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Early host–pathogen interactions in a marine bivalve: *Crassostrea virginica* pallial mucus modulates *Perkinsus marinus* growth and virulence

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ABSTRACT: *Perkinsus marinus* is an important protistan parasite of the eastern oyster *Crassostrea virginica*. Recent findings showed that oyster pallial organs (mantle, gills) are a major portal of entry for the parasite. Therefore, mucus covering these organs represents the first host effectors encountered by *P. marinus*. This study consisted of several experiments designed to investigate the effect of oyster pallial mucus on the growth, protease production and infectivity of *P. marinus*. In each experiment, *P. marinus* performance in cultures supplemented with pallial mucus (mantle, gill, or both) was compared to that of parasite cells grown in unsupplemented media or in cultures supplemented with oyster plasma or digestive extracts. *P. marinus* grown in media supplemented with *C. virginica* mantle mucus showed a significantly higher growth rate than cultures enriched with the other supplemental extracts, while cultures grown in gill mucus promoted higher protease production. Conversely, *P. marinus* grown in cultures supplemented with pallial mucus of the non-compatible host *Crassostrea gigas* (Pacific oyster) were dramatically inhibited. Challenge experiments showed a significant increase in *P. marinus* virulence in cultures supplemented with *C. virginica* pallial mucus as compared to unsupplemented cultures or to those supplemented with digestive extract or plasma. These results suggest that *C. virginica* mucus plays a significant role in the pathogenesis of *P. marinus* by enhancing the proliferation and the infectivity of this devastating parasite. The contrasting results obtained with both oyster species indicate that *P. marinus* host specificity may begin in the mucus.

KEY WORDS: Dermo · Virulence · Mucus · Host specificity

INTRODUCTION

Perkinsus marinus is a prevalent pathogen of the eastern oyster *Crassostrea virginica* along the east and Gulf coasts of the USA, causing massive mortality in oyster populations (Burreson & Calvo 1996). The 'perkinsiosis' or 'dermo' disease caused by this protist is characterized by several pathological changes in affected oysters, including emaciation and reduction in condition index, as well as a proteolytic degradation of tissues (particularly the gills) in the most severely infected individuals (Ford & Tripp 1996). Several aspects of this host–pathogen association have been described. For instance, *P. marinus* is known to produce extracellular products containing several enzymes (including serine proteases; La Peyre et al. 1995) that degrade proteins present in oyster hemolymph, reducing oyster defenses (Garreis et al. 1996) and causing cellular and tissular damages that aid parasite invasion of host tissues (La Peyre et al. 1995). The parasite is also capable of surviving phagocytosis by hemocytes through quenching of the oxidative cascade (Anderson 1999) using mechanisms involving dismutases and peroxidases (Schott & Vasta 2003, Schott et al. 2003a,b). Furthermore, *P. marinus* modulates the apoptosis of infected hemocytes as a way to favor its spread in host tissues (Sunila & LaBanca 2003, Goedken et al. 2005, Hughes

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et al. 2010). As a matter of fact, early histopathological investigations suggested an important role of hemocytes in the uptake of waterborne *P. marinus*: parasite cells are ingested and phagocytized by hemocytes present in the gut lumen that carry them inside tissues by diapedesis across epithelia (Mackin 1951, Mackin & Boswell 1956). Even though a role of the gut in the uptake of *P. marinus* is possible, subsequent studies using more sensitive and/or quantitative detection techniques have suggested that pallial organs (i.e. mantle and gill) represent an important portal of entry for *P. marinus* into host tissues (Dungan et al. 1996, Bushek et al. 1997, Winnicki et al. 2008, Allam et al. 2013). This was further supported by our recent work demonstrating that *P. marinus* cells are preferentially rejected by oysters as pseudofeces before ingestion and that early infections appear in mantle tissues (Allam et al. 2013), suggesting that the gut route is secondary for the initiation of infection.

It is not surprising that the pallial organs of oysters represent the main portal of entry for *Perkinsus marinus* in *Crassostrea virginica*. Because of its efficient mechanical and chemical processes, the bivalve digestive tract appears to be a strong barrier against infectious agents, and most fatal infections affecting bivalve mollusks are initiated in pallial organs (mantle and gills). This is the case for the protistan parasites *Haplosporidium nelsoni* and Quahog Parasite Unknown (QPX) which affect the oyster *C. virginica* and the clam *Mercenaria mercenaria*, respectively (Ragone Calvo et al. 1998, Smolowitz et al. 1998, Ford et al. 2002b, Burreson & Ford 2004, Dahl et al. 2010). Infections by the paramyxean *Marteilia sydneyi* are also initiated 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 infection advances, *Perkinsus* spreads from the pallial organs to other tissues using host hemolymph as a way of dispersal (Azevedo 1989, Navas et al. 1992, Rodriguez & Navas 1995, Villalba et al. 2004).

