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Histopathological alterations associated with Perkinsus spp. infection in the softshell clam Mya arenaria

SM McLaughlin

M Faisal
Virginia Institute of Marine Science

HISTOPATHOLOGICAL ALTERATIONS ASSOCIATED WITH *PERKINSUS* SPP. INFECTION IN THE SOFTSHELL CLAM *MYA ARENARIA*

McLAUGHLIN S.M.* & FAISAL M.**

Summary :

Softshell clams (*Mya arenaria*) collected from the Chester River in the upper Chesapeake Bay showed the presence of *Perkinsus* spp. in ~ 12 % (28/240) of clams examined. The infection seems to run a mild course with the host prevailing in encapsulating invading parasites. The gills appear to be the major site of infection ; however, the parasite was also found in the digestive gland, gonads, and kidneys and occasionally in the tissue and sinuses of adductor muscles. Typically, clusters of protozoal cells were embedded in an amorphous PAS-positive substrate and were surrounded by one or more layers of granulocytes. In heavy infections, both free and encapsulated *Perkinsus* spp. cells were observed in affected tissue forming aggregations of different sizes. Within the tissues of *M. arenaria*, the parasite propagated by schizogony. The presence of large encapsulations in vital organs such as the gills and gonads may adversely affect growth and fertility of affected clams.

KEY WORDS : Softshell clam, *Perkinsus* spp., histopathology, gills, encapsulation.

Résumé : MODIFICATIONS HISTOPATHOLOGIQUES ASSOCIÉES À L'INFECTION DE LA PALOURDE À COQUILLE LISSE, *MYA ARENARIA*, PAR *PERKINSUS* SPP.

La présence de Perkinsus *spp. a été constatée dans environ 12 % (28/240) des palourdes* (Mya arenaria) *récoltées dans le fleuve Chester, dans la partie haute de la Baie de Chesapeake. L'infection semble d'évolution bénigne chez l'hôte, avec encapsulation des parasites. L'infection paraît essentiellement localisée aux branchies ; cependant, le parasite a également été observé dans les glandes digestives, les gonades et le rein, et occasionnellement dans le tissu et les sinus des muscles adducteurs. Des grappes de cellules protozoaires sont typiquement incluses dans un substrat amorphe PAS positif et entourées d'une ou plusieurs couches de granulocytes. Dans les infections importantes, on observe à la fois des cellules de* Perkinsus *spp.encapsulées et des cellules libres dans le tissu atteint, formant des aggrégats de différentes tailles. Le parasite se propage dans les tissus de* M. arenaria *par schizogonie. La présence d'encapsulations de grande taille dans des organes vitaux comme les branchies et les gonades est susceptible d'affecter la croissance et la fertilité des palourdes atteintes.*

MOTS CLÉS : *palourde à coquille lisse,* Perkinsus *spp., histopathologie, branchies, encapsulation.*

INTRODUCTION

Significant losses of bivalves reported worldwide have been associated with infections by protozoa of the genus *Perkinsus*. For example, *Perkinsus marinus* causes deadly systemic infections in the eastern oyster *(Crassostrea virginica)* along the Atlantic and Gulf coasts of the United States (reviewed in Andrews, 1988). In tissues of infected oysters, division occurs by successive bipartitian of *P. marinus* cells in which karyokinesis is followed by cytokinesis (Perkins, 1996). *Perkinsus atlanticus* causes high mortalities in the Portuguese clam (*Ruditapes decussatus,* Azevedo, 1989). The parasite proliferates primarily in gill tissues causing death of infected clams (Chagot *et al.*, 1987). *Perkinsus olseni* destroys the adductor muscles of the black-lipped abalone *(Haliotis ruber)* leading to their death (Lester & Davis, 1981). Conversely, *Perkinsus* species have been identified in over 60 other mollusks worldwide with no reported mortalities (Perkins, 1993).

The softshell clam *(Mya arenaria)* is abundant from Maine to Virginia and is an important filter-feeder of the mesohaline portion of the Chesapeake Bay. Occasional occurrences of *Perkinsus* spp. have been reported in *M. arenaria* from as early as 1954 (Andrews, 1955). Epizootiological studies of 20 upper Chesapeake Bay sites by the Maryland Department of Natural Resources and the National Marine Fisheries Service between 1969-1989 also showed only rare occurrences of *Perkinsus* spp. infection in histologic sections of over 3500 softshell clams (Sara V. Otto,

*National Marine Fisheries Service, NOAA, Cooperative Oxford Laboratory, 904 South Morris St., Oxford, Maryland 21654-9724.
**Virginia Institute of Marine Science, School of Marine Science, The College of William and Mary, Gloucester Point, Virginia 23062.
Correspondence: Shawn McLaughlin. Tel. : 410/226-5193 – Fax: 410/226-5925.
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Oxford, Maryland, personal communication). Recent observations, however, indicate that the prevalence of *Perkinsus* spp. infection in *M. arenaria* is on the rise (McLaughlin *et al.*, 1995; McLaughlin, 1996). Hence, the objective of this study was to describe the most common histopathological alterations associated with *Perkinsus* spp. infection in *M. arenaria.*

MATERIALS AND METHODS

Sampling Sites and Clam Collection

Softshell clams were collected from four sites located in the mouth and lower Chester River, Maryland (Fig. 1) during 1995-1996 by hydraulic escalator dredge, kept on ice, and immediately transported to the Cooperative Oxford Laboratory, Oxford, Maryland. The clams averaged 66.1 ± 12.0 mm in length (ranging from 40 mm to 100 mm) and were held overnight in separate 76 l glass aquaria supplied with artificial seawater at temperatures between 10-16 °C and salinities adjusted to approximate those of the collection sites, i.e., between 2 and 14 ppt.

