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Spatial and temporal variability of disease refuges in an estuary: Implications for the development of resistance

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ABSTRACT

Although the concept of genetic refuge has long been employed in ecological and paleoecological context, it has only rarely been used to identify regions where organisms are protected from diseases that affect the rest of a population. The refuges harbor individuals that have not been exposed to selective mortality and remain susceptible to the disease. They represent a reservoir of susceptibility alleles that can mix with those from resistant survivors of disease and can retard the development of resistance in the population as a whole. Two water-borne protistan parasites affect oysters along the east coast of the United States: *Haplosporidium nelsoni* (MSX disease) and *Perkinsus marinus* (dermo disease). Both are sensitive to low salinity and their prevalence is reduced in the upper reaches of estuaries. We investigated the temporal and spatial structure and extent of putative refuges from these diseases in the upper Delaware Bay, USA and their potential to affect the development of resistance in the oyster population. Our results showed that refuges occurred as a continuum of zones, regions where a pathogen (1) was not present; (2) was present, but did not cause observable infections; and (3) caused infection, but neither disease nor mortality. The zones were transient, driven only partly by short-term climatic conditions, and differed according to parasite: *H. nelsoni* was often not present in the refuges, as inferred by the absence of polymerase chain reaction (PCR) – positive signals on the gills, and when it was present, it did not always cause lethal, or even histologically detectable, infections. In contrast, *P. marinus* was present in all upper estuary areas sampled, where it caused detectable, although not necessarily lethal-level, infections. Thus, a significant fraction of the oyster population is protected from selective mortality in these refuges even when the parasites are present. An incursion of *H. nelsoni* into the upper Bay in the 1980s left most of the surviving population highly resistant to MSX disease, although populations in the upper-most reaches are still susceptible. The lack of selection pressure in the refuges likely helps to retard the development of resistance to dermo disease, and theoretically could cause resistance to MSX disease to regress although there is no evidence to date that this has occurred.

1. Introduction

The concept of refuge has been the focus of many ecological and palaeoecological studies over several decades. Frequently, the term is used to describe a region where a species finds protection from some type of threat and serves as a genetic reservoir from which

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repopulation of a disturbed region can occur. Such threats or "disturbed environments" may be caused by short or long-term natural events such as droughts (Magalhaes *et al.*, 2002; Magoulick and Kobza, 2003), storms (Bongaerts *et al.*, 2010), or glaciation (Provan and Bennett, 2008; Vercken *et al.*, 2010). Anthropogenic pressures, particularly fishing, are often cited in arguing for the benefit of refuges (reserves) where the pressure is limited or relieved (Dugan and Davis, 1993; Guenette *et al.*, 1998). In a somewhat different vein, organisms may find protection from predation in refuges where the predator cannot survive (Covich *et al.*, 2009; Paterson and Whitfield, 2000; Vaughn and Fisher, 1988). The concept may even include refuges such as high or low temperature regimes that can protect species seasonally, as well as spatially (Moore *et al.*, 1996).

The idea of refuges has also entered the disease literature where it has been applied to sites that provide protection for (Cecere *et al.*, 2003; Lorenzo and Lazzari, 1999), as well as from (Caceres *et al.*, 2006; Glass *et al.*, 2007; Puschendorf *et al.*, 2009), pathogens and their vectors – and in the latter case, from disease outbreaks. For example, Springer (2009) demonstrated a 'pathogen refuge' evidenced by a reduction in rust disease in flax plants growing in a stressful, low-calcium soil environment. Caceres *et al*. (2006) examined the spatial and temporal differences in parasitic infection of *Daphnia dentifera* in a series of freshwater lakes, concluding that lake morphology was the primary predictor of variability, but that predation and weather might also be involved. Glass *et al*. (2007) used numerical modeling to predict sites that provided a refuge for the rodent vector of hanta virus, thus diminishing infection pressure on human hosts when the vector habitat became restricted. Using known spatial distributions of the amphibians infected with the chytrid fungus *Batrachochytrium dendrobatidis*, Puchendorf *et al*. (2009) also used models to predict areas, which seemed to be associated with temperature and rainfall, where amphibians might find refuge.

In estuarine systems, salinity is known to influence the distribution of species and may protect certain prey species that inhabit salinity regimes where a predator or competitor cannot survive (White and Wilson, 1996). Although the term "disease refuge" has been applied only recently to an aquatic environment (Hofmann *et al.*, 2009; Levinton *et al.*, 2011), low-salinity portions of estuaries have long been known to protect bivalve species such as oysters and clams from parasitic diseases (Andrews, 1983; Haskin and Ford, 1982; La Peyre *et al.*, 2003; Mackin, 1956; Ragone Calvo *et al.*, 1998).

Two of the most devastating diseases of an estuarine species affect the eastern oyster, *Crassostrea virginica*. They are MSX disease caused by the protistan *Haplosporidium nelsoni*, and dermo disease caused by the protistan *Perkinsus marinus*. *Haplosporidium nelsoni*, whose natural host is most likely the Pacific oyster, *C. gigas*, was introduced to the east coast of the United States (Burreson *et al.*, 2000). In *C. gigas*, the parasite causes no reported mortalities, but *C. viginica* is a highly susceptible host and suffered mass mortalities in the mid-Atlantic estuaries beginning in the late 1950s (Haskin and Andrews, 1988). *Perkinsus marinus* was for many years after its initial discovery in the Gulf of Mexico, restricted largely to the southern United States, but over several years beginning in the mid 1980s,

epizootic outbreaks occurred as far north as Cape Cod, Massachusetts (Ford, 1996). Only in the low-salinity portions of estuaries have oysters had any significant protection from these diseases (Andrews and Wood, 1967; Bushek *et al.*, this issue; Haskin and Ford, 1982; Levinton *et al.*, 2011; Mann *et al.*, 2009; Powell *et al.*, 2008). *Haplosporidium nelsoni* is particularly sensitive to salinity and although the low-salinity disease refuges enabled the persistence of relatively large numbers of oysters in the two most affected mid-Atlantic estuaries, Chesapeake Bay and Delaware Bay, they also impeded the development of resistance to MSX disease by the overall oyster population because they protected significant portions of that population from selective mortality (Carnegie and Burreson, 2011; Haskin and Ford, 1979). Not until a severe drought allowed *H. nelsoni* to invade the refuge of Delaware Bay, infecting and killing oysters over much of the upper bay, did the overall Delaware Bay oyster population develop substantial resistance to MSX disease (Ford and Bushek, this issue). *Perkinsus marinus* is more tolerant of low salinity than is *H. nelsoni* and infects oysters farther up estuary, but with little concrete evidence that resistance has developed (Bushek *et al.*, this issue).

In this paper, we report the results of an investigation to determine whether refuges from MSX disease still exist in the very upper-most reaches of Delaware Bay and its tributaries, and whether these potential refuges also protect oysters from dermo disease. We use the term "refuge," rather than "refugium," which in the context of conservation biology, refers to "an area of relatively unaltered climate that is inhabited by plants and animals during a period of continental climatic change (as a glaciation) and remains as a center of relict forms from which a new dispersion and speciation may take place after climatic readjustment" (http://www.merriam-webster.com/dictionary/refugium). Using these pathogens and diseases as examples, we also explore the question of how estuarine disease refuges are structured and maintained: is it because a parasite is unable to exist in the refuge, or is it present, but unable to develop infections and/or kill its host? Finally, we were interested in determining the temporal stability of refuges, in terms of both pathogen presence and its ability to develop infections. We used (1) polymerase chain reaction (PCR)-positive signals in gill samples to infer the presence of a parasite in the absence of detected infections, (2) traditional histological and culture methods to assess the ability of parasites to develop infections, and (3) infection intensity and mortality estimates to evaluate the selective pressure imposed in enzootic and putative refuge areas.

