

Haplosporidian Diseases of Imported Oysters, *Ostrea edulis*, in Dutch Estuaries

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Introduction

In The Netherlands, shellfish farming is restricted to one part of the Zeeland estuaries—the Oosterschelde. This is the only shallow, saltwater inlet which is suitable for commercial fattening and growing of mussels, *Mytilus edulis*, and oysters. Concerning the latter, it can be stated that apart from some small samples of Japanese oyster, *Crassostrea gigas*, and the Portuguese oyster, *Crassostrea angulata*, the European flat oyster, *O. edulis*, is the main interest and foundation of the Dutch oyster industry. The center of this oyster culture is situated in the easterly part of the Oosterschelde, near the town of Yerseke, on the Yerseke Bank. This is an area about 16 km long and 8 km wide. It can be characterized as a seawater basin with strong tidal movements and stable salinity values lying in the range from 29 to 30 ‰. This is because no rivers or other fresh water sources now enter the Oosterschelde. Water temperatures range from about 3°C in mid-winter to 20°C in summer, occasionally with extremes of -1.5°C and 22°C. Depths in the oyster farming area are from 0 to 5 m at low tide. The

oyster farming is a bottom culture in which oysters are planted and replanted on plots with optimal conditions to get marketable oysters of best quality.

Previously, spat were collected in the classical way on limed tiles and also on shells of the cockle, *Cardium edule*, and mussel, *M. edulis*. However, the extremely long and severe winter of 1962-63 had a catastrophic effect and threatened the Dutch oyster farming by killing nearly all the oyster stocks, both young and old. To survive economically, efforts were made to restock the Yerseke Bank with spat and young oysters from Brittany in France. Although these oysters are less resistant to winter conditions, the efforts proved to be economically successful and the Dutch oyster culture revived. It resulted in a close commercial relationship between the Dutch and French oyster growers, and also in a change to a more short-period culture in the Oosterschelde with total dependence upon the seed production in Brittany with its profitable

source of young oysters. No further attempts were made during that period to rebuild a large-scale oyster spat production in the Oosterschelde from the small oyster stock that had survived the severe winter of 1962-63.

In this system of balanced Dutch-French oyster production the "first clouds in the sky" came in the occurrence and extension of a mysterious oyster disease in 1967-68 in Brittany, first known as "Aber" disease. Although the Dutch oyster culture has had in the past serious problems with pests and diseases such as the shell disease caused by the fungus, *Ostracoblabe implexa*, and the pit disease due to the flagellate *Hexamita inflata*, it seemed that the Aber disease was a more serious threat.

In 1974 the Dutch Ministry of Agriculture and Fisheries became very worried by the increasing and violent extension of the Aber disease in most of the oyster centers of Brittany and it was decided to give special attention to the problem. It was too late to stop importations from Brittany because of lack of large-scale spat production in the Dutch oyster industry itself. No other source of young European flat oysters to use as seed oysters in suitable quantities, quality, and price, was found in Europe and the only choice was to continue with importations from Brittany as the sole chance of economic survival.

To minimize risks and to get a current view of disease activity in the Oosterschelde, it was decided to guide the oyster growers with respect to their oyster purchases outside The Netherlands and to check the oysters regularly after

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ABSTRACT—Examination of European flat oysters, *Ostrea edulis*, from Dutch oyster farms, but originating in France, revealed the occurrence of two haplosporidian oyster parasites in the Oosterschelde area. The most important, *Marteilia refringens*, seems to be no serious threat in Dutch waters since there is no extension of the parasite nor are there abnormal mortalities. This is in contrast to the situation in

France and Spain. The other haplosporidian, *Minchinia armoricana* van Banning, 1977, occurs only rarely and is currently no threat to the oyster farmers in The Netherlands. With both haplosporidian parasites, the main gap in knowledge is the inability to determine the full life cycle. Detection of the earliest stages and the method of infection of the oyster by the parasite have yet to be accomplished.

planting. The start of Dutch research into the Aber parasite, soon described as *M. refringens* by Grizel et al. (1974) and classified as haplosporidian by a detailed study of Perkins (1976), was in April 1974. With these discoveries, the disease was recognized as the first known haplosporidian parasite to threaten the Dutch oyster industry. The continuous survey of the Dutch oyster culture resulted also in the first observation, in 1974, of another haplosporidian oyster pathogen, which has been described as *M. armoricana* (van Banning, 1977). The occurrence and characteristics of both haplosporidians in the Dutch oyster culture are discussed separately in this paper.

Occurrence and Characteristics of *M. refringens* in Dutch Farming of the European Flat Oyster

Materials and Methods

The survey of *M. refringens* in Dutch oysters is in two parts:

1) A check by sampling all importations of oysters from France (initially of lots selected by the Dutch oyster growers while the oysters are still in France, followed by a second sampling of the oysters on arrival in The Netherlands). This procedure offers the advantage of selecting lots before purchase and reduces unexpected surprises after delivery and planting on Dutch oyster beds.

2) A check by weekly sampling of plots with French oysters and plots with oysters of the original Zeeland stock, which were free of *M. refringens* when the investigation started. This check should give an indication of the characteristics and intensity of any extension of the haplosporidian parasite in Dutch oyster culture.

