ALTERED BEHAVIOUR AND TRANSMISSION ECOLOGY OF FLUKE-INFECTED ZOMBIE ANTS

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ABSTRACT

Parasites can induce changes in the colour, morphology, and behaviour of their hosts. In some cases, the induced changes are extraordinary in their expression. In an iconic example, Formicid ants infected with the lancet liver fluke, *Dicrocoelium dendriticum*, attach their mandibles to a plant for hours or days. I described patterns of infection in two species of ants infected with *D. dendriticum* in a region of parasite emergence in southern Alberta and found that ants recruit parasites in clumps from their first intermediate host, terrestrial snails. Overall, infected ants contain, on average 27±25 (mean ± standard deviation) encysted metacercariae (n = 677). In a departure from classic examples of parasite manipulation, infected ants do not die on the plant. Rather, they detach, resume normal ant activities, then re-attach. This bizarre 'attach/detach/repeat' sequence of altered behaviour likely facilitates transmission into grazing mammals while also preventing desiccation of the ant hosts and their parasites. I conducted a field study monitoring the behaviour of infected ants and found that the timing of attachment and detachment of ants on plants is closely linked with environmental factors such as light intensity, relative humidity, temperature, and also with ant circadian rhythm. Additionally, I completed a spatiotemporal analysis of the distribution of infected ants on plants in the field and found that infected ants are often aggregated on plants adjacent to the nest that they originated from, representing the first reported case of "super aggregation" of parasites. Whereas a pattern of strong aggregation on plants was spatially and temporally consistent for one species of Formicid ant in this region of parasite emergence, the behavioural processes leading to aggregation are enigmatic, as are the consequences of aggregation for rates of fluke transmission from ants into definitive hosts.

CONTRIBUTIONS OF AUTHORS

Chapter 2-4 represent original unsubmitted work by Lauren Edison (LE; primary author) that is coauthored by Dr. Cam Goater (CG). LE contributed to field collections, dissections, data analysis, and writing. CG conceived the project and contributed supervisorship, project management, and editing.

USE OF GENERATIVE AI

OpenAI's generative artificial intelligence, ChatGPT-4, was used to fix warnings in the R script used in the statistical analyses of Chapters 2, 3, and 4 of this thesis.

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Chapter 1: General Introduction

The parasitic lifestyle is conspicuous on the Tree of Life and is thought to account for approximately 50% of animal diversity on earth (Poulin & Morand, 2000). Of this vast diversity of parasites, many are host specialist (Vazquez et al., 2005), many have multi-host life cycles (Rauch et al., 2005), and many can manipulate phenotypes of their host in some capacity (Heil, 2016). Indeed, the expression of 'extended host phenotypes' (Dawkins, 1982) following infection is a widespread phenomenon that spans a large range of diversity of both parasites and hosts. Alterations to host phenotypes such as colour, morphology, and behaviour are often associated with an increase in the development or reproduction of the manipulating parasite, often through enhanced facilitation of transmission between hosts. There are many well-known examples of host manipulation (Moore, 2002; Hughes et al., 2012; Ezenwa et al., 2022), including *Toxoplasma*-induced attraction to predators in rats (Berdoy et al., 2000), trematode-induced limb malformation in frogs (Johnson et al., 2002), and hairworm-induced water seeking behaviour in crickets (Thomas et al., 2002). Parasite alteration of host behaviour is one of the most notable types of manipulated host phenotypes. HBO's blockbuster series 'The Last of Us' (Mazin & Druckman, 2023) has garnered public interest for the behavioural manipulator, *Ophiocordyceps* spp. In this case, the fungus (*Ophiocordyceps* spp.) manipulates ants to leave their nest to die in a location that optimizes spore dispersal and infection of naïve ants (Anderson et al., 2009). The recent interest in this phenomenon of host manipulation from parasitologists, neurobiologists, ecologists, and evolutionary biologists, together with increased visibility in the public eye, has greatly emphasized both the complexity and our fascination with manipulated host behaviours by parasites.

Despite recent attention in this popular field of inquiry, fundamental aspects of host manipulation remain poorly understood, particularly the neurobiological mechanisms leading to altered host behaviours (Hasik et al., 2022; Nadler et al., 2023). Furthermore, the complexity and multi-dimensionality of host manipulation has only recently been recognized (Poulin, 2021; Hasik et al., 2022). For example, hairworm-infected crickets that move towards water are not simply 'sick and thirsty.' Rather, their brains express genes and contain neuro-modulating chemicals that influence host activity levels, orchestrate responses to polarized light, and reduce normal foraging and anti-predator responses (Obayashi et al., 2021). These results indicate that fundamental aspects of the general phenomenon of parasite-induced host manipulation are more complex than originally thought.

Some host manipulation systems are particularly well-studied and have contributed extensively to our understanding of the host manipulation phenomenon. These systems are generally of human importance (e.g., *Toxoplasma*, rabies virus, malaria, etc.), are those amendable to experimental manipulation (e.g., fungi in fruit flies, Elya et al., 2018), or have long traditions of study (e.g., larval acanthocephalans in arthropods; Moore, 1984). In combination, this targeted research effort has highlighted the complexity of alterations in host behaviour by parasites. In a pivotal study, Berdoy et al. (2000) showed that infection of brain tissue with encysted stages of the protist *Toxoplasma gondii* increased a rat's risk of being ingested by a cat definitive host. The mechanism somehow involved turning the rat's normal aversion to cat odour into an attraction (Berdoy et al., 2000). This result indicates that *T. gondii* evolved a strategy to manipulate its host's response to the risk of predation (Bramley et al., 2000). These findings inspired the neurological and genomic approaches to uncover the mechanistic components of this and other instances of host manipulation (Xiao et al., 2012; Dass & Vyas, 2014). Additionally, there have been several studies published regarding human *T. gondii* infection (Robert-Gangneux & Darde, 2012). These studies include findings of an increased risk of schizophrenia (Yolken et al., 2009), increased risk of being involved in traffic accidents (Flegr et al., 2002), and correlations with many other diseases (Flegr et al., 2014).

Another example of behavioural manipulation that highlights complexity is *Ophiocordyceps*-infected 'zombie' ants. This fungal parasite causes its ant host to leave its nest, climb up a plant, and then attach to the plant with its mandibles (Anderson et al., 2009). In this manipulated state, the fungus extends a stroma out of the thorax of the ant, killing it, and releasing spores to infect naïve ants that are foraging below (Mongkolsamrit et al., 2012). The *Ophiocordyceps*/ant system has been extensively studied, with particular focus on understanding neural mechanisms involved in the nest abandonment, trail-following, plant selection, summitting behaviour, and the action of the mandibles (de Bekker et al., 2017; de Bekker & Das, 2022). For example, the results of recent studies indicate that the manipulation of host circadian rhythm may play a role in leading infected ants out of their nests and onto a plant (de Bekker & Das, 2022). De Bekker & Das (2022) speculated that the fungus 'hijacks' the host's circadian rhythm, resulting in ants leaving their nest for attachment at the same time every day. Evidence in support of the circadian-rhythm hypothesis came from targeted transcriptomics approaches, revealing that certain circadian rhythm-related genes were expressed differently in infected versus uninfected ants, alongside lab and field studies that showed infected ants exhibiting the manipulated behaviour at the same time every day (de Bekker & Das, 2022). These results highlight the idea that the current hypotheses surrounding the key roles of factors such as temperature and day length in the expression of manipulated phenotypes may be too simplistic.

Another iconic host manipulator is the lancet liver fluke, *Dicrocoelium dendriticum*, in its second intermediate hosts, Formicid ants. Flukes are parasitic flatworms (Platyhelminths) in the class Trematoda. *Dicrocoelium dendriticum* has a unique and complex life cycle (Krull & Mapes, 1952; Krull & Mapes, 1953) that begins with hermaphroditic adults that live in the gall bladder and liver of grazing mammals, such as sheep, deer, and cows (Fig. 1.1). Adult flukes release fertilized eggs that are passed in the feces of the mammal. Terrestrial snails ingest eggs during foraging, each of which contains a ciliated miracidium that is released inside the snail's digestive tissue. Development then occurs, resulting in a mother sporocyst, then a daughter sporocyst that contains developing cercariae. Once the cercariae have matured, they leave the sporocyst and migrate to the respiratory tract of the snail. Here, the cercariae gather and become surrounded by snail mucus. These cercariae-filled 'slimeballs' are released into the environment through the respiratory pore of the snail. Slimeballs have been considered to bear a close resemblance to snail eggs (Krull & Mapes 1952; Krull & Mapes, 1953). Ants from the genus *Formica* collect the slimeballs and return them to their nests where their sisters can feed on the seemingly nutritious food source (Krull & Mapes, 1953). While feeding on the slimeball, an individual ant can ingest more than 150 cercariae (Manga-Gonzelez et al., 2001). Once inside the ant, the cercariae mature into metacercariae, most of which encyst in the hemocoel of the ant (Krull & Mapes, 1953). But in a curious exception to classical metacercariae development (Goater et al., 2014), one metacercaria remains unencysted and migrates anteriorly to the ant's 'brain' where it apparently makes contact with the suboesophageal ganglion (Martin-Vega et al., 2018). At this stage, the parasite manipulates the ant to leave its nest, ascend a plant, then firmly attach its mandibles, from which the ant can be ingested by a grazing mammal, completing the life cycle (Krull & Mapes, 1953). In a departure from the well-known, fungus-caused, 'zombie-ant' phenomenon, fluke-infected ants detach from their plant and are thought to resume *normal* activities within their nest (Moore, 2002; Goater et al., 2014). The 'attach/detach/repeat' manipulation, each stage of which is under the control of a single worm, is one of only a handful of known *reversible* host manipulations.

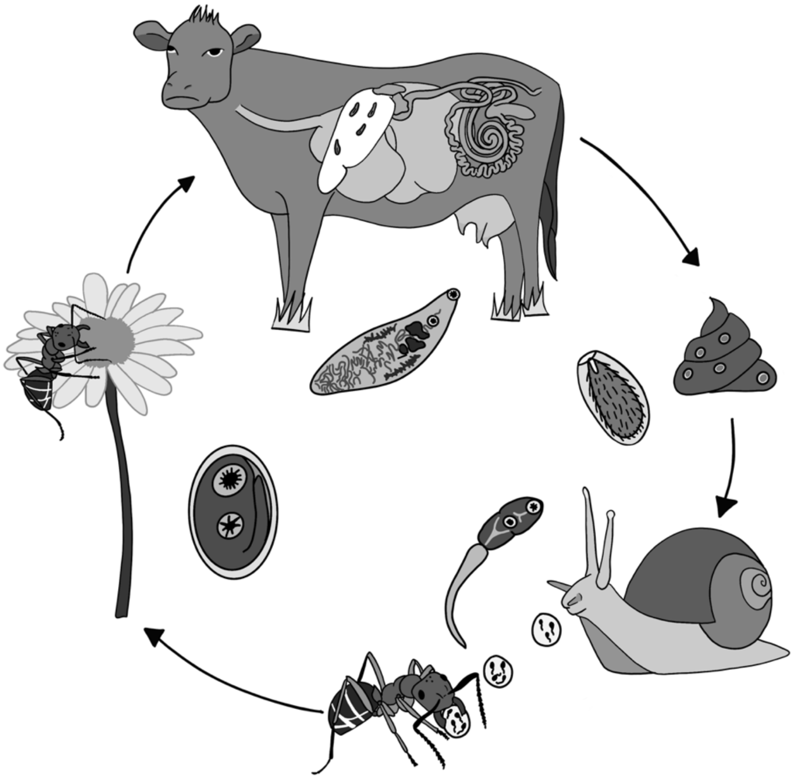

Figure 1.1. Life cycle of *Dicrocoelium dendriticum*. In this trematode life cycle, all three hosts, grazing mammals (definitive hosts), land snails (first intermediate hosts), and formicid ants (second intermediate hosts), are terrestrial. The parasite life stages include the adult flukes in the grazing mammal, the eggs in the mammal's feces, the cercariae in the land snail, and the metacercariae in the formicid ant. Metacercariae within the ant host induce a host behavioural manipulation, causing the ant to leave its nest, climb a plant, and firmly attach its mandibles, where it awaits ingestion by a grazing mammal.

The textbook explanation for reversibility of manipulated behaviour in *D. dendriticum*-infected ants is that attachment and detachment behaviours are determined, through unknown mechanisms, by temperature-dependent regulation of the muscles that open and close the mandibles (Moore, 2002; Goater et al., 2014). Although the temperature-dependent hypothesis is widely accepted, it has little empirical support. One of the first characterizations of reversibility involved *Formica polyctena* ants observed on pastures in southwest Germany (Spindler et al., 1986). Spindler et al., (1986) followed ants that originated from colonies that were known to be infected. They recorded the number of ants and the temperature at one-hour intervals over two days and found that most ants detached from plants during mid-day when temperatures were highest. However, approximately 10% of infected ants remained attached during the heat of the day, with no explanation, indicating a potential role for different environmental factors (Spindler et al., 1986). Botnevik et al., (2016) tested the effects of temperature, humidity, and light on the attachment behaviour of *D. dendriticum*-infected ants in a lab setting. They found that ant attachment and temperature had a strong negative relationship and concluded that temperature was the sole environmental factor driving the detachment of infected from plants (Botnevik et al., 2016). Unfortunately, this work was done on ants that were taken from the field and lacked a colony with a queen during the duration of the study (Botnevik et al., 2016). Ant behaviour is significantly different in the absence of a queen and colony (Vienne et al., 1998); therefore, it is hard to know if the same results would be obtained in a natural setting. The same research group then repeated this study in the field seven years later (Gasque & Fredensborg, 2023). They followed the behaviour of ants surrounding four infected ant nests, counting the number of ants attached and recording temperature, light intensity, and relative humidity six times a day over nine non-consecutive days. They came to the same conclusion, with temperature as the driving factor for ant attachment and detachment, despite their generalized linear model showing a role for relative humidity as well (Gasque & Fredensborg, 2023).

Dicrocoelium dendriticum was introduced to Cypress Hills Interprovincial Park (CHIP), Alberta, Canada from Europe in the late 1900's, most likely from livestock trade or other anthropogenic animal movement (van Paridon et al., 2017a). It has since become established in land snails (*Oreohelix* spp.; Dempsey et al., 2019), ants (*Formica* spp.; van Paridon et al., 2017b; Criscione et al., 2020), and grazing mammals (elk, deer, cattle, etc.; Colwell & Goater, 2010) in the park. Due to *D. dendriticum*'s recent introduction to and establishment within hosts in CHIP, it is considered an emerging parasite in the region, compared to Europe, where the parasite is native. Discovering the mechanisms responsible for the reversible manipulation in *D. dendriticum*-infected ants has been a central theme of inquiry in the Goater lab. While our lab and field studies have confirmed a general role for temperature in the diurnal activity patterns of infected ants in CHIP (Unrau, 2019; Li et al, 2025a; Li et al., 2025b), observations in 2023 suggested that additional factors may be involved. Results from imaging of the host-parasite interface (Unrau, 2019), metabolomics and genomics of the host and parasite (Li et al., 2025a; Li et al., 2025b), and assessing clonal diversity of metacercariae (Criscione et al., 2020) have given us extensive information on some components of the infection and manipulation in this region of fluke emergence, but foundational information about the system, especially regarding altered behaviour in the field, is lacking. To fully understand the results of complex mechanistic studies, like those listed above, we must first have an in-depth understanding of how the behavioural manipulation is expressed in nature. A study characterizing the reversibility of *D. dendriticum*-induced ant manipulation in an introduced system (e.g., in CHIP) has yet to be done. It is important to compare the results of behavioural studies in native and introduced systems to determine the mechanisms responsible for the altered behaviours.

Other features of the *Dicrocoelium*/ant manipulation also rest on anecdotal or incomplete information. For example, patterns of natural variation in transmission rates of *D. dendriticum* larvae into ants from snail intermediate hosts are poorly understood, as are data on rates of larval development within ant hosts (Manga-Gonzalez et al., 2001; van Paridon et al., 2017b). Furthermore, the extent to which patterns of infection in individual ants vary between sites, nests, species, and times of the year has not been evaluated in the unique case of fluke emergence in CHIP. These are critical knowledge gaps because the timing of exposure of ants to infective stages, and their rates of development within ants, may influence the timing and magnitude of host manipulation (Manga-Gonzalez et al., 2001). Additionally, understanding the basic epidemiology of this fluke infection will aid in understanding key rates of transmission, including rates of recruitment of parasites from snails to ants and the movement of metacercariae from ants to grazing mammals.

1.1 THESIS OBJECTIVES

My thesis focuses on describing the foundational aspects of the behavioural manipulation of ant hosts by *D. dendriticum* in this region of emergence in CHIP. My focus is on field surveys of samples of known infected ants, and detailed behavioural observations of infected ants as they attach and detach from plants adjacent to their nests. The main underlying objective of this thesis is to describe aspects of the behavioural manipulation of *Dicrocoelium*-infected ants that have theoretical support but little empirical support. In contrast to all published accounts of this iconic manipulation, I also compare key epidemiological patterns of infection between two sympatric species of ant in this region of emergence, and I contrast interspecific differences and similarities in the behavioural manipulation.

In Chapter 2, I use standard epidemiological host survey methods to determine how larval *D. dendriticum* parasite load varies within two species of ants. For this objective, I ask if changes in *D. dendriticum* burdens in individual ants (n>2000) are associated with variation in host species year, season, or host size. Despite the iconic nature of this host manipulation, this is the first effort to describe fundamental eco-epidemiological aspects of metacercariae transmission from snails into ants.

In Chapter 3, I use classical field monitoring methods to evaluate the timing of attachment and detachment of *D. dendriticum*-infected *Formica podzolica* and *Formica aserva* ants at 7 nests from May-August 2023 and 2024. I conducted field surveys to determine how the proportion of ants that are clinging to vegetation changes with time of day, temperature, relative humidity, light intensity, month, year, and species. Using these methods, I asked if the initiation of attachment and detachment of infected ants are associated with environmental cues, and I also monitored how the response to environmental cues varied across season, years, and host species. Current theory suggests that temperature is solely responsible for attachment and detachment of infected ants as to mitigate risks of desiccation, but this contention rests on limited evidence. Describing this enigmatic reversible manipulation will provide insight into how environmental cues and host circadian rhythm play a role in the initiation of manipulation by parasites.

In Chapter 4, I evaluate if variation in the utilization of the plant community by infected ants is associated with host species and/or plant diversity/plant density, and if the pattern of host utilization by individual ants is consistent within and between nests over a season of infection. Little is known about the spatiotemporal distribution of *D. dendriticum*-infected ants in their manipulated state. Other studies have found that different insects, infected with a wide variety of parasites, exhibit aggregated distributions during manipulation (e.g., *Xenos vesparum*-infected *Polistes dominulus* wasp system; Beani et al., 2011). Earlier anecdotal and serendipitous observations from researchers in the Goater lab indicate that infected ants tend to aggregate on plants that are available surrounding a nest. In an extreme case, I observed more than 200 infected ants aggregated on a single leaf of *Petasites frigidus var. sagittatus* (arrowleaf sweet coltsfoot). The distribution of *Dicrocoelium*-infected ants on plants has not been characterized. This is an important shortcoming because patterns of distribution will impact rates of metacercariae transmission into grazing mammals, with key influences on adult worm reproduction, host immunological responses, and host pathology. For the first time, I describe the pattern of plant utilization by infected ants. I used a field survey to characterize seasonal changes in plant utilization by infected ants and assessed how plant utilization was influenced by plant density, plant community structure, host species, and nest.

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Chapter 2: Eco-epidemiology of the host manipulator, *Dicrocoelium dendriticum*, in two species of Formicid ants in a region of parasite emergence

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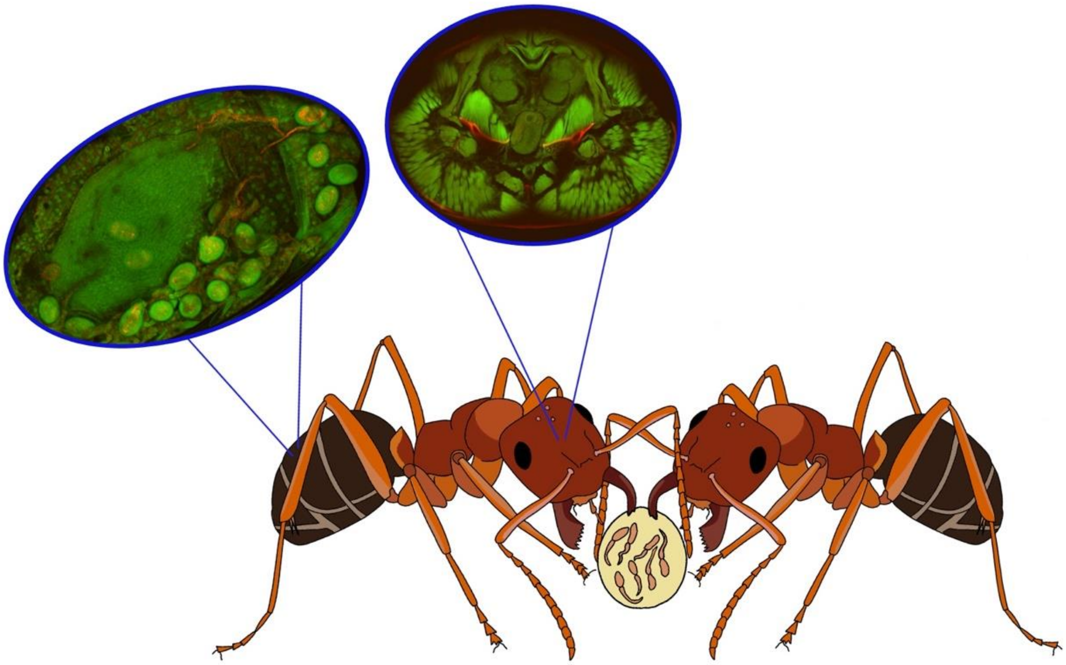
2.1 SUMMARY ABSTRACT

The transmission of many parasites involves the movement of ‘clumps’ of infective stages between hosts. Clumped transmission can result in complex and non-linear transmission dynamics, with important ecological and evolutionary consequences. But fundamental epidemiological characteristics that arise from clumped transmission are poorly characterized within natural host/parasite interactions. We report eco-epidemiological patterns of infection for metacercariae of the trematode *Dicrocoelium dendriticum* in samples of ants collected from a region of emergence in southern Alberta, Canada. In this terrestrial trematode, infection of ant intermediate hosts occurs when foraging workers encounter packets, or ‘slimeballs’, that are known to contain 10’s to 100’s of cercariae. In a total sample of 677 infected ants collected over two years from infected nests belonging to two host species, *Formica aserva* and *F. podzolica*, and the mean metacercariae intensity (± standard deviation) was 25.9±25.5 (range in intensity = 1-168) and 28.4±23.0 (range in intensity = 1-106), respectively. None of the observed variation

in metacercariae counts could be attributed to ant species (*Formica aserva*, *F. podzolica*), year (2021, 2022), month (May to August), or nest. Consistency in spatio-temporal patterns of infection sharply contrast patterns of high seasonality, high year-to-year variation, and high spatial variation typical of metacercariae/host interactions. These sharply contrasting results likely reflect restricted exposure to clumps of cercariae, possibly to a single exposure over a worker ant's lifetime. These eco-epidemiological patterns that highlight restricted, clumped transmission are consistent with the results of population genetic analyses involving metacercariae in ants collected from the same nests.