Bivalve pallial epithelia are covered with mucin secretions that provide an efficient physical barrier to help isolate and protect soft tissues (Simkiss & Wilbur 1977). These secretions act as a barrier to diffusion (Grimm-Jørgensen et al. 1986) and may function in selective ion transport (Ahn et al. 1988). Most prior work investigating bivalve pallial mucus was performed within the framework of investigations of the filter feeding process (see review by Ward & Shumway 2004). However, some studies particularly focused on the role of mucus in animal protection and specifically demonstrated the presence of defense-related factors in the mucus of terrestrial and aquatic mollusks. For instance, mucus produced by *Crassostrea virginica* contains several factors involved in innate immunity, such as hemolysins, lysozymes, proteases and lectins (McDade & Tripp 1967, Fisher 1992, Brun et al. 2000, Pales Espinosa et al. 2009, Jing et al. 2011). Our previous investigations showed the presence in oyster pallial mucus of lectins that facilitate the capture of suspended particles (Pales Espinosa et al. 2009, 2010, Jing et al. 2011). Despite the defensive role that it plays, the mucus of marine invertebrates can provide some pathogens with an advantage. For instance, mucus substrates are among the most common matrices colonized by microbes (Ofek & Doyle 1994). Microorganisms within such environments participate in the formation and maturation of a biofilm that further promotes growth and the persistence of some adapted (or specialized) microbes (Tuomola et al. 1999, Lee et al. 2000, Welsh et al. 2001). 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).

Surprisingly, there are no previous studies focusing on the interactions between *Perkinsus marinus* and oyster pallial mucus, despite the fact that pallial mucus is the first host component encountered by the parasite (and other waterborne microbes). This is particularly pertinent since *P. marinus* cells present in seawater are likely in a dormant stage (Villalba et al. 2004), and one would expect that contact with mucus may cause 'activation', allowing parasite cells to initiate the infection and facilitating the invasion mechanism. The overall objective of this study was to assess the effect of oyster pallial mucus on *P. marinus*. The main hypothesis was that contact between *P. marinus* and pallial mucus causes significant changes in the parasite's metabolism, leading to an increase in the expression of virulence factors and an overall increase in infectivity. A combination of *in vitro* and *in vivo* approaches was used to test this hypothesis using *Crassostrea virginica* and the resistant host *C. gigas* (Pacific oyster).

MATERIALS AND METHODS

Organisms

Adult *Crassostrea virginica* naïve for *Perkinsus marinus* were obtained from commercial sources in

Washington (CvWA, 87.1 ± 6.4 mm in length; Taylor Shellfish Farms) and Maine (CvME, 78.4 ± 5.6 mm in length; Pemaquid Oyster Company). Naïve Pacific oysters *C. gigas* (CgWA, 91.2 ± 6.1 mm in length) were also obtained from Taylor Shellfish Farms. Some experiments (see '*In vitro* culture') also used *C. virginica* (CvNY, 83.4 ± 7.7 mm in length) obtained from Frank M. Flower and Sons Oyster Company in New York. All oysters were shipped to the laboratory overnight. Upon arrival, they were cleaned of sediment and epibionts and maintained separately in 150 l aquaria filled with saline (salinity 28) well water (Flax Pond Marine Laboratory) maintained at 20 to 22°C. Oysters were fed daily using DT's Live Marine Phytoplankton (Sycamore, IL; Pales Espinosa & Allam 2006). They were acclimated to these conditions for 7 to 10 d before the beginning of each experiment.

Cultures of *Perkinsus marinus* (ATCC 50439) were grown at 23°C in sterile DME/F12-3 culture medium (Burreson et al. 2005). Exponentially growing cultures were gently centrifuged (400 × *g*, 15 min, room temperature), and pellets were resuspended in sterile artificial seawater (SAS, salinity 28, filtrated at 0.22 µm) and kept overnight before use in various experiments.

Effect of pallial mucus on *Perkinsus marinus* growth

These experiments evaluated the effect of mucus covering the pallial organs on the growth of *Perkinsus marinus in vitro*. The effects of oyster plasma and digestive extracts were also used to provide a comparative assessment of different oyster products.

Collection of mucus, plasma and digestive extracts

Oysters (i.e. CvNY, CvWA and CgWA) were carefully notched, and hemolymph was withdrawn from the adductor muscle using a syringe fitted with an 18-gauge needle. Hemolymph samples were centrifuged (400 × *g*, 15 min, 4°C), and the plasma supernatant was collected, filtered (0.22 µm syringe filters) and kept on ice until its use as a medium supplement, typically within the following 2 h. The right valve of each oyster was then carefully removed, and underlying pallial tissues were rinsed with SAS. Mucus from gills and mantle was collected separately using sterile cotton swabs following the procedure described by Pales Espinosa et al. (2009). Swabs were then immersed in 10 ml of ice-cold SAS and stirred at 4°C for 1 h on a rotating shaker. The resulting fluid (pallial mucus) was centrifuged (400 × *g*, 15 min, 4°C), and the supernatant was filtered (0.22 µm syringe filters) and maintained at 4°C until use, typically within 2 h. Following pallial mucus collection, the digestive gland of each oyster was dissected, finely minced using a razor blade and immersed in 5 ml of ice-cold SAS. Each tube was placed at 4°C for 1 h on a rotating shaker. The resulting fluid (digestive extract) was centrifuged twice (1000 × *g*, 4°C, for 15 and 30 min), filtered (1 µm and then 0.22 µm syringe filters) to remove debris and maintained at 4°C until use. A 25 µl aliquot of plasma, pallial mucus and digestive extract was used to determine protein concentrations using a Pierce BCA protein assay reagent kit as per manufacturer's recommendations. For each experiment, all samples were adjusted with SAS to equivalent protein concentrations before their use as supplements to culture media.

