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Study of perkinsosis in the carpet shell clam *Tapes decussatus* in Galicia (NW Spain). I. Identification of the aetiological agent and *in vitro* modulation of zoosporulation by temperature and salinity

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ABSTRACT: Morphological characters of zoosporulation stages and DNA sequence of the internal transcribed spacer (ITS) region and the small subunit ribosomal RNA (SSU rRNA) gene confirmed that the aetiological agent of perkinsosis in the clam *Tapes decussatus* from Galicia (NW Spain) was *Perkinsus atlanticus* Azevedo, 1989. *In vitro* modulation by temperature and salinity of the zoosporulation of the parasite was studied. The optimum temperature range for zoosporulation was 19 to 28°C. The temperature range allowing zoosporulation *in vitro* was 15 to 32°C, which is broader than previously reported (24 to 28°C) for *P. atlanticus*, and strongly suggests that zoospores can be produced in Galician Rías, where temperature ranges from 10 to 22°C. Prezoosporangia held at 10°C for 2 mo (similar to winter conditions in Galician waters) gave rise to viable zoospores after they were transferred to higher temperatures. This suggests that prezoosporangia could overwinter and zoosporulate in the next spring. Zoospores could survive for up to 22 and 14 d at 28 and 10°C, respectively. The optimum salinity range for zooporulation was 25 to 35‰. Zoospore production was abruptly reduced as salinity decreased. The lowest salinity at which zoosporulation was observed was 10‰. The effectiveness of different chlorine concentrations and exposure lengths to kill prezoosporangia and zoospores was tested. No survival of free zoospores, free prezoosporangia and prezoosporangia included in gill tissue was observed after incubation for 1 h with 50, 200 and 3000 ppm of chlorine, respectively.

KEY WORDS: *Perkinsus atlanticus* · *Tapes decussatus* · SSU rRNA gene · ITS region · Zoosporulation · Ultrastructure · Temperature · Salinity · Chlorine

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INTRODUCTION

Parasites of the genus *Perkinsus* (Mackin et al. 1950) are spread through marine mollusc populations worldwide, in association with extensive mortalities of commercially important molluscs. The following species (with their respective host type-species and locations) are included in this genus: *Perkinsus marinus* in the eastern oyster *Crassostrea virginica* (Mackin et al. 1950) along the Atlantic and Gulf coasts of the USA, *Perkinsus olseni* in the blacklip abalone *Haliotis ruber* from Australia (Lester & Davis 1981), *Perkinsus atlanticus* in the carpet shell clam *Tapes decussatus* from Portugal (Azevedo 1989), *Perkinsus qugwadi* in the Japanese scallop *Patinopecten yessoensis* from British Columbia, Canada (Blackbourn et al. 1998), *Perkinsus chesapeaki* in the softshell clam *Mya arenaria* from Chesapeake Bay, USA (McLaughlin et al. 2000), and *Perkinsus andrewsi* in *Macoma balthica* from Delaware Bay, USA (Coss et al. 2001b).

Perkinsus spp. have been discriminated on the basis of the morphology of their different stages, in

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particular the ultrastructure of the zoospore, host species, and host response to infection. Recent descriptions of the sequence of some regions of the *Perkinsus* spp. genomes (Goggin & Barker 1993, Goggin 1994, Reece et al. 1997, Kotob et al. 1999, Robledo et al. 2000, Coss et al. 2001b) and the development of a species-specifity PCR assay have proved useful in distinguishing among *Perkinsus* spp. (Marsh et al. 1995, Robledo et al. 1998, 2000, de la Herrán et al. 2000, Coss et al. 2001b). The taxonomic status of the genus *Perkinsus* has been controversial. Recent studies showed closer affinity with dinoflagellates than with apicomplexa (Goggin 1994, Siddall et al. 1997, Kotob et al. 1999, de la Herrán et al. 2000). Norén et al. (1999) suggested that *Perkinsus* spp. do not fit in either the dinoflagellate or the apicomplexa phyla, and created the new phylum Perkinsozoa.

In Europe, the occurrence of *Perkinsus*-like parasites has been reported in various bivalve species from Italy (da Ros & Canzonier 1985, Ceschia et al. 1991, Canestri-Trotti et al. 2000) and France (Goggin 1992). In Spain, they have been detected in bivalves from Andalucía (South Atlantic coast) (Villalba & Navas 1988, Navas et al. 1992), Cataluña (NE Mediterranean coast) (Sagristà et al. 1991, Santmartí et al. 1995), Galicia (NW Atlantic coast) (Figueras et al. 1992) and Asturias (North Atlantic coast) (Cigarría et al. 1997). Azevedo (1989) created the new species *P. atlanticus*, the only species of the genus reported from European coasts thus far. It was found in *Tapes decussatus* from the Algarve (Portugal), where heavy clam mortalities occurred (Ruano & Cachola 1986).

Known stages of the life cycle of *Perkinsus* spp. involve the occurrence of vegetative proliferation (palintomy) of the parasite in host tissues (Perkins 1996). Ray (1952) observed that when infected host tissues were incubated in fluid thioglycollate medium (FTM), trophozoites enlarged and developed a thick wall, thus becoming a new stage called hypnospore. When the hypnospores produced in FTM are isolated and placed in seawater, zoosporulation begins, culminating in the liberation of numerous free biflagellated zoospores (Perkins & Menzel 1966, Lester & Davis 1981, Azevedo 1989, McLaughlin et al. 2000). This is why hypnospores are called prezoosporangia also (Perkins 1996). Enlargement of trophozoites was also observed in moribund hosts (Ray 1954, Mackin 1962, Perkins 1968, Valiulis & Mackin 1969). Perkins (1968) and Valiulis & Mackin (1969) induced production of zoospores by isolating *Perkinsus* sp. prezoosporangia from decaying tissues of *Macoma balthica* and placing the parasite cells in seawater. Their observations led to the hypothesis that transformation of *Perkinsus* spp. trophozoites into prezoosporangia occurs naturally in moribund hosts and the latter begin zoosporulation when they are released into seawater. However, it is unclear whether prezoosporangia released in seawater from moribund and deceased oyster would zoosporulate in nature (Chu 1996). In the case of *P. qugwadi*, all these stages (trophozoites, prezoosporangia and biflagellated zoospores) can be observed in host tissues (Blackbourn et al. 1998). The 3 known stages of *P. marinus* (trophozoites, prezoosporangia and biflagellated zoospores) can cause infection in oysters, as has been showed experimentally. However, it is not known which stage is most effective and the principal stage for transmitting the disease in the field (Chu 1996). Viable *P. marinus* cells are released from live infected oysters in faeces (Bushek et al. 1996, Scanlon et al. 1997). In the case of *P. qugwadi*, experimental transmission by cohabitation was only possible when zoospores were present in the 'donors' (Bower et al. 1998). In the case of *P. atlanticus*, experimental transmission of the disease was performed with laboratory-induced zoospores (Rodríguez et al. 1994).

Field and laboratory studies have shown how temperature and salinity are environmental factors with a high influence on the disease caused by *Perkinsus marinus* in *Crassostrea virginica* (Andrews 1988, Ragone & Burreson 1993, Burreson & Ragone Calvo 1996, Chu 1996). Auzoux-Bordenave et al. (1995) studied the influence of environmental conditions on the zoosporulation process in *P. atlanticus* and concluded that the optimal range of temperature for its zoosporulation was 24 to 28°C, which is outside the normal temperature range of seawater along the Galician coast. However, perkinsosis is widespread in venerid clam populations of Galicia, and is a matter of concern for the clam industry in the region.

A research programme is being developed in our laboratory to evaluate the potential effect of this disease on Galician populations of the clam with the highest market value, *Tapes decussatus*. As a part of this programme, the objectives of this study were: (1) to determine what species is responsible for perkinsosis in *T. decussatus* in Galicia through morphological characterisation of the diagnostic stages (zoosporulation) and sequencing of specific DNA regions (at the beginning of this study, it had not been proved that *Perkinsus atlanticus* was the species occurring in Galician clams); (2) to evaluate the influence of temperature and salinity on the progression of zoosporulation and on the viability and life span of the different stages of the zoosporulation process, with emphasis on the environmental ranges of the Galician coast; (3) to establish an effective chlorine treatment to eliminate the parasite cells associated with zoosporulation, by evaluating different concentrations and exposure lengths.

