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Observations of a Protistan Disease Similar to QPX in *Mercenaria mercenaria* (Hard Clams) from the Coast of Massachusetts

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During the summer and fall of 1995, in clam aquaculture leases at two locations on the coast of Massachusetts, significant mortalities were observed to occur primarily in 1½- to 2-year old hard clams (*Mercenaria mercenaria*, quahog) planted in the leases. Examination of hard clams from those leases suggested a parasite similar to QPX (Quahog Parasite Unknown), as described by Whyte, S. K., Cawthorn, R. J., and McGladdery, S. E. (1994, *Can. Dis. Ag. Org.* 19, 129–136), was responsible for the poor condition of affected clams and the resulting high mortality. In clam tissues, the QPX-like parasite formed thalli surrounded by a mucofilamentous net. Larger thalli underwent endosporulation producing sporangia that contained approximately 40 endospores. Rupture of the sporangium released the endospores into the surrounding tissue. Inflammatory response by the clam to infection was chronic, active, and granulomatous. The occurrence both of phagocytic multinucleated giant inflammatory cells and inflammatory encapsulation of parasites were commonly identified as part of the response. Moderately to severely infected clams showed significantly reduced growth and a poorer condition index as compared to uninfected or mildly infected clams.

Key Words: *Mercenaria;* hard clam; disease; QPX; pathology; condition index; parasite; Labyrinthomorpha.

INTRODUCTION

Hard clam (*Mercenaria mercenaria*, quahog) culture in the northeast United States has enjoyed several decades of apparently disease-free status. However, in the late 1950s and early 1960s a protistan disease was reported in hard clams from the St. Lawrence River, Canada (Drinnan and Henderson, 1963). In 1989, the same protistan, then named Quahog Parasite Unknown (QPX), caused high mortality in hatchery-reared clams from Prince Edward Island, Canada (noted hereafter as CA QPX) (Whyte *et al.*, 1994). In that outbreak, CA QPX caused extensive mortalities in clams of 15 to 30 mm shell height and was observed to cause a significant inflammatory response. Within the clam tissues, the parasites were often surrounded by halos. Within the halos, the CA QPX organisms were spherical and were enclosed by a cell wall. Larger forms were multinucleate (Whyte *et al.*, 1994).

CA QPX has been tentatively identified as either one of the Thraustochytriales or Labyrinthulales (Whyte *et al.*, 1994), both of which are achlorophullous protists and are now placed in a separate phylum (Labyrinthomorpha) in the subkingdom Protozoa (Pokorny, 1985). Previously, they had been placed in the phylum Labyrinthulomycota of the kingdom Protista (Porter, 1990). Studies in molecular phylogeny, coupled with observations of zoospore structure, indicate that the Thraustochytrids and Labyrinthulids are members of the monophyletic assemblage known as the stramenopiles and probably diverged "before the separation of oomycetes and autotrophic stramenopiles" (Leipe *et al.*, 1994). Members of one or the other phyla appear to be ubiquitous saprophytic members of the marine and estuarine environments; however, disease-causing species have been observed. In 1980, a *Labyrinthula* sp. organism was identified as the cause of skin ulcerations in the octopus (*Eledona cirrhosa*) from Scotland (Hanlon and Forsythe, 1990). Yellow spot disease of the nudibranch (*Tritonia diomedea*) caused by a Thraustochytrid parasite was identified in 1982 (McLean and Porter, 1982). In 1983, a Thraustochytrid parasite was observed to be a cause of gill disease in wild caught squid (*Illex illecebrosus* Lesueur) maintained in a culture facility (Jones and O'Dor, 1983). In 1987, Bower described a severe parasitic infection of juvenile abalone, *Haliotis kamtschatkana*, caused by *Labyrinthuloi-*

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des haliotidis. Infectivity experiments in which juvenile abalone (about 1 mm shell diameter) were exposed to the flagellated zoospore stage of the parasite showed the zoospore was directly infective (Bower *et al.*, 1989).

In 1976, episodic mortality associated with a protist similar to QPX was identified in feral clams from Barnegat Bay, NJ (Dr. Susan Ford, Rutgers University, personal communication). In 1992, parasites similar to QPX were seen in a small sample of moribund feral clams from Mitchell River, Chatham, MA (R. Smolowitz, unpublished data). While organisms similar to QPX have reportedly been seen in apparently healthy populations (Whyte *et al.*, 1994), they have not been identified in other disease outbreaks until the present.

During the time interval of 1992 to 1995, local hard clam farmers in Provincetown, MA, noted annually, increasingly severe mortality of their clam stocks. While mortality was noted in all sizes of clams, and clams originating from several commercial shellfish hatcheries, the most severe losses occurred in clams just under market size (less than 1 in. valve width, approximate 2 years old). In 1995, anecdotal observations by the culturists indicated mortality was approaching 80% of their marketable stocks. The Provincetown culturists indicated that clams migrated to the sediment surface just prior to death. Clinical signs were frequently characterized by an inability of the hard clam to close it's valves tightly resulting in a 1- to 2-mm gape in the valves. In the fall of 1995, one bed in a Duxbury clam lease site experienced a sudden and severe onset of mortality in previously healthy and vigorously growing clams (approximately 2 years old).

This report describes two separate occurrences of QPX-like parasitic infections causing substantial mortality to 1½- to 2-year-old hard clams grown in clam lease sites along the southeastern coast of Massachusetts in the summer and fall of 1995 (noted hereafter as MA QPX).

MATERIALS AND METHODS

Sample Collection

Hard clams were collected for determination of the extent and cause of mortality on three different occasions and at two different locales in southeastern Massachusetts. The collection schedule and sample analyses conducted are described in Table 1. Clams were initially sampled at two lease sites in Provincetown, MA, on August 17, and October 19, 1995, with subsequent sampling at one lease site in Duxbury, MA, in October 24, 1995. All clams sampled were 1.5 to 2 years old.

Pathology

Cultured hard clams were examined pathologically as three different sample groups (Table 1). For sample 1, 7 hard clams, selectively collected for histopathologic examination by the investigators on one Provincetown shellfish lease site, were necropsied on August 17, 1995. Based on clinical appearance (see methods below), 5 of these clams were observed as being diseased (1D) and 2 as nondiseased (1ND) (control animals). For sample 2, 80 hard clams, 40 from each of two Provincetown clam leases, were selectively collected for pathologic examination by the investigators on October 19, 1995. Based on clinical appearance, 50/80 clams were designated as diseased (2D) (25 from each lease) and 30/80 clams as nondiseased controls (2ND) (15 from each lease).

Sample 3, consisting of a total of 50 hard clams, was submitted from a clam lease site in Duxbury, MA, on October 24, 1995. Sample 3A (25 clams) was randomly collected from a bed of clams observed to be suffering high mortalities. Sample 3B (25 clams) were randomly collected from a bed of clams at the same clam lease, but in which no significant mortalities were observed.

At necropsy, seasonal growth and amount of gaping

TABLE 1
Schedule of Hard Clams Collected and Analyses Conducted

Sample no.	Date of collection	Locale	Site	No. in sample	Analyses preformed
1	17 August 1995	Provincetown, MA	Lease A	5 diseased	Histopathology
				2 control	Histopathology
2	19 October 1995	Provincetown, MA	Lease A	25 diseased	Histopathology
				15 control	Histopathology
				0.07-m^2 cores	Mortality measurement
				10 diseased	Condition index measurement
				10 control	Condition index measurement
			Lease B	25 diseased	Histopathology
				15 control	Histopathology
				0.07-m^2 cores	Mortality measurement
				10 diseased	Condition index measurement
				10 control	Condition index measurement
3	24 October 1995	Duxbury, MA	Bed 1	25 randomly selected clams	Histopathology
			Bed 2	25 randomly selected clams	Histopathology

were measured with a metric ruler. Per clam weight was determined by weighing together 8 clams from each subgroup and determining average weight per clam. Squash preparations were made from mantle tissues in approximately 10 clams with large mantle nodules or swellings by removing a portion of the mantle, macerating it in a drop of sterile sea water on a microscope slide, and examining the preparation on a Olympus BH-2 photomicroscope. Oblique cuts of the soft tissue, including the digestive gland, gonad, kidney, heart, gill, mantle, mantle edge, and foot, were fixed in 10% formalin in sea water (Howard and Smith, 1983) and processed in paraffin. Standard 6 µm histologic sections were cut and stained with hematoxylin and eosin (H&E) (Humanson, 1979). Stained sections were examined with a BH-2 Olympus photomicroscope. The distribution and severity of infections were identified and recorded in each animal. Selected sections of tissue were stained with the following special stains and examined for staining of the parasite forms and surrounding tissue: periodic acid Schiff (PAS) with and without diastase digestion which stains polysaccharides and neutral mucosubstances, Gormori methenamine silver (GMS) (Humanson, 1979; Luna, 1992) which stains fungal elements and Alcian blue/PAS at pH 2.5 and 1.0 which stains mucins (Luna, 1992). In sample 2, a small portion of mantle edge was removed from each of the 80 clams prior to fixation. Each section of mantle tissue was macerated and held individually in fluid thioglycollate media for 5 days at room temperature. After 5 days, tissues were removed from the media, stained with Lugol's iodine solution, and examined for the presence of *Perkinsus marinus* or *Perkinsus* sp. hypnospores according to the technique of Ray (1954).

Determination of Overall Condition and Mortality

On October 19, 1995, samples were collected from both lease sites in Provincetown, MA to evaluate the level of mortality *in situ*. Two replicates of 0.07-m^2 core samples were collected from an affected bed at each lease site. Living and dead specimens were enumerated and the valve width, to the nearest 0.1 mm, was determined for each individual.