2. Methods

a. Oyster collection

Oysters were collected 6 times per year between April and November during 2007, 2008 and 2009 from 8 sites in Delaware Bay (Fig. 1, Table 1). Sampling times were chosen to reflect the high and low points in the annual prevalence cycles of the two pathogens in Delaware Bay and to span the seasons when they proliferate most rapidly in oysters (Bushek *et al.*, 1994; Ford and Haskin, 1982). Of the 8 sites, 5 were initially considered

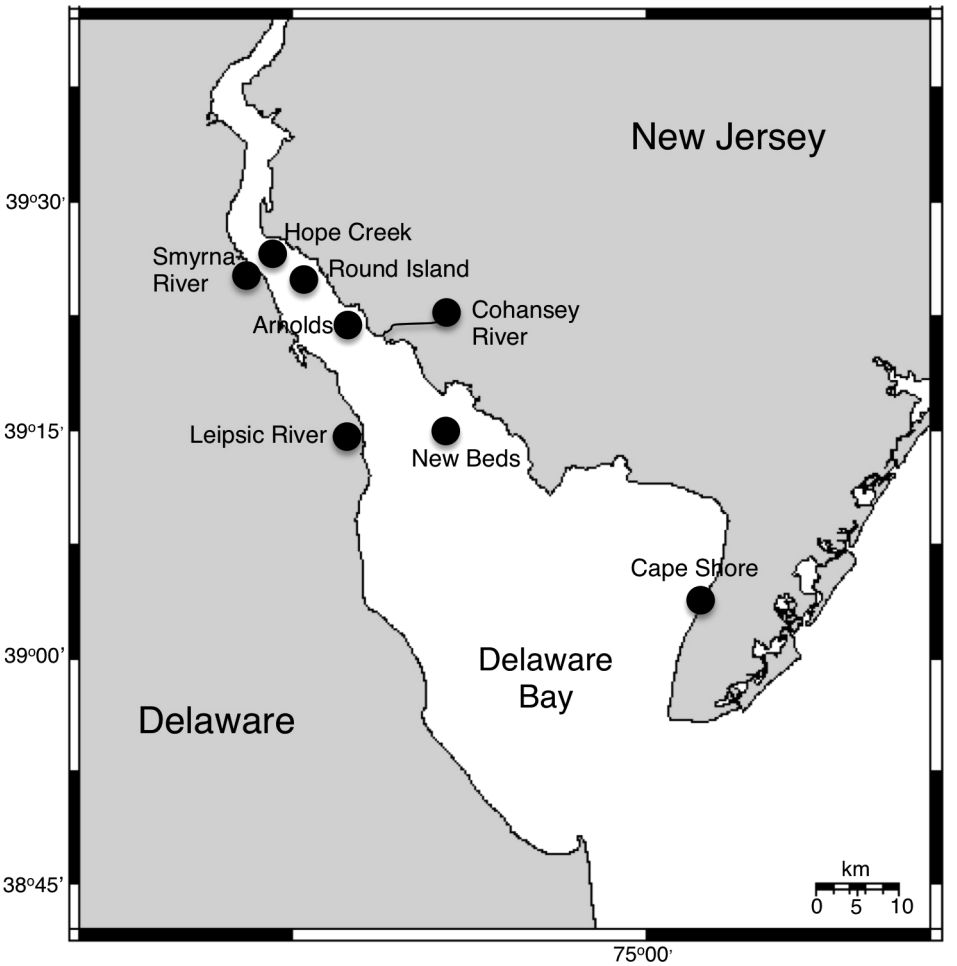

Figure 1. Delaware Bay chart showing locations where oysters were sampled to determine the presence of *Haplosporidium nelsoni* and *Perkinsus marinus* as determined from polymerase chain reaction (PCR), histology or culture methods.

to be potential disease refuges because of their low-salinity locations in (1) the very upper reaches of the main stem of the bay, or (2) the tributary rivers (Table 1). Three sites in the lower portion of the main stem were known to be sites where both *H. nelsoni* and *P. marinus* had been prevalent in previous years (Table 1). After the first year of sampling, it became clear that the Leipsic River, originally thought to be a likely refuge, was not. It was eliminated in favor of a site in the Smyrna River, 17.3 km upbay. At the same time, Arnolds Bed was discontinued as a sampling site for *H. nelsoni* and replaced by Hope Creek Bed, about 12.3 km upbay and the most upbay of the known oyster beds. Sampling for *P. marinus* at Arnolds Bed was continued for the duration of the study. All other sites were sampled

Table 1. Locations and number of samples collected for *Haplosporidium nelsoni* and *Perkinsus marinus* detection in the Delaware Bay main stem and tributaries, and time span sampled during the present study. Historical evidence of parasite prevalence is from long-term data sets (1958 to present for *H. nelsoni* and 1990 to present for *P. marinus*). (see Bushek *et al.*, this issue; Ford and Bushek, this issue).

	Present Study		Historical Levels		Site
Site Name	Time span sampled	N*	*H. nelsoni*	*P. marinus*	Description
Cape Shore (CS)	Apr 2007–Nov 2009	18	High	High	Intertidal reef
New Beds (NB)	Apr 2007–Nov 2009	18	Moderate	High	Commercial** oyster bed
Arnolds (AR)	Apr 2007–Nov 2007	6	Low	Moderate	Commercial oyster bed
Leipsic River (LR)	Apr 2007–Nov 2007	6	Unknown	Unknown	Scattered oysters near mouth
Round Island (RI)	Apr 2007–Nov 2009	18	Rare	Low	Commercial oyster bed
Hope Creek (HC)	Nov 2007–Nov 2009	13	Rare	Unknown	Commercial oyster bed
Cohansey River (CR)	Apr 2007–Nov 2009	18	Unknown	Unknown	Scattered oysters 8 km from mouth
Smyrna River (SR)	Nov 2007–Nov 2009	13	Unknown	Unknown	Scattered oysters 1.8 km from mouth

*Number of 20-oyster samples

**Commercial oyster beds are naturally occurring beds (sometimes called seed beds) that are managed for the commercial oyster fishery (see Powell *et al.*, 2008 for details)

for the entire study. In total, 2,580 oysters, in 131 samples, were collected and analyzed, including 380 (21 samples) assayed for *P. marinus* only at Arnolds Bed.

Oysters were obtained by dredging at all sites except the intertidal Cape Shore site (Fig. 1) where collections were made by hand. At each site, 22 to 25 live oysters (mean, SE shell height in mm = 74.5, 2.5; range = 46.2 to 88.0) were collected for disease analysis. At the river sites, where collections often required 6–8 dredge hauls, mortality was qualitatively estimated from the number of boxes (oysters without soft tissues, but with articulated shells) collected during the dredging for live oysters. Mortality was not assessed on the Cape Shore intertidal reef; however, in 2007 representatives of that year's set on the reef were placed in bags onsite and monitored for mortality through 2009. At commercial bed sites (Table 1), mortality was estimated from box and live oyster counts made during the fall oyster stock survey, which also provided an estimate of standing stock on the beds (Powell *et al.*, 2008).

b. Oyster processing

Oysters were opened with an oyster knife that was rinsed in 10% bleach and then tap water between oysters. A section of gill about 4 mm wide encompassing all four demibranchs and

approximately 6–7 mm posterior to the gill-palp junction was excised and preserved in 70% ethanol (EtOH) for PCR analysis (Ford *et al.*, 2011). Dissecting instruments were scraped clean in a bleach-sand mixture, dipped in EtOH and flamed between oysters. The rectum and a piece of mantle overlying the palps were then removed and placed in a tube containing 5 ml of Ray's fluid thioglycollate medium (RFTM) fortified with antibiotics (Ray, 1966). Dissecting tools were cleaned as above. Finally, a 5-mm section that encompassed the mantle, gill, and visceral mass was removed from each oyster and placed in a labeled cassette in Davidson's fixative (Shaw and Battle, 1957).

