STUDIES ON LIFE CYCLE STAGES OF THE OYSTER PARASITE *HAPLOSPORIDIUM NELSONI* (MSX)

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FINAL Report

to

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by

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Background

This project responded to priority **B** in the **Technical** category of the 1990 NOAA/NMFS **Request for Proposals on Disease Research on the American Oyster**: "Study selected aspects of life cycle and natural history stages of oyster parasites". It deals with selected aspects of the life cycle of *Haplosporidium nelsoni* (MSX), as described below.

Life cycle studies and transmission experiments on *Haplosporidium nelsoni* began soon after the parasite was first identified as the cause of epizootic mortalities of oysters, *Crassostrea virginica*, in Delaware and lower Chesapeake Bays in the late 1950s and early 1960s (Sprague 1962; Canzonier 1968). One outcome of these studies was that the spore stage, presumed to be a necessary part of the transmission process, was extremely rare in oysters. The general conclusion of these investigations was that an alternate or reservoir host was probably producing the stages that infected oysters, that the oyster might not be a natural host for *H. nelsoni* , and that the supply of infective particles might be totally independent of the supply of oysters (Sprague 1962; Farley 1967; Andrews 1968; Canzonier 1968; Ford and Haskin 1982).

Recent findings at this laboratory had challenged this hypothesis with the discovery that, in contrast to older oysters that had previously received most attention by pathologists, oyster spat (<1 yr) appeared to produce spores of *H. nelsoni* when they became infected (Barber et al. 1991). A second significant finding relevant to the life cycle of *H. nelsoni* occurred during examination of histological samples collected in Delaware Bay in October, 1989. We found operculated spores, resembling those of *H. nelsoni* in size and shape, in the digestive tract lumina (stomach, mid-gut, and intestine) of a number of mixed-age oysters. If these were spores of *H. nelsoni*, they should be an index of the pool of potential infective stages and, as such, might provide an important predictive measure of subsequent infection levels. Thus, the **first objective** of the project was to pursue these findings, which indicated that sporulation of *H. nelsoni* might occur regularly in spat, and that haplosporidan spores resembling those of *H. nelsoni*, and presumed to be ingested, were present in the water column).

Whereas spores are probably the transmission stage outside the oyster, the primary stage of *H. nelsoni* inside the oyster is the plasmodium. It is this stage that the oyster's defense mechanisms must recognize and eliminate if they are to be effective. In oysters, the hemocytes, or blood cells, are assumed to be the primary agents of defense against invading organisms (Stauber 1950; Tripp 1960; Cheng 1984). Hemocytes do aggregate in lesions containing the parasite (Farley 1968; Myhre 1973; Ford 1985)but their function is in doubt because evidence for phagocytosis or encapsulation is lacking from tissue sections (Douglass 1977; Ford 1988). Recent *in vitro* experiments at this lab had shown that fewer than 5% of plasmodia are phagocytosed, even by hemocytes from resistant strains. Thus, our **second objective** was to continue investigation of the response of host oysters to invasion by the parasite by *in vitro* methods.

From 1957, when *H. nelsoni* caused the first epizootic mortalities of oysters in Delaware Bay, through the 1970s, reports of the parasite were restricted largely to the mid-Atlantic states, from Connecticut south to Virginia. In the 1980s, however, *H. nelsoni* was reported from Maine to Florida, with epizootic outbreaks on Cape Cod, Long Island, and North Carolina (Haskin and Andrews 1988). A number of hypotheses existed to explain these events:

introduction of the parasite through oyster transplants; spread of an intermediate or alternate host; spread of spores directly by water currents, etc. Our **third objective** was to convene a workshop on the recent spread and intensification of *H. nelsoni*, to involve not only researchers and managers directly concerned, but also experts with knowledge about the distribution of other spore or cyst-forming marine organisms (e.g., myxosporeans, microsporeans, toxic dinoflagellates, etc.).

The accomplishments and results (and their significance) of this project are reported separately for each objective. Two manuscripts arising from this work are appended.

Major Objective 1: Develop information on the significance of sporulation of *H. nelsoni* in young oysters and on the findings of ingested haplosporidan spores in the digestive tract lumina of oysters.

1) Determine the temporal and spatial extent of sporulation of *Haplosporidium nelsoni* in spat and yearling oysters in Delaware Bay during 1991 and 1992.

Accomplishments:

At approximately weekly intervals between April 23 and October 1, 1991, 2810 spat of the 1990 year class were collected from the intertidal sand flats in lower Delaware Bay in front of the Rutgers Cape Shore Laboratory (Table 1). An additional 1634 spat from the same year class were collected from other locations in the Bay, mostly during June (Table 1). Also, 1043 spat, which had set during 1991, were collected from the Cape Shore flats between October 1 and December 13 (Table 1).

In 1992, 3102 spat (1991 year class) were collected at the Cape Shore between May 21 and September 28; and another 120 were collected from two other locations in the Bay in June (Table 2). Except at the Cape Shore, setting was sparse in 1991 (thus the small number of spat examined from other locations in 1992) and in 1992, setting was extremely light even at the Cape Shore. Because no native spat of the 1992 year class were available for sampling, we made two collections, during October 1992, from a group of hatchery-reared susceptible 1992 year class spat, which had been on the flats since June (Table 2).

Except for one collection date (June 6, 1991) when some spat with good recent shell growth were not shucked, all spat were opened and those with morphological indications of disease: lack of shell growth, pale digestive glands, emaciation, or a combination of the three ("SICK" designation in Tables 1 and 2) were selected for microscopic examination of digestive gland smears. Previous results had shown that spat with *H. nelsoni* spores always exhibited one of these conditions. Spat were scored as being patently infected or uninfected by *H. nelsoni* . Infected spat were categorized as having light or advanced infections and the presence of spores was also recorded.

Subsamples of 40 spat not examined by fresh smear were fixed for histological examination at each collection because we felt that accurate prevalence data could be obtained only from tissue slides. A total of 1485 spat fixed in 1991 were processed

into tissue sections and examined for the presence and intensity of *H. nelsoni* plasmodia and spore stages. Based on results of this examination, we were able to reduce the number of spat processed into tissue section in 1992 to 280, selected as representative of the infection cycle. Our final prevalence figures were a combination of those found in the fresh examination and those found by tissue slide histology of the apparently "healthy" spat (Tables 3 and 4).

Results:

In 1991, the maximum prevalence in previous-year spat at the Cape Shore was 50%; in 1992, it was 17% (Tables 3 and 4). In most samples from both years, it was 5% or less. Spores of *H. nelsoni* were found from early June through the third week in July in 1991. The following year, we found spores in our first sample, collected May 21, and continued finding them through July 21. Over all sampling dates, about 40% of the spat that had advanced plasmodial infections also had spores, although peak prevalence of spores in advanced infections was 75-100% (Tables 1 and 2). We did not find spores in current-year spat in either year (e.g., 1991 set in fall 1991). In addition to the Cape Shore, *H. nelsoni* spores were found in most other locations sampled in 1991 and 1992 (Fig. 1; Tables 1 and 2).

