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The Ultrastructural Study of *Nosema artemiae* (Codreanu, 1957) (Microsporidia: Nosematidae)

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Summary. Ultrastructure of microsporidium *Nosema artemiae* from the solar saltern located on the Southern Ukraine was studied. The parasite infects the musculature of brine shrimp *Artemia salina* (L.). All developmental stages of the parasite are diplokaryotic. Merogony occurs through binary division of the tetranucleate merogonial plasmodium. Sporogony is disporoblastic. Unfixed spores are broadly oval measuring 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4) µm. The layered exospore includes a coarse-grained basal layer, and an external coat resembling a double membrane. The polar filament is slightly anisofilar, making 13-16 coils (9-12 + 4). The polaroplast is composed of narrow anterior lamellae and posterior wide lamellae, tapered at the peripheral area. The obtained new data permits the elucidate of features which were incompletely characterized in the previous reports.

Key words: *Artemia salina*, Crustacea, Microsporidia, *Nosema artemiae*, ultrastructure.

INTRODUCTION

Six species of Microsporidia infecting *Artemia* spp. were recorded (Table 1). The descriptions were based on material collected in Romania (Codreanu 1957; Codreanu-Balcescu and Codreanu 1978, 1980), Spain (Martinez *et al.*1989, 1993, 1994) and Brazil (Martinez *et al.* 1992). The ultrastructural data of five of them were obtained, but detailed morphological descriptions were not given. Two species: *Vavraia anostraca* Martinez *et al.*, 1992 and *Endoreticulatus durforti* Martinez, Vivares *et* Bouix, 1993 were completely studied. The ultrastructural data of *Pleistophora myotropha* Codreanu, 1957 include some information concerning exospore construction and the merogony of the parasite. The ultrastructure of *Gurleya dispersa* Codreanu, 1957 is unknown. Three species with binary divided merogonial stages were noticed in *A. salina*. They are: *Unikaryon exiguum* (Codreanu, 1957), *E. durforti* and *Nosema artemiae* (Codreanu, 1957). The ultrastructure of *U. exiguum* was briefly observed, and only few data relating to sporogony and numbers of polar filament coils were presented (Codreanu-Balcescu and Codreanu 1978). *E. durforti* was completely studied by Martinez *et al.* (1993). *N. artemiae* was moderately studied by

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Codreanu-Balcescu and Codreanu (1978) in Romania, and more completely described by Martinez *et al.* (1994) in Spain, but the descriptions of the earliest stages of merogony were not presented.

The microsporidium identified as *N. artemiae* was newly found in South Ukraine. The peculiarities of the ultrastructure of the parasite are completely described, and the new data relating to early merogony and the spore construction are obtained. The systematic position of this microsporidium is discussed.

MATERIALS AND METHODS

The microsporidium was isolated from a brine shrimp, inhabiting the solar saltern located on the territory of Chernomorski Biosphere Reserve (46°35′ N, 32°16′ E). Infected tissues were prepared for light and ultrastructural study. The live spores and Giemsa stained slides were examined under a light microscope (phase contrast microscopy was used for observations of the live spores). For measurements, the software „Analysis Pro 2.11" in combination with Olympus BX50F4 microscope were used. One hundred spores were measured on every slide. For transmission electron microscopy, the infected tissues were fixed in a 2.5 % (v/v) glutaraldehyde in a 0.2 M sodium cacodylate buffer (pH 7.2) for 1-3 days. After washing and postfixation in 2.0 % (w/v) osmium tetroxide in cacodylate buffer for 1 h at 4°C, the pieces were dehydrated and embedded in Epon-Araldite as reported in previous papers (Ovcharenko and Wita 2001a, b). Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEM 100B electron microscope.

