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Cucumispora ornata n. sp. (Fungi: Microsporidia) infecting invasive 'demon shrimp' (*Dikerogammarus haemobaphes*) in the United Kingdom

Jamie Bojko[1,3,*] • Alison M. Dunn[1] • Paul D. Stebbing[2] • Stuart H. Ross[3,4] • Rose C. Kerr[3,4] • Grant D. Stentiford[3,4]

[1]Faculty of Biological Sciences, University of Leeds, Leeds, UK, LS2 9JT

[2]Epidemiology and Risk Team, Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Weymouth, Dorset, UK, DT4 8UB

[3]Pathology and Molecular Systematics Team, Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Weymouth, Dorset, UK, DT4 8UB

[4]European Union Reference Laboratory for Crustacean Diseases, Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth Laboratory, Weymouth, Dorset, UK, DT4 8UB

Author e-mails:

Jamie Bojko (*Correspondence) - bs09jb@leeds.ac.uk

Phone Number: N/A

Alison M. Dunn – A.Dunn@Leeds.ac.uk

Paul D. Stebbing – Paul.Stebbing@Cefas.co.uk

Stuart H. Ross – Stuart.Ross@Cefas.co.uk

Rose C. Kerr – Rose.Kerr@Cefas.co.uk

Grant D. Stentiford – Grant.Stentiford@Cefas.co.uk

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Abbreviations: Invasive non-native species = INNS

Abstract

Dikerogammarus haemobaphes, the ‘demon shrimp’, is an amphipod native to the Ponto-Caspian region. This species invaded the UK in 2012 and has become widely established. *Dikerogammarus haemobaphes* has the potential to introduce non-native pathogens into the UK, creating a potential threat to native fauna. This study describes a novel species of microsporidian parasite infecting 72.8% of invasive *D. haemobaphes* located in the River Trent, UK. The microsporidium infection was systemic throughout the host; mainly targeting the sarcolemma of muscle tissues. Electron microscopy revealed this parasite to be diplokaryotic and have 7-9 turns of the polar filament. The microsporidium is placed into the ‘Cucumispora’ genus based on host histopathology, fine detail parasite ultrastructure, a highly similar life-cycle and SSU rDNA sequence phylogeny. Using this data this novel microsporidian species is named *Cucumispora ornata*, where ‘ornata’ refers to the external beading present on the mature spore stage of this organism. Alongside a taxonomic discussion, the presence of a novel *Cucumispora sp.* in the United Kingdom is discussed and related to the potential control of invasive *Dikerogammarus spp.* in the UK and the health of native species which may come into contact with this parasite.

1. Introduction

The Microsporidia are a diverse group of obligate parasites within the Kingdom Fungi (Capella-Guitiérrez et al. 2012; Haag et al. 2014). They infect hosts from all animal phyla and from all habitats; are genetically diverse; use a variety of transmission methods; can infect a range of different tissue and organ types; and exhibit high developmental and morphological plasticity (Dunn et al. 2001; Stentiford et al. 2013a; Stentiford et al. 2013b). Plasticity in parasite morphology has led to the formation of polyphyletic taxa whose inter-relationships are now being clarified by application of molecular phylogenetic approaches (e.g. Vossbrinck and Debrunner-Vossbrinck, 2005; Stentiford et al. 2013b). Furthermore, similar approaches are being applied to increase the confidence in placement of the Microsporidia at the base of the Fungi (Capella-Guitiérrez et al. 2012). The discovery and description of novel taxa, such as *Mitosporidium daphniae*, emphasise this positioning by essentially bridging the gap between true Fungi, the Cryptomycota (e.g. *Rozella* spp.) and the Microsporidia (Haag et al. 2014). Novel taxonomic descriptions now combine data pertaining to ultrastructural features, lifecycle characteristics, host type and habitat type, and conclusively, phylogenetics (Stentiford et al. 2013b).

Microsporidia were first identified infecting members of the Gammaridae (a family of omnivorous amphipods found across the world in freshwater and marine habitats), specifically *Gammarus pulex*, by Pfeiffer (1895). Since this initial discovery, gammarids have been shown to play host to a wide diversity of Microsporidia (Bulnheim, 1975; Terry et al. 2003). Ten microsporidium genera are currently known to infect gammarid hosts including: *Dictyocoela* (unofficially presented by Terry et al. 2004); *Nosema* (Nägeli, 1857); *Fibrillanosema* (Johanna et al. 2004); *Thelohania* (Henneguy and Thélohan, 1892); *Stempillia* (Pfeiffer, 1895); *Pleistophora* (Canning and Hazard, 1893); *Octosporea* (Chatton and Krempf, 1911); *Bacillidium* (Janda, 1928); *Gurleya* (Hesse, 1903); *Glugea* (Thélohan, 1891); *Amblyospora* (Hazard & Oldacre, 1975) and *Cucumispora* (Ovcharenko and Kurandina, 1987). Based on phylogenetic analysis and tree construction, these gammarid-

infecting microsporidia appear alongside those infecting fish, insects and other crustacean hosts from marine and freshwater environments (Stentiford et al. 2013b). Members of these genera utilise either horizontal or vertical transmission pathways, or a combination of the two, to maintain infections within populations of target hosts (Smith, 2009). *Dictyocoela berillonum* (vertical transmission), *Pleistophora mulleri* (vertical and horizontal transmission) and *Gurleya polonica* (horizontal transmission solely) provide examples of these transmission methods (Czaplinska et al. 1999; Terry et al. 2003; Terry et al. 2004; Wattier et al. 2007).

Most organs and tissues of gammarids can become infected by microsporidia. Whilst some taxa cause systemic infections (e.g. *Cucumispora dikerogammari*), others target specific tissue types such as muscle fibres (e.g. *G. polonica* in *Orchestia sp.*). In general, vertically transmitted microsporidia infect gonadal tissues and often elicit only minor pathologies unless they are also capable of horizontal transmission (Terry et al. 2003). Horizontally transmitted microsporidia on the other hand can elicit negative effects on feeding and locomotion and often result in host mortality (Bacela-Spychalska et al. 2014). For these reasons, horizontally transmitted microsporidia are considered a useful target for biological control strategies against agriculturally-important insect pests (Hajek and Delalibera Jr, 2010).

