The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the thesis entitled:

**Ultrastructure of tapeworm-like parasites in marine dinoflagellates (Haplozoon)**

submitted by Philip Angel in partial fulfillment of the requirements for

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Abstract

Haplozoans are intestinal parasites of a specific group of marine annelids, called maldanids. Haplozoans are dinoflagellates, yet distinctly abnormal. Dinoflagellates are traditionally considered free-living, photoautotrophs, unicellular, and have two flagella. Yet somehow, Haplozoans are parasitic, and possess a mysterious “multicellular” trophont stage, with no flagella. Their life cycle is also largely unknown; while there is a well-observed adult trophont stage, but our understanding of other life stages is speculative at best. The trophont possesses three different types of compartments that create a multicellular appearance: (1) a trophocyte at the anterior of the cell, (2) gonocytes that compose most of the body length, and (3) sporocytes at the posterior. To explore the unique characteristics of Haplozoon, and provide clarity into the life cycle, I collected LM, confocal, and SEM data. The LM and confocal fluorescent data revealed that haplozoans are in fact unicellular. They possess a single plasma membrane and have compartmentalized their cell using amphiesma. New compartments are added behind the most anterior compartment of the cell and become more mature as they are pushed towards the posterior of the cell. This is a striking example of convergent evolution with a group of “strobilized” multicellular parasites, the Cestoda (Platyhelminthes). This pattern of compartmental maturity suggests that the posterior-most compartment of the cell produces the subsequent life history stage. The confocal data demonstrated that haplozoans do possess flagellar basal bodies in the membrane of each compartment, evidence that supports the existence of a hypothetical free-living dinospore stage. A novel finding from fluorescent tubulin staining was the existence of a complex network of microtubules, concentrated in the trophocyte of the cell. These microtubules of the trophocyte, dubbed the microtubular basket, allows the trophocyte to manipulate its shape and provides the dexterity for the suction cup and stylet to function. These haplozoan ultrastructure discoveries provide novel understanding of this enigmatic protist and provide a foundation from which to continue future research.
Lay Summary

Haplozoans are parasites that infect the intestines of a specific group of marine annelid worms. They are mysterious in several ways: (1) they look multicellular despite being part of a unicellular group of eukaryotes called dinoflagellates, (2) their ultrastructure and life cycles are poorly understood, and (3) they have several traits that are reminiscent of tapeworms, which are multicellular animals that diverged from dinoflagellates over a billion years ago. I used high-resolution light and electron microscopy to characterize novel ultrastructural traits in *Haplozoon*. The data show that haplozoans divide their cells using internal membranes, while possessing a single continuous membrane around their exterior. These compartments mature individually, with the most mature compartment positioned at the posterior of the cell. When the posterior compartment reaches maturity, it breaks away from the adult cell, forming a new juvenile stage.
Preface

All chapters from this thesis are original works by Phil Angel.

The protocol for Chapter 2 was designed by Phil Angel, Dr. María Herranz, and Dr. Brian Leander. *Haplozoon* samples for Chapter 2 were collected by Phil Angel. Molecular data from *Axiothella rubrocineta* was collected and analyzed with the help of Dr. Niels Van Steenkiste. Imaging was carried out by Phil Angel and Dr. María Herranz, with assistance from UBC Bioimaging Facility staff member Kevin Hodgson. Analysis of the image data was by Phil Angel and Brian Leander.

A version of Chapter 2 will be submitted for publication.
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List of Abbreviations

bp – base pairs
BSA – bovine serum albumin
DIC – Differential interference contrast
DNA – deoxyribonucleic acid
EM – electron microscopy
HIGS – heat inactivated goat serum
LM – light microscopy
m – meter(s)
ml – milliliter(s)
mm – millimeter(s)
mRNA – Messenger RNA
mtDNA – mitochondrial DNA
PBS – Phosphate-buffered saline
PBT – Phosphate-buffered Triton X-100
PCR – polymerase chain reaction
PFA – paraformaldehyde
rDNA – ribosomal DNA
SEM – Scanning electron microscopy
SSU – small subunit
TEM – transmission electron microscopy
µl – microliter(s)
µm – micron(s)/micrometer(s)
Acknowledgements

You know who you are.
Chapter 1: Introduction

1.1 Dinoflagellate morphology

Dinoflagellates are primarily unicellular organisms with two flagella – one transverse flagellum with multiple waves, and one longitudinal flagellum which is straighter with few waves. They are a major eukaryotic lineage within the monophyletic Alveolata, along with other major groups like ciliates and apicomplexans. The Alveolata are unified by the shared characteristic of alveoli; membranous sacs that sit below the plasma membrane of the cell. In the case of dinoflagellates, these alveolar sacs can be either empty, or filled with cellulosic material, and are typically referred to as amphiesma. This cellulosic material only appears in armored dinoflagellates, forming hard plates called theca for protection. Molecular data suggests theca arose once within dinoflagellates and defines a monophyletic group with 5 orders (Suessiales, Peridiniales, Gonyaulcales, Prorocentrales, Dinophysiales) (Janouškovec et al., 2017). Except Syndiniales, all other dinoflagellate orders also possess a unique nucleus called a dinokaryon. The chromosomes within a dinokaryon remain condensed throughout the entire cell, cycle, even when the cell is not dividing. Their chromosomes mostly lack histones, but instead have dinoflagellate-viral-nucleoproteins (DVNPs) (Saldarriaga & Taylor, 2017), which are used to tightly bind their genetic material. These DVNPs are viral in origin and are suspected to be the result of horizontal gene transfer from a virus.

1.2 Phylogeny

Modern dinoflagellate phylogenies recognize 8 orders: Syndiniales, Gymnodiniales, Noctilucales, Suessiales, Peridiniales, Gonyaulcales, Prorocentrales, and Dinophysiales. Syndiniales are the earliest branch of dinoflagellates, with the other 7 orders forming the Dinokaryota. Dinokaryotes are defined by the possession of a dinokaryon. Noctilucales and Gymnodiniales are also athecate, and the remaining 5 orders constitute the thecate dinoflagellates. These 5 orders can be differentiated based on molecular data, but also by their complex and distinct thecal plate patterns (Taylor, 1999).

This currently accepted phylogeny has not always been the case – early in the study of dinoflagellates, other orders were used such as Blastodiniales and Phytodiniales. The Blastodiniales was originally created to categorize all the parasitic dinoflagellates, and the
Phytodiniales was used for either photosynthetic dinoflagellates or those associated with algae. Unfortunately, parasitism and photosynthesis are not unique characteristics of either of these groups, which appear numerous times throughout the entire dinoflagellate lineage. In hindsight, these orders are viewed as being of convenience rather than accurately describing evolutionary history (Fensome, 1993). As more molecular data becomes available for members of the Blastodiniales or Phytodiniales, genera within these orders have been slowly moved to more suitable orders based on our modern understanding of molecular data and ultrastructure analysis (Fernando Gómez & Skovgaard, 2015; Hehenberger et al., 2018).

### 1.3 Parasitism within Dinoflagellata

Dinoflagellates are important components of marine planktonic communities – small organisms that live in the water column of the oceans, unable to resist the water current. Of the roughly 2,000 extant species of dinoflagellates, half are photosynthetic in the form of autotrophs and mixotrophs (Fernando Gómez, 2012). The remaining half are either predators, symbionts, or parasites. Parasites make up approximately 7% (Fernando Gómez, 2012).

Dinoflagellate parasites were first described as early as 1884 when *Oodinium pouchetti* was found in larvacean hosts (Pouchet, 1884). Slowly, more and more examples of dinoflagellate parasites were discovered, and taxonomically grouped based on light microscopy and observable life cycle similarities. By modern standards these criteria are both insufficient and inaccurate. Poorly defined orders such as the Blastodiniales and Phytodiniales have been the result. The Blastodiniales contains many groups of parasitic dinoflagellates, and whose members are often revisited in light of new molecular data.