RESULTS

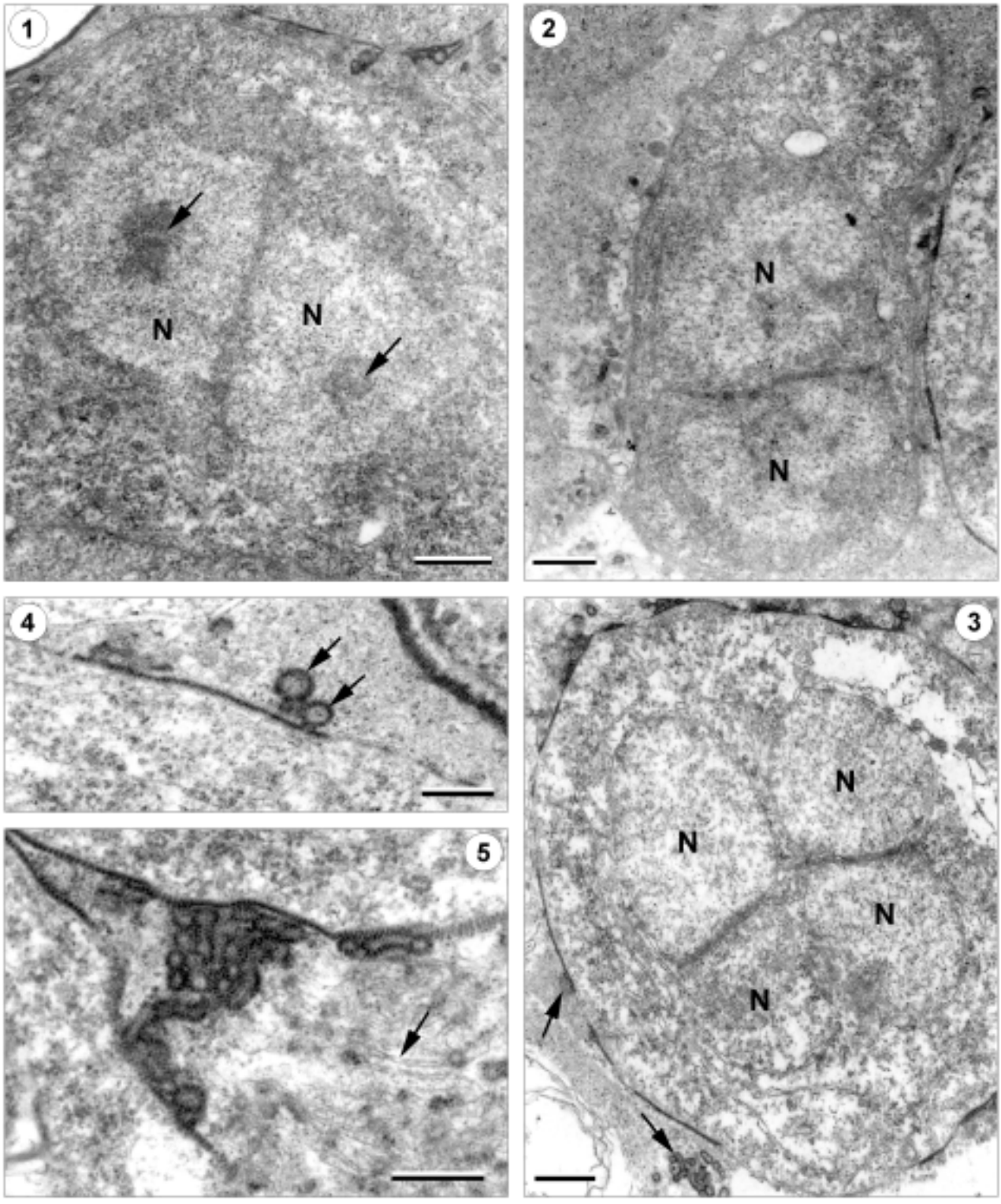
One of the 112 examined specimens of *A. salina* was found to be infected. The primary site of infections was the muscle tissue. Heavily infected muscles were hypertrophied and easily disrupted. All stages of the parasite were in direct contact with the host cell cytoplasm.