As expected, the total prevalence of H. nelsoni infections was found to be higher in the histological samples than in the fresh smears. Prevalences obtained from combined fresh and histological examinations ranged from 2 to 46 percentage points higher than those from the fresh exam alone (Tables 3 and 4). In 1991, we found a maximum of 10% prevalence by fresh exam and a maximum of 50% in the same sample by tissue section histology. In 1992, these figures were 8% and 17%, respectively. The greatest difference was in early and mid July in 1991 and in late July and early August in 1992, reflecting the onset of new, gill infection that would not have been found by fresh smears of the digestive gland. Only three cases of sporulation (all in 1991) were missed in the fresh screening (according to tissue slide results). These three appeared to be healthy (had abundant gonad) and were not selected for fresh smear examination. We also missed very few advanced infections by our fresh screening process. Our estimates of maximum spore prevalence (5% in 1991 and 3% in 1992) were essentially the same by both fresh smear and tissue slide examination.

2) Collect spores for *in vitro* studies and transmission experiments.

Accomplishments:

All spat in which spores were found in fresh examination were placed in regularly changed filtered sea water and allowed to rot. In some cases, shells were measured and these individuals were placed in separate tubes so we could determine the number of spores produced in spat of various sizes. The resulting spore preparations were held at 4^{o} C. The total number of spores present in 18 individual spat was determined from these samples by counting 4 aliquots from each in a hemocytometer.

Results:

There was no correlation between spat size (range:10x13mm to 28x31mm) and the number of spores present. In 1991, the highest number of spores in an individual was 1.1 million. In 1992, however, the highest count was 0.42 million and the mean for both years combined was about 0.16 million per individual, with large individual variability (Table 5). The rather large variance among replicate counts for individual oysters was caused by the tendency of spores to aggregate. Forcing suspensions through a hypodermic needle lessened this problem. These are counts from tissues remaining after sampling for diagnosis; an unknown number of spores were undoubtedly lost from each sample in diagnosing and processing. Interestingly, the spores were not fully mature in most of the spat.

3) Examine archived tissue sections for the presence of haplosporidan spores in digestive tract lumina. Note: The attached reprint "Occurrence and significance of ingested haplosporidan spores in the eastern oyster, *Crassostrea virginica* (Gmelin, 1791)" has detailed results and discussion of this segment of the project, highlights of which are provided here.

Accomplishments:

A total of 3292 archived slides was examined for the presence and abundance of haplosporidan spores in digestive tract lumina. The slides were selected to represent 1) series with continuous sampling over an entire year or more, 2) upbay-downbay gradients at periods of high ingested spore presence, and 3) years of high and low *H. nelsoni* infection prevalence.

Fresh gut contents of oysters suspended in the Maurice River, a tributary of lower Delaware Bay, were examined in November and December 1991.

Results:

Ingested haplosporidan spores were found at all locations examined in Delaware Bay, as well as in several other US east coast locations where *H. nelsoni*-infected oysters have been found. They predominated from May through October when they were present in 20% to 40% of the oysters examined, with a mean frequency of 0.5 spores per tissue section. We calculated that this would extrapolate to several thousand spores in the entire digestive tract and several hundred per liter in the water filtered by the oysters. Although the spores were present during the infection period for *H. nelsoni*, their frequency showed a weak, negative correlation ($r = -0.55$; $p < 0.02$; $N = 17$ years) with *H. nelsoni* prevalence the following year, suggesting that if they are a stage in the life cycle of that parasite, they are not directly infective to oysters, but may infect an alternate host.

Fresh spores were found in the gut contents of oysters suspended in the Maurice River during October and November, 1991. The mean size of these spores was similar to that of *H. louisiana*, a parasite of xanthid crabs (Sprague 1963; Perkins 1979), which are known to be present in the River. A total of 3108 spores was counted in a 70-μL sample of stomach contents from a single small oyster (shell dimensions 25 x

21.5 mm). Subsequent examination of tissue sections from 21 oysters in the same group revealed spore counts ranging from 0 to 9 per section, with a mean of 4.1. Apparently intact spores were found in feces of oysters brought from the River into the laboratory and placed in aquaria.

Significance:

It is clear from the extensive sampling in 1991 and 1992, along with more limited sampling from 1988 through 1990, that once a spat becomes infected, and especially if the infection becomes advanced (moderate to heavy systemic), there is a high probability that spores will be formed. The maximum proportion of sporulating cases among advanced infections (75-100%) is probably an accurate indication of the number of cases ending in sporulation. Smaller proportions preceding the peak may represent a phase in the development cycle that culminates in the maximum figures. The high frequency of sporulation in spat differs greatly from that in older oysters, in which far fewer than 1% of infected individuals have ever been found with spores. Spat are much smaller than adult oysters and have a higher metabolic rate; however, whether either of these conditions helps to support sporulation is presently not known. We noted that most of the spores we found did not appear to be fully mature. Similarly, Andrews (1978) observed that spores of *H. costale*, which are regularly produced in infected oysters, were not mature when oysters died. This might mean that spores (of both species) released from oysters, if they are critical to the life cycle, would have to mature in sea water or in another (intermediate) host or vector. On the other hand, it is possible that because we sampled live spat, we did not see the completion of the sporulation process for *H. nelsoni*, which may culminate just before death producing higher numbers of mature spores than we observed.

The spore prevalence figures and the counts of spores per spat, along with our calculations of the probable concentrations of *H. nelsoni*-like spores in the water column (see Barber and Ford 1992) allow us to make some very broad estimates as to whether spat could produce sufficient numbers of known *H. nelsoni* spores to account for the numbers of *H. nelsoni*-like spores in the water. Although prevalences of spores produced by infected spat have been low (5%) in the past three years, the number of spores formed in individual spat may be up to one million. It is difficult to estimate the total number of spat present in Delaware Bay during any given year, but based on extensive dredge sampling on Delaware Bay seed beds over the past 35 years, we believe that a figure of 100 spat per m^2 to be a reasonable average. From this, we calculate that there are 10^{10} - 10^{12} spat in Delaware Bay on an "average" year. We estimate that it would take 10^9 -10^{10} spore-producing spat, each releasing one million spores, to produce the concentration of spores in the water that we estimated from ingested *H. nelsoni*-like spores (Barber and Ford 1992). If we use the mean number of spores from our individual counts (1.6×10^5 spores per spat), we obtain a figure of 10^{10}-10^{11} spore-producing spat. We caution that these calculations make many assumptions, and do not take into account potential constant spore loss from the estuary in current outflow, loss from the water column through biodeposition (e.g., oyster feces), or destruction by microbes in the sediment. Because of these many sources of error, we think that if our comparative figures match within a couple of orders of magnitude, which they do, we must consider that *H. nelsoni* spores from infected oyster spat could be produced in sufficient quantity to be significant elements of the life cycle. Even if the ingested spores are not *H. nelsoni*, we can still use their abundance (several hundred per liter) as a gauge against which to measure *H. nelsoni* spore

production in spat. Spores are produced (and released) from spat during the period when oysters are becoming infected, and in some years at least, the potential number of spores produced in this manner may be very high. Spores from spat may directly infect other oysters or an intermediate host, or may mirror spore production in an alternate ("normal") host. On the other hand, because spores are not produced in spat in excess (i.e., many orders of magnitude greater than needed to match the calculated number of "ingested" spores in the water), we must continue to look for potential alternate or intermediate hosts.