Predators that consume their prey slowly and parasites that consume their hosts quickly can be difficult to distinguish. A common working definition for parasites is that they must either be endoparasites that consume their host from the inside, or ectoparasites which remain attached to their host for long periods of time (Saldarriaga & Taylor, 2017). Dinoflagellate parasites can be either endo- or ectoparasites, and have been discovered as parasites of many invertebrates, fish, other protists, and even other dinoflagellates.
1.4 Examples of dinoflagellate “multicellularity”

While there are no truly multicellular dinoflagellates, and few that compare to the complexity of *Haplozoon*, there are some examples of multinucleated dinoflagellates that can provide some context.

Some dinoflagellates form plasmodia. Rather than undergoing standard mitotic events to become multicellular, a plasmodium is multinucleated but does not undergo cytokinesis to separate into distinct cells. In this way they grow to become significantly larger than what might be expected of a single cell. In the case of *Syndinium turbo*, they infect the copepod *Paracalanus parvus* (Skovgaard, Massana, Balagué, & Saiz, 2005). The infection is universally fatal – once they have infected a host, the plasmodium grows to occupy the entire body cavity of the host. After killing the host, free-swimming zoospores are released to infect a new host. This life strategy is an example of one that blurs the line between uni- and multicellular organisms.

Another comparison to *Haplozoon* is *Amoebophrya*, which parasitizes other dinoflagellates (Taylor, 1968). *Amoebophrya* have two life stages: a free-living dinospore stage that seeks out new hosts, and a second intracellular parasite trophont stage. When the dinospore finds a potential host cell, it pierces the host’s plasma membrane and squeezes itself into the cytoplasm. As the trophont stage matures, it grows to occupy most of the host cell and eventually resembles a beehive in shape (Taylor, 1968). As it matures, the trophont undergoes many nuclear divisions, such that the mature trophont possesses many nuclei spread out throughout the beehive structure. The trophont compresses as it matures until it is flat and winding, eventually escaping from the host cell. At this point, the parasite has entered its “vermiform” stage and undergoes many cytokinetic events to produce daughter cells with single nuclei (Taylor, 1968). Once cytokinesis is complete, the vermiform breaks apart into hundreds of flagellated dinospores. The life cycle of *Amoebophrya* is like that of *Haplozoon* given the alternation of trophont and dinospore stages, and this similarity is a reason for the hypothesized existence of haplozoan dinospores. *Amoebophrya* aren’t quite a plasmodium because of their vermiform stage and production of dinospores. However, haplozoans are still unique in that they are a single cell that has functional multicellularity via differentiation of the cell into areas with dedicated functions.
1.5 Haplozoon

Haplozoon armatum was first described in 1906 (Dogiel, 1906), found parasitizing the intestinal tracts of the maldanid polychaete Travisia forbesii. Traditional dinoflagellates are photosynthetic, flagellated, and free-living. Haplozoans are none of these. Instead of flagella, their “adult” trophont stage appears multicellular with a differentiated body plan. To date, there are 11 described species of Haplozoon:

1. H. armatum, Dogiel, 1906 (host: Travisia forbesi)
2. H. lineare, Dogiel, 1907 (host: Clymene lumbricalis)
3. H. delicatulum, Dogiel, 1910 (Host: maldanid, gen sp?)
4. H. ariciae, Dogiel, 1910 (host: Aricia norvegica)
5. H. macrostylum, Dogiel, 1910 (host: maldanid, gen sp?)
6. H. obscurum, Dogiel, 1910 (host: Terebellides strömii)
7. H. clymenellae, Calkins, 1915 (host: Clymenella torquata)
8. H. dogieli, Shumway, 1924 (host: Leiochone clypeata)
10. H. praxillellae, Rueckert and Leander, 2008 (Host: Praxillella pacifica)
11. H. ezoense, Wakeman, 2018 (Host: Praxillella pacifica)

Seven of these species have only been described from European coastlines and with limited information (i.e., light micrographs and line drawings). H. clymenellae is the only described species from the Atlantic coast of North America, with H. axiothellae, and H. praxillellae being found on the Pacific coast. H. ezoense was discovered on the coast of Japan.

The state of knowledge of these organisms is extremely limited. The early characterizations of these species are very difficult to find, and only exist in their original German or French texts. Shumway (1924) was the first to produce scientific literature written in English. He provided two quality descriptions of the external morphology for both H. clymenellae and H. dogieli. These descriptions are limited by the light microscope technology of the time, so no images of these species exist but only scientific drawings. Cachon (1964) briefly mentioned a new species dubbed H. inerme, found within the larvacean tunicate Appendicularia.
sicula (Siebert, 1973). Unfortunately, Cachon failed to describe it sufficiently and so Haplozoon is still considered a group of parasites exclusive to maldanid polychaete hosts.

Only five modern scientific studies of Haplozoon exist (Leander, Saldarriaga & Keeling, 2002; Rueckert & Leander, 2008; Siebert, 1973; Siebert & West, 1974; Wakeman, Yamaguchi, & Horiguchi, 2018). A few studies from the 1970s explored early TEM technology (Siebert, 1973; Siebert & West, 1974). While they provided our first understanding of the internal morphology of H. axiothellae, the images produced do not meet modern standards and ought to be reproduced. The first high-resolution imaging comes from SEM data of H. axiothellae by Leander et al. (2002). Subsequently, Rueckert and Leander (2008) provided further SEM data during their initial description of H. praxillellae and provide the only LM data that exists for Haplozoon.

Wakeman, Yamaguchi, & Yamaguchi (2018) conducted TEM on H. ezoense, providing more informative TEM data than was previously available. They describe hair-like structures on the surface of haplozoans as “amphiesmal projections”, synonymous with the term “thecal barbs” used by Leander et al. (2002). They also refrain from using the term “suction cup”, in favor of “adhesive apparatus”. The granularity observed in the gonocytes and sporocytes was identified as starch granules. Wakeman et al. (2018) suggest that the gonocytes are separated by a single layer of amphiesma. Mitochondria had tubular cristae. Their primary discovery was the presence of non-photosynthetic plastids in the cytoplasm of gonocytes. This is consistent with the understanding that dinoflagellates have undergone multiple instances of plastid reduction (Saldarriaga et al., 2001).

The first molecular data for Haplozoon came from H. axiothellae in 2001 (Saldarriaga et al., 2001). Two subsequent sequences are also available from Rueckert and Leander (2008), and Wakeman, Yamaguchi, & Horiguchi (2018). These provide 18S rDNA sequences for H. axiothellae, H. praxillellae and H. ezoense and one 28S sequence for H. ezoense. As previously mentioned, these sequences failed to resolve the position of Haplozoon within the Dinoflagellata (Appendix A). Even when considering ultrastructure, current descriptions contradict each other. Siebert and West (1974) describe the alveolar sacs as containing thecal plates, however SEM by Leander et al. (2002) and Rueckert and Leander (2008) show the hexagonal alveolar sac arrangement characteristic of Gymnodiniales. Gymnodiniales are the “naked” dinoflagellate
group, and don’t have thecal armor. Recently, the origin of thecal plates was demonstrated to have occurred once only (Janouškové et al., 2017), and so there are no Gymnodiniales that contain theca. Therefore, the possession of thecal plates by *Haplozoon* is questionable, along with its phylogenetic position as a whole.

### 1.5.1 Body plan

What make *Haplozoon* unique among dinoflagellates is their functional multicellularity. Originally, haplozoans were described as having 2-26 “cells” (Shumway, 1924). They had single rows of cells, with the most mature cells containing a double row of sporocytes at the posterior of the cell. We now understand this to vary by species, but all haplozoans have “compartments” that are specialized for different functions, such as host attachment, feeding and reproduction. Having different parts specialized for different functions is a key feature of multicellularity.