Members of the genus Dikerogammarus are a group of freshwater amphipods, native to the Ponto-Caspian region. Within the genus, two taxa have received considerable attention as invasive non-native species (INNS) within Europe: the ‘killer shrimp’ *D. villosus* (Rewicz et al. 2014) and the ‘demon shrimp’ *Dikerogammarus haemobaphes* (Bovy et al. 2014). *Dikerogammarus villosus* is listed in the ‘top 100 worst invasive species in Europe’ (DAISIE, 2014) due to its widely documented detrimental impact on native invertebrate fauna and its ability to spread parasites to novel locations (Wattier et al. 2007). In 2010, populations of *D. villosus* were discovered in several locations within the UK where they have subsequently

caused significant issues to both native fauna and the environment (MacNeil et al. 2013). Subsequent to the invasion by *D. villosus*, in 2012, a second invader, *D. haemobaphes* was also detected in UK freshwater habitats and has since been detected at numerous sites across a wide geographic space (Bovy et al. 2014; Green-Etxabe et al. 2014).

An extensive survey of *D. villosus* using histopathology revealed a distinct lack of pathogens and parasites in populations of *D. villosus* in UK sites (Bojko et al. 2013). These data were reinforced in a subsequent study by Arundell et al, (2014) which demonstrated an absence of microsporidium pathogens in invasive *D. villosus* using a PCR-based surveillance approach. Parasites may alter the outcome or impact of invasions as they are either introduced into new communities along with invading species, or left behind in the host's ancestral range, affording the host "enemy release" (Dunn, 2009). In the case of *D. villosus*, its native microsporidium parasite, *C. dikerogammari*, was found to have hitchhiked along an invasion pathway in continental Europe, entering Poland (via the River Vistula), France and Germany (via the River Rhine) (Wattier et al. 2007; Ovcharenko et al. 2009; Ovcharenko et al. 2010). In these countries, *C. dikerogammari* has also been detected infecting native gammarids (Bacela-Spychalska et al. 2012), presumably via transmission from proximity to infected *D. villosus*. Conversely, studies of UK populations of *D. villosus* have found little evidence for the presence of this microsporidium, or indeed other pathogens; suggesting that at least in this location, *D. villosus* may be benefiting from enemy release (Bojko et al. 2013; MacNeil et al. 2013; Arundell et al. 2014).

In addition to *C. dikerogammari*, several microsporidia are known to infect *D. villosus* and *D. haemobaphes* across their invasive and native ranges (Table 1) (Bojko et al. 2013). It has been suggested that *C. dikerogammari*, may pose a significant risk to native range amphipods due to its potential for cross-taxa transmission (Bacela-Spychalska et al. 2012). In the current study we describe a novel microsporidium pathogen infecting *D. haemobaphes* collected from the River Trent, UK. Histological, ultrastructural and

phylogenetic evidence is used to propose a novel species within the genus *Cucumispora*. Our findings are discussed in relation to the invasion pathway for this pathogen to the UK, the relationship to sister taxa within the genus and the potential for the novel pathogen to spread to both native hosts, and to the invasive sister species *D. villosus*.

Table 1

Parasite:	Species:	Location	Reference
Microsporidia infecting *Dikerogammarus haemobaphes*	*Cucumispora* (=*Nosema*) *dikerogammari*	Goslawski Lake and Bug in Wyszków	Ovcharenko et al. 2010
	Thelohania brevilovum	Goslawski Lake, Poland	Ovcharenko et al. 2009
	Dictyocoela mulleri	Goslawski Lake, Poland	Ovcharenko et al. 2009
	Dictyocoela spp. ('Haplotype: 30-33')	Goslawski Lake, Poland	Wilkinson et al. 2011
	Dictyocoela berillonum	Unknown	Wroblewski and Ovcharenko (BLAST result)
		Wallingford Bridge and Bell Weir, UK	Green-Etxabe et al. 2014

2. Materials and Methods

2.1 Sample collection. Dikerogammarus haemobaphes (n=81) were sampled using nets from two sites on the River Trent, United Kingdom (grid ref.: SK3870004400 and SK1370013700) in March 2014. Animals were identified based on their morphology and placed on ice before dividing into three parts using a sterile razor blade. The 'head' and urosome were removed and placed into 100% ethanol for later DNA extraction. Sections 2 and 3 of the pereon, including the gnathopods, were dissected along with internal organs and placed into 2.5% glutaraldehyde for transmission electron microscopy (TEM). The remainder of the animal (pereon 4 to the pleosome) was fixed for histology in Davidson's freshwater fixative (Hopwood, 1996).

2.2 Histology. After 24 h, samples in Davidson's freshwater fixative were transferred to 70% industrial methylated spirit (IMS) before processing to paraffin wax blocks using an automated tissue processor (Peloris, Leica Microsystems, UK) and sectioned on a Finesse E/NE rotary microtome (Thermofisher, UK). Specimens were stained using haematoxylin