Histopathology

A total of 240 clams was processed for microscopical examination following the conventional histologic procedures of Howard & Smith (1983). Transverse pieces of tissues containing gills and most of the internal organs were fixed in 1 % glutaraldehyde-4 % formaldehyde in seawater of half the ambient salinity (McDowell & Trump, 1976; Farley *et al.*, 1986). Paraffin-embedded sections (4-6 µm) were stained with Mayer's hematoxylin and eosin (MHE). Additional slides were stained with Periodic Acid-Schiff procedure (PAS) and malt diastase digestion (MPAS) to demonstrate glycogen and various mucosubstances. Fixation and staining of tissues were performed as described by Howard & Smith (1983) from ingredients purchased from Fisher Scientific, Fairlawn, New Jersey, and Polyscientific, Bayshore, New York.

RESULTS

Examination of hematoxylin and eosin-stained tissue sections indicated a prevalence of *Perkinsus* spp. infection in 28 out of 240 (~ 12 %) softshell clams. In this study, ~ 71 % (20/28) of the clams were lightly infected with the parasites found predominately in gill tissue. Moderate infections were observed in ~ 21 % (6/28) of the clams examined and were characterized by increased parasitism in the gills and low to moderate numbers of parasites in other tissues. Only ~ 7 % (2/28) of the clams examined were considered to be heavily infected with most tissues being affected.

Gills appear to be the target tissue for *Perkinsus* spp. infections. In lightly infected clams, the protozoal cells were often seen between gill lamellae either free (Fig. 2.1) or within granulocytic hemocytes (Fig. 2.2). At sites where *Perkinsus* spp. cells were present, fusion between adjacent lamellae was often noticed. In some instances, hemocytes were observed encompassing a single protozoal cell (Fig. 2.2). Clusters of trophozoites were often seen in the gills embedded in amor-

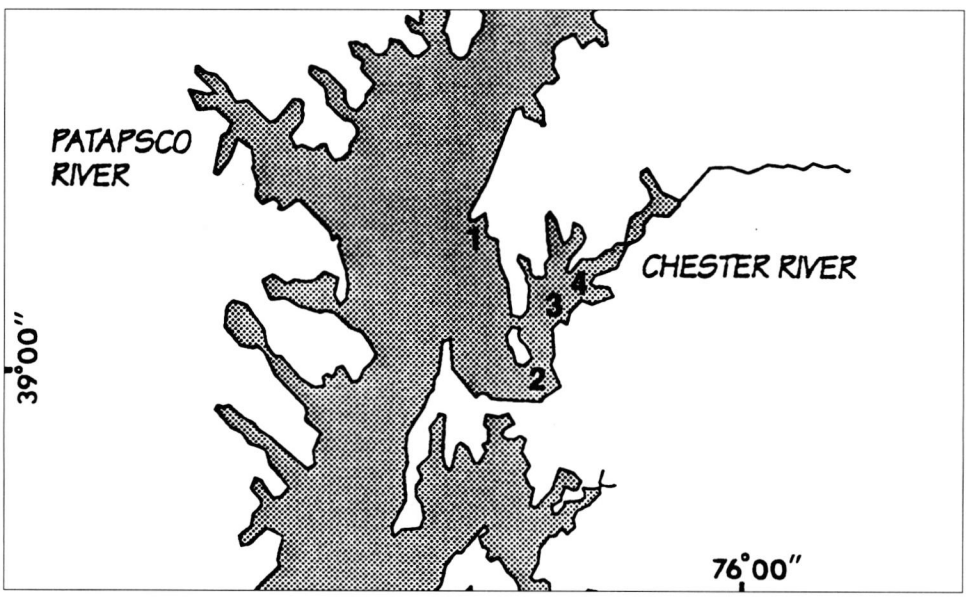

Fig. 1. – Softshell clam sampling sites in the Chester River, Maryland (1 = Swan Point, 2 = Cedar Point, 3 = Piney Point, 4 = Skip Point).

