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Ultrastructural Aspects of the Developmental Phases of *Perkinsus atlanticus/P. olseni* (Perkinsidae) Trophozoites Infecting the Gills of Clams, Including Encapsulation by Host Haemocytes

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Abstract

The genus *Perkinsus* (Alveolata, Perkinsidae) is comprised by intracellular pathogenic microparasites that infect several molluscs (mainly bivalves) worldwide, and are associated with high mortalities. The species *Perkinsus atlanticus* (later referred to as synonymy of *P. olseni*) infects the clam *Ruditapes decussatus* (Bivalve), a commercially important species in Mediterranean countries and in the Portuguese Atlantic coast. This study describes the several developmental phases of the trophozoites of this parasite in the gill tissues of *Ruditapes decussatus* from the Southern Portuguese Atlantic coast, based on light and transmission electron microscopy. Ultrastructural observations distinguish between three developmental phases of the trophozoites (early, middle, and late), during

Keywords: *Ultrastructure; Infection; Trophozoites; Perkinsus atlanticus (P. olseni); Haemocyte; Host Reaction; Encapsulation*

Abbreviations

Pre-Tz: Pre-Trophozoite; Tz: Trophozoite

Introduction

The microparasite genus *Perkinsus* Levine, 1975 of the phylum Apicomplexa [1] was originally described as belonging to the genus *Dermocystidium* [2], and currently, positioned within the recently established phylum Perkinsozoa of the superphylum Alveolate [3,4], despite this phylum the creation not being fully accepted by the scientific community.

Members of the genus *Perkinsus*, among which is the species *P. atlanticus* Azevedo, 1989 (synonym with *P. olseni*) [5,6], are microparasites responsible for disease outbreaks and high mortalities in several wild and farmed clam populations, including bivalves of great economic interest in European countries such as Portugal and Spain [5-13], and other geographical locations [1,14-31].

Perkinsosis are characterized by enlargement of the parasitized organs due to encapsulation of the trophozoites (Tz) by the host's haemocytes, which generally leads to the formation of whitish nodules or cysts, mainly in the gills of the infected molluscs [5,12-15,18-20,22-27]. This type of immune response to infection by *Perkinsus* spp. haemocytes has been frequently described [5,11,19,24,26,29,31-33]. A more complete understanding of many aspects of the mechanisms of invasion and transmission, as well as of the developmental stages of the *in vitro* zoosporulation, have also been studied in different species of the genus *Perkinsus* [33,36-44].

In this study, the ultrastructural features of the several developmental phases of the Tz of *P. atlanticus* are reported, emphasizing the host's immune response by haemocytic encapsulation.

Material and Methods

Collection of clams and trophozoite incubation

Several specimens of the clam *Ruditapes decussatus* (Mollusca, Bivalvia) (measuring 30 - 35 mm) parasitized with the perkinsozoan *Perkinsus atlanticus* were obtained from the Southern Portuguese Atlantic coast of Portugal, where this perkinsosis is known to be endemic, and from which the original description of this species was performed [5].

Trophozoites obtained from infected gills were incubated in Ray's thioglycollate medium, according to the procedure of Ray [37], and modified by Azevedo., *et al* [38]. This procedure was used to induce zoosporulation *in vitro*, so that hypnospores could develop and give rise to zoospores. Before their preparation for microscopic studies, clams were anaesthetized with MgCl2, and small fragments of the gill were observed by light microscopy - Differential Interference Contrast optics (LM-DIC) in order to confirm the presence of the parasites or the host reaction.

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Transmission electron microscopy

For transmission electron microscopy (TEM), fragments of infected gills were fixed in 4 - 5% glutaraldehyde in a 0.2M sodium cacodylate buffer (pH 7.4) for 8 - 10h, washed overnight in the same buffer, and post-fixed in 2% OsO_4 buffered with the same solution for 3h. All these steps were performed at 4°C. Samples were then dehydrated in an ethanol ascending series, followed by propylene oxide, and embedding in Epon. Semi-thin sections were stained with methylene blue-Azure II, and ultrathin sections were double contrasted with uranyl acetate and lead citrate before examination using a JEOL 100 CXII TEM operated at 60 kV.

Results

The Tz precursor cells in the gill tissues of the infected clams were identified as belonging to the species *Perkinsus atlanticus/P. olseni,* through means of LM and TEM observations. Some grouped and isolated Tz obtained from infected gills previously incubated in Ray's thioglycollate medium were observed in DIC. These cells, which were generally spherical with dimensions that could reach up to 20 - 22 µm in diameter, contained a central vacuole (Figure 1). The processes leading to zoosporulation were observed using the methodology in [38]. Trophozoites gave rise to numerous free zoospores, which displayed the morphological features previously described for *P. atlanticus*, i.e. two flagella, rhoptries, micronemes, a polar ring, and a coiled open-sided conoid (Figure 2).

