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Using bivalves as particle collectors with PCR detection to investigate the environmental distribution of *Haplosporidium nelsoni*

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ABSTRACT: Recently, PCR technology has been applied to search for marine microorganisms in environmental samples. Such sampling, however, has drawbacks, including the need to collect and filter large volumes of water, and the presence of substances in environmental samples that may destroy DNA or interfere with DNA isolation and amplification. We explored the possibility of using suspension-feeding bivalves in conjunction with PCR to investigate the environmental distribution of microparasites using the oyster pathogen *Haplosporidium nelsoni* (the MSX disease agent) with oysters and mussels in Delaware Bay, USA, as a model system. Delaware Bay oysters *Crassostrea virginica* have become very resistant to *H. nelsoni* infection development and rarely exhibit histologically detectable lesions. The ribbed mussel *Geukensia demissa* is also resistant. Infections were detected in only 6% of the histologically examined oysters in the present study, although PCR-positive signals for *H. nelsoni* were found in feces, gill or heart samples of up to 100% of oysters. Positive signals were found in the feces and gill (but not heart) samples of up to 50% of mussels. The temporal evolution of PCR signals suggested a wave-like pattern of putative infective particles, which mimicked a pattern documented in early mortality studies. The present study demonstrated that *H. nelsoni* persists throughout Delaware Bay, although it is rarely detected using standard histology. We propose that the use of suspension-feeding bivalves as particle collectors in combination with PCR could be a method for detecting the presence of marine microparasites and might help answer questions about factors that prevent outbreaks of epizootics in certain regions.

KEY WORDS: Detection methods · Histology · Resistance · Parasite distribution · MSX · Epizootic · Marine

INTRODUCTION

The presence of a marine parasite is typically determined by examining its known host(s) for infections by traditional histological or culture methods, or, with increasing frequency, by molecular methods. Recently, PCR technology has been applied to the search for microorganisms in environmental samples, including water, sediment and on organic surfaces (Vigneron et al. 2004, Lyons et al. 2005, Audemard et al. 2006, Blackstone et al. 2007). Such sampling, however, has certain drawbacks, including the need to collect and filter large volumes of water in order to obtain sufficient material for reliable detection, and the presence of substances in environmental samples that may destroy DNA or otherwise interfere with DNA isolation and amplification (Wilson 1997, Guy et al. 2003, Audemard et al. 2004, 2006).

Suspension-feeding bivalves filter material from large volumes of water and could be considered as collectors for water-borne particles. Loosanoff & Nomejko (1946) and Galtsoff (1964), for instance, estimated that large (100 to 120 mm shell height) eastern oysters *Crassostrea virginica* could filter up to 300 to 480 l d^{-1} (13 to

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20 l h^{-1}). The ability of bivalves to accumulate bacteria and viruses of concern to human health is well known (Canzonier 1988) and is also the basis for using mussels and oysters to measure water contamination (Kim & Powell 2006). Water-borne protistan parasites of the bivalves themselves are among the particles collected by the mollusks as they filter. If the bivalve is susceptible to infection by the parasite, the gills or mantle may be among the portals of entry (Farley 1968, Chintala et al. 2002, Ford et al. 2002). If the bivalve is not susceptible, the parasites may be carried toward the mouth with other particles and discarded in the pseudofeces, or consumed and, if not destroyed by digestive enzymes, voided in the feces. Ingestion and voiding of viable dinoflagellates by suspension-feeding bivalves has been demonstrated recently (Hegaret et al. 2008). The same might also be true of parasites that are unsuccessful in infecting a susceptible host. Thus, the presence of a parasite in an area might be detected by examining the feces or pseudofeces of bivalves in that area, even if a known susceptible host is not present.

The parasite *Haplosporidium nelsoni* was introduced to the east coast of the United States from the Pacific, where it infects the Pacific oyster *Crassostrea gigas* (Burreson et al. 2000). Although *H. nelsoni* has not been reported to cause mortalities in that species, the eastern oyster *C. virginica* is highly susceptible and has suffered heavy losses in many regions of the eastern United States since it was first detected in Delaware Bay, USA in 1957 (Haskin et al. 1966). The life cycle and mode of transmission of *H. nelsoni* are unknown. Direct oyster-to-oyster transmission has not been demonstrated experimentally and another host has been hypothesized (Sunila et al. 1999). The earliest infections are found in the gill epithelium (Farley 1968, Ford & Haskin 1982). Mortality ensues when infections become systemic, whereas resistance to mortality is associated with restriction of infections to the gill epithelium (Ford & Haskin 1987). In the late 1980s, the prevalence of *H. nelsoni* in Delaware Bay oysters, as determined by traditional tissue-section histology, declined dramatically. Annual peaks of 60 to 85% in the 1970s through the mid-1980s fell to 30% or less after 1987 (Burreson & Ford 2004). Ford (2002) hypothesized that the change resulted from a massive mortality that occurred in the mid-1980s when *H. nelsoni* moved upbay during a drought (Powell et al. 2008) leaving a bay-wide population with considerable resistance to *H. nelsoni* infection development. An alternative hypothesis is that the abundance of *H. nelsoni* has decreased, thus resulting in fewer infections.