Because the Dutch oyster growers buy individually, it was necessary to check every importation, even lots coming from the same area or grower in Brittany. Each sample consisted of 10-30 oysters, which may be considered statistically low, but the repeated character of sampling still gives wide-spread and useful information. Furthermore, it was accepted that with the enormous mass of imported oysters in-

involved, it would be impossible to keep out every diseased oyster and that no guarantee could be given of preventing an outbreak of the disease. Accepting this point, a level of infection of 10-20 percent, being the average acceptable annual loss in oyster culture, was regarded as permissible when the oyster growers were absolutely unable to get infection-free oysters.

All samples of oysters are examined histologically, for which small parts of the visceral mass are fixed routinely in Davidson's solution (Shaw and Battle, 1957) for 3-5 hours and embedded in Paraplast¹. For light microscopy, sections are cut at 7 μ m and stained in Mayer's hematoxylin with phloxine as counterstain. For electron microscope studies, small pieces of the visceral mass were fixed in 4 percent glutaraldehyde, made up in 3 percent NaCl at pH 7.2 (0.2 M cacodylate buffer), for 3 hours, and transferred to buffer for 12 hours, at a temperature of 4°C. Tissues were postfixed in 1 percent OsO₄ for 1 hour at room temperature, dehydrated, and embedded in Epon 812. Sections were stained in uranyl acetate and lead citrate, and examined with a Philips 200 or 300 electron microscope.

Experiments to check the possibilities of infection between oysters were carried out on trays in oyster pits (flow-through basins) at Yerseke as well as in aquaria in the laboratory.

Results

Situation on the Oyster Beds

In the first year of work, 1974, *M. refringens* was found to be imported into Dutch oyster farms. The most serious infections were observed in oysters imported for direct consumption trade, but these groups of oysters were not considered as important for extension of the disease because of the short stay of only a few weeks in pits during a period with decreasing temperatures

(October-December). This is also considered not to be the main infectious period of the parasite. These factors are considered to reduce the chance of a violent and mass extension of the parasite, together with the fact that these groups of oysters are kept in oyster pits which prevents close contact with oyster plots outside and offers the advantage that all are sold without the chance of any infected oyster remaining. For these reasons, but mainly for the absolute necessity for providing a commercial base for the oyster growers, no restrictions for importation of consumption oysters are made. Still this group of oysters is kept under examination, because there is some possibility of contact with the Yerseke Bank through the drainage locks of the pits.

Infected lots of seed oysters which are imported for culture purposes are considered to be a greater threat because of their long stay on the outside oyster beds, at least one summer, which offers by time span and season better chances for development and extension of *M. refringens*. However, the first results of 1974 showed that the first known and observed contact of the haplosporidian parasite and Dutch oyster farming was not of a serious character: No abnormal mortalities were observed, no increase of intensity of infection was found on the plot where infected oysters were planted, and no extension occurred into the still disease-free stock of original Zeeland oysters. This unexpected but encouraging result was observed again in 1975 (Table 1). In 1976 the following problem was encountered. The disease had so reduced the flat-oyster stock of Brittany that the Dutch oyster growers could not buy enough disease-free lots for their commercial needs. Based on the positive results of 1974 and 1975 it was decided to accept more infected lots, but still with a limit of 20 percent infection. Despite this increased importation of infected material on the Dutch oyster plots, the year 1976 showed the same character with no increase of incidence in the infected lots and the continuation of the disease-free condition of the original Zeeland oysters (Table 1).

¹Mention of trade names or commercial firms does not imply endorsement by the National Marine Fisheries Service, NOAA.

Table 1.—Prevalence of *Marteilia refringens* in European flat oysters imported from France and in native oysters of the Oosterschelde. Imported oysters included marketable, consumption oysters held in pits and seed oysters planted on open-water beds.

Item	1974	1975	1976
Imported oysters			
Consumption oysters			
Number of infected lots	8	16	2
Percentages of infection	30, 30, 40, 40, 50, 50, 50, 60	10, 20, 20, 20, 20, 20, 20, 20, 20, 30, 30, 30, 40, 60, 70, 80	10, 60
Number of infection-free lots	13	9	3
Seed oysters before planting			
Number of infected lots	1	2	14
Percentages of infection	10	10, 20	10, 10, 10, 10, 10, 10, 10, 10, 20, 20, 20, 20, 20, 30
Number of infection-free lots	37	119	112
Lots denied importation (over 20% infections)	0	19	32
Oysters in culture in Oosterschelde			
Plot planted with an imported, infected lot			
Number of samples	Neg. 22 Pos. 0	22 4	37 8
Percentages of infection	0	20, 20, 20, 20	20, 20, 20, 20, 20, 20, 20, 20
Plot of Zeeland oysters			
Number of samples	Neg. 23 Pos. 0	32 0	41 0

The Parasite in Oosterschelde Conditions

The parasite itself, observed structurally by light and electron microscopy during its stay in the Oosterschelde, appeared to be able to stay in good condition. Multinucleate plasmodia were observed as well as mature sporangia. Studies showed no significant differences in ultrastructure of the parasite compared with the results of Grizel et al. (1974) and Perkins (1976). Experiments carried out in 1975 and 1976 to try to establish the reasons why no extension occurred in the Oosterschelde gave no adequate explanation. Trays with infected oysters (incidence 90-100 percent infection) were placed between trays with healthy oysters in an oyster pit during the period October-December 1975, at temperatures from 10°C, decreasing to 5°C. On a small scale the same experiments were carried out in aquaria at 15°C. None of these experiments resulted in infection of the healthy oysters.

