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Journal of Natural History

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/tnah20>

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Published online: 21 Feb 2007.

To cite this article: Cristian A. Olivares (2005) Evidence of a parasite protist in *Eurhomalea lenticularis* (Sowerby, 1835) (Mollusca: Bivalvia): A case of intraoocytarian parasitism, *Journal of Natural History*, 39:23, 2073-2082, DOI: [10.1080/00222930500060447](https://doi.org/10.1080/00222930500060447)

To link to this article: <http://dx.doi.org/10.1080/00222930500060447>

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Evidence of a parasite protist in *Eurhomalea lenticularis* (Sowerby, 1835) (Mollusca: Bivalvia): A case of intraoocytarian parasitism

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(Accepted 18 January 2005)

Abstract

In the present paper a microsporidian parasitic protist is described and characterized; it was observed in oocytes of *Eurhomalea lenticularis* Sowerby. The clams were obtained in Algarrobo, Chile (33°20'S, 71°40'W). The observations were made on 5 µm sections of female clam gonads, processed by routine histological techniques with ARTETA trichromatic stain. According to the parasite's characteristics, it is established that the microsporidian protist is related to the genus *Steinhausia* (Chytridiopsidae). Microsporidian prevalence reached 20.6% ($n=34$) in summer months, but there was no evidences of cell damage in the oocyte of the host; in a total of 66 female clams, a 10.6% of infection was detected between December 1995 and August 1996. The infection intensity showed that 77.4% of the infested acini had single parasitized oocytes, and 88.0% of total oocytes parasitized contained only one cyst in the cytoplasm.

Keywords: *Eurhomalea lenticularis*, *intraoocytarian meroparasitism*, *microsporidian protist*, *parasitic protist*, *Steinhausia sp.*

Introduction

Bivalve molluscs, both in culture and in natural populations, have been severely affected by a wide range of microbial diseases. The causes of many mollusc diseases have diverse origins and in numerous cases their etiology remains undetermined. Among the diseases that attack bivalves, those produced by protists are most important (Sindermann and Rosenfield 1967; Figueras and Villalba 1988; Sindermann and Lightner 1988). In this respect, research on mollusc pathology began to be relevant during the 1950s, as a consequence of an epizooty by parasite protists that affected the American oyster, *Crassostrea virginica* Gmelin, on the North American east coast (Sindermann and Rosenfield 1967). As a result, several studies characterizing some protist pathogens have been produced. An apicomplexan protist disease, *Perkinsus marinus* Levine, has been reported in certain oyster grow-out areas in the middle Atlantic of the USA, the Gulf of México, Puerto Rico, Cuba, and Brazil (Sindermann and Lightner 1988; Ewart and Ford

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Published 12 May 2005

ISSN 0022-2933 print/ISSN 1464-5262 online © 2005 Taylor & Francis Group Ltd

DOI: 10.1080/00222930500060447

1993; Bower 2001). *P. marinus* parasitizes many bivalve molluscs, including clams and oysters (Lauckner 1983; Sinderman and Lightner 1988) and most researchers have concluded that oysters infected die primarily from extensive tissue lysis and blockage of major blood vessels (Ewart and Ford 1993).

Two ascetosporean protist diseases, *Haplosporidium nelsoni* Couch et al. and *Haplosporidium costale* Wood and Andrews, are both found in *Crassostrea virginica* along the Atlantic coast of the USA (Jones 2000). The earliest infections of *H. nelsoni* are observed in the oyster's gill, leading to the conclusion that the infective stage is water-borne. The high numbers of parasites damage tissues and interfere with normal functions such as respiration and feeding (Ewart and Ford 1993). *Bonamia* sp., a serious pathogen of oysters, has been reported in haemocytes of *Tiostrea chilensis* Philippi (Hine and Wesney 1992, 1994a, 1994b) and its role in metabolism of blood cells has been discussed.

