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Ultrastructural aspects of hepatic coccidiosis caused by *Goussia lusca* n. sp. (Apicomplexa: Coccidia) infecting *Trisopterus luscus* (Gadidae) from the NE Atlantic Ocean

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ABSTRACT: *Goussia lusca* n. sp. is described from the liver of pouting *Trisopterus luscus* from the NE Atlantic Ocean in Ibero-Atlantic Portuguese and Spanish waters. Mature oocysts were 31.7 (28.8 to 35.4) µm in diameter. Each oocyst contained 4 ellipsoidal sporocysts arranged in an aleatory position, and measuring ~13.7 × 9.2 µm. Each sporocyst contained 2 sporozoites. Ultrastructurally, the sporocyst wall consisted of a dense inner layer 115 nm thick, transversely striated, regularly intercalated by thin grooves with electron-lucent spaces, and separated from the outer layer by a thin, light (electron-lucent) space. The outer layer was multilamellated and consisted of parallel dense bands alternating with light spaces. These lamellae formed filamentous extensions of the wall. The dehiscence suture, a characteristic feature of the genus, was present in the sporocysts. No external clinical signs were observed in the host fish. Parasites observed in the liver tissue were often enveloped in a yellowish-brown matrix, generally known as 'yellow bodies'. Sometimes sporocysts were observed in direct contact with the liver cells. Parasites in degeneration and aggregations of amylopectin granules were frequently observed surrounded by host inflammatory cells. In severe infections, we observed large agglomerations of oocysts encapsulated by layers of concentrically arranged connective tissue forming large granulomas, which caused significant replacement of the host liver parenchyma by the parasite.

KEY WORDS: Coccidia · *Goussia lusca* · *Trisopterus luscus* · Liver · Ultrastructure · Ibero-Atlantic waters

INTRODUCTION

Species of the genus *Goussia* are coccidian parasites that have been found to cause significant damage in the digestive tract of teleost fish (Dyková & Lom 1981, Costa & Mackenzie 1994, Azevedo 2001, Gestal & Azevedo 2005). They are pathogens of considerable importance in both wild fisheries and fish farms (Anderson & Gordon 1982, Kent & Hedrick 1985). Severe infections affect fish condition and may cause mortalities. Intestinal *Goussia* species seem to be more numerous, and such infections have to date been studied in greater detail than those inhabiting extra-intestinal sites. Thus, the taxonomic description and pathology produced by extra-intestinal fish coccidia is incomplete, and the information available on this group of parasites in North Atlantic fish is particularly scarce (Costa & Mackenzie 1994, Gestal & Azevedo 2005).

Extra-intestinal coccidians infecting more than one host species of fish are common. *Goussia clupearum* has been found parasitizing the liver of at least 2 clupeid species (*Sardina pilchardus*, *Clupea harengus*), 2 scombroid species (*Scomber scombrus* and *S. japonicus*), 3 gadid species (*Micromesistius poutassou*, *Trisopterus esmarkii* and *T. minutus*), 1 carangid species (*Trachurus trachurus*) and 1 species of belonid (*Belone belone*). A further 5 *Goussia* species have also been described parasitizing marine fish at