In vitro culture

Supplemented cultures were prepared in 12-well plates by combining 1 ml of DME/F12-3 culture medium, 165 µl of *Perkinsus marinus* suspension at 10^7 cells ml^{-1} maintained overnight in SAS, experimental supplements (see previous subsection; CvNY: 0.15 mg protein ml^{-1}, CgWA: 0.15 mg protein ml^{-1}, and CvWA: 0.4 mg protein ml^{-1}) and were adjusted to 2.5 ml with SAS. Control cultures were prepared by replacing the experimental supplements with SAS. Culture plates were wrapped with paraffin tape to avoid evaporation and kept in the incubator at 23°C. Subsamples of 200 µl were taken on Days 0, 1, 4, 8 and 15 and were preserved in 33% ethanol at 4°C until processed for flow cytometry.

Flow cytometry analysis

Preserved samples were centrifuged (3000 × *g*, 5 min), and pellets were resuspended in SAS. Cells were labeled with SYBR Green I (stock solution at 10000×, Invitrogen) at a final concentration of 10× and incubated in the dark for 1 h. Samples were then analyzed and counted using a FACSCalibur flow cytometer (Becton Dickinson Biosciences). A minimum of 10^4 events were analyzed. *Perkinsus marinus* cells were identified according to their forward scatter (FSC) and side scatter (SSC) parameters. Polystyrene microbeads (3 µm diameter, Sigma-Aldrich) were used as internal controls for cell count calculation. Growth rates are noted as the percent of *P. marinus* growth (e.g. cell counts) in relationship to the unsupplemented control cultures measured at each sampling date.

Effect of pallial mucus on protease production by *Perkinsus marinus*

Protease activity was determined in *Perkinsus marinus* cultures supplemented with 0.4 mg protein ml^{-1} of different *Crassostrea virginica* (CvWA) extracts. Culture conditions followed the general design described above. Protease activity was measured spectrophotometrically in cell-free culture supernatants according to La Peyre et al. (1995). Briefly, azocasein substrate solution (3% w/v) was prepared by dissolving solid azocasein (Sigma-Aldrich) in phosphate buffer (pH 7.5) followed by centrifugation of the substrate (12 000 × *g*, 10 min, 4°C). Cell-free supernatant (30 µl) of *P. marinus* grown in the different experimental media was transferred in triplicate to a 96-well plate, and 50 µl of the azosubstrate was added to each well. Following incubation (24 h at 27°C), 200 µl of cold 10% trichloroacetic acid (TCA) was added to each well to stop the reaction. The plates were then shaken and centrifuged (2000 × *g*, 60 min). After centrifugation, 60 µl of the supernatant was added to a 96-well plate containing 70 µl of 1 M NaOH in each well, and absorbance was measured spectrophotometrically at 450 nm. The protease activity in the culture supernatant was normalized to the number of parasite cells per milliliter in each original sample, as determined by flow cytometry. Measurements were made on Day 0 (immediately after initiation of the cultures), Day 1 and Day 4. Data are presented as relative protease activity as compared to unsupplemented control cultures after subtraction of protease activity measured in each sample on Day 0, to eliminate activity originating from the experimental supplements themselves.

Effect of pallial mucus on *Perkinsus marinus* virulence

Perkinsus marinus cultures

Two separate experiments were performed to assess the effect of different oyster extracts on *Perkinsus marinus* virulence *in vivo*. Oysters were acclimated and maintained at 25°C (salinity 28) before the beginning of the experiments. In the first experiment, naïve CvME were used to generate experimental culture supplements and for *in vivo* challenge. Cultures of *P. marinus* were seeded at 10^6 cells ml^{-1} in 25 ml culture flasks containing DME/F12-3 culture medium supplemented with pallial mucus (mantle and gill mucus combined), digestive extracts, or plasma (0.6 mg ml^{-1}) pooled from 12 oysters. Cultures (including a control *P. marinus* culture supplemented with SAS) were incubated at 23°C. After 2 wk, aliquots were collected and used to enumerate *P. marinus* cells and remaining cultures were centrifuged (400 × *g*, 15 min, 22°C). The supernatant was then carefully aspirated and replaced with the same volume of SAS and *P. marinus* cultures replaced back in the incubator before being used for challenge on the morning of the next day.

In the second experiment, naïve CvWA were used to generate experimental supplements and for challenge. Based on the results of the first trial, plasma was not used in this experiment. Supplemented (all at 0.3 mg ml^{-1}) and unsupplemented cultures were subsequently handled as described for the first experiment.

Challenge experiments

Naïve *Crassostrea virginica* (CvME and CvWA for Expts 1 and 2, respectively) were carefully notched with bone shears avoiding damage to mantle tissues. Four days following notching, the oysters (20 to 24 oysters per treatment) were injected into the pallial cavity with *Perkinsus marinus* grown in supplemented or unsupplemented media (see preparation above; 2.5 × 10^6 per oyster in 1 ml SAS) through the shell notch using a 23-gauge blunt needle. Subsets of oysters were inoculated with SAS as negative controls. Following inoculation, oysters were covered with damp paper towels for 2 h at 22°C and subsequently returned to separate tanks (3 replicate tanks per treatment) maintained at 25°C. Oysters were fed and monitored daily for mortality. Moribund oysters were immediately removed, and their *P. marinus* loads in whole oyster tissues were determined using alternative Ray's fluid thioglycollate medium (ARFTM) following the procedures described by La Peyre et al. (2003). After 4 wk, the surviving oysters were also processed for *P. marinus* infections using ARFTM. To determine the combined effect of both time to death and *P. marinus* infection intensity (parasite load in wet tissue weight), a virulence index ranging from 0 (least virulent) to 10 (most virulent: short time to death combined with high parasite loads) was calculated as described by Chintala et al. (2002).