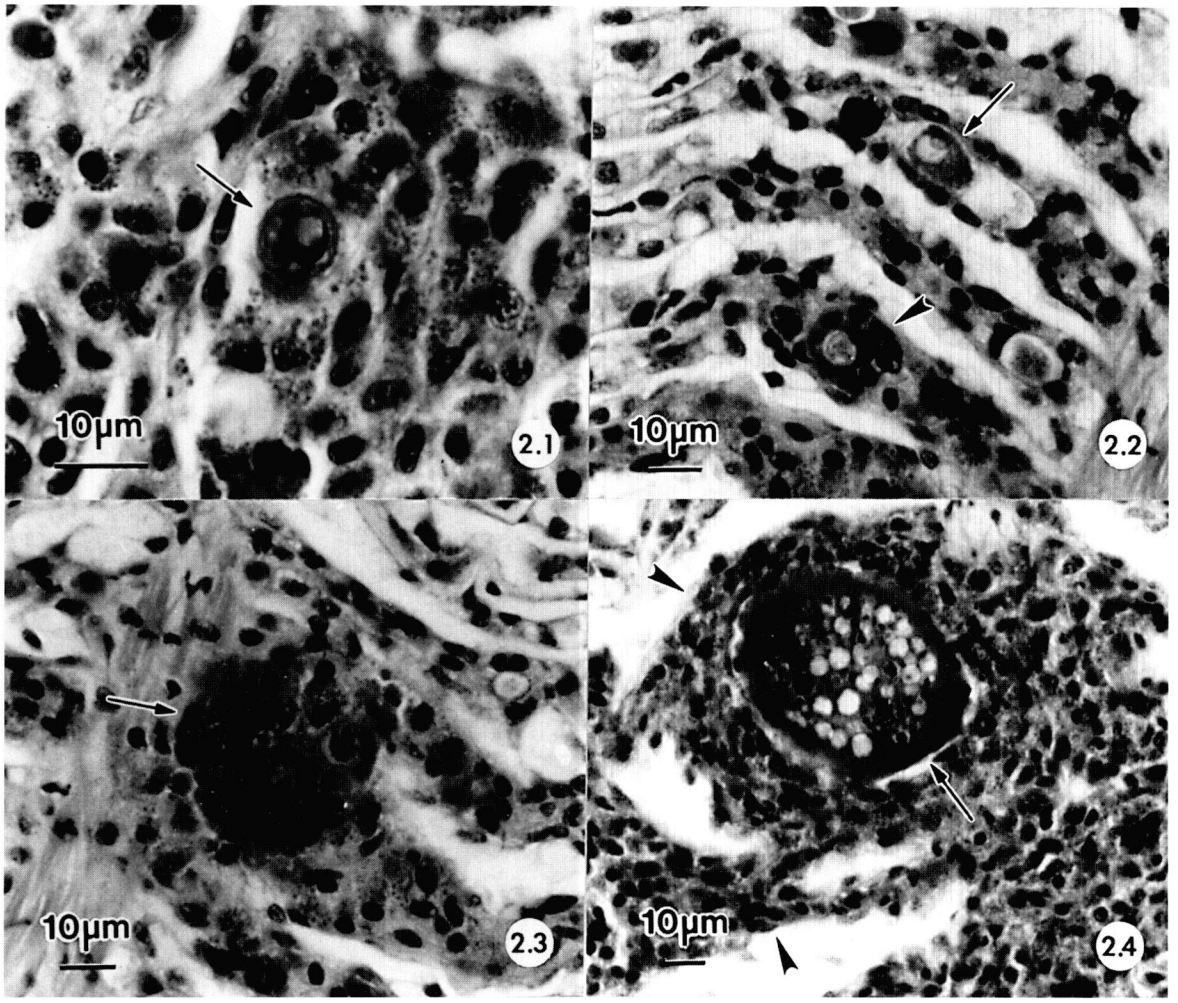

Fig. 2. – *Perkinsus* spp. in the gills of the softshell clam stained with MHE. 2.1: Unencapsulated trophozoites (arrow). 2.2: Protozoal cells within (arrow) or surrounded by (arrowhead) granulocytes. 2.3: Degenerated trophozoites embedded in amorphous eosinophilic material and tissue debris (arrow). 2.4: A typical *Perkinsus* spp. cyst (arrow) showing centrally located protozoal cells and acellular outer wall, notice fusion of adjacent gill lamellae (arrow head).

phous eosinophilic material and tissue debris (Fig. 2.3). Most *Perkinsus* spp. cell clusters were encapsulated in a well-circumscribed wall forming a cyst-like structure. These encapsulations constituted the most commonly observed lesion in gills of infected clams.

Figure 2.4 displays a typical *Perkinsus* spp. cyst with centrally located protozoal cells surrounded by eosinophilic amorphous material (0.5-6.0 µm in thickness) and a number of granulocytic hemocytes with darkly stained nuclei assembled around the outer wall of the encapsulated material. This wall was PAS-positive but negative for glycogen by the MPAS reaction. Cyst diameter averaged 17.8 ± 7.9 µm and ranged from 8 µm to 44 µm. The entrapped trophozoites were circular or oval, averaged 3.8 ± 1.4 µm in diameter, were uninucleated, and each contained a large vacuole that occupied most of the cell. Adjacent gill lamellae were occasionally fused (Fig. 2.4). With the increase in severity of infection, the number of cysts increased and their amorphous outer walls became more demarcated from surrounding tissues. In advanced infections, some cysts ranging in size from 24 to 68 µm in diameter (mean diameter = 47.6 ± 12.8 µm) were observed in addition to the smaller cyst-like structures observed in early infections. The details of granulocytic hemocytes bordering the cysts were often lost in the acellular material leaving atypical nuclei in the outer most layer of the cyst (Figs. 3.1 & 3.2). Some cysts fused forming larger cysts (Fig. 3.3) while in others, protozoal cells disappeared leaving irregular eosinophilic masses (Fig. 3.4). Fusion of gill lamellae was obvious in the vicinity of most cysts.

