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The effects of tidal elevation on parasite heterogeneity and co-infection in the eastern oyster, *Crassostrea virginica*

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ABSTRACT

Risk of infection by parasites can be driven by environmental heterogeneity, often at small scales. We quantified the effect of tidal elevation on infection patterns of two lethal parasites, *Perkinsus marinus* and *Haplosporidium nelsoni*, in an important coastal species, the eastern oyster, *Crassostrea virginica*. Within the southeastern US, oysters in Georgia and South Carolina are rarely found in the subtidal zone. Historically, it has been hypothesized that this pattern could be due to the increased exposure of hosts to waterborne parasites. We manipulated oysters at two tidal elevations (intertidal and subtidal) in a Georgia estuary to test if *P. marinus*, *H. nelsoni*, and co-infection by both parasites were different between tidal heights. We found that though *P. marinus* prevalence and co-infection prevalence of both parasites were not significantly different between tidal elevations, as has been found previously, *P. marinus* intensity and *H. nelsoni* prevalence were significantly higher intertidally than subtidally. These findings show that parasite infections can be higher in the host's natural (preferred) tidal height, and suggest that longer exposure to parasites in the subtidal is not a likely reason for the paucity of oysters at that tidal elevation in certain regions of the southeastern US. More broadly, our results provide further evidence that environmental effects on host-parasite interactions can vary by parasite species and across small (meter-long) spatial scales.

1. Introduction

Infection patterns of parasites and pathogens can be highly heterogeneous due to complex interactions between parasites, hosts, and their environment (e.g. Byers et al., 2008, 2015a). The environment can dictate geographic range, population abundance, and physiological response of both parasite and host species (Lafferty and Kuris, 1999; Altizer et al., 2006; Allen and 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 and Kuris, 1999; Soudant et al., 2005; Ford and 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. Thus, heterogeneous distributions of parasites throughout an ecological system can arise from environmental influences on parasites directly, their hosts, or their interactions.

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 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.

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. As an engineering species, oysters create complex habitat for other organisms in coastal systems, 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 through-

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out the host's East coast range. Independently, these parasites can cause high host mortality (Ford and Haskin, 1982; Andrews, 1984) and have exacerbated already low oyster population abundances caused by issues such as overfishing and habitat destruction (Rothschild et al., 1994).

Transmission of *P. marinus* occurs through the water column when infective stages of the parasite (trophozoites, zoospores, or hypnospores) are released from dead or moribund oysters, or through pseudofeces from live, infected oysters (Bushek et al., 2002). Uninfected oysters can contract infection by ingesting parasite spores while filter feeding (Ray, 1954). High parasite abundance within the host, through vegetative proliferation of *P. marinus* trophozoites, can lead to reduced host growth and reproduction, and eventually death if the energy needs of the parasite exceed the resources available for parasite and host maintenance (see Villalba et al., 2004 for detailed review). Unlike *P. marinus*, little is known about how *H. nelsoni* is transmitted between hosts (Burreson and Ford, 2004). Parasite spores can be observed in adult oysters, but are more often found in juvenile oysters less than a year old (Barber et al., 1991; Burreson, 1994). Because these spores are not directly infective to other oysters and experimental transmission has not been achieved in the laboratory, it is thought that an intermediate host is required to complete the life cycle (Burreson and Ford, 2004).

Shortly after *P. marinus* and *H. nelsoni* were discovered, water temperature and salinity were identified as the primary environmental factors that shape infection prevalence (proportion of infected individuals in a population) and intensity (severity of infection within an individual) (Ray, 1954; Haskins and Ford, 1982; Andrews, 1984; Soniat, 1996). Infections of both parasites tend to increase as water temperature and salinity increase from spring to summer, and peak in late summer-early fall (Ford and Tripp, 1996). Research has also suggested that diel-cycling hypoxia and air temperature may increase *P. marinus* transmission and intensity, respectively (Breitburg et al., 2015; Keppel et al., 2015; Malek and Byers, unpublished). However, *P. marinus* infections have not been found to vary across small spatial scales, such as within an intertidal-subtidal gradient, under field conditions (Burrell et al., 1984; O'Beirn et al., 1994; Ybanez, 2007; Malek and Breitburg, 2016). Comparatively, *H. nelsoni* is much less studied, with only a single experiment that tested for effects of environmental factors other than water temperature and salinity on infection patterns (Littlewood et al., 1992). Furthermore, studies that specifically address *H. nelsoni* infections in combination with *P. marinus* have been observational and are limited to the mid-Atlantic (Delaware Bay, Ford and Bushek, 2012), leaving an important gap in our understanding of patterns of parasite co-infection in *C. virginica* throughout much of the parasites' combined range.

