

Contribution to DAO Special 7 'Microcell parasites of molluscs'



# Ultrastructural comparison of *Bonamia* spp. (Haplosporidia) infecting ostreid oysters

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**ABSTRACT:** The ultrastructure of *Bonamia* from *Ostrea angasi* from Australia, *Crassostrea ariakensis* from the USA, *O. puelchana* from Argentina and *O. edulis* from Spain was compared with described *Bonamia* spp. All appear conspecific with *B. exitiosa*. The *Bonamia* sp. from Chile had similarities to the type *B. exitiosa* from New Zealand (NZ), but less so than the other forms recognized as *B. exitiosa*. Two groups of ultrastructural features were identified; those associated with metabolism (mitochondrial profiles, lipid droplets and endoplasmic reticulum), and those associated with haplosporogenesis (Golgi, indentations in the nuclear surface, the putative trans-Golgi network, perinuclear granular material and haplosporosome-like bodies). Metabolic features were regarded as having little taxonomic value, and as the process of haplosporogenesis is not understood, only haplosporosome shape and size may be of taxonomic value. However, the uni-nucleate stages of spore-forming haplosporidians are poorly known and may be confused with *Bonamia* spp. uni-nucleate stages. The many forms of NZ *B. exitiosa* have not been observed in other hosts, which may indicate that it has a plastic life cycle. Although there are similarities between NZ *B. exitiosa* and Chilean *Bonamia* in the development of a larger uni-nucleate stage and the occurrence of cylindrical confronting cisternae, the clarification of the identity of Chilean *Bonamia* must await molecular studies.

**KEY WORDS:** Review · *Bonamia* spp. · Haplosporidians · Ultrastructure · Taxonomy

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## INTRODUCTION

*Bonamia* spp., of the Phylum Haplosporidia, are obligate intrahaemocytic protistan parasites, primarily of ostreid oysters (Carnegie & Cochenneec-Laureau 2004, Engelsma et al. 2014, this DAO Special). Four species have been described. *B. ostreae* infects *Ostrea edulis* (Pichot et al. 1979), and possibly *O. angasi* (see Bougrier et al. 1986), *O. chilensis* (see Grizel et al. 1983)

and *O. puelchana* (see Pascual et al. 1991). It may also infect *Crassostrea* spp. (Carnegie & Cochenneec-Laureau 2004), and *C. gigas* may act as a reservoir for *B. ostreae* (see Lynch et al. 2010). It is reported from *O. edulis* in Atlantic and Mediterranean Europe, Morocco (A. Villalba pers. obs.; [http://web.oie.int/eng/info/hebdo/AIS\\_43.HTM#Sec2](http://web.oie.int/eng/info/hebdo/AIS_43.HTM#Sec2)), and western (Elston et al. 1986, Friedman et al. 1989, Marty et al. 2006) and eastern (Friedman & Perkins 1994) North America.

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It is an internationally notifiable OIE-listed pathogen. *Bonamia* (syn. *Mikrocystos*) *roughleyi* was described from *Saccostrea glomerata* (syn. *commercialis*) from Australia (Farley et al. 1988, Cochenneec-Laureau et al. 2003), but has not been subsequently isolated and its validity as a *Bonamia* species is doubtful (Hill et al. 2010, Carnegie et al. 2014).

*B. exitiosa* (syn. *exitiosus*) infects *O.* (syn. *Tiostrea*) *chilensis* (Hine et al. 2001, Berthe & Hine 2003) in New Zealand (NZ), *O. angasi* (see Corbeil et al. 2006) in Australia, *O. puelchana* in Argentina (Kroeck & Montes 2005, Kroeck et al. 2008, Kroeck 2010), *O. edulis* off the Atlantic (Abollo et al. 2008) and the Mediterranean (Narcisi et al. 2010) coasts of Europe and *O. stentina* in Tunisia (Hill et al. 2010), North and South Carolina (USA), NZ and Argentina (Hill et al. 2010). It also occurs in *C. ariakensis* at a port in North Carolina, into which it could conceivably have been introduced from Australia by shipping (Burreson et al. 2004, Bishop et al. 2006). Like *B. ostreae*, it is an OIE-listed and internationally notifiable pathogen. *B. perspora* infects *O.* (syn. *Ostreola*) *stentina* (syn. *equestris*) in the southeastern USA and differs from all other reported *Bonamia* spp. in having a spore stage (Carnegie et al. 2006). *B. exitiosa*-like isolates have been reported from *O. chilensis* in Chile (Balsero et al. 2006, Campalans & Lohrmann 2009, Lohrmann et al. 2009).

