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The Influence of Pallial Mucus from the Oyster, *Crassostrea virginica,* on the Virulence of its Pathogenic Alveolate, *Perkinsus marinus*.

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Abstract of the Thesis

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Perkinsus marinus, a protistan parasite of the Eastern oyster (*Crassostrea virginica*), has been a contributing factor to the severe decline of farmed and wild oysters on the East Coast of the United States and the Gulf of Mexico. Recent findings suggest that the pallial organs (mantle, gills) of the Eastern oyster are an important portal of entry for the parasite. In this case, the first contact *P. marinus* has with its host takes place in mucus covering the mantle and gill tissue. This study consisted of several experiments to investigate the effect of oyster pallial mucus on the growth, expression of virulence genes (*pm*SOD1, *pm*SOD2 and *pm*SUB), protease production and infectivity of *P. marinus*. In each experiment, *P. marinus* grown in pallial mucus (mantle, gill or both) was compared to *P. marinus* grown in media supplemented with seawater (control) and other experimental media such as oyster plasma or digestive extracts since the digestive tract is traditionally considered as the main portal of entry for the parasite. *P. marinus* grown in media supplemented with mantle mucus showed a significantly higher growth rate than cultures added with other supplemental extracts, while cultures grown in gill mucus

promoted a higher protease production. No differences were noted in the expression of virulence-related genes between cultures supplemented with mantle or gill mucus as compared to those added with seawater, however those grown in digestive extract or plasma showed a down-regulation compared to control (seawater) cultures. Lastly, challenge experiments showed that parasite cultures grown in pallial mucus caused severe early mortality of oysters coupled with high infection intensities, whereas oysters injected with cultures grown in seawater showed minimal mortalities and no mortalities were found in oysters injected with cultures supplemented with digestive extract during the four week experiment. These results demonstrate that oyster mucus plays a significant role in the pathogenesis of *P. marinus* by enhancing the proliferation and the infectivity of this devastating parasite.

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I. Introduction

The research presented in this study is aimed to better understand the early interactions between waterborne pathogens and their bivalve hosts, in particular the protistan parasite *Perkinsus marinus* and the oyster *Crassostrea virginica*. Specifically, the study focused on the potential role of mucus covering oyster pallial organs (mantle and gill) during early host-pathogen interactions. Pallial mucus is traditionally thought to serve as a physical and biochemical barrier playing protective roles for the organism, therefore limiting the deleterious effects of invading pathogens. The main hypothesis of this study is that specialized (or adapted) pathogens, such as *P. marinus*, are capable of diverting this barrier to their advantage facilitating entry into their hosts. This study is the first to address the role of bivalve pallial mucus directly on the physiology and infectiveness of waterborne pathogens. The eastern oyster, *Crassostrea virginica*, was used in this study because of its economical and ecological importance and because of the devastating impact of *P. marinus* on wild and aquacultured stocks. Although it would be likely difficult to develop methods to eradicate this disease, understanding early host-pathogen interactions and factors that facilitate infection (or resistance) could lead to the development of new mitigation strategies including the development of inhibitory drugs or therapeutics that are targeted to preventing the initial stages of disease development. Unraveling the role of mucus in the infection processes could also serve as a model for other host - pathogen models in invertebrates or vertebrates. Therefore, this research has implications beyond the interactions between *P. marinus* and *C. virginica*, and can thus serve as a model to eventually help treat other important disease problems.

1. *Perkinsus marinus*

a) History and life stages

Since the middle of the twentieth century, concern over introduction of disease has been stimulated by epizootic mortalities associated with pathogens in several species of oysters on the east coast of the United States and in Western Europe (Ford 1992). These epizootics, initially caused by transplantations and introductions of oysters, have been sustained in the environment by changes in temperature and salinity regimes (Ford 1992). Epizootics can have a devastating effect on wild oyster populations as well as commercially aquacultured oyster beds. One particular parasite, *Perkinsus marinus* (Dermo) has caused severe epizootics in the Eastern oyster (*Crassostrea virginica*) throughout the east coast of the United States. Dermo is a protistan parasite (Ragone Calvo *et al.* 2000) and is now widely distributed in *C. virginica* populations from the Gulf of Mexico to Maine (Bobo *et al.* 1997). In the 1950s, the distribution of *P. marinus* was limited to the Chesapeake Bay and the Gulf of Mexico (Andrews 1988); however transportation of infected Eastern oysters as well as winter warming linked to climate change has greatly extended the range of this parasite (Ford and Chintala 2006).

The life cycle of *Perkinsus marinus* is composed of three main stages including trophozoite, hypnospore and zoospore, all of which can be infective to *C. virginica*. The trophozoite stage occurs in the live tissues of the host where it reproduces using vegetative proliferation creating up to 32 daughter cells. These cells reside in live tissues or can be released through feces or diapedesis and shedding of infected hemocytes (Villalba *et al.* 2004, Bushek *et al.* 2002). Hypnospores are trophozoites that become enlarged under hypoxic conditions usually found in dead oysters or when host tissues are incubated in Ray's fluid thioglycollate medium (RFTM) (Villalba *et al.* 2004, Bobo *et al.* 1997, Andrews 1996). Hypnospores can survive "dormant" for long periods of time under unfavorable conditions such as high chlorine, low temperatures and low pH (Villalba *et al.* 2004). When conditions become favorable, hypnospores produce

flagellated zoospores that are released into the water column (Villalba *et al.* 2004, Perkins 1996). The most infective of these stages for oysters is still unknown (Villalba *et al.* 2004).

P. marinus is routinely cultured *in vitro* using standard cell culture media, however Ford *et al.* (2002) has demonstrated that the virulence of the cultures becomes attenuated over time compared to wild-type *P. marinus*. In addition, the life stages of *P. marinus in vitro* are present in different proportions to those found in the oysters' tissues (Ford *et al.* 2002). To better reflect *in vivo* host - pathogen interactions, researchers have developed methods to enhance the virulence of *in vitro* cultures by supplementing the growth media with oyster plasma or tissue extracts (Gauthier and Vasta 2002, MacIntyre *et al.* 2003, Earnhart *et al.* 2004, Brown *et al.* 2005). Although many of these studies had found a lower proliferation of *P. marinus* when cultures were supplemented with oyster extracts, several other culture attributes have improved such as infectivity (Earnhart *et al.* 2004), protease expression (MacIntyre *et al.* 2003, Brown *et al.* 2005) and a cellular morphology more closely resembling parasite cells found *in vivo* (MacIntyre *et al.* 2003, Earnhart *et al.* 2004).

b) Oyster - *Perkinsus marinus* interactions

Dermo has adopted several strategies allowing its proliferation inside the tissues of *C. virginica*. Gauthier and Vasta (2002) suggested that surface structures or, chemotactic factors produced by *P. marinus* cells, allow the parasite to initially gain entry into hemocytes after conducting a study where hemocyte uptake of live *P. marinus* was significantly greater than the uptake of glutaraldehyde-fixed *P. marinus*. Oyster hemocytes readily phagocytose *P. marinus*, however the parasite is able to evade intracellular destruction by employing mechanisms that allow it to either suppress the respiratory burst in infected hemocytes or tolerate resulting toxic reactive oxygen species (ROS) (Volety and Chu 1995, Ahmed *et al.* 2003, Schott *et al.* 2003). This ultimately allows Dermo to divide and proliferate inside hemocytes inducing their rupture and

release of daughter parasite cells (Villalba *et al.* 2004). A study involving *P. marinus* and plasma from the Pacific oyster (*C. gigas*), which is resistant to *P. marinus* infection, has also implicated that the virulence of Dermo to *C. virginica* is associated with the ability of the parasite to be phagocytosed and proliferate within *C. virginica's* hemocytes (Gauthier and Vasta 2002).

The capacity of *P. marinus* to suppress hemocyte respiratory burst was suggested to result from its ability to produce superoxide dismutases (SOD), a group of enzymes that protect intracellular parasites from oxidative damage (Ahmed *et al.* 2003) by converting the toxic reactive oxygen intermediates into less harmful products (Wright *et al.* 2002). Two Fe SOD genes, *Pm*SOD1 and *Pm*SOD2 have been identified from *P. marinus* (Schott and Vasta 2003, Wright *et al.* 2002). *Pm*SOD1 is directed to mitochondria and Schott and Vasta (2003) proposed that the gene product has a role in the virulence of *P. marinus* by allowing Dermo to resist the oxidative damage from hemocytes. Whereas the role of *Pm*SOD2 remains uncertain, Fernandez-Robledo *et al.* (2008) suggested it is directed to single-membrane subcellular compartments possibly relic of a plastid. Both of these *pm*SOD genes may represent virulence factors that help *P. marinus* disable hemocytes ROS defenses allowing parasite survival in infected cells.

Dermo also uses circulating hemocytes to disseminate throughout oyster tissues. Hemocyte motility *in vitro* is enhanced when hemocytes are exposed to *P. marinus* cells, however this is counteracted by extracellular products (ECP) released by *P. marinus* cells (Villalba *et al*. 2004). *P. marinus* ECP contain enzymes that degrade the proteins laminin, fibronectin and proteins present in oyster hemolymph. These protein degradations ultimately reduce oyster defenses (Garreis *et al.* 1996) and are thought to cause cellular and tissular damages that aid parasite invasion of host tissues (La Peyre *et al.* 1995). The best described components of *P. marinus* ECP are serine proteases (La Peyre *et al.* 1995) and a subtilisin serine protease gene (*Pm*SUB) has already been molecularly identified (Brown and Reece 2003). Phylogenetic analysis relates the *Pm*SUB to subtilisin-like serine proteases found in *Toxoplasma gondii* and *Plasmodium*

falciparum with functions suspected to be involved with tissue degradation as well as host immune suppression (Brown and Reece 2003, Brown *et al.* 2005). All of the above virulence factors help create conditions that allow the proliferation of Dermo within *C. virginica*. Although it is becoming better understood how *P. marinus* is able to create severe infections once inside its host, it is still unclear how the parasite initially gains access and invades *C. virginica*.

c) Initial parasite acquisition

The portal and mechanism of entry of *P. marinus* into oyster tissues remain unclear. Histological analyses of infected *C. virginica* had initially suggested that the gut is the principle portal of entry after *P. marinus* is ingested (Mackin 1951, Mackin and Boswell 1956). More recently, however, through an immuno-labeling study, *P. marinus* was found in pallial organs (mantle, gill and palp) during early infections suggesting an alternate route of entry into *C. virginica* (Dungan *et al*. 1996). Despite the fact that earlier studies reported most infections in the digestive epithelium, Dermo 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. This further supports that infection may be initiated in pallial organs.

Further evidence for initial infection in pallial organs was also shown in other *Perkinsus* species such as *P. olseni* which infect its host, *Tapes decusatus* (clam), through gill and palp tissue (Villalba *et al*. 2004). Initial infections in the pallial organs is also evident in the oyster *Saccostrea glomerata* by the paramyxean parasite *Marteilia sydneyi* that also infects the oyster through the palp and gills (Kleeman *et al*. 2002) and by the protozoan parasite *Haplosporidium nelsoni* that invades *C. virginica* through gill tissue (Burreson and Ford 2004). The specific mechanisms these parasites use to initially infect their host through pallial organs may be different, however they all demonstrate parasites' ability to breach pallial epithelial layers.

In addition to these previous findings, a recent study in our laboratory (Marine Animal Disease Lab) also demonstrated that the pallial organs of *C. virginica* are an important site of initial *P. marinus* infection (Winnicki *et al.* 2008). In this study, *C. virginica* were exposed to freely suspended or aggregated *P. marinus* cells and parasite loads were separately determined in visceral mass, gills, mantle, and the pseudofeces discharge area (PDA). The PDA, located ventro-laterally to the palps, is a specialized region of the mantle used for the accumulation and rejection of pseudofeces. Among all biopsies, the PDA displayed the highest parasite loads when infected by *P. marinus* in aggregates suggesting that *P. marinus* can be acquired in oysters through the pseudofeces discharge area and that parasite uptake is enhanced if presented in association with aggregates (Figure 1). In concordance with these findings, Ray (1966) also suggested that the anterior mantle located just lateral to the palps (also the PDA) showed more parasites than the mantle tissue near the adductor muscle.

2. Role of pallial mucus

The above results support that *Perkinsus marinus* invades its oyster host through the epithelial layers of oyster mantle and therefore the first contact Dermo has with *C. virginica* is with mucus covering this pallial organ. Mucus produced by bivalves plays an important protective role against 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, Pales Espinosa *et al*. 2009). Despite the defensive role mucus plays, mucus in marine invertebrates also appears to be used by some pathogens to their advantage. For example, *Vibrio shiloi*, a bacterial pathogen of corals, adheres to β-D-galactoside-containing receptors in corals' mucus in order to gain entry into the epidermal layers of the coral (Banin 2001).

Similarly, Dermo may also use oyster's mucus to its advantage. Recognition of *P. marinus* by host membrane-bound or soluble lectins has been hypothesized to involve surface determinants on the parasites cell and adherence of the parasite to host epithelial

cells and hemocytes may also involve these surface carbohyrates (Gauthier *et al*. 2004). Tasumi and Vasta (2007) recently discovered a galactose-binding lectin (called CvGal galectin for *C. virginica* galectin) which recognizes and binds exogenous ligands on microorganisms such as microalgae and *P. marinus*. These authors suggested that *P. marinus* uses CvGal recognition to its advantage by favoring phagocytosis of parasite cells by hemocytes to help its spread throughout oyster tissues.