Major Objective 2: Conduct *in vitro* experiments with plasmodia of *H. nelsoni* , which will clarify interactions between oysters and this life cycle stage at the cellular level. Note that our proposal indicated that the degree of effort to be expended on this objective would depend on whether spores were found while pursuing Major Objective 1. Because they were, especially in the "ingested spore" segment, we did concentrate on Objective 1 rather than Objective 2. A reprint "In vitro interactions between bivalve hemocytes and the oyster pathogen *Haplosporidium nelsoni* (MSX)" is appended. It details results and significance of some of the experiments supported on this project.

1) Determine whether the parasite produces an inhibitory substance or whether it is not recognized as foreign because it has surface receptors similar to those on oyster cells.

Accomplishments:

A number of in vitro experiments were conducted to examine the responses of oyster hemocytes to plasmodia treated with enzymes to cleave surface proteins. Plasmodia were enriched by panning (Ford et al. 1990) then incubated in several concentrations (1, 7, and 10 mg/mL) of trypsin (crude and purified) and proteinase K. The former is a rather specific enzyme that cleaves peptide bonds after the carboxyl group of (positively charged) lysine or arginine, whereas the latter is more general, cleaving peptide bonds after the carboxyl group of N-substituted hydrophobic, aliphatic, or aromatic amino acids. After enzyme treatment, the plasmodia were incubated with oyster hemocytes and the fraction of plasmodia that had been ingested by the hemocytes after an hour was recorded. In order to begin investigating the "real-time" interactions between plasmodia and hemocytes, we purchased a microscope-mounted video-camera (not on NOAA funds). Because this arrived at the end of the contract, we were able to make only very preliminary observations, including video taping, of interactions between oyster hemocytes and *H. nelsoni* plasmodia.

Results:

All enzyme treatments increased phagocytosis over controls, but the crude trypsin (which undoubtedly contained other enzymes) and the (nonspecific) proteinase K resulted in the highest rates (Fig. 2). Trypan blue dye uptake indicated that the treated plasmodia remained viable, or at least that their membranes remained intact. These results indicate that modifying surface protein structure on the parasites increased the ability of hemocytes to recognize them as "non-self". This, in turn, suggests that the intact surface proteins may have been instrumental in making the parasite appear to be "self".

We were particularly interested in documenting, by video tape, "retreat" type responses on the part of hemocytes after they contact plasmodia. We did see this type of response, but it did not occur in all interactions and it also appeared to happen in some hemocyte-hemocyte encounters. Clearly, a rigorous analysis of these interactions is necessary before any conclusions can be reached.

2) Determine whether soluble components of hemolymph or tissue extracts of resistant oysters kill or damage plasmodia making them more likely to be phagocytosed.

Accomplishments:

Based on our earlier findings that *H. nelsoni* plasmodia killed by immersion in low salinity water are immediately recognized and phagocytosed by oyster hemocytes, we had hypothesized that plasmodia entering the circulation of resistant oysters might be killed or damaged by a soluble component of the hemolymph. If this occurred, phagocytosis and intracellular degradation of parasites might occur too rapidly to be recorded in tissue sections, which would help explain the relative rarity of systemic infections in resistant oysters. Plasmodia collected from heavily infected oysters were enriched by panning and incubated in hemolymph from susceptible (James River, VA) and resistant oysters (6th generation selected), and in a filtered sea water control. Incubations were short term (1-2 h) and long term (20-24 h). Trypan blue dye was added as an indication of membrane integrity (i.e., viability). This experiment was performed during the fall and winter, and again in summer. Our rationale for examining the seasonal effect was that earlier studies at our laboratory indicated that the maximum expression of "resistance" occurs during the summer, when water temperatures are above 20^{o} C (see (Ford 1988)).

Results:

In our short-term experiments, dye uptake was identical in all three treatments indicating that there was no rapidly acting cytotoxic effect of serum from resistant oysters that would damage or kill plasmodia, causing them to be recognized and phagocytosed by hemocytes. There was some suggestion, however, of lower plasmodial survival after incubation of 20-24 h in serum of resistant oysters compared to serum from susceptible oysters (this experiment was performed in summer only). These results are preliminary, however, and somewhat difficult to interpret because overall plasmodial survival after the longer incubation was low in both sera (<50%). We intend to pursue this finding.

Significance:

Too few data were obtained on this project to make any concrete statements about the relative importance of surface receptors or inhibitor compounds in dictating the reaction of oyster hemocytes to *H. nelsoni* plasmodia or the mechanism of resistance against the parasite. In combination with information from other projects, however, they do provide some important information on host-parasite interactions. Although treatment with proteases such as trypsin

and proteinase K did enhance phagocytosis of plasmodia and supported the contention that hemocytes fail to recognize them as "non-self", other tests with inhibitors of aerobic or anaerobic metabolism resulted in a far greater phagocytic response. It is probable that metabolic inhibitors would also have affected surface components by halting production of host-like receptors or active uptake of host molecules from hemolymph, but because phagocytosis was several times greater than after surface modification alone, we are inclined to suspect that an inhibitory compound may well be involved. All available evidence so far indicates that phagocytosis, the most important mechanism typically assigned to defense, is not operating against *H. nelsoni*. Although admittedly crude and preliminary, our long-term incubation (20-24 h) results, in which viability was lower in resistant compared to susceptible oyster sera, hints that lowered susceptibility (susceptibility = providing proper environment for the parasite) rather than a better active defense, may be responsible for improved survival in "resistant" strains (see (Ford 1988).

Major Objective 3: Convene a workshop on the recent spread and intensification of *H. nelsoni*, which will include experts with knowledge about the distribution of other spore or cyst-forming marine organisms, model epizootics, and nearshore ocean circulation, as well as researchers and managers with direct knowledge of the oyster parasite .

Accomplishments:

Twenty researchers with expertise in *H. nelsoni* biology, life cycles and transmission of other parasites (marine and insect), oceanography (estuarine and nearshore circulation), epidemiology, and particle transport met at the Haskin Shellfish Research Laboratory for a day and a half workshop on March 18 and 19, 1992 (Table 6). The objective was to develop new ideas about how to investigate the *H. nelsoni* life cycle and mode of transmission.

Results:

The following is a summary of a number of ideas, deductions, and recommendations that were made by the "outside" experts. These are not necessarily new, but in many cases they are more refined by experience in other systems. They should not be considered the "final word" on the subject; rather, they are intended to stimulate future research.

1) It is likely that an alternate or intermediate host, or both, exists for *H. nelsoni*, but we should not give up on looking at the possibility of direct transmission via spores produced in oyster spat (< 1-yr old).