The root “troph” is of Greek origin and means “one who nourishes”. Many other biological terms use this root to indicate a relationship with nourishment (autotroph, chemotroph, etc.). Trophont is a term shared by many parasitic dinoflagellates to designate the stage that feeds on the host. The haplozoan trophont stage has 3 types of differentiated compartments: (1) a trophocyte used to attach to the host, (2) repeating gonocytes that compose the majority of the cell length, (3) and sporocytes which develop from mature gonocytes. The trophont attaches to the gut of the host by a suction disc located on the trophocyte of the cell. The trophont then pierces the host cell with a stylus also located on the trophocyte. The exterior of the cell is covered by barbs that present as fine hair-like structures that might function in surface mediated nutrition similar to the microtrichs of tapeworms (Brian Leander, 2008).

The anterior most compartment of the cell is the trophocyte. The trophocyte is larger than the gonocytes that are aligned immediately posterior to it. The rear most compartments are referred to as sporocytes, and depending on the species of *Haplozoon*, sporocytes may remain in single file (e.g. *H. lineare*), form double rows (e.g. *H. axiothellae*) or even multiple rows (e.g. *H. clymenellae*). It is presumed that the sporocytes are what allow the organism to reproduce, but this has not been shown definitively. All compartments contain their own nuclei, with some gonocytes being binucleate and some sporocytes being quadrinucleate.
1.5.1.1 Convergent evolution with tapeworms (Cestoda)

After SEM data from *Haplozoon* were obtained, it was pointed out that their body plan is reminiscent of Cestoda (Leander, 2008). Cestodes are parasitic animals that infect the digestive tracts of vertebrates (Scholz, Drábek, & Hanzelová, 1998). A scolex is located on the anterior end of a tapeworm; this is their attachment site which consists of hooks and suckers for attaching to the gut of their host (Scholz et al., 1998). The scolex is then followed by a series of proglottids, fundamentally identical units differing only in their maturity. New proglottids are generated and added behind the scolex of the tapeworm and create a maturity gradient towards the posterior of the animal (Loos-Frank, 1987). The most posterior proglottids are reproductively mature and eventually detach from the animal. These leave the host with the host’s feces and complete the life cycle when they attach to an intermediate host (e.g. arthropod) and successfully infect a new definitive host (vertebrate) (Loos-Frank, 1987).

Tapeworms have strobilized body plans that reflect their parasitic lifestyle. They cannot ingest food through a mouth because they have lost their digestive tract, and instead possess a thin tegument that allows nutrients to diffuse into their body (Andersen, 1975). Because they live inside the digestive tract of their host, the food consumed by their host is digested and the tapeworm simply needs to absorb those readily available nutrients. Tapeworms are covered with tightly packed microtriches that extend out from their body. These microtriches serve to increase the surface area of the tapeworm and increase the rate of nutrient absorption (Thompson, Hayton, & Sue, 1980).

Similarly, haplozoans inhabit the digestive tract of their maldanid host. Like tapeworms, they have an attachment apparatus (the stylet and suction cup of the gonocyte) that anchor to the gut wall of their host. Haplozoans present as a series of repeating subunits (gonocytes) that vary in maturity, and in the same way that proglottids detach from the posterior of the tapeworm, mature sporocytes detach from the posterior of haplozoans. In both these cases, the detachment of a mature subunit has the potential to become a new individual. Haplozoans are also covered in small protrusions (thecal barbs) that extend outward from the cell and increase the surface area of the cell, potentially for the purpose of absorbing nutrients.

It is worth noting a key difference between Cestoda and *Haplozoon* with respect to their reproduction. Cestoda are capable of both asexual and sexual reproduction, either with another
individual or self-fertilization. As with most dinoflagellates, haplozoans are only capable of asexual reproduction.

Despite being separated by a vast phylogenetic distance and existing on different scales of biological organization, the body plans of Cestoda and *Haplozoon* have converged on a strategy that is nearly identical. Because Cestoda are multicellular animals and *Haplozoon* are unicellular dinoflagellates, these analogous characters are in no way homologous and are therefore an outstanding example of convergent evolution over vast phylogenetic distances.

### 1.5.2 Life cycle

Very little is confirmed about the haplozoan life cycle. Given similarities with other dinoflagellate parasites, it is hypothesized that the known trophont stage is the “adult”. The sporocytes produced at the posterior end of the trophont would either encyst, or become motile dinospores, which would be released through the anus of the host. Once in the environment, the dinospores could be ingested by a new host, and subsequently develop into a trophont and attach themselves to the gut of the host. The youngest stage of the trophont has a single compartment (trophocyte), which develops gonocytes as it matures (Shumway 1924).

Dinospores were described by Shumway (1924) in *Haplozoon dogieli*. He observed that sporocytes easily detached from the cell and would develop into a small flagellated dinospore (Shumway 1924). They were 12 µm in length and similar to the dinospores of *Oodinium*, *Apodinium*, and *Blastodinium*. He also observed that dinospores quickly encyst if disturbed. However, Siebert (1973) was unable to reproduce the dinospores or cyst stages in *H. axiothellae*. Thus, no LM or EM images of this stage exist, and while their existence is likely, they have not been observed in almost 100 years.

### 1.5.3 Phylogenetic Position

Dogiel originally described *Haplozoon* as mesozoan animals (Dogiel, 1906), however their resemblance to other known parasitic dinoflagellates led to their placement within Blastodiniales. Small subunit rDNA sequences have been obtained from *H. axiothellae*, *H. praxillellae* and *H. ezoense*, but these data failed to resolve their phylogenetic position within dinoflagellates (Rueckert & Leander, 2008, Appendix A). They are currently placed within the
order Blastodiniales, family Haplozoaceae, genus *Haplozoon*. As with many other dinoflagellates still classified within Blastodiniales, their specific phylogenetic position remains unresolved.

1.6 **Annelid Hosts**

*Axiothella rubricincta* was originally described as *Clymenella rubricincta*, first discovered in 1901 near Puget Sound, Washington. They belong to the phylum Polychaeta; the taxon of annelid worms that inhabit marine environments. Polychaetes are distinctive for their segments with paired parapodia that include bristles called chaetae. Polychaetes are found extensively throughout the marine environment, inhabiting a wide range of temperatures, from the coldest trenches of the ocean to the highest temperatures associated with geothermal vents. *A. rubricincta* is a member of the Maldanidae, the so-called “bamboo worms”, so named due to their repeating segmental stripes that are reminiscent of bamboo. Maldanids secrete mucus to collect particles of sediment that ultimately forms a tube within which they reside. They are found typically in sandy or muddy sediment, with foreign debris entering the tube either consumed or incorporated into the wall.

1.6.1 **Host specificity**

Our understanding of haplozan host specificity is limited by the small amount of data available. Currently, the three 18S rDNA sequences available come from *H. axiothellae*, *H. praxillellae*, and *H. ezoense*. Until the discovery of *H. ezoense*, the limited data available suggested that *Haplozoon* were host-specific, with one parasite species associated with one host species exclusively. The recent discovery of *H. ezoense* calls this into question because its host is *Praxillella pacifica*, which is the same host as *H. praxillellae*. Multiple haplozoan species have never been found co-parasitizing the same host, which suggests biogeography may play a role in their host-parasite relationship. Although this recent finding indicates otherwise, host specificity is supported by early studies finding only one haplozoan species per host. Unfortunately, these studies involved no molecular data and limited morphological description (Shumway, 1924; Siebert, 1973; Siebert & West, 1974), and so the accuracy of these species identifications is questionable.
1.7 *Haplozoon axiothellae*

*H. axiothellae* is the most studied species of *Haplozoon*, following its discovery by Siebert (1973) in the Pacific Northwest of the United States. Siebert (1973) conducted an early LM study to stain the nuclei and cytoplasmic contents, while Siebert and West (1974) conducted a TEM study, and Leander et al. (2002) conducted an SEM study.

*H. axiothellae* consist of 10-20 compartments, that are 40-175 µm in length, and 15-40 µm in width. The variability in length depends on how many repeating gonocytes the cell has, which is thought to be related to its stage of development. The smallest cell observed in this study was a trophocyte and a single gonocyte. Most cells have a single row of compartments, while the larger cells may have double rows of sporocytes. *H. axiothellae* is comparable to *Haplozoon lineare* which only ever has a single row of compartments. In contrast, *H. delicatulum* and *H. clymenellae* can have 4 or more rows of compartments.

