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ORIGINAL PAPER

Characteristic Light and Electron Microscopic Features of *Adelina melolonthae,* a Coccidian Pathogen of the European Cockchafer, *Melolontha melolontha* (Coleoptera/Scarabaeidae)

Mustafa Yaman[1] · **Renate Radek**[2]

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Abstract

Purpose The aim of this study was to investigate and document the characteristic electron microscopic features of the oocyst of *Adelina melolonthae,* a coccidian pathogen of the European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae).

Methods Larvae of *M. melolontha* were collected at Ordu, Turkey. Each larva was dissected in insect Ringer solution. Adeleid oocysts from the tissue that is suspected to Contain coccidian infections were examined under a light microscope (Zeiss), a scanning electron microscope (FEI Quanta 200) and transmission electron Microscope (Philips EM 208).

Results Spherical to ellipsoidal oocysts measure ca. 24–44.5 µm (mean 35.6 µm) in diameter ($n = 50$) and include up to twelve sporocysts. The oocyst wall has double layers; the outer layer measured 400–500 nm in thickness and the inner one 10–25 nm. Sporocysts including two sporozoites are rounded ca. 11–12.5 µm (mean 11.7 µm) in diameter. The sporocyst wall consisted of only one 70–80-nm-thick layer. The sporozoites are ellipsoidal and measured 9–11 µm length and 3–4 µm in width. Sporocysts include residual bodies.

Conclusion In the present paper, the morphology and ultrastructure of the oocyst of *A. melolonthae* is documented for the first time and compared with other *Adelina* and coccidian species infecting insects. The results in this study confirm his identification and justify the classification as a separate species *A. melolonthae*, which differs from other *Adelina* species.

Keywords *Adelina melolonthae* · *Melolontha melolontha* · Biological control · Oocyst · Sporocyst · Ultrastructure

Introduction

The European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae) L., is one of the most serious insect pest species and causing considerable damage in all countries of Europe, where it is very common [1]. *M. melolontha* is frequently infected by entomopathogenic organisms [2–7]. An infection with the coccid *Adelina melolonthae* (Apicomplexa/Adeleidae) has been known already for many years [8]. Coccidia are mainly parasites of vertebrates less than 1% of the species infect insects [9]. Species of eight coccidian genera, *Adelina, Barrouxia, Chagasella, Legerella, Ithania, Rasajeyna* and *Ganapatiella,* have insects as sole or primary hosts, and all of them are rare and not known well. One of them is the genus *Adelina*, which exclusively infects insects. It includes several species that are commonly encountered. Members of the genus *Adelina* infect several insect orders such as Blattaria, Embioptera, Orthoptera, Diptera, Lepidoptera and Coleoptera [9]. Most of these insect-infecting *Adelina* species are transmitted by ingestion of contaminated food.

There is not much information on *Adelina melolonthae.* After its first description from *M. melolontha* by Tuzet *et al.* [8], it was further reported by Kharazi-Pakdel and Amargier [10]. Recently, Yaman *et al.* [7] recorded this pathogen from the same host in Turkey. Tuzet's [8] original description of *A. melolonthae* and all other descriptions of this coccidian pathogen so far are based on light microscopic morphology of the oocyst. In

✉ Mustafa Yaman
muyaman@hotmail.com

[1] Department of Biology, Faculty of Arts and Science, Bolu Abant İzzet Baysal University, 14030 Bolu, Turkey

[2] Institute of Biology/Zoology, Freie Universität Berlin, Königin-Luise-Str. 1–3, 14195 Berlin, Germany

all coccidian parasites infecting insects, the diagnostic stage is the oocyst. In the present paper, the morphology and ultrastructure of the oocyst of *A. melolonthae* is documented for the first time and compared with other *Adelina* and coccidian species infecting insects.

Material and Methods

Light Microscopy

Larvae of *M. melolontha* were collected at Ordu, Turkey. Each larva was dissected in insect Ringer solution. Wet smears of the larvae´s fat body, i.e., the tissue that is suspected to contain coccidian infections, were examined under a light microscope (Zeiss) at a magnification of 200–1000x. When an infection was found, the slides were air-dried and fixed in methanol for 2–5 min. The slides were then washed with distilled water and stained for approximately ten hours in freshly prepared 5% solution of Giemsa stain (stock solution, Carloerba, No. 6B712176C). Afterward, the slides were washed in running tap water, air-dried and re-examined under the microscope. Detected vegetative stages and oocysts were measured and photographed as described in Yaman *et al.* [11].