MATERIALS AND METHODS

Prezoosporangia isolation and zoosporulation assays. Clams *Tapes decussatus* were collected from an intertidal bed affected by perkinsosis, located in Vilalonga (Ría de Arousa, Galicia, NW Spain). They were shucked, and those with abundant white pustules (a sign of moderate to heavy infection) on the gill surface were selected for induction and isolation of prezoosporangia, following a procedure set up by Navas (pers. comm.), who modified previously reported methods (Perkins & Menzel 1966, Chu & Greene 1989). Briefly, gills of selected clams were excised and placed in 10 ml of fluid thioglycollate medium (FTM; Ray 1966) for 5 to 7 d at 20°C in darkness. Then, the gills were trypsinized (0.25%) for 60 to 90 min and prezoosporangia were separated from tissue remains by passing the suspension successively through sieves of 300, 160, 100 and 20 µm. Only material retained in the 20 µm sieve was kept, since no significant occurrence of prezoosporangia was detected in the other sieves. Prezoosporangia in the 20 µm sieve were washed with sterile filtered (0.22 µm) seawater (SFSW) plus antibiotics (200 U ml^{-1} Penicillin G and 400 µg ml^{-1} Streptomycin) and transferred into a 50 ml tube, ready to use in zoosporulation assays. Prezoosporangia isolated from 5 to 10 clams were used in each assay. The assays were performed in 12-well culture plates, which were kept in the dark. Except for the chlorine experiment, 15 000 prezoosporangia were suspended in 4 ml of SFSW plus antibiotics in each well; 3 replicates (3 wells) were run for each tested value of temperature, salinity, and chlorine concentration. The mean values of the 3 replicates are given throughout the paper.

Temperature assays. To study the influence of temperature on the viability of prezoosporangia, the zoosporulation process, and the resulting zoospores, prezoosporangia from a pool were divided into lots and incubated at 32, 28, 22, 15, 10 and 4 ± 1°C until no viable cells were detected. SFSW plus antibiotics adjusted to 30‰ was used. Because of the lack of zoosporulation in the case of prezoosporangia incubated at 4 and 10°C, partial volumes of each replicate were transferred to 22 or 28°C after 16 d of culture in order to determine if the ability to zoosporulate was maintained. In addition, partial volumes of prezoosporangia suspension incubated at 10°C were transferred to 19 or 28°C after 67 d of culture. Prezoosporangia continuously held at 22 and 28°C were used as controls in the transference assays. One 100 µl sample of the suspension was taken daily from each well in every assay. Fifty microlitres of the sample were set on a slide and examined with light microscopy to count the number of dead (DP) and viable (VP) prezoosporangia, dead (DZ) and viable (VZ) zoosporangia (cells which had entered the zoosporulation process but had not yet liberated zoospores), and empty (EZ) zoosporangia with an open discharge pore (zoosporangia from which the mature zoospores had been liberated). In the case of wells with zoospores, the remaining 50 µl of the sample were used to count the number of the latter (viable and dead) with a Malassez haemocytometer. The vital stain neutral red was added (10 mg l^{-1}) to every sample to determine prezoosporangium, zoosporangium and zoospore viability. In order to evaluate the synchronicity of zoosporulation in the well population, the following zoosporulation index (ZI) was calculated for every sample: $ZI = (EZ \times 100)/(VP + VZ + EZ)$.

In addition, a percentage of mortality (M) defined as the percentage of cells (prezoosporangia and zoosporangia) which died without accomplishing zoosporulation, was calculated as: $M = [(DP + DZ) \times 100]/(DP + DZ + VP + VZ + EZ)$.

The influence of temperature on the life span of the zoospores was also studied. For this purpose, prezoosporangia were kept in 30‰ SFSW plus antibiotics at 20°C. As soon as initial stages of zoosporulation were detected, zoosporangia were transferred to 24, 20 or 15°C. Zoospores liberated at these temperatures were isolated by passing the culture through 2 sieves of 75 and 10 µm. Zoospores from zoosporangia held at 24°C were transferred to 28°C, those from zoosporangia at 20°C were kept at the same temperature, and those from zoosporangia at 15°C were distributed between 15 and 10°C. In every case, zoospores were held in 30‰ SFSW plus antibiotics. Zoospore viability was estimated daily by counting the number of viable cells using vital stain neutral red, as described above. A step-by-step temperature transition was performed to avoid abrupt changes.

Salinity assays. The influence of salinity on the viability of prezoosporangia, the zoosporulation process and the resulting zoospores was studied at 2 temperatures, 19 and 28°C. The salinity values tested were 35, 30, 25, 20, 15, 10, 5 and 3‰ at 28°C, and 35, 30, 25, 20, 15, 9, 5 and 3‰ at 19°C. To avoid osmotic shock, gills were incubated in FTM with a salinity of 30, 15 and 10‰ for 7 d. Then, prezoosporangia were isolated in 30‰ SFSW for the 35, 30 and 25‰ tests, at 15‰ for the 20 and 15‰ tests, and at 10‰ for the 10 or 9, 5 and 3‰ tests. Prezoosporangia were incubated until no viable cells were detected. Cultures were set up as described for the temperature assays. The viability of prezoosporangia, zoosporangia and zoospores, the zoosporulation index and the percentage of mortality were estimated as described in the preceding subsection.

Chlorine assays. To determine if the chlorine treatment guaranteed the elimination of the stages

associated with zoosporulation, isolated prezoosporangia from a pool were divided into lots and incubated in 0 (control), 50, 100, 200 and 300 ppm of chlorine in 35‰ SFSW for 24 h. One 100 µl sample of the suspension was removed from each well and the number of live (stained with neutral red) and dead prezoosporangia were counted after 1, 3, 5, 8, 12 and 24 h. Similarly, free zoospores were exposed to concentrations of 10, 25, 50 and 100 ppm of chlorine in 35‰ SFSW for 24 h. The occurrence of live zoospores was assessed after 1, 2, 4, 12 and 24 h. In addition, infected gill lamellae were incubated in FTM to induce development of prezoosporangia. Then, gill lamellae were exposed to chlorine concentrations of 0, 600, 1000, 2000 and 3000 ppm in 35‰ SFSW for 1 and 3 h. Finally, the gills were fragmented with a scalpel and the number of live (stained with neutral red) and dead prezoosporangia were counted.

Transmission electron microscopy. Zoosporangia and zoospores obtained after incubation of prezoosporangia for 5 and 7 d at 20°C were washed and centrifuged. The resulting pellets were fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2, for 2 h at 4°C. Then, the pellets were washed twice with the same buffer, and postfixed in buffered 2% OsO_4 for 2 h at 4°C. The cells were included in 1.5% agar solution. The agar blocks were dehydrated in an ethanol series and embedded in Epon. Ultra-thin sections were double-stained with uranyl acetate and lead citrate, and observed in a JEOL 1010 transmission electron microscope (TEM) operated at either 40 or 60 kV.

DNA sequencing. DNA was isolated from *Perkinsus*-infected gill tissue of 5 *Tapes decussatus* individuals using a CTAB-based DNA extraction method (Carlini & Graves 1999). The small subunit ribosomal RNA (SSU rRNA) gene was amplified from the isolated DNA in the PCR using 'universal' primers (Medlin et al. 1988). Amplifications were done with 10 to 50 ng of genomic DNA in 25 µl reactions using the reaction conditions recommended for the BRL PCR Reagent System (Life Technologies) with the addition of 20% BSA (bovine serum albumin; 1 mg ml^{-1}). Cycling parameters for the SSU rRNA gene were as follows: an initial denaturation of 4 min at 94°C followed by 35 cycles (30 s at 94°C, 30 s at 45°C, 2 min at 65°C) and a final extension of 5 min at 65°C.

PCR primers were designed to specifically target the internal transcribed spacer (ITS) region of the ribosomal RNA gene unit of all *Perkinsus* species except *P. qugwadi* (Table 1). These primers were used in various combinations with each other and with previously published ITS region primers (Goggin 1994). The *Perkinsus*-specific primers were designed by aligning *Perkinsus* spp. ITS sequences previously published and deposited at GenBank (Goggin 1994: Accession Nos. PAU07697, PMU07700, POU07701, PSU07698, PSU07699; Robledo et al. 2000: Accession No. AF140295; Coss et al. 2001b: Accession Nos. AF252288, AF102171; Kotob et al. unpubl. data: Accession Nos. AF091541, AF091542, AF126022, AF150988, AF150989, AF150990; Brown et al. unpubl. data: Accession Nos. AF149876, AF150985, AF150986, AF150987) and those available for several different *Perkinsus* species in the laboratory of one of the authors (Reece unpubl. data) to identify conserved regions. The ITS sequence of *P. guqwadi* (Hervio et al. unpubl. data: Accession No. AF151528) was significantly different from that of other *Perkinsus* species, and therefore was not included for the purpose of PCR primer design. In addition, *Perkinsus* spp. ITS sequences were aligned to those ITS sequences that were closely matched in BLAST searches (Altschul et al. 1990) of the National Center for Biotechnology Information (NCBI) GenBank database (Accession Nos. AF131074, AF201747, AF208244, AF208245, AF208246, AF208249, AF318223, AF318224, AF318225, AF318226, AF318247, AF352363, AF352364, AF352365, AF352366, AF352370) and to available *Perkinsus* host species and other molluscan ITS sequences (Accession Nos. AF348987, AF348990, AF348993). PCR primers were designed to target regions that were conserved among the *Perkinsus* species yet were different from the ITS sequences of other organisms. The primers designated PerkITS85 and PerkITS750 were designed to target all *Perkinsus* species except *P. qugwadi* and the PerkITS585 primer targets *P. marinus*, *P. atlanticus* and *P. olseni* ITS sequences. The primers were designed and tested with the aid of MacVector DNA sequence-analysis software (Oxford Molecular). Each primer sequence was also