c. PCR assays

i. Tissue processing. The EtOH-fixed gill tissue from each oyster was minced and mixed. Dissecting instruments were cleaned as above. DNA was extracted from approximately 20 mg of the minced gill tissue using a Qiagen DNeasy® Blood & Tissue Kit according to manufacturer's directions. At least two randomly chosen tubes from each 20-oyster sample were analyzed on a GeneQuant Pro spectrophotometer (Amersham Biosciences). DNA concentrations averaged between 150 and 250 ng μl^{-1} and the 260/280 nm absorbance ratio was typically between 1.8 and 2.0.

ii. Haplosporidium nelsoni assay. The PCR assay for detection of genomic DNA from *Haplosporidium nelsoni* was modified from that published by Stokes *et al*. (1995) using the primers MSX A' (5′-CGA.CTT.TGG.CAT.TAG.GTT.TCA.GAC.C-3′) and MSX B (5′-ATG.TGT.TGG.TGA.CGC.TAA.CCG-3′) (Renault *et al.*, 2000). These primers amplify a 573-bp section of the small subunit SSU rRNA gene. One µl of extracted DNA was added to a master mix consisting of 2.5 µl of 10x PCR buffer II (Applied Biosystems), 1.5 µl $MgCl_2$ (1.5 mM final concentration), dNTPs (200 µM each), 25 pmol each of the primers, 0.6 units of AmpliTaq® DNA polymerase and enough nuclease-free water for a 25-µl total reaction volume. Cycling parameters were 2 min at 94°C for initial denaturation, then 35 cycles at 94°C for 30 s, 59°C for 30 s, and 72°C for 1.5 min for the denaturation, annealing, and extension steps, respectively, and a final elongation at 72°C for 5 min.

iii. Perkinsus marinus assay. The PCR assay of Audemard *et al*. (2004), as modified by Moss *et al*. (2006), was used to detect genomic DNA from *P. marinus* using the primers PmarITS-70F (5′-TTG.YTW.GAG.WGT.TGC.GAG.ATG-3′) and PmarITS-600R (5′-CGA.GTT.TGC.GAG.TAC.CTC.KAG.AG-3′). These primers amplify an approximately 509-bp section of the SSU rRNA gene. One µl of extracted DNA was added to a master mix consisting of 1.25 µl of 1 mg ml^{-1} bovine serum albumin (BSA), 2.5 µl of 200 mM Tris-HCl (pH 8.4), 2.5 µl of 500 mM KCl, 1.5 µl of 25 mM $MgCl_2$, dNTPs (200 µM each), 100 pmol each of the primers, 0.125 units of AmpliTaq® DNA polymerase, and enough nuclease-free water for a 25-µl total reaction volume. Cycling parameters were 4 min at 95°C for initial denaturation, then 40 cycles at 95°C for 1 min, 53°C for 1 min, and 65°C for 3 min for the denaturation, annealing, and elongation steps, respectively, and a final extension at 65°C for 10 min.

For both *H. nelsoni* and *P. marinus* assays, 10 µl of the amplification product was applied to a 2% agarose gel using SYBR® Green (5 µl in 40 ml of buffer, pH 8.0) to stain the product, which was then photo-documented as a digital image. Positive and negative controls were included in each PCR run and consisted of 24 µl of master mix with 1 µl of DNA extract from an *H. nelsoni* or *P. marinus*-infected oyster, or 1 µl of nuclease-free water, respectively.

Samples collected during the first 4 sample periods showed little difference between the PCR and RFTM assays for *P. marinus* (see Results). Therefore, the PCR assay was discontinued after August 2007 in favor of the RFTM method, which provides intensity, as well as prevalence data. Both histology and PCR assays were employed for *H. nelsoni* during the entire study.

d. Infection assays

The sections in Davidson's fixative were processed into tissue slides for detection of *H. nelsoni* infections and tissues in RFTM were assayed for *P. marinus* using standard procedures (Howard *et al.*, 2004; Ray, 1966). Infection intensities of *H. nelsoni* were scored according to whether they were localized or dispersed as well as by the relative numbers of parasites – rare to heavy (Ford, 1986). Infection intensities of *P. marinus* were scored on a scale from 0 to 5 (Ray, 1954). A mean intensity, which included only oysters in which parasites were detected, was then calculated for each sample. Infections were categorized as advanced or lethal based on previous studies relating intensity to gametogenesis (Dittman *et al.*, 2001; Ford and Figueras, 1988) and mortality (Bushek *et al.*, this issue; Ford and Haskin, 1982).

e. Environmental parameters

Surface water was sampled with a bucket and near-bottom water was collected using a Niskin-type bottle. Salinity and temperature were recorded using a YSI model 85 meter. Because salinity was measured only monthly at best, additional insight into salinity fluctuations was obtained from examining river flow data. Delaware River flow, measured at Trenton, New Jersey, and Cohansey River flow measured at Seeley, New Jersey were obtained from http://waterdata.usgs.gov/nj/nwis/. Delaware River flow is highly correlated with bay salinity (Wang *et al.*, this issue). No river flow data are available for the Smyrna River, so data for the St. Jones River, gauged at Dover, Delaware, about 17 km south, were used as a proxy.

3. Results

a. Temperature and salinity

Water temperature varied only slightly among sites visited on the same day (data not shown), a result consistent with both long-term records (Fegley *et al.*, 1994) and simulations of Delaware Bay water properties (Wang *et al.*, this issue). Salinity followed a seasonal pattern, with peaks in the fall and lows in the spring and early summer (Fig. 2). During 2008 and 2009, when salinity was measured at all sites except the Leipsic River, the two

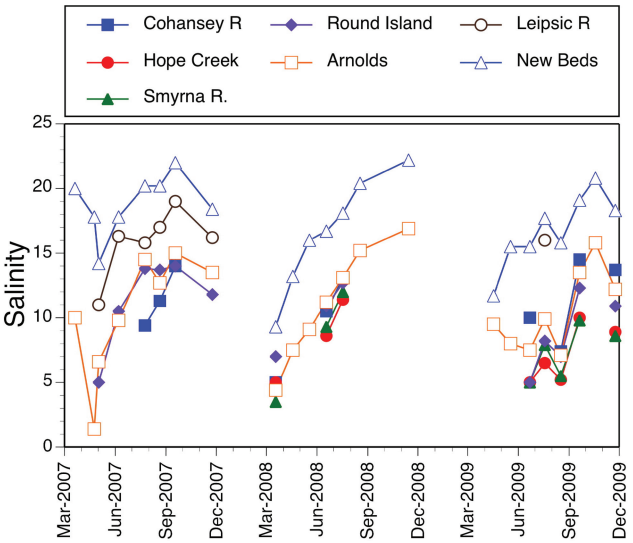

Figure 2. Seasonal bottom water salinity values by sampling site during the study period, 2007–2009. Samples were not collected during the winter.

Table 2. Salinity means and extremes measured on collection dates at sample locations. All means, except that for Leipsic River, were calculated from measurements made at the different sites on the same day or within a day of each other, in 2008 and 2009 (N = 9 dates). The Leipsic River salinity was measured in 2007 only. In that year, mean salinity at that site was halfway between those of Arnolds and New Beds (N = 4 dates) and was estimated to fall halfway between the values for those sites in 2008/2009. Minimum and maximum values span the study period from April 2007 to November 2009. Values are not corrected for stage of tide.