2.2 GRAPHICAL ABSTRACT



2.3 KEY FINDINGS

- Fluke larvae cause two species of sympatric Formicid ant to attach to plants with their mandibles in a region of parasite emergence in southern Alberta, Canada.
- In a total sample of 677 infected ants belonging to two host species, *Formica aserva* and *F. podzolica*, the mean metacercariae intensity (± s.d.) was 25.9±25.5 (range in intensity = 1-168) and 28.4±23.0 (range in intensity = 1-106), respectively.
- Variation in metacercariae counts between samples was not associated with season, year, ant species, or nest.
- The clumped pattern and high variation in metacercariae loads within individual ants can be explained by restricted encounters with clumps of cercariae originating from infected land snails.

2.4 INTRODUCTION

The transmission of many parasites involves the movement of infectious larvae within discrete packages or 'clumps.' This transmission mode has been demonstrated to influence patterns of parasite aggregation within exposed hosts (Walker et al., 2010), the distribution of clonal populations of worms in hosts (Criscione et al., 2020), and the rate of evolution of drug resistance (Cornell et al., 2003). Examples of clumped transmission include the penetration of infectious stages via vectors, transmission of aggregated tick and nematode larvae from vegetation to grazing herbivores, and trophic transmission of infected intermediate hosts into definitive hosts. Relative to the more typical mode of transmission involving the movement of single infectious larvae through 'trickle' type processes, there has been much less theoretical and empirical attention devoted to understanding the dynamics of clumped transmission (Walker et al., 2010). Even less attention has been devoted to fundamental field-based assessments of eco-epidemiological patterns of infection arising from clumped transmission.

The lancet liver fluke, *Dicrocoelium dendriticum*, has a three-host life cycle that includes an obligate clumped transmission stage. Following reproduction of hermaphroditic worms located within the bile ducts of grazing mammals, embryonated worm eggs are passed into the external environment via host feces. Terrestrial snails encounter fluke eggs during foraging bouts on the soil surface or on vegetation. Following ingestion, miracidia contained within an egg hatch, penetrate the gut, and then undergo cycles of asexual reproduction, ultimately leading to the production of cercariae. These infective, motile larvae are packaged into discrete, fluid-filled, mucous balls (= slimeballs) within the snail's lung that are secreted onto substrate (Krull & Mapes, 1952; Krull & Mapes, 1953). To continue the life cycle, slimeballs must be encountered by an individual ant and then broken apart to release individual cercariae prior to ingestion.

The lancet liver fluke is a recent introduction within a parkland region in South-Central Alberta, Canada (van Paridon et al., 2017a). van Paridon et al. (2017a) compared the population genetic structure of flukes sampled from throughout its global geographical distribution to infer a complex pattern of worm colonization into this region, with likely sources from coastal populations in Canada and direct introduction from European homeland sites. Within the confines of this fenced park, the life cycle has become established within various grazing herbivores (Beck et al., 2015), three species of Oreohelid land snails (Dempsey et al., 2019), and two species of Formicid ants (van Paridon et al., 2017b). Although spatio-temporal patterns of *D. dendriticum* infection within this region have been described in snail intermediate host (Dempsey et al., 2019), there is little information available on how epidemiological factors such as season, location, year, host species, or host size contribute to variation in worm counts in samples of ants.

Results from previous studies in the Goater laboratory indicate that clumped cercariae transmission has important eco-evolutionary implications for ant/*D. dendriticum* interactions (Criscione et al., 2020; Criscione et al., 2022). These authors used a population genetics approach to characterize the genetic (= clonal) structure of metacercariae populations within samples of infected ants collected from this region of fluke emergence. Their results showed that clonal diversity of *D. dendriticum* metacercariae populations within ants was lower than for any other species of trematode that has been assessed. A plausible explanation for this extreme pattern is that asexually produced cercariae within a slimeball are clonally related and that an individual ant rarely encounters slimeballs during its lifetime. Such a pattern of restricted, clumped exposure of ants to cercariae will be reflected in the temporal patterns of metacercariae infection in consecutive seasonal samples of ants. This prediction has not been tested.

In this study, we assessed infection patterns of *D. dendriticum* metacercariae within samples of two species of ants, *F. aserva* and *F. podzolica*, in Cypress Hills Interprovincial Park, Alberta. Our first aim was to assess how variation in metacercariae counts in ant samples was associated with variation in host species, nest, ant size, year, and month. A second aim was to characterize the timing of larval fluke recruitment in ants during the annual cycle of ant activity. By characterizing metacercariae cohorts based on morphological criteria, we can test the predictions described by Criscione et al., (2020) for restricted transmission of clones into ants. Lastly, we utilized our collection of a total of >2500 ants to report general infection characteristics for other endoparasites in *F. aserva* and *F. podzolica* at these sites.

2.5 MATERIALS AND METHODS

2.5.1 Study location

Cypress Hills Interprovincial Park (CHIP; Fig. 2.1; 49° 30’ to 49° 40’ N; 110° 00’ W) covers approximately 2,590 km^2 in the southeastern and southwestern borders of the western Canadian provinces of Alberta and Saskatchewan, respectively. The park is oriented along an east-west axis located approximately 50 km north of the Canada-United States border. CHIP comprises a high-elevation plateau that was protected from the extensive erosion that lowered the bedrock surface of the northern Great Plains during the Quaternary Period (Beaty, 1975). As an elevated plateau (maximum 1,465 m above sea level that rises 430-600 m above the surrounding arid prairie), CHIP is a unique, non-glaciated ‘sky island’ (Dempsey et al., 2019) that comprises a complex mosaic of forest, grassland, and aquatic habitats. The elevated plateau is dominated by fescue grasses and shrubs, characteristic of the mixed grass and montane natural sub-regions of Alberta (Downing & Pettapiece, 2006), whereas the forested slopes that rise from the surrounding prairie to the plateau are dominated by lodgepole pine (*Pinus contorta*), white spruce (*Picea glauca*), balsam poplar (*Populus* spp.), and trembling aspen (*Populus tremuloides*).

2.5.2 Site selection

Samples of *F. aserva* were collected from two sites on the Alberta side of CHIP (Fig. 2.1) that were chosen from among 32 known locations in the park where *F. aserva* have been observed attached to vegetation (Beck, 2016). Two sites (Staff Camp – SC and Ski Hill - SH) were selected for this study because background infection data was available from samples of attached ants (van Paridon et al., 2017b) and *Oreohelix* land snails (Dempsey et al., 2019) collected at these sites. Furthermore, our previous and ongoing studies on behavioural and population genetic aspects of the larval lancet fluke/ant interaction involve ants collected from these two sites (e.g., Criscione et al., 2020; Li et al., 2025a; Li et al., 2025b). Sites SC and SH are approximately 2 km apart. Each of these sites is located on gently sloped, South-facing forest stands under canopies dominated by mature balsam poplar, lodgepole pine and white spruce. *Formica aserva* nests are commonly observed in this habitat type, typically located within woody downfall and/or at the base of tree stumps.

Samples of *F. podzolica* were collected from a third site (Trans Canada Trail - TCT; Fig. 2.1) on the same day as the *F. aserva* samples. This site is located approximately 12 km southeast of the SC and SH sites. In contrast to the forested *F. aserva* sites, TCT is open prairie dominated by grasses and shrubs that are characteristic of the rough fescue/parry oat grass plant community type. The site is South-facing, with a gently sloping aspect, and sandy substrate.

2.5.3 Ant collection

Samples of *F. aserva* and *F. podzolica* were collected from the three sites during the last week of each month during the summers of 2021 and 2022. Samples of *F. aserva* were collected from SC and SH; samples of *F. podzolica* were collected from TCT (Fig. 2.1). There were multiple *Formica* spp. nests observed at each site. At SC and SH, approximately 10 large (20 – 100 cm diameter) *F. aserva* nests were conspicuous within the approximately 100 m X 100 m site. At TCT, there were 15 conspicuous *F. podzolica* nests (10 – 30 cm diameter) located within the 20 m X 10 m plot. Individual nests at the three sites tended to contain hundreds to thousands of active workers. For monthly *F. aserva* collections at SC and SH, we sampled ants that were associated with a single nest opening. These were the largest nests at the two sites and were located approximately 10 m from the next visible nest. These were the same nests sampled in our previous studies (van Paridon et al., 2017b; Unrau, 2019; Li et al., 2025a; Li et al., 2025b). In contrast, *F. podzolica* collections each month involved pooling samples from multiple nest entrances within the 20 m X 10 m site.

We collected a maximum of 400 ants from each site each month. A portion of these were sampled from plants located within 2 m of a nest entrance. These ants were easily observed with their mandibles firmly attached to the petals of flowers. Attached ants were gently removed from their flower and immediately fixed in ethanol. Concurrently, an additional sample of ants was collected directly from the nest. We assumed that these ants originated from the same nest as the adjacent sample of attached, infected ants. The purpose of collecting ants directly from a nest was to provide a crude estimate of total *D. dendriticum* prevalence within an individual nest. For collection, we applied gentle pressure to the surface of a nest with an open-topped, plastic, 1 L container. After approximately 200-400 workers had entered the container, ethanol was added for fixation.

2.5.4 Enumeration of metacercariae

The haemocoel of each ant was opened with the aid of a dissecting microscope (van Paridon et al., 2017b) to determine metacercariae count/host (= parasite intensity). Following worm counts, metacercariae from individual ants were pooled, removed from solution with a pipette, mounted on a microscope slide, and then observed under a compound microscope.

Individual metacercaria were classified as mature versus immature using morphological criteria described in Faltynkova et al. (2011). Metacercariae were classified as mature if they were enveloped by a thick, double-layered cyst wall; immature if they were not (Fig. 2.2).

Concurrent with our collection of metacercariae count data, we recorded the presence, identity, and number of other parasites. We also estimated the size and age of individual ants by measuring the length of the right hind tibia with a micrometer at 4X under a compound microscope.

2.5.5 Analyses

Statistical analyses were completed in R Statistical Software (v4.4.1; R Core Team, 2024). Due to marked heterogeneity in sample sizes between *F. aserva* and *F. podzolica* each month and because site and host species are confounded, we analyzed worm count data separately for the two species. For *F. aserva* counts, we used a generalized linear mixed model (GLMM) fit to a negative binomial distribution using a log-link function to assess differences in mean metacercariae counts between samples. Month, year, and host size were fixed effects; site was a random effect. For *F. podzolica* counts, we used a generalized linear model (GLM) fit to a negative binomial distribution using a log-link function with month, year, and host size as fixed effects. Non-significant 2-way interactions were removed from each model after a likelihood ratio test concluded that they do not increase the power of the models.

The low number of metacercariae observed with rudimentary cyst walls (5.3 % of 17,909 total observed metacercariae) limited our ability to evaluate temporal patterns of transmission from snails into ants. We used Fisher exact tests to evaluate differences in the proportion of ants within a sample that contained immature metacercariae. We also report the proportion of non-encysted metacercariae for each infrapopulation within those ants that contained at least one immature larva.

2.6 RESULTS

2.6.1 S*urvey of parasites in ants*

Metacercariae of *D. dendriticum* were the most commonly encountered macroparasite within the total sample of *F. aserva* and *F. podzolica* (Table 2.1). Overall, 26% of *F. aserva* (total mean ± standard deviation = 6.8 ± 17.4 metacercariae/host) and 20% *F. podzolica* (total mean ± standard deviation = 5.6 ± 15.2 metacercariae/host) collected were infected with *D. dendriticum* metacercariae, respectively. Three other parasites were encountered, but only in 0.07-1.2% of the total sample of ants. In the case of the unidentified larval mermithid nematode and an unidentified larval cestode, only two hosts within the total sample were infected (Table 2.1). Single larvae of the parasitoid wasp, *Elasmosoma* sp. were found in 27 of the total sample of 2,766 ants, representing 0.2-1.2% of *F. aserva* samples and 0-1.2% of *F. podzolica* samples.

2.6.2 Factors affecting metacercariae intensity

Metacercariae intensity in the total sample of attached *F. aserva* ranged between 0 and 168 metacercariae/host (n = 490, 25.5 ± 25.4). The results of the GLMM analysis indicated that none of this extensive variation could be explained by month, site, or year. Overall, there was a significant positive regression between ant size and metacercariae intensity (Fig. 2.3a; Table 2.2).

Metacercariae intensity in the total sample of attached *F. podzolica* ranged between 0 and 106 metacercariae/host (n = 142; mean = 29.4 ± 23.3). The results of the GLM analysis indicated that none of this variation could be explained by month or year. Overall, there was no significant relationship between *F. podzolica* size and metacercariae intensity (Fig. 2.3b; Table 2.2).

2.6.3 Patterns of metacercariae recruitment

95% of the 17,909 metacercariae assessed in this study were enveloped by thick cyst walls. The remaining 5% of metacercariae lacked the characteristic cyst walls and were considered new recruits. Counts of thin-walled metacercariae were highly variable and inconsistent between months and years for both species (Table 2.3). With a single exception (on *F. aserva* in June 2022), observations of new recruits were restricted to ant samples collected in mid to late summer. For samples of *F. aserva* collected in 2021, only 2 of 223 infected ants contained infrapopulations with non-encysted metacercariae (Table 2.3). In contrast, there was a significant increase in metacercaria recruitment between May and July samples in 2022 (p = 0.007). There was a significant increase in metacercariae recruitment in samples of *F. podzolica* collected in 2021 (p = 0.020), with a single peak in July samples (Table 2.3). The observed peak in July 2022 was not significant (p = 0.720**).**

Mean immature metacercariae intensity in ants that contained them was 32.6 ± 32.8 (Table 2.3). Of 26 ants that contained non-encysted metacercariae, 23 (89%) contained only immature metacercariae and were collected from nests and not attached to plants. The remaining

11% of ants that contained non-encysted metacercariae were collected while they were attached to plants. In these latter cases, infected ants contained a mixture of mature and immature metacercariae. In ants with mixed infections, an average of 33±43% of the total numbers of metacercariae in an ant were non-encysted.

2.7 DISCUSSION

Our results show that the life cycle of *D. dendriticum* has become established in this region of emergence within two species of Formicid ants, *F. aserva* and *F. podzolica*. Of the >600 ants collected during their attachment to plants, each was infected with *D. dendriticum* metacercariae. We rarely observed instances of attachment to plants by ants that contained no metacercariae in their hemocoel. Following the results of our previous surveys of infected *F. aserva* at these and other sites in this region (van Paridon et al., 2017b; Criscione et al., 2020), these observations are not surprising. However, the larger spatial and temporal scale of collections in the present study, the addition of a second species of ant intermediate host (*F. podzolica*), and the higher sample sizes confirm that parasite manipulation of host behaviour is a fundamental component of eco-epidemiological patterns of infection for this introduced parasite of veterinary and wildlife importance.

The mean numbers of *D. dendriticum* metacercariae counted within the haemocoel of ants (n = 677; mean ± standard deviation = 27±25) is comparable with the results of an earlier study completed on samples of *F. aserva* collected from other sites in Cypress Hills Park (van Paridon et al., 2017b). Similar mean intensities were reported in a comparable field survey involving four species of *D. dendriticum*-infected Formicid ants collected over two years from sheep pastures in northwest Spain (Manga-Gonzalez et al., 2001; mean intensities across different samples ± SE = 26±16; 28±9; 42±20; 97±21). Although the numbers of metacercariae in individual *Formica* spp. can reach into the hundreds in both emerging and homeland contexts (review in Manga-Gonzalez et al., 2001), mean intensities are not significantly different at approximately 25-30 per host.

One striking result of our field surveys is the lack of significant differences in mean *D. dendriticum* metacercariae intensity in samples of infected ants over time. A similar lack of pattern was reported for the study involving four species of Formicid collected from pastures in Spain (Manga-Gonzalez et al., 2001). These results contrast those of numerous host surveys involving trematode metacercariae in which significant differences in mean counts are frequently reported between samples. Indeed, high seasonal, spatial, and annual variation in prevalence and abundance is a hallmark of cercariae transmission into second intermediate hosts (Goater et al., 2014). This common pattern often arises from high variation in the rates that cercariae 'trickle' into second intermediate hosts and accumulate over a host's lifetime. High variation in rates of cercariae transmission in second intermediate hosts is determined by factors such as snail life history, snail density, and snail resource availability. Variation in this suite of factors can be expected for the three species of *Oreohelix* spp. land snails that are known intermediate hosts of *D. dendriticum* in this region (Dempsey et al., 2019), so other factors must determine these highly atypical patterns of metacercariae intensity.

An absence of cercariae accumulation over time in ants can explain the lack of significant differences of mean metacercariae intensity observed in this study. This absence, or at least reduction, can in turn be explained by the transmission of cercariae within clumps into individual ants. In a laboratory study, Krull & Mapes (1952) showed that infected snails packaged 100-200 cercariae into individual slimeballs. Furthermore, the environmental conditions (e.g., temperature, humidity) required for snails to package and deposit slimeballs were highly restricted. The authors concluded that exposure of ants occurred during infrequent foraging bouts on slimeballs, during which ants had the opportunity to simultaneously ingest multiple cercariae. Unfortunately, we have not observed slimeballs at our study sites, and our efforts to stimulate known infected *Oreohelix* spp. to releases slimeballs in the lab have been unsuccessful. In the absence of data on cercariae counts in individual slimeballs, the density of slimeballs on substrate, and data on the rates at which ants encounter slimeballs, we cannot know the details of cercariae transmission from snails to ants. However, infrequent exposure to clumps of *D. dendriticum* cercariae can explain the lack of significant differences of mean metacercariae intensity between samples of ants observed in this study.

The recruitment of *D. dendriticum* cercariae into ants appears to only occur during a narrow temporal window. Although the numbers of infected ants that contained immature metacercariae was low within the total sample, instances in which this occurred in 2021 in samples of *F. aserva* and *F. podzolica* were restricted to a single sample collected in July. A single July peak occurred in *F. podzolica* samples the following year. These results indicate that the deposition of slimeballs onto pasture, and thus opportunities for cercariae transmission into ants, typically only occurs during restricted periods in summer. This is despite the observation that *Oreohelix* spp. with apparently mature, mobile cercariae are observed at these same sites throughout summer (Dempsey et al., 2019). The implication of our survey results, together with the results of laboratory studies (Krull & Mapes, 1952), is that whereas infected snails may contain life-long infections, they only release slimeballs under specific environmental conditions. Like many land snails, Oreohelids frequently enter periods of metabolic quiescence (aestivation) in summer to reduce the risk of desiccation (Rees and Hand, 1993). We have observed this phenomenon in each of the three sympatric *Oreohelix* spp. located at our study sites in Cypress Hills Park (Dempsey et al., 2019). Yet we have also observed high densities of motile Oreohelids at our study sites during periodic rains in mid-summer, especially when the rains are followed by periods of high relative humidity. Perhaps it is environmental conditions such as these the trigger infected snails to package cercariae into slimeballs and then release them onto substrate (Krull & Mapes, 1952). These results emphasize the idea that ants likely encounter slimeballs, and the cercariae within, infrequently over their lifetimes.

The eco-epidemiological patterns that we report here can also be used to inform aspects of the iconic behavioural manipulation in ants. As expected, only *D. dendriticum*-infected ants were found attached to plants. Even in rare cases in which metacercariae were not recovered in the gaster, these ants contained a metacercariae in the brain. Thus, our results confirm the assumption (Manga-Gonzalez et al., 2001) that at least one metacercariae is required to orchestrate the complex attach/detach/repeat sequence of altered behaviours in ants. A further striking observation is that in those few ants that contained a single cohort of immature metacercariae, none were found attached to plants. The implication is that the onset of ant manipulation requires a period of development for the gaster metacercariae to reach infectivity, for the brainworm to complete development within the suboesophageal ganglion, or both. Other manipulative parasites require an obligate period of development prior to the onset of host manipulation (Moore, 2002; Hughes et al., 2012). Taken together, our results support the idea that eco-epidemiological surveys of manipulated hosts can lead to a more complete understanding of complex host manipulations.

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2.11 COMPETING INTERESTS

The authors declare none.

2.12 AUTHORSHIP AND CONTRIBUTORSHIP

LE contributed to field collections, dissections, analyses, and writing. CG conceived the project and contributed supervisorship, project management, writing, and editing.

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2.14 FIGURES AND TABLES

Figure 2.1. A map of study sites in Cypress Hills Interprovincial Park, Alberta, Canada. Monthly samples of the ant *Formica aserva* were collected from 'Staff Camp' (red circle; 49º39'27"N 110º16'50"W) and 'Ski Hill' (square; 49º39'24"N 110º15'40"W); samples of *F. podzolica* were from 'Trans Canada Trail' (triangle; 49º39'41"N 110º07'03"W). Figure created in Google Earth.

