cells incorporated into aggregates or cells freely suspended. Control oysters were exposed to seawater without added *P. marinus* cells. Different letters indicate statistically significant differences between treatments (Holm-Sidak post hoc test, $p < 0.05$).

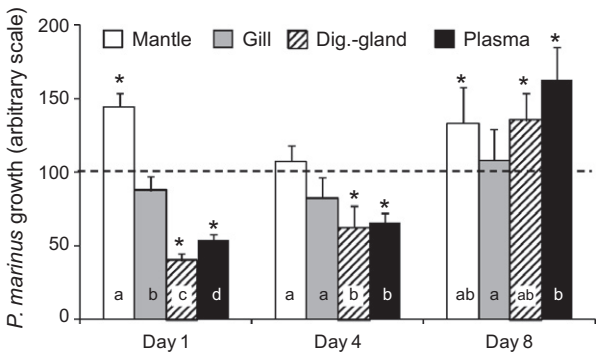

Fig. 5. Relative *P. marinus* cell counts (arbitrary scale, mean ± SD) in cultures supplemented with extracts from different oyster tissues, shown as % of cell counts measured on each sampling date in unsupplemented control cultures (dashed line). Letters (a, b, c and d) indicate which experimental treatments shared statistically equivalent (same letters) or different (different letters) cell counts within each sampling time. * indicates (see Fig. 3) statistically significant inhibition or enhancement of growth as compared to unsupplemented controls (Holm-Sidak post hoc test, $p < 0.05$, $n = 12$).

with mantle mucus, however, remained significantly higher than cell counts measured in cultures supplemented with digestive-gland extracts or plasma. The inhibitory effect of digestive-gland extracts and plasma on growth of *P. marinus* also decreased on Day 4, although growth of these cultures remained significantly lower than those of the unsupplemented controls. This inhibitory effect disappeared at Day 8 and cultures supplemented with mantle mucus extract, digestive-gland extract, and plasma had higher parasite counts than unsupplemented controls. Overall, gill mucus did not induce a significant change in parasite growth compared to the controls throughout the entire experiment.

4. Discussion

Parasitism is thought to represent the most common mode of feeding in nature, and parasites have developed myriad strategies to reach and infect their specific hosts (Price, 1980; Thompson, 2001). One of the most common mechanisms for non-vector transmitted parasites to reach an internal host environment is through ingestion. Results from the current work demonstrate that the alveolate parasite *P. marinus* also gains access to its host, *C. virginica*, by way of host feeding activities. The initial route of infection, however, seems to occur not through the gut after ingestion, but rather (or also) through the mantle on the pseudofeces rejection pathway. While the acquisition of *P. marinus* through the epithelia of the digestive tract after ingestion is possible, our findings combined with observations from previous studies support the concept that an important alternate route is through the principal pseudofeces discharge area (PDA) of the mantle (Fig. 6).

Because of its efficient mechanical and chemical processes, the bivalve digestive tract seems to represent a good barrier to infectious agents, and most fatal infections affecting bivalve mollusks

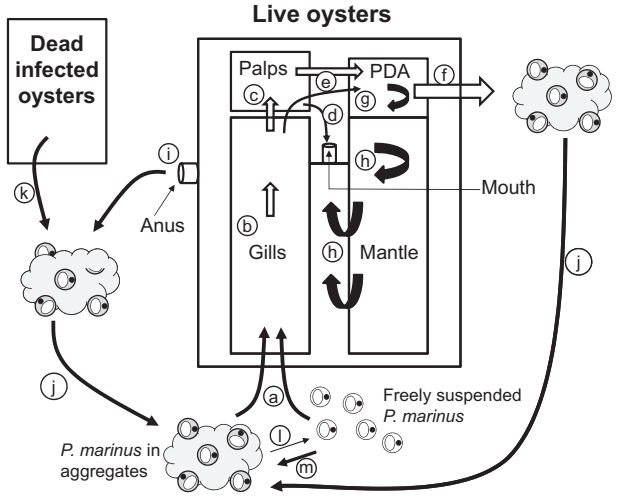

Fig. 6. Summary and conceptual scheme of the proposed infection model for *P. marinus* in the eastern oyster. Environmentally available *P. marinus* cells, in aggregates or freely suspended, are pumped into the pallial cavity (a), captured on the gills (b) and transferred to the labial palps (c) where a minor fraction is ingested (d). The bulk of captured parasite cells is transferred to the principal pseudofeces discharge area (PDA, e) where it is processed for rejection as pseudofeces (f). During the accumulation of pseudofeces on the PDA, a fraction of parasite cells initiates infection (g). Parasite cells subsequently spread to the remainder of the mantle and other organs (h). In advanced infections, parasite cells are shed into the feces (i) and contribute to the environmental pool of *P. marinus* cells in aggregates (j). Dead infected oysters also contribute to the pool of *P. marinus* cells (in aggregates and freely suspended, k) through decay and the action of scavengers (see text for more details). Exchange between pools of *P. marinus* cells (in aggregates vs. freely suspended) is likely (l and m) even though previously described physical and biological forces favor the formation of aggregates (m).