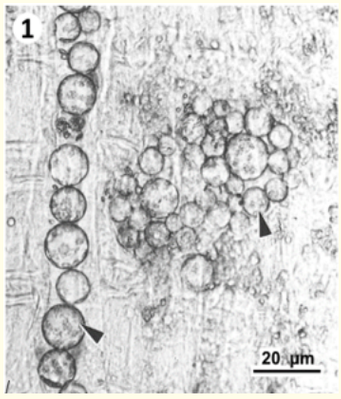

Figure 1: *Cluster with variable number of living trophozoites (arrowheads) of Perkinsus atlanticus/P. olseni observed in a small fragment of highly parasitized gill tissue of the clam Ruditapes decussatus.*

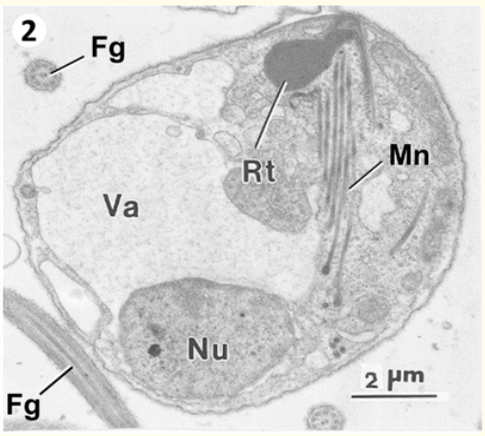

Figure 2: *Ultrastructural aspect of longitudinal sections of P. atlanticus/P. olseni zoospore obtained after zoosporulation, showing their typical organelles and structures: Nu-nucleus; Va-vacuole; Rt-rhoptries; Mn-micronemes, Fg-flagella/axonema (in transverse and longitudinal section).*

Ultrastructural observations allowed the differentiation of three distinct phases in the development of the Tz (Figure 3-14), which are here characterized.

Early phase (Figure 3): The development of the initial phases of the parasite, corresponding to pre-Tz, was difficult to observe using LM. The presence of clusters of parasitic cells could, however, be observed by TEM. Clusters were formed by five to nine cells, and contained an irregular outline externally surrounded by a thin homogenous and dense material that was very evident between these cells and in close contact with the plasmalemma of the pre-Tz. The cytoplasm of the pre-Tz was constituted by a homogeneous granular material with few organelles. The nuclei showed a homogeneous matrix where it was possible to observe a small dense mass, probably corresponding to the initial phase of the nucleolus development.

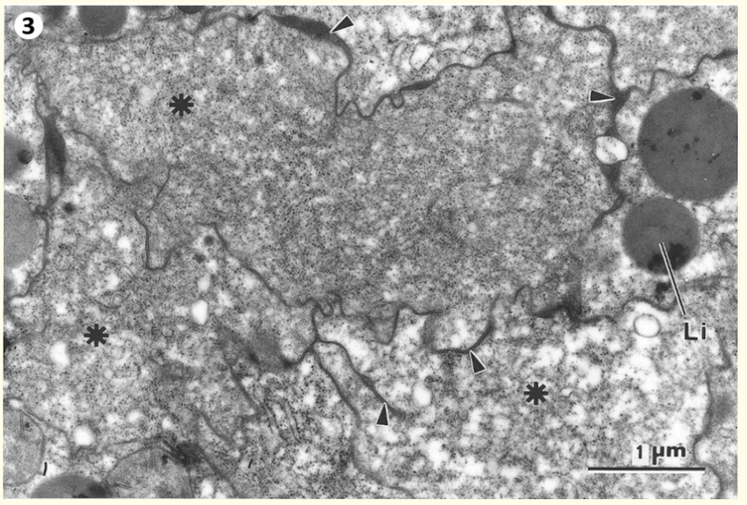

Figure 3: *Ultrastructural aspects of the parasitic cells (youngest pre-trophozoites) obtained from a small fragment of infected clam gill. Few organelles are present in the homogeneous granular matrix (*). The periphery of the parasitic cells show an irregular contour with the intercellular space occupied by dense material (arrowheads). The surrounding host cells show some lipid droplets (Li).*

Middle phase (Figure 4-8): The ultrastructural alterations that occurred during the previously phase may permit to consider the parasitic cells (pre-Tz) as Tz characterized by ellipsoidal to spherical morphology, and presented a denser and thicker cellular wall than in the anterior phase. The cytoplasm was mainly occupied by a granular matrix, small vacuoles, some lipidic droplets, and mitochondria. Each nucleus contained a prominent nucleolus (Figure 4-6). Some groups comprised by a variable number of Tz appeared surrounded by a second wall (Figure 6). The nucleus became spherical and displayed a more voluminous nucleolus formed by fibrillar and granular components, with an evident fibrillar center (Figure 6-8). The cytoplasm displayed several small vacuoles of variable diameter, which coalesced to form a larger vacuole (Figure 8), while the nucleus became eccentric (Figure 9).