Experiments carried out from June to October 1976 in aquaria at temperatures of 19°-25°C to check various organisms as possible intermediate hosts were also without success. Hepatopan-

creas of infected oysters was fed to brown shrimp, *Crangon crangon*, and shore crabs, *Carcinus maenas*, to study the result of crustacean digestion, but the parasite was not recognizable histologically. An experiment with the gammarid amphipod, *Marinogammarus marinus*, which occurs far more abundantly in some Brittany areas compared with the Oosterschelde, was also without a clear and positive result.

Discussion

The fact that the oyster pathogen *M. refringens* has not shown any violent character or extension in the Dutch oyster farming area—in contrast with the situation of most areas in Brittany and Northwest Spain—offers an interesting point of discussion. Known physical circumstances cannot be considered as an explanation for the difference, because temperature range, salinities, and the way of farming the oysters (except for Spain, using rafts with hanging culture) are similar. Also the fact that imported and infected oysters can show a well-developed parasite after some months of stay in the Oosterschelde, indicates that the physical conditions are unlikely to be the limiting factors preventing the extension of the disease.

These facts, together with the impossibility of infecting healthy oysters by the experiments carried out in the oyster pits and aquaria, do suggest dependency of another factor. Theoretically it could be explained by assuming the occurrence of a special reservoir or an intermediate host, which is not or is rarely present in the fauna of the Oosterschelde. Information from Brittany as well as from Spain (personal communications) indicate that the factors for extension and virulence can be different and even change to being ineffective in the more open- and deep-farming plots. It seems, therefore, that the characteristics of the area will limit the success of *M. refringens* by the occurrence of the intermediate host, rather than in the existence of diseased oysters. The combined impressions of the situations in Brittany and Spain do suggest an organism, living on or between the oysters which has no or a very restricted migrating behaviour, belonging very probably to the Invertebrata. Many invertebrates can fulfill the conditions, but a rough separation can be made in terms of organisms occurring more or less frequently in Brittany but which are rare or absent in the Oosterschelde. Many possibilities must be checked but, for the moment, it appears that knowledge of the haplosporidian, *M. refringens*, is in the same restricted phase as with most other haplosporidian studies: Much morphological information is available including ultrastructural studies, but the life cycle and the initial infection route are still unknown.

Occurrence of a New Haplosporidian Parasite, *M. armoricana*, in the Oyster Culture of the Oosterschelde

Materials and Methods

In the material studied for the Dutch oyster growers some peculiar and apparently diseased European flat oysters were observed. They provided material for the description of a new haplosporidian oyster pathogen, *M. armoricana* (van Banning, 1977), ob-

served in: 1) An oyster taken 19 August 1974 from a Dutch oyster plot planted for 3-4 months with oysters originating from Brittany, 2) an oyster sampled 21 July 1975 from a comparable situation, 3) another on 26 January 1976 from Brittany (St. Philibert), and 4) another on 14 February 1977 also directly from Brittany (Binic). The oysters were thin and glassy looking, and the July and August specimens showed a peculiar brown-colored meat and were of a tough consistency. The four infected specimens were observed from ca. 5,400 oysters studied histologically. Methods for study with light electron microscopy are the same as used in describing *M. refringens*.

Results

The first study of the brown-colored oyster showed the occurrence of a mass of spores in the connective tissue throughout the oyster. Squash preparations of fresh material showed that spores form two long projections of 70-100 μm . Electron microscope studies of the mature spores (Fig. 1) indicated a classification in the genus *Minchinia* and to a new species for which the name *M. armoricana* has been proposed (van Banning, 1977).

Spores

The spores measure $5.0-5.5 \times 4.0-4.5 \mu\text{m}$ in squash preparation of fresh material and $4.0-4.5 \times 3.0-4.0 \mu\text{m}$ in sections prepared for light and electron microscopy. The spores have an orifice covered by a hinged lid and are surrounded by an extraspore cytoplasm or outer spore coat (Fig. 1, 2, 3). In the sporoplasm are observed a nucleus (90-93 nm), a so-called "spherule" or Golgi apparatus (Perkins, 1969) near the orifice, and bodies characterized by Perkins (1969, 1976) as haplosporosomes. Fully mature spores were most numerous in the July and August oysters. The January and February specimens show more developing sporocysts with several stages of spore development. The earliest recognizable phase of spore development is represented by unicells without walls and contained in a sporont.

Sporocyst

The spores occur in sporocysts of 35-50 μm in diameter, present specifically in the connective tissue of the oyster. The wall of the sporocyst in young stages is very thin and folded in sections for electron microscopy. Between the developing spores numerous particles and organelles are enclosed (Fig. 4), some resembling mitochondria.

Sporonts

Stages of the parasite with sporont characteristics and with spindle-type nuclei were clearly observed only in the January oyster, from which no material for electron microscopy was taken. Light microscope study indicated that the size of the sporonts was in the order of 30-45 μm .

Plasmodia

Plasmodial stages were present in the January oyster in which no ultrastructural studies were possible. In light microscope preparations, the plasmodial stages measured 17-25 μm in diameter. Some material from the February oyster was fixed for electron microscope study and showed possible plasmodial stages of *M. armoricana*. Their identity as plasmodia is uncertain because of the lack of comparable data from other infected oysters. These results are further considered in the discussion.