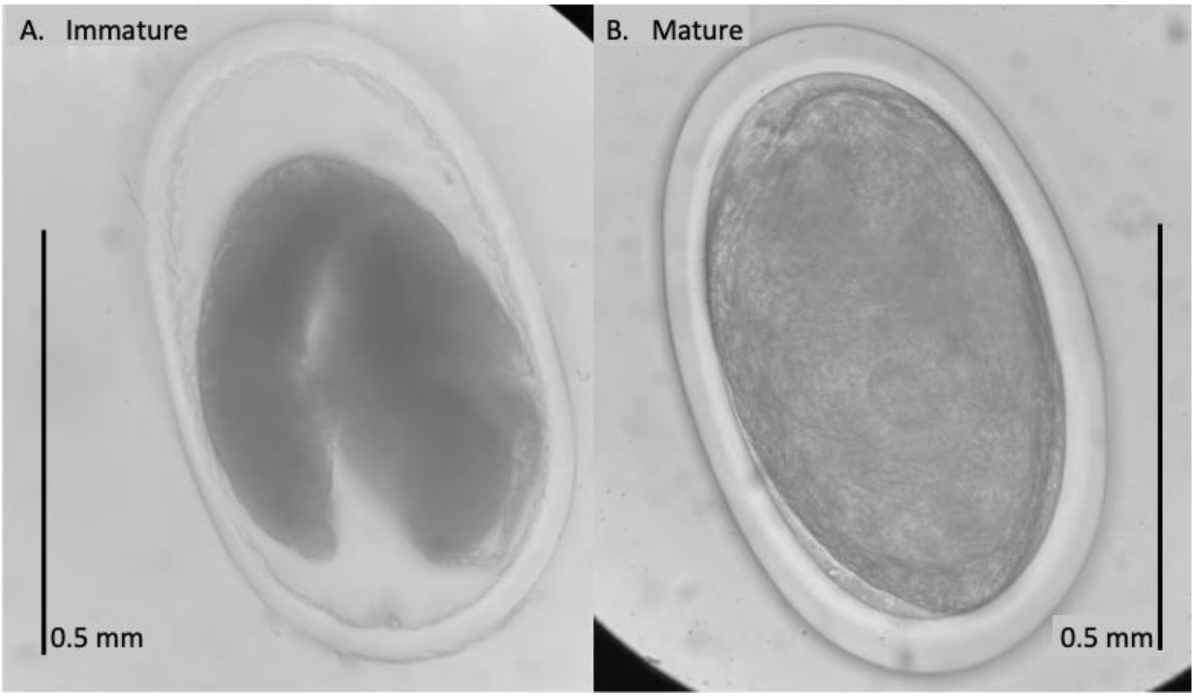


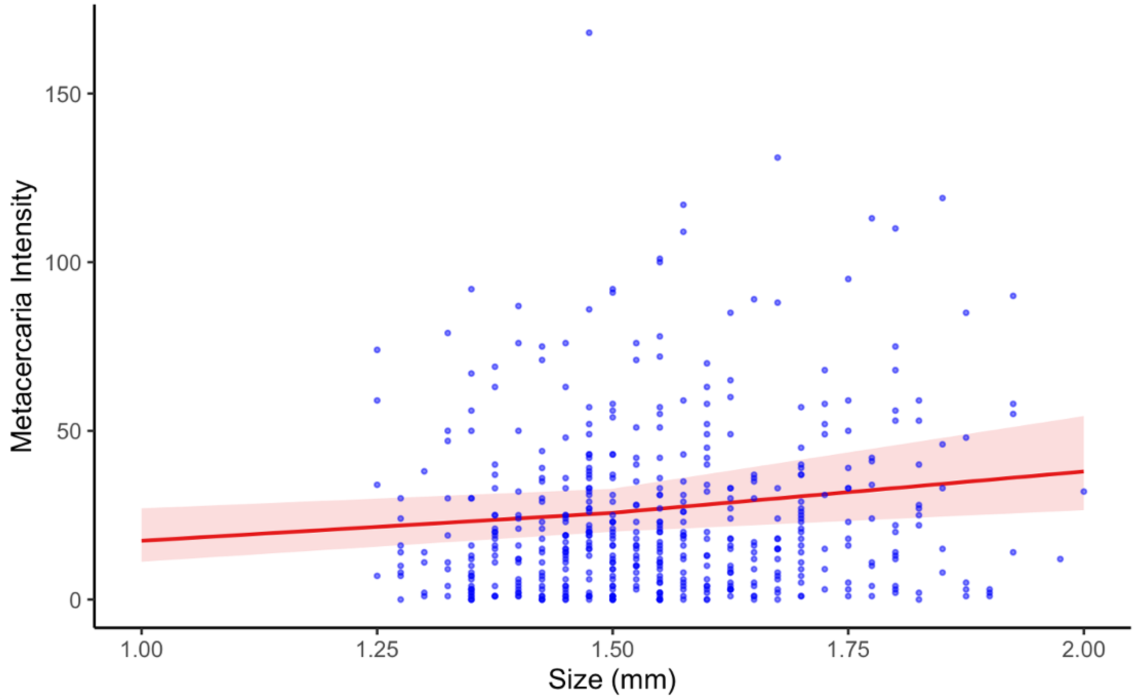
Figure 2.2. Contrasting phases of development (immature in A; mature in B) of *Dicrocoelium dendriticum* metacercariae dissected from the gaster of the ant, *Formica aserva* using a light microscope at 10X magnification.

Table 2.1. Summary host survey data for macroparasites recovered from samples of ants (n = 2766), *Formica aserva* and *F. podzolica*, collected from Cypress Hills Interprovincial Park, Alberta. x̄ represents the mean number of parasites per infected individual.

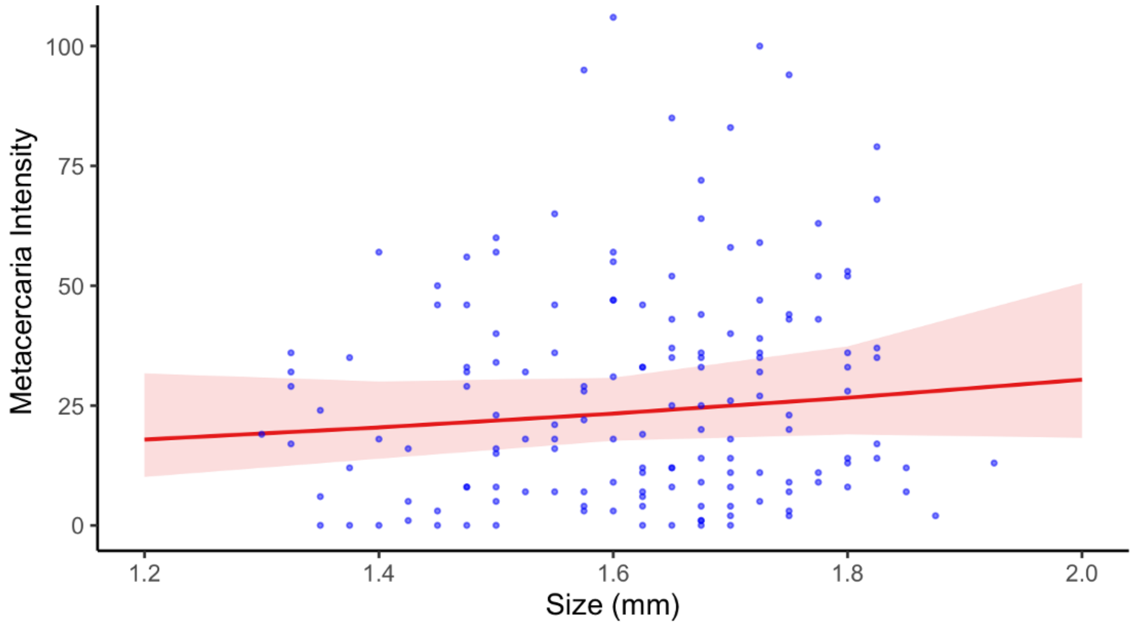
Species	Status	Larval *Dicrocoelium dendriticum*	*Elasmosoma sp.*	Mermithid nematode	Dilepidid cestode larvae
Formica aserva	Attached (n = 490)				
	n	477	1	0	1
	prevalence	97.3%	0.2%	0	0.2%
	x̄ ± s.d.	25.5 ± 25.4	1±0	0	4±0
	range	0-168	0-1	1	0-4
	Unattached (n = 1529)				
	n	67	19	3	1
	prevalence	4.4%	1.2%	0.2%	0.07%
	x̄ ± s.d.	26.7 ± 30.3	1±0	1±0	1±0
	range	0-161	0-1	0-1	0-1
Formica podzolica	Attached (n = 142)				
	n	133	0	0	0
	prevalence	93.7%	0	0	0
	x̄ ± s.d.	29.4 ± 23.3	0	0	0
	range	0-106	0	0	0
	Unattached (n = 605)				
	n	13	7	0	0
	prevalence	2.1%	1.2%	0	0
	x̄ ± s.d.	18.2 ± 16.9	1±0	0	0
	range	0-59	0-1	0	0

Table 2.2. Results of a generalized linear mixed model (*Formica aserva*) and of a generalized linear model (*Formica podzolica*) investigating the effects of month, year, and host size on the mean numbers of *Dicrocoelium dendriticum* metacercariae in samples of two species of ants collected from Cypress Hills Interprovincial Park, Alberta.

Species	**Fixed Effects**				
Formica aserva	Predictor	df	Estimate	x^2	p-value
	Month	3	-0.143	2.44	0.486
	Year	1	0.114	0.16	0.691
	Size	1	0.780	5.78	0.016
	Random Effects				
	Effect	N	Observations	Variance	
	Site	2	481	0.76	
	Marginal $r^2 = 0.026$			Conditional $r^2 = 0.027$	
Formica podzolica	Fixed Effects				
	Predictor	df	Estimate	x^2	p-value
	Month	3	-0.578	3.83	0.281
	Year	1	0.196	0.53	0.468
	Size	1	0.662	1.12	0.289
	Nagelkerke's $r^2 = 0.052$				



A. *Formica aserva*



B. *Formica podzolica*

Figure 2.3. Correlation between host size (hind tibia length) and metacercariae intensity in *Formica aserva* (A) and *Formica podzolica* (B). The red line and confidence intervals represent the predicted response from a GLM assessing the differences in metacercariae intensity in relation to site, year, month, and size. The blue dots represent counts of *D. dendriticum* metacercariae in individual ants. n = 464 (A) and n = 133 (B).

Table 2.3. Seasonal changes in the percentage of immature *Dicrocoelium dendriticum* metacercariae in two species of ants, *Formica aserva* and *F. podzolica*, collected from three sites in Cypress Hills Interprovincial Park, Alberta.

	Percent ants/sample with immature metacercariae*		Mean (±s.d.) percentage immature metacercariae/host	
	2021	2022	2021	2022
Formica aserva				
May	0 (7)	0 (55)	0	0
June	0 (48)	2.3 (87)	0	100 (±0)
July	2.1 (96)	7.1 (141)	32.5 (±30.5)	100 (±0)
August	0 (72)	17.6 (17)	0	95.8 (±4.2)
Formica podzolica				
May	0 (0)	0 (29)	0	0
June	0 (29)	0 (40)	0	0
July	24.2 (33)	11.1 (9)	100 (±0)	100 (±0)
August	0 (6)	0 (0)	0	0

*Numbers in brackets indicate sample size

Chapter 3: Environmental conditions trigger a reversible host manipulation in fluke-infected zombie ants

3.1 ABSTRACT

Many parasites orchestrate changes in the colour, morphology, and especially behaviour of their hosts. In an iconic example, Formicid ants infected with even a single larva (brainworm) of the fluke, *Dicrocoelium dendriticum*, attach their mandibles to a plant for hours or days, then detach, resume normal ant activities, then re-attach. This bizarre 'attach/detach/repeat' sequence of fluke-induced altered behaviour likely facilitates transmission into obligate grazing mammals while also preventing desiccation of the ant hosts. In the quest to uncover the mechanisms leading to this reversible manipulation, I monitored the timing of attachment and detachment by two species of infected ants on plants at seven infected nests while simultaneously monitoring changes in air temperature, relative humidity, and light intensity. Four *Formica aserva* nests were monitored for 5 consecutive days during three intervals in summer 2024 and three *F. podzolica* nests were monitored for 5 consecutive days during two intervals in summer 2024, resulting in 25 observation days. I found that 60-80% of attaching ants attached and detached every day, often in a pattern that appeared to be correlated with maximum/minimum temperature ranges. Overall, my results indicate that variation in the timing of ant attachment on plants was best explained by variation in temperature, light intensity, relative humidity, and time of day. I also observed increased rates of ant attachment at the same time most days, regardless of temperature, indicating the potential for a complex association between infection and ant circadian rhythm. There was a highly significant negative association between the proportion of ants attached to plants and light intensity. These results indicate that key environmental factors trigger both the attachment and detachment phases of host manipulation. This work provides the

foundation for definitive manipulative lab and field experiments to pinpoint how factors such as light intensity and temperature interact to cue host manipulations.

3.2 INTRODUCTION

Parasitologists, ecologists, and behaviourists have been interested in host manipulation for decades, particularly behavioural manipulation and the mechanisms that are responsible for its expression (Nadler et al., 2023; Poulin & Maure, 2015; Thomas et al., 2005). Host manipulation is common amongst host-parasite systems (Lafferty & Shaw, 2013). Some well-known examples include *Toxoplasma*-infected rats attracted to their cat predators (Berdoy et al., 2000), crickets infected with hairworms attracted to water (Thomas et al., 2002), and *Ophiocordyceps*-infected ants ascending plants and clamping onto them with their mandibles (Anderson et al., 2009). In each of these examples, researchers have demonstrated that the manipulation increases the parasite's rate of transmission to its next host. However, even in iconic host manipulations such as these, underlying mechanisms are poorly understood (Hasik et al., 2022; Nadler et al., 2023).

I study the host manipulator, *Dicrocoelium dendriticum* in its ant intermediate hosts in a region of emergence in southern Alberta, Canada. Its life cycle, general biology, and pattern of emergence in Southern Alberta is described in Chapters 1 and 2 and in Criscione et al., (2020), Criscione et al. (2022), Li et al., (2025a), and Li et al., (2025b). Grazing mammals (definitive host), terrestrial snails (first intermediate host), and Formicid ants (second intermediate host) are obligate hosts in the life cycle of this fluke. In the ant stage, cercariae mature into metacercariae, one travelling to the suboesophageal ganglion (SOG), and the rest (sometimes hundreds) encysting in the haemocoel (Chapter 2; Manga-Gonzalez et al., 2001; Krull & Mapes, 1953). In this host manipulation, infected ants leave their nest and then ascend a plant onto which they clamp with their mandibles. In this manipulated state, the ant (and its larval flukes) awaits accidental ingestion by grazing mammals to complete the life cycle. What sets this host manipulation apart from others (e.g., the *Ophiocordyceps*-ant system), is that infected ants do not die on the plant to facilitate transmission into the next host. Instead, fluke-infected ants detach from the plant, return to normal activities within their nest, and then re-attach to a plant (Manga-Gonzalez et al., 2001; Spindler et al., 1986). The attach/detach cycle is considered a "reversible manipulation" – one of very few known in nature. This reversibility is one aspect that makes this system unique, together with its fully terrestrial life cycle (trematode life cycles usually have obligate free-swimming larval stages; Galaktionov & Dobrovolskij, 2003) and that this is an introduced, and emerging, system in Canada (Chapter 1; van Paridon et al., 2017).

The temperature hypothesis has frequently been invoked to explain the characteristic attachment/detachment behaviours of *Dicrocoelium*-infected ants as they attach and detach from plants (Spindler et al., 1986; Manga-Gonzalez et al., 2001; Botnevik et al., 2016; Gasque & Fredensborg, 2023). The key prediction to arise from this hypothesis is that cool temperatures provide the cue for ant attachment to plants, whereas high temperatures that could lead to fatal desiccation provide the cue for detachment. There is little empirical support for either prediction (Gasque & Fredensborg, 2023). Furthermore, early anecdotal observations have noted cases in which infected ants remain attached to plants during the heat of the day, and still other cases in which infected ants do not attach during the cool hours of the day (Spindler et al., 1986; Manga-Gonzalez et al., 2001). The results of two European studies have shown that the proportion of infected ants attached to plants was significantly negatively correlated with temperature

(Botnevik et al., 2016; Gasque & Fredensborg, 2023). Although these studies found a strong relationship between attachment and temperature, there was also evidence that other environmental factors, such as relative humidity, play a role. For example, Gasque and Fredensborg (2023) concluded that temperature was the sole factor responsible for the intitiation of attachment and detachment in infected ants, although their model indicated that relative humidity also had a significant relationship with the proportion of ants attached. Additional studies are required to further examine the relative roles of key environmental covariates and their interactions in this reversible manipulation. It is especially important to continue testing this hypothesis to uncover the environmental influence on this manipulation to inform future mechanistic studies.

In this chapter, I characterize daily patterns of attachment and detachment onto plants by two species of sympatric, fluke-infected ants in a region of parasite emergence in Cypress Hills, Alberta. By combining data on the movement of infected ants to and from plants with matched data on time of day, temperature, humidity, and light intensity, I evaluate associations between host manipulation and potential environmental triggers. I am especially interested in using this field-based monitoring approach to test the hypothesis that temperature alone (Spindler et al., 1986; Botnevik et al., 2016; Gasque & Fredensborg, 2023) is responsible for turning this reversible manipulation on and off.

3.3 MATERIALS AND METHODS

3.3.1 Study site

This study took place from 2023-2024 in Cypress Hills Interprovincial Park (CHIP), Alberta, Canada. Geographical coordinates, together with general biological and geological characteristics of CHIP and the study sites are described in Chapters 1 and 2.

A total of seven ant nests were selected for monitoring in this study. Four of these had large *F. aserva* colonies and three contained large *F. podzolica* colonies. The four *F. aserva* nests (SC1, SC2, SC3, and SC4) are located within the SC site described in Chapters 2 and 4. For ants at this site, background information was available on eco-epidemiological patterns of larval *D. dendriticum* infection at the level of individual ants (Chapter 2), at the level of the individual nest (Chapter 2), and at the level of first intermediate host snails (Dempsey et al., 2019). The nests were approximately 75 m apart from each other. The nests were selected due to ease of accessibility relative to the field base and because my preliminary observations indicated it contained high densities of infected (= attached) ants on adjacent vegetation. The habitat between the four nests was composed of domestic lawn and pavement. There is little information on polydomy (multiple nest entrances for one colony) in *F. aserva* colonies. At SC, the nests were 10-75 m apart with no nest entrances between them; therefore, I considered each of the *F. aserva* nests as separate and independent colonies.

The three *F. podzolica* nests (TC1, TC2, and TC3) were located within the TCT site described in Chapter 2. This site contained approximately 10 mounds, each with a distinctive entrance. Individual mounds were approximately 20 m apart. The general characteristics of TCT, together with general eco-epidemiological patterns of larval *D. dendriticum* infection at this site, are described in Chapter 2. Because *F. podzolica* exhibits low incidence (<3% of all colonies) of polydomy (DeHeer & Herbers, 2004), I treated each nest entrance as an independent colony.

3.3.2 Pilot study – summer 2023

Due to time and resource constraints, monitoring the behaviours of all infected ants surrounding each of the nests was not feasible. Furthermore, seasonal changes in the availability of attached ants on plants at these sites was unknown prior to this study. Thus, I did not know if infected nests that contained either *F. aserva* or *F. podzolica* that had individuals attached to plants in early summer, would continue to have individuals that attach in later summer. To develop a full sampling regime that was consistent between nests, ant species, days, and months, I monitored attachment/detachment behaviours of *F. aserva* at four of the selected nests in a pilot study in 2023. For this study, I selected six plants within a 2 m radius of each nest, each of which had three or greater attached ants at the beginning of each monitoring period (June 16th-20th, July 18th-22nd, and August 14th-18th). This protocol meant that I could monitor a total of 18-40 ants per nest per survey. Once the six plants were identified, I marked each plant with different-coloured nail polish (Sally Hansen INSTA-DRI®). Additionally, attached ants on marked plants were marked with the colour corresponding to the plant that they were attached to. As the summer progressed, some plants did not have three or more ants. In these cases, plants with one to two ants were included. On each observation day, I visited each marked plant at 6:00 a.m., 10:00 a.m., 2:00 p.m., 6:00 p.m., and 9:00 p.m.

At each observation time, the surface of each marked plant was scanned for the number of attached ants. An ant was considered attached if its mandibles were biting into plant tissue and did not release following a gentle touch stimulus. Finally, during the pilot study, air temperature at each nest entrance was measured with a thermometer. Results of the pilot study can be found in Figure S3.1.

3.3.3 Monitoring infected ants on plants – summer 2024

One key outcome of the pilot study was that the four selected infected nests continued to have attached ants throughout consecutive months. I therefore monitored the same four *F. aserva* nests in the follow-up trials in 2024, alongside three additional *F. podzolica* nests (seven nests total). Furthermore, I continued to select six plants surrounding each nest, each of which had at least three ants attached during their selection at the beginning of each observation period.

My experience with the pilot study led to two methodological modifications that were implemented during the main study in 2024. First, additional, and more precise monitoring of key environmental covariates was required to potentially explain the extensive variation in attachment and detachment that were observed at the four nests in 2023. Following methods described in Gasque and Fredensborg (2023), I used shaded data loggers (SensorPush HT.w) placed approximately 1-m from each ant nest entrance to log temperature and relative humidity. In addition, light intensity at each marked plant was recorded using a handheld light meter (Lutron LX 1108). At each observation time, the light intensity from each of the six plants was averaged to represent a single estimate.

Second, I found that accurate assessment of the timing of attachment and detachment from plants did not require that ants be marked. While I recognize that marking ants to distinguish the timing of attachment and detachment of known individuals would provide valuable information, this individual-level approach was beyond the scope of my study. As in 2023, I selected a similar 5-day observation window to monitor the timing of attachment and detachment of ants, but at seven nests. Observations of infected *F. podzolica* occurred on May 14th-17th and June 3rd-7th, whereas observation of infected *F. aserva* took place on June 21st-25th, July 15th-19th, and August 9th-13th. The times of observation at each nest on each observation day were the same as in 2023.

