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INVESTIGATING THE LIFE CYCLE OF *HAPLOSPORIDIUM NELSONI* (MSX): A REVIEW

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ABSTRACT Attempts to decipher the life cycle of *Haplosporidium nelsoni* began almost immediately after it was identified as the pathogen causing MSX disease in eastern oysters, *Crassostrea virginica*. But transmission experiments failed and the spore stage, characteristic of haplosporidans, was extremely rare. Researchers concluded that another host was involved: an intermediate host in which part of the life cycle was produced, or—if the oyster was an accidental host—an alternate host that produces infective elements. A later finding that spores were found more often in spat (<1 y old) than in adults revived the idea of direct transmission between oysters. The new findings and the availability of molecular diagnostics led us to revive life cycle investigations. Over several years, oyster spat were examined for spores and searched for *H. nelsoni* in potential non-oyster hosts using both histological and polymerase chain reaction (PCR) methodologies. Although spores occurred in a high proportion of spat with advanced infections, it was concluded that they were unlikely to be a principal source of infective elements because naïve oysters used as sentinels to assess infection pressure became highly infected even after native oysters developed resistance, and infected spat could no longer be found. A histological survey of zooplankton and small bivalves in Delaware Bay found few recognizable parasites and nothing resembling a haplosporidan. A subsequent PCR study of water, sediment, and macroinvertebrates from Chesapeake, Delaware, and Oyster bays resulted in many positive samples, but *in situ* hybridization failed to identify any recognizable structures. PCR analysis of potential intermediate hosts for other molluscan pathogens has also resulted in many species yielding positive results but required *in situ* hybridization to verify infections. It is suggested that any future search for a nonoyster host of *H. nelsoni* be conducted in a relatively confined system and/or target specific phyla, strategies that have been successful in other life cycle studies. It is noted that candidate phyla could include those known to host haplosporidans and species whose abundance or distribution may have changed in concert with outbreaks of MSX disease in the northeastern United States in recent years.

KEY WORDS: parasite, transmission, host, spore, oyster, bivalve, histology, DNA, PCR, *Haplosporidium nelsoni*, marine disease

INTRODUCTION

In January 1959, a group of researchers met at Rutgers University in New Brunswick, NJ, to discuss a new and alarming subject. Catastrophic mortality had swept through the Delaware Bay oyster (*Crassostrea virginica*) beds during the preceding 2 y. A hitherto unknown parasite, ''Organism X,'' had been found the previous spring in the tissues of dead and dying oysters and the researchers were anxious to know more about it—and specifically about the likelihood that other oyster-producing regions along the coast would be affected. Transmission experiments initiated by Rutgers in the summer of 1958 showed no difference in mortality of presumed uninfected oysters regardless of whether they were mixed with infected individuals.

Oyster mortality conferences, as they came to be known, were held annually over the next decade at Rutgers, the Virginia Institute of Marine Science (VIMS), the Cooperative Oxford Laboratory (Maryland), and other locations. By the 1960 meeting, the organism had been provisionally identified by its plasmodial stage as a haplosporidan, but the lack of a spore stage delayed species designation and the parasite came to be known as MSX for ''Multinucleated sphere unknown.'' In 1966, the organism was officially described and named *Minchinia nelsoni* (Haskin et al. 1966). By that time, the spore stage had been found in a small number of oysters and was linked to the plasmodial stage by fluorescent antibody methodology (Barrow & Taylor 1966, Couch et al. 1966). Many years later, and after a change of genus to *Haplosporidium*, the parasite was determined to have been introduced from the Asia-Pacific region, where it infects the Pacific oyster *Crassostrea gigas* (Burreson et al. 2000).

During the first few years after it was discovered, reports of the mortality conferences mention attempts to transmit the parasite by proximity, feeding, injection, and tissue transplantation (http://hsrl.rutgers.edu/HSRL%20documents/MortalityConferences/index.MortalityConf.htm). Nothing was successful, and by the 1962 meeting it seemed everyone had concluded that the parasite was not directly transmissible and that another host must be involved. A presentation that Victor Sprague of the Chesapeake Biological Laboratory at the University of Maryland prepared for the 1962 Mortality Conference listed 28 organisms that his laboratory had screened as possible hosts without finding evidence of a haplosporidan (Sprague 1962). He also noted failure of ''crude'' proximity experiments using organisms from areas in which oysters became infected with the MSX organism. Two articles and one abstract are all that was published of those early transmission trials (Canzonier 1968, 1974, Andrews 1979), and only the abstract focuses solely on transmission attempts (Canzonier 1968).

The failure of transmission experiments and the scarcity of spore stages in infected oysters led to another argument, also made in the 1962 report by Sprague: ''…claiming no monopoly

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on any theory, we at Chesapeake Biological Laboratory favor the idea that MSX is a typical haplosporidan normal to another host, occurring sometimes in oysters but being usually incapable of developing to the spore stage in this unnatural host.'' The supposition that the eastern oyster was an aberrant host was challenged by later findings that the spore stage was produced regularly in juvenile oysters that became infected by *Haplosporidium nelsoni*, most frequently in spat (those less than a year old) (Andrews 1979, Barber et al. 1991, Burreson 1994).

In 1992, as part of a project funded by the Oyster Disease Research Program of NOAA, a group of 20 researchers with expertise in *Haplosporidium nelsoni* biology; in life cycles and transmission of other parasites (marine and insect); in oceanography (estuarine and nearshore circulation); epidemiology; and particle transport met at Rutgers University's Haskin Shellfish Research Laboratory (HSRL) for a life cycle Workshop (Ford et al. 1993). The objective was to develop new ideas about how to investigate the *H. nelsoni* life cycle and the pathogen's mode of transmission. Suggestions advanced at this meeting stimulated a new round of investigations, using both histological and molecular methodologies, the results of which are reported in this article.

Despite what was a massive effort encompassing several different projects over more than a dozen years, it was difficult to identify another host or describe a life cycle for *Haplosporidium nelsoni*—and thus we never published a report of the work. Nevertheless, it is believed that there is value in providing details of what was carried out and what was found in these investigations. Described here are several separate investigations covering (1) additional information on the frequency and seasonal distribution of *H. nelsoni* spores in oyster spat in Delaware Bay; (2) a histological survey for *H. nelsoni* in zooplankton and small bivalves in lower Delaware Bay; and (3) polymerase chain reaction (PCR)-based molecular surveys for *H. nelsoni* DNA in water, sediment, and benthic invertebrates in the lower York River (Chesapeake Bay), the lower Delaware Bay, and Oyster Bay (off Long Island Sound) (Fig. 1).

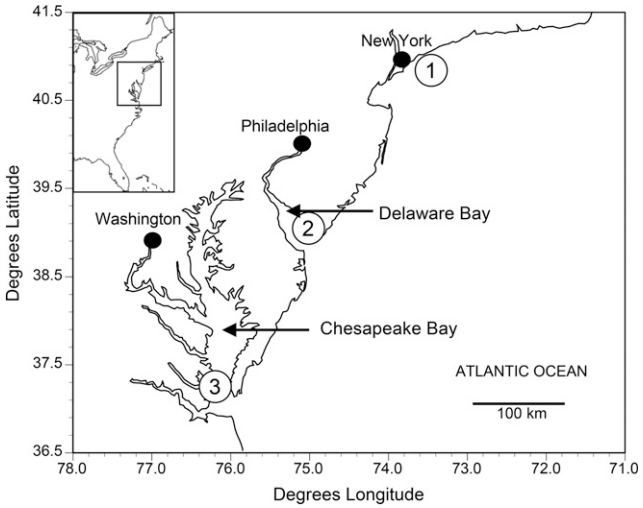

Figure 1. Locations along the mid-Atlantic coast of the United States where invertebrates were collected and examined for evidence of *Haplosporidium nelsoni*. (1) Oyster Bay, NY; (2) Lower Delaware Bay, NJ; (3) Lower York River, VA.