Discussion

Features of the haplosporidian, *M. armoricana*, of European flat oysters, are very similar in the basic systematic unit, the spore, to *Minchinia costalis* (see table 1 in van Banning, 1977). The latter has been known since 1959 in *Crassostrea virginica* along the Virginia coast in the United States. Resemblances are in shape and construction of the spore wall as well as in the order of size of the spore, whose mean size, however, is somewhat smaller than that of *Minchinia armoricana*. *Minchinia chitonis* and *M. nemertis* have far larger spores (table 1 in van Banning, 1977). However, a clear difference from *M. costalis* can be seen in

the ability of *M. armoricana* to form two long projections and to discolor the oyster brown at time of mass sporulation, features which are not observed or reported in *M. costalis*. On this point *M. armoricana* closely resembles *M. chitonis*, which also shows two projections in exactly the same position and also the ability to color the host brown (Pixell-Goodrich, 1915; Debaisieux, 1920). *Minchinia chitonis* occurs in the same area but in a different host (chitons) from *M. armoricana*.

Unfortunately, the study of the youngest stages of *M. armoricana* is incomplete in that suitable ultrastructural material and repetitive observations are lacking. Only the February oyster permitted some investigations of what is thought to be a plasmodium of *M. armoricana*. It was observed as an amoeboid cell (Fig. 5), measuring 5-9 μm , in which were present a number of vesicles or small nuclei, 0.7-1.3 μm in diameter, and characterized by electron-dense material. This electron-dense material is asymmetrically divided, giving the impression of "caps." In some presumptive plasmodia a large nucleus measuring 3.6-5.2 μm is also observed. At this point the author is uncertain whether the observed cell type is a granular hemocyte of the oyster or a plasmodium of *M. armoricana*. As regards the latter possibility, the observed cell agrees with some characteristics of the plasmodium of *Minchinia nelsoni*, as described by Haskin et al. (1966): "Plasmodium, 4-30 μm , occasionally 50 μm , with one to more than 60 nuclei from 1.5 to 7.5 μm in diameter." Of the group of smallest nuclei in *M. nelsoni*, 1.5-1.6 μm in diameter, it is noted that they have densely staining caps. In this the presumptive plasmodia of the *M. armoricana*-infected oyster do show a resemblance. However, the occurrence of one large nucleus resembling the type of nucleus found in oyster granulocytes or connective tissue cells and without any resemblance to the large nuclei (2.5-3.0 μm) of the *M. nelsoni* plasmodia with prominent intradesmoses (Haskin et al., 1966; Farley, 1967), makes it disputable. If the cell must be considered as an oyster