Living hard clams retrieved in the core samples were used to assess the overall condition of the hard clams. Following dehydration of soft tissue in a 60°C oven for 48 hr, the condition index was calculated as the soft tissue dry weight normalized to the width of the valve at the hinge, using the following formula

$$CI = DW_{soft}/W_{shell} \times 1000,$$

where CI is the condition index, DW_{soft} is the dry weight of the soft tissue (grams), and W_{shell} is the width of the clam at the hinge (mm).

Comparisons were made in condition index between each bed, and between clams demonstrating significant growth (>2 mm increase in valve length) during the summer season, and those demonstrating no or little growth (<2 mm increase in valve length). The extent of new shell laid down during the spring–summer growing season was easily observed. Growth during this interval was measured as the total valve width minus the valve width of the clam inside of the new growth area (mm). The comparisons of condition index and mortality were made using standard analysis of variance techniques (Zar, 1974).

RESULTS

Field Observations

In August, 1995, sample collection trips to Provincetown leases showed that a high proportion of clams dug out of clam beds (estimated at 50%) were gaping. Abundant sand granules, mixed with mucus, were lodged between the gaped valves. Numerous empty shells were present on the surface of the flats. Interestingly, clam baskets filled with recently dug clams emitted a obvious grinding, crunching sound due to the attempts of the clams to close their valves on sand granules.

In October, 1995, during the sample collection trip to Provincetown, lease holders reported that mortality and morbidity had decreased with the onset of fall weather. While numerous empty shells were still present on the surface of the flats, upon examination of clams in the flats, the number of gaping clams had markedly decreased from the August time period. Summer growth was 5 mm or greater on most clams remaining in the flats.

Sample 3 clams (submitted for pathologic evaluation) were not examined in the field by the authors. However, it was reported by the culturists (Richard Kraus, Aquaculture Research Corporation, Dennis, MA, and William Bennet, Duxbury, MA, personnel communications) that the clams generally did not migrate to the

TABLE 2

Summary of Level of Mortality in 0.07-m^2 Replicated Cores Collected from Leases (A and B) in Provincetown Harbor

Sample no.	Core no.	No. live clams	No. dead clams	% Dead clams
2A	1	17.5	11.5	39.66
	2	9.5	3.5	26.92
Subtotal (2A)		27.0	15.0	35.71
2B	1	13.5	7.5	35.71
	2	27.0	3.0	10.00
Subtotal (2B)		40.5	10.5	20.59
Total		94.5	40.5	30.00

TABLE 3

Comparison of Valve Length Increase during Growing Season and Condition Index between Hard Clams Showing Clinical Signs of MA QPX (without Growth) and Clams Not Showing Signs of MA QPX (with Growth)

		Valve length at start of growing season (mm)	Growth during season (mm)	Condition index
Clams with growth ($N = 10$)	Mean ± SE	6.12^{ns} 0.95	14.33* 0.45	34.66* 0.83
Clams without growth ($N = 10$)	Mean ± SE	5.55^{ns} 1.59	2.58* 0.60	18.77* 1.80

Note. ns, no significant difference ($P > 0.05$).
* Highly significant difference ($P < 0.001$).

surface when moribund and that most empty shells were found still buried in the sediment.

Mortality and Condition Index

The level of *in situ* mortality within leases at two quahog culture sites in Provincetown, with demonstrated presence of MA QPX, are presented in Table 2. Mortality, evaluated as the number of empty paired valves compared to the number of living clams, was relatively consistent between sites. Thirty percent (overall) of the clams randomly sampled in the core samples were empty valves.

A comparison of the condition index between groups of clams showing gross pathology consistent with the QPX infection and groups of clams not showing QPX symptoms, identified as clams without growth and clams with growth respectively, are presented in Table 3. Although the valve length of all clams at the start of the current growing season was not significantly different between infected and noninfected hard clams, the new growth of the quahog valves (calculated as total length − length at the start of the current growing season) was significantly ($P < 0.001$) greater for the clams showing no signs of QPX (14.33 mm ± 0.45) compared to clams showing signs of QPX (2.58 mm ± 0.60). This supports the selection criteria of growth versus no growth. The condition index of clams demonstrating growth (34.66 ± 0.83) during the current season was significantly higher ($P < 0.001$) than the condition index of those clams demonstrating signs of QPX infection (no growth, 18.77 ± 1.80).

Pathology

Gross Pathologic Examination

Table 4 lists average estimated growth (±SE) and number of clams with gaping and chipping at the valve margins for all samples for which data was collected.

Sample 1. Upon necropsy, gaping (between 1 and 2 mm) and chipping of the valve margins (two to five chips per animal) were noted in diseased animals. Shucked, diseased clams showed light tan discoloration of the mantle, mantle swelling, and mantle regression. Variable amounts of sand was embedded in abnormally increased amounts of mucus between the mantle edges. Shell chips on the valve margins extended as V-shaped losses of shell with the apex of the V occurring at the internal shell surface and the wings extending outwardly and laterally to the external surface of the shell.

Sample 2. In the diseased clams of sample 2 (2D), gaping varied between 0.25 and 1 mm and chipping was present on edges of most shells of diseased clams. Thickened retracted, light tan, swollen mantle edges were noted (Fig. 1A). Occasionally, yellow/tan nodules, 1–4 mm in diameter, were noted along the mantle edges or in the mantle especially in areas adjacent to the anterior adductor muscle (Fig. 2B). Rarely, erosion of the internal shell surface was noted in foci underlying nodules and swollen discolored portions of the mantles. In addition to tissue cells, debris, and hemocytes, squash preparations of swollen discolored mantle tissues showed variable numbers of round organisms that were 10–25 µm in diameter and were surrounded by a clear symmetrical halo. Each organism's cell wall contained either a large nucleus with variable cytoplasmic

TABLE 4

Results of the Gross and Histopathologic Examination of Hard Clams Sampled in this Study (D, Diseased; ND, Nondiseased)

Sample no.	No. of clams in the sample	Weight (grams)	Mean seasonal increase in valve length		Gaping (no. of clams)	Chipping of valve margins (no. of clams)	No. of clams infected	% of infected clams
			Mean (mm)	SE				
1D	5	—	2.6	0.9	4	5	3	60
1ND	2	—	10	0.0	0	0	0	0
2D	50	25	3.6	2.1	17	23	45	90
2ND	30	28	6.8	2.3	1	3	3	10
3A	25	50	14.4	3.1	7	1	9	36
3B	25	55	18.3	2.2	2	3	0	0