MATERIALS AND METHODS

Examination of Oyster Spat for Haplosporidium nelsoni *Spores (1988 to 1994)*

A preliminary account reporting the finding of significant numbers of spores in spat in Delaware Bay was published in 1991 covering data collected from 1988 through 1990 (Barber et al. 1991). That survey, in which more than 2,700 spat were examined, was subsequently extended and amplified to determine spore prevalence, to estimate the numbers of spores and to describe seasonal patterns.

At approximately weekly intervals, during the spring, summer, and fall of 1991 and 1992, spat were collected in lower Delaware Bay, mostly from intertidal sand flats in front of the HSRL Cape Shore Station (Fig. 1), a location that typically receives heavy oyster sets. In 1993 and 1994, spat were collected approximately every 7–10 days from May through August and every 2 wk in April and September. Over these 4 y, a total of 11,451 spat were collected from 5 year classes (1990 through 1994) and examined for the presence of *Haplosporidium nelsoni* spores. Oyster setting typically occurs in midsummer so a particular year class was sampled from October into December of its first year and then from April into September of the following year. Although most spat were collected from the Cape Shore site, approximately 18% of the total was collected from other sites in lower Delaware Bay (Ford et al. 1993).

All spat were shucked and a smear of the digestive gland was examined microscopically. Based on the results of the fresh smears, spat were scored as being patently infected or uninfected by *Haplosporidium nelsoni*. Infected spat were categorized as having light or advanced infections and the presence of spores was recorded. On most collection dates, subsamples of 25–40 spat in which spores were not found in fresh smears were fixed for tissue section examination as a check on the results of the smears. Final spore prevalence figures were a combination of those found in the fresh examination and those found by tissue slide histology of the apparently ''healthy'' spat.

To estimate the spore abundance in individual spat, 18 spat in which spores were found in fresh examination were measured (hinge to bill) and placed in individual tubes with regularly changed filtered seawater, with the tissues allowed to rot so as to liberate the spores. The resulting spore preparations were held at 4°C. The number of spores present in each spat was estimated from these samples by counting four aliquots from each in a hemocytometer.

Searching for Nonoyster Hosts: Tissue-Section Histology (1993 to 1994)

Based on suggestions made at the Life Cycle Workshop, it was hypothesized that an intermediate host for *Haplosporidium nelsoni* (necessary to the parasite life cycle, in which some development, such as spores, occurs) would be a mobile species, probably a zooplankter that would produce spores at about the time that oysters are first becoming infected in early summer. Zooplankton samples were collected at approximately weekly intervals on 18 dates from May through August in 1993 ($n = 72$) and on 10 dates from April through September in 1994 ($n = 40$). On each date, four stations in lower Delaware Bay were visited. At each station, a 500-μm plankton net with a 0.5-m opening was towed about 1 m below the water surface for 15–20 min, sampling a volume of 85–115 m^3 (Ford & Barber 1995).

In the laboratory, the samples were fractionated as well as possible to remove fish eggs, debris, and ctenophores. The remainder of each sample was scanned under a binocular scope for evidence of discoloration in zooplankton that could be caused by a large number of spores. A subsample was retained for species identification and enumeration. Species abundance was roughly estimated and categorized as rare to abundant in 1993 and by count for each sample in 1994. The bulk of the sample was embedded in paraffin blocks, sectioned, stained, and examined microscopically. Any recognizable parasites or pathology were recorded.

It is also possible that *Crassostrea virginica* is not the definitive host for *Haplosporidium nelsoni*, and an alternate host may be involved in which the complete life cycle is achieved and sporulation is regular. Again based on recommendations from the Life Cycle Workshop, it was hypothesized that *H. nelsoni* infections in an alternate host would resemble, epizootiologically, those in oysters, and given the preponderance of spores in small oysters, the sampling efforts were on concentrated on bivalves in the ≤30 mm size range.

Invertebrate samples were collected from the intertidal flats at or near the Cape Shore Station. Collections were made at low tide every 2 wk from May to September in 1993 and 1994. Sediment was dug to a depth of about 10 cm and passed through a 1-mm sieve. All live organisms were removed, identified, and fixed in Davidson's fixative. Soft tissues of molluscs large enough to shuck were removed from their shells before fixation; smaller shelled individuals were left in the fixative to decalcify. The intertidal sampling effort was not quantified, but at each date, two individuals spent the entire low tide period (1–3 h) collecting organisms. Tissues were embedded in paraffin, sectioned, stained, and examined microscopically for parasites and pathological conditions.

Searching for Nonoyster Hosts: Molecular Screening (1996 to 1998, 2010)

Sample Collection

Organisms, water, and sediment samples were collected in the lower York River, VA by the VIMS and in Delaware Bay, NJ, and Oyster Bay, NY, by the Haskin Shellfish Research Laboratory (Fig. 1). All are locations known to have experienced heavy *Haplosporidium nelsoni* infection pressure at the time of sampling.

Lower York River, VA. Sampling was conducted weekly in 1996 from March to December, every other week throughout the year in 1997 and 1998 in front of the VIMS campus. One hundred–liter water samples taken from 1.5 m below the surface were pumped from the VIMS dock through a series of screens (250 μm, 75 μm, 35 μm, and 10 μm). Material from each screen was fixed separately in 95% EtOH. Sediment was collected from a vessel a short distance offshore using a box corer. When present, the top, aerobic layer was scraped off into a 50-mL tube, and a subsample of the remainder was scooped into a second 50-mL tube. The remaining sediment was sieved through 500- and 250-μm screens. Individual organisms were picked from the 500-μm screen and sorted into general phylogenetic categories; those on the 250-μm screen were processed as a group. All sediment and organism samples were fixed in 95% EtOH.

A second sampling effort was conducted in May and June 2010 and focused on a variety of small crustaceans collected from a tray of oysters at the VIMS site and from eel grass beds at Goodwin Island, about 9 km southeast of VIMS at the mouth of the York River.

Lower Delaware Bay, NJ. Sampling was conducted once each month in January–March 1997 and then approximately weekly into early December 1997, and every other week from mid-March to mid-July in 1998. Sampling of sediment and macroinvertebrates in Delaware Bay was concentrated on the tidal flats in front of the HSRL Cape Shore Station. Macroinvertebrates were collected from the surface and sediment at low tide among trays of oysters. Sediment was dug by hand from locations on the inner and outer portions of the tidal flats.

One hundred–liter water samples collected by vessels from various sites in the lower bay were pumped from near bottom through 64- and 177-mesh screens. At the same time, a sample was collected using a 500-μm plankton net with a 0.5-m opening that was towed about 1 m below the surface for 15–20 min. All material were fixed in 95% EtOH.

Oyster Bay, NY. Macroinvertebrates were collected from samples of dredged oysters at Oyster Bay, NY, on two dates in November and December of 1997 and four dates in April, May, and June 1998. Organisms were fixed in 95% EtOH.