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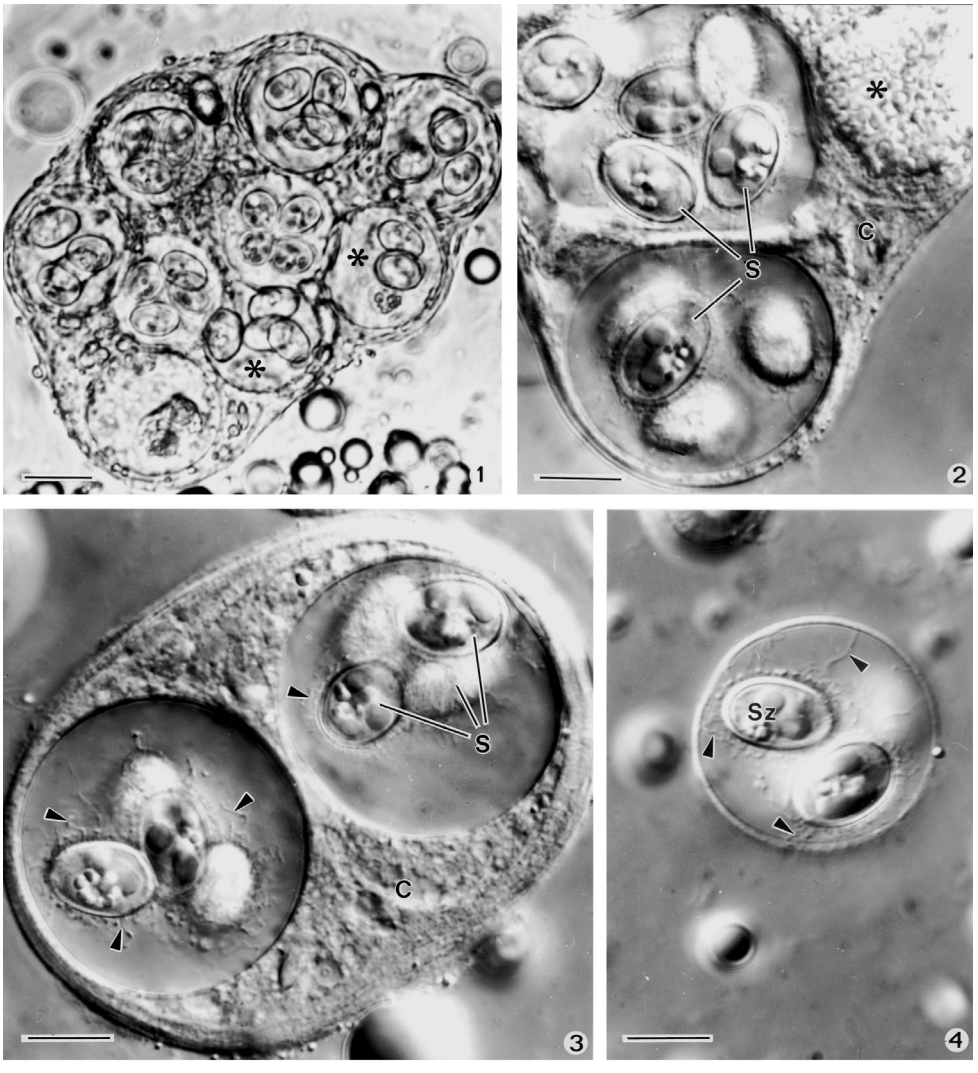
extra-intestinal locations, namely *Goussia cruciata* in the liver of *T. trachurus*, *Goussia gadi* in the swimbladder of 2 gadid species (*Gadus morhua* and *Melanogrammus aeglefinus*), *Goussia caseosa* in the swimbladder of the gadid *Macrourus berglax*, and *Goussia spraguei* and *Goussia auxidis* in the kidney of *G. morhua*, *M. aeglefinus*, *Thunnus albacares* and *Scomber australasicus*.

Until recently, there was increasing evidence that piscine coccidians do not have the narrow host specificity characteristic of mammalian and avian coccidians (Dyková & Lom 1983). However, Molnar et al (2005) have proved that *Goussia* spp. of fish have relatively strict host specificity. Host–parasite interactions, environmental conditions and geographical separation may combine to influence host specificity and host response (Fournie & Overstreet 1993).