156 and alcoholic eosin (H&E) and slides examined using a Nikon Eclipse E800 light microscope
157 at a range of magnifications. Images were obtained using an integrated LEICA^{TM} (Leica, UK)
158 camera and edited/annotated using LuciaG software (Nikon, UK). Animal processing
159 protocol here is identical to that described in Bojko et al. (2013).
160
161 *2.3 Transmission Electron Microscopy* (TEM)*.* Samples fixed for TEM (present in 2.5%
162 Glutaraldehyde) were processed through 2 changes of 0.1M sodium cacodylate buffer over
163 15 min periods. Secondary fixation was performed using osmium tetroxide (OsO4) (1 hour)
164 followed by two 10 minute rinses in 0.1M sodium cacodylate buffer. Samples were
165 dehydrated through an ascending acetone dilution series (10%, 30%, 50%, 70%, 90%,
166 100%) before embedding in 100 Agar resin using a resin:acetone dilution series (25%, 50%,
167 75%, 100%) (1 h per dilution). The tissues were placed into plastic moulds filled with resin
168 and polymerised by heating to 60˚C for 16 h. Blocks were sectioned using a Reichart
169 Ultracut Microtome equipped with glass blades (semi-thin sections (1µm)) or a diamond
170 blade (ultra-thin sections (around 80nm)). Semi-thin sections were stained using toluidine
171 blue and checked using standard light microscopy. Ultra-thin sections were stained using
172 uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Ultra-thin sections were
173 observed using a Jeol JEM 1400 transmission electron microscope (Jeol, UK).
174
175 *2.4 DNA extraction, PCR and sequencing.* The head and urosome of each amphipod, fixed
176 in ethanol, underwent DNA extraction using the EZ1 DNA tissue kit (Qiagen, UK).
177 Amplification of the partial SSU rRNA gene was accomplished using two previously identified
178 PCR primer sets (Vossbrinck et al., 1987; Baker et al. 1995; Tourtip et al. 2009) (see Table
179 2). V1F/530r and MF1/MR1 primer protocols were used in a GoTaq flexi PCR reaction
180 including 1.25U/reaction of Taq polymerase, 100pMol/reaction of each primer,
181 0.25mM/reaction of each dNTP, 2.5mM/reaction $MgCl_2$ and 2.5µl/reaction of DNA extract
182 (10-30ng/µl) in a 50µl reaction volume. Thermocycler settings for V1F/530r were; 95˚C (5
183 min), 95˚C (50 sec)-60˚C (70 sec)-72˚C (90 sec) (40 cycles), 72˚C (10 min). Thermocycler

settings for MF1/MR1 were; 94˚C (5 min), 94˚C-55˚C-72˚C (1 min per temperature) (40 cycles), 72˚C (10 min). Amplifications were run on a 1.5% agar gel (120V / 45 minutes) and products were excised from the gel and purified using freeze-and-squeeze purification before sequencing on an ABI PRISM 3130*xl* Genetic Analyser (Applied Biosystems, UK) or sequencing via Eurofins (Eurofins Genomics, UK).

Table 2

Forward Primer		Reverse Primer		Approx. fragment size	Reference
V1F	5'-CACCAGGTTGATTCTGCCTGAC-3'	530r	5'-CCGCGGCTGCTGGCAC-3'	530bp	Vossbrinck et al. 1987; Baker et al. 1995
MF1	5'-CCGGAGAGGGAGCCTGAGA-3'	MR1	5'-GACGGGCGGTGTGTACAAA-3'	900bp	Tourtip et al. 2009

2.5 Phylogenetic analysis. Gene sequences retrieved from microsporidium-infected demon shrimp were analysed using CLC Main Workbench (7.0.3) where a neighbour joining tree was produced, incorporating our own acquired sequences with other closely related microsporidium sequences, and in particular, those used in the analysis by Ovcharenko et al. (2010). The analysis included 1000 bootstrap replicates and utilised the Jukes-Cantor evolution model (Jukes and Cantor, 1969). Similar BLAST hit sequences from several undetermined "*Microsporidium sp.*" were also incorporated in to the phylogenetic analysis. The tree underwent 100 bootstrap replicates to test robustness. *Basidiobolus ranarum* (AY635841), *Heterococcus pleurococcoides* (AJ579335.1) and *Conidiobolus coronatus* (AF296753) were used as a fungal out-group.

3. Results

3.1 Pathology and ultrastructure. Prior to fixation, live animals did not display obvious clinical signs of infection. Despite this, histology revealed a microsporidium infection in 72.8% of animals obtained from the River Trent population. Infection was observed in the skeletal musculature (located mainly within the space immediately beneath the sarcolemma), nervous tissues, oocytes and connective tissues. Infections by spore life-stages of the

microsporidia were clearly visible via light microscopy, and often seen to begin infection in the sarcolemma of muscle blocks (Fig. 1a). In advanced infections, the majority of the skeletal musculature was replaced with microsporidia life stages, moving from the sarcolemma to infect the rest of the muscle block (Fig. 1b). Under high magnification, spores appeared somewhat elongate and were apparently in direct contact with the host cell cytoplasm (Fig. 1c). Infections in connective tissue cells appeared to lead to formation of cysts (multi-nucleated syncitia), potentially due to fusion of adjacent infected host cells (Fig. 1d). In female hosts, the gonad was sometimes targeted by the parasite, with microsporidia spores occasionally visible within oocytes. Limited host encapsulation of parasite life stages was observed, although in advanced infections, presumably related to host cell rupture, small melanised haemocyte aggregates were seen. In other cases, liberated spores were seen to be phagocytised by host haemocytes (Fig. 1e).

Transmission electron microscopy (TEM) of infected muscle tissues revealed merogonial and sporogonial life stages of a microsporidium pathogen developing in direct contact with the host cell cytoplasm. In early stages, the pathogen occupied the sub-sarcolemmal region at the periphery of infected muscle fibres with progression to the main muscle fibre in later stages of infection. The lifecycle began with a diplokaryotic meront (Fig. 2a) which followed one of two possible pathways; the first involving direct development to the diplokaryotic sporont, depicted by regional, and eventually complete, thickening of the cell membrane and darkening of the cell cytoplasm (Fig. 2b, c). The second pathway involved nuclear division to form a tetranucleate (2 x 2n) meront plasmodium which then divided through binary fission to form two diplokaryotic sporoblasts (Fig. 2d,e, f) (as seen by *C. dikerogammari* in Ovcharenko et al. 2010). In rare cases, unikaryotic meronts were observed however they were assumed to be non-representative cross-sections of diplokaryotic cells (cross-sections through a diplokaryotic meront due to the use of TEM gives the appearance of a unikaryotic cell). No sporophores vesicles were observed throughout this study.

The second pathway which involves a tetranucleate meront plasmodium stage, served as a multiplication step for the parasite (Fig. 2d,e,f) which is skipped during direct formation of the 2n meront to the 2n sporont, seen in pathway one (Fig. 2c, d). Both of these pathways appear to lead to the same eventual spore type. In both cases, diplokaryotic sporonts, with thickened cell wall and increasingly electron dense cytoplasm initiate development of spore extrusion precursors which mark the transition to the diplokaryotic sporoblast (Fig. 3a).