Table 1. *Perkinsus* spp. Primer pairs used in internal transcribed spacer (ITS) region PCR reactions. Previously published primers (Goggin 1994) and primers designed for specific amplification of the ITS region DNA of *Perkinsus* species were used in the combinations shown

Primers	Pair designation	Approx. size of amplification product (bp)
ITS-5 (Goggin 1994) ITS-585 (aggaacacgtgatatcacc)	A	594
ITS-5 ITS-750 (acatcaggccttctaatgatg)	B	755
ITS-85 (ccgctttgtttgga/ctccc) ITS-585	C	512
ITS-85 ITS-750	D	703
ITS-85 ITS-3 (Goggin 1994)	E	720

subjected to an independent BLAST search to help ensure specific amplification of *Perkinsus* DNA, while avoiding amplification of host or other contaminating DNAs.

PCR amplifications were done using the same reaction buffer conditions described above for the SSU rRNA gene, and the following cycling parameters were found to be optimal for amplification with each ITS region primer pair: an initial denaturation of 4 min at 95°C, followed by 40 cycles (1 min at 95°C, 1 min at 53°C, 3 min at 65°C), with a final extension of 5 min at 65°C. Optimal annealing temperatures were tested with the aid of a gradient thermal cycler (Biometra). Primer pairs were tested in PCR amplifications for specificity against uninfected clam *Tapes decussatus* tissue and against a variety of protistan DNAs.

Amplified products were cloned into a plasmid vector pCR2.1 (Invitrogen) using the TA cloning kit 'Invitrogen' following the manufacturer's protocol. The cloned DNA inserts were sequenced on an automated sequencer by simultaneous bi-directional cycle-sequencing using the Thermo Sequenase Sequencing Kit (Amersham Life Science) and infrared labelled primers (LI-COR) following the manufacturer's protocol. Sequencing reactions were electrophoresed and detected on a LI-COR automated sequencer (Model 4200L). The resulting ITS region sequences were subjected to BLAST searches (Altschul et al. 1990) of the NCBI GenBank database. Representative *Perkinsus* sp. (or *P. atlanticus*) ITS region sequences from each primer pair amplification were deposited in GenBank (Accession Nos. AF369967, AF369968, AF369969, AF369970, AF369971, AF369972, AF369973, AF369974, AF369975, AF369976, AF369977, AF369978, AF369979). Parsimony analysis of *Perkinsus* ITS sequences was performed using PAUP*4.0 (Swofford 2001).

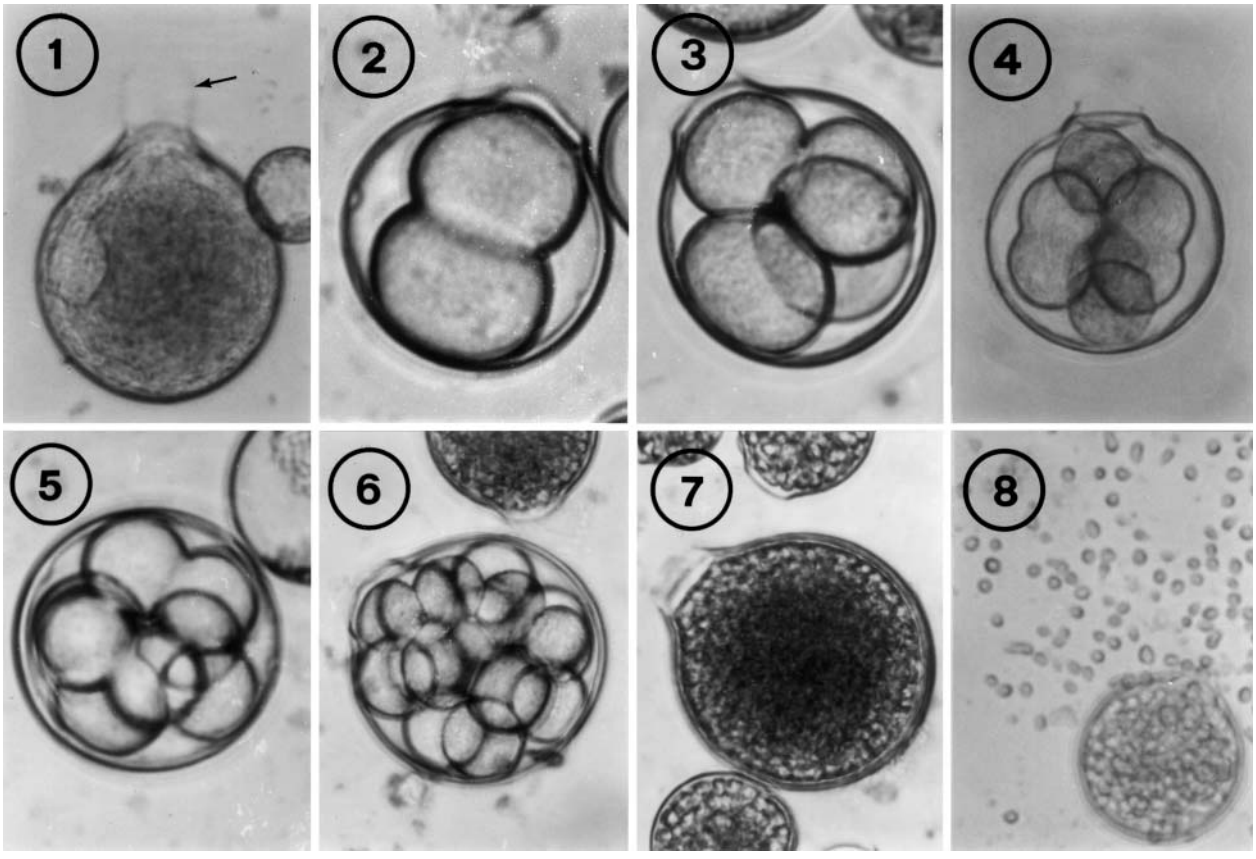
RESULTS

Morphology throughout zoosporulation

Under the light microscope, isolated prezoosporangia appeared spherical, with a large vacuole and athin layer of cytoplasm pressed against the cell wall. They ranged from 19.72 to 64.09 µm in diameter (mean ± SE: 38.9 ± 1.00 µm; N = 109). Zoosporulation started when prezoosporangia were placed in seawater. A single discharge tube appeared on each prezoosporangium early in the process, before any cell division occurred (Fig. 1). The mean ratio between the length of the discharge tube and the diameter of the zoosporangial body was 0.30 (N = 20). No zoosporangia with 2 discharge tubes were ever observed. Zoosporulation progressed with successive karyokinesis and cytokinesis giving rise to intermediate stages of 2, 4, 8, 16, 32, 64... and more cells. Finally, hundreds of zoospores were formed within the original cell wall (Figs. 2 to 7). Biflagellated ellipsoidal zoospores left the zoosporangium through the discharge tube. Free zoospores in live preparations ranged from 3 to 5 µm in length (mean ± SE: 3.69 ± 0.05 µm; N = 190) (Fig. 8).

Under the TEM, prezoosporangia showed a thick cell wall, a large vacuole, and cytoplasm with lipid droplets, numerous vesicles and mitochondria. The nucleus had a prominent nucleolus. Before cell division began, the cytoplasm contracted and intravacuolar fluid was released into the space between the cell wall and the protoplast, and the cytoplasm became granular with vacuole division. Division gave rise to cells with abundant vacuoles enclosing vacuoplasts, mitochondria, vesicles and lipid droplets. The nuclear membrane persisted during karyokinesis (Fig. 9). Two layers were distinguished in the zoosporangium wall, the inner layer being more electrondense (Fig. 10). Lomosomes were present in some wall regions (Fig. 11). Wall thickness ranged from 0.5 to 1.5 µm; it thickened progressively with increasing proximity to the discharge tube. The discharge pore and tube were formed by rupture of the cell wall and subsequent unfolding of the tube from the inner part of the cell wall. A plug blocked the pore, which was a thickened section of the inner layer of the cell wall (Fig. 10).

