Sporulation of Minchinia nelsoni (Haplosporida, Haplosporidiidae) in Crassostrea virginica (Gmelin)

Abstract. Minchinia nelsoni, *which Haskin, Stauber, and Mackin found in connective tissue and digestive diverticula of the American oyster, is characterized by a distinct peripheral endosome in its plasmodial and spore nuclei and by spores of unique dimension (7.5 by 5.4 μ). This species is believed to be the etiologic agent, formerly reported as "MSX," responsible for extensive oyster mortalities along the Middle Atlantic Coast.*

Plasmodial stages of a haplosporidian parasite were found in oysters from the Middle Atlantic States. The organism was designated "MSX" (multinucleate sphere X). Since the discovery of the organism, studies of its life history, pathology, and epizootiology have been made (*1*). Plasmodial stages of this organism have been described and named *Minchinia nelsoni* by Haskin, Stauber, and Mackin (*2*). The organism is believed to be the pathogen responsible for "Delaware Bay Disease" (*1*) which has caused extensive oyster mortalities since 1957 along the Middle Atlantic Coast of the United States, particularly in the more saline (greater than 15 parts per thousand) estuarine waters of Delaware and Chesapeake bays.

In our studies of the distribution, prevalence, and pathological manifestations of this and other shellfish parasites, we have sampled oyster grounds from several areas in Chesapeake Bay for the past 6 years (*3*). Recently, prespore and spore stages of a haplosporidian parasite were found. We consider these stages to be part of the life cycle of *Minchinia nelsoni* in the oyster.

Spores and prespore stages were found in July 1965 in 12 of 266 oysters from Chincoteague Bay, the Manokin River, and Tangier Sound on the eastern shore of Chesapeake Bay. Sporocysts and spores were first observed in fresh-stained and unstained squash preparations of connective tissue and digestive diverticula of live oysters that were refrigerated at 5°C for 1 to 3 weeks. The squash preparations were stained either with toluidine blue (0.5 percent aqueous) or with Quensel's stain (*4*); other preparations were fixed in Davidson's fixative (*5*) and stained with iron hematoxylin. Remaining portions of the oysters were fixed in Davidson's fixative, sectioned, and stained with iron hematoxylin or with Harris' hematoxylin–Ziehl's fuchsin-eosin (*6*). Thirty fresh mature sporocysts and 200 fresh spores were measured. Spores were studied in detail, since their morphology presented important diagnostic characteristics of the genus *Minchinia* (*7*). Descriptions were based on fresh squashes and on sections stained with iron hematoxylin. All drawings were made with the aid of a camera lucida; measurements are in microns. Photomicrographs were made of fresh and fixed sporocysts and spores.

In the plasmodial stages (Fig. 1*A*) the organism is generally between and intimately associated with connective tissues of body, gills, and mantle; it is also between and displaces epithelial cells of the digestive diverticula. It is rarely intracellular. The shape is variable, but usually spheroid, 4 to 25 μ in greatest width. The organisms contain varying numbers of spherical nuclei, 1.5 to 2.0 μ in diameter. Each interphase nucleus contains a small endosome at, or near, nuclear membrane.

In the prespore stages, at the onset of sporulation, small plasmodia (Fig. 1*B*) and their nuclei increase in size preceding karyokinesis. Nuclear division (Fig. 1*C*) continues after the nuclei in the enlarged plasmodia have increased in size approximately threefold to fourfold. A conspicuous bar or spindle ("Kernstab" of Jirovec) (*8*) appears within the nuclear membrane; the compact endosome becomes associated with the spindle and divides. The