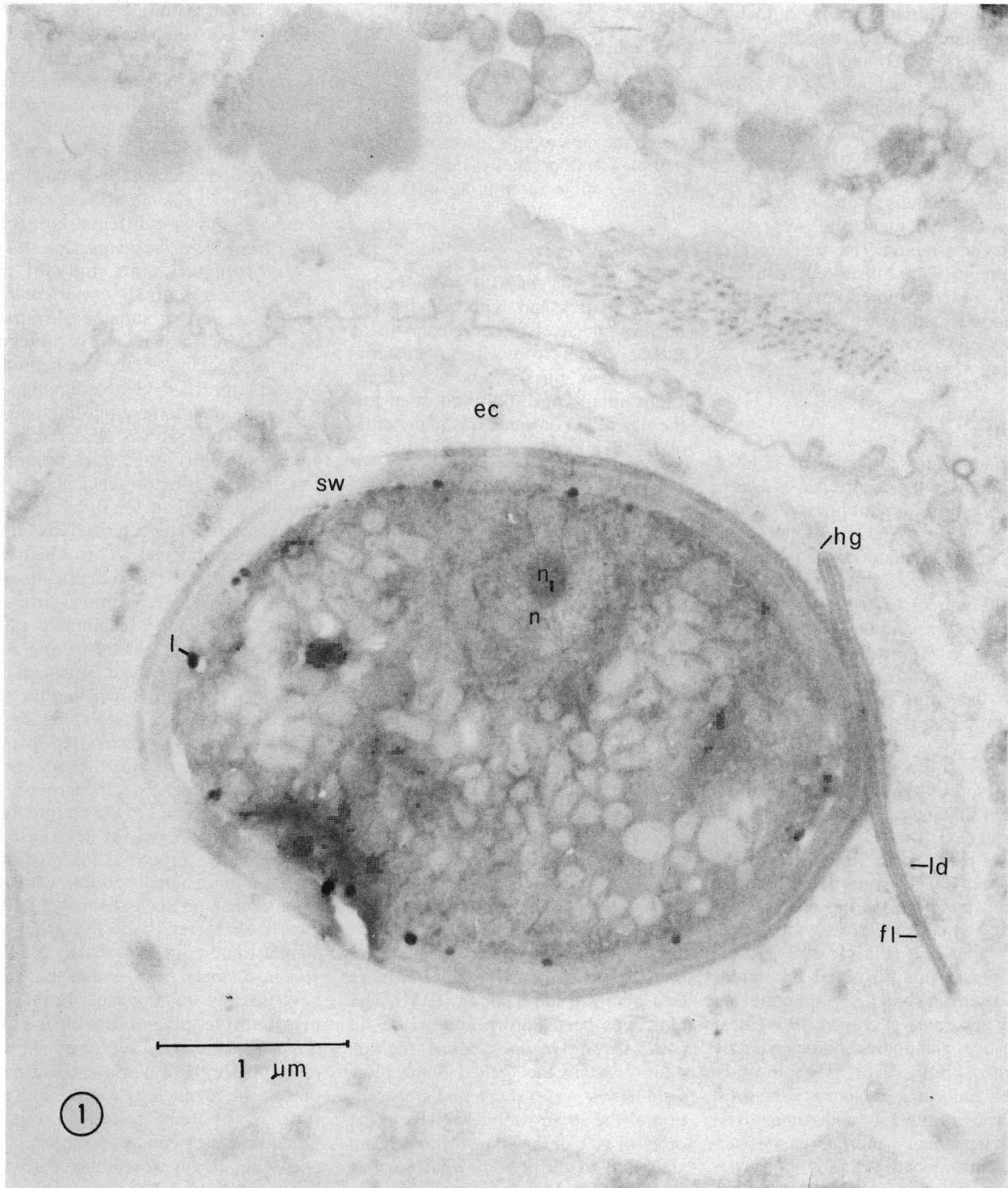


Figure 1.—*Minchinia armoricana*. Spore showing extraspore cytoplasm (ec), spore wall (sw), lid (ld) with hinge (hg) and flange (fl), lipoid inclusion bodies (l), and nucleus (n) with nucleolus (n₁). (From van Banning, 1977; reprinted with permission of Academic Press.)

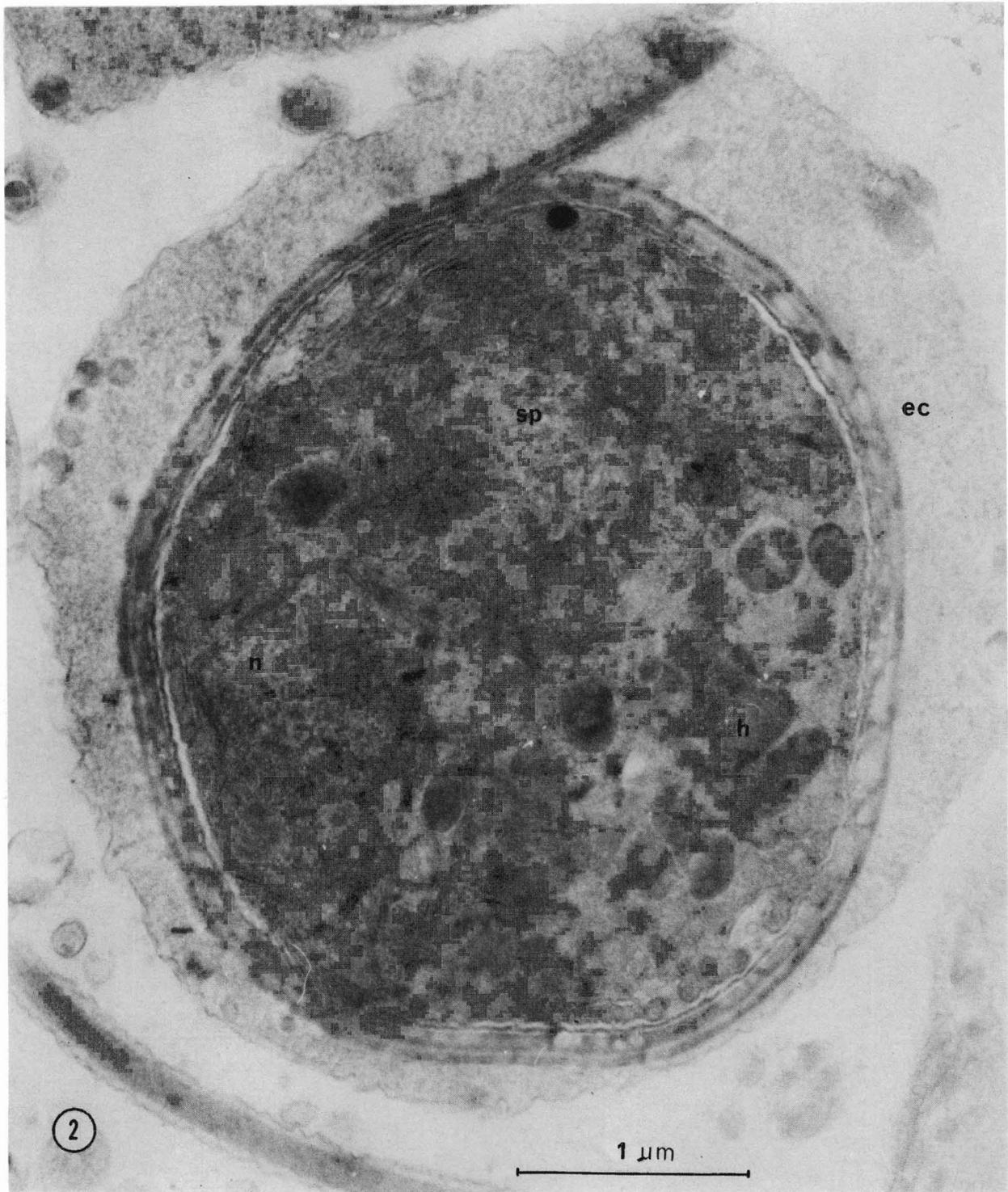


Figure 2.—*Minchinia armoricana*. Spore, with nucleus (n), spherule (sp), haplosporosomes (h), and extrasporoplasm cytoplasm (ec).