Some haemocytes were observed close to the parasitic cells (Figure 8). Some appeared to be surrounding the Tz (Figure 10), and few showed ultrastructural disorganization, appearing lysed and forming dense and irregular masses (Figure 8).

Late phase (Figure 9-14): The most evident cellular structure of the Tz was the central vacuole surrounded by several small vacuoles (Figure 9). The nucleus was eccentric and contained a voluminous nucleolus showing the typically nucleolar structures (Figure 9,10,13,14). In this late phase, the Tz frequently appeared encapsulated by numerous haemocytes, and surrounded by degraded dense material (Figure 9-14).

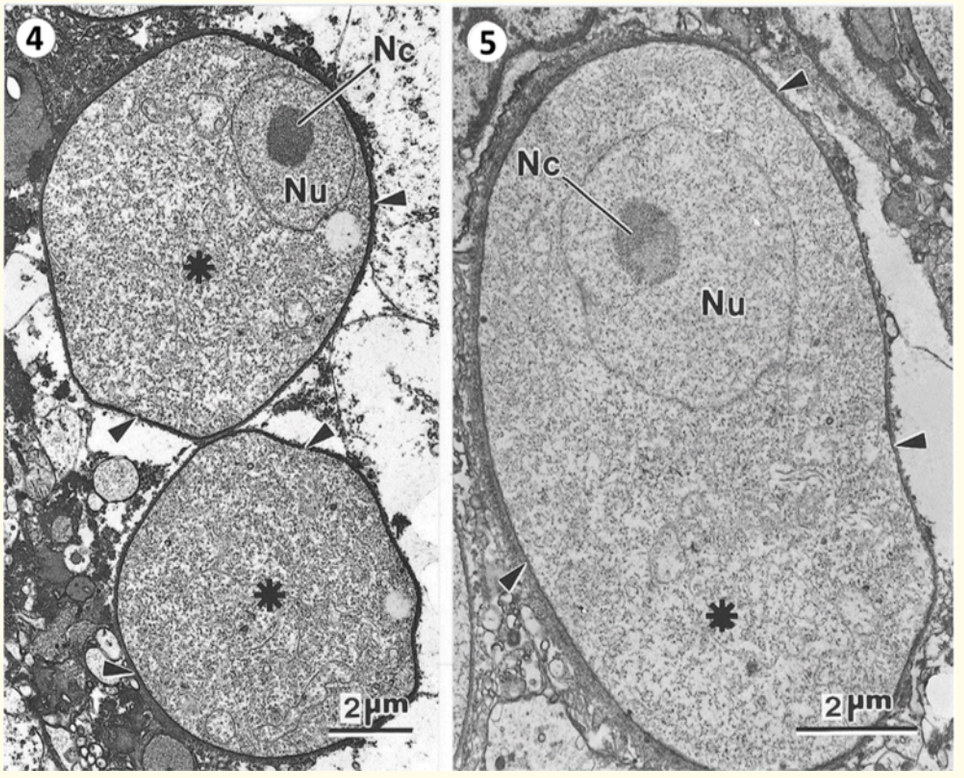

Figure 4 and 5: *Pre-trophozoites (*) infecting clam gills. The nucleus (Nu) contains a prominent nucleolus (Nc), and the cytoplasm is constitute by a granular hyaloplasma. A thin dense wall (arrowheads) surrounds these cells.*