Organelles including the anchoring disk, polar filament and condensed polaroplast began to form during development of the sporoblast (Fig. 3a). This was followed by thickening of the endospore (Fig. 3b) and eventual development of the mature spore (Fig. 3c). The mature spore was diplokaryotic, contained an electron dense cytoplasm and 7-9 turns of an isofilar polar filament, arranged in a linear rank at the periphery of the spore (Fig. 3c). The polar filament was 115.03nm +/- 3.4nm (n=4) in diameter and comprised of concentric rings of varying electron density (Fig. 3d). The manubrial region of the polar filament passed through a bilaminar polaroplast and terminated at an anchoring disk (Fig. 3e). The bilaminar polaroplast at the anterior of the spore contained an electron dense outer layer in contact with the plasmalemma, and an electron lucent, folded layer surrounding the polar filament. The polar vacuole occupied approximately 20% of the spore volume at the posterior end and was contained within an electron lucent membrane. Mature spores measured approximately 4.24µm +/- 0.43µm (n=19) in length and 2.03µm +/- 0.19µm (n=23) in width using histologically fixed material and TEM. The spore wall was comprised of a plasmalemma, endospore, exospore and external protein beading (Fig. 3f). The endospore was electron lucent, measuring 186.33nm +/- 33.5nm (n=115 (23 spores measured 5 times)) around the majority of the spore, however at the anchoring disk the endospore thinned to a third of its normal thickness (Fig. 3e). The exospore measured 39.9nm +/- 11.2nm (n=115 (23 spores)) and the external beads extended approximately 29.05nm +/- 4.5nm (n=15) from the exospore into the host cell cytoplasm (Fig. 3f).

On occasion small, electron dense, diplokaryotic cells, often attached to an undefined remnant were observed (Fig. 4a, b). Remnants seen in figures 4a and 4b are only ever present once on these unknown cells and have the appearance of type 1 tubular secretions (as seen in Takvorian and Cali, 1983). Takvorian and Cali (1983), state these secretions are associated with the sporoblast life stage; however these unknown cells in figure 4a and 4b lack the relevant organelles to be sporoblasts. The cells depicted here (Fig. 4a,b) and their accompanying remnants could be an early sporoplasm with a remnant of the polar filament, aberrant stages of development or possibly degraded life stages.

3.2 Molecular phylogeny. Molecular phylogeny of the microsporidium parasite infecting *D. haemobaphes* was based upon a partial sequence of the SSU rRNA gene retrieved from histopathology confirmed infected host material. A 1186bp sequence of the SSU rRNA gene retrieved BLAST (NCBI) comparisons with 98% similarity to "*Microsporidium sp.* JES2002G" (AJ438962.1) (query cover = 99%, ident.= 98%), a parasite infecting *Gammarus chevreuxi* from the UK, and to *Cucumispora dikerogammari* (91% sequence identity), a microsporidium parasite infecting *D. villosus* from continental Europe (Ovcharenko et al. 2010) - a close taxonomic relation to *D. haemobaphes*. Phylogenetic assessment using a neighbour joining analysis grouped this parasite (to be named *Cucumispora ornata*) with closely related BLAST hits (*Microsporidium sp.*) and *C. dikerogammari* (Fig. 5) (bootstrap value of 100). The phylogenetic analysis presented here utilised the majority of the microsporidium sequences presented by Ovcharenko et al. (2010) in their description of *Cucumispora dikerogammari*. The closely related *Microsporidium sp.* JES2002G (98% sequence identity) is distanced from *C. ornata* by a short branch length of 0.009 (relative genetic change), highlighting their similar sequence identity. *Cucumispora dikerogammari* and the parasite observed here are parted by a distance of 0.086 on the phylogenetic tree, with the closest member outside this group being *Spraguea lophii* (AF056013) with a branch distance, from the parasite, of 0.222.

4. Taxonomic Summary

Genus: *Cucumispora* (Ovcharenko et al. 2010)

In all developmental stages the nuclei are diplokaryotic and develop in direct contact with the host cell cytoplasm. Merogonic and sporogonic stages divide by binary fission. Each sporont produces 2 elongate sporoblasts which develop into 2 elongate spores with thin spore walls, uniform exospores and isofilar polar filaments arranged in 6–8 coils. The angle of the anterior 3 coils differs from that of subsequent coils. A thin, umbrella-shaped, anchoring disc covers the anterior region of the polaroplast, which has 2 distinct lamellar regions, occupying approximately one fourth of the spore volume. The parasite infects gammaridean hosts and infects primarily muscle tissue but can also occur in other tissues (adapted from Ovcharenko et al. 2010).

Type species: Cucumispora ornata n. sp. (Bojko, Dunn, Stebbing, Ross, Kerr, Stentiford, 2015)

Species description: Using histology and TEM, spores appear ellipsoid (4.24µm +/- 0.43µm in length and 2.025µm +/- 0.19µm in width), with an endospore (186.33 nm +/- 33.5nm) and externally beaded (decorated) exospore (40nm +/- 11.2nm). The polar filament turns between 7-9 times. The spores are diplokaryotic with a diplokaryotic lifecycle except for the putative presence of a unikaryotic meront. The lifecycle follows closely that of the initially described species *C. dikerogammari* but is morphologically dissimilar in some aspects, including a shorter spore length, coil turns and external beading. Relation by SSU rDNA phylogeny to *C. dikerogammari* is 91%. No transmission information is currently available. *Dikerogammarus haemobaphes* is currently the only known host but falls within the Gammaridae.

Type host: Dikerogammarus haemobaphes (Eichwald, 1841) (common name: demon shrimp)

Type locality: The River Trent (United Kingdom) and adjacent, connected waterways (SK3870004400 and SK1370013700). A confirmed site of an invasive population of *Dikerogammarus haemobaphes*. It is unknown whether this parasite exists in populations of *D. haemobaphes* in their native range.

Site of infection: Infections appear systemic, but infecting the musculature primarily. Connective tissues between the gut and gonad, musculature, nervous system and carapace are often infected in advanced cases.