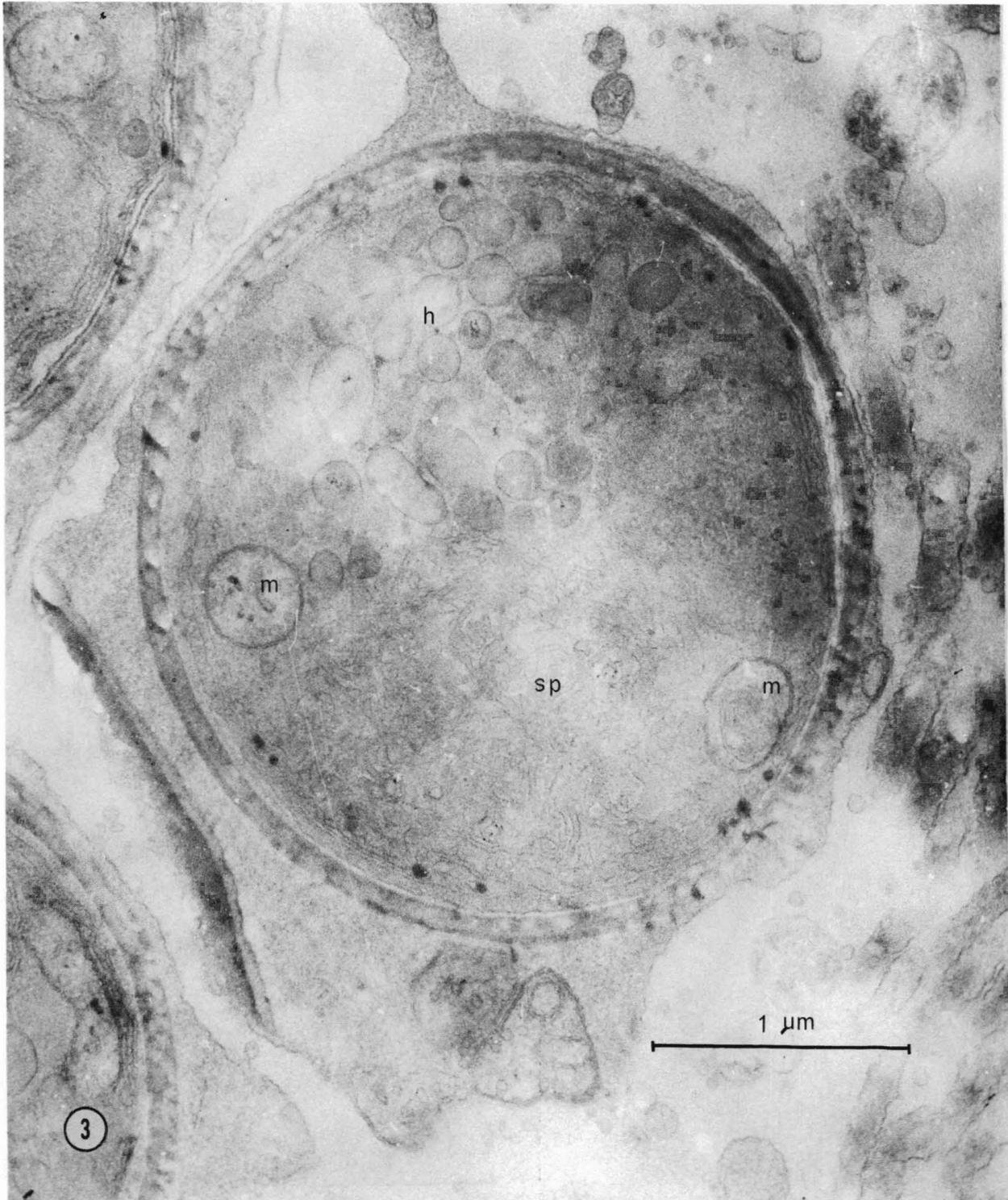


Figure 3.—*Minchinia armoricana*. Developing spore with spherule (sp), haplosporosome initials (h), and presumptive mitochondria (m).