	Salinity		
Location	Mean	Minimum	Maximum
Hope Creek	8.2	5.0	11.4
Smyrna River	8.3	3.5	12.0
Round Island	9.9	5.0	13.8
Arnolds	10.4	4.4	16.9
Cohansey River	10.9	5.0	14.5
Leipsic River	~13	11.0	19.0
New Beds	16.4	11.7	22.0

most upbay sites, Hope Creek and Smyrna River, had the lowest mean values at slightly more than 8; Round Island, Arnolds and Cohansey River were between 10 and 11, and New Beds was somewhat greater than 16. The Leipsic River was estimated to be about 13 (Fig. 2, Table 2). The minimum values recorded at all sites except the Leipsic River and New Beds were 5 or less. Maximum values ranged from 11 to 12 at Hope Creek and Smyrna River to

19 to 22 at Leipsic River and New Beds (Table 2). Salinity at the Cape Shore site is typically between 20 and 23 (Haskin Shellfish Research Laboratory, Cape Shore Hatchery records).

Time-line plots of streamflows for the Delaware, Cohansey and St. Jones Rivers showed somewhat different patterns. All flows spiked during the late winter and spring of 2007, but during most of the rest of the study period, only the Delaware River experienced above average flows, primarily from fall 2007 through spring 2008, with smaller spikes later in 2008 and 2009 (Fig. 3A). After the early 2007 spike, flows measured for the Cohansey and St. Jones rivers were slightly below the long-term means until August 2009, when they increased dramatically (Fig. 3B&C).

b. Haplosporidium nelsoni (MSX)

We inferred the presence or absence of *H. nelsoni* at a site and time from the combined results of the PCR and histology assays. Of the 110 20-oyster samples analyzed for *H. nelsoni* by both PCR and histology, all oysters in 45 (41%) were negative by both assays, which we interpreted as pathogen absence (Table 3). In 38 (35%) samples, at least one oyster was positive by PCR and at least one oyster, possibly the same individual, was positive by histology; in another 24 (22%) samples, at least one oyster was positive by PCR, but none by histology; and in 3 (3%) samples at least one oyster was positive by histology (all infections were rare or very light epithelial lesions), but all were negative by PCR (Table 3). All of the last three categories indicated pathogen presence at the site. Thus, the PCR assay results were highly correlated with the presence or absence of *H. nelsoni* in histological sections, but the assay was also able to detect pathogen DNA in the absence of histologically detected infections.

Temporal variation of *H. nelsoni* infections observed in histological sections was modest. Prevalences were never more than 25% and were generally below 15% (Fig. 4). Spatial variation was more pronounced, especially in 2007 and 2009 when samples with few or no detectable infections predominated in oysters from the putative refuges: Round Island, Hope Creek, and the Smyrna and Cohansey rivers. Most infections were rare to light and localized in the gill. The only infections classified as systemic were found in oysters from Cape Shore, New Beds and the Leipsic River, and only one of these, at New Beds in August 2008, was intense enough to be classified as moderate.

Compared to its occurrence as visible infections in oysters, the presence of *H. nelsoni*, as inferred from PCR-positive signals in gill samples, varied considerably according to the time of sampling. In addition to peaks, which reached 40 to 65%, in the November 2008 to March/April 2009 samples, high prevalences were recorded in July of 2007 in the Cape Shore, New Beds and Leipsic River samples, and in the July and August 2009 samples from New Beds (Fig. 4). Spatial variation was also pronounced. From April 2007 through August 2008, PCR-positive samples (those in which at least one oyster was positive) were restricted to the main stem of the bay as far upbay as Arnolds Bed (Fig. 5A). In September 2008, the distribution of PCR-positive samples expanded rapidly to include all sampled

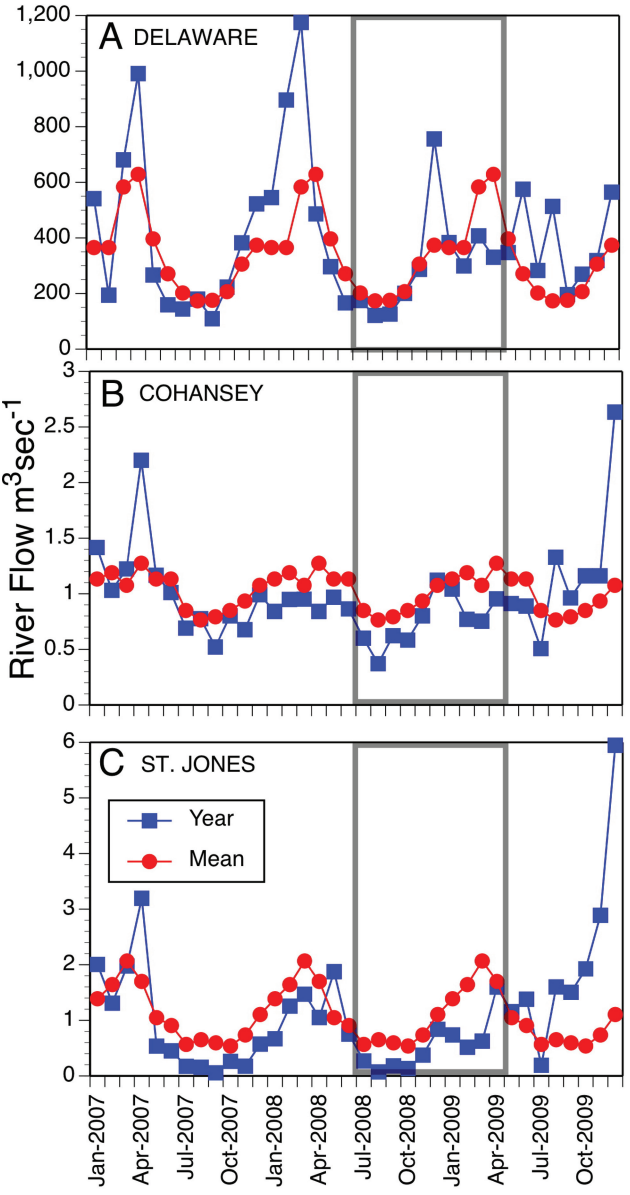

Figure 3. Stream flows in $m^3\ sec^{-1}$ for (A) the Delaware River at Trenton, New Jersey; (B) the Cohansey River at Seeley, New Jersey; and (C) the St. Jones River at Dover, Delaware. Samples were not collected in the St. Jones River, but it was used as a proxy for flow in the adjacent Smyrna River, which was sampled. Mean river flow refers to the entire record (Delaware: 1913–2009; Cohansey: 1977–2009; St. Jones: 1958–2009). Year refers to the year in the X-axis. The outlined regions represent the period during which the upstream refuges from *Haplosporidium nelsoni* disappeared.

Table 3. *Haplosporidium nelsoni*. Comparison of PCR with histology assay results according to sampling site (all dates pooled) in Delaware Bay and its tributaries. Percentages in parentheses are based on the total number of 20-oyster samples collected during the 2007–2009 study period. An assay result was considered positive if at least one oyster in the sample was recorded as positive. An assay result was considered negative if all 20 oysters in the sample were recorded as negative.

	Samples				
Location	Total	PCR+/ Histo+	PCR+/ Histo−	PCR−/ Histo+	PCR−/ Histo−
Hope Creek (HC)	13	2 (15%)	2 (15%)	1 (7%)	8 (62%)
Smyrna River (SR)	13	2 (15%)	2 (15%)	0	9 (50%)
Round Island (RI)	18	3 (17%)	4 (22%)	2 (11%)	9 (50%)
Arnolds (AR)	6	3 (50%)	1 (16%)	0	2 (33%)
Cohansey River (CR)	18	1 (5%)	3 (17%)	0	14 (78%)
Leipsic River (LR)	6	4 (67%)	0	0	2 (33%)
New Beds (NB)	18	13 (72%)	5 (28%)	0	0
Cape Shore (CS)	18	10 (56%)	7 (39%)	0	1 (6%)
Total	110	38 (35%)	24 (22%)	3 (3%)	45 (41%)

sites and this range persisted through April of 2009 (Fig. 5B). In these samples, prevalences reached as high as 50 to 65%, even in upbay and upriver sites where the PCR assay had earlier produced mostly negative results (Fig. 4). After April 2009, the range contracted again to include the only sites that were positive before September 2008 (Fig. 5C).