In the present study, we used PCR technology and the concept of using suspension feeders as collectors of *Haplosporidium nelsoni* to investigate the parasite's temporal and spatial distribution in Delaware Bay, and to examine the potential of these tools for answering questions about the parasite that cannot be answered by traditional histological methods.

MATERIALS AND METHODS

Bivalves. Eastern oysters *Crassostrea virginica* were collected from several sites on the New Jersey side of Delaware Bay (Table 1, Fig. 1). Native oysters were obtained from natural beds and one leased ground

Table 1. *Crassostrea virginica* and *Geukensia demissa*. Samples analyzed for *Haplosporidium nelsoni* by PCR and histology. Check marks (✓) indicate the comparisons made or individual components examined in the samples indicated. Naïve oysters *C. virginica* originated in the Damariscotta River, Maine. Native oysters and ribbed mussels *G. demissa* were from Delaware Bay

Date(s)	Location	Species	N	PCR assay						Histology
				Feces	Pseudo-feces	Gill pieces			Heart	Gill and visceral mass
						Washed vs. unwashed	Adjacent unwashed	Single unwashed		
1999										
June & July	Cape Shore	Naïve oyster	40	✓	✓	✓			✓	
June & August	Maurice River	Native oyster	21	✓		✓			✓	
	Maurice River	Native mussel	21	✓		✓			✓	
May, June, July, November	Delaware Bay Beds	Native oyster	140[a]	✓			✓	✓	✓	✓
October	554D	Native oyster	20				✓		✓	✓
2000										
April, May, June, July	Cape Shore	Native oyster	50	✓				✓	✓	
	Cape Shore	Naïve oyster	40	✓				✓	✓	
	Cape Shore	Native mussel	50	✓				✓	✓	
September	Cape Shore	Native oyster	20							✓
	Cape Shore	Naïve oyster	20							✓

[a]20 oysters collected in June were not processed for histology

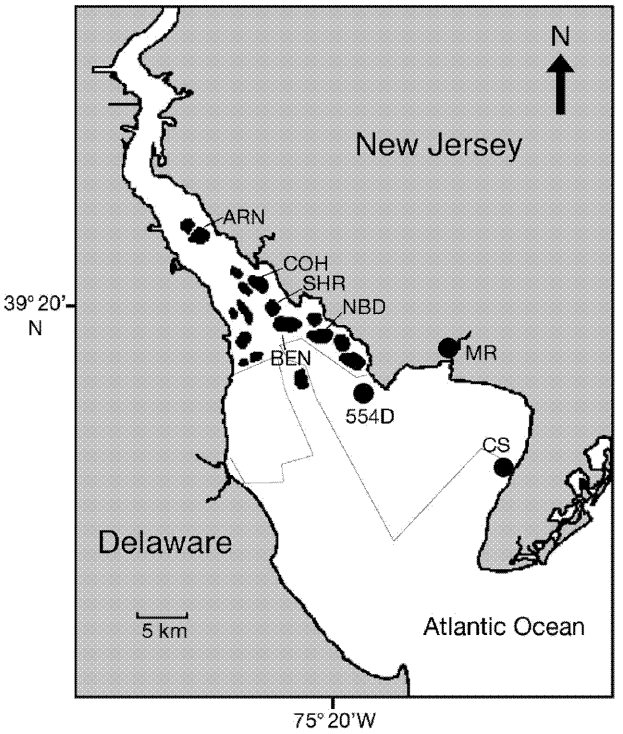

Fig. 1. Sampling locations in Delaware Bay. Upbay sites: Arnolds (ARN) and Cohansey (COH) natural beds; mid-bay sites: Shell Rock (SHR), Bennies (BEN) and New Beds (NBD) natural beds; lower bay sites: leased ground (554D), Maurice River (MR), and Cape Shore (CS). Thin grey lines: leased grounds

from May through November 1999. Both *C. virginica* and the ribbed mussel *Geukensia demissa* were collected from the Maurice River at Bivalve in June and August 1999. At the Cape Shore intertidal flats in lower Delaware Bay, oysters were collected in June and July 1999, and both oysters and ribbed mussels were sampled from April through July 2000. Salinity at mid-tide and mean Delaware River flow ranges from about 20 to 24 psu in the lower bay sites to 10 psu at Arnolds Bed, the uppermost natural bed sampled. The tidal variation is typically 2 to 3 psu above and below the mean. Salinity at the Maurice River site is highly variable, ranging from 0 to 20 psu depending on tide and precipitation.