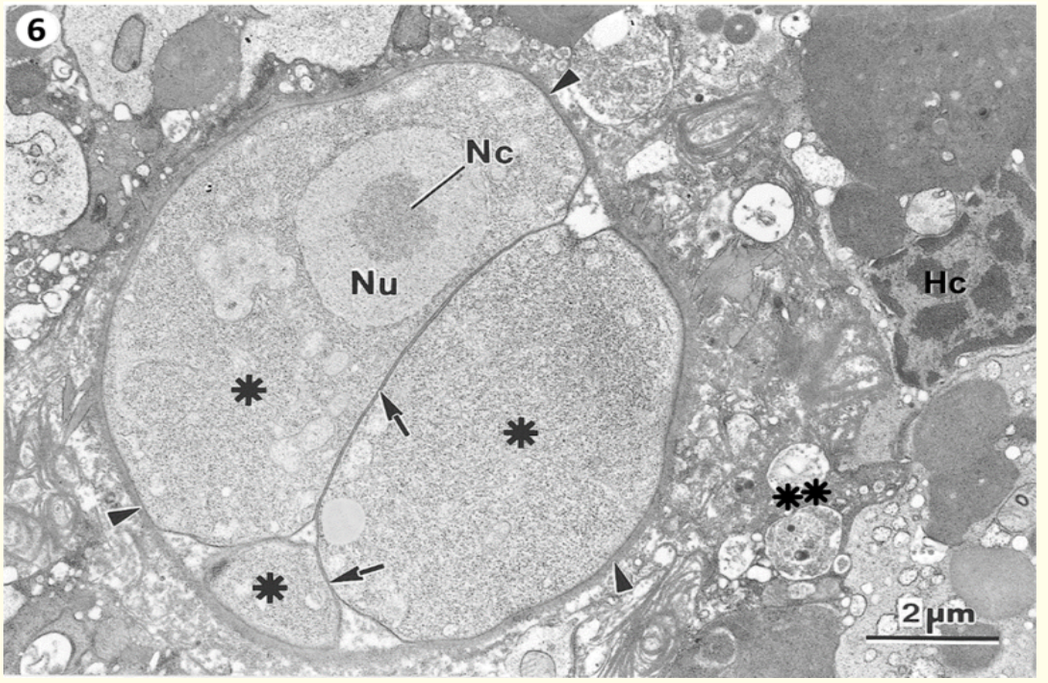

Figure 6: *Cluster formed by three pre-trophozoites (*) surrounded by a primary internal dense thin wall (arrows) and a secondary external thick wall (arrowheads). A haemocyte (Hc) and cell debris (**) can be observed nearby the cluster.*

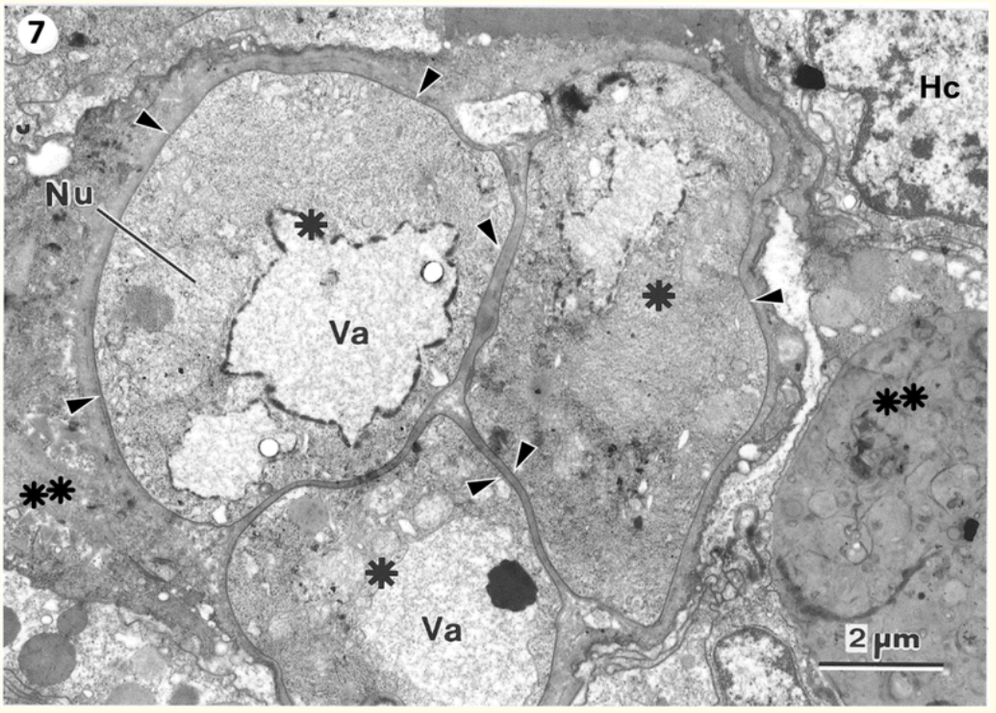

Figure 7: *Some pre-trophozoites (*), each showing its characteristic internal vacuole (Va), one of which containing a dense structure. The nucleus (Nu) and its nucleolus are visible. Surrounding the trophozoites, amorphous masses of cell debris (**) and a haemocyte (Hc) may be observed. A wall (arrowheads) externally surrounded each trophozoite.*