Sample Preparation

In both laboratories, samples collected in the 1996 to 1998 study were prepared and processed similarly. Macroinvertebrates were identified when possible and placed individually in 1.5-mL microfuge tubes with 95% EtOH, and weighed. Typically macroinvertebrates were processed as individuals; but if the individuals were too small for efficient DNA extraction, multiple organisms of the same species were processed together in the same tube. One half of each macroinvertebrate sample was set aside in neutral buffered formalin for *in situ* hybridization (ISH) of organisms that were positive by PCR (VIMS only). Organisms from water samples were processed as a group for each screen size. Small samples were processed in 1.5-mL tubes; larger samples in 15-mL tubes. Samples were homogenized with a sterile grinder in Tris–EDTA buffer. The DNA extraction protocol was based on 0.25 g of tissue and sample weights were typically between 0.20 and 0.30 g. Extraction of DNA from sediment was based on a 5-mL sample. Autoclaved tubes and pipets were used with fresh tips for each individual and at each stage of the procedure.

Shrimps and isopods collected in 2010 were placed on ice for several minutes until they stopped moving and were then sectioned longitudinally with a sterile scalpel blade. One section was placed in a tube containing 70% ethanol, the other in a tube containing Davidson's fixative in case molecular results signaled the need for histological examination. Amphipods and caprellids were too small to cut so intact animals were preserved in groups of 60 at the VIMS site and individually at Goodwin Island.

DNA Extraction, Purification, and Amplification

The 1996 to 1998 project was carried out during the very early stages of the PCR assay development and before DNA

extraction kits were available. Macroinvertebrate DNA extraction and purification was based on the guanidine thiocyanate tissue lysis procedure of Hill et al. (1991). The microwave preparation method of Goodwin and Lee (1993) was used to extract DNA from water and sediment samples. Initial difficulties with the removal of *Taq* DNA polymerase inhibitors in the environmental samples, especially in sediment, were overcome with ethidium bromide/high salt purification of the DNA (Stemmer 1991) and addition of bovine serum albumin to the PCR reactions (Kreader 1996). After extraction, DNA pellets were resuspended in Tris–EDTA buffer. DNA quantity and quality were not measured; however, because these were large-scale preparations, pellets were easily visible in most samples.

By 2010, when the small crustacean study was conducted, commercial kits were available for sample preparation. For these samples, EtOH was decanted and tubes allowed to air-dry before overnight lysis. Tissue lysis and DNA extractions were performed using the QIAamp DNA Mini Kit (Qiagen) as per manufacturer's instructions, except that elution volumes varied (100 μL–200 μL) depending on the size of the organism. Organisms were processed individually, except for VIMS oyster tray amphipods and caprellids that were pooled five per tube resulting in 12 extractions from each. Twenty of the *Palaemonetes* spp. contained eggs which were initially resistant to lysis, so sterile disposable tissue homogenizers were used to break them open before another overnight lysis. Genomic DNA was quantified using a NanoDrop 2000 (Thermo Scientific).

In the first study, extracted DNA was amplified for *Haplosporidium nelsoni* SSU rDNA using a heminested PCR protocol as described by Ford et al. (2009a), which was based on the primers MSX A′ and MSX B of Renault et al. (2000) in the first amplification with the addition of MSX C (Burreson et al. 2000) along with MSX A′ in the second. In 2010, PCR was performed on each sample for *H. nelsoni* SSU rDNA using primers MSX-A′ and MSX-B and on a subset of each sample group to demonstrate amplifiability using general SSU rDNA primers CS1 and CAS1 as described previously (Cochennec et al. 2000, Renault et al. 2000). Reactions contained approximately 200 ng DNA.

In situ Hybridization (ISH) of Positive Samples

Subsamples of macroinvertebrates collected at the VIMS site that were positive in the PCR reaction were subjected to ISH with the probe MSX 1347 using the protocol described and found to be specific for *Haplosporidium nelsoni* by Stokes and Burreson (1995). Fifteen samples from 1996 and 10 from 1997 were assayed. The organisms tested were mostly various species of worms, although two amphipod samples were included.

DNA Sequencing of Positive Samples

Four *Haplosporidium nelsoni* PCR-positive samples were subjected to DNA sequencing to confirm the identity of the amplified products. These were from the VIMS 1998 collections and included two sediment and two water samples. The MSX-A′ and MSX-C PCR products were cloned and four clones from each sample were sequenced using a LI-COR automated sequencer as described previously (Reece & Stokes 2003). The resulting sequences were analyzed using the GeneJockeyII software package (Taylor 1993).

RESULTS

Examination of Oyster Spat for Haplosporidium nelsoni *Spores*

Prevalence of *Haplosporidium nelsoni* infections in spat was the greatest at the start of the sampling period (1988) and gradually declined over the next 6 y (Table 1). Prevalence of spores followed the same pattern. Maximum infection prevalence in the 1987 year-class sampled in the spring and summer of 1988 as they approached yearling size was 50%. The maximum prevalence of advanced infections and those with spores was 30% and 25%, respectively. The 1988-year-class became infected that summer, and a peak of 28% in spore production occurred in December (Fig. 2). The maximum infection prevalence fell in subsequent years to between 0% and 29% and no individual from the 1992- through 1994-year-classes had detectable spores. In general, the highest total and advanced infection prevalence, and most spores, were found in the spring and summer of the calendar year after birth, although the date of maximum detected sporulation ranged from late May to late August, depending on year. The date of highest spore prevalence typically coincided with the peak in advanced infections (Table 1). Over all sampling dates, spores were found in an average of about 40% of the spat that had advanced plasmodial infections, although peak prevalence of spores in advanced infections was 75%–100% (Ford et al. 1993).

There was no correlation between spat size (shell height 16–28 mm) and the estimated number of spores recovered from tissue ($r^2 = 0.008$, $n = 18$). Estimated numbers ranged from just under 2×10^3 to 1.1×10^6, with a mean of 1.6×10^5 (SD = 2.6×10^5), although these would be minimal numbers because a portion of the digestive gland (containing spores) was removed for fresh-smear detection.

Searching for Non-oyster Hosts: Tissue-Section Histology

Despite examining tens of thousands of zooplankters, mostly shrimp and crab larvae and copepods collected during two summers, no recognizable haplosporidans were observed (Table 2). Only two recognizable parasites, both microsporidians, were found. The spore stage of an unidentified microsporidian was observed in sections of copepods (probably *Pseudodiaptomus pelagicus*) in June and September 1994. Another microsporidian was seen in a single nereid worm (probably *Nereis virens*) collected from the plankton in June 1993. In this case, only meront and sporont stages were present and the host tissue was very disrupted. Polychaetes were extremely rare in plankton samples leading us to believe that this individual may have been moribund because of the heavy infection.

More than 1,200 individual bivalves and other benthic organisms were collected and examined histologically during the study (Table 3). Although the species representation was far from equal (e.g., 586 *Tellina* sp. versus 2 *Lyonsia* sp.), it did represent the relative frequency and the total abundance of these species at the collection site. Trematodes and cestodes were common in the bivalves and were also found

TABLE 1.

Oyster spat collections from lower Delaware Bay by year class and collection period with maximum total, advanced, and spore prevalence of *Haplosporidium nelsoni*, 1988 to 1994.