Data treatment and statistical analysis

Statistical comparisons of the effect of the different oyster supplements in the *Perkinsus marinus* growth and protease activity experiments were performed using 1-way repeated-measures ANOVA (followed with Holm-Sidak post hoc pairwise tests when applicable) to comparatively assess different extracts from each oyster and eliminate the impact of overall inter-individual variability. Statistical analysis of infection intensities was performed using a 1-way ANOVA followed by Holm-Sidak post hoc test as needed. For categorical data (virulence indices), a 1-way ANOVA on ranks and Dunn's post hoc tests were used. Mortality data, consisting of time of death (i.e. day of experiment) for individual oysters, were compared by Kaplan-Meier log-rank survival analysis with Holm-Sidak post hoc testing for multiple comparisons (Kleinbaum & Klein 2005). Differences were considered significant when $p < 0.05$.

RESULTS

Effect of pallial mucus on *Perkinsus marinus* growth

Oyster *Crassostrea virginica* supplements differentially modulated *Perkinsus marinus* growth. In the CvNY (0.15 mg protein ml^{-1}) experiment, mantle mucus induced a rapid and significant increase (56 % increase after 1 d, $p < 0.01$, Holm-Sidak post hoc test) in the growth of *P. marinus* compared to unsupplemented control cultures (Fig. 1A). In contrast, digestive-gland extracts and plasma were inhibitory and caused a significant reduction (44 and 40 % decrease, respectively) in the growth of the parasite compared to controls on Day 1 ($p < 0.01$), while gill mucus supplements did not show any effect on *P. marinus* growth. On Day 4, *P. marinus* growth remained signifi-

