# ENVIRONMENTAL EFFECTS ON HOST-PARASITE INTERACTIONS IN AN ECOSYSTEM ENGINEER

by

### JENNAFER CHRISTINE MALEK

(Under the Direction of James E. Byers)

#### ABSTRACT

Host-parasite interactions can shape ecosystems, from individual species to entire communities. These interactions are highly heterogeneous due to complex relationships between hosts, parasites, and their environment. Environmental effects on host-parasite interactions are especially important in systems built around ecosystem engineers as negative parasite effects can be amplified onto entire communities. Additionally, as our climate changes, many of the environmental factors that shape host-parasite interactions are predicted to change as well, making it critical for us to identify the factors that effect host-parasite dynamics. To better understand the relationships between hosts, parasites, and the environment, we used a combination of field and laboratory studies to evaluate the affects of tidal elevation, air temperature, and predation on interactions between the ecosystem engineer Crassostrea virginica (eastern oyster) and two of its most lethal parasites, Perkinsus marinus and Haplosporidium nelsoni. The probability and intensity of parasite infections, and the probability of co-infection by both parasites, was significantly higher at higher (intertidal) than lower (subtidal) tidal elevations. This demonstrates that environmental factors can shape host-parasite interactions across small spatial scales and physical gradients. Because environmental conditions can fluctuate rapidly over short periods of time in the intertidal, we assessed the effects of increasing air temperature during air-exposure on oyster survival and immune response, and *P. marinus* infection intensity. We found that the parasite may benefit from increases in air temperature until its optimal temperature is reached, but the host has a higher capacity to survive at temperatures above the parasite's optimum. However, if temperatures exceed the host threshold, then neither species will benefit. Lastly, we evaluated how biotic factors (predation) affect oyster-parasite interactions. Though its been documented that predators can affect host-parasite interactions in other systems, we found that predators do not affect oyster-parasite interactions. Through our research, we have increased our understanding of the relationship between the environment and host-parasite interactions. This knowledge will be beneficial for creating research and management initiatives in the future that protect not just this engineering species but also the ecosystems that it creates.

INDEX WORDS: Crassostrea virginica, Perkinsus marinus, Haplosporidium nelsoni, co-infection, predator, immune response, climate change

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### JENNAFER CHRISTINE MALEK

B.S., University of Maine, 2005

M.S., University of Maryland, 2010

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

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Major Professor: James E. Byers

Committee:

Sonia Altizer James W. Porter Jonathan H. Grabowski Craig W. Osenberg

Electronic Version Approved:

Suzanne Barbour Dean of the Graduate School The University of Georgia December 2015

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#### CHAPTER 1

#### INTRODUCTION AND LITERATURE REVIEW

Host-parasite interactions can influence species abundance and distribution as well as the structure of entire communities. For example, in the last several decades, the fungal pathogen Batrachochytrium dendrobatidis has contributed to severe population declines or extinction of over 200 species of frogs around the globe (Skerratt et al. 2007). The 1980s die-off of the sea urchin Diadema antillarum due to an unknown bacterial agent led to an increase in algal cover on many Caribbean coral reefs (Lessios et al. 1984, Carpenter 1990). In systems structured around ecosystem engineers, host-parasite interactions that affect engineering host abundance may have amplified effects on the entire system. Engineers affect their surrounding community through alteration of physical habitat (Crain & Bertness 2006) and modification of biological and physical conditions (Hastings et al. 2007, Altieri et al. 2010). Reductions in the abundance of these species due to negative effects of parasites may change the role of the engineer within a community as well overall community structure. Severe reductions in the abundance of reef-building corals in the Caribbean and the ecological extinction of the American chestnut from North American forests due to parasites have led to reduced habitat for other species and lower biodiversity (Moberg & Folke 1999, McClanahan et al. 2002, Wang et al. 2013). In such systems, identifying factors that shape host-parasite interactions should be a research priority.

Interactions between hosts and their parasites can be highly heterogeneous in natural systems due to the complex relationships between not just hosts and parasites but also the environment. The environment can influence geographic range, population status, and physiological response of both host and parasite species, in turn affecting how these species interact with each other (Allen & Burnett 2008, Altizer et al. 2006, Lafferty & Kuris 1999). For instance, physiological stress caused by high temperatures in intertidal organisms or seawater acidification can suppress host immune responses, such as respiratory burst or phagocytosis (Allen & Burnett 2008, Bibby et al. 2008, Chu & La Peyre 1993). Suppression of these responses alters how the host reacts to parasite challenge. Alternatively, parasites may have less severe impacts on host species if parasites themselves are experiencing environmental stress such as exposure to pollutants or unfavorable temperature conditions (Lafferty & Kuris 1999).

Many of the abiotic and biotic environmental factors that are known to shape host-parasite interactions are predicted to be altered by changes in climate (IPCC 2014a). For example, it has been suggested that a warmer world may be a sicker world as some parasites have exhibited positive responses to increasing temperature (Harvell et al. 2002, 2004, Lafferty 2009). Parasite range expansions (Ford & Smolowitz 2007), higher parasite transmission between hosts (Mouritsen & Jensen 1997, Poulin 2006), and suppressed host immune response (Rigby & Jokela 2000, Navarro et al. 2003) have already been observed in response to increases in temperature. However, host immune responses such as fungal inhibition activity may be stimulated by increases in temperature (Ward et al. 2007). Additionally, some temperature-driven systematic responses in hosts may also help to mount resistance to parasite infection as climate

changes (Mydlarz et al. 2008). To understand how host-parasite interactions and potentially entire communities may be affected as the environment changes in the future, we need to understand the relationships between hosts, parasites, and their environment. The research described in the following dissertation focuses on identifying environmental factors that shape host-parasite interactions in a coastal ecosystem engineer, the eastern oyster, *Crassostrea virginica*.

#### Study System

Along the Atlantic and Gulf of Mexico coasts of the United States, the eastern oyster, *C. virginica*, is an ecologically and economically important species. As ecosystem engineers, oysters create complex reefs that provide habitat for numerous fish and invertebrate species (Lenihan et al. 2001, Coen et al. 2007, Newell et al. 2007). These reefs also protect shorelines from storms and wave action (Grizzle et al. 2002). Oysters improve water quality and contribute to benthic-pelagic coupling in coastal areas through filter feeding (Newell 1988, Newell et al. 2007). Additionally, oysters support an important commercial fishery. However, reduced population abundance due to overfishing, habitat destruction, and parasite epizootics has severely decreased the value of this fishery in some areas such as Georgia (Rothschild et al. 1994).

In the 1950s, two protistan parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, were identified as contributors to mass oyster mortalities in the Gulf of Mexico and Delaware Bay, respectively (Mackin et al. 1950, Haskin et al. 1965). By the 1980s, *P. marinus* had moved up the Atlantic coast to the Delaware and Chesapeake Bays. Currently *P. marinus* and *H. nelsoni* can be found as far North as Maine (Soniat 1996) and Nova Scotia (Stephenson et al. 2003), respectively. Infection patterns are primarily

driven by water temperature and salinity, and the parasites can be limited to certain geographic areas by low temperatures and salinities (Ford & Tripp 1996). Recent studies have demonstrated that diel-cycling hypoxia influences *P. marinus* through increased acquisition and progression of infections (Breitburg et al. 2015). These daily oxygen fluctuations can also affect oyster immune response through stimulation of phagocytosis and suppression of apoptosis (Keppel 2014). Additionally, laboratory research has indicated that several common oyster reef scavengers (crabs, snails, fish) may increase the transmission of *P. marinus* compared to passive parasite spread (Diamond 2012). The transmission mechanism for *H. nelsoni* has yet to be identified. Thus, much less is known about infection dynamics of *H. nelsoni* as there have been limited studies looking at environmental drivers other than water temperature and salinity (Littlewood et al. 1990).

Due to the destructive nature of both *P. marinus* and *H. nelsoni* on not only their host but also their community through loss of ecosystem services, we need to identify if other environmental factors control oyster-parasite dynamics. A stronger understanding of how changes in climate may affect oyster-parasite interactions will help us to more effectively shape research initiatives for oysters and their ecosystems. This dissertation evaluates the relationships between oyster-parasite interactions and three environmental factors that are predicted to be affected by change in climate: tidal elevation, air temperature, and predators.

#### **Summary of Chapters**

In Chapter 2 we evaluated if tidal elevation (intertidal vs. subtidal zones) drives heterogeneity in *P. marinus* and *H. nelsoni* infections. Through a manipulated field experiment, we assessed parasite infections between intertidal and subtidal oysters. We

found that the probability of infection (proportion of infected individuals) of *P. marinus* and *H. nelsoni* infections, co-infection by both parasites, and the intensity (severity of infection within an individual) of *P. marinus* infections were significantly higher in intertidal than subtidal oysters. We suggested that the duration of emersion or the variability of environmental conditions, particularly air temperature, during emersion may be the explanatory mechanism behind these patterns.

Expanding on the findings of Chapter 2, we conducted two laboratory experiments in Chapter 3 to test for effects of increasing air temperature on host survival and immune response, and *P. marinus* infection intensity. These experiments demonstrated that increases in air temperature favor the parasite to a certain point, but increases above the parasite's optimal temperature favor the host in terms of the capacity to survive at higher temperatures. These results also suggest that air temperature conditions during emersion may explain the significantly higher intensity of *P. marinus* infections in intertidal compared to subtidal oysters that we found in Chapter 2.

Lastly, in Chapter 4, we tested for effects of predators on *P. marinus* and *H. nelsoni* infection patterns and host immune response. Using a combination of field and laboratory studies, we evaluated the effects of multiple oyster predators (mud crabs, *Panopeus herbstii* and blue crabs, *Callinectes sapidus*) on parasite (*P. marinus* and *H. nelsoni*) prevalence and intensity, and host immune response. Across both experiments and predator species, we found that crab predators do not affect oyster-parasite interactions.

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## CHAPTER 2

# ENVIRONMENTAL EFFECTS ON PARASITE HETEROGENEITY AND CO-INFECTION IN AN ECOSYSTEM ENGINEER $^{\rm 1}$

<sup>&</sup>lt;sup>1</sup> Malek, JC and JE Byers. To be submitted to *Oecologia* 

#### <u>Abstract</u>

Environmental factors can affect parasites both directly and indirectly through their influence on host physiology. Such effects may be particularly pronounced if hosts are ecosystem engineers, and negative effects of parasites can be magnified within the larger ecosystem. We quantified the effect of tidal elevation on infection patterns of two lethal parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, in an important engineering species, the eastern oyster, Crassostrea virginica. In the southeast US, Georgia in particular, oysters are rarely found in the subtidal zone where waterborne parasites would have longer periods of access to the host. We manipulated oysters at two tidal elevations (intertidal and subtidal) in a Georgia estuary, hypothesizing that parasite infections in oysters would be more abundant and more intense subtidally than intertidally. Strikingly, we found that parasite prevalence, co-infection prevalence, and infection intensity were significantly higher in intertidal than subtidal oysters thus falsifying our hypothesis that the intertidal could be a refuge from parasitism. This pattern could stem from physiologically challenging conditions for the host due to the duration of emersion or the variability in environmental conditions, such as air temperature, during emersion. Our study identifies tidal elevation as a factor that shapes parasite infection patterns and helps to explain distributional patterns in an important engineering species. Additionally, we demonstrate that environmental factors can influence host-parasite interactions across small spatial scales.

#### **Introduction**

Infection patterns of parasites and pathogens can be highly heterogeneous due to complex interactions between parasites, hosts, and their environment. The environment can dictate geographic range, population abundance, and physiological response of both parasite and host species (Lafferty & Kuris 1999, Altizer et al. 2006, Allen & Burnett 2008). Parasite physiology, for example, can be influenced by factors such as temperature, salinity, and pollutants, which can affect parasite transmission and virulence (Lafferty & Kuris 1999, Soudant et al. 2005, Ford & Chintala 2006). Similarly, host characteristics such as immune response, reproductive status, age, and overall body condition can cause variability in parasite infection and these characteristics are in turn affected by environmental conditions. For instance, host immune responses such as respiratory burst and phagocytosis can be suppressed by physiologically stressful conditions (Cheng et al. 2004, Bibby et al. 2008. Such changes in immune response may increase host susceptibility to parasite challenge. To understand why parasite infections are heterogeneously distributed throughout ecological systems, we need to identify the environmental factors that influence parasite-host interactions. Such knowledge will help us comprehend how large-scale infection patterns may respond to environmental shifts and alterations in host or parasite populations.