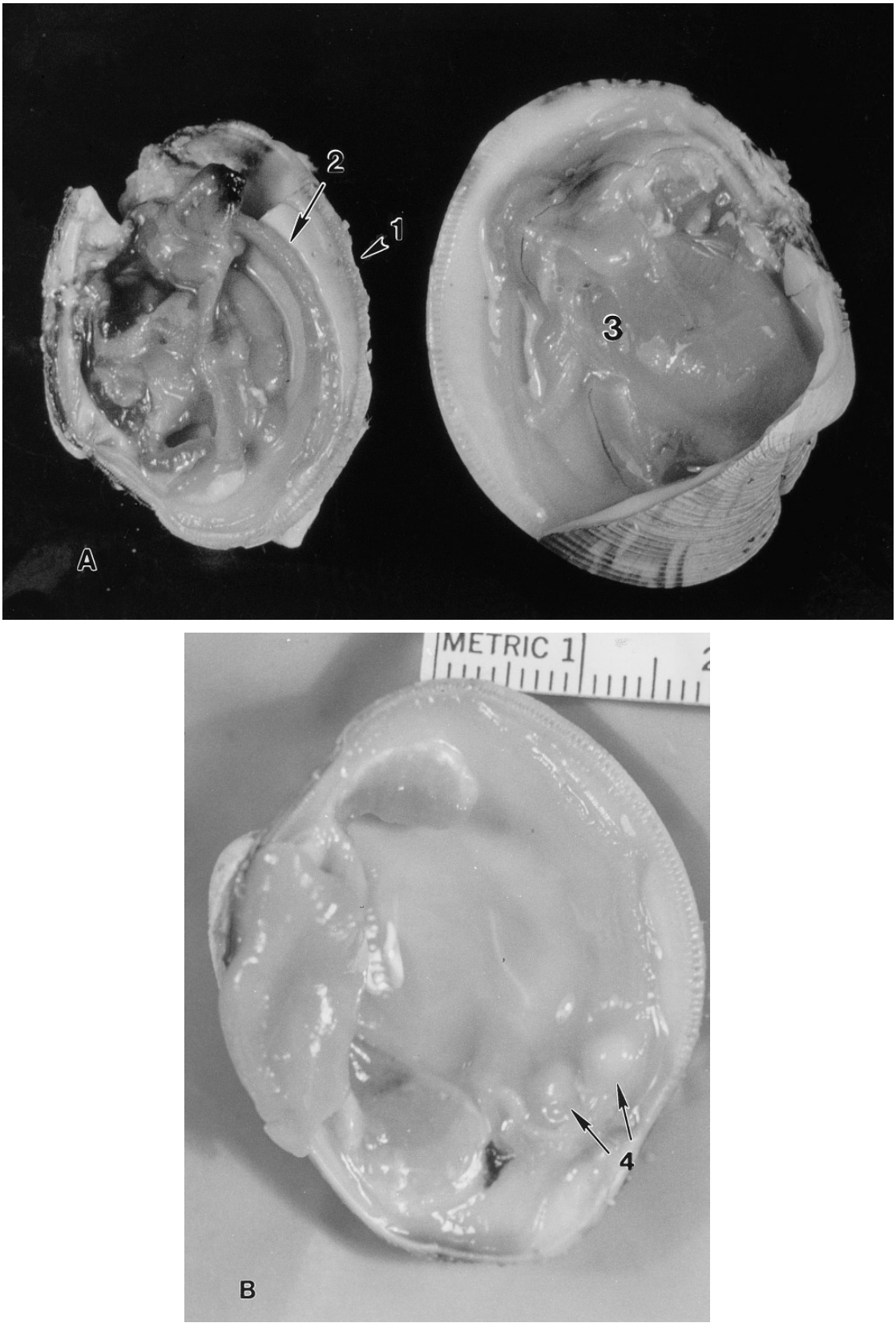
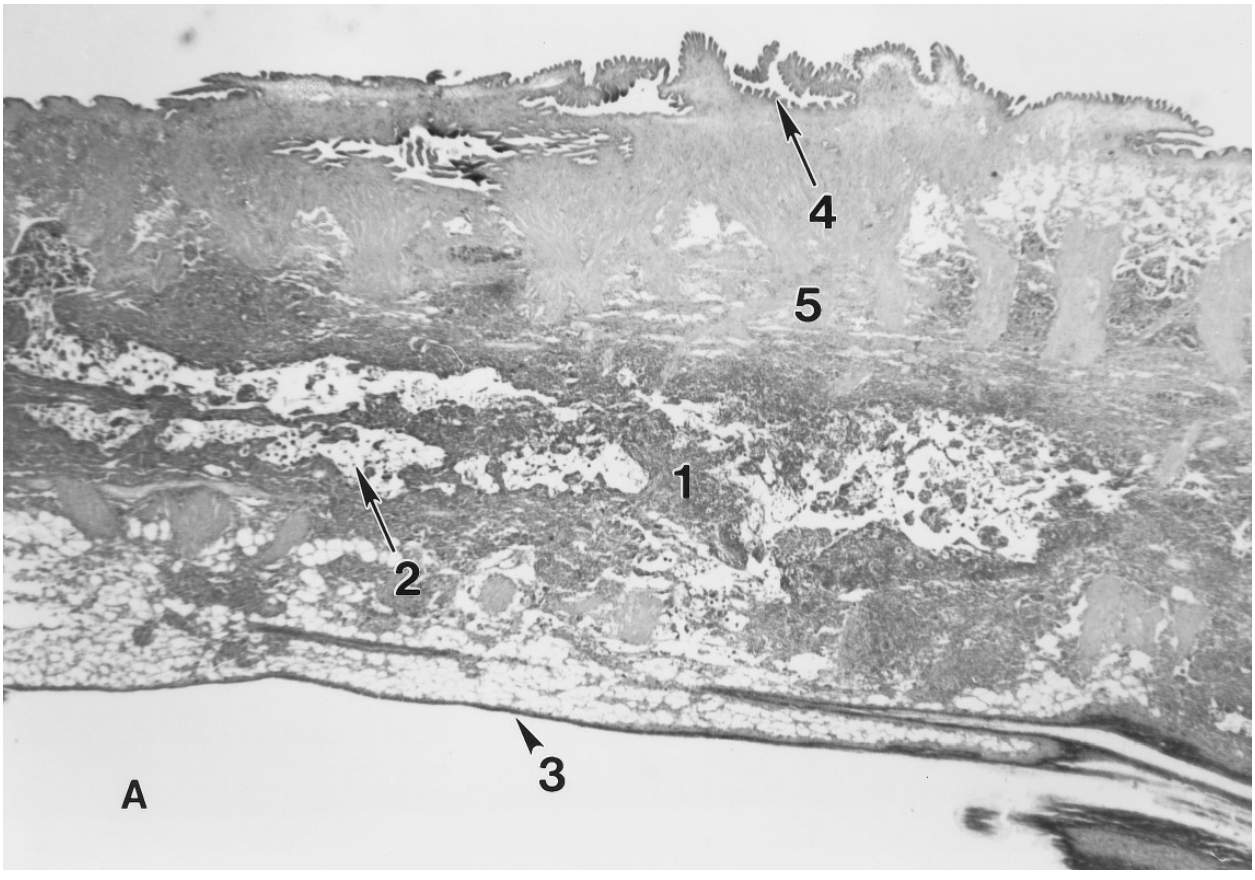
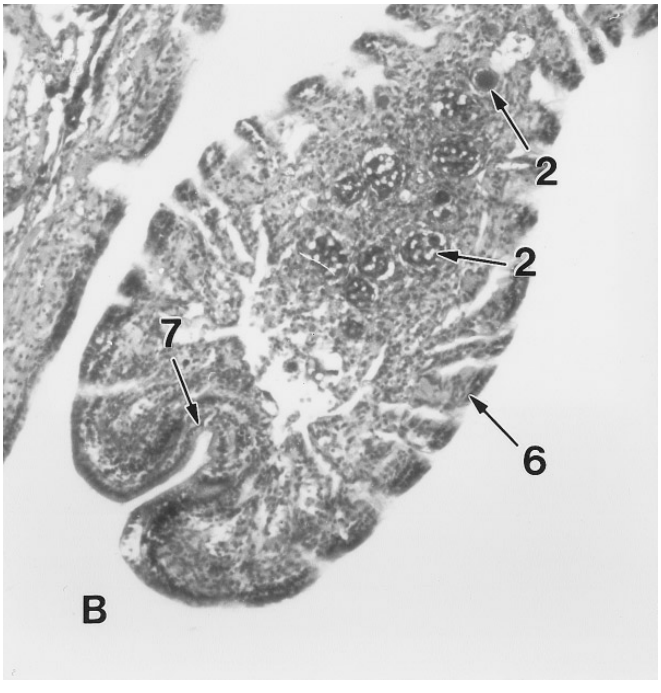


FIG. 1. Hard clams (*Mercenaria mercenaria*) from infected leases with one valve removed. (A) The infected animal shows chipping of the shell margins (1) and diffusely swollen retracted mantle edges (2) when compared to control animals (3). (B) Occasionally, distinct inflammatory nodules (4) resulting from QPX infections were noted in the mantle.





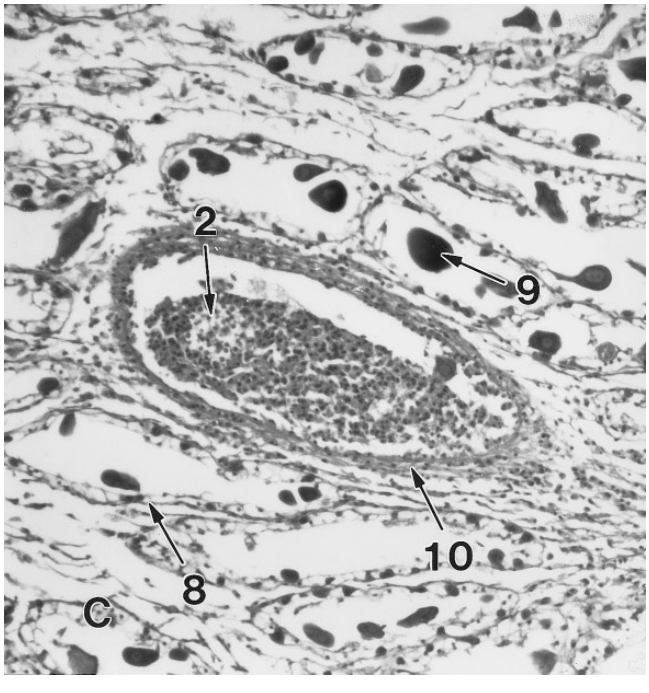


FIG. 2. Photomicrographs of paraffin-imbedded tissues from QPX-infected hard clams (H&E). Severe diffuse granulomatous inflammation resulting from QPX infection swells the mantle edge (A, 4.5×) and obstructs vascular channels in the interlamallar septa of the gill (B, 25×). A vessel (C, 25×) adjacent to the gonadal tubues shows partial thrombosis resulting from QPX infection and accompanying inflammation (1, granulomatous inflammation; 2, QPX parasites; 3, mantle cavity epithelium; 4, shell epithelium of the mantle; 5, muscle of the mantle edge; 6, gill filaments; 7, terminal groove; 8, gonadal tubule; 9, developing egg; 10, inflammed vessel).

volume (thalli) or many small spore-like organisms (sporangia). These organisms were not identified in the control clams (2ND).

Sample 3. Although gaping (<0.25 mm) was noted in Sample 3A clams, marked gaping, as identified in Sample 2D, was not observed. Irregular blackening of the mantle edge was identified in several animals in both samples 3A and 3B. Rarely, small chips (not obviously V shaped) were seen on the edges of the shells of 3A. Other findings in sample 3A were similar to those described in sample 2D above. Sample 3B did not show nodules or swollen mantles.

Histopathologic Examination

MA QPX parasites were primarily localized in the sinusoidal spaces and surrounding connective tissues of the infected organs/tissues. Percentage of clams from each sample in which the parasite was detected are listed in Table 4. MA QPX was identified in 90% (45/50) of the affected clams in sample 2D while only 10% (3/30) MA QPX-infected clams were identified in the control clams in sample 2ND.

The most commonly infected organs/tissue (Table 5) in the infected clams from Provincetown were the mantle (91%) (Fig. 2A) and the gill (63%) (Fig. 2B). The vascular spaces and adjacent connective tissues surrounding major organs of the body were markedly infected (24%) (Fig. 2C). The most common location of infection within the vascular spaces and adjacent connective tissues of the body were in areas bordering the style sac and intestinal profiles. The kidneys and pericardial gland, both blood filtering organs, were less commonly infected. Aggregations of hemocytes, mixed with parasitic forms, were often seen in the water tubules of gills from clams with heavy infections.