Etymology: "*Cucumispora*" (Ovcharenko et al. 2010) is so named due to the elongated, "cucumiform" spore morphology of initially described species *Cucumispora dikerogammari* (Ovcharenko and Kurandina, 1987; Ovcharenko et al. 2010). The specific epithet "*ornata*" is derived from the Latin word "ornatum" which means "adorned" in English. This refers to the external beading covering the exterior of the spore life stages of this organism.

Type material: Histological sections and TEM resin blocks from the UK specimens are deposited in the Registry of Aquatic Pathology (RAP) at the Cefas Weymouth Laboratory, UK. *Cucumispora ornata* SSU rRNA gene sequences from samples collected in the United Kingdom have been deposited in Gen-Bank (accession numbers to be assigned).

5. Discussion

In this study we describe a novel microsporidium parasite infecting an invasive gammarid, *Dikerogammarus haemobaphes*, from UK fresh waters. The parasite is herein named as *Cucumispora ornata* n. sp. based upon host ecology, histological and ultrastructural pathology, and partial sequencing of the SSU rRNA gene of the parasite. Given that *C. ornata* has not previously been described infecting gammarids (or other hosts) from UK waters, or elsewhere, it is presumed that it was similarly introduced during the invasion of its host after 2012. Whether it exists in *D. haemobaphes* within its native range has yet to be

346 determined but given its relatively close relationship to sister taxon *C. dikerogammari*
347 (Ovcharenko et al. 2010), which has been detected in native and continental invasive range
348 *D. villosus* (Wattier et al. 2007) it is assumed that *C. ornata* is also a native parasite of hosts
349 from the Ponto-Caspian region.
350
351 *5.1 Taxonomy of Cucumispora ornata n. sp.* Sequencing of the partial SSU rRNA gene of *C.*
352 *ornata* revealed a closely related branch containing this parasite, three unassigned
353 '*Microsporidium*' species infecting other Crustacea ('*Microsporidium'* is a holding genus
354 according to Becnel et al. 2014 until further information is acquired) and *C. dikerogammari*
355 infecting the sister gammarid *D. villosus* (Fig. 5). On this branch, *C. dikerogammari* and *C.*
356 *ornata* shared 91% sequence identity, with higher similarity between *C. ornata* and the
357 unassigned *Microsporidium* taxa available in BLAST. Although we acknowledge the
358 relatively low similarity between the partial SSU rRNA gene sequence between *C. ornata*
359 and *C. dikerogammari*, since both have a similar lifecycle, are muscle-infecting parasites of
360 congeneric hosts, with an additional three unassigned parasites (also in gammarids and
361 copepods) as branch relatives, we have elected to assign the parasite described herein to
362 the genus *Cucumispora*. A quickly evolving SSU rRNA gene may account for the relatively
363 low genetic similarity between *C. ornata* and *C. dikerogammari*. Relative gene sequence
364 evolution, primarily in the SSU genes, is known to vary between microsporidia (Philippe,
365 2000; Embley and Martin, 2006). Considering this, we propose that the remaining three
366 *Microsporidium* taxa described in studies by Terry et al. (2004), Jones et al. (2010) and
367 Krebes et al. (2010) are also likely to be members of this genus given their (relatively) close
368 SSU sequence identity and shared choice of crustacean hosts.
369
370 The placement of our novel parasite in to the genus *Cucumispora* is largely supported by
371 ultrastructural and lifecycle characteristics such as a diplokaryotic spore, development in
372 direct contact with the host cell cytoplasm, some similar spore features (bilaminar
373 polaroplast and thin anchoring disk) and predilection for similar host tissues and organs are

shared between *C. dikerogammari* (Ovcharenko et al. 2010) and the parasite described herein. Although we report putative uninucleate (1n) meronts in *C. ornata* (a feature not observed in *C. dikerogammari*), our confidence in reporting this trait is low given the limitations of TEM for detection of uninucleate life stages. However, diplokaryotic stages predominate the lifecycle and follow the development process observed for *C. dikerogammari*. The morphology of *C. ornata* does differ from *C. dikerogammari* in respect to spore length, the presence of a beaded exospore and a thicker endospore however morphology is often not a reliable tool for microsporidian taxonomy (Stentiford et al. 2013a). Differing features, such as the beaded exospore, when taken together with reasonable genetic variation in the SSU rRNA gene (9% difference between *C. ornata* and *C. dikerogammari*) may eventually be revealed to be sufficient for the erection of a novel genus to contain this parasite, but further information may be needed from other members of the *Cucumispora* before this can be reassessed. Concatenated phylogenies, based upon non-ribosomal protein coding genes and studies on fresh (live) material (not histologically processed) have the potential to assist definition and answer developmental queries of novel taxa in such instances and may prove fruitful for further study of this parasite (Stentiford et al. 2013b).

5.2 Cucumispora ornata n. sp. as an invasive species. Parasites that are transferred from 'exotic' locations can also be deemed as invasive (Dunn, 2009). Just like their hosts, invasive parasites have been shown in the past to cause negative effects on native fauna and ecosystems by either infecting native species or facilitating their hosts' invasive capabilities (Prenter et al. 2004; Dunn et al. 2009). The ecological impact of this new parasite is likely to be of considerable interest for the invasion of the host, and for the invaded freshwater community. The parasite reaches high burden in the host and causes a systemic pathology, primarily targeting the muscle tissues. Prevalence was also relatively high (72.8%). It is probable therefore that this parasite has a regulatory effect on the *D. haemobaphes* host population which may, in turn, moderate the potential impact of the

invader. Alternatively, *C. ornata* could have a detrimental impact on native species should transmission to new species occur. High spore densities were observed in the muscle of infected individuals suggesting that intraguild predation may provide opportunities for zoonotic transmission. The related microsporidium species, *C. dikerogammari* preferentially infects Ponto-Caspian amphipods but has been found to infect a variety of other amphipod species at low prevalence (Ovcharenko et al. 2010; Bacela-Spychalska et al. 2012; Bacela-Spychalska et al. 2014), and it is possible that *C. ornata* may be similarly generalist. It is important therefore that future work investigates the specificity of *C. ornata* and its virulence should it infect native hosts.