Scanning Electron Microscopy

For scanning electron microscopy, tissue samples stored in distilled water were crushed in a drop of water, slightly spread on a cover glass and air-dried. After sputtering with gold in a Baltec SCD 040 sputter coating device, the samples were observed in a FEI Quanta 200 scanning electron microscope.

Transmission Electron Microscopy

Samples for transmission electron microscopy were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h, rinsed in cacodylate buffer, post-fixed in 1% OsO_4 for 2 h, and rinsed in cacodylate buffer. After dehydration in an increasing ethanol series, the infected beetles were embedded in Spurr's resin [12]. Ultra-thin sections were mounted on Pioloform-coated copper grids which were stained with saturated uranyl acetate and Reynolds lead citrate [13]. They were then examined with a Philips EM 208 transmission electron microscope.

Results

A cocccidian infection was observed in the fat body of the host *M. melolontha*. In all coccidian parasites infecting insects, the diagnostic stage is the oocyst. Members of the family Adelidae are characterized by polysporocystic oocysts, which form several sporocysts within one oocyst. We were able to find such polysporocystic oocysts in the fat body, the typical tissue of adelaide infections (Figs. 1–12). Oocysts are spherical to ellipsoidal (Figs. 2, 3, 10) and measured 35.62 ± 4.04 (23.97–44.56) µm in diameter (n = 50). The oocyst wall has two layers (Figs. 1, 3, 6, 12–14). While the outer wall is considerably thick (Figs. 4, 7–9, 12–14), measuring approximately 400–500 nm, the inner wall is very thin (Figs. 13, 14) measuring only 10–25 nm in thickness. When viewed with SEM microscopy, the outer layer of the oocyst wall is generally smooth (Fig. 10) with some spheroid bodies, which appear in some areas (Figs. 10, 12, 13), while the inner surface of the outer layer is completely smooth (Fig. 9). Oocysts do not contain any residual cytoplasmic bodies (Figs. 2, 3, 5, 6, 7, 12, 14). The oocysts of *A. melolonthae* include up to twelve rounded sporocysts (Figs. 1–6, 11, 14), which measured 11.70 ± 0.42 (11.02–12.52) µm in diameter. The thickness of the sporocyst wall was measured from ultra-thin sections and is 70–80 nm (Fig. 14). Within one sporocyst, two sporozoites were observed (Figs. 5, 6). The sporozoites are ellipsoidal and measured 9–11 µm length and 3–4 µm in width. Sporocysts include cytoplasmic residual bodies (Figs. 5, 6). The studied species was identified as *Adelina melolonthae.*

Discussion

According to Malone and Dhana [14], twelve species belonging to the genus *Adelina* infect insects. Later, one more species, *Adelina grylli,* was identified from *Gryllus bimaculatus* by Butaeva [15]. Additionally, some more adeleid species have been re-recorded and studied [9, 16, 17]. A recent list of insect-infecting *Adelina* species and their morphological and ultrastructural characteristics is presented in Table 1.

In all coccidian parasites infecting insects the diagnostic stage is the oocyst. The shape and size of the oocysts, the number of sporocysts within an oocyst and the number of sporozoites in a sporocyst are the diagnostic features according to Undeen and Vavra [18]. These features have been used as taxonomic characters for describing species [19]. Five genera of coccidia, *Adelina, Chagasella, Legerella, Ithania* and *Barrouxia,* are

Table 1 *Adelina* species that infect insects and their morphological and ultrastructural features (modified and improved from [14])