*Bonamia* spp. have different patterns of development, and of developmental stages. *B. perspora* appears to have very few uni-nucleate, bi-nucleate, diplokaryotic and small multi-nucleate plasmodial vegetative stages. Plasmodia develop into sporonts, sporoblasts, sporocysts and spores, as do *Haplosporidium* spp. and *Minchinia* spp. (Carnegie et al. 2006). NZ *B. exitiosa* has many uni-nucleate, fewer bi-nucleate and diplokaryotic stages and no sporulation stages, and many uni-nucleate cells grow into large forms (Hine 1991a, Hine et al. 2001). Chilean *Bonamia* sp. is similar to *B. exitiosa* in developing large uni-nucleate stages during winter, but bi-nucleate stages are rare and multi-nucleate plasmodia unreported (Lohrmann et al. 2009). *B. ostreae* has dense and light forms of the uni-nucleate stage, which may become bi-nucleate or rarely diplokaryotic (Pichot et al. 1979), but plasmodia are small and very rarely encountered. Large plasmodia reported from *O. edulis* (Brehélin et al. 1982, Vivarès et al. 1982, Bonami et al. 1985) at one site in Brittany were probably vegetative stages of *H. armoricanum*. Therefore, initial uni-nucleate stages may be directly compared, but the development of large uni-nucleate forms in *B. exitiosa* and the Chilean *Bonamia* may not be directly compa-

rable with the uni-nucleate forms of *B. perspora* and *B. ostreae*. There are also 2 forms of bi-nucleate cell: those with separated nuclei after karyokinesis and before cytokinesis, common in *B. ostreae* (see Balouet et al. 1983); and those with 2 apposed nuclei in a diplokaryon, which are common in *B. perspora* (Carnegie et al. 2006) and *B. exitiosa* (Hine 1991a, Hine et al. 2001), but rare in *B. ostreae* (Pichot et al. 1979).

For brevity, the ultrastructures of published *Bonamia* spp. will not be illustrated here as they are already well illustrated (Pichot et al. 1979, Hine et al. 2001, Carnegie et al. 2006, Lohrmann et al. 2009). Also, previous studies nicely illustrate important features in this study, such as nuclear membrane-bound Golgi (NM-BG), indentations in the nuclear surface (INS), perinuclear granular material, a reticulated structure which is the trans-Golgi network (TGN) and haplosporosome-like bodies (H-LBs) (Hine 1991a, 1992, Hine & Wesney 1994a, Hine et al. 2001). Here we compared *B. exitiosa* from *C. ariakensis* on the US east coast, *O. puelchana* in Argentina and *O. edulis* in Spain, and considered what features permit their discrimination from other *Bonamia* species. Initially in this study, all cellular features were recorded, but subsequent analysis of nuclear shape, size and endoplasmic reticulum failed to show any patterns and are therefore not included herein.

## MATERIALS AND METHODS

The fixation and processing of tissues infected with *Bonamia* spp. for transmission electron microscopy (TEM) has been reported for *B. exitiosa* (Hine et al. 2001), *B. perspora* (Carnegie et al. 2006) and Chilean *Bonamia* sp. (Lohrmann et al. 2009). It should be noted that there was variation in methods of fixation and processing, particularly the use of reduced osmium tetroxide ( $\text{OsO}_4$ ) instead of normal  $\text{OsO}_4$  in the Chilean study, which enhanced membranes, possibly making NM-BG more apparent. The oysters infected with *B. ostreae* in our study were sampled from Lake Grevelingen, The Netherlands, and fixed for 1 h in 2.5% glutaraldehyde in 0.22  $\mu\text{m}$  filtered seawater (FSW), washed 2x in FSW, post-fixed for 1 h in 1%  $\text{OsO}_4$ , stained en bloc with 5% uranyl acetate in 0.1 M sodium acetate for 45 min, dehydrated through 50 to 100% ethanol, cut and stained with 5% uranyl acetate for 10 min and 5% lead citrate for 5 to 6 min.

*Ostrea puelchana* infected with *B. exitiosa* were sampled from San Matias Gulf, northern Patagonia, Argentina. Hearts were fixed for 1 h in 2.5% glutaraldehyde in 0.45  $\mu\text{m}$  FSW and washed 3x in FSW,

post-fixed in 1% OsO<sub>4</sub> buffered with 0.1 M sodium cacodylate at pH 7.2, dehydrated through a graded ethanol series including en bloc staining with 1% uranyl acetate at the 70% ethanol stage, and embedded in Spurr's resin. Ultrathin (~90 nm) sections were mounted on carbon-stabilised formvar-coated 1-hole grids, stained with Reynold's lead citrate, and examined on a Zeiss CEM 902 TEM. *Crassostrea ariakensis* infected with *B. exitiosa* from North Carolina (USA) were processed following methods used earlier for observation of *B. perspora* (Carnegie et al. 2006) and examined on a Zeiss CEM 902 TEM.

The measurements given are from sections through the centre of the cell in which the bilaminar nuclear and plasma membranes are clearly defined. Cell size, nuclear size and the size of haplosporosomes given in the following tables were calculated as the means of the longest dimension multiplied by the dimension at right angles to it. The nucleus:cytoplasmic (N:C) ratio was the mean of nuclear dimensions expressed as a percentage of the mean size of the cell. The term 'multi-nucleate' is used for cells with more than 2 nuclei, rather than 'plasmodium', which is reserved for a pre-sporulation stage.

