SCI. MAR., 60 (2-3): 283-288 SCIENTIA MARINA 1996

Ultrastructural data on the life cycle of the parasite, *Perkinsus atlanticus* (Apicomplexa), on the clam, *Ruditapes philippinarum*, in the Mediterranean*

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SUMMARY: An Apicomplexan *Perkinsus* species has been found parasitizing the clam *Ruditapes philippinarum* (= *Tapes semidecussatus*) collected on the Mediterranean coast in the region of the Ebro Delta (Tarragona, Spain). Light and transmission electron microscopy were used to study different stages of this parasite during zoosporulation induced by incubation in thioglycollate medium and seawater. During incubation the trophozoites began zoosporulation, which originated prezoosporangia and zoosporangia at different developmental stages. Successive cytokinesis and nucleokinesis gave rise to prezoospores, which became elongate and differentiated in biflagellated zoospores. The latter presented large mitochondria and an apical complex formed by a conoid, polar ring, micronemes, rhophtries and subpellicular microtubules. The zoosporangium wall showed some typical lamosomes and a discharge tube developed in early phases of incubation. Ultrastructural data were compared with the only four species of the genus *Perkinsus* previously described. The morphological data, the host and the geographic proximity suggest that the species located on the Mediterranean coast was *Perkinsus atlanticus*.

Key words: *Perkinsus atlanticus*, apicomplexa, *Ruditapes philippinarum*, zoosporulation, lamosomes.

RESUMEN: *Perkinsus*, (Apicomplexa) ha sido hallado parasitando almejas de la especie *Ruditapes philippinarum* (= *Tapes semidecussatus*), recolectadas en la costa Mediterranea, en la zona del Delta del Ebro (Tarragona, España). Se ha inducido el proceso de zoosporulación incubando las branquias parasitadas en un medio de tioglicolato, posteriormente han sido aislados los trofozoitos y cultivados tres dias en agua de mar. Durante el periodo de incubación los trofozoitos inician una esporulación que da lugar a prezoosporangios y zoosporangios. Se ha realizado un estudio al microscopio óptico y electrónico de los diferentes estadios observando como en el interior de la pared del prezoosporangio se suceden nucleoquinesis y citoquinesis que dan lugar a prezoosporas. Las prezoosporas se diferencian en zoosporas biflageladas. Estas zoosporas maduras presentan grandes mitocondrias con crestas tubulares y un complejo apical formado por un conoide, un anillo polar, micronemas, roftries y microtubulos subpeliculares, estructuras típicas del phylum Apicomplexa. La pared del zoosporangio está bien desarrollada y presenta un tubo de descarga y lamosomas. Los lamosomas, estructuras presentes en la pared de los zoosporangio de los Apicomplexa, dan positivo a las tècnicas de Thièry y ácido fosfotúngstico lo que nos indica la presencia de glucoproteinas en esta estructura. Los datos ultraestructurales obtenidos han sido comparados con las cuatro especies del gènero *Perkinsus* previamente descritos. Las características morfológicas estudiadas, la similaritud del huésped y la proximidad geográfica nos permiten concluir que se trata de *Perkinsus atlanticus*.

Palabras clave: *Perkinsus atlanticus*, apicomplexa, *Ruditapes philippinarum*, zoosporulación, lamosomas.

INTRODUCTION

The *phylum* Apicomplexa contains numerous pathogenic parasites of molluscan species. The most recent addition to the *phylum* Apicomplexa was the class Perkinsea, established to accommodate the genus *Perkinsus* (Levine, 1978). This genus is a severe pathogen in molluscan species (Lauckner, 1983), and was the only one described in this class. It is now represented by only four species: *Perkinsus marinus* (formerly named *Dermocystidium mari-*

*Received December 5, 1994. Accepted May, 25, 1995.
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num) (Mackin *et al.*, 1950), described as a bivalve pathogen in the American oyster, *Crassostrea virginica* from the USA (Perkins, 1969), *P. olseni* found parasitizing some bivalves from Australia (Lester and Davis, 1981), *P. atlanticus* parasitizing the clam *Ruditapes decussatus* from Portugal (Azevedo, 1989; Azevedo *et al.*, 1990), and *P. karlssoni* parasitizing the bay scallop *Argopectens irridians* from Canada (Mcgladdery *et al.*, 1991).