Year class	Collection period*	No. Samples†	No. Examined	Max inf prevalence	Date	Max adv prevalence	Date	Max spore prevalence	Date
1987									
	Spring/summer 1988	8	195	50%	15 July	30%	31 May	25%	20 June
1988									
	Fall 1988	8	181	nd	–	nd	–	28%	7 December
	Spring/summer 1989	10	109	5%	30 August	5%	30 August	5%	30 August
1989									
	Fall 1989	5	115	2%	nr	1%	nr	1%	nr
	Spring/summer 1990	11	1,703	15%	17 June	4%	21 June	3%	27 June
1990									
	Fall 1990	4	701	2%	9 October	1%	17 September	0%	–
	Spring/summer 1991	38	4,115	21%	8 July	8.5%	9 June	6%	9 June
1991									
	Fall 1991	9	1,253	5%	13 December	1%	25 November	0%	–
	Spring/summer 1992	21	3,222	8%	21 May	7%	21 May	3%	21 May
1992									
	Fall 1992	2	198	0%	–	0%	–	0%	–
	Spring/summer 1993	14	397	29%	14 September	14%	14 September	0%	–
1993	Spring/summer 1994	11	740	4%	19 July	0%	–	0%	–
1994	Fall 1994	7	527	0%	–	0%	–	0%	–

Max adv, maximum advanced infection prevalence; Max inf, maximum infection prevalence; Max spore, Maximum spore prevalence.

* Fall, October through December; spring/summer, April through September.

† These mostly represent different sampling dates; a few represent different sample sites collected on the same date.

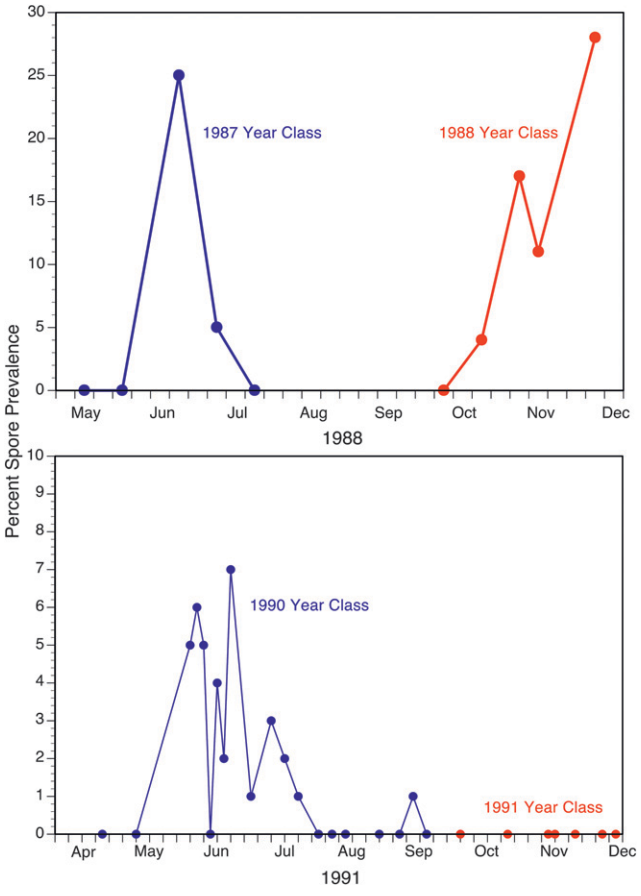

Figure 2. Seasonal pattern of *Haplosporidium nelsoni* spore production in spat along Cape May shore of lower Delaware Bay during the summers of 1988 and 1991.

in *Diopatra* sp. and *Balanus* sp. No recognizable protozoans were found.

Searching for Non-oyster Hosts: Molecular Screening

On 35 of the 86 (41%) water sampling dates at the York River site, the *Haplosporidium nelsoni* PCR product was produced in at least one of the four size fractions (Table 4). Positive signals were present in all months and in most fractions of water samples collected in 1996, but especially from March through July. Positive samples were far fewer in 1997 and 1998 when they were concentrated in June–July and September–October, respectively. Positive signals were present among size fractions in no particular pattern.

Most of the consistently PCR-positive sediment samples from the York River were macroinvertebrates caught on the 500-µm screen (Tables 4 and 5). In a temporal pattern very similar to that of the water samples, most positive sediment samples were collected in the first half of 1996. During that period, most samples scraped from the sediment surface and those retained on the 250-µm screen were positive. All surface and 250-µm samples collected after October 1996 were negative except on two sampling dates in 1998. Most of the 49 PCR-positive macroinvertebrates were polychaetes, including spionid, nereid, capitelid, and orbiniid worms (33 of 226 worm samples, 3.0%); however, a variety of other organisms including amphipods, isopods, and gastropods also yielded the *Haplosporidium nelsoni* PCR product (Table 5).

In lower Delaware Bay, four of 147 water samples collected in 1997 and two of 133 collected in 1998, and consisting of material retained on either or both 177-and 500-µm screens, yielded a PCR-positive reaction. None of the 108 sediment samples was PCR-positive; however, three samples of mixed and unidentified microbenthic organisms picked from 24 of these samples yielded PCR product (Table 5). Eleven macroinvertebrate samples yielded PCR-positive results. Among there were six polychaetes (of 126 worm samples, 4.7%), and three mud snails (of 27 mud snails, 11.1%). Three samples collected at Oyster Bay, NY, a mud snail, a mud crab, and a red beard sponge, gave positive PCR signals (Table 5).

Overall, positive samples were found in all seasons, although they predominated in the spring (Table 5). The most notable pattern was the abundance of positive samples of all types at the York River site during the first half of 1996, followed by a relative scarcity in the second half of the year, even fewer in 1997 and 1998. Interestingly, the correspondence between water samples and 500-µm sediment (i.e., macroinvertebrate) samples that was apparent in early 1996 was no longer evident in 1997: positive water samples were concentrated from May to July whereas positive macroinvertebrates were also found in winter and spring. Forty species from nine phyla collected from at least one site never yielded a *Haplosporidium nelsoni* PCR product (Table 6).

TABLE 2.

Estimated numbers of zooplankters collected from Delaware Bay and examined by tissue-section histology in 1994.

Date collected	Shrimp larvae	Copepods	Amphipods	Cladocera	Ostracods	Crab larvae	Fish eggs*
11 April	2,639	3,347	0	211	168	0	40
15 April	2,174	15,229	0	261	94	0	383
3 May	3,479	5,868	0	0	32	0	561
23 May	1,466	4,134	0	0	0	0	2,405
1 June	1,466	4,134	0	0	0	0	2,405
8 June	891	2,213	0	0	0	930	23,940
22 June	387	43	0	0	0	8,711	4,194
24 August	13	26	364	0	0	6,843	1,006
13 September	301	63	57	0	0	525	7
20 September	77	217	13	0	0	961	697
Total	12,893	35,274	434	472	294	17,970	35,638

* July samples were nearly all fish eggs and were not counted.

TABLE 3.

Small bivalves and benthic invertebrates from lower Delaware Bay examined by histology in 1993 and 1994.