2) It is not surprising that spores would be produced in only one life stage (spat) of the host (oyster); similar occurrences are known in mosquitoes.

3) Because spores are so infrequent in adult oysters, the oyster may be an abnormal/adventitious host. In this case, an alternate (normal) host would exist that would most likely be very similar to the oyster (i.e., a sessile bivalve) and the seasonal infection cycle would probably also be similar.

4) 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 (i.e., zooplankton, including larval forms). The parasite must have some mechanism to maintain itself near potential hosts (oyster or other similar estuarine species) in the estuary. The potential host is not likely to be a commercially valuable fish species because these have been examined extensively for parasites. Small non-commercial species are candidates, but haplosporidans have never been found in a vertebrate host.

5) An *H. nelsoni* spore produced in another host might not exactly resemble that produced in oysters, but would be in the classified in same phylum. (*Myxobolus cerebralis*, the agent of whirling disease in salmon, for instance, forms two distinct spores in two different hosts: a myxosporean spore in the salmon and a triactinomyxon spore in tubificid worms.) Spore size should not be considered an immutable criterion for differentiating between species.

6) Potential intermediate hosts should match the geographical distribution of *H. nelsoni* and should be producing spores just before oysters become infected. Such intermediate hosts may experience a rapid die-off at this time; also, sporulation may be extensive in the tissues, causing discoloration that may be evident macroscopically or in fresh squashes.

7) Water transport mechanisms could have moved spore-sized particles from Delaware to Chesapeake Bays; currents can transport particles 40 km offshore in the mid-Atlantic and then return them to estuaries. The general drift pattern is from north to south along the coast.

8) Current speeds in Delaware Bay are too great (14-km tidal excursion) to postulate that the delay (measured in weeks) in the onset of *H. nelsoni* infections in an up-estuary direction in Delaware Bay is caused by dispersion of infective particles (spores) by water movements from a downbay source. But the vertical position of the particles in the water column would influence their distribution.

9) Spores could be looked for in water and sediment samples, employing centrifugation and sieving to concentrate appropriate-size particles. Using oysters themselves as trapping and concentrating mechanisms (i.e., search of gut contents) may be the best way.

10) The viability of *H. nelsoni* spores in the environment may not be great because ultrastructurally the attachment of the spore lid does not appear to be very strong. On the other hand, dye exclusion studies suggest that they could be long-lived. Also, spores that settled to the bottom would likely be attacked by bacteria and bottom feeders. Conversely, ingested spores might survive the digestive process or even be made infective in the gut of a vector species, which might also transport them.

11) Previous transmission studies with *H. nelsoni* and *H. costale* spores may have been compromised by lack of freshness of spores. Experience in other in other host-parasite systems indicates that fresh spores are needed for transmission.

12) A detailed, ultrastructural comparison of *H. nelsoni* stages in spat and adult oysters during the sporulation period may provide clues as to why spores are so rarely formed in adults. Comparisons should also be made of physiological differences (e.g., enzyme activities or concentrations of metabolic substrates/products).

13) The use of haplosporidans that regularly produce spores (*H. costale* or *M. teredinis*) as models would be a valuable avenue of research.

14) A combination of settling and filtration of water before passing it over oysters would help determine infective particle characteristics.

Significance:

The suggestions and conclusions of the workshop, especially as they relate to the possibility of an alternate or intermediate host(s) stimulated us to write a follow-up proposal to the present one, entitled "Life cycle studies of *Haplosporidium nelsoni* (MSX): Spores and non-oyster hosts". It has been funded by the Oyster Disease Research Program.

LITERATURE CITED

Andrews, J. D. 1968. Oyster mortality studies in Virginia. VII. Review of epizootiology and origin of *Minchinia nelsoni*. Proc. Nat. Shellfish. Ass. 58: 23-36.

Andrews, J. D., and M. Castagna. 1978. Epizootiology of *Minchinia costalis* in susceptible oysters in seaside bays of Virginia's Eastern Shore,1959-1976. J. Invertebr. Pathol. 32: 124-138.

Barber, R. D., and S. E. Ford. 1992. Occurrence and significance of ingested haplosporidan spores in the eastern oyster, *Crassostrea virginica* (Gmelin, 1791). J. Shellfish Res. 11: 371-375.

Barber, R. D., S. A. Kanaley, and S. E. Ford. 1991. Evidence for regular sporulation by *Haplosporidium nelsoni* (MSX) (Ascetospora: Haplosporidiidae) in spat of the American oyster, *Crassostrea virginica*. J. Protozool. 38: 305-306.

Canzonier, W. J. 1968. Present status of attempts to transmit *Minchinia nelsoni* under controlled conditions. Proc. Nat. Shellfish. Ass. 58: 1.

Cheng, T. C. 1984. A classification of molluscan hemocytes based on functional evidences. p. 111-146. *In* [ed.] Invertebrate Blood. Cells and Serum Factors. Vol. 6. Plenum, New York.

Douglass, W. R. 1977. *Minchinia nelsoni* disease development, host defense reactions, and hemolymph enzyme alterations in stocks of oysters (*Crassostrea virginica*) resistant and susceptible to *Minchinia nelsoni*-caused mortality. Ph.D. Dissertation. Rutgers University. 232.

Farley, C. A. 1967. A proposed life cycle of *Minchinia nelsoni* (Haplosporida, Haplosporidiidae) in the American oyster *Crassostrea virginica*. J. Protozool. 14: 616-625.

Farley, C. A. 1968. *Minchinia nelsoni* (Haplosporida) disease syndrome in the American oyster *Crassostrea virginica*. J. Protozool. 15: 585-599.

Ford, S. E. 1985. Chronic infections of *Haplosporidium nelsoni* (MSX) in the oyster *Crassostrea virginica*. J. Invertebr. Pathol. 45: 94-107.

Ford, S. E. 1988. Host parasite interactions in oysters, *Crassostrea virginica*, selected for resistance to *Haplosporidium nelsoni* (MSX) disease: survival mechanisms against a natural pathogen. p. 206-224. *In* W. S. Fisher [ed.] Disease Processes in Marine Bivalve Molluscs. Vol. 18. American Fisheries Society, Bethesda, MD.

Ford, S. E., and H. H. Haskin. 1982. History and epizootiology of *Haplosporidium nelsoni* (MSX), an oyster pathogen, in Delaware Bay, 1957-1980. J. Invertebr. Pathol. 40: 118-141.

Ford, S. E., S. A. Kanaley, M. Ferris, and K. A. Ashton-Alcox. 1990. "Panning", a technique for enrichment of the parasite *Haplosporidium nelsoni* (MSX) from hemolymph of infected oysters. J. Invertebr. Pathol. 56: 347-352.

Myhre, J. L. 1973. Levels of infection in spat of *Crassostrea virginica* and mechanisms of resistance to the haplosporidan parasite *Minchinia nelsoni*. M.S. Rutgers University, New Brunswick, N. J. 102 pp.