In addition to interacting with their hosts and the environment, parasites also are often simultaneously interacting with other parasite species. Within a host, co-infecting parasites can have synergistic or antagonistic interactions that may influence parasite effects on the host, such as stimulation or weakening of host immune responses (Cattadori et al. 2008, Ezenwa et al. 2010). Co-infecting parasites can also be influenced

by environmental factors if certain conditions reduce host immune function (Supali et al. 2010) or favor the transmission or reproduction of one parasite species over the other. Additionally, interactions between multiple parasite species may affect how environmental factors influence individual host-parasite dynamics.

Identifying and understanding interactions between parasites, hosts, and their environment is important for all ecological systems, but may be especially important for host species that are ecosystem engineers. Engineering species affect community structure by altering physical habitat (Crain & Bertness 2006), modifying biological and physical conditions (Hastings et al. 2007, Altieri et al. 2010), and directly or indirectly controlling resource availability for other species (Jones et al. 1996). Parasites may therefore have amplified impacts on the entire ecosystem when hosts are ecosystem engineers. For example, the ecological extinction of the American chestnut from North American forests in the 1900s due to chestnut blight led to reduced canopy cover, habitat, and food for other species (Wang et al. 2013). In the Caribbean, parasites have caused severe reductions in the abundance of important reef-building corals, such as Acropora *palmata*, resulting in reduced community biodiversity and decreased habitat for other reef-dwelling species (Moberg & Folke 1999, McClanahan et al. 2002). In systems with large amounts of such biogenic structure (e.g., forests and coral reefs) it is vital to identify the environmental factors that drive heterogeneity in parasite infection, as these factors have the potential to alter ecosystem structure and composition.

In this study, we investigated environmental influences on parasite infection heterogeneity, including parasite co-infection, in the eastern oyster, *Crassostrea virginica*. This species is a well-studied, ecologically and economically valuable

ecosystem engineer along the Atlantic and Gulf of Mexico coasts of the US. In coastal systems oysters create complex habitat for other organisms, protect shorelines from erosion, and improve water quality (Lenihan et al. 2001, Grizzle et al. 2002, Coen et al. 2007, Newell et al. 2007). Since the 1950s, *C. virginica* has been plagued by two protistan parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, which co-occur throughout much of the host's range. Independently, these parasites can cause high host mortality (Ford & Haskins 1982, Andrews 1988) and have exacerbated already low population abundances caused by issues such as overfishing and habitat destruction (Rothschild et al. 1994).

Shortly after the discovery of these parasites, water temperature and salinity were recognized as the primary environmental factors that shaped infection prevalence (proportion of infected individuals in a population) and intensity (severity of infection within an individual) (Andrews 1988, Soniat 1996, see Ford & Tripp 1996 for a detailed review). Other research has suggested that diel-cycling hypoxia and air temperature may also influence *P. marinus* infections (Breitburg et al. 2015, Keppel et al. 2015, Malek & Byers Chapter 3). However, the directionality of these effects is not entirely clear as recent studies of *P. marinus* infections across intertidal air-exposure gradients have yielded equivocal results (Ybanez 2007, Malek & Breitburg *in review*). Only a single study has tested for effects of other environmental factors on *H. nelsoni* (Littlewood et al. 1990). There are no studies specifically describing *H. nelsoni* infections in combination with *P. marinus*, leaving an important gap in our understanding of *H. nelsoni* infection patterns, and ultimately patterns of co-infection in *C. virginica*.

To better understand how the environment can influence oyster-parasite interactions, we experimentally manipulated oysters within a Georgia estuary, in the Southeastern US. Our study addressed whether environmental conditions in the Southeast may differentially affect heterogeneity of parasite infections. Specifically, we hypothesized that parasite infection prevalence (P. marinus and H. nelsoni), co-infection prevalence, and infection intensity (P. marinus) would increase with decreasing tidal elevation due to longer durations of waterborne parasite exposure. The natural absence of oysters in the subtidal zone in the study region suggests that this environment compromises oyster success, potentially through physiological stresses that could make the host more vulnerable to infection. Previous work on P. marinus focused on the mid-Atlantic US (Malek & Breitburg in review) found no effect of air-exposure on parasite infections. Environmental differences between the mid-Atlantic and Southeast, including tidal range, air-exposure duration, air temperature, and oyster spatial distribution (mostly intertidal-subtidal mix vs. mostly intertidal, respectively), could affect parasite-host interactions. Thus we hypothesized that these earlier findings may be region-specific because parasite activity and host condition are highly sensitive to specific conditions during air-exposure (Burnett 1997, Milardo 2001, Allen & Burnett 2008, Keppel 2014).

#### <u>Methods</u>

#### **Oyster Collection and Processing**

We hand-collected wild oyster clusters containing multiple generations of oysters and a combination of living and dead shell material from Romerly Marsh Creek in the Wilmington River, Savannah, GA (31°55'21.78"N, 80°59'20.85"W) in May 2012. We broke clusters into smaller, 200-400 g clumps (~8 oysters >25 mm clump<sup>-1</sup>) and

defaunated the clumps by soaking them in fresh water for  $\sim$ 1 h to remove unwanted epifauna or predators. We then weighed clumps, measured the length of all individual oysters >25 mm, counted all individuals < 25 mm, and stored clumps in flow-through seawater tanks free of predators until deployment. We randomly selected and processed 110 oysters for baseline *P. marinus* infection prevalence and intensity.

#### **Experimental Design and Field Setup**

Oysters throughout Georgia are typically found exclusively in the intertidal zone. To test for effects of tidal elevation on *P. marinus* and *H. nelsoni* infections in the field, we used two experimental treatments: oysters were either kept at their natural intertidal elevation where they were exposed to air twice a day or kept at a lower, subtidal elevation where they were continuously submerged. We applied each tidal elevation treatment to 0.09 m<sup>2</sup> oyster reefs built inside 0.027 m<sup>3</sup> plastic crates (0.3 m x 0.3 m x 0.3 m) lined with Vexar mesh (0.635 mm) to exclude most large oyster predators. Crate bottoms were covered with large, individual oyster shells to create a reef base and 10 randomly selected experimental oyster clumps were placed on top. We sealed the crate tops with fine mesh bird netting to further exclude predators. Crates were attached to cinder block anchors buried in the mud in either the intertidal mud flat (1.5 m above the mean low water MLW mark) or in a parallel line in subtidal sediment (1.5 m below the MLW mark). Within a given tidal elevation, individual reefs were set 1 m from each other. In the intertidal, reefs were in line with wild oyster clumps that were interspersed throughout our study site.

We deployed 16 replicates of each tidal elevation treatment for 19 weeks (01-June through 13-Oct 2012). On the sediment next to experimental reefs, we deployed pressure

gauges (Onset HOBO, U20-001-04) in waterproof cases at each tidal elevation to record water temperature and air pressure every 15 min from which we calculated the duration of air-exposure for intertidal reefs. Upon retrieval, all oyster clumps were weighed and measured as described above. We selected a random subset of ~30 oysters per reef for parasite infection assessment.

#### **Parasite Infection Assessment**

P. marinus assessment

We tested for *P. marinus* infections using the Ray's Fluid Thioglycollate Media (RFTM) method (Ray 1954), which provided measures of both the probability of infection and infection intensity. Rectal tissue samples were incubated in thioglycollate media for six days, then stained with Lugol's Iodine and assessed microscopically for *P. marinus* hypnospores, which stain blue-black. We scored *P. marinus* intensity using the Mackin scale (Mackin 1962), a 6-point scale ranging from 0 (no infection) to 5 (heavy/lethal infection). We calculated the prevalence of infection by dividing the number of infected oysters (intensity score of 0.5 or higher) by the number of oysters sampled. We also calculated the mean infection intensity (i.e., RFTM score) of infected individuals on each reef (Soniat et al. 2006).

H. nelsoni assessment

We used common polymerase chain reaction (cPCR), a fast and highly sensitive diagnostic method, to assess *H. nelsoni* infections. This method detects the existence of the parasite in an oyster, but not the intensity of the infection. We collected gill and mantle tissue from all dissected oysters using sterile instruments to eliminate cross-contamination and stored tissue in 95% ethanol until the time of DNA extraction. From the ~30 oysters per replicate reef that had been sampled for *P. marinus*, we randomly

selected 8 individuals for *H. nelsoni* assessment. Qiagen DNEasy Blood and Tissue kits were used according to the manufacturer's protocol for animal tissue to collect a minimum of 200 µl of oyster DNA that was stored at -20°C until tested.

We used a *H. nelsoni* detection primer set from Stokes et al. (1995a) and Renault et al. (2000) (Table 1). The PCR mixture of 23 µl consisted of 12.5 µl GoTaq 2X Green Master Mix (Promega), 8 µl nuclease free water, 1.5 µl BSA, 0.5 µl each of MSX A' and B primers, and 2 µl of template DNA. Amplification was conducted on an Eppendorf Mastercycler following a program of 30 cycles of 94°C for 30 sec, 59°C for 30 sec, and 72°C for 1.5 min, with an initial holding step at 94°C for 4 min and final extension of 72°C for 5 min. Positive controls for *H. nelsoni* were obtained from N. Stokes (VIMS). We ran amplified products through a 1.5% agarose gel containing GelRed nucleic acid stain (Biotium) and viewed them with a UV transilluminator. All experimental samples and positive and negative controls were run in duplicate for *H. nelsoni* detection.

#### Statistical Analyses

We used mixed effects logistic regression models ('lme4' package in R) to analyze the probability of infection for individual oysters with a binary distribution as influenced by the fixed effect of tidal elevation. Replicate was treated as a random effect as multiple oysters were sampled from the same experimental reef. We analyzed *P*. *marinus* infections using data from all of the individuals tested with RFTM, including individuals from the subsample that were also tested for *H. nelsoni* infection. With a separate model, we analyzed the probability of *H. nelsoni* infections using all individuals in the subsample tested with PCR (regardless of co-infection status). For co-infection by both parasites, we conducted an identical third mixed effects logistic regression analysis,

with co-infection as a binary response (0=not infected or infected with only 1 parasite, 1=infected with both parasites). We also used co-infection data to determine if the two parasites randomly associate with separate Chi Square Tests of Independence for each tidal elevation. For these tests, we used the observed and expected number of individuals co-infected by both parasites.

We analyzed *P. marinus* infection intensity data based on Mackin scores with mixed effects Poisson regression, including only infected individuals from each experimental reef. As with the analyses on the probability of infection, we used tidal elevation as a fixed effect and replicate as a random effect. To determine if infection intensity had increased from the beginning of the experiment, we compared intensity scores from infected oysters from the initial sampling to intensity scores in the intertidal and the subtidal using a Poisson regression model. We further assessed the intensity data to determine if the proportion of lethal-level infections differed with tidal elevation by calculating the proportion of infections with Mackin scores  $\geq 3$  at each tidal elevation. Lastly, to examine the influence of *H. nelsoni* on the intensity of *P. marinus* infections, we used a mixed effects Poisson regression model to test the fixed effects of *H. nelsoni* infection status, tidal elevation, and their interaction on *P. marinus* infection intensity and included replicate as a random effect. If the interaction term was not significant, it was removed and the model was reanalyzed for main effects.

To determine if differential mortality occurred between tidal elevations, as it could have affected parasite infection results, we analyzed mortality using a mixed effects Poisson regression model identical to the one previously described for *P. marinus* infection intensity, including the number of dead oysters on each reef as our response

variable. All analyses were conducted in R Studio, version 0.98.1087 using R version 3.2.0 (R Core Team 2015).

#### **Results**

The probability of *P. marinus* infection was significantly higher in the intertidal than the subtidal, with ~25% more infections in the intertidal (Fig. 2.1, Table 2.2a). The proportion of oysters infected with *P. marinus* increased from the initial population baseline (32%, based on naturally intertidal oysters) in both tidal locations over the 19 wk experimental period, but a significantly greater number of individuals became infected in the intertidal than in the subtidal (Fig. 2.1).

The probability of *H. nelsoni* infection was significantly higher in the intertidal than in the subtidal as well, with approximately 50% more infected oysters in the intertidal (Fig. 2.1, Table 2.2b). The probability of co-infection with both *P. marinus* and *H. nelsoni* was also significantly higher in the intertidal than in the subtidal (Fig. 2.1, Table 2.2c). Additionally, Chi Square analysis indicated no non-random association of *P. marinus* and *H. nelsoni* at either tidal elevation (intertidal:  $X^2$ =0.036, df=1, p=0.85; subtidal:  $X^2$ =0.298, df=1, p=0.59).

