Investigating hotspots of *Parelaphostrongylus* spp. transmission to moose (*Alces alces*) in Western Manitoba

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Abstract

Moose (*Alces alces*) are a conservation concern for managers in areas of Manitoba due to slower than anticipated recovery from population declines. One factor potentially affecting their recovery is infection with pathogenic protostrongylid parasites. Meningeal worm (*Parelaphostrongylus tenuis*) and muscle worm (*Parelaphostrongylus andersoni*) infect moose, but only meningeal worm is known to cause severe pathology. These parasites also infect white-tailed deer (WTD, *Odocoileus virginianus*), which are thought to be the primary hosts spreading larval parasites in the environment. In Chapter 1, we investigated the spatial and temporal variation of protostrongylid infections in WTD to determine transmission hotspots. In four game-hunting areas in Western Manitoba, we found that prevalence of *Parelaphostrongylus* spp. in WTD feces was higher in two areas where managers are concerned for moose populations. Genetic analyses of the partial cytochrome c oxidase I gene revealed that parasite species co-occurred in three areas, which extended the southern range of muscle worm in Manitoba. In Chapter 2, we measured host density and habitat type to predict where and when transmission risk was highest. We assessed the spatial and temporal variation of host density (WTD fecal pellets and gastropods) and gastropod species richness. In addition, we determined whether larval parasite prevalence in WTD feces was associated with host density or habitat type. Only gastropod species richness was associated with areas where moose populations are of conservation concern. In addition, gastropod densities were higher in late summer, particularly in grasslands and forests. Although parasites tended to be found in mixed-wood and coniferous forests, there was no association with habitat. Our results suggest that moose have higher risk of protostrongylid transmission in areas with conservation concern due to higher *Parelaphostrongylus* spp. larval prevalence in WTD and higher gastropod host species richness. Moose have a higher risk of infection in late summer due to higher gastropod host densities and species richness. Further, WTD are transmitting not one, but two parasites suggesting the importance of determining the pathology of muscle worm to moose. Conservation

efforts aimed at preventing moose declines should incorporate these higher risk areas into their management plans.

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Introduction

Pathogenic infections, including those caused by parasites, display heterogeneity in transmission across individuals, species, and environments (Paull et al. 2012). Recognizing spatial and temporal variation in wildlife disease may aid conservation efforts by helping to identify hotspots of transmission. Hotspots occur where or when transmission rate, parasite prevalence (percentage of infected hosts) and/or intensity of infection is high (parasites per infected hosts, Paull et al. 2012), but may be difficult to locate as they can be influenced by host and environmental factors. For instance, contact rates, host susceptibility, and community structure may vary depending upon the environment and season (Paull et al. 2012). Locations or seasons associated with mating, overwintering, and feeding can increase transmission by aggregating individuals and increasing contact, especially where these areas are sparse or temporally ephemeral (Altizer et al. 2003). Additionally, transmission hotspots are frequently associated with anthropogenic environmental changes, such as fragmentation and eutrophication, that alter habitat quality and territory size (Patz et al. 2004; Johnson et al. 2010).

One host-related factor that has been suggested to predict hotspots is host density (Paull et al. 2012). In particular, changes in the density of specific hosts may lead to the formation or dissolution of a hotspot. Further, particular host species may disproportionally contribute to transmission by being more susceptible to infection, having increased contact rates with parasites, and/or by being highly competent (a host wherein the parasite can reproduce efficiently, Paull et al. 2012). These species are defined as amplification hosts in disease ecology (Paull et al. 2012). Some amplification hosts can be superspreaders if they distribute parasites further than most hosts (Kilpatrick et al. 2006). Superspreaders are characterized by increased shedding rates of parasites, longer duration of infection, increased contact rates with parasites and by having a wide home range which allows them to spread parasites further than other species (Paull et al. 2012). Studying transmission heterogeneity via spatial

and temporal variation of environments and host densities may reveal where hotspots are more likely to occur and aid conservation efforts for a host population of conservation concern.

In North America, moose (*Alces alces*) populations have declined or have been extirpated in many areas such as Minnesota, North Dakota, Nova Scotia, Ontario and Manitoba (Karns 1967; Whitlaw and Lankester 1994a; Lankester 2010, 2018). There are several presumed causes of the declines including climate change, habitat loss or degradation, and parasitism. Moose are aberrant hosts for the nematode parasite meningeal worm (*Parelaphostrongylus tenuis*), also referred to as brainworm. Infections of meningeal worm in moose causes a neurological disease often referred to as "moose disease", "moose sickness", "moose neurological disease" or "cerebrospinal nematodiasis (*P. tenuis*)" (Anderson 1971). This neurological disease causes ataxia, lameness, stiffness, general and lumbar weakness, circling associated with blindness and abnormal positions of the head and neck, and finally paraplegia (Anderson 1964). Infections of meningeal worm in moose result in mortality of infected individuals directly or may predispose them to other means of mortality such as predation or vehicle collisions (Anderson 1964). Interestingly, many reports of moose population decline also noted increasing densities of deer with meningeal worm (Lankester 2018). White-tailed deer (WTD, *Odocoileus virginianus*) are the common host of meningeal worm, and the parasite is innocuous in this host making them suitable amplification hosts (Anderson 1963). The spread of meningeal worm by WTD is a concern for other ungulates as the parasite also causes neurologic disease in mule deer (*Odocoileus hemionus*), wapiti (*Cervus canadensis*), caribou (*Rangifer tarandus terraenovae*), reindeer (*Rangifer t. tarandus*), goats (*Capra aegagrus hircus*), and domestic sheep (*Ovis aries*) as well as many others (Anderson 1971). It is thus important to investigate meningeal worm infections and the role of amplification hosts such as WTD when considering causes of moose declines.

Unfortunately, studying meningeal worm comes with some challenges. One method used to determine whether WTD are infected with meningeal worm, is to directly investigate the meninges for

evidence of the adult stage. This method is invasive and must be performed post-mortem by trained personnel (i.e., not always feasible). It can also result in false negatives as the parasite is difficult to find. Further, it does not take into consideration if WTD are shedding larvae and acting as amplification hosts. Another method to determine if WTD are infected and spreading meningeal worm is to investigate their fecal samples for larvae. This method is non-invasive and ensures that WTD are acting as amplification hosts and releasing parasites into the environment. However, identifying meningeal worm at this stage of development is challenging because it is morphologically indistinguishable from other dorsal-spined elasphostrongyline larvae such as *Parelaphostrongylus andersoni* and *Parelaphostrongylus odocoilei* (Lankester and Hauta 1989). These congeneric parasites, both known as muscle worm, also infect WTD in North America, and it is often unclear whether these parasites overlap geographically with meningeal worm (Lankester 2001). *Parelaphostrongylus andersoni* is thought to have a mostly northern distribution as it is commonly associated with caribou (*Rangifer tarandus caribou*, Lankester and Fong 1989; Lankester 2001). However, some researchers claim that the distribution of *P. andersoni* occurs across the entire range of WTD as well (Lankester and Fong 1989; Gajadhar et al. 2000; Lankester 2001). Meanwhile, *P. odocoilei* infects wild cervids and bovids in Western North America but is not present in Manitoba (Lankester and Fong 1989; Gajadhar et al. 2000; Lankester 2001; Jenkins et al. 2005). Of these parasites, only meningeal worm causes neurological disease and thus far, no one has reported severe pathology from infections of *P. andersoni* or *P. odocoilei* in moose (Lankester and Fong 1989). As a result, when concerning ourselves with the health of moose, it is important to accurately identify parasites as only *P. tenuis* infection is known to be a concern for moose populations.

Knowledge of the life cycle of elasphostrongyline parasites is important to consider when making predictions about where hotspots of meningeal worm infection may occur. In Manitoba, it is most important to recognize the differences in host use between meningeal worm and muscle worm. In this study, we refer to *P. andersoni* as muscle worm as it is the only muscle worm known to be present in

WTD in Manitoba. Both species occur as adults in WTD but differ in their site specificity (Figure I). The adult stage of meningeal worm normally inhabits the nervous system, while muscle worm is found primarily in the longissimus dorsi muscle of WTD (Anderson 1963; Lankester and Hauta 1989). The first-stage larvae (L1) for both parasites are passed into the environment in the feces of the WTD (Figure I, Anderson 1971; Lankester and Hauta 1989). In the environment, these L1s must colonize terrestrial gastropods (snails or slugs) wherein they molt several times before they are infective to the next host in the life cycle (Figure I, Lankester and Anderson 1968). WTD become infected with both meningeal worm and/or muscle worm when a terrestrial gastropod, infected with a third-stage larva, is consumed (Figure I, Anderson 1963). Meningeal worm larvae penetrate the WTD's stomach wall and migrate along spinal nerves to the spinal cord (Anderson 1971). The larvae develop within the dorsal horns of the spinal column and migrate into the subdural cavity where they mature in the meninges (Anderson 1963; Anderson et al. 1966). Similar to WTD, when moose consume the infective third stage larvae of meningeal worm, the larvae migrate to the central nervous system and seem to develop at the same rate as in WTD (Figure I, Anderson 1964). In moose, the larvae remain in the spinal cord rather than moving to the meninges (Anderson 1964, 1965; Anderson et al. 1966). The larvae also coil upon themselves and cause significant traumatic damage to the surrounding nervous system (Anderson 1963, 1965; Anderson et al. 1966). In contrast, muscle worm causes both muscle and lung damage in WTD experimentally infected with large doses of third-stage muscle worm larvae (Nettles and Prestwood 1976). While moose are suitable hosts of muscle worm, It is unknown whether this parasite causes any pathology (Lankester and Hauta 1989; Verocai et al. 2020).

Meningeal worm and muscle worm transmission to moose requires several hosts and specific environmental conditions. First, an infected WTD must be present to introduce the parasites to an area. The first study of meningeal worm proposed that the occurrence of neurologic disease in moose was associated with the northward spread of WTD to areas occupied by moose (Anderson 1965). Multiple

studies were undertaken to test whether WTD density was associated with meningeal worm prevalence (Karns 1967; Behrend and Witter 1968; Gilbert 1973; Saunders 1973; Bogaczyk et al. 1993; Whitlaw and Lankester 1994b, 1994a; McGraw et al. 2021). They predicted that as WTD density increased, there would be more WTD to become infected and thus more larvae shed into the environment. More larvae in the environment could lead to an increased chance of gastropods in the area becoming infected, which would consequently infect subsequent WTD and moose. Indeed, a positive relationship was found between the incidence of meningeal worm in WTD and WTD population density in Minnesota and Maine (Karns 1967; Behrend and Witter 1968). Further, several anecdotal studies suggest that moose populations decline when infected WTD density increases (Karns 1967; Saunders 1973; Gilbert 1974; Lankester 2001, 2018). In some areas, moose densities have an inverse relationship with mean intensity and prevalence of meningeal worm larvae in WTD fecal pellets (Saunders 1973; Whitlaw and Lankester 1994b). This result suggests that perhaps WTD infection, rather than WTD density can better predict moose infection patterns. These trends, however, were not always found suggesting that the relationship between WTD density, meningeal worm infections in WTD, and moose mortality is more complex (Gilbert 1973; Bogaczyk et al. 1993; Whitlaw and Lankester 1994a; McGraw et al. 2021). An alternative hypothesis is that the geographic distribution of suitable terrestrial gastropod hosts is limiting the transmission of meningeal worm (Gilbert 1973; Whitlaw and Lankester 1994a). Unfortunately, studies comparing gastropod density to WTD infection patterns are lacking.

Both WTD and moose become infected with meningeal worm or muscle worm when an infected intermediate host, a terrestrial gastropod, is consumed (Figure I). These terrestrial gastropods become infected when they encounter the first stage larvae of either species from infected WTD feces or the surrounding soil (Figure I, Anderson 1963). Meningeal worm larvae develop within gastropods from first to third third-stage larvae in approximately 3-4 weeks with constant 18˚C temperatures, however, development would be considerably slower in natural environments due to lower overnight

temperatures (Figure I, Anderson 1963). Unfortunately, similar studies with muscle worm have not been undertaken though due to the many similarities in the life cycle, the timing of development within gastropods is likely similar. Further, comparatively less is known about suitable hosts for muscle worm. Muscle worm is known to use two potential intermediate hosts, the snail *Triodopsis multilineata* in the lab and the slug *Deroceras laeve* in nature (Pybus and Samuel 1984). In contrast, natural infections of meningeal worm have been found in several snail and slug species such as *Arion circumscriptus, Deroceras laeve, Deroceras reticulatum, Deroceras gracile, Philomycus carolinianus, Discus whitneyi, Zonitoides arboreus, Zonitoides nitidus, Succinea ovalis, Cochlicopa spp., Anguspira alternata, Strenotrema fraternum, Pallifera dorsalis, Ventridens collisella, Ventridens intertextus, Triodopsis tridentata,* and *Triodopsis albolabris* (Anderson 1963; Lankester and Anderson 1968; Rowley et al. 1987; Platt 1989; Nankervis et al. 2000; Maze and Johnstone 2008). The native common slug, *D. laeve*, is a known host for both parasites and may be an amplification host as it is thought to be especially important for transmission due to its widespread abundance and ability to be active in a wide range of temperatures (Lankester and Anderson 1968). As many gastropod species are limited by environmental conditions, transmission of meningeal worm to moose is thus limited to conditions suitable for terrestrial gastropods.

Investigating habitat and microhabitat conditions that support high densities of terrestrial gastropods may reveal where transmission is more likely to occur. Overall, gastropod abundance seems to be highest in natural wood-lands compared to other habitats, likely due to their cooler temperature (Lankester and Anderson 1968; Hawkins et al. 1997a; Maze and Johnstone 2008; Maskey et al. 2015). Consistent with previous hypotheses, Lankester and Anderson (1968) found higher gastropod abundance in low, damp forest habitats compared to dry, elevated forest or open grasslands. Alternatively, Maze and Johnstone (2008) found highest gastropod abundance in a grassy opening within a dry, elevated forest. It was hypothesized that the high calcium and neutral pH of the soil in the

area may be conducive to high numbers of gastropods (Maze and Johnstone 2008). However, differences in gastropod abundance were found at three locations with similar pH (~6) and high calcium, suggesting that pH and calcium alone cannot be used to predict gastropod numbers (Platt 1989). More work is thus needed to discern areas where high densities of gastropod hosts may occur.

Environmental conditions also affect the transmission of meningeal worm, causing hotspots for transmission, by mediating larval abundance and survival. Decreased winter duration and severity, as well as increased growing season wetness and length, facilitate the transmission of meningeal worm (Lankester 2018). Decreased winter duration and severity is associated with increased WTD survival, especially fawns and yearlings which pass double to triple the number of larvae into the environment (Slomke et al. 1995; Peterson et al. 1996). Increased numbers of WTD may increase density and larval parasite output as well as possibly increase the overlap between moose and WTD (Lankester 2018). Further, infected WTD pass triple the number of larvae in spring than other times of the year (Slomke et al. 1995; Peterson et al. 1996). These larvae can survive constant freezing temperatures for several months, with 70% of larvae surviving freezing for six months, unless frequent freeze-thaw cycles occur (Lankester and Anderson 1968; Shostak and Samuel 1984). Cool, wet conditions in spring and fall are associated with higher larval survival and they facilitate gastropod abundance and activity both of which may increase chances of intermediate host infection (Burch 1962; Lankester and Peterson 1996; Hawkins et al. 1997a). WTD and moose are thus most likely to become infected in spring, from larvae that overwintered in a gastropod or in fall when gastropods infected in spring would be active (Lankester and Anderson 1968). Cool, wet conditions thus influence the temporal variation in transmission by increasing shedding rates and contact rates between hosts. These conditions may also influence the spatial variation in transmission.

More WTD are infected with *Parelaphostrongylus* spp. larvae in habitats with cooler upland mixed conifer forests and shrubland than warmer upland deciduous forests (Vanderwaal et al. 2015). The

results of this study were compared with gastropod habitat preferences of the same area, which revealed that the same habitats with more infected WTD also had higher gastropod abundance (Cyr et al. 2014; Vanderwaal et al. 2015). While this study claims these larvae were meningeal worm, no genetic work was done to identify larvae. Further, few studies have concurrently collected gastropods and investigated WTD infection in the same locations at the same time while documenting habitat cover differences. These investigations could shed light on areas where hotspots for transmission occur and where moose are most vulnerable to these parasitic infections.

The aim of this research was to investigate risk of meningeal worm and muscle worm infection to moose in Western Manitoba. Moose populations became a concern in areas of Manitoba when aerial surveys found indicators of declines in moose population such as reduced bull/cow and calf/cow ratios, indicating that moose populations were under stress (Blaikie 2011). The province then implemented moose hunting restrictions and cancelled hunting in several areas. Since then, there is still concern for moose populations in these areas (Wildlife and Fisheries Manitoba 2020). Moose hunting closures remain because although populations are stable, or are increasing in some areas, moose numbers are still low, and recovery has been slower than anticipated (Wildlife and Fisheries Manitoba 2020). It is therefore important to determine whether parasites are associated with these areas of concern and possibly contributing to the slow recovery of moose in these areas. The life cycles of these parasites are complex and investigating one or two variables has not been sufficient to reliably predict where transmission is likely to occur. In this study, we sampled meningeal worm and muscle worm in amplification hosts; WTD and gastropods and investigated the corresponding host densities and habitat composition of the sampled areas to determine transmission hotspots.

In the first chapter, we investigated the spatial and temporal variation of DSL prevalence in WTD in areas of Western Manitoba. We determined whether DSL prevalence in WTD were associated with areas of moose population concern. Further, we used the genetic identification of the DSL species to

update the known geographic range of meningeal worm and muscle worm in Manitoba. This work will provide novel insights into the geographic ranges of *P. andersoni* in Manitoba as this has previously been overlooked. If we know where and when moose are most at risk for DSL transmission, we can aim management initiatives to protect moose where they are most vulnerable.

In Chapter 2, we delve into the host and habitat conditions that contribute to transmission risk. We assessed the spatial and temporal variation of host density (WTD fecal samples and gastropod) to explain where and when transmission risk to moose is highest. We also determined the habitat that gastropods and DSL were more likely to be found in. Last, we determined whether host (WTD or gastropod) density or various habitat types could predict prevalence of DSL in WTD. The results of this study will give insight into how to predict infections of meningeal worm to moose. With this information, management initiatives aimed at conserving moose can take proactive approaches to protect vulnerable moose populations.