Species	Dates		No. examined	Parasites			
				Bucephalus	Other Trem & cestodes	% Buceph.	%Other Trem & cestodes
Bivalves	1993	1994					
Tellina agius & versicolor	8	10	586	6	170	1%	29%
Gemma gemma	5	2	141	1	5	1%	4%
Haminea solitaria	2	0	31	0	9	0%	29%
Ensis directus	6	5	157	0	56	0%	36%
Mullinea lateralis	8	4	56	1	19	2%	34%
Anadara ovalis	0	1	4	0	0	0%	0%
Tagelus plebeius	0	2	6	0	4	0%	67%
Nucula proxima	0	1	13	0	0	0%	0%
Lyonsia hyalina							
Polychaetes							
Diopatra cuprea	0	2	16	0	3	0%	19%
Glycera dibranchiata	0	6	15	0	0	0%	0%
Hydroides diathus & Filograna implexa	0	1	132	0	0	0%	0%
Platynereis dumerilii	0	3	15	0	0	0%	0%
Nemerteans							
Cerebratulus lacteus	0	7	15	0	0	0%	0%
Anemones							
Actinothoe modesta & Edwardsia elegans	0	6	21	0	0	0%	0%
Barnacles							
Balanus sp.	0	1	15	0	5	0%	33%

None of the 287 crustaceans from the two York River sites collected in 2010 yielded the *Haplosporidium nelsoni* PCR product, although DNA from a subsample of 111 of these organisms was successfully amplified using general SSU rDNA primers verifying the amplifiability of the samples (Table 7).

Sequencing and ISH were conducted on PCR-positive samples collected in the York River by VIMS. In every case, amplicons yielded the *Haplosporidium nelsoni* SSU RNA gene sequence, demonstrating that positive samples were not the result of cross-reactivity. *In situ* hybridizations with the *H. nelsoni*–specific DNA probe were conducted on 26 PCR-positive macroinvertebrate samples to discriminate between true infections and those where *H. nelsoni* or *H. nelsoni* DNA simply adhered to the external surface or passed through the gut. None of the samples revealed structures that reacted positively with the *H. nelsoni* probe.

DISCUSSION

Because the early failures to describe a life cycle and means of transmission of *Haplosporidium nelsoni*, two lines of thought have emerged: (1) that the eastern oyster is an accidental, dead-end host in which the pathogen does not complete its life cycle and that another organism—either an alternate host or an intermediate host—is necessary to maintain parasite populations; or (2) the pathogen can complete its life cycle by producing spores in juvenile (spat) eastern oysters but that these have been underrepresented in sampling programs over the years and thus have not been considered in a direct life cycle scenario (Haskin & Andrews 1988).

Spores in Spat

Between 1988 and 1994 more than 13,000 spat were examined, representing 8-y classes, from lower Delaware Bay. During that period, the overall prevalence of *Haplosporidium nelsoni* infections in spat decreased, as did the prevalence of spores, which declined from a high of 28% in 1988 to 0% after summer 1992. Despite the decreased infection prevalence, however, the proportion of advanced infections that contained spores remained high, often greater than 70% and up to 100% in many samples (Ford et al. 1993). Although they did not report spore prevalence as a proportion of advanced infections, Andrews (1979) found a 39% prevalence in 4-mo-old spat held in the York River in 1976 and Burreson (1994) found a 36% prevalence in spat at the same location in 1993. These were isolated instances, however, among many years of oyster sampling at this site.

In 1991, Barber et al. (1991) reported that of the 198 cases of *Haplosporidium nelsoni* sporulation found in Delaware Bay oysters since 1958, when the pathogen was first identified, only 1% had been found in adult oysters. Andrews (1979) and Burreson (1994) also remarked on the absence of spores in adult oysters deployed at the same locations where they found high spore prevalence in spat. This differential was not simply a matter of unselected spat undergoing initial exposure because the examination of adults undergoing first exposure had not found similar spore prevalence. Barber et al. (1991) speculated that some element of the spat metabolism provided a substance necessary for spore development which was not present, or was not present in sufficient quantities, in older oysters.

TABLE 4.

Water and sediment samples collected in the lower York River (Chesapeake Bay), Virginia, 1996 to 1998, and assayed by PCR for *Haplosporidium nelsoni* SSU rDNA.

		Water				Sediment		
	Date*	250 (μm)	75 (μm)	35 (μm)	10 (μm)	Top Layer	250 (μm)	500 (μm)†
1996	March (3)	++	+++	+	++	+	+++	++
	April (3)	+	+	+++	++	nd	+++	+++
	May (5)	++	–	+	++	++++	++++	+
	June (4)	++	+	++	++++	+	+++	++
	July (5)	+++	++	+	++	++	++	++
	August (4)	+	+	+	+	–	–	+
	September (4)	++	–	–	+	+	+	++
	October (5)	+	–	+	+	+	–	++
	November (3)	+	–	–	–	–	–	–
	December (3)	+	+	–	+	–	–	–
1997	January (2)	—	—	—	+	—	—	+
	February (2)	–	–	–	–	–	–	+
	March (2)	–	+	–	–	–	–	+
	April (2)	–	–	+	+	–	–	++
	May (2)	–	+	–	–	–	–	–
	June (2)	–	+	+	+	–	–	–
	July (3)	++	+	++	+	–	–	+
	August (2)	–	–	+	–	–	–	+
	September (2)	–	–	–	–	–	–	–
	October (2)	–	–	–	–	–	–	+
	November (2)	–	–	–	–	–	–	–
	December (2)	–	–	–	–	–	–	–
1998	January (2)	–	–	–	–	–	–	+
	February (2)	–	–	–	–	–	–	+
	March (2)	–	–	–	–	–	–	+
	April (3)	–	–	–	–	–	–	+
	May (2)	–	+	+	–	–	–	–
	June (2)	–	–	–	–	–	–	–
	July (2)	–	–	–	–	+	+	–
	August (1)	+	–	–	+	–	–	–
	September (2)	+	++	+	++	–	–	+
	October (2)	+	+	–	–	+	+	–
	November (2)	–	–	–	–	–	–	–
	December (2)	–	–	–	–	–	–	–

* Numbers in parentheses are the number of sampling dates in the month. Number of "+" indicates number of positive samples in that month.
† These samples are the macroinvertebrates listed in Table 5.

In Delaware Bay, prevalence in spat declined in concert with the decline in infections of native adult oysters, which began in 1987 (Ford & Bushek 2012). Before 1987, peak prevalence in adults was between 70% and 90% and many infections were advanced (Ford & Haskin 1982). Since then, prevalence has rarely reached 30% and is mostly 10% or less in adult oysters, and infections are typically light. Over the last two decades, only three cases with spores (all spat size, 30–40 mm, and collected in 2005, 2008, and 2013) have been found during histological examinations of oysters sampled for regular monitoring and for special projects, the latter of which included spat (HSRL unpublished records). Prevalence has likewise declined in lower Chesapeake Bay (Carnegie & Burreson 2011) and sporulation there too is relatively uncommon. In samples from the four wild oyster populations around Chesapeake Bay studied monthly from spring through fall of 2007 and 2008 by Carnegie and Burreson (2011), just 12 of 461 observed *Haplosporidium nelsoni* infections (2.6%) had proceeded to sporulation, most of these in spat less than 50 mm in size. Is this prevalence decline associated with diminished recruitment so that there are fewer spat available to produce spores? This seems unlikely. Not only have lower disease levels been associated experimentally with the development of resistance to MSX disease (Carnegie & Burreson 2011, Ford & Bushek 2012), but also recruitment has not diminished significantly (e.g., Fig. 9 in Powell et al. 2008).

The most compelling argument against direct transmission of *Haplosporidium nelsoni* from spat, however, is that naive oysters deployed as sentinels at the Cape Shore site as well as in the York River experience very heavy MSX infection pressure (Carnegie & Burreson 2011, Ford & Bushek 2012), arguing that, even in the absence of spore production in spat, there continues to be a quantity of infective particles sufficient to cause abundant and heavy infections in naive individuals. Further evidence of the abundance of infective particles is the detection of *H. nelsoni* DNA in association with oyster gills without histologically detected infections throughout Delaware Bay in

TABLE 5.