Tidal elevation was also a significant predictor of *P. marinus* infection intensity (Fig. 2.2, Table 2.3a), with significantly higher intensity infections in the intertidal than the subtidal. Compared to the initial baseline, the intensity of infections tended to be higher in the intertidal (z=1.777, P(z)=0.075), but not in the subtidal (z=0.704, P(z)=0.481). Further evaluation of infection intensity indicated that intertidal oysters had twice as many lethal infections (3-5 on the Mackin scale) as subtidal oysters (proportion lethal:  $p_{intertidal}=0.20$ ,  $p_{subtidal}=0.09$ ). The proportion of lethal infections increased from the

baseline ( $p_{baseline}$ =0.09) by ~120% in the intertidal but did not increase at all in the subtidal. We found no interactive effect of *H. nelsoni* infection and tidal elevation on *P. marinus* infection intensity (z=0.031, P(z)=0.975) and no individual effect of *H. nelsoni* (Table 2.3b).

Lastly, there was a significant effect of tidal elevation on mortality (estimate=-0.374, z=-2.001, P(z)=0.045), with higher mortality in the intertidal than the subtidal. However, mortality was very low across both tidal elevations (mean intertidal= $2.1 \pm 0.2$ ; mean subtidal= $1.5 \pm 0.2$ ). The differential mortality between tidal elevations likely did not affect the observed parasite infection patterns as average mortality was too low overall to cause detectable changes in the probability or intensity of infection.

#### **Discussion**

Differences in tidal elevation significantly affected the prevalence, intensity, and co-occurrence of parasite infections in the eastern oyster, *C. virginica*. The probability of infection by *P. marinus* and *H. nelsoni* individually, as well as co-infection by both combined, was significantly greater at intertidal than subtidal elevations. Intensity of *P. marinus* infections was also significantly higher in the intertidal, with a higher proportion of lethal infections compared to the subtidal. These findings indicate that significant differences in parasite infection can occur over relatively small spatial scales (~3m) and, in contrast to previous findings (Burrell et al. 1984, Malek & Breitburg *in review*, O'Beirn et al. 1994), suggests that differences in tidal elevation may shape parasite infection heterogeneity in this system.

Durations of emersion vary based on tidal range and geographic location. In our study, intertidal oysters were exposed to air an average of  $\sim$  4-4.5 h per tidal cycle.

O'Beirn et al. (1994) exposed oysters to a similar duration of air-exposure in another Georgia estuary but found no difference in *P. marinus* prevalence or intensity between tidal elevations. Additionally, Malek & Breitburg (*in review*) found no difference in *P. marinus* infections across a wide range of exposure durations (0.78-5.95 h) in the mid-Atlantic. Their results suggest that the duration of emersion may not be the primary driver of differences in parasite infections across tidal elevation.

The extreme variability in air temperature, oxygen, and CO<sub>2</sub> conditions over small spatial scales in the intertidal zone ( $\sim 1$  m) during periods of emersion may have negative impacts on host physiology and subsequently affect interactions with parasites (Burnett 1997, Boyd & Burnett 1999, Kuchel et al. 2010). Our experimental oysters experienced temperatures ranging from 16°C during submersion to spikes up to 45°C on the mud flat during low tide when exposed to air, with frequent temperature fluctuations  $\geq 15^{\circ}$ C over just a few hours. When exposed to air and fluctuations in temperatures, bivalves tend to cut off circulation with the atmosphere, leading to potentially toxic internal conditions (low O<sub>2</sub> and high CO<sub>2</sub>) (Burnett 1997), which can alter the host immune response (Allen & Burnett 2008, Keppel 2014). Periods of suppressed or stimulated immune response could increase host susceptibility to either acquisition of new infections or intensification of established infections. Though we did not test for host immune response in this study, it is possible that altered immune function due to the variability of conditions during air exposure could have contributed to the differences in parasite infection between intertidal and subtidal oysters (Boyd & Burnett 1999, Harvell et al. 2007).

Both *P. marinus* and *H. nelsoni* have been well studied individually, but little is known about how infection with one parasite influences infection by the other. We

observed that the probability of infection with one parasite was independent of infection by the other at both tidal elevations (as seen in results of the Chi Square analysis). Though multiple parasite infections can affect the host immune response, ultimately reducing the host's ability to mediate established infections (Cattadori et al. 2008, Graham 2008), we observed that infection with *H. nelsoni* did not affect the intensity of *P. marinus* infections (Table 2.3b). Overall, our analysis of co-infection suggests that *P. marinus* and *H. nelsoni* randomly associate in terms of the probability and intensity (*P. marinus*) of infection.

Interestingly, we found that parasite infections are significantly less prevalent and less intense in the subtidal than the intertidal. However, oysters in the southeastern US naturally live intertidally and are almost entirely absent from the subtidal zone in Georgia. It has been hypothesized that higher rates of parasitism, sedimentation, or predation keep oysters from populating the subtidal zone. In the subtidal, the average P. *marinus* intensity score was 1.3 (compared to 1.7 in the intertidal; Fig. 2.2), more than half of the infections were scored as light-moderate (Mackin scores of 0.5-1; Fig. 2.2), and there was a smaller proportion of lethal intensity infections (Mackin score  $\geq$  3) compared to the intertidal (data not shown). In the subtidal, oysters have twice the amount of exposure to parasites compared to the intertidal. The significantly lower probability of infection we observed in the subtidal further supports that the duration of exposure to parasites may not be as important as the difference in physical conditions in the intertidal in terms of shaping infection patterns. Alternatively, the constant movement of water in the subtidal may actually reduce oyster exposure to water-borne parasites such as *P. marinus*. If parasites suspended in the water column are removed from an area

before they can be consumed by new hosts, we could expect to see lower local parasite transmission in subtidal habitats. Additionally, the light to moderate intensity infections in the subtidal zone, and the absence of other lethal parasites that infect oysters, suggests that heightened *P. marinus* and *H. nelsoni* prevalence and intensity can be eliminated as factors explaining the lack of subtidal oysters in this region.

Though we observed that environmental conditions make the intertidal zone a more favorable habitat for parasite infections compared to the subtidal, Southeastern oysters thrive intertidally. The resiliency of intertidal reefs may be the result of high recruitment of juvenile oysters compensating for parasite-related mortality. In areas of low recruitment, parasites can have devastating effects on already low oyster abundances (Rothschild et al. 1994) and make it difficult for populations to rebound from epizootics (Mann & Powell 2007). Byers et al. (2015) found that across a biogeographic range from Cape Hatteras, NC to St. Augustine, FL, South Carolina and Georgia estuaries had the highest oyster recruitment, density, biomass, and the most rugose reef structure. Additionally, Malek (*unpublished data*) found that oyster recruitment was significantly higher in the intertidal than the subtidal at our specific study site. High recruitment and addition of reef biomass may counterbalance the effects of environmentally driven parasite patterns and resultant host mortality, allowing intertidal reefs to remain healthy overall.

Our findings are in conflict with other studies conducted in the same region that found no effect of tidal elevation on *P. marinus* infection. O'Beirn et al. (1994) observed close to 100% *P. marinus* prevalence in both the intertidal and subtidal. High values across the board afford very low resolution to detect differences between tidal elevations.
Infection prevalence was much lower in our study (~40-50%; Fig. 2.1), possibly due to decreased *P. marinus* transmission or lower ambient parasite levels (salinity and water temperature were very similar between all studies). Burrell et al. (1984) specifically sampled the largest oysters at their sites for *P. marinus* infection, which may have created a size bias, as larger oysters have a higher probability of being infected. We sampled oysters ranging from  $35-100^+$  mm as small oysters can also be infected by *P. marinus* under the right environmental conditions (McCollough et al. 2007). Lastly, both of the earlier studies sampled parasite infection throughout the course of an entire year. We focused on infections during the summer, when variability in environmental conditions is likely to be the most extreme and populations experience high parasite-related mortality and parasite transmission. Seasonal infection patterns could have been diluted in previous studies when parasite data were considered across seasons, resulting in no detectable effect of tidal elevation on *P. marinus* infection.

As an ecosystem engineer, *C. virginica* is a critical species in coastal habitats along the east and Gulf coasts of the US. In some areas, parasites have affected the abundance of this engineering species and altered overall community structure through loss of habitat, reduction of water quality, and increased shoreline erosion (Rothschild et al. 1994, Newell et al. 2007). We have identified another environmental variable that influences parasite infections in this system, even across small spatial scales. This knowledge will be beneficial for management or conservation efforts aimed at restoring oyster populations. In areas where oysters are primarily subtidal, building reefs in the intertidal to help combat parasite-related mortality may seem like a logical solution. Our data indicate that the intertidal may be a more beneficial habitat for parasites and

therefore efforts focused on intertidal restoration may not be successful. Overall, by identifying the environmental factors and corresponding mechanisms that shape infection patterns, we can better understand how parasites affect oysters and other ecologically and commercially important engineering species.

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Primer	Sequence (5'-3')
MSX A' (Renault et al. 2000)	CGACTTTGGCATTAGGTTTCAGACC
MSX B (Stokes et al. 1995a)	ATGTGTTGGTGACGCTAACCG

Table 2.1. Primer set for *H. nelsoni* assessment (Stokes et al. 1995a, Renault et al. 2000).

Table 2.2. Results of mixed effects logistic regression analyses testing the fixed effect of tidal elevation and the random effect of replicate on: **a.** probability of *P. marinus* infection, **b.** probability of *H. nelsoni* infection, and **c.** probability of co-infection. The reference tidal elevation for analyses was the intertidal treatment; thus, negative estimates for tidal elevation indicate that a subtidal oyster had a lower probability of infection than an intertidal oyster.

a. Probability of <i>P. marinus</i> infection						
Fixed Effect	Estimate	SE	z-Value	Pr(z)		
Intercept	< 0.001	0.109	-0.008	0.99		
Tidal Elevation	-0.330	0.154	-2.146	0.032		
Random Effect	Variance	SD				
Replicate	0.026	0.161				
b. Probability of	<i>H. nelsoni</i> infe	ction				
Fixed Effect	Estimate	SE	z-Value	Pr(z)		
Intercept	0.541	0.225	2.407	0.016		
Tidal Elevation	-1.040	0.332	-3.128	0.002		
Random Effect	Variance	SD				
Replicate	0.233	0.482				
c. Probability of	Co-infection					
Fixed Effect	Estimate	SE	z-Value	Pr(z)		

Intercept	-0.717	0.188	-3.807	< 0.001
Tidal Elevation	-0.611	0.305	-2.005	0.045
Random Effect	Variance	SD		

Table 2.3. Results of mixed effects Poisson regression analyses of *P. marinus* infection intensity including **a.** tidal elevation as a fixed effect and replicate as a random effect (intertidal was the reference tidal elevation) and **b.** *H. nelsoni* infection status and tidal elevation as fixed effects and replicate as a random effect (uninfected with *H. nelsoni* was the reference infection status and intertidal was the reference tidal elevation).

a. <i>P. marinus</i> intensity by tidal elevation					
Fixed Effect	Estimate	SE	z Value	P(z)	
Intercept	0.945	0.044	21.383	< 0.001	
Tidal Elevation	-0.133	0.067	-1.991	0.046	
Random Effect	Variance	SD			
Replicate	0	0			
b. <i>P. marinus</i> inter	nsity by <i>H. nel</i>	s <i>oni</i> infection s	status and tidal ele	evation	
Fixed Effect	Estimate	SE	t Value	P(z)	
Intercept	0.905	0.111	8.128	< 0.001	
H. nelsoni Status	0.097	0.121	0.798	0.425	
Tidal Elevation	-0.105	0.121	-0.866	0.386	
Random Effect	Variance	SD			
Replicate	0	0			



Figure 2.1. Mean proportion of *P. marinus* (based on all individuals assessed with RFTM, including subsample also assessed with PCR, n=425 Intertidal, n=420 Subtidal) and *H. nelsoni* infections (based on all individuals assessed with PCR, including co-infections, n=128 Intertidal, n=105 Subtidal) and co-infected oysters by tidal elevation (n=128 Intertidal, n=105 Subtidal). Error bars represent standard error calculated using replicate for each treatment. Solid black lines represent the estimated proportion of co-infection by *P. marinus* and *H. nelsoni* if infections are independent. Baseline *P. marinus* prevalence at the start of the experiment was 32%.



Figure 2.2. The distribution of Mackin scores (an index of infection intensity) among *P*. *marinus* infected oysters. The arrows on the x-axis represent the average Mackin score for baseline (black), intertidal (dark gray), and subtidal (light gray) oysters.

