

**INTERACTIONS BETWEEN ANTS AND LARVAE OF THE HOST-MANIPULATING PARASITE, *DICROCOELIUM DENDRITICUM***

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## **ABSTRACT**

Many parasites manipulate their host's behaviour to facilitate their own transmission. The phenomenon is complex, requiring multi-disciplinary approaches. I evaluated host decision making and utilized modern imaging techniques to understand how larvae of the fluke, *Dicrocoelium dendriticum*, can so radically alter the behaviour of their ant, *Formica aserva*, hosts. My results showed that infected ants make decisions regarding substrate that uninfected hosts never make. They preferentially attached with their mandibles to flower blossoms that were familiar to them and they preferred flowers that contained attached nestmates. Site-selection by larva occurred within the ventral-anterior-most region of the sub-esophageal ganglion of the ant brain, proximal to the control centres that regulate the action of the mandibles, feeding behaviours, and temperature sensing. My results provide the key foundation for further studies designed to determine whether the complex manipulation of ant behaviour requires direct physical and/or neurochemical modulation by the brain worm.

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## LIST OF ABBREVIATIONS

AdM	Primary mandibular closer muscle fibres
AL	Antennal lobe
AM	Antennal muscles
AN	Antennal lobe diffuse neuropil
ANG	Antennal lobe glomeruli
AP	Apodemes
C	Central body
CB	Cell body rind
CE	Compound eye
CLSM	Confocal laser scanning microscopy
CHP	Cypress Hills Inter-Provincial Park
CNS	Central nervous system
CW	Cyst wall
DD	<i>Dicrocoelium dendriticum</i>
DS	Dense secretory bodies
E	Esophagus
FC	Flame cell
H	Host tissue
IC	Inner cyst layer
LM	Light microscopy
MB	Mushroom body calyx
Micro-CT	Micro computed tomography
ML	Muscle layer
N	Nucleus
OC	Outer cyst layer
OL	Optic lobe
P	Peduncle

PDM	Pharynx dilator muscles
PPG	Postpharyngeal gland
SEG	Sub-esophageal ganglion
SGA	Secretory granule aggregations
SPG	Supra-esophageal ganglion
T	Tegument
TEM	Transmission electron microscopy
TS	Tegument spines

## Chapter 1: General Introduction

### 1.1 General Background

Parasites require host resources to support their development, survival, and reproduction. It follows that by exploiting their hosts, certain phenotypes of infected hosts will differ from hosts that are not infected. Phenotypic traits associated with host immunity, wound-healing, and tolerance are the best known (Schmid-Hempel, 2013). Yet the results of studies completed since the 1970's, and especially over the past 20 years, have indicated that host phenotypes in addition to those linked to host defense can also be profoundly altered by parasites. Indeed, some of those alterations can be spectacular in their expression. The abdomens of ants infected with the nematode *Myrmeconema neotropicum* turn bright red, a feature that is thought to mimic the berries that are common within the high canopy of tropical forests (Yanoviak, Kaspari, Dudley, & Poinar, 2008). The tentacles of semi-terrestrial snails that are infected with the larval stages of the trematode, *Leucochloridium* sp., are brightly coloured and pulsate (Wesołowska & Wesołowski, 2014). Extra and deformed limbs form when larval amphibians are infected with the flatworm *Ribeiroia* sp., a morphological change that increases the likelihood of predation after metamorphosis (Johnson & Sutherland, 2003). Each of these parasite-induced alterations to host phenotype function to increase parasite fitness. Thus, alterations in host phenotype are often driven by the parasite themselves, serving to increase rates of transmission, and thus reproduction, into obligate final hosts (Lafferty, 1999).

Another class of parasite-altered phenotypes that has gathered research

momentum over the past 20 years involve those associated with host behaviours. Several of the examples indicated above involve alterations of host behaviours, in addition to alteration in host conspicuousness (Adamo, 2002; Van Houte, Ros, & Van Oers, 2013). The results of behaviour studies indicate that the berry ants are less aggressive to potential predators (e.g. birds) and their mobility is altered to ‘wave’ their conspicuous abdomens, presumably to better mimic the berries that are common in the upper canopy (Yanoviak et al., 2008). Similarly, *Leucochloridium*-infected snails are reported to ascend higher onto vegetation in well-illuminated microsites on plants, where the flashes of their pulsating, coloured tentacles are most likely to be visible to avian predators that are required definitive hosts (Wesołowska & Wesołowski, 2014). The results of studies such as these are important because they indicate that parasite-induced alterations in behaviour traits can be as influential on parasite transmission and reproduction as those on host morphology, brightness, and colouration (Moore, 1995).

In another well-known example of parasite-induced manipulation of host behaviour, encysted stages of the protist parasite *Toxoplasma gondii*, reside within the central nervous system (CNS) of mice, causing them to be attracted to cat urine (Berdoy, Webster, & McDonald, 2000). This alteration in behaviour is thought to increase rates of predation by cats that are required to complete the life cycle. If this alteration is adaptive to the parasite, a reduction in fear and anxiety can be considered a specific alteration that acts to exploit sensory stimuli associated with foraging and predatory evasion responses specifically to cats (Adamo, 2012). The results of subsequent studies involving rats and mice infected with encysted stages of *T. gondii* have shown that these hosts responded the same to fear stimuli (learned danger) and anxiety stimuli (open spaces) as uninfected

hosts, except in the presence of feline urine (House, Vyas, & Sapolsky, 2011). Further, in enclosures with feline and rabbit urine on either side, uninfected rats preferentially went to the rabbit side, while infected animals went to the feline side (House et al., 2011). Thus, the typical fear/anxiety responses of the rats remained unchanged unless the hosts were presented with stimuli specific to infection. As typical fear and anxiety responses remained intact, this favours the hypothesis that *T. gondii* specifically alters hosts behaviour(s) that enhance parasite transmission, and thus, parasite reproduction.

The results of these and other studies involving *T. gondii* support the idea that the types of host behaviours that are altered by parasites is often tightly linked to the location within the host where the parasite resides (Adamo, 2012). As *Toxoplasma* encysts within the brains of its intermediate hosts, the targeting of brain structures associated with innate fear response, anxiety, and host-to-host communication might be expected. The results of studies involving experimentally-infected mice show that host neural activity increases in the amygdala, a structure in the brain that is responsible for sexual stimulation (Gonzalez et al., 2007; House et al., 2011), and that this neural spike mimics the response of uninfected male mice to female mice stimulant. *Toxoplasma* cysts that reside in the brain are increasingly found within the amygdala compared to other structures in the CNS (Gonzalez et al., 2007). Thus, site-selection of the cysts for the amygdala may contribute to the efficacy of the regulation in neural activity and thus the potential for parasite-mediated interference with the neurochemicals that underlie altered behaviour.

Within-host site selection is an important aspect of host-parasite interactions, with consequences for host immune responsiveness (Koella et al., 1998), the avoidance of interspecific competition with other parasites (Holmes, 1973), and parasite-mediated host

pathology (Sandland & Goater, 2001). As indicated in the examples above, site selection is also integral to the potential for the manipulation of host phenotypes, especially those associated with behaviour (Barber & Crompton, 1997). In particular; residence within the central or peripheral nervous systems can provide direct access to the structures that regulate complex suites of behaviours in animals (Gold & Shalden, 2007; Gronenberg, 1996; Ligasová, Bulantová, Ka, Koberna, & Mike, 2011). Experimental studies involving trematode metacercariae that encyst within the optic lobes have shown that infection is associated with reduced visual acuity of minnow intermediate hosts (Shirakashi & Goater, 2005). Likewise, metacercariae that encyst within the lens of the eye of the common bully are known to block the retina which distorts host vision (Stumbo & Poulin, 2016). Thus, for parasites that alter host behaviours to facilitate transmission, there is strong evidence that site-selection within the peripheral or central nervous systems plays a key role.

Several examples involving parasitoid wasps and their insect hosts demonstrate the tight connections between parasite site-selection and host behaviour manipulation. Adult female jewel wasps insert their ovipositor into the head region of their obligate cockroach host, specifically targeting precise regions of the brain and the sub-esophageal ganglion (SEG) (Gal & Libersat, 2008; Libersat, Delago, & Gal, 2009; Libersat & Gal, 2013); this structure in the insect CNS controls motor function (Gronenberg, 1996; Paul & Gronenberg, 2002). The female injects a potent venom that reduces the ability of the host to walk (Gal & Libersat, 2008). Cockroaches injected with calcium-channel blockers into their SEG exhibit similar immobility as cockroaches injected with the wasp venom (Gal & Libersat, 2010). The venom-injected cockroaches then remain unable to move as

the larva of the wasp develop, eventually killing its host (Gal & Libersat, 2008). In this example, site-selection within the host's SEG is integral to the wasp limiting the movement of its host.

The examples listed above all showcase the spectacular nature of parasite-induced alterations in host phenotypes – behavioural and otherwise. Within recent years, research involving parasite behaviour modifications have garnered more attention (Libersat et al., 2009; Thomas, Adamo, & Moore, 2005), however, empirical studies are few in comparison to theoretical studies and reviews (Poulin & Maure, 2015). As pointed out by a multitude of reviews, the need for empirical studies, and beyond that, mechanistic studies, are needed to advance the field of host manipulation (Hughes & Libersat, 2018; Poulin & Maure, 2015). Fundamentally, to understand the mechanisms parasites use to alter host behaviour, a multidisciplinary approach is necessary as parasites that reside within their hosts, especially the CNS, cannot be solely explained by pathology (Hughes & Libersat, 2018). For advances in this area, experimental and multi-disciplinary studies are required that involve hosts infected with CNS-residing parasites that demonstrate behaviours that differ from hosts that are uninfected.

## **1.2 Model System**

Larva of the lancet liver fluke, *Dicrocoelium dendriticum*, reside within the SEG of their ant intermediate hosts, causing a spectacular alteration in behaviour that is well described in introductory biology and parasitology texts (Krull & Mapes, 1953; Manga-González, González-Lanza, Cabanas, & Campo, 2001). This trematode is now cosmopolitan in its global distribution, particularly in areas containing domestic ruminant populations (Manga-González & González-Lanza, 2005; Manga-González et al., 2001;

van Paridon, Colwell, Goater, & Gilleard, 2017). Adult *D. dendriticum* reside in the bile ducts of ungulates - both domestic and wild. Eggs are produced and released in the feces of this final host, where they are consumed by the first intermediate host, terrestrial snails. Hundreds to thousands of asexually-produced larvae (cercariae) develop within snail hosts. During spring and summer in temperate locations, the larvae are emitted from snails within a mucous-coated 'slime ball' onto the substrate (Schuster, 1993). These packages of free-swimming cercariae are infective to a wide range of ants in the family Formicidae, which are required second intermediate hosts. Following ingestion by ants, larval *D. dendriticum* penetrate the gut and then encyst within the hemocoel where they become infective to the final host as metacercariae (Spindler, Zahler, & Loos-Frank, 1986); one or more of the ingested larvae migrate to the ant's brain following ingestion, where they tend to reside within the SEG (Romig, Lucius, & Frank, 1980).

Infected ants undergo an alteration in their typical behaviours. Even a single larva in the SEG causes its host to leave its nest, select a plant adjacent to its nest, ascend it to a certain height, and then latch onto it with its mandibles (Carney, 1969). Most typically, the infected ant will attach to flower petals; more rarely onto the leaves or stem of the flower, or onto grass blades (Spindler et al., 1986). Infected ants later detach from the plant and return to their nests. They then repeat this sequence of attachment, followed by detachment, the next day. The 'attach-detach-repeat' sequence of behaviours is highly temperature dependent (Botnevik, Malagocka, Jensen, & Fredensborg, 2016; Spindler et al., 1986). Through unknown mechanisms, the initiation of behaviours associated with the decision to leave the nest, select a flowering plant, ascend it, and then attach to it only occurs during the cooler hours of the day. In contrast, detachment and descent of the

plant occurs as temperatures rise above approximately 16°C (Spindler et al., 1986). This sequence of temperature-dependent behaviours means that infected ants remain attached to plants from late afternoon, throughout the night, until late morning. Overall, this pattern of altered behaviours is repeated each day in the areas immediately adjacent to infected nests until late summer when ants tend to cease foraging (Goater, unpublished observations).

One of the unique features of the larval *D. dendriticum*/ant interaction, and one that makes it ideal as a study system, is that key components of the altered behaviour are reversible. Thus, the manipulation turns on-and-off without requiring the death of the host. The reversible nature of the alteration involving attachment/detachment is unlike any known parasite-induced alteration in nature. In the case of the berry ants described above, the reddening of the abdomen is permanent. In the case of *T. gondii*-infected rats and mice, there is no evidence that changes in response to felines change over time. On the one hand, the complex and reversible nature of the attachment/detachment sequence of behaviours makes the discovery of underlying mechanisms more challenging. On the other hand, reversibility means that researchers can ‘turn on’ and ‘turn off’ the behaviour simply by exposing naturally-infected ants to contrasting temperatures. This latter feature allows researchers to study various aspects of the alterations under laboratory conditions.

The local availability of *D. dendriticum*-infected ants and the background information available on this local system provides an excellent foundation for studies focused on the altered behaviour. Previous studies have identified 32 known locations in Cypress Hills Park (CHP), Alberta where ants have been observed attached to vegetation (Beck, 2015). At three of these sites, we have observed attached ants adjacent to a well-

defined nest each year since 2009. At these sites, background information is available on patterns of *D. dendriticum* infection in ant (van Paridon, Gilleard, Colwell, & Goater, 2017) and snail (Dempsey et al., 2019) intermediate hosts. Ongoing studies that involve monitoring the behaviour of marked, infected ants as they leave and enter their nest also occur at these sites (Goater, unpublished observations), as do the imaging studies that have been completed at the brain worm/brain interface (Martín-Vega et al., 2018). Background information is also available that describes the history of *D. dendriticum* invasion and emergence into the Park (van Paridon, Gilleard, et al., 2017), its life-cycle within known hosts in the park (van Paridon, Gilleard, et al., 2017) and its pattern of utilization of definitive hosts (Beck, Goater, & Colwell, 2015).

### **1.3 Thesis Objectives**

My thesis uses the local *D. dendriticum*/ant model to better understand various aspects of the ‘attach-detach-repeat’ sequence of altered behaviours in infected ants. My thesis combines behavioural choice assays in laboratory settings, field surveys of infected ants on plants, and modern imaging techniques. One underlying premise of my work is that a detailed understanding of site-selection in the brain of infected ants and how the brain worm interacts with proximal host structures is a fundamental requirement for future studies aimed to uncover mechanisms. A second premise is that, because previous studies involving the behaviours of infected ants are observational, experimental studies designed to evaluate the decisions that infected hosts make are needed to understand the range of potential behaviours that *D. dendriticum* manipulates in its host.

Chapter two uses imaging tools to characterize site-selection by larval *D. dendriticum* in the SEG of naturally-infected ants, *Formica aserva*, collected from sites in

CHP. In this chapter, I image infected and uninfected *F. aserva* heads by combining, for the first time, light microscopy, serial histology, confocal laser-scanning microscopy (CLSM) and transmission electron microscopy (TEM) at the host-parasite interface. For this component of my thesis, I ask whether the morphology, orientation, architecture, and location of the brain worm in the SEG of infected ants can help us understand how *D. dendriticum* might influence behaviours associated with the action of the mandibles, overall host mobility, feeding behaviours, and/or behaviours associated ant-to-ant communication.

The third chapter evaluates flower choice behaviour of ants infected with *D. dendriticum*. For transmission to occur, the infected ants must make their way up vegetation and attach to it when temperatures drop. This atypical behaviour is not seen by their uninfected counterparts, which spend much of their time within the nest or foraging. The process of host decision-making when choosing which specific flower to select for attachment, and how it may be influenced by infection, is unknown. First, I conducted field surveys to quantify the manner in which infected ants utilize the population of available plants that are adjacent to a nest. Here, I ask whether infected ants are distributed at random across this resource or are aggregated on individual flowers. Second, I conducted behavioural experiments in which infected ants were offered a binary choice between substrate for attachment. Using these experiments, I ask if infected ants prefer to attach to familiar vegetation, the vegetation they were attached to upon collection, compared to vegetation that had never contained an infected ant. Following this, I tested aggregation within the experimental containers by introducing an additional infected ant, as well as by giving a single ant the choice between a flower that previously had an

infected ant attached. Through these experiments, I seek to clarify the nature of decision-making and overall mobility in hosts infected with a parasite that resides permanently and precisely within regions of the host's CNS.