The earliest observed stages of investigated microsporidium were rounded and elongated meronts approximately 2.6 × 4.4 µm in size (Figs 1, 2). They possessed two nuclei in diplokaryotic arrangement. The diameter of each nucleus measured 1.5 µm. The cytoplasm of the meronts was homogenously granular. Electron-dense nucleoli were registered in the nucleoplasm of early merogonial stages (Fig. 1). Merogony occurs by binary fission of tetranucleate rounded stages about 4.6 µm in diameter (Fig. 3).

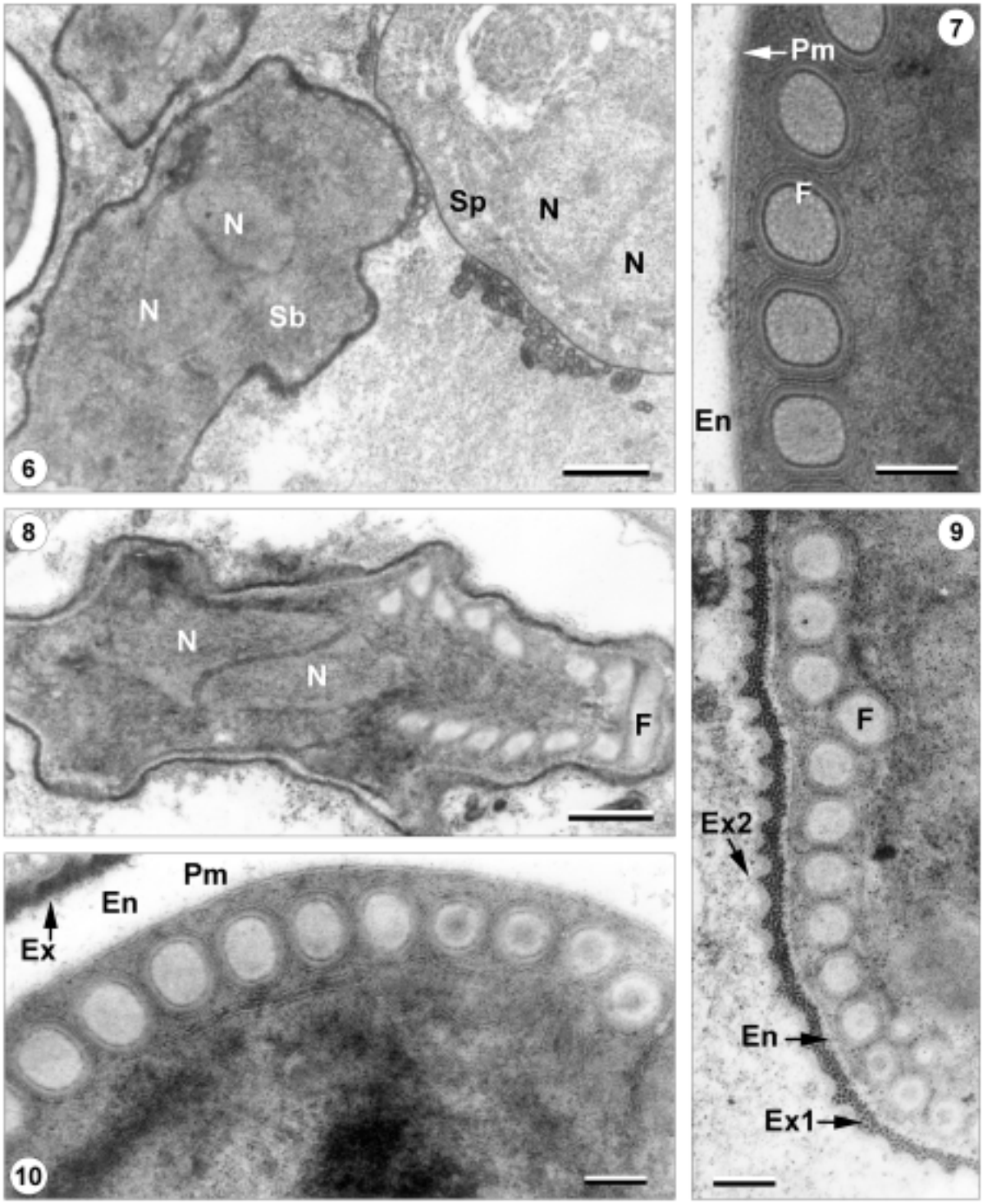
The beginning of the sporogony was marked by the structural transformation of the envelope surrounding the late meronts. The thickening of the late meronts surface was accompanied by appearance of electron-dense granules which was connected with vesiculo-tubular secrets, adhering to the plasma membrane (Figs 3-5). The vesiculo-tubular structures occur between the developing parasite cells. It can be present as clusters of irregularly arranged vesicles to strands of vesicles, covered with electron-dense granules (Fig. 5). Finely the sporont surface is covered with electron-dense granular material about 40 nm thick, connecting with a tubular caverns and ridges (Fig. 6). Few membrane profiles and vacuoles were visible inside of the cytoplasm of the sporont. Diplokaryotic sporonts were rounded to broadly oval. Each sporont gives rise by binary fission to two diplokaryotic sporoblasts. The rough episporontal coat developed into the exospore of the future spore wall (Figs 8, 9).