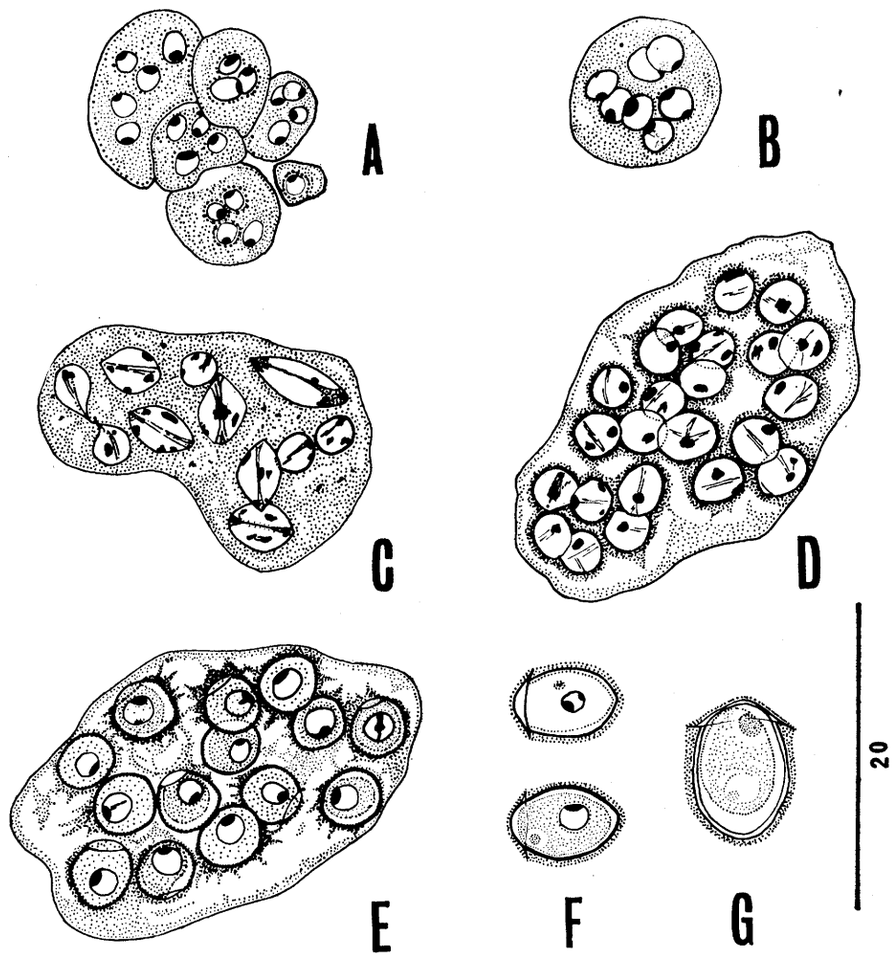

Fig. 1. Prespore and spore stages of *M. nelsoni*. *A*, Multinucleate plasomodia from connective tissue. *B*, Plasmodium with enlarged nuclei. *C*, Nuclear division within enlarged sporont. *D*, Sporont containing sporoblasts. *E*, Early sporocyst. *F*, Fixed and stained spores. *G*, Fresh spore. Measurements are in microns.

daughter nuclei (Figs. 1*D* and 2*A*), produced through karyokinesis, are still relatively large (3 to 4 μ), but in subsequent stages the size diminishes.

Sporonts (18 to 30 μ) formed after nuclear division contain the developing sporoblasts (Figs. 1*D* and 2*A*). Cytoplasmic condensation is apparent around the 3- to 4-μ sporoblast nuclei. The nuclei often possess remnants of the spindle. Sporoblasts rarely contain more than one nucleus. Nuclei are larger in early sporoblasts than in the more advanced. The reconstituted endosome is often adjacent to, or near, the nuclear membrane.

Early sporocysts (28 to 54 μ) are usually filled with a variable number of incompletely formed spores (Figs. 1*E* and 2*B*). Developing spores (6 to 8 μ) are round, with remnants of cytoplasm attached to the outside spore wall. An orifice is often apparent, but fully formed opercula are rare at this stage. Nuclei (1.5 to 2.0 μ) are smaller than those in sporoblasts. Remnants of a spindle are still present in a few nuclei.

Definitive sporocysts (28 to 54 μ) contain from 8 to about 50 spores, and rarely more (Fig. 2*D*). Sporulation is rare in the oyster, but when present, sporocysts are almost exclusively in the epithelium of the digestive diverticula. The oysters which contained sporocysts were 6 to 10 cm long, had orange to white diverticula, and were in poor condition. Histologically, the hosts showed extremely heavy plasmodial infection and a strong pathologic response.

The length of spores range from 5.3 to 10.7 μ and the width from 4.8 to 7.5 μ; modal size is 7.5 by 5.4 μ (Fig. 1, *F* and *G*, and Fig. 2, *E* and *F*). Fixation and staining cause about 10 percent shrinkage. The spore is surrounded by a refractive capsule (1 μ thick) without projections or appendages. It is rotund, with an operculum that extends laterally beyond the margin of the orifice to the margin of the capsule. The operculum is approximately 1 μ high. The wall is birefringent in fresh spores and opaque in optical section when stained (Fig. 1*F*). Sporoplasm is uninucleate and has a darkly staining spherule in various positions relative to the nucleus. The sporoplasm is apparently attached to the inner spore wall at or near the orifice. Upon maturing it becomes acid-fast, only partially fills the spore case, and the spherule disappears. The spore nucleus is small (1.5 to 2.0 μ) and has a peripheral endosome of identical appearance to those in smaller plasmodia (Fig. 1, *A* and *F*, and Fig. 2, *B* and *C*).