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## **Chapter 2: Parasites in the brain: Imaging tools reveal potential mechanisms of behavioural manipulation in zombie ants**

### **2.1 Abstract**

Parasites that reside within the central nervous system (CNS) of their hosts have the potential to alter a wide range of behaviours. The extent to which that potential is realized is poorly known, in part, because there is little information available on the physical, mechanical, and architectural nature of the host/parasite interface for CNS-dwelling parasites. Larva of the iconic manipulating trematode, *Dicrocoelium dendriticum*, reside within the brain of ants, leading to radical alterations in key host behaviours. Due to the complexities of behavioural alteration at such a small scale, I combined tools in light microscopy, transmission electron microscopy and confocal laser scanning microscopy to characterize microsite selection within the brain of field-collected *Formica aserva*, to describe the functional ultrastructure of the brain-dwelling larva, and to evaluate characteristics of the host-parasite interface. Microsite-selection of individual larva occurred within the ventral- and anterior-most region of the subesophageal ganglion, a precise location that strongly supports behaviour-function relationships. The brain-dwelling larva were enveloped by a thin and flexible 2-layered cyst wall that is always in direct contact with host tissue. The structure of the larval tegument and the nature of the parenchyma and muscle tissue was consistent with other encysted trematode metacercariae. However, the anterior-most regions of some brain-dwelling larva extended through the cyst wall and appeared to have direct contact with dorsal regions of the host SEG, directly proximal to regions of the brain that likely play a role in host feeding responses. These results hint at potential mechanisms used by larval

*D. dendriticum* within its host to cause behavioural changes, while confirming micro-site selection and identifying parasite ultrastructure.

## **2.2 Introduction**

For parasites that require transmission between hosts that occur on different trophic levels, parasite-induced alteration of host behaviour is a common outcome. Indeed, natural selection will favour this outcome if the alteration facilitates the transmission of infective stages between trophic levels (Lafferty, 1999). Parasite-induced alteration of behaviour has now been demonstrated in many host-parasite interactions (Poulin & Maure, 2015; Poulin & Morand, 2000). Yet despite the ubiquity of this phenomenon across many host and parasite systems, the underlying mechanisms of parasite-induced behavioural changes remain poorly known. Parasites located within the host's CNS have the strongest potential to alter host behaviour, however, due to the complex nature of the CNS these host-parasite systems are often challenging to study (Hughes & Libersat, 2018). One well studied system involves the encystment stages of the Apicomplexan protist, *Toxoplasma gondii*, that reside within the host's hypothalamus (Adamo, 2012) or amygdalar structures (Vyas, Kim, Giacomini, Boothroyd, & Sapolsky, 2007). In rat hosts, encystment within tissue of the brain leads to alterations in behaviours associated with anxiety, fear-responses and intraspecific attraction (Afonso et al., 2017; House, Vyas, & Sapolsky, 2011). Further, unencysted metacercariae of the trematode *Tylodelphys* sp. that occur within the eyes of fish undergo daily migrations to block the retina (Stumbo & Poulin, 2016). This behaviour leads to reduced visual acuity in infected fish, a behaviour that is thought to enhance the transmission of larvae into avian definitive hosts. Similar behavioural outcomes have been demonstrated to be caused by

larval trematodes that occur in the lens of the eye and in the optic lobes of fish (Karvonen, Seppälä, & Valtonen, 2004; Matisz, Goater, & Bray, 2010). These results imply that for several behaviour-manipulating parasites, residence within the CNS provides a blunt tool to facilitate transmission between trophic levels.

Further evidence for a direct linkage between altered host behaviours and infection comes from some parasitoid-host interactions. Female jewel wasps, *Ampulex compressa*, require cockroaches as a food source for their offspring. During the oviposition process, females target the sub-esophageal ganglion (SEG) for the injection of potent neurotoxins (Gal & Libersat, 2008). In this case, the outcome is direct inhibition of the ability of the cockroach to walk, thereby reducing host predation rates during larval development. Furthermore, the injection of sodium channel blockers into the SEG of uninfected cockroaches mimicked the same behavioural change as injection of the wasp venom into the SEG (Gal & Libersat, 2010). In this example, direct physical (and chemical) interference with a key motor control centre in the brain led to specific altered host behaviours that enhanced parasite survival. It is currently unknown whether other manipulating parasites that reside within the host CNS require a similar level of direct physical and or chemical manipulation of key control centres.

Larva of the trematode *Dicrocoelium dendriticum* also reside within the SEG of their ant intermediate hosts, leading to radical alterations in host behaviour (Romig, Lucius, & Frank, 1980; Spindler, Zahler, & Loos-Frank, 1986). Ants are infected following the ingestion of packets of free-living larvae (cercariae) that are deposited in the slime trails of infected land snails. Once ingested, the larvae penetrate the ant's gut wall and enter the hemocoel where they develop into fully-encysted, resting metacercariae

(Krull and Mapes 1953). Up to hundreds of metacercariae can occur in the hemocoel of a single ant (van Paridon, Gilleard, Colwell, & Goater, 2017). However, at least one larva migrates to the head region, where it resides within the sub-esophageal ganglion of the brain. This “brain worm” is responsible for the ant’s altered behaviour (Romig et al., 1980), which includes leaving the nest, selecting a plant, and attaching to it with the mandibles (Spindler et al., 1986). During attachment, infected ants do not feed or protect themselves from predators. Attachment occurs over hours or days, until temperatures exceed approximately 24°C when they return to their nests. They leave the nest again the next day when temperatures drop, typically retuning to the same plant (Botnevik, Malagocka, Jensen, & Fredensborg, 2016; Spindler et al., 1986; Chapter 3). The ‘attach-detach-repeat’ sequence continues depending on temperature or until an attached ant is ingested by a grazing mammal or dies of other causes (Carney, 1969).

Through the use of various microscopy techniques, the results of previous research have demonstrated that at least one larval *D. dendriticum* resides within the SEG of infected formicid ants (Martín-Vega et al., 2018; Romig et al., 1980). Whereas the total numbers of metacercariae in an ant can vary over two orders of magnitude (van Paridon, Gilleard, et al., 2017), the ‘brain worm’ appears to be required for the expression of the attach-detach-repeat sequence of behaviours. Although direct evidence for this contention from experimentally-exposed ants is not available, the results from field studies on marked ants show that ants that have a single larva in the brain, but no larvae in the abdomen, still attach to plants (Goater and van Paridon, unpublished observations). Location, size, orientation and morphology of the brain worm could all potentially be key components of the radical manipulation; however, characterization of these features has

not been completed, nor has it been done at a resolution that allows visualization of key elements of the host-parasite interface (Martín-Vega et al., 2018; Romig et al., 1980). It therefore remains unknown to what extent the brain worm is directly associated with, for example, host neurons involved in the action of the mandibles. Likewise, it is unknown to what extent the brain worm is associated with structures involved with other key aspects of the manipulation, such as feeding inhibition, predator avoidance, or ant-to-ant communication (Chapter 3).

In this chapter, I use a range of modern imaging techniques to visualize and characterize the ant-brain worm interface in naturally-infected *F. aserva*. Ants used for my imaging were collected from a location in southern Canada where the worm has become established following its introduction from continental Europe and its subsequent emergence (Goater & Colwell, 2007; van Paridon, Colwell, Goater, & Gilleard, 2017). At this location in Cypress Hills Inter-Provincial Park (CHP), Alberta, the life-cycle, history of emergence, host utilization and transmission dynamics are well described (Beck, Goater, & Colwell, 2015; Goater & Colwell, 2007; van Paridon, Colwell, et al., 2017; van Paridon, Goater, Gilleard, & Criscione, 2016). The purpose of this chapter is to combine light microscopy, confocal laser scanning microscopy (CLSM), and transmission electron microscopy (TEM) tools to characterize, for the first time, the host-parasite interface in the brains of *D. dendriticum*-infected ants. My overall aim is to use these visualization tools to better understand the nature of the interactions between a CNS-dwelling parasite and the suite of behaviours that it influences.

### **2.3 Materials and Methods**

### 2.3.1 *Source of D. dendriticum-infected ants*

*Dicrocoelium dendriticum*-infected ants were collected from two sites in CHP from May-August 2016-2018 between 5:00-11:00 AM. Ongoing work in our laboratory has previously established that *D. dendriticum*-infected ants, *F. aserva*, have been present each year at these two sites since at least 2010 (van Paridon, Gilleard, et al., 2017). Hundreds to thousands of ants can be observed attached to flower blossoms located adjacent to the nests at these two sites throughout the summer. Both infected and uninfected ants were collected from one or both sites when the air temperature was below 16°C. Uninfected ants (n = 10) were collected while they were either returning or leaving their nest and were immediately preserved in modified Karnovsky's solution for light and electron microscopy or acetic- zinc formalin fixative for CLSM. In some cases, infected ants were carefully removed from their flower blossom and either kept alive and transported to the University of Lethbridge in a cooler with ice or preserved at the site of collection. In the latter case, individual infected ants were fixed in acetic-zinc formalin if they were targeted for CLSM (n = 22) imaging, or in modified Karnovsky's solution if they were targeted for light and electron microscopy (n = 5).

### 2.3.2 *Tissue processing*

The procedures used to process host and parasite tissue for light microscopy and transmission electron microscopy (TEM) were adapted from Matisz, et al. (2010). First, the mandibles of ants were removed to allow improved penetration of Karnovsky's solution for at least 24 hours. Samples were then rinsed twice in 0.1M sodium cacodylate buffer (pH 7.2) for 10 minutes and fixed in cacodylate buffer and 1% OsO<sub>4</sub> for 1 hour. The samples were dehydrated in an ethanol series and embedded in a frontal (n = 4) or

sagittal ( $n = 1$ ) orientation (Fig. 2.1) in Spurr's resin, followed by polymerization at 60°C for at least 24 hours. Samples were then cut into 1  $\mu\text{m}$  sections using a Reichert OM-U2 ultramicrotome for light microscopy, and 70-150 nm sections for TEM. Sections were mounted onto stubbed slides and stained with toluidine blue for visualization using light microscopy.

Preparation for CLSM imaging also used frontal sectioning of ant heads (Fig. 2.1), with the mandibles and back of the head removed so that the thickness of the sample allowed for maximum light penetration. The samples were washed in distilled water prior to dehydration using the same ethanol series as described above. Sections were transferred into diluted pianeese Iib stain for approximately 24 hours then transferred to methyl salicylate for at least 12 hours to clear tissue. Following tissue clearing, samples were placed in new methyl salicylate until imaging.

### 2.3.3 *Imaging*

For light microscopy, samples were viewed using either a Zeiss Axioskop Imager MI viewed using StereoInvestigator software, or a Hamamatsu NanoZoomer 2.0-HT viewed on NDP software for colour images. For visualization using TEM, sections were placed onto plastic grids, stained with 4% uranyl acetate for 20 minutes, and then Reynolds lead citrate for 5 minutes. Images were taken with a Hitachi H-7500 TEM at an acceleration voltage of 100keV.

An Olympus FLUOVIEW FV1000 microscope was used for CLSM imaging. The Olympus confocal microscope was fitted with diode lasers, HeNe(G) laser, and a mercury laser. Components of the microscope that were standardized were the objective lens (ULSAPO 10X2 NA:0.40), laser 1 wavelength (488 nm) and laser 2 wavelength (635

nm). Alexa Fluor 488 and Alexa Fluor 633 dyes were used, with excitation wavelengths set at 488 nm and 635 nm, respectively. Images were obtained as sequential scans, analyzed on Olympus FLUOVIEW viewer. All images were lightly edited for clarity on Adobe Photoshop CC.

## 2.4 Results

### 2.4.1 Site selection by *D. dendriticum* in the ant brain

Each of the 27 infected *F. aserva* sampled from the 2 sites had at least one metacercariae within the SEG of the brain. Of these 27, a single metacercaria always resided within the anterior and ventral-most region of the SEG (Fig. 2.2a, 2.3). In one of the 27 infected brains, three metacercariae were enclosed within the SEG. One of these was located within the anterior and ventral-most region of the SEG, whereas the additional two were more posterior in the SEG.

At the location in the SEG where the parasite is visible (Fig. 2.2a), the antennal lobes are dorsal and they run the entire length of the anterior-posterior oriented larva (Fig. 2.3). The cell body rind of the antennal lobes and SEG are seen as large nucleated cells relative to adjacent neural tissue (Fig. 2.2a, 2.5). A large portion of the interior of the ant head is comprised of mandible adductor closer muscles, whereas the antennal muscles are located immediately below the antennal lobes (Fig. 2.5). One lobe of the post-pharyngeal gland is located dorsally, between the ant's esophagus and the dorsal exoskeleton; the other is located more centrally, immediately ventral to the SEG. The host's esophagus is always located centrally between the two antennal lobes (Fig. 2.3). A network of apodemes is located throughout the ant head, two of which are prominent on

either side of the ventral region of the SEG where the single larval *D. dicrocoelium* is located (Fig. 2.2a, Fig. 2.3). Results from the sequence of images in Fig. 2.3 indicate that the anterior/posterior-oriented apodeme that connects to the pharynx dilator muscle directly below the esophagus/pharynx runs immediately adjacent to the worm along its full length.

In all infected ants, the larva was surrounded by host neural tissue associated with the SEG (Fig. 2.3, 2.4). However, the distribution of host tissue immediately adjacent to the larva was highly heterogenous. Dorsally and laterally, the larva was embedded within relatively thick layers of host neuropil and/or cell bodies of the SEG. In contrast, neural tissue associated with the anterior- and ventral-most region of the worm was typically restricted to 1-2 cell layers (Figs. 2.2, 2.3, 2.4a, 2.5). Highly nucleated cell body rind tissue of the SEG was most prominent in the anterior and ventral portions (Fig. 2.5).

#### *2.4.2 Metacercariae morphology and ultrastructure*

The ventral sucker of the brain worm was consistently visible as a large, concentric, heavily muscled structure located within approximately the middle third of the worm. An oral sucker was located anterior-most in the larva, although it was not visible in all preparations. The larva was clearly bipartite, with the region anterior to the ventral sucker being heavily nucleated (Fig. 2.4a and b) and the region posterior being comprised of contrasting parenchyma tissue that was dominated by large, nucleated cells. Although the dorsal versus ventral orientation of the oral and ventral suckers was not consistent in all brains, the larva was consistently curled in its' orientation within the cyst wall.

The larva was enveloped by a complex tegument layer comprised of unidentified

carbohydrate and protein material. A complex layer of muscle was located immediately below the basement membrane (Fig. 2.6a and b). Secretory granule aggregations and increasingly nucleated cells were observed near the anterior end of the larva and the tegument, with flame cells apparent in the parenchyma tissue (Fig. 2.6c, d and e).

The wall of the cyst that enveloped the larva was bi-layered (Fig. 2.7). Larvae that were imaged via TEM demonstrated a visible cyst wall within a single plane ( $n = 5$ ). The inner cyst layer ranged in width from  $0.133 - 0.345 \mu\text{m}$  ( $n = 3$ ) and had a highly variable and inconsistent structure. The larval tegument was not always in contact with the innermost cyst layer. In cases where there was no direct contact, the tegument and inner cyst layer were digitiform, indicating that the thin and highly flexible cyst wall could mirror the shape of the tegument (Fig. 2.7). The region between the cyst wall and the tegument was filled with cellular debris, with projections from the inner cyst wall extending into the space (Fig. 2.6a and e; Fig. 2.7). The outer cyst wall layer ranged in width from  $0.0568 - 0.135 \mu\text{m}$  ( $n = 3$ ). The outer layer was always in direct contact with host tissue and maintains the same structure around the entire larva.