The elongated sporoblasts were diplokaryotic, measuring about 2.1 × 3.9 µm. Their cytoplasm contained cisternae of the endoplasmic reticulum, free ribosomes and the polar filament primordia (Figs 6, 8). The wall of the future spore consists of electron-transparent endospore about 30 nm thick and double-layered exospore (Figs 8, 9). The electron-dense internal layer of the exospore contains regular granular protrusions which may represent in tangential view as a circular rows (Fig. 9). The outer coat of the exospore resembled double membrane (Fig. 9). During spore maturation the endospore thickness increased, except the apical pole (Figs 12, 14).

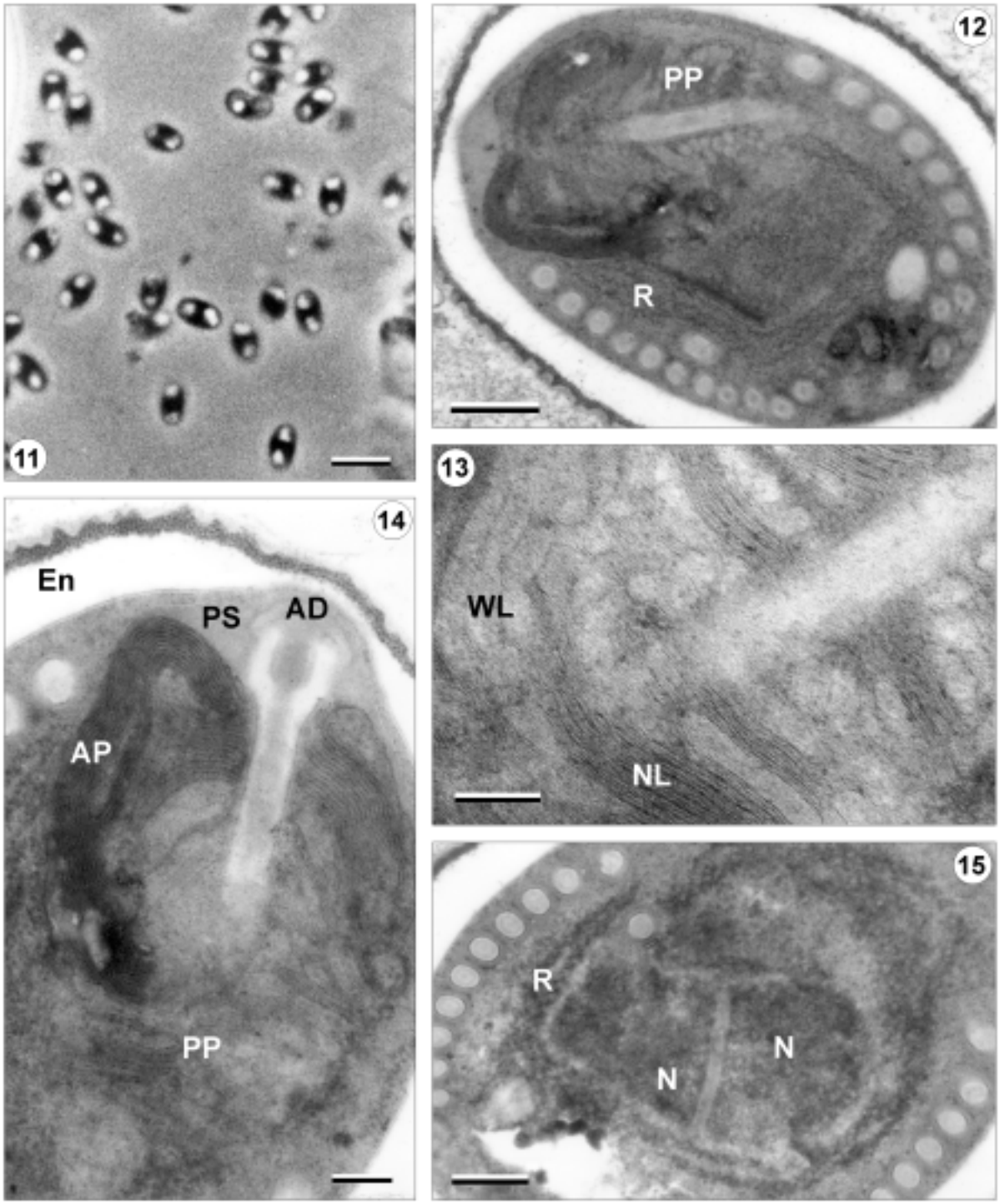
Unfixed spores were broadly oval (Fig. 11) measuring 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4) µm. The Giemsa stained spores were 2.3 ± 0.3 (2.0-3.2) × 3.5 ± 0.2 (3.1-4.6) µm in size. The spore envelope was composed of the 150-200 nm thick electron-transparent endospore, and the electron-dense exospore 75-90 nm wide (Figs 7, 10, 12, 14). The endospore was greatly thinned over the anchoring disc (Fig. 14). The exospore was layered with a coarse-grained basal layer 50-60 nm wide, and a thin external coat resembling a double membrane (Figs. 12, 14). The polar filament was slightly