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Ultrastructure of early stages of *Rozella allomycis* (*Cryptomycota*) infection of its host, *Allomyces macrogynus* (*Blastocladiomycota*)

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ABSTRACT

This study reconstructs early stages of *Rozella allomycis* endoparasitic infection of its host, *Allomyces macrogynus*. Young thalli of *A. macrogynus* were inoculated with suspensions of *R. allomycis* zoospores and allowed to develop for 120 h. Infected thalli at intervals were fixed for electron microscopy and observed. Zoospores were attracted to host thalli, encysted on their surfaces, and penetrated their walls with an infection tube. The parasite cyst discharged its protoplast through an infection tube, which invaginated the host plasma membrane. The host plasma membrane then surrounded the parasite protoplast and formed a compartment confining it inside host cytoplasm. The earliest host-parasite interface within host cytoplasm consisted of two membranes, the outer layer the host plasma membrane and the inner layer the parasite plasma membrane. At first a wide space separated the two membranes and no material was observed within this space. Later, as the endoparasite thallus expanded within the compartment, the two membranes became closely appressed. As the endoparasite thallus continued to enlarge, the interface developed into three membrane layers. Thus, host plasma membrane surrounded the parasite protoplast initially without the parasite having to pierce the host plasma membrane for entry. Significantly, host-derived membrane was at the interface throughout development.

1. Introduction

The objective of our research is to determine the mode of infection of the obligate endoparasite, *Rozella allomycis*, into its blastoclad host, *Allomyces*. Although ultrastructural studies demonstrate that the unwalled parasite thallus is located within host cytoplasm (Held, 1973a; Powell et al., 2017), how it gains entry into the host is unknown. *Rozella* parasitizes chytrids, oomycetes, and green algae and was once classified within the *Chytridiomycota* because of its production of posteriorly uniflagellate zoospores (Barr, 1980; Sparrow, 1960). Molecular analyses have revealed that phylogenetically *Rozella* lies outside the *Chytridiomycota* (James et al., 2006) and is now classified in the phylum *Cryptomycota* (Karpov et al., 2014a). In molecular phylogenetic studies, *R. allomycis* places together with other *Rozella* species (James et al., 2006; Letcher et al., 2017a, b) and commonly within a clade with two other groups of plasmodial endoparasites, the algal parasite *Aphelida* and the animal parasite *Microsporidia* (Letcher et al., 2013, 2015; Karpov et al., 2013, 2014b). These three groups have been named the *Opisthosporidia* (Karpov et al., 2014a), but their consistent phylogenetic placement in a monophyletic lineage is controversial (Bass et al., 2018; Tedersoo et al., 2018). It is however clear that they are related. As a clade sister of free-living nucleariids and *Chytridiomycota*, this lineage is widely viewed as an early-diverging branch of fungi (Berbee et al., 2017; James et al., 2013; Tedersoo et al., 2018), and as such can reveal adaptations in the evolution of fungi. The three groups have in common a stage in which chitin-containing walls are produced, but they are diverse in other ways. *Rozella* and aphelids reproduce with posteriorly uniflagellate zoospores, whereas *Microsporidia* reproduce with a walled spore containing a specialized structure for infection, the polar filament. In addition *Rozella* and aphelids are phagotrophic whereas *Microsporidia* are osmotrophic.

Of particular interest in reconstructing the evolutionary divergence of these groups from free-living nucleariids (Berbee et al., 2017) is understanding the evolution of nutritional modes. Key to understanding nutrition of obligate endoparasites is understanding how the parasite gains entry into host cells without damaging and

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killing the host at the onset of infection. An ultrastructural developmental study of *R. allomycis* in its blastoclad host, *Allomyces anomalous*, demonstrated that the parasite enters the host cytoplasm and totally phagocytizes the host from within the host cytoplasm (Powell et al., 2017). How the parasite gains entry directly into the host cytoplasm is not known. In another endoparasite of *Allomyces*, the blastoclad *Catenaria allomycis*, the parasite's thallus is totally immersed within host cytoplasm (Powell, 1982; Sykes and Porter, 1980). Unlike the thallus of *R. allomycis*, the thallus of *C. allomycis* appears to be walled at the earliest stage within the host (Sykes and Porter, 1980), even from the round-cell stage (Powell, 1982). Sykes and Porter (1980) concluded that during penetration into the host and entry into the host cytoplasm, the parasite ruptured the host plasma membrane, but did not immediately kill the host cell.

The purpose of this study is to reconstruct the early infection process of *R. allomycis* in its host *Allomyces macrogynus*. Held (1974) demonstrated that *Rozella* was a biotrophic obligate endoparasite because *Rozella* was not capable of infecting dead host hyphae. Consequently, the host-parasite interface is critical because this is the site of molecular and nutritional exchange necessary for maintaining the relationship between an obligate endoparasite and its living host required for the parasite's survival. Mode of entry into a relationship with the host cytoplasm is important because if the obligate parasite damages the host plasma membrane during the initial entry process, it could cause immediate death of the host cell and the inability of the parasite to continue development.