Organisms that yielded *Haplosporidium nelsoni* SSU rDNA product during sampling 1996 to 1998.

PHYLUM/class		Common name	Totalno. samples	No. PCR-positive samples	Season(s)*
York river†					
ANNELIDA					
Polychaeta	Spionidae	Mud worm	67	12 (10)	W, Sp, S, F
	Capitellidae	Thread worm	47	8 (5)	W, Sp
	Nereidae	Clam worm	42	4 (3)	F, W, Sp
	Orbiniidae	Orbiniid worm	26	3 (1)	Sp, S
	Glyceridae	Blood worm	22	1	W
	Ampharetidae	Ampharetid worm	7	1	S
	Maldanidae	Bamboo worm	6	1 (1)	Sp
	Cirratulidae	Fringe worm	4	1	Sp
	Pectinariidae	Trumpet worm	3	1	F
	Phyllodocidae	Paddle worm	2	1	F
ARTHROPODA					
Malacostraca		Hooded shrimp	–	–	–
	Gammaridae	Gammarid amphipod	39	2 (2)	Sp
	Corophiidae	Corophid amphipod	1	1 (1)	Sp
	Idoteidae	Isopod	2	1	S
	Hippolytidae	Grass shrimp	1	1	Sp
CHORDATA					
Leptocardii	Branchiostomidae	Lancelet/Amphioxus	1	–	F
MOLLUSCA					
Gastropoda	Retusidae	Barrel bubble	28	2	Sp, S
	Atyidae	Solitary glassy bubble	9	2 (1)	Sp
	Pyramidellidae	Turbonille	18	1	Sp
Bivalvia	Tellinidae	Clam	14	3	W, Sp
NEMERTEA					
	Lineidae	Nemertean worm	16	1	W
Phoronida					
		Phoronid worm	22	2 (1)	S, F
Delaware Bay‡					
ANNELIDA					
Polychaeta	Nereidae	Clam worm	116	4	Sp, S, F
	Spionidae	mud worm	2	1	S
	Unidentified		6	1	Sp
ARTHROPODA					
Amphipoda	Gammaridae	Gammarid amphipod	1	1	F
Crustacea	Pinnotheridae	Pea crab	2	1	F
Gastropoda	Nassariidae	Mud snail	27	3	S, F
Others	Mixed microbenthos		24	3	Sp, S
Oyster Bay§					
ARTHROPODA					
Crustacea	Panopeidae	Mud crab	10	1	Sp
MOLLUSCA					
Gastropoda	Nassariidae	Mud snail	13	1	Sp
PORIFERA					
Demospongiae	Microcionidae	Red beard sponge	2	1	Sp

* F, fall (9/21–12/20); S, summer (6/21–9/20); Sp, spring (3/21–6/20); W, winter (12/21–3/20).
† From 86 sampling dates (March 14, 1996 to December 16, 1998).
‡ From 59 sampling dates (March 28, 1997 to October 10, 1998).
§ From 6 sampling dates (November 11, 1997 to July 24, 1998).
Numbers in parentheses refer to samples subjected to ISH.

two studies (1999–2000 and 2007–2009) conducted after infection prevalence declined (Ford et al. 2009a, 2012). Clearly, *H. nelsoni* can produce spores, which are presumed necessary for its life cycle, in juvenile eastern oysters, but current evidence does not support this as the principal source of infective elements.

Searching for an Alternate or Intermediate Host

If *Haplosporidium nelsoni* transmission does not occur directly between oysters but requires another host, what might that host look like? Attendees at the 1992 workshop suggested that an alternate host producing infective stages independently

TABLE 6.

Macroinvertebrates, from all sites, examined by PCR that yielded no *Haplosporidium nelsoni* DNA product.

PHYLYM/Class	Family	Common name	Number of sample dates		
			York River	Delaware Bay	Oyster Bay
ANNELIDA					
Polychaeta	Nephtyidae	Cat worm	2	–	–
	Oenonidae	Opal worm	1	–	–
	Aphroditidae	Scale worm	2	–	1
	Onuphidae	Diopatra tube worm	–	2	–
	Oenonidae	Arabellid thread worm	–	2	–
	Sabellariidae	Reef worm	–	3	2
	Syllidae	Syllid worm	–	1	–
	Magelonidae	Rosy magelona	–	1	–
	Serpulidae	Hydroides/limey tube worm	–	2	–
	Opheliidae	Opheliid worm	1	–	–
Clitellata	Oligochaete (subclass)	Unidentified oligochaete	2	–	–
ARTHROPODA					
Branchiopoda	Paguridae	Hermit crab	–	9	4
	Panopeidae	Mud crab	–	8	3
	Portunidae	Blue crab	–	1	
	Paguridae	Long-clawed fiddler crab	–		1
Cirripedia	Balanidae	Barnacle	1	4	2
CNIDARIA					
Anthozoa	Cerianthidae	Burrowing anemone	8	–	–
	Cerianthidae	Anemone	–	6	–
Tentaculata	Bolinopsidae	Ctenophore	1	1	–
HEMICHORDATA					
Enteropneusta	Enteropneusta (class)	Acorn worm	3	–	–
MOLLUSCA					
Bivalvia	Mytilidae	Ribbed mussel	–	5	–
	Mytilidae	Hooked mussel	–	1	–
	Mytilidae	Blue mussel	–	2	2
	Pandoridae	Rounded Pandora	–	2	2
	Arcidae	Blood ark	–		2
	Tellinidae	Northern dwarf tellin	–	1	–
Gastropoda	Epitoniidae	Wentletrap	2	–	–
	Muricidae	Mottled dog whelk	2	–	–
	Muricidae	Northeast dog whelk		–	3
	Muricidae	Oyster drill	1	3	–
	Hydrobiidae	Seaweed snail	2	–	–
	Nudibranchia (order)	Nudibranch	1	–	–
	Naticidae	moon snail	–	3	–
	Calyptraeidae	Common slipper limpet	–	3	5
	Calyptraeidae	White slipper limpet	–	1	5
	Columbellidae	Mitrella snail	–	1	–
NEMERTEA					
Anopla	Lineidae	Milky nemertean/ribbon worm	–	3	–
PLATYHELMINTHES					
Rhabditophora	Stylochidae	Oyster flatworm	–	2	–
SIPUNCULA					
Sipuncula	Sipuncula (phylum)	Sipunculid/peanut worm	1	–	–
TUNICATA					
Ascidiacea	Molgulidae	Sea squirt	–	1	–

of oysters would most likely be very similar to the oyster (i.e., a sessile bivalve), and the seasonal infection cycle would probably also be similar. The production of spores in small oysters further suggests that such a host might be a small or juvenile bivalve. On the other hand, workshop attendees postulated that if an intermediate host exists, it is likely to be quite different from the oyster and possibly one that is itself highly mobile or is dispersed by water currents (e.g., zooplankton, including larval forms of larger fauna). The parasite must have some mechanism to maintain itself near potential hosts within the estuary. The conference participants pointed out that a potential intermediate host is not likely to be a commercially valuable fish species because these have been examined extensively for parasites. Small noncommercial fish species are

TABLE 7.

Small crustaceans collected in the lower York River, VA, in May (VIMS) and June (GI, Goodwin Island) 2010 and processed for *Haplosporidium nelsoni* SSU rDNA. All samples were negative.