anisofilar, making 13-16 coils of 130-140 nm in diameter. The last four coils were slightly narrower, 120-125 nm wide (Fig. 10). The transversal sectioned polar filament exposed classic ultrastructural organization with 19-20 electron-transparent longitudinal threads (Fig. 7). The polaroplast was composed of two lamellar parts. The anterior polaroplast contained densely packed narrow lamellae (Figs 12, 14). The posterior polaroplast had wide lamellae in central



Figs 1-5. Merogony and early sporogony of *Nosema artemiae*. **1 -** rounded early merogonial stage with the nucleoli (arrows) and diplokaryotic nuclei; **2 -** elongated merogonial stage; **3 -** meront/sporont transitional stage with two diplokarya. Patches of electron-dense granules (arrows) appear outside the membrane of parasite cell; **4 -** structural transformation of the parasite cell surface. Electron-dense conglomerates located on the cisternae (arrows), appearing in the host cytoplasm adjacent to parasite cells forms an outer layer; **5 -** modification of the host cytoplasm adjoining to sporogonial stages of the parasite. Electron-dense secretions on the surface of vesiculo-tubular formations are visible. The host endoplasmic reticulum is arrowed. N - diplokaryotic nuclei. Scale bars 0.5 µm (1-3); 0.2 µm (4, 5).