2. Materials and methods

2.1. Culture

R. allomycis (UM690) parasitic in *A. macrogynus* was obtained from Gerald Benny (University of Florida) who obtained it from the culture collection of R. K. Benjamin (Rancho Santa Ana Botanic Gardens, California). Single-membered cultures of *A. macrogynus* and dual-membered cultures of *A. macrogynus* infected with *R. allomycis* were maintained at 24 °C on 1/8 strength Emerson YPSS agar (Difco 273910) with a thin layer of water coating the agar surface.

2.2. Microscopy

On day 11 after inoculation of nutrient agar plates with the dual culture, *R. allomycis* zoospores were numerous and easily distinguished from the larger host spores. Twenty-four hr old thalli of *A. macrogynus* were inoculated with suspensions of *R. allomycis* zoospores and allowed to infect host hyphae and develop for 120 h. Infected hyphae were observed with a Nikon Eclipse E200 light microscope either directly on the agar surface or in a slide mount. For transmission electron microscopy, infected thalli at 2.5 h, 3.5 h, 4.0 h, 24 h, 72 h, 96 h, and 120 h post-infection were fixed for electron microscopy (Letcher and Powell, 2005; Powell et al., 2013). Material was thin embedded so that infected thalli could be selected, cut out, and mounted on plastic stubs for sectioning. Thin sections were cut with a Leica ultramicrotome, mounted on 300 mesh grids for random sections or coated slot grids for serial sections, and stained with uranyl acetate and lead citrate. Sections were observed on a Hitachi 7650 transmission electron microscope at 60 kV.

3. Results

3.1. Light microscopy of parasite's initial penetration into host

Twenty-four hour old host thalli consisted of a branched rhizoidal system and a single, unbranched hypha (Fig. 1A). Zoospores of *R. allomycis* mixed with young host thalli were attracted to the host and clustered around the host (Fig. 1A). At 2.5 h after inoculation, parasite zoospores encysted and attached to the host thalli (Fig. 1B). Although attachment to the rhizoids could occur (Fig. 1C), the hyphal portion of the 24hr-old host thallus was most densely infected (Fig. 1B). By 4.0 h after inoculation, small protoplasts of the parasite were visible in the host cytoplasm (Fig. 1C). At 24 h after inoculation, cultures consisted of a range of developmental stages because infection continued. From this stage multiple infections could be observed within a single host thallus (Fig. 1D).

3.2. Electron microscopy

3.2.1. Parasite infection of the host

Parasite zoospores contacted the host wall, rounded-up and produced a cyst wall (Fig. 2A). The cyst then produced an appressorium, which attached to the host wall (Fig. 2A, B). A large vacuole (the posterior vacuole) appeared in the cyst (Fig. 2B). The host cell wall changed in density (Fig. 2B) as an infection tube extended from an appressorium and penetrated through the host wall (Fig. 2C, D). The infection tube was peg-like at first (Fig. 2C). As the infection tube grew, it invaginated the host plasma membrane, which thickened at the area of contact. Granular material appeared between the host and parasite at this stage (Fig. 2C). The infection tube continued to elongate into the host, pushing the host plasma membrane inward as the parasite discharged its protoplast through an opening in the infection tube (Fig. 2D). The infection tube wall was thickened in the region it penetrated into the host cytoplasm (Fig. 2D). The cyst vacuole appeared to expand during this process (Fig. 2D) until the parasite protoplast was discharged, leaving only remnants of its cytoplasm in the cyst (Fig. 2E). The empty cysts (Fig. 2A, E) eventually collapsed (Fig. 2A).

3.2.2. Early host compartmentalization of parasite

At 3.5 h after inoculation, parasite spherical protoplasts lay in compartments within the host cytoplasm and near the host cell wall (Fig. 2A, F). The host-parasite interface at this stage was two layered with a wide space between the two membranes (Figs. 2D–F; 3A–C), the outer membrane layer being derived from the host plasma membrane and the inner layer being the parasite plasma membrane. The space between the two membranes was devoid of visible contents (Figs. 2F; 3A, C). There was variability in the width of space between the two membranes at the host-parasite interface within a compartment (Fig. 2F). The lumen could be wide in one area of the compartment while the parasite might be adjacent to the host membrane in another region of the compartment (Fig. 2F).

At this early stage of infection, host cytoplasm showed no signs of deterioration, such as excessive host vacuolization or organellar degeneration (Fig. 2A, F). As in parasite zoospores (Fig. 4E), mitochondria in the parasite early in infection are well developed with numerous plate-like cristae and dense matrices (Fig. 4A). Host mitochondria were sometimes closely appressed to the surface of the compartments (Figs. 2F; 3B), but this positioning may have only been the result of the presence of host mitochondria at the region of infection rather than host mitochondrial recruitment observed later in development (Fig. 4C, D).