Perkins, F. O. 1979. Cell structure of shellfish pathogens and hyperparasites in the genera *Minchinia, Urosporidium, Haplosporidium*, and *Marteilia* - taxonomic implications. Mar. Fisheries Rev. 41: 25-37.

Sprague, V. 1962. An alternate host for MSX. 61-44. Chesapeake Biological Laboratory.

Sprague, V. 1963. *Minchinia louisiana* n. sp. (Haplosporidia, Haplosporidiidae), a parasite of *Panopeus herbstii.* J. Protozool. 10: 267-274.

Stauber, L. A. 1950. The fate of india ink injected intracardially into the oyster, *Ostrea virginica* Gmelin. Biol. Bull. 119: 273-282.

Tripp, M. R. 1960. Mechanisms of removal of injected microorganisms from the American oyster, *Crassostrea virginica* (Gmelin). Biol. Bull. 119: 273-282.

Figure 1. Delaware Bay locations where *Haplosporidium nelsoni* spores were found in oyster spat during 1991 and 1992 sampling. Location are identified in Tables 1 and 2.

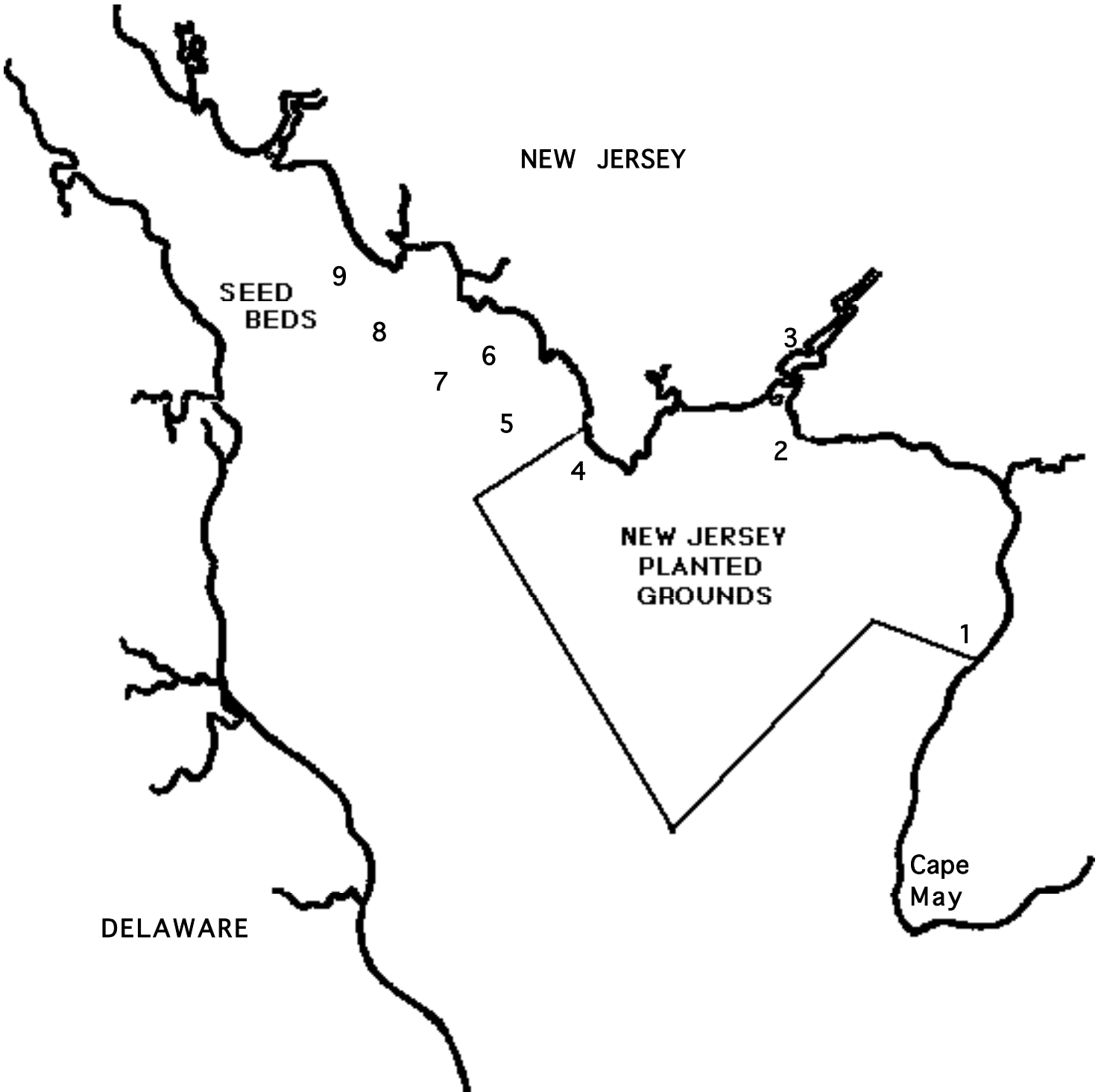

Figure 2. Percent of *Haplosporidium nelsoni* (MSX) plasmodia found in categories of interaction with oyster hemocytes after treatment with proteinases and incubation with the oyster cells for one hour. Single - plasmodia are alone; Touching - hemocytes are in contact with plasmodia; Ingested - hemocytes have ingested plasmodia. Vertical bars are the standard error of the mean of 2-4 replicates. In each replicate, 300-600 plasmodia were counted and classified.