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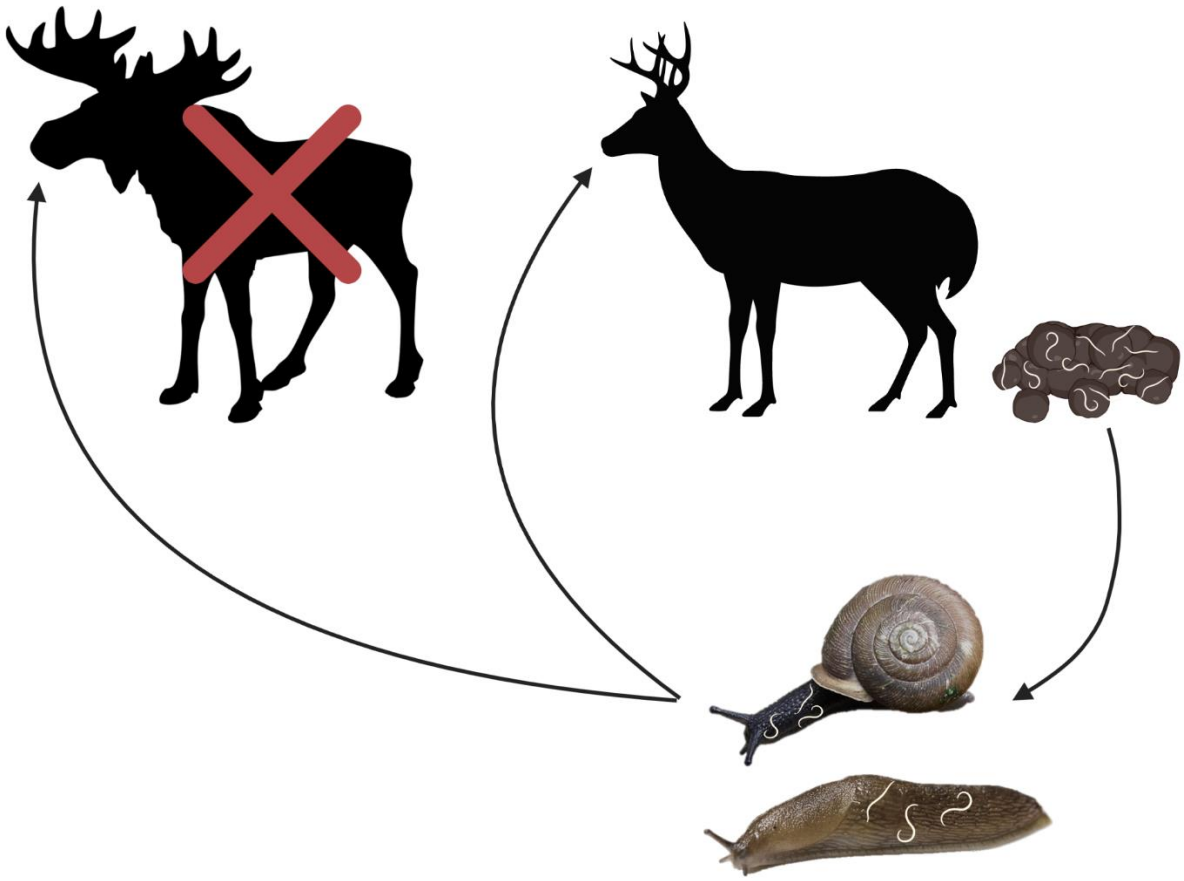

Figure I Simplified life cycle of meningeal worm (*Parelaphostrongylus tenuis*) in white-tailed deer (*Odocoileus virginianus)* and moose (*Alces alces*). White-tailed deer become infected when gastropods infected with stage 3 larvae are consumed. Adult worms lay eggs within the meninges of the deer and first stage larvae are released in the feces. Gastropods become infected when they encounter stage 1 larvae on feces or the surrounding soil. Infections of meningeal worm in moose cause severe morbidity and mortality. Created with BioRender.com.

Chapter 1: *Parelaphostrongylus* spp. larvae prevalence is associated with moose population declines in Western Manitoba

Abstract

In areas of Manitoba, moose (*Alces alces*) populations are a conservation concern and infections of meningeal worm (*Parelaphostrongylus tenuis*) may be contributing. Moose are aberrant hosts and experience severe pathology whereas white-tailed deer (WTD, *Odocoileus virginianus*), the common host, experience less pathology and are responsible for spreading larvae into the environment. Meningeal worm prevalence from hunter-harvested WTD heads suggests that moose populations are at higher risk of infection where populations are a conservation concern. However, the shedding rates of larval meningeal worm from WTD in these areas is unknown, particularly because the dorsal-spined larvae (DSL) are morphologically indistinguishable from muscle worm (*Parelaphostrongylus andersoni*). To determine transmission hotspots in Western Manitoba, we investigated the spatial and temporal variation of DSL prevalence in WTD feces (i.e. shedding rate) from four game hunting areas (GHA); two where management has concern for moose populations and two where there is no concern. We hypothesized that moose would be at higher risk of infection where and when there are higher shedding rates. Further, we expected to only recover meningeal worm, as muscle worm has only been reported from more northern areas of Manitoba. Over two years, we collected WTD feces three times throughout the summer along two 700 m transects from six locations in each GHA. We obtained larvae through fecal sedimentation and sequenced the partial cytochrome c oxidase I gene to confirm species identity. Zero-inflated models showed that DSL prevalence did not differ temporally but was higher in GHAs where there is conservation concern for moose. Genetic analyses of a subset of DSL collected (n = 19) revealed that meningeal worm and muscle worm were both present in Western Manitoba and co-occurred at three of the four GHAs. Our results reveal novel insights for the geographic distribution of muscle worm and emphasize the importance of using genetic analyses to identify DSL in WTD fecal

pellets. Our results suggest that risk of parasite infection is higher where there is conservation concern for moose populations so conservation efforts aimed at preventing moose declines in Western Manitoba should incorporate transmission risk of meningeal worm into management plans.

1.1 Introduction

In Manitoba, there is growing concern about the sustainability of moose (*Alces acles*) due to population declines in 2010 and slower than anticipated recovery (Blaikie 2011; Wildlife and Fisheries Manitoba 2020). Moose populations may be negatively impacted by predation, hunting, poaching, increased roads that facilitate higher hunter and predation success, as well as parasites and disease associated with the presence of white-tailed deer (WTD, Committee for Cooperative Moose Management 2017). In particular, meningeal worm (*Parelaphostrongylus tenuis*) is a parasite of concern due to it's potential to severely impact moose health (Anderson 1964). Infections of meningeal worm cause cerebral-spinal nematodiasis, often referred to more simply as neurological disease or "moose sickness" (Anderson 1964). Infected moose exhibit weakness of the hind limbs, blindness and a circling behaviour with infections often ending in paralysis of the hind limbs (Anderson 1964). As a result, infections of meningeal worm result in mortality and contribute to moose population declines in some areas (Anderson 1964). Another parasite, muscle worm (*Parelaphostrongylus andersoni*) is also present in Manitoba and is morphologically indistinguishable from meningeal worm larvae (Lankester and Hauta 1989). The L1 stage of both parasites have morphologically similar dorsal-spines and are therefore referred to as dorsal-spined larvae (DSL, Lankester and Hauta 1989). Currently, much less is known about muscle worms and their effects on moose health although moose are suitable hosts for muscle worm (Lankester and Hauta 1989; Verocai et al. 2020). As both parasites are present in Manitoba, and potentially contributing to moose population conservation concerns, it is important that we understand where they occur to determine where moose are most at risk of infection.

Given the severe pathology of meningeal worm and unknown pathology of muscle worm in moose, a clear understanding of the parasite species distributions is important. However, at present the extent of overlap in their distributions is unclear (Lankester 2001). The geographic distribution of meningeal worm is more well known, especially where the parasite overlaps with moose, and is found throughout Southeastern North America (Lankester 2001). On the other hand, muscle worm has a patchy distribution throughout North America with some conflicting reports of its geographic extent (Lankester 2001). Some researchers claim that its distribution is consistent with the range of white-tailed deer (*Odocoileus virginianus*, WTD), while others speculate that its distribution is more consistent with caribou (*Rangifer tarandus terraenovae*) and found inconsistently throughout the range of WTD (Lankester and Fong 1989; Gajadhar et al. 2000; Lankester 2001). Concurrent infections of meningeal worm and muscle worm in WTD have been found, although rarely (Prestwood et al. 1974; Lankester and Hauta 1989). It's unknown whether this rare co-occurrence is due to inadequate sampling of muscle worm, lack of genetic work to confirm morphologically indistinguishable larvae in ungulate feces, or as a result of competition between the parasite species. Overall, comparatively little is known about muscle worm in comparison to meningeal worm. Indeed, meningeal worm larvae are known to develop within 18 gastropod host species while muscle worm has only been found in two (Anderson 1963; Lankester and Anderson 1968; Pybus and Samuel 1984; Rowley et al. 1987; Platt 1989; Nankervis et al. 2000; Maze and Johnstone 2008). However, we do know that WTD disproportionally release both species of DSL throughout the year with more larvae released in spring and summer (Lankester and Hauta 1989; Peterson and Lankester 1991; Slomke et al. 1995). Overall, further research is needed to determine whether these parasites have overlapping distributions and different transmission dynamics. One way to address these issues is to investigate the spatial and temporal variation of DSL output of WTD.

Both meningeal worm and muscle worm are commonly found in WTD. Relative to its negative pathology in moose, meningeal worm is an innocuous parasite within WTD (Anderson 1963; Anderson

1964). In contrast, muscle worm only causes clinical illness in WTD with very high intensity infections (Nettles and Prestwood 1976). Therefore, for moose to become infected with meningeal worm and/or muscle worm, there must be infected WTD present shedding larvae in their feces as moose are aberrant hosts. Moose are therefore more at risk of infection with DSL where WTD are present and at an even higher risk if prevalence of meningeal worm and/or muscle worm in WTD is high and many larvae are being passed into the environment (Saunders 1973; Whitlaw and Lankester 1994b; Lankester 2018). In Western Manitoba, the edge of where meningeal worm has previously been documented, WTD heads collected (2018-2019) from the southern border with the US to as far north as The Pas (49.0024°N - 53.8263°N) have confirmed the presence of meningeal worm with prevalence ranging from 7-55% (10/138 – 17/31) in management areas (Province of Manitoba Fish and Wildlife Branch, unpublished data). In northern Manitoba, near Norway House (53.9821°N), caribou (*Rangifer tarandus*) fecal samples contained DSL identified as muscle worm using ITS-2 DNA sequences (Verocai et al. 2020). Muscle worm is therefore present in northern Manitoba, although no genetic testing has confirmed its presence further south than 53.9821°N. Thus, using DNA sequences to confirm the species of DSL present in WTD feces will be key in confirming the geographic extent of these two species and in estimating the frequency of co-occurrence. While the geographic extent of these parasites helps predict where the parasites occur, it provides little information about when hotspots of transmission are of greatest concern. Understanding the temporal variation (within and across years) in parasite shedding rates and contact rates with the hosts also helps to understand when transmission risk to moose may be highest.

In this chapter, we assessed the regional spatial and monthly temporal variation of DSL infections in WTD fecal samples from Western Manitoba. Specifically, we determined whether DSL prevalence was associated with areas where moose are of conservation concern to investigate whether risk of parasite transmission is associated with conservation status. In Manitoba, moose populations are estimated and managed within the bounds of game hunting areas (GHA). GHAs are defined under the

Hunting Areas and Zones Regulation (220/86) of The Wildlife Act (CCSM c. W130). In several GHAs all licensed moose hunting has been cancelled because populations are not at the desired levels (Blaikie 2011). We predicted that there would be higher DSL prevalence in WTD fecal samples where moose populations are a concern (GHA 13 and 18), as an indication that parasites may be negatively affecting moose populations in these areas. We also determined whether DSL prevalence differed temporally throughout collection in the summer season (~June – September) over two years. Differences in shedding rates between seasons of the year (i.e., between spring, summer, fall and witner) have been found with higher rates in spring and summer than fall and winter, but information is lacking about differences in DSL prevalence within summer months (Lankester and Hauta 1989; Peterson and Lankester 1991; Slomke et al. 1995). This information will demonstrate that WTD are not just infected with adult parasites, but where they are actively shedding the larvae into the environment. Further, we used genetic analysis of the cytochrome c oxidase 1 gene (*CO1*) to determine the species identity of the DSL present in WTD feces. DNA sequencing of DSL will confirm whether two species of *Parelaphostrongylus* are present in WTD in Western Manitoba and provide novel insights into the southern extent of the geographic ranges of *P. andersoni* in Western Manitoba. We predicted that DSL would be genetically confirmed as meningeal worm as adult meningeal worm was present in WTD sampled in 2018-2019 in all the areas we sampled (Province of Manitoba Fish and Wildlife Branch, unpublished data). We did not expect to find muscle worm as it has previously only been documented in Northern Manitoba (53.9821°, Lankester 2001; Verocai et al. 2020). This research will provide essential information about the transmission risk to moose in Manitoba. If we know where and when moose are most at risk for DSL transmission, we can aim management initiatives to protect moose where they are most vulnerable to infection.

1.2 Methods

1.2.1 Field collection

To determine DSL prevalence in WTD, fecal pellets were collected in four game hunting areas (GHA) in Western Manitoba. In game hunting area 13 and 18, moose are a conservation concern as their populations are below desired levels according to provincial managers (Wildlife and Fisheries 2020). As a result, all licensed hunting has been restricted in these GHAs (Blaikie 2011). In contrast, there is no restricted license hunting of moose in GHA 22 and 27, so we denote them as GHAs of no conservation concern. In our study, one game hunting area of concern and no concern were sampled in each year. GHA 18 and 22 were sampled in 2020 followed by 13 and 27 in 2021, respectively (Figure 1.1). Both GHAs of conservation concern, GHA 13 and 18, are more northern and encompass Porcupine Provincial Forest (52.5250° N) and Duck Mountain Provincial Park (51.8366° N) respectively. These GHAs are mostly composed of forested land with estimated proportions of 70.3% and 80.4% forested land respectively (Annual Crop Inventory 2019). In contrast, the GHAs of no conservation concern are more southern, GHA 22 (50.1956° N) and 27 (49.5513° N) and consist of 65.3% and 66.7% developed land (Annual Crop Inventory 2019). The prevalence of DSL in WTD feces was compared between GHAs where moose populations are a conservation concern and not a concern. This comparison indicated whether the risk of infection to moose is associated with the status of the moose populations (concern and not a concern) and sheds light on where risk of transmission is highest. To evaluate whether the time of year influences DSL prevalence and therefore when infection risk to WTD and moose is highest, each GHA was sampled three times; from June to September in 2020 and from June to August in 2021.

Within each GHA, six locations were sampled (Figure 1.1). When sampling a provincial park or provincial forest, locations were picked randomly *a priori* using the function “Create random point” in ArcGIS pro 2.7.3 (ESRI 2020). If a location was inaccessible, it was adjusted to be situated nearest to the closest road. Alternatively, when sampling outside of a provincial or national park, we sampled within

wildlife management areas. We numbered all the wildlife management areas in the GHA and used a random number generator to select six wildlife management areas to sample. If a management area was a lake or had no road access, we picked another number. Within each location, there were two transects for WTD pellet sampling (Figure 1.1). Terrestrial transects were preferentially set where there were WTD trails to ensure adequate samples of WTD feces. Terrestrial transects were ~714 meters and consisted of four WTD pellet plots (21m x 3m, Vanderwaal et al. 2015). We aimed to have a minimum of 200m between WTD pellet plots to decrease chances of sampling the same WTD. When possible, there was at least 100m between transects. Within WTD pellet plots, all fecal pellet groupings of >10 pellets were collected for analysis of parasites (Vanderwaal et al. 2015). Each pellet grouping was placed in a separate plastic bag and kept cool or frozen until analysis.

1.2.2 Processing field collections

The collected WTD fecal pellets were examined for DSL using the modified Baermann technique from Forrester and Lankester (1997). All fecal samples collected in 2020 were examined for DSL (n = 55) and in 2021 we assessed 72% (239/333) of the fecal pellet samples for parasites. Due to the large number of fecal samples in 2021, if a single quadrat had more than 10 fecal samples collected, we examined at least half of them for DSL. First, all pellets per fecal sample were counted and weighed. Ten pellets (or approximately 10 when fecal samples were clumped) were weighed and placed in a mesh envelope (made with window screening and staples) and submerged in a 300 mL beaker of water for 24 h (Forrester and Lankester 1997). After 24 h, the envelope with fecal pellets was removed and the solution was left to sit for 1h to allow the nematodes to sink to the bottom (Forrester and Lankester 1997). After 1 h, nine 1 mL aliquots of the water from the bottom of the beaker were examined with a stereomicroscope at 1.6 or 2.5x objective (Forrester and Lankester 1997). When nematode larvae were found, they were moved to a slide and examined with a compound microscope and a 10x objective to

determine if a dorsal spine was present. All DSL were preserved in 80% ethanol and stored at -20 ˚C prior to molecular identification.

We digitally imaged up to 3 DSL per fecal sample using a Zeiss AxioImager M2 (Zeiss Canada Ltd., Toronto, Canada) with the 40X objective. After image vouchering, each DSL was washed three times in 300 µl of homemade lysis buffer (50 mM KCL, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% Nonidet p-40, 0.45% Tween-20, 0.01% gelatin Verocai et al. 2020) to remove ethanol from the tissue. To degrade DSL sheaths before lysis, individual larva in 300 µl homemade lysis buffer were heated at 95°C for 15 min in a thermomixer set at 1000rpm followed by incubation at -80°C for one hr. Lysis was performed by adding proteinase K (1.2 mg/ml) and incubating at 55°C at 700rpm overnight. Proteinase K was inactivated with a 25 min incubation 95°C and DNA was purified by following the directions of the QiaAMP kit (Qiagen). We amplified ~800 bp of the cytochrome c oxidase I gene (*CO1*) using the forward primer PtCO1-F (5'-GGTTGGAGAGTTCTAATCATAAAGA-3') and a degenerate reverse primer aVeCo1 (5'-CAACAGTATAYATATGRTGRGCC-3'). We used a similar PCR protocol described in Verocai et al. 2020, but with a 20µl reaction that consisted of 11.6µl of sterile water, 4µl of 5X Buffer + 7.5mm $MgCl_2$, 0.4µl dNTPs, 0.4µl of both primers, 0.2µl of Phusion Taq and 3µl of DNA sample. The amplification was performed in a thermocycler with the following conditions: 98°C for 2 min, 35 cycles of 98°C for 20 s, 50.5°C for 30 s, 68°C for 40 s and, a final 68°C for 7 min. We used electrophoresis to visually confirm single bands in a 2% agarose gel. If bands were not very bright, we redid the PCR using the same procedures as described above except with 5µl DNA or performed PCR on the same DNA three times and combined the products during PCR clean-up. We purified PCR products with the GeneJet PCR purification kit (Thermo Fischer Scientific, Waltham, Massachusetts) and sequenced the DNA at the DNA sequencing and Synthesis Facility at Sick Kids Hospital. Contigs were constructed in Sequencher® 5.4.6 (Gene Codes Corporation, Ann Arbor, MI USA), aligned in MEGAX (Kumar et al. 2018), and compared to GenBank accessions using the blastn algorithm.

1.2.3 Statistics

Parasite prevalence often has an aggregated distribution with many hosts having no parasites or low parasite intensity (number of parasites per infected host), while a few hosts have high parasite intensity, resulting in zero-inflated distributions (Zuur et al. 2009). Accordingly, we used a zero-inflated regression model with a log link function to determine if DSL prevalence differed by conservation status (concern or no concern for moose populations), sampling year, and collection trip (early, middle and late summer). We included an interaction term between year and trip to determine whether the trend across collection parts of the summer differed by year. We used DSL abundance as the dependent variable as zero-inflated models require count data. We then offset the variable by the log-transformed number of fecal samples processed. Adding the offset to the model allows us to use zero-inflated regression to model count data with Poisson or negative binomial distributions as rates (offset by time) or densities (offset by area Zuur et al. 2009). In this case, we are modelling prevalence (the proportion of infected samples) rather than abundance of DSL as the number of fecal samples processed differed by transect and is important to account for. We analyzed according to collection trip, rather than by month of collection, because the length of the field sampling differed for each year; in 2020 we sampled from June – September, while in 2021 we sampled June – August. In both years, trips were spaced out evenly according to the length of the field sampling and thus differed for each year. Nonetheless, "Early summer" for both years was June, "Midsummer" was in July/August 2020 and July 2021, and "Late summer" was in September 2020 and August 2021. In the final dataset, we removed transects where no fecal samples were collected. We ran zero-inflated models with Poisson and negative binomial distributions then used a likelihood ratio test to determine which distribution was a better fit (Zuur et al. 2009). Chi-square tests were run to determine which variables were significant in the final models (Mangiafico 2016), we calculated the variance inflation factor to ensure that factors had no multicollinearity (i.e. values <5, Zuur et al. 2007). We calculated McFadden's pseudo-R^2 to determine

how well the model explained the variability in the response variable, using a value of 0.2-0.4 as an indicator of good fit (Louviere et al. 2000). All statistics were done in R version 4.1.2 “Bird Hippie” (R Core Team 2021). The zero-inflated models were run with the packages “readxl”, “multcompView”, “emmeans”, “rcompanion”, “car”, “pscl” and “lmtest” (Fox and Weisberg 2019, Graves et al. 2019, Lenth 2022, Mangiafico 2022, Wickham and Bryan 2019, Zeileis et al. 2008, Zeileis and Hothorn 2002). Column plots to visualize the data were made using “ggplot2” (Wickham 2016).