3.2.3. Development of host-parasite interface

By 24 h after inoculation, the parasite enlarged into a plasmodium, and the two membranes of the compartment were close to each other with little space between the two membranes (Fig. 3D, E). At this stage, the parasite plasmodium outline was irregular as it produced lobed extensions (based on serial section analysis)

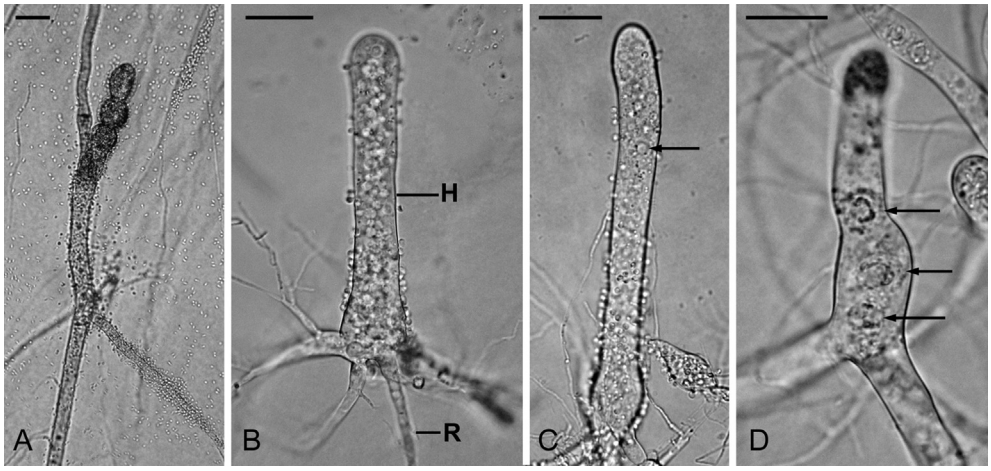

Fig. 1. Light microscopy of *R. allomycis* parasitizing *A. macrogynus* on 1/8 YPSS agar. (A) Ten minutes after twenty-four hr old host thalli were inoculated with parasite zoospores, zoospores cluster around host thalli. (B) Encysted parasite zoospores cover hyphal portion of host thallus 2.5 h after inoculation. (C) Parasite protoplast visible (arrow) in host 4.0 h after inoculation. Encysted parasite zoospores attach to both hyphal and rhizoidal portions of thallus. (D) Several parasite plasmodia (arrows) in hyphal portion of host thallus 24 h after inoculation. Scale bars in A–D = 30 μm. Abbreviations: H, hypha; R, rhizoids.

(Fig. 3D). No specific associations with host mitochondria were apparent. By this stage however, the parasite mitochondria have a depauperate appearance with few cristae and diffuse matrix (Fig. 4B) (Powell et al., 2017).

At 96 h the host-parasite interface consisted of three membranes (Fig. 4C, D). The outer two-membrane layers of the interface are interpreted as a host cisterna enveloping the parasite plasmodium and the inner membrane layer as the parasite's plasma membrane. Host mitochondria aligned along the surface of the host cisterna (Fig. 4C, D) and parasite mitochondria had depauperate appearances as illustrated at an earlier stage (Fig. 4B). Host cytoplasm still appeared normal (Fig. 4D), including the presence of the conspicuous concentric bodies (Fig. 4C).

3.2.4. *Parasite's total consumption of host*

Following this stage, the parasite began to phagocytize host cytoplasm (Powell et al., 2017). At 120 h after inoculation, the parasite plasmodium was transformed into a zoosporangium and totally filled the host. Inside of the host wall, zoospores were cleaved and no host contents or plasma membrane remained (Fig. 4E). In contrast to the parasite plasmodium at an earlier stage, mitochondria in zoospores had well developed cristae and matrices (Fig. 4E).

4. Discussion

4.1. *Development of the host-parasite interface in* R. allomycis

This study demonstrates that *R. allomycis* does not directly penetrate the host plasma membrane. We have now reconstructed the parasite's total vegetative development. The parasite zoospore encysts at the surface of the host and produces an appressorium. A large vacuole appears in the upper portion of the cyst cytoplasm. The host wall adjacent to the appressorium changes in texture, possibly due to enzymatic breakdown by the parasite. The appressorium then produces a peg-like structure which penetrates the host cell wall; and at this point, there appears to be deposition of material at the interface between the peg-like structure and host plasma membrane. The peg then elongates into an infection tube with a thickened wall and invaginates the host plasma membrane. The tip of the infection tube opens and through it the parasite protoplast is deposited into the space between the host cell wall and plasma membrane, possibly aided by the expansion of the cyst vacuole. Only fragments of cytoplasm remain in the emptied cyst, and the cyst wall eventually collapses.

The host then compartmentalizes the parasite protoplast in its own cytoplasm, enveloping the parasite with host-derived plasma membrane. Thus, early in infection the host-parasite interface is two-membrane-layered, with the outer membrane originating from the host plasma membrane and the inner membrane belonging to the parasite. When first formed there is a wide space between the host membrane and the parasite protoplast. With time the protoplast enlarges into a plasmodium and fills the lumen of the compartment, and the two membranes of the interface lie close to each other. It appears that the two-membrane layered host-parasite interface is maintained during the initial growth of the parasite plasmodium (~24 h), when host mitochondria are not regularly associated with the surface of the parasite plasmodium. The fact that the lumen of the space in the compartment becomes filled as the parasite protoplast expands, suggests that the outer membrane (derived from host plasma membrane) does not keep pace with the expanding parasite.