3.3.4 Statistical Analyses

I used the proportion of ants attached to plants at each nest as a response variable (Gasque and Fredensborg, 2023). 'Proportion of maximum ants attached' was calculated as the number of ants attached at one nest at each observation time divided by the maximum number of ants attached to plants at that nest over the five consecutive days. Data on air temperature, relative humidity, light intensity, and time of day were collected at the same time that ants were counted on plants. All environmental factors were scaled to a mean of zero and standard deviation of one. All analyses were completed in the statistical software R (R Core Team, 2024). Figures were made in R, using packages ggplot2 (Wickham, 2016) and sjPlot (Ludecke, 2024), and in Microsoft Excel (Microsoft Corporation, 2024).

I followed Gasque & Fredensborg (2023) and used generalized linear mixed models (GLMMs) that were fit to a binomial distribution using a logit-link function to evaluate the effects of temperature, humidity, and light intensity on the proportion of ants attached to the six plants surrounding a nest (lme4 package in R; Bates et al., 2015). Data on the proportion of ants attached were analyzed in separate models for the two species. Including species as a fixed effect in a global model was inappropriate because attached *F. podzolica* were only present on plants in May and June (Chapter 4) and because sample sizes for *F. podzolica* were small and heterogenous.

In preliminary models, temperature, relative humidity, light intensity, and time of day were included as fixed effects, with nest and date as random effects. However, following the results of Pearson's correlation analyses (Fig. 3.1), temperature was found to be highly significantly positively correlated with both light intensity and relative humidity. Due to this high collinearity, temperature was removed from subsequent models. The removal of temperature as a predictor resulted in a lower AIC value, residuals that were normally distributed, and no multicollinearity within the final model (DHARMa package in R; Hartig, 2024). Although temperature was removed from the model, I acknowledge that any results in relation to light intensity and relative humidity are also associated with temperature due to high correlation. All interactions were nonsignificant and were removed from the final models. Next, a type III ANOVA was completed to assess the significance of the effect of the predictors (car package in R; Fox & Weisberg, 2019). Following this procedure, a pairwise comparison post-hoc test was done, using Tukey's adjustment, to assess the difference between each of the time categories (emmeans package in R; Lenth, 2024). This sequence of steps was repeated in the final models for proportion attachment data for both species.

The results of the GLMM for *F. aserva* showed that ants tended to re-attach at 2:00 p.m., usually at the hottest temperature of the day. To uncover potential interactive effects between temperature and light intensity during this consistent 2:00 p.m. re-attachment phase, a two-way ANOVA was done on the effects of light intensity, temperature, and their interaction on the proportion of ants attached. For the ANOVA, light intensity and temperature were converted to categories of high and low, representing equal to or greater than the mean and less than the mean temperature, respectively. Then, each sample of ants at 2:00 p.m. was placed in the appropriate light intensity and temperature categories. Pairwise comparisons for these effects on proportion of ants attached were done using a Tukey's post-hoc test.

Binomial fits were chosen for the GLMMs following Gasque & Fredensborg (2023) and due to heterogeneity in sample sizes between nests and sampling periods. Traditionally, when analyzing insect counts, the GLMM would be fit with a Poisson or negative binomial distribution (Candy, 2000). Following these more traditional modelling methods used for count data, I developed two additional models, and the results are included in the supplementary material (Table S3.1; Table S3.2). Two GLMMs, one for *F. aserva* counts (Table S3.1) and one for *F. podzolica* counts (Table S3.2), were fit to a negative binomial and Poisson distribution, respectively (glmmTMB package in R; Brooks et al., 2017). For these analyses, I used the number of ants attached as the response variable with relative humidity, light intensity, time, and an offset term of maximum observed number of ants attached as fixed effects. Nest and date were included as random effects. An offset term was used to account for large differences in the maximum number of ants observed between nests.

3.4 RESULTS

3.4.1 Environmental factors associated with attachment

<u>*Formica aserva*</u>

Time of day was a significant predictor of the proportion of ants attached (Table 3.1). A pairwise comparison with Tukey adjustments showed that 10:00 am had a significantly lower proportion of ants attached than 2:00 p.m. ($p = 0.023$) and 6:00 p.m. ($p = 0.0054$). No other time comparisons were significant ($p > 0.05$). Relative humidity (Fig. 3.2) and light intensity (Fig. 3.3) were also significantly associated with the proportion of ants attached (Table 3.1). As relative humidity increased, an increased proportion of ants were attached to plants (Fig. 3.2). In contrast, as light intensity increased, ants were less likely to be attached (Fig. 3.3). Light intensity explained most of the variation in the model (Table 3.1). The fixed effects in the model explained 44% of the variance of the response variable (Table 3.1) and 89% of the variance in the response variable was explained when including both fixed and random effects (Table 3.1).

Because 2:00 p.m. tended to coincide with peak rates of return of ants to their plants, and light intensity was the strongest predictor in the GLMM, the interactive effects of light intensity and temperature on the proportion of attached ants was assessed at this time interval. Temperature (ANOVA; $F = 5.05$, $df = 1, 56$, $p = 0.029$) and light intensity (ANOVA; $F = 25.42$, $df = 1, 56$, $p = 5.16e-06$) were significantly negatively associated with the proportion of ants attached at 2:00 p.m., but the interaction of temperature and light intensity was not significant (ANOVA; $F = 1.56$, $df = 1, 56$, $p = 0.22$; Fig. 3.4). A Tukey HSD test for multiple comparisons revealed a significant difference in the proportion of ants attached between low and high light intensity when the temperature was high (Fig. 3.4; $p = 0.000038$). There was also a significant difference between low light intensity at low temperature and high light intensity at high temperature (Fig. 3.4; $p = 0.000081$). All other interactions were non-significant (Fig. 3.4; $p > 0.05$).

Formica podzolica

Time of day was a significant predictor of the proportion of *F. podzolica* attached (Table 3.2). A pairwise comparison with Tukey adjustments showed that 2:00 p.m. had an almost significantly lower proportion of ants attached than 7:00 a.m. ($p = 0.068$) and 5:00 p.m. ($p = 0.076$). No other time comparisons showed significant differences in the proportion of ants attached ($p > 0.05$). Relative humidity and light intensity were not significantly associated with the proportion of ants attached (Table 3.2). The fixed effects in the model explained 27% of the variance of the response variable (Table 3.2) and 84% of the variance in the response variable was explained when including both fixed and random effects (Table 3.2).

3.4.2 Timing of attachment and detachment

Formica aserva

Infected ants come and go from their plants through the course of a single day (Fig. 3.5). The lowest percentage of *F. aserva* attached was on the fifth day in August at 10:00 a.m. when only 18% of attaching ants were attached (Fig. 3.5). In this case, there were no other days that had less than 20% of attaching ants attached (Fig. 3.5). This result indicates that in general, at least 20% of attaching *F. aserva* remained attached to their plant, regardless of environmental conditions. In stark contrast, the highest percentage of ants attached was on the first day in July at 6:00 a.m. when on average, 100% of attaching ants were attached (Fig. 3.5). Overall, the higher the maximum daily temperature, the greater the decrease in the proportion of attached ants (see second, third, and fifth day in July, and the fourth day in August; Fig. 3.5). The highest range of percentage of attaching ants attached in one day was 0.86-0.28 and 1-0.42 on July second and first day in July when the highest temperature was 31.6°C and 27.0°C respectively (Fig. 3.5). Additionally, the lower the temperature, the less the decrease in percentage of attaching ants attached (see the first and fifth day in June, and the first day in August in Fig. 3.5). The smallest range of proportion of attaching ants attached in one day was 0.60-0.45 on the fifth day in June when the highest temperature was 20.4°C, which was also the lowest 'high' temperature observed in this study (Fig. 3.5).

I saw a consistent increase in proportion of attaching ants attached at 6:00 p.m., except for the first day in June and the fourth day in July (Fig. 3.5). Additionally, there was a small increase at 2:00 p.m. on 8 out of 15 of the observation days (the second and fourth day in June, the second, third, fourth, and fifth day in July, and the first and fifth day in August; Fig. 3.5). Again, this increase occurred regardless of temperature, indicating a potential role for other environmental factors (Fig. 3.5).

Formica podzolica

Formica podzolica demonstrated similar patterns of attachment to plants as *F. aserva* throughout the day, but there was high variation in the timing of both attachment and detachment. The lowest percentage of attaching ants attached was at 2:00 p.m. on day four with a mean of 29%, which was also the day with the highest temperature (Fig. 3.6). There were only two other instances where the percentage of attachment was less than 40% (Day three at 10:00

a.m. and Day four at 10:00 a.m.; Fig. 3.6). The highest percentage of attaching ants attached was 97% on day one at 7:00 a.m., which was also the day with the lowest 'high' temperature of the day (Fig. 3.6). The largest variation in proportion of attaching ants attached occurred on day four with ants varying from 0.73 to 0.29, showing that 44% of attaching ants left between the highest point (7:00 a.m.) and the lowest point (2:00 p.m.; Fig. 3.6). The smallest variation in percent of attaching ants attached was on day two with the proportion varying from 0.43 to 0.59, showing that 16% of attaching ants returned between the lowest point (2:00 p.m.) and the highest point (5:00 p.m.; Fig. 3.6).

Overall, 2:00 p.m. represented the lowest percentage of attaching ants attached on most days, only excluding day three (Fig. 3.6). There was also a general trend of ants returning to plants at 5:00 p.m. on all the observation days (Fig. 3.6). These trends are present regardless of temperature ranges, indicating a potential role for other environmental factors (Fig. 3.6).

3.5 DISCUSSION

The phenomenon of *Dicrocoelium*-infected ants clinging to plants at some times of the day and then resuming normal activity during other parts of the day has been a topic of interest for decades (Spindler et al., 1986; Gasque & Fredensborg, 2023). My study was the first to characterize the timing of attach/detach behaviours in *D. dendriticum*-infected ants in an introduced region. Consistent with previous studies completed in Europe, (Spindler et al., 1986; Botnevik et al., 2016; Gasque & Fredensborg, 2023), I found that temperature is likely associated with the initiation of attachment and detachment behaviour. I showed that most attaching ants (60-80%) follow the traditionally described pattern in Europe, attaching in the evening and early morning while the temperature is low and then detaching mid-day as temperatures rise (Goater et al., 2014). Additionally, my results are the first to show that the attach/detach manipulation persists over consecutive days and over an entire season. To date, the hypothesis that has received the most support is that temperature is closely associated with the reversibility of this manipulation. Presumably, the risk of ant desiccation increases as temperature increases, ultimately leading to a rapid reduction in transmission of fluke larvae from ants to grazing mammal. Although two recent studies (Botnevik et al., 2016; Gasque & Fredensborg, 2023) have found support for this hypothesis in both the lab and the field, there is little information on the influence of environmental factors on attachment/detachment in the *Dicrocoelium*-ant system in other locations, especially where this parasite has been introduced. In a departure from previous studies, I found a potential role for light intensity, relative humidity, and time of day, in addition to temperature, in the pattern of attachment and detachment observed, all of which support the desiccation hypothesis.

Light intensity was the strongest indicator of attachment/detachment of infected ants on plants in the model, which aligns with the idea that in direct, high light intensity, an insect would be at highest risk of desiccation (Law et al., 2019). Light intensity is also a known regulator of ant activity (Narendra et al., 2010). In *Formica polyctena*, a species known to be infected with *D. dendriticum* in Europe, high light intensity is correlated with the absence of foragers (Narendra et al., 2010). When Narendra et al., (2010) manipulated the presence of light, ants did not forage during periods of light, and vice versa. They suggest that this is due to light intensity regulating the circadian rhythm of ants, therefore light intensity is the indicator that ants use to determine the onset of foraging (Narendra et al., 2010). This is important to the *Dicrocoelium*-ant system as a recent study involving ants from the same nests I have monitored found that circadian rhythm-related transcripts were either upregulated or downregulated depending on the stage of manipulation (i.e., attached vs unattached; Li et al., 2025b). Additionally, studies completed on the *Ophiocordyceps*-ant system, in which the manipulated phenotype is similar to the *Dicrocoelium*-ant system minus the reversibility, have found that the initiation of manipulation may be due to the parasite 'hijacking' the ant's circadian rhythm (de Bekker & Das, 2022). This take-over of host circadian rhythm is hypothesized to result in the manipulated phenotype being expressed at the same time everyday within different individual hosts (de Bekker & Das, 2022). This opens the possibility that the manipulation of host circadian rhythm by *D. dendriticum* may play a role in the timing of attachment and detachment on plants.

Another environmental factor that was associated with timing of attachment/detachment was relative humidity, aligning with the results of studies on desiccation risk in insects in the context of pathogens (high humidity = low risk, low humidity = high risk; Brown et al., 2023). In a study that examined the relationship between temperature and relative humidity in the context of mosquito-borne diseases, they found that high relative humidity increased lifespan and activity of mosquitos and concluded that at high temperatures, mosquitos were more of a risk of transmitting human disease if the relative humidity was also high (the transmission cycle of mosquito-borne illness is sensitive to changes in humidity; Brown et al., 2023). These findings are supported by the results of various studies showing that insects have longer lifespans and less mortality at temperature extremes when the humidity is high (Kobey & Montooth, 2013; Giri et al., 2024; Botsch et al., 2024). If I want to continue with the idea that the risk of host desiccation is behind the attachment/detachment behaviours that are seen in the ant host of *D. dendriticum*, I need to acknowledge that various key environmental factors are involved in creating conditions that risk desiccation.

In a study completed on German pastures, Spindler et al., (1986) observed ants leaving and returning to their plants throughout the day. Results from their study showed that approximately 10% of infected ants remained attached throughout the hottest temperatures of the day, noting further that in some cases, ants initiated attachment to plants at high temperatures (Spindler et al., 1986). Similarly, Manga-Gonzalez et al. (2001) observed variation in individual behaviours of infected ants on Spanish pastures, specifically in the length of time individuals remain attached at high temperatures. In general, my results are consistent with these earlier findings. Thus, while approximately 60-80% of attaching ants detached at hot daytime temperatures, a small subset of ants remained attached, regardless of how unfavourable the temperature was to their survival, and to the parasite's survival. In addition, I observed ants returning to or arriving to the plants during unfavourable conditions (high temperature, high light intensity, and low relative humidity). These results confirm that there are individual differences in the expression of manipulated behaviour, perhaps linked to an ants' current state of nutrition, or to other factors such as age, caste, and size.

Not all host species will respond to environmental factors equally, resulting in different patterns in environment-regulated manipulation. In this study, I showed differences in patterns of attachment between two ant species, one that is red/brown and nests in the stumps of fallen trees (*F. aserva*), and another that is black and nests in sandy soil (*F. podzolica*). *Formica podzolica* showed no environmental influence on attachment, while *F. aserva* showed significant influence of environmental factors. This may be explained by the differences in the time of year that data was available (May-June for *F. podzolica* and June-August for *F. aserva*) or by the lower number of replicates and sample sizes in *F. podzolica*. Another potential explanation for these differences could be that the two species are adapted to different conditions, therefore one may be less susceptible to extreme environmental conditions than the other. Law et al., (2019) found that the lighter an ant’s cuticle, the less tolerant it is to temperature, humidity, and light intensity extremes. They also showed that overall ant condition was associated with its cuticle colour, and as a result, its tolerance of environmental extremes (Law et al., 2019). Ants that live under a canopy are lighter and less tolerant of extreme conditions compared to ants that do not live under a canopy that are darker and more tolerant of extreme conditions (Law et al., 2019). This could explain why environmental factors had less of an influence on the manipulated behaviour of the darker ant, *F. podzolica*, when compared to the lighter ant, *F. aserva*. It would be beneficial to conduct further studies on the differences in environmental influence on the manipulated behaviour of infected ants to provide insight into host-species differences, and potentially into adaptive differences in the host’s response to the parasite.

In my study, due to high correlation with the predictor variables, temperature may have been the underlying driver of changes in manipulated behaviour, but instances of ants re-attaching at high temperatures or remaining attached throughout the day suggest that there may be other key environmental factors playing a role (such as light intensity or relative humidity). Lab studies are required to assess the independent effects of each environmental factor, along with their potential interactive effects. Previous lab studies (Botnevik et al., 2016: Unrau, 2019; Li et al., 2025a; Li et al., 2025b) have used individual ants, in the absence of a colony and queen. This is detrimental to behavioural studies due to stark behavioural differences of individual ants in the presence or absence of a queen and sister workers (Vienne et al., 1998). Therefore, future lab studies that assess the effects of environmental factors on manipulated behaviours must be done on colonies as opposed to individual workers.

3.6 CONCLUSION

Avoidance of desiccation by the ant and ultimately the parasite is likely behind this complex attach-detach sequence, but I reject the idea that temperature is solely responsible for this phenomenon. Instead, I suggest that this reversibility of host manipulation is due to a complex combination of extreme, desiccation related, environmental factors, such as high temperature, high light intensity, and low relative humidity, with a potential role for host circadian rhythm. Understanding the basics of what parasites are manipulating their hosts to do and under what conditions will provide insight into the precise mechanisms related to this and other manipulations.

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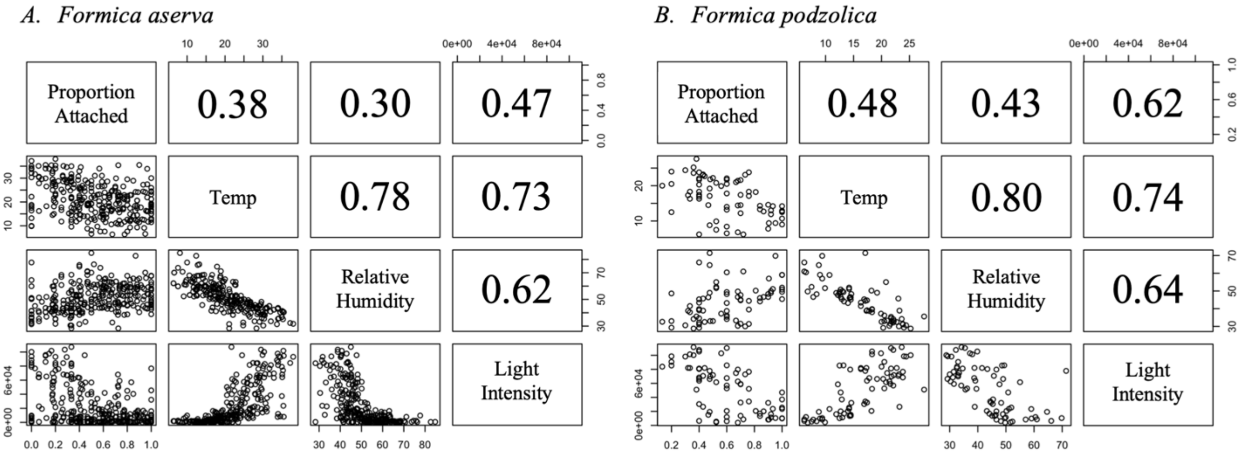
3.8 AUTHORSHIP AND CONTRIBUTORSHIP

LE contributed to field observations, data analysis, and writing. CG conceived the project and contributed supervisorship, project management, writing, and editing

3.9 FIGURES AND TABLES


A. *Formica aserva*

B. *Formica podzolica*

Figure 3.1. Pearson's correlation matrix showing associations between the proportion of infected A) *Formica aserva* and B) *F. podzolica* attached to plants and temperature, relative humidity, and light intensity.

Table 3.1. Summary GLMM results for the effects of time of day, relative humidity, and light intensity on the proportion of *Dicrocoelium dendriticum*-infected *Formica aserva* attached to plants in Cypress Hills, Alberta.

Fixed Effects				
Predictor	df	Estimate	x^2	p-value
Time of Day	4	0.18	17.93	0.0013
Relative Humidity	1	0.18	13.44	0.00025
Light Intensity	1	-0.65	169.94	< 2.2e-16
Random Effects				
Effect	N	Observations	Variance	
Nest	4	296	0.42	
Date	15	296	0.21	
Marginal $r^2 = 0.44$			Conditional $r^2 = 0.89$	

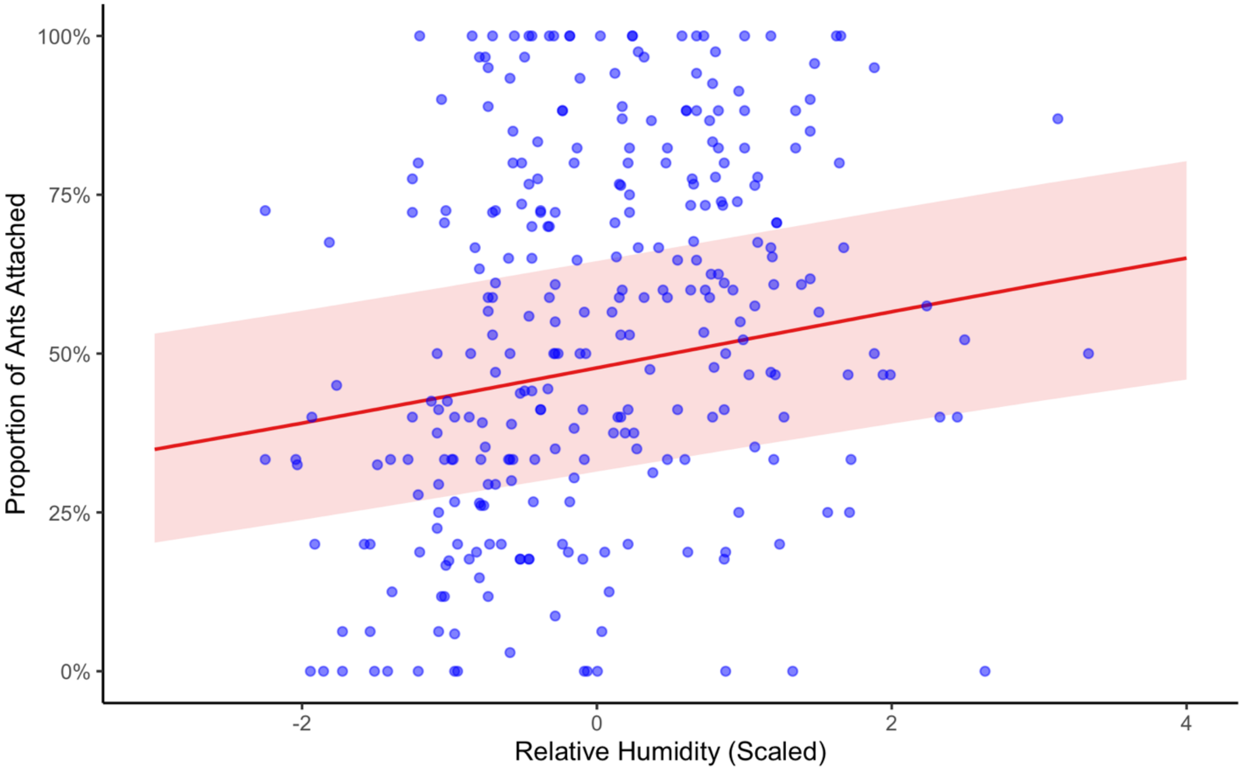


Figure 3.2. The relationship between scaled relative humidity and the proportion of *F. aserva* attached to plants. The red line and effect size is the predicted response from a GLMM assessing the effect of time of day, relative humidity, and light intensity on the proportion of *Dicrocoelium dendriticum*-infected *Formica aserva* attached to plants in Cypress Hills, Alberta. Blue points represent raw data collected from June-August 2024 (n = 296).