Extracellular proteins released by *P. marinus* can also modify oyster defenses (Garreis *et al.* 1996) and contribute to an increased infection in *C. virginica* (La Peyre *et al.* 1995). Earnhart *et al.* (2004) showed that the infectivity of *P. marinus* increases when cultures are supplemented with oyster tissue homogenate, therefore suggesting the presence of factors in oyster tissues modulating the parasite's virulence. Based on this result and on the fact that the first contact between *P. marinus* and *C. virginica* occurs in mucus, it is relevant to evaluate the effect of pallial mucus on *P. marinus* cells. 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 could cause an "activation" allowing parasite cells to produce and release virulence factors like proteases or modify the expression of other virulence-related genes. Such virulence factors could facilitate the survival of parasite cells in host cells and tissues therefore enhancing the invasion mechanism.

Objective and Hypothesis

The overall objective of this study is to assess the effect of oyster pallial mucus on *P. marinus*. The main hypothesis is that contact of *P. marinus* with pallial mucus causes significant changes in parasite's metabolism, leading to an increase in the expression of virulence factors and an overall increase in infectivity.

II. Methods

1. Materials

a) Oysters

The oysters used in the following experiments were obtained from several sources. Large *C. virginica* used in Experiment 1 were obtained from Oyster Bay (NY). The Pacific oyster (*C. gigas*) also used in Experiment 1 were obtained from Taylor Shellfish Farm (WA). Taylor Shellfish Farm also provided the remaining naïve *C. virginica* oysters that were utilized in Experiments 2, 3 and 4. Upon arrival to the lab all oysters were scrubbed and placed into seawater (28 ppt) maintained at 25°C.

b) *Perkinsus marinus*

Perkinsus marinus (ATCC-50439) was provided by Christopher Dungan (Maryland Department of Natural Resources). Parasite cultures were maintained in DME/F12-3 growth medium (see composition in Appendix 1) in an Ambi-Hi-Lo® Chamber (Lab-Line Instruments Inc) incubator at 23°C. All experiments used exponentially growing cultures (8 to 10 days old). The day prior to their use, cultures were centrifuged (400 x *g*, 23°C), media aspirated and replaced with sterile 0.22 μm filtered artificial seawater (28 ppt). Replacing the media with seawater the day prior to experimentation was performed to mimic natural conditions facing waterborne *P. marinus* cells.

2. Collection of plasma, pallial mucus and digestive extract

In experiments using plasma, oysters were carefully notched with bone shears close to the adductor muscle. Hemolymph was withdrawn from the adductor muscle using a syringe fitted with an 18-gauge needle. The extracted hemolymph was placed into 2 mL Eppendorf tubes on ice until ready for centrifugation. The hemocytes were removed by centrifugation at 900 x *g* for 5 minutes at 4°C. The plasma supernatant was

collected and filtered through 0.22-μm syringe filters. Sterile plasma was stored on ice until its use as a media supplement, typically within the following four hours.

To obtain gill and mantle mucus, the oysters were carefully opened with an oyster knife and tissues were rinsed with seawater (28 ppt). Mucus was collected separately (Experiments 1, 2 and 3) or combined (Experiment 4) from gills and mantle by using cotton swabs following the general procedures described by Pales Espinosa *et al*. (2009). The tips of each cotton swab were removed and placed into 15 mL Falcon tubes containing 7 mL ice-cold seawater (28 ppt). Each tube was placed at 4°C and gently shaken. After one hour, the liquid was poured (excluding tips) into another 15 mL Falcon tube for centrifugation at 900 x *g* for 15 minutes at 4°C. The supernatant was collected and filtered through 0.22-μm syringe filters. Sterile mucus extracts were stored on ice until their use as supplements to culture media.

Following the collection of mucus extract, the digestive gland of each oyster was dissected and finely minced using a razor blade. The minced tissue was suspended in 15 mL Falcon tubes containing 5 mL of ice-cold seawater (28 ppt). Each tube was placed at 4°C and gently shaken. After one hour the liquid was carefully poured (excluding minced tissues) into another 15 mL Falcon tube for centrifugation at 1,000 x *g* for 30 minutes at 4°C. To ensure removal of all tissues, this supernatant was poured off and centrifuged again at 1,000 x *g* at 4°C for 30 minutes. Following centrifugation the extract was filtered through 1-μm syringe filters immediately followed by 0.22-μm syringe filters. Sterile digestive extracts were also stored on ice until their use as media supplements.

Protein concentrations of plasma, pallial mucus extracts and digestive extracts were determined using a bicinchronic acid assay (BCA; Pierce, Rockford, Illinois). All samples within each experiment were adjusted with sterile seawater to equivalent protein concentrations before their use as supplements to culture media.

3. Effect of oyster supplements on *P. marinus* growth

a) Experiment 1: Comparison of *C. virginica* and *C. gigas*

This experiment compared the effect of mucus from two oyster species shown to have differing resistance to *P. marinus*, namely the eastern oyster *Crassostrea virginica* (susceptible) and the Pacific oyster *C. gigas* (resistant). Plasma, mantle mucus, gill mucus and digestive extract were collected from twelve large *C. virginica* (NY) using the procedure described above. Following extraction, supplemented cultures were separately prepared in 12-well plates by combining 1 mL of filtered DME/F12-3 culture medium, 165 μL of exponentially grown culture maintained overnight in sterile seawater at 1.2 x 10^7 cells mL^{-1}, experimental supplements (0.15 mg protein mL^{-1}) and were adjusted to 2.5 mL with sterile artificial seawater (SAS, sterilized by filtration through a 0.22 μm filter). Two series of control cultures were prepared by replacing the experimental supplements with SAS or with standard culture medium. Culture plates were wrapped with paraffin tape to avoid evaporation and kept in the incubator at 23°C. Subsamples of 200 μL were taken at days 0, 1, 4, 8 and 15 and were preserved in 33% ethanol at 4°C until processed for flow cytometry (FCM). This same procedure was followed using ten large *C. gigas* (WA).

FCM analyses of *P. marinus* cell counts in the 200 μL subsamples were carried out using a BD FACSCalibur™ Flow Cytometer. Prior to FCM, samples were labeled with SYBR Green I (stock solution at 10,000x) at a final concentration of 10x and were incubated in the dark for 1 hour. SYBR Green I binds to DNA producing a green fluorescence signal that allows for differentiation of cells from cell debris or instrument noise. Data acquisition was set to 30 sec at the low flow rate (12 μL min^{-1}). FCM data was analyzed using BD Cell Quest™ Pro software (version 5.2.1) and *P. marinus* cells were identified according to forward scatter (FSC) and yellow-green fluorescence (FL-1).

b) Experiment 2: Effect of high levels of oyster extracts on *P. marinus* growth

Plasma, mantle mucus, gill mucus and digestive extract were collected from twelve large *C. virginica* (WA) as described above. Following extraction, supplemented cultures were prepared in 12-well plates by combining 1 mL of filtered DME/F12-3 culture medium, 1 mL of exponentially grown culture at 2.5 x 10^6 cells mL^{-1}, experimental supplement added at 0.4 mg protein mL^{-1} and samples were adjusted to 4 mL with SAS. As a control, cultures were also prepared by replacing oyster extracts with SAS or DME/F12-3 culture medium. Culture plates were wrapped with paraffin tape to avoid evaporation and kept in the incubator at 23°C. Subsamples of 100 μL were taken at days 0, 1, 4, 8 and 15 and were preserved in 33% ethanol at 4°C until processed for FCM.

FCM analyses of *P. marinus* cell counts were determined as described above with slight modifications. To minimize instrument noise due to the presence of ethanol, the supernatant was aspirated from the subsamples after centrifugation at 3,000 x *g* for 5 minutes and was replaced with SAS. Unlike Experiment 1, samples were not labeled with SYBR Green I. Instead, 3 μm diameter beads were utilized as internal size control and *P. marinus* cells were identified according to their forward scatter (FSC) and side scatter (SSC).

4. Effect of oyster extracts on *P. marinus* virulence and infectivity

a) Experiment 3: Effect of oyster extracts on the production of virulence factors

Sample preparation

Plasma, mantle mucus, gill mucus and digestive extract were collected from 12 large *C. virginica* (WA) as described above. In order to obtain a high initial concentration of *P. marinus* cells, the cultures were concentrated five fold by

centrifugation at 400 x *g* and a portion of the seawater supernatant was removed. Supplemented cultures were prepared in 12-well plates by combining 1 mL of filtered DME/F12-3 culture medium, 1 mL of exponentially grown culture at 1.2 x 10^7 cells mL^{-1}, experimental supplement added at 0.4 mg mL^{-1} and samples were individually adjusted to 4 mL with SAS. As a control, cultures were also prepared by replacing oyster extracts with SAS or DME/F12-3 culture medium. Culture plates were wrapped with paraffin tape to avoid evaporation and kept in the incubator at 23°C.

Subsamples of 1 mL and 100 µL were taken at days 0, 1 and 4. The 1 mL subsamples were centrifuged at 400 x *g* for 10 minutes at room temperature. Culture supernatant was poured off into 1.5 mL Eppendorf tubes and was stored at -80°C until use for protease activity determination. The remaining pellets were also stored at -80°C until use for gene expression analysis. The 100 µL subsamples were preserved in 33% ethanol at 4°C for determination of cell counts using FCM as described in Experiment 2.

Gene expression

Three *P. marinus* genes were selected from GenBank for further investigations including superoxide dismutase 1 (*Pm*SOD1, AY095212), superoxide dismutase 2 (*Pm*SOD2, AY095213) and subtilisin (*Pm*SUB, AY340234). Total RNA was extracted from the *P. marinus* pellets retained from the virulence growth experiment using the TRIzol reagent system (Invitrogen Corporation). RNA grade glycogen (Fermentas) was added to each pellet at 5 µL before adding the Trizol reagent in order to enhance the precipitation of RNA. RNAs were resuspended in RNAase-free water and quantity and quality were assessed spectrophotometrically using a NanoDrop (ND-1000).

For each sample, 2 µg total RNA was individually submitted to reverse transcription using the oligo dT anchor primer (5'-GACCACGCGTATCGATGTCGAC$T_{(16)}$V-3') and Moloney murine leukaemia virus (M-MLV) reverse transcriptase. The real-time PCR assay was performed with 2 µL

cDNA (1/20 cDNA dilution) in a total volume of 10 μL, using an Eppendorf Mastercycler ep Realplex. The reaction components of the real-time PCR were made up of 1x Absolute QPCR SYBR Green ROX Mix (ABgene, UK) and 100 nM of each primer. The PCR program used began with an activation at 95°C for 10 minutes and 50 cycles of an initial denaturation step at 95°C for 30 sec, followed by annealing and extension at 60°C for 1 min. Primer sequences of the primer pairs used (*pm*SOD1, *pm*SOD2 and SUB) are listed in Table 1. All experiments also used the *P. marinus* housekeeping gene 18S (AF324218) primer pair (Table 1) as a control. All gene transcription levels were normalized to the level of 18S transcription for each particular sample to account for sample-sample variability, differences in spectrophotometry readings and pipetting error.

Protease activity

Protease activity was determined spectrophotometrically for the cell-free culture supernatants according to the procedure used by La Peyre *et al*. (1995). Specifically, azocasein substrate (3% w/v) was prepared by dissolving solid azocasein (Sigma, A2765) in phosphate buffer (pH 7.5) followed by centrifugation of the substrate at 12,000 x *g* for ten minutes. Cell-free supernatant (30 μL) was transferred in triplicate to a 96-well plate and 50 μL of the azosubstrate was added to each well. After quickly vortexing and centrifugation at 500 x *g* for 5 minutes, the plates were incubated for 24 hours at 27°C. Following incubation, 200 μL of cold 10% trichloroacetic acid (TCA) were added to each well to stop the reaction. The plates were shaken and centrifuged at 2,000 x *g* for 60 minutes. After centrifugation, 60 μL of the supernatant was added to a plate containing 70 μL of 1 M NaOH in each well. The plate was then read on a Wallac Victor^2 1420 multilabel counter (Perkin Elmer life sciences) at 450 nm.

b) Experiment 4: Effect of oyster extracts on *P. marinus* infectivity

Culture preparation

Cultures of *P. marinus* (ATCC-50439) were seeded at 10^6 cells mL^{-1} in 25 mL culture flasks containing DME/F12-3 culture medium supplemented with pallial mucus or digestive extract from *C. virginica* oysters (WA) as well as an un-supplemented treatment as a control. The pallial mucus and digestive extract supplements were added to the cultures with a final protein concentration of 0.3 mg mL^{-1}. The cultures were maintained and grown in an incubator at 23°C for 17 days. After this growth period the cultures were enumerated using a hemocytometer and centrifuged at 400 x *g* for 15 minutes at room temperature. The media was carefully aspirated and replaced with 25 mL of SAS. Cultures in seawater were kept overnight to be used as inoculums the following day.

