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Ultrastructural characterization of the host–parasite interface between *Allomyces anomalus* (Blastocladiomycota) and *Rozella allomycis* (Cryptomycota)

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ABSTRACT

Rozella allomycis is an obligate endoparasite of the water mold *Allomyces* and a member of a clade (= Opisthosporidia) sister to the traditional Fungi. Gaining insights into *Rozella's* development as a phylogenetically pivotal endoparasite can aid our understanding of structural adaptations and evolution of the Opisthosporidia clade, especially within the context of genomic information. The purpose of this study is to characterize the interface between *R. allomycis* and *Allomyces anomalus*. Electron microscopy of developing plasmodia of *R. allomycis* in host hyphae shows that the interface consists of three-membrane layers, interpreted as the parasite's plasma membrane (inner one layer) and a host cisterna (outer two layers). As sporangial and resting spore plasmodia develop, host mitochondria typically cluster at the surface of the parasite and eventually align parallel to the three-membrane layered interface. The parasite's mitochondria have only a few cristae and the mitochondrial matrix is sparse, clearly distinguishing parasite mitochondria from those of the host. Consistent with the expected organellar topology if the parasite plasmodia phagocytize host cytoplasm, phagocytic vacuoles are at first bounded by three-membrane layers with host-type mitochondria lining the inner membrane. Thus, *Rozella's* nutrition, at least in part, is phagotrophic in contrast to osmotrophic nutrition of traditional fungi.

Introduction

Species of *Rozella* Cornu are obligate endoparasites of Oomycetes, Chytridiomycetes, Monoblepharidomycetes, Blastocladiomycetes, and green algae (Sparrow 1960) in which the unwalled, multinucleate sporangial plasmodium eventually completely fills the host. At maturity the sporangial plasmodium uses the host wall as its own as it cleaves into numerous posteriorly uniflagellated zoospores, which are discharged through one to multiple discharge pores or tubes. Thick-walled resting spores may also be produced, and they lie loosely within the host.

Because *Rozella* reproduces asexually with the formation of posteriorly uniflagellate zoospores, this genus has in general

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been considered a member of the Chytridiomycetes (Barr 1980; Held 1975; Sparrow 1938, 1960), but its placement has been controversial (reviewed in Karling 1942). Based on zoospore ultrastructure of *Rozella allomycis* Foust (Held 1975), Barr (1980) classified *Rozella* in the Spizellomycetales. However, the shape, flagellar apparatus organization, and organellar arrangements of *R. allomycis* zoospores are quite distinct from these features in zoospores of core Spizellomycetales members, and Barr (1980) in fact questioned the validity of classifying it in the Spizellomycetales.

The discovery that *R. allomycis* placed in a clade sister to all other Fungi was an unexpected result of the first broad-based phylogenetic analysis of zoosporic Fungi (James *et al.* 2006). Following this study, phylogenetic analyses of molecular environmental surveys from diverse habitats have revealed numerous phylotypes that form a clade with *R. allomycis*, *Rozella* ex. *Rhizoclosmatium* JEL 347, *Rozella* ex *Pythium*, and *Rozella rhizoclosmatii* Letcher and Longcore (Corsaro *et al.* 2016; James & Berbee 2012; Jones *et al.* 2011; Lara *et al.* 2010; Lazarus & James 2015; Letcher *et al.* 2016b). To recognize the distinctive nature of the clade containing *Rozella* species and environmental phylotypes, Jones *et al.* (2011) erected the new phylum, Cryptomycota (Jones *et al.* 2011; Karpov *et al.* 2014) (= Rozellomycota, Corsaro *et al.* 2014).

Cryptomycota is phylogenetically related to two other groups of plasmodial endoparasites, which once were considered disparate groups: Aphelida, parasites of algae which phagocytize host cytoplasm (Karpov *et al.* 2013; Letcher *et al.* 2013, 2015; Schnepf *et al.* 1971), and Microsporidia, parasites of animal cells which exhibit an osmotrophic mode of nutrition (Corradi 2015). Because these three groups share characteristics with both fungi and protists, there is debate as to which kingdom they belong (Grossart *et al.* 2016). Suggesting that they share characteristics with both kingdoms, Karpov *et al.* (2014) erected the superphylum Opisthosporidia for these three groups. Because of their pivotal phylogenetic position in the evolution of fungi and divergence from protists, it is important to characterize this group more fully. Characterizing the nature of the interaction between *R. allomycis* and its blastocladialean host, *Allomyces anomalus* R. Emers., at the cellular level is vital to an understanding of fungal evolution (Stajich *et al.* 2009).

Foust (1937) demonstrated that *R. allomycis* induces its host hyphae to produce septa across their coenocytic hyphae and to compartmentalize infection, which suggests a great degree of host manipulation in which the parasite maintains major host function solely for its own reproduction. Held (Held 1973, 1974, 1975, 1980, 1981) made extensive developmental studies of *R. allomycis* in *Allomyces* sp. but never described details of the host–parasite interface. The purpose of this study is to explore the ultrastructure of the interface between *R. allomycis* and *A. anomalus*.

Materials and methods

Culture

Rozella allomycis (CSF 55) on *Allomyces anomalus* was isolated by Timothy James from a sample collected from a roadside ditch in Hattiesburg, MS. Cultures were maintained on 1/8 strength Emerson YPSS agar (Difco 273910).

Transmission electron microscopy

Infected hyphae were collected at 2–6 d after inoculation of host with zoospores of the parasite. Host hyphae infected with the parasite were prepared for electron microscopy as described (Letcher & Powell 2005) and thin embedded so that infected regions could be selected, excised, and mounted on plastic stubs for sectioning. Thin sections were cut with a Leica ultramicrotome and mounted on 300 mesh grids. Serial sections were mounted on 30 nm coated slot grids. Sections were stained with uranyl acetate and lead citrate and observed on a Hitachi 7650 transmission electron microscope at 60 kV.

Results

Light microscopy of Rozella allomycis development

Uninfected hyphae of *Allomyces* are typically aseptate, except for the septal delimitation of their reproductive structures (vegetative sporangia, resting sporangia or gametangia). However, the parasite induces host hyphae to produce septa, which compartmentalize the parasite plasmodia as they develop into unwalled sporangial plasmodia (Fig 1A and B) or walled resting spores (Fig 1C–E). Often times these host compartments that result from the parasite's induction of septation are swollen (Fig 1D). At maturity, sporangial plasmodia and resting spores occupy separate host compartments (Fig 1E).