Figs 6-10. Electron micrographs of the late sporogonial stages and polar filament structure of *Nosema artemiae*. **6 -** sporont and sporoblast with diplokaryotic nuclei. The late sporogonial stages are bounded by a rough coat which develops into the exospore of the future spore; **7 -** part of a longitudinal section of the spore. Endospore, plasma membrane and ultrastructure of transversal sectioned polar filament are visible; **8 -** the late sporoblast with diplokaryon and developed polar filament; **9 -** electron micrographs of a part of longitudinal section of immature spore. The exospore contains basal layer with a regular protrusions and the double membrane like external coat. The narrow electron-transparent endospore and sixteen transversely sectioned polar filament coils are also visible; **10 -** electron micrographs of a part of longitudinal sectioned mature spore. The spore wall and one row of polar filament coils are visible. The diameter of polar filament of four last coils is narrower. The spore wall consists from the plasma membrane, endospore and two layered exospore. En - endospore, Ex - exospore, Ex1 - exospore with a regular protrusions, Ex2 - exospore with double membrane like external coat, F - polar filament, N - diplokaryotic nuclei, Pm - plasma membrane, Sb - sporoblast, Sp - sporonts. Scale bars 1.2 µm (6, 8); 0.1 µm (7, 9, 10).



Figs 11-15. Light and electron micrographs of the spores of *Nosema artemiae*. **11 -** phase contrast image of the live spores; **12 -** diagonal section of the middle part of the mature spore. The structure of the spore wall, the construction of the posterior polaroplast and the rows of ribosomes are observable; **13 -** electron micrograph of the peripheral polaroplast construction. The polaroplast cisternae are formed for the narrow and wide lamellae; **14** - ultrastructure of the apical part of the spore. The construction of the spore wall, anchoring disc, polar sac, anterior and posterior parts of polaroplast are shown. The endospore is hardly thinner over the anchoring disk. The anterior polaroplast was constructed of densely packed narrow lamellae. The posterior polaroplast has wide lamellae in the central parts, transforming into narrow lamellae and tubules where it has been sectioned peripherally; **15 -** diagonal section of the posterior part of the spore. The cytoplasm of the spores contains numerous ribosomes forming chains around the nuclei. AD - anchoring disc, AP - anterior polaroplast, En - endospore, N - nuclei, NL - narrow lamellae, PP - posterior polaroplast, PS - polar sac, R - ribosomes, WL - wide lamellae. Scale bars 5 µm (11); 0.5 µm (12); 0.2 µm (15); 0.1 µm (13, 14).

Table 1. Comparative data of Microsporidia described from *Artemia* spp.

Prominent features	*Nosema artemiae* (Codreanu, 1957)	*Endoreticulatus durforti* Martinez Vivares *et* Bouix, 1993	*Gurleya dispersa* Codreanu, 1957	*Pleistophora myotropha* (Codreanu, 1957)	*Unikaryon exiguum* (Codreanu, 1957)	*Vavraia anostraca* Martinez *et al.*, 1992
1	2	3	4	5	6	7
Distribution	Romania, Spain, Brasil, Ukraine•	Spain	Romania	Romania	Romania	Brasil
Site of infection	Musculature, haemocytes, hypoderm	Intestinal epithelium	Haemocel	Musculature	Intestinal epithelium	Musculature, Intestinal epithelium, haemocel
Infected cells	Hypertrophyed. Giant nuclei are produced. Cytoplasmic organelles are destructed•	Hypertrophyed	Destructed	No data	Not hypertrophyed	Destructed
Parasitophorous vesicle	Absent	Appears during merogony	No data	No data	Appears during sporogony	Absent
Meront size (µm)	3.0-6.0 (Martinez *et al.* 1994); 2.6 × 4.4•	2 in diameter	2.3-3.0	No data	2.0-4.0	No data
Meront nuclei	Diplokaryotic	Isolated	Punctiform	No data	Isolated	Isolated
Merogonial plasmodium	1-2 nucleate (Codreanu 1957), tetranucleate•	2-4 nucleate	No data	No data	No data	2-11 nucleate, with folded coat
Mode of division	Binary fission•	Binary fission, rosette-like fragmentation	No data	No data	Binary fission	Plasmotomy
Sporont shape	Elongate, thick walled, with protuberances	Round	Round	Lobulated (early)	No data	Round
Sporont size (µm)	2.1 × 3.9•	Up to 19	No data	8.6-11.6 µm to 16.0-20.3 µm	No data	No data
Sporont nuclei	Diplokaryotic	Isolated	No data	No data	No data	Isolated
Mode of division	Binary fission	Rosette-like fragmentation	No data	No data	Binary fission	Stepwise and rosette-like fragmentation
Sporophorous vesicle	Absent	Subpersistent	Covered by fine membrane	No data	No data	Merontogenetic
Number of sporoblasts	-	8-128	4, 2 (rare)	4-32, mostly 8, 16	No data	16-128, mostly 32, 64, exceptionally 8
Inclusions	-	No data	No data	Fine granules and tubules	No data	Tubules
Size of sporophorous vesicle (µm)	-	Up to 19	7.5-9.4	No data	No data	11-21