1.3 Results

In 2020 we found 5 fecal samples with DSL. There was 7% (1/15) prevalence from GHA 22 and 10% (4/40) prevalence from GHA 18. In 2021, we found 23 samples with DSL. GHA 13 had 33% (9/27) prevalence and GHA 27 had 7% (14/212) prevalence of DSL. The zero inflated model revealed that DSL prevalence differed by conservation status (P = 0.02, Table 1.1). DSL prevalence was higher where there is concern for moose populations (Figure 1.2, red). DSL prevalence did not differ by collection trip, by year sampled, or the trip*year interaction (Table 1.1). McFadden’s R^2 was 0.21 indicating that our model adequately explained variation in DSL prevalence.

We found infected WTD fecal pellets in all GHAs sampled and in 50% (12/24) of all locations sampled within the GHAs (Figure 1.3). From our DNA sequencing, we obtained *CO1* sequences (502-843 bp) for 19 DSL collected from 11 fecal samples (Table 1.2). The blastn search indicated a >90percent identity to *P. tenuis* and *P. andersoni* accessioned in GenBank (Table 1.2). We found 6 DSL were most genetically similar to *P. tenuis* (97-100 percent identity with 90-99% query coverage). The GenBank accessions originated from Maryland, USA (e.g., EF173722-EF173723). Thirteen DSL were most genetically similar to *P. andersoni* (93-94 percent identity with 95-99% query coverage. The GenBank accessions were collected from Oregon and Washington, USA (e.g., EU052277- EU052280, EU029987-EU029987). We found *P. tenuis* inall GHAs and *P. andersoni* from GHA 13, 18 and 27 (Figure 1.3).

Further, in 2/11 fecal samples from which worms were sequenced, we found co-infections of *P. andersoni* and *P. tenuis* (Table 1.2).

1.4 Discussion

Our study shows that DSL prevalence was positively associated with areas of moose population concern in the four GHAs examined in Western Manitoba, likely due to the higher prevalence of DSL in GHA 13, located in Porcupine Provincial Forest. These trends are consistent with the higher prevalence of adult meningeal worm in WTD heads in northern GHAs (13 and 18) compared to southern GHAs (22 and 27). Our results confirm that WTD are shedding more DSL into areas where managers are concerned about moose populations. At present, more work is needed to determine whether there are differences in the shedding rates between the species of DSL. Our current sampling can only confirm the presence of both species as more than three samples per fecal pellet would need to be processed to estimate the abundance of either species. For conservation efforts, quantifying the risk of infection of *P. tenuis* to moose is important given the severe pathology that occurs in moose. At present, less is known about the pathology of *P. andersoni* in moose, so how much of a risk *P. andersoni* is to the conservation status of moose is unclear.

We did not find a temporal difference in DSL prevalence across trips in the summer. The lack of a temporal variation within a summer and across years indicates that infected WTD shed larvae into the environment consistently during this season. Although the life-span of muscle worm is unknown in WTD, meningeal worms can live at least 3.7 years in WTD creating a stable population of adult worms that shed their larvae into the environment in the summer (Duffy et al. 2002). Some studies have found differences in DSL prevalence among years though this difference has been attributed to fawns that first become infected in the Fall, and then increase DSL prevalence in the subsequent seasons in the next year (Peterson et al. 1996). The lack of temporal variation in DSL prevalence could be due to the low prevalence of DSL in fecal pellets in our study (7-33%). As a result, we may not have been able to detect

differences among the parts of the summer. One factor that could affect DSL prevalence is the age of the fecal pellet though to our knowledge there are no studies quantifying this effect in summer (Forrester and Lankester 1998). However, it is predicted that prevalence of older feces will have fewer DSL than fresh feces. Regardless of age, we collected all fecal pellets in our plots to obtain as many fecal samples as possible. DSL prevalence, as estimated from fecal pellets, was lower than the prevalence of adult meningeal worm found within WTD heads from Agriculture and Resource Development, Manitoba in the same areas. The difference in prevalence between the L1 stage and adult stage is likely biological, rather than a sampling error. The shedding rate of DSL by WTD is not consistent throughout the life of the adult parasite or even throughout the year (Peterson et al. 1996). Further, these parasites are dioecious and require at least two individuals to be present in WTD for reproduction to occur. The WTD head results may thus over-estimate the impact that meningeal worm is having in these areas as not all parasites may be shedding larvae into the environment increasing risk for subsequent hosts.

Although we found a positive association between DSL prevalence and GHA status, not all DSL collected were meningeal worm, the cause of moose neurological disease. Genetically confirming the presence of muscle worm in WTD was surprising as there were no reports of muscle worm in the areas we sampled. Our results improve several aspects of our knowledge about *P. andersoni* as it has not been well studied (Lankester and Hauta 1989; Nettles and Prestwood 1976). First, we found muscle worm in more southern locations in Manitoba than previously reported, including as far south as Melita, MB (49.1548° N, Lankester and Hauta 1989; Lankester 2001). Second, it appears that meningeal worm and muscle worm have similar distributions in Western Manitoba as we found the two species co-occurred at 58% (4/7) of locations from DSL with w genetic sequences. These locations include our most northern location (52.7215° N) and one of our most southern locations (49.1548° N). At present, inferences about co-occurrence from genetically identified individuals are limited as DNA sequencing from more individuals per pellet is needed to determine whether a species is truly absent. Third, they

are both found in WTD suggesting that moose may become infected if they are consuming the same pool of infected gastropod hosts as WTD. Our results confirm that researchers and managers using WTD fecal pellets to estimate prevalence of DSL should confirm species identity using genetic sequences. Our study exemplifies that although muscle worm may not have been previously reported in an area, it may be present and if unrecognized leads to overestimation of the shedding rate of meningeal worm.

Documenting the co-occurrence of these two parasites at GHAs suggests more investigations are needed to estimate co-infection. Co-infection of meningeal worm and muscle worm was reported to be rare (Prestwood et al. 1974; Lankester and Hauta 1989). It is unknown whether these parasites were rarely found together because of competition or due to muscle worm being overlooked as it is labor intensive to locate the worms in the musculature (Nettles et al. 1974). However, our study found that 18% (2/11) of the fecal samples that had DSL sequenced had co-infections of meningeal worm and muscle worm. To estimate co-infection, more worms from each fecal pellet should be sampled to determine if co-infections are indeed rare or just previously overlooked by focusing on locating adults. Our work only sequenced up to 3 DSL per fecal sample, which is insufficient to determine if both larval species were present in all samples as we found between 0.02-5.04 larvae/mL/g WTD feces. Future work should be done to identify more larvae per fecal samples to determine the relative abundance of each species that are shed in fecal samples. The presence of one species may be masked by higher quantities of the other in fecal samples. Future studies could estimate species accumulation curves for individual fecal samples or geographic areas to determine the ratios at which each parasite is shed and how many larvae should be genetically identified to determine whether both species are present.

It is currently unknown whether muscle worm infections cause symptoms in moose. Future research is therefore needed to determine whether muscle worm causes pathology in moose and whether management should account for this risk when assessing the sustainability of moose populations. Higher DSL prevalence in WTD fecal samples from areas where moose populations are a

conservation concern may suggest that moose are at higher risk of meningeal infection in these areas. In addition, increased shedding rates could result in increased contact rates with gastropod hosts, which would increase the chances of moose infection. Meningeal worm and muscle worm appear to use at least one of the same gastropod hosts (*D. laeve*), so if transmission risk is high for one parasite, it could also be high for the other if the parasite is introduced to the area (Lankester and Anderson 1968; Lankester and Hauta 1989). As moose are directly infected by consuming infected gastropod hosts, estimating contact rates between the L1 stage in the fecal pellets and the gastropod is required to predict when and where there are hotspots of meningeal worm and muscle worm transmission Western Manitoba.

1.5 Conclusions

This study highlights the need to genetically identify DSL found in WTD fecal pellets to confirm whether moose are at risk for *P. tenuis* transmission. We demonstrated that WTD in Western Manitoba are also infected with muscle worm and that this parasite has a more southern distribution throughout Manitoba than previously reported. Further, we confirm that meningeal worm and muscle worm co-occur in most of the GHAs we sampled including the most northern and southern locations. It is currently unknown how muscle worm affects moose health, but high intensity infections in WTD result in muscle and lung damage. Meningeal worm on the other hand, causes severe morbidity and mortality to moose. Understanding the distributions of these parasites throughout Manitoba is essential when planning management initiatives to conserve moose populations. We conclude that parasite prevalence in WTD fecal samples is associated with areas sampled with moose population concern in Western Manitoba and conservation efforts should focus initiatives on areas where moose are most at risk of infection.

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Table 1.1 Dorsal-spined larvae prevalence differed by game hunting area status (concern vs. no concern for moose populations) according to a zero-inflated negative binomial model. Bolded *P* are significant (<0.05)

	Degrees of freedom	X^2	*P*
Status	**1**	**5.31**	**0.02**
Year	1	0.17	0.68
Trip	2	1.10	0.58
Trip*Year	2	0.67	0.71

Table 1.2 *Parelaphostrongylus tenuis* and *Parelaphostrongylus andersoni* co-occur at three of the four game hunting areas (GHA) sampled in Western Manitoba. Results of blastn search conducted on 2/23/2022 to genetically identify *Parelaphostrongylus* L1s from white-tailed deer feces by comparisons to GenBank accessions (our cytochrome c oxidase I gene sequence lengths in base pairs (bp) specified in brackets). The ranges for query coverage and percent identity are reported for accessions that were most similar to sequences obtain in this study.

Sampling area	Parasite ID [sequence length bp]	GenBank Match	Query coverage (%)	Percent Identity (%)
GHA 13*	*Parelaphostrongylus* sp. 1 [843 bp]	*Parelaphostrongylus andersoni*	90-96	99-100
	Parelaphostrongylus sp. 2[502 bp]	*Parelaphostrongylus tenuis*	99-99	93-93
	Parelaphostrongylus sp. 3[836 bp]	*Parelaphostrongylus andersoni*	90-96	99-100
	Parelaphostrongylus sp. 4[811 bp]	*Parelaphostrongylus andersoni*	90-96	99-99
GHA 18*	*Parelaphostrongylus* sp. 5[778 bp]	*Parelaphostrongylus andersoni*	92-98	99-99
	Parelaphostrongylus sp. 6[778 bp]	*Parelaphostrongylus andersoni*	92-97	99-100
	Parelaphostrongylus sp. 7[805 bp]	*Parelaphostrongylus tenuis*	95-95	93-93
	Parelaphostrongylus sp. 8[798 bp]	*Parelaphostrongylus andersoni*	91-97	97-97
	Parelaphostrongylus sp. 9[787 bp]	*Parelaphostrongylus andersoni*	94-99	99-100
	Parelaphostrongylus sp. 10[782 bp]	*Parelaphostrongylus andersoni*	93-98	99-100
	Parelaphostrongylus sp. 11[772 bp]	*Parelaphostrongylus andersoni*	93-99	97-97
GHA 22	*Parelaphostrongylus* sp. 12[796 bp]	*Parelaphostrongylus tenuis*	95-95	93-94
	Parelaphostrongylus sp. 13[775 bp]	*Parelaphostrongylus tenuis*	98-98	93-93
GHA 27	*Parelaphostrongylus* sp. 14[806 bp]	*Parelaphostrongylus andersoni*	90-96	99-100
	Parelaphostrongylus sp. 15 [807 bp]	*Parelaphostrongylus tenuis*	94-94	93-93
	Parelaphostrongylus sp. 16[841 bp]	*Parelaphostrongylus andersoni*	90-96	99-100
	Parelaphostrongylus sp. 17[806 bp]	*Parelaphostrongylus andersoni*	90-96	99-99
	Parelaphostrongylus sp. 18[808 bp]	*Parelaphostrongylus tenuis*	95-95	93-93
	Parelaphostrongylus sp. 19[836 bp]	*Parelaphostrongylus andersoni*	90-96	99-100

*Game hunting areas (GHA) with conservation concern for moose populations

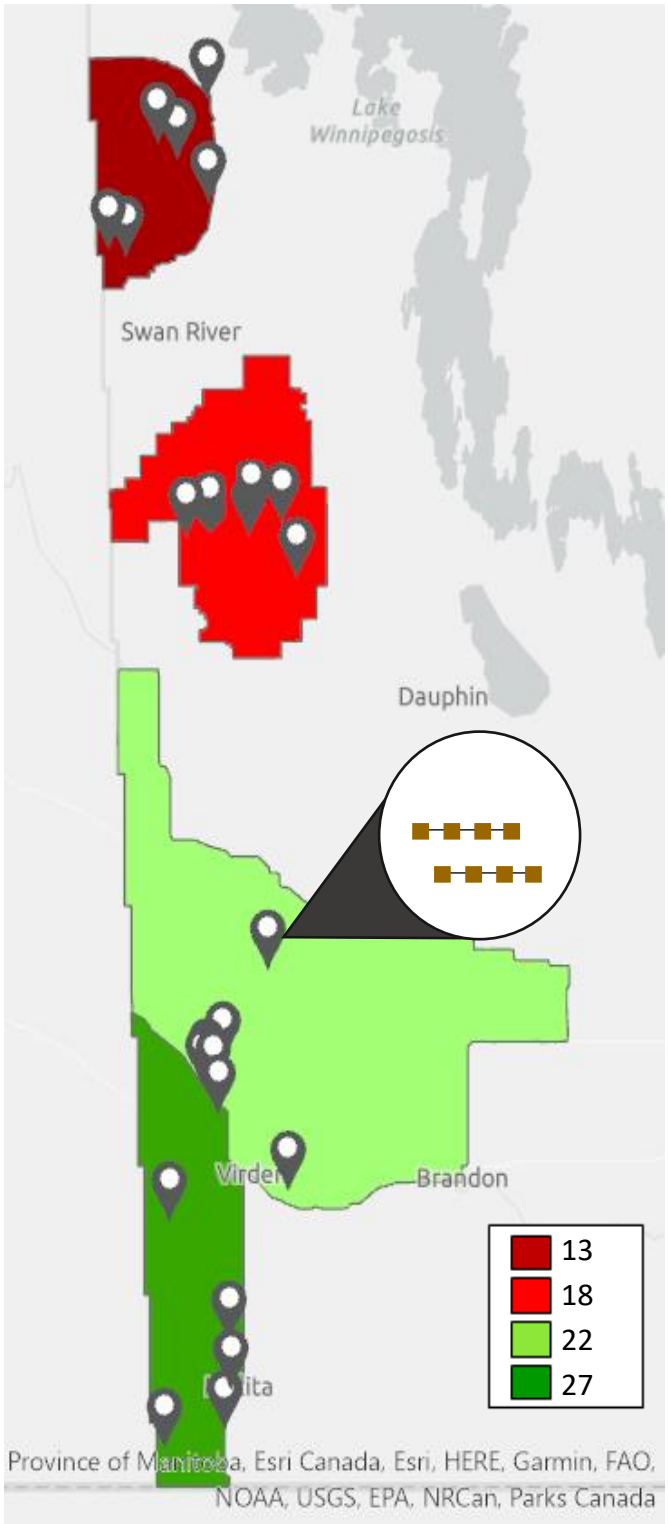

Figure 1.1 Sampling locations for each game hunting area sampled in Western Manitoba 2020-2021**.** Areas in red are where there is concern for moose populations. Areas in green are where there is no concern for moose population. Within each location, we sampled 2 transects consisting of 4 quadrats. Map made in ArcGIS 2.7.3 (ESRI 2020).

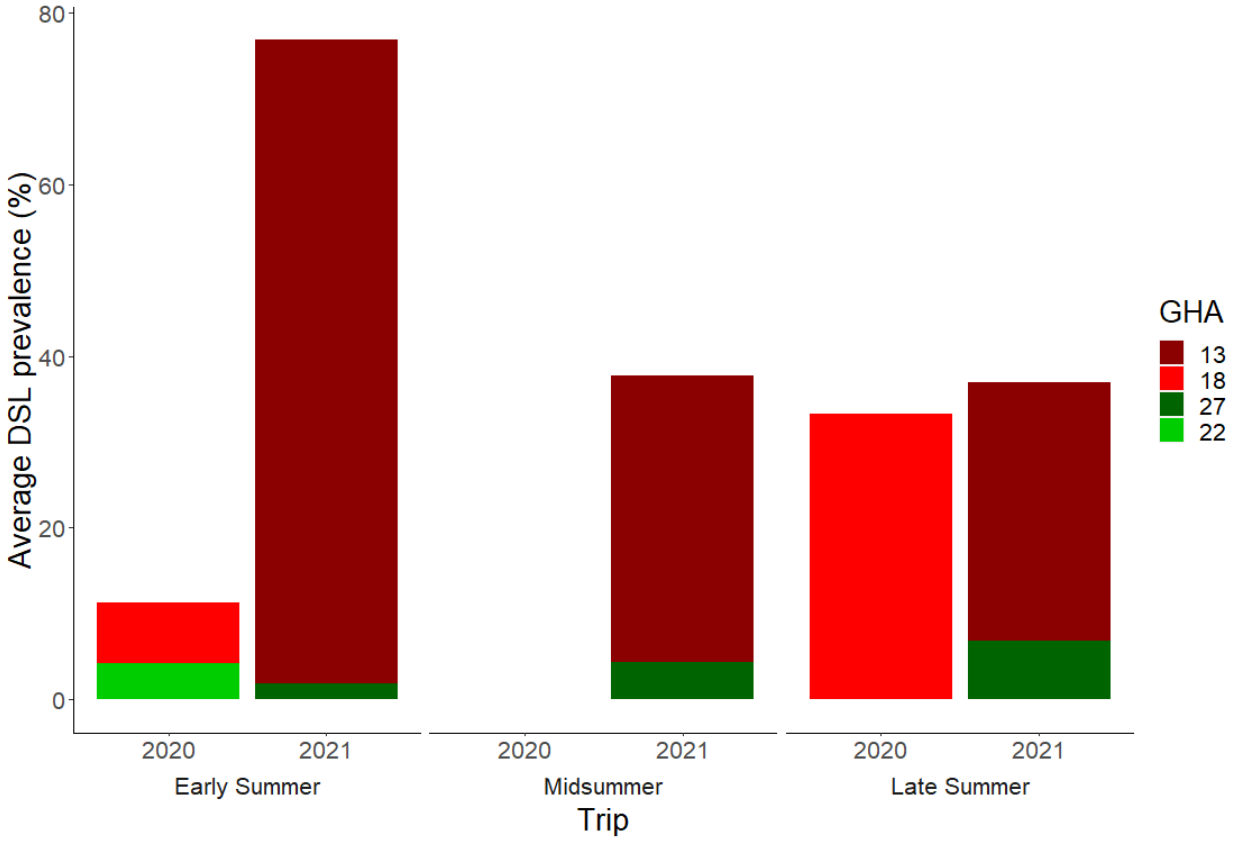

Figure 1.2 Average dorsal-spined larvae (DSL) prevalence in white-tailed deer fecal samples in each game hunting area (GHA) collected in 2020 and 2021. For trips, early summer collection was in June, mid-summer was July-August and late summer was August-September. GHAs 18 and 22 were sampled in 2020 (bright colours) while GHAs 13 and 27 were sampled in 2021 (dark colours). GHA 13 and 18 (red) are areas where moose populations are a conservation concern whereas GHAs 22 and 27 (green) are areas where moose populations are not a conservation concern.