Overall, the prevalence of histologically detectable infections followed the PCR prevalence, but with variation in the ratio of PCR to histology that varied with site and collection date (Figs. 4, 6). The fraction of samples that were positive by both PCR and histology generally decreased in an upbay and up river direction and the fraction of samples that were negative by both, increased (Table 3). The ratio of the overall PCR to histology prevalence for all samples varied from 1.6 at the Leipsic River to 4.3 at the Cohansey River and to 10.5 in the Smyrna River (Fig. 6), with a slight trend towards higher values in the upstream regions.

c. Perkinsus marinus (dermo)

The prevalence of *P. marinus*, as determined by PCR or RFTM assays, and usually both, was low or zero at the first sampling in April 2007 and increased to reach peaks of ∼80–100% by August at all sampled sites except Round Island Bed and the Cohansey River (Fig. 7). Among the 24 20-oyster samples collected from 6 sites during this period, 19 were positive by both assays (i.e., at least one oyster was positive by each assay), oysters in 3 samples were entirely negative by both assays, while the remaining 2 samples were positive by PCR (at least one oyster was positive) and negative by RFTM. In contrast to the

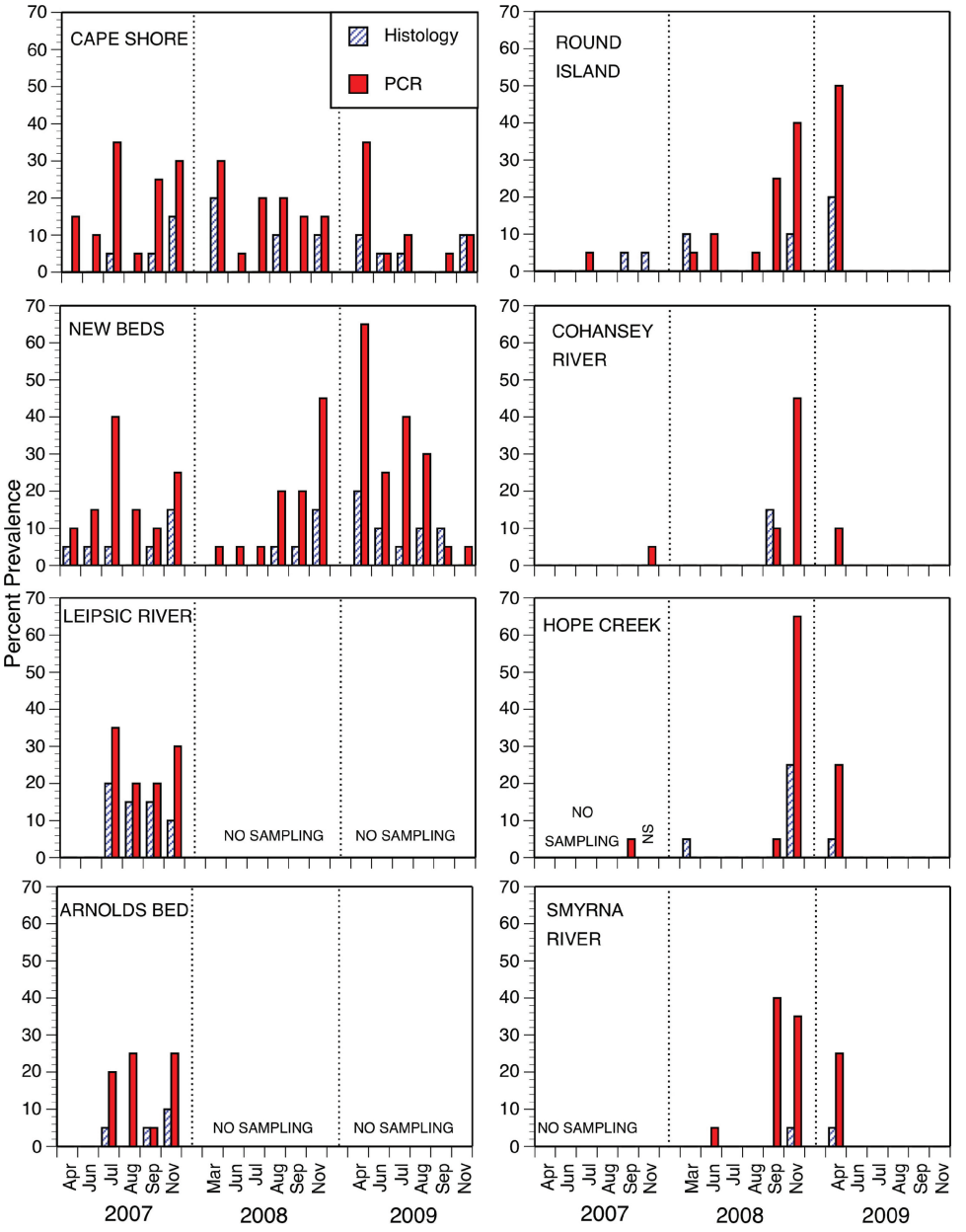

Figure 4. Time-line prevalence of *Haplosporidium nelsoni* detected as PCR-positive signals and as infections observed in tissue sections, at sampling sites in Delaware Bay and tributaries during 2007–2009. Dotted lines demarcate years. Unless designated by "no sampling" or "ns," absence of bar represents a negative sample for that assay.

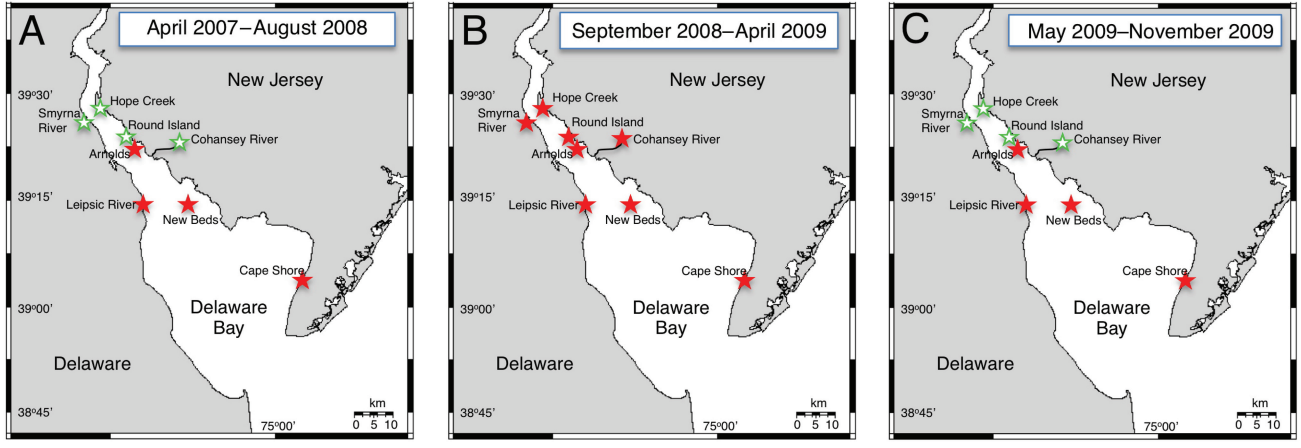

Figure 5. Delaware Bay chart showing the transient nature of refuges from *Haplosporidium nelsoni*, as inferred by the presence or absence of PCR-positive signals. (A) April 2007 to August 2008; (B) September 2008 to April 2009; (C) May 2009 to November 2009. Filled (red) stars represent sites where PCR-positive oysters were persistent and empty (green) stars, where PCR-negative oysters predominated during segments of the study period.