Instead of apicomplexa terminology, the terminology proposed by Siddall et al. (1997) for typifying dinoflagellates will be used to describe the fine structure of the zoospores. Thus, the terms apical ribbon instead of conoid, ribbon-associated vesicles instead of conoid-attached micronemes, and toxicysts instead of rectilinear micronemes will be used. Zoospores were uninucleated, biflagellated, ellipsoidal. Usually, the nucleus was situated in the posterior part of the zoospore, lacked a patent nucleolus, and chromatin was more condensed at the periphery (Fig. 12). Several vacuoles were located in the cytoplasm (Figs. 12 to 15). Occasionally, vacuoplasts were observed within the vacuoles (Fig. 12). The mitochondria (or mitochondrion) were in a lateral position close to the flagellar insertion; most of them were bar-shaped and were extended from the apical ribbon to the posterior cellular region (Figs. 12 & 13). Peripheral tubular cristae were observed in the mitochondria (Figs. 12 & 13). The flagella had the typical organization, 9 doublets + 2 central microtubules. Occasionally, images suggesting the occurrence of mastigonemes arising from flagella were observed (Fig. 14). Insertion points of the 2 flagella were



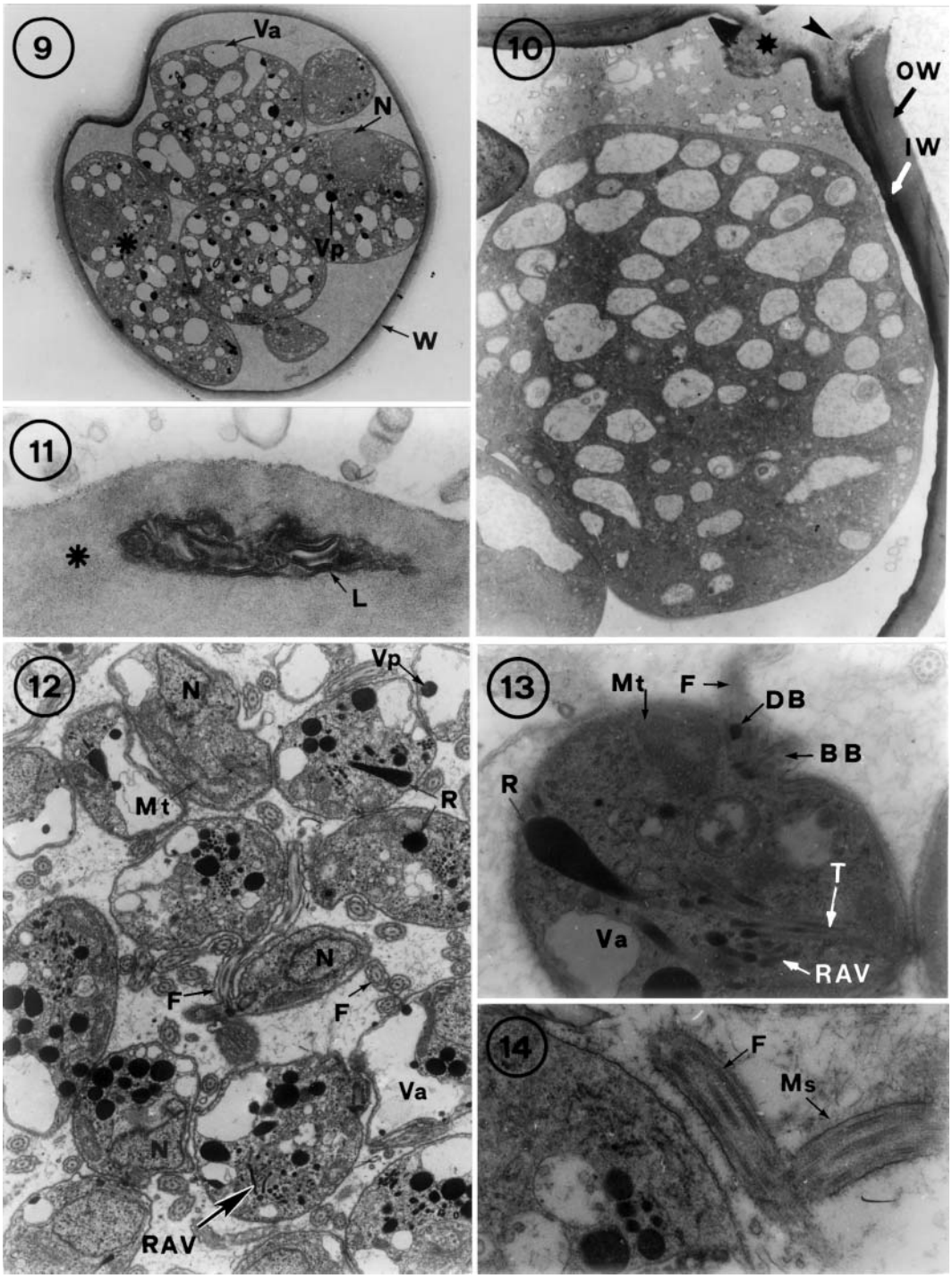
Figs. 1–8. *Perkinsus atlanticus*. Bright-field micrographs showing progression of zoosporulation. Fig. 1. Zoosporangium at single cell stage, showing the discharge tube (arrow) slightly out of focus (550×). Fig. 2. Zoosporangium with 2 cells (1000×). Fig. 3. Zoosporangium with 4 cells (775×). Fig. 4. Cell division during transition from 4- to 8-cell zoosporangium (550×). Fig. 5. Zoosporangium with 8 cells (900×). Fig. 6. Zoosporangium with 16 cells (525×). Fig. 7. Zoosporangium enclosing hundreds of motile zoospores (525×). Fig. 8. Free zoospores swimming around a zoosporangium which still encloses zoospores (550×)

located laterally, not far from each other, on the anterior third of the zoospores. The longitudinal axis of the 2 basal bodies formed an angle of 64° (Fig. 13). There was a large dense granular body in the lumen of the basal bodies (Fig. 13). The apical ribbon was open-sided, and located at the apical pole (Fig. 15). The rhoptries were electron-dense, vase-shaped, extending from the apical ribbon area to the posterior portion of the zoospore (Figs. 12 to 15). The ribbon-associated vesicles were a bundle of moderately electron-dense, elongated, vesicular structures of wavy appearance. They extended backwards from the apical ribbon (Figs. 12 & 13). In cross-section, each vesicle had a pale matrix, with a small electron-dense central core and a thin electron-dense coat (Fig. 16). A bundle of toxicysts extended from the apical ribbon to the posterior part of the cell and terminated in a small knob-like structure (Figs. 13 & 15). Cortical alveoli were observed in the anterior part of the cell (Fig. 15). Subpellicular microtubules were also seen.

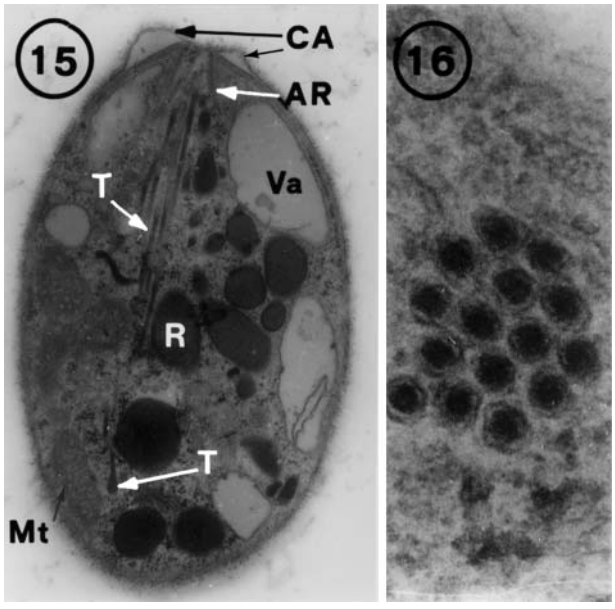
DNA sequences

Sequences of the SSU rRNA gene amplification products were found to be of 2 different types. One set of sequences closely matched those of molluscan bivalves in BLAST searches of GenBank, and a portion of the sequence was identical to the GenBank-deposited partial 18S rRNA gene sequence of *Tapes decussatus* (AF295121). These sequences were presumed to be from host DNA. The other set of sequences (4 DNA clones) was found to have 99.5 to 99.8% sequence identity, representative of 4 to 2 nucleotide differences, to a GenBank deposited sequence for the SSU gene of *Perkinsus atlanticus* (AF140295: Robledo et al. 2000).