Later (96 h) the parasite plasmodium becomes extensive, and the host-parasite interface is composed of three-membrane layers. As the parasite plasmodium enlarges, it is possible that the outer-membrane layer (derived from the host plasma membrane) of the earlier formed two-membrane layered interface degenerates and is replaced with what we interpret as host cisterna (composed of two membrane layers), which envelopes the parasite plasmodium. Other origins of additional membrane could be plausible, such as merely an additional membrane (of either parasite or host origin) is added to the interface. The fact that the space between the initial investing host plasma membrane and parasite plasma membrane becomes narrower as the parasite plasmodium enlarges, however, suggests that the investing host plasma membrane does not continue to expand commensurately with the enlargement of the parasite. At this developmental stage, parasite mitochondria appear depauperate with poorly developed cristae and matrices (Powell et al., 2017) and host mitochondria cluster along the surface of the parasite plasmodium. Interestingly our study has shown that parasite mitochondria in the zoospore and early invading protoplast do not have the depauperate mitochondrial appearance as found at later stages and when host mitochondria are recruited to the surface of the parasite. This suggests that the

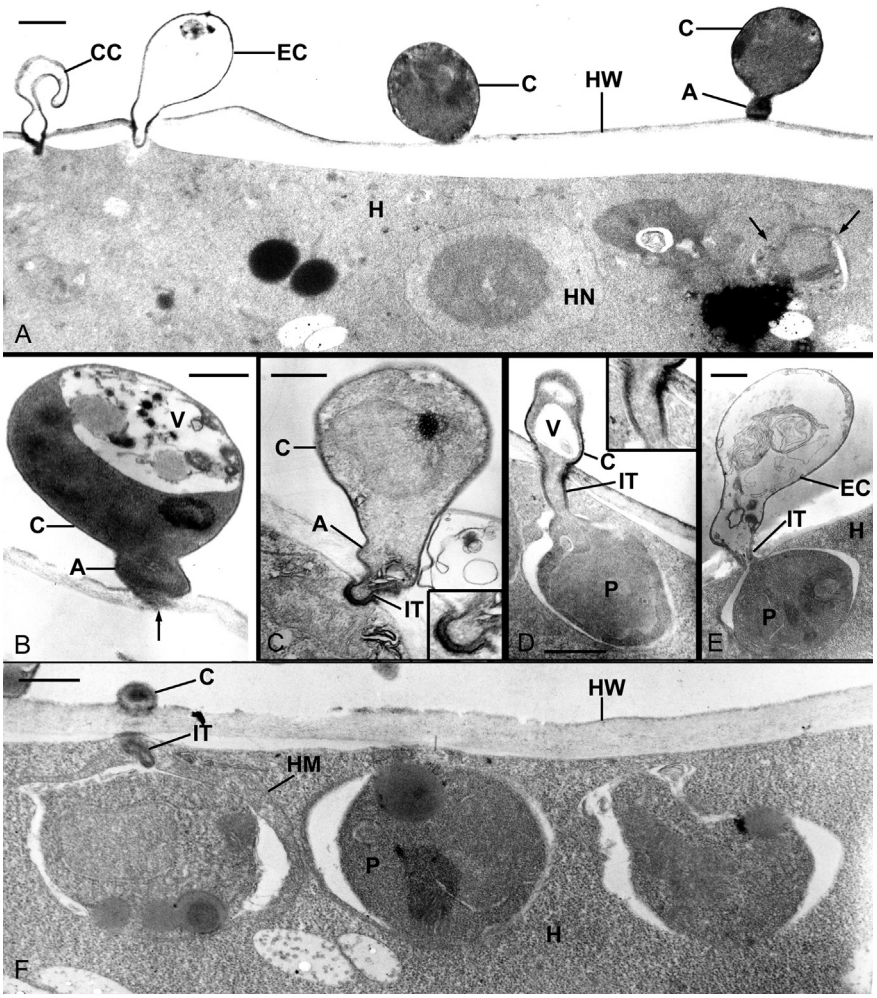

Fig. 2. Ultrastructural features of early parasite infection of host. A, B, D–F. 3.5 h; C. 2.5 h after inoculation. (A) Parasite cysts at different stages of development attached to host hypha wall. Host cytoplasm appears normal, including the nucleus. Parasite in compartment within host cytoplasm (arrows). Two-membrane layers (arrows) compose host-parasite interface, the outer membrane derived from host plasma membrane and the inner membrane the parasite plasma membrane. (B) Host cyst with posterior vacuole and appressorium. The host wall changes in appearance next to the infection tube, which is beginning to develop from the appressorium (arrow). (C) Parasite cyst with infection tube extending from the appressorium. Infection tube peg-like and invaginates host plasma membrane. The inset shows that the host plasma membrane appears thicker at this point and granular material is at the interface with the parasite. (D) Parasite cyst with vacuole, discharging protoplast into host. Infection tube penetrates host cell wall; end of tube opens allowing discharge of protoplast. Discharged parasite protoplast invaginates host plasma membrane, setting up the two-membrane layered host-parasite interface. Inset shows thickened infection tube wall where it extends into the host. (E) Empty parasite cyst with end of infection tube open and protoplast surrounded by host plasma membrane, forming a two-membrane layered interface. (F) Three parasite protoplasts near host cell wall and in host compartments. Variable width of space between two membranes of the host-parasite interface; lumen may be wide or the parasite may be adjacent to the host membrane. Host mitochondrion adjacent to outer-membrane layer of the compartment. Portions of the cyst and infection tube visible. Scale bars in A = 1.0 μm; B–F = 0.5 μm. Abbreviations: A, appressorium; C, cyst; CC, collapsed cyst; EC, empty cyst; H, host; HM, host mitochondrion; HN, host nucleus; HW, host wall; IT, infection tube; P, parasite; V, parasite vacuole.

parasite becomes progressively dependent upon the host for growth, supporting the hypothesis that the position of host cisterna and mitochondria at the surface of the parasite plasmodium has a functional role in the transfer of host lipids and energy to the parasite as it develops (Powell et al., 2017).