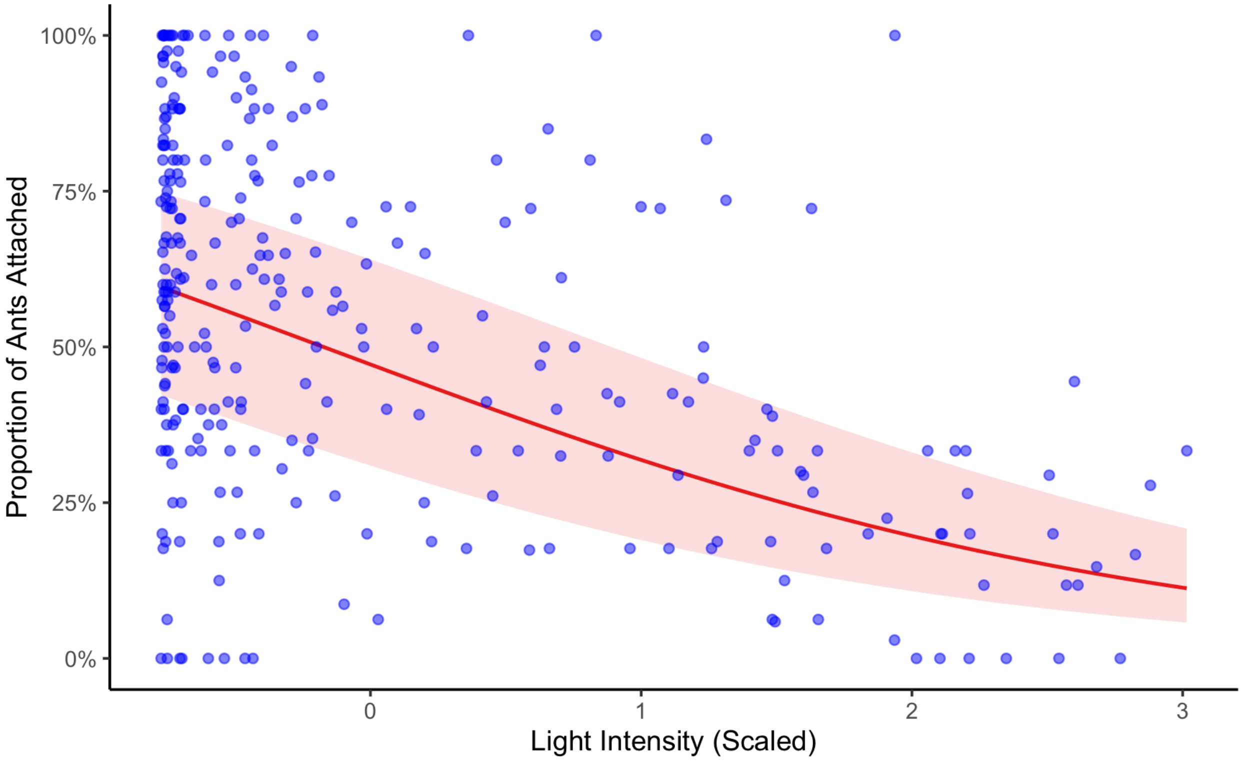


Figure 3.3. The relationship between scaled light intensity and the proportion of *Formica aserva* attached to plants. The red line and effect size represent the predicted response extracted from a GLMM assessing the effect of time of day, relative humidity, and light intensity on attachment of *Dicrocoelium dendriticum*-infected *Formica aserva* in Cypress Hills, Alberta. Blue points represent raw data collected from June-August 2024 (n = 296).

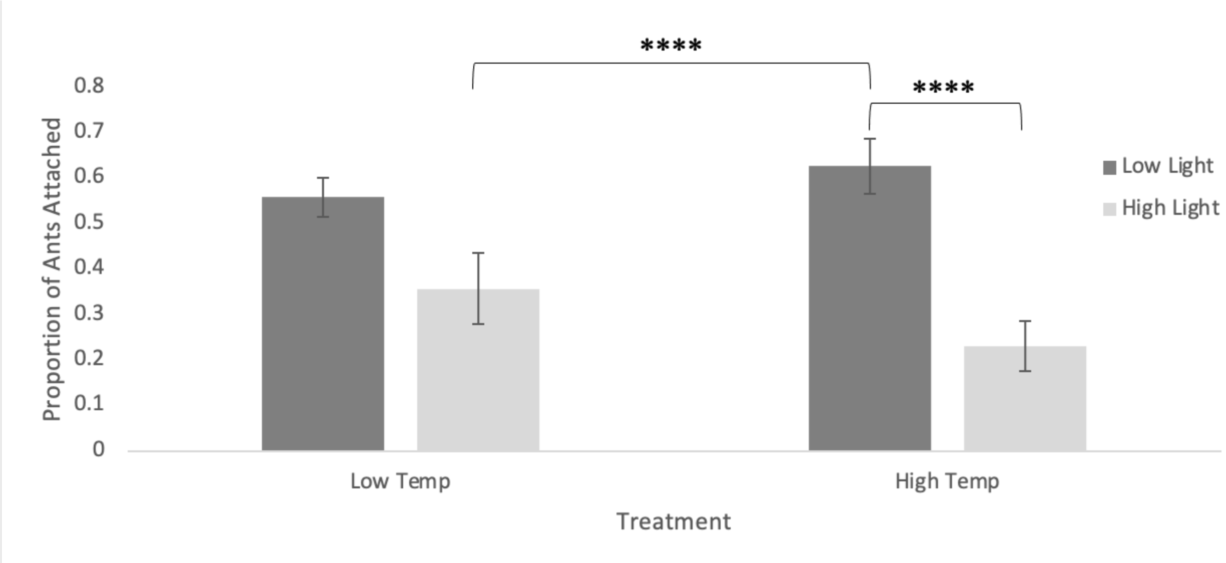


Figure 3.4. Interaction between temperature and light intensity on the proportion of *Dicrocoelium dendriticum* infected *Formica aserva* attached to plants at 2:00 p.m. in Cypress Hills, Alberta. The stars represent significant differences between treatments. Values are means +/- s.e.

Table 3.2. Summary GLMM results for the effects of time of day, relative humidity, and light intensity on the proportion of *Dicrocoelium dendriticum*-infected *Formica podzolica* attached to plants in Cypress Hills, Alberta.

Fixed Effects				
Predictor	df	Estimate	x^2	p-value
Time of Day	3	0.095	8.21	0.042
Relative Humidity	1	0.13	2.50	0.11
Light Intensity	1	-0.20	2.48	0.12
Random Effects				
Effect	N	Observations	Variance	
Nest	3	71	0.09	
Date	9	71	0.59	
Marginal $r^2 = 0.27$			Conditional $r^2 = 0.84$	

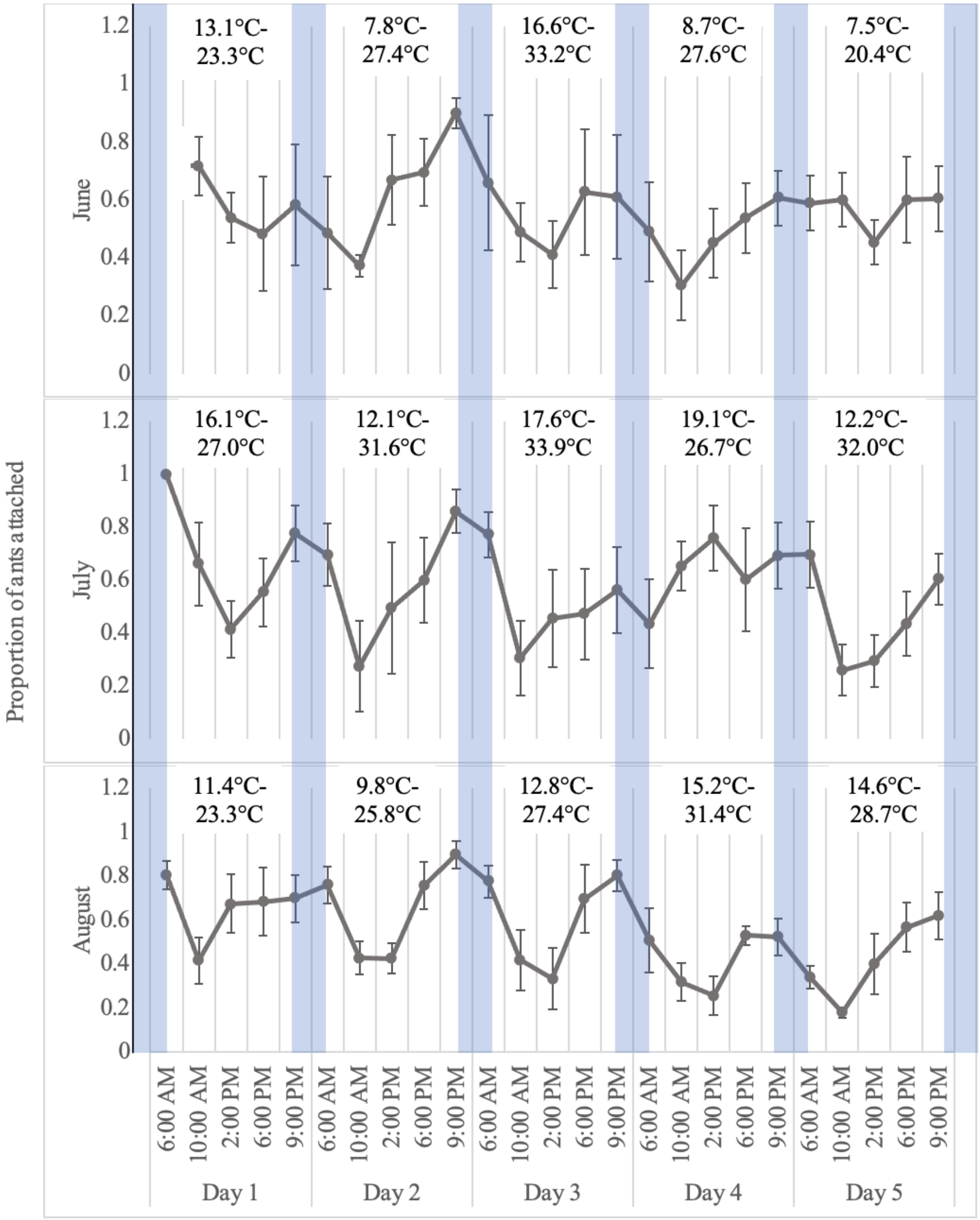


Figure 3.5. Diurnal changes in mean (±s.e.) proportion of *Dicrocoelium dendriticum*-infected *Formica aserva* attached to plants in June, July, and August 2024. Data were collected in Cypress Hills, Alberta. The maximum and minimum temperatures recorded during each day are indicated at the top of each daily time series.

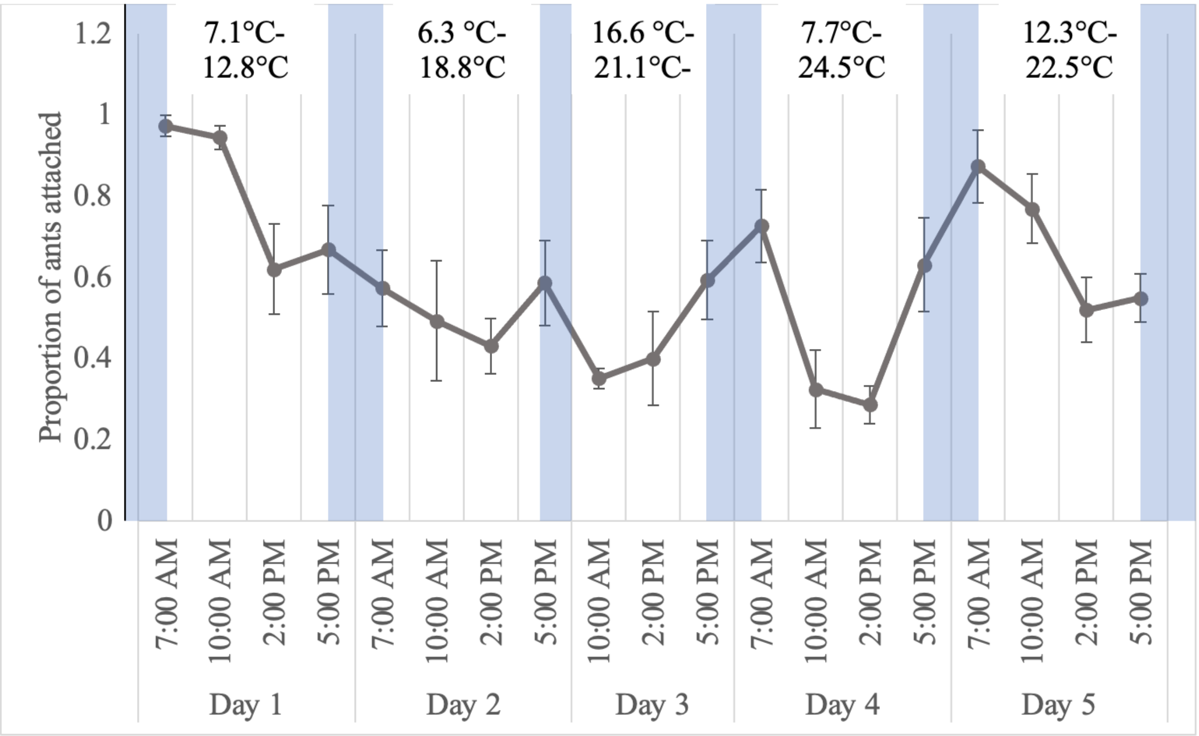


Figure 3.6. Diurnal changes in mean (±s.e.) proportion of *Dicrocoelium dendriticum*-infected *Formica podzolica* attached to plants in June 2024. Data were collected in Cypress Hills, Alberta. The maximum and minimum temperatures recorded during each day are indicated at the top of each daily time series.

3.10 SUPPLEMENTARY MATERIAL

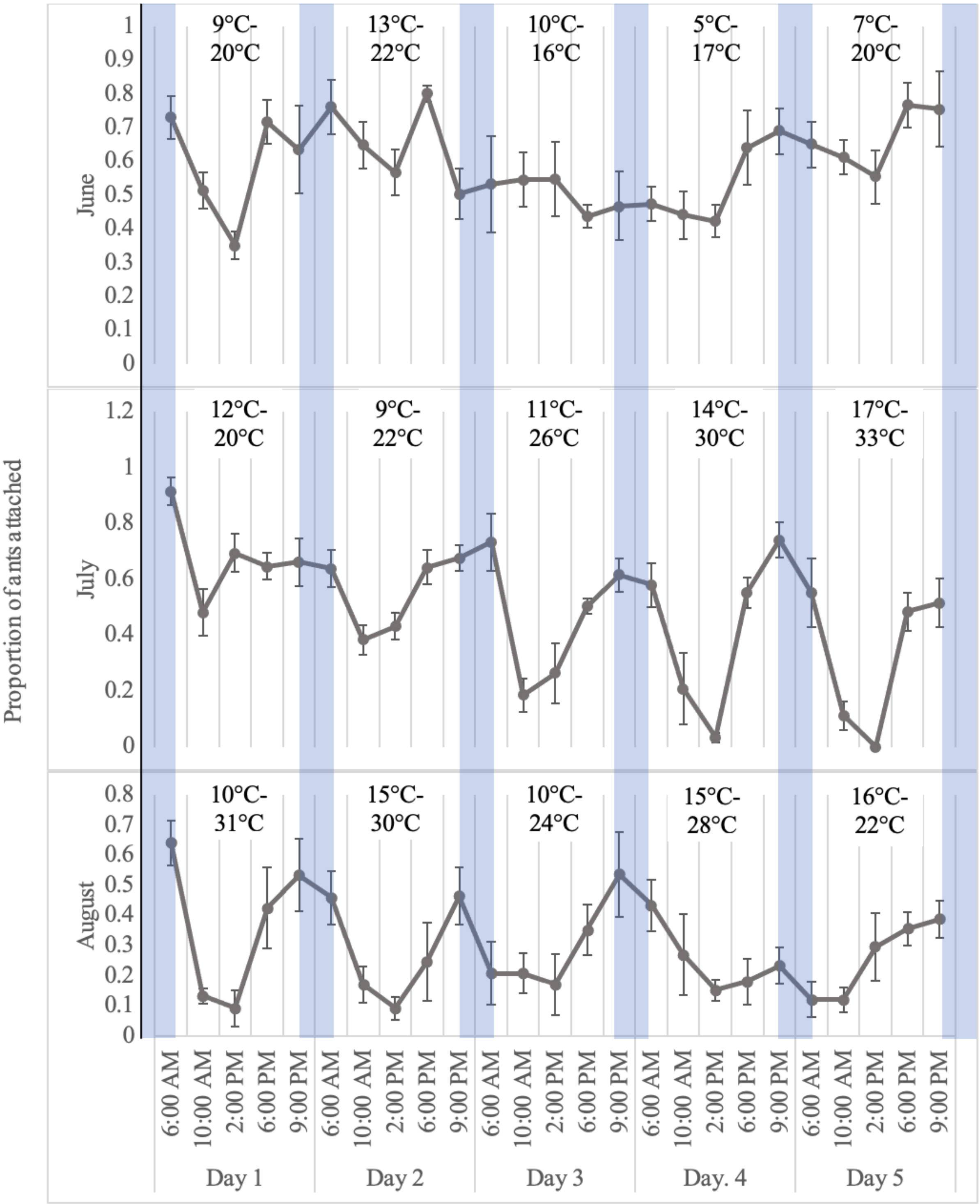


Figure S3.1. Diurnal changes in mean (±s.e) proportion of *Dicrocoelium dendriticum*-infected *Formica aserva* attached to plants in June, July, and August 2023. Data were collected in Cypress Hills, Alberta. The maximum and minimum temperatures recorded during each day are indicated at the top of each daily time series.

Table S3.1. Summary GLMM results for the effects of time of day, relative humidity, and light intensity on the number of *Dicrocoelium dendriticum*-infected *Formica aserva* attached to plants in Cypress Hills, Alberta.

Fixed Effects				
Predictor	df	Estimate	x^2	p-value
Time of Day	4	0.032	6.91	0.14
Relative Humidity	1	0.068	4.85	0.028
Light Intensity	1	-0.33	90.30	< 2.2e-16
Random Effects				
Effect	N	Observations	Variance	
Nest	4	296	0.08	
Date	15	296	0.03	
Marginal $r^2 = 0.10$			Conditional $r^2 = 0.18$	

Table S3.2. Summary GLMM results for the effects of time of day, relative humidity, and light intensity on the number of *Dicrocoelium dendriticum*-infected *Formica podzolica* attached to plants in Cypress Hills, Alberta.

Fixed Effects				
Predictor	df	Estimate	x^2	p-value
Time of Day	3	0.014	1.56	0.67
Relative Humidity	1	0.044	0.43	0.51
Light Intensity	1	-0.15	4.09	0.043
Random Effects				
Effect	N	Observations	Variance	
Nest	3	71	0.00	
Date	9	71	0.02	
Marginal $r^2 = 0.044$			Conditional $r^2 = 0.068$	

Chapter 4: Fluke-infected zombie ants aggregate on plants adjacent to their nests

4.1 ABSTRACT

Behavioural manipulation of hosts by parasites is a common strategy for a parasite to increase its transmission rates. Many parasites change the colour, morphology, and behaviour of their hosts, often as a strategy to facilitate their own transmission. In a few rare cases when the manipulated behaviours of infected hosts can be monitored under natural conditions, infected hosts have been observed to aggregate into clumps. Examples include ants infected with fungal parasites and wasps infected with parasitoid flies. Our understanding of the frequency and consequences of host aggregation for manipulative parasites, and its potential adaptive significance, is poor. Formicid ants infected with larvae of the fluke, *Dicrocoelium dendriticum* are manipulated to leave their nest, climb a plant, and then attach to it with their mandibles. I have anecdotally observed instances of extreme aggregation of infected ants on plants, in which hundreds of infected ants can be observed on a single plant, but few or none on adjacent plants. I evaluated patterns of aggregation on plants adjacent to fluke-infected nests of two species of Formicid ant, *Formica aserva* and *F. podzolica*. My results showed that infected *F. aserva* were significantly and consistently aggregated on available plants near their nests over the course of a summer, whereas infected *F. podzolica* tended to be distributed at random among available plants. The magnitude of aggregation of infected *F. aserva* varied with month, nest, and the nature of the plant community. Infected ants did not attach or aggregate onto the most common or dominant plant adjacent to their nests, but instead appeared to actively select a particular species. These results provide an empirical foundation to help understand the consequences of aggregation to parasite transmission, parasite reproduction, and host manipulation.

4.2 INTRODUCTION

Animal aggregation has been observed extensively in the natural world, ranging from flatworms to mammals (Allee, 1931). Aggregation can be defined as the clustering of individuals in space (Fig. 4.1). Often, these aggregations are associated with characteristic social behaviours such as grooming, but animal assemblages also have a multitude of functions in non-social species, ranging from resisting extreme environmental conditions to protection from predators (Allee, 1931). Although living in an aggregation can have many benefits for individuals, there are also costs such as increased risk of infection with parasites/pathogens (Cote & Poulin, 1995; Patterson & Ruckstuhl, 2013) and resource competition (Krause & Ruxton, 2002). On the other hand, some instances of aggregation may not be associated with a function at all; instead, clustering may be simply a pattern of animals gathering in stable groups (Parrish & Edelstein-Keshet, 1999).