At the Cape Shore, 2 stocks of oysters were sampled: (1) 'naïve' oysters from the Damariscotta River, Maine, which have experienced little or no previous MSX-disease pressure, had no detectable *Haplosporidium nelsoni* infections when examined histologically at the time of importation (Haskin Shellfish Laboratory records), and are highly susceptible to infection by *H. nelsoni*; and (2) native oysters that had set on a nearby intertidal reef. The Maine oysters were held in a rack and bag system about 100 m from the reef. In 1999, the Maine oysters were deployed on 27 May and the first sample was collected on 24 June. In 2000, they were deployed on 5 May and first sampled on 12 May. Mussels were collected from the same reef as the native oysters, with which they were intermingled. Although the bivalves were not measured, they were all adults, between about 60 and 80 mm in shell height and from 2 to 3 yr old. Bivalve numbers ranged from 5 to 20 individuals per sample, with 2 to 5 tissue, feces and/or pseudofeces samples assayed per individual. A total of 382 individual bivalves was examined and 1108 PCR reactions were run.

Feces and pseudofeces collection. Several of the collections included sampling of feces, and one of these (Cape Shore, July 1999) also involved sampling of pseudofeces. To accomplish this, oysters and mussels were brought into the laboratory, scrubbed under running tap water and placed individually in plastic tubs containing approximately 1 l of seawater that had been filtered through a 1 µm cartridge filter. The bivalves were maintained in the tubs overnight without aeration to prevent disturbance of the feces and pseudofeces, which were collected the following morning using a Pasteur pipette and placed in separate 15 ml centrifuge tubes. The tubes were centrifuged at 13 000 to 14 000 × *g* for 2 min and the pellet transferred to 95% EtOH.

Tissue collection. The bivalves were carefully opened at the hinge with an oyster knife that was rinsed in tap water and then immersed in a 10% hypochlorite solution between individuals. Special care was taken not to disrupt the pericardial membrane when the adductor muscle attachment was severed. Dissecting instruments were dipped in 70% EtOH and flamed between bivalves and tissues. One or 2 adjacent sections of the gill were then removed and placed in individual 1.5 ml microfuge tubes containing 95% EtOH. Each section was approximately 5 to 7 mm long and encompassed the entire proximal to distal length of at least 2 demibranchs. Two adjacent gill pieces were assayed to help evaluate the spatial variability in PCR-positive signals on this organ. In some trials, to assess whether the DNA detected by PCR on the gill was in the tissue (implying an infection of *Haplosporidium nelsoni*) or enmeshed in mucus, as opposed to being loosely attached, one of the gill pieces was rinsed in a stream of 0.22 µm filtered seawater before fixation. Finally, to identify systemic infections, the heart was removed and fixed in a separate tube.

PCR assay. DNA extraction was generally accomplished within 1 to 2 d after collection. The 95% EtOH was replaced with TE buffer (10 mM Tris, pH 8.0, 1.0 mM EDTA) and the tissue, feces or pseudofeces was homogenized in a 1.5 ml microfuge tube using a sterile plastic pestle. Approximately 25 to 50 mg of

the homogenate was then lysed with guanidine thiocyanate (GTC) (Hill et al. 1991). The DNA was precipitated with 3 M sodium acetate (1:10 v/v, final concentration 176 mM) and isopropanol (1:6 v/v, final concentration 35%) and extracted using an ethidium bromide/high salt procedure (Stemmer 1991). At the final step, EtOH was decanted and the volume of precipitated material was roughly estimated as ranging from 'very small' (≤1.5 µl) to 'large' (~10 to 12 µl). The tube was then air dried and the resulting DNA pellet was resuspended in a volume of TE buffer that ranged from 5 µl for 'very small' to 20 µl for 'large' precipitate volumes. The purified DNA was stored at 4°C until it was amplified (usually within a week). Although DNA concentration was not measured, every effort was made to use roughly similar amounts of tissue for each sample.

