Original Article

Insights into the Morphology of Haplozoan Parasites (Dinoflagellata) using Confocal Laser Scanning Microscopy

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Keywords
Axiothella; Blastodiniales; centrin; dinoflagellate; Haplozoon; Maldanidae; spindle; syncytium; tubulin.

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Received: 8 February 2021; revised 26 March 2021; accepted April 16, 2021.
doi:10.1111/jeu.12855

ABSTRACT
We describe new insights into the morphology and life history of the bizarre parasite Haplozoon axiothellae (Dinoflagellata) using light microscopy (LM), scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). Trophonts were isolated from the intestines of host maldanid polychaetes, Axiothella rubrocincta, collected from San Juan Island, Washington, USA. LM and SEM confirmed features previously observed, such as amphiesmal projections, mature and immature junctions between the nucleated compartments of the vermiform syncytium and visible polygonal alveoli. CLSM of adult trophonts fluorescently stained for DNA, tubulin, centrin, and plasma membrane demonstrated several new ultrastructural traits: (1) an extensive basket of parallel microtubules within the trophomere used for host attachment, (2) two physically separated MTOCs (i.e. putative pairs of basal bodies) beneath pores on the ventral side of each compartment, (3) robust mitotic and/or meiotic spindles associated with one to four nuclei in each compartment, (4) spindles with polar bodies that are disconnected from the MTOCs, (5) a centrin-stained fibril within the trophomeres that potentially functions to retract the motile stylet, and (6) cytokinesis in the posterior-most compartments. This study renames haplozoan compartments using the suffix “-mere” rather than “-cyte” (i.e. trophomere, gonomere, sporomere) to reflect their status within a single syncytium.

Haplozoans are an unusual group of parasitic, vermiform dinoflagellates that were first described in 1906 with the species Haplozoon armatum collected from the intestines of the maldanid polychaete, Travisia forbesii (Dogiel, 1906). The vast majority of haplozoans occur in maldanid hosts (bamboo worms) and were largely unstudied for most of the 20th century (Cachon 1964, Shumway 1924). Since the early 2000s, four new species have been discovered (H. praxillellae, H. ezoense, H. gracile, and H. pugnus) and described with modern microscopy and small ribosomal DNA sequences, providing new insights into the ultrastructural organization and biogeographical distribution of haplozoans as a whole (Leander et al. 2002; Rueckert and Leander 2008; Wakeman et al. 2018; Yamamoto et al. 2020). Although dinoflagellates typically consist of a single cell, haplozoans are complex multinucleated organisms consisting of traits that have converged with the overall life style and morphology of tapeworms (Cestoda), such as strobilized bodies, an anterior host attachment apparatus of hooks and suckers, surfaces covered with dense arrays of microtrich-like projections, and posterior reproductive units that exit the intestines of one host to infect another one (Leander 2008a). This case of convergent evolution involving animals and dinoflagellates reflects phylogenetic distances over a billion years old and fundamentally different levels of biological organization (Leander 2008a, 2008b).

Current data suggest that amphiesmal (alveolar) sacs are used to compartmentalize a single haplozoan cell into distinct nucleated units that are morphologically and functionally differentiated from one another (Leander et al. 2002; Wakeman et al. 2018). The trophonts of Haplozoon are composed of three distinct functional regions: (1) an anterior “trophocyte,” (2) a mid-region...
composed of multiple “gonocytes,” and (3) posterior “sporocytes”. However, to reflect their status within a single syncytium, we rename the haplozoan compartments using the suffix “-mere” rather than “-cyte” from this point forward: trophomere, gonomere, and sporomere. The trophomere possesses a motile stylet, accessory nonmotile stylets, and an adhesive (suction) disk that anchors the parasite to the intestinal lining of the host (Siebert 1973; Yamamoto et al. 2020). The gonomeres and sporomeres form a chain of repeating subunits that comprise the majority of the trophont’s length. In 1924, the posterior sporomeres of H. dogielii were observed to produce a flagellated dinospore, indicating they have a reproductive function (Shumway 1924); however, this observation has not been made since, which underscores the many gaps in our understanding of the ultrastructure and life cycle of haplozoans (Siebert 1973; Yamamoto et al., 2020).