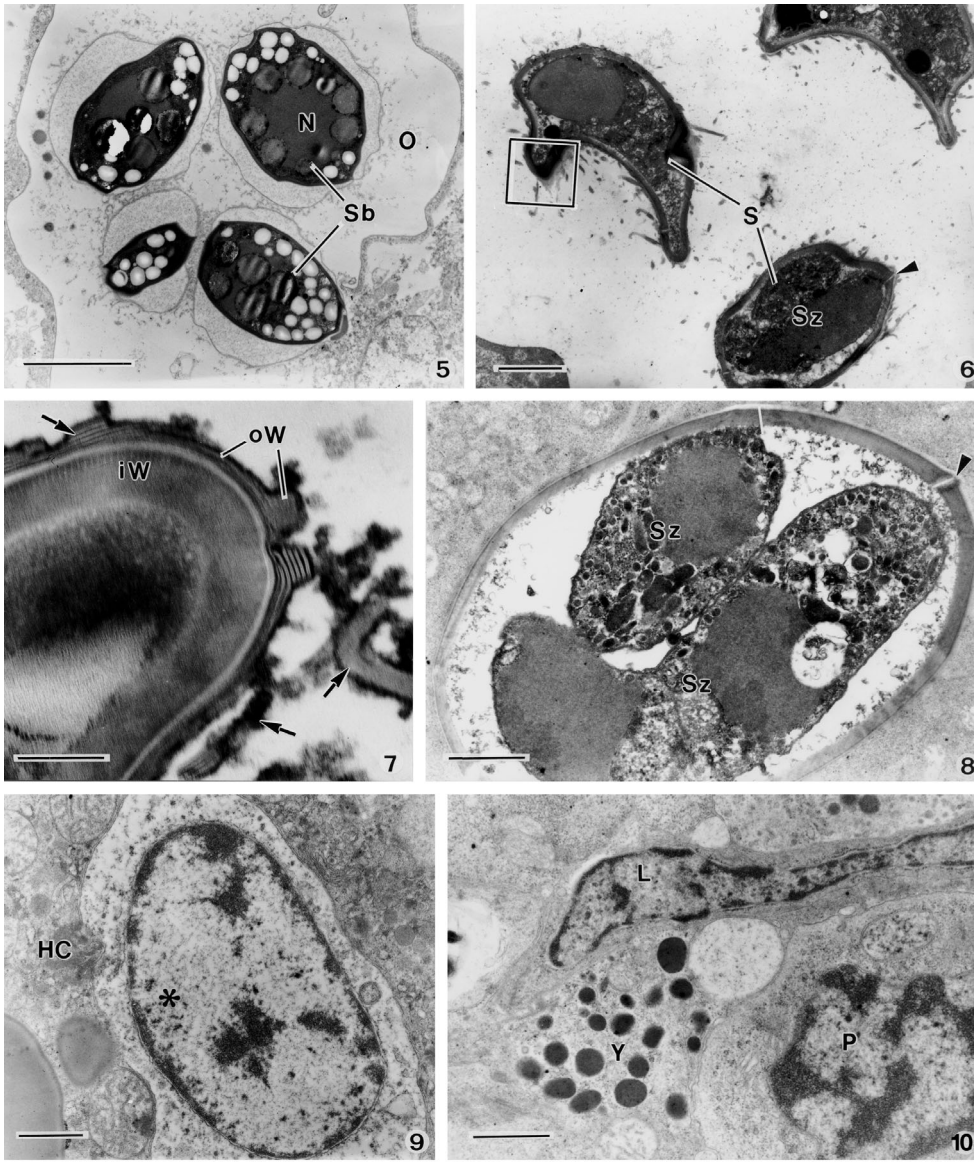
The present paper describes some light microscopy and ultrastructural aspects of morphology and pathology induced by a new extra-intestinal *Goussia* species, *Goussia luscus* n. sp., the agent that causes hepatic coccidiosis in one of the most commercially exploited fish populations in temperate waters of NE Atlantic, the pouting *Trisopterus luscus*.

MATERIALS AND METHODS

Specimens of the pouting *Trisopterus luscus* Linnaeus, 1758 (Teleostei: Gadidae) were sampled from commercial vessels off Galicia (NW Spain) and the Portuguese North Atlantic coast. Small fragments of parasitized fresh liver were examined by light microscopy. Sporulated oocysts were observed and photographed using Nomarski differential interference-contrast (DIC) optics. Measurements were made either directly on living oocysts using a calibrated micrometer or from photographs obtained from living oocysts.



Figs. 1 to 4. *Goussia lusca* n. sp. infecting *Trisopterus luscus*. Light micrographs (DIC) of sporogonial stages of *G. lusca* in the liver of *T. luscus* showing oocysts with 4 sporocysts. Fig. 1. Large agglomerations of sporulated oocysts (*) encapsulated by layers of concentrically arranged connective tissue forming large granulomas; scale bar = 20 µm. Figs. 2 & 3. Aggregation of oocysts in the liver tissue enveloped in a thin fibrotic capsule, showing the rest of the necrotic host cell, yellowish-brown matrix or 'yellow body' (C), and remains of an aborted oocyst inside the 'yellow body' (*). Each oocyst contains 4 sporocysts (S). The sporocyst wall shows the filamentous extensions (Fig. 3, arrowheads); scale bar = 10 µm. Fig. 4. Isolated oocyst showing the sporocysts inside, and large filamentous extensions in the sporocyst wall (arrowheads). Each oocyst contains 2 sporozoites (Sz); scale bar = 10 µm