Perkinsus or *Perkinsus*-like infestations in clams were first reported on the coast of Portugal (Chagot *et al.*, 1987; Azevedo, 1989) and later in other regions. Infestation was detected on the Atlantic coast: in Galicia (NW Spain) (Gonzàlez Herrero *et al.*, 1987; Figueras *et al.*, 1992), in Huelva (SW Spain) (Navas *et al.*, 1992), and on the west coast of France (Arcachon) (Goggin, 1992). On the Mediterranean coast *Perkinsus* infestation was noticed in Laguna Veneta (NE Italy) (Da Ros and Canzonier, 1985), in the Delta Ebro (NE Spain) (Sagristà *et al.*, 1991), and on the Sete coast (France) (Goggin, 1992). Localization of *Perkinsus* in these studies was established by the thioglycollate method and the parasite was not identified to species level.

High mortality in clams, *R. decussatus* and *R. philippinarum* was detected in clam farms in "Bahia dels Alfacs" (Delta Ebro, Tarragona, Spain) in summer 1990. The gills of clams from this zone were cultured in fluid thioglycollate medium according to the procedure of Ray (1952), which revealed an infestation by *Perkinsus* sp. in clams *R. decussatus* and *R. philippinarum* (Sagristà *et al.*, 1991; Santmarti *et al.*, 1995). The percentage of infestation was between 80% and 90% in summer 1991. The prophylaxis and therapy measurements (collection of moribund or dead samples, reduction of population density, leaving the beds to lie fallow for a period) led to a decrease in infestation to about 15% in 1993 (Santmarti *et al.*, 1995).

In our laboratory we examined the infestation present in *Ruditapes philippinarum* located in this zone of the delta of the river Ebro. Ultrastructural studies of the host gill tissue response induced by the presence of *Perkinsus* sp. in clams were carried out (Sagristà *et al.*, 1995, Montes *et al.*, 1995a) and a polypeptide specifically involved in the defence mechanisms was identified and characterized (Montes *et al.*, 1995b).

The purpose of this study was to describe ultrastructural details of the trophozoites and some life cycle stages obtained *in vitro* by zoosporulation of *Perkinsus* sp. found in clams from the Ebro Delta Region in order to identify them.

MATERIALS AND METHODS

Infected live specimens of *R. philippinarum* (= *T. semidecussatus*) (Mollusca: Bivalvia) were obtained from the Ebro Delta region (Tarragona, Spain). Clams were collected from the "Bahia dels Alfacs" (in culture fields belonging to the "Cofradia de Pescadors de Sant Carles de la Ràpita"). Trophozoites of *Perkinsus* sp. (Phylum Apicomplexa, Class Perkinsea) were obtained from infected clam gills and cultured in fluid thioglycollate medium according to the procedure of Ray (1952). Additional identification was provided by staining in Lugol-Iodine solution. Consecutive purification and culture of the trophozoites in sea water were performed as described previously (Chu and Greene, 1989, Azevedo *et al.*, 1990). Some stages of zoosporulation, including free zoospores obtained from cultures *in vitro*, were washed and centrifuged and prepared for light (LM) and transmission electron microscopy (TEM). For TEM the pellet was fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8, for 2 h at 4º C, washed for 2 h at 4º C in the same buffer and post-fixed in buffered 2% OsO_4 for 2 h at the same temperature. After dehydration in an acetone series, the material was embedded in Epon. Ultrathin sections were double-stained with uranyl acetate and lead citrate and observed in a JEOL 100CXII or in a Philips 200 TEM operated at 60 KV.

Some sections were placed on gold grids and stained by Thièry's method (Thièry, 1967) and by phosphotungstic acid (PA) at pH 0.3 (Rambourg, 1971) in order to stain glucids and glycoproteins.

RESULTS

Macroscopic observations of the gills of *R. philippinarum* revealed the presence of the several milky-white nodules in the gill of the parasitized gaper clams. Histological sections observed under the light microscope revealed different sized nodules in the connective tissues of gill, foot, gonads and mantle (Fig. 1A).