Parasite manipulators can facilitate their own transmission between hosts, often expressed in extraordinary extended host phenotypes. Intriguingly, parasite manipulators have been observed to induce host aggregation, with implications for parasite transmission and parasite mating. For example, in *Ophiocordyceps*-infected ants, the ants aggregate at their "final destination," high points on vegetation (Imirzian et al., 2020). The hypothesis proposed by these researchers is that the ants aggregate in high-traffic areas, increasing the risk of transmission to naïve ants foraging below (Imirzian et al., 2020). Another example of altered host distribution occurs in the *Xenos vesparum*-infected *Polistes dominulus* wasp system (Beani et al., 2011; Hughes et al., 2004). In this case, the parasitoid larvae induce host clumping, perhaps to increase mating opportunities for the metamorphosed adults (Hughes et al., 2004).

Host aggregation is likely to have implications for the manner in which parasites are transmitted among hosts. When host distributions are clumped, it follows that parasite distributions will also be clumped. Parasite aggregation within host populations is a common finding (Shaw et al., 1998; Poulin, 2007) with key implications for patterns of parasite-induced host pathology, the regulation of parasite populations, and the expression of host immunity. The phenomenon is so common that Poulin (2007) identified parasite aggregation as a general law in parasite population biology. Although parasite aggregation, and its consequences, have been well studied, the phenomenon of host clumping, and concomitant parasite clumping, is far less described.

It is difficult to assess potential linkages between host aggregation and parasitism when parasites are often small and cryptic. Yet in certain cases, infective stages of parasites, or at least the hosts they reside in, are conspicuous on the landscape. The bright red abdomens of certain nematode-infected 'berry' ants, the swollen abdomens of sticklebacks infected with a larval cestode, and the bright-coloured cysts of trematode-infected coral polyps are conspicuous to human observers (Goater et al., 2014). Larvae of the iconic manipulating trematode, *Dicrocoelium dendriticum*, cause a well-known alteration in the behaviours of their ant intermediate hosts (review in Chapter 1-3). Infected ants leave their nests during the cool, dim, and humid hours of the day and then affix their mandibles onto inflorescences located within a few meters of their nest (Chapter 3; Botnevik et al., 2016; Gasque & Fredensborg, 2023). Ants remain attached to the inflorescence, until the next morning when temperatures rise, light intensifies, and air becomes drier (Chapter 3; Botnevik et al., 2016; Gasque & Fredensborg, 2023). Their attachment to vegetation facilitates the transmission of the encysted larvae, via accidental ingestion, into a grazing mammal. Attachment to vegetation also provides an unambiguous snapshot of the distribution of infected ants on the plants that are available to them in the vicinity of a nest because uninfected ants never exhibit this behaviour.

I have observed ants infected with *D. dendriticum* aggregated on plants surrounding their nests in a region of fluke emergence in Cypress Hills, Alberta (e.g., Unrau, 2019; Fig. 4.2) similar to the clumping reported in O*phiocordyceps*-infected ants (Imirzian et al., 2020). Although my observations are anecdotal, the aggregated nature of infected ants on plants has supported earlier sampling activities (e.g., Chapter 2; Chapter 3; Martin-Vega et al., 2018; Criscione et al., 2020) because aggregations on plants facilitates the detection and collection of infected ants. However, the distribution of infected ants on available plants has not previously been described. Furthermore, the manner in which time of year, host species, colony, and plant community structure influence the use of available plants for attachment is unknown.

My field-based study aims to describe the spatiotemporal distribution of fluke-infected ants on plants adjacent to four infected nests in Cypress Hills Interprovincial Park (CHIP; Chapter 2; Chapter 3) during the period they are attached to plants and thus available for ingestion by grazing definitive hosts. I first test whether individuals of two species of infected ant, *F. aserva* and *F. podzolica*, are distributed at random among plants available to them surrounding a nest. I next test whether overall patterns of distribution on available plants change over the course of the season and whether such changes are influenced by colony or host species. Lastly, I test whether patterns of distribution of ants on plants can be attributed to the nature of the plant community adjacent to infected nests.

4.3 MATERIALS AND METHODS

4.3.1 Selection of ant nests

Patterns of infected ant distributions on plants were studied at four nests in CHIP during the summer of 2024. I monitored the distribution of both *Formica aserva* and *F. podzolica* on plants at the same four nests (SC1, SC2, TC1, and TC2) where I assessed eco-epidemiological patterns of infection (Chapter 2) and diurnal patterns of attachment (Chapter 3). Detailed descriptions of these sites, and of CHIP in general, are provided in Chapters 1, 2, and 3. *The F. aserva* nests (SC1 and SC2) were approximately 75 m apart, whereas the *F. podzolica* nests (TC1 and TC2) were approximately 10 m apart.

4.3.2 Plant community characterization

Extensive sampling efforts at numerous nests in CHIP indicated that approximately 90% of attaching ants attach to plants that are within 1.5 m of the nest entrance (Goater & Colwell, 2007; Unrau, 2019). To ensure that I counted the greatest number of attached ants (Gasque & Fredensborg, 2023), I demarcated a 3 m X 3 m quadrat around each nest, with the nest located approximately in the centre (Fig. 4.3). Three field workers thoroughly searched each quadrat to identify and count each plant stem, excluding trees, grasses, and sedges. Trees, grasses, and sedges were excluded as observations of infected ants in CHIP have indicated that ants prefer to attach to forbs and shrubs (Unrau, 2019).

I selected ‘stem count’ as the unit of analysis (rather than flower count or inflorescence count) because the ants were often attached to plants that lacked flowers. The utilization of stem counts helped to keep the methods consistent throughout the season and between the four nests.

Additionally, it was not possible to determine plant separation of individual plants because many developed through underground rhizomes (e.g., *Solidago* and *Trifolium* spp.). Thus, I counted stems that were not connected above ground as separate (Sankovitz et al., 2018).

For each plant species found in each plot, I recorded its identity and development phase (i.e., vegetative, budding, flowering, in seed, senescent). At each survey, I collected the following metrics of plant community structure: species richness (total number of plant species in the quadrat), plant density (number of stems in the quadrat), and plant dominance. The plant surveys were repeated biweekly at each nest from May to August 2024, for a total of 7 plant surveys per nest. The area of the plot was kept consistent throughout the season by marking the four corners with flags.

4.3.3 Ant observations

The plant surveys took place in the morning (7:00 -10:00 a.m.) or in the evening (6:00 - 9:00 p.m.) to maximize the number of ants attached to plants (Chapter 3; Gasque & Fredensborg, 2023; Spindler et al., 1986). While conducting the plant surveys at each nest, I identified plants that had ants attached and recorded the plant species, its developmental phase, how many ants were attached to it, and its coordinates within the quadrat on an x-y coordinate system (origin in the SE corner; Fig. 4.3). I surveyed for ants biweekly, for a total of seven ant surveys per nest. At TC1 and TC2, infected *F. podzolica* were only present between late May - early July, resulting in a total of four surveys. At SC1 and SC2, ants were present in late May-early August, resulting in six surveys.

4.3.4 Statistical analysis

During each survey, all ants on plants within the quadrat were counted. In an initial analysis, I tested whether the attached ants available in a quadrat at each survey were distributed at random among the stems available. I constructed frequency distributions of ant counts on stems, including plants with no ants attached, to provide an assessment of the overall distribution of infected ants on available plants. A chi-square goodness of fit test was used to test if the distribution of points varied significantly from a Poisson (random) distribution. Low-frequency tails were binned to ensure that all expected frequencies were greater than five. To evaluate the magnitude of potential aggregation and how it changed with time, species, and nest, I compared the variance-to-mean ratios (VMR). This aggregation index (Whitlock & Schluter, 2020) was calculated from the mean number of infected ants on the total sample of stems in a quadrat and the variance in ant counts. Surveys with fewer than five attached ants were not included in the analysis. If the VMR was greater than one and the chi-squared test was significant, the ants were classified as aggregated on available plants. If the VMR approached a value of one or lower and the chi-squared test was not significant, the ants were classified as randomly distributed (Whitlock & Schluter, 2020).

I used Monte-Carlo simulations, following methods described in Imirzian et al. (2020) to evaluate whether the plants that the ants were attached to were spatially aggregated within a quadrat (spatstat package in R; Baddeley et al., 2015). I ran 500 simulations of points (= number of plants with attached ants) randomly distributed in a 3 m X 3 m area (the same size as the sample quadrats). For each of the 500 simulations, the nearest neighbour distance of each point was calculated and then the mean nearest neighbour distance in one simulation was calculated, resulting in 500 mean nearest neighbour distances, which represent the null distribution. I calculated a one-way p-value by taking the number of simulated mean nearest neighbour distances that the observed nearest neighbour distance was greater than and dividing that by the total number of simulations. If $p<0.05$, the mean nearest neighbour distance is less than what was expected by chance, meaning that the distribution is clumped. This was repeated at all sampling times at each nest, except for those that had fewer than five attached ants. All statistical analyses were completed in R Statistical Software (v4.4.1; R Core Team, 2024).

4.4 RESULTS

4.4.1 Patterns in the distribution of attached ants

The distribution of infected *F. aserva* on plants was significantly different from a Poisson distribution during four of the six sampling times at SC1 and five of the six sampling times at SC2 (Fig. 4.4). At both nests, the distribution was closest to random at the end of the season, in late July/early August (Fig. 4.4). In contrast to the results for *F. aserva*, the distribution of infected *F. podzolica* on plants was never significantly different from a Poisson.

4.4.2 Variance-to-mean ratios

The variance-to-mean ratio (VMR) values for *F. aserva* distributions were always greater than one when the distribution varied significantly from a random distribution, indicating that the distribution of infected *F. aserva* on plants was aggregated (Fig. 4.5A). At SC1, peak aggregation occurred in late May, when 63 ants were attached to 16 plants, half of which (52%)

were attached to a single plant (Fig. 4.5A). The lowest aggregation at SC1 occurred in late July when 16 infected ants were distributed among 10 plants (Fig. 4.5A).

Peak aggregation of *F. aserva* at SC2 occurred in early July when 54 ants were distributed across 24 plants (Fig. 4.5A). Of those 54 ants, 33% were attached to a single plant. The lowest aggregation at SC2 occurred in early August when 14 ants were distributed across 9 plants (Fig. 4.5A).

The VMRs for *F. podzolica* distributions were always close to 1 (Fig. 4.5B). At TC1, the closest to an aggregated distribution that I saw was in late June when VMR was 2.2, when 22 ants were distributed among 16 plants (Fig. 4.5B). At TC2, the VMR never exceeded 1.5, indicating that the ants were approximately randomly distributed on plants (Fig. 4.5B).

4.4.3 Spatial Analysis

Figures 4.6 and 4.7 show the distribution of attached ants on plants at each of the 6 (SC1 and SC2), 4 (TC1), or 3 (TC2) time periods at the four nests. In general, these figures demonstrate the aggregated nature of attached ants on plants adjacent to nests, particularly between May and early July, confirming the aggregated pattern demonstrated in the frequency distributions (Fig. 4.4). At SC1, the ants demonstrated a distinct handedness with almost all individuals attached on the South half of the x-axis (Fig. 4.6A). At SC2, the ants showed the opposite pattern with most ants attaching on plants on the North side of the x-axis (Fig. 4.6B). A pattern of distinct spatial grouping was not apparent at either *F. podzolica* nest (Fig. 4.7).

During some of the sampling times involving *F. aserva* counts, the plants used for attachment were, themselves, clustered, differing from a random distribution. At SC1, the plants used for attachment were aggregated during 67% of the sampling times (Fig. 4.8A). The plants were closest together in late May with a mean nearest-neighbour distance (NND) of 0.16 m and they were farthest apart in early August with a mean NND of 0.49 m (Fig. 4.8A). On the other hand, at SC2, the plants used for attachment were only aggregated during 33% of the sampling times (Fig. 4.8A). The plants were closest together in early June with a mean NND of 0.23 m and they were farthest apart in late July with a mean NND of 0.38 m (Fig. 4.8A).

The spatial distribution of the plants used for attachment by infected *F. podzolica* was rarely aggregated. At TC1, plants used for attachment were clustered during 50% of the sampling times (Fig. 4.8B). The plants were closest together in early June with a mean NND of 0.25 m and they were farthest apart in early July with a mean NND of 0.83 m (Fig. 4.8B). On the other hand, at TC2, the plants used for attachment were never clustered, with the mean NND ranging from 0.2719 m (June 8th; Fig. 4.8B) to 0.5236 m (June 26th; Fig. 4.8B).

4.4.4 Plant preference

Overall, ants attached to plants that were not the dominant plant in the plot in 14 of the 19 samples. For *F. aserva*, ants attached to a non-dominant plant in 67% and 100% of the samples at SC1 and SC2, respectively (Table 4.1). For *F. podzolica*, the ants attach to the non-dominant plant in 25% and 33% of the samples at TC1 and TC2, respectively (Table 4.2).

Plant selection by ants changed over the season at SC1 (Table 4.1), switching back and forth between common dandelion, cow parsnip (*Heracleum maximum*), and Missouri goldenrod

(*Solidago missouriensis*). Regardless of the plant selected for attachment, ants attached disproportionally to the plants that were available in the plot. In one extreme example, 78% of 63 *F. aserva* attached to cow parsnip but cow parsnip only represented 14.5% of plants available within the plot (Table 4.1; SC1; May 27th). In another example, 56% of 16 *F. aserva* attached to dandelion but dandelion only represented 5% of plants available within the plot (Table 4.1; SC1; July 23rd).

A similar pattern of seasonality and plant switching occurred for *F. aserva* at SC2 (Table 4.1). Ants attached to common dandelion (*Taraxacum officinale)* in May and early June, then shifted to common snowberry (*Symphoricarpos albus*) for the remainder of the summer. This change in plant choice aligns with the shift to common snowberry as the dominant plant in the plot, but the ants were still using common snowberry disproportionally to its availability in the plot. In the most extreme case, 100% of 14 ants were attached to common snowberry, but it only represented 22.8% of the plants available for attachment (Table 4.1; SC2; August 6th). Additionally, common dandelion was flowering but then moved to seed when ants made the transition to common snowberry.

Infected *F. podzolica* also exhibited plant choice (Table 4.2). In the most extreme case, 58% of 29 ants attached to field chickweed (*Cerastium arvense*) at TC1, but field chickweed represented only 29.4% of plants available for attachment (Table 4.2; TC1; June 8th). In this case, *F. podzolica* selected the dominant plant in the plot. Field chickweed was flowering from May 27th-June 26th. On June 26th, ants switched to northern bedstraw (*Gallium boreale*), which is when it became dominant in the plot, and when its flowers began budding (Table 4.2). The only time that ants did not choose the dominant plant was on July 8th, when 43% of ants preferred shrubby cinquefoil (*Dasiphora fruticosa*), which represented only 0.4% of plants that were available for attachment (Table 4.2; TC1; July 8th). It is notable that shrubby cinquefoil was flowering during the July 8th survey, whereas field chickweed was in seed.

Formica podzolica exhibited plant choice at TC2 similar to that observed at TC1, with field chickweed selected at the beginning of the summer (Table 4.2). The most extreme case occurred when 40% of 30 ants attached to field chickweed, representing only 6% of available plants (Table 4.2; TC2; June 8th). Then, similar to TC1, plant choice switched to golden bean (*Thermopsis rhombifolia*) and bastard toadflax (*Comandra umbellata*), with 18% of 12 ants attaching to each of these plant types, respectively (Table 4.2; TC2; June 26th). This switch is notable because golden bean and bastard toadflax only represented 3% and 0.3%, respectively, of plants available for attachment. Golden bean was in seed, while bastard toadflax was flowering when the switch happened.

4.5 DISCUSSION

In a similar plant and ant survey, Spindler et al. (1986) showed that infected ants prefer to attach to plants that are flowering. The results of my assessments are comparable, with ants changing their choice of plant for attachment based on plant phenology. The preference for inflorescences indicates that plant choice was more closely linked with plant phenology than with plant dominance within a plot. It is well recognized that carbohydrates comprise a key component of *Formica* spp. diets (Arganda et al., 2014). Flowers provide a key source of carbohydrates through nectar, meaning that ants tend to prefer to forage, and perhaps attach, on flowering plants (Das & Das, 2023). Ants are selective when foraging on plants, only foraging on a small proportion of plant resources available to them (Rockwood, 1976; Englicky & Sera, 2019). The selective nature of foraging could be applied to the plants they choose to attach to when infected. Additionally, the parasite may be inducing the ants to attach to plants that are more likely to be grazed by the definitive host. Although grazers tend to prefer herbaceous plants, like grass, when browsing, they often consume flowers (DeBano et al., 2016; Hesselmann et al., 2025). At SC, I have seen evidence of browsing on dandelion flowers around the nest. I found that the plants that ants were attached to were aggregated within the plot. Without knowing the spatial distribution of all plants in the plot, it is unclear whether the ants chose to attach to plants that were closer together because their preference plants were restricted to these areas, or if they were choosing plants that were spatially aggregated for a different reason such as a handed bias (Hunt et al., 2014). Nevertheless, one intriguing possibility that can explain the general observation that infected ants select flowers for attachment is that larval *D. dendriticum* somehow exploit the ant's preferences for foraging and converts it to a preference for attachment. Over time, the density of plants in the plot increased, as demonstrated in Figure 4.9, introducing the possibility that ants were highly aggregated in May (at SC1) due to the low number of living plants available (Fig. 4.9).

Dicrocoelium-infected *F. aserva* tended to aggregate on available plants, whereas *F. podzolica* tended to be randomly distributed on plants. These stark differences in distribution were unexpected. However, interspecific differences in patterns of host manipulation by a single parasite are commonly reported. For example, in *Gammarus* spp. infected with larvae of the acanthocephalan *Pomphorhynchus laevis*, one host species, *G. pulex*, exhibits manipulated drifting behaviour when infected, whereas a different host, *G. roeseli*, does not experience the same behavioural changes when infected (Lagrue et al., 2007). Understanding the functional significance of the observed differences in distribution on plants between infected *F. aserva* and infected *F. podzolica* will require follow-up studies. Although the metacercariae intensity has been compared across various ant host species in Europe, there are no studies assessing interspecific behavioural differences (Manga-Gonzalez et al., 2001). Studies designed to evaluate differences in rates of encounter, and thus transmission, between grazing mammals (definitive hosts) and the clumped vs randomly distributed species of intermediate hosts would be especially valuable.

The two species of ant host also differed in the duration of time throughout the summer that infected individuals expressed attachment. *Formica podzolica* were only observed on plants during four of seven surveys, while *F. aserva* were observed for nearly the entire summer (six of seven surveys). These differences were likely linked to the contrasting life histories of the two host species. Ants are known to have different life history strategies ranging from the number of queens per colony to nuptial flight timing, and many other aspects (Talbot, 1948; Tsuji & Tsuji, 1996). Fully black-coloured wood ants (e.g., *F. podzolica*) have different life histories compared to lighter-coloured ants (e.g., *F. aserva*), including reduced sensitivity to harsh environmental conditions (Law et al., 2019). They also differ in their foraging and nursing habitats (Law et al., 2019), which could contribute to the differences observed in the distribution of ants on plants. It is important to acknowledge that these congeneric hosts have stark differences, and the way the parasite manipulates them may be different. Therefore, making blanket conclusions about host manipulation can be misleading when presenting ideas about how, where, and when parasites, like *D. dendriticum*, are transmitted. In areas where *F. podzolica* is the only ant species infected with the parasite, the transmission window from ants to cattle is restricted to the end of May-end of June. On the other hand, in areas where *F. aserva* is infected, the transmission window is broader, from the end of May to the end of August. As discussed above, understanding the functional significance of interspecific differences, in this case in the seasonal availability of infected ants on plants, will require follow-up studies on relative rates of encounter between grazing mammals and the two species of infected ants.

My study represents the first time that aggregation behaviour has been reported in the *Dicrocoelium*-ant system. I have also shown that this system may be the first report of a unique case of "super-aggregation," with parasites clumping within their host and hosts clumping on plants. The ingestion of a "super-aggregation" of hosts and parasites would result in extreme levels of infection that have already been observed in calves and yearlings of Wapiti cattle in CHIP (Goater & Colwell, 2007). Goater and Colwell (2007), hypothesized that high numbers of parasites in the cattle were likely acquired though repeated ingestion of metacercariae, but this study reveals the possibility that a cow could achieve these high infection intensities (>2000 worms; Goater & Colwell, 2007) from the ingestion of a single plant with high numbers of attached ants. Super-clumped transmission could have differing implications on the dispersal of *D. dendriticum* across the landscape, potentially relying on few heavily infected mammals (super-spreaders; Paull et al., 2011; Johnson & Hoverman, 2014) to disperse the parasite.

Although the pattern of aggregation on plants in infected *F. aserva* is clear, the functional significance of aggregation remains unknown. Current proposed explanations for host aggregation in other systems include increased mating success (Hughes et al., 2004; Allahverdipour et al., 2019) and increased intraspecies transmission success (Imirzian et al., 2020). However, these explanations cannot be directly applied to *D. dendriticum* because the adult flukes are hermaphroditic, easily reproducing in the definitive host (Beck et al., 2015), and because *Dicrocoelium* cannot be transmitted to sister ants. Additionally, each ant host has an average of 26.5±25 (mean ± standard deviation) infective metacercariae (and up to >150 metacercariae; Chapter 2), meaning that the definitive host already experiences clumped transmission following the ingestion of even a single ant. Perhaps 'super-aggregation' provides a benefit to individual worms that are being transmitted through ants. In my most extreme case, I observed 33 infected *F. aserva* on a single plant. Based on my results from Chapter 2, a potential grazing mammal would ingest approximately ~ 800 infective metacercariae with a single encounter. Clumped transmission of this magnitude would be likely to enhance rates of reproduction in the liver of definitive hosts (Beck et al., 2015). An alternative explanation is that simultaneous ingestion of large numbers of larvae may provide a strategy to overwhelm host immunity in the gastro-intestinal system of definitive hosts (Colwell and Goater, 2010). Alternative hypotheses for the functional significance of ant aggregation on plants (and thus, parasite aggregation on plants) could be tested under laboratory conditions, perhaps using feeding experiments on model definitive hosts such as hamsters (Brode & Geyer, 1981).