In this study, we describe novel ultrastructural features in haplozoans using confocal laser scanning microscopy (CLSM) on adult trophonts fluorescently stained for DNA, tubulin, centrin, and plasma membrane. This approach allowed us to visualize the organization and distribution of (1) condensed chromosomes, (2) cortical microtubules, (3) robust mitotic and meiotic spindles, (4) putative pairs of basal bodies (syn. centrioles) separated from spindle pole bodies, (5) centrin-stained fibrils associated with the stylet in the trophomeromes, and (6) the plasma membrane. Implications of these observations for understanding the life cycle of haplozoans are also discussed.

MATERIAL AND METHODS

Collection of hosts and isolation of Haplozoon axiothellae

Individuals of the maldanid polychaete Axiothella rubrocincta were collected in Argyle Lagoon (San Juan Island, WA; 48.521068, –123.014626) in March 2002, June 2018, July 2018, and June 2019. At low tides of –0.26 m maximum, sediment from the intertidal zone was sampled from which A. rubrocincta was isolated by hand. The host worms were placed in falcon tubes of seawater and transported back to the University of British Columbia (UBC) in a cooler with ice. In the laboratory, A. rubrocincta were stored in a New Ocean® Cabinet Style Tank NO-2L. The host worms were dissected within 1–3 days of sampling with forceps and depression microscope slides filled with seawater to release the contents of their intestinal tract. Haplozoon trophonts were isolated from the gut contents by glass pipette micromanipulation and washed twice with seawater. The isolated trophonts were then prepared for differential interference contrast (DIC) light microscopy (LM), scanning electron microscopy (SEM), and confocal laser scanning microscopy (CLSM). As noted in the relevant figure legend, one SEM image presented here was sampled from which H. dogielii were isolated by forceps and placed in a metal basket, ensuring that the filter paper was always submerged. The metal basket containing the filter paper was critical point dried with CO2 using a Tousimis Autosamdri 815B critical point dryer. After critical point drying, the filter paper was mounted on an aluminum stub and sputter coated with gold-palladium using a Cressington 208HR high-resolution sputter coater. SEM images were captured using a Hitachi S-4700 Field Emission SEM. High-resolution images were taken at 5,000X magnification and stitched together as a montage using Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA).

Confocal laser scanning microscopy

One hundred Haplozoon trophonts were prepared for CLSM. The parasites were placed in a chamber slide with filtered seawater and fixed with 4% paraformaldehyde (PFA) for 20 min at 4 °C. After fixation, each chamber was washed several times with phosphate-buffered saline (PBS) 1× buffer for 40 min at 4 °C. To begin preparation for fluorescent staining, a 1 ml blocker was prepared as follows: 890 µl of phosphate-buffered Triton X-100 (PBT) 0.2%, 60 µl of heat inactivated goat serum (HIGS), and 50 µl bovine serum albumin (BSA). PBS 1× buffer was removed from each chamber, replaced with the blocker mixture, and stored at 4 °C for 30 min. To inhibit non-specific binding of antibodies, the parasites were incubated in blocking solution (PBS 1× + Triton X-100 0.2% (PBT) + 0.5% bovine serum albumin (BSA) + 6% heat inactivated goat serum (HIGS) at 4 °C for 30 min. The cells were then treated with the primary antibodies rabbit
anticentrin and mouse anti-acetylated alpha tubulin (Sigma-Aldrich, cat# T6793) at a concentration of 1:100 in blocking solution at 4 °C for 12 h. After incubation, the primary antibodies were removed with multiple washes of PBT 0.2% at 4 °C for an hour. The trophonts were then treated with a secondary antibody mix consisting of goat antirabbit Alexa Fluor 488 (Invitrogen, cat# A11070) and donkey-antimouse Alexa fluor 647 (Invitrogen, cat# A31571) at a concentration of 1:100 at 4 °C for 12 h. The samples were co-labeled for DNA by treatment with 10% Hoechst solution (Molecular Probes, cat # 33342) together with the secondary antibody mix. Stained trophonts were washed with multiple exchanges of PBT followed by three 10 min exchanges of 1x PBS at 4 °C.