#### *2.4.3 Structure of the host-parasite interface*

TEM imaging indicated that gross pathology was not obvious in host tissue immediately adjacent to the outer cyst wall of the larvae. Thus, host nervous tissue adjacent to the worm contained intact neuropil and intact cell bodies and the cells themselves contained intact endoplasmic reticulum and mitochondria (Fig. 2.6a and b). Although not consistently observed, there were specific instances in which tissue pathology was evident. In these cases, pathology in the form of increased vacuolization of cell body rind in the SEG occurred within the highly restricted region between the dorsal-

most region of the worm (and its' associated cyst wall) and the distal-most point of the dorsal apodeme (Fig. 2.8). Other regions of the SEG that lay directly adjacent to the outer cyst layer showed inconsistent signs of pathology, with vacuolization within other regions of the SEG close to the parasite being observed in only one of the brains imaged for TEM.

Frontal sectioning showed that the brain larva was entirely enveloped by the bi-layered cyst wall at high magnifications. However, in other cases, particularly involving CLSM images, direct physical contact between the larvae and host neural tissue was observed. The apodeme directly dorsal to the parasite extends down from the esophagus towards the parasite (Figs. 2.3, 2.9). In several brains imaged by CLSM, the apodeme appeared to be overlain and in contact with the dorsal portion of the parasite (Fig. 2.9). At the dorsal interface, extension of the parasite outside of the traditional cyst shape and towards the apodeme and antennal lobes was observed (Figure 2.9).

## 2.5 Discussion

Of the 27 *Dicrocoelium dendriticum*-infected *F. aserva* brains that were imaged in this study, all had at least one metacercariae within the anterior- and ventral-most region of the SEG. My observation of site selection within this region of the SEG is in line with previous work involving larval *D. dendriticum* in the SEG of *F. aserva*, *F. polyctena* and *F. rufa* (Martín-Vega et al., 2018; Romig et al., 1980). Since each of these imaged ants was collected and fixed while they were firmly attached to plants, site-selection within the anterior and ventral region of the SEG may be a fundamental requirement for this behavioural manipulation. The sub-esophageal ganglion is well-documented as the control centre for motor function of the mouthparts and is composed of three neuromeres associated with the mandibles,

maxillae, and labium where information is received from each respective nerve (Eichmüller, Hammer, & Schäfer, 1991). The mandibular motor neurons originate in the mandible neuromere, located in the anterior-ventral region of the SEG. The cell bodies of these neurons reside within the cell body ring of the SEG (Paul & Gronenberg, 2002). Thus, site-selection of larval *D. dendriticum* within the anterior-ventral most portion of the SEG places it in direct proximity to both the cell bodies and the mandibular nerves.

To cause the complex sequence of altered behaviour in infected ants, the brain worm must influence behaviours that are independent of the action of the mandibles. These include mobility associated with leaving and returning to the nest, inhibition of feeding, ant-to-ant communication (Chapter 3) and temperature sensing. My imaging results indicate that microsite selection by the single brain worm includes sites that are proximal to the antennal lobes, antennal muscles, the esophagus, pharynx dilator muscles, and the post pharyngeal gland. Although brain ultrastructure and function of *F. aserva* has not been characterized, inferences regarding structure-function relationships can be made based on research conducted on other ant species (Gronenberg, 2008; Nishikawa et al., 2008; Paul & Gronenberg, 2002) and closely related insects such as bees (Eichmüller et al., 1991; Ito et al., 2014; Knebel et al., 2018; Schachtner & Bräunig, 1995).

As described previously, female jewel wasps inject venom directly into the SEG of their cockroach hosts. The injection of venom has been shown to severely restrict the host's ability to walk (Gal & Libersat, 2008). Both gamma aminobutyric acid (GABA) and dopamine have been attributed to the behaviour changes in the host, with the increase in GABA directly linked with inhibition of movement of the front legs (Hughes & Libersat, 2018). A reduction in movement is integral in the *D. dendriticum*-ant system, particularly during the period when the ant is attached with its mandibles to a plant. Thus,

inhibition of movement of infected ants via the regulation of biogenic amines or neurotransmitters such as GABA, which has been shown to occur in other parasite-insect interactions, is a distinct possibility. FMRFamides, a class of neuropeptides, have also been identified as a neurotransmitter within the CNS and are associated with GABAergic interneurons (Eichmüller et al., 1991; Homberg, Kingan, & Hildebrand, 1990). The close linkage between FMRFamides and muscle contraction provides another possibility for a mechanism leading to altered movement in *Dicrocoelium*-infected ants (Homberg et al., 1990).

Altered ant behaviour is stimulated by a change in temperature, with colder temperatures (below 16°C) starting and maintaining attachment behaviour (Botnevik et al., 2016; Spindler, Zahler, & Loos-Frank, 1986). Environmental stimulation is typically communicated through the antennae in many species of ants, where sensilla receptors on the antennae receive information associated with humidity, CO<sub>2</sub> concentrations, and temperature (Nakanishi, Nishino, Watanabe, Yokohari, & Nishikawa, 2010). These external conditions are routed to the antennal lobes and communicated to other parts of the CNS depending on the response. Neural connections between the antennal lobes and SEG, as well as the presence of FMRFamide immune-reactive cells within the antennal lobes and proximity to the brain worm all provide potential avenues for the regulation of the attach-detach-repeat sequence of behavioural manipulations (Eichmüller et al., 1991; Homberg et al., 1990).

Visualization of the interface between the brain worm and the host SEG shows that the parasite is surrounded by a two-layer cyst wall, which can be seen in both TEM and light microscopy images. The cyst wall encircles the worm at both the anterior-most

and posterior-most positions. Even when positioned in its characteristic “c” shape, where the anterior and posterior parts of the parasite are separated by SEG tissue, the parasite remained enclosed. The bi-layered cyst wall appears physically different from the layer that surrounds the exterior of the SEG, thus we can infer that at least one of the layers in the cyst is not derived from host tissue. The inside layer of the cyst is not always in direct contact with the parasite. In instances where the cyst and parasite are not touching, the cyst maintains the shape of the parasite within.

The orientation of larval *D. dendriticum* within the brains of infected ants was not consistent. Thus, the position of the oral and ventral suckers varied extensively between samples. Mechanical manipulation of host neural tissue using either sucker is unlikely since the same region of the larva is not adjacent to a specific region of the SEG, nor are the suckers oriented towards a particular structure within the brain. In the anterior region of the larva, around the oral sucker and extending down towards the ventral sucker, the parenchyma tissue appears increasingly nucleated. Furthermore, secretory granule aggregations and increasingly nucleated cells are observed near the anterior regions of the worm and the tegument, with specialized flame cells being apparent in the parenchyma tissue (Goater, Bray, & Conn, 2009; Mitchell, 1974). Increased nuclei and ribosomes could indicate the presence of secretory tissue, which is important in both the formation of the cyst wall as well as the release of molecules that may contribute to host behaviour changes (Goater et al., 2009; Mitchell, 1974).

Tissue damage appeared to be restricted to a specific region of the SEG between the larval and the ant apodeme that extends ventrally from the pharynx. Host cell vacuolization, a known marker of cell apoptosis (Shubin, Demidyuk, Komissarov,

Rafieva, & Kostrov, 2016), was observed within the SEG and parts of the cell body rind. The release of neurochemicals or a physical change in either the apodeme or the parasite may result in damage to the SEG of the ant host. Direct contact between the metacercariae and the SEG was also visualized in a study involving ants collected from the same sites but using micro-CT technologies (Martín-Vega et al., 2018). My results, especially those that incorporate confocal imaging, confirmed the possibility of direct contact between larval tissue and tissue in the host SEG. In these images, there was strong evidence that the larvae were not completely enveloped by the cyst wall. It is conceivable, as suggested in the CT study by Martín-Vega et al. (2018) that incomplete encystment would allow for direct physical/mechanical contact between the larva and SEG tissue, enabling the parasite to physically interact with the brain or release potential neuromodulating molecules. However, in contrast to the results of both the micro-CT and confocal imaging, TEM images showed total encystment. The relatively low-resolution capabilities of these two imaging systems might explain why the cyst wall would not be easily differentiated from either host or parasite tissue and thus could be assumed to be absent. Despite this, these higher magnification images were not taken through the entirety of the parasite/host interface so perforations within the cyst wall could have been missed.

A flexible host apodeme runs the entire length of the larva in the brain, extending between the antennal lobes down from the esophagus. This cord-like structure was observed in both infected and uninfected *F. aserva* ants. Pharynx dilator muscles that control the action of the pharynx attach to this apodeme (Paul, Roces, & Hölldobler, 2002). Overlap between the larva and this extended apodeme were especially clear in

CLSM images, including instances where direct physical contact between the two structure was evident. These early results present the intriguing possibility that for the direct influence by larval *D. dendriticum* on the pharynx dilator muscles, and thus an influence on behaviours associated with ant feeding. Infected ants that attach to plants are unable to feed because their mandibles are firmly attached to plants. Thus, inhibition of normal host feeding responses must play a role in the sequence of attach-detach-repeat behaviours induced by the larva in the SEG. My results provide the first indication that the manipulation of host feeding responses may play a role leading to the attach-detach-repeat behaviours observed in *D. dendriticum*-infected ants. This hypothesis requires testing.