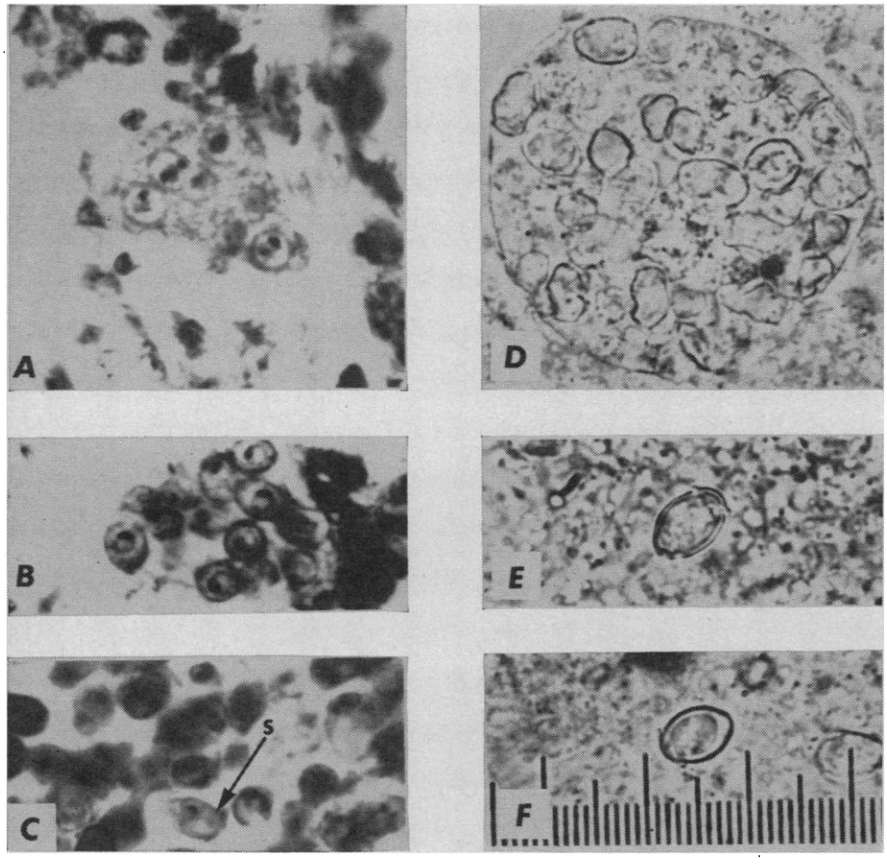

Fig. 2. *A*, Early sporont. *B*, Immature spores showing characteristic nucleus, opercula not yet formed. *C*, Maturing and mature operculate spores. Lighter-stained spore exhibits operculum, nucleus, and spherule (*s* arrow). *D*, Sporocyst in vivo. *E*, Spore in vivo, capsule and operculum in focus. *F*, Spore in vivo, wall and sporoplasm in focus. Nucleus and spherule visible. *A*, *B*, and *C* were stained with iron hematoxylin; *D*, *E*, and *F* show fresh material. Scale is the same for all photomicrographs; 1 micrometer unit equals 1 μ.

In addition to *Minchinia nelsoni*, two haplosporidians occur in oysters. One is an undescribed hyperparasite of the trematode *Bucephalus* (*9*); the other is a known species, *Minchinia costalis* (Wood and Andrews, 1962) (*1*).

The *Bucephalus* hyperparasite cannot be considered congeneric with *M. nelsoni* because it does not possess a spore operculum that originates from the capsule (*7*). Furthermore, stages of *M. nelsoni* have not been found in *Bucephalus* tissues in mixed infections. The trematode and particularly its hyperparasite occur rarely even in areas where *M. nelsoni* is abundant.

The spore of *M. nelsoni* is much larger than the spore of *M. costalis* (3.09 by 2.58 μ). The endosome of the interphase nucleus is always near or adjacent to the nuclear membrane and very distinct in *M. nelsoni*, but is often less distinct and usually not peripheral in *M. costalis*. Sporulation in *M. nelsoni* occurs in the epithelia of the terminal lobes of the digestive diverticula, whereas in *M. costalis* it occurs throughout the tissues. Another significant difference between *M. nelsoni* and *M. costalis* is the lack of antigenic similarity between the two species as found by Barrow and Taylor (*10*) with a fluorescent-antibody technique.