Figure 1 Light micrographs (LM) showing the general morphology of Haplozoon axiothellae. (A) A trophont in lateral view showing the stylet (st), ventral adhesive disk (ad), internal accessory stylets (arrowheads), nuclei (n) within each gonomere (G), and starch granules (gr) within the sporomeres (S). (B and C) The same trophont in dorsal view taken at different times under different magnifications: B was captured with the 20X objective lens; C was captured with the 63X objective lens. The LMs show the trophomere (T), gonomeres (G), and 12 sporomeres (S) arranged as three tetrads (te). As shown in C, the sporomeres begin to detach from the trophont at their junction with the gonomeres (double arrowhead) over time. Scale bars: 40 μm.
Plasma membrane staining was performed on living trophonts with dye FM4-64 (Thermo Fisher, cat # F34653). The trophonts were extracted from hosts \((n = 20)\), placed within a chamber slide and stained in a working solution composed of 50 \(\mu\)l of FM4-64 stock solution and 950 \(\mu\)l of seawater.

The chambers of the slide were removed, and the area of the slide containing trophonts was covered with a 1.5 mm thick 20 × 20 mm coverslip. Slides were kept in the dark to avoid photobleaching until imaging. CLSM data were captured under recommended excitation and emission settings using an Olympus FV1000 confocal laser scanning microscope equipped with a 60X objective at the UBC Bioimaging facility. Z-stack projection images for the tubulin and centrin staining, but not for the live-cell plasma membrane staining, were compiled with Fiji, version 2.00 (Wayne Rasband, National Institutes of Health). Figure plates were prepared with Adobe Illustrator CS6 (Adobe Systems Incorporated, San Jose, CA).

Figure 2 Scanning electron micrographs (SEM) showing the surface details of *Haplozoon axiothellae*. (A) Low magnification SEM of an immature trophont in lateral view showing the stylet (st), the ventral adhesive disk (ad), developing gonomeres (G), and developing sporomeres (S). Scale bar: 25 \(\mu\)m. (B) Higher magnification SEM of the ventral surface of a trophont showing pores near the anterior junctions (arrows pointing left) and posterior junctions (arrows pointing right) of each compartment. This image was reported previously in Leander et al. (2002). Scale bar: 10 \(\mu\)m. (C) Higher magnification SEM of the posterior end of an immature trophont showing the relatively deep junction (double arrowheads) between the developing gonomeres and sporomeres. Scale bar: 10 \(\mu\)m.
RESULTS

Light microscopy
Parasites were found in every dissected host (n = 50), a rate of infection higher than that found in the same location by Leander et al. (2002). The LM data of *H. axiothellae* showed the three distinct regions as follows: the trophomere, gonomeres, and sporomeres (Fig. 1). Trophont length varied proportionally to the number of compartments and ranged from 55 to 200 µm long. Trophomeres were 30–40 µm wide, and gonomeres were

Figure 3 Confocal laser scanning micrographs (CLSM) of an immature trophont of *Haplozoon axiothellae* stained for DNA (blue) and tubulin (white). (A) Differential interference contrast light micrograph showing the nuclei (n) in the developing sporomeres and the ventral adhesive disk of the trophomere (ad) oriented upward. (B) DNA staining showing the condensed chromosomes within each nucleus. (C) Tubulin staining showing accumulations of microtubules associated with the inner lining of the adhesive disk (ad), the ventral side of each compartment (oriented upward) and robust spindles. (D) Superimposed DNA and tubulin staining showing the robust spindles associated with each nucleus in different stages of division. Scale bars: 20 µm.
usually about 30 μm wide and up to 40 μm wide if a double row of sporomeres was present (n = 20). The sporomeres in intact trophonts were usually arranged as tetrads (Fig. 1). Accessory nonmotile stylets were observed in the trophomere (Fig. 1). The gonomeres were relatively flattened along the anterio-posterior axis, whereas the sporomeres were more spherical in shape (Fig. 1). The nuclei of the gonomeres occupied most of the compartment, and the granularity of cytoplasm increased from anterior to posterior, with the sporomeres being the most granular in appearance (Fig. 1).