## 2.6 References

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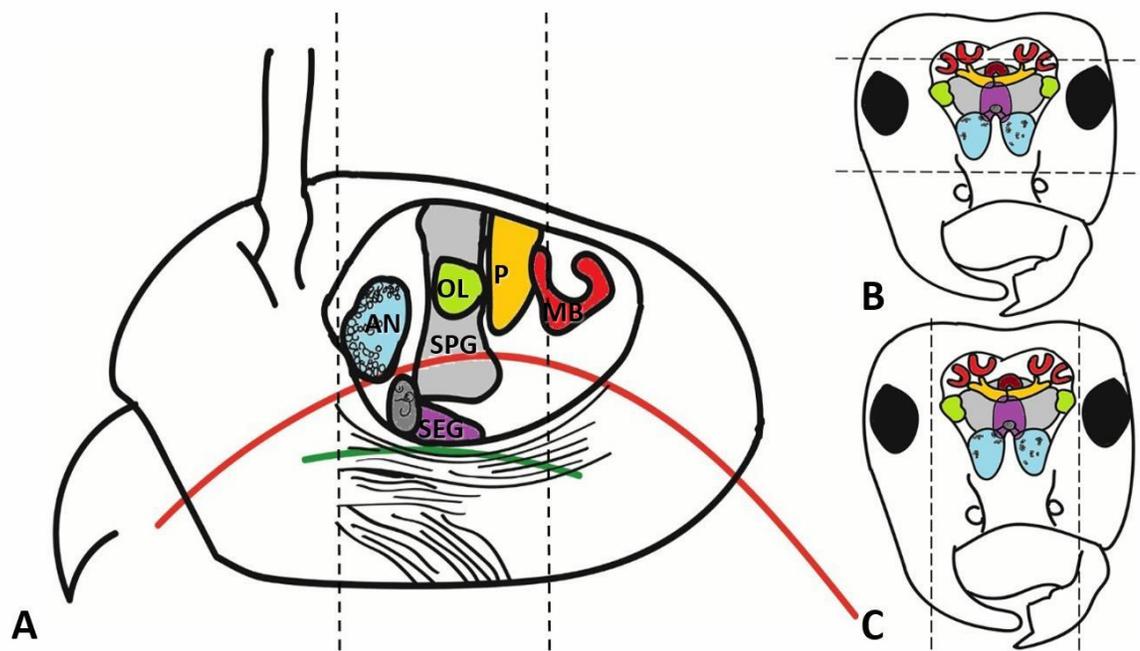
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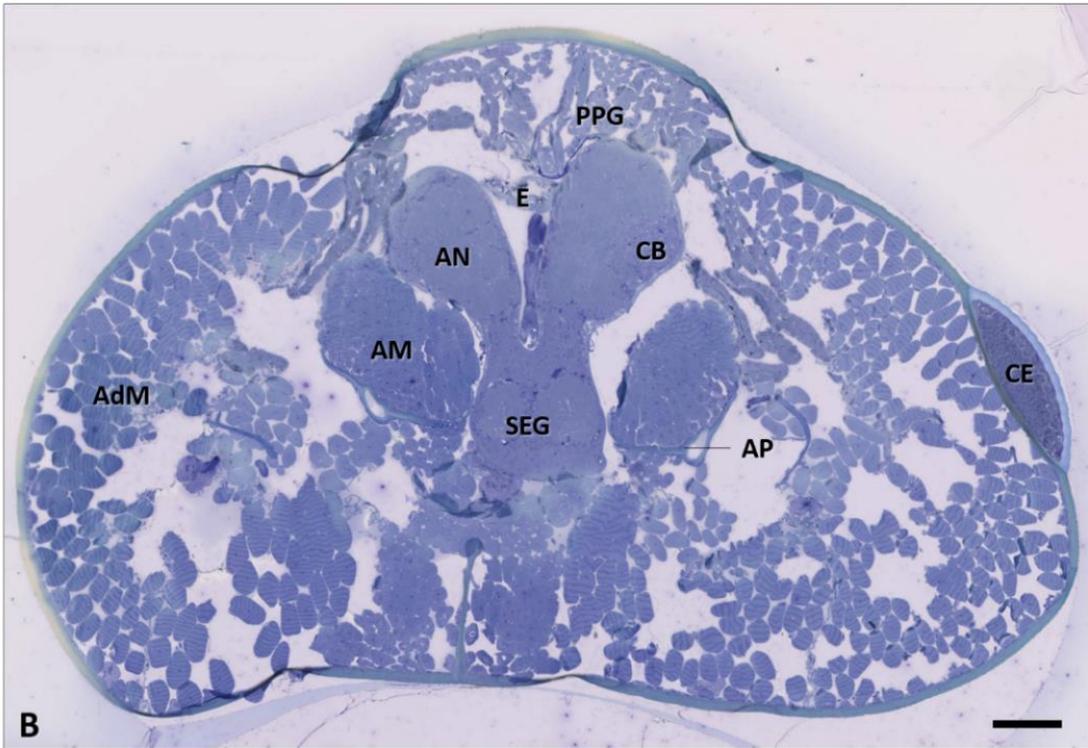
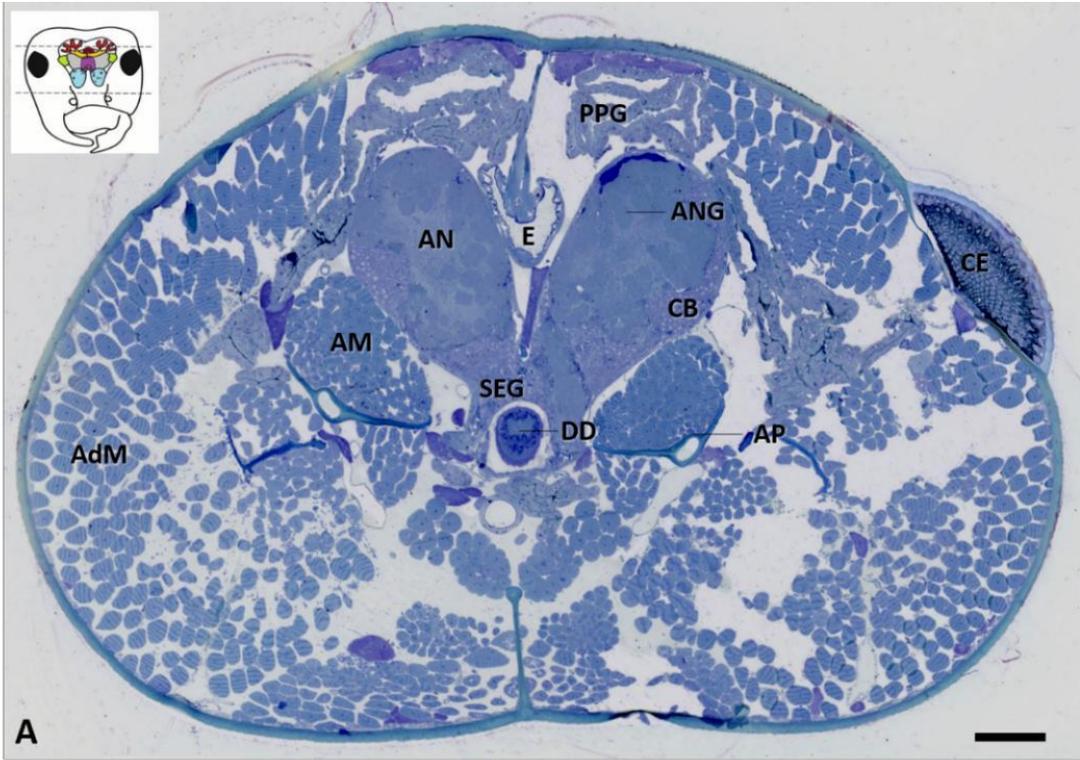
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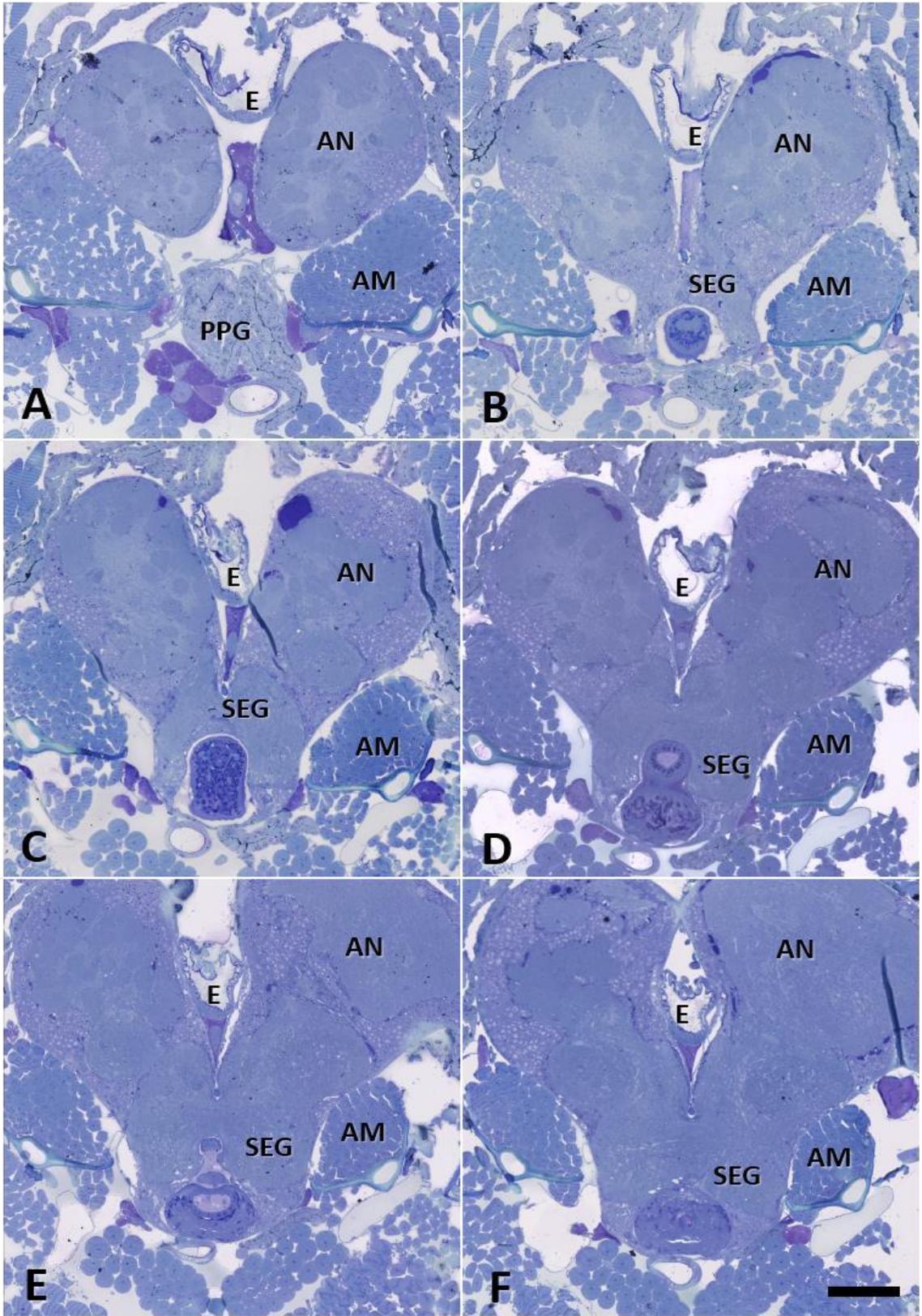
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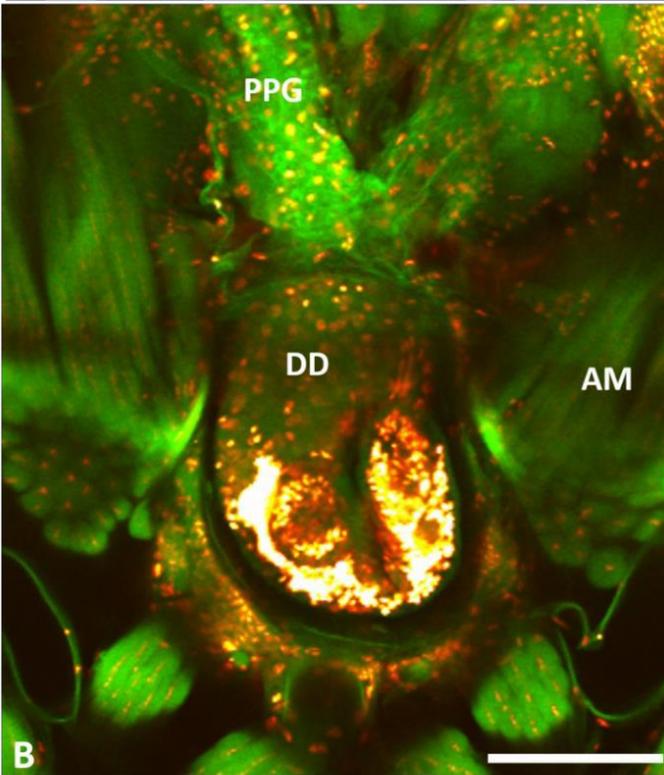
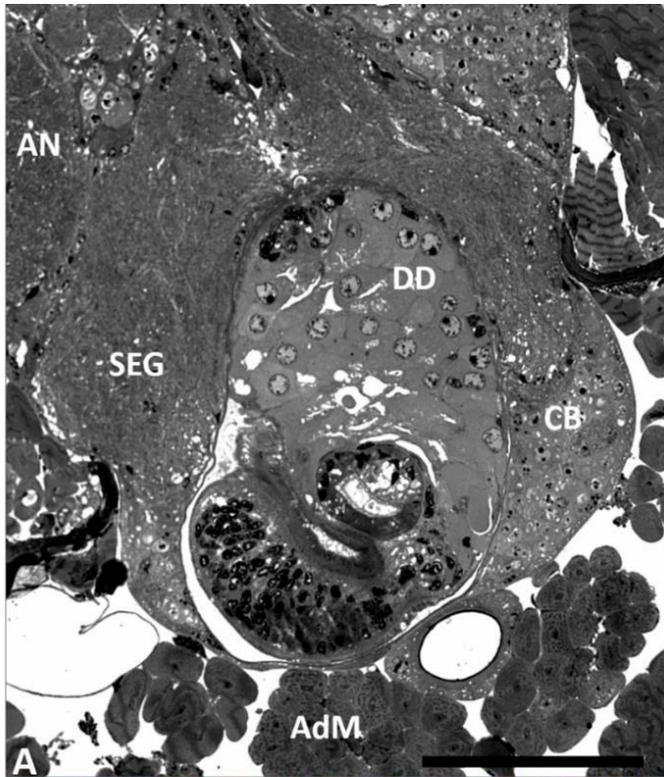
**Figure 2.1.** Selected planes of orientation of ant brains that were used to visualize larval *D. dendriticum*. Images (A) and (B) demonstrate sections with frontal orientation, whereas image (C) is sagittal in orientation. The dashed lines show where the ant head was sliced for preparation with confocal microscopy; only the anterior line was cut for light and electron microscopy. Abbreviations: AN, antennal lobes; C, central body; MB, mushroom body calyx; P, peduncle; OL, optic lobe; SEG, sub-esophageal ganglion; SPG, supra-esophageal ganglion.



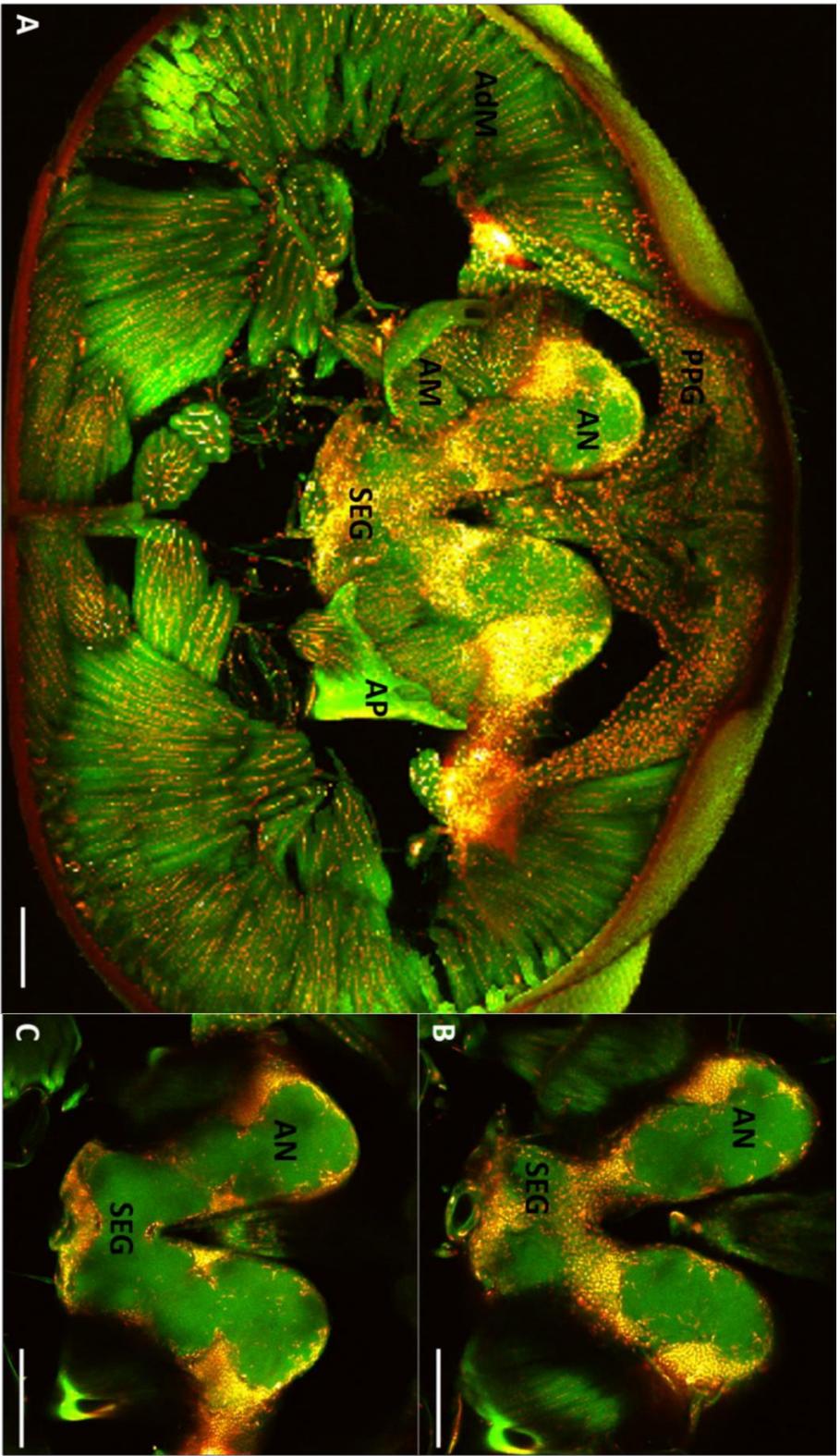
**Figure 2.2.** Frontal sections of the heads of *D. dendriticum* (DD)-infected (A) and uninfected (B) ants, *Formica aserva*. A single larva is located within the sub-esophageal ganglion (SEG) of the brain, located ventral to the antennal lobes, observed in these sections as antennal lobe diffuse neuropil (AN), glomeruli (ANG), and cell body rind (CB). Two prominent apodemes (AP) are located laterally on both sides of the larva, with antennal muscles (AM) attached. Dorsal to the larva is the esophagus (E) with pharynx dilator muscles (PDM) attached above. Surrounding the main brain structure is the postpharyngeal gland (PPG), with the remainder of the head filled with mandibular muscles, primarily mandibular closer muscle fibers (AdM). One compound eye (CE) is visible in the top right corner (scale bar = 100 $\mu$ m).



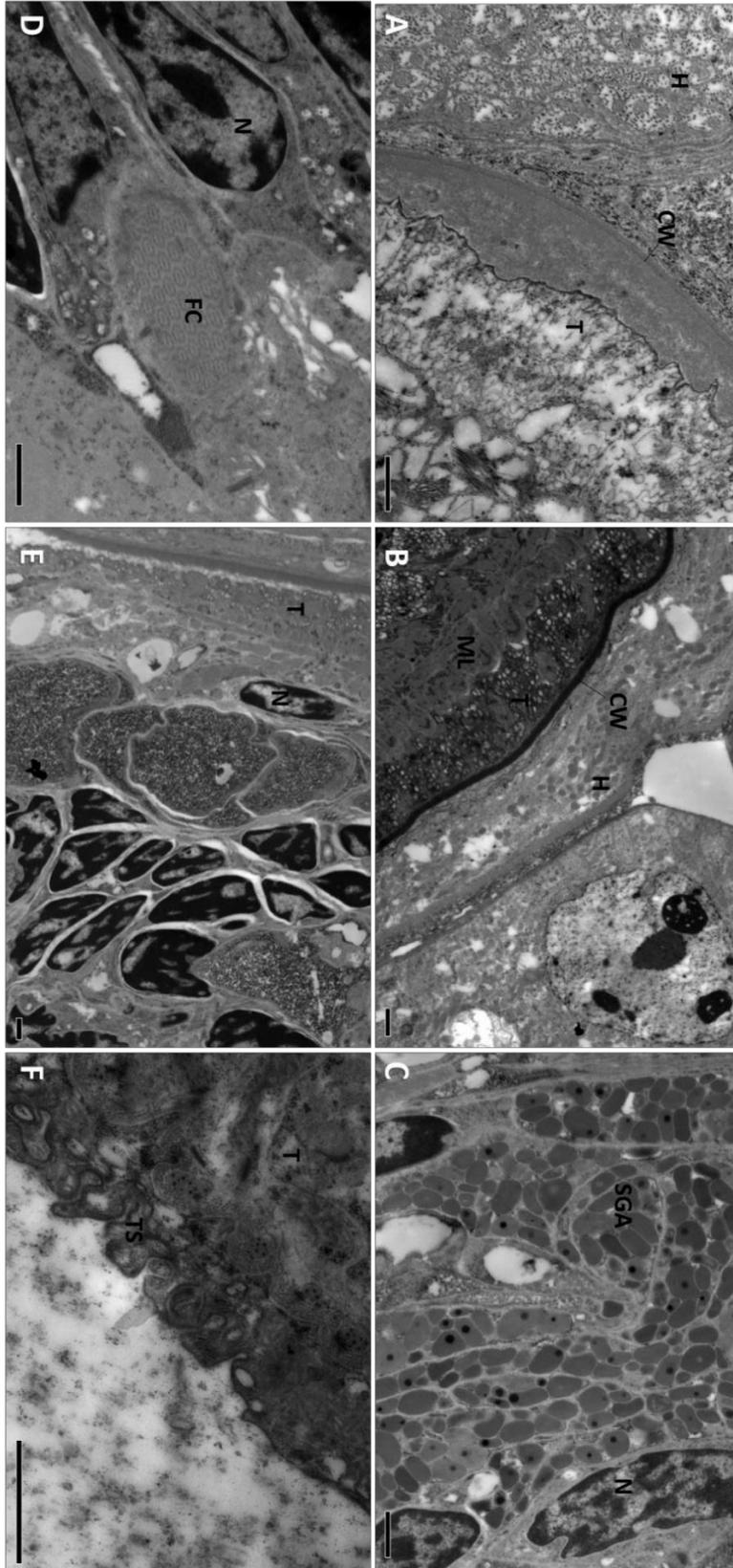
**Figure 2.3.** Light microscopy images of a frontally-sectioned ant head infected with larval *D. dendriticum*. The images are ordered to demonstrate aspects of the larva as it extends anteriorly-posteriorly through the SEG. The first section is the anterior-most region just before the SEG becomes visible. Images A-F proceed posteriorly through the ant head until only the posterior-most end of the worm is visible. The oral and ventral suckers are visible in images D and E, with the area between the two (C) showing an increasingly nucleated region. A single metacercaria is visible in the ventral portion of the SEG, extending from the anterior-most part further posterior into the SEG (scale bar = 100µm). Abbreviations: AM, antennal muscles; AN, antennal lobe diffuse neuropil; E, esophagus; PPG, post- pharyngeal gland; SEG, sub-esophageal ganglion.