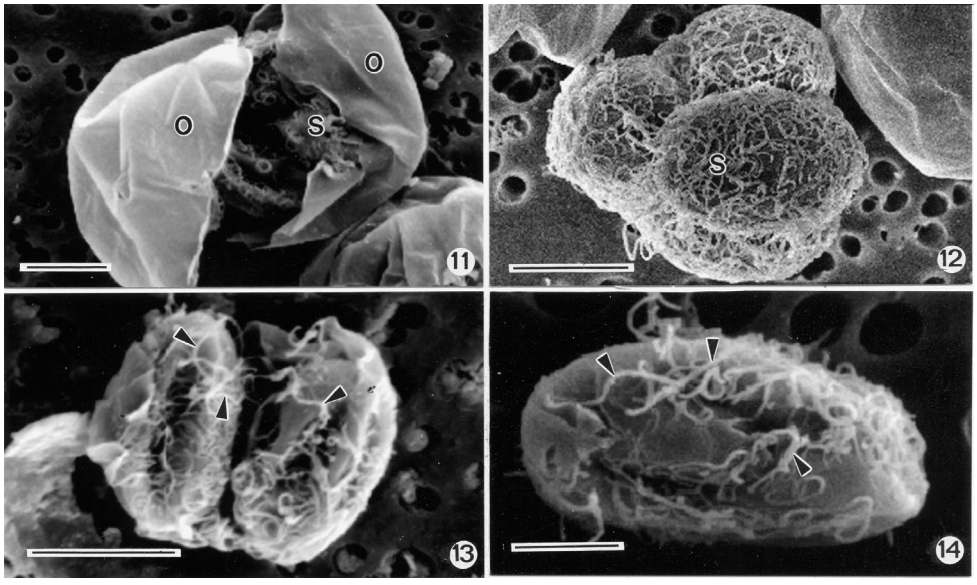


Figs. 5 to 10. *Goussia lusca* n. sp. infecting *Trisopterus luscus*. Ultrathin section of different aspects of sporogonic stages of *G. lusca* infecting the liver of *T. luscus*. Fig. 5. Oocyst (O) containing 4 aleatory disposed sporoblasts (Sb) in the developmental phase and showing a prominent nucleus (N); scale bar = 5 µm. Fig. 6. Mature sporocysts (S) containing 2 sporozoites (Sz) showing the symmetrically opposite disposition of the dehiscence suture (arrowhead). The boxed area showing a completely formed sporocyst wall is enlarged in Fig. 7; scale bar = 2 µm. Fig. 7. Detail of the sporocyst wall (Fig. 6), showing regular transverse striation of the inner wall (iW), and the multilamellated outer wall (oW) with external projections (arrows); scale bar = 0.2 µm. Fig. 8. Isolated sporocyst in direct contact with the liver tissue of *T. rachurus* showing the dehiscence suture of the sporocyst wall (arrowhead), and 2 sporozoites inside (Sz); scale bar = 1 µm. Fig. 9. Degenerate gamogonic stage (*) in the host cell (HC); scale bar = 1 µm. Fig. 10. Detail of the yellowish-brown matrix (Y) surrounding degenerated young stage of the parasite (P) with a lymphocyte (L) adjacent; scale bar = 1 µm

Isolation and purification of the parasite. Parasitized liver tissue extracts containing *Goussia* oocysts were homogenized, and tissue homogenates were filtered through 2 layer nylon gauze sieves of 100, 60 and 40 µm pore diameter (Millipore) to remove large pieces of host tissue. Purification of the parasite was performed following protocols based on Percoll gradients (Gestal et al. 1999). Samples of isolated and purified sporocysts were collected and fixed in glutaraldehyde for later scanning electron microscopy studies.

Transmission and scanning electron microscopy. For transmission electron microscopy (TEM) analysis, small fragments of the infected tissues was fixed in 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4) for 4 h at 4°C, washed for 24 h at 4°C in the same buffer and post-fixed in buffered 2% OsO_4 for 2 h at the same temperature. After dehydration in a graded ethanol series and propylene oxide, the infected tissue was embedded in Epon. Semithin sections were stained with methylene blue. Ultrathin sections were double stained with uranyl acetate and lead citrate, and observed using a JEOL 100CXII TEM operated at 60 kV.

For SEM study, isolated and purified oocyst suspension was fixed for 4 h in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.3) at 4°C and washed for 30 min in the same buffer. Samples were then dehydrated in an ethanol series, critical point dried in CO_2 using a Polaron E3000 and sputter-coated in a Polaron SC 500 using 60% gold-palladium. They were examined with a JEOL JSM 6700F scanning electron microscope operated at 5 kV.