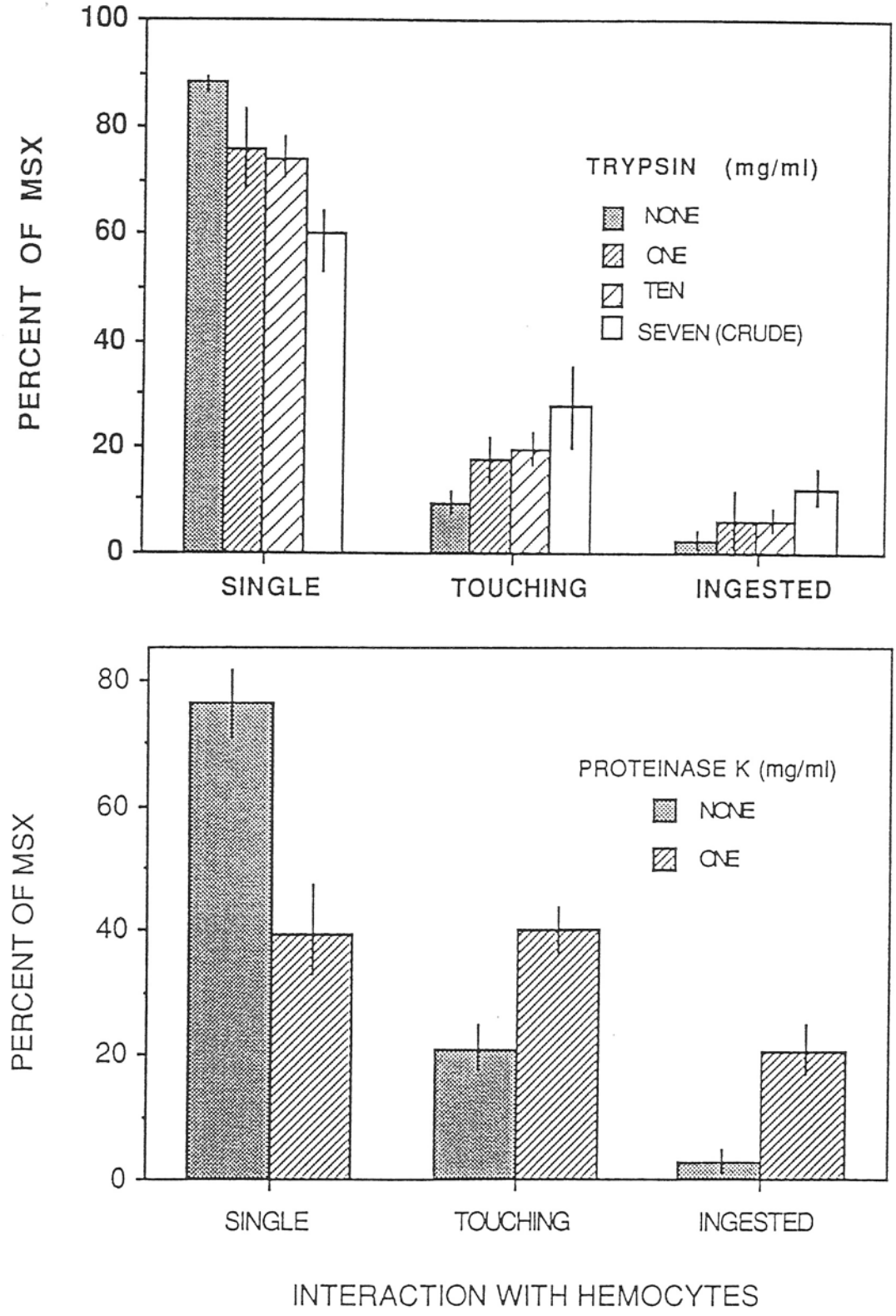

Table 1. Prevalence of all *Haplosporidium nelsoni* infections and infections with spores in oyster spat collected in 1991.

CHT LOC	GRD	COLL. DATE	TOT COLL.	TOTAL SHUCK	TOT SICK	LT. PLAS.	ADV PLAS.	TOTAL PLAS.	TOTAL PRE-SP	TOT SP	TOT INF.	PREVALENCE TOT	SP	LT INF	ADV INF	SP/INF	SP/ADV
	1990 SET																
1	CAPESHORE	4/23	78	78	13	5	3	8	0	0	8	10%	0%	63%	38%	0%	0%
1	CAPESHORE	5/10	61	61	12	0	0	0	0	0	0	0%	0%	0%	0%		
1	CAPESHORE	6/3	61	61	4	0	4	4	0	3	4	7%	5%	0%	100%	75%	75%
1	CAPESHORE	6/6	247	117	19	2	10	12	1	7	12	10%	6%	17%	83%	58%	70%
1	CAPESHORE	6/8	55	55	5	0	3	3	0	3	3	5%	5%	0%	100%	100%	100%
1	CAPESHORE	6/9	134	134	15	5	2	7	0	0	7	5%	0%	71%	29%	0%	0%
1	CAPESHORE	6/12	152	152	11	1	10	11	1	6	11	7%	4%	9%	91%	55%	60%
1	CAPESHORE	6/17	243	243	11	3	6	9	1	6	9	4%	2%	33%	67%	67%	100%
1	CAPESHORE	6/20	27	27	5	0	2	2	0	2	2	7%	7%	0%	100%	100%	100%
1	CAPESHORE	6/30	73	73	3	0	2	2	0	1	2	3%	1%	0%	100%	50%	50%
1	CAPESHORE	7/8	67	67	3	0	3	3	0	2	3	4%	3%	0%	100%	67%	67%
1	CAPESHORE	7/15	196	196	6	1	3	4	0	3	4	2%	2%	25%	75%	75%	100%
1	CAPESHORE	7/22	224	224	14	4	3	7	1	3	7	3%	1%	25%	75%	43%	100%
1	CAPESHORE	7/29	228	228	12	3	1	4	0	0	4	2%	0%	25%	75%	0%	0%
1	CAPESHORE	8/5	204	204	10	1	2	3	0	0	3	1%	0%	25%	75%	0%	0%
1	CAPESHORE	8/13	215	215	5	1	0	1	0	0	1	0%	0%	25%	75%	0%	
1	CAPESHORE	8/27	264	264	4	1	2	3	0	0	3	1%	0%	25%	75%	0%	0%
1	CAPESHORE	9/4	129	129	12	2	5	7	0	0	7	5%	0%	25%	75%	0%	0%
1	CAPESHORE	9/11	117	117	7	0	3	3	0	1	3	3%	1%	25%	75%	33%	33%
1	CAPESHORE	9/19	120	120	6	1	0	1	0	0	1	1%	0%	25%	75%	0%	
1	CAPESHORE	10/1	54	54	1	0	0	0	0	0	0	0%	0%	25%	75%		
		TOT	**2949**	**2819**	**178**	**30**	**64**	**94**	**4**	**37**	**94**	**3%**	**1%**	**32%**	**68%**	**39%**	**58%**
	1991 SET																
1	CAPESHORE	10/1	138	138	1	1	0	1	0	0	1	1%	0%	100%	0%	0%	
1	CAPESHORE	10/24	233	233	12	5	0	5	0	0	5	2%	0%	100%	0%	0%	
1	CAPESHORE	11/12	185	185	20	3	1	4	0	0	4	2%	0%	75%	25%	0%	0%
1	CAPESHORE	11/19	166	166	9	2	0	2	0	0	2	1%	0%	100%	0%	0%	
1	CAPESHORE	11/25	134	134	8	1	1	2	0	0	2	1%	0%	50%	50%	0%	0%
1	CAPESHORE	12/5	120	120	18	2	1	3	0	0	3	3%	0%	67%	33%	0%	0%
1	CAPESHORE	12/13	67	67	ALL	0	0	0	0	0	0	0%	0%	0%	0%		
		TOT	**1043**	**1043**	**68**	**14**	**3**	**17**	**0**	**0**	**17**	**2%**	**0%**	**82%**	**18%**	**0%**	**0%**

Table 1 (con't). Prevalence of all *Haplosporidium nelsoni* infections and infections with spores in oyster spat collected in 1991.