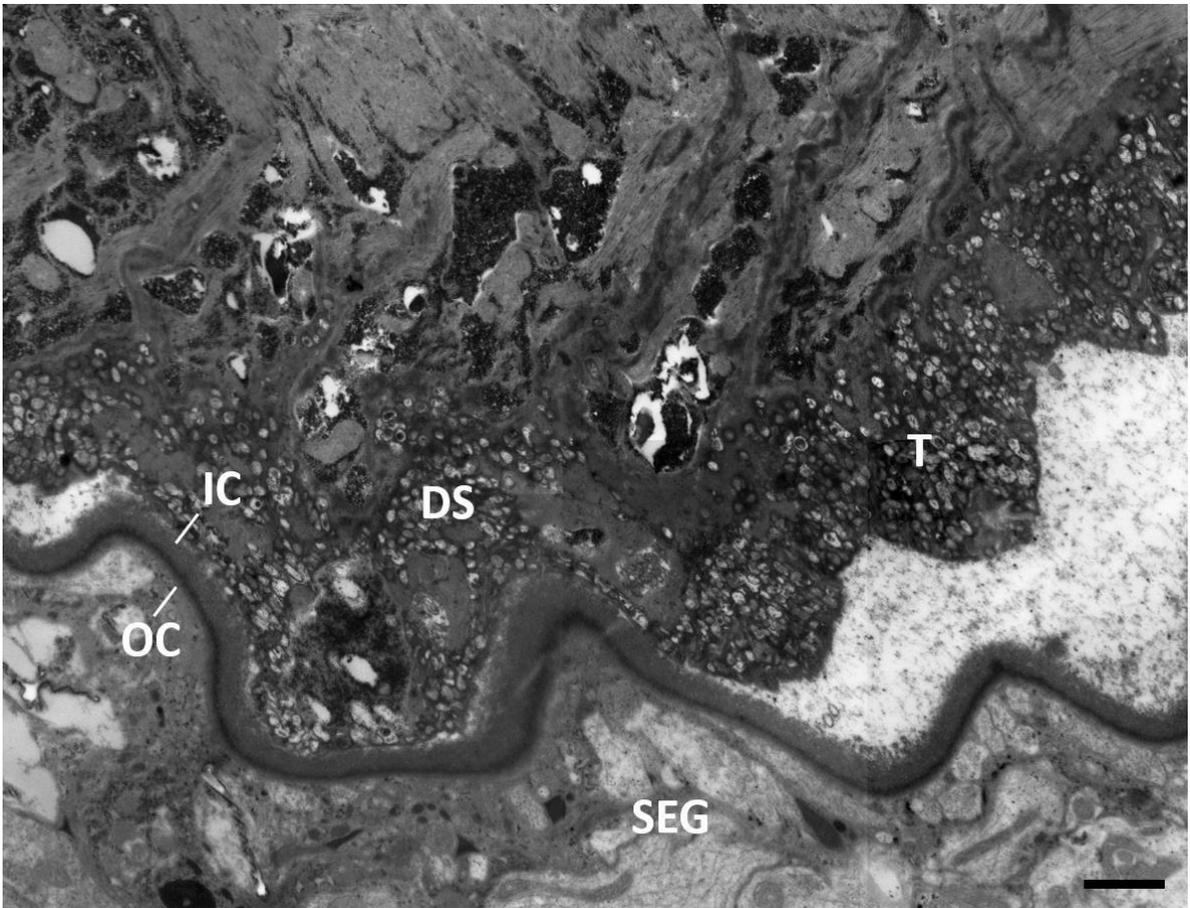
**Figure 2.4.** Light microscopy (A) and CLSM (B) images of frontally-sectioned ant head infected with a single *D. dendriticum* larva. The oral and ventral suckers of the larva, which make up the anterior portion of the worm, are ventral within the SEG. Areas with a high number of nucleated cells appear as darkly-stained red/orange/white areas of the worm in CLSM (scale bar = 100µm). Abbreviations: AM, antennal muscles; CB, cell body rind; E, esophagus; PPG, post-pharyngeal gland; SEG, sub-esophageal ganglion.



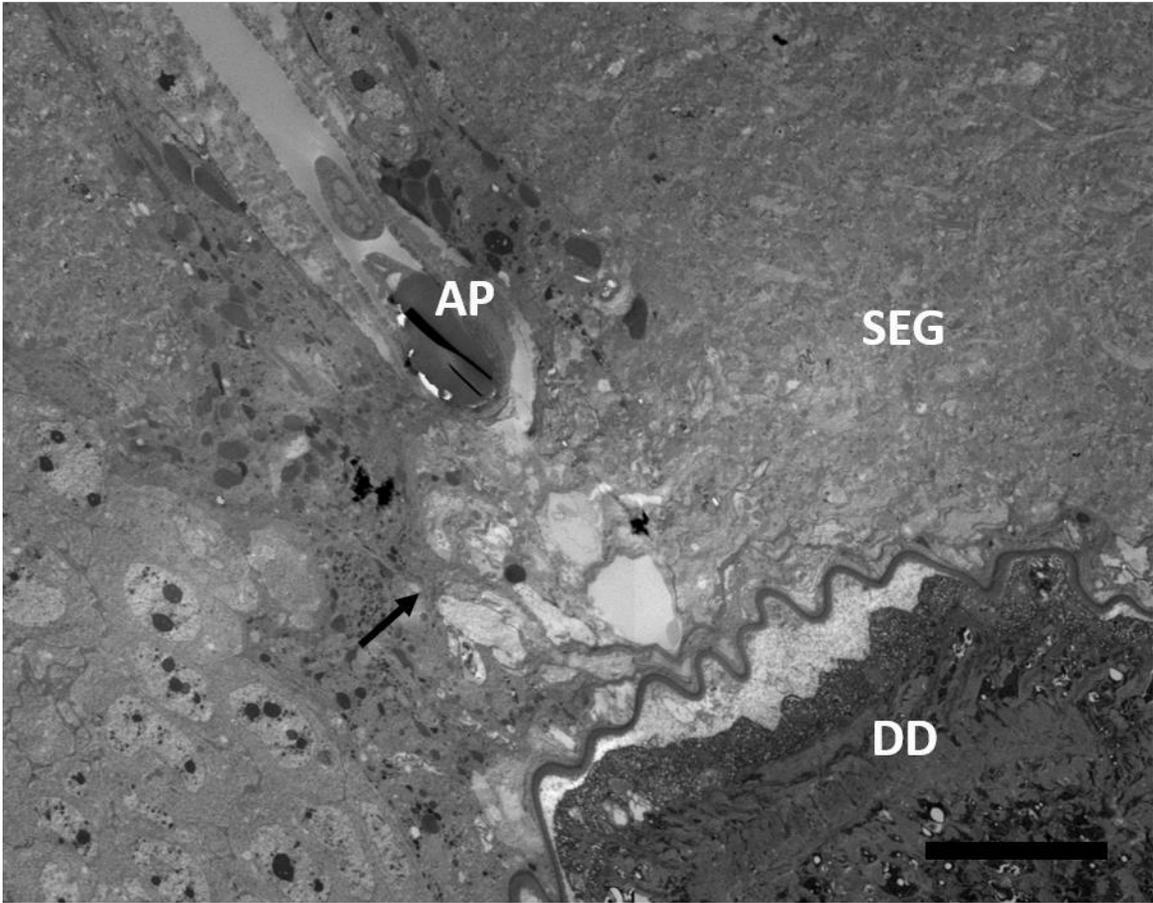
**Figure 2.5.** CLSM images of a frontally-sectioned uninfected ant infected head. A z-stack image was generated (A) to view an overlap of all structures present within the region of the brain that harbours larva of *D. dendriticum*. Image (B) denotes the anterior of an ant head where *D. dendriticum* larva would first become visible, whereas image (C) is further posterior at the location where the SEG becomes integrated into the supra-esophageal ganglion. The extent of highly nucleated regions within the SEG increase in both the anterior and ventral portions (scale bar = 100µm). Abbreviations: AP, apodeme; AdM, mandibular closer muscle fibers; AM, antennal muscles; AN, antennal lobe diffuse neuropil; PPG, post-pharyngeal gland; SEG, sub-esophageal ganglion.



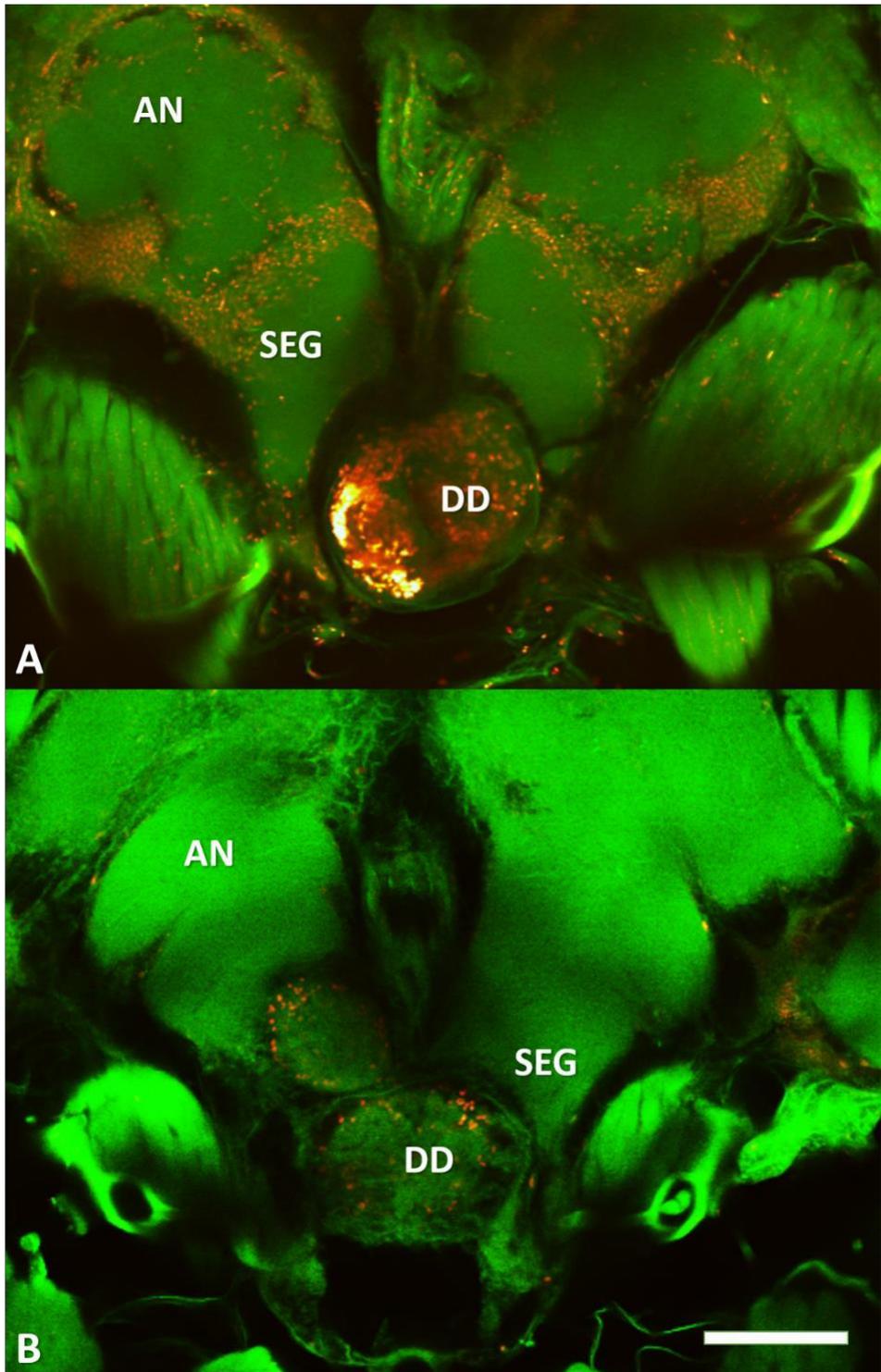
**Figure 2.6.** TEM images of larval *D. dendriticum* within the brain of infected ants. Orientation is sagittal (A) and frontal (B-F). Mitochondria and endoplasmic reticulum are visible in the host tissue adjacent to the parasite's outer cyst wall (A and B). The tegument of the worm is not always in contact with the innermost layer of the cyst wall (F). Secretory granule aggregations and increasingly nucleated cells are visible near the anterior portion of the worm and the tegument, with specialized flame cells apparent in the parenchyma tissue (C, D, E). (scale bars = 1 $\mu$ m). Abbreviations: CW, cyst wall; FC, flame cell; H, host tissue; ML, muscle layer; N, nucleus; SGA, secretory granule aggregations; T, tegument; TS, tegument spines.



**Figure 2.7.** TEM image at the host-parasite interface in a *D. dendriticum*-infected ant. A bi-layered cyst wall separates the parasite tegument from host tissue in the ant's SEG. Within the tegument, dense secretory bodies make up the outer edge (scale bar = 1 $\mu$ m). Abbreviations: DS, dense secretory bodies; IC, inner cyst layer; OC, outer cyst layer; SEG, sub-esophageal ganglion; T, tegument.



**Figure 2.8.** TEM image of the interface of *D. dendriticum* and *Formica aserva* brain tissue at the dorsal end of the parasite within the SEG. Host tissue damage in the SEG (arrow) is evident in the region between the dorsal-most part of the cyst wall and the tip of the host apodeme that extends ventrally from the esophagus. (scale bar = 10 $\mu$ m). Abbreviations: AP, apodeme; DD, *Dicrocoelium dendriticum*; SEG, sub-esophageal ganglion.



**Figure 2.9.** CLSM images of frontally sectioned *Formica aserva* ants infected with *Dicrocoelium dendriticum*. Contact between the apodeme that extends ventrally down from the esophagus, which connects to the pharynx dilator muscles, and the parasite is visible (A). The larva is not fully enveloped by cyst wall but extends into the SEG and potentially the SPG (B). The cyst layer that is visible does not appear to surround the extension (scale bar = 100µm). Abbreviations: AN, antennal lobe diffuse neuropil; DD, *Dicrocoelium dendriticum*; SEG, sub-esophageal ganglion.