Taxon	Common name	Location	Number of individuals
Palaemonetes spp.	Grass shrimp	GI	60/80*
Crangon sp.	Brown shrimp	GI	43
		VIMS	2
Isopods	Isopods	GI	25
		VIMS	32
Amphipods	Amphipods	GI	5
		VIMS	60 (12)†
Caprellids	Skeleton shrimp	VIMS	60 (12)†

* Eighty total reactions run; 60 were the initial runs and 20 were repeated runs from eggs of gravid females.
† Numbers in parentheses represent pooled samples of five individuals each.

candidates, but haplosporidans have never been found in a vertebrate host.

Following these suggestions, tens of thousands of zooplankters were examined histologically and more than a 1,000 small bivalves and other invertebrates collected in the lower Delaware Bay over a 2-y period, during the warm season when oysters become infected by *Haplosporidium nelsoni*, without finding anything resembling a haplosporidan—and only two instances of recognizable microparasites, both microsporidians—one in a copepod and one in a nereid worm.

As the histology project was nearing an end, the availability of PCR technology was becoming more widespread and offered another methodology for the search. Partnering between the Rutgers and VIMS laboratories expanded the search area from Delaware Bay to the lower York River, a tributary of Chesapeake Bay—both areas that experience heavy *Haplosporidium nelsoni* infection pressure. In contrast to the lack of histological evidence, PCR methodology using primers specific for *H. nelsoni* yielded positive results in both locations. Positive signals were found at the VIMS site in the lower York River in all types of samples: water, sediment, and macroinvertebrates.

Far fewer positive PCR signals were found in Delaware Bay than in the York River. Although the reason for the disparity in PCR signals between the two sites is not known, they are very different physically and have different species composition. For instance, the Delaware Bay site is intertidal, very high energy, and has a sandy substrate. The York River site is subtidal, lower energy, and has a muddy substrate. Importantly, the disparity in PCR results between sites, and among years at the York River site, seems not to have translated into differential infection pressure at the two locations. Prevalence of *Haplosporidium nelsoni* in naïve "sentinel" oysters was equally high at both locations during and right after the sampling period (83%–90% at the York River site and 70%–100% at the Delaware Bay site) (Carnegie & Burreson 2011, Ford & Bushek 2012). Prevalence reached 84% at the York River site in 2010 when no *H. nelsoni* DNA was found in a study focused on crustaceans. Thus, detection of PCR-positive signals for *H. nelsoni* in environmental samples does not appear to be a good predictor of subsequent infection levels in oysters.

In both locations, most positive macroinvertebrates were polychaete worms, mostly nereids, capitellids, and spionids, although a high proportion of mud snails was positive in Delaware Bay. Polychaetes and mud snails live in close proximity to oysters. The worms inhabit the crevasses in clumps of oysters and can be found inside the shells of gaping oysters. Nereids are omnivores whereas spionids and capitellids can be deposit feeders. All three are likely to ingest feces and pseudofeces of live infected oysters [which can contain *Haplosporidium nelsoni* DNA (Ford et al. 2009a)], and nereids may feed on the flesh of moribund or dead infected oysters. Similarly, mud snails may well ingest feces and pseudofeces of infected oysters.

The failure of ISH applied to PCR-positive organisms to identify any recognizable structures strongly suggests that these positive reactions were not to a true infection, but to *Haplosporidium nelsoni* cells, pieces of cells or fragments of DNA either passing through the gut or adhering to external surfaces. The widespread occurrence of positive signals in many different organisms in several phyla reinforces the argument that most, if not all, of the PCR-positive signals did not represent true infections.

Searching for Hosts of Other Molluscan Pathogens

Since the 1996 to 1998 PCR-based search for a *Haplosporidium nelsoni* host, a number of similar studies have been published that used molecular tools to search for intermediate and alternate hosts for other molluscan pathogens. It is instructive to examine the results in the light of these more recent studies.

One of the results that stands out in all of these surveys is the large number of different species that reacted positively to the PCR primers used for the particular organism under consideration. Audemard et al. (2002) sampled 62 species of mostly invertebrates living in French oyster ponds ("claires") during a search for potential intermediate hosts of *Marteilia refringens*, the parasite of flat oyster *Ostrea edulis* and mussels *Mytilus edulis* and *Mytilus galloprovincialis*. Thirteen species yielded the *M. refringens* PCR product. They were mostly crustacean zooplankters, molluscans, an ascidian, and an annelid, but only two had consistently high numbers of positive responses: a cnidarian, *Cereus pedunculatus* (48 of 273 positive = 17.6%), and a copepod, *Paracartia (Acartia) grani* (five of six pooled samples positive = 83.3%.) *In situ* hybridization failed to detect the pathogen in the cnidarian tissues, but heavy staining occurred in the ovary of the female copepod. The copepod became infected on being exposed to infected oysters; however, transmission from the copepod back to the oyster was not achieved. This suggests the possibility of a second intermediate host or need for a longer period to produce infective stages in the copepod (Audemard et al. 2001).

Following the 2002 Audemard et al. study, Carrasco et al. (2007a,b) used PCR to assay zooplankton for *Marteilia refringens* DNA in a more open-water system encompassing two bays on the Mediterranean coast of Spain. They obtained positive reactions in six copepod species—none being *Paracartia grani*—and a brachyuran larva. They did not perform ISH to confirm infection, although they subsequently confirmed the experimental results of Audemard et al. (2002) by showing,

using ISH and electron microscopy, the presence of *M. refringens* in *P. grani* after the copepods were allowed to feed on feces and pseudofeces of infected *Ostrea edulis* (Carrasco et al. 2008).

Lynch et al. (2007), searching for other hosts of another pathogen of *Ostrea edulis*, *Bonamia ostreae*, in Ireland, sampled macroinvertebrates in 11 taxa encompassing 20 species and 1,154 individuals, and zooplankton encompassing an additional 36 identified species. Eight macroinvertebrate species in a variety of taxa gave positive reactions to PCR analysis: annelids, crustacea, acidians, anthozoa, porifera, and an echinoderm. The prevalence ranged from 3% (annelids) to 50% (porifera). Although this study did not include confirmation of infection by ISH or other microscopical methods, the authors conducted proximity experiments using three of the positive invertebrates: anthozoan, polychaete, and echinoderm. After exposure to the echinoderm (a brittle star), *B. ostreae* was found in heart smears of two of 30 oysters. Oysters exposed to the other two species and to controls did not become infected. The authors cautioned, however, that they had no proof that the brittle star was actually infected with *B. ostreae* because they were unable to recover material for histological examination. In this study, 19 of 80 pooled zooplankton samples provided PCR-positive reactions but individual species from positive grouped samples failed to react.

In a recent survey to detect *Haplosporidium nelsoni* DNA in invertebrates on or near oyster cages in the Damariscotta River, ME, an area that had recently experienced an MSX-disease epizootic, Messerman and Bowden (2016) found qPCR product in three tunicate, two gastropod, and one crustacean species, but no polychaetes. Twenty-six percent of plankton samples were qPCR positive. Tunicates, which are fouling organisms on the oyster cages and have high filtration capacity, had the highest prevalence (30% to nearly 70%), probably the result of ingestion. Nearly all positive samples amplified only weakly.

Adlard and Nolan (2015) followed a different sampling plan in their search for an intermediate host or hosts for *Marteilia sydneyi*, a pathogen of the Sydney rock oyster, *Saccostrea glomerata*. Rather than sample representatives of many species in a location, they targeted polychaetes based on a postulated correlation between the abundance of these benthic invertebrates and *M. sydneyi* prevalence during disease outbreaks in Australia. Using a combination of PCR and ISH they screened 1,247 individuals in 21 taxonomic groups of polychaetes. Individuals in eight of the 21 were PCR-positive with prevalences of 2%–33%. Individuals from five of the groups were then subjected to ISH. Organisms within sections of only two of 116 individuals tested reacted with the probe. The polychaete was subsequently identified as *Nephtys autraliensis*. The two positive individuals represented 4% of the individuals of that species tested by ISH.