Like other haplozoans, the cell attaches to the surface of the host's intestinal tract by a depression at on the trophocyte called a suction disc. The trophocyte has a curved needle-like organelle called a stylet that protrudes from the trophocyte. Within the trophocyte are other sessile stylets with unknown function. The nucleus is at the posterior of the trophocyte. Siebert (1973) points out that the trophocyte of *H. axiothellae* is smaller and more strongly curved than in other described species. Gonocytes follow the trophocyte. They are smaller in size, with each possessing nuclei that fill most of the compartment. Division occurs transversely, and most cells are either undergoing mitosis or are binucleate. Sporocytes are usually quadrinucleate but are not present in all colonies depending on their stage of development.

The SEM work by Siebert and West (1974) briefly describes 3 layers of the cellular membranes. They describe a single continuous outer layer equivalent to a cell membrane, with another layer below of compressed “thecal vesicles”. This is prior to our current understanding of alveoli (Cavalier-Smith, 1991); these “thecal vesicles” would now be known as alveoli. The divisions between gonocytes are described as consisting of outer continuous membranes and flattened vesicles but pressed so closely together they are not easily distinguished. Mitochondria are located below the alveoli towards the outside of the cell, with a few found towards the center.

High-resolution detail of the thecal barbs was observed under SEM (Leander et al. 2002), building on the TEM description from Siebert and West (1974). Mature and immature
junctions were described, with mature junctions being deep separation between gonocytes, and immature junctions being shallower indentations. When thecal barbs were absent, pentagonal or hexagonal alveolar sacs were visible. Examination of the junctions between gonocytes suggested the outer membrane is continuous. This would mean that haplozoans are not multicellular or colonial, but rather a compartmentalized, multinucleated, unicellular organism. Alveolar sacs were not continuous across junctions, and this suggests the divisions between compartments are formed by amphiesma instead. A ventral line of pores was discovered with one pore per gonocyte, towards the anterior junction. It is possible these pores are a vestigial remnant of flagellar pores present in most dinoflagellates. However, TEM studies showed no evidence of flagellar systems (Siebert & West, 1974).

1.7.1 Speculative epibionts

Leander et al. (2002) observed flat, oval, bodies covering much of the parasites’ surface. They were mostly distributed over areas that were void of thecal barbs. Their length ranged from 0.4-1.5µm, with a mean of 0.5 µm. Some of the bodies were observed undergoing apparent binary fission, while others were connected by bridge-like structures in what could be evidence of conjugation. These bodies were not previously observed in TEM. Their identity remains unknown but Leander et al. (2002) suggests they may be bacterial epibionts of the dinoflagellate parasite. They were on the small side with a mean length of 0.5 µm, but compliant within the accepted range of bacteria size (Schulz & Jørgensen, 2001). If these truly are bacterial epibionts, this would constitute a tiered parasitic relationship in which the epibionts live on H. axiothellae, which in turn live inside the maldanid host.

1.8 Statement of research question

There is much to explore given the general lack of knowledge about Haplozoon. Their life cycle remains unknown and is mostly speculative based on the life cycles of other parasitic dinoflagellates. Observations under LM, with information from confocal fluorescent microscopy, will shed additional light on haplozoan structure and development.

Perhaps the most interesting question surrounds their apparent multicellularity. The earliest research indicated haplozoans consists of many cells, but SEM data from Leander et al.
(2002) indicates the cell has a single external plasma membrane. A full suite of microscopy techniques involving LM, SEM, and confocal staining for tubulin, centrin, DNA, and plasma membranes should provide clarity into haplozoan cellular organisation. This will also provide more information to compare the body plans of *Haplozoon* and tapeworms.

With a unique cellular arrangement, understanding their compartmentalization will require visualization of their cytoskeleton. Yet, previous studies indicate the absence of microtubules within *Haplozoon* (Siebert & West, 1974; Wakeman et al., 2018). To this effect, confocal microscopy using tubulin stain will address whether microtubules exist and what role they may play.

In addition to their cytoskeleton, they have a unique membrane configuration that allows *Haplozoon* to compartmentalize their cell. They appear to be multicellular in this respect. Confocal microscopy using a plasma membrane stain will visualize the exact configuration of the plasma membrane of the cell and confirm the structure of the intracellular compartment membranes.

While some research found no flagellar systems in their TEM data (Siebert & West, 1974; Wakeman et al., 2018), Leander et al. (2002) found suspected flagellar pores with SEM data. As dinoflagellates, it is likely that haplozoans have not completed lost their flagellar apparatus. This is especially true if flagellated dinospores observed by Shumway (1924) are part of the life cycle. Confocal microscopy using centrin stain should visualize any flagellar basal bodies if they are present.

In summary, I will conduct LM, SEM, and confocal microscopy with DNA, tubulin, centrin, and plasma membrane staining. These will all clarify the cellular organisation of the cell with respect to its membranes, cytoskeleton, and nuclei. It will also potentially provide insight into haplozoan development and its life cycle.
Chapter 2: Characterization of the ultrastructure of *Haplozoon axiothella*

2.1 Synopsis

Despite being originally described in 1906, there is little literature on *Haplozoon*. What we do know highlighted the importance of investigating *Haplozoon* morphology further, and it was clear that our understanding would benefit from modern microscopy techniques. I sampled Argyle Lagoon on San Juan Island for *H. axiothellae*. Using LM, SEM, and confocal microscopy, I collected data from *H. axiothellae* for the purpose of clarifying its internal morphology. Confocal microscopy revealed a complex network of microtubules along the inner membrane of the anterior trophocyte. The previous observations of nuclei undergoing consistent mitosis were confirmed, with DNA and microtubule staining clearly demonstrating nuclei at various stages of mitosis with their associated microtubules. Centrin staining was used to explore the flagellar pores seen by Leander et al. (2002) and revealed two flagellar basal bodies present in each compartment. Plasma membrane staining determined that haplozoans are surrounded by a single plasma membrane, demonstrating they are unicellular, and truly a compartmentalized syncytium as suggested by Leander et al. (2002). In particular, the data collected from the tubulin and plasma membrane stains provided further understanding of the haplozoan life cycle, reinforcing this example of convergent evolution over a vast phylogenetic distance with tapeworms.

2.2 Methods

2.2.1 Sampling and isolation of parasites from host

*Axiothella rubrocincta* was collected in Argyle Lagoon on San Juan Island (48.521068, -123.014626), at low tides of -0.26 m maximum. I dug up the sediment with a shovel, with special attention to anthill-like mounds of sediment that indicated presence of annelids. *A. rubrocincta* were then separated from the sediment by hand, and placed in falcon tubes of seawater, and transported back to UBC in a cooler with ice.

In the lab, *A. rubrocincta* were stored in a New Ocean® Cabinet Style Tank NO-2L. They were generally dissected within 1-3 days of sampling but could survive up to 1 week. Worms were occasionally dissected up until 1 week after sampling, but no parasites were extracted if the host was dead.
A. rubrocincta were teased apart with forceps to release the contents of their intestinal tract in depression microscope slides filled with seawater. Cells were isolated by glass pipette micromanipulation and twice washed with seawater. They were then ready for preparation for LM, SEM, or confocal microscopy, depending on their intended purpose.

2.2.2 Host DNA barcoding

One individual of A. rubrocincta was set aside for DNA barcoding. DNA was extracted from host tissues using the Quiagen DNeasy® Blood & Tissue Kit. CO1 sequences were amplified with the forward primer polyLCO (5'-GAYTATWTTCACAAATCATAAAGATATTTG-3’) and reverse primer polyHCO (5'-TAMACTTTCWGGGTAGCAAAARAATCA-3’) (Carr, Hardy, Brown, Macdonald, & Hebert, 2011). PCR samples were amplified using illustraTM PuReTaq™ Ready-To-Go™ PCR beads, 23µl of molecular grade water, 1µl of template host DNA, and 0.5µl each of polyLCO and polyHCO. The PCR protocol was as follows: denaturation at 94°C for 2 min, annealing at 45°C for 45 seconds, and then elongation at 72°C for 2 min. This cycle occurred 5 times. At this point, 35 more cycles were completed with an annealing temperature of 50°C instead of 45°C. This modification of annealing temperature is referred to as “touchup” cycles. With primers that are not binding well to the template DNA, having an initial annealing temperature that is higher promotes higher binding at the site of interest, afterwards the annealing temperature is reduced to ensure binding site specificity through the majority of the PCR amplification.