# CHAPTER 3

# DIFFERENTIAL RESPONSES OF HOST AND PARASITE TO TEMPERATURE: IS THERE AN ADVANTAGE IN A WARMING WORLD?<sup>2</sup>

 $<sup>^{2}</sup>$  Malek, JC and JE Byers. To be submitted to *Functional Ecology* 

#### <u>Abstract</u>

Changes in climate are predicted to influence infection patterns of parasites and pathogens. In particular, increases in temperature may greatly alter host-parasite interactions in several ways. Parasites could benefit from increased virulence, elevated transmission rates, or weakened host immune response. Alternatively, hosts could benefit from decreased parasite growth (if parasites respond negatively to increasing temperature) or from stimulated immune responses that help to mediate or even eliminate parasite infections at higher temperatures. In the Southeastern United States, the Eastern oyster, Crassostrea virginica, is infected by the lethal parasite, Perkinsus marinus. Under field conditions, intertidal (air-exposed) oysters have significantly higher P. marinus infection prevalence and intensity than subtidal (submerged) oysters. However, it is unclear what mechanism drives this pattern. We used laboratory experiments to determine how host and parasite respond to increasing air temperature during low tide. We simulated intertidal exposure in the lab using air temperature chambers ranging from 27-53°C and assessed host survival and immune response (phagocytic activity), and parasite infection intensity. Host survival decreased slightly between 27-39°C and then dropped off drastically above 39°C. Host phagocytic activity was only negatively affected at air temperatures above the host's thermal threshold. Finally, parasite infection intensity increased between 27-35°C, but significantly decreased between 35-39°C. Overall, our results suggest that host and parasite will differentially respond to increases in air temperature. Temperatures up to 35°C may favor the parasite while the host may have a higher capacity to survive than the parasite at temperatures between 35-39°C.

However, depending on the degree of warming that occurs, it may be that neither species benefits from a warming world.

#### **Introduction**

Anthropogenically driven changes in climate are influencing terrestrial and marine ecosystems, from individual species to entire communities (Janzen 1994, Brown et al. 1997, Loeb et al. 1997, Crick & Sparks 1999). Environmental factors including air and water temperature, seawater acidification, and sea level are all projected to increase due to climate change (IPCC 2014a). Climate models predict that global mean surface air temperature will increase anywhere from 0.3°C to upwards of 4.5°C by 2090 with larger, region-specific increases expected (IPCC 2014a, b). Such changes in temperature will likely drive considerable variation in biological processes such as metabolic rate (Gillooly et al. 2001, Ganser et al. 2015) and immune function. Temperature, for example, can alter both innate and adaptive immune functions including phagocytic and phenoloxidase activity (Cheng et al. 2004, Wang et al. 2008), and the expression of acquired immune genes (Raffel et al. 2006, Dittmar et al. 2014). Stimulation or suppression of these biological processes by changes in temperature can affect organismal responses to other factors.

One of the primary uncertainties about the expected shifts in temperature is how such changes might alter interspecific interactions such as host-parasite interactions. If parasites respond more favorably to increases in temperature than their hosts, through higher parasite growth and reproduction, a warmer world may be a sicker world (Harvell et al. 2002, Harvell et al. 2004, Lafferty et al. 2004). Some parasites have already demonstrated positive responses to climate-induced increases in temperature, including increased geographic ranges (Pounds et al. 2006, Ford & Smolowitz, 2007) and increased transmission between hosts (Mouritsen & Jensen 1997, Moore et al. 2000, Poulin 2006). For example, in the Caribbean, increases in both the prevalence (proportion

of infected individuals in a population) and growth rate of lesions of yellow band disease in corals covaried significantly with increases in annual mean water temperature from 1999 to 2007 (Harvell et al. 2009). Such positive parasite responses to increasing temperature may be connected to temperature-induced changes in host-related mechanisms including increased physiological stress (Lesser & Farrell 2004, Allen et al. 2008) or suppressed immune response (Chisholm & Smith 1994, Cheng et al. 2009, Al-Zahraa 2008).

Alternatively, increasing temperature may benefit hosts more than parasites by stimulating immune responses (Ward et al. 2007, Mydlarz et al. 2008, Mydlarz et al. 2009). For example, Cheng et al. (2004) observed that Taiwan abalone exposed to increases in water temperature had significantly higher respiratory burst activity compared to abalone at lower temperatures. Mydlarz et al. (2009) found that the coral Montastraea faveolata had higher prophenoloxidase activity (an important precursor to other immune functions used in parasite resistance) when exposed to higher than normal water temperature. Increasing temperature has also been found to stimulate amoebocyte production (and subsequently melanosome production) in sea fans which can concurrently help fight parasite infection (Mydlarz et al. 2008). Though a range of host immune responses have been found to be stimulated by increasing temperature, the larger implications of these effects are still uncertain. Because effects of increasing temperature on host immune response, and thus host-parasite interactions, can be varied and are often based on observational studies, we sought to experimentally examine how both hosts and parasites will be influenced by increasing temperature.

Along the East and Gulf of Mexico coasts of the US, the eastern oyster,

Crassostrea virginica, is an ecologically and economically important ecosystem engineer that creates complex reef habitat and supports a commercial fishery. Throughout most of its range, C. virginica is infected by the protistan parasite Perkinsus marinus, which can cause considerable host mortality. First discovered in the Gulf of Mexico in the 1950s (Mackin et al. 1950), P. marinus has expanded its range from the mid-Atlantic to the Northeast in the last several decades, primarily due to climate-driven increases in winter water temperatures (Ford & Chintala 2006, Ford & Smolowitz 2007). Environmental drivers of *P. marinus* infection patterns are numerous, including water temperature, salinity, diel-cycling hypoxia, tidal elevation, and weather events such as El Niño/Southern Oscillation (ENSO) and the North Atlantic Oscillation (NAO) (Ford & Tripp 1996, Soniat et al. 2006, Soniat et al. 2009, Breitburg et al. 2015, Keppel et al. 2015, Malek & Byers Chapter 2). Additionally, the activity and function of oyster hemocytes, which are the primary line of oyster immune defense, are also affected by water temperature, salinity, and diel-cycling hypoxia (Hégaret et al. 2003, Keppel 2014). Thus, characteristics of both host and parasite in this system are affected by factors that are predicted to be altered by climate change.

Many of the environmental drivers of both *P. marinus* infection patterns and *C. virginica* immune response are related to temperature. Malek & Byers (Chapter 2) observed that intertidal oysters had significantly higher prevalence and intensity (severity of infection within an individual) of *P. marinus* infections than subtidal oysters. They suggested that the mechanism driving this pattern could be either the duration of emersion (~4-4.5 h) or the variability of conditions, primarily air temperature during

periods of emersion. In this study, we further investigated the role of air temperature in the *C. virginica - P. marinus* system. Specifically, we evaluated if increasing air temperature, including temperatures that could occur with climate change, affects host survival and immune response, and parasite infection intensity. As many biological processes tend to be affected by changes in temperature, we hypothesized that host survival and immune response would be negatively affected as air temperature increased above a certain thermal threshold. Additionally, as *P. marinus* responds favorably to increases in water temperature (Ford & Tripp 1996), we hypothesized that infection intensity would also increase with increasing air temperature, until an optimal air temperature was reached. Above this optimal temperature, we expected that infection intensity would decrease as a result of negative temperature effects on the parasite.

#### Materials and methods

#### **Oyster Collection, Preparation, and Maintenance**

To achieve a more comprehensive understanding of the effects of increasing air temperature on host survival and immune response, we wanted to measure these variables in both the presence and absence of *P. marinus* infection. The intertidal oyster population at the Skidaway Institute of Oceanography's (SkIO) Priest Landing had a baseline *P. marinus* prevalence of ~ 60% based on destructive quantitative PCR assessment (qPCR, see Parasite Assessment below) in early May 2014. Based on this prevalence, we assumed that oysters collected from this population would provide roughly equal numbers of infected and uninfected hosts (we were unable to determine infection status until the conclusion of experiment due to the destructive nature of parasite assessment). We randomly collected 225 individual oysters (30-100mm) from intertidal reefs in late

May and cleaned them of sediment and fouling agents such juvenile oysters. We tagged individuals with bee tags (The Bee Works), measured shell heights, and allowed 72 h of acclimation to laboratory water conditions of 27°C (average summer water temperature in GA) prior to beginning air temperature treatments.

To prevent *P. marinus* transmission during the experiment, we held oysters individually in labeled, independent 32 oz containers that corresponded to individual oyster ID tags. We filled containers with artificial seawater (salinity ~25, Instant Ocean dissolved in tap water) and changed the water every 2-3 d to limit accumulation of feces and nutrients. Each container had its own air supply run through a hole in the container lid, with average dissolved oxygen concentrations ranging from 2.9-4 mg/L. We fed each oyster daily with 1 ml of a 2:500 ml dilution of Shellfish Diet 1800 in deionized water (Reed Mariculture, 2 billion cells ml<sup>-1</sup>).

## *General Experimental Design Elements Temperature Treatments*

To determine if host survival and immune response and parasite infection intensity decrease with increasing air temperature, we selected eight air temperatures ranging from 27-53°C at 3-4°C intervals. This range bracketed both average ambient water (27°C) and average maximum air (36°C, with occasional spikes up to ~45°C) temperatures in Georgia during summer, as well as extreme air temperatures that oysters could experience based on climate change scenarios (IPCC 2014b). We also included a subtidal treatment that received no air exposure and was held at a constant water temperature of 27°C to ensure experimental survival data was not the result of laboratory holding conditions. We constructed air temperature chambers (1 per temperature treatment) using 30 gal plastic bins (Sterilite) wrapped on the exterior with fiberglass insulation to better maintain and stabilize target temperatures. We transferred oysters from their water-filled individual holding containers to air temperature chambers for 4.5 h (calculated from field data collected in GA, Malek & Byers Chapter 2) to simulate intertidal exposure, 6 d wk<sup>-1</sup>.

Temperature chamber bottoms were lined with sand to create more natural environmental and heat absorption conditions. We suspended a clamp lamp with an infrared heat bulb ranging from 75-150 watts from a hole in the center of the chamber lid to heat the chambers to target temperatures. In the bottom corner of each heat chamber we put a temperature sensor (Dallas OneWire DS18B20 Maxim), which was wired to an Arduino Micro micro-controller attached to a breadboard. We controlled the power supply of chamber lamps using 8A solid state relays which were also wired to the Arduino through the breadboard. Code was developed to keep the chambers within  $\pm 0.5^{\circ}$ C of the target temperature by turning lamps on and off via the relays in response to the temperatures reported by the sensors. Temperature data was logged every 10 seconds for each chamber to monitor treatment integrity.

#### Host Survival Experiment

To develop survival curves for the 25 oysters exposed to each air temperature treatment, we recorded oyster mortality daily prior to starting air temperature exposure and upon return of oysters to individual containers. We collected tissue for *P. marinus* assessment from oysters once mortality was visually observed. The experiment ran for 3 weeks and we sampled all remaining live oysters for *P. marinus* infection at the conclusion of the experiment.

We assessed *P. marinus* infections using the Ray's Fluid Thioglycollate Media (RFTM) method which detects live *P. marinus* spores in oyster tissue. The specific methods for RFTM assessment can be found in Malek & Byers (Chapter 2), modified in the current study only by the inclusion of gill and mantle tissue in addition to rectal tissue. We found that this methodology was limited in detecting infections at high temperatures ( $\geq$ 43°C) as we observed lower infection prevalence in high compared to low temperature treatments ( $\leq$ 39°C) that had comparable prevalence to our initial baseline of ~60% (data not shown). It is possible that the parasite died or was too degraded at higher temperatures to be identified with the RFTM method. Parasite intensity data in this experiment was limited to 27-39°C because the tissue quality from deceased individuals was not sufficient for testing by other methodologies (qPCR, see below).

To determine the thermal threshold of oyster survival and if survival was affected by *P. marinus* infection, we analyzed oyster survival by air temperature, *P. marinus* infection status (infected vs. uninfected), and their interaction with the 'survival' package in R (Therneau 2015), which uses Kaplan-Meier estimation to evaluate survival and generate survival curves. However, because *P. marinus* detection from RFTM assessment was limited to lower temperatures, in this analysis we included infection data only for the lower temperature treatments (27-39°C) in which *P.* marinus detection was reliable. Using a separate survival model, we also examined the effect of air temperature, *P. marinus* infection intensity (RFTM Mackin score), and their interaction on oyster survival, including both infected and uninfected oysters in the analysis. If interactions were not significant, they were removed and the model was run again to evaluate main effects. We also wanted to determine the thermal threshold of *P. marinus* and know if infection intensity could be predicted from air temperature and host survival status (i.e., if the host lived until the end of the experiment or died during the experiment). To test this we used a 2-way analysis of variance (ANOVA) to test the fixed effects of air temperature (27-39°C), survival status, and their interaction on *P. marinus* infection intensity. If the interaction was not significant, it was removed and the model was run again. We did not include the 27°C subtidal treatment in the formal survival or parasite analyses, but use it as an informative basis of comparison.