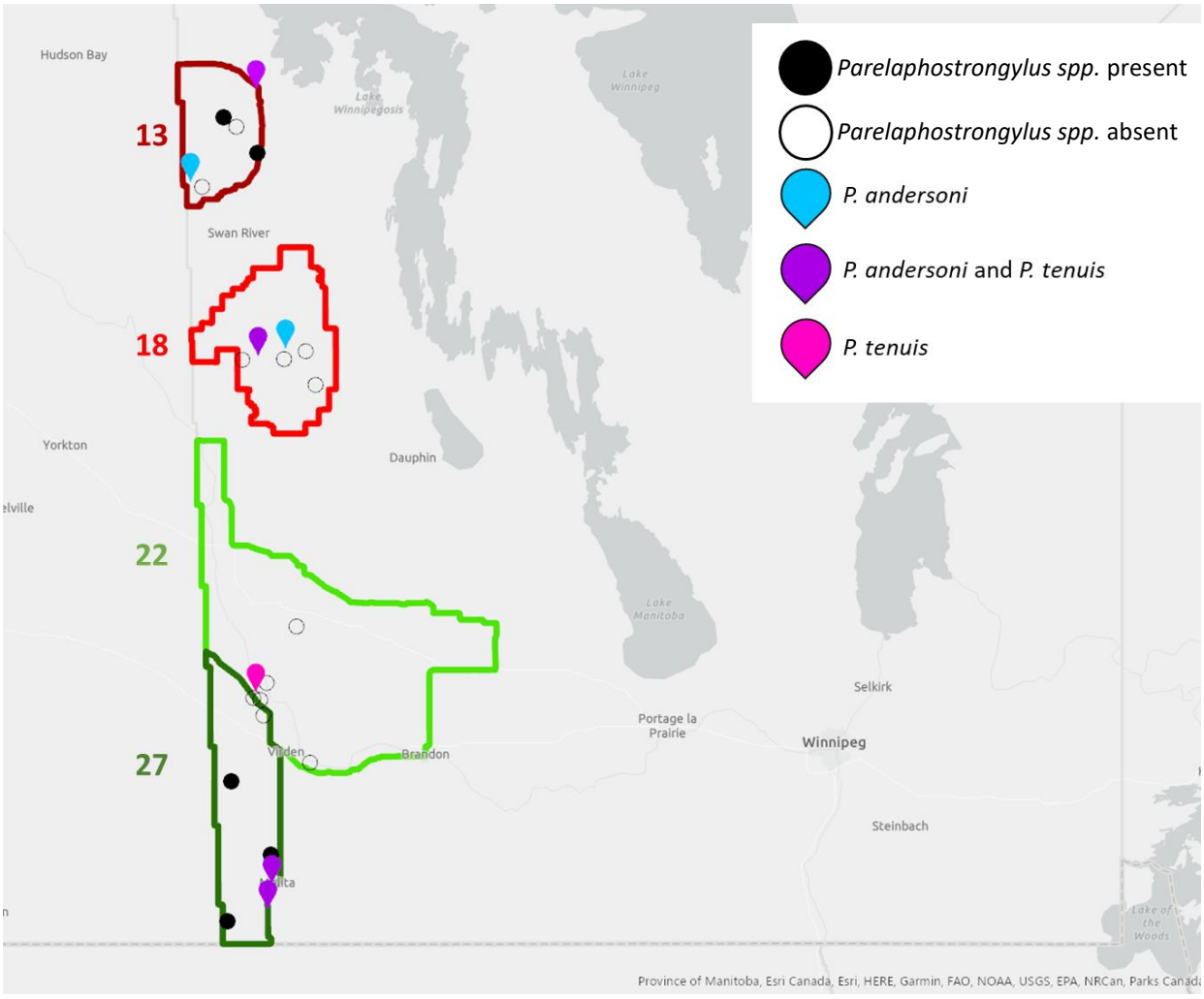

Figure 1.3 Summary of *Parelaphostrongylus* spp. infection in white-tailed deer (*Odocoileus virginianus*) fecal samples collected in Western Manitoba. Partial *COI* genetic sequence results found white-tailed deer fecal samples with *Parelaphostrongylus andersoni* and *Parelaphostrongylus tenuis* in game hunting area (GHA) 13, 18 and 27 and only *P. tenuis* infected fecal pellets in GHA 22.

Chapter 2: Investigating the host and habitat factors that contribute to hotspots for *Parelaphostrongylus* spp. transmission

Abstract

In areas of Western Manitoba, managers are concerned for moose (*Alces alces*) due to low population numbers and there is concern that meningeal worm (*Parelaphostrongylus tenuis*) infections may be contributing. Meningeal worm causes severe pathology and morbidity in moose and moose are at risk of infection where white-tailed deer (WTD, *Odocoileus virginianus*) disperse larvae into the environment and gastropod vectors are present. In Chapter 1, we determined that shedding rates were higher in game hunting areas (GHA) with concern for moose populations suggesting an increased risk of parasite transmission in these areas. However, transmission risk is also affected by contact rates between hosts. Thus, higher density of hosts (WTD and gastropods) may increase contact rates with the parasites and further increase transmission risk. Assessing host density and the habitats they are found in may reveal hotspots for parasite transmission to moose. To test this hypothesis, we first assessed the spatial and temporal variation of host densities to estimate where and when transmission risk is highest. Second, we investigated whether habitat could predict gastropod host density. Third, we determined whether host densities or habitat could predict infection prevalence in WTD feces to estimate hotspots for transmission to moose. We sampled WTD feces and gastropods along transects in four GHAs; two where there is concern for moose populations and two where there is no concern. First, zero-inflated models showed that host densities did not differ spatially but differed temporally with higher fecal density in early summer and higher gastropod host density in late summer. Additionally, we found more gastropod host species in areas where moose populations are a concern. Second, gastropod density was higher in forests (e.g. *Discus whitneyi*) and grasslands (e.g. *D. whitneyi* and *Deroceras laeve*). Third, parasite prevalence was not predicted by host densities or habitat type. The results suggest that contact rates between gastropods and moose are highest in late summer indicating transmission risk to moose

is highest during this period. Further, this study revealed that hotspots for transmission are complex and difficult to determine thus finer scale studies are likely needed to predict hotspots of transmission.

2.1 Introduction

There are several species of protostrongylid parasites that cause morbidity in ungulates (Lankester 2001). In Manitoba, meningeal worm (*Parelaphostrongylus tenuis*) and muscle worm (*Parelaphostrongylus andersoni*) are both present and infect white-tailed deer (WTD, *Odocoileus virginianus*, Lankester 1974; Verocai et al. 2020). Meningeal worm presence is cause for concern because this parasite causes severe pathology and mortality in moose (*Alces alces*, Anderson 1964). Moose infected with these parasites exhibit lumbar weakness, ataxia, blindness, and finally paraplegia (Anderson 1964). Due to these severe symptoms, infections of meningeal worm in moose can result in mortality (Anderson 1964). Concerns about moose population in areas of Manitoba could therefore be impacted by the presence of meningeal worm and the severe symptoms infections cause (Committee for Cooperative Moose Management 2017). Comparably much less is known about muscle worm but its presence is important to note because it is morphologically indistinguishable from meningeal worm at one of its larval stages (e.g., L1 stage, Lankester and Hauta 1989). While we know that meningeal worm causes severe morbidity and mortality to moose, it is unknown whether muscle worm may cause morbidity in moose (Anderson 1964; Lankester and Hauta 1989). In Chapter 1 we determined that protostrongylid larvae prevalence in WTD feces was higher in areas where moose are a conservation concern relative to other areas in Western Manitoba. However, the host and habitat factors that explain this association were not investigated. More detailed information is required to determine whether host density or habitat can be used to estimate hotspots of protostrongylid transmission to moose.

The density of the hosts of the *Parelaphostrongylus* spp. life cycle affect whether an area is a hotspot for transmission. Both meningeal worm and muscle worm have complex life cycles where the common definitive host is a WTD. The L1 stage, also termed dorsal-spined larvae (DSL) in

protostrongylid nematodes, are released in the feces of this host (Anderson 1963). The parasite must then contact, penetrate, and successfully develop to the L3 stage within a terrestrial gastropod intermediate host (Lankester and Anderson 1968). Subsequent WTD, and moose, become infected when they consume infected gastropods (Lankester and Anderson 1968). Therefore, for moose to become infected, WTD must be present to introduce the parasite into the ecosystem and terrestrial gastropods must be present for the parasite to develop and infect subsequent WTD and moose. Thus, it is important to assess the temporal and spatial variation of both vertebrate and invertebrate hosts to understand when and where moose are more at risk of infection.

Investigations of the role of WTD dynamics in infection patterns suggest that their population density predicts the prevalence of the L1 stage in the environment (Karns 1967; Behrend and Witter 1968). These results suggest that moose may be more at risk of protostrongylid infection in areas with higher densities of WTD. Indeed, anecdotal reports suggest that moose population declines coincided with increases in infected WTD density (Karns 1967; Saunders 1973; Gilbert 1974; Lankester 2001, 2018). Further, moose densities decreased with increase prevalence of meningeal worm larvae in WTD fecal samples (Saunders 1973; Whitlaw and Lankester 1994b). However, trends linking WTD density to infection prevalence and moose neurological disease are not found consistently (Gilbert 1973; Bogaczyk et al. 1993; Whitlaw and Lankester 1994a; McGraw et al. 2021). Lankester (2018) proposed that these inconsistent trends could be due to difficulties in censusing clinically ill and minimally compromised moose, as well as difficulties in estimating WTD densities that vary seasonally and annually. However, another factor to consider is the spatial and temporal variation of gastropods given that parasites must develop in these hosts before they are infective to moose or WTD.

Host specificity to gastropods has been explored more for meningeal worm compared muscle worm. At least 18 gastropod species are suitable hosts for meningeal worm suggesting that this species is a generalist for intermediate hosts (Anderson 1963; Lankester and Anderson 1968; Rowley et al. 1987;

Platt 1989; Nankervis et al. 2000; Maze and Johnstone 2008). In particular, the slug *Deroceras laeve* is considered an important host for transmission due to its widespread distribution and tolerance of a wide range of temperatures (Lankester and Anderson 1968). Although the slug is commonly infected, *Zonitoides arboreus* and *Discus whitneyi* have been found to have high meningeal worm prevalence (Kearney and Gilbert 1978; Lankester and Anderson 1968; Lankester and Peterson 1996; Upshall et al. 1986). Few studies have investigated gastropod hosts for muscle worm, although it is known that *D. laeve* is a suitable host (Lankester and Fong 1989). Terrestrial gastropods are more active and abundant in spring and fall when conditions are cool and wet (Lankester and Peterson 1996). As a result, WTD and moose are more likely to become infected in spring, from larvae that overwintered in their gastropod host or in fall when gastropods infected earlier in the year would be active and the infective stage (L3) developed (Lankester and Peterson 1996). Only one study has investigated gastropod hosts in Manitoba (Nicolai et al. 2019). This study investigated gastropods in southeastern Manitoba and found 23 species of terrestrial gastropods in Manitoba's Tall Grass Prairie Preserve (Nicolai et al. 2019). Gastropod hosts have an essential role in infecting moose so it's important that we know which gastropods are in Manitoba and their relative densities to estimate moose infection risk. If we can determine where there are increased densities of gastropod hosts and therefore increased contact rates, we may be able to predict hotspots for meningeal worm and muscle worm transmission.

To predict terrestrial gastropod host occurrence, habitat is important to consider. Both gastropod hosts, and the L1 larvae that infect them, are directly influenced by environmental conditions. Overall, decreased winter duration and severity, as well as increased wetness and length of growing season, facilitates the transmission of meningeal worm (Lankester 2018). The cool, wet conditions that coincide with spring and fall are associated with higher L1 larval survival and facilitate gastropod abundance and activity, both of which may increase chances of intermediate host infection (Burch 1962; Peterson et al. 1996; Hawkins et al. 1997a). Gastropod abundance seems to be highest in

wood-lands compared to other habitats, likely due to their cooler temperature and higher moisture (Lankester and Anderson 1968; Hawkins et al. 1997b; Maskey et al. 2015). Consistent with previous hypotheses, Lankester and Anderson (1968) found higher gastropod abundance in low, damp forest habitats compared to dry, elevated forest or open grasslands. Similarly, DSL prevalence is likely also dependent on low temperature and high moisture. To determine hotspots for transmission, DSL presence in WTD fecal samples was compared to different habitat variables (Vanderwaal et al. 2015). More infected WTD were found in habitats with more upland mixed conifer forests and shrubland than in areas with upland deciduous forests (Vanderwaal et al. 2015). The results of this study were compared with gastropod habitat preferences of the same area, which revealed that habitats with higher gastropod abundance also had more fecal samples with DSL present (Cyr et al. 2014; Vanderwaal et al. 2015). Unfortunately, DSL are morphologically indistinguishable to species and this study did not identify the DSL found using genetic analysis (Lankester and Hauta 1989). So, while they assume all DSL collected were meningeal worm, muscle worm may also have been present. Further, when investigating habitats that predict *Parelaphostrongylus* spp. transmission, this study investigates one area thoroughly without diverse habitat types such as open grasslands present. More studies are needed across wide geographic areas that incorporate locations with diverse habitat compositions to get a better picture of where moose are more likely to become infected and inform management initiatives aimed at conserving moose across a broad geographic range. It's important that we study the host and habitat factors in Manitoba, as meningeal worm is present, and DSL prevalence is positively associated with areas where moose populations are a conservation concern.

In Chapter 2, our first objective was to assess the spatial and temporal variation of the hosts (WTD and gastropod) to explain where and when transmission risk to moose is highest in Western Manitoba. Our second objective was to determine whether WTD fecal pellet density, gastropod density or habitat type could be used to predict DSL prevalence in WTD fecal pellets or gastropods as an

indication of moose infection risk. For the first objective, we predicted there would be higher WTD fecal pellet density, gastropod host density and gastropod host species richness in areas where moose populations are a conservation concern because of the higher infections of DSL in WTD in these areas (Chapter 1). In addition, we predicted higher gastropod density in these areas because they are comprised of mostly forested land, which is associated with cooler, more moist environments conducive to gastropods and as an indication of increased transmission rates (Lankester and Anderson 1968). We also determined if gastropod density was associated with habitat type. We predicted gastropod density would be positively associated with forested areas such as broadleaf, coniferous, or mixed-wood forests that are conducive to large gastropod populations due to the cool, moist environment they offer (Lankester and Anderson 1968; Cyr et al. 2014).

For the second objective, we determined whether prevalence of *Parelaphostrongylus* spp. larvae could be predicted by host factors such as WTD fecal pellet density or gastropod density as well as habitat type. We tested for these relationships for L1 larvae found in WTD fecal samples as well as L3 larvae found within terrestrial gastropods. We predicted that larva prevalence would be positively associated with higher WTD fecal pellet density and gastropod host density, with gastropod density explaining relatively more of the variation in parasitism as consumption of these hosts is required for infection of WTD or moose. With respect to parasite habitat preference, we predicted larva prevalence to follow a similar trend as gastropod density and be positively associated with conifer or mixed-wood forests areas that offer cool, moist environments and negatively associated with drier, warmer cover types such as grasslands (Lankester and Anderson 1968; Vanderwaal et al. 2015). The overall goal of the project was to estimate hotspots of parasite infection by determining where and when there were high densities of hosts, the habitat they were associated with and whether these hosts and habitats predicted DSL infection. With this information, management initiatives can expand their efforts and have a more pro-active approach to prevent moose population declines.

2.2 Methods

2.2.1 Field collection

To assess vertebrate and invertebrate host densities, WTD pellets and gastropods were collected in four game hunting areas (GHA) in Western Manitoba. We used WTD pellets as a proximate for WTD density, as higher density of WTD fecal pellets likely indicates higher WTD density. Direct methods for estimating WTD density, such as spotlighting from road transects, are less reliable in forested areas due to WTD in forests attracted to roadsides whereas indirect methods such as counting pellets are more consistent in both forested and open areas (Anderson et al. 2013). Further, larvae are transferred from the host to the environment on fecal pellets so fecal pellet density may reflect contact rates between larvae and gastropods. As in Chapter 1, we collected samples from GHA 18 and 22 in 2020 and 13 and 27 in 2021. We chose GHAs with differing habitat compositions; GHA 13 and 18 comprise Porcupine Provincial Forest and Duck Mountain Provincial Park respectively, and the GHAs are composed of 70.3% and 80.4% forested land respectively (Figure 2.1). In contrast, GHA 22 and 27 are mostly prairie and agriculture land and thus consist of 65.3% and 66.7% developed land respectively (Figure 2.1). Further, we chose GHAs that differ in the conservation status of moose populations; GHA 13 and 18 are closed to licensed moose hunting because their populations are not at levels desired by provincial managers, while moose populations are not a conservation concern in GHA 22 and 27 (Blaikie 2011, Wildlife and Fisheries 2020). WTD fecal pellet density, gastropod density and gastropod species richness were compared between GHAs where moose populations are a conservation concern and not a concern. This comparison indicated whether these vertebrate and invertebrate host densities are associated with areas where we found higher DSL prevalence in Chapter 1 and where moose populations are a concern. To assess whether the time of summer influenced gastropods density and fecal pellet density, and therefore when there was increased contact rates and greater infection risk to

WTD and moose, each GHA was sampled three times a year; from June to September in 2020 and from June to August in 2021.

As outlined in Chapter 1, we sampled 6 randomly selected locations within each GHA and within each location we sampled two ~714m transects for WTD pellets and terrestrial gastropods (Figure 2.2). We sampled WTD fecal pellets from four plots per transect (21m x 3m, similar to Vanderwaal et al. 2015). Within WTD pellet plots, all fecal pellet groupings of >10 pellets were counted to determine relative WTD abundance (Vanderwaal et al. 2015). We also had 68 0.25m^2 cardboard squares for gastropod collection along each transect with 10m between cardboard pieces (Cyr et al. 2014, Figure 2.2B). To collect gastropods along the transect, 68 dampened cardboard squares (0.5m x 0.5m) per transect were set out the morning prior to collection (Cyr et al. 2014). The cardboard squares were checked for gastropods the following morning, ideally between 6:00 and 9:00am (McCoy 1999). All snails and slugs were collected from the cardboard squares and stored in small plastic bags until they were identified to species and analyzed for parasite infections. When possible, one voucher of each species was imaged, preserved in 80% ethanol, and stored at -20˚C for genetic confirmation of morphological identification.

2.2.2 Processing field collections

As in Chapter 1, we used a modified Baermann technique to examine the collected WTD fecal pellets for DSL (Forrester and Lankester 1997). Nematodes that were recovered were examined with a compound microscope using a 10x objective to determine if a dorsal spine was present. Meningeal worm and muscle worm are morphologically indistinguishable as L1, and thus DSL must be identified using genetics (Lankester and Hauta 1989). For analyses in this chapter, DSL of both species will be included as transmission to WTD and moose occur through the same pathways.

To determine if the collected terrestrial snails were infected with the L3 stage of *Parelaphostrongylus* spp., the gastropods were examined within five days to ensure we were dissecting live specimens. We identified all gastropods to species, measured shell or body length with digital calipers to the nearest 0.01 mm and dissected under stereoscope (Burch 1962; Getz et al. 2017). Given that our field trips were approximately 9 days, some snails and slugs were transported to the laboratory at the University of Manitoba. Prior to dissection in the laboratory, some of these individuals were artificially digested to ensure a more thorough investigation for nematodes. Individual gastropods were cut into pieces, put in 1.5mL tubes filled with a digestive solution (0.6% pepsin and 0.7% HCl) and left in a water bath at 37˚C for 2-4 h. After 2-4 h, the resulting mixture was examined for L3s that should have separated from the gastropod tissues during dissection (Ballantyne and Samuel 1984). We used morphological features to identify the L3s of *Parelaphostrongylus* to species (Ballantyne and Samuel 1984).