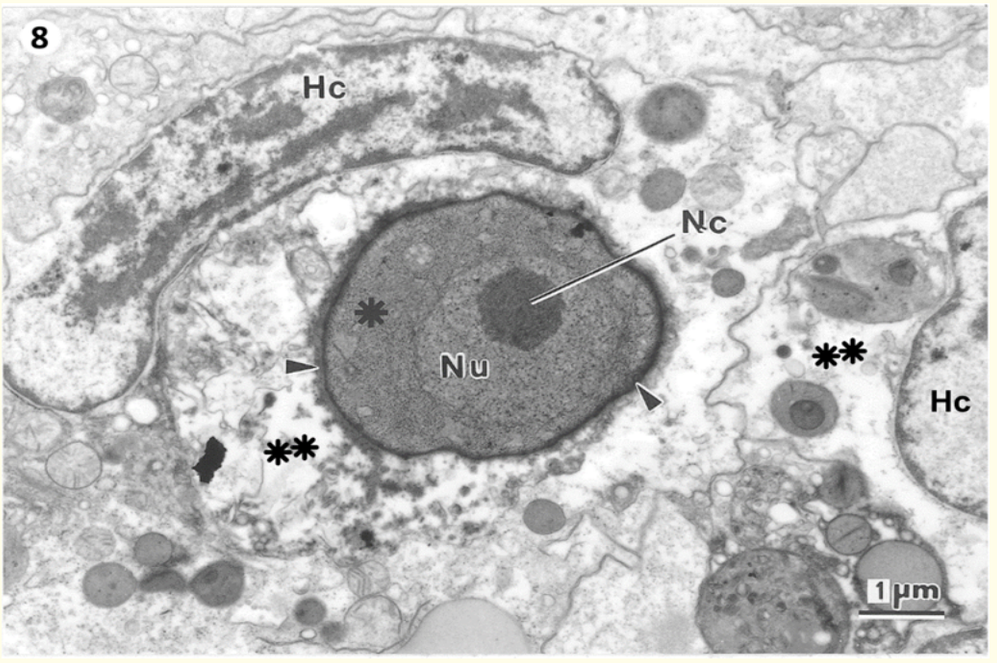

Figure 8: *A pre-trophozoite (*) partially engulfed by a haemocyte (Hc), showing the nucleus (Nu) containing a prominent nucleolus (Nc). The nucleus of the haemocyte shows evident heterochromatin masses. In the surrounding area, heterogeneous cell debris (**) are in closed contact with trophozoite wall (arrowheads).*