Infected clam gills maintained for 3 days in thioglycollate medium showed enlargement and maturation of the trophozoites. These were easily identifiable when stained dark blue by Lugol-Iodine solution.

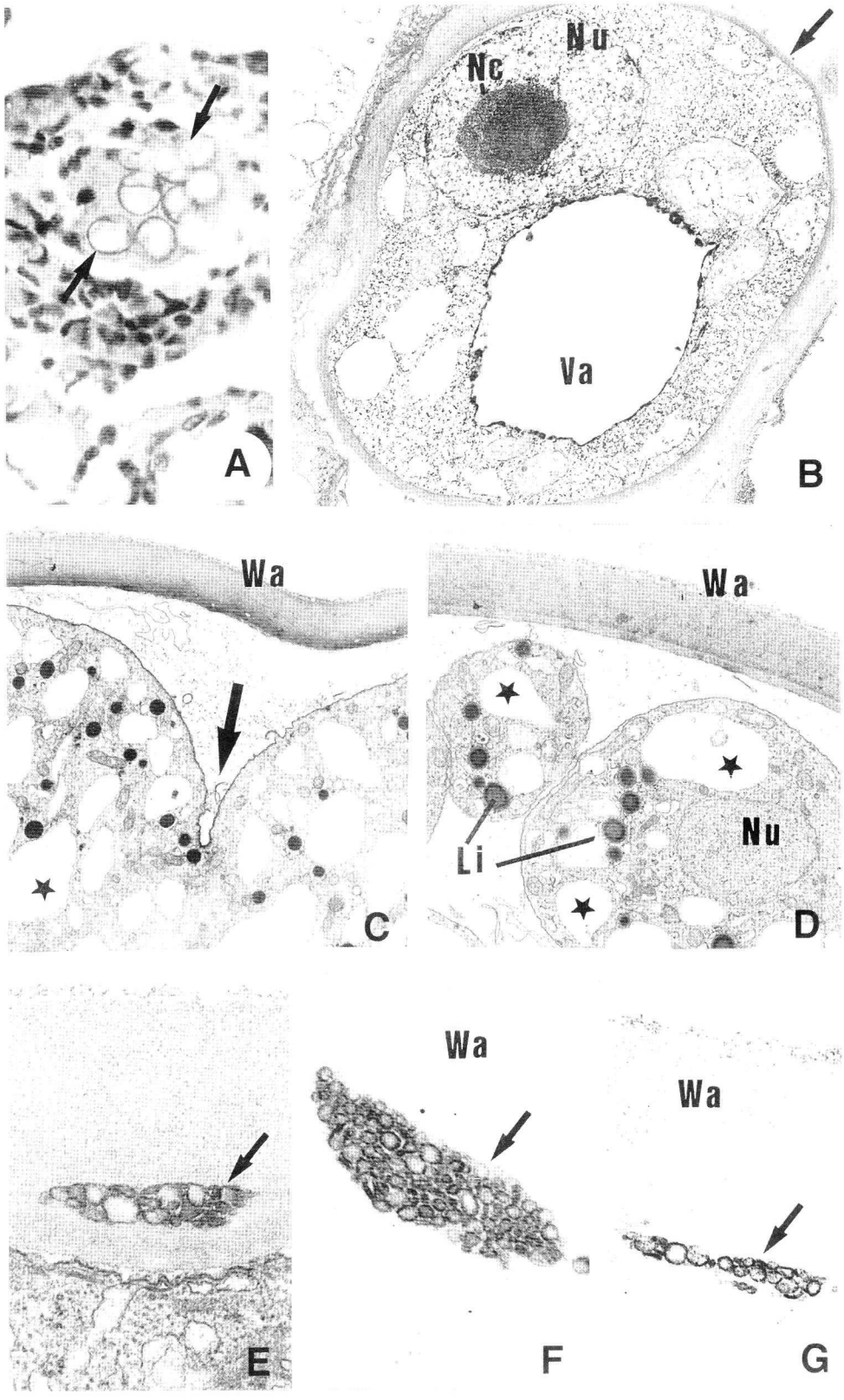

FIG. 1. – **A**. Connective tissue of the clam gills showing some trophozoites of *Perkinsus* (*arrows*). x 640. **B**. Ultrathin section of a trophozoite in gill of clam showing thin wall (*arrow*), nucleus (*Nu*), nucleolus (*Nc*) and vacuoles (*Va*). x 27,000. **C**. Ultrathin section of a prezoosporangium showing some details of the wall (*Wa*) and a prezoospore division (*arrow*). x 4,200. **D**. Ultrathin section of some prezoospores shows nucleus (*Nu*), lipids (*Li*) and vacuoles (★) within the prezoosporangium wall (*Wa*). x 8,100. **E**. Details of prezoosporangium wall (*Wa*) showing the internal layers of the wall and a lamosome (*arrow*). x 42,200. **F** and **G**. Ultrathin sections of the zoosporangium wall (*Wa*) and the lamosomes (*arrows*) showing a PA and Thièry positive reaction. **F**. PA method. x 46,750. **G**. Thièry method. x 29,750