In the infection process, a single parasite sporangial plasmodium completely fills a host compartment (Fig 1A). In basipetal progression, each sporangial plasmodium cleaves into numerous zoospores (Fig 1A). Zoospores undergo a characteristic period of roiling before leaving the host compartment as a mass through discharge papillae in the host wall (Fig 1A). After zoospore discharge, host compartments are empty, and the irregular configuration of septa is visible (Fig 1B).

Unlike sporangial plasmodium development, during resting spore development multiple plasmodia occupy a host compartment (Fig 1C). Plasmodia developing into resting spores are irregular in outline at first but eventually become spherical (Fig 1C). The spherical plasmodia then become more compact, and walls appear around the resting spore plasmodia (Fig 1D). Resting spore wall formation is not synchronous within a compartment (Fig 1D) and some rounded plasmodia abort before maturing into resting spores (Fig 1E). Remnants of host cytoplasm remain in host compartments after resting spores are formed (Fig 1C–E).

Transmission electron microscopy of host–parasite interface

Sporangial plasmodial stage

The developing sporangial plasmodium is lobed and irregular in outline (Fig 2A). There are numerous nuclei which are distinctive from host nuclei because the nucleolus is crescent-shaped and adpressed to the inner surface of the nuclear envelope (Fig 2A), rather than oval and peripherally located in

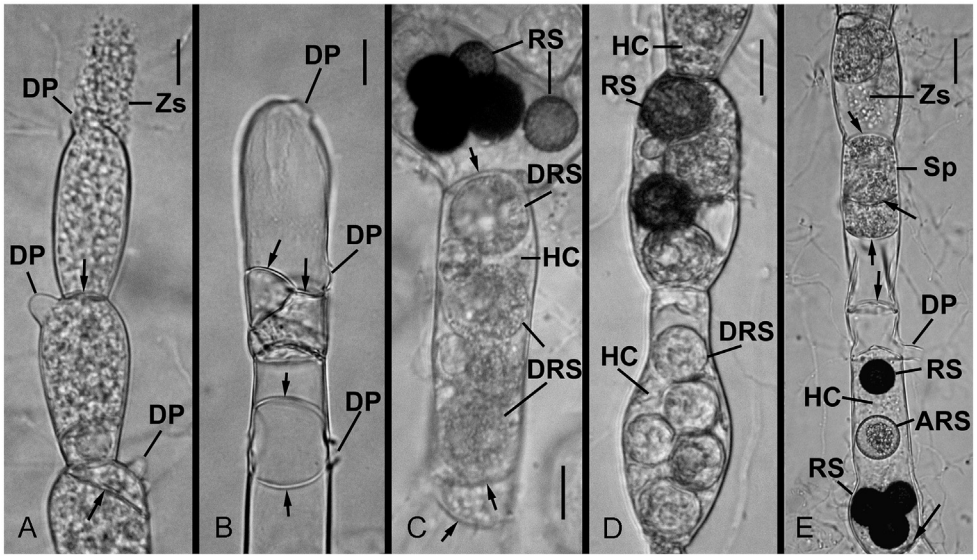

Fig 1 – Light microscopy of developmental stages of *R. allomycis* parasitizing *A. anomalus* on 1/8 YPSS agar. (A) Discharge papillae form as parasite's sporangial plasmodia develop basipetally within host hypha. Septa (arrows) separate individual plasmodia. Parasite zoospores seen discharging from apical host compartment. (B) Empty host hypha after parasite zoospore discharge. Two discharge papillae are visible on the apical host compartment. Septa (arrows) are irregularly organized in host, which contains no residual cytoplasm. (C, D) Multiple parasite resting spore plasmodia form within a single host compartment. Host hypertrophy is more pronounced than with sporangial plasmodium infection. Residual host protoplasm remains as resting spores develop and mature with the production of spiny walls. (E) More apically-produced sporangial plasmodia release zoospores, as resting spore plasmodia form basipetally in succession. Within a compartment one resting spore appears abortive. Septa delimit compartments (arrows). Bars in A–E = 10 μm. Abbreviations: ARS, abortive resting spore; DP, discharge papilla; DRS, developing resting spore; HC, host cytoplasm; RS, resting spore; Sp, parasite sporangial plasmodium; Zs, zoospores.

the nucleoplasm (Fig 2A). Multiple vacuoles are present in the parasite (Fig 2A). Clusters of lipid globules are scattered in the cytoplasm (Fig 2A and B); but unlike lipid globules in the host, where microbodies associated with lipid globules are prominent, microbodies were never observed associated with the parasite's lipid globules (Fig 2B). The parasite cytoplasm is densely packed with ribosomes (visible as scattered electron-dense granules) (Fig 2A and B). Endoplasmic reticulum is sparse, and there is no evidence of a Golgi apparatus with regularly stacked cisternae. Host mitochondria are abundant, and some align along the surface of the developing sporangial plasmodium (Fig 2A, B, D). Mitochondria are scattered in the parasite but appear depauperate, with few cristae and a diffuse matrix (Fig 2A, C). In contrast host mitochondria have well-developed plate-like cristae and a dense matrix (Fig 2A, B, D). The interface between the host and sporangial plasmodium consists of three-membrane layers, which we interpret as the parasite plasma membrane (inner-one layer) and host cisterna (outer-two layers) with the innermost membrane of the host cisterna closely juxtaposed to the parasite's plasma membrane (Fig 2D). No ribosomes were observed on the host cisterna.

Resting spore plasmodial stage

Each resting spore plasmodium contains a single nucleus (Figs 3A and 4A), distinctive from sporangial plasmodia with multiple nuclei (Fig 2A). The contours of developing resting spore plasmodia are irregular (Fig 3A) but assume an overall spherical shape toward maturity (Fig 4A). Numerous lipid globules surround the nucleus (Figs 3A and 4A), and cored vesicles (vesicles containing an electron-dense globule) are scattered throughout the cytoplasm (Figs 3B and 4A). Multivesicular vesicles (smaller vesicles contain small spheres) (Fig 3B) and vacuoles are also present. Mitochondria in resting spore plasmodia have few cristae and a diffuse matrix (Fig 3B), which distinguishes them from host mitochondria with numerous well-developed cristae and dense matrices (Fig 3B). Host mitochondria align along the surface of resting spore plasmodia (Figs 3A, B, C, 4A, B), essentially surrounding each within the host cell. As with the host-sporangial plasmodium interface, the interface between host and resting spore plasmodium consists of three membranes (Fig 3B, C, D), which we interpret as a host cisterna surrounding the parasite plasma membrane. Remnants of host protoplasm, including concentric granules (Fig 4B), remain as resting spore plasmodia develop (Figs 3A and 4A, B).