The remaining eight species in the genus *Minchinia* differ from *M. nelsoni* in the following aspects.

1) *M. chitonis* (Lankester, 1885) (*7*, *11*) has capsular spore projections and a central nucleolus with radiating chromatin strands in the spore nucleus. Reported only from chitons.

2) *M. limnodrili* (Granata, 1913) (*7*, *12*) has a reticulated spore nu-

cleus and is reported from the fresh-water annelid *Limnodrilus udekemianus*.

3) *M. nemertis* (Debaisieux, 1919) (*7, 13*) has a spore with an excentric nucleolus and a conical operculum. It is found in the nemertean *Lineus bilineatus*.

4) *M. cernosvitovi* (Jirovec, 1936) (*7, 8*) has a central chromatin mass in the spore nucleus and was described from the fresh-water oligochaete *Opistocysta flagellum*.

5) *M. dentali* (Arvy, 1949) (*7, 14*) has a spore with a conical operculum and is found in the scaphopod *Dentalium* from Europe.

6) The spore of *M. pickfordae* (Barrow, 1961) (*7, 15*) possesses a collar and an excentric nucleolus and is found in fresh-water snails.

7) *M. louisiana* Sprague 1963 (*16*) has a larger spore than *M. nelsoni* and 3 to 4 peripheral chromatin granules in the nucleus. It was described from one specimen of the mud crab, *Panopeus herbstii*.

8) *M. ascidiarum* (Duboscq and Harrant, 1923) (*17*) has a central or slightly excentric nucleolus in its spore nuclei and was described from the ascidian *Amaroucium proliferum*.

Association of spores with vegetative stages formerly known only by the cryptic label "MSX" provides information about the sequence of life-cycle stages of *Minchinia nelsoni* in the oyster and further strengthens the placing by Haskin *et al.* of the organism in the genus *Minchinia*. However, the possibility of an alternate or intermediate host has not been eliminated, and spores identified in this study may provide workers with another stage to be used in future experiments on transmission. Such experiments and the determination of ecological conditions necessary for sporulation should enhance understanding of the epizootiology of this serious disease of American oysters.

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Fluorescent-Antibody Studies of Haplosporidian Parasites of Oysters in Chesapeake and Delaware Bays

Abstract. *A fluorescent antibody was produced against a plasmodial oyster parasite thought to be a haplosporidian,* Minchinia nelsoni, *which was termed MSX. The antibody reacted with this type of plasmodia from oysters,* Crassostrea virginica *(Gmelin), from all sources, and it also reacted with its sporulating and spore stages. This reaction indicates cospecificity of the stages. No reaction occurred with any stage of another oyster haplosporidian,* Minchinia costalis, *indicating that the* M. nelsoni *and the* M. costalis *are antigenically distinct species.*

A multinucleate, usually spherical organism, MSX (*1*), has been studied during the past 8 years because of its incrimination in considerable mortalities of oysters, *Crassostrea virginica*, of the Middle Atlantic Coast. The observed stages have suggested that the organism is a haplosporidian, and it was named *Minchinia nelsoni* on the basis of plasmodial morphology (*2*). When the spore stage, necessary for morphological characterization of the organism, is known, the organism can be readily identified (*1*). When the taxonomic position of the organism is not known, all but epizootic and pathological studies are hampered—particularly when the parasites have not yielded to laboratory transmission. Recently, however, Couch *et al.* (*3*) described a spore other than *Minchinia costalis* (*1*) in the oyster, which they associated with the plasmodial infections of *M. nelsoni*.

We have prepared a fluorescein-conjugated antibody from horse serum which is highly specific for *M. nelsoni*. This reagent can be used to diagnose and relate microorganisms from different hosts in the life cycle, to seek new stages, and to improve recognition of the parasites, at the same time eliminating other known parasites and associates from involvement in the study.

Cape Cod oysters (thought to be free of *M. nelsoni*) were homogenized, dehydrated, and frozen to produce a powdered oyster antigen that included antigenic substances and organisms from the mantle cavity and digestive system as well as from the oyster. It was used (i) as an antigen to produce antiserum for removal of nonspecific antigens from an extract of homogenized oyster tissue infected with *M. nelsoni*, providing a relatively pure antigen for a second host reaction; and (ii) to absorb oyster antibodies from antiserum to *M. nelsoni*. Six heavily infected oysters from Pocomoke Sound, Chesapeake Bay, were the source of the *M. nelsoni* antigen. The parasite anti-