are initiated in pallial organs (mantle and gills). This is the case of the protistan parasites *Haplosporidium nelsoni* and Quahog Parasite Unknown (QPX) which affect the oyster, *C. virginica* and the clam, *Mercenaria mercenaria*, respectively (Burreson and Ford, 2004; Dahl et al., 2010; Ford et al., 2002b; Ragone Calvo et al., 1998; Smolowitz et al., 1998). The paramyxean *Marteilia sydneyi* has also been shown to initiate infections in the pallial organs of oysters (Kleeman et al., 2002). This route of infection seems to be common for other members of the genus *Perkinsus*, which infect clams and oysters worldwide. As the infection advances, *Perkinsus* spp. spread from the pallial organs to other tissues using host hemolymph as a way of dispersal (Azevedo, 1989; Navas et al., 1992; Rodriguez and Navas, 1995; Villalba et al., 2004). Whereas the specific mechanisms these parasites use to initially infect their host may differ, all demonstrate the parasites' ability to breach the epithelial layers of pallial organs. Therefore, it is not surprising that the mantle seems to represent a major portal of entry for *P. marinus* in *C. virginica*. What is particularly intriguing in the case of *P. marinus* is the apparent involvement of the PDA, a specific area of the mantle where material captured by the gills but not ingested is accumulated before being rejected as pseudofeces. For instance, parasite counts were 1–2 orders of magnitude higher in the PDA as compared to the other tissues. While the development of some levels of infection in control oysters is not surprising (rather expected) since these animals were exposed to seawater from an enzootic area (Long Island Sound), it is interesting to note that parasite counts in this group were also highest in the PDA (Fig. 4C). It should be noted that the overall parasite levels found in this study are relatively low (advanced infections often exceed 10^6 *P. marinus* cells g^{-1} wet oyster tissue, Bushek and Allen, 1996; Bushek et al., 1994), suggesting that the disease was in the early stages among our experimental oysters. Therefore, higher counts in the PDA reflect higher initial acquisitions in that area rather than differential post-acquisition growth and/or accumulation of the parasite in various tissues. It is possible that the higher infection intensity at the PDA, as compared to other tissues, results from longer contact time between the parasite and host tissues at this site during the accumulation of pseudofeces. This intriguing distribution of *P. marinus* cells in oyster mantle has been noted by Ray (1966) who revealed that mantle tissues located just lateral to the palps (i.e., the PDA) showed many more parasite cells than other areas of the mantle among naturally infected oysters.

Oysters can filter large quantities of water (up to 10 L h^{-1} g^{-1} dry tissue; Jordan, 1987) in their quest to secure particulate food. They are also capable of post-capture sorting of particles based on biochemical cues from living cells (Beninger and Decottignies, 2005; Pales Espinosa et al., 2010a, 2009, 2010b; Ward et al., 1998). In this study, we demonstrated that freely suspended *P. marinus* cells are preferentially rejected in pseudofeces when compared to the nutritious microalgae *I. galbana*. Rejected particles are entrapped in a mucous matrix as pseudofeces and are transported to the PDA where they accumulate until being ejected intermittently from the pallial cavity (Newell and Langdon, 1996; Ward et al., 1994). Therefore, the results of the transmission study showing higher infection prevalence and higher parasite loads in the PDA as compared to other tissues suggest that *P. marinus* is commonly acquired across the mantle epithelium, especially at the PDA where pseudofeces that contain infectious cells accumulate. To our knowledge, this is the first time such a unique pathogen-uptake mechanism (acquisition post capture but prior to ingestion) has been reported for an invertebrate.

Another important finding of this study is the role of aggregates in facilitating *P. marinus* transmission. Higher disease prevalence and parasite loads (particularly in the PDA) were measured in oysters exposed to cells of *P. marinus* incorporated in aggregates compared to freely suspended cells. Aggregates (aka marine snow,