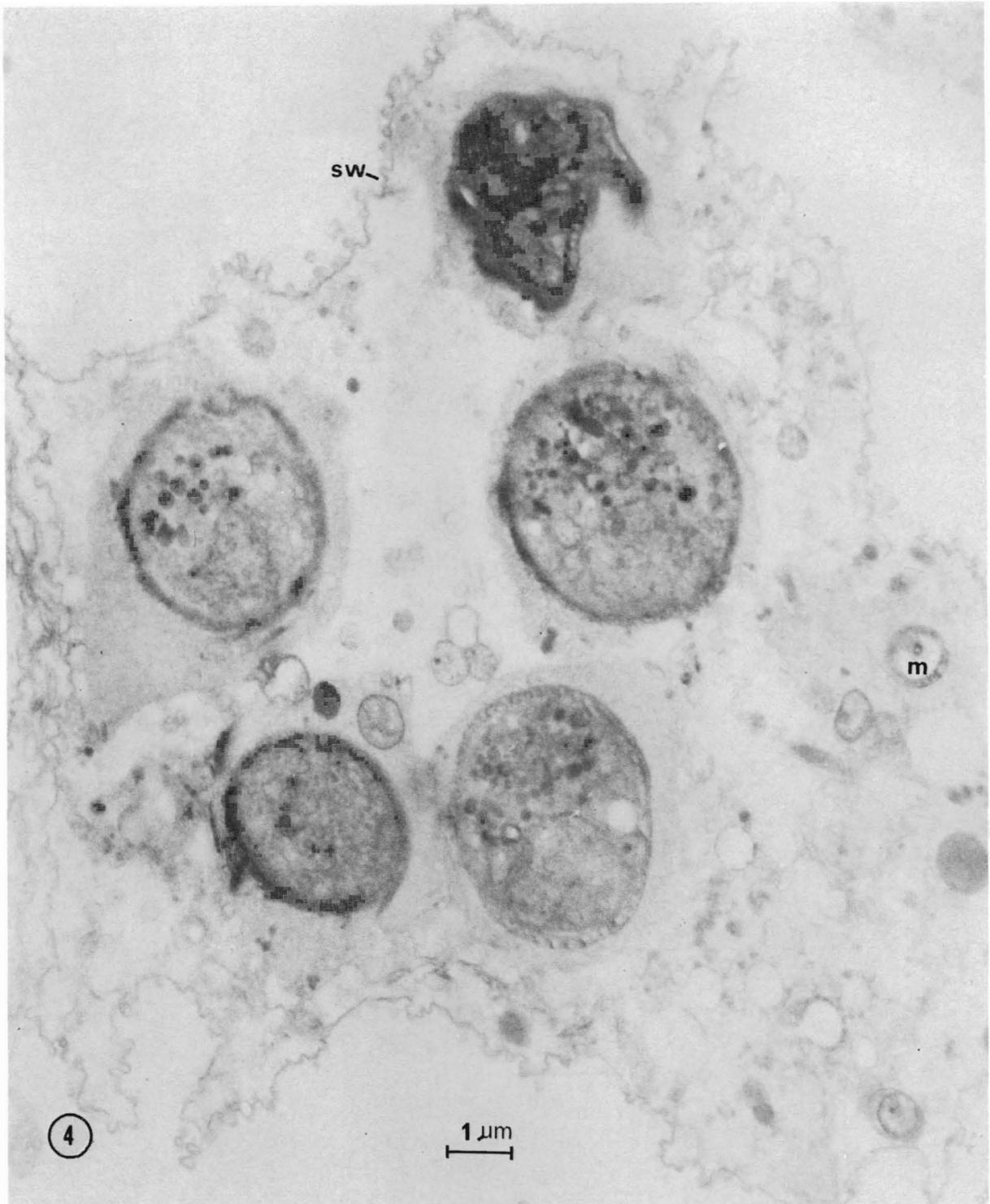


Figure 4.—*Minchinia armoricana*. Early sporocyst stage with very thin sporocyst wall (sw) surrounding developing spores and organelles, some of which resemble mitochondria (m).

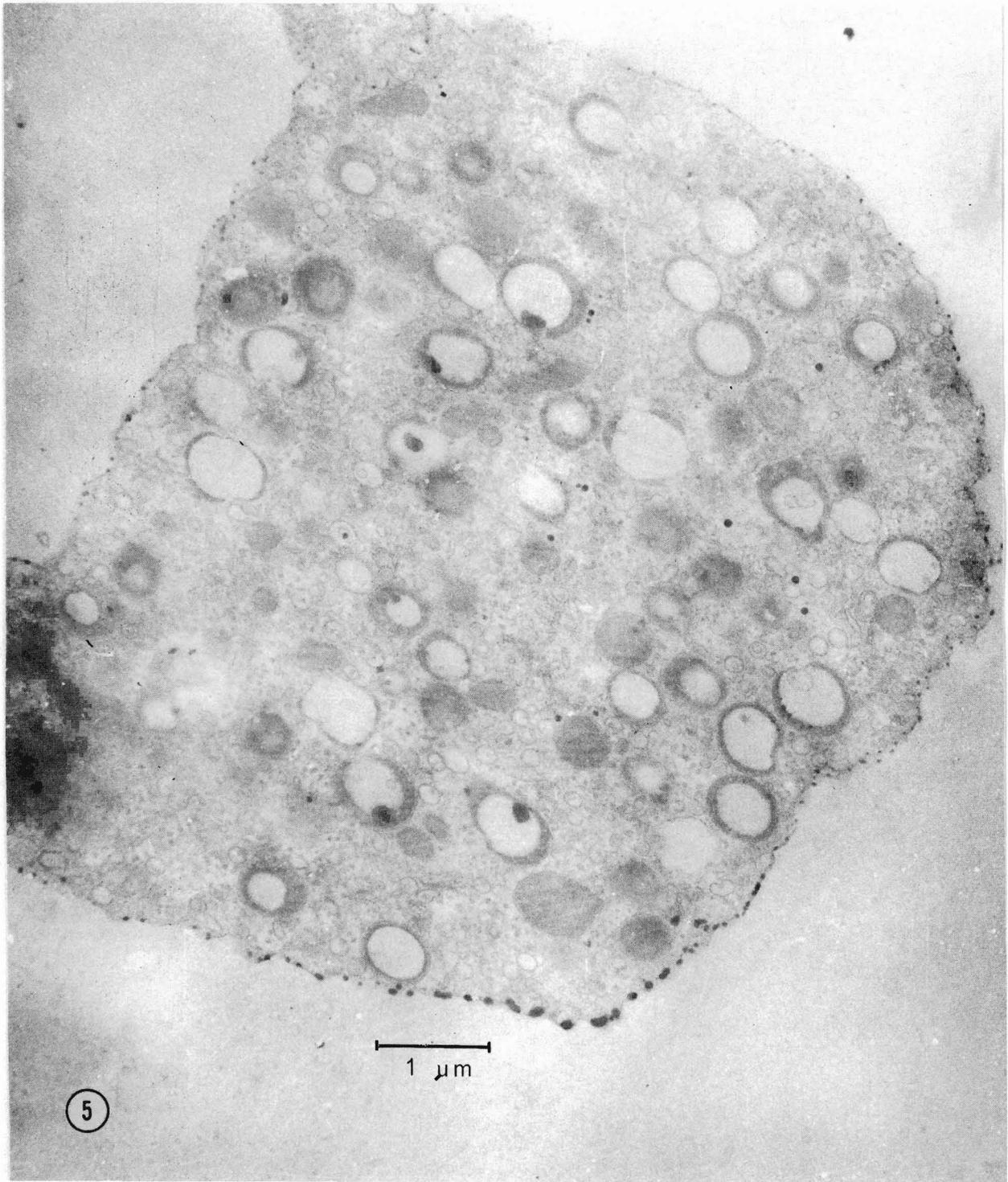


Figure 5.—*Minchinia armoricana*. Cell observed in infected European flat oyster which may be a plasmodium-like stage of the parasite.