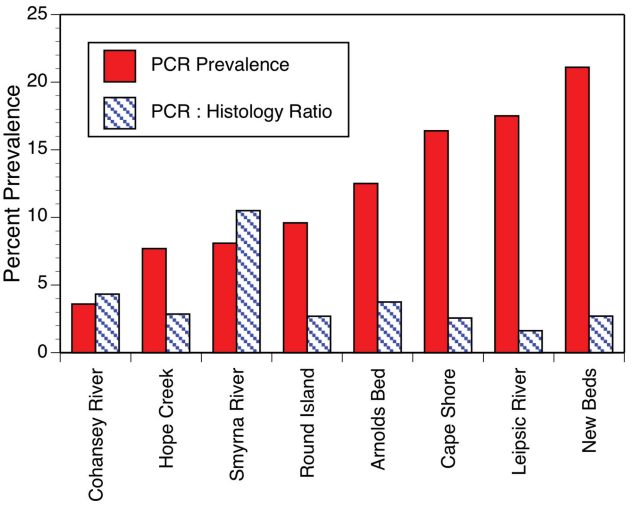

Figure 6. Mean prevalence of all PCR-positive signals from oyster gill, and the ratio of this value to the prevalence of all histologically detected infections of *Haplosporidium nelsoni* at Delaware Bay sampling sites during the study period (2007–2009).

results for *H. nelsoni*, the PCR prevalence of *P. marinus* in positive samples was higher than that obtained by RFTM in only slightly more than half the samples. More importantly, the average prevalence difference between the two methods was only 10 percentage points or less except at the Cohansey River site, where the PCR prevalence was 28 percentage points higher than the RFTM prevalence. Because there was so little overall difference between the two methods, we eliminated the PCR assay for *P. marinus* for the rest of the study in favor of the RFTM assay, which provides an estimate of infection intensity as well as prevalence.

At all sites, *P. marinus* prevalence and intensity followed the typical pattern of increasing from a spring low to a late summer peak (Fig. 7). In the region from the Cape Shore to Arnolds Bed and including the Leipsic River, prevalence remained high through the fall, whereas it declined in the more upbay and upriver sites. Peak prevalence was ∼80% or higher at all sites except the Smyrna River, where it did not exceed 25%. Infection intensity followed prevalence and was consistently higher downbay (Figs. 7, 8), where mean intensity approached or exceeded 3 each year. At upstream sites - Hope Creek and Round Island, and the Smyrna River and Cohansey Rivers - intensity varied considerably and did not always follow the prevalence pattern (Fig. 7). At times only a small fraction of oysters were infected at these sites, but the infections could be heavy in those individuals. At these sites, the mean prevalence of infections in the most advanced stages, 4 and 5, ranged from 0% at the Smyrna River to 6% at Round Island (Fig. 8). Most advanced and lethal infections occurred during the late summer (August and September) when dermo disease-caused mortality predominates.

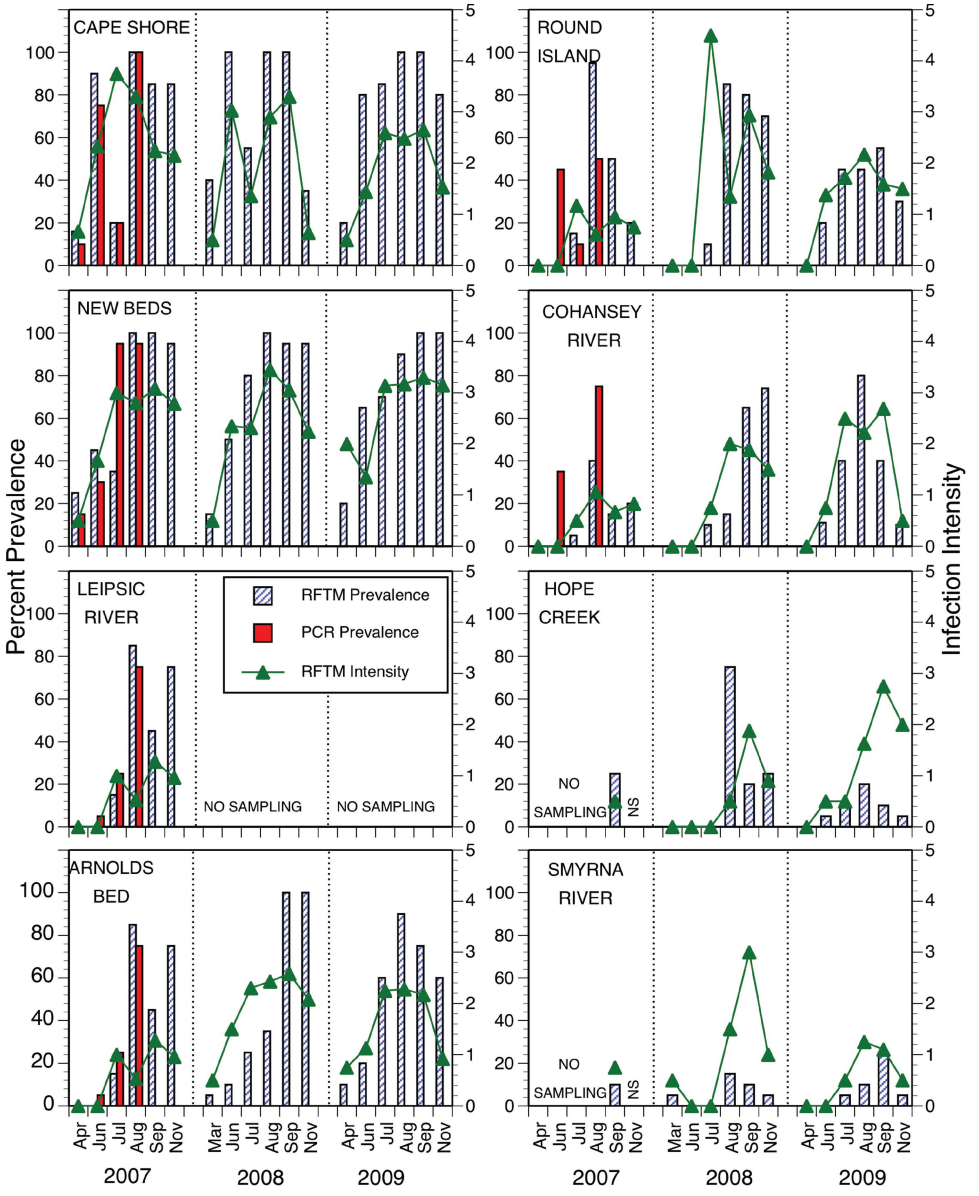

Figure 7. Time-line of *Perkinsus marinus* presence detected as PCR-positive signals from oyster gill (discontinued after August 2007) and as infections (prevalence and intensity) observed after culture of rectal and mantle tissue samples in Ray's fluid thioglycollate medium, at sampling sites in Delaware Bay and tributaries during 2007–2009. Infection intensity was rated on a scale of 0.5 to 5 and includes infected oysters only. Dotted lines demarcate years. Unless designated by "no sampling" or "ns", absence of symbol or bar represents a negative sample for that assay.