Oyster inoculation

Naïve *C. virginica* (WA) were maintained in 10 L tanks at 25-26°C in 28 ppt well water free of *P. marinus* from Flax Pond, NY. Each tank had its own re-circulating carbon filtration unit as well as an air stone. Following a three day acclimation period the oysters were carefully notched with bone shears avoiding damage to mantle tissues. Four days following notching, the oysters (24 per treatment) were injected using a 23-gauge blunt needle through the notch into the pallial cavity with the cultures enhanced with pallial mucus, digestive extract and the un-supplemented cultures at a concentration of 2.5×10^6 per oyster in a volume of 1 mL. Twelve oysters were also inoculated with artificial seawater as a negative control. Following inoculation, the oysters were covered with damp paper towels for 2 hours at room temperature and subsequently returned to separate tanks (3 replicate tanks/treatment excluding the negative control injected with artificial seawater where there was only 1 replicate tank, 8 oysters/tank, Figure 2).

For four weeks the oysters were fed and monitored daily for gaping (moribund) behavior or mortality. Moribund oysters were removed from tanks and *P. marinus* infections were determined using alternative Ray's fluid thioglycollate medium (ARFTM) (Nickens *et al.* 2002). After four weeks, the surviving oysters were also processed for Dermo infections using ARFTM.

Optimized P. marinus body burden protocol

Dermo infections were assessed following the general procedures described by Nickens *et al*. (2002). Oysters were shucked and the mantle tissue was dissected and separated from the remainder tissue (gill and visceral tissue). The wet weights (g) of the mantle and remainder tissue were separately determined and a volume of ice-cold 1X PBS (Phosphate Buffered Saline 10X Ready Concentrate; Fisher BioReagents BP665-1) was added at ten times the weight of the tissue. Tissues were then homogenized using a Polytron homogenizer (Brinkman Instruments) for 5-10 seconds or until the tissue was well minced. One mL of tissue homogenate was added to a 15 mL Falcon tube containing 9 mL of ARFTM (Nickens *et al.* 2002) supplemented with 10 μL of chloramphenicol (50 μg/mL), 50 μL of lipid mixture (0.5% final, Sigma L5146) and 50 μL of nystatin (10,000 units/mL) was gently layered on top of the suspension. Following incubation (6-8 days in the dark at room temperature) the tubes were centrifuged at 1,500 x *g* for 10 minutes and the ARFTM was aspirated and replaced with 10 mL NaOH (2 N). The tubes were then incubated in a water bath at 60°C for 2 – 6 hours and vortexed every 30 minutes until all tissue was digested. After digestion was complete, pelleted parasite cells were washed three times by centrifugation (1,500 x *g* for 10 min) and resuspension, first in 10 mL of deionized water (DI) then twice with a phosphate buffer/bovine serum albumin (PB/BSA) solution (0.1 M, 0.5 mg mL^{-1} BSA, pH 7.8). After the final washing with phosphate buffer the supernatant was aspirated carefully leaving the pellet in 100 μL PB/BSA.

The prevalence and infection intensity of *P. marinus* in each sample was determined by splitting the 100 μL parasite suspension, which represents 0.1 g of the original tissue, into 2 wells (50 μL/well) on a 96-well clear flat bottom plate. First, to stain the hypnospores, 50 μL of Lugol's iodine working solution (1:5 diluted stock; 5%, LabChem Inc LC15675) was added to one of the two replicate wells before counting enlarged hypnospores under an inverted microscope (10 x). The second series of replicate wells containing samples with greater than 200 hypnospores were serially (2-fold) diluted before counting by adding 50 μL of the PB/BSA solution before staining. The wells within the dilution having a concentration of 50 – 200 hypnospores were enumerated. The counts in these wells were multiplied by their dilution factor to determine the number of cells that were present in the original 100 μL. The samples in which the initial well had less than 200 hypnospores were treated by adding 50 μL of iodine to the second replicate well without further dilution and the total count of hypnospores between both wells represented the number of hypnospores in the original 100 μL. The total number of hypnospores per gram was determined by multiplying the number of hypnospores determined in the 100 μL by 10.

5. Data treatment

Statistical comparisons of the treatments in the *P. marinus* growth experiment, protease activity and the gene expression analysis were all performed using one-way repeated measures ANOVA (Holm-Sidak post-hoc test). In Experiment 4, statistical analysis on infection intensities and the virulence index used one-way ANOVA (Holm-Sidak post-hoc test).

a) *P. marinus* growth

Growth rates were calculated by subtracting the starting cell counts (day zero) from cell counts determined on each sampling day and then dividing the difference by those at day zero.

b) Gene expression

The gene expression analysis was based on four oysters from Experiment 3. In order to account for variance between samples caused by RNA extraction or cDNA synthesis, each sample was normalized to the housekeeping gene 18S by substraction and those values are referred to as the normalized gene expression. To further standardize the samples, values were calculated to be relative to the un-supplemented control and are referred to as the relative gene expression or the fold change in gene expression given as the equation: $\Delta\Delta CT = 2^{-(\ [\ Cttest\text{-}CT18s\ ]\text{-}\{\ Average\ [Ctctrl\text{-}Ct18S]\ \}\ )}$.

c) Protease Activity

The protease activity in the culture supernatant was normalized to the number of parasite cells mL^{-1} in each original sample as determined by FCM in Experiment 3.

d) Infection Experiment

To determine the combined effect of both time to death during Experiment 4 and *P. marinus* infection intensity, a virulence index was calculated based on methods described by Chintala *et al.* (2002) (Table 2). Ratings were given from 0 – 5 for each individual oyster based on how long the oyster survived and the number of hypnospores $gram^{-1}$ wet weight after it was processed for ARFTM. These ratings were added together to obtain the virulence index ranging from 0 (least virulent) to 10 (most virulent).

III. Results

1. Effect of oyster supplements on *P. marinus* growth

a) Comparison of *P. marinus* supplemented with *C. virginica* and *C. gigas* extracts

In both the *C. virginica* and *C. gigas* experiments, *Perkinsus marinus* grew over time, however growth rates varied significantly between different time points and among treatments. Most notably, in the *C. virginica* experiment, the growth rates at days 1 and 4 for *P. marinus* supplemented with mantle mucus were 0.78 and 1.59, respectively, significantly higher than cultures supplemented with digestive extract (0.28 and 0.85 respectively; $P < 0.001$) or gill mucus (0.49 and 1.03 respectively; $P < 0.001$) (Figure 3). At day 8, there were no significant differences in growth rates of *P. marinus* cultures supplemented with mantle or digestive extract. Cultures supplemented with plasma displayed a significantly lower growth rate than those added with mantle mucus at day 1, however by days 4 and 8 the growth rates were not found to be significantly different. By day 15, no significant differences in growth rate existed between any of the treatments.

In the *C. gigas* experiment, growth rates at day 1 for digestive extract and mantle mucus supplemented cultures were 0.47 and 0.39, respectively, significantly higher than those measured in cultures supplemented with gill mucus (0.14; $P = 0.007$) (Figure 4). Although it was not statistically significant, growth rates in cultures supplemented with *C. gigas* mantle and gill mucus extracts were lower at days 1 and 4 than those measured in cultures supplemented with digestive extract. By days 4, 8 and 15 the growth rate of cultures supplemented with *C. gigas* plasma was significantly higher than all the other treatments (3.30, 6.16 and 11.3 respectively; all $P < 0.001$). By day 15, cultures supplemented with digestive extract displayed significantly higher growth rates (7.32) than those added with either mantle or gill mucus extracts (2.29 and 3.00 respectively; $P < 0.001$).

b) Effect of high levels of oyster extracts (0.4 mg protein mL^{-1}**) on *P. marinus* growth**

At day 1, the growth rates of cultures supplemented with mantle and gill mucus were 0.61 and 0.69 respectively, which were both significantly higher than those measured in cultures added with digestive extract (0.42; $P < 0.001$) or plasma (0.13; $P < 0.001$) (Figure 5). The growth rate at day 4 was significantly higher in cultures added with mantle mucus (1.61) as compared to cultures supplemented with plasma (0.80; $P < 0.001$), while the growth rate in cultures supplemented with gill mucus was significantly higher than all of the other treatments (2.35; $P < 0.001$). At day 8 there were no significant differences in growth between cultures supplemented with mantle mucus and the other treatments, however by day 15 the growth rate of *P. marinus* supplemented with plasma was significantly lower than all the other treatments (3.18; $P < 0.001$).

2. Effect of oyster extracts on *P. marinus* virulence and infectivity

a) Effect of oyster extracts on the production of virulence factors

Effect of oyster supplements on the growth rate

Although not statistically significant at day 1, plasma supplemented *P. marinus* had a lower growth rate than all the other oyster supplemented treatments (0.113, $P = 0.052$). These differences became significant at day 4 ($P < 0.001$). Additionally, by day 4 cultures supplemented with mantle and gill mucus displayed higher growth rates (1.82 and 1.58, respectively) than those added with digestive extract (1.29) although differences were not statistically significant (Figure 6).

Effect of oyster supplements on gene expression

Normalized gene expression in oyster supplemented cultures were found to be significantly different from each other for all genes at day 1, ($P < 0.05$) (Figure 7) while

no significant differences were observed for any treatments at days 0 and 4. At day 1, *P. marinus* cultures supplemented with mantle and gill mucus showed significantly higher *Pm*SOD2 expression levels (lower CT value) (CT of 9.53 and 9.33 respectively) than those supplemented with digestive extract (11.88; P = 0.013, One-Way Repeated Measures ANOVA followed by Holm-Sidak post-hoc test). Similarly, *Pm*SUB expression was also higher in cultures supplemented with mantle and gill mucus (9.08 and 8.91) compared to those added with digestive extract (10.45, P = 0.006). Although significant differences were not observed in *Pm*SOD1 at day 1, results did show that the expression of this gene was higher in cultures added with mantle and gill mucus as compared to those supplemented with digestive extract or plasma.

The fold change in gene expression was also calculated to determine the gene expression in *P. marinus* cultures supplemented with oyster extracts relative to those added with seawater (control). A One-Way Repeated Measures ANOVA indicated significant differences in *pm*SOD2 and *pm*SUB expression at day 1 among cultures added with different oyster supplements (P = <0.05) (Figure 8). Pair-wise comparisons did not show significant differences between the treatments for any gene at day 1, however the expression of all three genes tested was similar in cultures supplemented with mantle and gill mucus and those added with seawater (represented by the X-axis). In contrast, down-regulation of all three genes was evident when *P. marinus* was supplemented with digestive extract or plasma.

Effect of oyster supplements on protease activity

Significant differences were observed between the different treatments at day 4 with higher protease activity in cultures supplemented with gill mucus and plasma (3.49 and 3.40 arbitrary unit, respectively) compared to those grown in either mantle mucus or digestive extract (1.56 and 1.90, respectively; P < 0.001) (Figure 9).

b) Effect of oyster extracts on *P. marinus* infectivity

Moribund oysters (gaping, no reaction to physical stimuli) were most prominent in oysters injected with *P. marinus* cultures supplemented with pallial mucus (Figure 10). By day 3, there were three moribund oysters (one from each separate tank) in the pallial mucus treatment. Moribund oysters in this treatment reached over 50% by day 10 and peaked at 67% (16 oysters) at the end of the four-week experiment. Two moribund individuals were also removed on days 13 and 20 from oyster batches injected with parasite cultures supplemented with seawater. In addition, the negative control treatment (unchallenged) had one gaping oyster on day ten. There were no moribund oysters in the digestive extract treatment.

All of the moribund oysters removed before the end of the four-week experiment were processed immediately (mantle and remainder of oyster processed separately) for prevalence and intensity of *P. marinus* by ARFTM. *P. marinus* was detected in all 16 moribund oysters from the pallial mucus treatment. The parasite was also found in one of the two oysters removed from the batches injected with *P. marinus* cultures supplemented with seawater, while the single oyster removed from the unchallenged control treatment was negative for *P. marinus*. After the four-week period, all surviving oysters were processed for *P. marinus* prevalence and intensity. All of the oysters remaining in the pallial mucus (8 oysters) treatment were found to be negative for *P. marinus* infection, resulting in an overall infection prevalence of 67% for this treatment (Figure 11). Although there were no moribund oysters removed from the digestive extract treatment during the course of the experiment, 70% (16 out of 23 oysters) were infected with *P. marinus* (Figure 11). Among oysters injected with parasite cultures supplemented with seawater, 44% (10 out of 23 oysters) were infected, and no infections were detected in the negative control treatment (12 oysters).

The average numbers of hypnospores gram^{-1} for the pallial, digestive extract and seawater treatments were 37,060, 1,218,491 and 308 respectively in mantle tissues and

15,162, 2,947,780 and 123 respectively in the remainder oyster tissues (Figure 12). The individual oyster infection intensities varied between the pallial mucus, digestive extract and seawater supplemented treatments as well as varied within each infected oysters mantle or remainder tissue. Parasite loads in moribund oysters from the pallial mucus treatment at day three ranged from 2,000 to 14,200 hypnospores gram^{-1} in the mantle tissues and 400 to 12,000 hypnospores gram^{-1} in the remainder oyster meat (Figure 13). Severe infections were detected in the mantle tissues of moribund oysters at day 5, 6, 8 (2 oysters), 9 and 10 with parasite loads greater than 72,800 hypnospores gram^{-1}. The highest parasite load in mantle tissue from the pallial mucus treatment was from a moribund oyster collected on day 5 with 478,133 hypnospores gram^{-1}. In the remainder tissues, high infections with intensities greater than 73,600 hypnospores gram^{-1} were present in moribund oysters collected on days 6, 8, 9 and 10 with the highest value in a moribund oyster at day 6 with 108,800 hypnospores gram^{-1}. The only infected moribund oyster from the batch injected with *P. marinus* cultures supplemented with seawater (moribund on day 13) had 330 and 950 hypnospores gram^{-1} in mantle and remainder tissue respectively. In the digestive extract treatment, 25% had severe infections in the mantle tissue with parasite loads greater than 112,400 hypnospores gram^{-1} (Figure 13) and a maximal load of 12,420,000 hypnospores gram^{-1}. Likewise, in the remainder tissue of oysters from the digestive extract treatment, 25% were severe infections starting at 146,800 hypnospores gram^{-1} with the highest intensity at 44,200,000 hypnospores gram^{-1}. Parasite loads were much lower among surviving positive oysters injected with parasite cultures supplemented with seawater with a maximal load of 3,980 hypnospores gram^{-1} in mantle tissue and 680 hypnospores gram^{-1} in the remainder tissue.