Scanning electron microscopy

The SEM data of *H. axiothellae* were consistent with the morphological characteristics previously described (Leander et al. 2002; Rueckert and Leander 2008; Wakeman et al. 2018; Yamamoto et al., 2020). The cell possessed dense arrays of the amphiesmal projections (syn. thecal barbs, spines) over the cell surface, an adhesive (suction) disk and a single stylet that extended from the apical end of the trophomere (Fig. 2A). Unless obscured by amphiesmal projections, the ventral surface of trophonts had pores positioned near the anterior junctions and posterior junctions of each compartment (Fig. 2B; reported previously in Leander et al. 2002). Putative epibionts first reported by Leander et al. (2002) were visible on the parasite’s surface, and areas lacking amphiesmal projections and putative epibionts contained polygonal amphiesmal (alveolar) sacs that were about 0.7 μm wide (Fig. 2). Relatively, deep junctions were evident between the gonomeres and sporomeres (Fig. 2C).
Confocal laser scanning microscopy

Tubulin staining demonstrated a complex superficial basket of microtubules running in parallel along the longitudinal axis of the trophomere that were most concentrated in the adhesive disk and around the motile stylet (Fig. 3, 4). Microtubules were also concentrated on the ventral side of the gonomeres and sporomeres (Fig. 3, 4). However, the densest staining for tubulin revealed robust microtubular spindles associated with the condensed chromosomes in every gonomere and sporomere (Fig. 3, 4). The combination of DNA and tubulin staining illustrated nuclei within
different compartments in different stages of either mitosis or meiosis (Fig. 3, 4). The trophomerces had two nuclei in either interphase or early prophase (Fig. 3). The gonomeres had one or two nuclei in different stages of mitosis, and binucleated gonomeres were positioned toward the posterior end of the trophont (Fig. 3, 4). The sporomeres contained two or four nuclei with associated microtubular spindles (Fig. 3, 4). No evidence of DNA fluorescence was observed from putative epibionts on the parasite’s surface.

The centrin antibody staining demonstrated two distinct bodies on the ventral side of each trophomere and gonomere; these centrin-stained bodies were separated near the anterior and posterior junctions of each compartment (Fig. 5). In the trophomere, the two centrin-stained bodies were located at the anterior and posterior sides of the ventral adhesive disk (Fig. 5). The trophomere also contained a centrin-stained fibril in the general position of the motile stylet and oriented nearly in parallel to the adhesive disk. The position of the anterior centrin-stained body in the two posterior-most gonomeres was shifted toward the dorsal side (Fig. 5). Tubulin antibody staining was concentrated near the centrin-stained bodies, and putative microtubules radiated away from these points and throughout the compartments (Fig. 5).

Plasma membrane staining demonstrated a single outer membrane that enveloped the entire trophont (Fig. 6). The stylet region was also stained suggesting that the motile stylet sits within an apical invagination of the plasma membrane (Fig. 6). The higher concentrations of fluorescent staining along the margin of the trophonts are consistent with the position of amphiesmal projections (spines) on the surface of each compartment (Fig. 2). The alveolar (amphiesmal) vesicles beneath the plasma membrane and that form the junctions between the trophomere and gonomeres were unstained, presumably because the lipophilic stain we used (FM™ 4-64FX) only binds to the outer leaf of the plasma membrane. However, the junction between the most posterior gonomere and the sporomeres contained stained plasma membrane, reflecting early stages of cytokinesis (Fig. 6B, C).

**DISCUSSION**

The CLSM data showing a single plasma membrane around the entire compartmentalized trophont is...
consistent with the SEM and TEM data from previous studies of *Haplozoon* (Leander et al. 2002; Seibert 1973; Wakeman et al. 2018; Yamamoto et al. 2020). The functionally differentiated compartments within the syncytium are separated by internal septa composed of amphiesmal (alveolar) vesicles, so we have replaced the original suffix “-cyte” with “-mere” when referring to the different compartments as follows: trophomeres, gonomeres, and sporomeres. The suffix “-cyte” stems from their original discovery in which *Haplozoon* was described as a possible transition stage from single-celled to multicellular organisms and less accurately implies that haplozoan compartments are physically intact cells (Dogiel, 1906).