MA QPX was identified histologically in 36% (9/25) of the clams sampled from a clam bed in the Duxbury lease that experienced high mortality (3A). No parasitic infection was noted in clams from the control bed (3B). Tissue location of the parasitic infection in the Duxbury animals showed similarities to that of the Provincetown clams (Table 5), except that a higher percentage of animals showed infections of the vascular and connective tissues of the body (66%) than in the Provincetown clams. However, the most common locations of QPX infection within the clam's bodies were similar to those seen in the Provincetown clams.

Three of the five clams identified as having parasites in tissue section in sample 1 also showed either branchial, digestive gland, or gastric necrosis associated with a mixed population of bacterial rods. Only rarely did bacteria occur in MA QPX-diseased clams from other samples (sample 2D, 2%; sample 3A, 4%).

Only two other infectious agents were identified in these samples of clams. In sample 2, two clams also showed a mild rickettsia-like infection of the digestive gland epithelia similar to that observed by Bower *et al.* (1994). In sample 3A, two clams showed a mild rickettsia-like infection of the epithelial cells of the gill filaments (Bower *et al.*, 1994). In none of these four animals was any inflammation or disfunction of the organs noted in association with these infected cells.

Description of the QPX organism in tissues. The classical mycological terminology used by Moss (1985) is adopted herein with the recognition that appropriate classical protozoological terminology can also be used (e.g., thallus = trophozoite; sporulation = schizogony, etc.). Our choice is arbitrary since the QPX organism is a protist. It is not appropriate to call it a fungus or a protozoan species.

The smallest thalli of MA QPX parasites seen in H&E-stained tissue sections measured 4 μm in diameter. Larger thalli showed a central 2–5 μm round well-defined amphophilic nucleus surrounded by a 1 to 2 μm width lighter amphophilic cytoplasm enclosed withing a cell wall that was often intensely basophilic (Fig. 3A). Intimately covering the outer surface of the cell wall was a filmy material that extended dendritic (and sometimes sheet-like) processes into the surrounding areas. Dendritic-like, filmy to flocculent, deeply blue stained (hematoxylin and eosin) material has been termed an ectoplasmic net in other studies when discussing Labyrinthomorphids (Jones and O'Dor, 1983; Perkins, 1972, 1973; Whyte *et al.*, 1994) (Fig. 3A). However, the occurrence of a mucoid component (identified with special stains) and a filamentous component (F. Perkins, unpublished electron microscopic data) of

TABLE 5
Results of the Histopathologic Examination of Organs from Two Groups of MA QPX-Infected Clams

	Sample 2D		Sample 3A	
	No. organs infected/ total no. of organs examined in infected clams (45/50)	% Organs infected	No. organs infected/ total no. of organs examined in infected clams (9/25)	% Organs infected
Mantle	41/45	91	7/9	77
Gill	25/40	63	6/9	67
Connective tissues/sinusoids of the soft body	11/41	27	6/9	66
Kidney	3/15	20	1/4	25
Pericardial gland	6/22	27	1/4	25
Adductor muscle	1/18	6	0/5	0
Foot	1/31	3	1/8	13
Digestive gland	0/28	0	0/7	0
Ganglions/major nerves	0/13	0	0/2	0
Palps	0/10	0	0/0	0

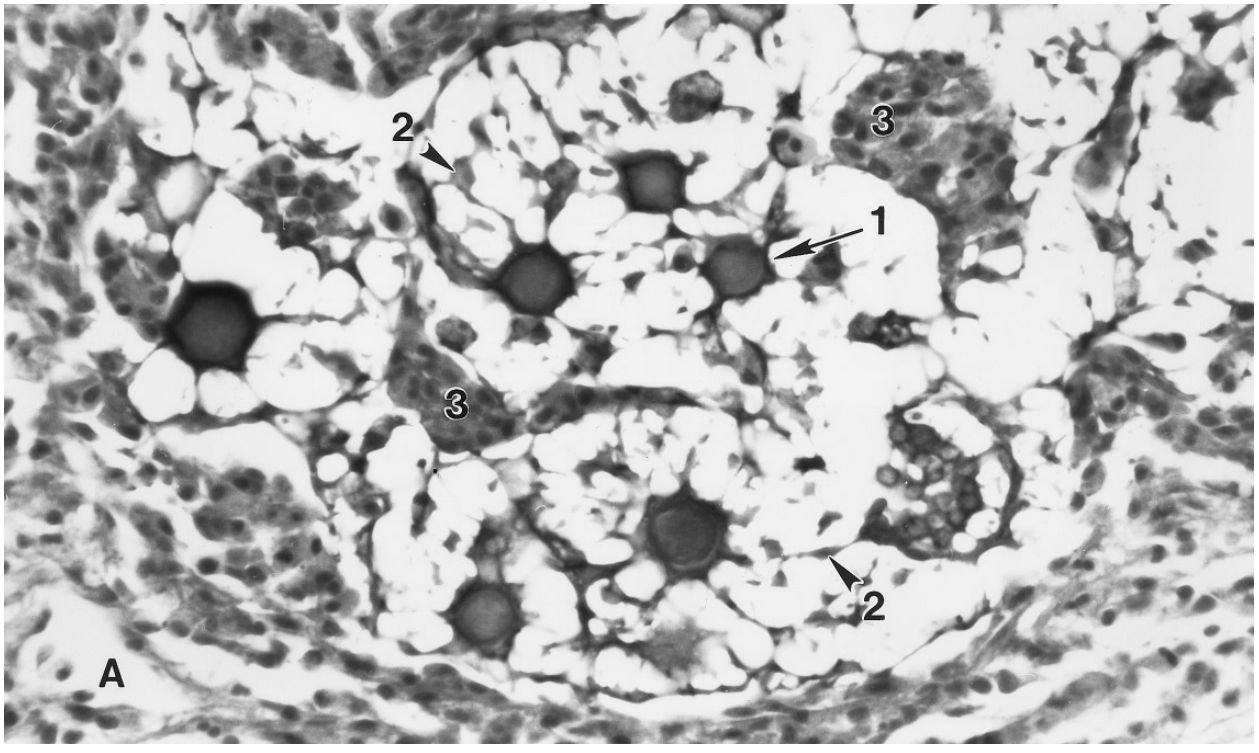


FIG. 3. Photomicrographs of various QPX forms in paraffin-embedded hard clam mantle and gill tissues (100×, H&E). Various sized thalli (A) and sporangia (B and C) of QPX are surrounded by mucofilamentous net within lucent areas and by granulomatous inflammation. Hemocytes, some of which are fusiform in outline, are migrating between the thalli and surrounding sporangia (A and C). In some foci, thalli, which are partially degraded, are present within the cytoplasm of hemocytes (B and C). Wispy mucofilamentous net is also present between thalli that have expanded away from each other after lysis of the sporangia (D) from which they originated. Occasionally mucofilamentous net is not identified in the lucent areas (E) (1, thallus; 2, mucofilamentous net; 3, granulomatous inflammation; 4, sporangia; 5, fusiform hemocytes; 6, partially degraded thallus in a hemocyte; 7, lysed sporangia; 8, lucent areas without mucofilamentous net).

the net found associated with this MA QPX organism has not been identified previously in Labyrinthomorphids. Therefore, we will call this substance mucofilamentous net instead of ectoplasmic net.

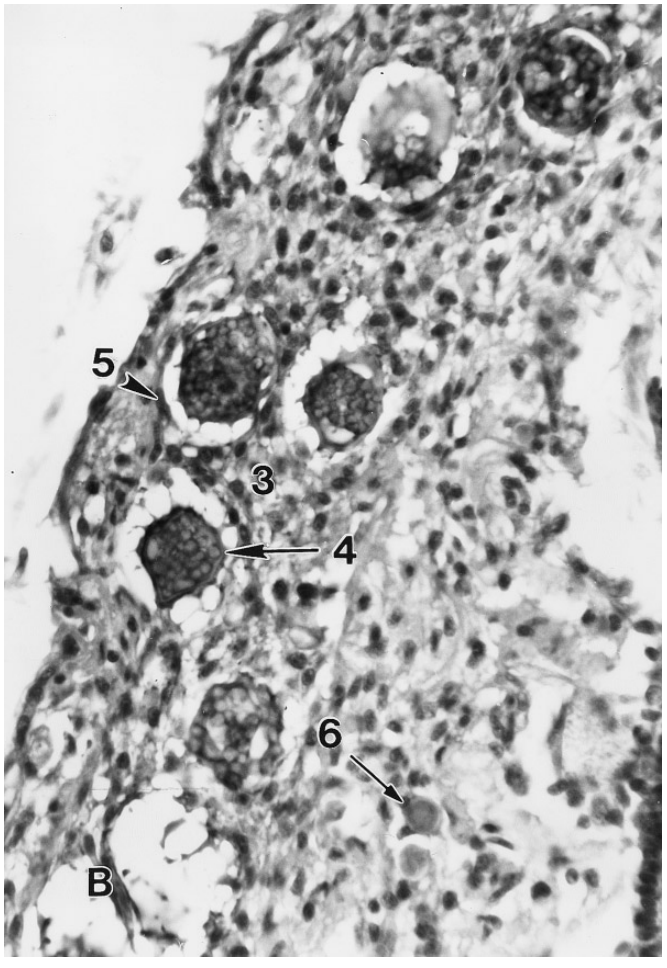
Sporangia of approximately 10–15 μm in diameter showed a loss of nuclear definition and instead contained small round (each approx 2 μm in diameter) daughter cells or immature thalli (endospores) (Fig. 3B). Between 30 and 40 immature thalli (endospores) could be seen in sporangia which were 18–25 μm in diameter. Deep blue cell walls were noted around each of the developing thalli within the sporangia.