CHT LOC	GRD	COLL. DATE	TOT COLL.	TOTAL SHUCK	TOT SICK	LT. PLAS.	ADV PLAS.	TOTAL PLAS.	TOTAL PRE-SP	TOT SP	TOT INF.	PREVALENCE TOT	SP	LT INF	ADV INF	SP/INF	SP/ADV
	1990 SET																
	LOWER BAY																
3	MAURICE R.	6/16	235	87	13	0	2	2	0	0	2	2%	0%	0%	100%	0%	0%
4	381D	5/3	28	28	0	1	0	1	0	0	1	4%	0%	100%	0%	0%	
2	EAST POINT	5/10	40	40	6	0	0	0	0	0	0	0%	0%				
2	EAST POINT	6/6	137	117	12	2	2	4	0	2	4	3%	2%	50%	50%	50%	100%
4	455D	4/24	32	32	4	1	0	1	0	0	1	3%	0%	100%	0%	0%	
4	455D	6/10	32	32	3	0	0	0	0	0	0	0%	0%				
4	455D	6/21	51	51	2	0	1	1	0	1	1	2%	2%	0%	100%	100%	100%
		TOT	**555**	**387**	**40**	**4**	**5**	**9**	**0**	**3**	**9**	**2%**	**1%**	**44%**	**56%**	**33%**	**60%**
	UPPER BAY																
5	EGG ISL	6/21	49	49	2	1	1	2	0	1	2	4%	2%	50%	50%	50%	100%
6	STRAWBERRY	6/7	84	84	7	1	4	5	1	2	5	6%	2%	20%	80%	40%	50%
6	STRAWBERRY	6/10	111	111	5	1	0	1	0	0	1	1%	0%	100%	0%	0%	
6	STRAWBERRY	6/21	87	87	3	0	0	0	0	0	0	0%	0%				
7	NEW BEDS	6/7	138	138	8	3	4	7	0	1	7	5%	1%	43%	57%	14%	25%
7	NEW BEDS	6/21	77	77	0	0	0	0	0	0	0	0%	0%				
8	BENNIES	6/7	104	104	4	0	1	1	0	0	1	1%	0%	0%	100%	0%	0%
8	BENNIES	6/21	90	90	3	2	0	2	0	0	2	2%	0%	100%	0%	0%	
9	SHELL ROCK	6/7	68	68	2	0	0	0	0	0	0	0%	0%				
9	SHELL ROCK	6/21	101	101	5	0	0	0	0	0	0	0%	0%				
		TOT	**909**	**909**	**39**	**8**	**10**	**18**	**1**	**4**	**18**	**2%**	**0%**	**44%**	**56%**	**22%**	**40%**
	1991 SET																
3	MAURICE R.	11/6	160	160	7	3	1	4	0	0	4	3%	0%	75%	25%	0%	0%
4	455D	11/6	50	50	1	0	0	0	0	0	0	0%	0%				
			210	**210**	**8**	**3**	**1**	**4**	**0**	**0**	**4**	**2%**	**0%**	**75%**	**25%**	**0%**	**0%**

Table 2. Prevalence of all *Haplosporidium nelsoni* infections and infections with spores in oyster spat collected in 1992.

CHT LOC.	GRD	COLL. DATE	TOT COLL.	TOTAL SHUCK	TOT SICK	LT. PLAS.	ADV PLAS.	TOTAL PLAS.	TOTAL PRE-SP	TOT SP	TOT INF.	PREVALENCE TOT	SP	LT INF	ADV INF	SP/ADV
	1991 SET															
1	CAPESHORE	5/21	103	103	8	1	7	8	3	3	8	8%	3%	13%	88%	43%
1	CAPESHORE	5/27	116	116	7	3	4	7	2	2	7	6%	2%	43%	57%	50%
1	CAPESHORE	6/4	102	102	7	2	4	6	2	2	6	6%	2%	33%	67%	50%
1	CAPESHORE	6/11	171	171	15	1	9	10	1	4	10	6%	2%	10%	90%	44%
1	CAPESHORE	6/18	191	191	4	1	2	3	0	1	3	2%	1%	33%	67%	50%
1	CAPESHORE	6/25	226	226	14	1	6	7	0	3	7	3%	1%	14%	86%	50%
1	CAPESHORE	7/1	178	178	7	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	7/8	187	187	9	3	6	9	1	5	9	5%	3%	33%	67%	83%
1	CAPESHORE	7/14	211	211	15	2	2	4	0	2	4	2%	1%	50%	50%	100%
1	CAPESHORE	7/21	263	263	11	0	4	4	0	3	4	2%	1%	0%	100%	75%
1	CAPESHORE	7/27	168	168	5	1	0	1	0	0	1	1%	0%	100%	0%	
1	CAPESHORE	8/4	181	181	14	0	2	2	0	0	2	1%	0%	0%	100%	0%
1	CAPESHORE	8/10	183	183	4	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	8/19	159	159	6	0	1	1	0	0	1	1%	0%	0%	100%	0%
1	CAPESHORE	8/25	149	149	3	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	8/31	161	161	6	2	1	3	0	0	3	2%	0%	67%	33%	0%
1	CAPESHORE	9/10	119	119	2	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	9/17	125	125	4	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	9/28	109	109	4	0	0	0	0	0	0	0%	0%			
		TOT	**3102**	**3102**	**145**	**17**	48	**65**	**9**	**25**	**65**	**2%**	**1%**	**26%**	**74%**	**52%**
	1992 SET															
1	CAPESHORE	10/6	99	99	4	0	0	0	0	0	0	0%	0%			
1	CAPESHORE	10/28	99	99	2	0	0	0	0	0	0	0%	0%			
		TOT	**198**	**198**	**6**	**0**	**0**	**0**	**0**	**0**	**0**	**0%**	**0%**			
	1991 SET UP BAY															
8	NEW BEDS	6/25	55	55	3	0	3	3	2	2	3	5%	4%	0%	100%	67%
9	SHELL ROCK	6/18	65	65	8	1	7	8	1	1	8	12%	2%	13%	88%	14%
		TOT	**120**	**120**	**11**	**1**	**10**	**11**	**3**	**3**	**11**	9%	3%	9%	91%	30%

Table 3. Comparison of prevalence data from preliminary (fresh) and final (combined fresh/tissue section) examination of 1990 set collected in 1991.

COLL. DATE		PREVALENCE* ALL INFECTIONS	LIGHT INF.	ADV INF.	SPOR. INF.	ADV. W/ SPORES	ALL SPOR. INFECTIONS
6/3	PRELIMINARY	7%	0%	100%	75%	75%	5%
6/3	FINAL	11%	36%	64%	36%	56%	4%
6/9	PRELIMINARY	5%	71%	29%	0%	0%	0%
6/9	FINAL	16%	46%	54%	7%	14%	1%
6/17	PRELIMINARY	4%	33%	67%	67%	100%	2%
6/17	FINAL	9%	72%	28%	28%	100%	2%
6/30	PRELIMINARY	3%	0%	100%	50%	50%	1%
6/30	FINAL	12%	59%	41%	11%	26%	1%
7/8	PRELIMINARY	4%	0%	100%	67%	67%	3%
7/8	FINAL	50%	86%	14%	6%	43%	3%
7/15	PRELIMINARY	2%	25%	75%	75%	100%	2%
7/15	FINAL	24%	94%	6%	6%	100%	2%
7/22	PRELIMINARY	3%	57%	43%	43%	100%	1%
7/22	FINAL	35%	96%	4%	4%	100%	1%
7/29	PRELIMINARY	2%	75%	25%	0%	0%	0%
7/29	FINAL	21%	98%	2%	0%	0%	0%
8/5	PRELIMINARY	1%	33%	67%	0%	0%	0%
8/5	FINAL	9%	89%	11%	0%	0%	0%
8/13	PRELIMINARY	0%	100%	0%	0%	0%	0%
8/13	FINAL	17%	100%	0%	0%	0%	0%
8/27	PRELIMINARY	1%	33%	67%	0%	0%	0%
8/27	FINAL	20%	73%	27%	0%	0%	0%
9/4	PRELIMINARY	5%	29%	71%	0%	0%	0%
9/4	FINAL	25%	75%	25%	0%	0%	0%
9/11	PRELIMINARY	3%	0%	100%	33%	33%	1%
9/11	FINAL	12%	79%	21%	7%	33%	1%
9/19	PRELIMINARY	1%	100%	0%	0%	0%	0%
9/19	FINAL	21%	100%	0%	0%	0%	0%
10/1	PRELIMINARY	0%	0%	0%	0%	0%	0%
10/1	FINAL	15%	100%	0%	0%	0%	0%