Sometimes among resting spore plasmodia, there are plasmodia that appear to degenerate and abort development (Fig 4A), just as observed with light microscopy (Fig 1E). In these situations, host mitochondria surround the surface of developing resting spore plasmodia, but not the aborting resting spore plasmodium (Fig 4A). In other cells developing resting spore plasmodia and sporangial plasmodia are found in the same host compartment, but the sporangial plasmodium appears to degenerate as evidenced with swollen nuclear envelopes of its multiple nuclei (Fig 4B). As with the aborting

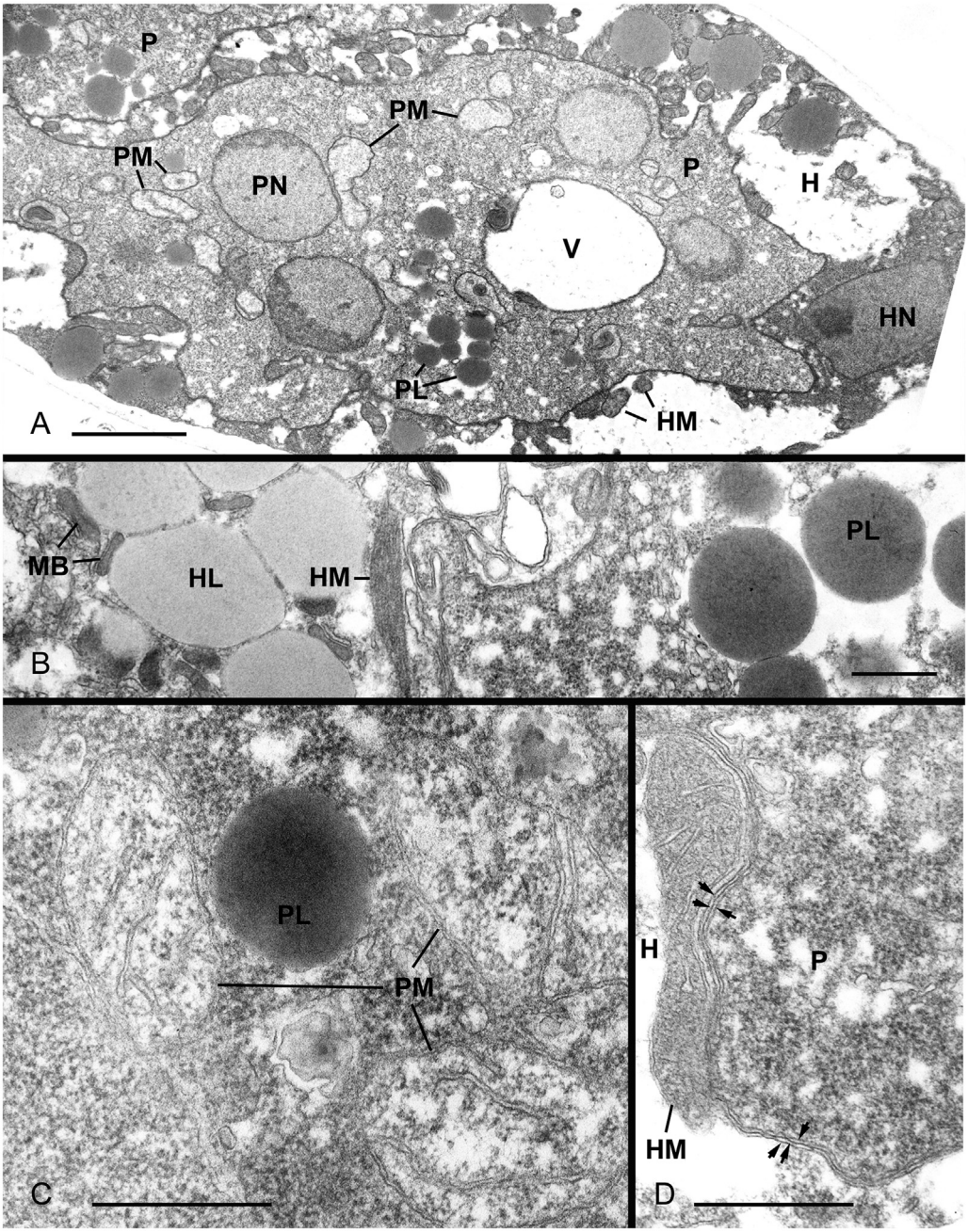

Fig 2 – Ultrastructural features of parasite sporangial plasmodia in host. (A) Developing sporangial plasmodium with irregular outline partially fills a host compartment. It contains multiple nuclei, a prominent vacuole, mitochondria with few cristae, and clusters of lipid globules, but no microbodies are evident. The nucleolus is crescent-shaped and adjacent to the parasite nuclear envelope. Host nuclei are larger than those of the parasite and contain spherical nucleoli. Host mitochondria align along the surface of the parasite. (B) Microbodies are associated with host lipid globules but not those of the parasite. (C) Parasite mitochondria with few cristae and diffuse matrix. (D) Host mitochondria with numerous cristae and dense matrix. Three layers of membranes form the host–parasite interface consisting of the parasite's plasma membrane (single arrow) and presumed host cisterna forming the other two layers (double arrows). Bars in A = 2 μm, B–D = 0.5 μm. Abbreviations: H, host; HL, host lipid globule; HM, host mitochondrion; HN, host nucleus; MB, host microbody; P, parasite; PL, parasite lipid globule; PM, parasite mitochondrion; PN, parasite nucleus; V, parasite vacuole.

resting spore (Fig 4A), the degenerative sporangial plasmodium had few mitochondria at its surface while the resting spore plasmodium was encircled with host mitochondria (Fig 4B).