2.2.3 DNA extraction and PCR amplification of snail tissue

To confirm shell-based identifications of the snails, we chose a subset of individuals for DNA sequencing. We digitally imaged the shells of ethanol-preserved snails to create vouchers before DNA extraction. Up to 30 mg of preserved tissue (80% ethanol) from the head-foot region or the whole body was twice soaked in water to remove the ethanol. Each tissue sample was pulverized in liquid nitrogen and the DNA was extracted using the E.Z.N.A. Mollusc kit according to the manufacturer's instructions (Omega Bio-Tek). We amplified the cytochrome *c* oxidase subunit 1 gene (*CO1*) using primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3' and TAA ACT TCA GGG TGA CCA AAA AAT CA (Folmer et al. 1994). PCR amplification was performed in 25 µL reactions containing 50 ng of DNA, 1X buffer, 1.5 mM MgCl2, 0.4 µM of each primer, 0.2 mM of each dNTP and 0.05unit/µL Taq polymerase. The amplification was completed with a thermocycler profile with an initial denaturation of 95˚C for 5 min, followed by 40 cycles of 95˚C for 30 s, 50˚C for 45 s, and 72˚C for 1 min, and concluding with 5 min at 72˚C. PCR

products were visualized in 2% agarose gels and products with single bands were purified with the GeneJET PCR Purification Kit (Thermo Fischer Scientific, Waltham, Massachusetts). Purified products were sequenced in both directions at the Hospital for Sick Children, Toronto, Ontario, Canada using an ABI 3730XL Sanger Sequencer (Thermo Fischer Scientific, Waltham, Massachusetts).

2.2.4 DNA analysis

Contigs were constructed and manually edited by eye in Sequencher v. 5.4.6 (Gene Codes Corporation, Ann Arbor, Michigan). Sequences were queried using the blastn algorithm on GenBank to compare to shell-based identifications. Sequences considered to be the closest matches had an e-value of 0 and percent identity >90. For all our sequences, if percent identity was <90, more than one species or genera was included.

2.2.5 Habitat composition

To identify habitats in which moose were more at risk for infection, the proportion of forest, grassland, water and developed land along each transect was determined using the Annual Crop Inventory and ArcGIS Pro (ESRI, 2020). The Annual Crop Inventory digital maps were made for all of Canada using satellite imagery (Open Government Licence – Canada, Government of Canada; Agriculture and Agri-Food Canada; Science and Technology Branch). Decision Tree method was used to determine the habitat type of the area from optical (Landsat-8, Sentinel-2) and radar (RADARSAT-2) satellite images (Earth Observation Team of the Science and Technology Branch (STB) at Agriculture and Agri-Food Canada (AAFC)). Ground-truth information was provided by provincial crop insurance companies in Manitoba to accompany satellite images (STB at AAFC). The spatial resolution of the maps was 30m. To determine the type of habitat within our transects, we created a line from the start and end points of each transect then made a buffer area around each transect using the “Create Buffer” tool (ArcGIS Pro 2.7.3, ESRI 2020). To investigate which habitats terrestrial gastropod hosts were most likely to be found in, a 100m radius buffer was made around the transect to encompass turns in the transect

but limit the habitat encompassed. To investigate which habitats were associated with higher prevalence of DSL in WTD, buffers of 1.7 km radius were made to encompass the range of WTD (McCance 2014; Vanderwaal et al. 2015). Using the "Summarize within" tool, we found the habitat composition within the buffered areas. We combined urban and developed land, green houses, pasture and forages and all crop lands into the category of developed land. Other habitats of interest included broadleaf forest, mixed-wood forest, coniferous forest, grassland, shrubland, water and wetland. The habitat composition was then compared to the DSL prevalence in WTD or terrestrial gastropod density to determine whether certain habitats predict infection risk of WTD and therefore potentially moose, or gastropod density as these hosts are the vector that infects WTD and moose.

2.2.6 Statistics

Similar to Chapter 1, we used zero-inflated models to determine if the density (n/m^2) of WTD pellets differed by status (concern for moose populations or no concern for moose populations), sampling year or collection trip (early, middle and late summer). We included an interaction term between year and trip to determine whether the trend across collection trips differed by year. In addition, we used the same modelling approach to determine if gastropod host density and species richness was explained by the same set of independent variables. We decided to use species richness, rather than another diversity index, because in many locations few host species and few individuals were collected, and many indices are dependent on abundance as well as the number of species collected. Each of the three response variables was offset by the log-transformed area (m^2) that was sampled as some transects varied in length for a variety of reasons (e.g., area getting too steep, too wet, too many fallen trees, presence of bear cubs, etc.). By adding the offset of area to the model, we can use zero-inflated models to investigate count data as densities (Zuur et al. 2009). Therefore, in the models we used WTD fecal pellet abundance and gastropod abundance as the dependent variable, as zero-inflated models require count data but offset the data by area to analyze density instead. We

analyzed according to collection trip because the length of the field sampling differed for each year; in 2020 we sampled from June – September, while in 2021 we sampled June – August. In both years trips were spaced out evenly according to the length of the field sampling and thus differed slightly between years. Nonetheless, "Early summer" for both years was June, "Midsummer" was in July/August in 2020 and July in 2021, "Late summer" was in September in 2020 and in August in 2021. We ran the models with a Poisson and a negative binomial distribution then used likelihood ratio tests to determine which distribution was a better fit (Zuur et al. 2009). Chi-squared tests were performed following zero-inflated models to determine whether GHA status, trip, year, or the trip year interaction was associated with WTD fecal pellet density or gastropod density (Mangiafico 2016). For each zero-inflated model, we calculated McFadden's pseudo-R^2 to determine how well the model explained the variability in the data. For McFadden's pseudo-R^2, a value of 0.2-0.4 indicates a good fit (Louviere et al. 2000). We also validated the models by calculating the variance inflation factor (VIF) to ensure no multicollinearity (VIF <5, Zuur et al. 2007). All statistics were done in R version 4.1.2 "Bird Hippie" (R Core Team 2021). The zero-inflated models were run with the packages "readxl", "multcompView", "emmeans", "rcompanion", "car", "pscl" and "lmtest" (Fox and Weisberg 2019; Graves et al. 2019; Lenth 2022; Mangiafico 2022; Wickham and Bryan 2019; Zeileis, Kleiber et al. 2008; Zeileis and Hothorn 2002). Column plots to visualize the data were made using "ggplot2" (Wickham 2016).

To investigate whether the density of any of the gastropod host species collected were associated with habitat type, a redundancy analysis (RDA) followed by an analysis of variance (ANOVA) was performed. Host density (n/m^2) was log(x+1) transformed to account for an abundance of zeros in the data. We included habitat type categories of developed land, forest (broadleaf, coniferous and mixed-wood forests), water (water and wetlands) and grass (grassland and shrublands). These analyses were run with packages "vegan" and "tidyverse" (Oksanen et al. 2020, Wickham et al. 2019). Zero-inflated models were also performed to determine if the gastropod host density of each gastropod host

species collected (abundance offset by log of the area, in m^2, examined) was influenced by certain habitats. We included broadleaf forest, coniferous forest, mixed-wood forest, grassland, shrubland and developed land to get a more in-depth view of the habitat preferences. We followed the zero-inflated models with chi-square tests to determine which habitat types were associated with gastropod density (Mangiafico 2016). We calculated McFadden's pseudo-R^2 to determine how well each model explained the variability in the data. VIF was calculated to ensure no multicollinearity (VIF <5, Zuur et al. 2007).

Zero-inflated models were also run to determine if gastropod density, WTD fecal pellet density or habitat type predicted L1 *Parelaphostrongylus* spp. prevalence (abundance offset by the number of fecal samples examined). Unfortunately, L3 *Parelaphostrongylus* spp. prevalence in gastropods was too low to include in statistical analyses as either a dependent or independent variable. We followed the zero-inflated models with chi-square tests to determine whether the variables in question were associated with DSL prevalence (Mangiafico 2016). We calculated McFadden's pseudo-R^2 to determine how well the model explained the variability in the data. The models included the independent factors of gastropod density, WTD fecal pellet density, as well as the habitat types: broadleaf forest, mixed-wood forest, coniferous forest, grassland, shrubland, developed land, water and wetland. Due to multicollinearity (VIF >5), we ran univariate models and if the habitat cover was significant in the univariate model it was included in the final model (Vanderwaal et al. 2015). The zero-inflated models were done in R version 4.1.2 "Bird Hippie" (2021) with the packages "readxl", "multcompView", "emmeans", "rcompanion", "car", "pscl" and "lmtest" (R Core Team 2021, Zeileis, et al. 2020, Zeileis 2002, Fox and Weisberg 2019, Wickham and Bryan 2019, Graves et al. 2019, Lenth 2022, Mangiafico 2022).

2.3 Results

In 2020, we collected 55 WTD fecal samples from June – September with 70.9% (39/55) of the fecal samples collected from GHA 18. In 2021, we collected 333 WTD fecal samples from June – August

2021 and 7%, (23/333) from GHA 13 and 93% (310/333) from GHA 27. We found fecal density (n/m^2) differed by year sampled (P <0.01), across trips sampled (P = 0.01) and the interaction between trip and year was significant (P <0.01, Table 2.1). There was no difference in WTD fecal pellet density between areas where there is concern for moose populations compared to where there is no concern (Table 2.1). McFadden's pseudo-R^2 was 0.15. We found higher density of fecal samples in 2021, especially from GHA 27 (Figure 2.3). In 2021, fecal pellet abundance was similar across trips while in 2020 we collected higher density of WTD fecal samples in early summer (June) compared to other months (Figure 2.3).

In 2020, we collected 1,164 terrestrial gastropods. Of those, 41.4% (482/1,164) were known hosts of meningeal worm and 29.5% (343/1,164) was *D. laeve*, the only host found that is a host for muscle worm (Table 2.2). In 2021, we collected 679 gastropods, 50.0% (339/679) of which were known hosts of meningeal worm and 10.0% (66/679) were *D. laeve*. We found that gastropod density (n/m^2) differed across trips (P <0.01) and the interaction between trip and year was significant (P = 0.03, Table 2.3). The model had a McFadden's pseudo-R^2 of 0.144. The density of terrestrial gastropods collected increased with trips for GHA 22 and 13 whereas GHA 18 and GHA 27 had more even densities throughout (Figure 2.4). Across trips, there was high density of gastropod hosts in early and midsummer in 2020 but higher density of gastropod hosts in late summer in 2021 (Figure 2.4).

We collected 15 gastropod species in 2020 and 12 gastropod species in 2021. Of those, 5 were known hosts of meningeal worm; *Deroceras laeve, Discus whitneyi, Zonitoides arboreus, Succinea ovalis* and *Cionella lubrica*. *Deroceras laeve* was the only gastropod host of muscle worm collected. Gastropod host species richness differed by moose population status (P = 0.02) and the interaction between trip and year was significant (P = 0.045, Table 2.4). The model McFadden's pseudo-R^2 of 0.243. As seen in Figure 2.5, the GHAs with a status of concern for moose populations (red) had higher gastropod host species richness.

From our DNA sequencing, we obtained 19 total *CO1* sequences (557-689 bp) identified as 10 species using shell-based identification (Table 2.5). The blastn search indicated >90 percent identity to 9 snail taxa in GenBank. For 8 of those species there was high similarity (>95 percent identity) to species that were accessioned in GenBank. However, several of the sequences we recovered were similar to more than one species or an unidentified species making taxonomic identifications from *CO1* sequencing less clear. First, our sequence identified as *Columella* sp. had high similarity with *Columella edentula* and *Columella simplex* as well as many species labelled *Columella* sp. in GenBank. Second, our sequence identified as *Vertigo tridentata* had high similarity with *Vertigo* sp. and a few species labelled *Vertigo coloradensis* in GenBank. Third, *Vitrina pellucida* had high similarity to sequences from both *V. pellucida* and *Vitrina angelicae* in GenBank. In particular, our species identifications for *V. tridentata* and *V. pellucida* were mismatched to taxa in GenBank indicating that further study of species diversity in these genera is needed. We also found that our DNA sequence initially labeled as *Striatura ferrea* had high similarity with sequences from *Zonitoides arboreus* in GenBank (96-99 percent identity). After reviewing the digital image voucher for *S. ferrea* we decided that the few individuals identified as *S. ferrea* were likely *Z. arboreus*. Using this finding, we updated our dataset that included gastropod species richness before conducting statistical analyses.

A redundancy analyses followed by an ANOVA were performed to determine whether any of the habitats (forest, grass, water or developed land) could predict the density (n/m^2) of different host species collected in each year of collection. The RDA plot explained 18.7% of the variation in the data (RDA1 = 0.12, RDA2 = 0.07, Figure 2.6). An analysis of variance (ANOVA) revealed that habitat type did not predict host density for the host species ($F_{(4,19)} = 1.31$, $P = 0.25$). The first axis (RDA1) explained the variation in gastropod density (Figure 2.6). The second axis (RDA2) explained the variation in ratio of *D. laeve* slug to the other snails collected (Figure 2.6). Although habitat did not predict gastropod density, the snails, especially *Z. arboreus* and *D. whitneyi*, clustered with forest habitat whereas *D. laeve* slugs

were between forest and grass habitat (Figure 2.6). Further, the snails cluster with areas where there is concern for moose populations (red) while *D. laeve* slugs did not (Figure 2.6).

A more in-depth view of habitat preference found that habitat composition had strong effects on the density (n/m^2) of two of the gastropod hosts collected. We found that the slug *D. laeve* was collected in locations with more grassland ($P = 0.01$, Table 2.5). The snail *D. whitneyi* was found in locations with lower proportions of developed land ($P < 0.01$), and higher proportions of mixed-wood forests ($P = 0.02$), broadleaf forests ($P = 0.01$), coniferous forests ($P = 0.01$), and grasslands ($P = 0.01$, Table 2.6). There were no habitat types that were associated with *Z. arboreous* or *S. ovalis* densities (Table 2.6).

We found 0.2% (2/821) L3 *Parelaphostrongylus* spp. prevalence in terrestrial gastropods collected. One nematode was from *D. whitneyi* and the other from *D. laeve* collected in August from the area where moose are a conservation concern.

Univariate zero-inflated models revealed that DSL prevalence in WTD fecal samples was positively associated with mixed-wood ($P = 0.01$, McFadden's pseudo-$R^2 = 0.148$) and coniferous forests ($P = 0.01$, McFadden's pseudo-$R^2 = 0.119$). However, a model including both of these habitat types found no association between mixed-wood forests or coniferous forests with DSL prevalence (McFadden's pseudo-R^2 of 0.164, Table 2.8).

2.4 Discussion

2.4.1 White-tailed deer fecal density

Contrary to our prediction, WTD fecal pellet density was not associated with the conservation status of the GHAs. Assuming that fecal pellet density is a proximate for WTD density, our results suggest that WTD density was similar across GHAs and that WTD density was not a predictor of parasite transmission risk. This point is further supported by results from Chapter 1, in which we found that DSL

prevalence was higher in GHAs with conservation concern for moose. The difference in DSL prevalence based on conservation status occurred despite collecting considerably more fecal pellets from GHAs with no moose conservation concern. These discrepancy between WTD density and DSL prevalence in WTD suggests that *Parelaphostrongylus* spp. prevalence in WTD was not associated with WTD density. The results are not surprising as other studies found a lack of association between WTD density and meningeal worm infection prevalence as well (Gilbert 1973; Bogaczyk et al. 1993). These results could be due to the complex life cycle of *Parelaphostrongylus* spp. that require multiple hosts. Although infected WTD are present, the larval parasites must develop in terrestrial gastropods to infect subsequent WTD and moose (Lankester and Anderson 1968). The intermediate gastropod host may be more strongly influencing transmission risk as WTD and moose infection requires consumption of infected gastropods.

2.4.2 Gastropod host density and species richness

By assessing the temporal variation in gastropod host density, we found that transmission risk is likely highest in late summer (August-September) due to increased densities of gastropod hosts in these months. Higher gastropod density in these months could lead to higher contact rates with parasites in the WTD feces or soil, or increased consumption rates thus creating hotspots for transmission in these months. Areas with more infected gastropods would be areas where the risk of transmission to WTD and moose would be greatest. Our results are similar to previous studies that suggested that WTD and moose are most likely to become infected in fall when gastropods infected earlier in the season would be active (Lankester and Anderson 1968). Indeed, Peterson et al. (1996) found that prevalence of DSL in fawn fecal samples were correlated with the duration of the previous autumn transmission period. This idea is further supported by the appearance of sick moose in spring suggesting infection occurred the previous autumn (Lankester 2001).

Gastropod host density also did not differ with GHA conservation status, but we found that gastropod host species richness was associated with GHA status and more gastropod species were found in areas where there is concern for moose populations and higher DSL prevalence. GHA conservation status may not have explained variation in gastropod host density because one of the species, the common slug *D. laeve*, was found in all GHAs and therefore it's inclusion in the model may be masking trends for other hosts. *Deroceras laeve* is thought of as an important host for protostrongylid larvae because its widely distributed and more commonly infected than other gastropod hosts (Lankester and Anderson 1968). Our results were similar as *D. laeve* was the most common gastropod species collected in most areas and habitat types in our study. Perhaps *D. laeve* is the cause of some transmission risk in a variety of locations but the presence of more host species in some areas results in higher DSL prevalence in WTD. In Western Manitoba, there are at least 17 species of terrestrial gastropods, 5 that are known hosts in Western Manitoba. It was previously unknown which species occurred in Manitoba outside of the Tall Grass Prairie Preserve in Eastern Manitoba. The study in Eastern Manitoba found over 20 species of terrestrial gastropods (Nicolai et al. 2019). Future studies should compare Eastern and Western Manitoba for differences in terrestrial gastropod density, DSL prevalence in WTD fecal pellets and habitat types to determine whether transmission risk is higher in Eastern Manitoba and whether moose are more at risk of meningeal worm infections in the east.

Contrary to our predictions, most of the gastropod host species were not strongly associated with habitat type. However, significant associations with habitat type were found for two gastropod host species. First, *Discus whitneyi* was positively associated with broadleaf, mixed-wood and coniferous forests. This habitat use by *D. whitneyi* is similar to what other studies have found with overall gastropod density (pooling all species in the study area). For example, higher gastropod density occurred in mixed coniferous-deciduous forests in northern Michigan and Minnesota (Nankervis et al. 2000; Cyr et al. 2014)., Second, *D. laeve* was positively associated with grasslands rather than forested

areas, which is similar to the results of Kearney and Gilbert (1978). Our study shows the importance of using a wide geographic range and sampling areas with diverse habitat conditions to get initial information of where gastropods occur in higher densities. These studies should be followed with targeted sampling at a finer spatial scale to obtain more in-depth information of the habitat types and abiotic conditions associated with higher densities of infected gastropods.

Despite sampling over 1,654 individuals in 17 gastropod species over 2 years, we found low prevalence (0.2%) of *Parelaphostrongylus* spp.. The low prevalence of meningeal worm L3 larvae in gastropods is common with <0.1% - 5% in most studies (Lankester and Anderson 1968; Gleich et al. 1977; Kearney and Gilbert 1978; Upshall et al. 1986; Pitt and Jordan 1995; Lankester and Peterson 1996). Although our results and those of others suggest that the prevalence of meningeal worm in gastropods is low, Lankester and Peterson (1996) determined accidental ingestion of infected gastropod was a feasible explanation for high prevalence of *P. tenuis* in fawns by autumn based on the amount of vegetation consumed and the density of gastropods in the area, assuming infection occurred after consuming a single infected gastropod (mean +/- SD of 3.2 +/- 2.5 larvae). The two L3s recovered from infected gastropods (*D. laeve* and *D. whitneyi*) were not identified to species using morphology despite suggestions in the literature that tail morphology can distinguish between *P. tenuis* and *P. andersoni* (Ballantyne and Samuel 1984). Given the level of expertise that may be required for morphological species identification, DNA sequencing could be used to more objectively determine the species present.