A 2-stage hemi-nested PRC protocol (Zimmerman et al. 1994) was used to amplify *Haplosporidium nelsoni* DNA in the samples. The first stage, which employed primers MSX A and MSX B, amplified a 564 bp region of the *H. nelsoni* small subunit rDNA (SSU rDNA) (Stokes et al. 1995). The second stage, which used primers MSX A and MSX C amplified a 251 bp segment of the first region (Burreson et al. 2000). For the first amplification, 1 µl of the template DNA was added to 24 µl of a solution containing buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 M $MgCl_2$, 10 µg ml^{-1} gelatin); 25 pmol of each primer; 200 µM each of dATP, dCTP, dGTP and dTTP; 10 µg bovine serum albumin (BSA); and 0.6 units of AmpliTaq DNA polymerase (Perkin-Elmer). The mixtures were placed in a Delta-Cycler II thermal cycler (Ericomp), denatured for 5 min at 94°C, then cycled 35 times at 95°C for 55 s, 59°C for 1 min and 73°C for 3 min, with a 5 min final extension at 73°C. In the second amplification, all ingredients of the reaction mixture were the same except the buffer, which consisted of 60 mM Tris-HCl, pH 10, 15 mM $(NH_4)_2SO_4$ and 1 mM $MgCl_2$. The thermal cycler program was also the same except that the annealing temperature was 53°C rather than 59°C. For both amplifications, a positive control (genomic *H. nelsoni* DNA) and a negative control (water) were included. Approximately 2.5 µl of each second-stage amplification product was electrophoresed on a 2% agarose gel and stained with ethidium bromide to visualize bands.

Histology. Tissue sections were made from 140 native oysters that were also processed for PCR analysis (Table 1). An additional 20 oysters each from the naïve and native groups were examined histologically after the PCR sampling at the Cape Shore was completed in 2000. All were examined and scored using previously described methods (Farley 1968, Ford & Haskin 1982). Slides from May and July collections from the natural beds were made by sectioning the oysters frontally approximately two-thirds of the distance from the distal to the proximal ends of the demibranchs and again in the same direction, but angled into the visceral mass. The 2 outside pieces were embedded cut-face down so that 2 frontal sections of the gill, as well as some of the visceral mass, were examined for each individual. All other slides were made from a single cross section that also included gill and visceral mass. The extra gill tissue of the May and July sections increased the chances of finding small, localized gill lesions during the early infection period.

RESULTS

PCR assays

Gill-sample and feces vs. pseudofeces comparisons

On a sample basis, the prevalence of PCR-positive signals on adjacent pieces of gill was comparable (Table 2). On an individual oyster basis, however, adjacent pieces did not always give the same result. That is, in the same oyster, a piece of gill with a positive signal might be adjacent to a piece with a negative result. Thus, the combined prevalence from both pieces was higher than that from only a single piece (Table 2). The greatest disparity between pieces from a single oyster was evident in samples from the uppermost bay site and the Maurice River, both relatively low-salinity sites compared to the mid-bay and Cape Shore sites. In the low-salinity sites, only 50 to 67% of the results of one piece (positive or negative) matched that of the other. In the high-salinity sites, the matches were 80 to 90%. Washing had no significant effect on the results (paired sign test: $p = 0.250$) (Table 3). In the single 20

Table 2. *Crassostrea virginica*. Percent prevalence of *Haplosporidium nelsoni* PCR-positive signals on adjacent pieces of unwashed *C. virginica* gill. The Cape Shore sample consisted of naïve oysters; all others were natives. Dates given as mm/dd/yy. N = total number of oysters sampled. Matches = percent of paired samples with the same result, positive or negative

Sample location	Date	N	Prevalence (%)			
			Gill 1	Gill 2	Combined total	Matches
Upper Beds	07/14/99	10	80	70	100	50
Central Beds	07/14/99	10	90	90	100	80
Cape Shore	07/26/99	20	90	80	90	90
Maurice River	08/24/99	10	10	30	40	67
554D	10/29/99	20	50	55	60	85

Table 3. *Crassostrea virginica* and *Geukensia demissa*. Percent prevalence of *Haplosporidium nelsoni* PCR-positive signals on adjacent pieces of washed and unwashed oyster *C. virginica* and mussel *G. demissa* gill. The Cape Shore sample consisted of naïve oysters; Maurice River oysters and mussels were natives. Dates given as mm/dd/yy. N = total number of bivalves sampled. Matches = percent of paired samples with the same result, positive or negative

Species	Sample location	Date	N	Prevalence (%)			
				Washed	Un-washed	Combined total	Matches
Oyster	Cape Shore	06/24/99	20	100	90	100	90
Oyster	Maurice River	06/21/99	11	36	27	64	45
Mussel	Maurice River	06/21/99	11	9	0	9	91

oyster sample in which both were analyzed, pseudofeces had a PCR-positive prevalence of 89% and feces had a prevalence of 71%. These values were not statistically different (chi-square: df = 1, p = 0.638).