## RESULTS

All *Bonamia* spp. occurred as uni-nucleate and bi-nucleate forms, and a multi-nucleate stage may occur in all species but was not encountered because of rarity. NZ *B. exitiosa* and *B. perspora* also have a diplokaryotic stage. TEM observations on moribund *B. roughleyi* showed a multi-nucleate stage (Cochennec-Laureau et al. 2003) that appeared to undergo schizogony to form the uni-nucleate stage, unlike *Bonamia* spp. and other known haplosporidians (P. M. Hine pers. obs.), suggesting that it may not be a *Bonamia* species.

Uni-nucleate *B. exitiosa* from *Ostrea angasi* in Australia, *Crassostrea ariakensis* from the eastern USA (Fig. 1B), *O. puelchana* from Argentina, and Spanish *O. edulis* (Fig. 1A) (Table 1) resemble NZ 'type' *B. exitiosa* (Table 2), being larger than *B. ostreae* (Fig. 2), with smaller haplosporosomes, and slightly larger than Chilean *Bonamia*, with larger haplosporosomes. Haplosporogenesis was less often observed in *B. exitiosa* from *C. ariakensis* than in other species (Table 1), except *B. ostreae* (Table 2). The uni-nucleate stage of *B. perspora* was larger but with smaller chromosomes (Table 2) (Fig. 3A) than the other *Bonamia* spp., with subsequent multi-nucleate plasmodia (Fig. 3B) developing into spores (Fig. 4). Few data were available on

Table 1. Comparison of the uni-nucleate stage of *Bonamia* spp. Haplorporogenesis in this and the following tables is calculated on the number of individuals having nuclear membrane-bound Golgi (NM-BG), trans-Golgi network (TGN), indentations in the nuclear surface (INS), or haplosporosome-like bodies (H-LBs). Size is length × width; N/C: nucleus/cytoplasm ratio; Prev.: prevalence; NR: not recorded; C. ariakensis; *Crassostrea ariakensis*. Data are means  $\pm$  SD

Species	Origin	n	Host	Size (μm <sup>2</sup> )	N:C (%)	Haplosporogenesis						Mitochondrial profiles No.	Lipid droplets No. (%)			
						Prev. (%)	NfM-BG (%)	TGN (%)	—INS— (%)	Haplosporosomes No.	Size (μm <sup>2</sup> )	%	H-LBs No.	Range		
<i>B. exitiosa</i>	Australia	45	<i>Ostrea angasi</i>	2.8 ± 0.4	NR	NR	NR	NR	10 ± 4	156 ± 15	NR	4 ± 1	0.5 ± 0.8	30		
<i>B. exitiosa</i>	Argentina	27	<i>O. puelchana</i>	3.1 ± 0.3	49 ± 6	61	43	25	31	Slight	148 ± 12	40	0.53 ± 0.8	0–2	3 ± 2	
<i>B. exitiosa</i>	East USA	45	<i>C. ariakensis</i>	2.8 ± 0.4	50 ± 4	18	9	0	5	Flattened	18 ± 10	152 ± 8	13	0.2 ± 0.5	0–7	3 ± 1
<i>B. exitiosa</i>	Spain	4	<i>O. edulis</i>	2.7 (2.8, 53–57)	50	25	0	25	25	17, 12, 154 ± 20	0	11, 7 (n = 12)	7, 5	0–7	2–7	1, 1, 1, 0
<i>Bonamia</i> sp.	Chile	59	<i>O. chilensis</i>	2.5, 2.6, 2.7 2.1 ± 0.4	58 ± 12	48	41	2	0	13 ± 7	138 ± 19	20	0.37 ± 1.06	0–7	2 ± 1	0–5
													0.3 ± 0.4	26		

Table 2. Comparison of the uni-nucleate stage of the 3 described *Bonamia* spp. NM-BG: nuclear membrane-bound Golgi; TGN: trans-Golgi network; INS: indentations in the nuclear surface; H-LBs: haptosporosome-like bodies; N/C: nucleus:cytoplasm ratio; Prev.: prevalence; NG: not given. Data are means  $\pm$  SD

Species	Origin	n	Host	Reference	Size ( $\mu\text{m}^2$ )	N:C Prev.	Haplosporogenesis			H-LBs			Mitochondrial profiles No.	Lipid droplets No. Prev. (%)					
							NM-BG (%)	TGN (%)	INS (%)	Haplosporosomes No.	Size ( $\mu\text{m}^2$ ) (%)	No.	Range						
<i>B. ostreeae</i>	Netherlands	23	<i>Ostrea edulis</i>	Pichot et al. (1979)	2.1 ± 0.4	55 ± 7	14	27	0	9 ± 4	188 ± 21	26	0.30 ± 0.47	0-1	1 ± 1	0-4	0.4 ± 0.6	30	
<i>B. extitiosa</i>	New Zealand	60	<i>O. chilensis</i>	Hfine et al. (2001)	3.2 ± 0.5	52 ± 6	52	11	32	5	29 ± 17	148 ± 11	21	0.47 ± 0.96	0-3	4 ± 1	2-6	0.5 ± 0.9	32
<i>B. perspora</i>	East USA	NG	<i>O. stentina</i>	Carnegie et al. (2006)	4.1 ± 0.6	NG	NG	NG	NG	3 ± 3	137 ± 19	NG	6 ± 2	NG	NG	NG	NG	38	