flocs) are an important component of aquatic ecosystems (see review by Simon et al. (2002)). They are composed of organic and inorganic constituents which can include senescent phytoplankton, microzooplankton, fecal pellets, detritus, suspended sediment, and infectious microbes (Bochdansky and Herndl, 1992; Lampitt et al., 1990; Riebesell, 1991a, 1991b). Oysters that are heavily-infected by *P. marinus* shed viable parasite cells into the environment via their feces (Bushek et al., 2002; Villalba et al., 2004). Parasite cells can also be released from oyster tissues via decomposition and scavenging following mortality (Diamond, 2012; Hoese, 1962). Parasite cells released into the environment in feces and in tissue fragments would be subject to physical processes that lead to the production of aggregates (e.g., collisions with other particles during settling/resuspension). How aggregates enhance *P. marinus* infection prevalence and/or intensity remains unclear although two distinct mechanisms could be involved. Firstly, incorporation of *P. marinus* into aggregates might increase the likelihood that the parasite cells are transported to the PDA in pseudofeces, enhancing their uptake. Whereas some aggregated material is disaggregated and ingested (Kach and Ward, 2008; Ward and Kach, 2009), large agglomerations of material are more likely to be transported to rejection tracts of the gills and labial palps. Such agglomerations might carry large numbers of *P. marinus* cells to the PDA, increasing the local infective dosage to a degree that overwhelms the oyster's ability to fight the infection at this site. Previous studies comparing the uptake of microalgae showed a higher rejection in pseudofeces of cells that were clumped into larger aggregates compared to species of similar size that remain freely suspended (Barillé et al., 2003).

Another possibility is that *P. marinus* infectivity increases when parasite cells are incorporated into aggregates. Given their organic-rich composition, aggregates can be sites of intensive heterotrophic activity such that a single aggregate can be viewed as a microbial "hot spot" and aggregates have been previously shown to significantly concentrate microbial pathogens (Lyons et al., 2007, 2010). The speculation of an increase in *P. marinus* virulence in aggregates is supported by the findings of the culture-supplementation experiment where significant changes in *P. marinus* physiology were measured when pallial (mantle) mucus was added to the culture media. Indeed, it is well established that through the feeding process marine bivalves release significant quantities of transparent exopolymer particles (TEPs), which are important constituents of the organic-matter matrix of aggregates in estuarine environments (Heinonen et al., 2007; Li et al., 2008). Parasite cells that are incorporated into aggregates could come in contact with bivalve-generated TEP and respond accordingly. Oysters that capture these aggregates would then be exposed to the highly infective *P. marinus* cells.

Surprisingly, there are no previous studies focusing on the interactions between *P. marinus* and oyster pallial mucus, despite the fact that pallial mucus is the first host mechanical and chemical barrier encountered by the parasite (and other waterborne microbes). Mucus produced by bivalves plays an important protective role against some microorganisms (Fisher, 1992), and mucus in *C. virginica* contains several biochemical barriers such as hemolysins, lysozymes, lectins and proteases (Brun et al., 2000; Fisher, 1992; Jing et al., 2011; McDade and Tripp, 1967; Pales Espinosa et al., 2009). Despite the defensive role that mucus plays, mucus of marine invertebrates can also provide some pathogens with an advantage. For example, *Vibrio shiloi*, a bacterial pathogen of corals, adheres to β-D-galactoside-containing receptors in coral mucus in order to gain entry into the epidermal layers of the polyps (Banin et al., 2001). Results presented here showed significant and rapid increase in *P. marinus* replication following contact with mucus from the mantle tissue, indicating changes in the physiology of the parasite. Interestingly, in contrast to mantle mucus, digestive-gland extracts significantly inhibited *P. marinus* growth, further highlighting the efficiency of the digestive barrier in neutralizing invasive microbes. Additionally, our preliminary studies suggest that exposure of *P. marinus* cells to mantle mucus significantly enhances virulence in vivo (Pales Espinosa et al., submitted) to levels usually seen in "wild-type" *P. marinus* (Ford et al., 2002a). Together, these findings suggest that pallial mucus not only facilitates the parasite's access to a suspected major portal of entry (PDA) through aggregation processes but may also enhance *P. marinus* pathogenicity as well.

The study of the ecology and evolution of infectious diseases has developed over the last few decades with the emergence of multidisciplinary approaches, which allow research on the ecological factors that affect the dynamics and spread of diseases. Progress of this field in marine invertebrates is often limited by a lack of understanding of the detailed mechanisms as well as the ecological factors that facilitate infection. We presented here an ensemble of findings that highlight the intricate interactions between physiological and ecological processes affecting *P. marinus* transmission in oysters. For instance, we provide strong evidence suggesting that *P. marinus* is commonly acquired by the oyster when parasite cells are transported to the PDA and are awaiting rejection as pseudofeces. The results also supported a significant role for marine aggregates as potential reservoirs and vehicles for *P. marinus* infection in oyster-rich estuarine environments. These results fit well with the known mechanisms contributing to *P. marinus* release and abundance in the environment (Fig. 6), despite the lack of understanding of other aspects potentially affecting Dermo disease dynamics (such as the possible role of zoospores for example, Burreson and Ragone Calvo, 1996; Villalba et al., 2004). More attention should be given to early host-pathogen interactions taking place in the pallial cavity of bivalve mollusks, an environment that shares many similarities to the vertebrate oral cavity (i.e., semi-confined compartment, with highly regulated fluid circulation, and containing significant defense factors). Overall, the success or failure of infection establishment in bivalves may lie in the outcome of the interactions that take place at the pallial interfaces.

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