To evaluate the infection progression within the tissues of moribund oysters from the pallial mucus treatment, the percent difference of parasite loads in mantle and remainder tissue was calculated (Figure 14). A level of 100 indicates equal parasite loads (g^{-1}) in the mantle and remainder tissue while values below or above 100 represent higher or lower parasite loads in mantle as compared to remainder tissues, respectively. Moribund oysters collected on or before day 5 had systematically higher parasite loads in

the mantle while those sampled after day 5 primarily had higher parasite loads in the remainder tissue.

In order to better evaluate the overall virulence of parasite cultures with different oyster supplements, a virulence index was calculated taking into account both the mortality throughout the course of the experiment as well as parasite load in each moribund oyster according to the method of Chintala *et al.* (2002). The virulence index was significantly higher for *P. marinus* cultures supplemented with pallial mucus for both the mantle and remainder tissue (4.4 and 4.3 respectively) as compared to cultures supplemented with digestive extract (1.6 and 1.5 respectively; $P = 0.001$ and $P < 0.001$ respectively) or those supplemented with seawater (both 0.9 with $P < 0.001$) (Figure 15).

IV. Summary and Discussion

Unlike previous studies, which focused on using oyster tissue homogenate or plasma as supplements to *P. marinus* cultures, this is the first study investigating the effect of oyster pallial mucus on the parasite. Additionally, other studies focused on long-term effects of the supplements, most with incubations for four or six weeks (Brown *et al.* 2005, Earnhart *et al.* 2003, Earnhart *et al.* 2004), with the shortest being an eight day incubation investigating the effect of oyster plasma on *P. marinus* (Gauthier and Vasta, 2002). After a long incubation period it is likely that proteins in the extracts would degrade and therefore lose any pro or anti- *P. marinus* activity present in the extracts. Therefore, in this study, I focused on the short-term effects of the mucus and examined the same cultures subsequently over a short period of time so that early changes in the culture dynamics could be noted. Although in some cases, to effectively compare our data to other investigations, long-term trends were also assessed.

1. Host mucus enhances *Perkinsus marinus* growth

P. marinus was first exposed to supplements from susceptible and resistant oysters and the parasite growth rates were measured to determine promoting or inhibiting effects of each supplement. Results demonstrated that growth was enhanced in cultures supplemented with pallial mucus from susceptible *C. virginica* as compared to digestive extract or plasma, while both mucus and digestive extracts from resistant *C. gigas* inhibited *P. marinus* growth. Additionally, *P. marinus* cell proliferation was observed in all treatments. These results are unlike previous tissue homogenate supplementation experiments, in which parasite cell proliferation was maintained or decreased (Brown *et al*. 2005; Earnhart *et al.* 2003, 2004; Gauthier and Vasta 2002). In 2004, Earnhart *et al.* suggested the reduced *P. marinus* proliferation to result from signal molecules that might be released from degrading tissues so that the parasite does not overwhelm its host too quickly. Disparities between results presented here and other supplementation studies may be attributed to the nature of supplement used (mucus *versus* tissue homogenate for

example) or other differences in experimental parameters such as initial *P. marinus* cell concentrations, overall length of the experiments (time of sampling points) and added growth medium components such as fetal bovine serum (FBS).

Mantle mucus from *C. virginica* promoted parasite growth as compared to the other treatments. Specifically at day 1, the growth rate of cultures supplemented with mantle mucus was 2.5 fold greater than that of cultures added with either digestive extract or plasma and 1.6 fold greater than that of cultures added with gill mucus. Results at day 4 continued to show the promoting effect of mantle mucus with parasite growth rates 1.5 fold higher than those obtained in cultures supplemented with gill mucus and 1.87 fold higher than those added with digestive extracts. It is possible that mantle mucus contains promoting factors that enhance the growth of the parasite, but there may also be inhibitory factors in those supplements where growth was minimal, particularly digestive extract. Interestingly, growth rates at day 1 in *P. marinus* cultures added with seawater (negative control) were generally lower than those from the mantle mucus treatment but higher than those from cultures added with digestive extracts or plasma (although differences were not statistically significant). This further suggests the presence of factors in mantle mucus promoting an early increase in parasite growth rate, while inhibitory factors appear to be present in digestive extracts and plasma. However, differences between the oyster supplements or with control cultures disappeared by days 8 and 15. This demonstrates the importance of investigating the early effects of these supplements on *P. marinus* growth, especially since it is likely that any growth promoting or inhibitory factors are quickly degraded after their introduction into the culture medium. Overall, these results show that *C. virginica* mantle mucus has a measurable and significant promoting effect on the growth of *P. marinus* within the first 24 hours and lasting up to four days compared to the digestive extract.

a) *C. gigas* mucus supplements inhibit *P. marinus* growth

The overall results from the *C. gigas* (resistant oysters) experiment show very dissimilar trends from that of the *C. virginica* experiment. Specifically, the growth rates of *P. marinus* cultures supplemented with both mantle and gill mucus as well as digestive extract were lower than those measured in cultures added with seawater (negative controls). Therefore, mantle mucus, gill mucus and digestive extract from *C. gigas* appears to contain factors that inhibit the growth of *P. marinus*. Additionally, unlike previous *C. virginica* experiments, the early growth rates (days 1 and 4) of *P. marinus* added with mantle mucus were often not significantly different from other oyster supplemented treatments suggesting a non-specific response of *P. marinus* to the different *C. gigas* supplements.

Interestingly, this strong inhibitory effect observed in mantle mucus, gill mucus and digestive extract was not evident in the plasma of *C. gigas* since parasite growth in plasma supplemented cultures was similar to that of cultures added with seawater, and significantly higher than growth rates measured using other oyster supplements (Figure 4). This suggests that *C. gigas* plasma does not contain the inhibitory factors that the pallial mucus or digestive extracts contain. Gauthier and Vasta (2002) obtained similar results when exposing *P. marinus* to *C. gigas* plasma after eight days of growth. They concluded that resistance of *C. gigas* to *P. marinus* infection derives from cellular and not humoral factors. However, the present study suggests an alternative scenario by demonstrating that both *C. gigas* mantle and gill mucus inhibits growth of *P. marinus*, while *C. virginica* pallial secretions often enhance *P. marinus* growth. Therefore, it appears that host specificity starts at these pallial interfaces.

b) High levels of oyster extracts prolong growth promoting effects

The growth rates in Experiment 3 continued to show that *C. virginica* mantle mucus promotes the early growth of *P. marinus*. Additionally, compared to cultures

added with seawater, growth of *P. marinus* added with mantle mucus was promoted to a larger extent and the growth promoting effects lasted throughout the entire 15 day experiment. These results are likely caused by the increased level of protein in the oyster supplements providing more available nutrients for the parasite's growth. While previous supplementation experiments only assessed long-term effects of oyster supplements, they usually found *P. marinus* proliferation to be inhibited compared to the un-supplemented growth mediums and attributed this to limited availability of nutrients in the culture medium or inhibitory agents likely present in the whole oyster homogenate (Brown *et al* 2005). Studies have shown that *in vivo P. marinus* under limiting nutrients tends to decrease its replication rate while increasing its size or doubling time to prevent overcoming its host too quickly (Choi *et al.* 1989, Earnhart *et al.* 2004, Brown *et al.* 2005), which is in agreement with trends from the previous supplementation experiments. However, in our experiment, the increase in protein levels of mantle or gill mucus supplements clearly provided the necessary nutrients and promoting factors to elicit a strong growth response. Interestingly, Saunders *et al.* (1993) noted that *P. marinus* epizootics may be initiated by environmental conditions tipping the nutrient balance in favor of the parasite, which may have been the case in Experiment 4 where high early mortality in the pallial mucus treatment was incurred. Although it has not yet been documented, the pallial mucus layer of the oyster likely contains high protein levels. It would be interesting to test the effects of using higher protein mantle or gill mucus supplements on *P. marinus*.

Contrary to the results from Experiment 1, cultures added with digestive extract were not significantly different from the mantle supplemented cultures, nor did those cultures added with digestive extract elicit an inhibiting effect compared to cultures added with seawater. Perhaps at this increased protein level the nutrient benefits from the digestive extract outweigh the inhibitory factors. Studies have found that whole oyster homogenate added at protein levels greater than 1.0 mg mL^{-1} caused decreased cell proliferation and in some cases cell death likely due to proteases and inhibitory factors present in the host (Earnhart *et al.* 2004, Brown *et al.* 2005). Lastly, although inhibition

by the digestive extract was not apparent, *P. marinus* added with mantle mucus continued to promote a higher early growth rate than *P. marinus* supplemented with digestive extract. Therefore, these growth experiments collectively provide overwhelming evidence that *C. virginica* mantle mucus is beneficial to *P. marinus*, promoting its early growth. These results also demonstrate the complex relationship that exists between the differing host products and their effects on the metabolism and physiology of the parasite.

2. Mucus enhances the production of virulence factors by *Perkinsus marinus*

Growth rates obtained from Experiment 4, which used the same supplement protein levels as Experiment 3, showed that mantle mucus promoted early growth of *P. marinus* as compared to plasma or seawater (control). Additionally, similar to Experiment 3, results indicated that cultures grown in mantle mucus and digestive extract were not significantly different from each other. Therefore, results appear to be consistent under similar experimental conditions.

a) *P. marinus* virulence gene expression

Although the results included a limited number of samples, significant differences in gene expression were found at day 1 between oyster supplemented treatments for *Pm*SOD2 and *Pm*SUB. Additionally at day 1, results indicated that treatments added with digestive extract and plasma caused an immediate down-regulation of all three genes, while those treatments added with mantle or gill mucus had levels of gene expression similar to that of *P. marinus* added with seawater (value of 1). The down regulation response elicited by digestive extract is similar to results found by Brown *et al.* (2005) who demonstrated that oyster tissue homogenates suppress *Pm*SUB gene transcription in comparison to un-supplemented controls. They suggested the suppression is due to the possibility of inhibitory agents found in the host products. It is also possible that once *P. marinus* is inside the oyster and in contact with plasma and

digestive tissues, transcription of these genes may be down-regulated to conserve energy expenditures by the parasite.

Although supplementation with mantle or gill mucus did not induce an up-regulation of any of the genes, the gene expression levels remained similar to that of the treatment added with seawater, which provides evidence that mantle and gill mucus do not suppress gene expression. These results do not support the hypothesis that virulence-related genes studied here are up-regulated during early phase contact of *P. marinus* with mantle mucus.

b) Protease activity increases in *P. marinus* supplemented with gill mucus

Results showed higher protease activity at day 4 in cultures added with gill mucus and plasma as compared to those supplemented with mantle mucus or digestive extract. ECP are implicated in the degradation of tissues and La Peyre *et al.* (1995) noted an observation initially made by Ray, Mackin and Boswell (1953) that *P. marinus* infected gill tissues rapidly disintegrated, which is congruent to observations made in the infection experiment performed in this study. The higher protease activity observed in the gill mucus supplemented treatment may indicate the presence of specific signal molecules in the gill mucus, which might function to initiate and therefore cause secretion of proteases by *P. marinus*. This release of proteases may be initiating the rapid degradation of gill tissue, therefore enabling *P. marinus* to invade the tissues of *C. virginica*. In concordance with these observations, Brown *et al.* (2005), from a personal communication with Earnhart, noted that mantle or gill tissue increased the protease activity of *P. marinus*, but only at doses with 1.50 mg mL^{-1}. It is interesting to note that 0.5 mg protein mL^{-1} of gill mucus was sufficient to induce an increased protease activity, which may suggest that the response Earnhart had observed was from the presence of gill mucus included in the tissue homogenate.

On the contrary, *P. marinus* added with mantle mucus and digestive extract did not cause a significant increase in *P. marinus* protease activity. Brown *et al.* (2005) also notes, from a personal communication with Earnhart, that there was a decrease in protease activity of ECP from *P. marinus* grown in homogenate than fully defined media. They suggest that at late stages of infection proteases may not be needed; therefore *P. marinus* may only be initiating protease production upon contact with specific receptors. Tissue degradation by proteases may not be ideal once the parasite enters oyster tissues, especially if *P. marinus* is using host components (hemocytes) to spread infection throughout the oyster. Additionally, it is important to note that significant differences in protease activity were observed between the treatments supplemented with mantle and gill mucus, which demonstrates that there are different factors present in the mucus of these organs.