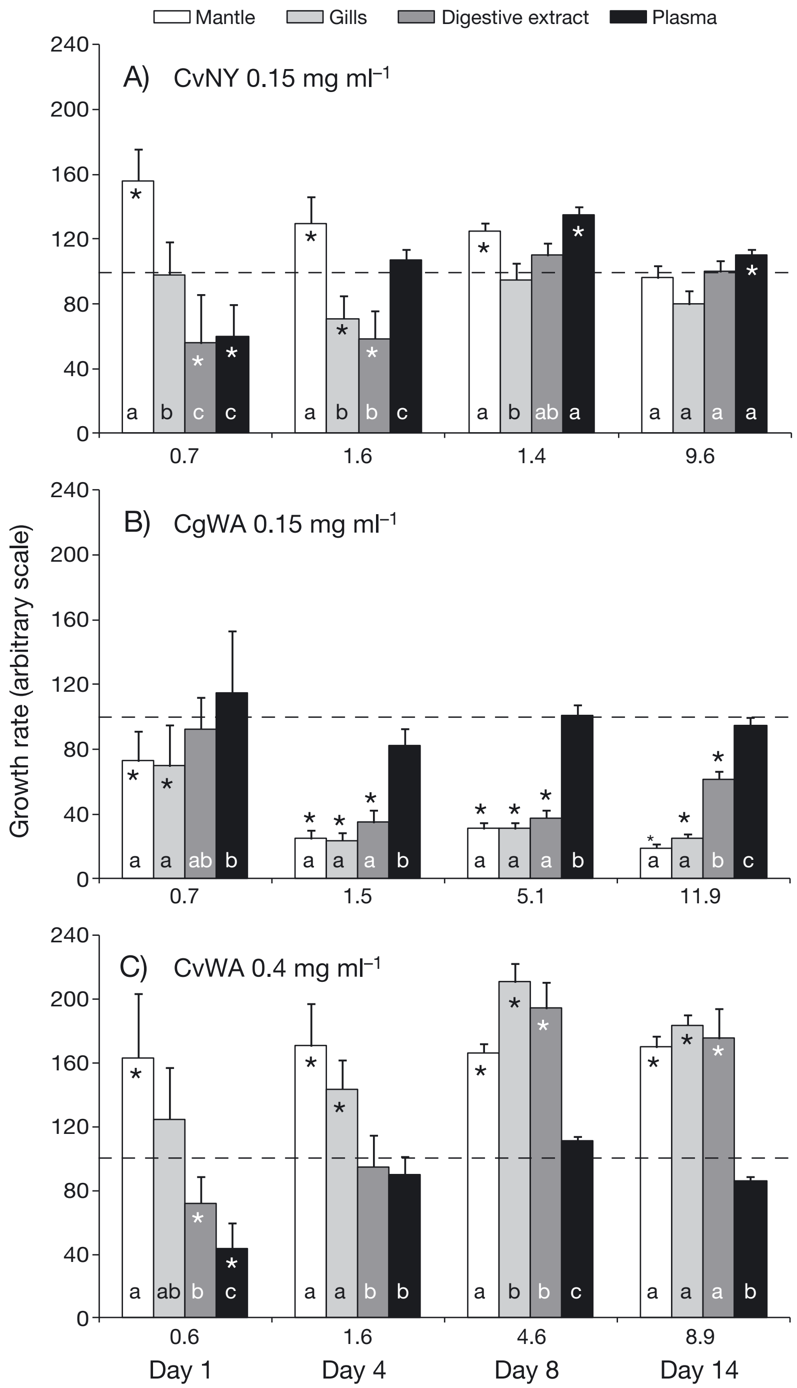

Fig. 1. *Perkinsus marinus*. Replication (arbitrary scale, mean ± SD) in culture media supplemented with different *Crassostrea virginica* extracts (mantle, gills, digestive extract and plasma) as compared to unsupplemented control cultures (100, represented by the dashed line; actual cell counts in control cultures at each sampling time [$\times 10^6$ ml^{-1}] are given below the *x*-axis). For each experiment (CvNY: *C. virginica* from New York; CgWA: *C. gigas* from Washington; CvWA: *C. virginica* from Washington), different letters indicate statistically significant differences between cultures supplemented with different extracts within each sampling time (Holm-Sidak post hoc test, $p < 0.05$, n = 12 oysters per data point). *: statistically significant inhibition or enhancement of growth as compared to unsupplemented controls

cantly higher in cultures supplemented with mantle mucus as compared to those supplemented with gill mucus, digestive-gland extracts, or plasma. Differences between treatments leveled off on Day 8 and disappeared completely on Day 14. Interestingly, different trends were revealed in the second experiment which used culture supplements obtained from *C. gigas* (also 0.15 mg protein ml^{-1}). For instance, mantle and gill mucus, as well as digestive-gland extracts, were strongly inhibitory to *P. marinus* growth, while plasma supplements did not affect parasite growth (Fig. 1B). Throughout this experiment, the highest inhibitory activity was systematically measured in pallial organ mucus. Results from the CvWA experiment (0.4 mg protein ml^{-1}) followed the same trend on Day 1 as the CvNY experiment, with induction of *P. marinus* growth in cultures supplemented with mantle mucus and inhibition of parasite growth in cultures supplemented with digestive-gland extracts or plasma (Fig. 1C). On Day 4, higher parasite growth remained detectable in cultures supplemented with mantle mucus (and to a lesser extent with gill mucus) as compared to the remaining treatments. A marked difference with the first experiment is that digestive-gland extracts in the CvWA experiment induced a higher growth rate of the parasite on Days 8 and 14 as compared to controls.

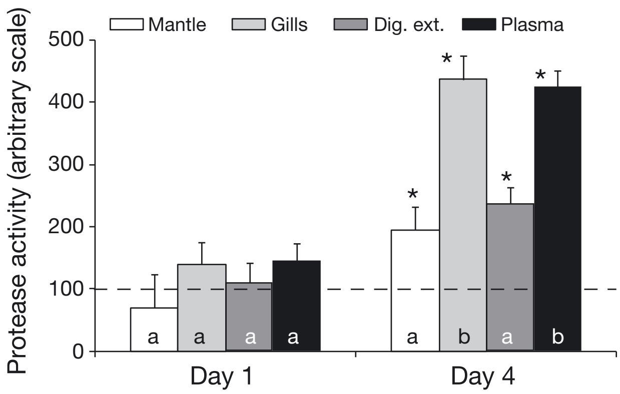

Fig. 2. *Perkinsus marinus*. Protease activity (arbitrary scale, mean ± SD) in supernatants of *P. marinus* cultures supplemented with different oyster *Crassostrea virginica* extracts (mantle, gills, digestive extract and plasma) as compared to unsupplemented control cultures (100, represented by the dashed line). Different letters indicate statistically significant differences between cultures supplemented with different extracts within each sampling time (Holm-Sidak post hoc test, $p < 0.05$, n = 12 oysters per data point). ⋆: statistically significant increase or decrease of protease activity as compared to unsupplemented controls

Effect of pallial mucus on protease production by *Perkinsus marinus*

Protease production by *Perkinsus marinus* was generally similar in all cultures on Day 1, but displayed different trends according to the experimental supplement on Day 4 (Fig. 2). For instance, a higher protease activity was measured in the supernatants of cultures supplemented with gill mucus and plasma as compared to cultures supplemented with mantle mucus or digestive-gland extracts (~100% increase; $p < 0.001$, Holm-Sidak test) or to unsupplemented controls (~300% increase). Among supplemented treatments, protease activity was highest in cultures supplemented with gill mucus and lowest in cultures supplemented with mantle mucus.

Effect of pallial mucus on *Perkinsus marinus* virulence

For both experiments (CvME and CvWA), mortality was most prominent in oysters injected with *Perkinsus marinus* cultures supplemented with pallial mucus (Fig. 3, $p < 0.01$ Holm-Sidak test). In this treatment, mortality reached 20% (CvME) and >50% (CvWA) by Day 10 and peaked at 40% (CvME) and 67% (CvWA) at the end of the 4 wk experiment. Mortality was lower in oysters injected with unsupplemented parasite cultures (20 and 10% for CvME and CvWA, respectively) or with *P. marinus* supplemented with plasma or digestive extracts (5 to 10%) and was comparable to that found in unchallenged oysters (5%). All of the moribund oysters removed before the end of the 4 wk experiment were processed immediately for prevalence and intensity of *P. marinus* by ARFTM. *P. marinus* was detected in all moribund oysters from the pallial mucus, the plasma and digestive tract treatments, but in none of the moribund oysters removed from the unchallenged control treatments (for both CvME and CvWA).