Figs. 11 to 14. *Goussia lusca* n. sp. infecting *Trisopterus luscus*. Scanning electron microscopy photographs of sporogonic stages of *Goussia lusca*. Fig. 11. Oocyst (O) containing sporocysts (S); scale bar = 5 µm. Fig. 12. Sporocysts (S) joined in aleatory position and covered by a multi-lamellated outer wall showing external projections; scale bar= 2 µm. Fig. 13. Arrangement of sporocysts inside the oocysts; scale bar = 5 µm. Fig. 14. Detail of the sporocyst wall showing the outer multilamellated filamentous extensions (arrowheads); scale bar = 2 µm

RESULTS

Description of *Goussia lusca* n. sp.

(Figs. 1 to 15, Table 1)

Using light microscopy, we were able to observe different phases of the coccidian life cycle disseminated in the liver tissues of the pouting *Trisopterus luscus*. Using interference contrast optics (Nomarski) we observed numerous unsporulated oocysts and sporocysts in parasitized tissue samples of the host's cell) (Figs. 1 to 4). In some cases, walls of several oocysts abutted each other, suggesting that they had been formed by macrophages into groups and later surrounded by a connective tissue capsule (Figs. 1 to 3).

Mature oocysts were 31.69 (28.8–35.4) µm (n = 25) in diameter. Each oocyst contained 4 ellipsoidal sporocysts arranged in an aleatory position and measuring 13.7 (13.1–14.4) × 9.24 (8.52–9.84) µm. Each sporocyst contained 2 sporozoites (Table 1, Figs. 1 to 4).

Ultrastructural analysis revealed the presence of different developmental phases of the coccidian disseminated in the liver tissue. Sporoblasts or immature sporocysts were observed limited by a single membrane and containing a prominent nucleus and numerous electron-lucent polysaccharide granules (Fig. 5). Mature sporocysts containing 2 sporozoites or infective cells (Fig. 6) revealed a sporocyst wall consisting of a dense inner layer 115 nm thick, transversally striated at 15 nm intervals, regularly intercalated by thin grooves 10 nm in size with 5 nm electron-lucent spaces, and separated from the outer layer by a thin, light space. The outer layer was multilamellated and consisted of 6 nm thick parallel dense bands alternating at 12.5 nm intervals with 6.5 nm thick light spaces. These lamellae formed filamentous extensions of the wall delimited by external dense bands of 7.5 nm thickness (Figs. 7 & 12 to 15). The dehiscence suture, a characteristic feature of the genus, was present in the sporocysts (Figs. 6, 7 & 15).

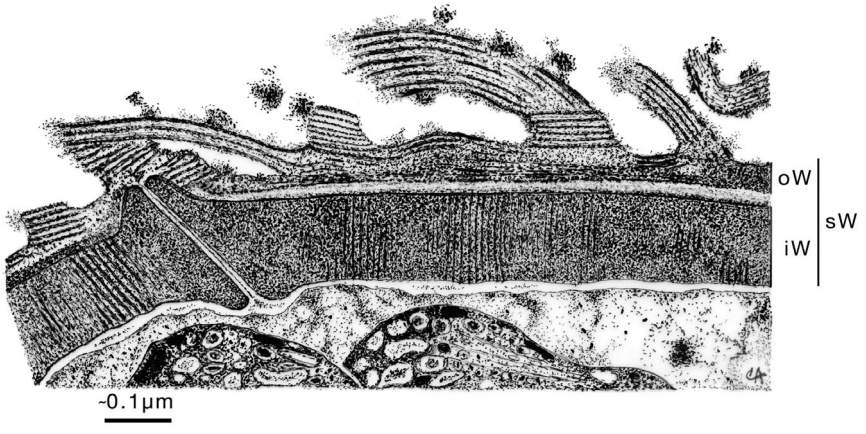


Fig. 15. Schematic drawing of the sporocyst wall (sW) consisting of an inner (iW) and outer wall (oW)

Unsporulated and sporulated oocysts observed in the liver tissue were often enveloped in a yellowish-brown matrix or 'yellow body' composed of amylopectin granules from the parasite and necrotic or aggregated host liver cells and oocysts (Figs. 2 & 10). Sometimes it was also