Table 1 (contd.)

Spore shape	Oval (Codreanu 1957), oval with blunt ends (Martinez *et al.* 1994), broadly oval•	Oval	Pyriform	Ovoid	Ovoid	Oval (stained)
Spore size (µm)	4.2-5.7 long (Codreanu 1957); 3.0 (2.8-3.5) × 4.6 (3.9-5.1) (Martinez *et al.* 1994); 3.0 ± 0.2 (2.7-3.5) × 4.9 ± 0.3 (4.5-5.4)•	0.9 ± 0.12 × 1.7 ± 0.15	5.0-5.9 long	5.3-6.9 long	2.6-3.2 long	Macrospores 2.0-3.0 × 3.5-5.0; microspores 1.5-2.0 × 2.8-3.5
Spore nuclei	Diplokaryotic	Single,with additional envelope	Single	Single	Single	Isolated
Spore wall wide (nm)	156-213 (Martinez *et al.* 1994); 215-290•	140-150	No data	No data	No data	94-97
Exospore	Two layered, the surface layer resemble double membrane. Coarse-grained basal layer and double membrane shaped coat•	Uniform	No data	No data	No data	With a dense surface coat
Exospore thickness (nm)	75-90; 57 (Martinez *et al.* 1994)	30	No data	No data	No data	35
Polar sac	Umbrella-shaped, 1/5 of the spore length	Expanded	No data	No data	No data	Laterally prolonged
Polaroplast	Bipartite	Bipartite	No data	Voluminous laminate	No data	Bipartite
Anterior polaroplast	Tightly packed lamellae	Few lamellae	No data	No data	No data	Tightly packed lamellae
Posterior polaroplast	Wide lamellae (Martinez *et al.* 1994); wide lamellae, narrower in peripheral part•	Membraneous saccules	No data	No data	No data	Expanded lamellae
Polar filament	Isofilar, or lightly anisofilar (Martinez *et al.* 1994)	Isofilar	No data	No data	Isofilar	Anisofilar
Polar filament coils	13-16 with more narrow four last coils; 15-17 (Codreanu-Balcescu and Codreanu 1980); 11-13 (Martinez *et al.* 1994); 13-16 with more narrow four last coils•	8-11	No data	11-12, the 3 distal being constricted	6	10-12+5–6; 8-9+3
Polar filament rows	1	2	No data	No data	No data	2; 1
Diameter of polar filament (nm)	110-130 (Martinez *et al.* 1993); 120-140•		No data	No data	No data	120+92; 90+70

(• - authors data)

part, transforming into narrow lamellae where it has been sectioned peripherally (Figs 13, 14). The cytoplasm of the spores contained numerous ribosomes sometimes forming chains around the diplokaryon (Figs 12, 15).

DISCUSSION

Generally, the ultrastructure of the investigated microsporidium was similar to these of *Nosema* and *Brachiola* genera. A few details of the cytology need comments: the ultrathin organization of the exospore, the ultrastructural features of the polar filament and vesiculo-tubular structures along the parasite cell surface.