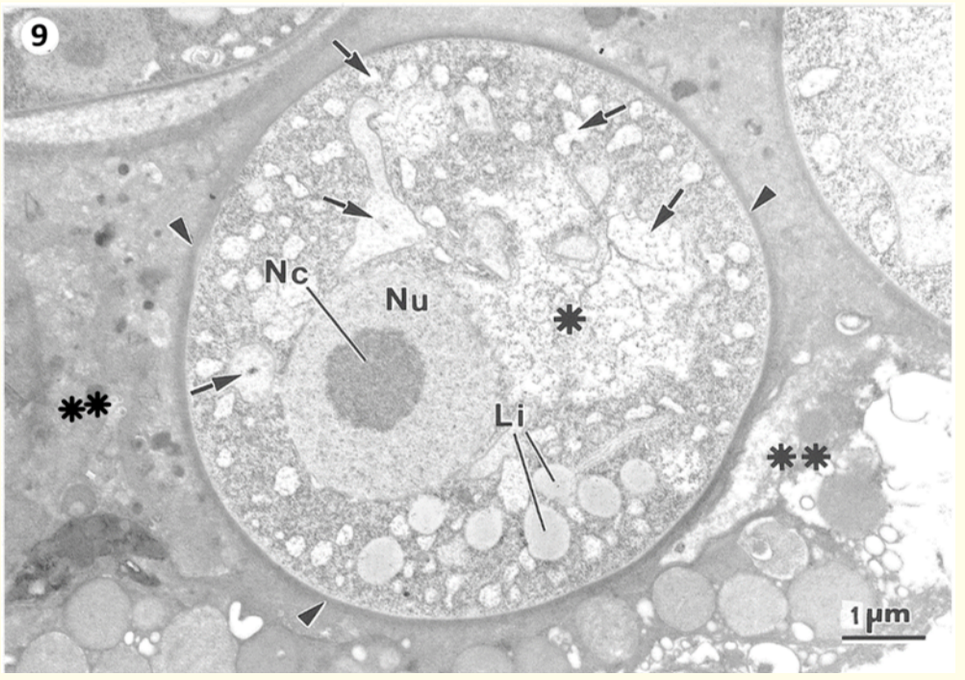

Figure 9: *Middle trophozoite phase (*) showing a prominent nucleus (Nu) and it nucleolus (Nc). The cytoplasm contains several lipidic droplets (Li), and some small vacuoles (arrows), some of which seem to be in the process of fusing (*). Externally, these trophozoites are surrounded by a dense wall (arrowheads) in contact with the heterogeneous cell debris resulting from the haemocytic reaction (**).*

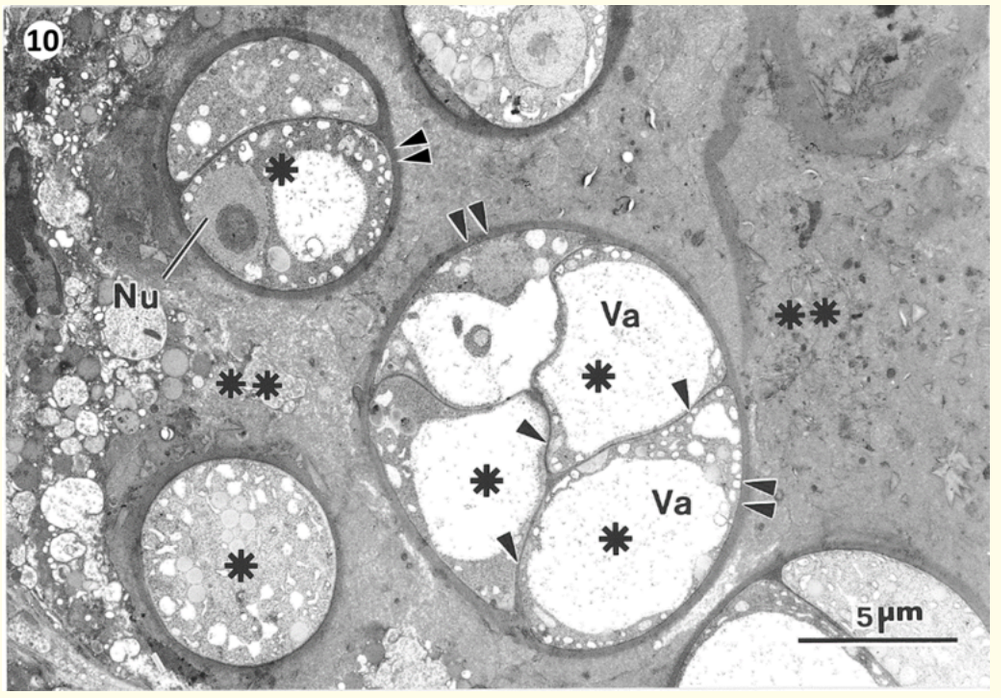

Figure 10: *Single and clustered trophozoites (*) showing both the primary (arrowhead) and the secondary wall (double arrowheads) incarcerated by homogenous amorphous mass (**) resulting from the host reaction.*