PCR products were held at 4°C following PCR amplification. I used gel electrophoresis to ensure the PCR product was of appropriate length (~650bp). ExoSAP-IT® was used to digest small non-target DNA fragments and clean up the PCR product for sequencing. The PCR product was then sequenced by using an Illumina MiSeq™ next generation sequencer (http://illumina.com) at the Sequencing + Bioinformatics Consortium at the University of British Columbia. Sequencing results were then identified using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm species ID.
2.2.3 Light microscopy

For LM images, 3-5 clean parasites were transferred to a glass slide with a small amount of filtered seawater. Specimens were covered with a cover slip edged in Vasoline. LM was done with live specimens, photographed at 63x magnification using a Zeiss Axioplan 2 microscope equipped with a Zeiss-Axiocam 503-color camera. Differential interference contrast (DIC) optics was used. Video was also captured demonstrating distinctive movements of the trophocyte.

2.2.4 Scanning electron microscopy

Twenty parasites were isolated from *H. axiothella* and prepared for SEM. A Swinnex filter holder with a 10 µm polycarbonate filter was submerged in a 10 mL cannister of autoclaved seawater. Isolated parasites were deposited via glass pipette into the filter holder such that they are never exposed to the air and remain wet at all times. A piece of Whatman filter paper was mounted on the bottom of a slightly larger canister. This filter paper was saturated with 4% osmium tetroxide (OsO₄), and the larger canister was placed over the smaller canister that contained the filter holder with the isolated parasites. The parasites were fixed by OsO₄ vapor for 10 min. After this 4-5 drops of OsO₄ were dropped into the smaller canister containing the filter holder with parasites, and then covered again by the larger canister for 5 min.

To wash out the fixative, a syringe with 10 mL of seawater was screwed onto the filter holder and then removed from the small cannister containing OsO₄ and seawater. Using the syringe, the filter holder was washed with 10 mL of seawater twice. The filter holder was then washed with a graded series of ethanol concentrations (50%, 70%, 85%, 100%) of 10 mL each. A second wash with 100% ethanol was performed, and then the filter paper were removed from the Swinnex filter holder, placed in a metal basket, and critical point dried with CO₂ using a Tousimis Autosamdry 815B Critical Point Dryer. After critical point drying, the filter papers were mounted on aluminum stubs, 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. Images were taken at 5000x and stitched together using Adobe Photoshop. The composite image is then presented without a background, which was removed using Adobe Photoshop.
2.2.5 Confocal microscopy

One hundred parasites were isolated from *H. axiothella* and prepared for confocal microscopy. 16% PFA was diluted to 4% using autoclaved seawater. Using a chamber slide, parasites were evenly distributed between 4 chambers. They sat for 10 min to settle to the bottom of the chamber. Under a Leica microscope, each chamber was approximately 1 mL and filled with 800 µl of 4% PFA. Parasites underwent fixation for 20 minutes. After fixation, each chamber was washed with PBS 1x buffer for 40 min at 4 °C. If staining immediately, I proceeded to the next step. If I wanted to store the fixed parasites for a later date, a small amount of sodium azide was added to each chamber as an antibiotic and stored at 4 °C. If using stored parasites later on, I washed each chamber 3 times with PBS 1x buffer for 10 min each wash at 4 °C.

In preparation of fluorescent staining, a 1 ml blocker mixture was prepared: 890 µl of PBT 0.2%, 60 µl of HIGS, and 50 µl BSA. PBS 1x buffer was removed from each chamber, replaced with the blocker mixture and stored at 4 °C for 30 min. After this a new mixture was created with the addition of primary antibodies for staining. The composition was 10 µl centrin antibody (rabbit), 10 µl tubulin antibody (mouse), 870 µl of PBT 0.2%, 60 µl of HIGS, and 50 µl of BSA. This mixture was vortexed, used to replace the blocker mixture in the chambers, and incubated at 4 °C for 12 hours.

After 12 hrs of incubation, the antibodies were wash twice quickly with PBT 0.2%, and then 5 times for 10 minutes each, at 4 °C. A new mixture was created with the addition of secondary antibodies for staining. The composition was 10 µl secondary antibody (rabbit), 10 µl secondary antibody (mouse), 870 µl of PBT 0.2%, 60 µl of HIGS, and 50 µl of BSA. This mixture was vortexed, used to replace the primary antibody mixture in the chambers, and incubated at 4 °C for 12 hrs.

After 12 hrs of incubation, the chambers were washed twice consecutively with 1x PBS buffer, then 3 more times each for 10 min at 4 °C. The samples were incubated a third time with a hoescht stain mixture of 990 µl PBS and 10 µl hoescht, again at 4 °C for 12 hrs.

After the third incubation of 12 hrs, each chamber was 3 times for 10 min each with 1x PBS buffer. Animal fibers from HIGS and BSA were manipulated if they were obstructing imaging of the cells. The chambers of the slide were removed, and the area of the slide
containing cells was covered in a 20 x 20 mm coverslip (1.5 mm thickness). Slides for imaging were kept in the dark until to avoid photobleaching the samples until they were imaged.

Fluorescent confocal images were captured using an Olympus FV1000 Laser Scanning/Two-Photon Confocal Microscope at 60x magnification.

2.3 Results
2.3.1 Light microscopy

The LM data of *H. axiothellae* showed the three cellular compartments known as the trophocyte, gonocytes, and sporocytes (Figure 1a, b). Haplozoan cells were found in every dissected worm, indicating a 100% rate of infection. Stylet movement of the trophocyte was observed and captured on video. Additional secondary immobile stylets were also observed in the trophocyte (Figure 1a). The nuclei of the gonocytes took up most of the compartment as described by Shumway (1924). Granularity of the gonocyte cytoplasm increased with the maturity of the gonocyte, with the sporocytes have the most granular cytoplasm (Figure 1a). The most mature gonocytes were binucleate (Figure 3a).

Cell size varied proportionally to the number of compartments it had. Cells with few or no rows of gonocytes could be as small as 40 μm in length, or as long as 175μm with multiple rows of sporocytes. A cell with multiple gonocytes and up to one sporocyte were measured as 100μm in length. Cells were on average 20μm in width, but up to 40μm if a double row of sporocytes was present. Cells consisting of only a trophocyte were not observed.

2.3.2 Scanning electron microscopy

The SEM data of *H. axiothellae* contained many external morphological characteristics previously described by Leander et al. (2002) and Rueckert and Leander (2008). The suction cup of the trophocyte was clearly visible (Figure 1c). The cell possessed thecal barbs protruding from the cell membrane, and the stylet was extended out of the trophocyte (Figure 1c). Along some of the gonocytes where there were no thecal barbs, the potential epibionts first reported by Leander et al. (2002) were visible (Figure 1c). Where there were no thecal barbs or suspected epibionts, a gymnodinoid alveolar sac pattern was visible. The flagellar pore reported by Leander et al. (2002) was not visible, due to the angle of the SEM data that were captured.
Fig. 1 – LM and SEM showing general morphology of *Haplozoon axiothellae*. a. Light micrograph demonstrating trophocyte internal stylets (st), gonocyte nuclei (g), and starch granules present in the gonocytes and sporocytes (sc). b. Light micrograph of a mature trophont demonstrating the trophocyte (t), gonocytes (g), double rows of sporocytes (sp), and junctions between gonocytes (j). c. Scanning electron micrograph with a protruding stylet (st). Bar = 25 µm.
2.3.3 Confocal microscopy

The confocal fluorescent data of *H. axiothellae* contained information never visualized in previous studies. Centrin antibody staining showed every compartment possesses two flagellar basal bodies despite a lack of flagella (Figure 4a). This includes the trophocyte, and all gonocytes and sporocytes.