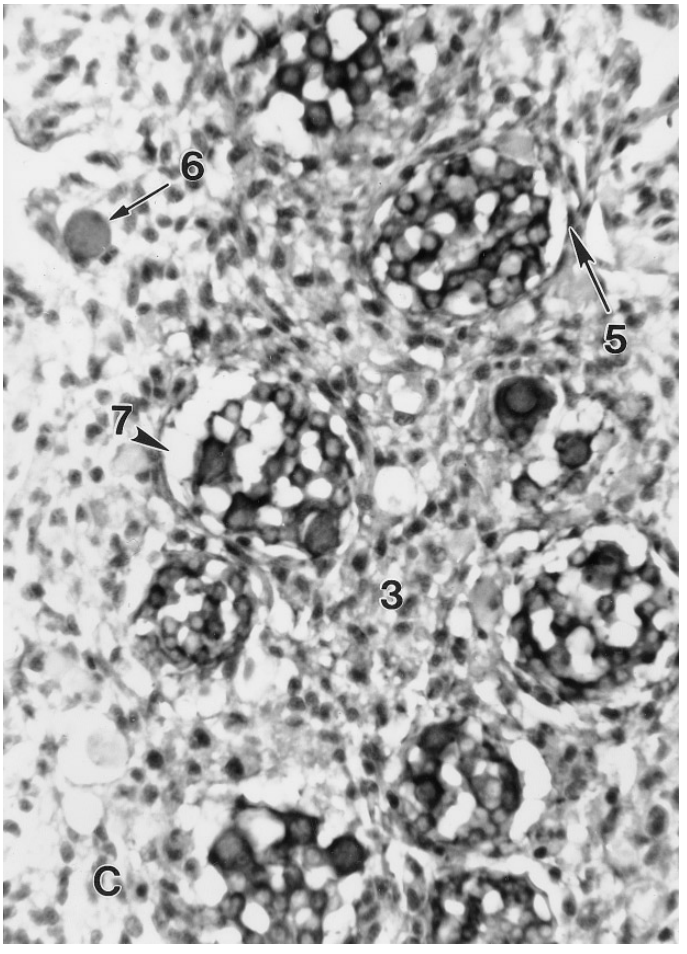
Rupture of the sporangia resulted in disruption and disappearance of its cell wall (and its surrounding mucofilamentous net) and release of numerous small thalli into the surrounding tissue (Figs. 3C and 3D). The newly liberated thalli often occurred in an expanded circular formation and were interconnected by extensions of the mucofilamentous net. In some areas, mixtures of various sized thalli and sporangia were noted. No other forms of the parasite were noted in tissue sections.

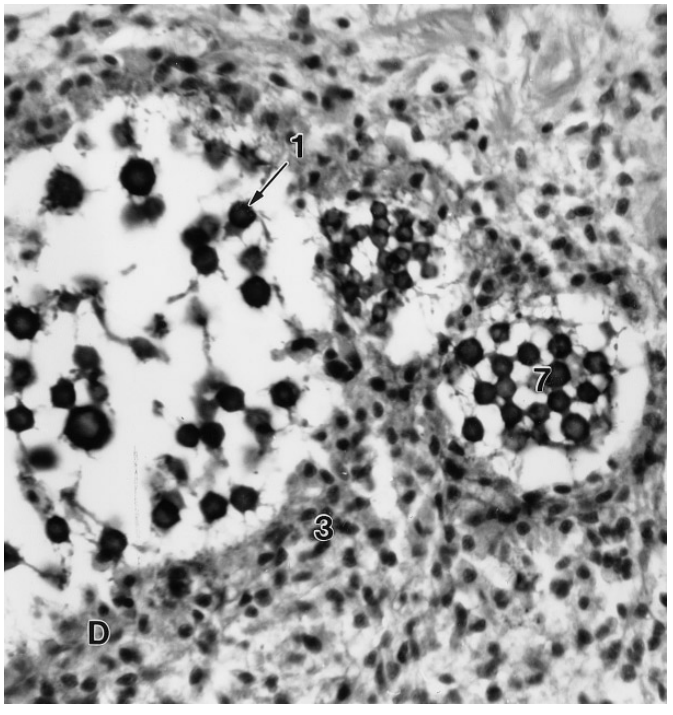
In most areas, the thalli and sporangia, enveloped by the mucofilamentous net, were embedded in an otherwise lucent (clear) area. In some infected foci the net was abundant in the lucent areas, and in others, it was only rarely observed (Fig. 3E).

Description of the clam's inflammatory response. The inflammatory response to the parasitic invasion of the clam tissues was chronic, active, multifocal, and granulomatous with encapsulation. Granulomatous inflammation, consisting of an abundant mixed population of phagocytic hemocytes, both granular and agranular, had migrated to areas of parasitic invasion in the sinusoids and the surrounding loose connective tissues and expanded those tissues. Such infected tissue foci were sometimes very large (up to 300 × 500 μm) and were often extensively distributed within the infected tissue.

Hemocytes usually showed an inability to effectively surround the individual parasites or collections of parasites when extensive lucent areas, containing mucofilamentous nets, surrounded the parasite forms (Fig. 4A). However, even when large lucent areas with nets surrounded cells of the parasite, hemocytes, many of which had become fusiform in outline (transformed), often had migrated between mucofilamentous strands







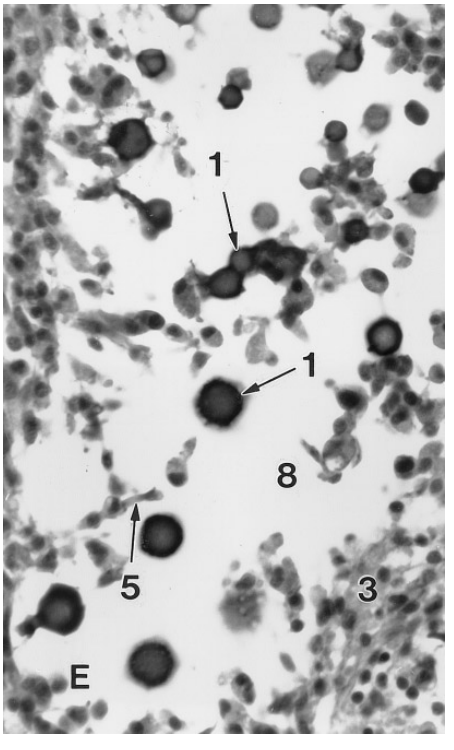


Fig. 3—*Continued*

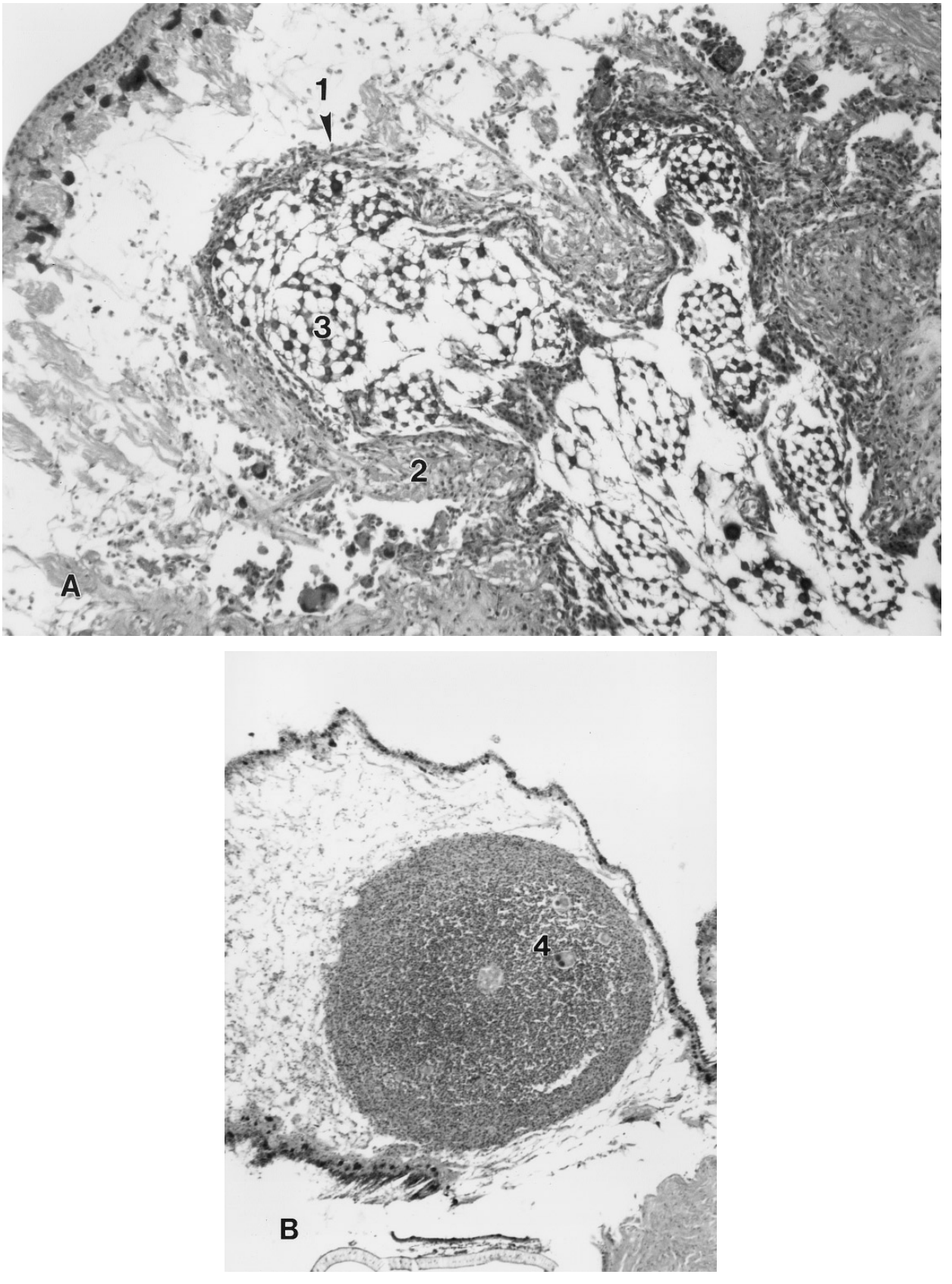


FIG. 4. QPX infection of hard clams resulted in a granulomatous inflammation as seen in these photomicrographs of paraffin-embedded tissue sections of the mantle (H&E). (A) This extensive granulomatous lesion resulted in poor (1) to moderate (2) encapsulation of the proliferating QPX organisms (3) in the mantle tissues (25×). (B) Occasionally, formation of distinct inflammatory nodules (4) containing only rarely visible parasites were noted in the mantle tissues (10×).

in the lucent areas to the cell wall of the developing vegetative cyst (Figs. 3A, 3B, and 3C). The resulting invading hemocytes incompletely divided the thalli and sporangia from each other.