Table 3. Comparison of bi-nucleate and diplokaryotic stages of *Bonamia* spp. NM-BG: nuclear membrane-bound Golgi; TGN: trans-Golgi network; INS: indentations in the nuclear surface; H-LBs: haplosporosome-like bodies; N:C: nucleus:cytoplasm ratio; Prev.: prevalence

Species	Origin	n	Stage	Size ( $\mu\text{m}^2$ )	N:C (%)	Haplosporogenesis				Mitochondrial profiles	Lipid droplets No.	Prev. (%)	
						Prev. (%)	NM-BG (%)	TGN (%)	INS (%)	No. of haplo- sporosomes	H-LBs		
<i>B. exitiosa</i>	New Zealand	5	Diplokaryon	3.5–3.9	81–86	100	40	20	20	13–37	0–1	2–6	0–2 40
<i>B. exitiosa</i>	Argentina	5	Bi-nucleate	3.5–3.9	73–76	40	20	0	0	11–46	0–2	4–6	0 0
<i>B. exitiosa</i>	East USA	3	Bi-nucleate	2.6–3.4	60–79	33	0	0	0	Too few	0–1	3–5	0–1 33
<i>Bonamia</i> sp.	Chile	6	Bi-nucleate	2.4–3.7	75–92	33	17	0	0	13–27	0–1	2–4	0–1 67
<i>B. ostreae</i>	Netherlands	5	Bi-nucleate	2.0–2.6	84–94	40	20	0	0	3–13	0–1	1–2	0–1 20

Table 4. Comparison of ultrastructural features of *Bonamia* spp. in relation to size groups. Data are means  $\pm$  SD. Prev.: prevalence

Size group ( $\mu\text{m}$ )	Prev.	Haplosporogenesis No. of haplo- sporosomes	Mitochondrial profiles	Lipid droplets No.	Prev.
<b><i>B. exitiosa</i> from New Zealand</b>					
2.6–3.0	40	30 $\pm$ 21	4.4 $\pm$ 0.9	0.4 $\pm$ 0.9	20
3.1–4.0	56	27 $\pm$ 14	4.0 $\pm$ 1.3	0.7 $\pm$ 0.9	40
4.1–5.0	82	63 $\pm$ 23	8.2 $\pm$ 4.6	1.3 $\pm$ 1.4	73
<b><i>B. exitiosa</i> from Argentina</b>					
2.6–3.0	71	14 $\pm$ 4	4 $\pm$ 1	0.3 $\pm$ 0.7	25
3.1–4.0	50	19 $\pm$ 10	4 $\pm$ 1	0.5 $\pm$ 1.2	25
<b><i>B. exitiosa</i> from <i>Crassostrea ariakensis</i></b>					
2.1–2.5	17	17 $\pm$ 8	1.8 $\pm$ 1.0	0.6 $\pm$ 0.7	39
2.6–3.0	9	18 $\pm$ 10	3.1 $\pm$ 1.2	0.9 $\pm$ 1.0	59
3.1–4.0	0	11 $\pm$ 6	5.2 $\pm$ 1.1	1.4 $\pm$ 1.3	80
<b><i>Bonamia</i> sp. from Chile</b>					
1.6–2.0	55	11 $\pm$ 7	1.8 $\pm$ 1.3	0.2 $\pm$ 0.4	16
2.1–2.5	38	12 $\pm$ 5	1.9 $\pm$ 1.0	0.3 $\pm$ 0.5	33
2.6–3.0	100	20 $\pm$ 4	2.5 $\pm$ 1.7	½ = 0.5	50
<b><i>B. ostreae</i></b>					
1.6–2.0	27	7.7 $\pm$ 3.0	1.3 $\pm$ 1.2	0.3 $\pm$ 0.5	27
2.1–2.5	33	9.8 $\pm$ 4.8	1.5 $\pm$ 1.0	0.2 $\pm$ 0.4	17
2.6–3.0	50	12.8 $\pm$ 4.8	1.5 $\pm$ 1.0	0.8 $\pm$ 1.0	50

diplokaryotic and bi-nucleate forms because of the difficulty in getting central sections through both nuclei (Table 3). The diplokaryon of NZ *B. exitiosa* was similar in size to the bi-nucleate forms in Argentinian oysters, but had a higher N:C ratio, haplosporogenesis and lipid droplets. Haplosporosome numbers were higher in NZ and Argentinian *B. exitiosa* and Chilean *Bonamia* sp. than in *B. ostreae*, but haplosporogenesis was more prevalent in NZ *B. exitiosa* than in the other species, which showed similar levels of haplosporogenesis (Table 3).

With increase in size, each *Bonamia* showed a general increase in haplosporogenesis, number of mitochondrial profiles and lipid, except in Argentinian *B. exitiosa* and *B. exitiosa* from *C. ariakensis* (Table 4). The size groups of *B. exitiosa* in *C. ariakensis*, and more so Chilean *Bonamia* sp. and *B. ostreae*, were smaller than those of Argentinian and NZ *B. exitiosa*. In relation to seasonal differences, *Bonamia* from *O. chilensis* in NZ and Chile sampled at different times of the year showed that while NZ *B. exitiosa* showed a great increase in all parameters, Chilean *Bonamia* showed the same trend, but less so (Table 5).