In our study, randomly choosing locations within GHAs for transect sampling may have led to underestimates of DSL prevalence in gastropods. For instance, selective sampling of gastropods has led to higher (though still relatively low) estimates of DSL prevalence. For instance, Parker (1966) found that 2.6% (13/509) of snails and slugs were infected with meningeal worm larvae though most of the infected gastropods were selectively sampled from deer feces and only 0.8% (3/397) of the randomly

collected gastropods were infected. In addition to their proximity to deer feces, DSL prevalence in gastropods seems to vary according to gastropod host species. Maze and Johnstone (2008) found the highest prevalence of meningeal worm with 9.0% (73/808) though the prevalence was lowest in *D. laeve* (1.6%, 1/61) and highest in *D. whitneyi* (14.7%, 21/114), *Ventridens intertextus* (20.3%, 24/118), and *Triodopsis albolabris* (20.0%, 14/70). This study also illustrates that spatial heterogeneity in gastropod abundance can be high as 81% of all snails and 91.7% of infected snails were found at a single collection site, a likely hotspot for meningeal worm transmission. This site was located on a hillside with dense interwoven ground cover and soil with high calcium and neutral pH. In our sampling, we randomly chose areas to sample to assess gastropod density and species richness in a variety of habitat types. However, future worked aimed at finding areas with infected gastropods could assess whether characteristics like slope, ground cover, soil type, or proximity to white-tailed deer feces predict where infected gastropods are found.

Another aspect that may have influenced our estimates of gastropod density and diversity is the collection method. Similar to most other studies, we used cardboard traps set in transects (Kearney and Gilbert 1978; Upshall et al. 1986; Lankester and Peterson 1996; Oggier et al. 1998). However, it is unclear whether restricting collection to the cardboard trap method may under-sample some arboreal gastropods (McCoy and Nudds 1997). In this study, *D. whitneyi* and *S. ovalis* spent equal amounts of time on the ground and climbing suggesting that they could be important hosts for protostrongylid parasites because they spend time on the ground where they can become infected with L1s and then climb where they (and their L3s) are more vulnerable for consumption by ungulate definitive hosts. In contrast, *Z. arboreus* and *D. laeve* climbed infrequently resulting in a higher abundance of these species on cardboard relative to above ground vegetation. Both findings are consistent with the idea that cardboard samples may be biased toward terrestrial species. Indeed, our results from cardboard trap sampling found high numbers of *D. laeve* (n > 300), although we also found relatively high numbers of *S.*

ovalis (n > 150) and *D. whitneyi* (n > 100). Currently, it is unclear whether a bias in gastropod sampling affects estimate of DSL prevalence in gastropods. Future work should assess whether the prevalence of DSL larvae in gastropods differs between gastropods collected from the ground-dwelling and arboreal gastropods. Perhaps these climbing gastropods are more important to the transmission of meningeal worm, as they may have a higher risk of consumption by deer and moose as both primarily feed on browse (Franzmann and Schwartz 1997; Hewitt 2011).

The results from genetic sequencing of gastropods confirmed most of our species identifications using shell morphology. However, there were a few exceptions. For example, we identified a few individuals as *S. ferrea*, but the genetic sequence similarity to *Z. arboreus* suggested we had misidentified those individuals. In addition, morphologically identified *V. tridentata* and *V. pellucida,* had high genetic similarity with a few species or with individuals identified to genus and therefore we were unsure of our identifications to species. Many gastropods are difficult to identify with shell morphology, this study demonstrates the benefits of using genetic sequences to verify species identification based on shell morphology. The wide range of percent identity (93-100) within some of the gastropod species suggests that our sequences could contribute to future work on the phylogeography and speciation of terrestrial gastropods.

2.4.3 Dorsal-spined larvae prevalence

The univariate models showed that DSL prevalence was positively associated with mixed-wood and coniferous forests. This habitat association is consistent with our prediction and previous findings from Vanderwaal et al. (2015). We expected DSL prevalence to be positively associated with cooler more wet environments of mixed-wood and coniferous forests. However, the parameter estimates for the single response variable models were low and had pseudo-R^2 between 0.12-0.15 indicating that the univariate models did not explain much variation in DSL prevalence. Further, when two significant habitat variables from the individual models were analyzed together in one model, we found that DSL

prevalence was not associated with habitat composition. More data is likely needed to determine which habitats DSL infections are most likely to occur in. Selectively collecting exclusively fresh samples from May – October, or frozen samples from December – April may increase the chances that larvae will be found in feces. It is still unknown how long larvae remain in WTD fecal pellets and once they leave how far they travel from the pellets. Answers to these questions could aid in using fecal samples to assess WTD infection as well as how likely gastropods are to be infected by these L1 larvae.

2.5 Conclusions

Our results give novel insights into the transmission patterns in Western Manitoba. In our study, neither fecal pellet density, gastropod density nor habitat type predicted DSL prevalence in white-tailed deer fecal samples. However, increased gastropod species richness in late summer in areas with conservation concern for moose suggested that the occurrence of multiple gastropod species contributes to increased infection prevalence of WTD. We found 17 gastropod species, 5 of which are known hosts to meningeal worm. The most common host found was *D. laeve*, which is known to be an intermediate host for meningeal worm and muscle worm. Although our random sampling approach was useful in determining where and when gastropod species were present, it did not reveal areas with hotspots of transmission to gastropods (areas with high DSL prevalence in gastropods). Future studies should focus on testing which host and habitat factors support high densities and diversity of gastropods to better predict where and when there are hotspots of *Parelaphostrongylus* transmission to moose.

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Table 2.1 White-tailed deer (*Odocoileus virginianus*) fecal density (n/m^2) differed by year collected, trip sampled and the interaction between trip and year was significant according to a zero-inflated Poisson model. Bolded *P* are significant (<0.05).

	Degrees of freedom	χ^2	*P*
Status	1	0.035	0.85
Year	**1**	**58.29**	**<0.01**
Trip	**2**	**12.96**	**<0.01**
Trip*Year	**2**	**15.55**	**<0.01**

Table 2.2. Composition of terrestrial gastropods collected in Western Manitoba, June-September 2020-2021. Gastropod species were identified to the species using morphology and keys. Does not include individuals that were crushed or were otherwise unable to be identified.

Group	Family	Species	Count	Percent total
Parelaphostrongylus spp. vectors				
Slug	Limacidae	*Deroceras laeve*	381	46
Snail	Endodontidae	*Discus whitneyi**	128	16
Snail	Zonitidae	*Zonitoides arboreus**	99	12
Snail	Vallonidae	*Cochlicopa lubrica**	16	2
Snail	Succineidae	*Succinea ovalis*	197	24
Total			821	
Non vectors				
Slug	Arionidae	*Arion fasciatus*	4	<1
Slug	Arionidae	*Arion intermedius*	1	<1
Snail	Succineidae	*Catinella avara**	52	6
Snail	Pupillidae	*Columella* sp.*	79	9
Snail	Zonitidae	*Euconulus fulvus**	115	14
Snail	Succineidae	*Oxyloma retusa*	81	10
Snail	Valloniidae	*Vallonia gracilicosta*	1	<1
Snail	Vallonidae	*Vallonia parvula*	1	<1
Snail	Pupillidae	*Vertigo arthuri**	5	1
Snail	Pupillidae	*Vertigo* sp.*	2	<1
Snail	Zonitidae	*Vitrina* sp.*	283	34
Snail	Valloniidae	*Zoogenetes harpa**	209	25
Total			833	

*Digital picture vouchers and genetic vouchers provided.

Table 2.3 Gastropod host density (n/m^2) differed across trips sampled and the interaction between trip and year was significant according to a zero-inflated negative binomial model. Bolded *P* are significant (<0.05).

	Degrees of freedom	χ^2	*P*
Status	1	0.20	0.65
Year	1	3.33	0.07
Trip	**2**	**22.07**	**<0.01**
Trip*Year	**2**	**6.83**	**0.03**

Table 2.4 Gastropod host species richness differed by GHA moose population status and the interaction between trip and year was significant according to a zero-inflated Poisson model. Bolded *P* are significant (<0.05).

	Degrees of freedom	χ^2	*P*
Status	**1**	**5.82**	**0.02**
Year	1	1.06	0.30
Trip	2	4.60	0.10
Trip*Year	**2**	**6.22**	**0.045**

Table 2.5. The blastn search conducted on 2/18/2022 indicated a >90% percent identity to 9 snail taxa. Results of blastn search were used to compare shell-based identifications with GenBank accessions (our cytochrome c oxidase I gene sequence lengths in base pairs (bp) specified in brackets). The ranges for query coverage and percent identity are reported for accessions that were most similar to sequences obtain in this study. Mismatches between shell-based identifications and GenBank matches are bolded.

Shell-based ID [sequence length bp]	GenBank Match	Query coverage (%)	Percent Identity (%)
Cochlicopa lubrica-1 [686 bp]	*Cochlicopa lubrica*	90-97	93-94
Cochlicopa lubrica-2 [686 bp]	*Cochlicopa lubrica*	90-97	93-94
Cochlicopa lubrica-3 [646 bp]	*Cochlicopa lubrica*	89-99	92-94
Cochlicopa lubrica-4 [686 bp]	*Cochlicopa lubrica*	84-97	93-94
Columella sp. [683 bp]	*Columella* spp.	90-97	93-100
Discus whitneyi-1 [684 bp]	*Discus whitneyi*	68-96	98-100
Discus whitneyi-2 [683 bp]	*Discus whitneyi*	68-96	98-100
Euconulus fulvus [557 bp]	*Euconulus fulvus*	86-100	94-99
Vertigo arthuri-1 [686 bp]	*Vertigo arthuri*	82-95	99-100
Vertigo arthuri-2 [632 bp]	*Vertigo arthuri*	73-97	99-100
Vertigo tridentata [686 bp]	***Vertigo* spp.**	81-95	96-100
***Vitrina pellucida*-1** [687 bp]	***Vitrina* spp.**	74-97	99-100
***Striatura ferrea*-1** [683 bp]	***Zonitoides arboreus***	80-99	96-99
Zonitoides arboreus-1 [683 bp]	*Zonitoides arboreus*	80-99	95-100
Zonitoides arboreus-2 [686 bp]	*Zonitoides arboreus*	80-99	95-100
Zonitoides arboreus-3 [683 bp]	*Zonitoides arboreus*	80-99	96-99
Zoogenetes harpa-1 [683 bp]	*Zoogenetes harpa*	95-95	100-100
Zoogenetes harpa-2 [684 bp]	*Zoogenetes harpa*	95-95	100-100
Catinella sp.-1 [689 bp]	NA*	NA*	NA*

*Not found in blastn search, closest match <90 percent identity

Table 2.6 Zero-inflated models with a negative binomial distribution for host density (n/m^2) of each gastropod host species given proportion coverage of different habitat types. *P* are significant (<0.05). Contains information licensed under the Open Government Licence – Canada.

Gastropod host species	Habitat type	Degrees of freedom	Estimates	χ^2	*P*
Deroceras laeve	Mixed-wood forest	1	-0.014	0.06	0.59
McFadden's pseudo-R^2: 0.124	Broadleaf forest	1	0.018	1.72	0.19
	Coniferous forest	1	0.022	1.50	0.21
	Grassland	**1**	**0.064**	**6.14**	**0.01**
	Shrubland	1	0.074	2.40	0.12
	Developed land	1	0.018	0.41	0.52
Discus whitneyi	**Mixed-wood forest**	**1**	**0.131**	**5.37**	**0.02**
McFadden's pseudo-R^2: 0.215	**Broadleaf forest**	**1**	**0.111**	**6.80**	**0.01**
	Coniferous forest	**1**	**0.129**	**6.90**	**0.01**
	Grassland	**1**	**0.166**	**7.80**	**0.01**
	Shrubland	1	0.164	3.42	0.06
	Developed land	**1**	**-0.269**	**13.92**	**<0.01**
Zonitoides arboreus	Mixed-wood forest	1	-0.105	0.45	0.50
McFadden's pseudo-R^2: 0.122	Broadleaf forest	1	0.021	0.07	0.80
	Coniferous forest	1	0.005	<0.01	0.95
	Grassland	1	0.020	0.05	0.83
	Shrubland	1	0.074	0.29	0.59
	Developed land	1	-0.027	1.53	0.22
Succinea ovalis	Mixed-wood forest	1	-0.034	0.57	0.45
McFadden's pseudo-R^2: 0.210	Broadleaf forest	1	0.036	2.56	0.11
	Coniferous forest	1	-0.023	0.63	0.43
	Grassland	1	-0.042	1.03	0.31
	Shrubland	1	0.167	3.65	0.06
	Developed land	1	-0.006	0.05	0.83

Table 2.7 Univariate zero-inflated Poisson models for dorsal-spined larvae prevalence (% infected) in white-tailed deer fecal pellets given proportion coverage of different habitat types and host density (n/m^2). Bolded *P* are significant (<0.05). Contains information licensed under the Open Government Licence – Canada.

Habitat type	Degrees of freedom	Estimates	χ^2	*P*	McFadden's pseudo-R^2
Mixed-wood forest	**1**	**0.06**	**6.88**	**0.01**	**0.15**
Broadleaf forest	1	0.01	1.90	0.17	0.11
Coniferous forest	**1**	**0.04**	**6.23**	**0.01**	**0.12**
Grassland	1	-0.03	2.94	0.09	0.12
Shrubland	1	0.09	1.07	0.30	0.09
Developed land	1	-0.01	2.44	0.12	0.10
Water	1	0.01	0.31	0.58	0.08
Wetland	1	0.04	0.52	0.47	0.09
Gastropod host density	1	0.62	1.42	0.23	0.09
White-tailed deer fecal density	1	-4.70	0.24	0.62	0.08

Table 2.8 Habitat type did not predict prevalence of dorsal-spined larvae prevalence. Zero-inflated Poisson models of protostrongylid prevalence and two habitat types. Contains information licensed under the Open Government Licence – Canada.

Habitat type	Degrees of freedom	Estimates	χ^2	*P*
Mixed-wood forest	1	0.04	2.13	0.14
Coniferous forest	1	0.03	1.60	0.21

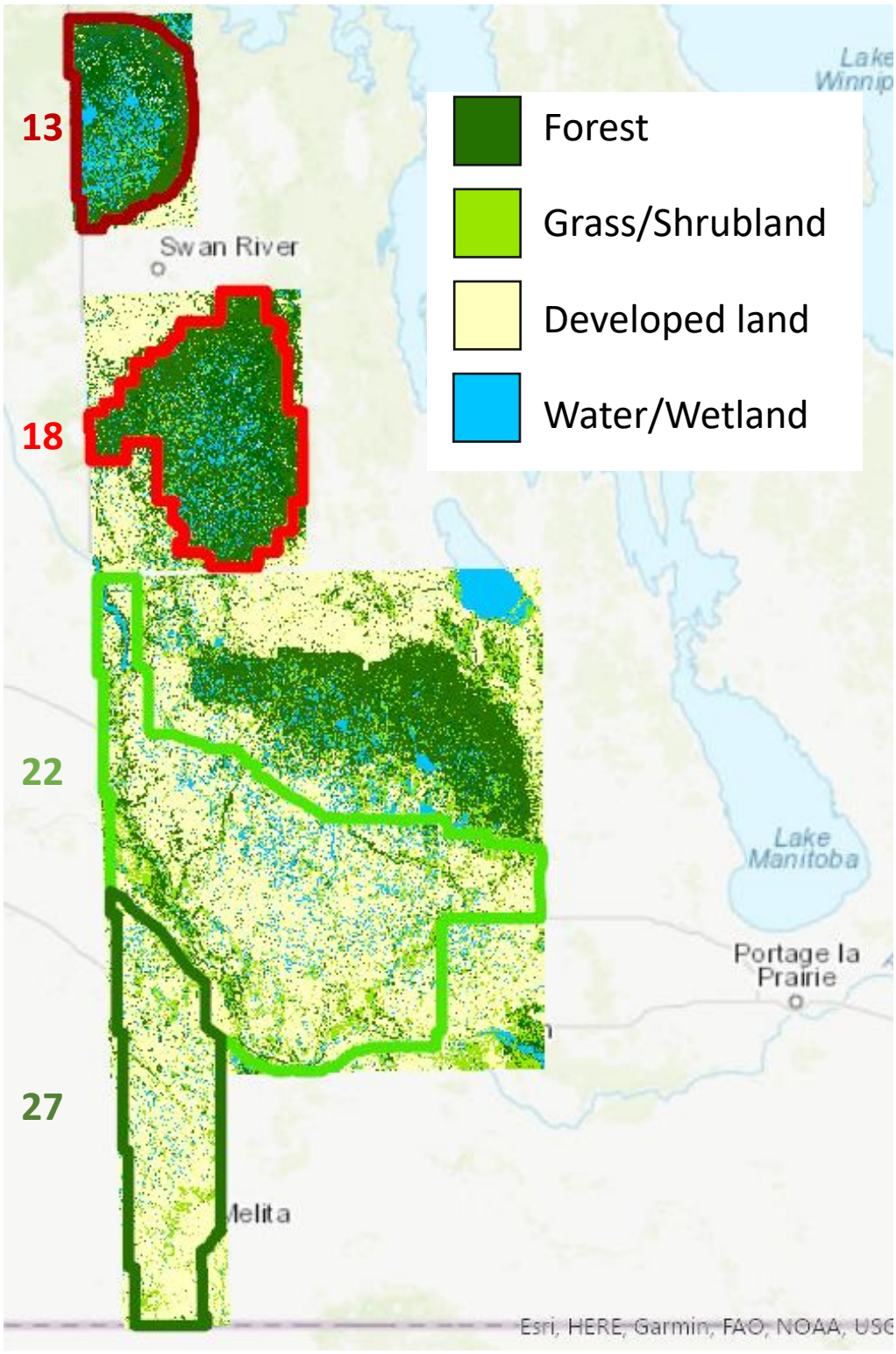

Figure 2.1 The habitat composition (proportions of each habitat type) of each game hunting area in the study. Data from Annual Crop Inventory 2019 and summarized within each GHA using ArcGIS Pro 2.7.3. Contains information licensed under the Open Government Licence – Canada.