Spatial and temporal distribution on natural beds

PCR-positive signals were present in the feces, gill and heart samples of oysters from each of the natural bed locations at each sampling date in 1999. Samples were grouped into upbay and mid-bay beds to (1) simplify presentation (Fig. 2), (2) because, historically, the beds within each group had similar *Haplosporidium nelsoni* prevalences (Haskin & Ford 1982), and (3) because no significant differences in PCR signals were detected among the beds within each designated region (chi-square: $p > 0.05$). Eighty to 100% of feces and gill samples from both regions were PCR-positive in the May sample, but prevalence was only 20% in heart samples. In both regions, the prevalence of signals on the gill dropped to ≤50% in early June (feces were not sampled then), but was again 80 to 90% by mid-July. In July, positive signals were found in the feces of half the oysters sampled on the mid-bay beds, but none was detected in feces from the upbay beds. By early November, the prevalence of gill signals had again dropped to ≤50%. At no sample date did the prevalence of positive signals in the heart exceed 30%. All oysters with PCR-positive heart samples also had PCR-positive gill samples.

Oysters vs. ribbed mussels

Sampling at the Cape Shore site in 2000 detected PCR-positive signals in the feces (40%) and gills (80%) of the native oysters at the first collection, on 15 April (Fig. 3). At the same time, positive signals were found in the feces and gills of 20% of the ribbed mussels collected on the same reef. A month later, in mid-May, naïve (Maine) oysters, which had been deployed just 7 d earlier, were already showing PCR prevalences equal to those of the native oysters: 70 and 90% in the feces and gill samples, respectively. Positive signals were also found in the feces (20%) and gill (50%) samples of ribbed mussels collected at the same time. In early June, observed prevalences were unchanged in the naïve oysters and the mussels, but had fallen to 40–50% in the native oysters. Little additional change was found in gill samples of all groups at the final 2 collection dates, 29 June and 20 July. Importantly, no positive signals were detected in the feces of any of the groups in the late June sample, but they were again present in all 3 groups in the July sample, at which time they were abundant: 50 and 80% in the native and naïve oysters, respectively. Positive results in the hearts of the native oysters, which were sampled at all 4 times, never rose above 30%, whereas those of the naïve group were already 60% when first sampled at the end of June and rose to 80% by 20 July. No positive signals were detected in mussel hearts, which were sampled in April and July.

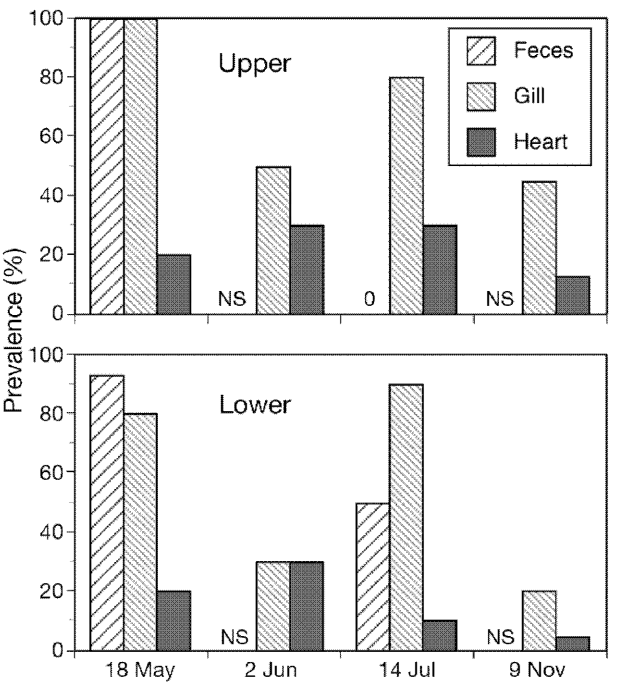

Fig. 2. *Crassostrea virginica*. Prevalence (%) of *Haplosporidium nelsoni* PCR-positive signals in tissues and feces of native oysters *C. virginica* collected in 1999 from 2 regions of the natural beds of Delaware Bay. May: N = 5 oysters for upbay, 15 for mid-bay; June & July: N = 10 for both areas; November: N = 20 for both areas. Feces and tissue samples on a given date were collected from the same individuals. NS: not sampled