Amplification products of expected sizes were obtained with all ITS region PCR primer pairs (Table 1). Primer Pairs A, B, C, D, and F demonstrated specificity to DNA isolated from *Perkinsus* sp.-infected host tissue, while with the C primer pair a weak amplification product was observed from uninfected *Tapes*



Figs. 9–14. *Perkinsus atlanticus*. Transmission electron micrographs throughout zoosporulation process. Fig. 9. Zoosporangium with a thick wall (W) enclosing a few prezoospores; prezoospores display a prominent nucleus (N), numerous vacuoles (Va) in the cytoplasm, and vacuoplasts (Vp) inside some vacuoles; some prezoospores are in the process of cytokinesis (*) (2100×). Fig. 10. Detail of the zoosporangium at the base of the discharge tube; wall has 2 layers, outer (OW) and inner (IW); the outer layer is discontinuous; the base of the discharge tube (arrowhead) and pore plug (*) arise from the inner layer (4100×). Fig. 11. Detail of an area of the zoosporangium wall (*) showing lomosome (L) (40 500×). Fig. 12. Zoospores inside zoosporangium; F: flagellum; Mt: mitochondrion; N: nucleus; R: rhoptry; RAV: ribbon-associated vesicle; Va: vacuole; Vp: vacuoplast (8500×). Fig. 13. Zoospore inside zoosporangium; detail of the area of flagellum insertion; BB: basal body; DB: electron-dense body; F: flagellum; Mt: mitochondrion; RAV: ribbon-associated vesicle; R: rhoptry; T: toxycist (17 500×). Fig. 14. Zoospore flagella (F) showing possible mastigonemes (Ms) arising from flagellum surface (32 000×)



Figs. 15 & 16. *Perkinsus atlanticus*. Transmission electron micrographs of zoospore. Fig. 15. Free zoospore showing apical ribbon (AR), cortical alveoli (CA), mitochondrion (Mt), rhoptry (R), toxicyst (T) and vacuole (Va) (20000×). Fig. 16. Detail of zoospore cytoplasm showing cross-sections of ribbon-associated vesicles (91000×)

decussatus tissue (Fig. 17). Two to 5 amplification products from PCR with each ITS region primer pair were sequenced from 3 different infected host-tissue DNA isolations. In BLAST searches (Altschul et al. 1990), the sequence of all amplification products most closely matched ITS sequences from *P. atlanticus*, *P. olseni* and *Perkinsus* spp. from *Chama pacificus* and *Anadara trapezia* (Goggin 1994, Robledo et al. 2000). The tree resulting from parsimony analysis is shown in Fig. 18. All *Perkinsus* spp. sequences from this study grouped with the ITS sequences of *P. atlanticus*, *P. olseni* and the *Perkinsus* spp. from *C. pacificus* and *A. trapezia*.

Influence of temperature

The results of the temperature assay are shown in Fig. 19. Zoosporulation occurred over a wide range of temperatures, from 15 to 32°C. At 32°C, a very low number of free zoospores were seen. The zoosporulation index was very low and most cells died before zoosporulation ended. Mortality reached 98% by Day 16 at that temperature. The optimum tested temperatures were 22 and 28°C. Prezoosporangia kept at 22 and 28°C gave rise to an early (Day 3) and intense peak of zoospore liberation. Free zoospores were seen from Day 3 until Day 14 at 28°C and from Day 3 until Day 26 at 22°C. Zoosporulation index exceeded 75% by Days 3 and 5, respectively, which indicates a high level of synchrony. This index reached 100% by Day 16 at 28°C and by Day 25 at 22°C, with a low percentage of mortality. At 15°C, the process began later and lasted longer. The zoosporulation index increased more slowly. The maximum number of free zoospores occurring simultaneously at this temperature was much lower than at 28 and 22°C. However, free zoospores were seen for a longer time, from Day 14 to Day 53, thus indicating lack of synchrony. On the latter day, the wells became empty

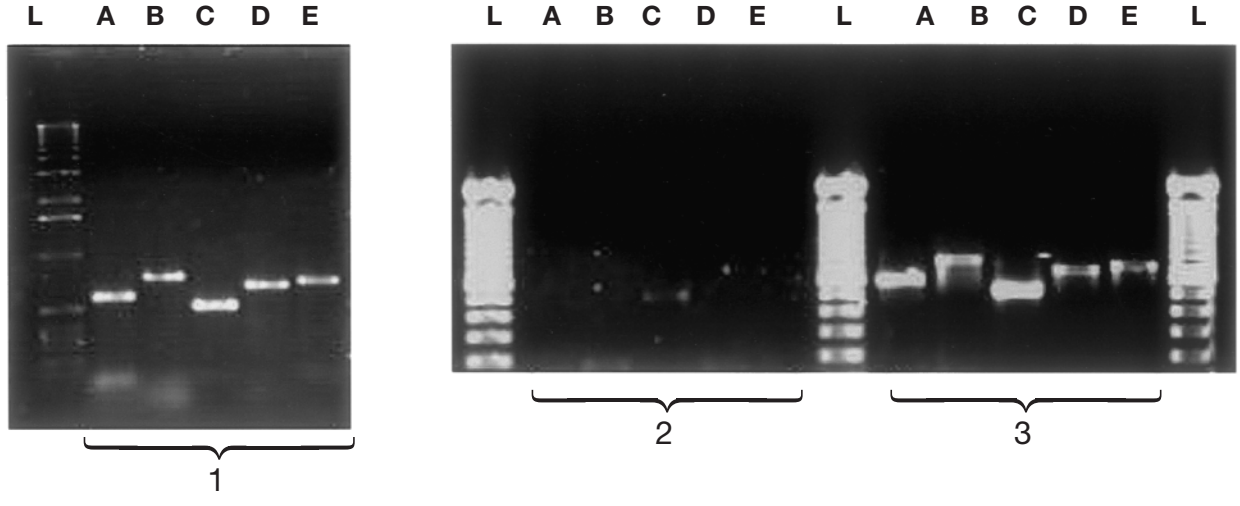


Fig. 17. *Perkinsus* spp. Agarose gel (2%) stained with ethidium bromide and viewed on a UV transilluminator. PCR amplification products obtained with the ITS region primer pairs A, B, C, D, and E (gel lanes labelled) used in reactions with (1) DNA isolated from *P. marinus*-infected *Crassostrea virginica* tissue, (2) DNA isolated from uninfected *Tapes decussatus* tissue and (3) *Perkinsus* sp.-infected *T. decussatus* tissue. Lanes labelled 'L' contain either the 1 kb ladder or 100 bp ladder size standards (1 kb ladder: Life Technologies)

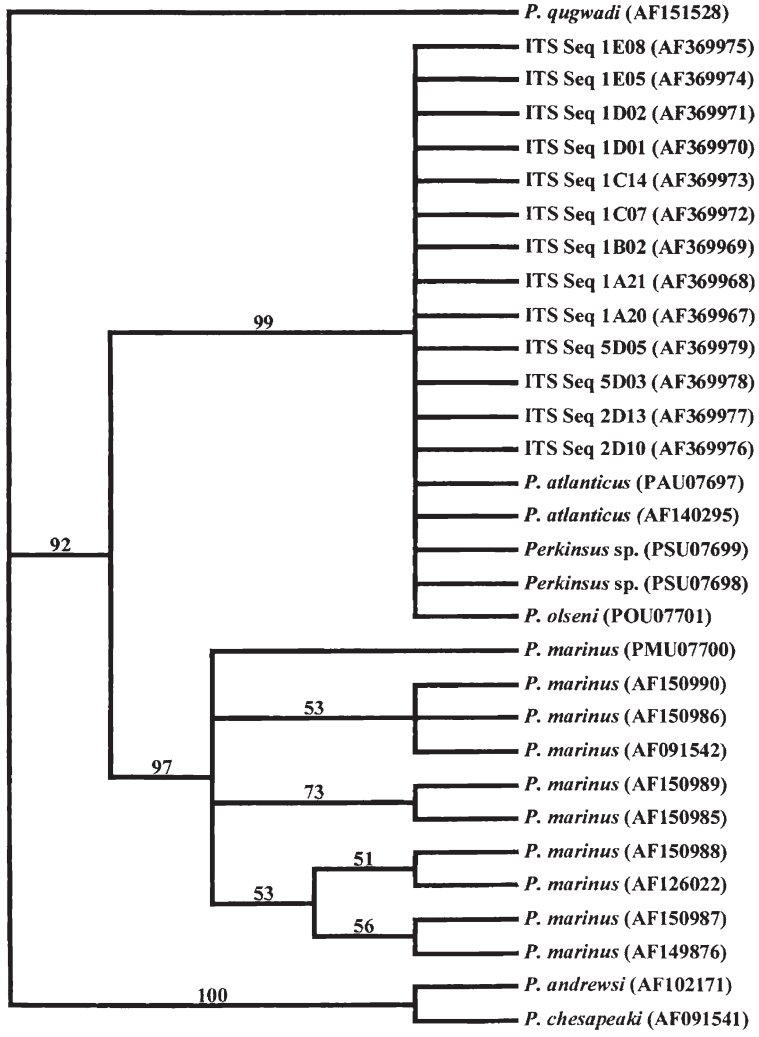


Fig. 18. *Perkinsus* spp. Tree generated by phylogenetic analysis of *Perkinsus* spp. ITS sequences. Parsimony jackknife analysis was performed with 100 jackknife replicates of 100 random additions. Jackknife support values are indicated on branches. Sequences from this study are labelled 'ITS Seq' with the parasite-infected *Tapes decussatus* individual number from which DNA was isolated, followed by the letter designation of the primer pair used in PCR, followed by the DNA clone number. GenBank accession numbers for each sequence are given in parentheses