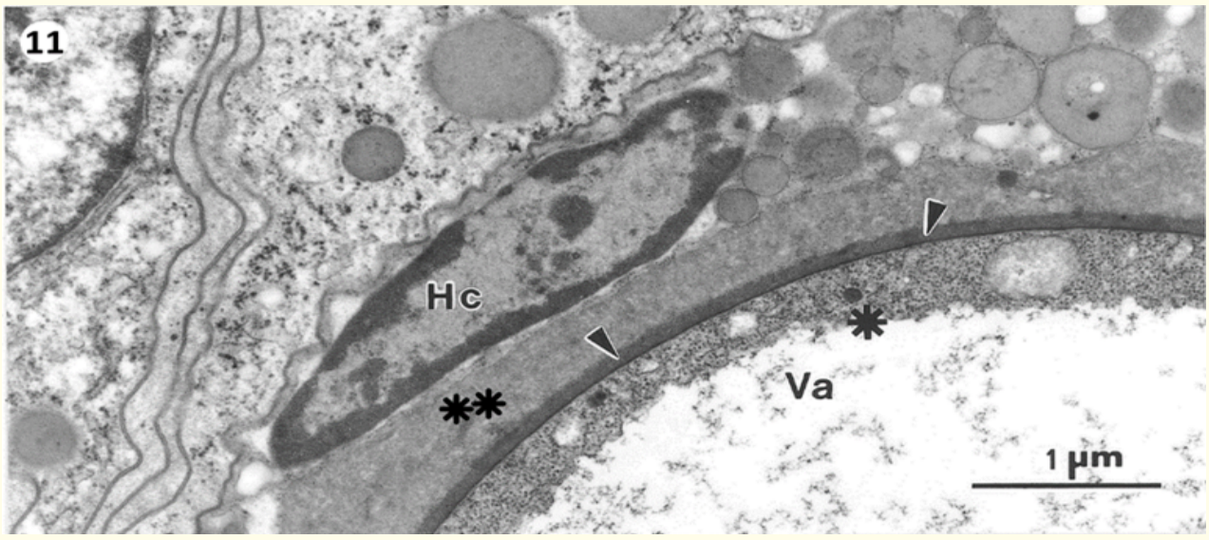

Figure 11: *Ultrastructural detail of the periphery of a trophozoite (*) showing part of the internal voluminous vacuole (Va) and the dense wall (arrowheads). Externally, a haemocyte (Hc) is in close contact with the trophozoite wall. Between the haemocyte and the wall, a homogenous amorphous mass (**) appears adherent to wall.*

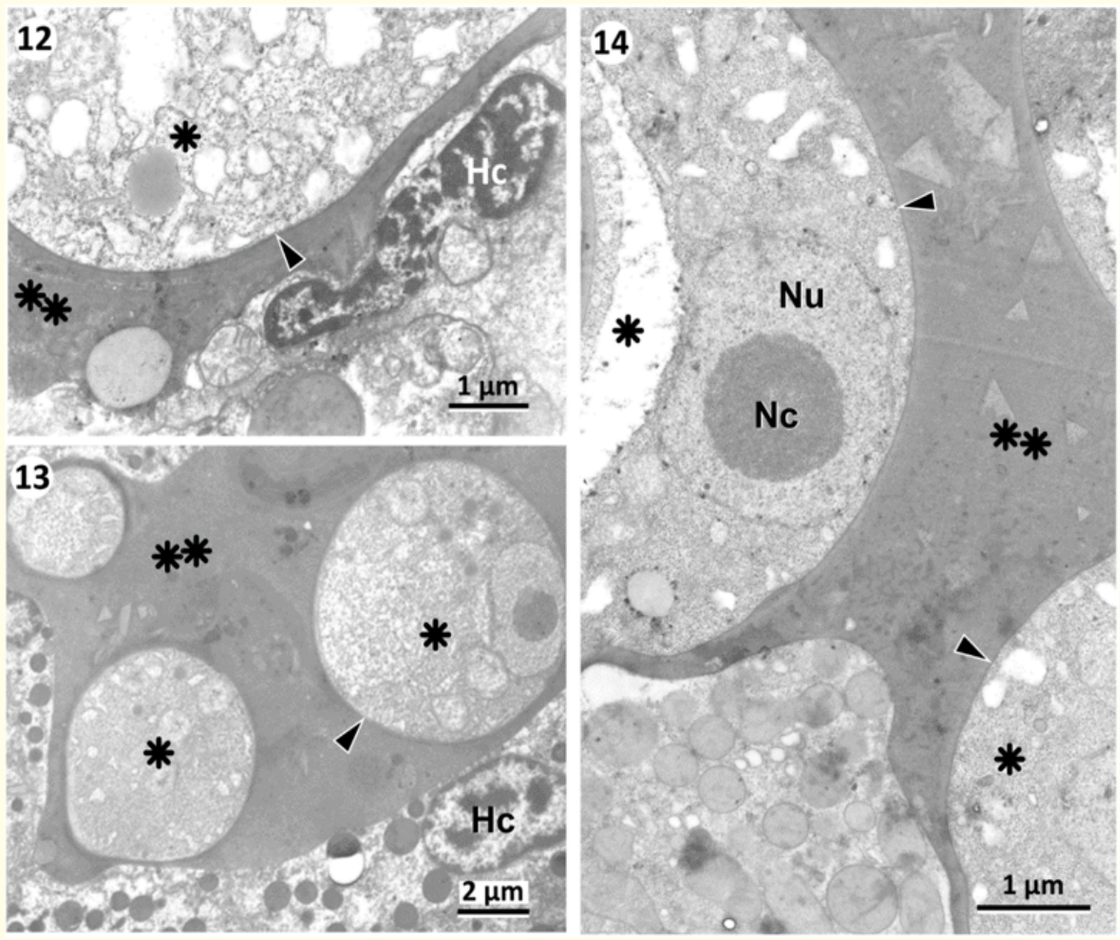

Figure 12-14: *Ultrastructural aspects of the sequential stages of trophozoite (*) encapsulation showing the parasitic cells surrounded by the engulfing haemocytes (Hc) and the dense homogenous amorphous masses (**) resulting from de host cell reaction. In this late phase, the trophozoite wall (arrowheads) appears ultrastructurally similar to the heterogeneous amorphous mass (**) enveloping the trophozoites.*