Microsporidian etiological agents that are known to parasitize marine bivalves include *Steinhausia ovicola* (= *Chytridiopsis ovicola*, Léger and Hollande) which attacks the ova of *Ostrea edulis* L. from Marennes, France (Léger and Hollande 1917; Lauckner 1983; Figueras and Villaba 1988), but the host appeared to be affected only slightly. Some cases of multiple infestation were accompanied by a diminution of cytoplasmic yolk and lysis of the nucleus. *Steinhausia mytilovum* (= *Chytridiopsis mytilovum*, Field) parasitizes the ova of *Mytilus edulis* L. from the Atlantic and West Coasts of the USA (Field 1923; Sprague 1965, 1970; Hillman 1991). This protist has also been found in oocytes of *Mytilus galloprovincialis* Lam. from Ría de Vigo, Spain (Robledo et al. 1994) and the Gulf of Naples, Italy (Vincentis and Renzoni 1963). This parasite occurs in the cytoplasm and also in the nucleus of the oocyte host. Field (1923) and Sprague (1965) restudied *S. mytilovum* and recognized amoeboid organisms in the follicles and genital canals of female mussels, and uninucleate cysts in the cytoplasm of infested ova as the earliest developmental stage of the microsporean. According to Field (1923), thousands of eggs in a mussel may be parasitized and destroyed by *S. mytilovum*. However, Sprague (1965) established that the proportion of infested eggs was quite small in *M. edulis* collected near Ocean City, Maryland, USA. Sprague (1964) described *Nosema dollfusi*, which is a hyperparasite of *Bucephalus cuculus* McCrady, in *Crassostrea virginica* L. and established the importance of considering whether *N. dollfusi* is an actual or potential oyster pathogen, since there are pathological conditions associated with the escape of the *Nosema* spores into the oyster tissues.

Becker and Pauley (1968) described a *Protista incertae sedis* in the ovary of *Crassostrea gigas* L. from California. The authors conclude that the organism differed in many aspects from parasites of bivalve molluscs, but presumably belonged among the Sporozoa. There is little evidence about its capacity for killing adult oysters.

In Chile, parasitological research on molluscs is sparse and there are no specific studies on pathogenic protists.

The objective of this paper is to describe and to characterize a microsporidian parasitic protist, observed in oocytes of *Eurhomalea lenticularis* Sowerby from Algarrobo, Chile. Further studies are required to determine whether the parasitic state involves the pathogen's whole life (holoparasitism) or only part of it (meroparasitism).

Material and methods

Clams were collected by scuba diving from a subtidal sand bottom to a depth of 8 m, in Algarrobo, Chile (33°20'S, 71°40'W), between 26 June 1995 and 29 August 1996. From a

sample of 336 adult clams, *Eurhomalea lenticularis* (males=132; females=204), 66 females were analysed and seven infected specimens were obtained.

Sections of tissue approximately 5 mm thick were cross cut from the medial portion of the gonad, and fixed in ALFAC (ethanol, formalin and acetic acid) (López et al. 1982). The sections were dehydrated and embedded in Paraplast, sectioned at 5 µm and stained using the Trichromic ARTETA Method, as described by López et al. (1982). Observations and the photomicrographs of the distinct developmental stages of the protistan parasite were made with a Leitz Orthoplan microscope with Orthomat photographic equipment. Measurements were obtained with a 1.0 µm precision ocular micrometer.

Quantitative data on the number of individual acini and infected oocytes were used to calculate prevalence (%) and intensity of infection (%). Results of infection intensity were compared using an analysis of variance test (one-way ANOVA with the *F* value significant at the 99% level of confidence) and *t* test (Sokal and Rohlf 1979).

The parasite protistan was described and identified according to Manwell (1968), Kudo (1969), Lee et al. (1985), Larsson (1986) and Corliss (1994).

Results

Description

The parasite protistan affected only a few females of *Eurhomalea lenticularis*. In the same way, few gonadal acini contained the cysts of the parasite. The specific site of infection is the cytoplasm of pedunculated and free vitellogenic oocytes (PVO, FVO) of female clams. No cysts were observed in the germinal vesicles (GV), in ovarian tissue, or in other regions of the host.

Cysts (sporocysts, sporophorous vesicles) (Figures 1–5). Located in the cytoplasm of PVO and FVO. Generally ovoid in cross-section, clearly delimited from cytoplasm of the egg, but the cyst membrane indistinct as a separate structure. About 15.5 µm (standard deviation, SD 5.0) in diameter. The cysts, which were closely associated with the host-oocyte GV, have an apparent elastic membrane. The cyst may cause invagination of the host-GV membrane. In a few cases, there was a tendency to lysis of the GV. Oocyte infections consist of a single or various cysts containing sporulate and vegetative stages. The interior of the cysts appear with light microscopy as hyaline areas with developmental stages located centrally.