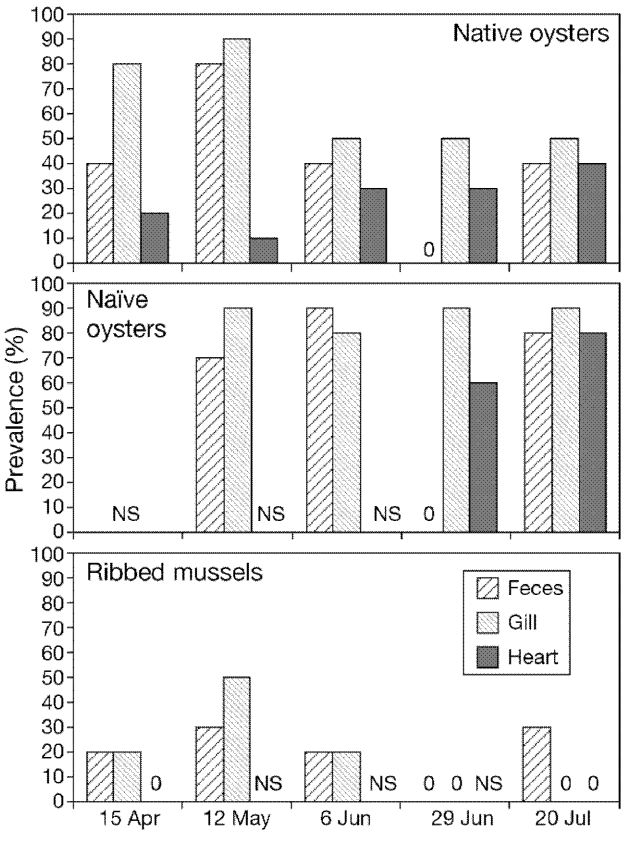

Fig. 3. *Crassostrea virginica* and *Geukensia demissa*. Prevalence (%) of *Haplosporidium nelsoni* PCR-positive signals in tissues and feces of native and naïve oysters *C. virginica* and ribbed mussels *G. demissa* collected in 2000 at the Cape Shore in lower Delaware Bay. N = 10 bivalves at each date. Feces and tissue samples on a given date were collected from the same individuals. NS: not sampled

Histology

Of the 140 native oysters from Delaware Bay beds that were examined by both histology and PCR, only 10 (5.7%) had *Haplosporidium nelsoni* that was detected by histology. Eight of these infections were localized in the gill epithelium; the other 2 were light systemic infections. All 10 oysters were positive by PCR from their gill tissue DNA and 3 were positive from heart tissue DNA. Of the remaining 130 oysters in which no histologically detected infections were found, 65 were positive using PCR on gill tissue and 15 of these were also positive in heart tissue. In early September 2000, about 5 wk after the last PCR assay, 20 each of the naïve and native oysters from the Cape Shore were examined histologically. All of the former had histologically detected infections. Half were systemic, and half of these were already very heavy. Only one native oyster was found to be infected by histology and it had a rare infection (<10 plasmodia in the section) localized in the gill epithelium.

DISCUSSION

Using PCR technology and oysters and mussels as particle collectors, we were able to describe some spatial and temporal patterns of the oyster pathogen *Haplosporidium nelsoni* in Delaware Bay that were not apparent using traditional histological methods. We inferred the parasite's distribution based on PCR-positive signals in tissues and feces. In assessing these results, it is important to stress that PCR detected a segment of DNA from a single gene, not the parasite itself. A PCR-positive signal does not necessarily indicate an infection or even a viable parasite (Burreson 2008). Further, since it is impossible to test primers against all potentially similar organisms, cross-reactions could result in false positives. Even though we found very few infections by histology in the oysters we assayed by PCR, we conclude that the PCR signals we detected represent *H. nelsoni* DNA for 3 reasons: (1) the parasite has been present in Delaware Bay for decades, (2) all oysters determined to be infected by histology were also PCR positive, and (3) the primers that we employed amplify a sequence that, to date, has been found to be unique and specific for *H. nelsoni* among the haplosporidians whose SSU rDNA has been sequenced (Bearham et al. 2007), including *H. costale* and *Minchinia teredinis*, which may co-occur with *H. nelsoni*.