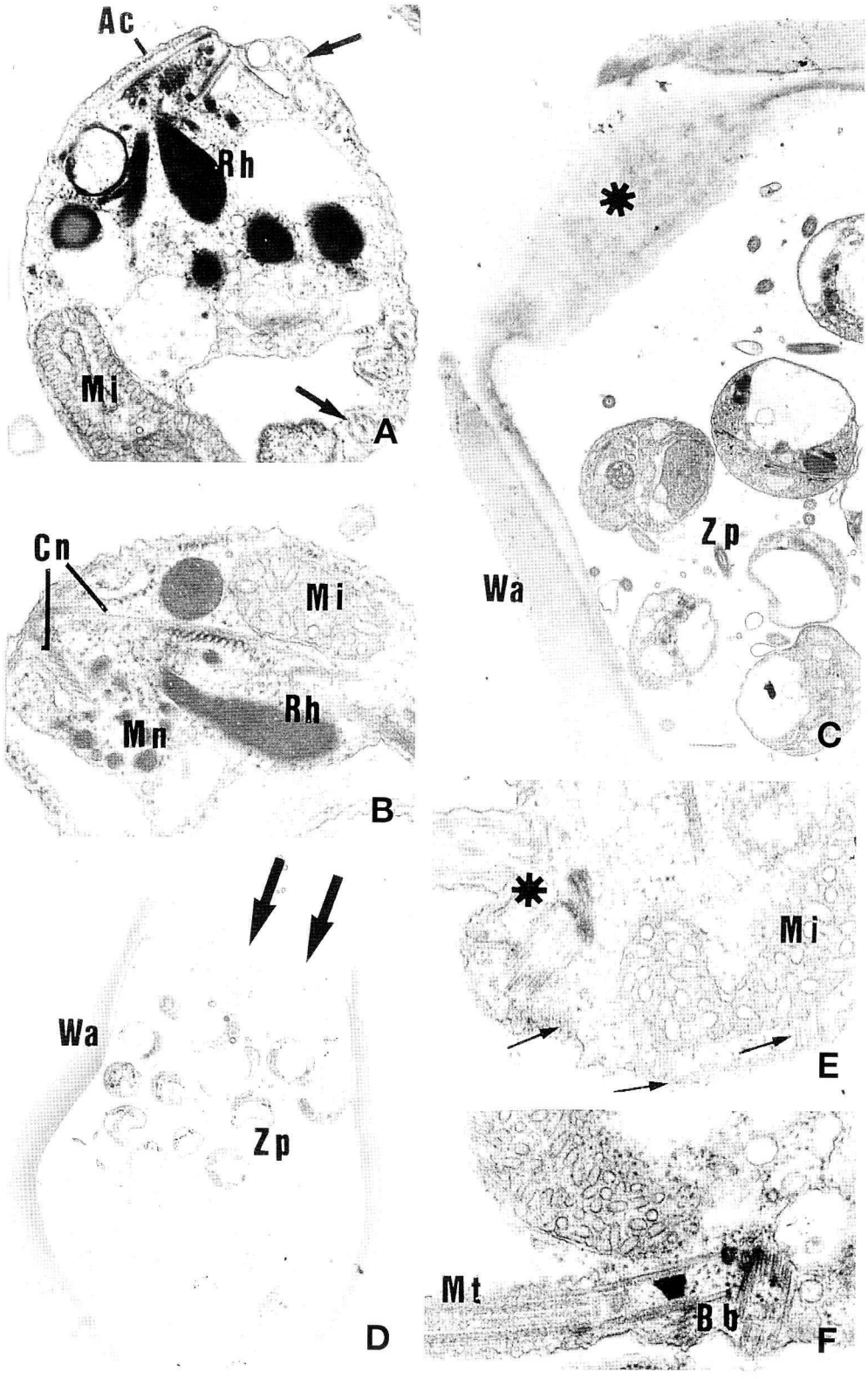

FIG. 2. – **A**. Ultrathin section of a prezoospore showing some structures of the apical complex (*Ac*); rhoptries (*Rh*). A large mitochondrion (*Mi*) and some axonema sections are present (*arrows*). x 22,750. **B**. Detailed of the apical complex of a zoospore. Rhoptries (*Rh*); micronemes (*Mn*) conoid (*Cn*) and mitochondrion (*Mi*). x 39,000. **C**. Ultrathin section of the apical pole of a zoosporangium showing the wall (*Wa*), discharge tube (*) and some zoospores (*Zp*). x 5,600. **D**. Ultrathin section of a zoosporangium showing the wall (*Wa*) and the opening of the discharge tube (*arrows*) and some zoospores (*Zp*) within the zoosporangium. x 3,200. **E**. Ultrathin section of the insertion region of the flagella (*). Near a mitochondrion (*Mi*), some subpellicular microtubules (*arrows*) are present. x 24,750. **F**. Detail of the insertion zone of the flagella, showing the longitudinal section of the basal body (*Bb*) and the microtubular axoneme (*Mt*). x 42,500.

At the ultrastructural level these large cells were delineated by a thin fibrogranular wall. The trophozoites of different sizes (5-10 µm in diameter) had an eccentric nucleus and a large vacuole containing several vacuoplasts. One or two voluminous nucleoli with fibrillar and granular components were evident. The cytoplasm was closely apposed to the inner surface of the wall and contained numerous vesicles and some lipid droplets (Fig. 1B).

The trophozoites were isolated and cultivated in sea water, where they began a prezoosporulation process. After 12 hours, the trophozoites grew and successive nucleokinesis and cytokinesis were seen (Fig. 1C). These successive divisions occurred within the prezoosporangium wall, giving rise to some hundreds of prezoospores, which contained numerous vacuoles, vesicles, lipid droplets and small mitochondria (Fig. 1D).