Table 5. Comparison of *Bonamia* infecting *Ostrea chilensis* in New Zealand and Chile in relation to season. Data are means  $\pm$  SD. Prev.: prevalence

Date	n	Size ( $\mu\text{m}^2$ )	Haplosporo- genesis at Golgi (%)	No. of haplo- sporosomes	Mitochondrial profiles	Lipid droplets No.	Prev. (%)
<b>New Zealand</b>							
Jan 1987	106	3.1 $\pm$ 0.4	39	15 $\pm$ 7	3 $\pm$ 2	0.7 $\pm$ 0.9	44
Apr 1990	61	3.0 $\pm$ 0.4	44	21 $\pm$ 9	4 $\pm$ 2	1.1 $\pm$ 1.5	56
Jun-Jul 1990	109	3.5 $\pm$ 0.7	85	37 $\pm$ 18	7 $\pm$ 3	1.1 $\pm$ 0.5	48
Aug 1990	147	4.3 $\pm$ 0.6	82	58 $\pm$ 24	9 $\pm$ 4	2.1 $\pm$ 2.0	73
<b>Chile</b>							
Mar 2005	11	2.0 $\pm$ 0.3	36	12 $\pm$ 7	2 $\pm$ 1	0.3 $\pm$ 0.5	22
Apr 2005	24	2.2 $\pm$ 0.5	36	13 $\pm$ 5	1 $\pm$ 1	0.3 $\pm$ 0.6	28
May 2005	20	2.2 $\pm$ 0.4	59	11 $\pm$ 6	2 $\pm$ 1	0.2 $\pm$ 0.4	15
Jul 2005	49	2.2 $\pm$ 0.7	43	16 $\pm$ 7	3 $\pm$ 2	0.4 $\pm$ 0.9	30
Aug 2005	10	2.4 $\pm$ 0.6	Poor fixation	Poor fixation	Poor fixation	Poor fixation	Poor fixation

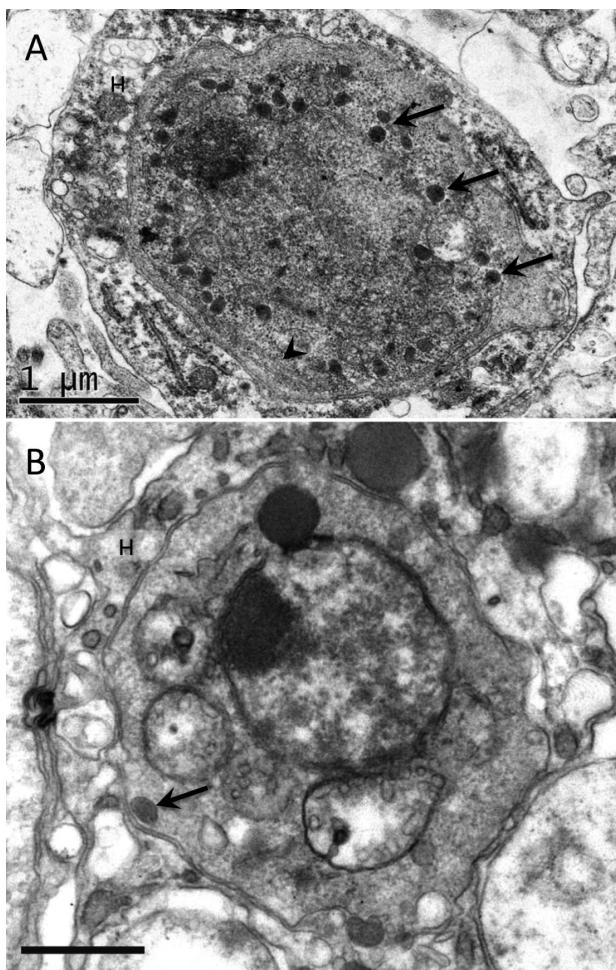


Fig. 1. Uni-nucleate stages of *Bonamia exitiosa* in (A) *Ostrea edulis* from Spain and (B) *Crassostrea ariakensis* from the USA. Host haemocyte cytoplasm indicated in both cases (H) to mark the exterior of the *B. exitiosa* cells. Note the widely varying abundance of haplosporosomes (arrows), and the parallel arrays of smooth endoplasmic reticulum (arrow-head) in (A). Both scale bars = 1  $\mu\text{m}$

## DISCUSSION

The problem with comparative ultrastructural studies on congeneric undescribed species, as in molecular studies showing DNA sequence divergence among such species, is deciding how much difference constitutes a separate species. While molecular studies reveal genotypes and are quantifiable in base transitions, ultrastructural studies reveal phenotypes which may be subject to many variables. These include host factors such as the species concerned, its age, physiological state, reproductive state and the presence of other infections (Hine 2002), environmental factors such as water temperature and salinity (Audemard et al. 2008, Carnegie et al. 2008), and methods of sam-