## **Chapter 3: Flower choice by zombie ants infected with larvae of the brain-encysting trematode, *Dicrocoelium dendriticum***

### **3.1 Abstract**

*Formica* sp. ants infected with larvae of the trematode *Dicrocoelium dendriticum* attach with their mandibles onto flower blossoms adjacent to their nests. Uninfected ants never demonstrate this behaviour. Perhaps most remarkably, infected ants also detach from their flower blossoms when temperatures rise each day. The mechanisms underlying this ‘attach-detach-repeat’ sequence of behaviours are unknown. Determining the nature of attachment and detachment decisions made by infected ants is one line of inquiry that can help us understand the neural centers in the brain that may be influenced by infection. I used binary choice chambers within laboratory growth chambers to stimulate attachment (10°C) and detachment (25°C) behaviours in *D. dendriticum*-infected ants, *F. aserva*, collected from the field. My results showed that when infected ants were offered a choice between a familiar flower blossom and an unfamiliar one, they significantly preferred the former. In addition, significant attachment preference occurred onto flower blossoms that currently, or previously, contained an infected nestmate compared to those that did not. The latter results help to explain the observed pattern of aggregation of ants on flowers at two naturally-infected field sites. These findings indicate that infected ants recognize, process, and respond to, chemical signals emanating from flower blossoms to preferentially attach to a particular substrate. Although the ultimate mechanisms behind the attach-detach-repeat sequence of altered behaviours remain elusive, my results support the notion that they must involve a complex, temperature-dependent interaction with neural centers involved with the detection of environmental cues and/or ant-to-ant

communication.

### **3.2 Introduction**

Many animal behaviours are traditionally thought to be motivated by an individual's requirement to optimize its fitness. Changing environmental conditions requires that simple actions, such as foraging for food and avoiding predators, require constant decision-making by an animal to maximize the probability that it will survive and reproduce (Koprivnikar & Penalva, 2015). A large range of environmental factors including temperature, humidity, the presence of predators, and food availability, are well known to influence decision-making in animals (Houston, Clark, McNamara, & Mangel, 1988; Koprivnikar & Penalva, 2015; Milinski & Heller, 1978). Although much less recognized, parasite infection can also alter an animal's decision-making, directly influencing its fecundity, nutrient up-take, and ability to avoid predators (Milinski, 1990). Parasite-induced behaviour manipulation, which in many cases can modify host behaviours to enhance rates of parasite transmission and reproduction, is one common consequence of parasitism (Adamo, 2012; Libersat, Delago, & Gal, 2009; Poulin & Maure, 2015). Mosquitos infected with *Plasmodium* sp., the causative agent of malaria, increase their biting frequency relative to uninfected ones, thereby increasing rates of transmission of infective stages (Koella et al., 1998). Similarly, bees infected with conopid fly larvae (*Physocephala rufipes* and *Sicus ferrugineus*) spend less time foraging at individual flowers, while also changing plant species more frequently (Schmid-Hempel & Stauffer, 1998). Thus, convincing evidence exists that the alteration of common host behaviours can facilitate the development and transmission of parasites (Milinski, 1990).

Studying host behavior alteration by parasites is an emerging field in animal

behaviour and evolutionary biology, particularly now that the techniques that allow researchers to detect changes at the genomic and transcriptomic level are possible (Herbison, Lagrue, & Poulin, 2018; Hughes & Libersat, 2018; Libersat, Kaiser, & Emanuel, 2018). A detailed understanding of the behaviours being altered is key in deciphering what aspects of the host the parasite can manipulate, especially for parasites that interact with the host's central nervous system (CNS) (Herbison et al., 2018; Libersat et al., 2018). In these cases, the altered behaviours likely arise due to direct or indirect interactions between the parasite and the host that occur at the host-parasite interface (Adamo, 2012; Hughes & Libersat, 2018). Invertebrates are often excellent model organisms for studies of this nature, since they can act as both hosts and parasites (Libersat et al., 2009), they are often amenable to manipulation under lab and field conditions, and their neural systems are often well characterized (Libersat et al., 2009; Libersat & Gal, 2013). Thus, parasites that alter host behaviours by targeting the CNS of insects are ideal model systems.

Ants in the family Formicidae are known hosts for larvae (metacercariae) of the trematode fluke, *Dicrocoelium dendriticum*. Individual ants are exposed to infection when they encounter packets of cercariae that have been deposited onto substrate by infected terrestrial snails (Krull & Mapes, 1953). Once ingested by an ant, at least one of the larvae migrates into the anterior- and ventral-most region of the sub-esophageal ganglia of the brain (Martín-Vega et al., 2018, Chapter 2), where it resides for the rest of the ant's life. The remaining larvae encyst within the abdomen, where they await ingestion by a definitive host (grazing mammals). The larva that reaches the brain is believed to be responsible for the radical manipulation of the ant's behaviour (Botnevik, Malagocka,

Jensen, & Fredensborg, 2016; Carney, 1969; Romig, Lucius, & Frank, 1980; Spindler, Zahler, & Loos-Frank, 1986). Infected ants leave their nest during the cool hours of the day to ascend vegetation that is usually within a few metres of the nest entrance. Following plant selection, infected ants attach firmly with their mandibles and remain attached throughout the evening or as long as temperatures remain below approximately 16°C (Spindler et al., 1986). When temperatures exceed this threshold, infected ants relax their mandibles, detach from the plant, and then return to their nest or seek shade (Carney, 1969; Spindler et al., 1986).

The ‘attach-detach-repeat’ sequence of behaviours requires that *D. dendriticum*-infected ants make a series of key decisions that their uninfected nestmates do not make: when to leave and return to the nest, where to attach on a plant, and so on. The results of previous studies have shown that infected ants prefer to attach to flower blossoms in comparison to other parts of a flowering plant (Spindler et al., 1986; Mitchell et al., 2017). However, attachment and detachment behaviours may be based on a variety of other environmental conditions that can arise from subtle cues emanating from individual plants, such as species of plant, blossom age, and the presence of other infected, attached ants. At present, it is unknown whether infected ants show attachment preferences when they are provided with alternatives, and if so, whether such choices provide fitness benefits to the parasites, their hosts, or both. Further understanding of the altered behaviour can contribute to what we know about how the parasite is manipulating its host. For instance, if infected ants select blossoms for attachment that have, or had, infected nestmates, then the larva must manipulate regions of the brain that are involved in ant-to-ant recognition and/or communication.

Native to continental Europe, *D. dendriticum* is now found in many regions of the world. The results of host surveys and DNA sequence variation of adult worms show that the fluke was introduced into the Cypress Hills region of southern Alberta, Canada prior to the 1980's, then emerged approximately 20 years later (Goater and Colwell, 2007; van Paridon et al., 2017). Within this region of emergence, adult worms reach reproductive maturity in large herbivores such as beef cattle, elk, and deer (Beck, Goater, & Colwell, 2015), whereas three species of terrestrial snail in the genus *Oreohelix*, and the ant, *Formica aserva*, are the primary first and second intermediate hosts, respectively (Dempsey, 2017; van Paridon, Gilleard, Colwell, & Goater, 2017). Infected *F. aserva* have been documented to occur throughout Cypress Hills Park, often at densities > 300 attached ants/nest on any one day between June and mid-August (Beck, 2015). The results of our ongoing and long-term field studies at three selected sites indicate that marked infected ants tend to return to the same flower blossoms each day, for up to one week (Goater, unpublished observations).

The purpose of this study is to evaluate choice behaviours of individual *D. dendriticum*-infected ants that are offered contrasting flower substrates for attachment. A central motivation for this line of inquiry is to better understand the mechanisms by which a single *D. dendriticum* larva orchestrates the 'attach-detach-repeat' sequence of behaviours in infected ants. I hypothesize that infected ants prefer attachment to certain substrates (flower blossoms) over others, and they do so based on their recognition and response to chemical signals and/or visual cues. I tested this hypothesis by offering individual infected ants' choices between two flower types in binary choice chambers. In an initial test, I offered infected ants a choice between a familiar and a novel flower to

determine if infected ants recognize and select substrates with which they have had prior experience. I then offered another group of infected ants a choice between a flower blossom that already contained an attached worker from the same nest and a novel flower to determine if infected ants preferred to aggregate with other infected ants. Lastly, I offered infected ants a choice between a flower that previously had an unfamiliar infected ant attached to it and a novel flower to test if infected ants use only vision to detect the presence of other infected ants. To complement the choice experiments, field surveys were conducted to assess the distribution of sub-populations of infected ants on flowering plants adjacent to their nest.

### **3.3 Materials and Methods**

#### *3.3.1 Study sites*

Field surveys and host collections were completed at two sites located on the Alberta side of Cypress Hills Inter-Provincial Park (CHP) in the southeastern corner of Alberta, Canada. Infection characteristics of larval *D. dendriticum* in snails and ants, respectively, at these two sites have previously been described (Beck, 2015; van Paridon et al., 2017; Dempsey et al., 2018). Site 1, known as Staff Camp (49°39'49.5"N 110°16'75.4"W; 1289 m a.s.l.) is 1.4 km from Site 2, known as Ski Hill (49°39'50.6"N 110°15'66.1"W; 1306 m a.s.l.). Tree cover at the two sites is characteristic of boreal habitats located approximately 500 km to the north and in the foothills of the Rocky Mountains located approximately 400 km to the west. Both sites were located under mixed-wood canopy dominated by balsam poplar (*Populus balsamifera*) and white spruce (*Picea glauca*).

Flowering herbs, such as common dandelion (*Taraxacum officinale*), vetch (*Vicia*

*cracca*), goldenrod (*Solidago canadensis*), and clover (*Trifolium hybridum*), are dominant in the understories. We have observed ants attached to the flowers and stems of each of these species of plant at both sites (Beck, 2015; van Paridon et al., 2017) and rarely, to the blades of various species of grasses. The results of our ongoing studies at these two sites indicate that marked infected ants tend to attach to dandelion flowers between May and mid-June, then to clover until approximately mid-August (Goater, unpublished observations).

### 3.3.2 *Distribution of infected ants on plants*

To evaluate the distribution of infected ants among available flowers that were adjacent to nests at the two sites, I demarcated one 5m X 5m sub-site with flagging tape within each site. The presumed nest entrance was located at the approximate center of the sub-site. Each sub-site was surveyed twice, once on June 6, 2017 and again on June 14, 2017. During each survey, each sub-site was assessed for the total number of dandelions and the total number of infected ants per plant. Each plant was first assessed from above and then the ventral and dorsal surfaces of the petals and stems were manually handled to assess for attached ants that could not be viewed from above. Individual stems with an attached flower head were considered an individual inflorescence. Each of the four surveys was completed between 6-9 a.m. when temperatures ranging between 9°C-12°C.

### 3.3.3 *Source of ants for laboratory trials*

Infected, attached ants were collected from the two sites in the summers of 2016-2018 by cutting the stem of the plant (dandelion or clover) several centimeters below the

inflorescence. Samples of uninfected ants that were used in preliminary pilot studies were collected by hand from around each of the two nests. Attached ants and the stems they were attached to were placed into individual plastic containers within a cooler for transportation to the laboratory at University of Lethbridge. The containers were placed into a CMP 6050 growth chamber (Controlled Environment Limited, Winnipeg Mb) at 10°C to maintain attachment behaviours and to limit ant mortality.

#### *3.3.4 Design of choice chambers*

Plastic choice chambers were designed that could contain one-two infected ants for up to five days and one-two live flowers that could be used for potential attachment (Fig. 3.1). The chambers were also designed to permit observation of ant behaviour at different temperatures within the growth cabinets with minimal human disturbance. The lids were perforated to allow air flow within the top section of the container. The bottoms of each container were also perforated such that when the containers were placed into 3 L plastic tubs, the choice chambers would fill with approximately 1 cm of water. The purpose of adding water was to facilitate maximum turgor pressure in each flower during the behaviour assays. Two holes were drilled into the plexiglass stage (Fig. 3.1), each of which contained a 2 cm-long plastic straw. The stem of each flower was placed through the straw so that its base was in contact with water for the duration of the experiment. The straws were colour-coded to facilitate the randomized positioning (right versus left) of the two flowers within each container.

#### *3.3.5 Choice trials: General methods*

Experiments were designed to test binary preferences of individual infected ants. The typical design involved two flowers, one or both of which was familiar to the ant (i.e. the flower from which the ant was collected at one of the two field sites) or unfamiliar. In the latter case, the novel flower was of the same species as the familiar flower but was collected from area within the park that did not contained infected ants. At the start of each trial, the two flowers were added simultaneously and at random to one of the two straws and then left undisturbed until the trial's completion. Attachment and detachment temperatures were chosen to minimize mortality and to be outside of the transition zone (15-24°C; Badie et al. 1973). Each trial proceeded as follows: Ants were removed from the stock container at 10°C and placed at random into a choice container. Each container was then placed into a growth chamber at 10°C for 30 min. These ants invariably remained firmly attached to their flower. To stimulate detachment from their flowers, the containers were removed from the 10°C chamber and placed into an adjacent chamber set at 25°C for a minimum of 40 min. Finally, the choice containers were returned to the 10°C chamber to stimulate an additional bout of attachment. The first cool/warm cycle was considered an acclimation period that would allow each ant to explore the container and its component flowers. At the completion of their second exposure to 10°C, the position of the ant on one of the two plants was assessed. 'Attachment' was defined as the active 'biting' of plant material by the mandibles. Infected ants never use their legs for attachment. Detachment was defined as the release of the plant by the mandibles, a process that typically required 45-70 min once the container was placed at 25°C. Thus, if an ant was observed moving over a plant at the end of the second cooling period or was stationary on a plant, but not attached with the mandibles, it was designated as

‘detached’. The proportion of individual ants selecting one plant or another at the end of the second trial was compared to an expected random proportion.

### 3.3.6 *Choice trials: Novel attachment*

An initial experiment was designed to test if attachment would occur if the ant was removed from its initial dandelion flower and offered two completely novel dandelion flowers. An infected ant ( $n = 16$ ) could select between two novel dandelion flowers or not attach to either. The ants were placed in the container while still attached to a flower and then subjected to warming until detachment. The initial flower was then removed, and two novel flowers were placed in the container. The ants were then subjected to two rounds of alternating warm and cool temperatures. Attachment during the second round of cooling was monitored to see if an infected ant would return to the same attachment substrate after the initial attachment.

### 3.3.7 *Choice trials: Familiar versus novel flowers*

The experiment was designed to determine if infected ants preferentially attached to a familiar or novel plant. Thus, each ant ( $n = 16$ ) could select its familiar dandelion flower, a novel dandelion flower, or not attach to either. The containers were subjected to the two rounds of alternating warm and cool temperatures described above. The position of each ant was assessed at the end of each of the cool periods. As a further test, the same experiment was conducted to determine if there was a side bias within the container. The containers were subjected to the two rounds of alternating warm and cool temperatures described above, and then the positions of the familiar plant and novel plant were switched. The containers were then subjected to two more rounds of warm/cool

temperatures. The position of each ant was assessed at the end of each of the cool periods.

### 3.3.8 *Choice trials: Aggregation of infected ants*

The purpose of this set of trials was to evaluate if infected ants attached to flowers that currently or previously contained another infected ant. The first experiment included two infected ants, both of which were collected from one of the two sub-sites described above. Since both ants were collected within close proximity to a known infected nest, we assumed that the two ants in each container were nestmates. As well, *Formica* sp. ants typically display aggressive behaviours in the presence of ants from another nest which was not observed (Martin et al., 2011). One dandelion was familiar to one of the nestmates, whereas the second was a novel flower to both ants. The first cool/warm period was restricted to the ant that was collected from the familiar flower. The purpose of this trial was to establish the preference for familiarity that was demonstrated in the experiment described above. Following the second period of cooling, the detached infected nestmate was added to the container. After 30 minutes, the positions of the two ants on one flower, both flowers, or neither, was assessed.

A second trial was designed to distinguish whether the preference for the occupied dandelion that was observed in the previous experiment was due to visual or chemical signals. We used the same procedures as described above, with the exception that following the second period of cooling, the attached ant was manually removed from its familiar flower. Thus, when the second ant was added to the container, it had the opportunity to choose between a novel dandelion and a novel dandelion that previously had an infected nestmate attached to it. Following the 30-minute period of cooling, the

position of the ant on the novel dandelion, the novel dandelion that had contained an infected nestmate, or neither, was assessed.