Granulomatous inflammation resulted in varying degrees of encapsulation of infecting MA QPX organisms. Encapsulation by the hemocytes making up the granulomatous inflammation varied from poor to good (Fig. 4A). The term encapsulation is used herein to denote a circumferential accumulation of hemocytes, some of which transform (become elongated) and align themselves in parallel/circumferential bundles (Sparks and Morado, 1988).

In clams containing parasites that were located in small lucent areas or that had no delimiting lucent areas and in which there were sparse amounts of mucofilamentous net, the clam's granulomatous inflammation more effectively encapsulated and degraded the parasites, resulting in formation of distinct nodules (Fig. 4B). These foci often contrasted with the more extensive, poorly defined infections usually present in animals containing parasites surrounded by larger lucent areas with variable amounts of net.

Within the granulomatous inflammation, primarily in those foci populated by parasites with a predominance of small or no lucent areas, individual hemocytes were noted with phagocytosed and partially digested vegetative spores within them (Figs. 3B and 3C). In other foci, small aggregates of hemocytes (3–10) had encapsulated a single thallus or sporangium. Such encapsulated parasites were in various stages of degeneration. Many granulocytes within inflamed parasitic foci contained abnormally large, abundant, irregular, eosinophilic granules (3–5 µm diameter) within their cytoplasm. Rarely, individual hemocytes contained even larger, round, dense eosinophilic granules (sometimes up to approximately 10 µm in diameter) (Fig. 5).

Appreciable necrosis (lysis) of clam tissues or infiltrating hemocytes was not identified in the tissues. Only rarely was individual hemocyte necrosis identified associated with the parasitic infections. Occasionally small areas of muscle or connective tissue necrosis were seen when tissues were entrapped in inflamed foci. Such necrosis, however, appeared to be the result of the inflammatory process and not directly due to the parasites.

Multinucleated giant host cells of various sizes, containing from 3 to 25 nuclei, were present in some clams in the parasitic-induced inflammation. Multinucleated giant cells were especially prominent when adjacent to large sporulating cells or in animals containing parasites with small or no lucent areas or mucofilamentous nets. In addition to the large, 20-µm, round, eosinophilic granules occasionally occurring within them, some multinucleated giant cells also contained

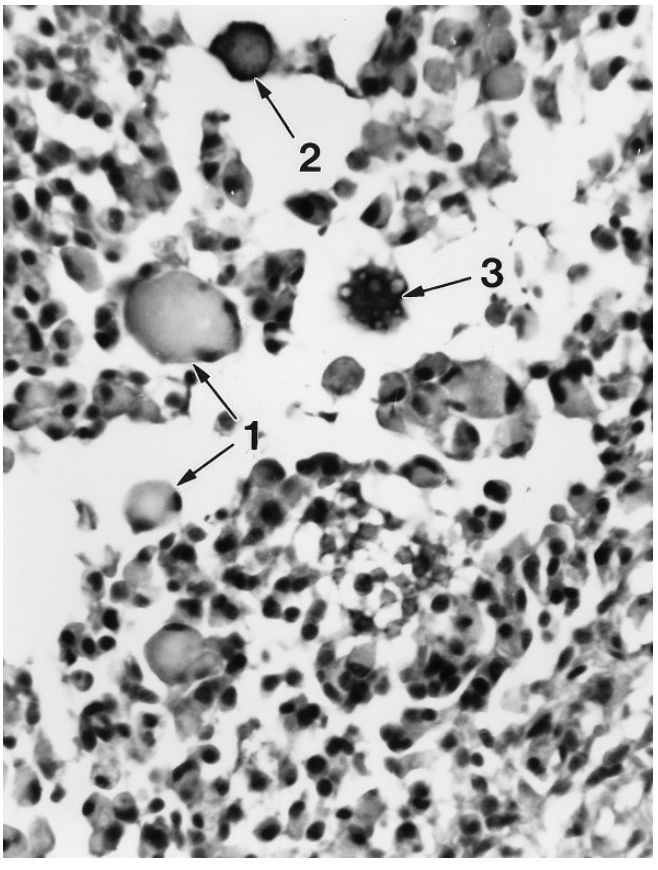


FIG. 5. Often hemocytes in QPX-infected clams contained large irregular granules within their cytoplasm (1, irregular granules of hemocytes; 2, thallus; 3, sporangia) (photomicrograph of a paraffin-embedded tissue section, 100×, H&E).

recognizable forms of degraded sporangia (Fig. 6A) and thalli (Fig. 6B).

Special Histologic Stains

Special stains are routinely used to identify common staining characteristics of groups of organisms resulting in more accurate histologic detection and identification of those organisms. Special stains were used in this study to compare staining characteristics of the MA QPX organism to special staining results reported for the CA QPX organism and are listed below.

Aqueous PAS with and without digestion. PAS stained the spore wall and the cytoplasm of the thalli but not the nucleus or net (Fig. 7A). Sporangia with delimited daughter cells (immature thalli) showed staining of the sporangial wall and of individual walls of the daughter cells. Organisms remained positive after diastase digestion followed by PAS staining, indicating the PAS positive materials were not glycogen. Very mild to no staining of the mucofilamentous net was noted.

GMS. In tissues sections stained with GMS, thalli showed strong positive staining of the nucleus and

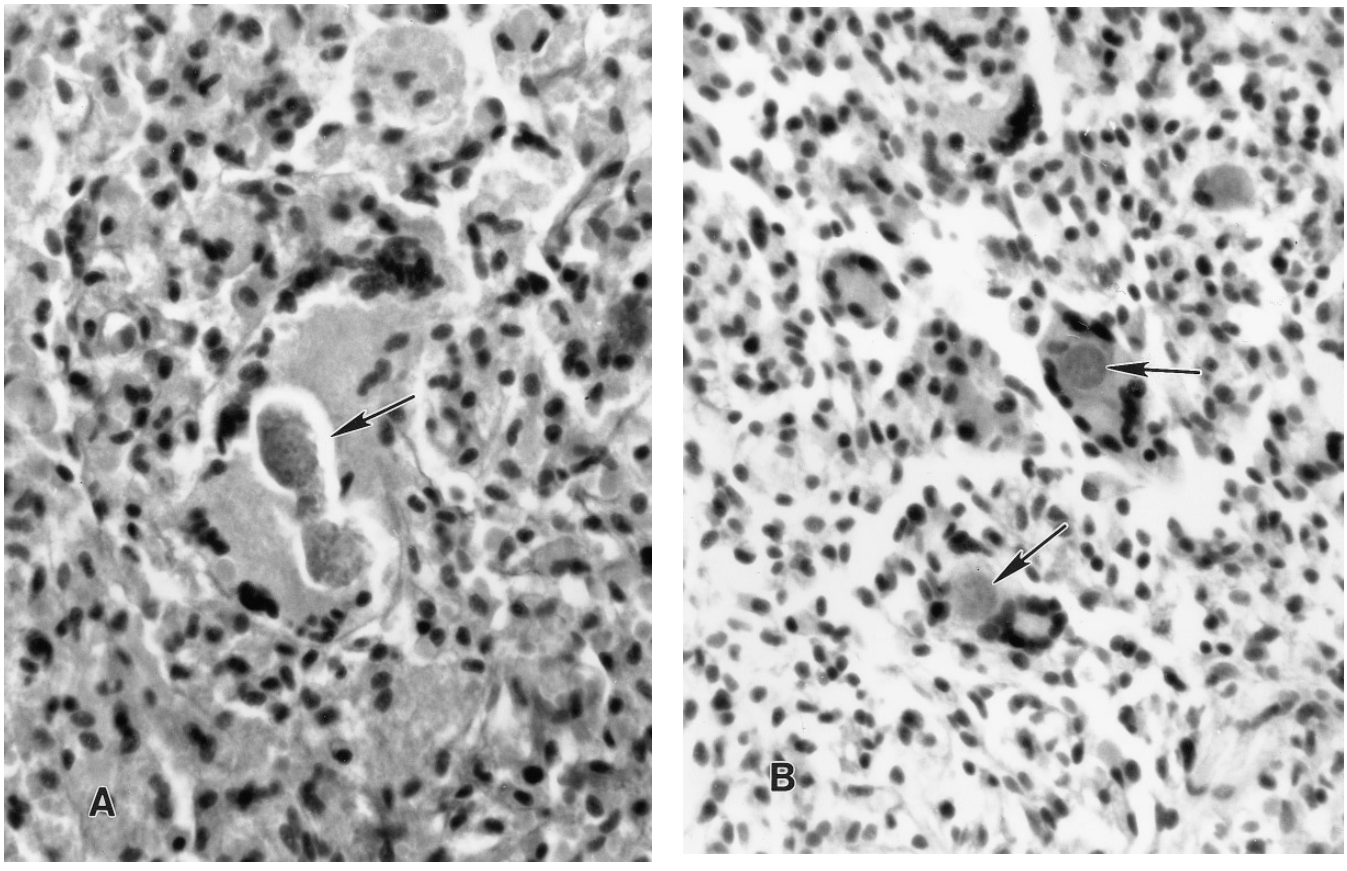


FIG. 6. In some tissues, multinucleated giant cells of host origin were identified in the granulomatous inflammation associated with QPX infections as illustrated in these photomicrographs of paraffin-embedded tissue sections. (A) This large multinucleated giant cell contains a partially degraded sporangium (arrow) within its cytoplasm. (B) Two multinucleated giant cells in this photomicrograph contain recognizable thalli in the cytoplasm (2). (100×, H&E)

appeared as a fine black granular staining (Fig. 7B). No other portions of the parasite were stained.