Adelina species	Tissues infected	Num. sporocyst per oocyst	Oocyst diam. (µm)	Sporocyst diam. (µm)	Sporozoite size (µm)	Ultrastructural features		Reference
						Oocyst wall (nm)	Sporocyst wall (nm)	
A. akidium	Fat body	12–20	30–40	10	ND	ND	ND	[35]
A. collembolae	Fat body	24	40	7.5–8	ND	ND	ND	[36]
A. eryptocerci	Various	5–21	24–51	10–12	ND	ND	ND	[39]
A. mesnili	Fat body	6–8	–	15	ND	ND	ND	[40]
A. riouxi	–	8–18	30–40	7–10	ND	ND	ND	[38]
A. sericesthis	Fat body/ Connective	4–8	30–40	10.8–11.9	8.7–9.7	ND	ND	[41]
A. simplex	Gut	8–16	25–40	–	ND	ND	ND	[42]
A. tenebrionis	Fat body	2–12	20–35	10–12	ND	ND	ND	[43]
A. tenebrionis	Fat body	3–13	29.2–45	12.3–14	ND	ND	ND	[14]
A. tipulae	Gut	4–10	35–40	-	ND	ND	ND	[42]
A. transita	Various	6–20	30–40	10–11	ND	ND	ND	[37]
A. zonula	Fat body	8	–	–	ND	ND	ND	[44]
A. grylli		4–22	–	–	ND	ND	ND	[15]
A. triboli	Fat body	4–16	40	10–13	11.3–12.5 × 1.7–2.7	ND	140–200	[21]
A. melolonthae	Fat body	6–14	30–35	11	ND	ND	ND	[8]
A. melolonthae	Fat body	4–12	35.62 ± 4.04 (23.97–44.56)	11.70 ± 0.42 (11.02–12.52)	9–11 × 3–4	Outer: 400–500 Inner: 10–25	70–90	In this study

ND No Data

entirely entomoxenous. Our *Adelina* isolate contains up to twelve sporocysts in an oocyst and always two sporozoites in a sporocyst. *Adelina* species may contain different numbers (3–30) of sporocysts within an oocyst but they always have two sporozoites within a sporocyst and thus differ in the sporocyst/oocyst and sporozoite/sporocyst ratios from the genera *Chagasella* (3 sporocysts / 2 sporozoites), *Legerella* (0 sporocyst / 15–40 sporozoites), *Ithania* (1–4 sporocysts / 9–33 sporozoites) and *Barrouxia* (many sporocysts / 1 sporozoite) [18].

So far, all descriptions on *A. melolonthae* have been based on the light microscopic morphology of its oocyst [7, 8, 10]. As shown in Table 1, the coccidian pathogen described here shows close similarities with *A. melolonthae* recorded from *M. melolontha* by Tuzet *et al.* [8]. This concerns the shape of oocysts, sporocysts and sporozoites, the number of sporocysts within an oocyst, and the number of sporozoites in a sporocyst. However, there are some minor variations in oocyst and sporocyst size. The oocysts (24–44.6 µm, mean 35.6 µm) recorded in this study are longer than the originally described oocysts (30–35 µm) from *M. melolontha* by Tuzet *et al.* [8]. Similar slight differences are present in sporocysts. We measured a diameter of 11–12.5 µm (mean 11.7 µm), while Tuzet's value is 11 µm.

It is often difficult to compare descriptions of *Adelina* species because early characterizations were based mainly on light microscopic oocyst morphology. Ultrastructural details are not available for the majority of the *Adelina* species (Table 1). These restricted methods resulted in an unnecessary creation of new species of *Adelina* [20]. Also Tuzet's [8] description of *A. melolonthae* was completely based on light microscopic morphology. The results in this study confirm his identification and justify the classification as a separate species *A. melolonthae*, which differs from other *Adelina* species.