Evidence for phagocytosis of host cytoplasm

Differences in mitochondrial structure aid in distinguishing parasite mitochondria and host mitochondria (Figs 2C and D, 3B). Lobes of the developing sporangial plasmodium

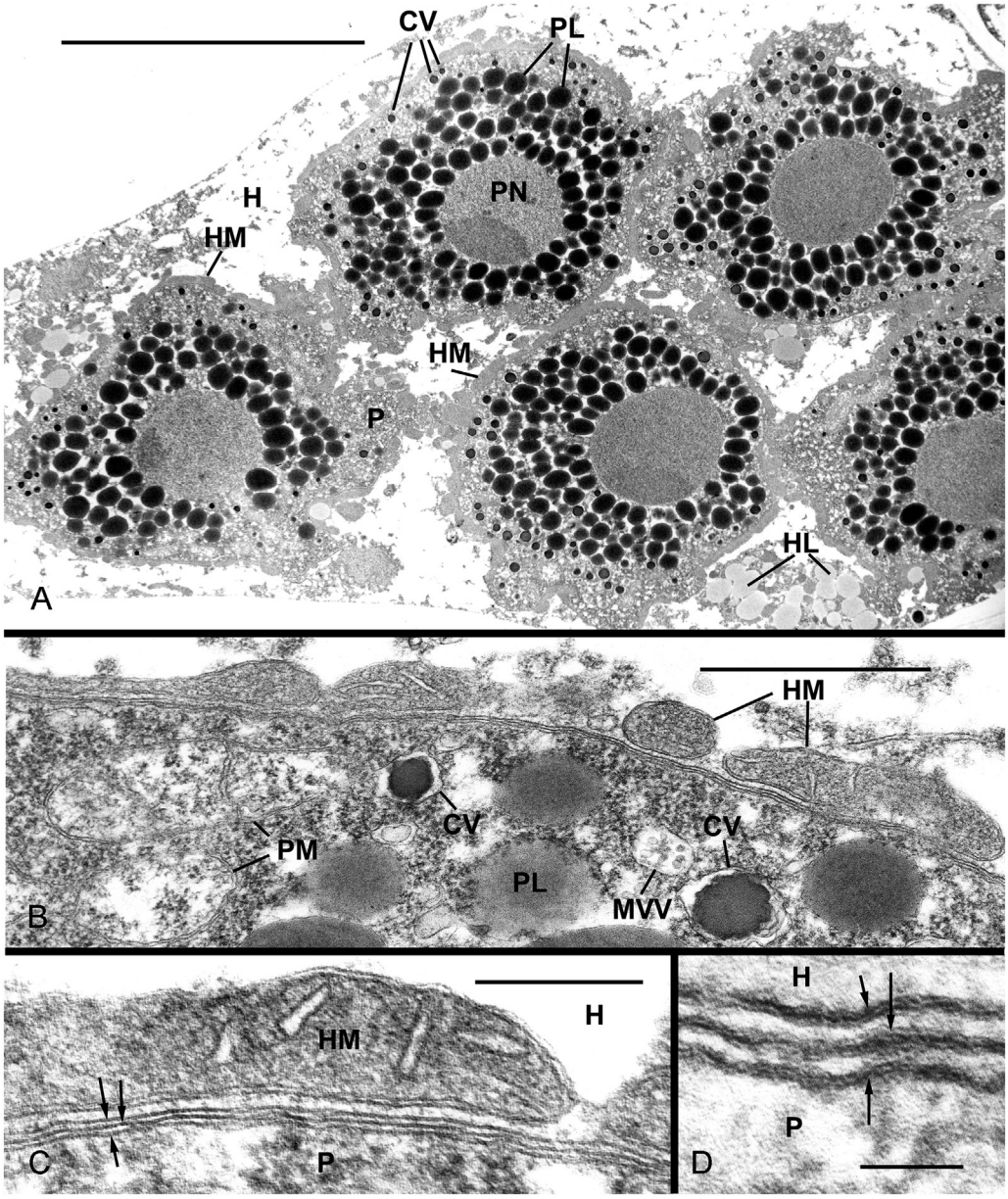

Fig 3 – Ultrastructural features of parasite resting spore plasmodia in host. (A) Multiple resting spore plasmodia in a single compartment, each plasmodium containing a single nucleus and numerous lipid globules and cored vesicles. (B) Host mitochondria align along the surface of the parasite resting spore plasmodium. Compare morphology of host *versus* parasite mitochondria and observe cored vesicles and multivesicular vesicle in parasite. (C, D) Notice the three-layered interface consisting of parasite plasma membrane (single arrows) and host cisterna (double arrows). The tri-lamellar structure of the unit membrane is visible in D. Bars in A = 10 μm, B = 1 μm, C = 0.25 μm, D = 50 μm. Abbreviations: CV, cored vesicle; H, host; HL, host lipid globule; HM, host mitochondrion; MVV, multivesicular vesicles; P, parasite; PL, parasite lipid globule; PM, parasite mitochondrion; PN, parasite nucleus.

partially encompass segments of the host cytoplasm (Fig 2A). The sporangial plasmodium eventually completely fills the host compartment (Fig 5A). Within the sporangial plasmodium, numerous phagocytic vacuoles contain host-type mitochondria lining their membranes (Fig 5A, B, F). These vacuoles contain additional organelles including lipid globules, nuclei, and vesicles (Fig 5B), and the contents appear to be breaking down (Fig 5A). We tracked the appearance and disappearance of these vacuoles in serial sections and found that these vacuoles are not merely in pockets of host cytoplasm into the sporangial plasmodium; rather they are discrete vacuoles within the plasmodium. Phagocytic vacuoles with abundant cellular contents have three-layered membranes (Fig 5C and D), but in some regions two-layered membranes (Fig 5E). The vacuolar membrane is two-layered or one-layered in vacuoles with less dense matrices (Fig 5F). A summary of stages in the formation of the phagocytic vacuole is illustrated in Fig 6.