3. Mucus causes increased infectivity in *Perkinsus marinus*

Results showed that oysters exposed to *P. marinus* enhanced with pallial mucus exhibited a significantly higher early phase mortality compared to oysters exposed to *P. marinus* cultures added with digestive extract or un-supplemented media. Interestingly, although experimental conditions were not identical, Ford *et al.* (2002) also observed early phase mortality in oysters infected with wild-type *P. marinus*. In their experiment, oysters infected with wild-type *P. marinus* produced mortalities earlier than cultured *P. marinus*, however, they attributed the earlier mortality to dosing trauma, carried over elements like bacteria from the donor oysters or potent virulence factors secreted by the wild-type parasites. In our experiment, although dosing trauma is a reasonable explanation for early mortality, all oysters in each treatment were dosed identically and still only oysters in the pallial mucus enhanced treatment exhibited significant early phase mortality. Bacterial introduction is also unlikely since antibiotics were present in the culture medium and cultures were kept sterile up until the inoculation. Therefore, it can be speculated that the early phase mortality in our experiment is attributed to powerful virulence factors produced by *P. marinus* that has been enhanced with pallial mucus.

Although *P. marinus* cultures supplemented with pallial mucus increased the early phase mortality of oysters, survivors collected from this treatment at the end of the experiment (4 weeks) were not infected with *P. marinus*. This finding is unexpected and does not support the hypothesis of higher virulence in mucus supplemented *P. marinus*, however there may be a plausible explanation for this result. Earnhart *et al.* (2004) suggested that *C. virginica* obtained from Washington might be more resistance to *P. marinus* compared to naïve oyster populations in Maine although disease prevalence data in this study were unclear. Ford *et al.* (2002) reported 100% disease prevalence in experimentally challenged *C. virginica* originating from Maine. Since the *P. marinus* cultures supplemented with pallial mucus and the digestive extract supplemented cultures led to similar levels of cumulative prevalence (66-70% after 4 weeks), perhaps the uninfected oysters remaining in both of these treatments represent oysters that show more resistant to *P. marinus*.

It is clear from the results that both pallial mucus and digestive extract have a significant effect on the infectivity of the parasite when compared to un-supplemented cultures. The parasite prevalence and infection intensities of oysters inoculated with either of the oyster supplemented cultures were greater than those of the oysters inoculated with the seawater supplemented cultures. These results are consistent with those of Earnhart *et al.* (2004), which also found that oyster homogenate increases the infection intensities. It is important to note that the oyster mucus can elicit a similar response to that of tissue homogenate. This further proves the hypothesis that there are factors present in the pallial mucus of the oyster that are capable of increasing the virulence of *P. marinus*.

There were no statistically significant differences between the infection intensities in the pallial mucus enhanced and digestive extract supplemented treatments. However, several oysters in the digestive extract enhanced treatment exhibited extremely severe infections without causing mortality, while infection intensities in oysters exposed to

parasite enhanced with pallial mucus were consistent between individuals and an early phase mortality was apparent. The severe infections present in the digestive extract treatment may actually mean a lower virulence of parasite in this treatment since the oyster is capable of sustaining high *P. marinus* loads without mortality. On the other hand pallial mucus may be important in initiating infection and causing mortality. From these results it is evident that parasite-host interactions are complex and more than one virulence factor may be involved in causing both early mortality and the ability to cause severe infections.

The results from this experiment clearly show two different trends in terms of early mortality and infection intensities. In order to compare these two important virulence traits, a virulence index taking into account both time to death and infection intensities was calculated. This virulence index indicates that *P. marinus* enhanced with pallial mucus is significantly more virulent than both *P. marinus* enhanced with digestive extract or un-supplemented media (Figure 15). Similarly, Ford *et al.* (2002) found that wild-type *P. marinus* were more virulent than cultured parasites based on their virulence index. The same authors also noted 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 show that pallial mucus is able to activate cultured *P. marinus* and thereby restoring the virulence of the parasite.

Lastly, infection intensities in each oyster indicated that early infections (prior to day five) were higher in the mantle tissue (Figure 14). This strongly suggests that infections are initiated in the mantle tissue and subsequently *P. marinus* is spread throughout the remaining tissues of the oyster.

V. Conclusions

This study strongly suggests that pallial mucus play a major role in the interactions between the oyster *C. virginica* and its alveolate parasite *P. marinus*. In particular, mantle mucus appears to facilitate the colonization by *P. marinus* as indicated by the rapid (1 to 4 days) increase in parasite growth rates after contact with mantle mucus. The inhibition of *P. marinus* growth by *C. gigas* pallial mucus shows that host specificity may be initiated at the first contact the parasite has with the oyster. Most importantly, data from the infection study demonstrated that mucus contains yet unknown components capable of "turning on" the virulence of the parasite. Lastly, the higher infection intensities present in mantle tissue within the first five days following injection strongly suggests that the mantle represents the major portal of entry for this parasite into its oyster host.

Tables and Figures

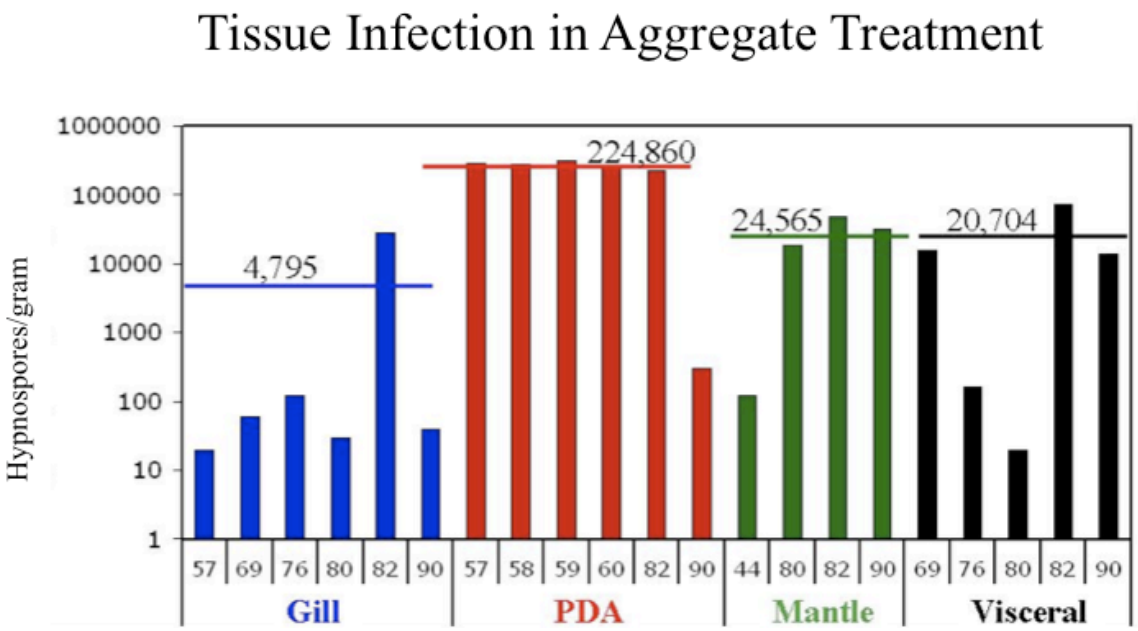

Figure 1. Infection intensities (hypnospore g^{-1} wet tissue) in oysters experimentally infected with *P. marinus* laden aggregates. Infection intensities in each oyster were separately determined for gills, the pseudofeces discharge area (PDA), mantle and visceral tissue. The PDA displayed infection intensities an order of magnitude higher than the mantle and visceral tissue and two orders of magnitude higher than the gill tissue.

Virulence Genes

Purpose	Primer name	Sequence from 5' to 3'
Superoxide dismutase 1	*pm*SOD1 for	GCT CAT TGC TGA CGG CAA ACT
	*pm*SOD1 rev	TGT CGT TGA CGA AGT CCC AGT GTA
Superoxide dismutase 2	*pm*SOD2 for	TGC AAT ACC CTC TGG TAA AGG GCT
	*pm*SOD2 rev	AAT GGA AAT CCA CAG TTG CTG CCG
Subtilisin	SUB for	CTG CTA ACG CTG GCC AT
	SUB rev	CAA TAT TAA CCA CAG AAC CGA TGT
18S	18S for	TAA TAC ATG CGT CAA GGC CCG ACT
	18S rev	AGA AAC TTG AAT GGT CCA TCG CCG

Table 1. Primer sequences used in this study. The 18S gene was used as housekeeper.

Virulence Index

Survival Time		Infection Intensity	
Day to Death	Rating	$\log_{10}$ *P. marinus* g^{-1} wwt	Rating
Survivor	0	0-0.999	0
20-28	1	1.000-2.349	1
16-20	2	2.350-3.699	2
11-15	3	3.700-5.049	3
6-10	4	5.050-6.399	4
1-5	5	6.400-7.750	5

Table 2. Rating used for determination of the virulence index (adapted from Ford *et al*. 2002).

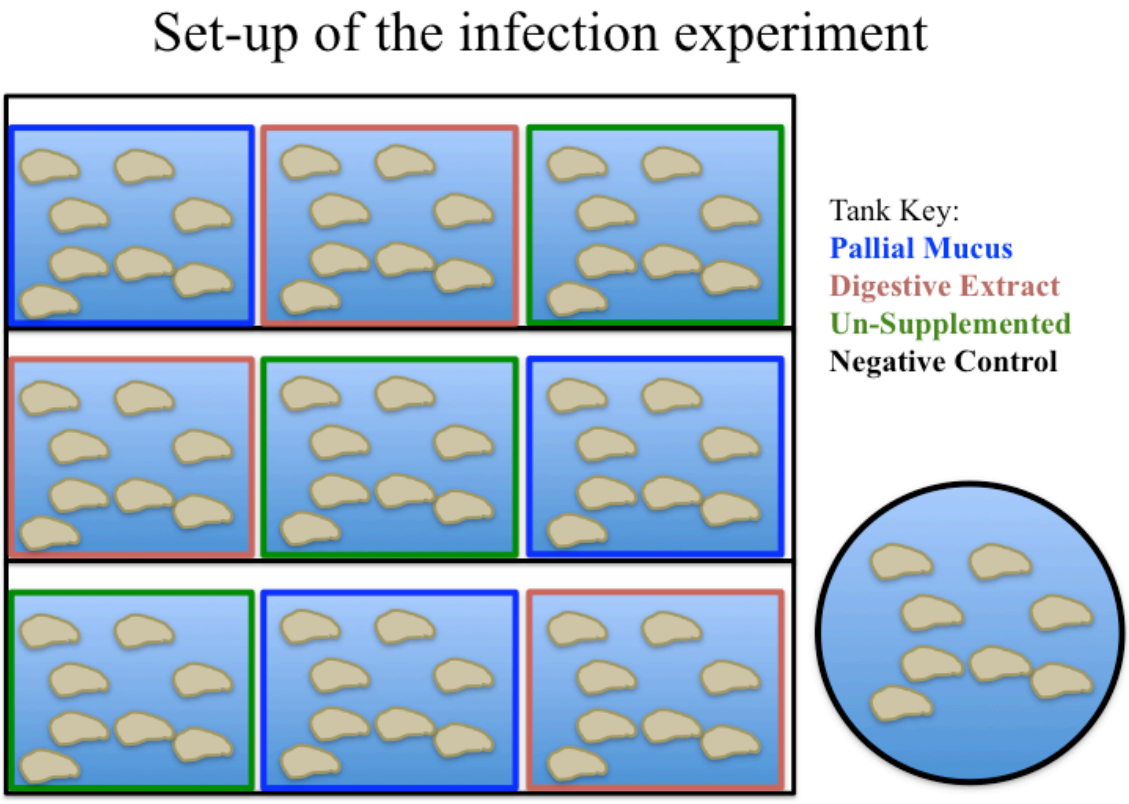

Figure 2. Set-up of Experiment 4. Oysters in each experimental treatment were split into three separate replicate tanks (N = 27/treatment; e.g. 9/replicate tank).