After the 4 wk period, all surviving oysters were processed for *Perkinsus marinus* prevalence and intensity. In the CvME experiment, the overall prevalence (including moribund oysters) was 100% for the pallial mucus, digestive extract and plasma treatments. Among oysters injected with unsupplemented *P. marinus* cultures, 90% were infected, and no infections were detected in the negative control treatment. In the CvWA experiment, all survivors from the pallial mucus treatment were found to be negative for *P. marinus* infection, resulting in an overall infection prevalence of 67%. In contrast, even though mortality in oysters injected with *P. marinus* supple-

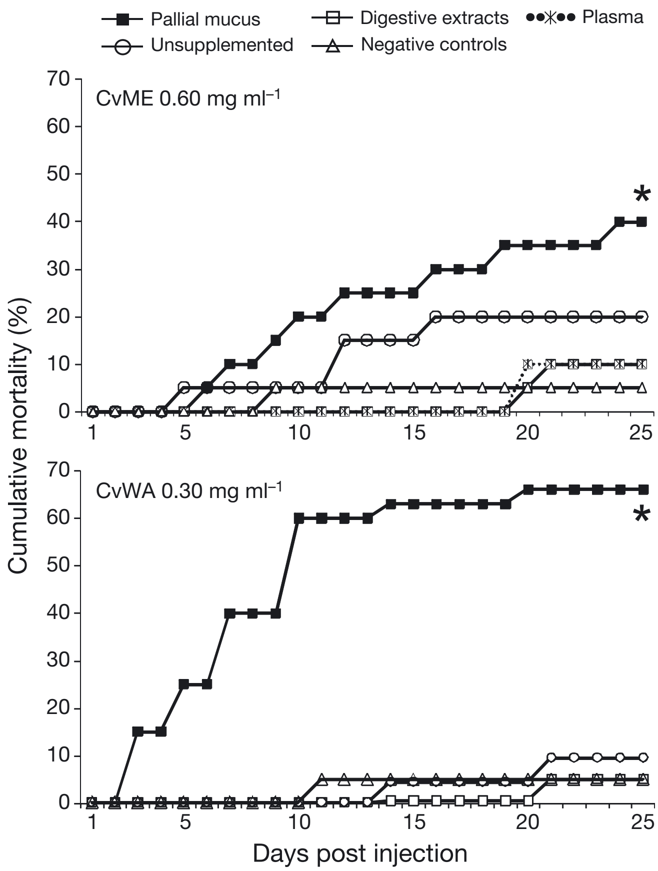

Fig. 3. *Perkinsus marinus*. Cumulative mortality (%) in *Crassostrea virginica* (CvME or CvWA: from Maine or Washington) challenged with *P. marinus* grown in media supplemented with different oyster extracts (mucus, digestive extracts and plasma [CvME only]). Negative control oysters were injected with seawater only. *: statistically higher mortality levels than all other treatments (log-rank test, $p < 0.05$)

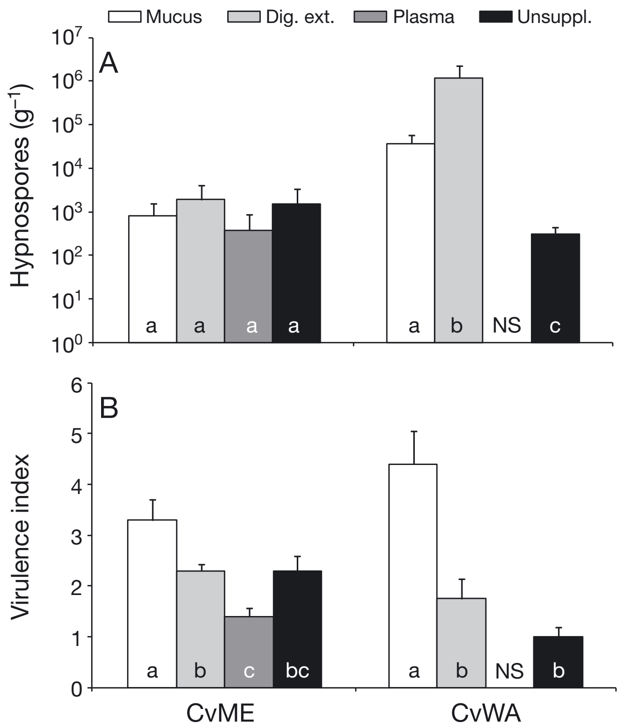

Fig. 4. *Perkinsus marinus*. Mean ± SD (A) loads and (B) virulence indices in *Crassostrea virginica* (CvME or CvWA: from Maine or Washington) challenged with parasite cells grown in media supplemented with different oyster extracts (mucus, digestive extract and plasma). Different letters indicate statistically significant differences (Holm-Sidak post hoc test, $p < 0.05$; $n = 20$ to 24 oysters per treatment) between treatments within each experiment. NS: not sampled

mented with digestive extracts was very low, 70 % of survivors in this batch were infected with *P. marinus*. Among oysters injected with unsupplemented parasite cultures, 44 % were infected, and no infections were detected in the negative control treatment.