Table 1. *Goussia* spp. Comparative data on the morphology of the sporogonic stages of *Goussia* species with an extra-intestinal site in marine fish. Measurements of oocysts and sporocysts are in mm, inner and outer sporocyst wall in nm. s.p.: striation period (nm); pb: parallel bands (nm); ls: light spaces (nm)

Goussia spp.	Host	Organ parasitized	Locality	Oocysts	Sporocysts			Sporocyst wall		Source
					Length	Width	Arrangement	Inner wall s.p.	Outer wall pb/ls	
G. cruciata	*Trachurus trachurus*, *T. mediterraneus*, *T. picturatus*	Liver, pyloric caeca	West coast of France, Aegean Sea	–	7.8	5.7	Cross	–	–	Lom & Dyková (1992)
	Trachurus trachurus	Liver	NE Atlantic Ocean	18.9 (17–20)	8.5 (8–9)	6.1 (6–6.5)	Cross	150–180 s.p. 14.4	4/6.25	Gestal & Azevedo (2005)
G. gadi	*Gadus morhua*, *Melanogrammus aeglefinus*	Swimbladder	North and Baltic Seas, N Atlantic Ocean	33	15.8 (13.8–17.9)	10.9 (8.3–13.8)	Aleatory	Total spore wall 480	Smooth	Morrison et al. (1993)
G. caseosa	*Macrourus berglax*	Swimbladder	Newfoundland	42	19.2	13.6	Aleatory	–	–	Lom & Dyková (1982)
G. spraguei	*Gadus morhua*, *Melanogrammus aeglefinus*	Kidney	NW Atlantic Ocean	16.6	12	7.6	Aleatory	–	–	Morrison & Poynton (1989)
G. auxidis	*Thunnus alalunga*, *T. albacares*, *Scomber australicus*	Kidney	SW Pacific Ocean	20 (18–28)	8–12	6–7	Aleatory	160–180 s.p. 14	100–120	Jones (1990)
G. clupearum	*Clupea harengus*	Liver	NW Atlantic Ocean	20	–	–	Aleatory	200	40	Morrison & Hawkins (1984)
	Clupea harengus, *Scomber scombrus*, *Trisopterus esmarkii*, *T. minutus*	Liver	NE Atlantic Ocean, Scottish waters	20.5–29.5	–	–	Aleatory	–	–	Costa & Mackenzie (1994)
	Alosa alosa, *Clupea harengus*, *Sardina pilchardus*, *Sprattus sprattus*, *Scomber scombrus*, *Micromesistius poutassou*, *Trachurus trachurus*	Liver	North Atlantic and, Pacific Oceans, Mediterranean and North Sea	18–25	8–12	4–10	Aleatory	–	–	Lom & Dyková (1992)
	Scombridae, Carangidae, Sparidae, Clupeidae	Liver	SE Atlantic Ocean	17.5 (14–21)	9–11	5–9	Aleatory	–	–	Diouf & Toguebaye (1993)
	Micromesistius poutassou, *Trachurus trachurus*,	Liver	NE Atlantic Ocean, Galician waters	22.5	10.5	7	Aleatory	–	–	Abollo et al. (2001)
	Belone belone	Liver	NE Atlantic Ocean, Portuguese waters	21.2	10.5	6.3	Aleatory	25	8/6	Azevedo (2001)
G. lusca	*Trisopterus luscus*	Liver	NE Atlantic Ocean, Iberian Peninsula	31.7 (28.8–35.4)	13.7 (13.1–14.4)	9.2 (8.5–9.8)	Aleatory	115 s.p. 15	6.0/6.5	Present study

possible to observe sporocysts outside the oocysts in direct contact with the liver cells (Fig. 8). Parasites undergoing degeneration and aggregations of amylopectin granules were frequently observed to be surrounded by host inflammatory cells (Figs. 9 & 10).

No external clinical signs were seen in any of the fish. However, in severe infections, we observed large agglomeration of oocysts encapsulated by layers of concentrically arranged connective tissue forming large granulomes, replacing the host liver parenchyma (Fig. 1).

Taxonomic description

Specimens. Syntypes (ultrathin sections of fish liver containing sporogony stages with mature sporocysts) were deposited in the International Protozoan Type collection, Smithsonian National Museum of Natural History, Washington DC (Accession No.: USNM 1089 187).

Locality. NE Atlantic coasts off the Iberian Peninsula, 41° N, 7° W).

Host. *Trisopterus luscus* (Linnaeus, 1758).