The three-layered interface persists as host mitochondria are recruited to the plasmodium and cover the surface of the cisterna and as the plasmodium begins to phagocytize host cytoplasm (Powell et al., 2017). Because of the topology of the host-parasite interface, phagocytic vacuoles consist of three membranes (parasite plasma membrane as the outer membrane plus the two-layered host cisterna), with mitochondria lining the inner layer and other host organelles occupying the lumen of the vacuole (Powell et al., 2017). With time contents in the phagocytic vacuoles are digested, including the two members of the host cisterna, leaving only the outer membrane of the vacuole, which originated from the parasite's plasma membrane (Powell et al., 2017). At the time the parasite's zoospores are produced, it is evident that the parasite sporangial plasmodium totally consumes host cytoplasm as well as the host plasma membrane.

4.2. *Development of the host-parasite interface in other* Rozella *strains*

We found as did Held that *R. allomycis* zoospores are rapidly attracted to young thalli of *Allomyces* (Held, 1974) and multiple sites of penetration occur on a single hypha resulting in multiple parasite protoplasts within host cytoplasm early in infection (Held, 1981). Held (1972, 1973a, 1973b) proposed that host wall appositions assisted the endoparasite in penetration into the host and failure to infect was because a wall apposition was not induced. Although we observed wall appositions at some sites of infection, in our study they were not necessary for the parasite's successful penetration of the host. Although not illustrated, Held (1973a) suggested that early

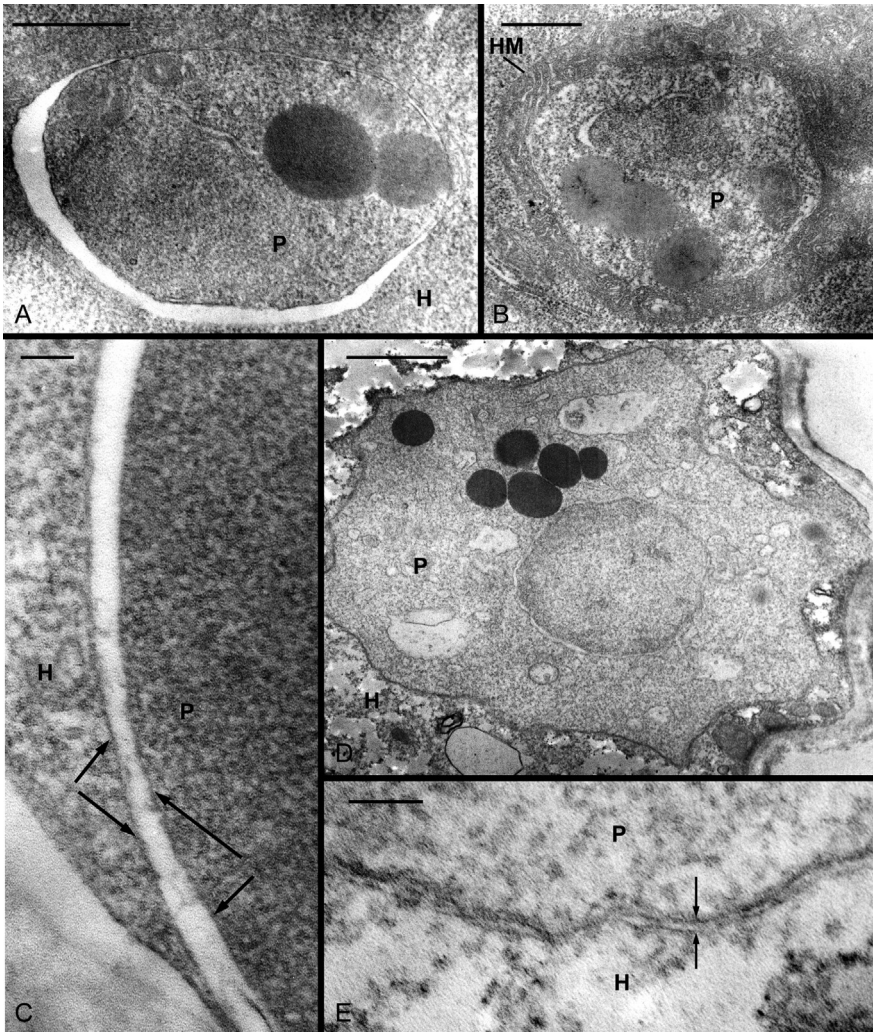

Fig. 3. Ultrastructural features of early parasite infection of host. A–C. 3.5 h; D–E. 24 hr after inoculation. (A) Detail of parasite protoplast within compartment in host cytoplasm. The host parasite interface consists of two membrane layers with space between the two layers. (B) Tangential section of host mitochondrion surrounding a portion of compartment. (C) Detail of the two membrane-layered host-parasite interface (arrows) and intervening space. (D) Parasite plasmodium with irregular outline expanding within the host compartment, with no specific interactions with host mitochondria. Little intervening space remains between two membranes of host-parasite interface. (E) Detail of two appressed membranes (arrows) forming the host-parasite interface as the parasite plasmodium enlarges within the host compartment. Scale bars in A, B = 0.5 μm; C, E = 0.1 μm; D = 1.0 μm. Abbreviations: H, host; HM, host mitochondrion; P, parasite.