Staining for plasma membrane and SEM also demonstrated early stages of cytokinesis between the most posterior gonomere and the most anterior sporomere (Fig. 2C, 6). This finding is consistent with the inference that sporomeres eventually separate from the posterior end of the adult trophont and develop into a motile life stage, originally proposed by Shumway (1924) as a dinospore with conventional transverse and longitudinal flagella that leaves the intestines of one host to infect another one. TEM data show that the cytoplasmic granularity of gonomeres reflects the presence of energy reserves, such as starch granules (Siebert and West 1974; Wakeman et al. 2018). Our data show that in *H. axiothellae*, a distinct gradient of cytoplasmic granularity increases from anterior to posterior and eventually becomes heavily concentrated in mature sporomeres. This observation is consistent with the inference that sporomeres produce freeliving life stages, which would benefit from a yolk-like reserve of energy to transiently navigate the marine environment prior to entering a new host.

One novel finding from CLSM of trophonts fluorescently stained for tubulin was the existence of an elaborate basket of parallel microtubules around the trophomere, which was not noticed in earlier TEM studies (Siebert and West, 1974; Wakeman et al., 2018). This basket of microtubules explains the mechanism underlying the dynamic pulsating behavior of the trophomere and the operation of the adhesive disk, where microtubules are especially concentrated. Microtubules were also concentrated on the ventral side of each gonomere and sporomere and radiated within each compartment from two distinct points along the ventral midline: one near the anterior junction and one near the posterior junction. These two positions within each compartment are entirely congruent with the CLSM data of adult trophonts fluorescently stained for centrin. Centrin is typically localized to the fibers that link pairs of basal bodies (centrioles) together, which collectively form the microtubule organizing center (MTOC) of the cytoskeleton (Aubusson-Fleury et al. 2017; Höhfeld et al. 1994; Yubuki and Leander 2013).

The consistent localization of microtubules and centrin in our CLSM data suggest that two pairs of basal bodies exist within every compartment of the trophont and are separated in the gonomeres and sporomeres near their respective anterior and posterior junctions. The two putative pairs of basal bodies in the trophomere are also positioned on the ventral side, specifically on the anterior and posterior edges of the adhesive disk and appear to organize the dense array of microtubules supporting the concave lining of the adhesive disk and the elaborate superficial basket. The ventral localization of the tubulin and centrin staining near the anterior and posterior junctions of each compartment is also consistent with the position of the longitudinal row of ventral pores observed previously with SEM on *H. axiothellae* (Leander et al. 2002) (Fig. 2B). Moreover, the shifted position of the anterior centrin-stained body in the posterior-most gonomeres (Fig. 5) reinforces the SEM data showing that some of the pores divert from the midventral line and off to the side. This overall congruency in the position of pores, centrin and microtubules supports the hypothesis that the ventral pores in each compartment of *Haplozoon* reflect vestiges of the flagellar pores present in most dinoflagellates, especially the monokinetid found in some multinucleate dinoflagellates, such as *Polykrikos* (Hoppenrath and Leander 2007a, 2007b; Leander et al. 2002). This is further supported by SEM data from *H. ezoense* showing a flagellum-like fibril extending from the tip of the stylet, suggesting that the opening of the trophomere through which the stylet protrudes is also a flagellar pore (Wakeman et al. 2018). The general position and orientation of the centrin-stained fibril we observed within the trophomeres in *H. axiothellae* are consistent with a possible centrin-based contractile mechanism, known in many other eukaryotes (e.g. *Chlamydomonas, Oxyrrhis, Vorticella, Gogendeau et al. 2008; Höhfeld et al. 1994; Maciejewski et al. 1999; Salisbury et al. 1988*), that functions to retract the motile stylet.