To better understand environmental influences on oyster-parasite interactions, and parasite co-infection, we experimentally manipulated oysters within a Georgia estuary, in the Southeastern US. Our study addressed the effects of tidal elevations on the spatial heterogeneity of *P. marinus* prevalence and intensity, *H. nelsoni* prevalence, and the co-occurrence of both parasites within the oyster host. We expected, based on previous studies, that *P. marinus* infections would not differ between tidal elevations (Burrell et al., 1984; O'Beirn et al., 1994; Ybanez, 2007; Malek and Breitburg, 2016), but effects of tidal elevation on the prevalence of *H. nelsoni* and co-infection by both parasites have not been tested experimentally. Our holistic approach allowed us to examine the oyster-parasite relationship of each parasite individually as well as in combination.

2. Methods

2.1. 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^{-1}) and defaunated the clumps by soaking them in fresh water for ~1 h to remove unwanted epifauna or predators. We then weighed the defaunated 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. Analytical constraints prevented simultaneous collection of baseline *H. nelsoni* prevalence.

2.2. Experimental design and field setup

Oysters throughout Georgia are found nearly exclusively in the intertidal zone, approximately 1.5 m above mean low water. 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^2 oyster reefs built inside 0.027 m^3 plastic crates (0.3 m × 0.3 m × 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, in line with natural oyster reefs) 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, experimental reefs were deployed in an area largely devoid of naturally occurring oysters (at least 5 m from the closest reef on either side).

We deployed 16 replicates of each tidal elevation treatment for 19 weeks (01-June through 13-October 2012) in Romerly Marsh Creek (31 55′23.41″N, 80 59′21.29″W). This time frame encompassed the primary seasonal cycle of increased infection parasite prevalence and intensity (Andrews and Hewatt, 1957; O'Beirn et al., 1994). We conducted our experiment within the same site to standardize variability in environmental factors that can occur at larger scales, including the waterborne concentration of parasite spores (*P. marinus* in particular), which can be heterogeneous over spatial and temporal scales (Craig et al., 1989; Audemard et al., 2006; Tarnowski, 2015). We also deployed pressure gauges (Onset HOBO, U20-001-04) in waterproof cases on the sediment next to experimental reefs at each tidal elevation. These recorded 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 and measured the shell height of a random subset of ~30 oysters per reef that were then assessed for parasite infection.

2.3. Parasite infection assessment

2.3.1. 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 from each reef (Soniat et al., 2006).

Table 1
Primer set for *H. nelsoni* assessment (Stokes et al., 1995; Renault et al., 2000).

Primer	Sequence (5′–3′)
MSX A′ (Renault et al., 2000)	CGACTTTGGCATTAGGTTTCAGACC
MSX B (Stokes et al., 1995)	ATGTGTTGGTGACGCTAACCG

2.3.2. *H. nelsoni* assessment

We used common polymerase chain reaction (cPCR, following methods from Andree et al., 2000) 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 (n = 128 per treatment). 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. (1995) and Renault et al. (2000) (Table 1). The PCR mixture of 23 µl consisted of 12.5 µl GoTaq 2 × 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 s, 59 °C for 30 s, 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.

2.4. Statistical analyses

We used mixed effects logistic regression models ('lme4' package in R, Bates et al., 2015) to analyze the probability of infection for individual oysters with a binary distribution (0 = not infected, 1 = infected) as influenced by the fixed effect of tidal elevation (intertidal vs. subtidal). Because parasite infection can be correlated with oyster size (large oysters filter more water and have been filtering for longer than small oysters, potentially increasing their exposure to and accumulation of parasites (Ford and Tripp, 1996)), we also included shell height (in mm, measured from umbo to bill) as a random effect in our models. To ensure that oysters included in parasite infection analyses were part of our initial experimental population and not newly recruited juveniles, we excluded oysters < 40 mm from the data set. Replicate (experimental reef) was treated as a random effect because 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, identical 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 using 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 and shell height as random effects. To determine if infection intensity had increased from the beginning of the experiment, we compared intensity scores from infected oysters in the initial sampling to final intensity scores using Poisson regression models. We used a separate model for intertidal and subtidal oysters. We further assessed the intensity data to determine if the proportion of intense infections differed with tidal elevation by calculating the proportion of infections with Mackin scores ≥ 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 and shell height as random effects. 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, which could have affected parasite infection results, we analyzed oyster mortality using a mixed effects Poisson regression model. Specifically, we modeled the number of dead oysters on each experimental reef as a function of tidal elevation (fixed effect) with replicate as a random effect. All analyses were conducted in R Studio, version 0.98.1087 using R version 3.2.0 (R Core Team, 2015).