It is also important to acknowledge that social hosts tend to cluster regardless of infection status (Depickere e al., 2004; Depickere et al., 2008). Depickere et al., (2004) found that ants exhibit a natural tendency to aggregate regardless of external factors such as environmental conditions, and perhaps infection status. Therefore, the host aggregation that I observed in *F. aserva* could simply be a side effect of infecting a social host. Until studies are done to assess the adaptive significance of this behaviour, it is important to acknowledge both possibilities, parasite adaptive behaviour, and side effect of infecting a social host.

4.6 CONCLUSION

Ants infected with *D. dendriticum* tend to utilize a small proportion of plants available to them, resulting in aggregated distributions that differ temporally, spatially, and between species. Discovering the adaptive significance of aggregation behaviour in hosts infected with manipulative parasites will be key in understanding the role that host distributions play in parasite transmission and success.

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4.8 AUTHORSHIP AND CONTRIBUTORSHIP

LE contributed to field behavioural observations, data analysis, and writing. CG conceived the project and contributed to supervisorship, project management, writing, and editing

4.9 FIGURES

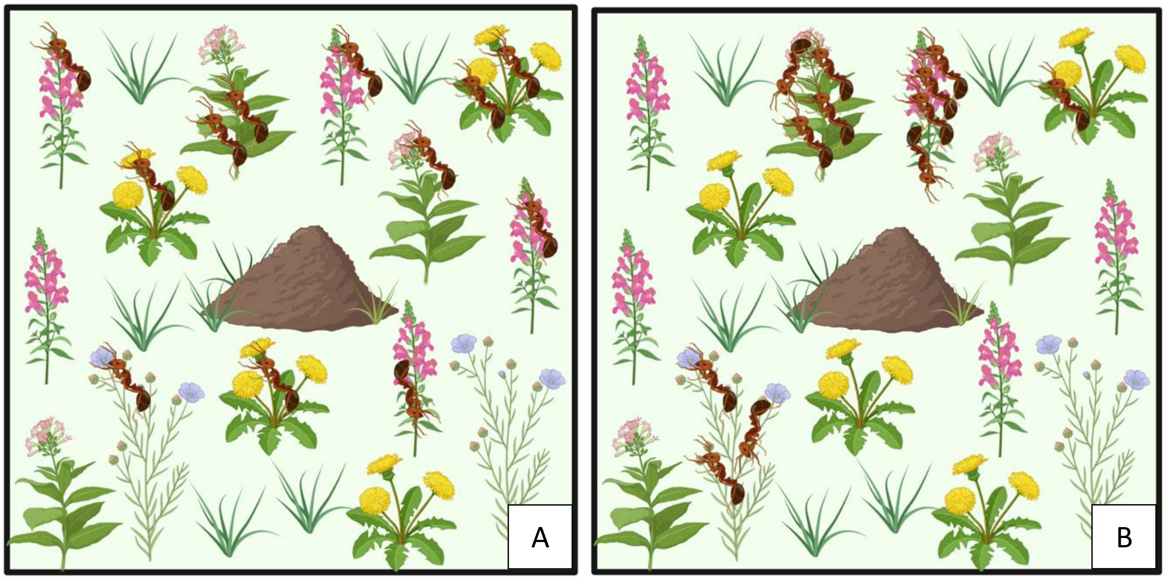


Figure 4.1. Schematic representation of two hypothetical patterns of distribution of 12 infected ants on 20 plants adjacent to a nest. 'A' describes an approximately random distribution of ants on plants; 'B' describes an approximately aggregated ant distribution. Figure created in BioRender.com.

Figure 4.2. Hundreds of *Formica aserva* workers infected with *Dicrocoelium dendriticum* aggregated on a single coltsfoot plant (*Petasites frigidus var. sagittatus*) in Cypress Hills, Alberta, Canada.

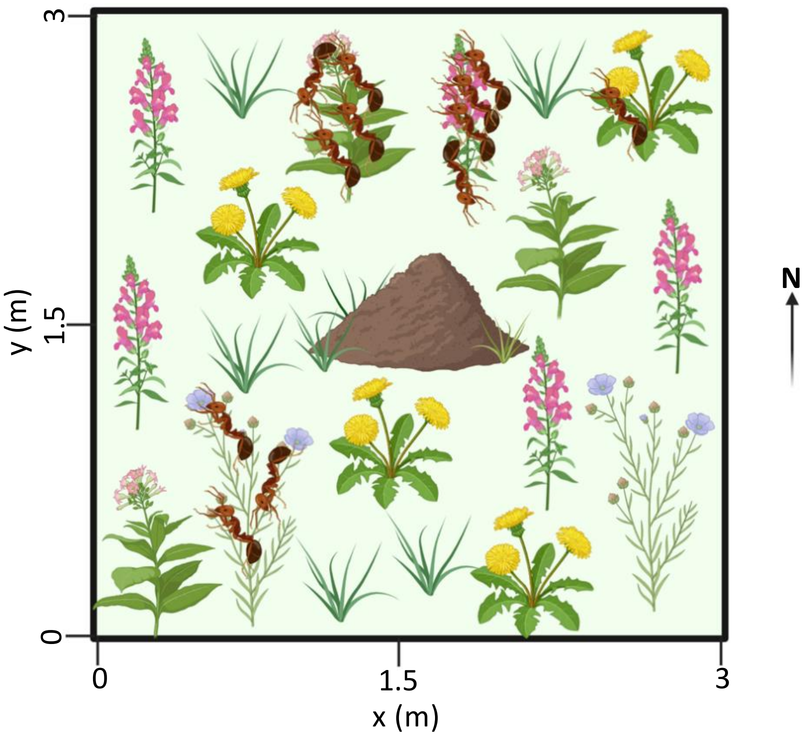


Figure 4.3. Schematic diagram of a sample quadrat surrounding an ant nest. The ants on the plants are worker ants infected with *Dicrocoelium dendriticum*. Figure created in BioRender.

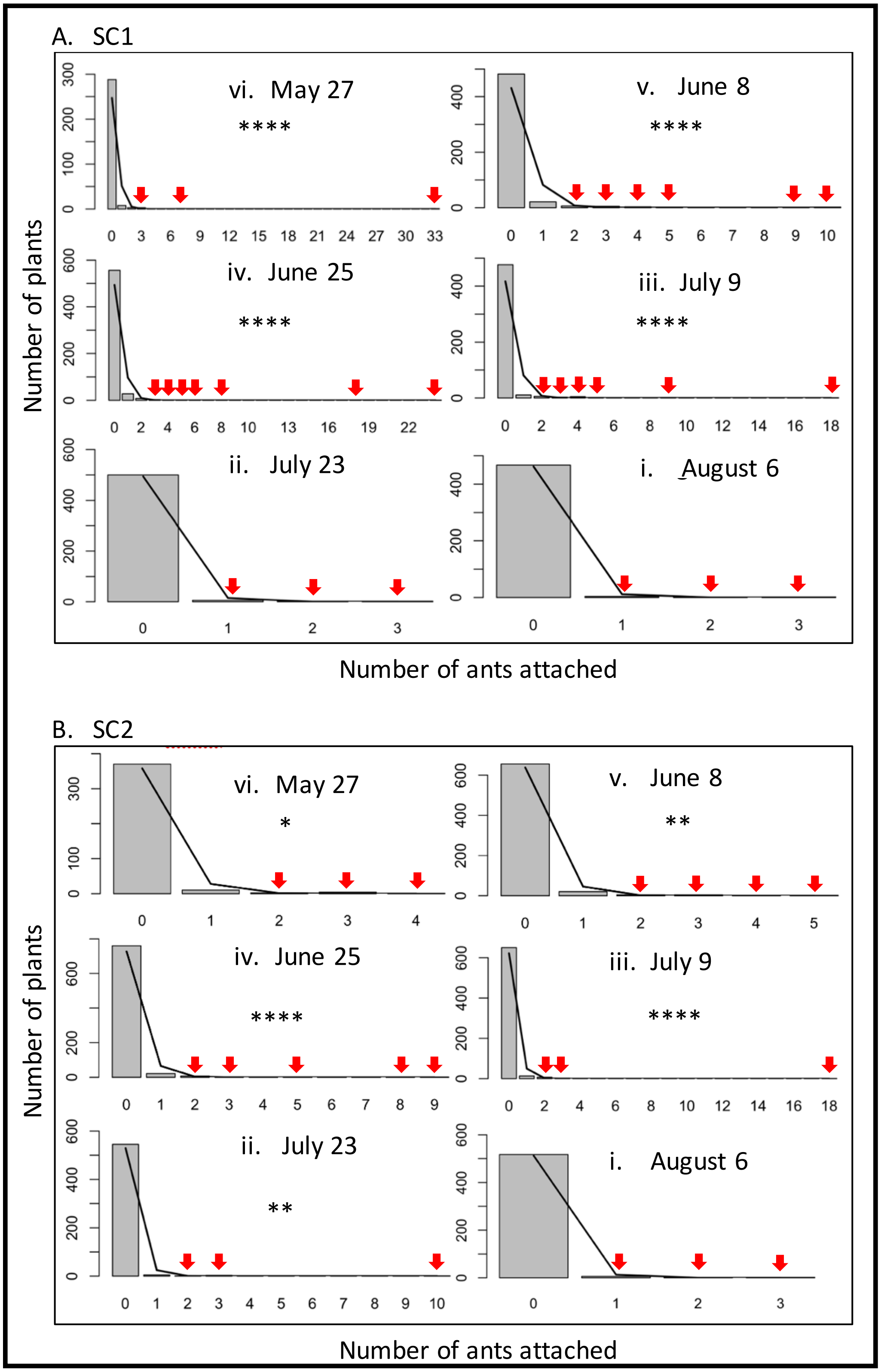
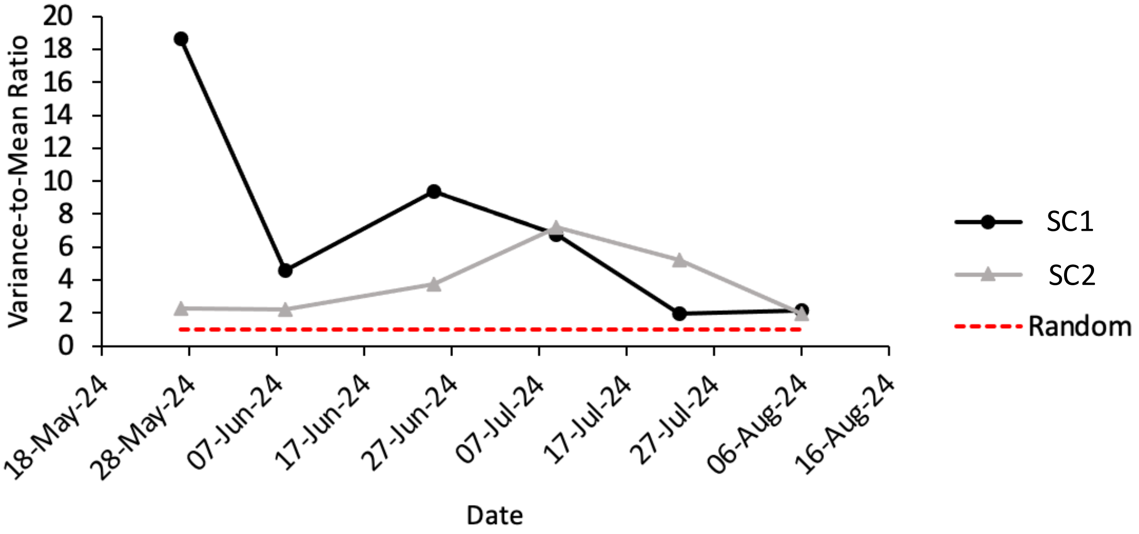


Figure 4.4. Frequency distributions of *D. dendriticum*-infected *Formica aserva* counts on plants surrounding two nests, SC1 (A) and SC2 (B), in Cypress Hills, Alberta. Roman numerals indicate frequency distributions constructed from ant counts at the two nests at different times

throughout summer, 2024. Bars represent the observed frequencies, and the black line represents the expected frequencies from a Poisson distribution. The asterisks represent significance from a chi-squared goodness of fit test comparing observed and expected distributions (*=p<0.05, **=p<0.01, ***=p<0.001, ****=P<0.0001). The red arrows represent instances where the number of ants on plants is greater than zero.

A. *F. aserva*



B. *F. podzolica*

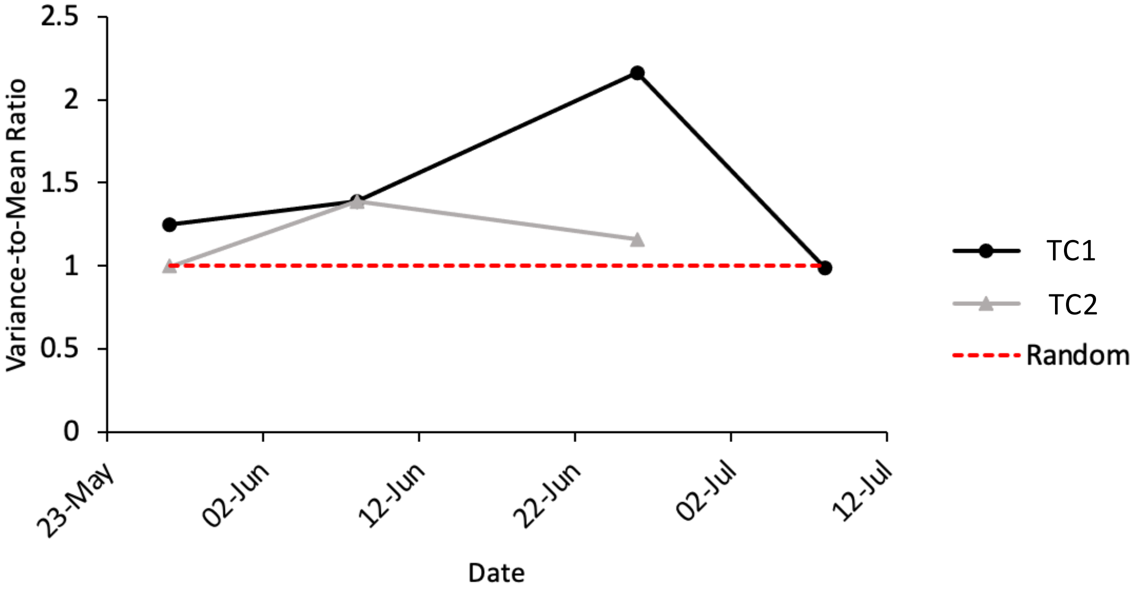


Figure 4.5. Seasonal changes in *D. dendriticum*-infected ant aggregation on plants surrounding two *F. aserva* nests (A) and two *F. podzolica* nests (B) in Cypress Hills, Alberta. Aggregation at each time interval was estimated as the variance in numbers of ants on the total sample of plants adjacent to nests, divided by the mean. The red line represents a random distribution (VMR = 1).

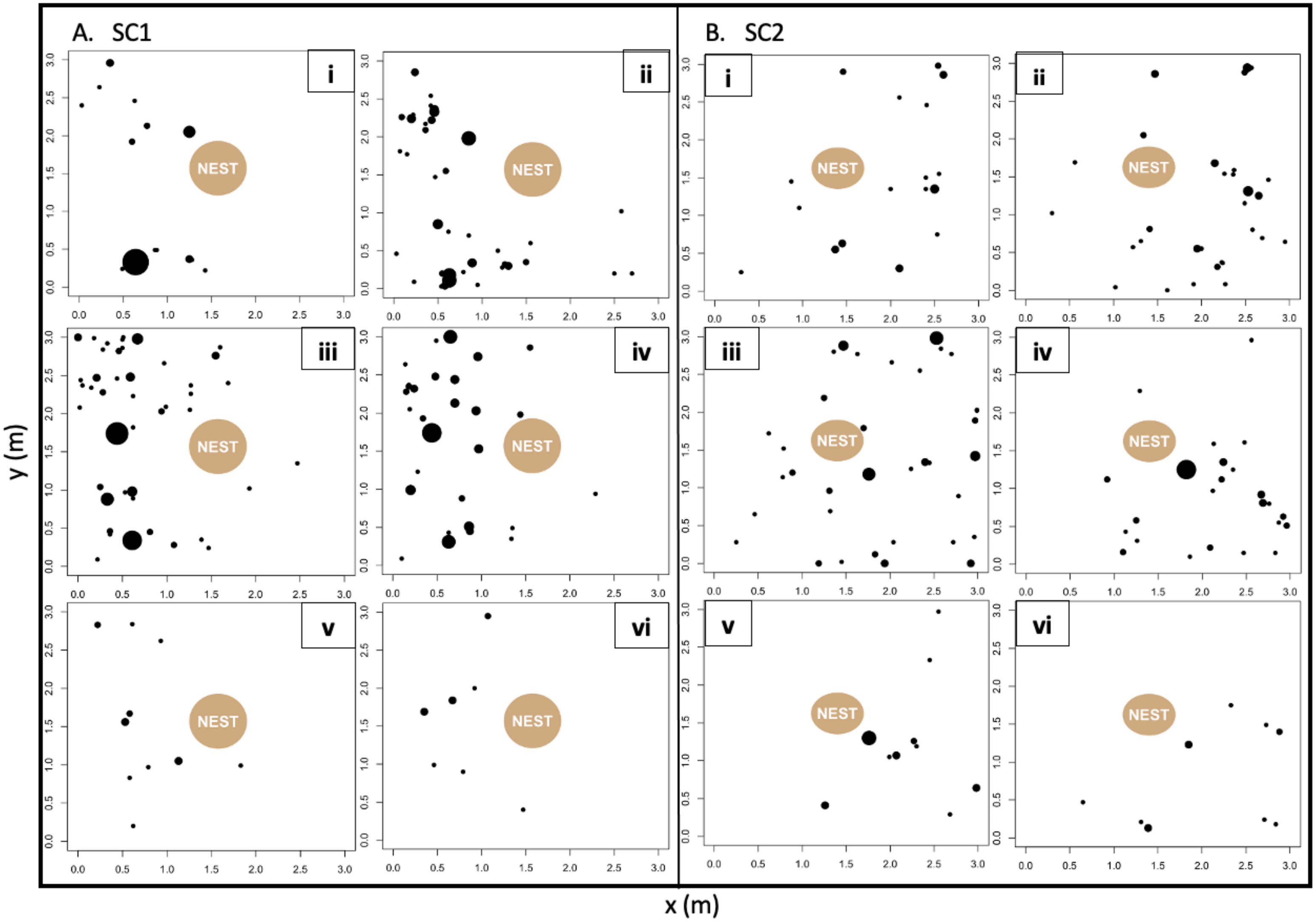


Figure 4.6. Spatial distribution of *D. dendriticum*-infected ants on plants surrounding two *F. aserva* nests, SC1 (A) and SC2 (B) in Cypress Hills, Alberta. Roman numerals represent different sampling times during summer, 2024 (i= May 27th, ii= June 8th, iii= June 25th, iv= July 9th, v= July 23rd, vi= August 6th). Black circles represent plants that had attached infected ants, with circle size representing the numbers of ants on that plant.

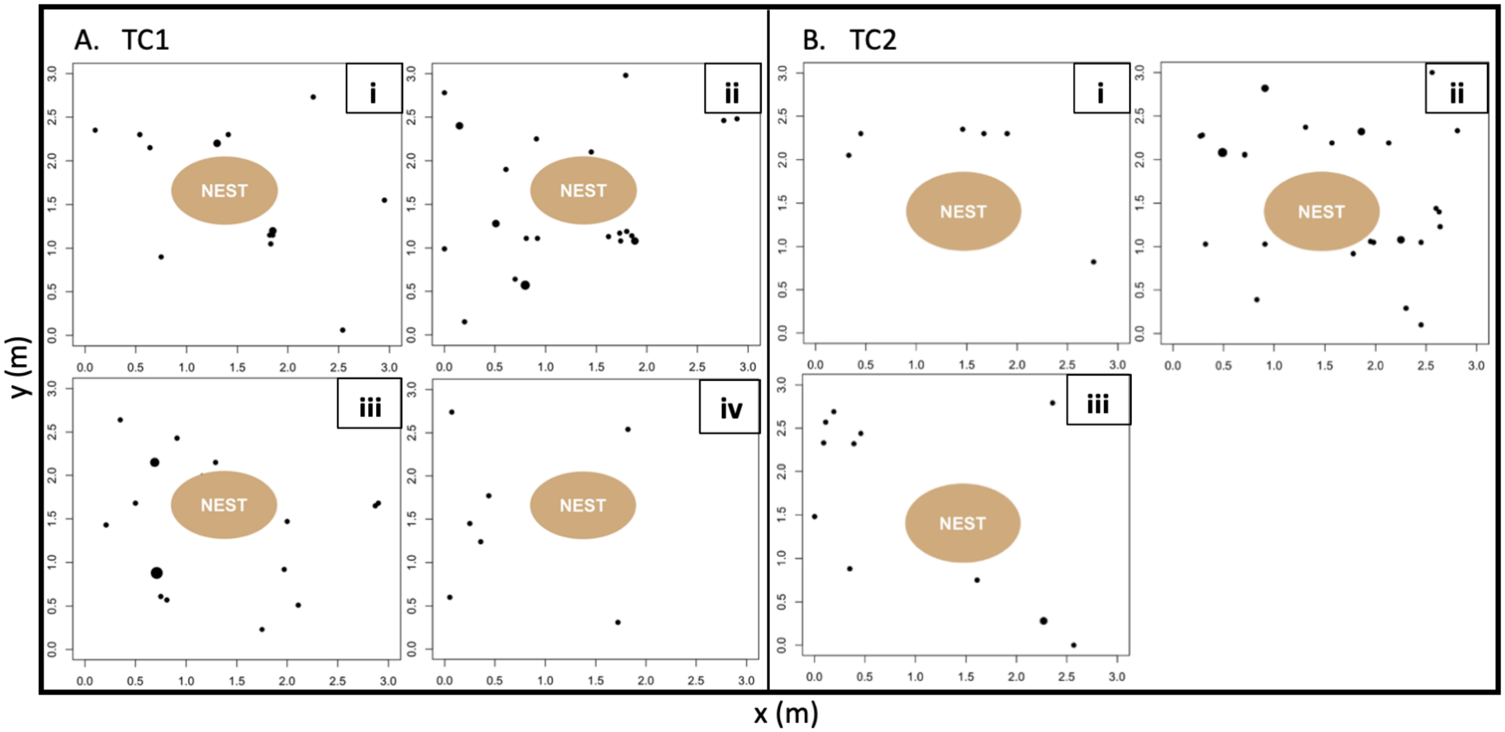


Figure 4.7. Spatial distribution of *D. dendriticum*-infected ants on plants surrounding two *F. podzolica* nests, TC1 (A) and TC2 (B) in Cypress Hills, Alberta. Roman numerals represent different sampling times during summer, 2024 (i= May 27th, ii= June 8th, iii= June 26th, iv= July 8th). Black circles represent plants that had attached infected ants, with circle size representing the numbers of ants on plants.