### 3.3.9 *Analyses*

The distribution of infected ants among available dandelion blossoms at the two sub-sites was semi-quantitatively assessed at early June (June 6, 2017) and mid-June (June 13, 2017). To test if ants were randomly distributed among available flowers, I compared the distribution of ants on flowers to a Poisson distribution using a Chi-squared test and Yates correction. To meet Chi-square assumptions, categories of two ants or more were grouped into a single group. The variance in ant counts divided by the mean was calculated to determine the variance to mean ratio where a value close to one indicates a random distribution, a value above one indicates over-dispersion, and a value below 1 indicates under-dispersion (Shostak and Scott, 1994).

The results of the binary choice experiments were analyzed with the exact binomial test in R (McDonald, 2013) (R version 3.3.3 (2017)) where a 50:50 ratio was assumed as the null model of random choice between the two available flowers in a container. For each experiment, the results of the two cooling periods were independently analyzed. Ants that did not attach or were attached to the container were excluded from analyses. I endeavored to use 16 individual ants for each trial, based upon the results of pilot studies completed in the same containers in summer, 2015. Variation in sample sizes was due to variation in the availability of infected ants on dandelions at specific times. Larger sample sizes were required for the multi-ant tests to account for the number of infected ants that I presumed would not attach.

### 3.4 Results

#### 3.4.1 Field survey

Approximately 9% and 14%, respectively, of the hundreds of available dandelion flowers at the two sites contained attached ants during the first survey in early June. At Site 1 in early June, 88 infected ants were distributed among 615 available flowers (Fig. 3.2). Of these, 46.6% were attached to a flower by themselves, whereas the remaining 53.4% attached to a plant that contained other ants. At Site 2 in early June, 65 infected ants were distributed among 332 available flowers (Fig. 3.2). Of these, 60.0% attached to a flower by themselves, whereas the remaining 40.0% attached to a plant that contained other ants. During both June surveys at each site, the numbers of available plants and infected ants remained approximately consistent. The observed distributions did not fit the expected Poisson distributions (Early June: Site 1  $X^2 = 38.4$ ,  $df = 2$ ,  $p\text{-value} < 0.001$  and Site 2  $X^2 = 6.18$ ,  $df = 2$ ,  $p\text{-value} < 0.05$ . Mid-June: Site 1  $X^2 = 27.2$ ,  $df = 2$ ,  $p\text{-value} < 0.001$  and Site 2  $X^2 = 10.4$ ,  $df = 2$ ,  $p\text{-value} < 0.005$ ). The variance to mean ratios showed that for each date and site, the frequency distribution was over-dispersed (early June: Site 1 VMR = 2.20 and Site 2 VMR = 2.10; mid-June Site 1 VMR = 1.70 and Site 2 VMR = 2.35). Overall, the distribution of infected ants among the available flowers was aggregated at both sites.

#### 3.4.2 Choice trials: General patterns

Over the course of two years, I completed 82 choice trials involving one infected ant and two flowers. Of these 82 trials of individual ants, 81.7% resulted in a clear choice for one flower over another by the end of the second period of cooling. Thus, in the trials where only one ant was used, 18.3% of the total number of infected ants used in the trials

did not exhibit typical attachment behaviour within the choice chambers. These ants continued walking around the container or remained motionless on the flowers (without attachment) or the walls of the container, or rarely, attached with their mandibles to the silicon used to make the container.

#### *3.4.3 Choice trials: Novel attachment*

During the first period of cooling, 15 of the 16 ants attached. This shows that the infected ants will attach onto the plant regardless of whether the plant is familiar. In the second cycle, 12 of the ants that previously attached returned to the same “novel” dandelion, with 14 total ants attaching (binomial test,  $n = 14$ ,  $p = 0.013$ ).

#### *3.4.4 Choice trials: Familiar versus novel flowers*

During the first period of cooling, seven of the 10 ants that attached did so onto their familiar plant (Fig. 3.3). In the second cycle, each of those seven ants returned to their familiar plant, as well as an additional four ants that initially went to the novel plant. By the end of the second period of cooling, there was a significant preference for the familiar plant (Fig. 3.3; binomial test, cycle 2  $n = 12$ ,  $p < 0.01$ ). Four of the 16 ants did not attach to a plant during either cycle.

Similar results were seen in the follow-up experiment that tested for a side-preference (Fig. 3.4). During the second cycle, ants preferentially attached to the familiar flower (binomial test, cycle 2  $n = 14$ ,  $p = 0.013$ ). After the side the flowers were oriented to was switched, nine infected ants went to the familiar plant, while four went to the novel plant (binomial test cycle 3  $p = 0.27$ ). However, in the following and final cycle, the infected ants significantly went to the familiar plant although it was in a different position

from when they attached during the second cycle (binomial test, cycle 4  $n = 15$ ,  $p = 0.035$ ). Thus, infected ants distinguished the familiar plant following the switch and continued to return to their familiar flower for attachment.

#### *3.4.5 Choice trials: Aggregation of infected ants*

Initial trials were conducted to establish a familiar flower within the container before the main experiment started in both the nestmate's familiar plant experiment and the aggregation experiment. In both experiments, the initial infected ant preferentially went to the familiar plant (binomial test, cycle 2  $n = 30$ ,  $p = 0.005$  and binomial test, cycle 2  $n = 34$ ,  $p < 0.001$ , respectively), thus establishing a home plant within the container. The ants significantly attached to the same plant as the nestmate (90.9% of the time) when given the choice between a novel flower and the novel flower that had the nestmate attached (binomial test,  $n = 22$ ,  $p < 0.001$ ). Although infected ants that did not attach were excluded from the analysis, 47.6% ( $n = 20$ ) of the ants added second did not attach. When presented with a nestmate's familiar plant or a novel plant, 15 of the 22 infected ants that attached went to the nestmate's familiar plant (Fig. 3.5). In the second cycle, an additional six ants attached to the familiar plant. Thus, 21 of the 25 attached ants selected the flower that had previously contained a nestmate (binomial test,  $n = 25$ ,  $p < 0.001$ ).

### **3.5 Discussion**

Taken together, 81.7% of the ants that I placed into an artificial choice chamber made a non-ambiguous choice to attach to one of the two flowers. In contrast, uninfected ants never demonstrated flower choice or attachment within the 16 trials. Thus, one of the

key results of these laboratory trials is that *D. dendriticum*-infected ants consistently undergo their unique ‘attach-detach-repeat’ sequence of behaviours in both natural and artificial laboratory conditions. The ability to consistently and predictably ‘manipulate the manipulator’ provides a powerful tool that can be used to dissect the overall alteration into isolated components.

One clear result that was consistent among several trials was that *D. dendriticum*-infected ants preferentially attached to their familiar flower (the flower they were found attached to in the field). Although fidelity to a certain flower for attachment is consistent with observations of marked infected ants (Goater, unpublished observations), these are the first results to document such a preference under controlled laboratory conditions. The initial experiment where both flowers are novel, yet in the second cycle there is still a significant preference for returning to the same flower, implies that signals for familiarity can be created/determined within one attachment cycle. The strongest evidence for a preference for familiarity arises from the results of the second experiment, in which the positioning of the familiar flower was switched between the choice trials. These results showed that once an ant had selected a familiar flower for attachment, particularly after the second round of cooling, it continued to select the same flower after it was moved to the other side of the container. Further evidence for a preference for familiarity comes from the other choice experiments, where although the flowers were not familiar to them initially, the infected ants typically returned to the same flower in the second cycle. Taken together, these results provide strong support for the idea that once a flower blossom is selected, an ant returns to that same flower.

My experiments facilitated two decision-based responses, at least one of which

must be manipulated by the larval *D. dendriticum*. First, an infected ant must decide on which flower blossom to select for initial attachment. Following the first period of detachment following warming, the ant must then make the decision whether to return to the same blossom, or to select an alternative. One possibility is that infected ants are being stimulated by the same signals that signal the availability of food resources. Depending on the species of ant, foraging behaviour utilize signals, such as tandem running (physical contact with another ant to signal resources to a nest mate), visual/spatial components (using landmarks), or pheromone trails (a chemical trail left on the ground to guide other ants from the nest to a food source) to re-establish locations of food in relation to resources (Gronenberg, 1996). In many instances, using pheromone trails in conjunction with other foraging signals is common, especially in colonies with larger nest sizes (Beckers, Goss, Deneubourg, & Pasteels, 1990). In my behavioural trials, infected ants showed preferential decision-making in favor of familiar flowers, despite being presented with the same species and condition of novel flower. Although I did not assess for the precise signals that ants might be detecting, the consistent preference for a familiar flower suggests that the ants were responding to a signal in the chamber that attracted them to the familiar flower. This preference for a familiar flower remained without the presence of the infected ant on its flower, thus the ant is not solely utilizing vision to determine flower choice. The results support the idea that *D. dendriticum* might be manipulating the detection of, and response to, signals associated with foraging and feeding behaviour.

Both the field survey and behaviour experiment showed that infected ants preferentially select blossoms that contain other infected ants. The manipulation of the

infected ant's foraging/feeding behaviours by the larva in the brain might also explain the preference for blossoms that already have an attached ant. The results of foraging trials involving honey bees have also shown a preference for flowers that already have pollinating or nectar-feeding individuals, although the exact motivation is unknown (Plowright & Orba, 2014). There is evidence that aggregation of ants is due in part to the recognition and use of pheromone trails (Jeanson, Deneubourg, Grimal, & Theraulaz, 2004). In this case, once a scout finds a resource, it lays an initial trail back to the nest, which allows its nestmates to efficiently collect food sources (Beckers et al., 1990; Morgan, 2009). These trails can be reinforced after each successful acquisition of food. When given a choice between a blossom with a nestmate or a novel flower, there was clear selection for the blossom with the nestmate. Furthermore, once that selection was made, the ant was more likely to return to that same blossom. One explanation for these results is that the initial ant in the container had already laid a pheromone trail to signal resources, which it then used to find its familiar flower. This trail was then subsequently used by the additional ant added into the container, resulting in aggregation on that blossom.

Site-selection within the sub-esophageal ganglion means that larval *D. dendriticum* is in close proximity to the antennal lobes (Chapter 2). Thus, direct interference with receptors for information gathered by the antennae, including temperature and nestmate recognition (Nakanishi, Nishino, Watanabe, Yokohari, & Nishikawa, 2010), is possible. The sub-esophageal ganglion itself is the center for motor control of the mouth parts, a crucial component for attachment to flower blossoms (Knebel et al., 2018; Paul & Gronenberg, 2002). Thus, physical manipulation could

conceivably occur as a result of the proximity of the larva to the antennal lobes and/or to its controlling musculature (Chapter 2), as well as its location within the SEG (Martín-Vega et al., 2018; Romig et al., 1980). The position of *D. dendriticum* within the CNS and the behaviours that are being manipulated are indicative that the parasite is influencing structures within the brain involved in the recognition of signals. There is also the possibility of indirect manipulation via the release of neurochemical modulators, which has been demonstrated to occur in systems such as *Toxoplasma gondii*-infected laboratory mice and caterpillars infected with a parasitoid wasp (Libersat et al., 2018; Parlog, Schlüter, & Dunay, 2015).

In conclusion, my experiments indicate that infection of *F. aserva* with the trematode *D. dendriticum* results in clear alterations of decision-making in ants. Infected ants consistently prefer to cling onto flower blossoms that are familiar and result in the aggregation of infected ants, a result which can help explain the observed pattern of aggregation of infection ants onto flowers under field conditions. Through the results of the sequence of experiments completed in this study, the exploitation of ant foraging behaviour by larval *D. dendriticum* through alterations to ant-to-ant communication becomes one clear possibility.

### 3.6 References

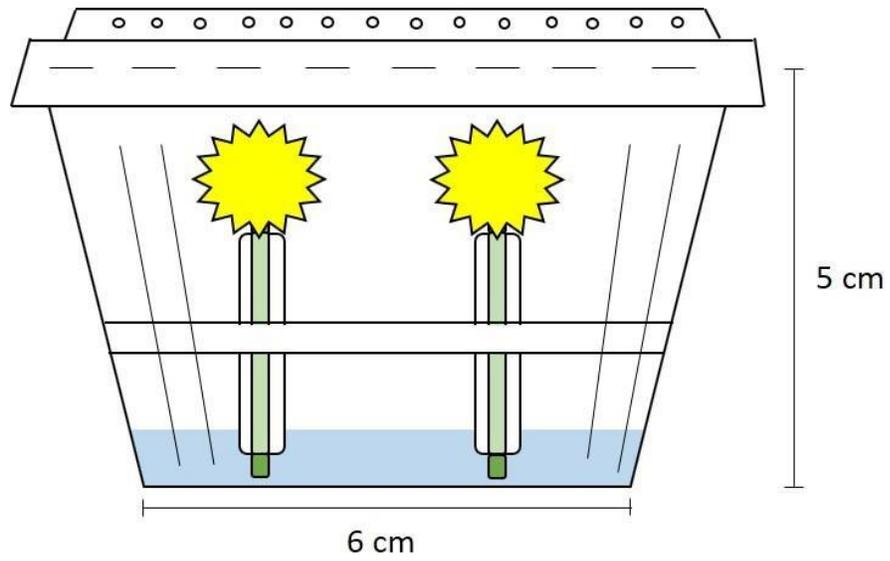
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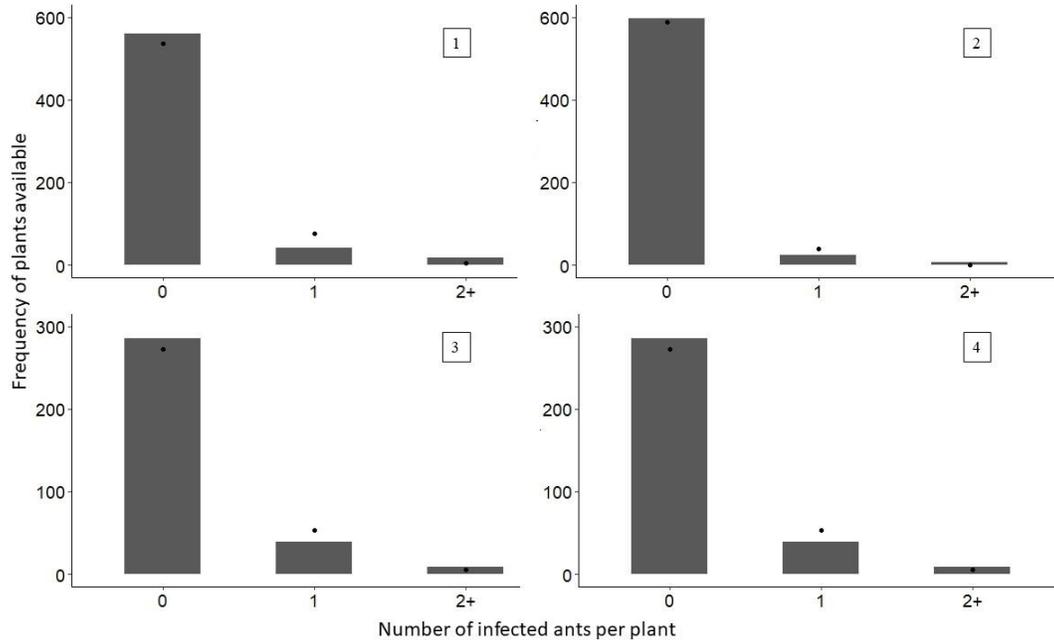
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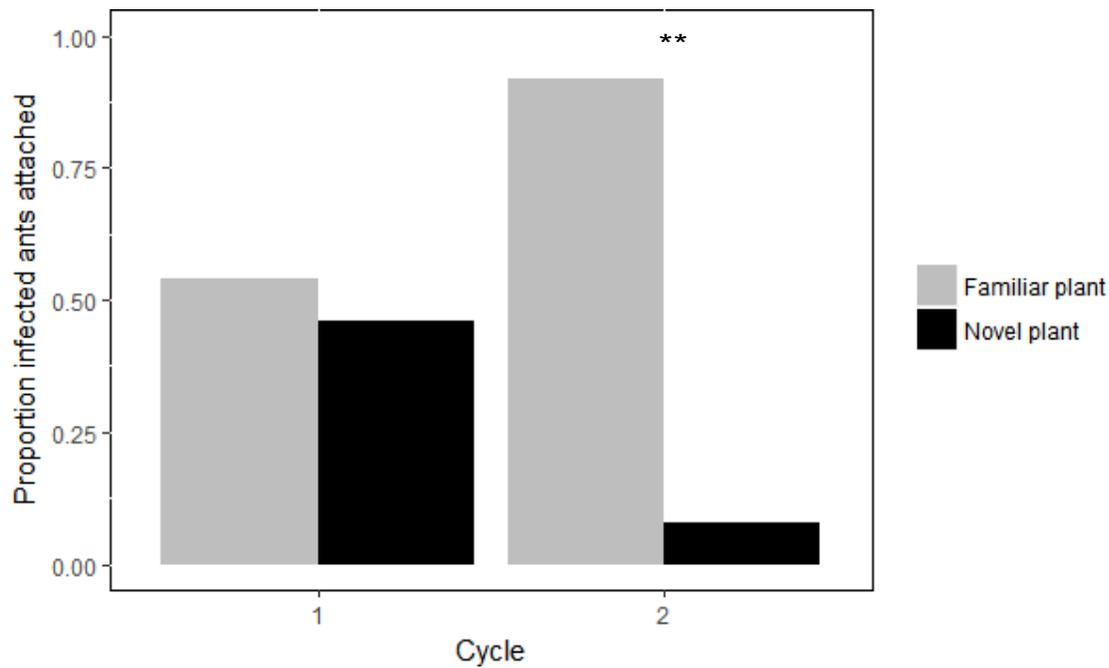
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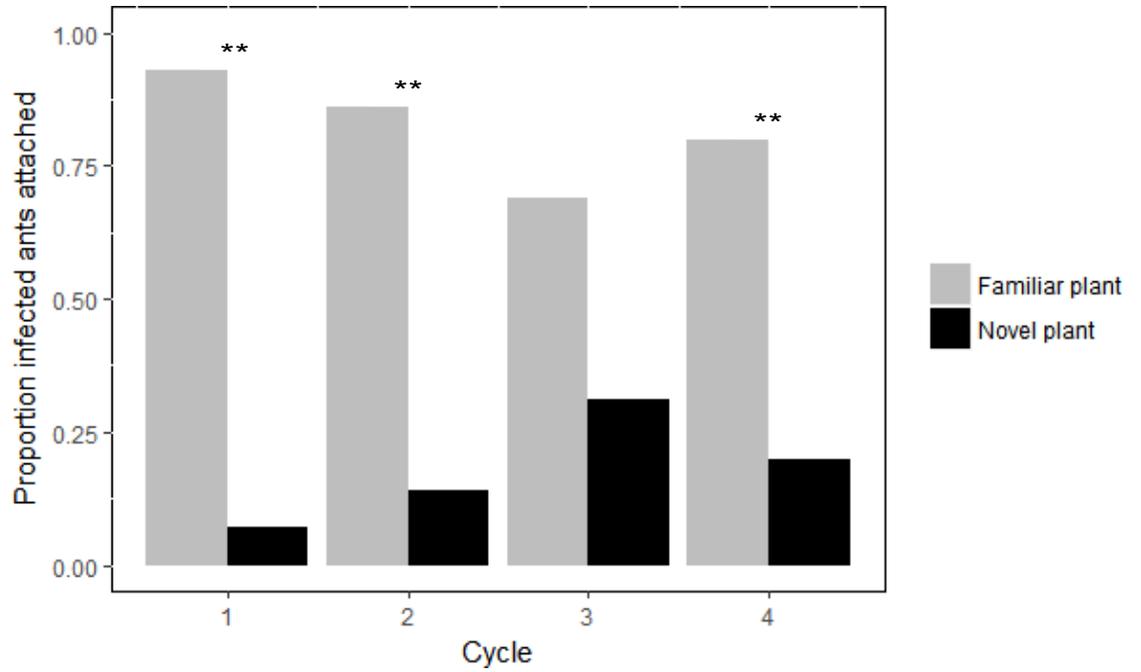
**Figure 3.1.** Design of the binary choice chamber used to assess flower choice in ants (*F. aserva*) infected with larvae of the trematode, *D. dendriticum*.