*Categories “ALL INFECTIONS” and “ALL SPORULATING INFECTIONS” are based on all oysters examined; Categories “LIGHT,’ ADVANCED” and “SPORULATING’ are based on infected oysters only; category “ADVANCED W/SPORES” is based on advanced infections only.

Table 4. Comparison of prevalence data from preliminary (fresh) and final (combined fresh/tissue section) examination of 1991 set collected in 1992.

		PREVALENCES*					
COLL DATE		ALL INF.	LIGHT INF.	ADV INF.	SPOR. INF.	ADV. W/ SPORES	ALL SPOR. INF.
5/21	PRELIMINARY	8%	13%	88%	38%	43%	3%
5/21	FINAL	17%	33%	67%	17%	26%	3%
6/4	PRELIMINARY	6%	33%	67%	33%	50%	2%
6/4	FINAL	8%	52%	48%	24%	50%	2%
6/18	PRELIMINARY	2%	33%	67%	33%	50%	1%
6/18	FINAL	9%	88%	12%	6%	50%	1%
7/1	PRELIMINARY	5%	33%	67%	56%	83%	3%
7/1	FINAL	17%	80%	20%	17%	83%	3%
7/14	PRELIMINARY	2%	50%	50%	50%	100%	1%
7/14	FINAL	12%	92%	8%	8%	100%	1%
7/27	PRELIMINARY	1%	100%	0%	0%	0%	0%
7/27	FINAL	18%	100%	0%	0%	0%	0%
8/10	PRELIMINARY	0%					
8/10	FINAL	18%	100%	0%	0%	0%	0%

*Categories “ALL INFECTIONS” and “ALL SPORULATING INFECTIONS” are based on all oysters examined; Categories “LIGHT,’ ADVANCED, and “SPORULATING’ are based on infections oysters only; category “ADVANCED W/SPORES” is based on advanced infections only.

Table 5. Counts of *Haplosporidium nelsoni* spores from tissues of individual oyster spat collected in 1991 and 1992. Tissues remaining after sampling for original diagnosis were allowed to rot in individual tubes (with frequent centrifugation to pellet the spores followed by a change of sea water). Means and standard deviations were calculated from 4 hemocytometer counts of each sample.

	SHELL SIZE(mm)		TOTAL SPORES	
COLLECTED	HT	LG	MEAN	STD DEV
Jun-91	18	15	1,650	1,100
Jun-91	25	16	10,450	15,282
Jun-91	31	28	63,250	18,700
Jun-91	13	10	39,050	14,300
Jun-91	22	13	1,130,250	340,156
Sep-91	26	17	33,550	7,269
Jun-92	23	18	205,150	97,916
Jun-92	24	13	155,100	38,797
Jun-92	19	13	48,950	50,882
Jun-92	25	16	52,800	29,680
Jun-92	19	15	197,450	19,211
Jun-92	23	13	116,600	24,825
Jun-92	27	24	37,950	6,569
Jun-92	16	12	33,000	20,948
Jun-92	21	17	415,250	103,258
Jun-92	23	15	148,500	26,552
Jun-92	19	11	56,100	9,920
Jun-92	28	24	191,400	41,080
		MEAN	163,136	
		ST DEV	261,881	
		ST ERROR	61,726	

TABLE 6
WORKSHOP ON THE LIFE CYCLE AND TRANSMISSION OF *HAPLOSPORIDIUM NELSONI* (MSX)
HASKIN SHELLFISH RESEARCH LABORATORY
RUTGERS UNIVERSITY

MARCH 18 AND 19, 1992

ATTENDEES

NAME	ORGANIZATION	EXPERTISE
DR. THEODORE ANDREADIS	CONNECTICUT AG. EXP. STATION	Life Cycles/transmission
DR. JAY ANDREWS	COLLEGE OF WILLIAM AND MARY	MSX/Epizootiology
MS. KATHRYN ASHTON-ALCOX	RUTGERS UNIVERSITY	MSX/Parasitology
MR. ROBERT BARBER	RUTGERS UNIVERSITY	MSX/Spores
DR. EUGENE BURRESON	COLLEGE OF WILLIAM AND MARY	MSX/Parasitology
MR. WALTER CANZONIER	MAURICE R. OYSTER CULT. FNDN.	MSX/Transmission
DR. JAMES CHURCHILL	WOODS HOLE OCEANOGRAPHIC INST.	Oceanography, Nearshore Currents
MR. C. AUSTIN FARLEY	NATIONAL MARINE FISHERIES SERVICE	MSX/Parasitology
DR. SUSAN FORD	RUTGERS UNIVERSITY	Convener
DR. JAMES FUXA	LOUISIANA STATE UNIVERSITY	Epizootiology/transmission
DR . HAROLD HASKIN	RUTGERS UNIVERSITY	MSX/Epizootiology
DR. ROBERT HILLMAN	BATTELLE MEMORIAL INSTITUTE	MSX/Haplosporidia
MR. FRED KERN	NATIONAL MARINE FISHERIES SERVICE	MSX/Epizootiology
DR. STEPHEN KLEINSCHUSTER	RUTGERS UNIVERSITY	Cell biology
DR. JAY LEVINE	NORTH CAROLINA STATE UNIVERSITY	Epidemiology/MSX locations
MR. JAY LEWIS	NATIONAL MARINE FISHERIES SERVICE	MSX/Oyster Diseases
DR. JAMES MILLER	RUTGERS UNIVERSITY	Climatology/Oceanography
DR. ROBERT OLSEN	OREGON STATE UNIVERSITY	Fish Parasites/Life Cycles
DR. FRANK PERKINS	COLLEGE OF WILLIAM AND MARY	MSX/Haplosporidia/Protozoans
DR. GARY TAGHON	RUTGERS UNIVERSITY	Benthic layers/organism-flow
DR. ALBERT UNDEEN	USDA,ARS	Parasite spores/sporulation