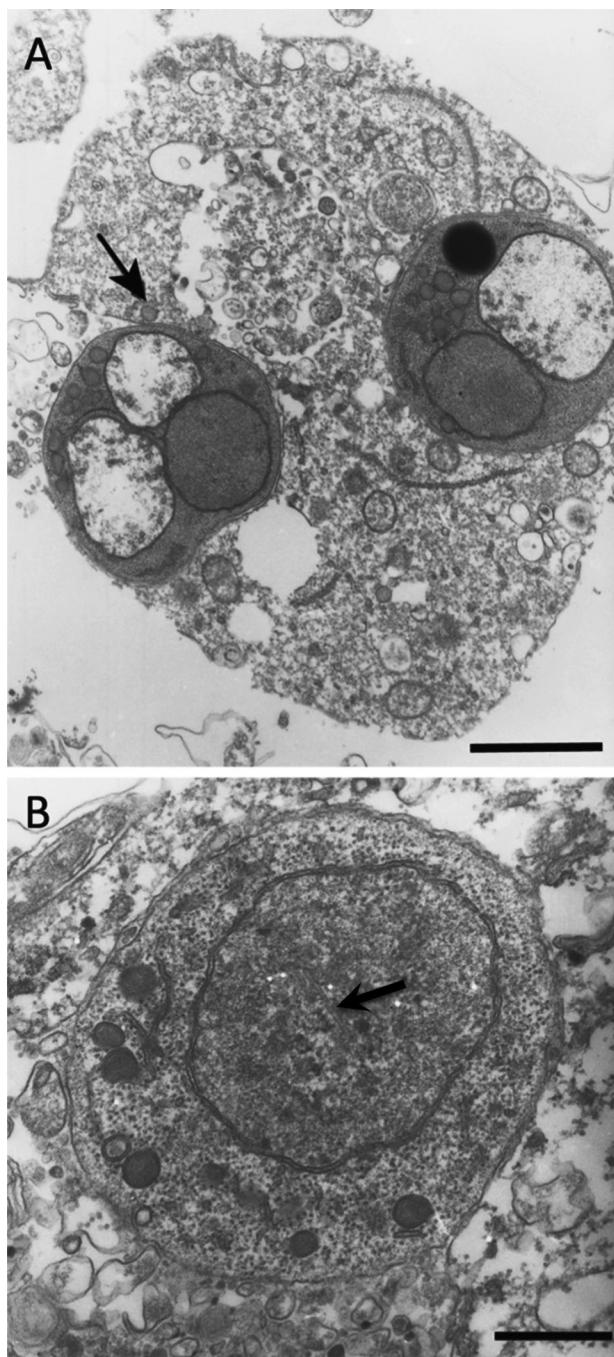


Fig. 2. *Bonamia ostreae* uni-nucleate stages in *Ostrea edulis*. (A) Low-power image of 2 cells infecting a single haemocyte. Note the displacement of *B. ostreae* nucleus to cell periphery, a common observation and distinct trait of this *Bonamia* species. Note also an extracellular haplosporosome (arrow), present along with numerous cytoplasmic haplosporosomes. Scale bar = 1  $\mu\text{m}$ . (B) High-power image of a single *B. ostreae* cell. Note again the abundant haplosporosomes, with smooth endoplasmic reticulum also displayed along with an intranuclear microtubule (arrow). Scale bar = 0.5  $\mu\text{m}$

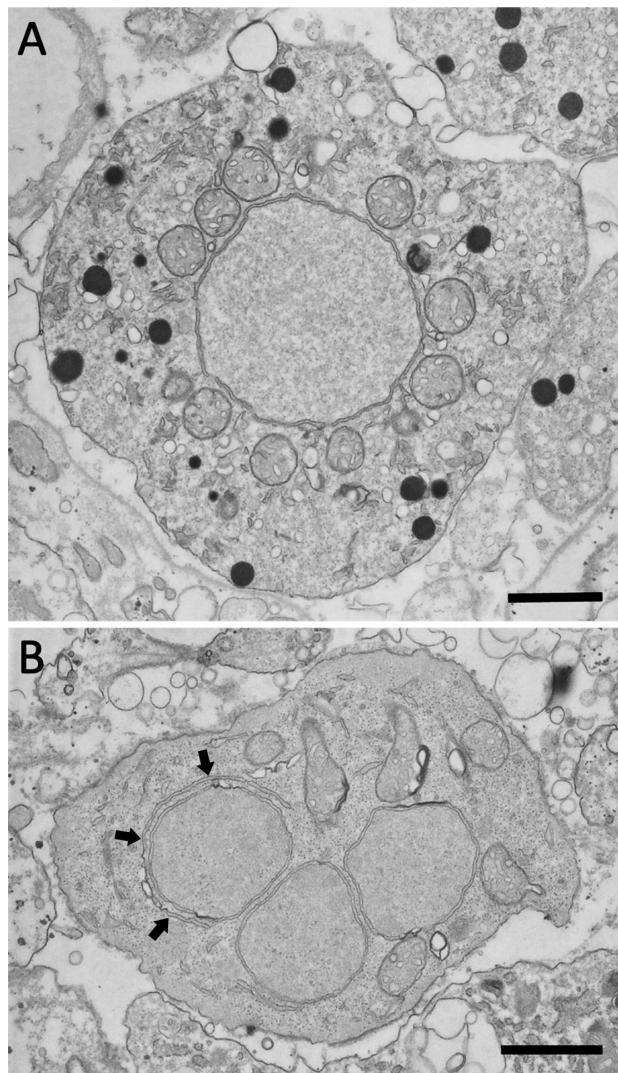


Fig. 3. Vegetative stages of *Bonamia perspora* in *Ostrea stentina* from the USA. (A) Uni-nucleate stage. (B) Small trinucleate plasmodium. Note the abundant mitochondria, particularly closely apposed to the nucleus in (A), the long stretch of endoplasmic reticulum head (arrows) in (B), and the general paucity of haplosporosomes. Dark bodies in (A) are lipid bodies. Both scale bars = 1 µm