Vegetative stages (Figures 1, 2). Less frequently, multinucleate sporogonial plasmodia, of similar size to the sporocyst, have been observed. They appear as deeply stained cytoplasmic masses, slightly granulate, with light areas without stain and with irregular smooth contours that seem to be amoeboidal prolongations of cytoplasm. Numerous basophile, small nuclei are distributed in the plasmodial cytoplasm. In a single cyst, these nuclei are cylindrical, ovoidal and spherical. Eritrosinophilic and spherical nuclei, grouped principally at the border of the plasmodial mass, are also observed. In general, only one cyst in the vegetative stage is observed in each oocyte (Table I; Figure 1).

Sporulate stage (Figures 3–5). The more frequent parasitic stage appears as a cyst that encloses 21.8 (SD 12.2) ovoidal or spherical spores (in cross-sections) within light areas without stain. This structure is near to the GV. Free spores have not been observed in the oocyte cytoplasm or caryoplasm. Generally, one cyst in the sporulate stage is present in

Table I. Infection intensity (%) in oocytes of *Eurhomalea lenticularis*.

Clam no.	No. of infested oocytes	No. of cysts per oocyte			
		1	2	3	4
E209	4	50.0	50.0	0	0
E212	32	96.9	3.1	0	0
E213	10	90.0	0	10.0	0
E217	14	100.0	0	0	0
E219	4	100.0	0	0	0
E220	12	90.9	9.1	0	0
E230	26	88.5	3.9	3.9	3.9
Mean		88.0	9.4	2.0	0.6
SD		18.0	18.5	3.8	1.5

Table II. Prevalence of intraoocytarian infection of *Eurhomalea lenticularis*.

Month	No. of examined individuals	No. of infected individuals	Frequency (%)
December 1995	10	4	40.0
January 1996	21	3	14.3
February 1996	3	0	0
July 1996	16	0	0
August 1996	17	0	0
Total	66	7	

each oocytarian cell (Table I; Figure 3), but it is possible to find up to four cysts in each oocyte, all in different sporulate developmental stages. The spores are grouped in lax masses. The size of the spores is 2.9 µm (SD 0.6). Each spore exhibits a peripheral nucleus as a gross chromatinic cluster, without a clear halo; at the opposite pole, the cytoplasm has a non-staining vacuolar space. The rest of the sporoplasm is homogeneous and eritrosinophilic. Other spores showed two parietal nuclei or traces of a second nucleus. The spores have similar dimensions, but one spore of 4.5 µm was observed, 1.7 times larger than normal spores.

Quantitative aspects

Prevalence (Table II). The data show clearly that the prevalence of intraoocytarian infection in the clam *Eurhomalea lenticularis* occurred only during summer months, reaching 20.6% in samples for December 1995, January and February 1996 (*n*=34). Considering the total sample (December,1995 to August 1996; *n*=66) the prevalence diminished to 10.6%.

Figures 1–5. (1) Acini with infested oocytes of *Eurhomalea lenticularis*. Vegetative stage of microsporidian protist. Arrows, cysts with multinucleate sporogonial plasmodia; Ac, gonadal acinus; n, oocyte nucleus. Scale bar: 50 µm. (2) Vegetative stage of microsporidian protist in oocytes of *E. lenticularis*. C1, cyst with plasmodium in early development; C2, cyst with plasmodium in later development; Oo, oocyte. Scale bar: 20 µm. (3) Acinus with infested oocytes of *E. lenticularis*. Sporulate stage of microsporidian protist. Arrows, cysts with spores; Ac, gonadal acinus; n, oocyte nucleus. Scale bar: 50 µm. (4) Sporulate stage of microsporidian protist in oocytes of *E. lenticularis*. n, oocyte nucleus; cs, cyst with spores of microsporidia; s, mature spores. Scale bar: 20 µm. (5) Spores of microsporidian protist in oocyte of *E. lenticularis*. s, immature spores; n, oocyte nucleus. Scale bar: 20 µm.

Infection intensity (Tables I, III). There was a significant difference between the amount of infested oocytes in each gonadal acinus of *E. lenticularis* (ANOVA, $F=14.8$; $P<0.01$). Overall, 77.4% of the acini with infected oocytes exhibited only one oocyte with protistan cysts. This value was highly significant compared to the acini with two to six infested oocytes ($t=9.5$; $P<0.01$). No significant differences were computed for acini with two to six infested oocytes (ANOVA, $F=2.3$; $P>0.01$) which did not exceed 22.5% of the total sample of infested acini. One acinus (0.8%) with six parasitized oocytes was an exceptional case of multiple infestation.