In the CvME experiment, overall parasite loads in oysters injected with *Perkinsus marinus* cultures supplemented with pallial mucus, digestive extracts and plasma were 8.3×10^2, 4.9×10^3 and 3.8×10^2 hypnospores g^{-1}, respectively (Fig. 4A). These levels were within the same range as parasite loads measured in oysters injected with unsupplemented cultures (1.5×10^3 hypnospores g^{-1}). In the CvWA experiment, parasite counts were 3.7×10^4, 1.2×10^6 and 3.1×10^2 hypnospores g^{-1} for oysters injected with *P. marinus* cultures supplemented with pallial mucus, digestive extract and unsupplemented cultures, respectively (Fig. 4A).

The virulence index was significantly higher ($p < 0.05$, Dunn's post hoc test) for *Perkinsus marinus* cultures supplemented with pallial mucus (3.3 for CvME and 4.4 for CvWA; Fig. 4B) as compared to cultures supplemented with digestive extracts (2.3 and 1.6, respectively) or plasma (1.4 for CvME, not sampled for CvWA) or to unsupplemented cultures (2.3 and 0.9, respectively).

DISCUSSION

Mucus covering bivalve pallial organs represents the first host factor encountered by waterborne microbes. In the case of *Perkinsus marinus*, increasing evidence suggests that pallial organs (mantle, gills) of *Crassostrea virginica* represent an important portal of entry for the initiation of infection (Dungan et al. 1996, Bushek et al. 1997, Winnicki et al. 2008, Allam et al. 2013). This study focused on the investigation of the effects of mucus covering pallial organs on *P. marinus* and showed significant changes in the physiology of the parasite (growth, metabolism and virulence) following exposure to oyster pallial mucus.

Host mucus enhances *Perkinsus marinus* growth

Perkinsus marinus growth *in vitro* was rapidly enhanced in cultures supplemented with pallial mucus from susceptible *Crassostrea virginica* as compared to unsupplemented cultures, but was inhibited when digestive extracts or plasma were used. Previous studies using *C. virginica* whole-tissue homogenates or plasma also showed a reduction in *P. marinus* proliferation rates (Earnhart et al. 2004, Brown et al. 2005) even though comparison between our study and previous work is difficult since previous reports only assessed long-term effects (4 to 6 wk) of oyster supplements. Results presented here are also in agreement with those of a preliminary study showing rapid enhancement of parasite growth in cultures supplemented with mantle mucus and a reduction of growth in cultures added with digestive extracts or plasma (Allam et al. 2013). Earnhart et al. (2004) demonstrated a reduction in *P. marinus* proliferation associated with an increase in cell size in cultures supplemented with oyster homogenates. The size of *P. marinus* cells was not determined in this study, but qualitative microscopic monitoring of the cultures showed generally larger parasite cells in culture supplemented with mantle mucus as compared to unsupplemented controls.

Collectively, these results show that oyster tissues and secretions contain factors that reduce (plasma and tissue homogenates in Gauthier & Vasta 2002, Earnhart et al. 2004, Brown et al. 2005; the present study for plasma) or enhance (mantle mucus in the present study) *Perkinsus marinus* growth. It is noteworthy that the growth enhancing or inhibitory effects of the different *Crassostrea virginica* supplements were temporary and progressively disappeared when a low protein concentration (0.15 mg ml^{-1}) was used, but remained more obvious at the end of the experiment that used a higher protein concentration (0.4 mg ml^{-1}). Despite the fact that these 2 experiments used oysters from different geographical origins, these results suggest a dose- and time-dependent response of *P. marinus* cultures to oyster supplements. Overall, the most interesting trend in both experiments is that oyster secretions contain factors that rapidly (<1 d) modulate the physiology of the parasite. This result emphasizes the importance of investigating the early effects of supplements on *P. marinus* growth, especially since it is likely that some growth-promoting or -inhibitory factors are quickly degraded after their introduction into the culture medium.

Results from the *Crassostrea gigas* (resistant oysters) experiment show very dissimilar trends compared to those obtained in *C. virginica*. For instance, pallial mucus (from both mantle and gill), as well as digestive extracts from Pacific oysters, inhibited *Perkinsus marinus* growth. This strong inhibitory effect was not observed in cultures supplemented with *C. gigas* plasma (comparable to seawater) in agreement with the results of Gauthier & Vasta (2002) who concluded that resistance of *C. gigas* to *P. marinus* infection may be derived from cellular and not humoral factors.