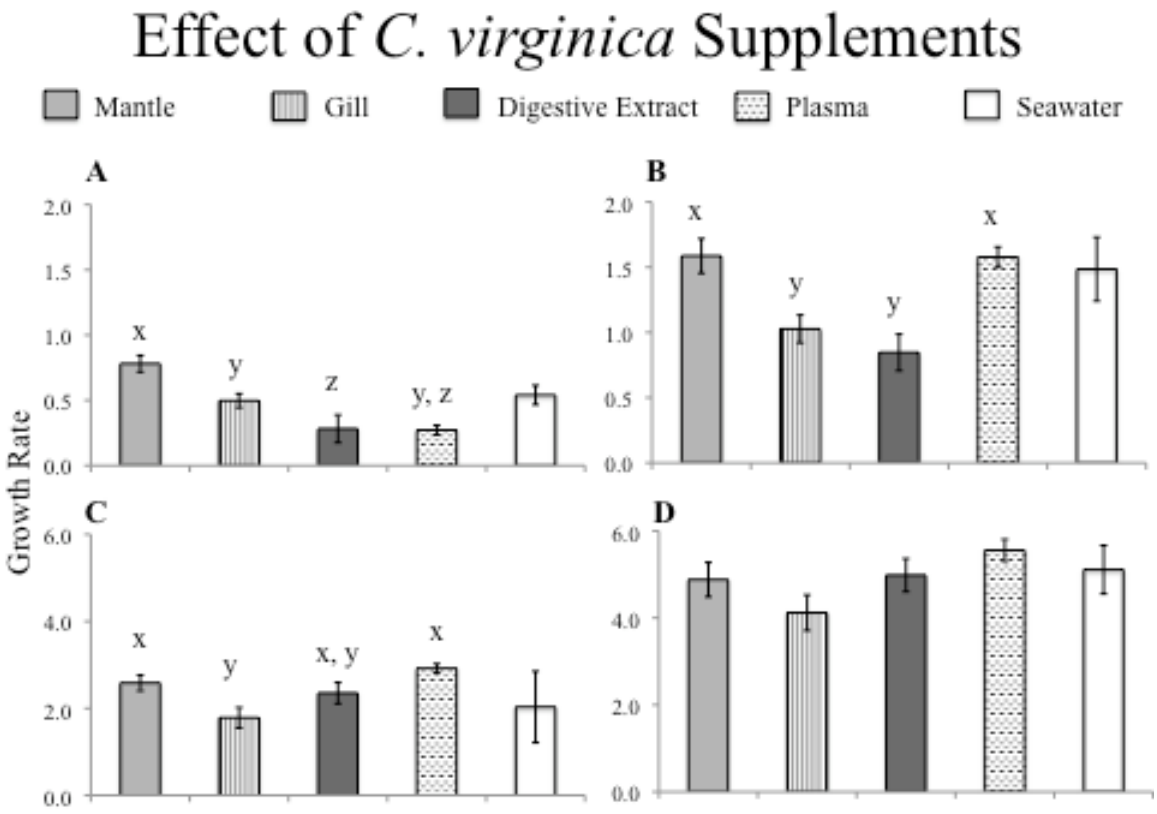

Figure 3. The growth rate of *P. marinus* cultures supplemented with 0.15 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma. A seawater supplement was also used as a control. Different letters indicate significant differences among different treatments (One-way repeated measures ANOVA followed by Holm-Sidak post-hoc test). N = 12. **A.** Growth rate at day 1 ($p \leq 0.001$) **B.** Growth rate at day 4 ($p \leq 0.001$) **C.** Growth rate at day 8 ($p = 0.002$) **D.** Growth rate at day 15 ($p = 0.079$)

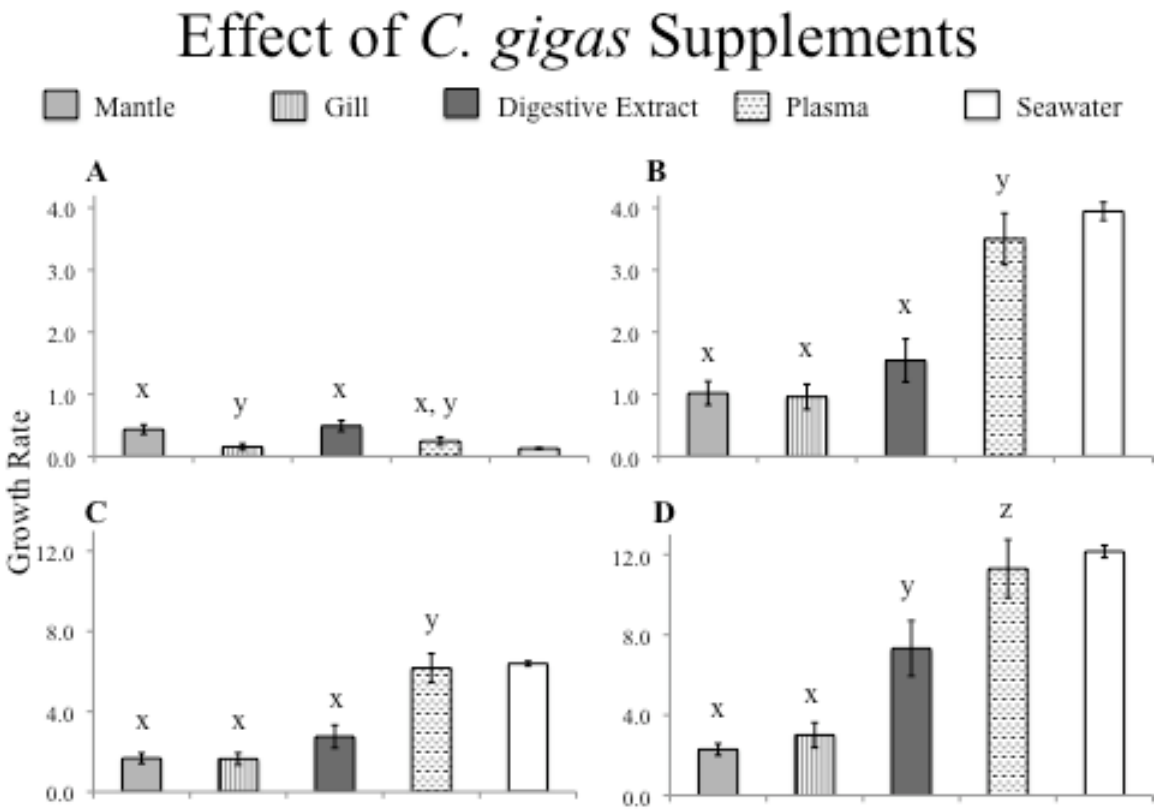

Figure 4. The growth rate of *P. marinus* cultures supplemented with 0.15 mg protein mL^{-1} *C. gigas* mantle mucus, gill mucus, digestive extract or plasma. A seawater supplement was also used as a control. Different letters indicate significant differences among different treatments (One-way repeated measures ANOVA followed by Holm-Sidak post-hoc test). N = 10. **A.** Growth rate at day 1 ($p = 0.007$) **B.** Growth rate at day 4 ($p \leq 0.001$) **C.** Growth rate at day 8 ($p \leq 0.001$) **D.** Growth rate at day 15 ($p \leq 0.001$)

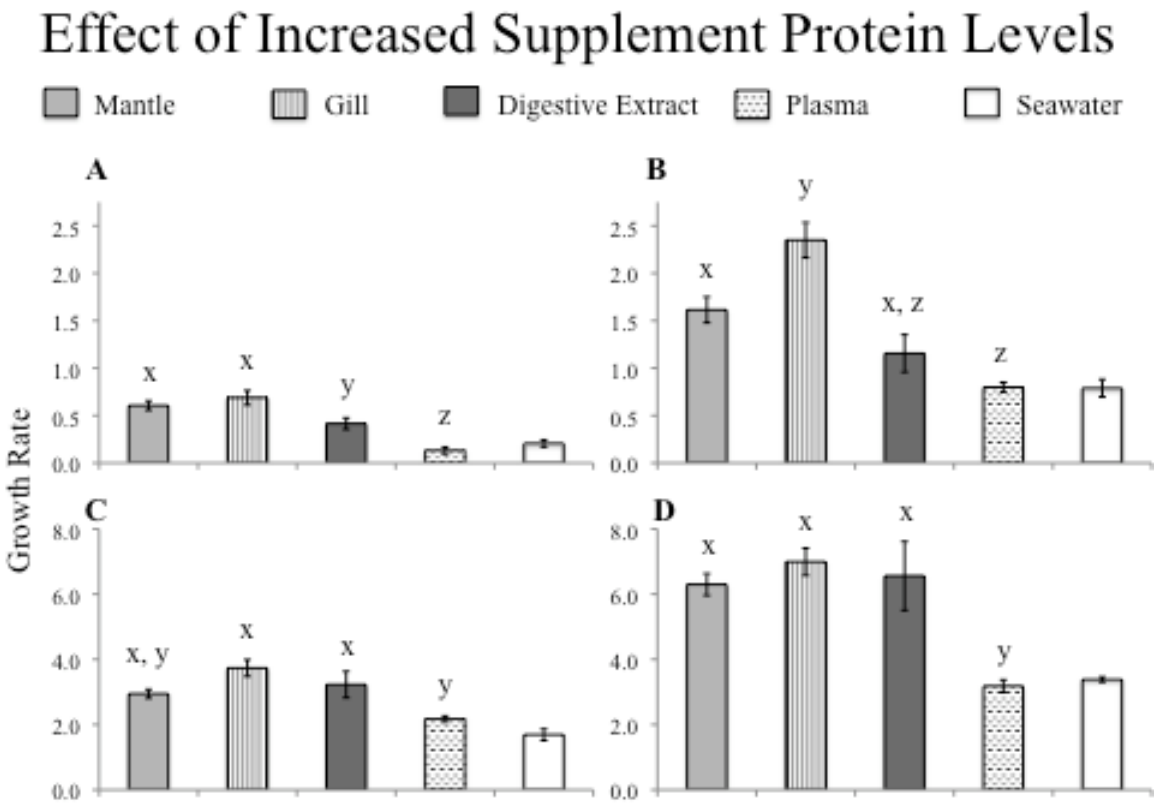

Figure 5. The growth rate of *P. marinus* cultures supplemented with 0.4 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma. A seawater supplement was also used as a control. Different letters indicate significant differences among different treatments (One-way repeated measures ANOVA followed by Holm-Sidak post-hoc test). N = 12. **A.** Growth rate at day 1 ($p \leq 0.001$) **B.** Growth rate at day 4 ($p \leq 0.001$) **C.** Growth rate at day 8 ($p = 0.001$) **D.** Growth rate at day 15 ($p \leq 0.001$)

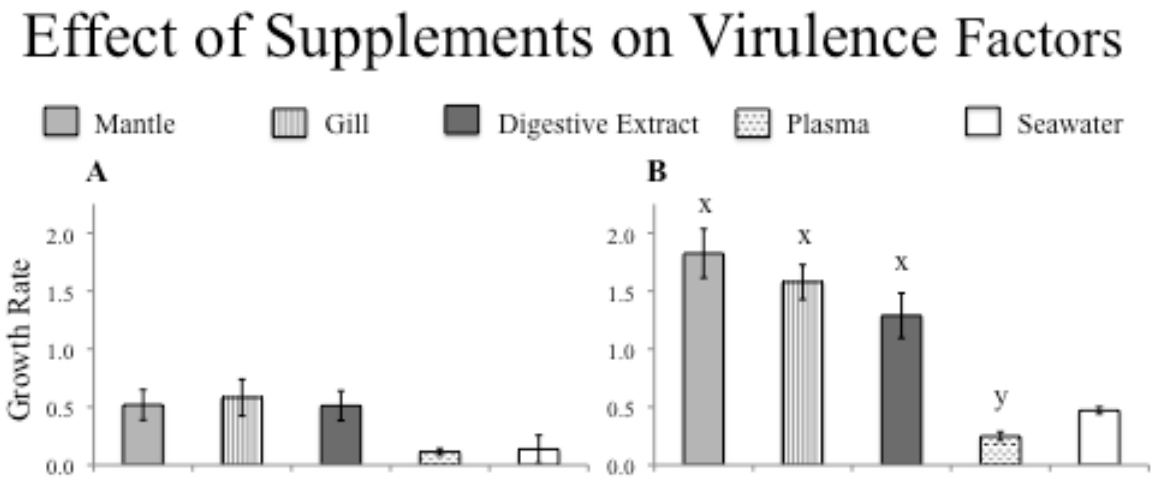

Figure 6. The growth rate of *P. marinus* cultures supplemented with 0.4 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma. A seawater supplement was also used as a control. Different letters indicate significant differences among different treatments (One-way repeated measures ANOVA followed by Holm-Sidak post-hoc test). N= 12. **A.** Growth rate at day 1 ($p = 0.052$) **B.** Growth rate at day 4 ($p \leq 0.001$)

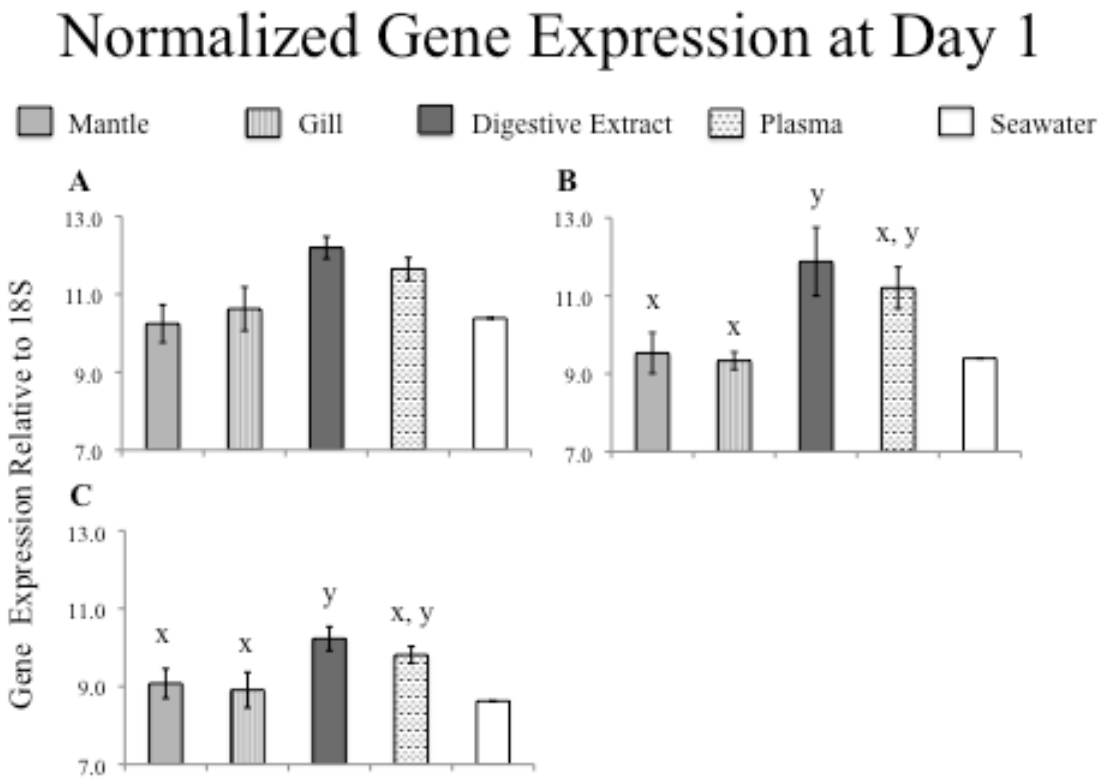

Figure 7. Gene expression in *P. marinus* supplemented with 0.4 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma normalized by subtraction to the expression of 18S. Different letters indicate significant differences in gene expression (One-way repeated measures ANOVA followed by a Holm-Sidak post-hoc test) N = 4. **A.** *Pm*SOD1 ($p = 0.029$) **B.** *Pm*SOD2 ($p = 0.013$) **C.** *Pm*SUB ($p = 0.006$)