pling, fixation and post-fixation. Parasite factors, such as the stages present at the time of sampling, which may be seasonal (Hine 1991a,b), the physiological state of the parasite and possible plasticity of life cycles, have also to be considered. In the latter case, although a spore stage is not known for *Bonamia exitiosa*, it does not necessarily mean that spores may not be formed under certain environmental conditions.

Interpretation of the ultrastructural features of *Bonamia* spp. must take these variables into account. These features fall into 3 groups: parasite metabolism, haplosporogenesis, and sporogony, the latter

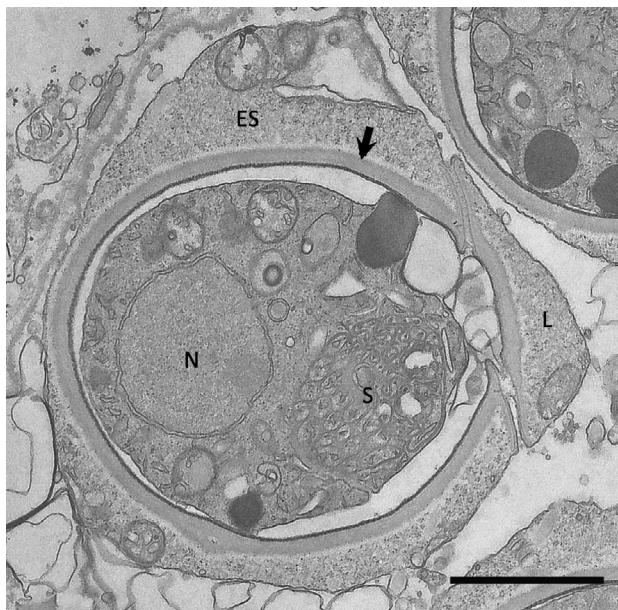


Fig. 4. Prespore of *Bonamia perspora* in *Ostrea stentina* from the USA. Note the developing wall (arrow), with episporic cytoplasm (ES) still present, and the well-defined lid (L), nucleus (N) and spherule (S). Scale bar = 1 µm

only being known in *B. perspora*. The metabolic state of the parasite is indicated by the mitochondria, lipid droplets and endoplasmic reticulum, which are most obvious in the development from a small intracellular uni-nucleate early stage to the large extracellular amoeboid feeding stage with large amounts of lipid, mitochondria and parallel arrays of endoplasmic reticulum in NZ *B. exitiosa* (see Hine & Wesney 1994a). High lipid content was linked to season and development in the lipid-rich ovary in autumn to early winter (April to June; Hine 1991b). Seasonal annual development may also be linked to temperature in *C. ariakensis* infections (Carnegie et al. 2008). The production of lipid vesicles from, and acid hydrolases in, lipid droplets are associated with formation of a parasitophorous vacuole (PV; Hine & Wesney 1994b), and 36% of *Bonamia* sp. in *Crassostrea ariakensis* were in PVs. Therefore, features reflecting metabolism are of little taxonomic value unless multiple variables are taken into account.

The taxonomic significance of haplosporogenesis, as indicated by NM-BG, TGN, INS, perinuclear granular material, and H-LBs (Hine & Wesney 1992), is difficult to assess, as the function of haplosporosomes is unknown. Whilst it has been suggested that their glycoprotein coat and putative DNA core resembles the structure of viruses, their release associated with lysis of surrounding cells suggests a lytic function (Hine et al. 2002), but subsequent re-entry of cores in

a haplosporidian infecting abalone (Hine et al. 2002) could represent horizontal gene transfer (Keeling & Palmer 2008). The size and shape of haplosporosomes may be taxonomically important, but their plasticity in size and shape in relation to fixation is unknown. However, as haplosporosomes are the only structures quantifiable in size and number, they may be taxonomically important. If so, the uni-nucleate stages of NZ, Australian, *C. ariakensis*, Argentinian and Spanish *B. exitiosa*, and Chilean *Bonamia* are distinct from *B. ostreae* and *B. perspora*. These relationships are reflected in molecular phylogenies with NZ, Australian, *C. ariakensis*, Argentinian and European *B. exitiosa* closely related to the Chilean *Bonamia* (Hill et al. 2014, this DAO Special). Therefore, haplosporosome shape and size may be taxonomically important, but not haplosporosome number, as that is related to the size or developmental stage of the parasite. However, studies on *Marteilia* spp. (Paramyxoviridae) (Villalba et al. 1993, Longshaw et al. 2001) suggest that haplosporosome shape and size may not be reliable as a taxonomic feature. Also, asporous *Bonamia* spp. are usually uni-nucleate and may be confused with the uni-nucleate stage of other haplosporidians, but most haplosporidian TEM studies have been on sporulation and the uni-nucleate stage is rarely described. The intra-haemocytic location of asporous *Bonamia* spp. is also unreliable because the vegetative stages of other haplosporidians may be phagocytosed by host haemocytes.