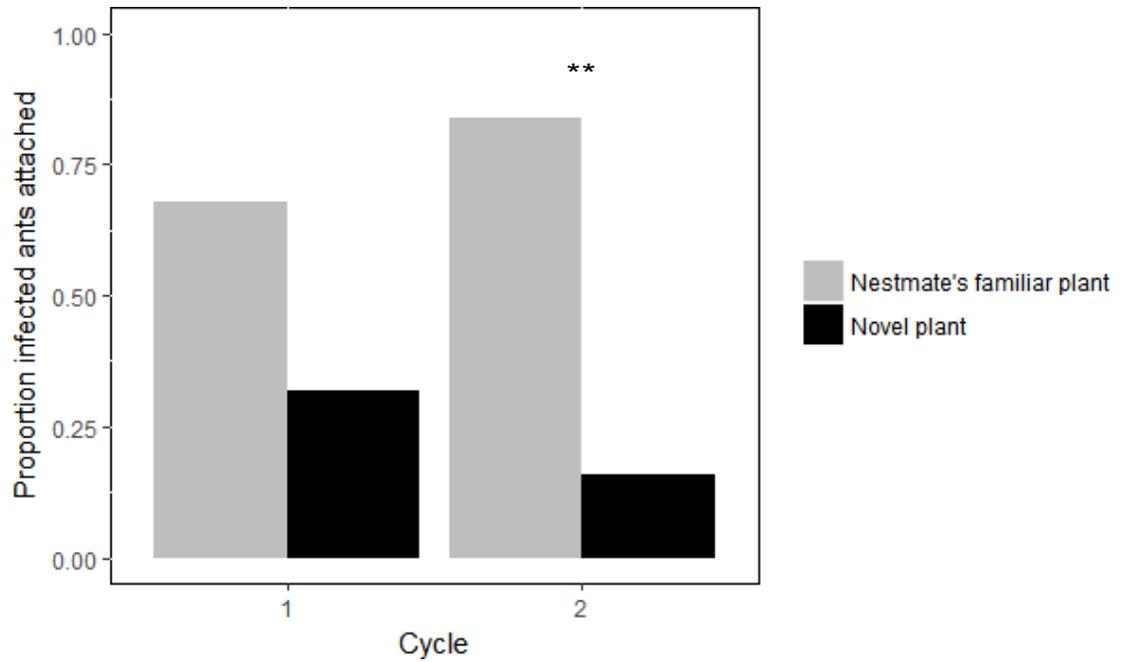
**Figure 3.2.** Frequency distributions of the number of *Dicrocoelium dendriticum*-infected ants on two populations of dandelions, surveyed at site 1 (early June: 1 and mid-June: 2) and site 2 (early June: 3 and mid-June: 4) known infected ants' nests located in Cypress Hills Park comparing the observed distribution (grey bars) and the predicted expected frequency (black dots) from a Poisson distribution.



**Figure 3.3.** The proportion of infected ants attached onto a familiar or novel plant over two cycles of attachment (10°C) and detachment (25°C) (cycle 1 n = 11, cycle 2 n = 12). Asterisks denote significant differences between the choices of ants made between familiar versus novel flower blossoms.



**Figure 3.4.** The proportion of infected ants attached onto a familiar or novel plant through four cycles of attachment (10°C) and detachment (25°C) where the position of the plants was switched after the second cycle (cycle 1 n = 14, cycle 2 n = 14, cycle 3 n = 13, cycle 4 n = 15). Asterisks denote significant differences between the choices made between familiar versus novel flower blossoms.



**Figure 3.5.** The proportion of ants infected with *Dicrocoelium dendriticum* attached onto a novel plant or a nestmate’s familiar plant throughout two cycles of attachment (10°C) and detachment (25°C) (cycle 1 n= 21, cycle 2 n = 25). Asterisks denote significant differences between the choices made between familiar versus novel flower blossoms.

## Chapter 4: General Discussion

Our understanding of the diverse and complex interactions that central nervous system (CNS)-inhabiting parasites have with their host remains incomplete. While the use of carefully-selected model systems, such as *Toxoplasma* in rodent intermediate hosts, and cockroaches infected with parasitoid larva, have provided remarkable advances, there are still important knowledge gaps. In the work reported here, I utilized a CNS-inhabiting model host-parasite interaction to evaluate aspects of the behaviours altered by larval *Dicrocoelium dendriticum* and to characterize the host-parasite interface using modern imaging tools.

Building upon previous imaging literature (Martín-Vega et al., 2018; Romig, Lucius, & Frank, 1980), I imaged the ultrastructure of the brain worm and for the first time, the interface between the brain worm and the sub-esophageal ganglion (SEG) of its ant host. Microsite selection was confirmed within the anterior- and ventral-most region of the SEG. Earlier literature has provided indication that the location of larval *D. dendriticum* in the SEG could provide a blunt mechanical tool to influence the mandibular nerves that are located within the anterior-ventral region of the SEG (Martín-Vega et al., 2018; Romig et al., 1980). Positioning within the SEG highlights important aspects that may be manipulated by the parasite to cause the behaviour alterations that I have observed in the field and in my suite of choice experiments described in Chapter 3. Thus, the mandibular nerves and motor neurons that are responsible for the action of the mandibles are located precisely within the anterior- and ventral-most region of the SEG (Eichmüller, Hammer, & Schäfer, 1991; Knebel et al., 2018; Paul, Roces, & Hölldobler, 2002). Neuropeptides such as GABA and FMRFamide have both been implicated in

immunoreactivity within the SEG and in behaviour manipulation in other host-parasite systems involving insects with CNS- inhabiting parasites (Eichmüller et al., 1991; Homberg, Kingan, & Hildebrand, 1990; Hughes & Libersat, 2018; Miles & Booker, 2000). The challenge for further studies is to combine molecular tools, immunohistochemistry tools and manipulation tools to 1) determine the linkage between infection, the action of the mandibles, and ambient temperature and 2) determine if manipulation requires physical contact with specific regions of the SEG (as indicated in my confocal laser scanning microscopy (CLSM) images), the production of neurochemicals that influence the action of the mandibular muscles, or both.

Microsite selection within the SEG in regions that are proximal to the mandibular nerves can help explain only a restricted set of the overall ‘attach-detach-repeat’ behaviours that characterize this manipulation. The temperature-dependent nature of attachment and detachment with the mandibles indicates that the brain worm must also influence the ant’s detection of temperature cues, its processing of these cues, or its response to these cues (Botnevik, Malagočka, Jensen, & Fredensborg, 2016). Sensillae located on the paired antennae are known to play a key role in the detection of environmental cues such as CO<sub>2</sub> concentrations and especially temperature (Nakanishi, Nishino, Watanabe, Yokohari, & Nishikawa, 2010; Ozaki et al., 2005; Renthal, Velasquez, Olmos, Hampton, & Wergin, 2003; Ruchty et al., 2009). However, the processing of these cues occurs within the antennal lobes, a distinct set of paired structures that lie immediately adjacent to the SEG. In several of my CLSM and TEM images, the brain worm was located immediately adjacent to the antennal lobes. Immunoreactive cells for FMRFamide are co-localized with GABA immune-reactive cells within

the antennal lobes of other insects (Homberg et al., 1990). Thus, there is potential for *D. dendriticum*-induced interference or manipulation of the temperature sensing neural apparatus in infected ants. If that potential is demonstrated in future studies, it would help explain the mechanism behind the temperature-dependent cycle of attachment and detachment that underlies this interaction.

Further, infected ants must actively leave their nest and they must forego typical foraging behaviours in favour of attachment, typically for hours at a time. One key finding of my imaging results is the proximity, and potential interaction, with the dorsal apodeme that connects to the pharyngeal dilator muscles, immediately ventral to the esophagus. This result provides the first indication that in addition to manipulation of the mandibles, interference with normal feeding structures, and in turn their associated behaviours, may play a fundamental role in the ‘attach-detach-repeat’ sequence of behaviours of infected ants. My results suggest that direct physical contact between the brain worm and this apodeme, and thereby the muscles that control the action of the esophagus, may play a role in this alteration. Results from my CLSM images indicate that the tip of this ribbon-like apodeme is almost in direct contact with the anterior of the worm. If further evidence confirms that the physical contact between the brain worm and the apodeme is responsible for, at least in part, an inhibition of feeding behaviours, this would be the first support for the ‘puppet-master hypothesis’ (Adamo, 2012) for the physical manipulation of host behaviours. Follow up studies must confirm the precise role of the dorsal apodeme in feeding behaviours, including in uninfected hosts.

Prior to the results presented in this thesis, experimental tests of *Dicrocoelium dendriticum* altered behaviour were primarily limited to field surveys and to tests of

conditions (e.g. temperature) that induce attachment/detachment (Botnevik et al., 2016; Carney, 1969; Manga-González & González-Lanza, 2005). My results presented in Chapter 3 are the first to show that larval *D. dendriticum* influences host decision-making of their ant host. Thus, infected ants preferentially approach, climb, and attach to specific flower blossoms. This result was observed in the field survey and especially in the binary choice trials utilizing growth chambers. None of these behaviours has been observed in uninfected ants. Further, infected ants preferred to attach to familiar flower blossoms over novel flowers, and preferred to attach to blossoms that have, or previously had, an infected nestmate attached to it. These results indicate that in addition to the obvious manipulations of the mandible muscles and to temperature-sensing, effects on host-decision making are also a key component of the overall manipulation. Presumably, infected ants detect certain cues from flower blossoms and use these cues to influence their decision regarding which substrate to use for attachment. There are two lines of evidence that these signals are reinforced by the infected ants following repeated visits to a flower blossom. First, in most of the binary choice trials, there was an improvement in the selection of the familiar flower between the rounds of attachment/detachment. Second, the preference for a naïve flower blossom that had previously contained an infected nestmate suggests that a signal had been deposited by the nestmate, detected, and then followed. These results strongly suggest that the utilization and selection of flower blossoms for attachment parallels the typical responses that ants have to food sources.

Thus, my results from Chapter 3 point to the manipulation of typical foraging behaviours to facilitate flower attachment. Intriguingly, this conclusion is in line with the results presented in Chapter 2 that made a link between site selection in the SEG and the

possible manipulation, via an apodeme, of the action of the ant esophagus. This conclusion needs to be confirmed with further study. One direction would be to characterize the possible pheromone signals (Barlin, Blum, & Brand, 1976; Mashaly, 2010; Vander Meer, Alvarez, & Lofgren, 1988) that infected ants use to mark a plant and then determine if infected and uninfected ants differ in their response to it. Given the proximity between the brain worm and the antennal lobes, and in turn antennae (Chapter 2), it is certainly conceivable that larval *D. dendriticum* may influence aspects of ant-to-ant communication and temperature sensing (Ozaki et al., 2005).

Although my results that focus on key altered behaviours and the physical interface between the brain worm and SEG has lent support to possible modes of host control, the gold standard to understand the mechanism of behaviour manipulation requires the use of molecular techniques. Further work should focus on aspects of gene regulation and the production of potential neuromodulators in infected individuals through modern genomics tools (de Bekker et al., 2015; Herbison, Lagrue, & Poulin, 2018; Hughes & Libersat, 2018). The quantification of differences in neuropeptide concentrations and inducing infected ant behaviour in uninfected individuals, using similar approaches in other host-manipulation systems (Cheeseman & Weitzman, 2015; Gal & Libersat, 2010; Parlog, Schlüter, & Dunay, 2015), will bring us closer to understanding how this brain worm influences the CNS of its host. Continued imaging, particularly after artificial infection, is also necessary to understand the migration route of brain worm and its course of development within the SEG. This thesis utilized multi-disciplinary techniques to help understand how *D. dendriticum* might alter the behaviour of its ant host. With further research that uses these results as a foundation, we will one

day have an all-encompassing view of this interaction.

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