The prezoosporangium wall was 0.8-1.0 µm thick and presented two layers of which the inner was more electrodense and 0.1-0.2 µm thick. Between the two layers of the wall some regions presented lamosomes (Fig. 1E). The lamosomes showed positive Thièry and PA staining reactions (Figs. 1F and 1G). The surface of the wall presented amorphous material that was positive to Thièry staining.

Two days after the beginning of the incubation in sea water, the largest zoosporangium frequently contained several hundred prezoospores. In these phases, the prezoospores became elongate and differentiated two flagella and a typical apical complex (Fig. 2A). The vacuoles became smaller and fewer and the vacuoplasts disappeared (Fig. 2A). The mitochondria in this phase showed good internal organization with numerous tubular cristae (Figs. 2A and 2B). After 60 hours of incubation in sea water the prezoospores became motile within the prezoosporangium. During this last phase of the maturation processes the discharge tube present in the zoosporangium wall reached the rupture point (Fig. 2C), at which time the flagella of the mature zoospores were completely projected out. The insertion point of the flagella was located laterally on the zoospores (Fig. 2E). The flagella presented a typical axoneme composed of 9 sets of microtubular doublets (Figs. 2E and 2F). At this stage, each zoospore possessed a well-organized apical complex (Fig. 2B). Finally after 72 hours of incubation numerous free motile biflagelated zoospores appeared outside the zoosporangium. The exit through the discharge tube, previously developed during the zoosporulation process, was clearly visible (Fig. 2D).