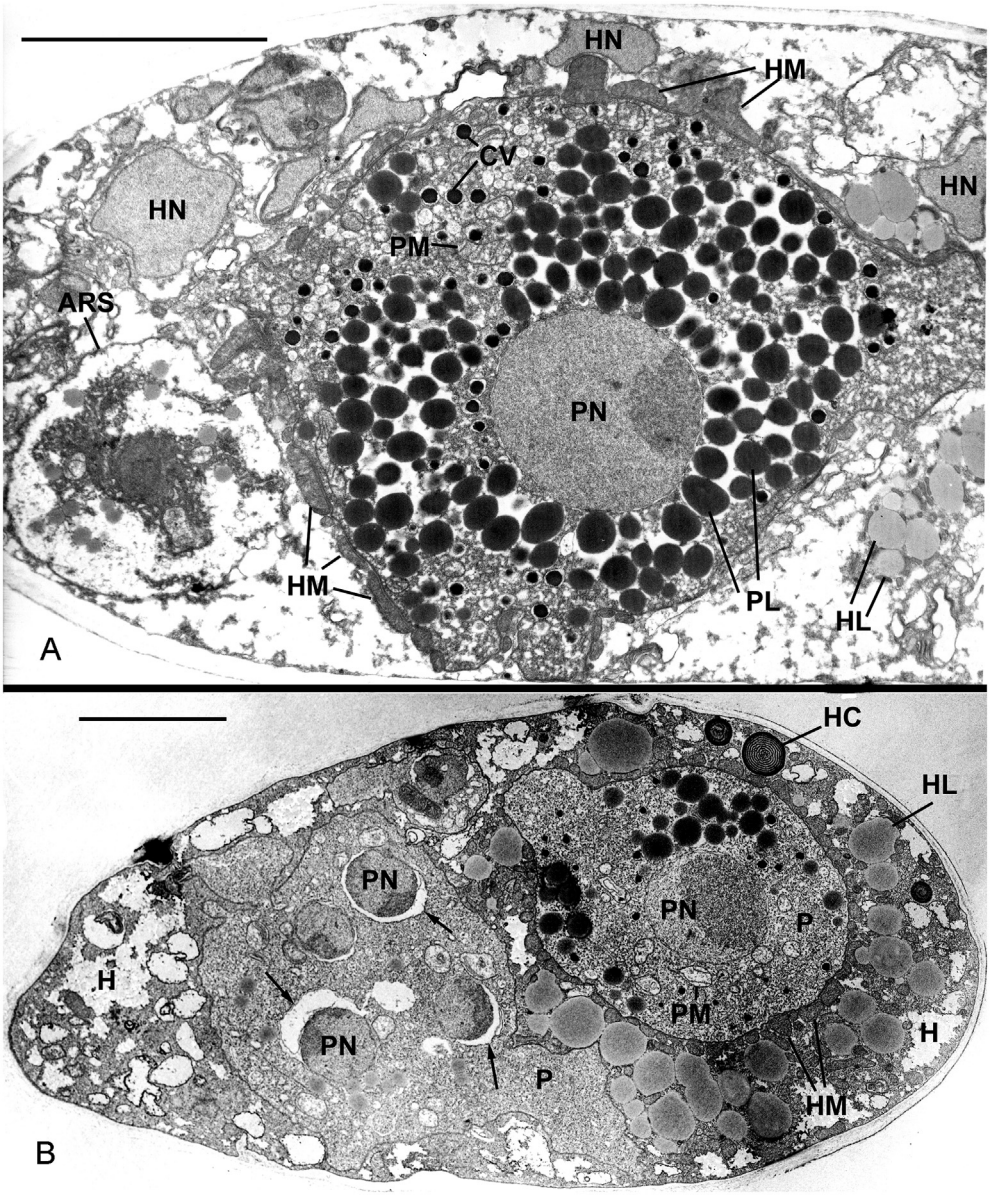

Fig 4 – Ultrastructural features of parasite in host. (A) Resting spore plasmodium at right contains a single nucleus, numerous lipid globules, and cored vesicles. Host mitochondria cover the surface of the resting spore plasmodium. An abortive resting spore at left lacks host mitochondria at its surface. Host cytoplasm contains host nuclei and lipid globules. (B) Host with resting spore plasmodium at right and an apparently abortive sporangial plasmodium at left. Host mitochondria cover the surface of the resting spore plasmodium but not the sporangial plasmodium. Swollen nuclear envelopes (arrows) suggest degradation of the sporangial plasmodium (based on several nuclei in plasmodium). A, B = 5 μm. Abbreviations: ARS, abortive resting spore; CV, parasite cored vesicles; H, host; HC, host concentric granule; HN, host nucleus; HL, host lipid globule; HN, host nucleus; HM, host mitochondrion; PM, parasite mitochondrion; PN, parasite nucleus.

Discussion

Development

The parasite induces host hyphae to produce septa, confining the parasite in compartments that are sometimes swollen. Host cell compartments contain either sporangial plasmodia or resting spores, but not both at maturity of the parasite stage. A single sporangial plasmodium completely fills a host compartment, but multiple resting spore plasmodia occur in a host compartment with residual host cytoplasm remaining. Sporangial plasmodia never produce a wall but rather use their host's walls; whereas, resting spore plasmodia ultimately generate multilayered walls (Karling 1942, 1977). Held (1981) discussed why it may be advantageous for