Tubulin staining revealed a complex network of microtubules within the trophocyte (Figure 2c, d). The inner surface of the trophocyte compartment is lined with parallel lines of microtubules (Figure 3b) that form a basket supporting the entire structure of the trophocyte (Figure 3a, c). The microtubules are most concentrated near the suction cup of the trophocyte, and near the stylet (Figure 3a). There are some microtubules in the gonocytes but not nearly as concentrated as in the trophocyte.

By staining for DNA, the confocal data show that gonocytes initially have a single nucleus (Figure 1), while the most mature gonocytes are binucleate (Figure 3a). After developing their first few gonocytes, the most posterior gonocyte would become a sporocyte whose cytoplasm is significantly more granular than the gonocytes (Figure 1a). This granularity has recently been identified as starch (Wakeman et al., 2018). At this point, while still in a single row, the sporocytes are quadrinucleate (Figure 3c). No trophonts with double rows survived the staining process to collect confocal data, only smaller trophonts with single rows were successfully stained (Figure 2b, c, d).

In conjunction with the hoescht staining of DNA, microtubules are clearly visible in the form of spindle fibers interacting with the chromosomes (Figure 2d, 3a). The nuclei of all gonocytes were observed in active mitosis or having completed mitosis and separated into two nuclei. Nuclei in the trophocyte were sometimes observed in mitosis but most often were not actively dividing (Figure 3a). Quadrinucleated sporocytes were present (Figure 3c). The nuclei of the cells presented as a gradient of stages of mitosis, with the earliest phases (e.g., interphase) occurring at the anterior of the cell, and the later phases (e.g., cytokinesis) occurring at the posterior of the cell (Figure 4c).

If the bodies observed under SEM by Leander et al. (2002) were epibionts it is expected that their DNA would also be stained be visible. No DNA fluorescence was observed on the surface of the haplozoan cell.
FM-464 plasma membrane stained the membrane surrounding the haplozoan cell. A single outer membrane was visible, with no staining of the membranes that compartmentalize the cell (Figure 4b). Mature cells that were visible in later stages of developed, showed evidence of cytokinesis at the posterior of the cell (Figure 4c). The plasma membrane appeared to be developing between the most posterior gonocyte and the most anterior sporocyte.

Fig. 2 – Confocal fluorescence staining for tubulin and DNA in *Haplozoon axiothellae*. a. Light micrograph with DIC, b. DNA stained fluorescent blue, c. Tubulin stained fluorescent white, d. Fluorescent staining of DNA and tubulin. Bar = 25 µm.
**Fig. 3** – Confocal fluorescence staining for tubulin and DNA in *Haplozoon axiothellae*. **a.** Two separate haplozoan cells, with a large arrow indicating the tubulin concentration in suction cup (white), and double arrows indicating a binucleate gonocyte (blue). Bar = 25 µm. **b.** A cross section of tubulin in the trophocyte of the haplozoan demonstrating the linear arrangement of the microtubule basket (white). Bar = 10 µm. **c.** A single haplozoan cell with small arrows indicating the four nuclei of a quadrinucleate sporocyte (blue). Bar = 10 µm.
Fig. 4 – Confocal fluorescence staining for tubulin, centrin, and plasma membrane in *Haplozoon axiothellae*. **a.** Confocal fluorescence with arrows indicating flagellar basal bodies embedded in the membrane of the cell, with microtubules visible (white). Bar = 25 µm. **b.** A cross section of plasma membrane fluorescence showing a single plasma membrane surrounding the cell. Bar = 25 µm. **c.** A different haplozoan showing cytokinesis occurring at the posterior of the cell. Bar = 15 µm.
2.4 Discussion

2.4.1 Life cycle and development

Given the complexity of the haplozoan cell, we suspect that the life cycle is not as simple as in other dinoflagellates. By collecting LM and confocal data, we hoped to gather deeper insight into the compartmentalization of the haplozoan cell and that this could inform our understanding of the haplozoan life cycle.

Under LM, various stages of trophont developed were observed. Trophonts, always possessing a trophocyte, have anywhere from 1 to 8 gonocytes, with the largest observed cell possessing 8 rows of gonocytes and 5 rows of double sporocytes. The dinospores observed by Shumway (1924) were not observed. If dinospores do play a role in their life cycle, its possible the life cycle of *Haplozoon* is similar to another dinoflagellate parasite – *Chytriodinium*. These are ectoparasites of copepod eggs and like *Haplozoon*, have an unresolved molecular phylogenetic position. In *Chytriodinium*, an infective dinospore stages lives in the water column, lasting up to a few hours while seeking a host (Gómez, Moreira, & López-García, 2009). After finding a host, they attach to the surface of the egg with a feeding tube and transform into a trophont stage as they begin to feed (Gómez et al., 2009). After absorbing the contents of the egg, the trophont reproduces asexually by producing dinospores internally via sporogenesis. After a short amount of time, the trophont membrane ruptures and releases dinospores into the environment. The dinospores then go on to infect new hosts.

There are certain elements of the *Chytriodinium* life cycle that do not inform our understanding of *Haplozoon*. The haplozoan host-parasite relationship is not a one-on-one as in the case of *Chytridinium*. Haplozoans also do not rupture their membrane to release their next life stage. However, a similarity does exist because of the alternation between a trophont parasite stage, and a free-living dinospore stage.

Shumway (1924) observed the presence of haplozoan dinospores. Hypothetically, after a haplozoan dinospore parasitizes a host, the dinospore then becomes an immature trophont. This stage would be limited to a trophocyte and perhaps 1 or 2 gonocytes. Trophonts with 1 or 2 gonocytes were frequently observed in *H. axiothellae*. Shumway (1924) reports seeing trophonts consisting of trophocytes only in *H. dogieli*; however, trophonts this small were not observed in
H. axiothellae. Slowly, the haplozoan trophont would begin to grow and develop gonocytes as it matures, eventually producing sporocytes and dinospores of its own.

It is unclear how many nuclei the sporocytes possess after they have separated into double rows because this stage of development is both rare and fragile. Haplozoan cells frequently broke apart due to the manipulations involved in staining, and the cells with double rows are especially fragile. Previous observations (Shumway, 1924), indicate quadrinucleate sporocytes ultimately become four unique dinospores, each with a single nuclei. Because the trophocyte compartments have a single nucleus, this would support the idea that the dinospore from which they developed also possessed a single nucleus. However, because no dinospores were observed, the number of nuclei a dinospores possesses is unconfirmed.

A novel finding was possible due to the simultaneous staining of DNA and tubulin. The tubulin staining clearly shows spindle fibers present in the center of almost all the nuclei. In conjunction with the DNA confocal data, it is apparent that the haplozoan nuclei are constantly undergoing division. The data illustrate a maturity gradient in which nuclei in the earliest phases of mitosis are most anterior, and the nuclei in the later phases of mitosis are more posterior. This implies that new gonocytes are produced from the trophocyte at the anterior end. Even the earliest LM data have suggested gonocytes can be binucleate, and that the sporocytes are ultimately quadrinucleate (Shumway 1924). This always suggested a certain linear progression of nuclear fission. But the clear existence of spindle fibers in nuclei throughout the entire cell paints a more complete picture.