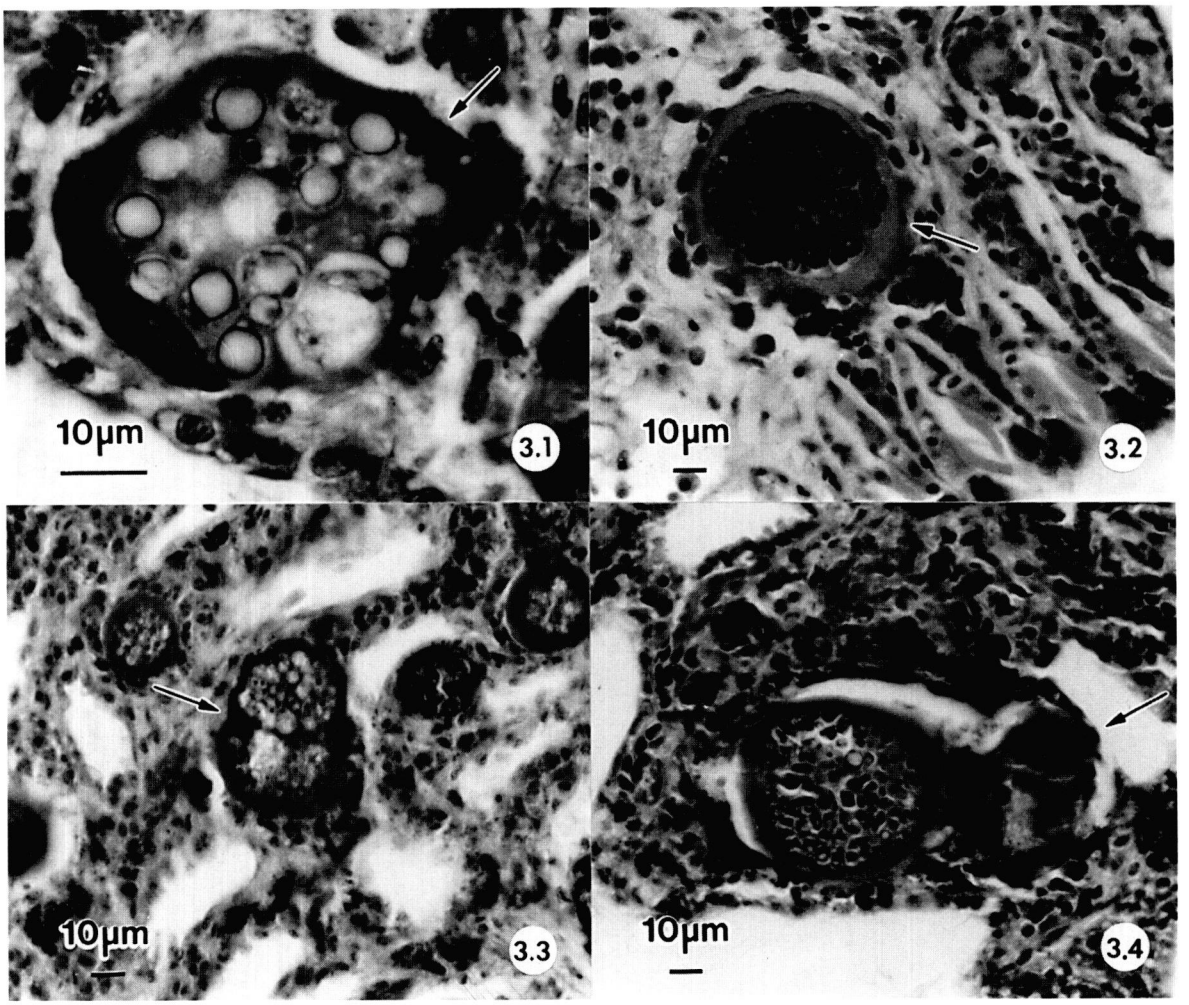

Fig. 3. – *Perkinsus* spp. in the gills of the softshell clam stained with MHE. 3.1: Cluster of protozoal cells embedded in amorphous eosinophilic material and surrounded by a layer of granulocytes (arrow). 3.2: Cluster of protozoal cells surrounded by a thick, acellular wall with the structure of surrounding hemocytes (arrow) lost in this wall. 3.3: Fusion of two adjacent cysts (arrow). 3.4: Eosinophilic, structureless material replaced protozoal cells in a cyst (arrow).

In advanced infections, *Perkinsus* spp. cells predominated the internal structure of gill lamellae (Fig. 4.1) and subepithelial connective tissue (Fig. 4.2). Large lesions were observed in the branchial connective tissues that consisted of encapsulated or free trophozoites, tissue debris, structureless amorphous acidophilic substrate, and hemocytes. Cyst formation was accompanied by loss of the underlying tissue structures including gill lamellae (Figs. 4.3 & 4.4). Cysts observed in the digestive gland were similar in size and morphology to those found in the gills (Fig. 5.1) and were distributed in the connective tissue underlying the mucosa or between the glandular tissue (Fig. 5.2). *Perkinsus* spp. cells were sometimes seen within the gut lumen. Figure 5.3 shows a large lesion in the digestive gland.

Perkinsus spp. cells and cysts were also noticed within the connective tissues of the gonads (Fig. 6.1). Multiple, hypercellular lesions were noticed in gonadal ducts (Fig. 6.2). These lesions varied in size and often were ruptured (Fig. 6.3). The lesions consisted of free and encysted protozoal cells and granulocytic hemocytes within an eosinophilic matrix (Figs. 6.4 & 6.5). Similar lesions were occasionally noticed in the kidneys. The parasite was found in the intertubular connective tissue (Fig. 7.1) with obvious aggregation of granulocytic hemocytes (Fig. 7.2).