Alcian blue/PAS (Ab/PAS) at pH 2.5 and pH 1.0. Tissue sections stained with Ab/PAS at pH 2.5 showed strong blue positive staining of the mucofilamentous net surrounding thalli and sporangia. Ab/PAS at pH 2.5 stains blue those mucins that are similar to hyaluronic acid and sialomucins (Luna, 1992). Only very mild blue staining was noted at pH 1.0 (indicates similarity to sulfated mucosubstances) (Luna, 1992).

Thioglycollate Culture of Tissues

No positively stained hypnospores were noted from tissues cultured from any of the 80 animals. However, large sporangia, sometimes with daughter cells, surrounded by halos of 5–8 µm in width were seen within the macerated tissues. These sporangia and their halos did not absorb the ink stain differentially from the tissues.

DISCUSSION

This study has shown a strong gross and histopathologic correlation between the prevalence and severity of QPX in clams and the high morbidity and mortality noted in clams from Provincetown and Duxbury leases in 1995. Animals selected from Provincetown leases on the basis of poor growth and gaping showed QPX histologically at a markedly higher prevalence than animals sampled from the same leases that showed good growth and no gaping. No other significantly infectious agents were identified. Such findings strongly indicate that QPX is the primary infectious organism responsible for the observed morbidity and mortality. It is probable that MA QPX was also responsible for the high morbidity and mortality in clams in Provincetown leases in the preceding years.

In the Provincetown leases, many more animals appeared to be gaping and dying in August than in October. Certainly, it is known that increased summer temperatures often increase the proliferation of para-

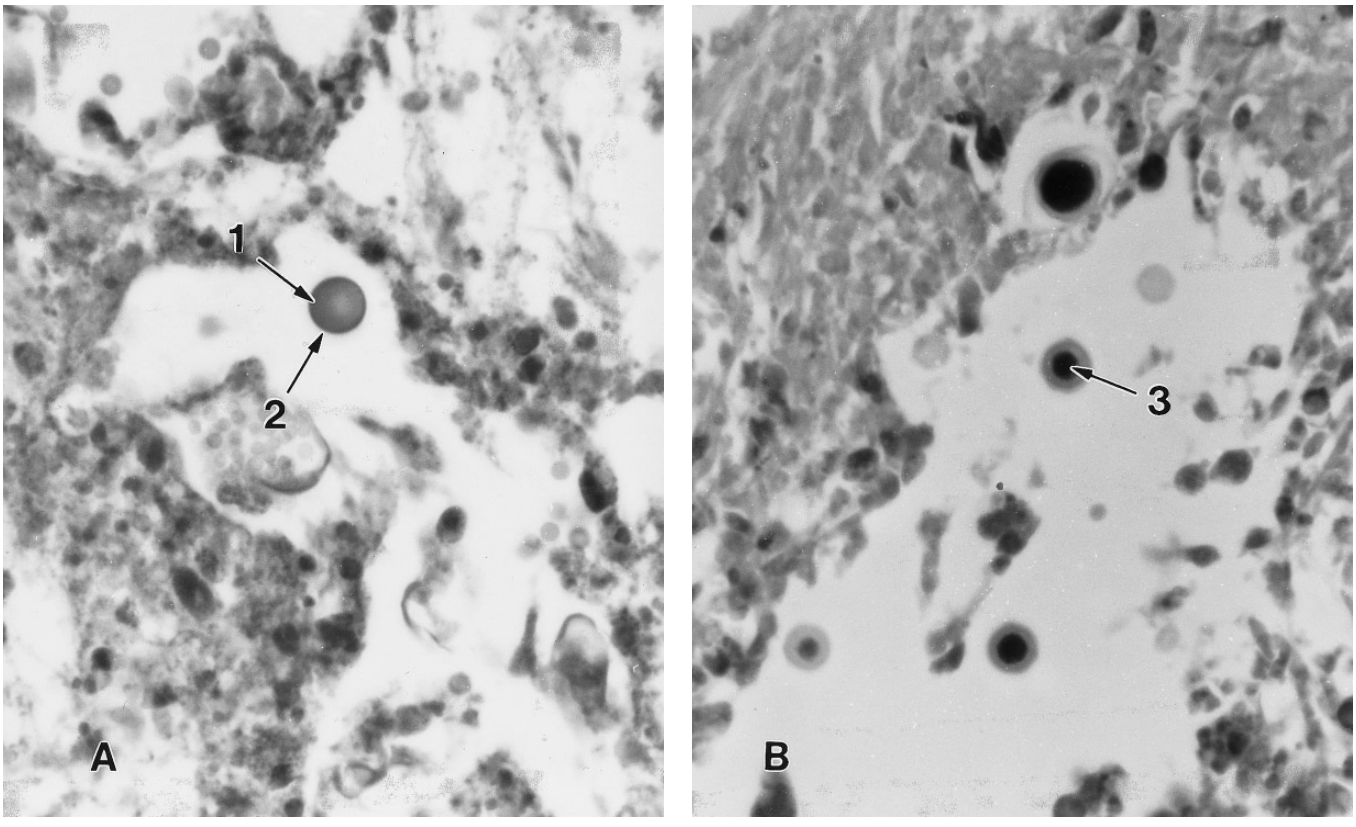


FIG. 7. Photomicrographs of sections of paraffin-embedded tissues of QPX-infected hard clam stained with special stains. (A) PAS (with diastase) stained the cytoplasm (1) and cell wall (2) of the QPX organisms. (B) GMS stained the only nucleus (3) of the QPX organisms. (100×).

sites, such as *P. marinus*, in the tissues of bivalves (Sparks, 1985). It is possible that a similar proliferation of MA QPX parasites, occurring as water temperature increases, could account for anecdotally reported higher mortality in the summer than in the fall months. Additionally, it is probable that secondary opportunistic infections due to bacteria (as seen in sample 1), fungi, or secondary predation of diseased clams by crabs and other organisms are more likely to be a complicating factor in the warmer waters of summer than in cooler waters of the late fall or winter.

Many of the clams from Provincetown had increased amounts of mucus with embedded sand present between the mantle edges. We postulate that many of the V-shaped notches in the shell edges resulted from pressure flaking by sand granules, from the sandy sediment, caught in this mucus. The chips occurred when the clams attempted to close their shells. Certainly poor summer growth, slight gaping with increased mucus and sand, and chipping were common signs of infection in Provincetown's clams.

Thickened, retracted, light tan mantle edges were the most common gross tissue lesions. Yellow-tan nodules in the mantle were less commonly observed in the clams. Well demarcated yellow nodules in the mantles correlated with histologically identifiable distinct foci of well encapsulated parasites, while diffuse swelling and light tan discoloration of mantle tissues correlated with the poorly defined, less well encapsulated areas of parasitic inflammation.

Sample 3 clams (Duxbury) did not show significant gaping with mucus and sand, shell chipping, or the noticeable surfacing of moribund clams. Sample 3A (clams showing mortality) did show mild black discoloration of the mantle edges and mild gaping. Sample 3 clams were buried in finer grain size substrate and it is likely that the different substrate may account for the lack of trapped sand, decreased chipping, and the decreased numbers of observed moribund clams surfacing compared to clams from Provincetown. Sample 3 clams, as a whole, showed much better growth than samples 1 and 2. No significant history of morbidity or mortality had been identified previously in Duxbury. It is probable that MA QPX infections in Duxbury clams represented a relatively acute onset of morbidity and mortality in previously healthy clams that contrasted to Provincetown's chronic but progressively increasing mortality occurring primarily in mid to late summer.

Field Results

Although mortality rates at the Provincetown lease sites have anecdotally been observed at levels approaching 90% (N. Meads, Provincetown aquaculturist, personal observation), the mortality observed during this study, using random core samples, averaged 30%. The lease holders routinely maintain their lease sites and remove empty valves within the clam plots. In addition, the behavior of the clams infected with MA QPX in Provincetown resulted in the dying clams rising to the sediment surface before death. The end result of this behavior is that the empty valves are washed to the edge of the netted plot following mortality thereby removing the valves from the site of the mortality. Therefore the mortality level observed in this study only reflects the mortality since the previous "clean-up" during the preceding spring and only includes those animals that remain buried in the site. Thus the mortality observed during this study should be interpreted as a very conservative estimate of the expected mortality due to a QPX infection.