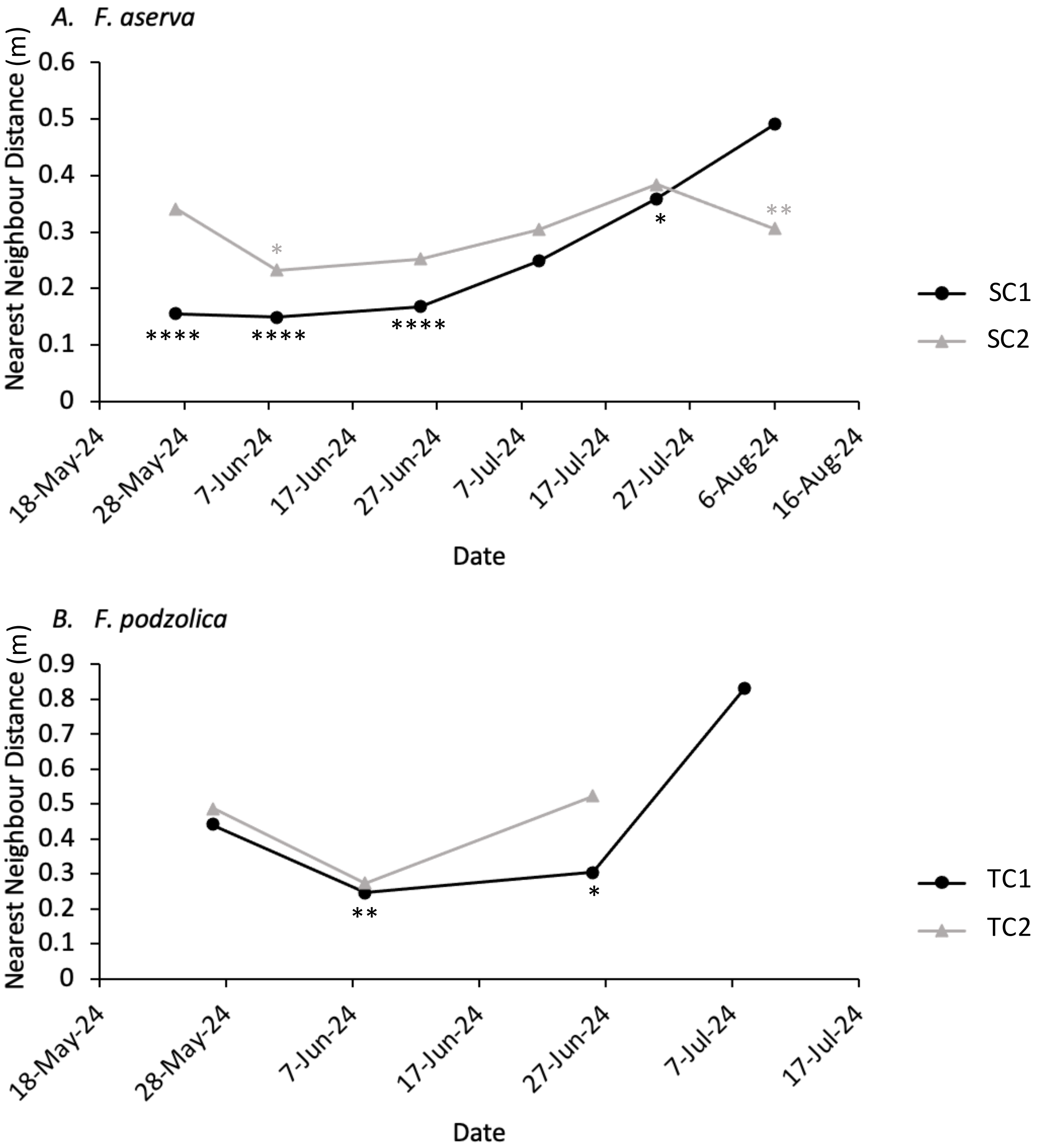


Figure 4.8. Seasonal changes in nearest neighbour distances (m) of *D. dendriticum*-infected *F. aserva* (A) and *F. podzolica* (B) over summer, 2024 in Cypress Hills Park, Alberta. Asterisks represent whether the plants are significantly aggregated compared to simulated random points (*=$p<0.05$, **=$p<0.01$, ***=$p<0.001$, ****=$P<0.0001$).

Figure 4.9. The plant community surrounding a *Formica aserva* nest that contains workers infected with *Dicrocoelium dendriticum* in May (A) and August (B), 2024.

Table 4.1. General characteristics of the plant communities surrounding two *Formica aserva* nests containing *D. dendriticum*-infected workers in Cypress Hills, Alberta, Canada.

Date	Plant Species Richness	Total Number Stems	Plant Dominance	Dominant Plant	Number of Ants	Plant Choice	Percent Plant Choice
SC1							
May 27, 2024	16	304	18%	Canada Violet	63	Cow Parsnip	78%
June 8, 2024	20	523	23%	Horsetail	99	Cow Parsnip/ Canada Violet	20%
June 25, 2024	19	601	20%	Missouri Goldenrod	118	Missouri Goldenrod	31%
July 9, 2024	17	506	17%	Horsetail	98	Cow Parsnip	23%
July 23, 2024	17	510	21%	Missouri Goldenrod	16	Dandelion	56%
August 6, 2024	17	474	19%	Missouri Goldenrod	12	Missouri Goldenrod	50%
SC2							
May 27, 2024	12	369	38%	Horsetail	30	Dandelion	40%
June 8, 2024	13	654	51%	Horsetail	49	Dandelion	21%
June 25, 2024	14	760	53%	Horsetail	71	Snowberry	37%
July 9, 2024	14	674	44%	Horsetail	54	Snowberry	38%
July 23, 2024	14	555	41%	Horsetail	26	Snowberry	77%
August 6, 2024	13	526	35%	Horsetail	14	Snowberry	100%

Table 4.2. General characteristics of the plant communities surrounding two *Formica podzolica* nests containing *D. dendriticum*-infected workers in Cypress Hills, Alberta, Canada.

Date	Plant Species Richness	Total Number Stems	Plant Dominance	Dominant Plant	Number of Ants	Plant Choice	Percent Plant Choice
TC1							
May 27, 2024	30	857	26%	Chickweed	15	Chickweed	39%
June 8, 2024	29	964	29%	Chickweed	29	Chickweed	58%
June 26, 2024	25	832	25%	Northern Bedstraw	22	Northern Bedstraw	25%
July 8, 2024	28	855	30%	Northern Bedstraw	7	Shrubby Cinquefoil	43%
TC2							
May 27, 2024	26	3071	79%	Northern Bedstraw	6	Chickweed	33%
June 8, 2024	26	2920	77%	Northern Bedstraw	30	Chickweed	40%
June 26, 2024	24	3071	82%	Northern Bedstraw	12	Golden Bean/ Toadflax	18%

Chapter 5: General Discussion

5.1 MAIN TAKEAWAYS

This thesis examined the ecoepidemiology and behavioural manipulation of Formicid ants infected with the iconic manipulator, *Dicrocoelium dendriticum*. This work made three major contributions to the study of *D. dendriticum* in its ant hosts and to the fields of parasitology, insect ecology, and behavioural ecology.

The first major contribution of this thesis is my demonstration that clumped transmission is a key component of some parasites with complex life cycles. In Chapter 2, I found that ants recruit metacercariae from slimeballs in clumps within restricted timeframes. I also confirmed that parasite load does not change between months or years, indicating that any seasonal changes in the expression of manipulated behaviour cannot be attributed to changes in parasite loads. In Chapter 4, I found that ant infected ants are clumped on plants during behavioural manipulation. These clumps of hosts, infected with clumps of parasites, represent the first reports of potential super-clumped transmission. Chapter 3 added context to the timing of transmission from ants to grazers. Results indicated that ant attachment was negatively correlated with light intensity and positively correlated with relative humidity, indicating that super-clumped transmission to mammals is most likely to occur when the temperature is low, light intensity is low, and relative humidity is high. These findings expand on the current understanding of clumped transmission, adding a new aspect of clumped hosts, as opposed to just clumped parasites.

The second main contribution lies in highlighting the importance of field studies in understanding altered host behaviour in natural systems that have yet to be replicated in the lab, demonstrated in Chapters 3 and 4. Chapter 3 revealed the importance of light intensity, relative humidity, and time of day, in addition to temperature in the expression of manipulated behaviour of *D. dendriticum*-infected ants. This added level of complexity counters the simplistic sole role of temperature that is so often described in the literature (Spindler et al., 1986; Manga-Gonzalez et al., 2001; Botnevik et al., 2016; Gasque & Fredensborg, 2023). Indeed, my results in Chapter 3 are the first to propose the idea that the enigmatic reversibility of *D. dendriticum*-induced manipulation may be due to complex interactions among key environmental factors. Additionally, Chapter 3 is the first field study to support the possibility of circadian rhythm disruption caused by the parasite that has been suggested through metabolomics and genomics studies of ants collected at these very same sites (Li et al., 2025; Li et al., 2025b). The results in Chapter 4 are the first to characterize the spatiotemporal distribution of *D. dendriticum*-infected ants during attachment to plants. I found that ant hosts are significantly aggregated on plants, consistent with other systems that have described this behaviour (*Xenos Vesparum*-infected *Polistes Dominulus*, Hughes et al., 2004; *Ophiocordyceps kniphofioides*-infected *Cephalotes atratus,* Imirzian et al., 2020). This behaviour was not previously described and unveiling new behaviours may aid in the quest for mechanistic explanations of behavioural manipulation and will likely contribute to the understanding of the transcriptomics, metabolomics, and proteomics findings (Li et al., 2025a; Li et al., 2025b).

The third main contribution is that Chapters 2, 3, and 4 are some of the first studies to contrast differences between two host species in the nature of host manipulation. Chapter 3 was the first study to indicate that different host species of the same genus (*Formica*) have differences in the times of year and length of the year that manipulated behaviours are expressed. Chapter 4 confirmed that *Formica podzolica* expressed manipulated behaviour earlier (early May) and for a shorter duration (4 weeks) than *Formica aserva* (late May for 6 weeks). These differences in behaviour could not be explained by metacercariae intensities as Chapter 2 showed that mean intensities did not change over the season in either species. Interestingly, I showed that host life history differences, in this case related to the timing of nest abandonment by workers, led to differences between the two species in the availability of infected ants on plants (Chapter 2). This is an interesting finding that paves the way for future studies on the transmission biology of generalist, emerging parasites.

5.2 SOCIAL CONTEXT OF MANIPULATION

Dicrocoelium dendriticum is a trematode with a complex life cycle, with metacercariae in Formicid ant intermediate hosts. Ants are eusocial animals, living in tight-knit colonies consisting of sister ants belonging to different worker classes (i.e., forager, brood-tender, etc.) and a queen (Wheeler, 1910; West & Purcell, 2020). This introduces a complex social component to any parasite that enters the colony (Schmidt-Hempel, 1998). Due to their social behaviour and large colony sizes, ants and other social insects are highly susceptible to parasitic, viral, and bacterial infections (Schmid-Hempel, 1998). An example is the microsporidian, *Varimorpha ceranae* in *Apis mellifera* (European honeybee) colonies (Highes et al., 2006). This parasite is transmitted between individuals of the same species and has negative consequences to the colony due to parasite-induced premature foraging (Gorblirsch et al., 2013). The costs associated with intraspecies transmission of parasites can be disregarded in *D. dendriticum*-infected ants because they cannot directly transmit infected stages to naïve ants. However, although the parasite cannot be directly transmitted between individuals, foragers may disperse the cercariae by exposing other workers to the source of exposure (i.e., slimeballs; Krull &

Mapes, 1952; Krull & Mapes, 1953). Additionally, the ants that are experiencing manipulated behaviour are abandoning their usual tasks within and outside of the nest. The implications of task abandonment due to fluke infection in infected Formicid ant colonies has yet to be studied.

The two ant species in this study (*F. aserva* and *F. podzolica*) have differences at the colony level that may be responsible for the intraspecific differences in the behavioural alteration caused by *D. dendriticum* infection. Black-coloured ants such as *F. podzolica* are less sensitive than lightly coloured ants such as *F. aserva* to environmental conditions that favour desiccation such as high temperature, high light intensity, and low humidity (Law et el., 2019). The differences in environmental tolerance could explain the findings in Chapter 3, indicating that *F. podzolica* attachment and detachment are not closely linked with changes in light intensity or relative humidity. At the colony level, *F. podzolica* and *F. aserva* have different nuptial flight times, different nesting materials, and different life history strategies. *Formica podzolica* nests in sandy soil, exposed to continuous direct sunlight. On the other hand, *F. aserva* nests in the stumps of fallen trees, usually under a canopy shaded from direct sunlight. The different nesting strategies are responsible for the differences in tolerance to extreme environmental conditions (Law et al., 2019).

Social parasitism in ants is a life history strategy that involves either nest take-over by a queen of a different species (temporary and permanent social parasitism) and/or workers kidnapping the brood of a different species (dulotic social parasitism); hence, in both cases, the worker outputs of the kidnapped individuals are not related to their own fitness (D'Ettorre & Heinze, 2001; Borowiec et al., 2021). This is a fascinating part of ant ecology that has implications for parasites that infect ants that are either the kidnappers or the kidnapped. In this thesis, I studied two species, one a brood-stealer, *F. aserva* (Scarparo et al., 2024), and one a victim of brood-stealing, *F. podzolica* (Savolainen & Deslippe, 2001). It is even more interesting as I have observed *F. aserva* as social parasites of the brood of *F. podzolica* in CHP. The chapters of this thesis did not include any nests that had evidence of brood-stealing; therefore, I did not directly study the effects of socially parasitic life cycles in intermediate hosts of trematodes. Chapters 3 and 4 showed that there are differences in the timing of manipulated behaviour expression, and an interesting question that stems from this finding is does this difference in timing persist in colonies that have kidnapped *F. podzolica* workers? Additionally, Chapter 4 found differences in the manipulated spatiotemporal distribution of the two species. It would be interesting to evaluate whether kidnapped, infected *F. podzolica* would join *F. aserva* aggregations. Nests with kidnapped infected workers have yet to be documented. Therefore, the implications of social parasitism to *D. dendriticum*-infected ants have yet to be discovered. The *F. aserva*/*F. podzolica* species pair in CHP would be an ideal model for studies interested in linking the phenomenon of host manipulation with the phenomenon of social parasitism.

Understanding the worker class system in ant colonies is essential for a complete understanding of the differences in altered behaviour between infected individuals. Chapters 3 and 4 revealed extensive differences in the expression of manipulated behaviour at the individual ant level. Formicid ants experience differences in worker class (i.e., forager vs brood tender) based on a size-based task division (morphological polyethism; West & Purcell, 2020). Although we know that smaller individuals belong to the brood-tending class of workers and larger ants belong to the forager class, we have no information on the size thresholds for these different worker classes (West & Purcell, 2020); Although, I found that larger ants had higher metacercariae intensities (Chapter 2). Chapters 3 and 4 revealed differences in the expression of manipulated behaviour at the individual ant level.

Chapter 3 indicated that 10-30% of ants remain attached during extreme environmental conditions (high temperature, high light intensity, and low relative humidity), unlike most ants that detach during these conditions. This introduces the potential that individuals that remain attached belong to a different worker subclass than those that leave their plants (i.e., nurse versus forager).

Chapter 4 showed that some infected ants aggregate in their manipulated state (attached to a plant), while others remain solitary. This could be due to an underdeveloped capacity for following scent trails, leading to ants not joining the aggregations. The reason for individual differences is unknown, but one possibility to consider is the role of worker class in the expression of manipulated behaviour. Although, because we cannot accurately distinguish brood-tenders from foragers, we cannot yet attribute behavioural differences to differences in worker class. Similar to morphological polyethism, European honeybees (*Apis mellifera*) exhibit age-based worker class division (temporal polyethism; younger workers are nurses and older workers are foragers; Robinson et al., 1994). In *Varimorpha ceranae*-infected *A. mellifera*, there have been reports of premature foraging (Goblirsch et al., 2013). In other words, *V. ceranae* disrupts the temporal polyethism of honeybees, changing their physiology to induce a premature switch from the nurse class to the foraging class (Gorblirsch et al., 2013). This parasitic interference with polyethism within a colony could be present in other social host systems, including in *D. dendriticum*-infected ants. Understanding the role of morphological polyethism in eusocial host manipulation is important and should be studied further.

5.3 CAVEATS

The results of my field studies reported in Chapters 2, 3, and 4 have provided important new data on the epidemiology and manipulated behaviours of infected ants in nature. Unfortunately, field studies cannot account for all confounding variables that may be associated with the response variable. Therefore, lab studies are required to address specifics of the environmental and spatial factors influencing the results in Chapters 2, 3, and 4. Lab reared colonies infected with *D. dendriticum* have yet to be successfully established. The Goater lab has attempted to raise infected and uninfected colonies in the lab but to limited success. The first limitation is that we must establish a lab colony that has large numbers of workers to ensure high sample sizes of infected workers while replicating the natural 4% prevalence within the nest (Chapter 2). This is exceptionally difficult when attempting to start a colony with a single *Formica* spp. queen (Mori & Le Moli, 1998). Despite this difficulty, we would also need replicates, meaning that we need to successfully rear two or more colonies in the lab. After we can establish a lab colony, we run into a new problem which is that *D. dendriticum* has a complex, three-host life cycle that is resource demanding, time consuming, and hard to maintain in the lab. Specifically, the production of slimeballs in a lab setting has rarely been observed. Therefore, finding specific methods to stimulate slimeball production from lab-reared snails is another hurdle to conducting experimental infections to create a lab-reared infected colony of ants.

Previous studies addressing manipulated behaviours and mechanisms of *D. dendriticum*-infected ants have taken samples of infected workers from the field and placed them in growth chambers that simulate environmental conditions (Botnevik et al., 2016; Li et al., 2025a; Li et al., 2025b). This method may be acceptable for short-term studies and mechanistic studies, but it is well known that ants exhibit different behaviours in the absence of a colony with a queen (Vienne et al., 1998). Therefore, when including small samples of workers without a colony in a lab experiment that spans multiple days, the behavioural results may be affected by the absence of a queen and large colony (Vienne et al., 1998). This could cause a disruption to the behaviour we would normally observe in the field especially in the circadian rhythm of the ants.

Chapters 2, 3, and 4 are foundational studies that provide significant background to the context of *D. dendriticum*-induced behavioural manipulation. Without foundational behavioural studies like those in Chapters 3 and 4, we cannot fully understand results from imaging, metabolomics, transcriptomics, and proteomics work that is aimed at discovering the mechanisms behind manipulated behaviour. For example, a previous member of the Goater lab, Chenhua Li, found that adenosine (a neurotransmitter that helps regulates sleep cycles) was released during certain stages of manipulation (Li et al., 2025a). She also found that circadian rhythm related transcripts were upregulated or downregulated during different stages of manipulation (i.e., attachment vs detachment; Li et al., 2025b). These findings lacked context from observational studies as prior studies (Botnevik et al., 2016; Gasque & Fredensborg, 2023) concluded that circadian rhythm was likely not playing a role in this manipulation. Contradictory to prior studies, the results from Chapter 3 revealed a strong role for light intensity in regulating attachment/detachment of infected ants and ants use light intensity to regulate their circadian rhythms (Narendra et al., 2010). This gives important context to the findings of varying findings about circadian rhythm genes and sleep-regulating neurotransmitters (Li et al., 2025a; Li et al., 2025b). Next, we should conduct lab studies to test the precise role of circadian rhythm in this instance of manipulated behaviour, and perhaps we can find evidence to support the idea that the parasite is ‘hijacking’ the ant’s circadian rhythm in the same way as *Ophiocordyceps spp.* (de

Bekker & Das, 2022). This is just one example of how results of field studies can be informative in mechanistic studies.

My thesis includes the first reports of a new behaviour in fluke-infected ants, aggregation of hosts on plants (Chapter 4). Although this behaviour has now been reported, there are several questions that remain unanswered. The main question that arises from this new behaviour is why do *D. dendriticum*-infected ants aggregate at all? This leads to the critical knowledge gap in this system, along with many others, that the adaptive significance is not usually tested for manipulated behaviours, instead hypotheses are proposed, and widely accepted, without supporting evidence. This phenomenon also occurs in the pursuit of mechanistic explanation of manipulated behaviours. For example, it has been long accepted that attachment/detachment of *D. dendriticum*-infected ants is regulated solely by temperature, but it is not until recently that this hypothesis has been tested in the lab and in the field (Chapter 3; Botnevik et al., 2016; Gasque & Fredensborg, 2023). It is important to test alternative hypotheses to provide empirical support for the proposed mechanisms and adaptive significance of manipulated behaviours.

Finally, Chapters 2, 3, and 4 all took place in CHIP, which is one of few places in Canada where this parasite has been introduced and is emerging (van Paridon et al., 2017). Conducting replicate studies in the parasite's native range (Europe) would provide a valuable comparison between an introduced and native parasite system. Additionally, conducting replicate studies in other areas where the parasite has been introduced, such as on Salt Spring Island (van Paridon et al., 2017), would provide us with more information on manipulated behaviours in introduced systems.

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