in infection there was a two-membrane host-parasite interface between *R. allomycis* and *Allomyces*, which is what our study has demonstrated. Held (1973a) also reported later in the infection process the *R. allomycis* and *Allomyces* interface was a single-membrane, that of the parasite, which is in contrast to the three-layered membrane interface we observed. If indeed the surrounding host plasma membrane breaks down as the parasite fills the compartment, it is possible that the one-membrane stage is a transition stage between the two and three-membrane interface stages we observed.

Electron microscopy of only three additional species of *Rozella* has been reported (Letcher et al., 2017a, 2017b; Powell, 1984). The interface between *Rozella rhizoclosmatii* and its chytrid host consisted of three membranes, as in *R. allomycis* with its blastoclad host (Letcher et al., 2017b; Powell et al., 2017). In contrast, the host-parasite interface between *Rozella polyphagi* and its chytrid host consisted of a single membrane, the parasite plasma membrane, and scattered patches of host smooth and rough endoplasmic reticulum (Powell, 1984). As in *R. allomycis*, *R. polyphagi* plasmodium also appeared to recruit host mitochondria to its surface and to phagocytize host cytoplasm (Powell, 1984). The interface of *Rozella multimorpha* in its oomycete host was not examined (Letcher et al., 2017a). How these other species of *Rozella* gain entry into their hosts is still not resolved.

4.3. Comparison of endoparasite mechanisms of host infection

Our study has demonstrated that *R. allomycis* gains entry into the host cytoplasm without piercing the host plasma membrane. The complex organization of endoparasite-host interfaces varies depending on parasite and host, and sometimes during the progress of infection in the same parasite. This is exemplified in other groups of endoparasites that reproduce with flagellated spores. For example when the holocarpic oomycete endoparasite *Eurychasma dicksonii* invades its brown algal host, a two membrane interface is established, one of host origin and the other the plasma membrane of the parasite. The space between the two membranes becomes filled with the "ribbon-like fibrillar cell coat" of the parasite (Sekimoto et al., 2008). This type of interface suggests that the host plasma membrane is involved in enveloping the parasite as it