Condition index is a general indicator of the physiological status of the bivalve and is commonly used to evaluate the overall condition of the organism (Lucas and Beninger, 1985). The calculation of the condition index used in this study normalized the soft tissue mass in the clam to the width of the valves at the hinge. The selection of the normalizing standard is an important component of the calculation (Cosby and Gale, 1990) and little information is available as to which constant is most appropriate for the hard clam. The normalizing standard of the width at the valve hinge was selected since it reflects the criteria established for the harvest and marketing of the quahog in Massachusetts.

In this study, the condition index of quahogs demonstrating little growth was significantly depressed in comparison to the condition index of the clams having good growth through the summer growing season. This suggests that the nongrowing hard clams were physiologically depressed. Histopathological examination of those clams showing depressed growth indicated a large proportion of those animals were infected with the QPX parasite (90% prevalence, Sample 2D). Hard clams infected with MA QPX are thought to be physiologically compromised but more data are required to ascertain the role the QPX parasite may play in impacting the physiological status leading to death in the hard clam.

Comparison of MA QPX to CA QPX

The disease caused by the MA QPX parasite in hard clams described in this paper is very similar to the disease caused by the CA QPX parasite identified in Canada (Whyte *et al.,* 1994) (S. E. McGladdery, personal communication). The size of the QPX organisms (5 to 71 µm for CA QPX and 4 to 25 µm for MA QPX), sporulation (in sporangia), number of daughter cells produced in the sporangia (approximately 40), occurrence of halos, and severity of the reactive inflammation were similar between the CA QPX and the MA QPX (Whyte *et al.,* 1994). Additionally, the animals most at risk from dying of this disease in the MA QPX and the CA QPX disease outbreaks were the 1½- to 2-year-old age group.

However, the following differences are noted. In MA QPX, infection and accompanying inflammation occurred primarily in the mantle. The gill was the second most commonly infected organ. The third most commonly infected loci were the sinusoids and connective tissue of the body adjacent to the intestine and style sac profiles. Unlike CA QPX disease (Whyte *et al.,* 1994), MA QPX parasites and inflammation were not normally seen in adductor muscle or muscles and connective tissues of the foot (except in the central vascular sinuses adjacent to or beneath the style sac).

PAS stained the cell wall and the cytoplasm of the MA QPX organisms in tissue sections. Whyte *et al.* (1994) reported that PAS stained only the cell wall in CA QPX organisms. GMS stained the nucleus only in MA QPX organisms as compared to CA QPX organisms in which Whyte *et al.* (1994) described cell wall staining only. Such findings may indicate that a slightly different labyrinthomorphid organism may be responsible for the MA QPX infections.

Whyte *et al.* (1994) reported that the halos around the CA QPX parasites in tissues from the 1989 hatchery outbreak were the result of lytic actions by the parasite. While lucent areas, similar to the lytic areas described in Whyte *et al.* (1994), were identified in tissue sections surrounding MA QPX parasites, mucofilamentous, dendritic-like material was regularly noted in variable but sometimes abundant amounts. Such material was only rarely seen around parasites in tissue sections of clams infected with CA QPX (Whyte *et al.,* 1994). Mucofilamentous material in the clear areas around the parasites could have been removed in histologic processing of CA QPX. Certainly at least partial removal was noted in MA QPX tissues processed for histology. Ab/PAS-stained tissue sections of MA QPX identified the mucofilamentous material as having a large mucoid component. Additionally, recent transmission electron microscopic (TEM) examination of nodules of QPX infection in hard clams from Provincetown have shown this material is not composed of membranous extensions as would have been expected if this material was ectoplasmic net (Perkins, 1973). However, small filaments did extend from the QPX cell wall (F. Perkins, unpublished data).

In both CA QPX and MA QPX, in addition to mucofila-

mentous net, the lucent areas often contained nonnecrotic, sometimes fusiform, hemocytes that appeared to be migrating to cells of the parasite between dendritic extensions of the net in the lucent areas. Lysis of adjacent inflammatory cells and connective tissues was not significant in the MA QPX-infected tissues. The lucent areas surrounding parasites did not collapse during processing or on squash preparations as would be expected in significant lysis (necrosis). Additionally, our observations strongly suggest that the mucofilamentous net physically produces a barrier that inhibits migration of hemocytes to the parasite and thus inhibits phagocytosis.

Inflammation

Localization of parasites and accompanying inflammation in sinusoids of the clam bodies was more commonly noted in the Duxbury disease outbreak. The high percentage of infected clams sampled from the Duxbury lease with parasites and accompanying inflammation in the vascular spaces and adjacent connective tissues surrounding the internal organs contrasted to the finding in Provincetown infected clams. Duxbury findings may be the result of examining a smaller infected sample population (9 vs 45 for Provincetown) or may be a characteristic of the acute disease in previously uninfected populations.

Inflammation associated with MA QPX organisms was chronic, active, and granulomatous and showed various degrees of encapsulation. The encapsulating phenomena observed in this study has been described before in bivalves (Sparks and Morado, 1988) and is similar to granuloma formation in fish. In fish, granuloma formation (the equivalent of bivalve encapsulation) can show various degrees of maturity ranging from large poorly organized foci containing some elongated macrophagic cells lying parallel or at angles to each other (chronic active inflammation) to mature quiescent granulomas with an onion skin-like layering composed of fusiform macrophages. Granulomas of the later type are sharply demarcated from the surrounding tissues. Noga *et al.* (1989) showed that fish macrophages take on squamous epithelial-like features when forming granulomas. It is probable that the agranular hemocytes of the clam function in a similar manner and may represent a phylogenetic precursor of fish macrophages. The parasitic inflammatory lesions seen in this study did not show the mature, onion skin-like encapsulation (granuloma formation), but did show a less organized encapsulating efforts. Such finding are consistent with a "chronic active" pathologic description for inflammation.

Multinucleated giant cells have been identified in healing oyster wounds where they were thought to be macrophagic and of hemocytic origin (Sparks and Morado, 1988). The occurrence of large multinucleated giant cells, seen in some foci in these infections, was not reported by Whyte *et al.* (1994). The multinucleated giant cells, seen in the parasitic inflammation in Massachusetts clams, appeared to phagocytize the larger vegetative and sporulating forms of the parasite and appeared to result from fusion of adjacent hemocytes. Multinucleated giant cells are sentinel cells for chronic granulomatous inflammation in mammals. They are part of the inflammatory response resulting from infection by biphasic fungus, such as *Coccidiodies immitis*, that produces sporangia in tissue and hyphae in culture (Jones and Hunt, 1983). Multinucleated giant cells are also identified in mammals with protozoal infections due to *Besnoitia* sp. (an Apicomplexan) which produces large multicellular cysts in tissues (Jones and Hunt, 1983). Thus, while the occurrence of multinucleated giant cells in MA QPX-induced inflammation is unusual for bivalves, multinucleated giant cells commonly occur in granulomatous inflammation induced by similar parasites in other animals.

Hemocytes in the inflammation occasionally contained recognizable smaller forms of the parasites within their cytoplasm. Many hemocytes and multinucleated giant cells contained numerous large irregular eosinophilic granules in their cytoplasm. These granules were not consistent with regular, smaller, 1- to 2-µm granules usually seen in the cytoplasm of granulocytes. It is possible that the large irregular granules seen in hard clam granulocytes in the QPX infections represent secretory granules. It has been reported that granulocytes of the clam *Tapes semidecussatus* produce variably sized, sometimes abundant secretory granules (Montes *et al.*, 1995). After secretion, this substance forms a fibrillar, acellular substance that surrounds the infecting parasites, resulting in a homogenous acellular barrier between the parasites and the clam's inflammatory cells. In *T. semidecussatus*, granulocytes with secretory granules were histologically most abundant in the inflammatory response in areas immediately adjacent to the parasites (Montes *et al.*, 1995). However, granulocytes containing large irregular granules in inflammation associated with QPX were not localized around the parasites themselves. Also, a homogenous acellular barrier did not occur in inflammation associated with QPX infections in hard clams. Since poorly degraded thalli were identified in the phagolysosomes of some hemocytes within the inflammation an alternate explanation for the unusual granules is that they represent indigestible products of phagocytosis (parasites and parasitic debris) that had been isolated in phagolysosomes and residual bodies (Hinsch and Hunte, 1990).

Method of Infection

Direct infection of adjacent animals via a flagellated zoospore has been shown to be the method of infection in a Labyrinthomorphid disease of abalone (Bower *et al.*, 1989). It is likely that direct infection of adjacent clams also occurs in CA QPX and MA QPX disease via flagellated or nonflagellated cells or both types of cells. However, it is not possible at this point to rule out either that clams are intermediate hosts for this parasite, that the QPX organism is a facultative parasite or that the clam is an aberrant host. Since MA QPX as reported in this work showed differences from CA QPX as reported by Whyte *et al.* (1994), further taxonomic identification of the parasites as well as an elucidation of the mechanism of transmission of infections is needed. Additionally, this study examined only 1½- to 2-year-old animals. The occurrence and severity of infection in younger clams from these leases is also not known.

Distribution of the Parasite

In addition to the occurrences of QPX in clams from Canadian waters, the parasite was identified in archived hard clam tissue from Barnegat Bay, NJ, collected during an incident of high mortality in the 1970s (Susan Ford, personal communication), and from four moribund clams in various stages of autolysis from Mitchell River in Chatham, MA in 1993. QPX-like parasites were not identified in subsequent examinations of clams from either of these locations. It is possible that the disease is more widespread than presently appreciated. The need for routine screening of seed and adult populations of hard clams before transporting to a new area is obvious.

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