because of the frequent sampling, thus preventing new sampling. At that time, the zoosporulation index was 64% and the mortality was 86%. At 10 and 4°C, zoosporulation did not start, although mortality did not reach 100% until Days 130 and 67, respectively. By the end of the assay, ca. 1% of the incubated prezoosporangia had accomplished zoosporulation at 32°C, 70% at 28°C, 62% at 22°C, and 9% at 15°C.

Prezoosporangia incubated for 16 d at 10°C and then transferred to 22 or 28°C gave rise to free zoospores. Those incubated at 4°C gave rise to an almost negligible zoospore production when transferred to 22 or 28°C (Fig. 20). Free zoospores were observed earlier at 28°C than at 22°C, and zoosporulation started earlier in prezoosporangia incubated at 10°C than at 4°C (Fig. 20). Prezoosporangia incubated for 67 d at 10°C and then transferred to 19 or 28°C gave rise to free zoospores in both cases.

The results for the zoospore life span can be summarised as follows: zoospores held at 28°C survived for up to 22 d; those held at 20°C survived for up to 21 d; zoospores held at 15°C survived for up to 15 d; those held at 10°C survived for up to 14 d. Zoospore motility decreased progressively during those time periods.

Influence of salinity

Results of the 28°C assay are summarised in Fig. 21. Zoosporulation occurred at 35, 30, 25, 20, 15 and 10‰. Free zoospores were observed as early as Day 2 in some cases. At 5 and 3‰, zoosporulation did not occur and mortality reached almost 100% by the first day. At 35, 30 and 25‰, the zoosporulation index reached 90% by Day 3, with low mortality, thus giving rise to maximum values of zoospore production. At 20, 15 and 10‰, mortality of zoosporangia was high; therefore fewer zoospores were produced. Zoosporulation began progressively later with decreasing salinity.

Fig. 22 shows the results of the 19°C assay. Zoosporulation occurred at 35, 30, 25, 20 and 15‰. Maximum production was reached at 35, 30 and 25‰ also, although in this case, differences among the salinity values were more pronounced than at 28°C. Liberation of zoospores started later (Day 4) than at 28°C and their number decreased more slowly until their complete disappearance during Days 32 to 35. The zoosporulation index increased more slowly. At 20 and 15‰, production of zoospores was lower and took place later than at higher salinities. At 9‰, a few zoosporangia filled with zoospores were observed, but neither empty zoosporangia nor free zoospores were found. Mortality was significantly higher from 9‰ downwards. As in the 28°C assay, percentage mortality reached almost 100% by the first day at 3‰. However, it took 30 d to reach 100% mortality at 5‰.

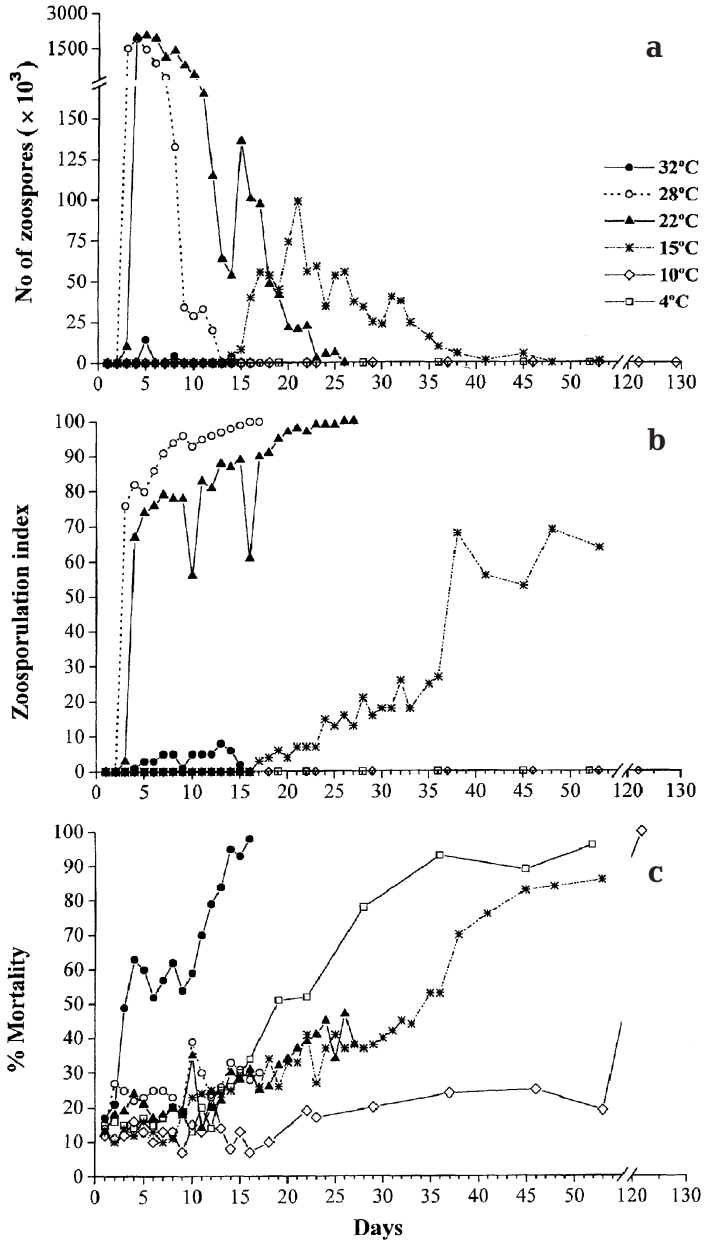


Fig. 19. *Perkinsus atlanticus*. Effect of temperature on zoosporulation. Tested temperature values were 32, 28, 22, 15, 10 and 4°C, in an assay performed at 30‰. Daily variation in the (a) mean number of zoospores, (b) mean value of the zoosporulation index, and (c) mean value of the mortality percentage, all calculated from 3 replicates, at each temperature. The period considered in each panel started when prezoosporangia were placed in the wells immediately after isolation

Effect of chlorine

The effect of chlorine exposure on parasite survival is shown in Tables 2 to 4. Exposure of free zoospores to 50 ppm of chlorine for 1 h produced 100% mortality (Table 2). In the case of free prezoosporangia, 100% mortality was reached by exposure to 200 ppm of chlorine for 1 h (Table 3). Death of prezoosporangia within host tissues occurred after exposure to 3000 ppm for 1 h (Table 4).

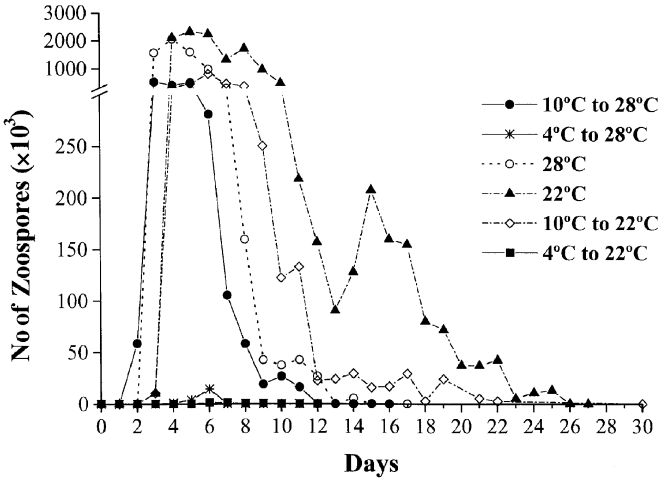


Fig. 20. *Perkinsus atlanticus*. Effect of pre-incubation of prezoosporangia at low temperature on zoosporulation. Daily variation in the mean number of zoospores, calculated from 3 replicates, in each treatment is shown. The different treatments involved incubation of prezoosporangia at either 4 or 10°C for 16 d and transfer to 22 or 28°C; controls involved incubation at 22 and 28°C from the beginning of the assay. The period considered started when prezoosporangia were transferred from the lower to the higher temperature

DISCUSSION

The morphology of the stages associated with zoosporulation, the DNA sequences of the SSU rRNA gene and ITS region and the host species of this parasite all support identification of the aetiological agent of perkinsosis in *Tapes decussatus* from Galicia as *Perkinsus atlanticus* Azevedo, 1989. PCR primers designed to specifically amplify *Perkinsus* spp. ITS region DNA from infected host tissue were successfully used in this study. All ITS region sequences obtained were from parasite DNA and grouped with previously published *P. atlanticus* sequences (Goggin 1994, Robledo et al. 2000) in phylogenetic analyses. Robledo et al. (2000) proposed that *P. atlanticus*, *P. olseni* and *Perkinsus* sp. from *Anadara trapezia* form a subgroup different from *P. marinus*, according to their ITS region sequences. Our results support this hypothesis. Goggin (1994) further suggested that *P. atlanticus* and *Perkinsus* spp. from Australia belong to a single species, according to their ITS region and 5.8S rRNA sequences.