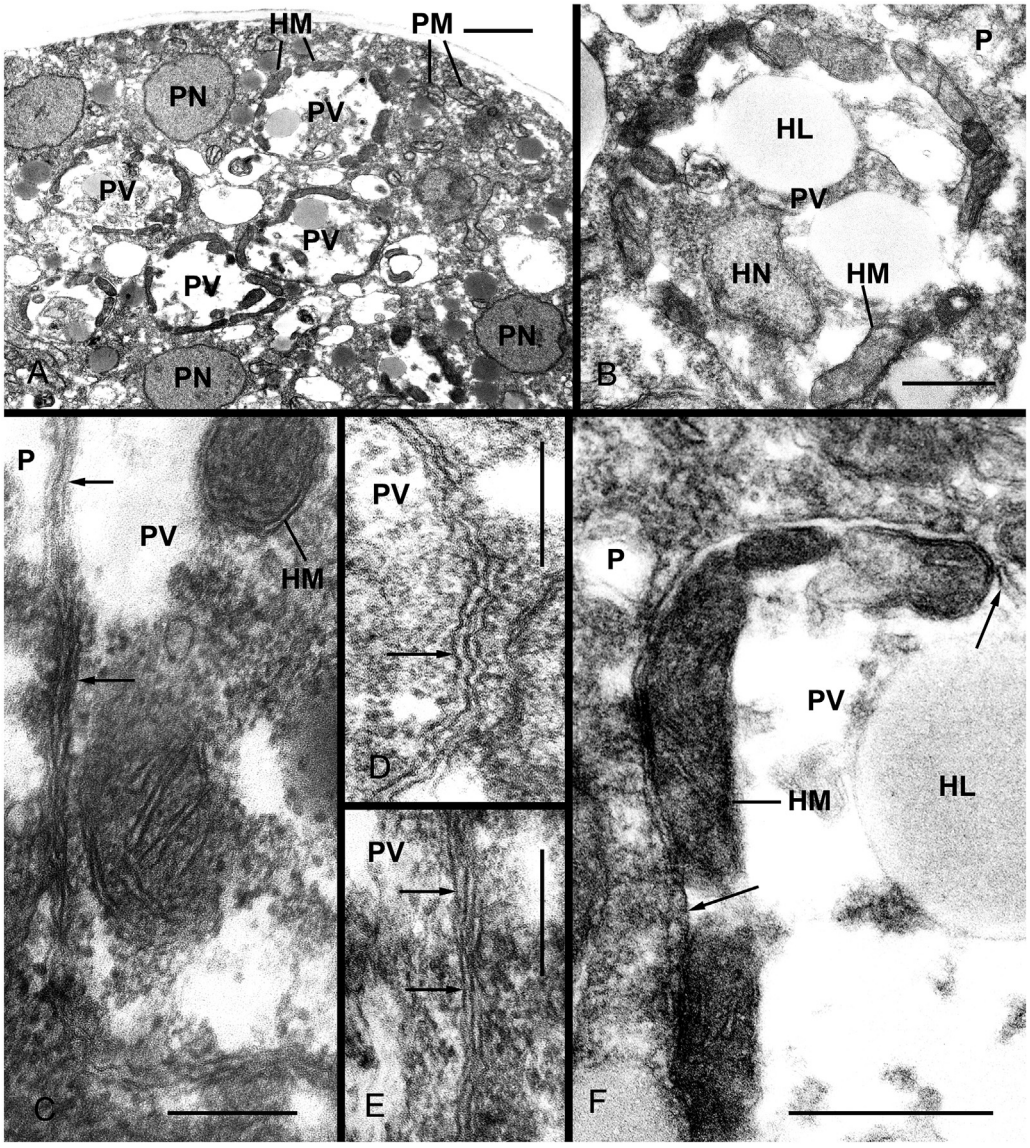

Fig 5 – Ultrastructural features of parasite phagocytosis of host cytoplasm. (A) Sporangial plasmodium completely fills host compartment (no host cytoplasm remains); contains scattered nuclei and mitochondria as well as numerous phagocytic vacuoles with host-type mitochondria lining the inner membrane surface of phagocytic vacuoles. (B) Detail of contents of phagocytic vacuoles. Host-type mitochondria line the inner membrane of the phagocytic vacuole. The vacuole also contains a host nucleus, lipid globules, and scattered ribosomes. (C, D, E) Details of the phagocytic vacuolar membrane, which initially consists of three-membrane layers (arrows). (F) Phagocytic vacuolar membrane consisting of two-membrane layers (arrows). A = 2 μm, B = 0.5 μm, C, D, E = 0.25 μm, F = 0.5 μm. Abbreviations: H, host; HL, host lipid globule; HM, host mitochondrion; HN, host nucleus; P, parasite; PM, parasite mitochondrion; PN, parasite nucleus; PV, parasite phagocytic vacuole.

Rozella allomycis to occupy a host compartment with a single sporangial plasmodium due to the need for adequate cytoplasmic pressure for zoospore discharge. In his hypothetical scheme (Held 1981), a single sporangial plasmodium completely filling the host compartment could produce the turgor pressure necessary for zoospore discharge with the aid of the host cell wall. This hypothesis forms the basis for understanding why at maturity, sporangial plasmodia and resting spores do not share a single host compartment, as sporangial plasmodia depend on their host cell walls for turgor and resting spores produce their own cell walls, not depending upon the host cell wall for turgor.

It is interesting that resting spore plasmodia in a single compartment may not all associate equally with host mitochondria; and those without host mitochondrial associations appear to degenerate. Similarly once resting spores begin to develop in a compartment, mitochondria are associated with their surfaces but not the surfaces of sporangial plasmodia, which may initially share a compartment with resting spore plasmodia, but which later degenerate. One possible

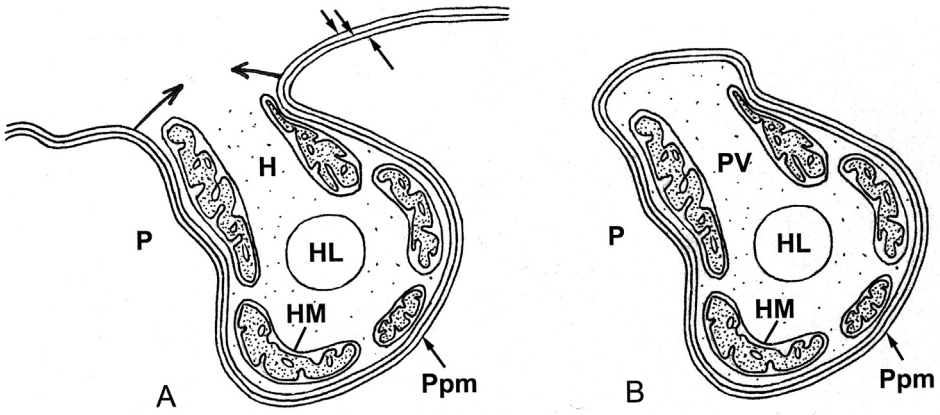

Fig 6 – Schematic interpretation of phagocytic vacuole formation. (A) Lobes of the plasmodium partially surround portions of host cytoplasm. Three-membrane layers form host parasite interface consisting of host cisterna (double small arrows) and parasite plasma membrane (small single arrow). Membranes come together (large arrows). (B) Membrane fusion results in a three-layered vacuolar membrane and host-type mitochondria lining the inner membrane of the phagocytic vacuole. Abbreviations: H, host; HL, host lipid globule; HM, host mitochondrion; P, parasite; Ppm, parasite plasma membrane; PV, parasite phagocytic vacuole.

explanation for this phenomenon is that normal plasmodia are able to recruit mitochondria, but abnormal plasmodia are not. An alternate hypothesis is that the association of mitochondria with the parasite's surface in *Rozella* is required for its survival, and plasmodia compete within the same host compartments for association with host mitochondria, which may be necessary for enhanced ATP acquisition from its host. This alternate hypothesis presents a mechanism for preventing resting spores and sporangia from occupying the same host compartment.

How *Rozella* stores nutrients for energy is an unexplored area. *Rozella* produces clusters of lipid globules, which might be used as a stored energy source, but the absence of microbodies adjacent to lipid globules in plasmodia, in contrast to the host, indicates the glyoxylate cycle is not the metabolic route for their utilization in the plasmodium (Powell 1976). It is significant that the zoospore stage, which presumably depends upon utilization of its own stored reserves, have mitochondria with well-formed cristae and a dense matrix (Held 1975), suggesting metabolic differences between the plasmodium and zoospore in ATP generation. Similarly, although microbodies were not apparently associated with plasmodial lipid globules, they are associated with zoospore lipid globules (Held 1975; Letcher *et al.* 2016b). The association of microbodies with lipid globules in zoospore of *Rozella* suggests that unlike the plasmodium the zoospore may utilize stored lipids by the glyoxylate cycle as in Chytridiomycota (Powell 1976).