From 1957, when *Haplosporidium nelsoni* was first associated with massive mortalities of native oysters in Delaware Bay, until 1987, fall prevalence peaks, determined by histology, were typically above 50% (Ford & Haskin 1982, Burreson & Ford 2004). During the decade and a half before 1988, prevalence maxima were 60 to 85%. In 1988, prevalence in native oysters dropped abruptly. Since then, oysters collected during the fall survey on the Delaware Bay natural beds, as well as yearly samples of natives from the Cape Shore taken during a period from late summer into early fall since 1998, have had prevalences ≤30%, and most samples have been histologically negative (S. E. Ford & D. Bushek, unpubl. Haskin Shellfish Research Laboratory records). Nevertheless, results of the present study indicate that, a decade after the sharp decline in histologically detectable infections, *H. nelsoni* was still prevalent in Delaware Bay and widespread along the salinity gradient. The latter result contrasts with the clear prevalence decrease, pre-1988, of histologically detectable infections along the same gradient (Haskin & Ford 1982) and the documented inability of *H. nelsoni* plasmodia to survive low salinity both *in vivo* and *in vitro* (Andrews 1983, Ford 1985, Ford & Haskin 1988).

The 'presence/absence' PCR methodology used did not permit us to estimate the quantity of *Haplosporid-*

ium nelsoni DNA in the individual samples, which might reflect the abundance of parasites in the water, but the prevalence of individual oysters with positive results was similar regardless of position along the gradient. Our data are consistent with another study in which Barber & Ford (1992) reported finding ingested haplosporidian spores, matching the size and shape of *H. nelsoni* spores, in the guts of oysters throughout Delaware Bay. Thus, the decline of histologically detectable infections measured along salinity gradients in both Delaware Bay and the James River, Virginia (Andrews 1964, Haskin & Ford 1982) may result more from a failure of *H. nelsoni* to successfully infect and proliferate in oysters at lower salinity than an absence of parasites in those waters. Because the complete life cycle of *H. nelsoni* is unknown, it may also be possible that some of the PCR-positive signals are evidence of a stage that is not infective to oysters.

Albeit limited, our attempts to wash putative *Haplosporidium nelsoni* from gills were not successful. Similarly, in a previous study, PCR-positive signals in seed oysters did not disappear after depuration in filtered, UV-treated water (Ford et al. 2001). At least some of these signals may well indicate parasites in very small, localized gill lesions. Others may represent parasites that are not in the tissue, but have become trapped in mucus on the gill surface. The PCR-positive signals in the mussel gill samples suggested parasites that could be trapped in mucus or even degraded parasites. Although histological sections of ribbed mussels were not examined in the present study, a previous *in vitro* investigation showed that *H. nelsoni* plasmodia were quickly phagocytosed by hemocytes of this species, implying that the parasite would not survive long if it did invade the mussel (Ford et al. 1993). The fact that no PCR-positive heart samples were found in mussels reinforces this argument.

Early investigations into the *Haplosporidium nelsoni*-caused epizootic included timed importation of naïve oysters into Delaware Bay and showed that mortality patterns depended on when the oysters were deployed and presumably when they became infected (Haskin et al. 1965). Ford & Haskin (1982, p. 124) interpreted these data as indicating an infective period for lower Delaware Bay 'that begins and is heaviest in June, decreases in mid-summer, then resumes with varying intensity later in the summer and may extend into November'. Our molecular data also support the concept of waves of infective particles. This is shown in the feces data from the 2 sequential sampling series. In 1999, PCR signals in feces declined from 100% in mid-May to 0% in mid-July on the upbay beds, and from 90 to 50% in the mid-bay samples. In the Cape Shore samples collected in 2000, all groups had positive signals (up to 90% in oysters and 30% in ribbed mussels) in May and early June, but by the end of June all feces samples were negative. Three weeks later, the feces of all groups once again showed positive signals, at prevalences up to 80% (naïve oysters). It could be argued that positive results in the feces represent parasites being shed from infected oysters rather than parasites collected from the water being voided directly in the feces or pseudofeces. In fact, in the present study, the prevalence of positive signals in feces, gill and heart was highest in the naïve oysters and lowest in the mussels, suggesting that the different sample results were not independent in all cases. However, the drop to 0% prevalence in feces occurred simultaneously in all 3 groups at the Cape Shore, despite the continued presence of positive signals in the gill and heart samples. Similarly, the 0% prevalence in feces of upper natural bed oysters was concurrent with 80% prevalence in the gill samples from the same individuals. Thus, infection — or at least the presence of parasites on the gill — does not necessarily mean that parasites are shed into the feces. We argue, therefore, that PCR-positive signals in the feces or pseudofeces can be decoupled from infections, particularly in relatively resistant bivalves with low infection levels, and certainly in non-hosts, and can serve as an index of parasite presence in the water.