3. Results

The probability of *P. marinus* infection, when accounting for oyster size, tended to be higher in the intertidal than the subtidal, but was not statistically significant (Fig. 1, Table 2a). The proportion of oysters infected with *P. marinus* increased in both tidal elevations over the 19 wk. experimental period, from 32% (based on naturally intertidal oysters) to 52% in the intertidal and 42% in the subtidal (Fig. 1).

The probability of *H. nelsoni* infection was significantly higher in the intertidal than in the subtidal, with approximately 50% more infected oysters in the intertidal (Fig. 1, Table 2b). However, the probability of co-infection with both *P. marinus* and *H. nelsoni* did not differ with tidal elevation (Fig. 1, Table 2c). Chi Square analysis of co-infection prevalence 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).

The intensity of *P. marinus* infections was higher in the intertidal compared to the subtidal (Fig. 2, Table 3a). When compared to the initial baseline infection data, the final intensity of infections tended to be higher in the intertidal (z = 1.777, P(z) = 0.075), but not in the

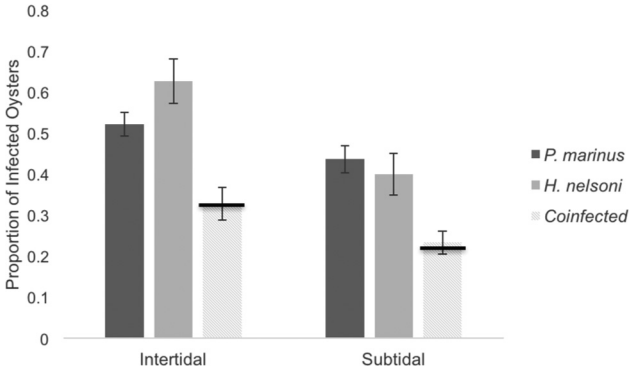

Fig. 1. Mean proportion of *P. marinus* (based on all individuals assessed with RFTM, including subsample also assessed with PCR, n = 425 Intertidal, n = 420 Subtidal) and *H. nelsoni* infections (based on all individuals assessed with PCR, n = 128 Intertidal, n = 105 Subtidal) and co-infected oysters by tidal elevation (n = 128 Intertidal, n = 105 Subtidal). Error bars represent standard error calculated across replicates 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%.

Table 2
Results of mixed effects logistic regression analyses testing the fixed effect of tidal elevation and the random effect of replicate and oyster shell height on: a. probability of *P. marinus* infection (n = 378 in intertidal across 16 replicates, n = 379 in subtidal across 14 replicates (2 reefs lost)), b. probability of *H. nelsoni* infection (n = 125 in intertidal across 16 replicates, n = 96 in subtidal across 14 replicates), and c. probability of co-infection (n = 125 in intertidal across 16 replicates, n = 96 in subtidal across 14 replicates). 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 *P. marinus* infection

Fixed effect	**Estimate**	**SE**	**z Value**	**Pr(z)**
Intercept	0.092	0.130	0.706	0.480
Tidal elevation	− 0.297	0.172	− 1.727	0.084
Random effect	**Variance**	**SD**		
Replicate	0.049	0.222		
Shell height	0.150	0.388		

b. Probability of *H. nelsoni* infection

Fixed effect	**Estimate**	**SE**	**z Value**	**Pr(z)**
Intercept	0.560	0.216	2.587	0.010
Tidal elevation	− 0.990	0.323	− 3.057	0.002
Random effect	**Variance**	**SD**		
Replicate	0.166	0.408		
Shell height	< 0.0001	< 0.0001		

c. Probability of co-infection

Fixed effect	**Estimate**	**SE**	**z Value**	**Pr(z)**
Intercept	− 0.717	0.191	− 3.765	< 0.001
Tidal elevation	− 0.496	0.309	− 1.606	0.108
Random effect	**Variance**	**SD**		
Replicate	< 0.0001	< 0.0001		
Shell height	< 0.0001	< 0.0001		