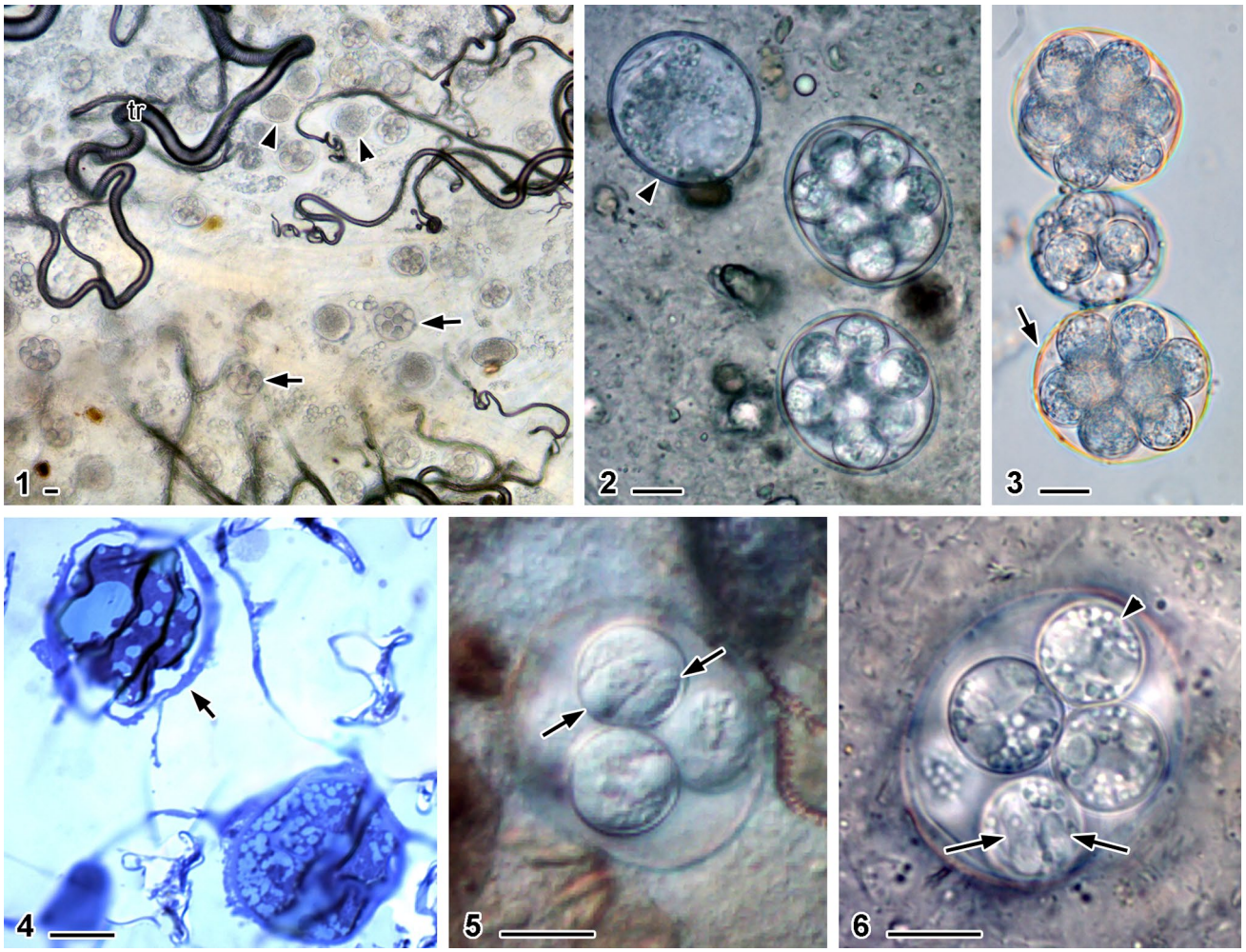


Fig. 1–6 Light microscopy of *Adelina melolonthae* oocysts. Scale bars: 10 µm**. 1** Infection site (fat body with tracheae) filled with oocysts of *A. melolonthae*. Unsporulated (arrowheads) and sporulated oocysts (arrows). **2–3** Sporulated oocysts with double membranes (arrow), including up to twelve sporocysts, and no residual bodies. **4** Giemsa-stained oocysts (arrow indicates the thick wall). **5–6** Two ellipsoidal sporozoites (arrows) per oocyst with residual bodies (arrowheads)

Ultrastructure

In recent identification keys for *Adelina* species, ultrastructural characteristics (especially of the oocysts) are always included [14, 21, 22]. Until now, fourteen *Adelina* species infecting insect have been recorded, but in only few of them the oocyst ultrastructure was studied by electron microscopy. Table 1 lists the morphological and ultrastructural characteristics of these *Adelina* species, and shows that *A. melolonthae* clearly differs from other *Adelina* species infecting insects. Our ultrastructural study of *A. melolonthae* reveals a single-layer sporocyst wall. Different numbers of wall layers of coccidian sporocysts have been reported in the literature [21]. Nyberg and Knapp [23] reported a single-layered wall for *Eimeria tenella*. Zizka [8] described a three-layered sporocyst wall for *Adelina tribolii,* Speer *et al.* [24] a two-layered wall for *Isospora canis*, Marchiondo *et al.* [25] and Duszynski *et al.* [26] a three-to-four-layered wall for *Eimeria nieshulzi* and *E. funduli,* respectively. Christie *et al.* [27] even reported a five-layered wall for *Toxoplasma gondii* [see 21]. The thickness of coccidian sporocyst walls may also vary. The sporocyst wall of *A. melolonthae* in this study measured only 70–80 nm. However, Źižka [21] reported 140 to 200 nm thickness of the oocyst wall for *A.*