DISCUSSION

An infestation in *R. philippinarum* was reported in the Delta del Ebro, on the Mediterranean coast (Sagristà *et al.* 1991). The cellular response of the host clam to infection by *Perkinsus* was studied (Sagristà *et al.*, 1992; Montes *et al.*, 1995a).

The ultrastructure of the trophozoites found in the gills of the clams *R. philippinarum* as well as of the trophozoites found in connective tissues of various organs (Sagristà *et al.*, 1992) correspond to the genus *Perkinsus* as shown when the parasitized host tissue was cultivated in Ray's fluid thioglycollate medium (Ray, 1952), where the trophozoites enlarged and were easily identifiable when stained dark blue by Lugol-Iodine solution.

The zoosporulation processes induced *in vitro* and the ultrastructural study of zoosporangium and zoospores performed in this study allows us to identify the species of *Perkinsus*. Life cycle stages observed in zoosporulation of *Perkinsus* obtained from the Mediterranean coast showed similarities with *P. atlanticus* localisated in clams of Portugal (Azevedo, 1989). Several morphological characteristics were also shared with the *P. atlanticus* which had been isolated from *Ruditapes decussatus* (Azevedo, 1989, Azevedo *et al.*, 1990). In our observations, however, the particular morphology of the mitochondria in zoospores differed markedly from the *P. atlanticus* collected on the Portugal coast. The peripheral tubular cristae observed during zoosporulation were not perceived in the mitochondria of the zoospores from Portugal. Such morphological variations may be due to environmental differences.

Other details observed in this study have not been previously described. The wall of zoosporangium had two layers and lamosomes were present. The presence of lamosomes between the wall and plasmalemma during prezoosporulation process seems to be a common structure to the species of the genus *Perkinsus* (Perkins, 1969; Azevedo, 1989; La Peyre *et al.*, 1993). However, little is known about the lamosoma present in zoosporangia wall of Apicomplexa. In the present ultrastructural study, the Thièry methods (Thièry, 1967) and phosphotungstic acid (PA) stain, pH 0.3 (Rambourg, 1971), revealed that the lamosome contained glucidic or glycoprotein material. However, the zoosporangium wall did not present positive reaction with the Thièry method or with phosphotungstic acid.

The present study was performed on *Perkinsus* infestation in *R. philippinarum*, but in the area, the

clam *Ruditapes decussatus* has the same rates of infestation (Santmarti *et al.* 1995). The genus *Perkinsus* was detected in 97 species of molluscs (Perkins, 1993). The infestation by *Perkinsus atlanticus* was initially detected in the clam *Ruditapes decussatus* (Azevedo, 1989; Azevedo *et al.*1990). Few reports about infestation by *Perkinsus* in *R. philippinarum* clams have been published (Sagristà *et al.* 1991, Goggin, 1992, Navas *et al.*, 1992). Recently an experimental infestation of *R. philippinarum* by *P. atlanticus* isolated from *R. decussatus* has been described (Rodriguez *et al.*, 1994). In natural conditions we have also detected that specimens of *R. philippinarum* that were not infected were rapidly parasitized when transfered to infested areas (Santmarti *et al.*, 1995). These studies confirm the possibility that both species of clam may have been infected by the same parasite.

On the basis of ultrastructural data reported in the present study there does not appear to be sufficient reason to justify the creation of a new species of genus *Perkinsus*. The morphological characteristics, the host similarity and the geographic proximity suggest that the species studied in this work was *P. atlanticus* which was first described in the clams *R. decussatus* (Azevedo, 1989).

ACKNOWLEDGEMENTS

This work was partially supported by grants from the "Integrated Actions" between Portugal and Spain. We thank Almudena Garcia and the staff of "Serveis Cientifico Tècnics (Universitat de Barcelona)" for technical assistance, Mrs Mercè Santmarti for providing specimens for this study and Robin Rycroft for linguistic advice.

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