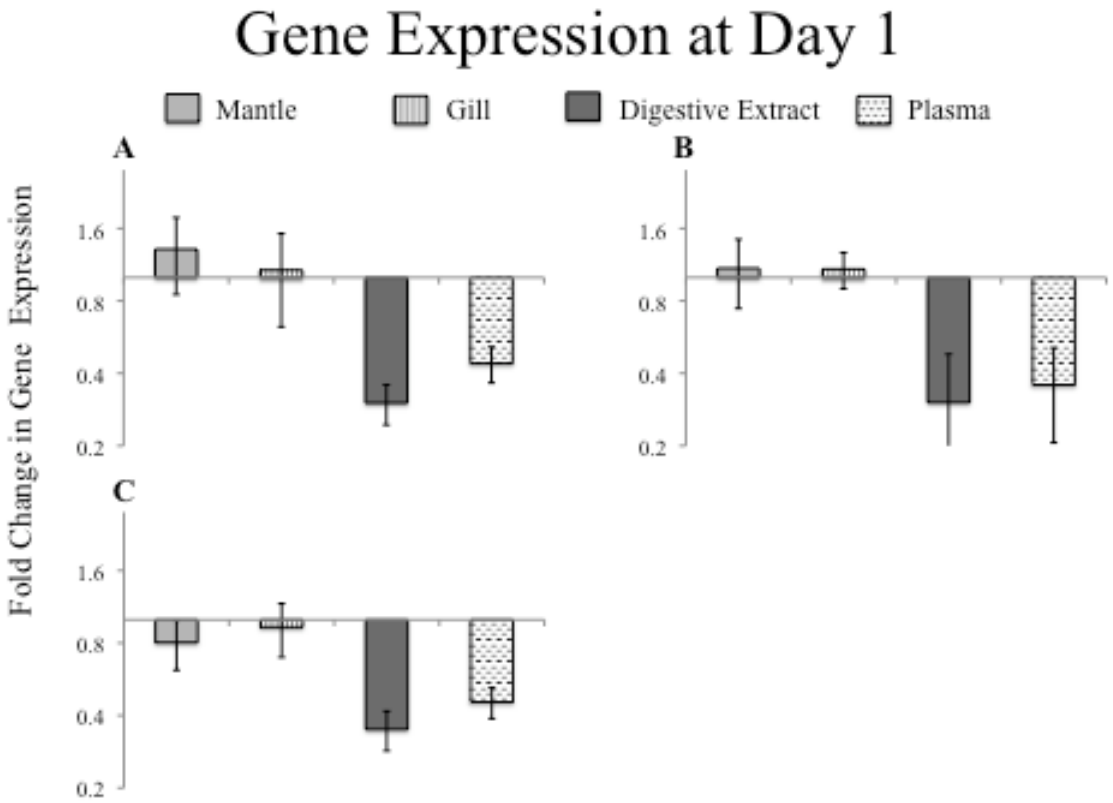

Figure 8. The fold change of gene expression in *P. marinus* supplemented with 0.4 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma. A One-Way Repeated Measures ANOVA indicated significant differences among cultures added with different oyster supplements in *pm*SOD2 and *pm*SUB. N = 4. **A.** *Pm*SOD1 ($p = 0.155$) **B.** *Pm*SOD2 ($p = 0.046$) **C.** *Pm*SUB ($p = 0.031$)

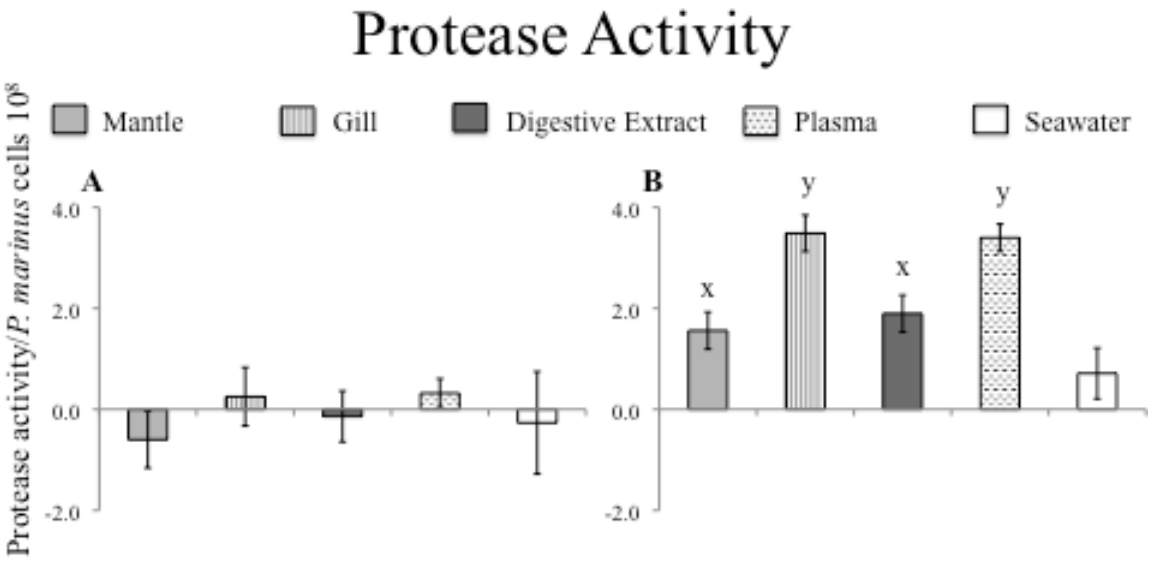

Figure 9. The protease activity (per 10^8 *P. marinus* cells) in the supernatant of *P. marinus* cultures supplemented with 0.4 mg protein mL^{-1} *C. virginica* mantle mucus, gill mucus, digestive extract or plasma. Different letters indicate significant differences among different treatments (One-way repeated measures ANOVA followed by Holm-Sidak post-hoc test). N = 12. **A.** Protease activity at day 1 ($p = 0.195$) **B.** Protease activity at day 4 ($p \leq 0.001$)

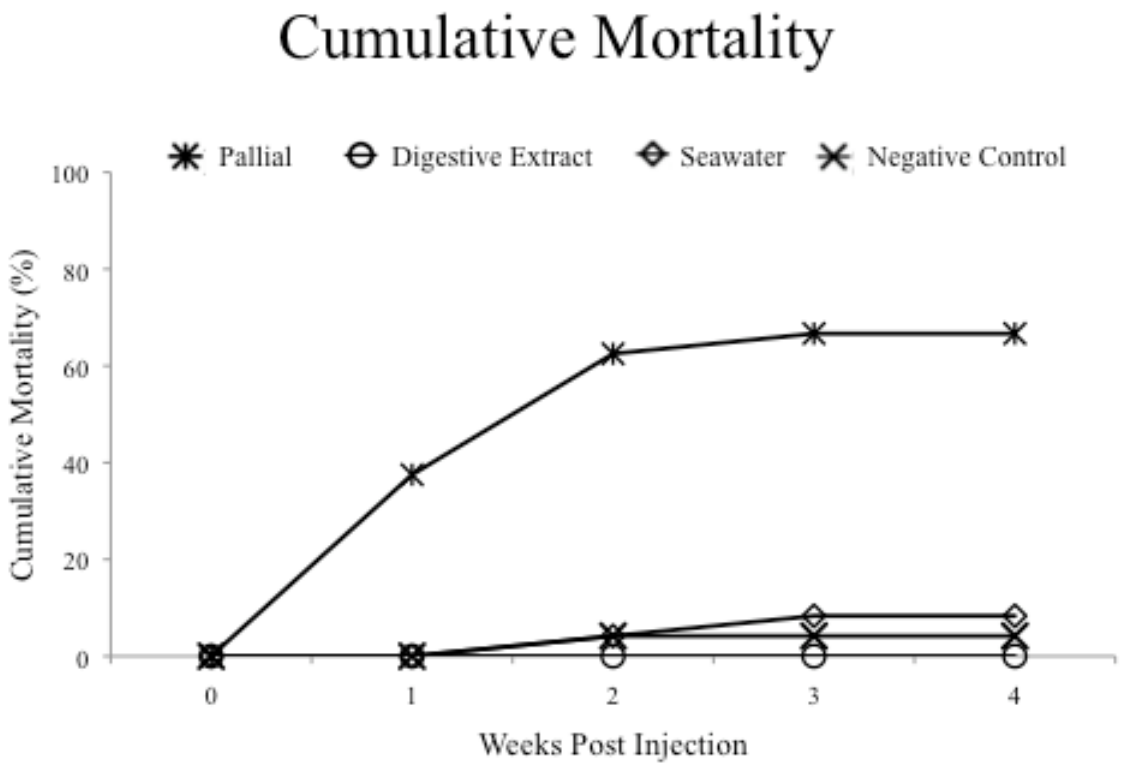

Figure 10. Cumulative percent mortality in the Experiment 4. Pallial supplemented *P. marinus* caused over 50% mortality after 2 weeks post injection while minimal mortality was observed in the seawater supplemented *P. marinus* treatment and no mortality in the digestive extract supplemented treatment. The one dead oyster in the negative control was found to be negative for *P. marinus* infection.

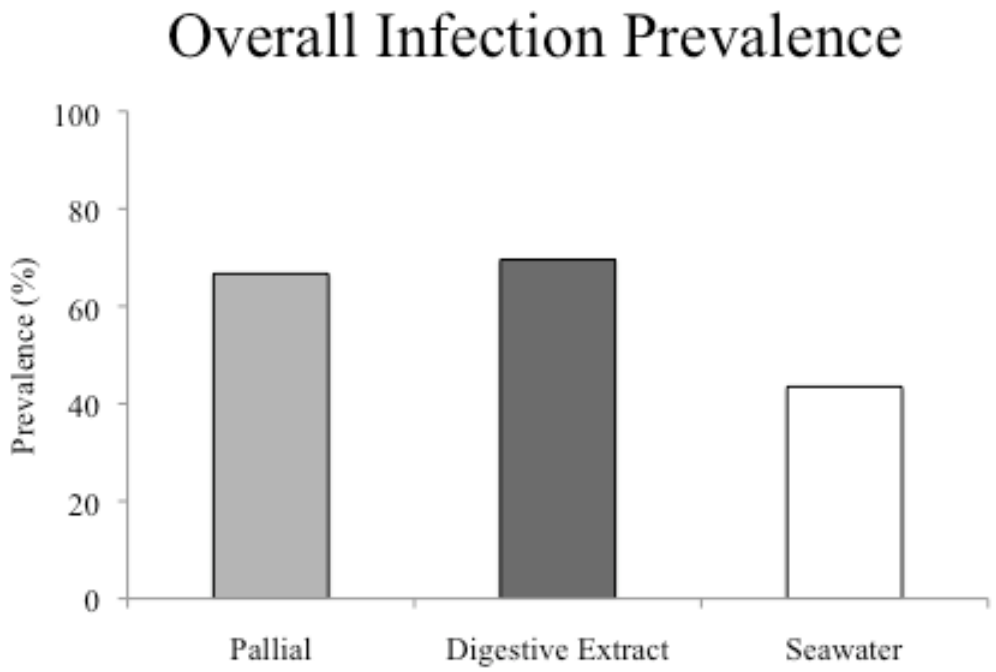

Figure 11. Overall infection prevalence in oysters processed in Experiment 4 both during and after the four-week incubation period. The pallial mucus supplemented *P. marinus* treatment had 66% hypnospores prevalence. The digestive extract supplemented *P. marinus* treatment had 70% hypnospores prevalence and the seawater supplemented *P. marinus* treatment had 44% hypnospores prevalence.