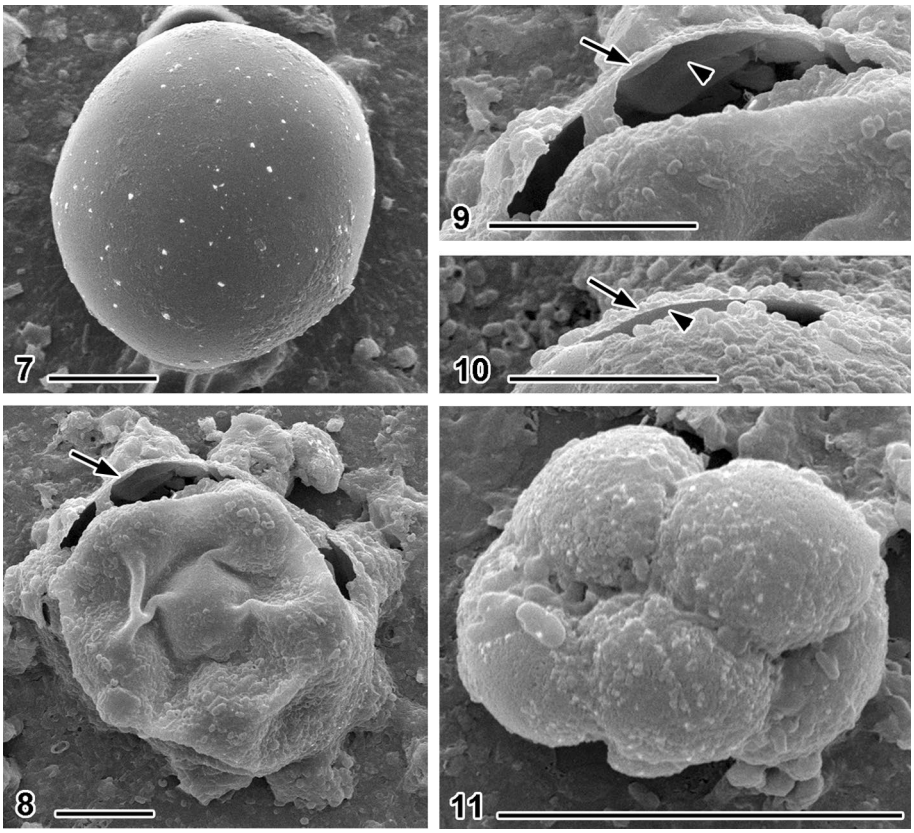
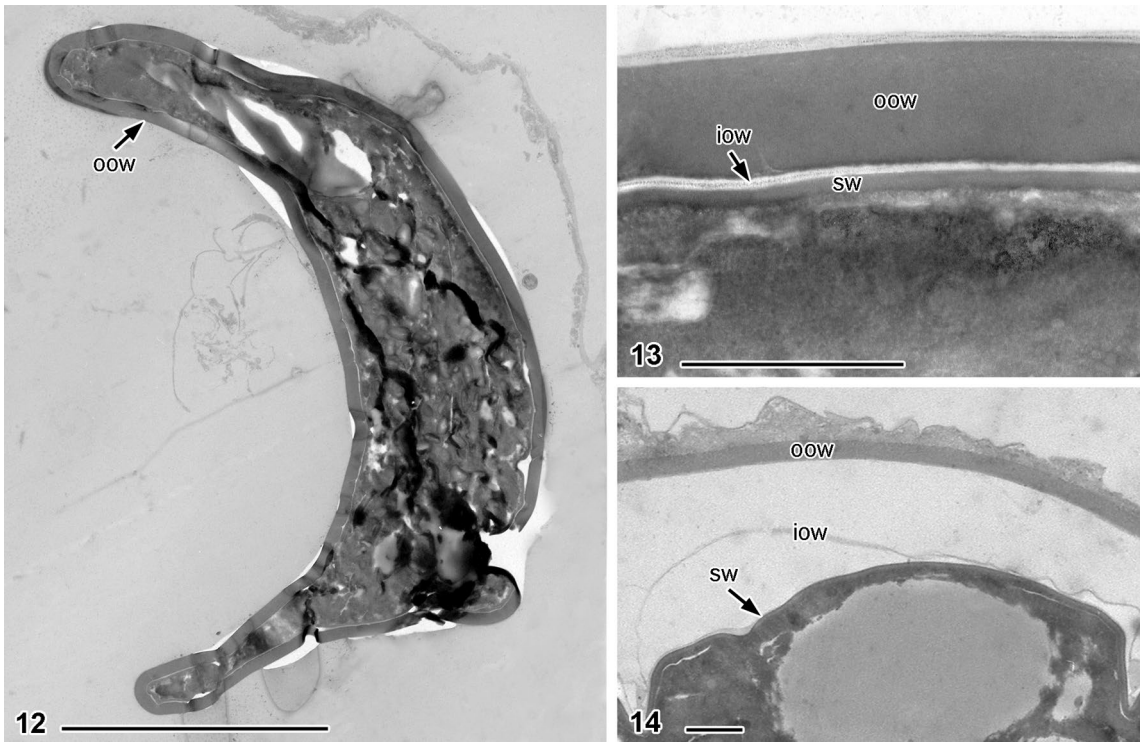