The prezoosporangial diameter of the Galician parasite was 38.9 ± 1.00 µm, 30 to 40 µm in *Perkinsus*

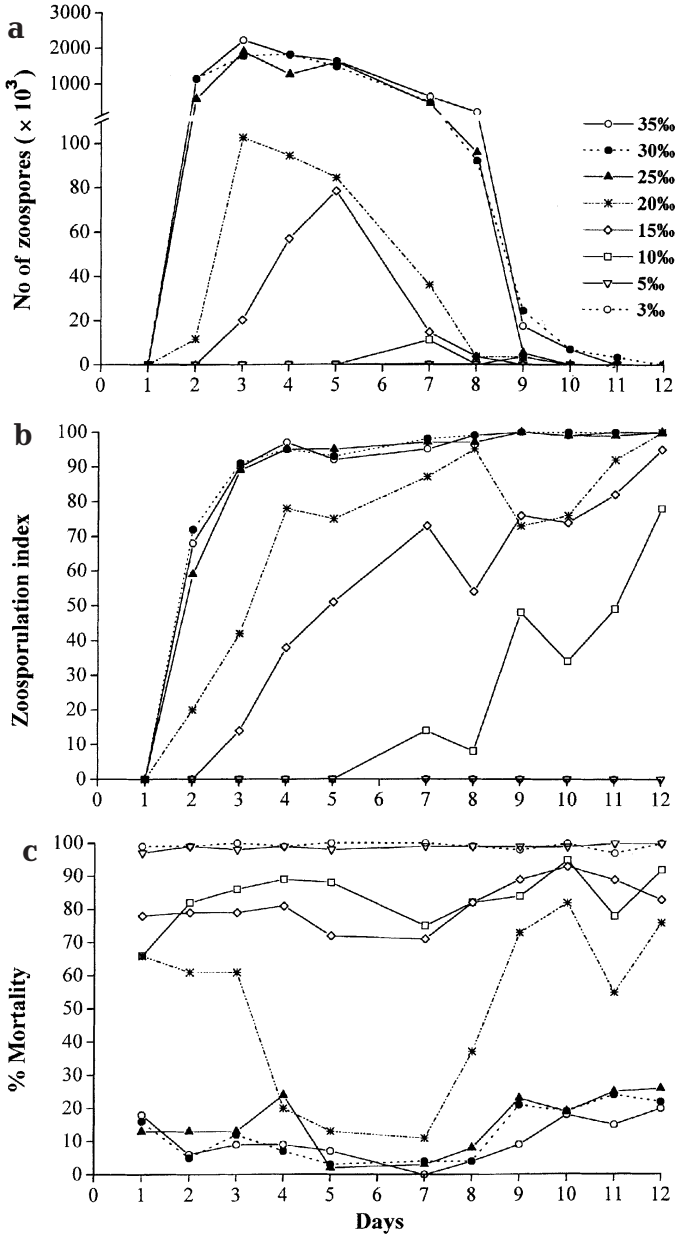


Fig. 21. *Perkinsus atlanticus*. Effect of salinity on zoosporulation at 28°C. Tested salinity values were 35, 30, 25, 20, 15, 10, 5, and 3‰, in an assay performed at 28°C. Daily variation in the (a) mean number of zoospores, (b) mean value of the zoosporulation index, and (c) mean value of the mortality percentage, all calculated from 3 replicates, at each salinity value. The period considered in each panel started when prezoosporangia were placed in the wells immediately after isolation

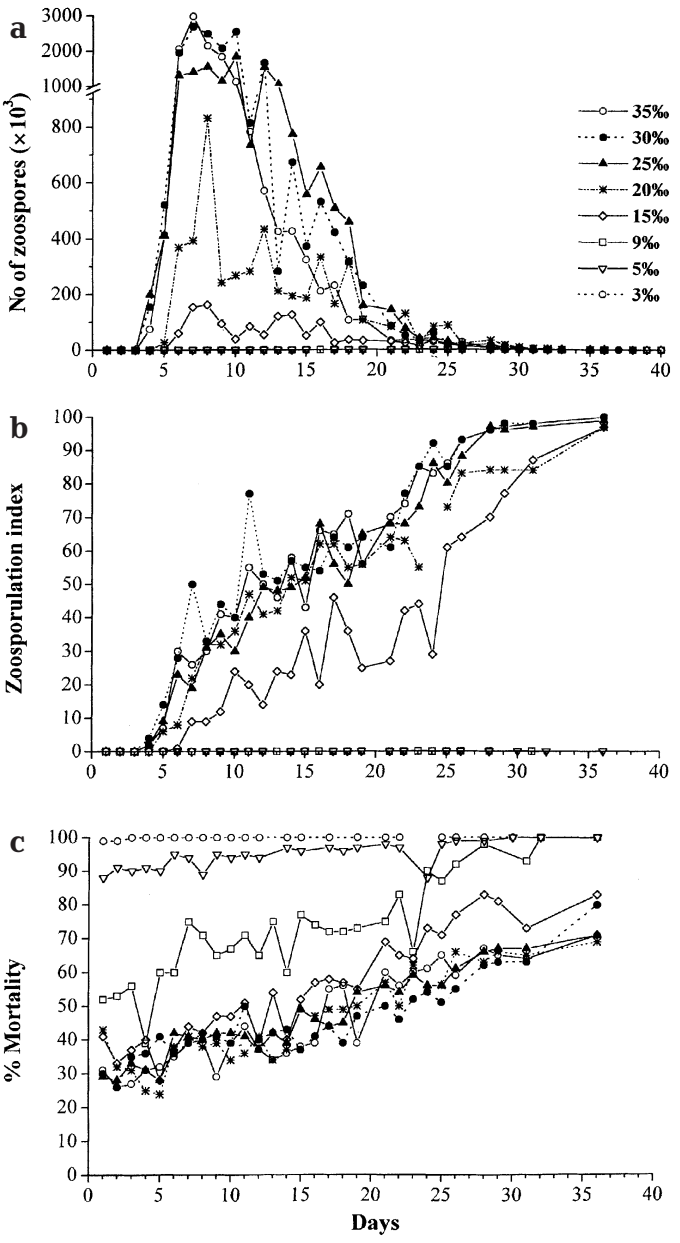


Fig. 22. *Perkinsus atlanticus*. Effect of salinity on zoosporulation at 19°C. Tested salinity values were 35, 30, 25, 20, 15, 9, 5, and 3‰, in an assay performed at 19°C. Daily variation in the (a) mean number of zoospores, (b) mean value of the zoosporulation index, and (c) mean value of the mortality percentage, all calculated from 3 replicates, at each salinity value. The period considered in each panel started when prezoosporangia were placed in the wells immediately after isolation

atlanticus (Azevedo 1989), 30 to 80 µm in *P. marinus* (Perkins 1996), 74 µm (56 to 94 µm) in *P. olseni* (Lester & Davis 1981), 69.3 ± 29.3 µm in *P. chesapeaki* (McLaughlin et al. 2000), 12.6 ± 1.18 µm in *P. qugwadi* (Blackbourn et al. 1998) and 67 ± 12 µm in *P. andrewsi* (Coss et al. 2001a). Nevertheless, accurate comparison of prezoosporangium size among *Perkinsus* spp. would require similar incubation conditions (period length and temperature). The zoosporangium wall showed 2 layers and presented lomosomes in the Galician parasite as in *P. atlanticus* (Azevedo et al. 1990, Sagristà et al. 1996) and in *P. chesapeaki*