Perkinsus spp. cells were also noticed in muscle tissues and sinuses. Except for the presence of hemocytic infiltration, the structure of the adjacent muscles seems to be unaffected (Fig. 8).

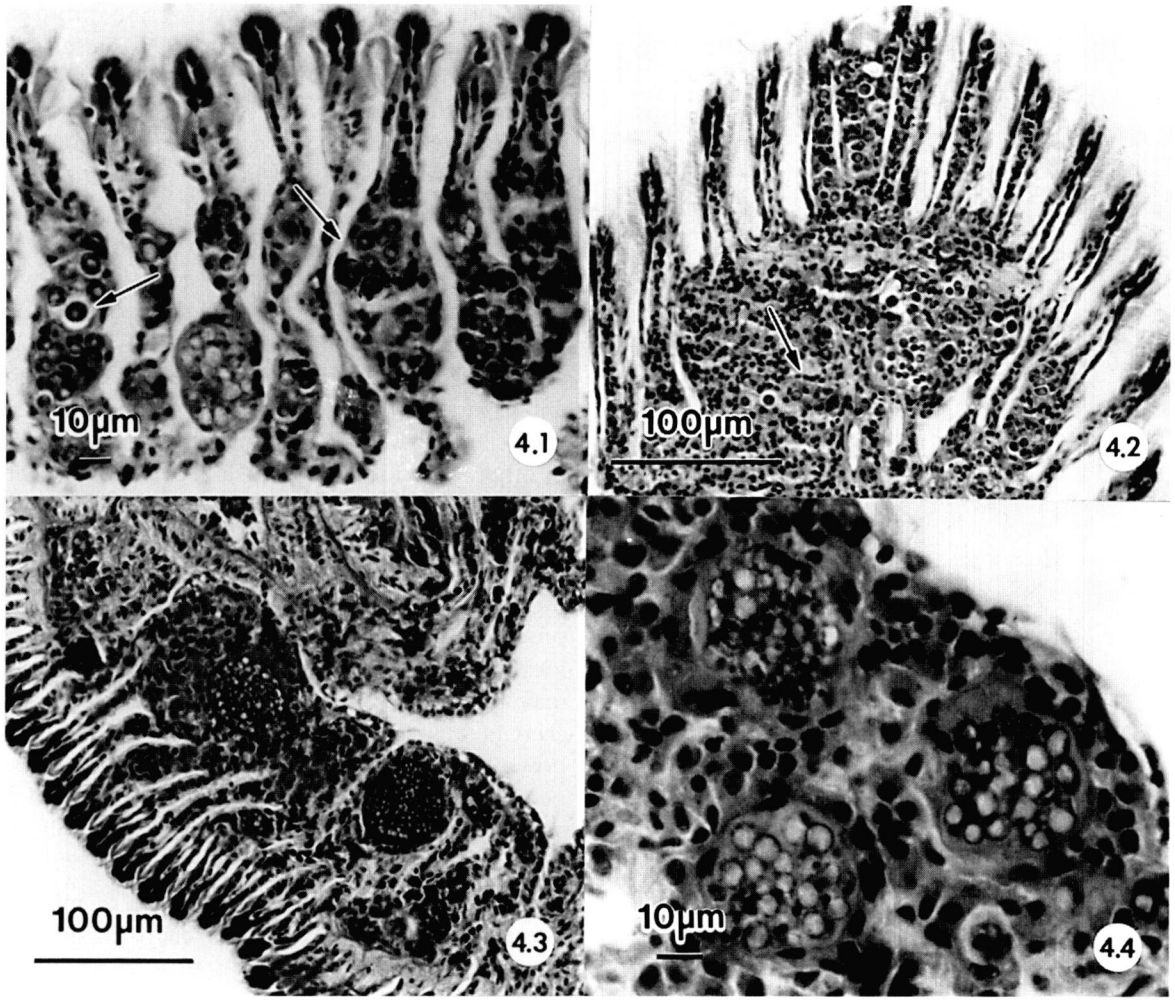

Fig. 4. – *Perkinsus* spp. in the gills of heavily parasitized softshell clam stained with MHE. 4.1: *Perkinsus* spp. cells predominated the structure of gill lamellae (arrows show two sites of many). 4.2: *Perkinsus* spp. cells (arrow) filling the subepithelial tissues of the gills. 4.3 and 4.4: Large cysts in the branchial connective tissues with the loss of underlying tissue structures.

It was apparent that the parasite propagated by schizogony in affected tissues. Schizonts containing up to four daughter cells were also noticed (Fig. 9). Sizes of the schizonts ranged from 6 µm to 12 µm and averaged 7.97 ± 1.6 µm in diameter.

DISCUSSION

Protozoal cells in clam tissue sections exhibited the typical morphology and division patterns of *Perkinsus* spp. The average diameter of trophozoites was larger than those reported for *P. marinus* in stained tissue sections (Mackin, 1951). Diagnostic thioglycolate assays as described by Ray (1952, 1963) confirmed the presence of *Perkinsus* spp. in tissues of infected clams in this study (data not shown). Moreover, analysis of the small subunit ribosomal RNA gene confirmed the relationship of softshell clam *Perkinsus* spp. to other members of the genus Perkinsus (Kotob *et al.*, 1998a, 1998b).