#### Host Immune Response Experiment

We ran a second experiment with a new collection of individual oysters and the same protocol described above to test for effects of increasing air temperature on *C*. *virginica* immune response. Based on the survival data obtained in the previous experiment, to achieve the best resolution and boost sample sizes, we eliminated the 31 and 53°C treatments. Higher air temperatures (43-50°C) had 35 individuals per treatment to try to increase the number of days that these treatments could be sampled and lower temperatures (27-39°C) had 25 individuals per treatment. Four times over the course of two weeks (at 2, 4, 7 14 d post start of experiment), we randomly selected six live individuals from each air temperature treatment for host immune response assessment. Several high air temperature treatments experienced complete oyster mortality within several days (43-50°C), so only the 27-39°C treatments could be sampled on all four days. Start time of air exposure was staggered on sampling days so that individuals were sampled immediately after removal from air temperature chambers.

We also wanted to test for effects of *P. marinus* infection status on host immune response. To address the limitation in parasite assessment using the RFTM method, we used qPCR (Gauthier et al. 2006, Stokes et al. *in prep*) to detect parasite infections in this second experiment, which detected any form of parasite DNA (live, dead, or degraded) and enabled us to evaluate *P. marinus* infection status across all temperatures. Detailed methods for qPCR assessment, adapted from Gauthier et al. (2006) and Stokes et al. (*in prep*), can be found in Appendix 1.

We used phagocytic activity as a measure of host immune response. Phagocytic activity was assessed by using fluorescent latex beads as a proxy for invading cells and measuring phagocytic consumption of beads by granular oyster hemocytes (granulocytes) with flow cytometry following the methods of Goedken & DeGuise (2004) and instruction of M. Levin (UConn, *personal communication*). From this assessment, we were able to calculate the proportion of highly active granulocytes (those that consumed  $\geq$ 3 beads) and the mean number of beads consumed by all granulocytes in each air temperature treatment. Please see Appendix 2 for specific assessment methods.

To determine the thermal threshold of phagocytic activity within the host and if this threshold was affected by *P. marinus* infection we used an ANOVA including air temperature, sampling day (2, 4, 7, or 14 d after the start of the experiment), and *P. marinus* infection status as fixed effects, as well as specific pairwise comparisons. The pairwise comparisons included air temperature with each fixed factor as the influence of these factors themselves may vary with air temperature. If the interactions were not significant, they were removed and the model was run again. We used 2 separate ANOVAs, one for each measure of phagocytic activity.

While we were confident in our analysis of phagocytic activity, we wanted to ensure that detected differences based on air temperature were not the result of the unequal sample size across sampling days as complete mortality occurred in several high air temperature treatments by sampling day 7. Because air temperature was our primary variable of interest, if both air temperature and sampling day had a significant effect on phagocytic activity, we conducted a residual analysis that first accounted for the effect of sampling day (linear regression with phagocytic activity as the response and sampling day as the fixed effect). Residuals from this model were then regressed against air temperature (linear regression with residuals from the previous model as the response and air temperature as the fixed effect). If air temperature was still found to be a significant predictor of phagocytic activity when sampling day was accounted for, we conducted post-hoc Tukey's HSD pairwise comparisons to identify differences between air temperature treatments. We arcsine square root transformed the proportion of highly active cells (consuming  $\geq 3$  beads) to meet the assumptions of normality. Non-significant interactions were not reported, only the results of analysis of main effects. As with the Host Survival Experiment, the 27°C subtidal treatment was not included in formal statistical analyses. All analyses were run in R version 3.2.0 (R Core Team 2015).

#### <u>Results</u>

#### Host Survival Experiment

There was no interactive effect of air temperature and *P. marinus* infection status (27-39°C) on *C. virginica* survival (Fig. 3.1, Table 3.1a), but there was a significant effect of air temperature. We observed a clear delineation in response to air temperature between 39 and 43°C. Air temperature treatments  $\leq$ 39°C maintained at least 50% oyster

survival over 21 days and treatments  $\geq$ 43°C reached 0% oyster survival within several days (Fig. 3.1). There was also no interactive effect of air temperature and *P. marinus* infection intensity and no individual effect of intensity on survival (27-39°C)(Table 3.1b).

Analysis of variance indicated that there was no interactive effect of air temperature and host survival status (live vs. dead) on *P. marinus* infection intensity (RFTM) at lower temperatures but we found that air temperature had a significant effect on infection intensity (Table 3.2). Tukey's HSD pairwise comparisons indicated that oysters exposed to 35°C had significantly higher infection intensities than oysters exposed to other temperatures, with the exception of 31°C (Fig. 3.2). We also found that host survival status had a marginally significant effect on infection intensity (Table 3.2), with oysters that died during the experiment tending to have higher intensity infections than those that survived the full duration of the experiment (data not shown).

#### Host Immune Response Experiment

Both the proportion of highly active cells (consumed  $\geq$ 3 beads) and the mean number of beads consumed tended to decrease with increasing air temperature (Fig. 3.3). There was no interactive effect of air temperature with sampling day or *P. marinus* infection status for either measure of phagocytic activity. When non-significant interactions were removed, we found that air temperature and sampling day had significant effects on both measures of phagocytic activity, but there was no effect of *P. marinus* infection status (Table 3.3 & 3.4). Due to the significant effects of both air temperature and sampling day on phagocytic activity, we ran a residual analysis for each measure of activity. These analyses suggested that when sampling day was accounted for,

there was still a significant effect of air temperature on the mean number of beads consumed (df=5, F=3.083, p=0.013) but not on the proportion of highly active cells (df=5, F=0.843, p=0.523). Tukey's HSD analysis indicated that oysters exposed to 50°C consumed significantly fewer beads than those exposed to lower air temperatures (Fig. 3.3b).

#### **Discussion**

Climate change is predicted to affect environmental factors that shape hostparasite interactions. We found that increasing air temperature will have different effects on the oyster *C. virginica* and its parasite, *P. marinus*. Our results indicated that the intensity of parasite infections peaked at 35°C and then began to decline (Fig. 3.2). Host survival was >50% until 39°C and host immune response (phagocytic activity) was largely unaffected until at least 50°C (Fig. 3.1, 3.3). The temperatures at which we observed changes in host survival and parasite infection intensity are well within the range of air temperatures projected to occur in the Southeast US within the next one hundred years (Mitchum 2011). Thus, increases in air temperature will favor the parasite below 35°C and the host between 35-39°C, when infections intensities are lower and the host has a higher capacity for survival. The question of whether a warmer world will be a sicker world will depend on the degree of warming in this system where we observed differential responses of host and parasite at moderate amounts of warming. Ultimately, this question becomes moot if temperatures exceed the host's thermal threshold.

The current average intertidal air temperature in mid-coast Georgia in the summer is ~32°C (Malek *unpublished data*), therefore increasing average air temperature in this region will likely cause an increase in *P. marinus* infection intensity. Higher intensity

parasite infections observed at 35°C may be detrimental to the oyster host through decreased shell and soft tissue growth (Ray et al. 1953, Menzel & Hopkins 1955), reduced reproductive development (Dittman 1993), and weakened adductor muscles (Mackin 1962). Such effects can eventually lead to host mortality. We did not find a significant relationship between host survival and parasite infection intensity (Table 3.1). However, it may take longer than the relatively short duration of our experiment for negative effects of the higher intensity infections observed to manifest in observable changes in host survival.

Interestingly, we also did not see an effect of parasite infection status or air temperature on phagocytic activity, except at very high temperatures (Fig. 3.3). Infection by *P. marinus* can cause an increase in hemocyte abundance as the host responds to the parasite (Anderson et al. 1995). However, an increased number of hemocytes may not translate to an overall increase of phagocytic activity. Similar to our study, Goedken et al. (2005) found no difference in the percentage of phagocytizing cells in oysters with and without *P. marinus* infections and suggested that other immune functions (apoptosis) may be more important for regulating infection. Additionally, Anderson et al. (1992) observed that reactive oxygen intermediate concentrations were higher in oysters with more intense *P. marinus* infections. If infection intensity is a strong predictor of changes in immune response, we would not have detected an effect of parasite infection on phagocytic activity as we analyzed activity by infection status, not intensity.

The absence of an effect of air temperature on phagocytic activity except at extreme temperatures (Fig. 3.3b) was also surprising. Oyster hemocytes tend to be very sensitive to environmental factors such as temperature, salinity, reproductive status, air

exposure, and season (Fisher et al. 1989, Hégaret et al. 2003, Duchemin et al. 2007, Kuchel et al. 2010, McKenzie et al. 2014). If hemocytes were more sensitive to other factors such as lab compared to field conditions (i.e., changes in water temperature and salinity), effects of air temperature on phagocytic activity at temperatures below 50°C may not have been detected. To gain a more thorough understanding of how increasing air temperature affects oyster immune response, we need to broaden the range of responses tested. In our study, we considered only phagocytic activity but other responses such as apoptosis or respiratory burst may be more important in regulating *P. marinus* infection (Goedken et al. 2005).

Although the parasite initially appears to have an advantage in the face of increasing air temperature, we observed that temperatures above the parasite's optimal conditions may favor the host in terms of parasite infection intensity and capacity for survival. Though oyster survival declined considerably at temperatures above 35°C (Fig. 3.1), host phagocytic activity remained steady (Fig. 3.3) and the parasite experienced a significant decrease in infection intensity (Fig. 3.2). Oysters at 39°C had similar infection intensities as those at 31°C, where the host maintained high survival (Fig. 3.1). Other studies have demonstrated that after peaking at 35°C *in vitro*, *P. marinus* begins to die between 36-40°C and substantial mortality occurs between 41-44°C (Dungan & Mamilton 1995, Soudant et al. 2005). In combination with these results, our study suggests that the host may have a higher capacity to survive at temperatures between 35-39°C than the parasite. The lower intensity infections at temperatures above 35°C would seemingly be beneficial for the host as temperature increases.

There is much speculation about how changes in climate will affect host-parasite interactions (Harvell et al. 2009, Lafferty 2009). Our study suggests, at least for oysters and one of their most detrimental parasites, that increasing air temperature may at first result in bursts of *P. marinus* activity, but as temperature continues to rise the host has a higher capacity to survive than its parasite. However, if temperature increases too much, the host's thermal threshold will also be crossed and neither species will benefit. As temperature is lower in more northern portions of the host's range compared to Georgia, it may take longer for the observed effects of air temperature to influence host-parasite interactions. However, increases in temperature are predicted to be larger in northern compared to southern regions (Christensen et al. 2007). Such differential changes in temperature between geographic regions may result in similar influences of air temperature on oyster-parasite interactions sooner than later. Overall, observed changes in C. virginica populations in the future may be more likely to be caused by parasite infection initially but as temperature continues to increase, biological responses of the host are more likely to affect populations than P. marinus infection. Recognizing that hosts and parasites can have differential responses to increasing temperatures will allow us to more effectively shape our future research initiatives for other ecologically and economically valuable species.

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Table 3.1. Results of Kaplan Meier analysis of *C. virginica* survival based on (**a**.) air temperature treatment and *P. marinus* infection status (RFTM) and (**b**.) and *P. marinus* infection intensity. Non-significant interactions are not reported.

a. Survival by air temperature treatment and P. marinus infection status (27-39°C)							
Source	Coef	Exp(coef)	SE(coef)	Z	p(z)		
Temperature	0.101	1.106	0.039	2.614	0.009		
Infection Status	0.123	1.131	0.336	0.367	0.713		
<b>b</b> . Survival by air temperature treatment and P. marinus infection intensity (27-39°C)							
Source	Coef	Exp(coef)	SE(coef)	Z	p(z)		
Source Temperature	Coef 0.101	Exp(coef)	<b>SE(coef)</b> 0.039	<b>z</b> 2.591	<b>p(z)</b> 0.010		
Table 3.2. Results of 2-way ANOVA testing for effects of air temperature (27-39°C) and survival status (live vs. dead) on *P. marinus* infection intensity (RFTM) in the Host Survival Experiment.

P. marinus infection	on inte	ensity by air temper	ature (27-39°C) ar	nd survival sta	tus
Source	Df	Sum of Squares	Mean Square	F Value	P(F)
Air Temperature	3	10.615	3.538	4.829	0.006
Survival Status	1	2.466	2.466	3.365	0.074

Table 3.3. Results of ANOVA testing for effects of air temperature treatment, sampling day, and *P. marinus* infection status on the proportion of highly active cells (cells that consumed  $\geq$ 3 beads) by in the Host Immune Response Experiment. Non-significant pairwise interactions were removed from the model and are not reported.