There were statistically significant differences in the amount of cysts per oocyte (ANOVA, $F=29.1$; $P<0.01$); 88.0% of the parasitized oocytes had a single cyst. Oocytes containing two, three and four cysts showed no differences between them (ANOVA, $F=1.86$; $P>0.01$) and represented 12% of the total sample of infested oocytes. Oocytes with four cysts were scarce (0.6%).

Discussion

Taxonomic position

The parasitic protist cannot be identified at the species level with the limited material available. Notwithstanding, the taxonomic characters observed place it in the phylum Microspora Sprague, 1977. The characteristics which relate it with the microsporidians are: intracellular parasitism; merogony, sporogony and sexual reproduction in the host cell (Miller 1997; Larsson 2004); spores small and simple, ovoid, spherical or cylindrical and refringent, with two light areas in the opposed poles (Lauckner 1983; Lee et al. 1985; Larsson 2004). A few members of the Microspora are known to parasitize marine bivalves. These intracellular protozoans are small (3–6 µm), completing their entire life cycle in a single host cell. Intermediate hosts are not known. Microsporean spores enclose an amoeboid sporoplasm (“amoebula”). Inside the host cell, the parasite undergoes merogony and sporogony (Lauckner 1983; Larsson 2004).

The oocyte as an infection site is uncommon among the observed parasites of bivalves (Becker and Pauley 1968). According to Villalba et al. (1993), a *Steinhausia*-like microsporidian was observed in oocytes of the clam *Venerupis pullastra* Montagu, from Galicia, Spain. A *Steinhausia*-like infection was also observed in histological sections of the gonads of female Sidney rock oyster *Saccostrea commercialis* Iredale and Roughley, obtained from a farm in Queensland, Australia (Anderson et al. 1995). Two species of *Steinhausia*

Table III. Infection intensity (%) in gonadal acini of *Eurhomalea lenticularis*.

Clam no.	No. of infested acini	No. of oocytes infested per acinus				
		1	2	3	4	6
E209	4	100.0	0	0	0	0
E212	19	63.2	21.1	10.5	0	5.3
E213	10	100.0	0	0	0	0
E217	8	37.5	50.0	12.5	0	0
E219	4	100.0	0	0	0	0
E220	11	90.9	9.1	0	0	0
E230	14	50.0	28.6	7.1	14.3	0
Mean		77.4	15.3	4.3	2.0	0.8
SD		26.6	18.9	5.6	5.4	2.0

with microsporidian affinities (Sprague 1965, 1967) have been recorded from the ova of marine shellfish: Léger and Hollande (1917) described a cnidosporidian, *Chytridiopsis ovicola* Léger and Hollande, infesting the oocytes of *Ostrea edulis* L., taken at Marennes, France. The protist was rare and occurred only in certain ovarian follicles of parasitized oysters. *Chytridiopsis mytilovum* Field was observed parasitizing oocytes in the mussel *Mytilus edulis*, from the US Atlantic coast (Field 1923; Sprague 1965), and has also been found in *M. galloprovincialis* from Italy (Vincentis and Renzoni 1963) and Spain (Robledo et al. 1994). Two species of *Steinhausia* were originally believed to belong to the Haplosporidia and have been described as *Chytridiopsis ovicola* Léger and Hollande, and *Haplosporidium mytilovum* Field, respectively. Dollfus (1921) considered *C. ovicola* as a coccidian but gave no indication that its systematic position might be in doubt (Lauckner 1983). Sprague (1963) felt that *H. mytilovum* had been erroneously classified. Manier and Ormières (1968) studied the ultrastructure of the type species of the genus *Chytridium* (*C. socius*), concluding that it is actually a microsporan. Later, Sprague et al. (1972) created the new microsporan family Chytridiopsidae, proposed the genus *Steinhausia*, and included the two protist species found in *Ostrea edulis* and *Mytilus edulis* (Lauckner 1983).