The pathological alterations in infected softshell clams are different from those described for eastern oysters infected with *P. marinus*, where the infection runs an aggressive systemic course (Mackin, 1951). Widespread tissue lysis often observed in oysters heavily infected with *P. marinus* was not observed in softshell clams infected with *Perkinsus* spp. This lysis is due to

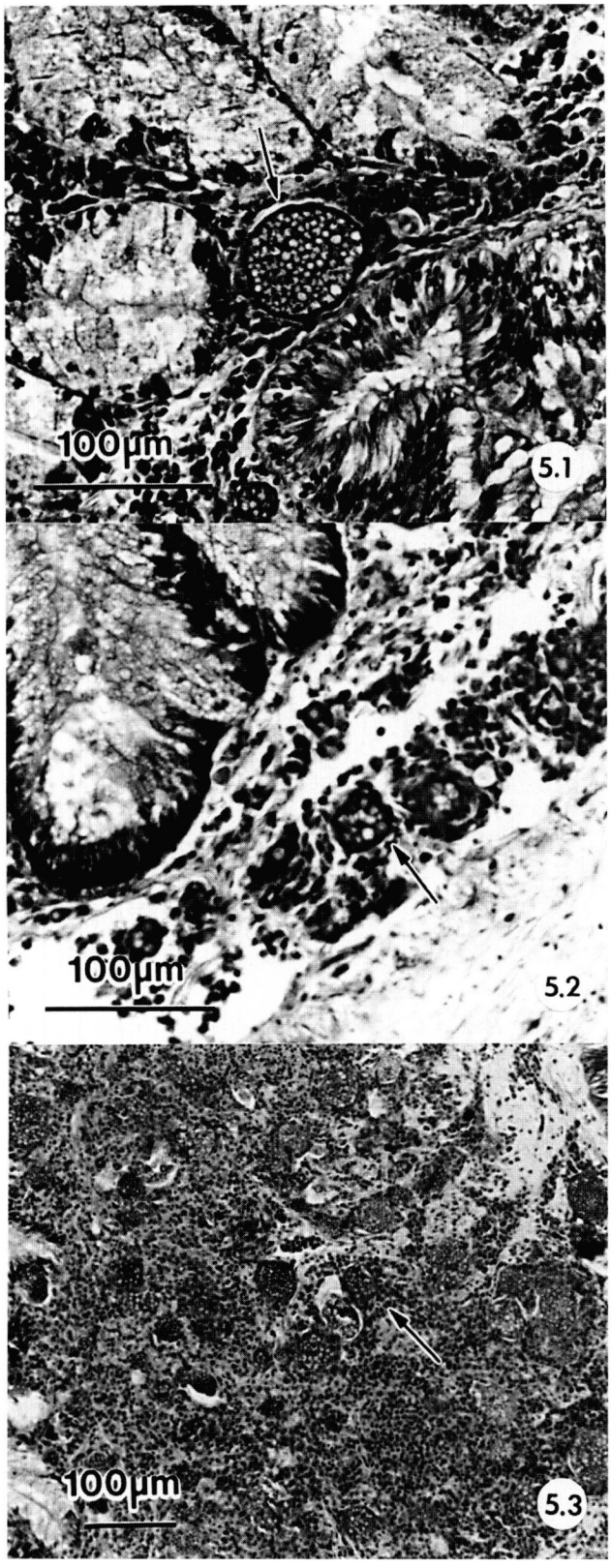

Fig. 5. – Section through portion of the digestive gland stained with MHE. 5.1 and 5.2: *Perkinsus* spp. cysts and clusters (arrows) in the connective tissues of the digestive gland. 5.3. A large lesion in the digestive gland containing several clusters of protozoal cells (arrow), hemocytes, and amorphous, eosinophilic material.

the production of extracellular serine proteases by *P. marinus* (La Peyre & Faisal, 1995; Faisal *et al.*, in press). In the softshell clam, the parasite was observed mainly in the connective tissue, mostly without damaging epithelial lining, which is in accordance with other *Perkinsus* spp. including *P. marinus* (Perkins, 1976, 1993).

A clear tissue response has been provoked in *M. arenaria* due to the presence of *Perkinsus* spp. with the infiltrating granulocytic hemocytes emerging as a major player. The three-layered cyst, outer hemocytic, middle structureless, PAS-positive material, and centrally entrapped trophozoites, typically seen in infected softshell clams share striking similarities to those described in other venerid clams parasitized by *P. atlanticus* or unidentified *Perkinsus* spp. (Chagot *et al.*, 1987; Comps & Chagot, 1987). The elegant studies of Montes *et al.* (1995, 1996) provided evidence that the structureless matrix of the cyst originates from secretory products released following the death of the granulocytic hemocytes. It is suspected that the cyst wall may function in blocking trophozoite dissemination (Navas *et al.*, 1992, Montes *et al.*, 1995, 1996); however, such a cellular reaction may also be suspected to disrupt normal functions of affected tissues such as the gills or block the gonad alveoli as seen in this study.