Haplozoan have a distinct pattern of nuclear fission: the trophocyte produces new gonocytes, these gonocytes then undergo mitosis creating a maturity gradient going towards the posterior of the cell, at which point gonocytes become binucleate, and further subdivide into sporocytes that are quadrinucleate and in which starch concentrates in the cytoplasm. If a trophont with double rows had survived the staining process, I suspect we would see that the quadrinucleate sporocytes have divided to a point where each compartment of the double row has a single nucleus. These would presumably become dinospores. Because of the granularity of the cytoplasm, it is difficult to tell the number of nuclei present per sporocyte at this stage using light microscopy alone. All of these observations are consistent with what has been previously described in H. axiothellae (Leander et al., 2002; Siebert, 1973; Siebert & West, 1974).
These observations in terms of the haplozoan life cycle are specific to *H. axiothellae*. It’s possible that other haplozoan species with a similar compartmentalization will follow a similar pattern in terms of nuclear division, for example *H. lineare*. But for other haplozoan species with a different compartmental arrangement, the pattern of nuclear division must be different. For example, *H. clymenellae* reportedly is not restricted to double rows of sporocytes and can have upwards of ten rows of sporocytes. In this case, the division of gonocytes into sporocytes will be unique given that the compartments are subdividing much further than in *H. axiothellae*. The same principles likely apply, but the order and number of nuclear divisions must ultimately differ between species.

2.4.2 Convergent evolution with Cestoda

This maturity gradient of gonocytes that runs anterior to posterior also solidifies the comparison between the body plans of *Haplozoon* and the Cestoda. As previously mentioned, new tapeworm proglottids are added to the neck of the animal, behind the scolex. Thus, the most mature proglottids are found towards the posterior of the tapeworm, eventually becoming reproductively mature and breaking away to infect a new host. The confocal data from *H. axiothellae* paints a similar picture. The trophocyte produces new gonocytes (i.e., the proglottid analogue) and these slowly develop until they are reproductively mature. As in tapeworms, haplozoans consist of repeating subunits that are progressively more and more mature, and eventually separate from their parent to infect a new host. This confirms another layer of similarity between *Haplozoon* and Cestoda, adding to what is already a striking example of convergent evolution over vast phylogenetic distances and fundamentally different levels of biological organization.

2.4.3 Trophocyte microtubule network

Beyond the microtubules making up the spindle fibers of the nuclei, microtubules were heavily concentrated in the trophocyte. Given the complexity of the stylet movement and the mobility exhibited by the trophocyte, it was suspected that this compartment would require microtubules. However previous TEM studies found no evidence of subthecal microtubules (Siebert & West, 1974; Wakeman et al., 2018). Given the suction cup apparatus requires some
strength and mobility to function, this high concentration of microtubules provides the parasite with the ability to latch onto the gut of the host. The microtubule basket of the trophocyte allows the compartment to be especially flexible, contorting as it attempts to latch onto host tissue, and insert its stylet.

2.4.4 Flagellar basal bodies

Despite our lack of information on a potential dinospore stage, if such a stage exists it would absolutely require flagella to navigate a marine environment. A single flagellar pore per gonocyte was previously identified in *H. axiothellae* (Leander et al., 2002). However, other previous studies indicate the absence of any flagellar system (Siebert & West, 1974; Wakeman et al., 2018). For this reason, centrin staining was performed to clearly label any flagellar basal bodies embedded in the plasma membrane of the cell. The confocal data from the centrin staining revealed two flagellar basal bodies per compartment embedded in the plasma membrane of the cell (Figure 4a). This adds to previous findings of a single flagellar pore per gonocyte, and conclusively demonstrates the presence of a flagellar apparatus that is necessary for the hypothetical dinospore stage to navigate through the environment and find a new host. A recent study identified a flagellar-like filament projecting near the base of the stylet (Wakeman et al., 2018), which is in agreement with the confocal data showing a flagellar basal body near the base of the stylet in *H. axiothellae*. This supports the idea that the flagellar system still functions at some stage in the haplozoan life cycle, likely a mobile stage prior to the trophont, which would go on to lose the flagella after developing into a trophont.

2.4.5 Membrane system and cellular compartmentalization

The most immediately striking haplozoan feature is their apparent multicellularity. Despite their multicellular appearance, there are no known dinoflagellates that are truly multicellular. For this reason, clarifying how *Haplozoan* relates to multicellularity was a key aspect of this study. SEM data indicates that the entire organism is surrounded by a single outer membrane (Leander et al., 2002), in contrast to being historically described as multicellular or colonial (Shumway, 1924; Siebert, 1973; Siebert & West, 1974). While the SEM data indicated that haplozoans are more accurately described as compartmentalized single cells, this study was
not conclusive (Leander et al. 2002). Determining the composition of the inner membranes was crucial, whether they were composed of plasma membranes, or of amphiesma as suggested by SEM and TEM (B Leander et al., 2002; Wakeman et al., 2018).

Fluorescent plasma membrane staining was conducted to better visualize the haplozoan membrane system and demonstrated a single outer membrane surrounding the cell. This supports Leander et al. (2002) in their classification of *Haplozoon* as a compartmentalized syncytium. The internal septa that separate the various compartments of haplozoans were not stained by the plasma membrane stain (Figure 4b). This indicates that the structure of the inner membranes used to compartmentalize the cell are different than the classically understood plasma membrane that surrounds it. This is further evidence that the compartmentalization is done by the amphiesma.

Plasma membrane staining also discovered cytokinesis occurring between the posterior most gonocytes and the anterior most sporocyte of multiple cells (Figure 4c). Multiple stages of cytokinesis were observed, ranging from the absence of a membrane (Figure 4b), the beginning stages of membrane formation, and the almost complete formation of a membrane separating two compartments (Figure 4c). It is suggested that this is the process by which sporocytes further divide into their double rows but was not confirmed because no confocal fluorescent data was collected from haplozoans with double rows. This builds on the description that haplozoans possess mature and immature junctions between compartments (Leander et al., 2002). Mature junctions were described as deep indentations between compartments, while immature junctions were shallow indentations that suggested an eventual cleavage. This description would be consistent with cytokinesis beginning to form where deep junctions were observed externally, and through fluorescence is visible as the formation of a new plasma membrane between cells.

### 2.4.6 A putative new species of *Haplozoon*

While dissecting *A. rubrocincta* in search of *H. axiothellae*, a haplozoan cell was found that was morphologically distinct from *H. axiothellae* (Appendix A.1). This was interpreted as a possible new species of *Haplozoon*. While a complete morphological description of a new species is a challenge when only a single individual is found, there are still some possible implications on host-parasite specificity.
To date, *Haplozoon* have only ever been found one species at a time. That is, every host has only ever contained a single species of *Haplozoon*. Recently, it was found that multiple haplozoan species are capable of infecting the same host (Rueckert & Leander, 2008; Wakeman et al., 2018). This finding suggests that haplozoan species are either: (1) not host-specific, or (2) host-specific, but mediated by geographical distribution. In this way, while *H. praxillellae* and *H. ezoense* are both established parasites of the annelid *Praxillela pacifica*, they wouldn’t co-parasitize the same host species due to their respective geographic distributions.

However, the discovery of a new haplozoan species co-parasitizing *Axiothella rubrocincta* along with *H. axiothellae* changes our understanding of host-specificity even further. Never have two haplozoan species been found co-existing within the same host. This finding is speculative provided only a single cell of the possibly new haplozoan species was found. But this suggests that when the geographic distribution of multiple haplozoan species overlaps, they may be capable of co-parasitizing.

It also raises another question: if this new haplozoan species is not a major parasite of *A. rubricincta*, then in which maldanid host does its life cycle typically take place? Presumably, if this new species is not commonly found in *A. rubricincta* (which is consistent with my observations), then it should be more commonly found in another host. Because the success rate of infection is presumably very low, the life cycle of these dinoflagellate parasites would rely on mass reproduction to ensure successful infection of a new host. This life strategy would require a primary host in which the parasite does reproduce to a high degree. This implies that another maldanid in the Pacific Northwest may be the primary host for this haplozoan species.
Chapter 3: Conclusion

By sampling for *Haplozoon axiothellae* on San Juan Island, WA, I was able to collect LM, and confocal data that provide new insights into our understanding of this mysterious parasite. The LM data provide updated micrographs for *H. axiothellae*, with the last published micrographs being from 1973. These micrographs, along with confocal data, suggest *H. axiothellae* develop repeating subunits linearly towards the posterior of the cell, with each compartment more mature than the last. These data illustrate an amazing example of convergent evolution between *Haplozoon* and Cestoda across significant phylogenetic distance.