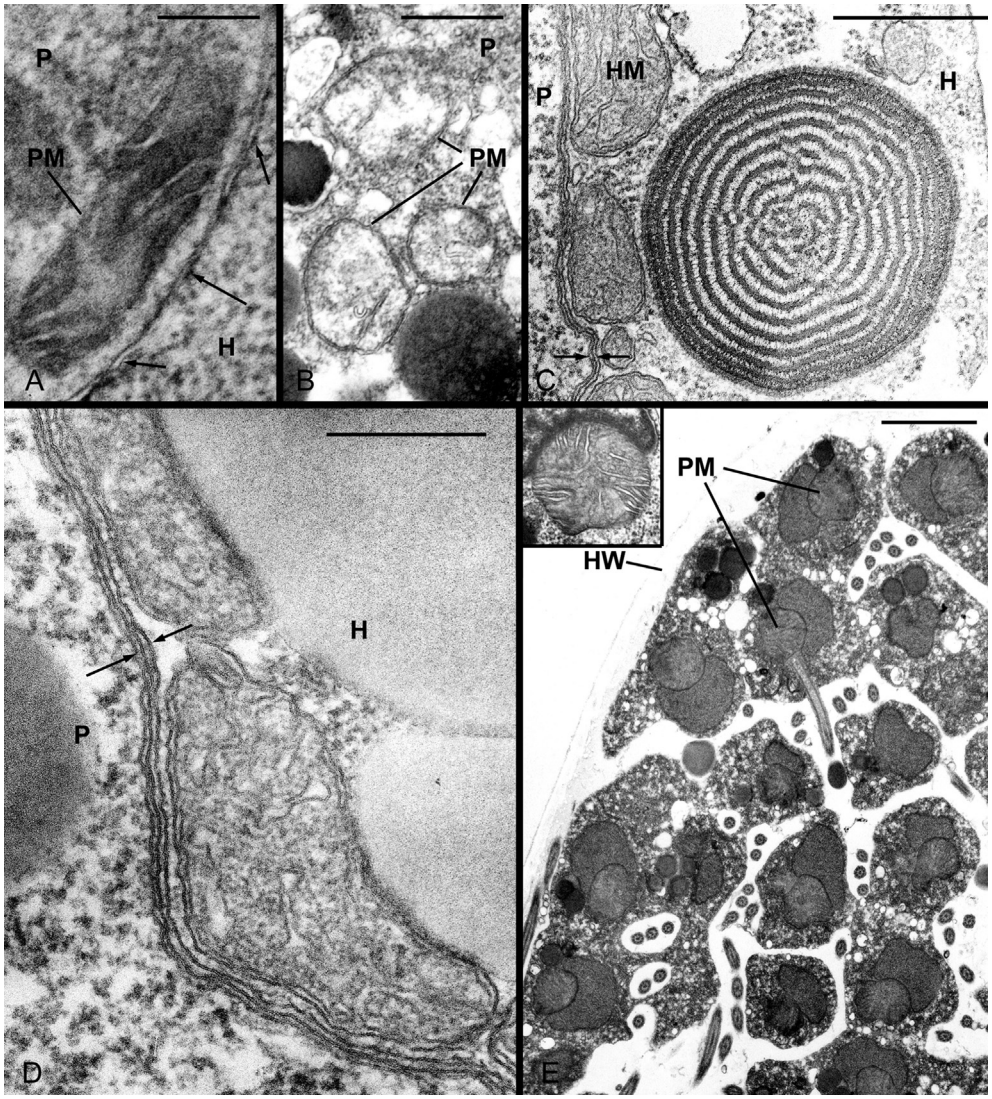

Fig. 4. Ultrastructural features of parasite infection of host. A. 2.5 h; B. 24 hr; C, D. 96 hr; E. 120 hr after inoculation. (A) Parasite mitochondrion early in development is well-developed with numerous plate-like cristae and a dense matrix. Arrows point out the two-membrane layered host-parasite interface in which, at this segment of the compartment, a portion of the parasite protoplast is adjacent to the surrounding host membrane. (B) Parasite mitochondria later in development are depauperate with few cristae and diffuse matrices. (C) Host parasite-interface 96 h after inoculation consisting of three-membrane layers (arrows). Host mitochondria align along surface of interface; host cytoplasm contains normal organelles, including conspicuous concentric bodies. (D) Detail of three-membrane layered host-parasite interface (arrows). (E) No host cytoplasmic contents remain 120 h after inoculation, and parasite zoospores cleave from the plasmodium, filling the space within the host wall. The inset at the upper left shows that parasite zoospore mitochondria have well-developed cristae and dense matrices. Scale bars in A, C = 0.5 μm; B = 0.2 μm; D = 0.25 μm; E = 2.0 μm. Abbreviations: H, host; HM, host mitochondrion; HW, host wall; P, parasite; PM, parasite mitochondrion.

enters the host cytoplasm. As another example, in the plasmodiophorids (another group of flagellates in the SAR supergroup) entry into the host was shown to be similar to entry of *R. allomycis* into its host. The unwalled, endoparasitic plasmodium of *Plasmodiophora brassicae* within the host cytoplasm is surrounded by two unit membranes, the inner one being the parasite plasma membrane and the outer one being the host plasma membrane (Aist and Williams, 1971; Williams and McNabola, 1970). Entry into the host cytoplasm is by insertion of the parasite protoplast through the host cell wall and invagination of host plasma membrane, which engulfs the invader. With this method of entry into host cytoplasm, the integrity of the host plasma membrane is not disrupted. Differences in host-parasite interfaces, however, are reported among plasmodiophorids.

Whereas *P. brassicae* interface with its plant host consists of two membranes, the host parasite interface between *Sorosphaera veronicae* and its plant host is a single membrane interface, the parasite's (Braselton and Miller, 1975). Similarly with the chytrid-like parasite of plants, *Olpidium brassicae*, the parasite protoplast is in direct contact with host cytoplasm, and there is a single membrane interface, the parasite's plasma membrane until the parasite produces a wall around itself (Lesemann and Fuchs, 1970; Temmink and Campbell, 1968, 1969). Thus, it is not known if the parasite actually breaks through the host plasma membrane to gain entry into the host cytoplasm. It is still possible that the host engulfs the parasite, which would result in a two-membrane interface with the host layer eventually breaking down, leaving a single membrane layer, the plasma membrane of the parasite.

This study also gives us a better understanding of differences in host-parasite interfaces and nutritional mechanisms among the three groups in the Opisthosporidia: *Rozella*, aphelids and Microsporidia. Whereas *Rozella* and aphelids both exhibit phagotrophic

nutrition, their interactions with host cells are different. Aphelid zoospores encyst at the surface of host algal cells and then produce a long infection tube, which penetrates the host cell wall and invaginates the host plasma membrane (Karpov et al., 2014b; Letcher et al., 2017c). The spore protoplast is discharged from the cyst through the infection tube, probably assisted by the expansion of the "posterior vacuole," and it invades the space between the host wall and host protoplasm. The amoeboid protoplast then phagocytizes host protoplasm from outside the host plasma membrane (Karpov et al., 2014a, 2014b). The parasite's phagocytosis of host protoplasm results in a two-membrane-layered food vacuole, with the inner membrane derived from the host plasma membrane and the other membrane derived from the parasite's plasma membrane (Karpov et al., 2014b). At maturity the parasite totally consumes the host (except for residual bodies), replacing the host's protoplasm with its own plasmodium and using the host wall as its own when zoospores are formed and discharged (Letcher et al., 2017c). Aphelid thallus development is similar to that found in *Rozella* in using the host wall as its own during spore formation and discharge. In contrast to *Rozella*, however, the unwalled protoplasts of aphelids do not directly enter into the host cytoplasm. With aphelids the mode of infection only requires penetration of the host cell wall. As a consequence the aphelid endoparasite does not have to cross the host plasma membrane in the infection process (Karpov et al., 2014a, 2014b) as does *Rozella*. Whereas *Rozella* consumes its host cytoplasm from the inside (Powell et al., 2017), aphelids consume host cytoplasm from the outside (Karpov et al., 2014a, 2014b).