Source	Df	MSE	F value	Pr(>F)
Air Temperature	5	0.028	4.082	0.002
Sampling Day	3	0.108	15.955	< 0.001
P. marinus Infection Status	1	0.013	1.924	0.169

Table 3.4. Results of ANOVA testing for effects of air temperature treatment, sampling day, and *P. marinus* infection status on the mean number of beads consumed by entire granulocyte population in the Host Immune Response Experiment. Non-significant pairwise interactions were removed from the model and are not reported.

Source	Df	MSE	F value	Pr(>F)
Air Temperature	5	1.263e <sup>+9</sup>	7.168	< 0.001
Sampling Day	3	2.455e <sup>+9</sup>	13.937	< 0.001
P. marinus Infection Status	1	8.570e <sup>+7</sup>	0.487	0.487



Figure 3.1. Total survival of all experimental oysters by air temperature, regardless of infection status.  $n\approx 25$  for each air temperature curve.



Figure 3.2. Mean *P. marinus* infection intensity (Mackin scores based on RFTM tissue assessment) at low temperatures (subtidal-39°C) in the Host Survival Experiment. Mean intensity was calculated using only infected oysters. Standard error bars calculated across individual oysters within each treatment. n=44 across air temperature treatments. Capital letters indicate results of post hoc Tukey's HSD analysis; different letters indicate statistically significant differences between treatments and similar letters indicate no statistical difference between treatments.



Figure 3.3. Phagocytic activity of oyster hemocytes in the Host Immune Response Experiment, measured as **a**. the mean proportion of highly active cells (indexed by the consumption of ≥3 fluorescent beads; left column) and **b**. the mean number of beads consumed (sum of beads consumed by all cells; right column) in response to air temperature. Data shown is from the second day of air temperature exposure which was the only sampling day that had an equal sample size for all air temperatures (n=6 for each treatment). Error bars represent standard error calculated across individual oysters within each air temperature treatment. Capital letters indicate results of post hoc Tukey's HSD analysis; different letters indicate statistically significant differences between treatments and similar letters indicate no statistical difference between treatments.

# CHAPTER 4

# PREDATOR EFFECTS ON HOST-PARASITE INTERACTIONS IN THE EASTERN OYSTER, *CRASSOSTREA VIRGINICA*<sup>3</sup>

<sup>&</sup>lt;sup>3</sup> Malek, JC and JE Byers. To be submitted to *Marine Ecology Progress Series* 

# <u>Abstract</u>

Environmental factors can be important drivers of species interactions, including host-parasite interactions. Predators in particular can influence host-parasite interactions in several ways. First, predators may directly influence these interactions by preferentially feed on infected (or uninfected) hosts, altering parasite prevalence patterns. Second, predators can indirectly alter host behavior in ways that increases host exposure or susceptibility to parasites. Such effects of predators have been observed across a variety of predator and host species on oyster reefs. In this study we tested if oyster predators influence interactions between the eastern oyster, Crassostrea virginica, and two of its most prevalent parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*. Using a combination of field and laboratory experiments, we tested for predatory effects of mud crabs (*Panopeus herbstii*) and blue crabs (*Callinectes sapidus*) on the prevalence and intensity of parasite infections, and on host immune response. Overall, our experiments suggest that crabs do not exert direct or indirect effects on ovsters that result in changes in oyster-parasite interactions or oyster immune response (phagocytic activity). Through this research we have identified an environmental factor that does not affect oyster-parasite interactions and increased our understanding of the role of the environment in shaping host-parasite patterns.

# **Introduction**

The environment plays a large role in shaping host-parasite relationships (Breitburg et al. 2015, Harvell et al. 2004, Keppel et al. 2015, Malek & Byers Chapter 2 & 3, Sadd 2011, Wolinska & King 2005) by affecting hosts, parasites, and their interactions (Mitchell et al. 2005, Wolinska & King 2005, Malek & Byers Chapter 2). Research addressing environmental effects on host-parasite interactions has largely focused on aspects of the physical environment such as temperature, salinity, and pollution (Studer et al. 2010, Malek & Byers Chapter 3, Haskin & Ford 1982, Reisser & Forward 1991, Studer & Poulin 2012, Hoole 1997, Koprivnikar et al. 2007, Marcogliese & Pietrock 2011). However, biological factors may also affect these interactions (Grosholz 1992, Schmitz & Nudds 1994, Hall et al. 2009). For example, interspecific competition can increase competitor vulnerability to predation (Connell 1961) or parasitism. It is also well established that parasites can make hosts more vulnerable to predation and even competition (Hudson et al. 1992, Grosholz 1992, Lafferty & Morris 1996, Hudson & Greenman 1998, Joly & Messier 2004). Yet, little is known about how these biological factors influence host-parasite interactions.

One of the most influential biotic factors in ecological systems is predation. Predators can reduce prey populations (Connell 1970, Messier & Crète 1985) and thus can substantially influence host-parasite interactions. For example, the 'healthy herd' hypothesis suggests that by removing weakened (infected) hosts from a population, predators can reduce parasite transmission and parasite epizootics (Packer et al. 2003a, Hall et al. 2005), providing positive feedbacks on host populations. Systems in which predators preferentially feed on infected hosts (Abey 2002, Duffy et al. 2005, Duffy &

Hall 2008), provide support for this hypothesis. Alternatively, predators can increase parasite prevalence within a host population either through preferentially consuming uninfected prey or by acting as 'predator spreaders' that aid in parasite dispersal when infected hosts are consumed (Cáceres et al. 2009). Though there are an increasing number of examples of such predator effects on host-parasite interactions, the outcome often differs between species and systems (Lafferty 2004, Duffy et al. 2005, Johnson et al. 2006, Duffy 2007, Cáceres et al. 2009).

Predators can also effect hosts (Grabowski & Kimbro 2005, Wirsing et al. 2008, Christanson & Creel 2010, Wirsing & Ripple 2010, Matassa & Trussell 2012) by changing host traits such as behavior, growth, and development (Blaustein 1997, Trussell et al. 2003). Predators can increase parasite transmission by altering host-host interactions (Stephenson et al. 2015) or by causing hosts to spend more time in habitats with high parasite density. For example, shoaling behavior by female Trinidadian guppies in response to predators increases direct contact between hosts, leading to higher transmission of monogenean parasites (Richards et al. 2010). Additionally, predators can alter host feeding behavior, reducing energy resources available for growth, development (Trussell et al. 2003), and other physiological functions (Rigby & Jokela 2000, Navarro et al. 2003). For instance, altered energy allocation in response to predators can suppress host immune responses and increase susceptibility to parasites (Rigby & Jokela 2000, Allen & Little 2011, Kerby et al. 2011, Janssens & Stoks 2013).

Predators effect prey species inhabiting reefs built by the eastern oyster, *Crassostrea virginica*, both through direct consumption and indirect changes in prey traits. Oysters inhabit coastal estuarine systems along the Atlantic and Gulf of Mexico

coasts of the US (Grabowski 2004, Grabowski & Kimbro 2005, Grabowski et al. 2008) and are subject to a suite of different predator species throughout this range. Exposure to predators can elicit morphological responses in oysters such as altered resource allocation and differential shell strength (Newell et al. 2007, Johnson & Smee 2012).

Oysters also interact with two lethal parasites, Perkinsus marinus and Haplosporidium nelsoni, which can have devastating lethal effects on oyster populations (Ford & Haskins 1982, Andrews 1988). Numerous abiotic factors have been identified as environmental drivers of parasite infection patterns (water temperature, salinity, intertidal air-exposure; Ford & Tripp 1996, Malek & Byers Chapter 2), but the role of biotic factors in this system is largely unknown (White et al. 1987, Diamond 2012). The specific transmission mechanism of *H. nelsoni* has not been identified (Burreson & Ford 2004), however we know that *P. marinus* is released from dead, infected oyster tissue or excreted by heavily infected oysters through pseudofeces. Parasites are then suspended into the water column and filtered by susceptible hosts (Ray 1954, Bushek et al. 2002). The transmission mechanisms of *P. marinus* may provide an avenue for predators to act as 'predator spreaders', increasing parasite transmission between hosts. Alternatively, predators may indirectly alter oyster morphological and behavioral traits such as filtration. The strong evidence of predator effects on oyster reefs in addition to the wellestablished role of environmental factors in shaping patterns of *P. marinus* and *H. nelsoni* infections make oyster reefs a tractable system for evaluating predator effects on hostparasite interactions.

Many oyster predators function as scavengers of dead oyster tissue as well as active hunters of live oysters. In the lab, Diamond (2012) observed that scavenger species

(crabs, snails, fish) presented with shucked, infected oyster tissue increased the rate of *P*. *marinus* transmission, regardless of any previously observed tissue preference (infected vs. uninfected with *P. marinus*). It is unknown if predators exhibit similar feeding and transmission behaviors in the field.

Predators may also affect oysters through reduction of filtration. This behavioral change could decrease oyster exposure to parasites. Alternatively, reduced filtration could lengthen the time that ingested particles remain in the host digestive tract and thus increase the probability that ingested parasites establish infection. While closed, oysters can experience hypoxic (low O<sub>2</sub>) or hypercapnic (high CO<sub>2</sub>) conditions in their shell cavity that decrease important immune defenses such as hemocyte activity and production of reactive oxygen intermediates (Boyd & Burnett 1999, Allen & Burnett 2008, Keppel 2014). Unfavorable internal conditions or changes in energy allocation (Johnson & Smee 2012, Newell et al. 2007) in response to indirect predator effects could suppress immune defenses in the oyster host, leading to higher parasite prevalence.

To determine if predators affect oyster-parasite interactions we conducted a combination of field and laboratory experiments. First, we conducted a field experiment that either exposed or protected oysters from mud crabs. We hypothesized that oysters exposed to mud crabs would have a higher prevalence of *P. marinus* and *H. nelsoni* infections due to crab-induced spreading of parasites (i.e., acting as 'predator spreaders'). Additionally, we expected oysters exposed to crabs to filter less than oysters protected from crabs, resulting in higher *P. marinus* infection intensity (severity of infection within an individual) and suppressed immune response (as measured by phagocytic activity).

Based on results from our field experiment, we also conducted a laboratory experiment using a larger predator, the blue crab, to parse out potential indirect predator effects using chemical and tactile predator cues. We hypothesized that oysters exposed to chemical predator cues would have higher prevalence and lower intensity of *P. marinus* infections than control oysters (no cue). Chemical cues are less likely than tactile cues to alter oyster filtration behavior but crabs may still be releasing parasites into the water column, spreading them to other oysters (i.e., acting as 'predator spreaders'). However, we expected that oysters exposed to a combination of chemical and tactile cues would have lower prevalence and higher intensity of *P. marinus* infections than control oysters. Tactile predator cues could reduce filtration, thus reducing exposure to parasites in the water column and trapping consumed parasites within the host.

# **Methods**

#### Field Experiment

To test for predator effects on parasite infections (*P. marinus* and *H. nelsoni*) in oysters under field conditions, we hand collected large wild oyster clusters from Romerly Marsh Creek in the Wilmington River, Savannah, GA (31°55'21.78"N, 80°59'20.85"W). For complete methods on oyster processing, please see Malek & Byers (Chapter 2). Briefly, we broke clusters (combination of live oysters and dead shell) into 200-400 g clumps that were cleared of predators and fouling agents. We then weighed clusters, counted the number of oysters <25 mm, and measured oysters >25 mm.

We used two experimental treatments: mud crab predators were either absent ('no predator') or added at a density of eight per 0.09 m<sup>2</sup> reef ('predator'), a realistic density for Georgia oyster reefs (J. Malek *personal observation*). We hand collected mud crabs

(carapace width range=20-40 mm, mean=28 mm) from the oyster reefs adjacent to our deployment site in Romerly Marsh Creek. We applied the predator treatments to experimental oyster reefs built inside plastic crates that retained the oysters and predators (see Malek & Byers Chapter 2 for specific reef construction details). We placed fifteen processed oyster clumps in each crate and sealed the tops with Vexar mesh lids to prevent immigration or emigration of predators. Lids were sewn on with 1.65 cm nylon line for easy reentry throughout the experiment. We secured crates flush with sediment by anchoring each to an embedded cinder block. Reefs were spaced 1 m apart along the creek bank in line with naturally occurring intertidal oyster reefs (~1.5 m above Mean Low Water). We randomly assigned experimental treatments to each of the 16 reefs deployed across the intertidal mud flat (8 replicate reefs treatment<sup>-1</sup>). To maintain treatment integrity, reefs were checked twice weekly to remove any predators that had entered 'no predator' treatments, mainly immature mud crabs, and to replenish dead or missing mud crabs in 'predator' treatments. Reefs were deployed for 17 wk in 2013 (June 21- October 18). At the conclusion of the experiment, we randomly selected 15 oysters from each reef for simultaneous assessment of parasite infection and immune response (n=120 for each predator treatment).