Fig. 7–11 Scanning electron microscopy of *Adelina melolonthae* oocysts. Scale bars: 10 µm. **7** Whole oocyst; the outer layer of the oocyst wall is generally smooth. **8** Content of an oocyst leaving from the broken outer layer (arrow). Bacteria and cell debris lie on the surface of the inner layer. **9–10** Details of broken oocysts show the thickness of the outer wall layer (arrows) and its smooth inner surface (arrowheads). **11** A group of sporocysts released from a broken oocyst. Bacteria and cell debris cover the surface of the sporocysts

tribolii and mentioned thicknesses of 70 nm or 110 nm in other coccidian species. Therefore, the description of the oocysts ultrastructure of *A. melolonthae* provided by this study will be useful for the identification and comparison of other *Adelina* species infecting insects.

Biological Impact

Several insects play beneficial roles in research, medical and agricultural fields [28, 29]. However, many insect species cause damage to plants in agriculture [30, 31]. Due to the harmful effects of chemical insecticides on the environment [32], different alternative control methods and entomopathogenic organisms are being researched for ecological pest control candidates [6, 33]. Infections caused by coccidians can be summarized in two main groups, namely desirable and undesirable infections. Coccidian parasites mainly infect humans and animals where they are generally undesirable since the infections may have a negative impact on health. However, some coccidian species occur as pathogens in various invertebrates, especially in insects. Infections of pest insects leading to diseases and death are desirable. The use of such pathogens in biological control would be safe for vertebrates and humans since they only infect insect hosts. Pathogens are a natural suppressing factor to increase mortality of insect hosts and may lead to greater sensitivity toward insecticides [18, 34]. Therefore, a correct identification of entomopathogenic pathogens is necessary as a basis for studies on their biological control potential. We have demonstrated that electron microscopy of the dispersive stages, i.e., the oocysts can deliver important information for a unequivocal identification and taxonomy of coccidian species.



Figs. 12–14 Transmission electron microscopy of *Adelina melolonthae* oocysts. Scale bars: 10 µm (**12**) and 1 µm (**13** and **14**). **12** Cross section of oocyst. **13** High magnification of oocyst wall. Note the translucent layer covering the thick outer oocyst wall (oow) and a very thin inner oocyst wall (iow) sw = sporocyst wall. **14** Deformed oocyst wall. Note that outer and inner walls were separated; sporocyst includes large, homogeneous residual body

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Declarations

Conflicts of interest The authors declare that they have no conflict of interest. We certify that this manuscript is original and has not been published and will not be submitted elsewhere for publication while being considered by Acta Parasitologica.

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