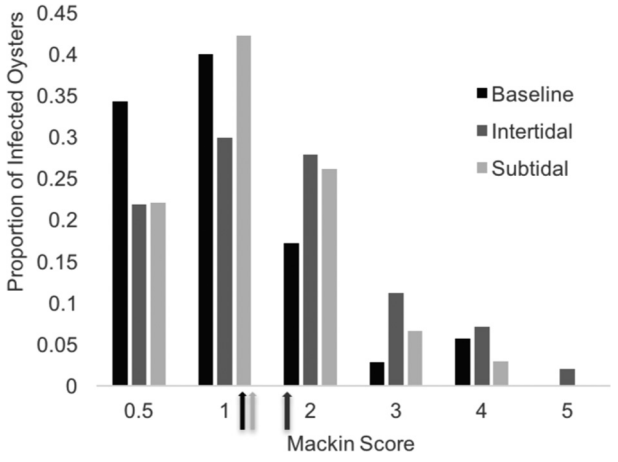

Fig. 2. The distribution of Mackin scores (an index of infection intensity) among *P. marinus* infected oysters. The arrows on the x-axis represent the average Mackin score for baseline oysters at the beginning of experiment (black), and intertidal (dark gray) and subtidal (light gray) oysters at the end of the experiment.

subtidal (z = 0.704, P(z) = 0.481). Further evaluation of infection intensity indicated that intertidal oysters had twice as many intense infections (3–5 on the Mackin scale) than subtidal oysters (proportion intense: $p_{intertidal}$ = 0.20, $p_{subtidal}$ = 0.09). The proportion of intense 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 = 0.147, P(z) = 0.883) and no individual effect of *H. nelsoni* (Table 3b).

Lastly, there was a significant effect of tidal elevation on oyster 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% ± 0.2 SE; mean subtidal = 1.5% ± 0.2 SE). The differential mortality between tidal elevations likely did not affect the observed patterns in parasite infection because average mortality rates were too low overall to cause detectable changes in the probability of infection. Additionally, the low mortality suggests that parasite infection was likely not a prominent cause of host mortality during the experiment.

Table 3
Results of mixed effects Poisson regression analyses of *P. marinus* infection intensity including a. tidal elevation as a fixed effect and replicate and shell height as random effects (intertidal was the reference tidal elevation; n = 199 in the intertidal and n = 175 in the subtidal as only infected oysters were included in analyses) and b. *H. nelsoni* infection status and tidal elevation as fixed effects and replicate and shell height as random effects (uninfected with *H. nelsoni* was the reference infection status and intertidal was the reference tidal elevation).

a. *P. marinus* intensity by tidal elevation

Fixed effect	**Estimate**	**SE**	**z Value**	**P(z)**
Intercept	0.947	0.044	21.351	< 0.001
Tidal Elevation	− 0.131	0.068	− 1.933	0.053
Random effect	**Variance**	**SD**		
Replicate	< 0.0001	< 0.0001		
Shell height	< 0.0001	< 0.0001		

b. *P. marinus* intensity by *H. nelsoni* infection status and tidal elevation

Fixed effect	**Estimate**	**SE**	**z Value**	**P(z)**
Intercept	0.900	0.116	7.718	< 0.001
H. nelsoni status	0.112	0.126	0.893	0.372
Tidal elevation	− 0.102	0.125	− 0.818	0.414
Random effect	**Variance**	**SD**		
Replicate	< 0.0001	< 0.0001		
Shell height	< 0.0001	< 0.0001		

4. Discussion

Differences in tidal elevation significantly affected the intensity of *P. marinus* and prevalence of *H. nelsoni* infections in the eastern oyster, *C. virginica*. Although prevalence of *P. marinus* infections trended higher in the intertidal zone, this pattern was not significant. Furthermore, the co-occurrence of the two parasites, did not differ with tidal elevation. At least in the case of *P. marinus*, we had expected to find similar results to other studies that showed little influence of tidal elevation on *P. marinus* infection (Burrell et al., 1984, O'Beirn et al., 1994, Ybanez, 2007 and Malek and Breitburg, 2016). However, our findings indicate that significant differences in oyster-parasite interactions do occur over a relatively small spatial scale (~3 m), but these differences can vary by parasite species and the metrics used to quantify their impacts on the host.