The gills appear to be the major site of infection by *Perkinsus* spp. in the softshell clam. In general, the infection seems to run a mild course with the host prevailing in encapsulating the invading parasites. In cases of moderate or heavy parasitism, however, substantial areas of the gills were occupied by *Perkinsus* spp. aggregates and hemocytes. These sometimes massive, parasitic aggregates may interfere with respiration and other physiologic processes. Similar involvement of gill tissue has been reported with *P. atlanticus* and other *Perkinsus* spp. infecting other bivalve molluscs such as *R. decussatus* (Azevedo, 1989, Rodriguez & Navas, 1995), *Tapes semidecussatus* (Azevedo, 1989, Montes *et al.*, 1995; Sagrista *et al.*, 1995), and *Macoma balthica* (Perkins, 1988).

In cases of heavier parasitism, free trophozoites, cysts of different sizes containing protozoal aggregates, and lesions have been observed in other vital organs such as the digestive gland, gonads, and kidneys. It is difficult to assess the impact of infection on functions of these organs; however, the presence of large lesions, particularly in the gonads, may adversely affect fertility and growth of affected clams. The low number of severely parasitized clams may either indicate few clams reach high parasite burdens or that the heavily infected clams die from *Perkinsus* spp. or from secondary invaders. The presence of *Perkinsus* spp. trophozoites in sections of the muscle of heavily parasi-

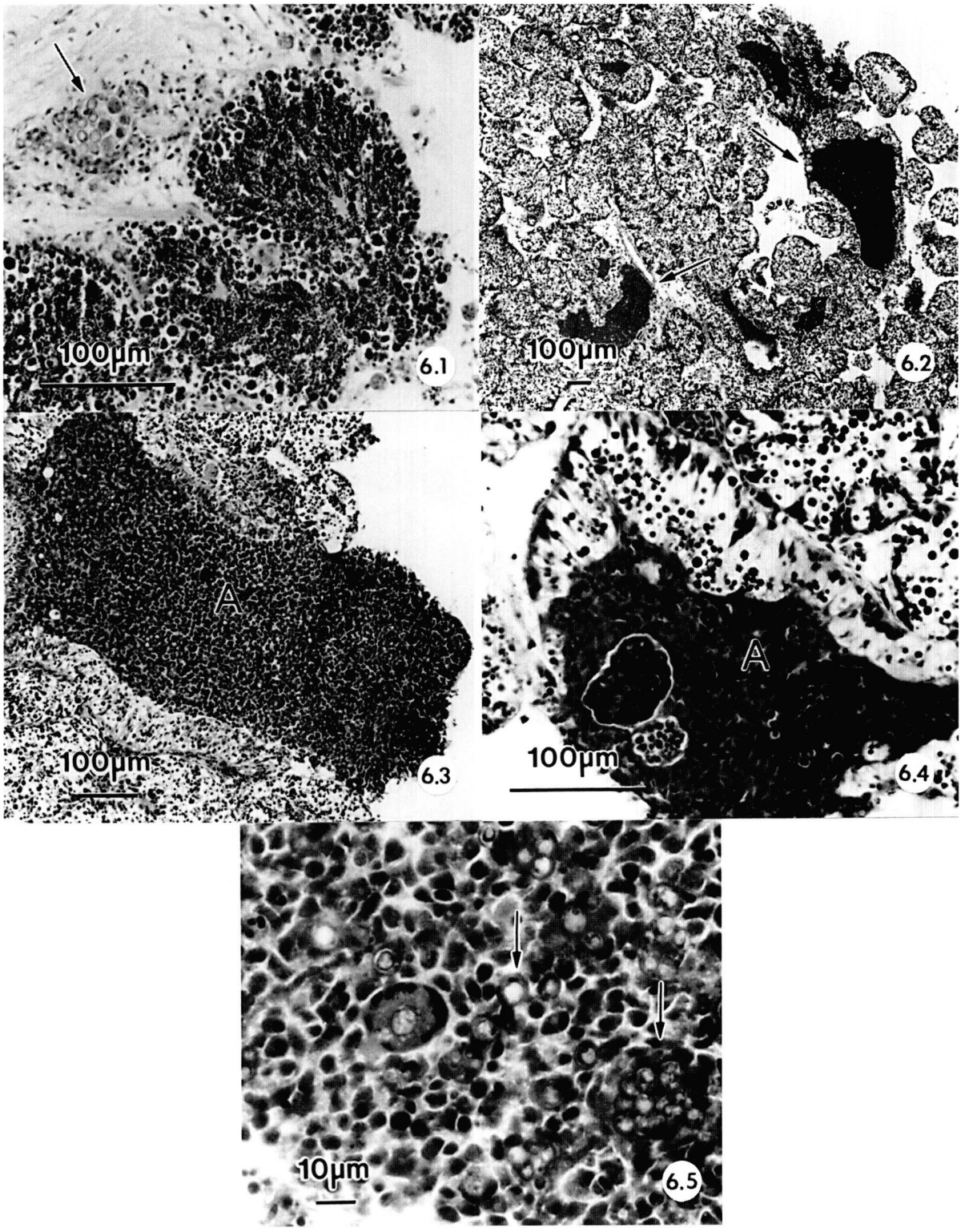

Fig. 6. – *Perkinsus* spp. in the gonads stained with MHE. 6.1: *Perkinsus* spp. cells (arrow) in the connective tissues of the gonads. 6.2: Multiple lesions (arrows) in and around gonadal alveoli. 6.3: A large lesion (A) that ruptured. 6.4 and 6.5: Larger magnifications of a lesion (A) showing protozoal cells and clusters (arrows).