Comparative host–parasite interfaces

We interpret the interface between *Allomyces* and *Rozella allomycis* sporangial plasmodia and resting spore plasmodia as consisting of the parasite plasma membrane surrounded by the host cisterna and mitochondria. *Allomyces* is the host for two zoosporic endoparasites, *R. allomycis* (Cryptomycota) and *Catenaria allomycis* (Blastocladiomycota) (Sparrow 1960). With both endoparasites of *Allomyces*, there is direct contact between the parasite surface and host cytoplasm. How these organisms breach the host plasma membrane without killing the host has not been resolved. *Rozella allomycis* and *C. allomycis* differ in that, although both begin their infection in host cytoplasm as unwalled protoplasts, *C. allomycis* early in development forms a cell wall and grows into a polycentric thallus (Powell 1982) whereas *R. allomycis* enlarges into an unwalled sporangial plasmodium filling the host. The host *Allomyces* responds differently to the two parasites in several ways. The induction of host septal formation which compartmentalizes the infection occurs with *R. allomycis*, but not *C. allomycis* (Powell 1982). In *R. allomycis* a cisterna with no signs of ribosomes surrounds a plasmodium, but in *C. allomycis* host rough endoplasmic reticulum clusters around the parasite forming a net rather than an envelope around the parasite (Powell 1982). Lipids sometimes clustered near the surface of *C. allomycis*, but host mitochondria do not align along the surface of the parasite as they do at the interface with *R. allomycis*. Thus, these two parasites of *Allomyces* induce different host responses.

The only other species of *Rozella* studied with transmission electron microscopy are *Rozella polyphagi*, a parasite of the Chytridiomycota *Polyphagus euglenae* (Powell 1984), and *Rozella rhizoclosmatii*, a parasite of the Chytridiomycete *Rhizoclosmatium globosum* (Letcher *et al.* 2016b). As with *R. allomycis*, *R. polyphagi*, and *R. rhizoclosmatii* plasmodium are in direct contact with host cytoplasm. In *R. polyphagi* patches of host smooth and rough endoplasmic reticulum cover portions of the plasmodium but do not envelope the plasmodium (Powell 1984), as does the host cisterna with *R. allomycis*. The host–parasite interface for *R. rhizoclosmatii* is more similar to that found for

R. allomycis because it consists of three-membrane layers (Letcher *et al.* 2016b).

The host–parasite interface between *R. allomycis* and *Allomyces anomalus* exhibits striking similarities to the interface between some microsporidia and their hosts. Microsporidia are obligate intracellular parasites of animal cells and are sister to Cryptomycota in most molecular phylogenetic analyses, indicating a shared common ancestor (Haag *et al.* 2014; James *et al.* 2013). The Microsporidia exhibit diverse host parasite interfaces (Cali *et al.* 2011), even within the same host, sometimes changing during parasite development (Mansour *et al.* 2005). As in *R. allomycis,* a host cisterna surrounds the plasmodium in some Microsporidia, lying close to the parasite's surface (Cali *et al.* 2011; Lom & Nilsen 2003; Mansour *et al.* 2005). In other microsporidia, plasmodia are confined within a parasitophorous vacuole (Rönnenbaumer *et al.* 2008; Scanlon *et al.* 2004); however, similar to the *R. allomycis* interface, host cisternae and mitochondria are closely appressed to the parasitophorous vacuole surface, an interface found among some Apicomplexa, a distantly related group of endoparasites (Sinai *et al.* 1997).

Evidence for parasite recruitment of host mitochondria

Recent genomic analyses have shown that the *Rozella allomycis* mitochondrial genome is reduced but present (James & Berbee 2012). The poorly developed cristae of *Rozella* mitochondria in the plasmodium indicate a reduction in the electron transport system and a resultant reduction in ability to generate ATP in the thallus mitochondrion. Ultrastructural demonstration of the proximal position of host cisternae and mitochondria at the surface of plasmodia of *R. allomycis* suggests a functional role in the transfer of host lipids and energy to the parasite.

From genomic analyses, James *et al.* (2013) identified nucleotide transport genes in *R. allomycis* like those found in Microsporidia and used to acquire ATP from their hosts (Corradi 2015; Tsaousis *et al.* 2008). Concordant with this discovery, it can be hypothesized that *R. allomycis* plasmodia recruit host mitochondria as a mechanism to acquire ATP from host mitochondria, with the nucleotide transporter proteins bringing ATP from the host cytoplasm into the parasite cytoplasm. Alternatively, it is also possible that nucleotide transporter proteins might bring ATP produced by the host mitochondria sequestered in phagocytic vacuoles in sporangial plasmodia into the parasite cytoplasm. The association of mitochondria with developing resting spore plasmodia is even more striking than for sporangial plasmodia. This may reflect the fact that resting spores represent a contraction of parasite cytoplasm that is no longer undergoing phagocytosis, and therefore more dependent upon transmembrane import of host ATP.

It is noteworthy, however, that the absence of a detectable ATP transporter gene from the microsporidian *Mitosporidium daphnia*, coupled with the aggregation of host mitochondria to the periphery of the parasitophorous vacuole (Haag *et al.* 2014), imply that the subcellular location of host mitochondria may have many roles (Heinz *et al.* 2014). It is easier to envisage a benefit to the parasite rather than to the host as the result of this spatial relationship; but the localization could support numerous functions in which ATP is required. Moreover, until biochemical analysis of the *Rozella* nucleotide transport genes related to Microsporidia nucleotide transport genes is performed (Tsaousis *et al.* 2008), it is premature to assume that the highest affinity nucleotide for uptake will be ATP.

As observed in *R. allomycis*, mitochondrial cristae and matrix of *Rozella polyphagi* (Powell 1984) and *Rozella rhizoclosmatii* (Letcher *et al.* 2016b) are poorly developed. It is significant, therefore, that as observed around plasmodia of *R. allomycis,* host mitochondria may be found along the surfaces of *R. polyphagi* (Powell 1984; see figs 4, 5, 10), however, not as compactly over the parasite surface as in *R. allomycis*. Thus, *Rozella* plasmodia in these two species appear to recruit host mitochondria to their cell surface, a phenomenon also observed in plasmodia of a related group, the Microsporidia (Corradi 2015; Hacker *et al.* 2014; Scanlon *et al.* 2004; Tsaousis *et al.* 2008), and distantly related groups of endoparasites such as the Apicomplexa (Sinai *et al.* 1997). One similarity between *Rozella* and Apicomplexa is the absence of complex 1 of the respiratory chain (Vaidya & Mather 2009). Reduced potential for energy production may be causally related to mitochondrial attraction by parasites.