To determine predator effects on oyster-parasite interactions we assessed *P*. *marinus* and *H. nelsoni* infections in oysters. We used sterile methods to collect gill and mantle tissue from oysters after retrieval from the field and froze samples at -20°C until time of processing. Following the protocol developed by Stokes et al. (*in prep*; adapted from Gauthier et al. 2006), we used quantitative PCR (qPCR) to assess of the probability and intensity of *P. marinus* infection. Complete details are reported in Malek & Byers

(Chapter 3). We assessed the probability of *H. nelsoni* infection using cPCR (adapted from Stokes et al. 1995, Reanault et al. 2000), full methods are reported in Malek & Byers (Chapter 2). We also sampled a subset of 48 oysters from the initial collection in June to determine the baseline parasite prevalence and intensity in our experimental population. However, baseline data for *P. marinus* was collected using the RFTM method (see next section) while final data was collected using qPCR so we were unable to make direct comparisons between baseline and final infection data.

We measured oyster immune response through phagocytic activity of oyster hemocytes using flow cytometry (Goedken & DeGuise 2004, M. Levin, pers. communication). See Appendix 2 in Malek & Byers (Chapter 3) for detailed methods. Briefly, we incubated oyster hemolymph with fluorescent latex beads and analyzed the samples with a FACSCalibur flow cytometer to detect the number of hemocytes that phagocytized beads and how many cells consumed a specific number of beads. From this data we calculated the proportion of granular oyster hemocytes (granulocytes) that had high phagocytic activity (i.e., consumed  $\geq$ 3 fluorescent beads (foreign cells)) and the mean number of beads consumed by all granulocytes.

To test for effects of predators on infection, we analyzed the probability of infection for each parasite and for co-infection with both parasites with three separate mixed effects logistic regression models ('lme4' package in R). Predator treatment was included as a fixed effect and replicate as a random effect to account for multiple oysters being sampled from the same experimental reef. The effect of predator treatment on the intensity of *P. marinus* infections (number of *P. marinus* DNA copies detected in a host based on qPCR amplification) was analyzed using a generalized linear mixed model

(GLMM), with predator treatment as a fixed effect and replicate as a random effect. Infection intensity data were log10 transformed to equalize the variance among individuals.

To determine whether mud crabs and *P. marinus* infection affected phagocytic activity of oyster hemocytes, we analyzed the proportion of highly active cells and the mean number of beads consumed by granulocytes, with separate GLMMs that included predator treatment, the probability of *P. marinus* infection, and infection intensity as fixed effects and replicate as a random effect. We arcsine square root transformed the proportion of highly active cells and log (ln) transformed the mean number of beads

#### Lab Experiment

Based on the results of the field experiment, we wanted to test if a larger predator that tends to consume more oysters and provide stronger physical cues (blue crabs) exerts indirect effects on oyster-*P. marinus* interactions. In May of 2015 we collected oyster clusters at the Skidaway Institute of Oceanography's (SkIO) Priest Landing lab in Savannah, GA. We cleaned clusters, removed dead shell, and broke them into ~200-400 g clumps that were stored in artificial seawater in the lab. We fished for blue crabs at SkIO and Romerly Marsh Creek using recreational crab traps. Blue crabs were held in individual wire mesh cages until time of use. All Lab Experiment organisms were transported in coolers to the Odum School of Ecology at the University of Georgia in Athens, GA where we held oysters and crabs in separate 30 gal plastic tanks filled with artificial seawater (Instant Ocean aquarium salt dissolved in tap water, maintained at 24 psu and ~22°C). We aerated tanks with airstones and maintained an average dissolved

oxygen of 4.0-6.0 mg/L. Oysters were fed daily with Shellfish Diet 1800 (5:1200 ml for  $\sim 8 \times 10^6$  cells ml<sup>-1</sup>) and we conducted water changes once a week for all experimental and holding tanks to limit nutrient and waste accumulation.

In the lab, we used three experimental treatments: an oyster-only control, a chemical cue treatment, and a combined chemical and tactile cue treatment. In each of 12, 30 gal tanks (n=4 tanks per treatment) we built a focal oyster reef in one half using 12 processed oyster clumps. A large wire mesh predator cage containing 5 oyster clumps was placed in the other half of the tank where the treatment factor was manipulated. The chemical cue treatment contained an adult *C. sapidus* confined in the predator cage that could spread cues of crushed food oysters and potentially *P. marinus* spores to focal oysters. The combined chemical and tactile cue treatment was the same, but also included a juvenile *C. sapidus* with its claws wrapped shut (Gorilla tape) on the focal oyster side of the tank to provide non-lethal tactile stimulus to focal oysters. Clumps in the predator cage were replaced as necessary in both of these treatments to provide a continuous supply of food. The oyster-only control treatment contained no predators. No crab mortality occurred during the course of the experiment and after 5 wk, we sacrificed 36 oysters tank<sup>-1</sup> for parasite infection assessment (n  $\approx 144$  treatment<sup>-1</sup>).

We tested for the probability and intensity of *P. marinus* infections using the Ray's Fluid Thioglycollate Media (RFTM) method. We followed the methods of Malek & Byers (Chapter 2) with the adjustment that we included gill and mantle tissue as well as rectal tissue for parasite assessment. We sacrificed 12 oysters from each experimental tank (1 oyster clump<sup>-1</sup>) prior to applying experimental treatments to get a baseline measure of the probability and intensity of *P. marinus* infection.

Due to the initial heterogeneity in *P. marinus* infection prevalence between treatment tanks detected through baseline sampling, we used the change in prevalence for each experimental tank as our response variable. We analyzed the change in prevalence with a GLMM including predator treatment as a fixed effect and replicate as a random effect. We did not adjust for initial infection intensity because initial prevalence was often low (1-2 oysters), which prevented us from being able to reliably estimate tankspecific intensities. We analyzed final intensity with a mixed effects Poisson regression model using predator treatment as a fixed effect and replicate as a random effect. All analyses were run in R ver 3.2.0 (R Core Team 2015).

# **Results**

## Field Experiment

Mud crabs did not have a significant effect on the probability of infection of either *P. marinus* or *H. nelsoni*, or the probability of co-infection by both parasites (Fig. 4.1, Table 4.1a-c). We were unable to compare the initial and final probability of *P. marinus* infection due to a change in assessment methods, but the proportion of oysters infected by *H. nelsoni* increased by ~250% from the initial population baseline (Fig. 4.1). The average number of *P. marinus* DNA copies (infection intensity) was ~60% higher on reefs with no mud crabs than those with mud crabs, however among-individual variation was very large so this difference in intensity was not statistically significant (Fig. 4.2).

Mud crabs also did not affect phagocytic activity of oyster hemocytes. Neither the proportion of highly active granulocytes (consumed  $\geq$ 3 beads) nor the mean number of beads consumed by granulocytes differed between 'no predator' (mean proportion=0.34  $\pm$  0.01; mean number of beads=109822  $\pm$  1524) and 'predator' (mean proportion=0.34  $\pm$ 

0.01; mean number of beads=111748  $\pm$  1470) reefs (Table 4.3). Additionally, there were no interactive effects between mud crabs and the probability or intensity of *P. marinus* infection on phagocytic activity and no individual effects of either parasite measure (Table 4.3).

#### Lab Experiment

We found that blue crabs had no effect on either the change in *P. marinus* infection prevalence or final infection intensity (Table 4.4 & 4.5). Based on our calculation of the change in prevalence, we found that a similar amount of parasite transmission occurred in all treatments (Fig. 4.3). Final mean infection intensities were similar across all treatments, falling between Mackin scores of 1-2, which are considered light-moderate infections (Fig. 4.4).

# **Discussion**

Predators can have multiple influences on host-parasite interactions through direct or indirect effects on host species (Werner & Peacor 2003, Pressier et al. 2005, Hall et al. 2009). Though such predator effects have been documented for various species on oyster reefs, including oysters (Grabowski 2004, Grabowski & Kimbro 2005, Grabowski et al. 2008, Johnson & Smee 2012), we found that predators, specifically crabs, do not affect host-parasite interactions in *C. virginica*. Additionally, there was no effect of predators on phagocytic activity of oyster hemocytes (Table 4.3). Our combination of field and laboratory studies using multiple oyster predators suggests that crab predators do not directly or indirectly shape host-parasite interactions in this system.

In our field experiment, the lack of support for predator effects on oyster-parasite interactions may be due to natural variation in other environmental factors in the field

that could reduce or counteract predator effects. For example, mud crabs may more actively feed on oysters during high tide when conditions are less physiologically challenging (i.e., lower physiological stress). If mud crabs do preferentially feed on infected oysters and release parasite spores into the water column, this effect could be counteracted by tidal currents that move the parasites before they can spread to neighboring oysters. Additionally, experimental reefs were in close proximity to other predators which could have indirectly altered mud crab behavior (Grabowski & Kimbro 2005, Grabowski et al. 2008), thus affecting mud crab influence on oyster-parasite interactions. Lastly, if mud crabs did cause behavioral changes in oysters, their effects may not have been strong enough to elicit a long term response in oyster-parasite interactions if the retention time of chemical cues in the water column is brief.

We expected that mud crabs could alter host-parasite interactions if they increase parasite transmission (Cáceres et al. 2009, Diamond 2012). Predator-related oyster mortality was visually observed on our predator reefs, indicating that crabs did consume oysters. However, the probability of infection for both parasites suggests that mud crabs do not increase parasite transmission when they consume infected individuals (Fig. 4.1) and thus do not act as 'predator spreaders'.

Predators can indirectly change host behavior. As a sessile species, the primary 'behavior' of oysters is the quantity and timing of their filtering the water column for food. We hypothesized that crabs would negatively affect this behavior but saw no indication of such effects by mud crabs in the field, as would have been suggested by differences in *P. marinus* infection intensity or phagocytic activity. This result prompted us to use larger blue crabs in the laboratory where oysters would be more inundated by

non-lethal cues in a controlled environment. However, we also saw no effects of blue crabs on oyster-parasite interactions. Our results corroborate studies by Byers et al. (2014) and Dodd (2015) who found that mud and blue crabs did not significantly affect chlorophyll *a* drawdown by oysters. In combination, these studies and ours indicate that even with intensified exposure to non-lethal cues under controlled lab conditions, crab predators do not indirectly affect oyster filtration.

Though crabs do directly and indirectly effect oysters, our results indicated that these effects do not influence oyster-parasite interactions. However, there may be other predator or pest species that can influence oyster-parasite interactions. For example, the hemolymph-sucking snail, *Boonea impressa*, has been shown to potentially act as a vector for *P. marinus* transmission and cause higher intensity infections in infected hosts (White et al. 1987). Additionally, sponges (*Cliona sp.*) can excavate into oyster shells (Hatch 1980), weakening the host's shell strength which is a primary defense against predators and reducing resources available for other physiological functions by causing energetically costly internal shell damage (Galtsoff 1964).

There is growing empirical and theoretical evidence indicating that predators can significantly affect host-parasite interactions (Packer et al. 2003, Duffy et al. 2005, Hall et al. 2005, Duffy et al. 2011). It's even been observed that predator effects can result in trophic cascades that influence entire communities (Duffy 2007). However, many of these studies have been conducted in freshwater plankton systems that may not be representative of interactions between larger species. We found that in an coastal estuarine system that has well documented examples of strong predator effects across a range of species, crabs do not affect host-parasite interactions between *C. virginica* and

two of its most destructive parasites. By evaluating predator effects on oyster-parasite interactions, we have helped expand our understanding of the relationship between biotic environmental factors and host-parasite systems.