A few Tz still displayed ultrastructural features more similar to those that characterized the middle phase. Each of the Tz in close contact with the haemocytes displayed a large vacuole with light matrix and dense masses adhering to the periphery of a thick wall (Figure 9-14). Some Tz were surrounded by an electron-dense and heterogeneous material originating from the host tissues (Figure 10-14). Large cysts or nodules were observed; Tz were encapsulated by dense and heterogeneous masses intermingled with peripheral haemocytes, some of which exhibited aspects of degradation (Figure 13-14). Ultrastructure showed that the cysts encapsulating the Tz were formed by lysed granular cells and granules that had coalesced. Some dense cellular debris encloses the Tz or was surrounding the nodules (Figure 10,12-14). The final stage of this phase was characterized by complete encapsulation of the Tz and rupture of the gill epithelium. A large number of degraded haemocytes and several cellular structures with lysed aspects were observed surrounding the Tz from ruptured cysts (Figure 12-14).

Discussion

The morphology and ultrastructure of the different developmental phases of the parasite obtained from the clam *Ruditapes decussatus* by Ray's technique using thioglycollate medium, followed by cultures *in vitro* to develop the zoosporulation, confirm its identity as *Perkinsus atlanticus* [5]. Prior to its synonymy with *P. olseni* [6], this species was described in detail from infected *R. decussatus* obtained from the same collection site chosen for this study [5,38].

The ultrastructural aspects of the Tz and the encapsulation described in the present study are consistent with those observed in other *Perkinsus* spp. [13,18,19,22,32,44]. The encapsulation of the Tz by host cells described in different *Perkinsus* spp. is more evident and attain biggest dimensions, when the Tz were located in the gill tissues, than in other organelles or structures [5,29,32,33,42].

The main objective of the present study was to describe the developmental phases that lead to the maturation of the Tz after infection by zoospores, as well as the host immune response by the haemocytes. Since the gills are the preferred site of infection [5,38], only infected tissues from this organ were ultrastructurally studied.

The appearance of the perkinsosis in the exposed clams, as observed by light and TEM in this study, resembled the morphological and ultrastructural aspects of the Tz obtained from parasitized clams collected directly in the field [20,24,25,27,30,33].

The current study provides a first-hand description of the ultrastructural development of the Tz and their encapsulation by haemocytes as a host immune response.

The ultrastructural aspects of the Tz revealed asynchrony in its development, in which the simultaneous presence of Tz in different phases of development was observed. We tried to analyse the different ultrastructural aspects of the sequential developmental phases based on the sequential evolution of different organelles and structures right through to the final phases of Tz maturation. In the present study it was observed that the developmental phases were not synchronized, however, this could be interpreted as result of a sequential and continuous period of infection. Comparing these developmental phases of the Tz, described in some *Perkinsus* sp. seem to show similar morphologic aspects to other species of this genus [5,23,26,29,32,33,42].

Infiltrating haemocytes phagocytose *Perkinsus* parasite and possible secreted the acellular encapsulating dense matrix or host cell debris is a common phenomenon that were described in several *Perkinsus* spp. phases characterized by defensive host reaction [5,13,14,27,29,32,33]. This encapsulation by haemocytes and or agglutinations of numerous cell debris, giving rise to the dense nodules, has been described prior to the rupture of these nodules, which causes the demise of infected hosts, as reported in several studies [5,19,23,26,31,32].

The nucleolus that develops its greatest volume in the final stages of its maturation is certainly important structure in the transcriptional activity for the production of rRNA/RNP that participate in the ribosome formation to produce the proteins in this phase of the incarceration of Tz by phagocytes and acellular host debris.

Conclusion

This ultrastructural study details the sequential developmental phases of *Perkinsus* zoospores from pre-Tz to mature Tz that appear encapsulated by the host haemocytes, as part of its immune response to the infection. The different described species of the genus *Perkinsus*, infecting the fauna from some geographic areas, are referred as of great economic importance, as negative results of their pathogenic

action. The microscopic studies of the evolutionary phase of the life cycles of the different species is of great importance in order to characterize the specific aspects of the incarceration and type of the host reaction and the mode of propagation of the perkinsosis.

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Conflict of Interest

All authors have declared that no conflicting interest exist.

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