5.3 *The future of Cucumispora ornata n. sp. in the UK*

Future assessment of *C. ornata* should include host range and capability for invasive species control. Movement of these invaders facilitates the movement of their pathogens so tracking the spread of this invasion is an important endeavour (Anderson et al. 2014). It may be interesting to consider that demon shrimp and killer shrimp do not currently co-exist in the UK. Were they to co-habit a location, it would provide the opportunity to transfer parasites. The introduction of microsporidia to killer shrimp populations in the UK has been suggested as a future possibility for controlling, otherwise unmanageable, populations that currently lack these parasites (Bojko et al. 2013). The presence of *C. ornata* in UK waterways may provide such an opportunity. Microsporidia have been adapted as biological control agents in the past and have shown to be effective in this role (Hajek and Delalibera Jr, 2010) however the application of microsporidia biological control agents to control an invasive species in an ecosystem setting has not been previously attempted.

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612
613 **Figure and Table Captions:**

614 **Figure 1:** *Cucumispora ornata* n. sp. associated histopathology in *D. haemobaphes*. a)
615 Microsporidian infection colonising the sarcolemma and muscle cells of available muscle
616 blocks (white arrow). Some muscle remains uninfected (*). Scale = 100µm. b) Large
617 infection replacing areas of the muscle block within the leg of *D. haemobaphes*. Scale =
618 10µm. c) A high magnification image of microsporidian spores under histology. The inset
619 sows both laterally and longitudinally sectioned spores. Scale = 10µm. d) Microsporidian
620 filled cells (white arrow) in the connective tissue between the gut smooth muscle (black
621 arrow) and gonad (white star) of *D. haemobaphes*. Individual nuclei are depicted with a white

triangle. Scale = 10µm. e) Granulocytes in the heart are present with phagocytised microsporidian spores (white arrow). The sarcolemma of the heart muscle also appears infected (black arrow). Scale = 10µm.

Figure 2: Merogony of *Cucumispora ornata* n. sp. in the musculature of *Dikerogammarus haemobaphes*. a) Diplokaryotic meront. Host mitochondria (M) appear in close association. Scale = 500nm. b) Diplokaryotic meront with initial wall thickening (white arrow). Scale = 500nm. c) Diplokaryotic meront to diplokaryotic sporont transition. White arrows indicate thickening cell membranes. Scale = 500nm. d) A tetranucleate cell. Scale = 500nm. e) Binary fission of a tetranucleate cell. The white arrow indicates where the division is occurring and the black arrow indicates the microtubules present. The white triangle highlights the ever thickening cell wall. Scale = 500nm. f) Post-separation of the tetranucleate sporont to two diplokaryotic sporonts. The white triangle highlights the thickness of the cell wall at this developmental stage. Scale = 500nm.

Figure 3: *Cucumispora ornata* n. sp. lifecycle progression from the sporoblast to final mature spore. a) The sporoblast, present with nuclei (N) and developing polar filament (white arrow). Scale = 500nm. b) Thickening of the sporoblast endospore (white arrow). Scale = 500nm. c) The final diplokaryotic spore life stage with darkened cytoplasm, polar vacuole (PV), nuclei (N), polar filaments (white arrow), polaroplast (P) and anchoring disk (A). Scale = 500nm. d) High magnification of individual turns of the polar filament. Scale = 20nm. e) High magnification image of the anchoring disk and associated thinning of the endospore (white arrow). Scale = 100nm. f) External beading on the exospore. Scale = 100nm.

Figure 4: Images of the commonly seen, unidentified cells. a) An example cell, present with nuclei (N) and electron dense cytoplasm, was commonly seen during the study. A currently undefined cytoplasmic extrusion is highlighted by a white arrow. Scale = 500nm. b) High magnification image of the cytoplasmic remnant (white arrow) attached to the cytoplasm (*) of the undefined cell. Scale = 500nm.

649

650 **Figure 5:** Neighbour joining phylogenetic tree using partial SSU rRNA gene sequences from
651 microsporidia. *Basidiobolus ranarum* (AY635841), *Heterococcus pleurococcoides*
652 (AJ579335.1) and *Conidiobolus coronatus* (AF296753) are used as out-group species.

653 **Table 1:** Microsporidian parasites known to infect *Dikerogammarus haemobaphes*.

654 **Table 2:** Primer sets used to partially amplify the microsporidian SSU rRNA gene.