Microsporidia is a group with heterotrophic nutrition that does not produce flagellated spores, but rather, a walled spore with a special structure for penetrating into the host, the polar filament. Because microsporidia are endoparasites of animal cells, their infection process does not have to deal with penetration through host walls as do *Rozella* and aphelids. Different methods of entry into host cytoplasm have been reported for microsporidia (Franzen, 2005). In the most widely reported mechanism, microsporidia walled spores attach to the surface of host cells and inject the spore's protoplast (sporoplasm) directly into the host cell cytoplasm through an everted, hollow polar filament, which pierces through the host plasma membrane (Cali and Takvorian, 2014; Franzen, 2005). Similar to the encysted zoospores of *Rozella* and aphelids (Letcher et al., 2015), discharge of the parasite's cytoplasm is associated with a large vacuole, the posterior vacuole, at the apex of the spore. Unlike *Rozella* and aphelid infection of hosts, the host plasma membrane is actually punctured (Franzen, 2005). The speed and force of insertion of the polar filament into host cells is believed to prevent immediate death of the host due to damage of the host plasma membrane (Cali and Takvorian, 2014). Alternative methods of entry into the hosts have been reported, including the host cell phagocytizing the parasite's spore or discharged protoplasts into phagosome vacuoles (=parasitophorous vacuoles) upon contact with the host's plasma membrane, thus, not having to break through the host plasma membrane (Cali and Takvorian, 2014; Couzinet et al., 2000; Franzen, 2005; Takvorian et al., 2005). In the host engulfing the parasite upon contact and sequestering it within a host vacuole, the mechanism of parasite entry into host cytoplasm is similar to *Rozella*.

4.4. Comparison of endoparasite host-parasite interfaces

With Microsporidia development within host cytoplasm, a wide range of host-parasite interfaces is found. Cali and Takvorian (2014) described four basic types of interfaces with variations among each type. Type III, "indirect contact by post-produced isolation," is most similar to the host parasite interface between *R. allomycis* and *A. macrogynus*. In *Endoreticulatus* infections (Cali and El Garhy, 1991), a double-membrane host cisterna surrounds the parasite (resulting in three membrane layers), which is similar to the interface of the enlarging *Rozella* plasmodium within its host (Powell et al., 2017).

The host of *R. allomycis*, *Allomyces*, appears to respond differently to two different obligate endoparasites. With the blastoclad endoparasite of *Allomyces*, *C. allomycis*, there is a wall around the parasite from the earliest stage of infection detected with electron microscopy (Powell, 1984; Sykes and Porter, 1980), and Sykes and Porter (Fig. 8; 1980) report that the host plasma membrane is ruptured in the infection process. This would mean that the host must have some way to repair its plasma membrane before death occurs to the cell. *Allomyces*, does respond to the presence of the *C. allomycis* and encases it within a net of rough endoplasmic reticulum, which might represent the parasite gaining proximity to host protein production (Powell, 1984). In contrast, *R. allomycis* does not rupture the host plasma membrane as it gains entry into the host cytoplasm. Rather the host first engulfs the endoparasite with its plasma membrane as the parasite is discharged through the infection tube and then it contains the parasite within a compartment in its cytoplasm. Thus, *Allomyces* as a host responds differently to the two parasites.

Given the phylogenetic relationship between *Rozella* and *Microsporidia* (Corsaro et al., 2016), it is interesting that, although their nutritional modes are different, mechanisms of infection may have some similarities. The *Microsporidia* is a much larger group of organisms than *Rozella* and exhibits a wide range of host-parasite interfaces, but the three-membrane interface found in *Rozella*, may occur in some microsporidia (Cali and El Garhy, 1991). There is evidence that one of several methods for the microsporidian parasite's entry into the host may be by host engulfment of the parasite (Couzinet et al., 2000; Franzen, 2005), which is similar to *Rozella*. On the other hand, whereas aphelids are phagotrophic as is *Rozella*, their host-parasite interfaces are different. Aphelids phagocytize host cytoplasm from outside the host cytoplasm, and *Rozell*a phagocytize host cytoplasm from within the host cytoplasm. As phylogenetic relationships among members of the *Opisthosporidia* become better resolved, we can begin to map the evolution of nutritional mode and host-parasite interface along a lineage in which parasites' genomes become more reduced and parasites become more dependent upon host for energy production (Berbee et al., 2017; James and Berbee, 2012; James et al., 2013; Powell et al., 2017). The *Rozella-Allomyces* interaction could be a model for future laboratory research exploring the effects of changes in environmental conditions on the host-parasite relationship.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2018.11.009.

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