Site of infection. Hepatic parenchyma.

Etymology. The specific epithet refers to the host species *Trisopterus luscus*.

DISCUSSION

Classification of the coccidian parasites (Apicomplexa: Eimeriorina) on the generic and family levels is traditionally based on characteristics such as the life cycle, infected organs, morphological structure and size of the sporogonic stages, number and disposition of sporocysts and sporozoites in the exogenous stages and host specificity (Levine, 1982). The genus *Goussia* was traditionally treated as a synonym of the genus *Eimeria* because oocysts contained 4 sporocysts, each having 2 sporozoites, even though the excystation system differed from that of the *Eimeria* species, since it lacked the Stieda body but showed a bivalved sporocyst structure with a sporocyst wall consisting of 2 jointed plates that are opened for the excystation process. Because of these typical characteristics, the genus *Goussia* Labbé, 1896 was revived by Dyková & Lom (1981), who placed all fish coccidians with these characteristics in this genus and described *G. clupearum* as the type species. Our ultrastructural study showed that the coccidian parasite in the liver of pouting described in the present study presented all these taxonomic characteristics, thus allowing us to identify it as belonging to the genus *Goussia.*

A large number of *Goussia* species have been described to date. However, among those described from marine teleosts, only a few have been reported from an extra-intestinal location (Table 1), and even fewer described using ultrastructural techniques. All of the latter differ in a number of morphological features from the species described in the present paper. The development of sporoblasts, sporocysts and sporozoites of the our *G. luscus* are similar to those of *G. clupearum* described in marine fish, and even to those described in other *Trisopterus* species, such as *T. minutus* and *T. esmarkii* in Scottish waters (Table 1). However, they differ in oocyst and sporocyst size. The results obtained by TEM also showed that they differ from *G. clupearum* at the striation period of the inner layer and the multilamellated outer layer of the sporocyst wall. In addition to the differences in oocyst and sporocyst size and the striation period of the sporocyst wall, the aleatory arrangement of the sporocysts inside the oocysts of *G. lusca* is also different from the characteristic cross arrangement observed in *G. cruciata.* The oocyst and sporocyst size and the internal arrangement of the sporocysts observed in *G. gadi* could be considered similar to those of *G. lusca*. However, the outer wall of the sporocysts is different, in that it lacks the filamentous extensions that were observed in *G. gadi*. Similarly, this new species differs from *G. auxidis*, *G. spraguei* and *G. caseosa* with respect to the measurements at the various sporogonic stages, and to the host and organ it infects.

The presence of isolated sporocysts in direct contact with host cells has previously been described by Azevedo (2001) in *Goussia clupearum* parasitizing *Belone belone* and also by Gestal & Azevedo (2005) in *G. cruciata* infecting the horse mackerel *Trachurus trachurus*. It is not known whether the life cycle of *G. clupearum* depends on an intermediate host (Morrison & Hawkins 1984). In fish, direct transmission without an intermediate host has been demonstrated only in piscine coccidians and also recently in intestinal coccidian such as *G. carpelli* (Lom & Dyková, 1992). However, direct transmission by auto-reinfection could occurs in extra-intestinal *Goussia* species, which justify the presence of free sporocysts in the host tissue, which was yet suggested by Azevedo (2001).

The hepatic coccidiosis observed in *Trisopterus luscus*, with the formation of large agglomerates of oocysts developing granuloms of connective tissue or fibrotic capsules and producing a partial replacement of the host liver parenchyma by the parasite, clearly supports previous reports that infection by *Goussia* species contributes to a deterioration in host condition due to loss of functional activity of the liver, leading to a reduction in total body weight and thus in the market value of infected fish (MacKenzie 1981, Kent & Hedrick 1985, Jendrysek et al. 1994, Abollo et al. 2001, Gestal & Azevedo 2005). Several authors (Morrison &

Hawkins 1984, Costa & Mackenzie 1994, Gestal & Azevedo 2005) have described the eventual destruction of the functional parenchyma and the development of inflammatory reactions close to degenerate parasites and 'yellow bodies' from fish infected with extra-intestinal *Goussia* species). This underlines the claim that this group of parasites could be considered as a significant pathogen with important effects on infected fish.

Future research should aim towards a better understanding of the life cycle, host–parasite interactions, and induced pathology and molecular phylogenetic studies using analysis of 18S rRNA sequences are needed to clarify many biological features of this interesting group of fish coccidia.

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