Figure 1

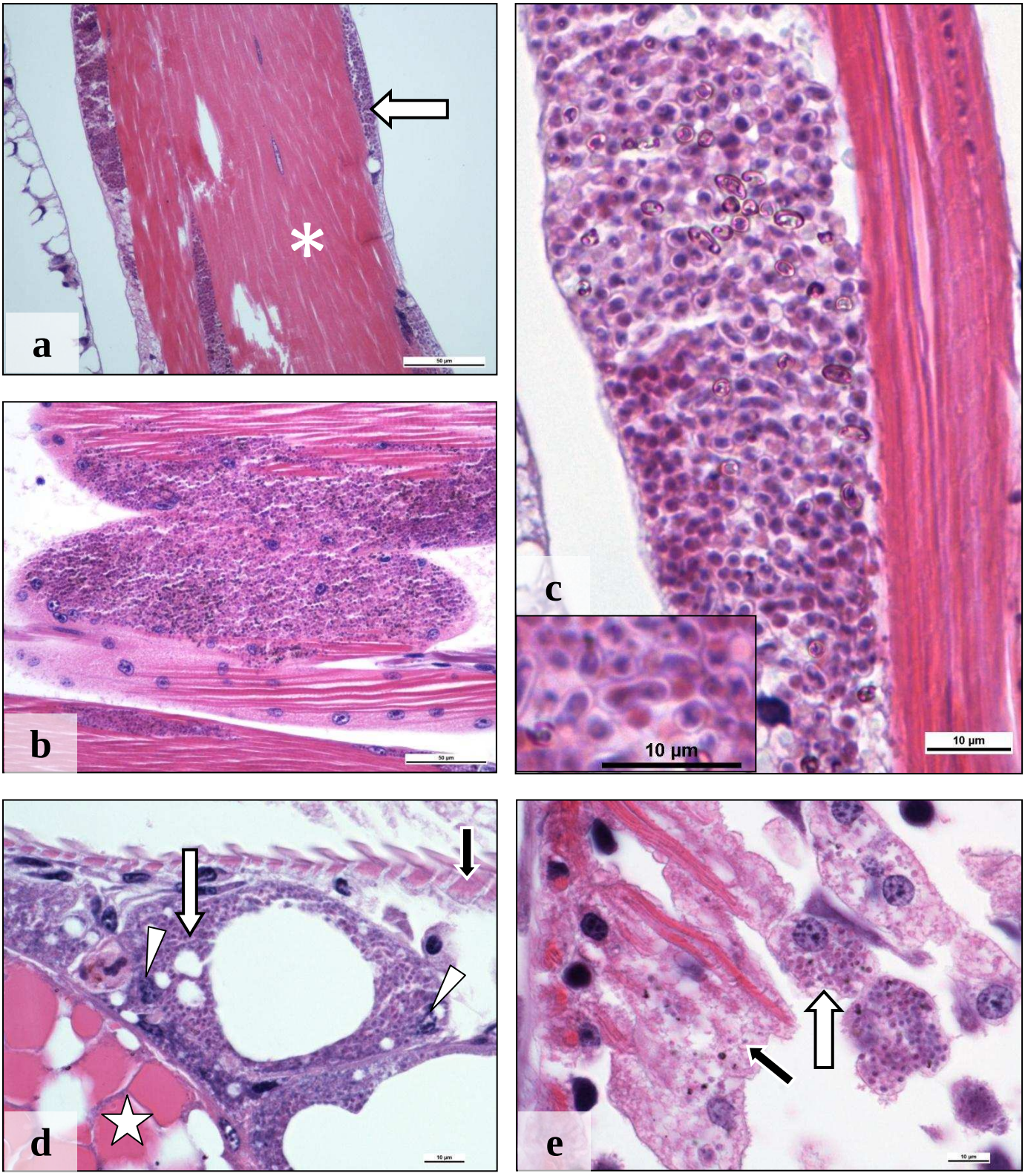

Figure 1

Figure 2

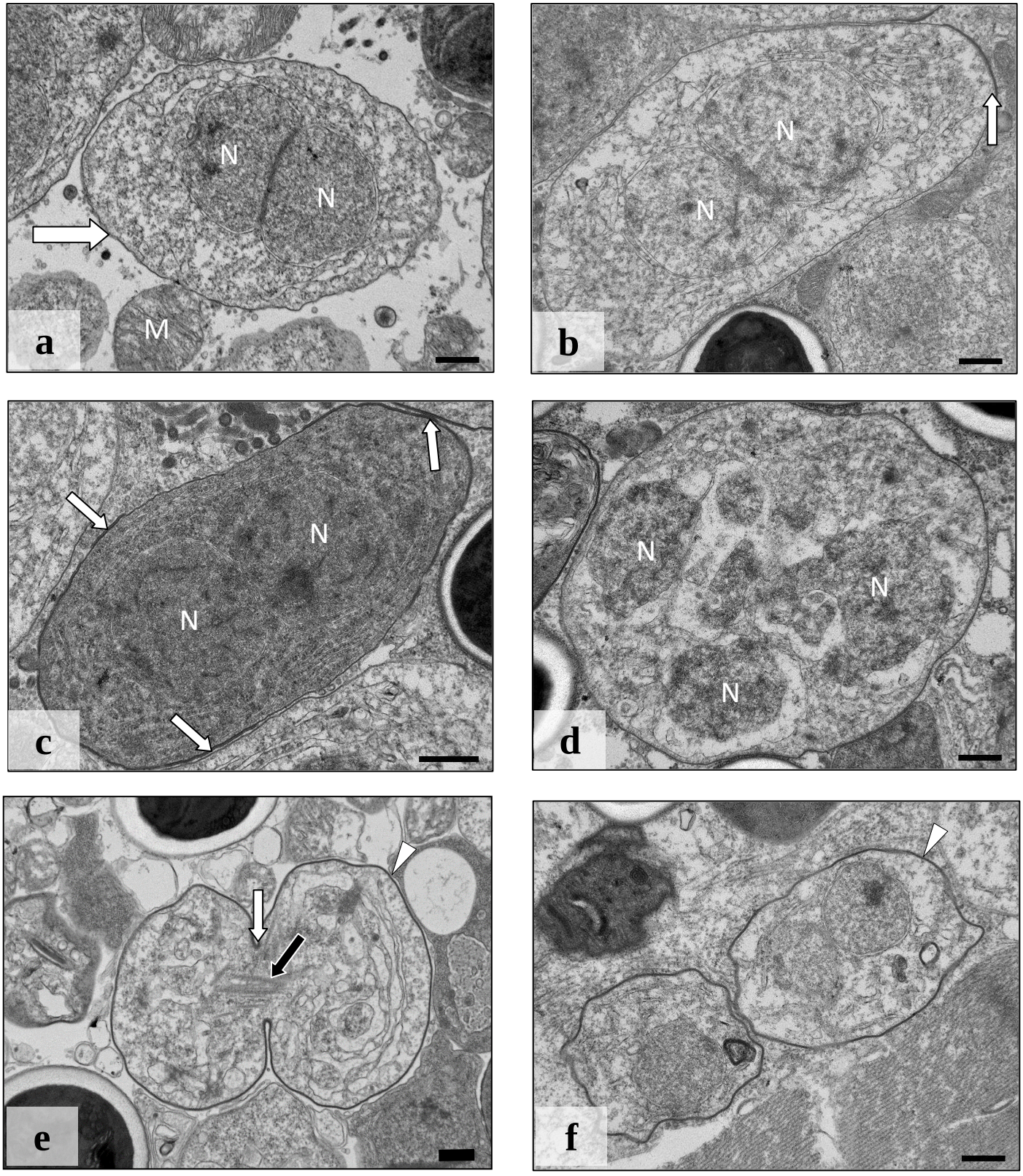

Figure 2

Figure 3

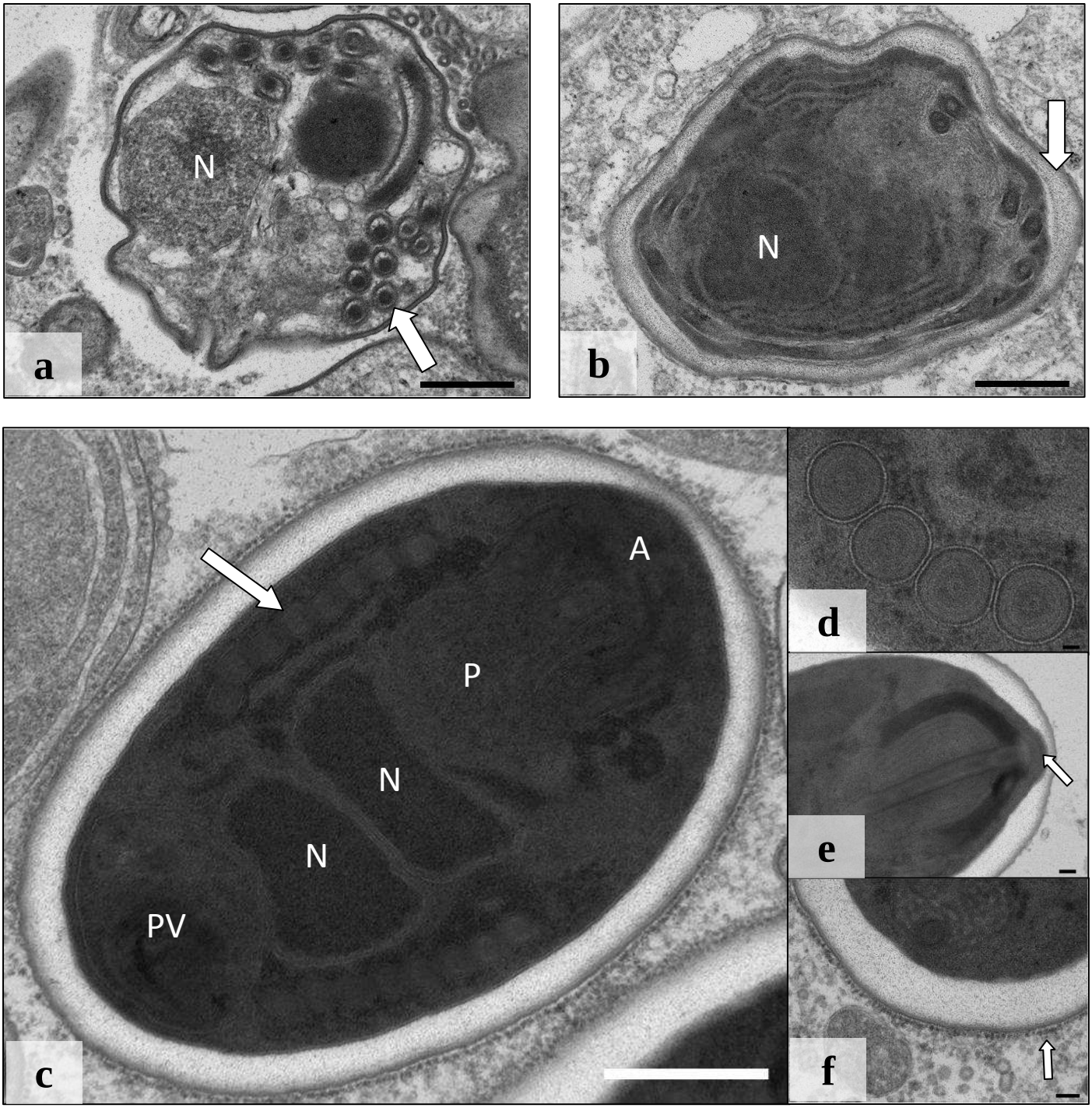