The confocal data provides the first fluorescent imaging of a haplozoan cell, and revealed several previously unknown features: (1) the unique microtubule arrangement with the trophocyte, (2) nuclei undergoing constant division in preparation for mitosis, (3) conclusive evidence of flagellar basal bodies in the plasma membrane, (4) a single continuous plasma membrane surrounding the cell, and (5) evidence of cytokinesis separating mature sporocytes with a their own plasma membranes.

This last finding is particularly important, because it confirms that haplozoans are not multicellular, and should not be referred to as such in the future. As Leander et al. (2002) suggested, they are a compartmentalized syncytium, using amphiesmal membranes to form junctions between compartments. However, despite being unicellular, they have evolved functional differentiation within their single cell.

3.1 Future Directions

There remains significant work to be done with respect to developing a more complete understanding of *Haplozoon*. Here I will suggest topics for future research, that would complement the work I have accomplished and the studies that came before me.

3.1.1 Life Cycle

Perhaps the most mysterious aspect of *Haplozoon* is their life cycle. Based on our understanding of other dinoflagellate parasites, and the observations of Shumway (1924), it is highly likely a flagellated dinospore stage exists in the life cycle of *Haplozoon*. However, it remains mysterious and understudied because it has only been observed once (Shumway, 1924),
while other studies have failed to reproduce these observations (Siebert, 1973). I did not observe these dinospores when isolating cells for my own purposes. I don’t think my lack of observations disproves the existence of a dinospore stage – my focus was isolating adult trophonts for the purpose of understanding their cellular compartmentalization. Isolating the relatively large trophonts for my microscopy protocols was challenging in and of itself, and so I am not surprised that I did not casually observe dinospores. I suspect it would require concerted effort during dissections of the host, and perhaps a cell culture, to ultimately isolate the dinospore stage.

Despite this, it is logical that such a stage exists. The haplozoan trophont is not equipped with flagella to navigate a pelagic environment, and so a flagellated dinospore stage that can act as a transition between hosts is likely. The eventual examination of the dinospore would complete our understanding of the haplozoan life cycle, but also flesh out our understanding of their morphology. For example, it will clarify the function of the basal bodies that I observed and indicate whether they are actively used by at least one life stage or are the remnant of a lost characteristic. A standard protocol of LM, SEM, TEM, and perhaps confocal microscopy will provide a robust description of the dinospore morphology.

There is also some confusion surrounding the haplozoan cysts that can form from dinoflagellates. Originally, the cysts were described as a mechanism for dinospores to survive a harsh environment (Shumway, 1924). However, SEM data show a bud-like growth on haplozoan trophonts that has also been labeled a cyst (Leander et al., 2002). So, in addition to the description of the haplozoan dinospore, also clarifying whether dinospores are capable of encysting would add to our understanding of the haplozoan life cycle even further.

### 3.1.2 Phylogeny

As previously mentioned, normal DNA barcoding techniques using 18S rDNA sequences, fail to resolve the phylogenetic position of many dinoflagellates, including *Haplozoon*. There has been success using transcriptomics to resolve the phylogeny within Dinoflagellata (Janouškovec et al., 2017), and other enigmatic groups (Lu et al., 2017). Transcriptomics analyze all the RNA present within a cell at a given time. Using this method, hundreds if not thousands of comparisons across many sequences of RNA can be made between individuals. Rather than relying on a single sequence such as in standard DNA barcoding, the
sheer number of comparisons used in transcriptomics. provides significantly more power for solving phylogenies. Often DNA barcoding using an 18S rDNA sequence is enough to discriminate one species from another, and a transcriptome would be a complex solution to a simple problem. But with these more difficult phylogenetic questions about deeper relationships, transcriptomics is a powerful tool. Given previous success resolving the dinoflagellate phylogeny (Janouškovec et al., 2017), a transcriptome from a haplozoan species should significantly improve inferences about its phylogenetic position.

This will also work towards the discrediting of the order Blastodiniales. Blastodiniales has been described as an “order of convenience” (Fensome, 1993), grouped together for their parasitic life cycles but not supported by molecular data. Phylogenetics confirm this is a polyphyletic group (Saldarriaga et al., 2004). Haplozoon, among other dinoflagellates currently classified as Blastodiniales (Oodinium, Amyloodinium), will inevitably be reclassified among other monophyletic dinoflagellate orders, and Blastodiniales will in time fall from use.

### 3.1.3 Updated species descriptions and further species discovery

To date, only three of the eleven species of Haplozoon have been adequately described using modern morphological and molecular data. Eight species of Haplozoon (H. armatum, H. lineare, H. delicatulum, H. ariciae, H. macrostylum, H. obscurum, H. clymenellae, H. dogieli) are almost mythical given the extremely limited information available. To broaden our understanding of Haplozoon, and to learn more about these enigmatic species that were discovered over 100 years ago, future work should include confirming the existence of these species using modern microscopy (TEM, SEM, LM) and molecular techniques.

Additionally, there is significant haplozoan species discovery potential. Although it is not certain that all maldanid worms serve as hosts for Haplozoon, very limited sampling has been done and it is highly likely that the dissection of further maldanid worm species will reveal the existence of new species of Haplozoon.

### 3.1.4 Further fluorescence staining for confocal microscopy

More staining of haplozoan cells is expected to yield even more information about their cellular organization. In particular, it would be ideal to stain for the amphiesma with the hope of
conclusively demonstrating the membranes that compartmentalize the haplozoan cell are in fact amphiesma. Current data suggests this is the case, but a successful staining would leave no doubt. Ciliate alveoli are often stained using silver staining techniques (Galigher & Kozloff, 1971), and it’s possible a variation of silver staining could stain dinoflagellate amphiesma as well.

Additionally, a plasma membrane stain that could survive aldehyde fixation, such as FM-464X, would allow for very detail confocal data to be obtained from the haplozoan plasma membrane. The staining I conducted was a live-cell staining, and while it clearly demonstrated a single plasma membrane that surrounded the haplozoan cell, image data from a fixed cell would be much higher detail. It would clearly define the external cellular membrane, as well as any points at which cytokinesis is occurring, in an image that is much better resolved than the ones I obtained.

It’s also likely that a successful actin staining would provide even more insight into the activity of the haplozoan cell. Specifically, staining of the trophocyte, which is very active and likely contains actin in some form.

3.1.5 Epibionts

Potential epibionts were described by Leander et al. (2002). Although my project did not involve TEM of Haplozoon, TEM of a gonocyte that possesses the suspected epibionts will reveal what they are, epibionts or otherwise.
References

Andersen, K. (1975). Ultrastructural studies on Diphyllobothrium ditremum and D. dendriticum (Cestoda, Pseudophyllidea), with emphasis on the scolex tegument and the tegument in the area around the genital atrium. Zeitschrift Für Parasitenkunde, 46(4), 253–264.


Appendix

A.1 18S Dinoflagellate Phylogenetic Tree

18S tree of Dinoflagellata including *Haplozoon*, but whose position in the tree is poorly resolved. This tree is from Rueckert and Leander (2008).

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**Perkinssus marinus** (AF126013)

**Syndiniaceae**

- **Syndinium sp.** (DQ149409)
  - Eukaryote clone OLI11010 (AJ402329)
  - Marine Alveolate Group I

- **Amoebophrya in Gymnodinium miniatum** (AF472553)

- **Haplozoon prattiae** n. sp. (EU594092)
- **Haplozoon siculothelae** (AF274264)

**Blastodiniaceae**

**Kryptoceratium foliaceum** (AF274268)
- **Peridiniales**
- **Dinophysiales**

**18S tree of Dinoflagellata including Haplozoon**, but whose position in the tree is poorly resolved. This tree is from Rueckert and Leander (2008).
A.2  *Haplozoon* sp. nov.

LM of a possible novel species of *Haplozoon* found while dissecting *A. rubrocincta.*