Evidence for phagocytosis

Serial section analysis of sporangial plasmodia demonstrates parasitic phagocytosis of host cytoplasm as the process whereby the sporangial plasmodium totally replaces the host cell contents within walled-off host compartments. A possible sequence of events in the phagocytosis of host cytoplasm and its digestion in phagocytic vacuoles is as follows (Fig 6):

a. Developing plasmodia produce many lobes which partially encompass host cytoplasm, allowing engulfment of portions of the host protoplasm. The host–parasite interface consists of the parasite plasma membrane (one layer), which a host cisterna (two-membrane layers) coats, and a layer of host mitochondria (Figs 3D and 6A). The fully expanded sporangial plasmodium contains numerous vacuoles enclosing organelles such as lipids, nuclei, ribosomes and host-type mitochondria.
b. Phagocytic vacuoles that are filled with contents and recently formed have a three-layered membrane. Because the plasma membrane of the developing sporangial plasmodium is surrounded by a host cisterna and mitochondria, engulfment of host contents results in a vacuole composed of three layers: an outer membrane layer (the parasite's plasma membrane) and two-inner-membrane layers (the host cisterna). Because of the topology of the host parasite interface, host mitochondria coat the inner surface of the vacuole (Fig 6B).
c. As contents of the phagocytic vacuoles are digested, their matrices become less dense, and the inner-two membranes (the host cisternae) are progressively broken down, reducing the membrane layers from three, to two and finally one. This sequence of events leaves the phagocytic vacuole with a membrane composed of parasite plasma membrane. When zoospore formation occurs, it is possible that the vacuole membrane contributes to delimitation of zoospores.

As was observed with *Rozella polyphagi* parasitizing the chytrid *Polyphagus euglenae* (Powell 1984), parasite mitochondria of *Rozella allomycis* have poorly developed cristae and matrices which distinguish them from host mitochondria. Host-type mitochondria were found in vacuoles in the plasmodium of both of these parasites. However unlike *R. allomycis* in *Allomyces*, host cisternae do not totally surround the parasite plasmodium, and at the site where the parasite appears to be engulfing host mitochondria, there is no investing host endoplasmic reticulum (Powell 1984). Consistent with this observation, we hypothesize that the phagocytic vacuole of *R. polyphagi* has a single membrane layer derived from the parasite's plasma membrane. Thus, our study of *R. allomycis* supports conclusions of an earlier ultrastructural investigation of *R. polyphagi* (Powell 1984) that *Rozella*, unlike traditional Fungi, is capable of phagotrophic nutrition. The behavior of resting spore plasmodia, where remnants of host cytoplasm remain, supports that osmotrophic nutrition may also be possible with *Rozella*.

Evolutionary transitions

Rozella exhibits shared as well as distinguishing characteristics with two related groups of obligate intracellular parasites, the Microsporidia and Aphelida, supporting the concept of a shared ancestry and later evolutionary divergence. Phagocytosis and flagellated-unwalled cells occur among Aphelida (Gromov 2000; Schnepf *et al.* 1971), but not Microsporidia. Whether phagotrophic behavior is limited to Cryptomycota and aphelids, and has been lost in more derived Microsporidia, however, remains to be tested. A number of other fungal intracellular parasites also have a naked protoplast infective stage (e.g., *Coelomomyces*), which is a prerequisite for phagotrophy.

Parasitic recruitment of host mitochondria occurs in *Rozella* and Microsporidia (Cali *et al.* 2011; Corradi 2015; Tsaousis *et al.* 2008) but not Aphelida. Aphelida plasmodia have mitochondria with well-developed cristae and dense matrices (Karpov *et al.* 2013, 2014; Letcher *et al.* 2013, 2015). In contrast mitochondria of *Rozella* plasmodia have poorly formed cristae and diffuse matrices. Stealing host ATP is a phenomenon documented in the Microsporidia with mitosomes, mitochondrial-like structures totally lacking cristae and a genome. The mitosome of Microsporidia represents extreme reduction in complexity and inability to generate ATP (Cali *et al.* 2011; Corradi 2015; Keeling *et al.* 2010; Tsaousis *et al.* 2008). *Rozella* mitochondria in turn are more developed than in *Paramicrosporidium*, another presumptive member of Cryptomycota, which is more closely related to Microsporidia (Corsaro *et al.* 2014). This gradation of complexity of mitochondria may represent transitional forms between the conventional structure of mitochondria and mitosomes of Microsporidia, indicating the evolution of an increasing dependency upon host by the parasite for energy acquisition (Corradi 2015).

Absence of a typical Golgi apparatus with stacked cisterna occurs in *Rozella* and Microsporidia (Beznoussenko *et al.* 2007; Cali *et al.* 2011; Corradi 2015) plasmodia, but Aphelida plasmodia have a typical Golgi apparatus (Letcher *et al.* 2013, 2015). Held (1975) labeled a tubular area surrounded by vesicles as the Golgi apparatus in *Rozella* zoospores, but never demonstrated cisternal stacking. A loss of stacking of cisternae in the Golgi apparatus is a transition observed among the fungi going from Chytridiomycota, with stacked cisternae (Powell 1994; Powell & Letcher 2014), to higher fungi with lack of stacking but with a single cisterna called Golgi equivalents (Bracker 1967; James *et al.* 2006). Even in a single fungal lineage, basally placed members of the Blastocladiomycota have a Golgi apparatus with stacked cisternae and more terminally placed members lack cisternal stacking of the Golgi apparatus (James *et al.* 2006; Letcher *et al.* 2016a). Interestingly in Microsporidia, Beznoussenko *et al.* (2007) cytochemically labeled single tubular cisternal networks or 'Golgi analogs' which resembled 'Golgi equivalents' in Fungi (Bracker 1967). These results imply that loss of cisternal stacking in the Golgi apparatus has occurred repeatedly and leads to diversification of secretory mechanisms in lineages.

Conclusions

From the sampling of *Rozella* species thus far, it is apparent that the host–parasite interface among species of *Rozella* varies. We have shown that the interface between *Allomyces* and *Rozella allomycis* sporangial plasmodia and resting spore plasmodia consists of three-membrane layers interpreted as the parasite plasma membrane surrounded by a host cisterna and mitochondria. Thus, *R. allomycis* plasmodia are in direct contact with host cytoplasm, enveloped by a host cisterna. Both sporangial and resting spore plasmodia may recruit host mitochondria to their surfaces, which align along the outer membrane of the host cisterna. This recruitment has also been observed in Microsporidia and may reflect the active uptake of ATP from the host by the parasite through the nucleotide transporter proteins whose genes were horizontally acquired from *Chlamydia* (Tsaousis *et al.* 2008). The sporangial plasmodium totally fills the host compartment with no host contents remaining after zoospore discharge. The topology of membranes and presence of host-type mitochondria in vacuoles of sporangial plasmodia support phagocytosis as a mode of nutrition for *Rozella* and the reason no host contents remain in host compartments after sporangial plasmodium zoospore discharge.

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