Figure 3

Figure 4

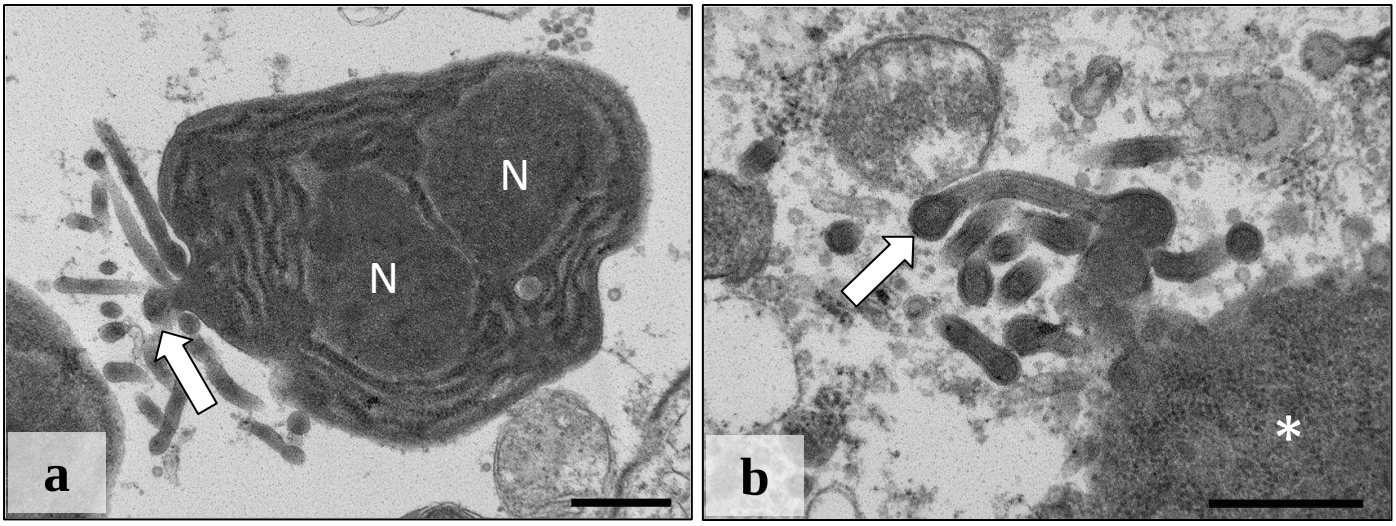

Figure 4

Figure 5

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