This preliminary research does not provide information about the nature of growth-inhibitory or -promoting factors. A variety of antimicrobial factors, including chlorinated acetylenes (Walker & Faulkner 1981), terpenes (Ireland & Faulkner 1978), indole derivates (Benkendorff et al. 2001), glycerol derivates (Gustafson & Andersen 1985) and glycoproteins (Yamazaki 1993) have been isolated from mollusks. Previous research identified specific proteases and protease inhibitors in oyster plasma that impact *Perkinsus marinus* (Romestand et al. 2002, Xue et al. 2006). On the other hand, mucus secretion can favor the growth of adapted (or specialized) microbes in marine organisms. For instance, bacterial growth is enhanced, including the opportunistic *Vibrio alginolyticus*, in media supplemented with coral mucus (Ducklow & Mitchell 1979, Ritchie 2006). Fish mucus contains factors that enhance or inhibit the growth of different bacterial species (Ebran et al. 1999, Nagashima et al. 2003, Vine et al. 2004). Mucus secretions of the squid *Euprymna scolopes* differentially regulate the dynamics of microbial communities of the light organ to favor the survival and growth of its symbiont *Vibrio fischerii* (Davidson et al. 2004). While the mechanisms of antimicrobial activity of mucus have been the subject of different studies, to our knowledge, no previous studies have focused on the characterization of mucus factors that promote microbial growth in marine organisms.

Host mucus enhances *Perkinsus marinus* virulence

Earnhart et al. (2004) showed increased infectivity (as determined by high hypnospore counts) in parasite cultures supplemented with whole oyster homogenates. Results obtained in the present study demonstrated that the modulation of parasite virulence depends upon the different oyster supplements used. For both challenge experiments, mortality was significantly higher in oysters injected with parasite cells grown in media supplemented with pallial mucus, but not in those supplemented with digestive extracts or plasma. Notably, oysters injected with *Perkinsus marinus* supplemented with mucus showed high early phase mortality, similar to that described by Ford et al. (2002a) who used wild-type *P. marinus* freshly isolated from infected oysters to inoculate naïve specimens. All oysters used in the present work were injected in the pallial (shell) cavity; hence, mortality caused by injection trauma is expected to be minimal and similar across treatments. Another noteworthy element is that infection intensity was generally higher in oysters injected with parasite cells from cultures supplemented with digestive extracts as compared to those supplemented with mucus even though mortality was higher in the latter group. In other words, *P. marinus* cells grown in media supplemented with digestive extracts proliferate well in oyster tissues causing heavy infections (as determined by high hypnospore counts) without causing mortality, suggesting a lower virulence of the parasite in this treatment. In contrast, rapid and high levels of mortality were measured in oysters exposed to parasites enhanced with pallial mucus, suggesting significantly enhanced virulence for the parasite under these conditions. The calculated virulence index supports this hypothesis and indicates that *P. marinus* enhanced with pallial mucus is significantly more virulent than both *P. marinus* enhanced with digestive extract and unsupplemented media (Fig. 4B). Similarly, Ford et al. (2002a) found that wild-type *P. marinus* was more virulent than cultured parasites based on their virulence indices, even though they found similar and relatively low infection intensities (hypnospore counts) between both treatments. Ford et al. (2002a) clearly demonstrated that cultured parasites lost their virulence immediately, most likely due to the inability of the culture environment to induce the parasite to produce virulence factors. Our results suggest that pallial mucus is able to activate cultured *P. marinus* to restore the virulence of the parasite. From these results, it is evident that parasite–host interactions are complex and that different virulence-related factors may control the balance between early phase mortality and the establishment of heavy infection without causing mortality. This suite of results suggests that the mechanisms involved in the proliferation of the parasite in oysters may be different from those causing the rapid deleterious impact leading to mortality of the host.

Results reported in Fig. 2 showed an increase in protease activity in extracellular products from all supplemented parasite cultures as compared to unsupplemented controls, in agreement with previous studies showing enhanced protease production in *Perkinsus marinus* cultures supplemented with oyster plasma or tissue homogenates (MacIntyre et al. 2003, Brown et al. 2005). Nevertheless, protease activity was lowest in cultures supplemented with pallial mucus as compared to the other supplemented treatments despite the fact that parasite cells from these cultures were highly virulent. These findings suggest that extracellular proteases may not be involved in the rapid oyster mortalities shown here, even though they have been reported as virulence factors capable of lysing oyster hemolymph proteins (La Peyre et al. 1995). Other factors thought to be involved in *P. marinus* virulence but that were not assessed in our study include anti-oxidant enzymes (Schott et al. 2003a) and metal-carrier proteins such as natural resistance-associated macrophage protein (Lin et al. 2011). While these factors may be involved in parasite survival and proliferation in host tissues, they are unlikely to be involved in the rapid oyster mortalities reported here with mucus-supplemented cultures or by Ford et al. (2002a) using wild-type parasite cells. Ford et al. (2002a) also reported high mortality levels in oysters displaying relatively low infection intensities (low hypnospore counts). These findings highlight the need for a more thorough investigation of the *P. marinus* virulence factors involved in rapid oyster death.

In conclusion, this study showed that *Crassostrea virginica* pallial mucus plays a significant role in the pathogenesis of *Perkinsus marinus*. These findings further support the infection model proposed for this parasite (Allam et al. 2013), which emphasizes the role of pallial organs and oyster mucus secretions in the pathogenesis of perkinsiosis in *C. virginica*. The contrasting results obtained here with the resistant oyster species (*C. gigas*) suggest that *P. marinus* host specificity may begin in the mucus. A characterization of molecular changes in *P. marinus* in response to mucus exposure is currently underway to identify the putative virulence factors involved in rapid oyster mortality. The identification of mucus factors involved in the rapid modulation of *P. marinus* physiology requires additional studies.

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