The PCR prevalence of gills from oysters in the 1999 upbay series also suggested a wave-like infective pattern with prevalences of 80 to 100% in mid-May, 30 to 50% in early June, 80 to 90% in mid-July and 20 to 40% in early November. This pattern is also a demonstration of resistance to infection development in the native oysters. Before 1988, gill epithelial lesions typically progressed into systemic infections in the late summer and most infections were systemic for the remainder of the fall and winter (Ford & Haskin 1982). This pattern was apparent in the PCR results from the naïve oysters at the Cape Shore in 2000, in which positive signals were prevalent in the heart within 2 mo of deployment. High infection prevalence was later confirmed by histology. In contrast, the pattern shown on the natural beds in the present study suggests that very light and localized gill infections (or perhaps just 'externally' adhering parasites), not detected in most histological sections examined from the same oysters, were acquired and then lost at least twice during the 1999 study period.

Our results were obtained in an estuary where *Haplosporidium nelsoni* has been observed in tissue sections since it was first identified in 1958; these results are consistent with mortality and ingested-spore data from earlier studies (Haskin et al. 1965, Barber & Ford 1992). Our finding of so few histologically detectable infections in the native oysters was beneficial because it allowed us to make inferences about the presence of

H. nelsoni in the environment without viewing the parasite in oysters. The question becomes whether we can draw the same inference from samples collected in regions where the parasite has never, or only rarely, been seen histologically. We should certainly be skeptical of equating PCR-positive findings with infections (Burreson 2008); however, we do know that *H. nelsoni* can exist in areas without causing epizootics, even though the oysters in these areas prove to be highly susceptible when tested in enzootic locations (Ford & Haskin 1987). For instance, the parasite was detected histologically in Long Island Sound oysters in 1960 and on Cape Cod in 1967, but the first epizootics in these locations were not recorded until the mid-1980s (Haskin & Andrews 1988, Matthiessen et al. 1990, Sunila et al. 1999). In southern Maine, *H. nelsoni* was detected as early as 1983 (Sherburne & Bean 1991), but the first reported epizootic did not occur until 1995 (Barber et al. 1997). Plasmodial stages of what is presumed to be *H. nelsoni* have been reported, although at relatively low prevalence, as far south along the US east coast as Florida without causing recorded epizootics or mortality (Hillman et al. 1988, Lewis et al. 1992, Dougherty et al. 1993, Bobo et al. 1996, Kim & Powell 2006). The recent report of high (30 of 41 oysters analyzed) *H. nelsoni* prevalence in the Gulf of Mexico, which is based on PCR results only (Ulrich et al. 2007), is intriguing because the parasite has not been detected in numerous histological surveys of eastern oysters throughout the Gulf (Winstead 1995, Winstead et al. 2004, Kim & Powell 2006, Y. Kim pers. comm.).

What has prevented epizootic outbreaks in these areas? Is it a paucity of infective stages, an environmental factor that inhibits infection development or a combination of these or other factors? The results of the present study suggest a way of distinguishing between these alternatives for *Haplosporidium nelsoni* and perhaps other marine parasites. PCR can clearly detect the presence of a parasite and quantitative PCR can provide an estimate of abundance (Yarnall et al. 2000, Audemard et al. 2004). Finding few or no PCR signals would suggest that the parasite is either not present in the environment or not abundant enough to result in histologically detectable infections. In the case of *H. nelsoni*, this scenario might be associated with the absence or scarcity of a hypothesized alternate or intermediate host. Finding PCR-positive signals to be prevalent without concomitant histologically detectable infections in a susceptible host would indicate that the environment is not favorable for the development of infections or that the PCR signals are detecting a stage of the parasite that is not infective for the host in question. A logical next step would be *in situ* hybridization (in the host species), which often locates parasites not obvious with traditional stains (Audemard et al. 2002, Carnegie et al. 2003, Bearham et al. 2008). The presence of a parasite could even be signaled by a non-host, as demonstrated by our finding of PCR-positive reactions for *H. nelsoni* in the feces and gill samples of ribbed mussels. Detection in the feces or pseudofeces is particularly attractive because it is a non-destructive technique and can be used with both host and non-host species. Looking for parasite DNA in the feces or the gills, particularly of a host in which infections do not become established, would also be a simple and inexpensive method of identifying periods when that parasite is present in the water and potentially infective. Although our protocol does not identify possible environmental factor(s) responsible for limiting infection development, it is an expedient way to suggest the directions that research might take.

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