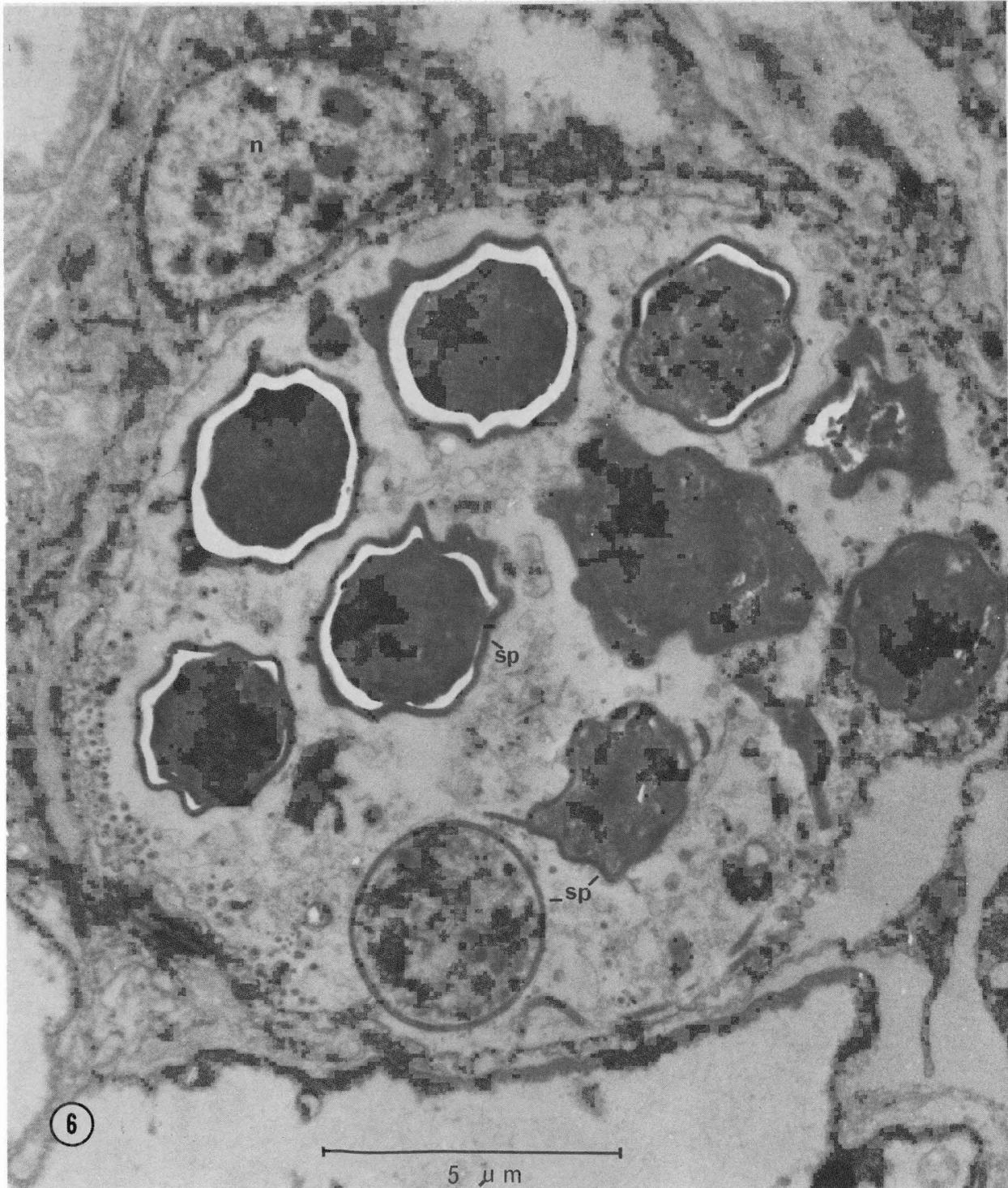


Figure 6.—*Minchinia armoricana*. Sporocyst with nearly mature spores (sp) enclosed by and developing in a cell with a large nucleus (n).

granulocyte, the observed situation in Figure 6 does at least indicate that *M. armoricana* can be intracellular in its host.

To close the gap in knowledge of the younger stages of *M. armoricana*, more specimens of diseased oysters need to be found. Attention should be given particularly during oyster sampling in the September-January period on the Dutch and French oyster beds. Fortunately, the rarity of this haplosporidian oyster parasite makes it of no significant threat at the moment for the Dutch oyster industry, but the serious problems caused in the American oyster, *C. virginica*, industry of the U.S. Middle Atlantic coast due to *M. cos-*

talis and *M. nelsoni* does give good reason to direct research attention to *M. armoricana* and its status in European oyster culture.

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