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Table 4.1. Results of mixed effects logistic regression analysis of predator treatment for **a.** probability of *P. marinus* infection, **b.** probability of *H. nelsoni* infection, and **c.** probability of co-infection, with replicate included as a random effect. The reference predator treatment for analyses was 'no predator', with negative estimates indicating a decrease in the probability of infection with the addition of predators.

a. <i>P. marinus</i> preval	ence by predato	r treatment		
Fixed Effect	Estimate	SE	z-Value	Pr(z)
Intercept	2.517	0.594	4.235	<0.0001
Predator Treatment	-0.168	0 4129	-0.407	0 684
Random Effect	Variance	SD		0.004
Replicate	1 (4)	1 220		
	1.002	1.209		
b. <i>H. nelsoni</i> prevale	ence by predator	treatment		
Fixed Effect	Estimate	SE	z-Value	Pr(z)
Intercept	-1 296	0 274	-4 725	< 0.0001
Dradatan Traatmant	-0.161	0.220	0.480	0.625
Random Effect	Variance	0.329 SD	-0.489	0.025
Replicate	1.68	0.410		
c. Co-Infection by p	redator treatmen	nt		
Fixed Effect	Estimate	SE	z-Value	Pr(z)
Intercept	-1.508	0.325	-4.641	< 0.0001
Dre later Tracture (	0.120	0.244	0.250	0.727
Predator Treatment	-0.120	<u> </u>	-0.330	0.727
Kandom Ellect	variance	50		
Replicate	0.338	0.582		

Table 4.2. Results of GLMM analysis of *P. marinus* infection intensity by predator treatment with replicate as a random effect. The reference predator treatment for analyses was 'no predator', with negative estimates indicating a decrease in the intensity of *P. marinus* infection with the addition of predators.

P. marinus Intensity	by predator tre	atment		
Fixed Effect	Estimate	SE	t-Value	Pr(t)
Intercept	3.192	0.231	13.843	< 0.0001
Predator Treatment	-0.139	0.211	-0.657	0.511
Random Effect	Variance	SD		
Replicate	0.245	0.495		

Table 4.3. Results of GLMM analysis of phagocytic activity by predator treatment, *P. marinus* infection intensity, and infection status. The reference predator treatment for analyses was 'no predator', with negative estimates indicating a decrease in phagocytic activity with the addition of predators. The reference *P. marinus* infection status was uninfected and higher *P. marinus* copy numbers indicated higher intensity infections, thus negative estimates indicated a decrease in phagocytic activity in the presence of higher intensity parasite infection.

a. Proportion of highly activ	e cells			
Fixed Effect	Estimate	SE	t-Value	Pr(t)
Intercept	5.648e <sup>-1</sup>	$1.884e^{-2}$	30.632	< 0.001
Predator Treatment	5.357e <sup>-3</sup>	1.342e <sup>-2</sup>	0.399	0.693
P. marinus Infection Status	$4.693e^{-2}$	2.633e <sup>-2</sup>	1.783	0.076
P. marinus Intensity	2.543e-3	5.365e-3	0.474	0.636
Random Effect	Variance	SD		
Replicate	< 0.001	0.016		
b. Mean number of beads co	nsumed			
Fixed Effect	Estimate	SE	t-Value	Pr(t)
Intercept	5.006	$1.306e^{-2}$	383.330	< 0.001
Predator Treatment	$1.012e^{-2}$	1.119e <sup>-2</sup>	0.904	0.375
P. marinus Infection Status	1.810e <sup>-2</sup>	$1.801e^{-2}$	1.005	0.316
P. marinus Intensity	4.156e <sup>-3</sup>	3.818e <sup>-3</sup>	1.088	0.278
Random Effect	Variance	SD		
Replicate	< 0.001	0.021		

Table 4.4. Results of GLMM analysis of the change in *P. marinus* prevalence (finalinitial) with predator treatment as a fixed effect and replicate as a random effect. The reference treatment for analysis was the control treatment, with negative estimates indicating a decrease in the change in *P. marinus* infection prevalence with the addition of chemical and then tactile predator cues.

Difference in <i>P. marinus</i> inf	ection prevale	nce		
Full Model	df	Mean Square	F	Pr(F)
Predator treatment	2	0.001	0.093	0.912
Fixed Effect	Estimate	SE	t-Value	Pr(t)
Intercept	0.197	0.056	3.489	0.007
Control vs. Chemical Cue	-0.020	0.074	-0.277	0.791
Control vs. Chemical + Tactile Cue	-0.031	0.074	-0.425	0.686
Random Effect	Variance	SD		
Replicate	0.002	0.043		

Table 4.5. Results of mixed effects Poisson regression of *P. marinus* infection intensity with predator treatment as a fixed effect and replicate as a random effect. The reference treatment for analysis was the control treatment, with negative estimates indicating a decrease in *P. marinus* infection intensity with the addition of chemical and then tactile predator cues.

P. marinus infection intensity					
Full Model	df	Chi Square	Pr(chisq)		
Predator treatment	2	0.172	0.918		
Fixed Effect	Estimate	SE	z-Value	Pr(z)	
Intercept	0.865	0.103	8.431	< 0.001	
Control vs. Chemical Cue	0.039	0.130	0.298	0.766	
Control vs. Chemical + Tactile Cue	0.055	0.134	0.410	0.682	
Random Effect	Variance	SD			
Replicate	0	0			



Figure 4.1. Mean final proportion of *P. marinus* and *H. nelsoni* infections and co-infected oysters by predator treatment in the field experiment ( $n\approx120$  for each treatment). Error bars represent standard error calculated across replicates for each treatment. Solid black line represents the baseline proportion of oysters infected with *H. nelsoni*. Baseline data for *P. marinus* (and thus co-infection) was measured using the RFTM method. Because final infection data status was measured using qPCR, baseline and final data are not comparable.



Figure 4.2. Mean *P. marinus* infection intensity by replicate by predator treatment as measured by qPCR (indexed by the number of copies of *P. marinus* DNA detected within a host sample) in the field experiment (n=99 for 'no predator' treatment, n=100 for 'predator' treatment). Standard error bars represent standard error calculated using replicate for each treatment.



Figure 4.3. Mean change in *P. marinus* prevalence of each replicate (final-initial) by predator treatment in the lab experiment ( $n\approx144$  for each treatment). Error bars represent standard error calculated across replicate for each treatment.


Figure 4.4. Mean final *P. marinus* infection intensity of each replicate by predator treatment in the lab experiment (n=40 for control, n=64 for chemical cue treatment, n=53 for chemical and tactile cue treatment). Error bars represent standard error calculated across replicate for each treatment. The black dashed bar represents the baseline average intensity.

### CHAPTER 5

#### CONCLUSIONS

The environment greatly influences species interactions, including host-parasite interactions. Both biotic and abiotic factors are powerful drivers of host-parasite patterns in terrestrial and marine systems. Many of these environmental factors are predicted to be affected by changes in climate. Identifying what factors shape infection patterns will help us to better understand how host-parasite interactions may change in response to shifting climate. As negative effects of parasites can by magnified on the entire ecosystem when hosts are ecosystem engineers, we wanted to ascertain which environmental factors control infection patterns of the parasites *P. marinus* and *H. nelsoni*, as well as patterns in host immune response, in the commercially important engineering species, *C. virginica*.

Historically, water temperature and salinity have been the primary factors that positively correlate with patterns in parasite infection in this system. In Chapter 2 of this dissertation, we identified tidal elevation as a driver of parasite heterogeneity over small spatial scales. Specifically, parasite infections are more prevalent and more intense in intertidal compared to subtidal habitats. Based on our results, we can rule out parasites as a factor that affects the tidal distribution of oysters in Georgia. In an effort to determine the explanatory mechanism behind the observed pattern between tidal elevations, in Chapter 3 we established that increasing air temperature in the intertidal will differentially affect oysters and *P. marinus*. The observed optimal air temperature for *P. marinus* may have contributed to the patterns of parasite infection found between

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intertidal and subtidal habitats in Chapter 2. Subtidal oysters do not experience the high air temperatures that resulted in increased *P. marinus* infection intensity in our lab study. Therefore, we can assume that exposure to such temperatures in the intertidal contributed to the significantly higher infection intensities we found in intertidal oysters in Chapter 2. We now have a better understanding of how both host and parasite will respond to predicted increases in air temperature due to climate change.

Lastly, despite compelling evidence that predators can directly and indirectly effect host species on oyster reefs, as well as host-parasite interactions in other systems, in Chapter 4 we found that predator effects on oysters do not control oyster-parasite interactions. The probability and intensity of parasite infection were not affected by predators in either the field or the laboratory. Host immune response was also not affected by predators under field conditions. Results from these studies suggest that biotic factors may not be as important in shaping oyster-parasite interactions as abiotic factors such as air or water temperature. By identifying predators as a factor that does not affect oyster-parasite interactions we can more clearly focus future research efforts on other environmental factors.

Overall, we have provided support that oyster-parasite interactions can be influenced by a wide variety of environmental factors, even at small spatial scales. Additionally, we found that some aspects of climate change will differentially affect this system. As oysters are the foundation of many coastal and estuarine ecosystems, knowing which factors do and do not affect host-parasite interactions will help us to develop more effective research initiatives to better understand not only oysters, but also the complex communities which they create.

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# APPENDIX 1: DETAILED METHODS FOR PARASITE ASSESSMENT USING qPCR

To test for *P. marinus* infection status with qPCR in the Host Immune Response Experiment, we sterilely collected gill and mantle tissue from oysters and froze these samples at -20°C until time of DNA extraction. E.Z.N.A.® Tissue DNA Extraction Systems by Omega Bio-tek were used according to the manufacturer's protocol to collect a minimum of 200  $\mu$ l of oyster DNA that was stored at -20°C until the time of analysis.

The primer and probe set for *P. marinus* quantification was adapted from Gauthier et al. (2006) and modified by Stokes et al. (*in prep*). Our 10 µl PCR reaction mixture consisted of 5 µl Taqman Fast Advanced 2X Master Mix (Life Technologies), 2.4 µl nuclease free water, 0.20 µl Bovine Serum Agglutinate (BSA, 10mg/ml; New England BioLabs), 0.90 µl each PMAR-F (TTGTTAACGCAACTCAATGCTTTGT) and PMAR-R (AAGCGCACATAACGAACCACC) primers, 0.50 µl PMAR MGB-probe (GCTTGAACTAACTCT), and 1 µl template DNA.

We ran all standards (gBlock generated (Stokes et al. (*in prep*)), negative controls, and experimental samples in duplicate. We used a BioRad CFX96 Touch Real-Time PCR detection System for amplification with a program of 40 cycles of 3 s at 95°C and 30 s at 60°C with an initial denaturation of 20 s at 95°C. Data were analyzed using the CFX Manager Software, version 3.1. Experimental samples with copy numbers below 32 were considered below the limit of detection (LOD). Samples with copy numbers between 32-100 were considered positive for *P. marinus* and included in calculations of the probability of infection. Samples with copy numbers below 100 were considered to be below the limit of quantification (LOQ) and were not included in calculations of intensity. There were very few individuals with copy numbers between 32-100 so intensity calculations were not significantly altered by the exclusion of these individuals.

## APPENDIX 2. DETAILED METHODS FOR ASSESSING HOST IMMUNE RESPONSE

We collected ~ 500  $\mu$ l of hemolymph from the adductor muscle of each oyster with a sterile syringe and stored hemolymph samples on ice in 2 ml microcentrifuge tubes until time of processing (no more than 3 h). In 96-well plates, we incubated 100  $\mu$ l of hemolymph with 100 µl of sterile seawater (Sigma) and 50 µl of fluorescent bead solution (200  $\mu$ l of bead stock (Life Technologies) into 4800  $\mu$ l DI; beads serve as proxy for foreign or invading cells) for 60 min in the dark at 28°C and a stir speed of 100 rpm. We stopped the reaction using 25  $\mu$ l of 6% buffered formalin and ran triplicate hemolymph samples for each individual oyster. We filtered each sample prior to running it through a FACSCalibur flow cytometer and samples were read for 100,000 individual events (cells). The flow cytometer was set to detect and record the number of events with fluorescence representative of  $\geq 1$ ,  $\geq 2$ , or  $\geq 3$  fluorescent beads to determine the proportion of cells that phagocytized a specific quantity of beads (i.e., foreign cells). With CellQuest software (ver 4.0.1), we used forward and side scatter readings for each sample to identify and gate cells fitting the characteristics of granular hemocytes (granulocytes, large and complex cells). The proportion of granulocytes consuming  $\geq 1$  and  $\geq 2$  beads was ~100% for all samples, so we conducted statistical analysis on the 'highly active' cells that consumed  $\geq 3$  beads.

We also calculated the mean number of beads consumed by all granulocytes for each individual oyster using the proportion of granulocytes that consumed a specific number of beads (1, 2, or 3) and the total number of gated granulocytes (i.e., those that

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consumed at least 1 bead and were recorded by the flow cytometer). Our calculations were capped at 3 beads as it became difficult to accurately distinguish beads that consumed  $\geq$ 4 from  $\geq$ 3. This cap made our upper estimates of bead consumption conservatively low as there was a small portion of the cells that consumed  $\geq$ 3 beads.