The exospore construction is the important taxonomic attribute for determination of the generic position of microsporidians (Larsson 1999). Usually the spores of the species of the genus *Nosema* possessed uniformly structured exospore (Sato *et al.* 1982, Sokolova and Lange 2002). The type species *N. bombycis* has "exospore thin, endospore moderately thick" (Sprague *et al.* 1992). Uniform dense exospore has also been reported in *N. granulosis*, infecting *Gammarus duebeni* (Amphipoda) (Terry *et al.* 1999). The layered exospore with a double outer coat is characteristic for the genera of the family Thelohaniidae (Larsson 1988), but an exospore with two distinct strata was also reported among several *Nosema* like microsporidians such as *Schroederella plumatellae* (Morris and Adams 2002) and *N. omaniae* (Diarra and Toguebaye 1995). Generally the layered exospore is not peculiar for representatives of the genus *Nosema*. The first ultrastructural data concerning exospore construction of *N. artemiae* were obtained on the material collected in Spain. The two-layered exospore with the surface layer resembling a double membrane was demonstrated on most of published photographs (Martinez *et al.* 1994). Identically structured exospore has the microsporidium studied (Figs 9, 12, 14), and we are disposed to believe that the exospore with an external double layer is characteristic feature of this species.

The first information about the number of polar filament coils (15-17) of *N. artemiae* was documented by Codreanu-Balcescu and Codreanu (1980). More complete records were presented by Martinez *et al.* (1994). According of them, the coiled part of polar filament of *N. artemiae* is arranged in 13-16 coils, where the last four coils were somewhat narrower, but the spores with 11-13 coils were also observed, mainly in the species coming from South East of Spain. The four coils of polar filament of the spores of microsporidium collected in South Ukraine and Spain were slightly narrower, than the others. The polar filament develops until the spore is perfectly mature, and immature coils are normally slightly narrower than completely mature coils (Larsson 1986). It does not seem that the last coils should be immature, because the number of narrow coils is always four (Fig. 10). A similarly constructed polar filament was described in *Nosema chaetocnemae*, from *Chaetocnema tibialis* (Coleoptera), but the authors defined this polar filament as isofilar (Yaman and Radek 2003).

The vesiculo-tubular secretions observed in the current study appear irregularly along the cell surface of late merogonial and sporogonial stages (Figs 5, 6). Similar structures were described in *Brachiola* spp. and some other microsporidians, but contrary to *N. artemiae* it were observed mostly as a protuberance of the sporont wall but not the sphaerular dense secretions adhering to the plasmalemma of late meront and forming the inner layer od the future exospore. It may be treated rather as episporontal inclusions than as appendages of the sporont wall. In case of *N. artemiae* as "episporontal space" should be defined a part of host cell cytoplasm adjoining to the parasite cells.

A comparison of *N. artemiae* with *Brachiola vesicularum*, *B. algerae* and *B. gambiae* brings some similarities. No multinucleate plasmodia or ribbon-like stages containing more than two diplokarya occur in *N. artemiae* similar to *B. vesicularum* and unlike to other *Nosema* species. The short polar filaments with the more narrower three (*B. vesicularum*), one (*B. algerae*) and four (*N. artemiae*) posterior coils are likewise constructed (Canning and Sinden 1973, Cali *et al.* 1998, Weiser and Žižka 2004). *Brachiola* related species are thermophilic, proliferating and sporulating at temperatures ≥30°C (Cali *et al.*1998). The same and even higher temperatures are characteristic for southern salters, inhabiting *Artemia* spp.

Based on obtained data we are disposed to believe that *N. artemiae* cannot be considered as *Brachiola* or classic *Nosema*-belonging species. More precise definition of taxonomic position of this microsporidium and other *Nosema*-related species with slightly anisofilar polar filament and double-layered exospore becomes possible after the further molecular analysis of these parasites. The achieved data confirms suggestion about heterogeneous character of the genus *Nosema*.

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