At least two strong reasons suggest why tidal elevation could theoretically affect parasite infection, both of which operate through differences in emersion during low tide. Increased duration of emersion, such as found in the mid-intertidal zone, can decrease how long oysters are exposed to parasites in the water column. This process could potentially decrease infection in intertidal oysters. Alternatively, increased exposure to air could intensify physiologically challenging conditions for oysters that can cause internal stress (Burnett, 1997) and increase susceptibility to infection (Allen and Burnett, 2008).

Because we observed heightened *P. marinus* intensity and *H. nelsoni* prevalence in the intertidal, we suggest this pattern could be the result of the conditions that the host faces during air exposure. The intertidal zone can experience extreme variability in air temperature, oxygen, and CO_2 conditions over small spatial scales (~1 m) that may have negative impacts on host physiology and subsequently affect interactions with parasites (Burnett, 1997; Boyd and Burnett, 1999). 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 ≥ 15 °C over just a few hours. When exposed to air, bivalves tend to cut off oxygen exchange with the atmosphere, leading to potentially physiologically challenging internal conditions (low O_2 and high CO_2) (Burnett, 1997) that can alter the host immune response (Allen and Burnett, 2008; Keppel, 2014) and may increase host susceptibility to parasite infections. Though we did not test for host immune response in this study, it is possible that altered immune function during air exposure could have contributed to the differences in *H. nelsoni* infection between intertidal and subtidal oysters (Boyd and Burnett, 1999). One other study in Delaware Bay has evaluated the effects of tidal elevation on *H. nelsoni* and found that placement within the intertidal zone (using 5 intertidal heights but no subtidal locations) did not influence parasite infection prevalence or intensity (Littlewood et al., 1992). Because physical conditions in the intertidal, such as air temperature, likely vary considerably between the mid-Atlantic and the Southeast, the sensitivity of oyster-parasite interactions to the effects of tidal elevation may depend on the magnitude of the variation in physical conditions in the intertidal zone and thus could vary geographically.

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 throughout much of the parasites' shared range (Ford and Bushek, 2012). Our experimental approach indicated 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 Tests of Independence). 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. 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.

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 (Ofiara and Stevens, 1987; Burrell et al., 1984). However, we found that parasite infections tend to be less prevalent (*H. nelsoni*) and intense (*P. marinus*) in the subtidal than the intertidal. In the subtidal, more than half of the *P. marinus* infections were scored as light-moderate intensity (Mackin scores of 0.5–1; Fig. 2), and there was a smaller proportion of high-intensity infections (Mackin score ≥ 3) compared to the intertidal. Burrell et al. (1984) also observed a higher proportion of more intense infections in the intertidal than the subtidal, but the differences between tidal elevations were not statistically significant. Additionally, our findings for *P. marinus* prevalence agree with other studies conducted in the same region that found no effect of tidal elevation on *P. marinus* infection prevalence (Burrell et al., 1984; O'Beirn et al., 1994). Thus, taken together, the evidence suggests that heightened parasitism by *H. nelsoni* and *P. marinus* can be eliminated as a factor explaining the lack of subtidal oysters in this region, and factors such as predation and sedimentation of reefs should be further considered as possible causes of this spatial pattern.

Though we observed that environmental conditions make the intertidal zone a more favorable habitat for *H. nelsoni* infections and *P. marinus* intensity compared to the subtidal, Southeastern oysters thrive intertidally. The resiliency of intertidal reefs to the moderate to high prevalence of both *P. marinus* and *H. nelsoni* that we observed (> 50% for both) may be the result of high recruitment of juvenile oysters compensating for any 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 and Powell, 2007). Byers et al. (2015b) 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 zone than the subtidal at our specific study site. High recruitment and addition of reef biomass may counterbalance the effects of higher *H. nelsoni* prevalence and more intense *P. marinus* infections and resultant parasite-related host mortality in the intertidal.

As an engineering species, *C. virginica* plays a critical role in coastal ecosystems along the East and Gulf coasts of the US. In some areas, parasites have reduced the abundance of this engineer 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). However, in the Southeast, high host recruitment and low parasite-induced mortality may compensate for moderate to high parasite prevalence and intensity. This study demonstrates that an environmental factor other than water temperature and salinity can affect *H. nelsoni* infection prevalence and *P. marinus* infection intensity, even across small spatial scales. By identifying the environmental factors and corresponding mechanisms that shape infection patterns, we can better understand how parasites affect ecologically and commercially important species.

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