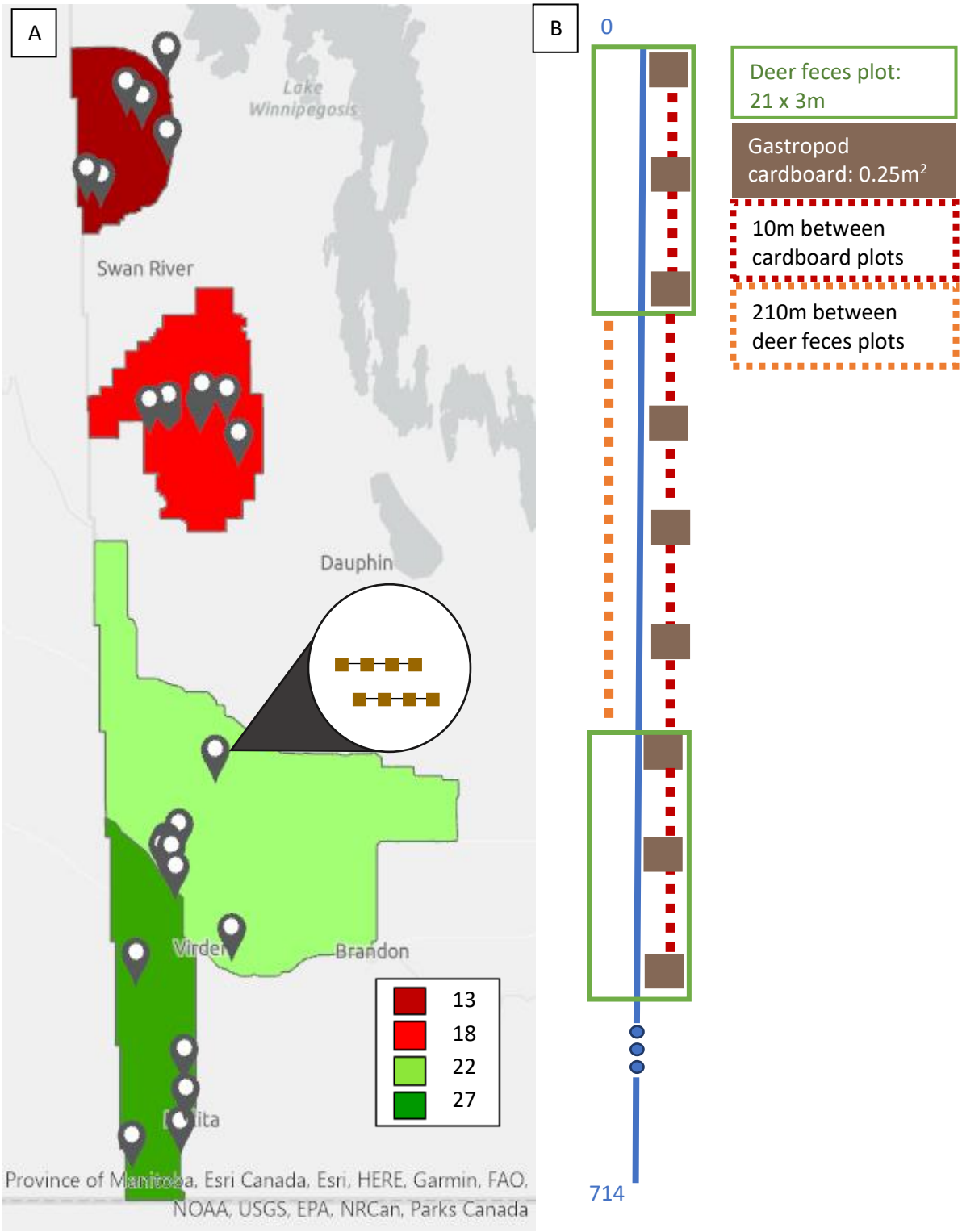

Figure 2.2 A. Sampling locations for each GHA sampled in Western Manitoba 2020-2021. Within each location, we sampled 2 terrestrial transects. Map made in ArcGIS 2.7.3 (ESRI 2020). B. Transect outline. 714m transects consisted of 4 plots where white-tailed deer fecal samples were collected and 68 cardboard squares that were checked for gastropods.

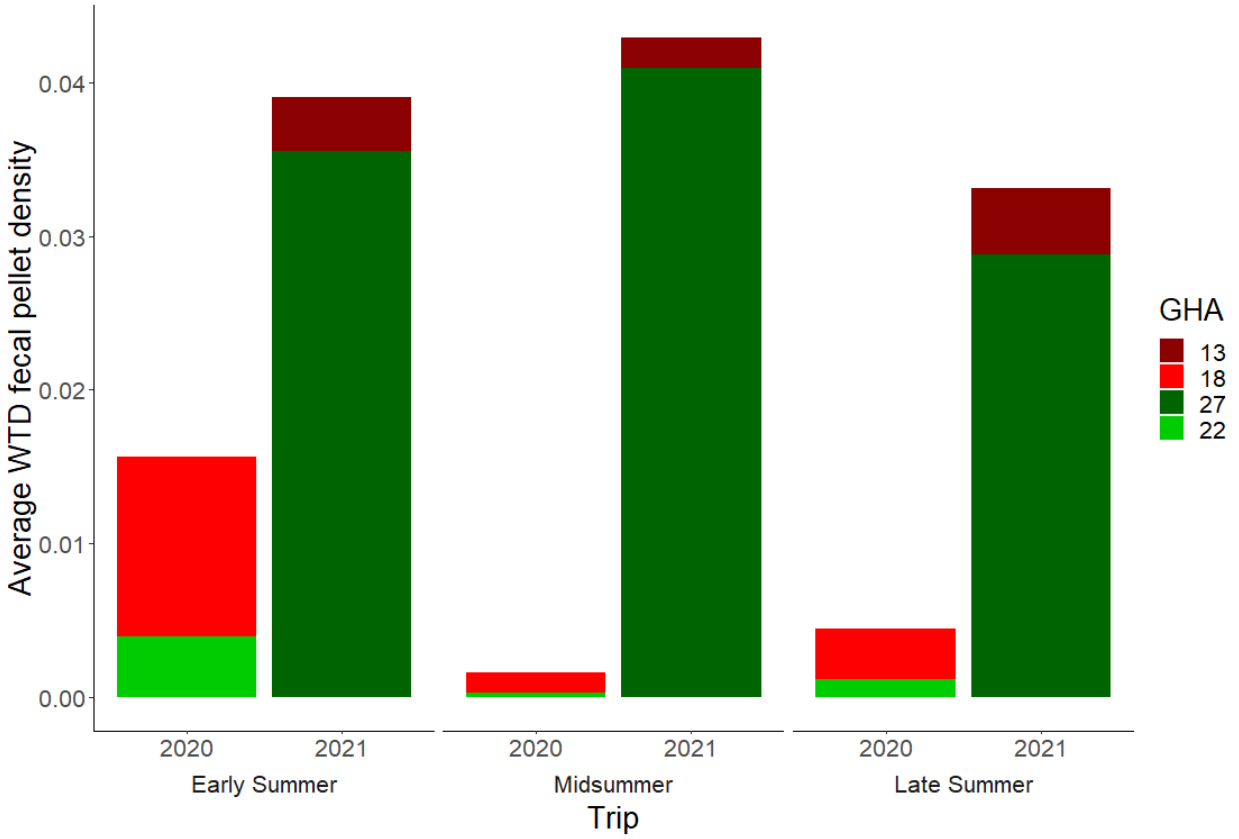

Figure 2.3 Average white-tailed deer (WTD, *Odocoileus virginianus*) fecal pellet density (pellets/m^2) for each game hunting area (GHA) collected in 2020 and 2021. For trips, early summer collection was in June, mid-summer was July-August and late summer was August-September. GHAs 18 and 22 were sampled in 2020 (bright colours) while GHAs 13 and 27 were sampled in 2021 (dark colours). GHA 13 and 18 (red) are areas where moose populations are a conservation concern whereas GHAs 22 and 27 (green) are areas where moose populations are not a conservation concern.

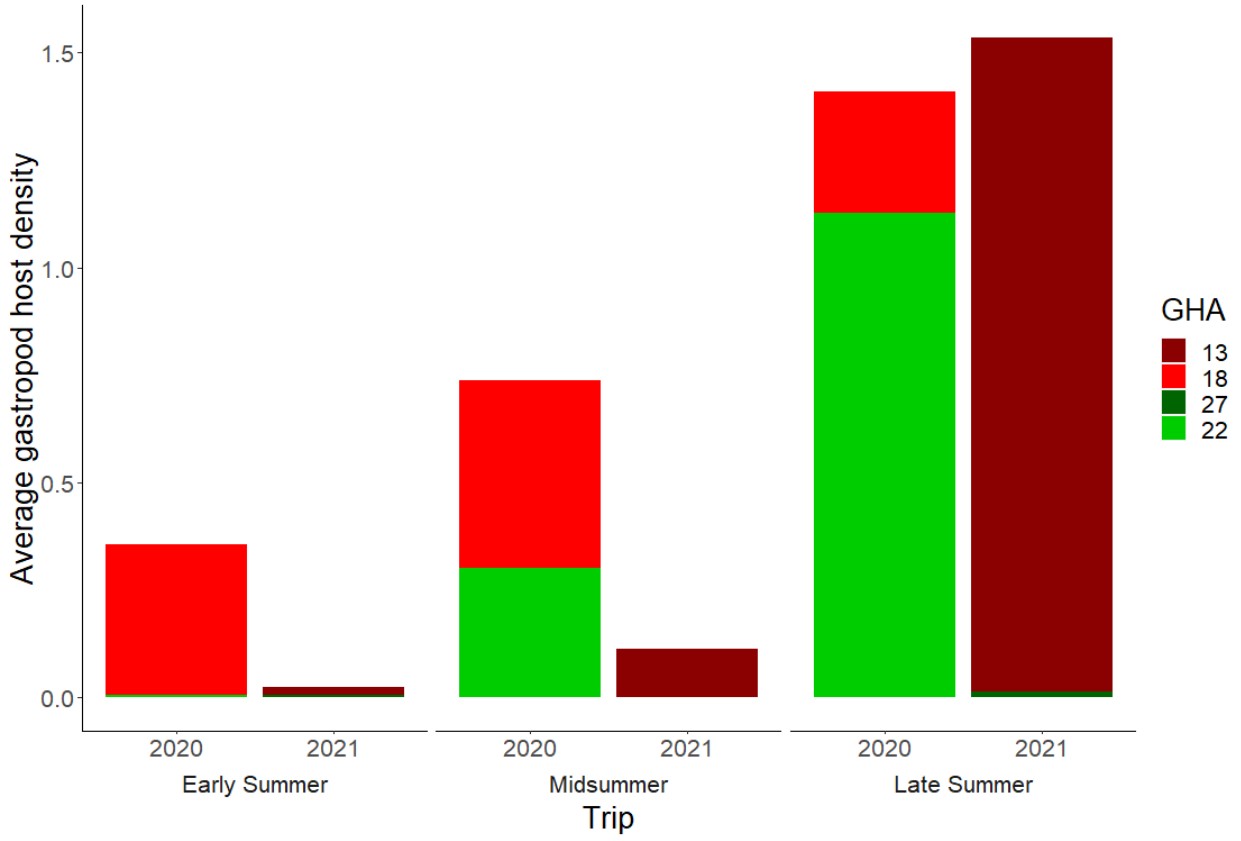

Figure 2.4 Average gastropod density (gastropods/m^2) for each game hunting area (GHA) collected in 2020 and 2021. For trips, early summer collection was in June, mid-summer was July-August and late summer was August-September. GHAs 18 and 22 were sampled in 2020 (bright colours) while GHAs 13 and 27 were sampled in 2021 (dark colours). GHA 13 and 18 (red) are areas where moose populations are a conservation concern whereas GHAs 22 and 27 (green) are areas where moose populations are not a conservation concern.

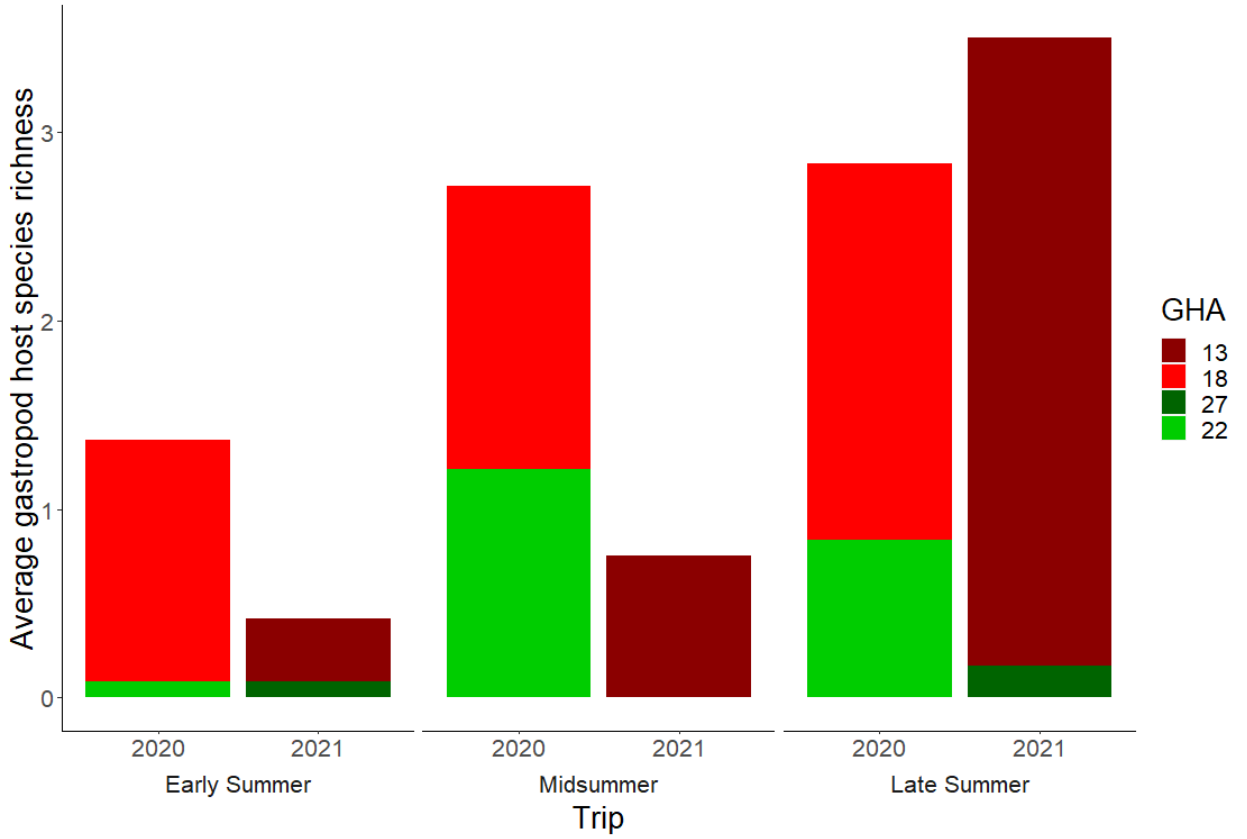

Figure 2.5 Average gastropod species richness for each game hunting areas (GHA) collected in 2020 and 2021. For trips, early summer collection was in June, mid-summer was July-August and late summer was August-September. GHAs 18 and 22 were sampled in 2020 (bright colours) while GHAs 13 and 27 were sampled in 2021 (dark colours). GHA 13 and 18 (red) are areas where moose populations are a conservation concern whereas GHAs 22 and 27 (green) are areas where moose populations are not a conservation concern.

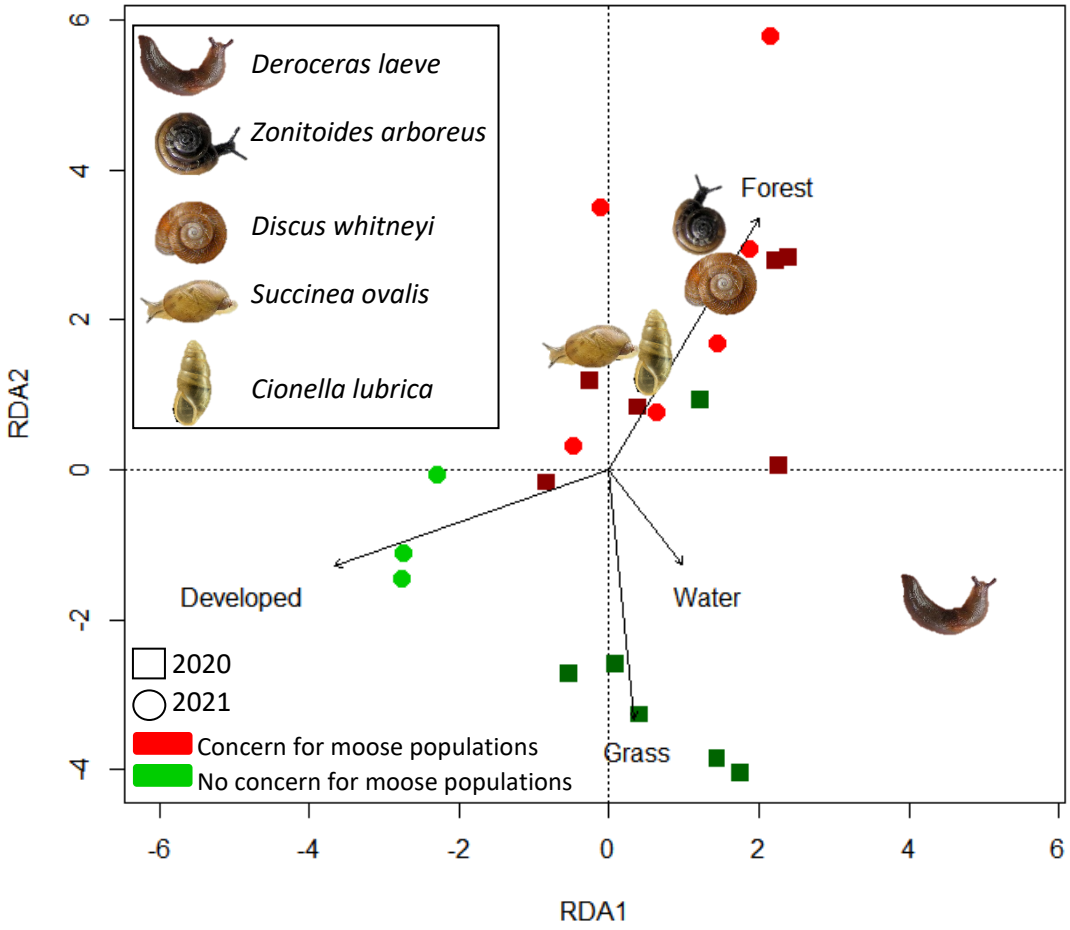

Figure 2.6 Redundancy analysis of the relationship between gastropod host species density (gastropods/m^2) and the habitat composition of the location they were found. Habitat variables include Forest (broadleaf, coniferous and mixed-woods), Grass (grassland and shrubland), Water (water and wetlands) and Developed (agriculture and developed areas). Green points are locations in game hunting areas (GHA) were there is no concern for moose populations, red points are locations in GHA were there is concern for moose populations. Squares are locations sampled in 2020, circles are locations sampled in 2021.

Thesis Conclusion

We investigated hotspots of *Parelaphostrongylus* spp. transmission to moose (*Alces alces*) in Western Manitoba. There are two *Parelaphostrongylus* parasites present in Manitoba, meningeal worm (*Parelaphostrongylus tenuis)* and muscle worm (*Parelaphostrongylus andersoni*) but only meningeal worm is known to cause severe pathology and morbidity in infected moose. Also, both parasites are spread by their primary hosts, white-tailed deer (WTD, *Odocoileus virginianus*). Previous work in Manitoba has found areas with higher prevalence of adult meningeal worm in WTD heads, but the shedding rates and thus risk to subsequent hosts was unknown. It is challenging to study the shedding rates of these parasites in WTD fecal samples because these parasites are morphologically indistinguishable at this stage of development. Few studies have genetically identified these parasites, so the relative geographic distribution of both parasites is unclear in Manitoba and its unknown where and when moose are more at risk of *Parelaphostrongylus* spp. infections.

In Chapter 1, we assessed the spatial and temporal prevalence of *Parelaphostrongylus* spp. larvae found in WTD fecal pellets to determine where and when risk of infection in moose was highest as well as determine whether moose population concerns were associated with higher prevalence of parasites. We found that prevalence of *Parelaphostrongylus* spp. larvae in WTD feces was positively associated with areas sampled where moose population are a conservation concern. There was no association with any of the variables included to assess temporal variation of parasite prevalence, suggesting that WTD shed larvae consistently throughout the summer and between years collected.

There was higher prevalence of the adult stage of meningeal worm in WTD from the areas where we collected in Western Manitoba from 2018-2019 (7-55% infected WTD) than the DSL prevalence we found in WTD fecal pellets (7-33%) which contains infections of muscle worm (Province of Manitoba Fish and Wildlife Branch, unpublished data). However, determining infection prevalence

from WTD heads may overestimate infection risk as not all infected WTD may be shedding larvae in their fecal pellets. Therefore, assessing infection prevalence in WTD fecal pellets may give a better indication of larvae in the environment and moose infection risk. The data from the WTD heads also gave no indication of the distribution of muscle worm in Western Manitoba. Finding muscle worm in WTD tissue is challenging, requiring post-mortem analyses of WTD by trained personnel, and is thus not always feasible. Therefore, another advantage of collecting WTD feces to investigate meningeal worm infection risk is the ability to determine muscle worm prevalence as well.