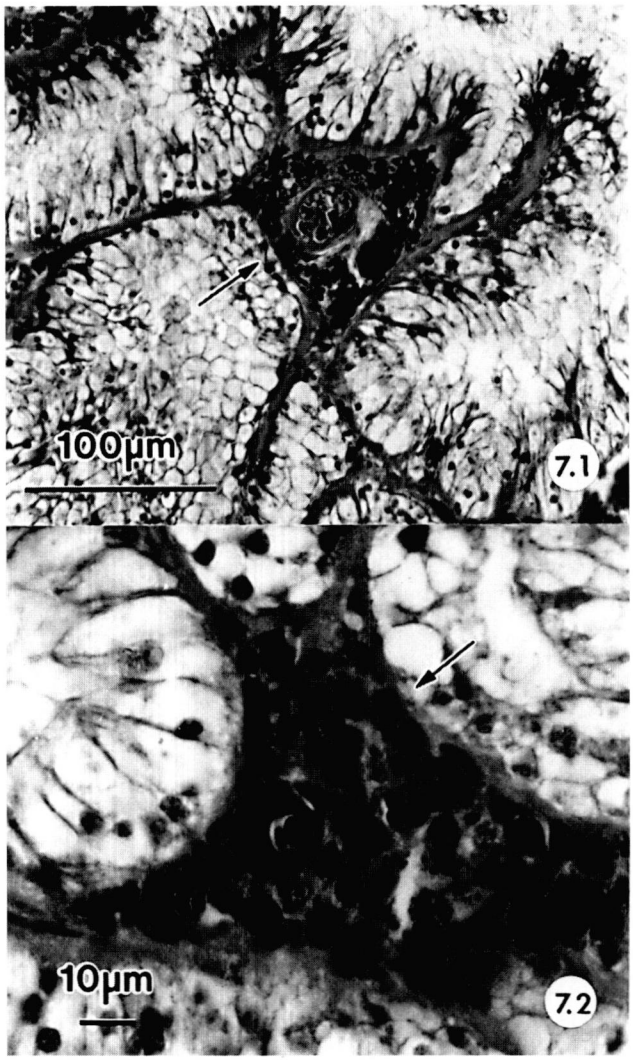

Fig. 7. – Sections through the kidneys stained with MHE. 7.1 *Perkinsus* spp. cyst in the connective tissue between the kidney tubules surrounded by aggregation of granulocytes. 7.2. Trophozoite surrounded by a massive aggregation of granulocytes (arrow).

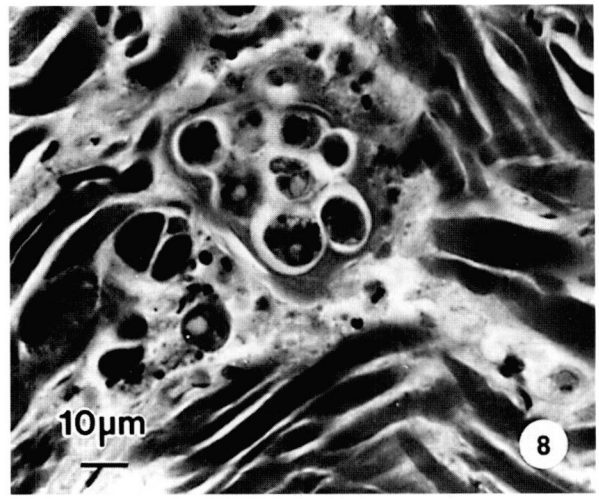

Fig. 8. – A stained section of muscle (MHE) showing trophozoites and clusters of *Perkinsus* spp.

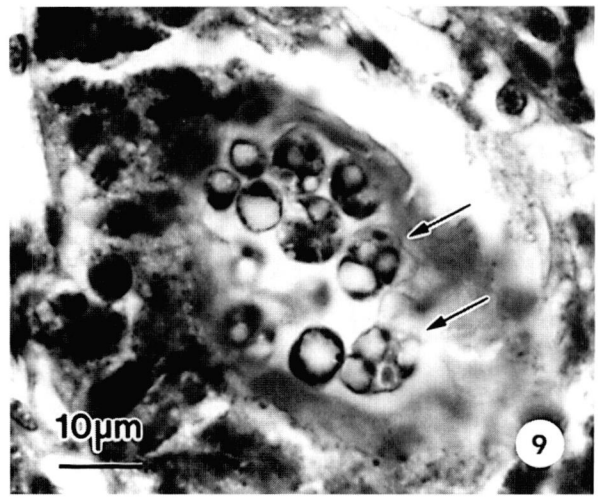

Fig. 9. – *Perkinsus* spp. schizogony in tissues of the softshell clam. Schizonts (arrows) showing multiple daughter cells.

tized softshell clams suggests that the protozoal cells could be disseminated to the entire body via hemolymph.

Isolation of *Perkinsus* spp. cells from infected softshell clams and *in vitro* propagation was successful (McLaughlin & Faisal, in press). Studies to determine species identification and host range of softshell clam *Perkinsus* spp. are being carried out.

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