An important aspect of the *Marteilia* spp. findings is that the ISH probes bound to organisms in potential intermediate hosts that were morphologically different from the stages found in oysters and mussels. They were clearly atypical structures in the host organism, although they resembled microparasites, including single-cells, plasmodia-like forms, and clusters of tiny cells (Carrasco et al. 2008, Boyer et al. 2013, Arzul et al. 2014, Adlard & Nolan 2015). Thus, it is believed that the histological survey, although it relied on conventional staining, would have found more than the two microparasites that were recognized had they been present.

From these studies it appears that finding PCR-positive signals in the environment, as in our search for *Haplosporidium nelsoni* hosts, is not particularly difficult. The very small proportion of PCR-positive species that were confirmed by ISH to have true infections in the above *Marteilia* work is a clear reminder that ''PCR-positive'' and ''infected'' are not equivalent (Burreson 2008). Also, the possibility of contamination in sampling gear and in the laboratory must be considered as a possible cause for the high PCR values. On the other hand, not obtaining a reaction among individuals of a species does not necessarily eliminate that species as a host (Audemard et al. 2002). In many of these studies, the prevalence of ISH-confirmed infections in intermediate hosts was relatively low, thus large sample sizes are necessary to find and confirm potential hosts, even though PCR-positive samples are typically more numerous and can point to candidate species for further study. Fortunately, advances in molecular diagnostics have greatly increased the capacity for processing large numbers of samples since the first molecular investigation 20 y ago.

The need for large samples is illustrated by the very low prevalence of at least four haplosporidians: *Bonamia perspora* in only 31 of 2,144 (1.4%) crested oysters *Ostreola equestris* (Carnegie et al. 2006); *Haplosporidium nelsoni* in 40 of 4,313 (0.9%) of Pacific oysters *Crassostrea gigas* (Kern 1976, Kang 1980, Friedman et al. 1991, Friedman 1996); *Haplosporidium armoricana* in just four of 5,400 (0.07%) flat oysters *Ostrea edulis* (van Banning 1979); and *Minchinia mercenariae* in two of several thousand hard clams *Mercenaria mercenaria* (Ford et al. 2009b).

The histological sampling of benthic invertebrates and zooplankton in Delaware Bay did involve large numbers of some species, was conducted during the warm season when oysters acquire *Haplosporidium nelsoni* infections, and spanned 2 y. Similarly, the PCR-based studies were conducted during the same period in four different years, but the numbers of individual organisms processed was limited by the relatively cumbersome processing protocol available in the early years, and involved far fewer organisms than have the more recent studies. The 2010 sampling of crustaceans using more modern and efficient methods allowed us to examine many more individuals, although the collection period was more limited. The protocol was designed to minimize the action of PCR inhibitors found in environmental samples, which would otherwise have diminished the chances of finding true positive reactions. Nevertheless, the results do not allow us to rule in or rule out any particular species as an alternate or intermediate host for *H. nelsoni*.

A final point that needs to be considered in the case of *Haplosporidium nelsoni* is that an alternate host is being looked for in which spores are formed regularly, whereas the *Marteilia* spp. search was for an intermediate host. In contrast to *H. nelsoni*, stages of *Marteilia* spp. do progress to spore formation in the bivalve hosts (Perkins 1976) even though direct transmission does not occur. The fact that an alternate host in which *H. nelsoni* sporulates regularly appears to be needed does not eliminate the possibility than an intermediate host is also required. Other haplosporidans do regularly produce spores but with a single possible exception (Barrow 1965), direct transmission has never been reported for any non-*Bonamia* haplosporidan species. It is, however, dubious that much serious effort has been expended to investigate transmission in these species.

Where to Go from Here

Successful searches for intermediate hosts of invertebrates have been conducted in confined systems (Andreadis 1985, Wolf et al. 1986, Audemard et al. 2002) or have targeted specific phyla using criteria from previous observations (Carrasco et al. 2007b, Adlard & Nolan 2015). Many of the areas in which *Haplosporidium nelsoni* is found are large estuaries, with hundreds of species that could be potential hosts. Smaller, more confined water bodies, such as the Atlantic coastal bays and rivers, would likely have fewer candidate species, but the *H. nelsoni* spore, presumably the transmission stage, is thick-walled and probably can survive for extended periods. The possibility that filaments projecting from the spore wall (Burreson & Reece 2006) assist with flotation provides further possibility for long-distance dispersal. Thus, focusing just on potential hosts in the immediate vicinity of infected oysters may be too limiting, and it may also enhance the likelihood of false-positive molecular signals resulting, not from true infections, but from ingestion or external adherence of the parasites or their DNA from those infected oysters (Messerman & Bowden 2016, and this study).

Most hypotheses link the introduction of *Haplosporidium nelsoni* to the east coast of the United States to transport of its Asian host, *Crassostrea gigas*, for commercial or experimental testing. Spores are found in *C. gigas* (Kern 1976, Friedman et al. 1991) so the presumed transmission stage could have been imported in those oysters; however, if another host is involved in its life cycle, that host would have had to be introduced and become naturalized, or have been native already. A number of different invertebrates have been infected by *Haplosporidium* spp., including molluscs, crustaceans and annelids, so members of the genus can clearly live in a range of host types. Although it may be difficult at this point to pinpoint when in the past alien species appeared in Delaware and Chesapeake bays, a more feasible approach might be to look for species that have migrated northward along the Atlantic coast of North America over the past three decades as epizootics caused by *H. nelsoni* have moved northward. Whereas it is possible that acceptable alternate hosts could already have been present along the northeast coast, and *H. nelsoni* could have eventually invaded these areas from a point source, it is noted that *H. nelsoni* was present in waters from New York to Maine (Haskin & Andrews 1988, Burge et al. 2014) well before the onset of major epizootics in the northeast, which suggests that a change in distribution or abundance of a key alternate host species may have contributed to the observed pattern of outbreaks in the north. A scarcity of potential alternate hosts has been hypothesized as an explanation for why epizootics have not occurred in the southeastern United States despite the known presence of *H. nelsoni* and what would seem to be favorable environmental conditions in the region (Hofmann et al. 2001). Lack of an appropriate alternate or intermediate host may also be a reason for the apparent absence of *H. nelsoni* in Gulf of Mexico oysters (Ford et al. 2011).

Recent publications have documented the development of resistance to *Haplosporidium nelsoni* in natural populations of oysters in areas of the lower Chesapeake Bay and much of Delaware Bay (Carnegie & Burreson 2011, Ford & Bushek 2012, Bushek & Ford 2016), therefore, searching for another host may now seem like an esoteric undertaking. But the parasite has by no means disappeared. Prevalence fluctuates from year to year, continuing to contribute to oyster mortality, even where resistance minimizes its impact. Furthermore, there are large regions where oysters are still susceptible, and recent epizootics in Maine and Canada (Stephenson et al. 2003, Messerman et al. 2014) clearly show that it is still a very destructive parasite. Finally, definitively showing that infective stages of *H. nelsoni* are produced in another host has obvious implications for the transfer, from enzootic waters, of oyster seed for aquaculture.

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