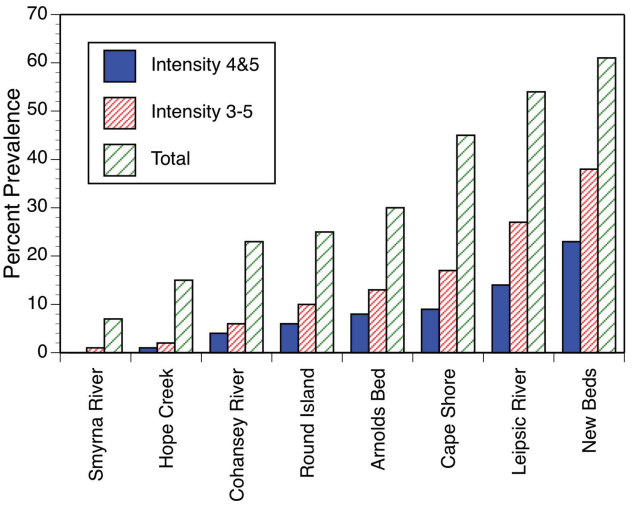

Figure 8. Mean total prevalence and mean prevalence of advanced (stages 3 to 5) and lethal (stages 4 and 5) infections of *Perkinsus marinus*, determined using Ray's fluid thioglycollate medium assay at Delaware Bay sampling sites during the study period (2007–2009).

d. Co-occurrence of the two parasites

The presence of the two parasites at the sampled sites (determined by PCR and histology for *H. nelsoni* and RFTM for *P. marinus* and averaged over the study period) was highly correlated ($p < 0.00001, r^2 = 0.99$), although the prevalence of *H. nelsoni* was only 1/3 that of *P. marinus* (Fig. 9).

e. Oyster mortality

Estimated annual mortality on the four commercial oyster beds sampled in the present study was at or below the mean for the period since dermo disease became enzootic in Delaware Bay (1990–2009) (Table 4). The three most upbay beds (Hope Creek, Round Island and Arnolds) had mortality that was below "background" levels (about 10%), having little or no association with MSX or dermo diseases, except for Arnolds Bed in 2009, when mortality was estimated to be 16%. The 36 to 43% mortality estimated for New Beds was clearly associated with dermo disease (see Fig. 6). Mortality was not assessed on the Cape Shore reef; however, spat collected from the site in late 2007 and placed in replicate bags suffered annual mortalities of 54 to 60% that was also associated with dermo disease (Table 4, Fig. 6). Mortality was not quantified in the rivers; however, no more than 2–3 boxes (dead oysters without soft tissues, but with articulated valves) were typically found during the collection of 20–25 live oysters. Given that valves remain attached for approximately a year in Delaware Bay (Ford *et al.*, 2006), mortality in the creeks could be estimated at about 10% annually.

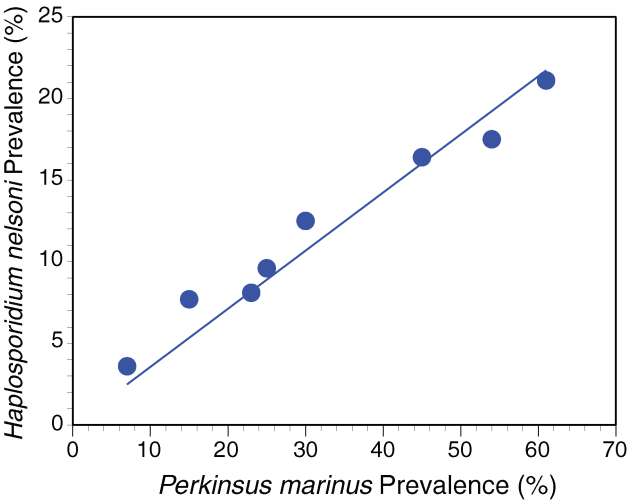

Figure 9. Spatial distribution (=presence) of two parasites of eastern oysters in Delaware Bay (r^2 = 0.99). Symbols represent the mean prevalence of each parasite at each of the 8 sites sampled during the study. Prevalence of *Perkinsus marinus* was determined using Ray's fluid thioglycollate medium analysis of mantle and rectal tissue; prevalence of *Haplosporidium nelsoni* was inferred from PCR analysis of gill tissue and tissue-section histology.

Table 4. Annual percent mortality estimated from total box counts obtained during the fall survey of 4 commercial oyster beds sampled in the present study. No long-term mean (SE) is provided for Hope Creek, which was not sampled before 2007. Mortality at the Cape Shore site was obtained from 2007-year class spat placed in bags onsite and monitored through 2009. N/A = not available.

	Percent Mortality			
Location	2007	2008	2009	1990–2009
Hope Creek	5	3	6	N/A
Round Island	7	6	10	10.9 (1.2)
Arnolds	7	9	16	11.5 (1.1)
New Beds	36	43	41	39.3 (3.0)
Cape Shore		54	60	N/A

4. Discussion

a. Disease refuges

Although the refuge concept has long been employed in ecological and paleoecological contexts, it has only recently been used to identify regions where organisms are protected from diseases (Caceres *et al.*, 2006; Glass *et al.*, 2007; Puschendorf *et al.*, 2009). To our knowledge, the term "disease refuge" was first employed for a marine ecosystem by Hofmann *et al.* (2009). The term was introduced in the context of local environmental

constraints on the two oyster pathogens that are the subject of the present study and how such refuges might contribute to the development of resistance to the pathogens and to the genetic structuring of the affected oyster population.

When considering whether to call an area a "refuge from disease" and especially when the object is to assess selection pressure that might result in resistance to disease, it is important to stress that the presence of a pathogen, or even infection, does not always equate with disease, which we define as damage to an organism that impairs the performance of its vital functions, including growth and reproduction. In an early study, Andrews and Wood (1967) classified oyster-growing regions of lower Chesapeake Bay according to whether *Haplosporidium nelsoni* was detected in histological sections and whether it caused mortality. The advent of molecular detection assays now permits us to detect the presence of a parasite that may not cause an observable infection. Thus, we can consider disease refuges to exist as a continuum of zones: (1) pathogen is not present; (2) pathogen is present, but does not cause observable infections; and (3) pathogen causes infection, but neither disease nor mortality. The endpoint of this continuum is where the refuge no longer exists and where the pathogen exerts selective pressure by causing disease severe enough to reduce fecundity or cause death, or both. Understanding where a pathogen exerts selective pressure along this continuum and where that pressure occurs along the distribution of the host species is critical to understanding the response of the host population.

In order to describe refuges from the diseases caused by oyster pathogens in Delaware Bay, we examined each of these possible conditions using (1) PCR to detect parasite presence in the absence of detectable infections, (2) standard histological or culture methods to demonstrate infections, (3) infection intensity to assess the likelihood of reproductive impairment and (4) mortality rates to further assess the strength of selective pressure. Our results show that each of these refuge zones do exist in the estuary, primarily for *H. nelsoni* (see Table 3, Figs. 4 and 5), the agent of MSX disease and to a lesser extent for *Perkinsus marinus*, which causes dermo disease. Furthermore, our results indicate that refuges can be temporally and spatially transient. In the case of MSX disease, the pathogen *H. nelsoni* was absent from upbay and upriver refuges (Cohansey and Smyrna Rivers, Hope Creek and Round Island Beds) most of the time during our study, as inferred from the lack of PCR-positive signals on the gills, but pulses of presumed infective particles appeared to penetrate the refuges at certain times of low river flow (Wang *et al.*, this issue). Even when this occurred, however, histologically detected infections remained rare, localized, non lethal and not even severe enough to impair reproduction (Barber *et al.*, 1988; Ford and Figueras, 1988).

Long-term data indicates that a high degree of resistance to MSX disease has developed in oysters from much of Delaware Bay (Ford and Bushek, this issue). It is consequently legitimate to ask whether the scarcity of histologically detectable infections in the refuges during these incursions of infective particles might be due to resistance rather than to environmental conditions that inhibit the development of infections. Common-garden

deployments at a site where they were exposed to *H. nelsoni*, of adult oysters and their hatchery-reared offspring from three sites (Cohansey River, Smyrna River and Hope Creek) that we have identified as refuges from MSX disease, show that, although they are more resistant than naïve stocks from outside the estuary, they still develop more and heavier infections, and die at a significantly higher rate, than oysters from the lower bay (Hofmann *et al.*, 2009), indicating that resistance alone cannot explain the failure of significant infections to develop in these regions of the estuary. Similarly, Caceres *et al.* (2006) were able to eliminate resistance of *Daphnia dentifera*, as a cause of variability in parasitic infection among lakes in southern Michigan, USA, because they could easily infect the host in laboratory trials.