Another novel finding from the CLSM of trophonts fluorescently stained for tubulin is the presence of robust mitotic or meiotic spindles associated with the condensed chromosomes within every nucleus. Each compartment typically contained either one, two, or four nuclei at different stages of either mitosis or meiosis, suggesting a dynamic and continuous proliferation of gonomeres and sporomeres along the entire length of the trophont. Although the ploidy of each nucleus along the length of the trophont is unknown, the different numbers of nuclei in the trophomeres, gonomeres, and sporomeres might offer clues. The trophomeres and gonomeres contain microtubular spindles associated with only one or two nuclei, indicating mitotic activity. By contrast, the sporomeres contain spindles associated with two or four nuclei, suggesting that haploid reproductive “dinospores” are being generated by meiosis to facilitate sexual reproduction.

Presumably, either haploid or diploid sporomeres are perpetually released from the posterior end of the trophont and replenished by the continuously dividing gonomeres. CLSM of trophonts fluorescently stained for tubulin and centrin demonstrate that the putative pairs of basal bodies (syn., centrioles or MTOCs) are physically separated from the “pole bodies” of the robust spindles; the likely presence of microtubular (syn. fibrous) ribbons that tether the nucleus to the basal bodies were not
Morphology of Haplozoon Using CLSM

Angel et al.

definitely detectable using the resolution available with tubulin staining under CLSM (Gavelis et al. 2019). Although the separation of centrioles from the spindle pole bodies seems unusual, especially when thinking about animal cells, it is the rule in dinoflagellates, a group of organisms that tends to challenge many conventions in cell biology (Lukes, Leander and Keeling 2009). In most species of dinoflagellates examined so far, now including Haplozoon, centrioles do not organize the spindle microtubules, which ultimately terminate in the cytoplasm (Barlow and Triemer 1988; Gavelis et al. 2019).

In summary, CLSM of haplozan trophonts fluorescently stained for plasma membrane, DNA, tubulin, and centrin demonstrated several novel traits associated with the nuclei and cytoskeletons of these strange intestinal parasites. The plasma membrane staining reinforced the interpretation that haplozan trophonts are uniquely organized as a compartmentalized syncytium of three functionally and morphologically differentiated regions. The tubulin staining demonstrated an elaborate basket of parallel microtubules around the trophomere that also supports the concave lining of a robust adhesive disk and enables the parasites to actively probe and attach to the intestines of their host. The tubulin staining also demonstrated high concentrations of microtubules along the ventral side of the trophonts and robust mitotic or meiotic spindles associated with the nuclei in every compartment. The centrin staining demonstrated two separate MTOCs, inferred to be pairs of basal bodies (centrioles), positioned beneath pores on the ventral side of each compartment near their respective anterior and posterior junctions. Like in dinoflagellates generally, these MTOCs are separated from the pole bodies of the microtubular spindles associated with each nucleus. The centrin staining also demonstrated a distinct fibril in the trophomerous, suggesting centrin-based contractility functions to retract the motile stylet. The presence of two or four nuclei and high concentrations of granular energy reserves in the sporomeres suggest that reproductive cells are being generated at the posterior end of the trophonts. Although the life cycle of haplozoans remains to be fully characterized, the CLSM data presented here combined with previous observations of putative dinospores suggests that reproductive units leave the intestines of one host to reinfect another host. Comparisons of the life cycle and morphological traits in haplozoans (dinoflagellates) and tapeworms (animals) continue to underscore an outstanding example of convergent evolution within the intestines of animal hosts that reflects vast phylogenetic distances and fundamentally different levels of biological organization.

ACKNOWLEDGMENTS

We would like to thank N. Okamoto and G. Gavelis for discussions about the CLSM data. This research was performed in partial fulfilment of an MSc degree at UBC by PA. This work was supported by grants to BSL from the National Science and Engineering Research Council of Canada (NSERC 2019-03986) and the Tula Foundation’s Hakai Institute.

AUTHOR CONTRIBUTIONS

BSL conceived, funded, and guided the study. PA and MH conducted sampling and performed the microscopy. PA and BSL built the figures and wrote the manuscript; PA, MH, and BSL made further revisions and approved the final version of manuscript.

LITERATURE CITED