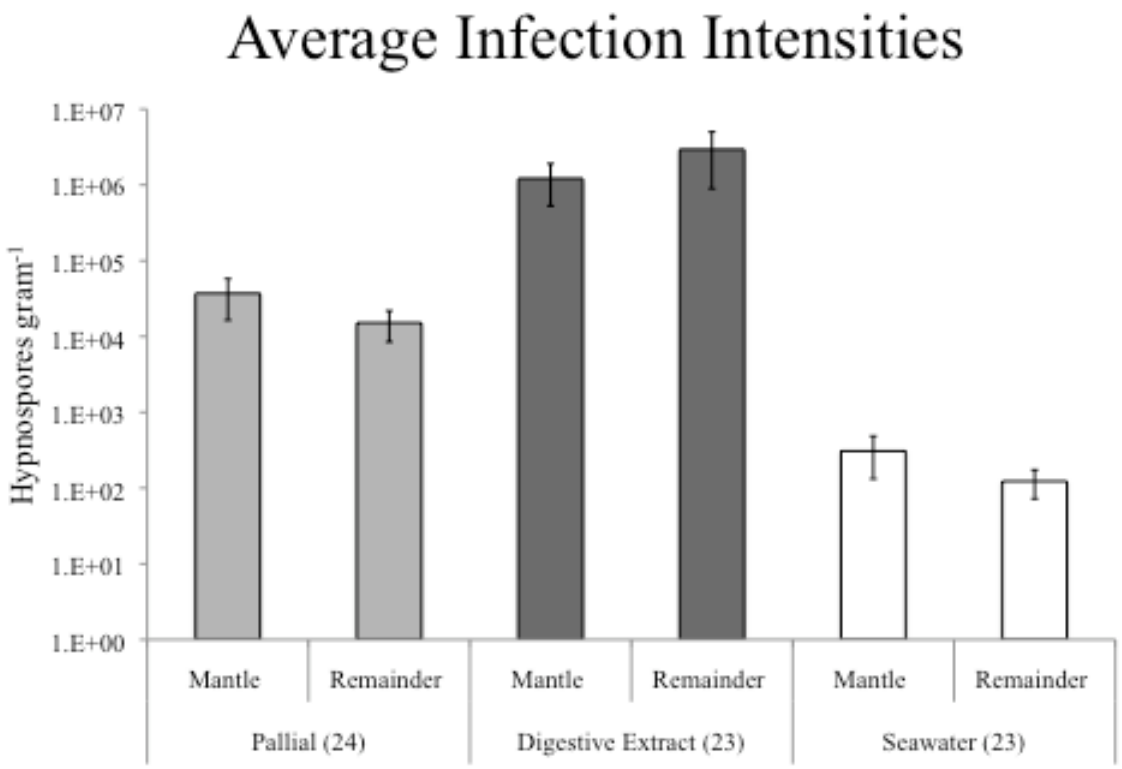

Figure 12. Average infection intensities of oysters processed in Experiment 4 both during and after the four-week incubation period. Each oyster was separately determined for hypnospores prevalence in mantle or remainder tissue. The pallial mucus supplemented *P. marinus* treatment had 37,060 and 15,162 hypnospores gram^{-1} in the mantle and remainder tissue respectively. The digestive extract supplemented *P. marinus* treatment 1,218,491 and 2,947,780 hypnospores gram^{-1} in the mantle and remainder tissue respectively and the seawater supplemented *P. marinus* treatment 308 and 123 hypnospores gram^{-1} in the mantle and remainder tissue respectively.

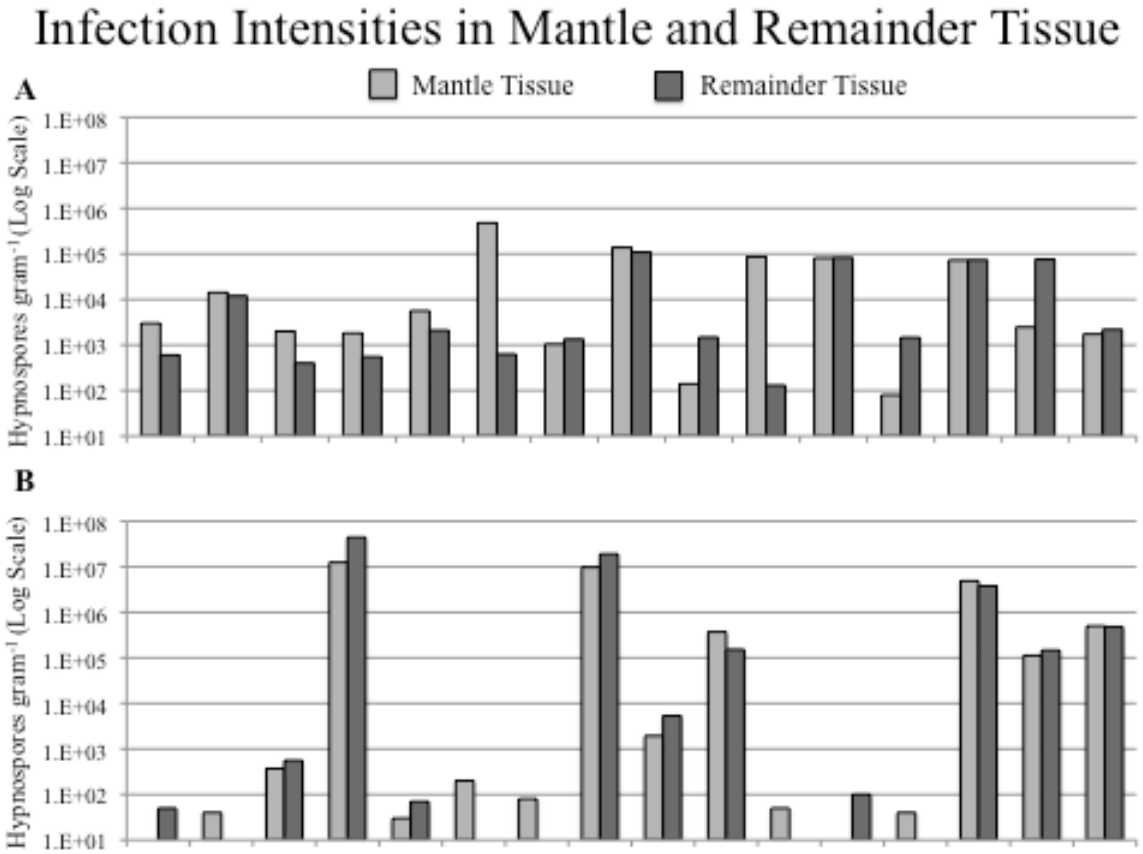

Figure 13. Infection intensities (hypnospores gram^{-1}) of each positive *P. marinus* infection in oysters in Experiment 4. Intensities are separately determined for the mantle or remainder tissues of each oyster. **A.** Pallial mucus supplemented *P. marinus* treatment. **B.** Digestive extract supplemented *P. marinus* treatment.

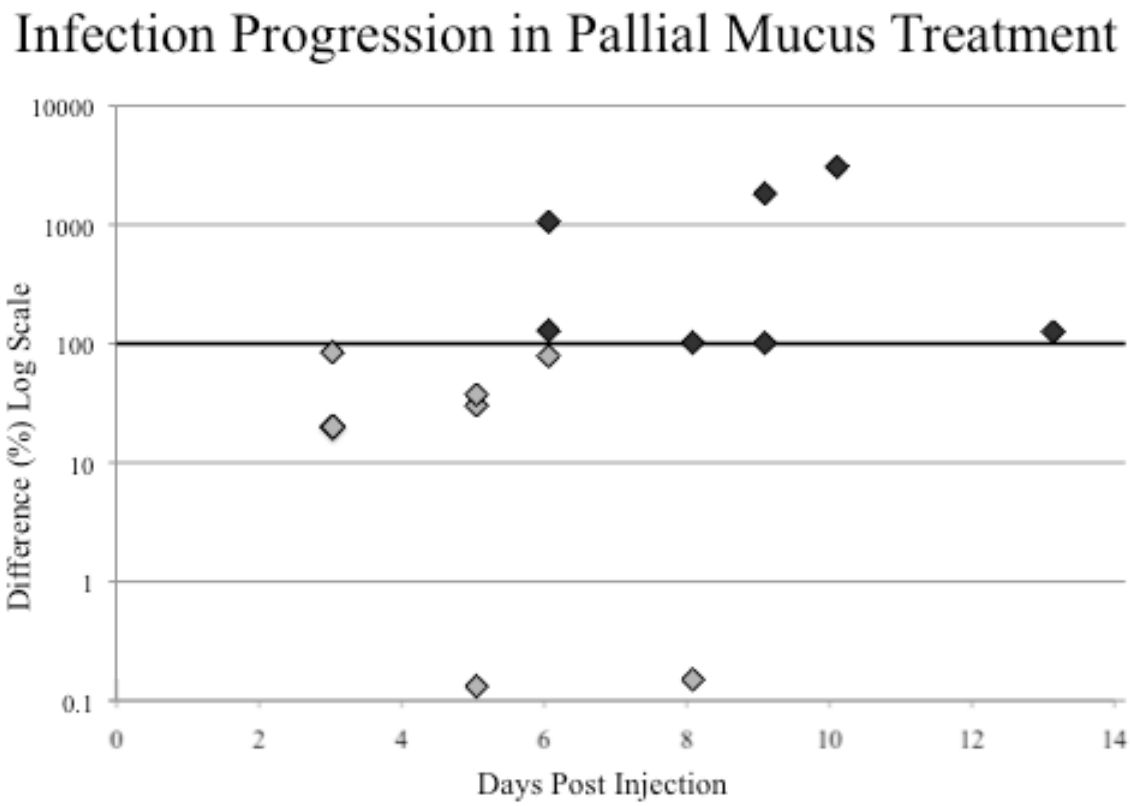

Figure 14. The percent difference of infection intensities in the mantle or remainder tissue of each moribund oyster in the pallial mucus treatment from days 3 to 14. A value of 100% indicates equal levels of infection in the mantle and remainder tissue, while values below 100% indicates higher infection in the mantle tissue and above 100% indicates higher infection in the remainder tissue. Prior to five days post injection infection intensities were higher in the mantle tissue and after five days the infection intensities were higher in the remainder tissue.

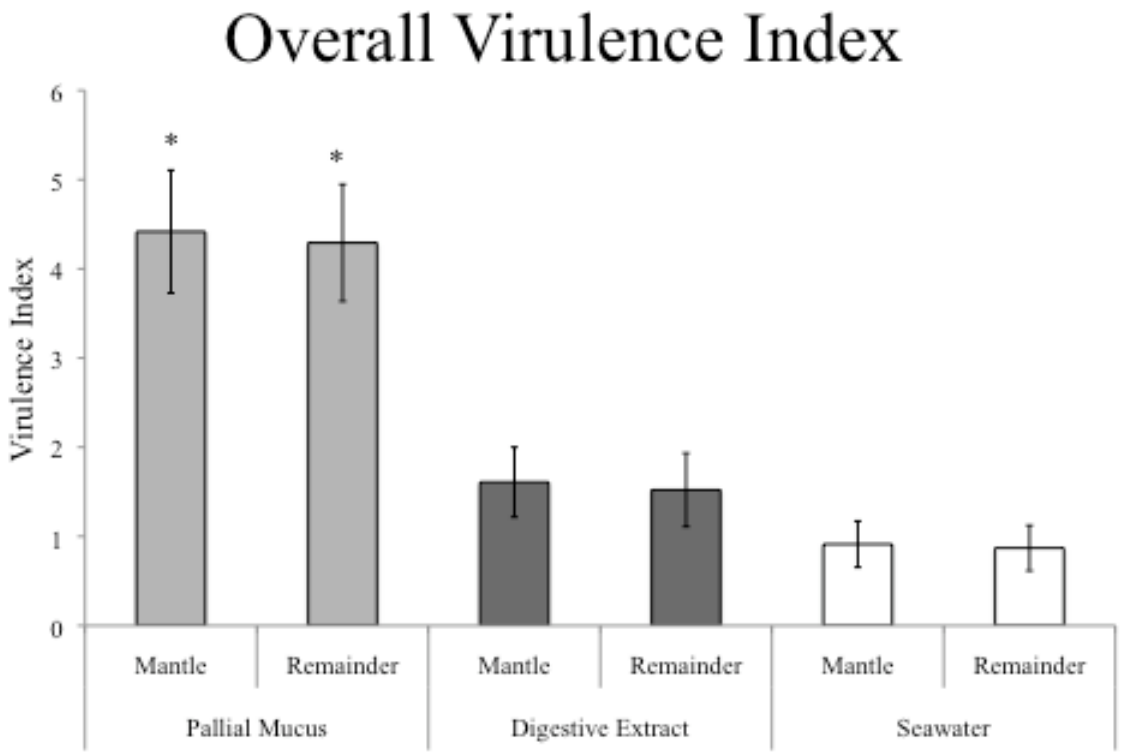

Figure 15. The virulence index for each oyster with separate indices determined for the mantle or remainder tissue. A one-way ANOVA followed by Holm-Sidak post-hoc test indicates that both the mantle and remainder tissue of the pallial mucus supplemented *P. marinus* treatment have a significantly higher virulence than it's respective tissue in the digestive extract supplemented *P. marinus* treatment or the seawater supplemented *P. marinus* treatment (all $p \leq 0.001$).

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Appendix

1. Medium Composition (Dungan and Hamilton 1995)

Media was supplemented to the following final concentrations with fetal bovine serum (FBS, 3% v/v), HEPES (25 mM), sodium bicarbonate (7 mM), yeast extract ultrafiltrate (Sigma Y4375, 0.2% v/v), lipid mixture (Sigma L5146, 0.1% v/v), glucose (0.05% w/v), trehalose (0.01% w/v), galactose (0.01% w/v), L-glutamine (2 mM), penicillin (200 U/mL), streptomycin (200 ng/mL), chloramphenicol (50 μg/mL) and nystatin (10,000 units/mL).