Table 2. *Perkinsus atlanticus*. Effectiveness of different chlorine concentrations and exposure lengths in eliminating zoospores. +: occurrence of live zoospores; –: no live zoospores detected

Chlorine conc. (ppm)	1 h	2 h	4 h	12 h	24 h
10	+	+	+	+	+
25	+	+	+	+	+
50	–	–	–	–	–
100	–	–	–	–	–

Table 3. *Perkinsus atlanticus*. Effectiveness of different chlorine concentrations and exposure lengths in eliminating free prezoosporangia

Chlorine conc. (ppm)	Exposure length (h)	No. of dead cells	No. of live cells	% mortality
Control: 0	3	280	610	31
50	1	127	273	32
	3	130	184	41
	5	132	192	41
	8	95	117	45
	12	82	28	74
	24	157	27	85
100	1	100	120	45
	3	289	46	86
	5	224	8	96
	8	192	0	100
	12	205	0	100
200	1	260	0	100
	3	235	0	100
300	1	67	0	100
	3	253	0	100

(McLaughlin et al. 2000). Three wall layers and lomosomes were observed in *P. marinus* (Perkins & Menzel 1967). In *P. qugwadi*, lomosomes were not observed (Blackbourn et al. 1998). Occasionally, 2 discharge tubes were observed in *P. marinus* (Perkins & Menzel 1966) and *P. chesapeaki* (McLaughlin et al. 2000). Two discharge tubes were never seen in the Galician parasite nor were they described for *P. atlanticus* (Azevedo 1989, Azevedo et al. 1990, Sagristà et al. 1996). No discharge tube was observed in *P. qugwadi* zoosporangia (Blackbourn et al. 1998). The mean ratio between the length of the discharge tube and the diameter of the zoosporangial body was 0.30 in the Galician parasite. It is one-third in *Perkinsus* spp. from *Tridacna gigas, Anadara trapecia*, and *Macoma balthica*; one-quarter in *P. olseni* from *Haliotis laevigata*; and one-fifth *in P. marinus* (Goggin & Lester 1995).

Table 4. *Perkinsus atlanticus*. Effectiveness of different chlorine concentrations and exposure lengths in eliminating prezoosporangia in gill tissues

Chlorine conc. (ppm)	Exposure length (h)	No. of dead cells	No. of live cells	% mortality
Control: 0	3	71	96	42
600	1	63	36	64
	3	118	63	65
1000	1	40	8	83
	3	20	1	95
2000	1	168	185	48
	3	238	12	95
3000	1	187	0	100
	3	246	0	100

The zoospore size was smaller (3.69 ± 0.05 µm) than that reported from *Perkinsus atlanticus* in Portugal: 4.5 ± 0.6 µm (Azevedo 1989). The zoospore size was 3.73 ± 0.48 µm in *P. chesapeaki* (McLaughlin et al. 2000), 4.4 ± 0.6 µm in *P. andrewsi* (Coss et al. 2001a), 4.5 ± 1.0 µm in *P. qugwadi* (Blackbourn et al. 1998), and 4 to 6 µm in *P. marinus* (Perkins & Menzel 1967). The basal bodies of the 2 flagella formed an angle of 64° in the Galician parasite and 65 ± 2° in *P. atlanticus* (Azevedo 1989). However, a right angle was described for *P. marinus* (Perkins & Menzel 1967) and *P. chesapeaki* (McLaughlin et al. 2000). In *P. qugwadi*, basal bodies do not appear to have a static relationship to each other (from parallel to nearly right-angle) (Blackbourn et al. 1998). Other structures, such as the apical ribbon, the ribbon-associated vesicles and toxicysts described from the zoospore of *Perkinsus* spp., were observed also in the Galician parasite. Cortical alveoli were always observed in an anterior position, as reported for *P. marinus* (Perkins 1996) and *P. chesapeaki* (McLaughlin et al. 2000), whereas in *P. qugwadi* (Blackbourn et al. 1998) they were observed over the entire body. In *P. andrewsi*, cortical alveoli were found in various positions (Coss et al. 2001a).

Our results have shown a great influence of temperature and salinity on the viability and longevity of the zoosporulation stages of *Perkinsus atlanticus*. *In vitro* zoosporulation was accomplished over a wide temperature range: 15 to 32°C. Considering the number of zoospores produced, the optimum temperature range was 19 to 28°C, with a low percentage of mortality and a high zoosporulation index. At 22 to 28°C, zoosporulation progressed synchronously in the well population, giving rise to an early, massive zoospore liberation. As temperature de-

creased, zoosporulation became slower and asynchronous, and thus zoospore liberation lasted longer and fewer zoospores occurred simultaneously in the well population. Moreover, percentage mortality increased as temperature decreased. At 10 and 4°C, some prezoosporangia could survive for up to 4 and 2 mo, respectively, without entering zoosporulation. Nevertheless, prezoosporangia kept at those temperatures did not lose the ability to zoosporulate, since this occurred when the temperature became favourable.

Auzoux-Bordenave et al. (1995) reported a temperature range of 24 to 28°C for *Perkinsus atlanticus* zoosporulation. They did not test any intermediate temperature between 24 and 15°C, and did not observe zoosporulation at the latter temperature. The annual pattern of temperature variation in the seawater of the clam beds of Galician Rías Bajas involves values of 14 to 22°C between April and November (above 19°C in summer only) and 10 to 14°C in the other months. Therefore, seawater temperature is always below the range required for zoosporulation reported by those authors. Chu & Greene (1989) reported a temperature range of 18 to 28°C for *P. marinus* zoosporulation after testing at 4, 18, 20 and 28°C. If our *in vitro* results were extrapolated to natural environmental conditions, zoosporulation of *P. atlanticus* could occur in Galician Rías from mid-spring to mid-autumn. If temperature prevented zoosporulation in the remaining months, prezoosporangia could overwinter and zoosporulate in the following spring. The annual pattern of variation of the weighted incidence of perkinsosis in Galician populations of *Tapes decussatus* displays minimum values in winter, with a peak in mid-spring and another peak in late summer-early autumn (Villalba et al. 2000). Therefore, the presumed zoosporulation bloom occurring in spring from overwintering free prezoosporangia could contribute to the increase in weighted incidence of perkinsosis in mid-spring, together with other factors such as a proliferation of very light infections which have overwintered and trophozoite transmission among clams.

Our results showed that the lower the temperature the shorter the life span of zoospores. Nevertheless, the lower the temperature the longer the period of zoospore production due to lack of zoosporulation synchrony among prezoosporanga/zoosporangia. The longest zoospore life span was 22 d at 28°C. Chu & Greene (1989) noted that *Perkinsus marinus* zoospores lived from 3 to 28 d in 4 to 32‰ at 20 to 28°C.

In vitro zoosporulation was accomplished over a wide salinity range: 10 to 35‰. The optimum salinity range for zoosporulation was 25 to 35‰. Zoospore production decreased abruptly as salinity decreased, and zoosporulation was delayed. Prezoosporangia held in 9‰ at 19°C began zoosporulation, but zoospore liberation did not occur. Very low salinity (3‰) caused rapid 100% mortality of prezoosporangia; thus exposure to freshwater seems to be an effective way of killing free prezoosporangia. Likewise, Auzoux-Bordenave et al. (1995) reported a salinity range of 10 to 35‰ at 24°C for *Perkinsus atlanticus* zoosporulation. In the case of *P. marinus* a salinity range from 6 to 32–34‰ at 28°C was reported by Chu & Greene (1989). Salinity below 25‰ is not common in Galician clam beds, and is only recorded in the winter months, when zoosporulation could be inhibited/prevented by low temperature.

A high chlorine concentration (3000 ppm for 1 h) was needed to kill prezoosporangia in gill tissues. *Perkinsus marinus* trophozoites within oyster tissues survived 2100 ppm chlorination (Bushek et al. 1997). Free prezoosporangia also showed high tolerance to chlorine, even higher than that of *Perkinsus* sp. from *Anadara trapezia* and *Haliotis laevigata*, for which exposure to 6 ppm of chlorine for 30 min and 2 h, respectively, assured 100% mortality (Goggin et al. 1990). High tolerance to chlorine, low temperature, and low pH (Auzoux-Bordenave et al. 1995) exposure suggest that prezoosporangia may be a dormant stage in the life cycle of *Perkinsus* spp. that allows it to overcome unfavourable conditions. Laboratory-induced zoospores were effective in transmitting *P. atlanticus* to healthy clams (Rodríguez et al. 1994). However, it is unclear whether prezoosporangia released in seawater from moribund and deceased clams would zoosporulate in nature, and it is not known which stage of *P. atlanticus* is most effective and the principal stage for transmitting the disease in the field. Further research is needed to assess the role of zoosporulation in the life cycle of this parasite.

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