Only two upbay sites, the Smyrna River and Hope Creek Bed, could be considered dermo disease refuges during the three years of our study. Although we found *P. marinus* infections at both sites, the mean prevalence of infections advanced enough to significantly impair reproduction or cause mortality (stages 4 and 5 in which parasites are found in moderate to heavy numbers in all tissues examined) (Dittman *et al.*, 2001) was 0 to 1%. The mean prevalence of stages 4 and 5 at the other sampled sites ranged from 4% in the Cohansey River to 23% at New Beds (Fig. 8). Even at the Cohansey River and Round Island sites, where 4% and 8% of oysters had potentially lethal infections, the total annual mortality rate was estimated at 10% or less, most of which can be considered "background" mortality, not associated with either disease (Bushek *et al.*, this issue). Even if it were, numerical simulations of the time required to develop resistance to dermo disease indicate that an annual selective mortality rate of 10% or less would be "profoundly slow" (Powell *et al.*, 2011b). Whether a catastrophic mortality associated with dermo disease, comparable to that which led to MSX disease resistance in the oyster population (Ford and Bushek, this issue) would have a similar outcome is unknown.

b. What are the mechanisms maintaining refuges?

Springer (2009) hypothesized that the fitness costs for organisms living in stressful environments might be counter-balanced by "reductions in…antagonistic species interactions," specifically with pathogens. Thus, he proposed, refuge from pathogens was a tradeoff for living in a stressful environment. In a study involving 13 flax species, Springer (2009), showed that low-calcium (stressful) soils were associated with lower prevalence of a pathogenic rust fungus than were higher calcium soils. He postulated several mechanisms to explain how a stressful environment might create a refuge from the rust pathogen. Effects of the environment on the host could include (1) smaller, less dense flax that would reduce transmission or (2) greater investment in defenses against the pathogen because of the high cost of replacing or repairing tissue. Alternatively, the pathogen itself might be stressed and become less virulent.

In many estuaries, growth rates and soft-tissue condition of oysters decrease along the decreasing salinity gradient (Kraeuter *et al.*, 2007), implying that low-salinity, or some

factor associated with it such as poor food, high turbidity and extremes associated with high freshwater runoff provides a stressful environment for oysters. In the case of the oyster-parasite relationship, however, low-salinity stress on the pathogens, rather than on the host, is the mechanism most consistent with available data. Salinity has long been correlated with the distribution of *H. nelsoni* and *P. marinus* infections in estuaries (Andrews and Wood, 1967; Burreson and Ragone Calvo, 1996; Haskin and Ford, 1982), and both *in vivo* and *in vitro* studies have demonstrated the sensitivity of the pathogens to low salinity (Burreson *et al.*, 1994; Chu *et al.*, 1993; Ford, 1985; Ford and Haskin, 1988). Histologically detectable *H. nelsoni* infections are rare at salinities below 10 and associated mortalities are rare at <15 (Andrews, 1983; Haskin and Ford, 1982). In contrast, *P. marinus* can infect and proliferate in oysters at salinities as low as 3 (Chu *et al.*, 1993), but has reduced virulence at salinities below about 10 (Ragone and Burreson, 1993). Thus salinity per se may be the only parameter needed to explain the existence of refuges in the upper bay and tributaries. On the other hand, dilution of infective stages, as suggested by Mackin (1956) and documented by Ellin (2000) for *P. marinus* may also play a role, particularly when freshwater runoff is high. Interestingly, the presence of the two parasites at the 8 sites sampled, as inferred from a combination of PCR signals and histological or culture methods was highly correlated. This result is unlikely to be caused by an interaction of the parasites within individual oysters; rather it suggests a common mechanism influencing the distribution of infective stages.

Arguing against low salinity as the sole mechanism maintaining refuges, at least from MSX disease, is that except for the slightly below average river flows in the two tributary rivers (St. Jones and Cohansey) during the late summer of 2008 when the first pulse of presumed *H. nelsoni* infective particles was observed in the refuges, there is not much evidence of either high salinity or exceptionally low river flow that could have facilitated such a marked pulse. Stream flows at that time, in fact, were not greatly different from 2007 when the refuges existed. Circulation modeling indicates that upbay transport of passive particles in 2008 was correlated with the pulse of *H. nelsoni* (histologically detected infections and PCR signals combined) in the upper bay refuges (Wang *et al.*, this issue). The absence of a similar pulse of *P. marinus* infections may be related to its persistence in oysters at low salinity. Acquisition of new infective particles would be masked by redevelopment of previously established infections under the influence of seasonal temperature increases. The probability that *H. nelsoni* requires an alternate or intermediate host to complete its life cycle (Haskin and Andrews, 1988) introduces an element that could well influence distributional patterns of this pathogen, but inclusion of this possibility into the *H. nelsoni* distributional model must wait until this putative organism(s) is identified.

c. Implications for selection for disease resistance

In addition to the variability in selection pressure on fecundity and survival that we have discussed, an important element in the overall effect of disease on the possible development

of resistance in an estuarine population is the relative sizes of the subpopulations that are affected by, or protected from, selection. An analogous example of genetic refuge and its impact on the acquisition of resistance by a wild population is the use of refuges to retard the development of pesticide resistance by crop-destroying insects (Carriere *et al.*, 2004; Gould, 1998). Resistance can develop very rapidly in insect pests that feed on crops sprayed with insecticide or that have been transgenically modified to contain a gene or genes responsible for producing toxin(s) that can kill more than 95% of the pests. Refuges, in this case, consist of fields that are not sprayed or are planted with non-modified crops, and are adjacent to the treated fields. The refuge delays the development of resistance by the pests because susceptible insects persist in the refuge and breed with the resistant survivors in the treated field, creating "hybrids" that are only intermediate in resistance – or may be totally susceptible if resistance is a recessive trait (Gould, 1998). The delay depends, among other things, on the strength of selection (toxin lethality), the relative fitness of the resistant and susceptible insects and their intermediates, the number of genes involved and the relative size of the refuge and its population (Gould, 1998). Numerical modeling indicates that the same factors are at play in the development of disease resistance by oysters (Powell *et al.*, 2011b). A major difference, however, is the transient nature of estuarine refuges, driven only partly by short-term climatic conditions. Temporary elimination of an estuarine disease refuge is unlikely to have an impact on the genetic structure of the resident hosts unless it results in a catastrophic event, such as the widespread and severe drought-associated *H. nelsoni* epizootic that caused extensive selective mortality in the upper Delaware Bay in 1985–86 (Ford and Bushek, this issue). The presence of refuges from selection likely helps to retard the development of resistance to dermo disease and theoretically could cause resistance to MSX disease to regress, although there is no evidence to date that this has occurred.

We do not know the abundance of oysters in the creeks and rivers flowing into Delaware Bay, although the number of dredge hauls that it typically took to find enough oysters for the samples suggests that they are not great. On the other hand, annual stock surveys by Rutgers University Haskin Shellfish Research Laboratory (Powell *et al.*, 2011a) on the commercial oyster beds show that over the past several years (2007–2010), nearly 40% of the estimated standing stock of oysters on the natural beds on New Jersey side of Delaware Bay is contained within the area where the population suffered minimal or no deleterious impacts of either parasite during our study – even though the parasites were present at least part of the time. The projected impact of climate change will likely push salt (and the parasites) farther into the estuary as sea level rises and summer droughts intensify, but increased seasonal precipitation and streamflow may counteract saltwater ingress to some extent (Najar *et al.*, 2000). The potential impact of these changes on disease resistance and the oyster population of Delaware Bay is currently being investigated through a series of climate change simulations (Wang *et al.*, this issue), and may ultimately depend upon the ability of oysters to find refuges further up the estuary.

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