Uni-, bi- and multi-nucleate stages are known from *C. ariakensis* and probably Argentinian and Spanish *B. exitiosa*, and visually they were indistinguishable from each other and Australian *B. exitiosa*. Chilean *Bonamia* could be distinguished by the more osmophilic membranes and NM-BG, but this could have been due to fixation and/or processing. NZ *B. exitiosa* also has a diplokaryotic stage, as does *B. perspora*, and the diplokaryon is a feature of multi-nucleate plasmodia of spore-forming haplosporidians, such as *Urosporidium crescents*, *Haplosporidium louisiana*, *H. nelsoni* and *H. costale* (Hine et al. 2009). Large NZ *B. exitiosa* in autumn and winter also contain cylindrical confronting cisternae (Hine & Wesney 1992), as do Chilean *Bonamia* (Lohrmann et al. 2009), which have been attributed to an underlying viral infection (Hine & Wesney 1992). As the NZ and Chilean *Bonamia* infect the same host (*O. chilensis*), but Chilean *Bonamia* are smaller with fewer, smaller haplosporosomes (Tables 1 & 2), Chilean *Bonamia* may have derived from NZ *B. exitiosa* but have been geographically isolated for sufficient time for the Chilean *Bonamia* to emerge as a distinct species. The *Bona-*

*mia* from Australia (Corbeil et al. 2006), *C. ariakensis* (see Carnegie et al. 2006), Chile (Balseiro et al. 2006) and Spain (Abollo et al. 2008) have all been reported as *B. exitiosa*, but ultrastructurally, the Chilean *Bonamia* is similar to, but different from, *B. exitiosa*. Ultrastructure supports the identification of Australian, *C. ariakensis*, Argentinian and Spanish forms as *B. exitiosa*, as in molecular phylogenies (Hill et al. 2010, 2014). They appear more similar to each other in the cycling of uni-nucleate and bi-nucleate stages, than to the multiple forms of NZ *B. exitiosa*, but this may be due to the smaller sample sizes taken on only 1 occasion. There is a possibility, however, that the type species, NZ *B. exitiosa*, is atypical of the species, or that it has an underlying viral infection indicated by its ultrastructure (Hine & Wesney 1992).

When studying phenotypic traits of an intracellular protozoan parasite, it is necessary to consider that the ultrastructure observed may be mediated by the host, as is histopathology, and possibly life cycles. Organisms are parsimonious and do not evolve to produce forms or stages that are of no benefit or waste energy reserves. *Bonamia* spp. may simply normally cycle through uni-nucleate and bi-nucleate stages, as seen in *B. ostreae* and the *C. ariakensis*, Argentinian and Spanish *B. exitiosa*. NZ *B. exitiosa* targets abundant host ovarian lipids during the female *O. chilensis* spawning cycle, to form a large actively feeding uni-nucleate stage not reported from elsewhere. NZ *B. exitiosa* also forms diplokarya which are only known from spore-forming species, and therefore NZ *B. exitiosa* may sporulate as a survival strategy under limiting conditions, suggesting that *Bonamia* spp. may have plastic life cycles. For example, the spore-forming species *H. armoricanum* may be able to go through its life cycle without sporulating, as suggested by the large plasmodia but absence of spores in *O. edulis* from St Philibert, Brittany (Brehélin et al. 1982, Vivarès et al. 1982, Bonami et al. 1985).

In conclusion, the interpretation of ultrastructure has to take into account multiple variables mediated by the parasite, the host and the environment. While this study on ultrastructure supports molecular phylogenies in that *B. exitiosa* is ultrastructurally distinct from *B. ostreae* and *B. perspora*, it shows considerable variability in the ultrastructure of *B. exitiosa* in different host species. These phenotypic differences reflect not only the *Bonamia* species concerned, but also the inter-relationships of the parasites with their hosts and the circumstances under which they were fixed, and variables in fixation. In comparison with the precise genotypic characterisation of organisms in molecular phylogenies, TEM studies are impre-

cise, and because of the many variables affecting the parasite, they are not often useful in identifying species. The ultrastructure of the Chilean *Bonamia*, in particular, does not adequately distinguish it from NZ *B. exitiosa*. However, while molecular studies can be used to classify the parasite and establish its phylogenetic affinities, they give no information on the structure of the parasite, its developmental stage, physiological state and its interactions with the host. Diagnosis should combine molecular techniques with, in the case of protists, ultrastructural characterisation. The latter must identify ultrastructural features of taxonomic, rather than physiological or developmental, importance. Consequently, for spore-forming haplosporidians, spore structure is most useful, but there is less certainty regarding non-sporous or pre-spore stages.

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