In this work, the microsporidian shows clear relations with the genus *Steinhausia* and in particular with *S. ovicola*. The main similarities between these species and the microsporidian detected in the clam *Eurhomalea lenticularis* were: occurrence within developed oocytes of the clams; the most frequently observed stage consisted of an intracytoplasmic, spherical or ovoidal cyst (sporocyst), 18–25 µm in dimension; cyst associated with host-GV. A single cyst per oocyte, but rare cases of multiple infestation of up to four cysts. Spores of 2–3 µm in size; 7–40 spherical or cylindrical spores per cyst; spore with one peripheral nucleus and opposite pole with vacuolar cytoplasm; mature spores of similar dimensions; immature spores, pale and with two nuclei. Less frequently, vegetative stages or multinucleate sporogonial plasmodia have been found, with the same cytological characters: cytoplasm granular, wavy borders and with some “pseudopodia”, later phases with alveolar cytoplasm, small and grouped nuclei; developed plasmodial stage without peripheral prolongations. The principal difference is: the spores do not have a nucleus with a light halo.

Steinhausia mytilovum differs from the microsporidian of *E. lenticularis* in having spherical cysts sometimes inside the nucleus and spores of 4.0 µm diameter. Furthermore, uninucleate amoeboid stages and uninucleate cysts have never been observed among the follicles, in the genital ducts or in the oocyte cytoplasm of female clams.

Therefore, the microsporidian of *E. lenticularis* is placed tentatively in the genus *Steinhausia*. This is believed to be the first record of the occurrence of the haplosporidian *Steinhausia* in Chilean molluscs.

Prevalence

A low prevalence (10.6%) of *Steinhausia* sp. infection was observed in histological sections of the gonads of female *Eurhomalea lenticularis*. These values agree with those recorded by Hillman (1991), who reported a prevalence of 5–10% for mussels infested with *Steinhausia mytilovum* in California, USA. On the other hand, Anderson et al. (1995) obtained a prevalence of 70% in *Saccostrea commercialis* females with *Steinhausia*-like infection in Queensland, Australia. Prevalence of *Steinhausia* sp. infection in *E. lenticularis* was markedly increased during the summer months. Apparently, ecological factors such as temperature determine the distribution of *Steinhausia* in the samples examined. The data

suggest that this agent proliferates readily in temperate months, while during winter months infested molluscs are not observed. This is perhaps due to decreased winter water temperatures. This could suggest that the microsporidian is dormant for almost half the year in the locality of Algarrobo, and is in accord with observations in Chesapeake Bay and the Gulf of Mexico for the apicomplexan protist *Perkinsus marinus* (Lauckner 1983). Therefore, prolonged low temperature may be a significant limiting factor for this species.

Infection intensity

The infection intensity was similar for acini and oocytes. The results show that it is common to observe a single infested oocyte for acini and a single cyst for an oocyte, while the cases of multiple infection are rare. The cause, in oocytes, could be that a high number of parasites might establish strong interspecific competition for space and food when they share an intraoocytarian microhabitat. Notwithstanding, it is necessary to increase the number of samples through a year in order to estimate accurately the full seasonal variation in the intensity of infection. Perhaps the results could be similar to the prevalence determined for *P. marinus* in the northern hemisphere (Quick and Mackin 1971; Ragone-Calvo and Bureson 1994).

In the literature it is emphasized that *S. ovicola* completes its total life cycle in a single host cell (holoparasitism). In this respect, *S. ovicola* and *S. mytilovum* affect only female hosts and a transovarial transmission has been suggested for both species (Lauckner 1983). As to an intraoocytic parasite of fish, merogonial stages occur in young oocytes and sporophorous vesicles with spores in older oocytes (Pekkarinen 1995). Since the effects of a single cyst on the viability of the ova is only slight, infested eggs probably develop normally and carry the microspore over to the offspring (Léger and Hollande 1917; Lauckner 1983; Comtet et al. 2003). In this research, it was also observed that the parasite does not affect the oocytarian cell, except in rare cases where nucleolysis is evident. Infection could be seasonal, corresponding to the gonadal cycle of the clam, but the reservoir of parasites during the non-proliferative gonadal phase is unknown (Bower 2001). An important observation was the diminution of spores inside the cyst as development progresses. Apparently, mature spores could continue their life cycle in other microhabitats (e.g. other tissues of the same host or another host). Notwithstanding, the possibility of a differential development of the more viable spores is not rejected. More studies are needed to test the working hypothesis of meroparasitism.

Acknowledgements

I wish to thank Donald Brown and Rodolfo Rivero for the infected clam specimens and for collaboration in the histological treatment of the samples. Funding was provided by Project DIUV 20/95, Universidad de Valparaíso, Chile.

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