We used the partial *CO1* gene to differentiate the L1 stage of *Parelaphostrongylus* from WTD fecal pellets to species and found both meningeal worm and muscle worm. Considering previous reports of the geographic range of muscle worm, we did not anticipate recovering muscle worm in the GHAs we sampled, especially for the more southern ones. We found both parasite species in GHA 13, 18, 27, and only meningeal worm at GHA 22, though more sampling in this GHA may reveal *P. andersoni*. Based on our results, it is likely that meningeal worm and muscle worm follow similar geographic distributions in the areas of Western Manitoba. Not only did the parasite species co-occur within a GHA, but we confirmed co-infection of meningeal worm and muscle worm within some individual WTD. Co-infections have been rarely reported, but that may be due to the challenges of diagnosing adult worms in the meninges and in the muscles. We only genetically identified up to 3 larvae per sample which is not sufficient sampling to estimate co-infection at the WTD population level, especially if one species is being shed at a higher rate than the other. As genetic identification is costly, time-consuming, and requires expertise and specialized equipment, species accumulation curves for individual fecal samples or geographic areas could be estimated to determine the ratios at which the different parasites species are shed and how many larvae should be genetically identified per sample to determine if both species are present. Alternatively, if morphological characteristics could be found to discriminate between the two species, we could eliminate the need for extensive genetic analyses. This

study highlights the need to genetically identify DSL found in WTD fecal samples as we cannot assume that DSL in fecal pellets from these areas are only meningeal worm. We illustrated that muscle worm is found more south in Manitoba than has been previously reported, overlapping with meningeal worm in at many locations. Further work is needed to expand our knowledge of the geographic ranges of both parasites in other parts of Manitoba.

There were discrepancies in the literature about whether muscle worm distribution primarily followed that of their caribou hosts or whether their distribution is consistent with the geographic range of WTD. While we cannot determine whether muscle worm is found consistently throughout the entire range of WTD, our research shows muscle worm infections in WTD hosts in areas where we do not expect to find caribou in Manitoba. Research investigating meningeal worm in WTD fecal samples should ensure adequate identification in larvae and report findings of muscle worm to broaden our understanding of its geographic distribution throughout North America.

In Chapter 2 we assessed the host and habitat factors that could contribute to *Parelaphostrongylus* spp. transmission to moose. To investigate the role of host density, we assessed the spatial and temporal variation of WTD fecal pellet density and intermediate host (terrestrial gastropod) density. Definitive ad intermediate host densities were not associated with DSL prevalence and or areas where there is concern for moose populations. Instead, gastropod host species richness was associated with areas sampled where moose populations are a conservation concern. This result suggests that areas with greater gastropod species diversity may be hotspots for meningeal worm transmission. Meningeal worm is a generalist for intermediate host specificity which may be advantageous for its transmission to WTD and moose. These gastropod hosts occur in different habitats (*D. laeve* is present in grasslands and *D. whitneyi* is found in forests, grasslands, and shrublands) and the hosts may also differ in their climbing abilities which may play a role in transmission. Some of the gastropod hosts, such as *D. laeve* and *Z. arboreus*, are primarily soil-dwelling. These hosts may have

increased risk of becoming infected by L1 larvae in feces or in soil from increased contact rates and may be consumed when WTD forage close to the ground from the grass-forb layer. Alternatively, other gastropod hosts, such as *D. whitneyi* and *S. ovalis*, spent equal time on the ground as they did climbing which makes these gastropods vulnerable to infection on the ground and vulnerable to consumption by WTD and moose when the gastropods climb. The presence of hosts with both movement patterns may increase transmission risk to WTD and moose.

The temporal analyses revealed higher gastropod density in late summer suggesting that infection risk to moose is likely highest in these months. Higher gastropod host density could increase contact rates with the larval parasites in the WTD feces or soil as well as increased consumption rates by WTD and moose once the gastropods are infected. However, both hypotheses need to be assessed especially given the potential influence of spatial and temporal heterogeneity on these variables. In our study, neither pellet nor gastropod density was directly associated with DSL prevalence. Perhaps this trend is because WTD were not infected in the area in which they were shedding fecal pellets. To investigate the role of habitat type, we determined whether gastropods or DSL were associated with habitat type and predicted that both would be positively associated with higher proportions of forest habitat types as these areas suggest cooler, more wet conditions. We did not find overwhelming evidence that either gastropods or DSL followed this pattern. Only one gastropod species, *D. whitneyi*, was associated with forest habitat types and habitat types did not predict DSL prevalence in our final model. Future work using methods of gastropod collection that consider gastropods that climb and identifying habitat characteristics such as canopy type, canopy cover, litter depth, or dominant vegetation from the exact location of collection could help distinguish hotspots for transmission.

This project also provides novel insights to the gastropod hosts present in Western Manitoba. Our study provided *CO1* sequences (557-689 bp) for 10 species with 19 total sequences to a public database, many of which will be one of only a few documented individuals. These sequencies will also

increase the known geographic range of the species for future studies investigating the role of gastropod hosts in *Parelaphostrongylus* spp. transmission.

This thesis provides a framework for future studies of meningeal worm in Manitoba. From random sampling, we determined that hotspots for *Parelaphostrongylus* spp. infection to moose are difficult to discern. Habitat type did not predict gastropod density or DSL prevalence. However, even if habitat predicted infection risk, gastropod activity is likely also highly dependent on daily temperature and precipitation variation, making hotspots temporally ephemeral as well as difficult to find spatially. Further, these ephemeral hotspots need to be in areas where moose are likely to consume an infected gastropod. Based on the results of our study, we suggest a mix of random and targeted sampling in areas with high prevalence of *Parelaphostrongylus* spp. in WTD feces. We suggest testing whether the prevalence of meningeal worm in gastropods is associated with elevation or canopy cover. In addition, future research should determine whether arboreal gastropod species collected from vegetation have higher prevalence of meningeal worm. These arboreal gastropods may be amplification hosts for meningeal worm as they may be consumed more frequently by WTD and moose.

We established that areas that have conservation concerns for moose populations are associated with *Parelaphostrongylus* spp., and these areas also have more gastropod host species present. While this may indicate that parasite infection is contributing to moose population concerns, more detailed studies investigating cause of moose mortality is needed to determine whether meningeal worm is significantly affecting moose population health. Nonetheless, management initiatives should be aware of higher prevalence of *Parelaphostrongylus* spp. larvae in WTD in these areas when planning initiatives to prevent moose population declines.

Appendix I: R Code

```
# Libraries

library(pscl)

library(lmtest)

library(car)

library(readxl)

library(multcompView)

library(emmeans)

library(rcompanion)


All_data <- read_excel("Moose_Project_Data.xlsx", sheet = "All_data")

All_data$GHA <-as.factor(All_data$GHA) #game hunting area

All_data$Status <- as.factor(All_data$Status) #status: concern vs no concern for moose populations

All_data$Year <- as.factor(All_data$Year) #year collected

All_data$Trip <- as.factor(All_data$Season) #Trip collection (A, B, C)

All_data$host_abund <- as.numeric(All_data$host_abund) #gastropod host abundance


# HOST ABUNDANCE #

host_abund.f <- formula(host_abund ~ Status + Year * Trip + offset(log(area)) |

                        Status + Year * Trip + offset(log(area)))

#test for multicollinearity using variance inflation factor (VIF)

test <- lm(host_abund ~ Status + Year + Trip, data = All_data)

vif(test)
```

```
#Zero-inflated mdoel with poisson distribution

Host_abund_Zip <- zeroinfl(host_abund.f, dist = "poisson", link = "logit", data = All_data)

summary(Host_abund_Zip)


#Zero-inflated mdoel with negative binomial distribution

Host_abund_Zinb <- zeroinfl(host_abund.f, dist = "negbin", link = "logit", data = All_data)


#Likelihood ratio test comparing poisson and negbin model

lrtest(Host_abund_Zip, Host_abund_Zinb)

#Zinb is significantly better (less negative, larger #)

summary(Host_abund_Zinb)

#model validation

plot(residuals(Host_abund_Zinb), fitted(Host_abund_Zinb))

plot(residuals(Host_abund_Zinb), All_data$Trip)

plot(residuals(Host_abund_Zinb), All_data$Status)

plot(residuals(Host_abund_Zinb), All_data$Year)

#check for over-dispersion (<1)

E2 <- resid(Host_abund_Zinb, type = "pearson")

N  <- nrow(All_data)

p  <- length(coef(Host_abund_Zinb)) + 1 # '+1' is due to theta

sum(E2^2) / (N - p)

#Calculate McFadden's pseudo-R2

Host_abund_Zinb_null <- update(Host_abund_Zinb, . ~ 1)
```

```
1-logLik(Host_abund_Zinb)/logLik(Host_abund_Zinb_null)

#Mangiafico 2016, Rcompanion method is to use chi-sq test to find chi-square and p-value

Anova(Host_abund_Zinb, type = "II", test = "Chisq") #http://rcompanion.org/handbook/J_01.html


#Verify that this is how to determine Pseudo-R2

nagelkerke(Host_abund_Zinb)

#Tukey test

marginal = emmeans(Host_abund_Zinb, ~ Status + Trip + Year)

pairs(marginal, adjust="tukey")

####### HOST SPECIES RICHNESS ####

host_spp.f <- formula(host_spp ~ Status + Year * Trip + offset(log(area)) |

                      Status + Year * Trip + offset(log(area)))

host_spp_Zip <- zeroinfl(host_spp.f, dist = "poisson", link = "logit", data = All_data)

summary(host_spp_Zip)

host_spp_Zinb <- zeroinfl(host_spp.f, dist = "negbin", link = "logit", data = All_data)

lrtest(host_spp_Zip, host_spp_Zinb)

Anova(host_spp_Zip, type = "II", test = "Chisq")

#McFadden's pseudo-R2

nagelkerke(host_spp_Zip)

#model validation

plot(residuals(host_spp_Zip), fitted(host_spp_Zip))
```

```
plot(residuals(host_spp_Zip), All_data$Trip)

plot(residuals(host_spp_Zip), All_data$Status)

plot(residuals(host_spp_Zip), All_data$Year)

#Check for over-dispersion

E2 <- resid(host_spp_Zip, type = "pearson")

N  <- nrow(All_data)

p  <- length(coef(host_spp_Zip)) + 1 # '+1' is due to theta

sum(E2^2) / (N - p)

#McFadden's pseudo-R2

host_spp_Zip_null <- update(host_spp_Zip, . ~ 1)

1-logLik(host_spp_Zinb)/logLik(host_spp_Zip_null)

#Tukey test

marginal = emmeans(host_spp_Zip, ~ Status + Trip + Year)

pairs(marginal, adjust="tukey")

####### FECAL SAMPLES ####

#Update data to omit Carly's extra transects where no fecal samples were collected

All_data_pellets <- na.omit(All_data)

#View(All_data_pellets)

Fecal_pellets.f <- formula(Fecal_pellets ~ Status + Year * Trip + offset(log(area)) |

                      Status + Year * Trip + offset(log(area)))

Fecal_pellets_Zip <- zeroinfl(Fecal_pellets.f, dist = "poisson", link = "logit", data = All_data_pellets)

summary(Fecal_pellets_Zip)
```

```
Fecal_pellets_Zinb <- zeroinfl(Fecal_pellets.f, dist = "negbin", link = "logit", data = All_data_pellets)

lrtest(Fecal_pellets_Zip, Fecal_pellets_Zinb)

summary(Fecal_pellets_Zinb)

#model validation

plot(residuals(Fecal_pellets_Zinb), fitted(Fecal_pellets_Zinb))

plot(residuals(Fecal_pellets_Zinb), All_data_pellets$Trip)

plot(residuals(Fecal_pellets_Zinb), All_data_pellets$Status)

plot(residuals(Fecal_pellets_Zinb), All_data_pellets$Year)

E2 <- resid(Fecal_pellets_Zinb, type = "pearson")

N  <- nrow(All_data_pellets)

p  <- length(coef(Fecal_pellets_Zinb)) + 1 # '+1' is due to theta

sum(E2^2) / (N - p)

Fecal_pellets_Zinb_null <- update(Fecal_pellets_Zinb, . ~ 1)

1-logLik(Fecal_pellets_Zinb)/logLik(Fecal_pellets_Zinb_null)

Anova(Fecal_pellets_Zinb, type = "II", test = "Chisq")



nagelkerke(Fecal_pellets_Zinb)

marginal = emmeans(Fecal_pellets_Zinb, ~ Status + Trip + Year)

pairs(marginal, adjust="tukey")

### spatial and temporal variation of DSL prevalence

All_data_DSL <- read_excel("Moose_Project_Data.xlsx", sheet = "All_data_DSL")
```

```
All_data_DSL <- na.omit(All_data_DSL) #take out transects where no fecal pellets were collected

All_data_DSL$Status <- as.factor(All_data_DSL$Status)

All_data_DSL$Year <- as.factor(All_data_DSL$Year)

All_data_DSL$Trip <- as.factor(All_data_DSL$Season)

All_data_DSL$host_dens <- as.numeric(All_data_DSL$host_dens)

All_data_DSL$DSL_abund <- as.numeric(All_data_DSL$DSL_abund)

All_data_DSL$pellets_baer <- as.numeric(All_data_DSL$pellets_baer)

All_data_DSL$Fecal_dens <- as.numeric(All_data_DSL$Fecal_dens)

test <- lm(DSL_abund ~ Status + Year + Trip, data = All_data_DSL)

vif(test)

DSL.f <- formula(DSL_abund ~ Status + Year * Trip +offset(log(pellets_baer)) |

                 Status + Year * Trip +offset(log(pellets_baer)))

DSL_Zip <- zeroinfl(DSL.f, dist = "poisson", link = "logit", data = All_data_DSL)

summary(DSL_Zip)

DSL_Zinb <- zeroinfl(DSL.f, dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_Zinb)

lrtest(DSL_Zip, DSL_Zinb)

#model validation

plot(residuals(DSL_Zip), fitted(DSL_Zip))

plot(residuals(DSL_Zip), All_data_DSL$Trip)

plot(residuals(DSL_Zip), All_data_DSL$Status)

plot(residuals(DSL_Zip), All_data_DSL$Year)
```

```
E2 <- resid(DSL_Zip, type = "pearson")

N  <- nrow(All_data_DSL)

p  <- length(coef(DSL_Zip)) + 1 # '+1' is due to theta

sum(E2^2) / (N - p)

DSL_Zip_null <- update(DSL_Zip, . ~ 1)

1-logLik(DSL_Zip)/logLik(DSL_Zip_null)



Anova(DSL_Zip, type = "II", test = "Chisq") #http://rcompanion.org/handbook/J_01.html

marginal = emmeans(DSL_Zip, ~ Status + Trip + Year)

pairs(marginal, adjust="tukey")

## Which host densities and habitat types influence DSL prevalence

#First, use univariate models

DSL_abund_null <- zeroinfl(DSL_abund ~ offset(log(pellets_baer))  |

                    offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)

#Mixedwood

DSL_abund_mix_ZIP <- zeroinfl(DSL_abund ~ Mixedwood_D + offset(log(pellets_baer)) |

        Mixedwood_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data =All_data_DSL)

summary(DSL_abund_mix_ZIP)


DSL_abund_mix_ZINB <- zeroinfl(DSL_abund ~ Mixedwood_D + offset(log(pellets_baer)) |

        Mixedwood_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_abund_mix_ZINB)
```

```
lrtest(DSL_abund_mix_ZIP, DSL_abund_mix_ZINB)


Anova(DSL_abund_mix_ZIP, type = "II", test = "Chisq")
nagelkerke(DSL_abund_mix_ZIP)


#Coniferous
DSL_abund_con_ZIP <- zeroinfl(DSL_abund ~ Coniferous_D + offset(log(pellets_baer)) |
        Coniferous_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_con_ZIP)

DSL_abund_con_ZINB <- zeroinfl(DSL_abund ~ Coniferous_D + offset(log(pellets_baer)) |
        Coniferous_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)
summary(DSL_abund_con_ZINB)
lrtest(DSL_abund_con_ZIP, DSL_abund_con_ZINB)
Anova(DSL_abund_con_ZIP, type = "II", test = "Chisq")
nagelkerke(DSL_abund_con_ZIP)
#Broadleaf
DSL_abund_BL_ZIP <- zeroinfl(DSL_abund ~ Broadleaf_D + offset(log(pellets_baer)) |
        Broadleaf_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_BL_ZIP)


DSL_abund_BL_ZINB <- zeroinfl(DSL_abund ~ Broadleaf_D + offset(log(pellets_baer)) |
        Broadleaf_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)
```

```
summary(DSL_abund_BL_ZINB)

lrtest(DSL_abund_BL_ZIP, DSL_abund_BL_ZINB)

Anova(DSL_abund_BL_ZIP, type = "II", test = "Chisq")

nagelkerke(DSL_abund_BL_ZIP)

#Grassland

DSL_abund_grass_ZIP <- zeroinfl(DSL_abund ~ Grass_D + offset(log(pellets_baer)) |
        Grass_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)

summary(DSL_abund_grass_ZIP)

DSL_abund_grass_ZINB <- zeroinfl(DSL_abund ~ Grass_D + offset(log(pellets_baer)) |
        Grass_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_abund_grass_ZINB)

lrtest(DSL_abund_grass_ZIP, DSL_abund_grass_ZINB)

Anova(DSL_abund_grass_ZIP, type = "II", test = "Chisq")

nagelkerke(DSL_abund_grass_ZIP)

#Developed

DSL_abund_Developed_ZIP <- zeroinfl(DSL_abund ~ Developed_D + offset(log(pellets_baer)) |
        Developed_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)

summary(DSL_abund_Developed_ZIP)


DSL_abund_Developed_ZINB <- zeroinfl(DSL_abund ~ Developed_D + offset(log(pellets_baer)) |
        Developed_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_abund_Developed_ZINB)

lrtest(DSL_abund_Developed_ZIP, DSL_abund_Developed_ZINB)
```

```
Anova(DSL_abund_Developed_ZIP, type = "II", test = "Chisq")

nagelkerke(DSL_abund_Developed_ZIP)

#Shrubland

DSL_abund_Shrub_ZIP <- zeroinfl(DSL_abund ~ Shrub_D + offset(log(pellets_baer)) |
        Shrub_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)

summary(DSL_abund_Shrub_ZIP)


DSL_abund_Shrub_ZINB <- zeroinfl(DSL_abund ~ Shrub_D + offset(log(pellets_baer)) |
        Shrub_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_abund_Shrub_ZINB)

lrtest(DSL_abund_Shrub_ZIP, DSL_abund_Shrub_ZINB)

Anova(DSL_abund_Shrub_ZIP, type = "II", test = "Chisq")

nagelkerke(DSL_abund_Shrub_ZIP)

#Water

DSL_abund_Water_ZIP <- zeroinfl(DSL_abund ~ Water_D + offset(log(pellets_baer)) |
        Water_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)

summary(DSL_abund_Water_ZIP)


DSL_abund_Water_ZINB <- zeroinfl(DSL_abund ~ Water_D + offset(log(pellets_baer)) |
        Water_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)

summary(DSL_abund_Water_ZINB)

lrtest(DSL_abund_Water_ZIP, DSL_abund_Water_ZINB)

Anova(DSL_abund_Water_ZIP, type = "II", test = "Chisq")
```

```
nagelkerke(DSL_abund_Water_ZIP)

#Wetland
DSL_abund_Wetland_ZIP <- zeroinfl(DSL_abund ~ Wetland_D + offset(log(pellets_baer)) |
        Wetland_D + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_Wetland_ZIP)


DSL_abund_Wetland_ZINB <- zeroinfl(DSL_abund ~ Wetland_D + offset(log(pellets_baer)) |
        Wetland_D + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)
summary(DSL_abund_Wetland_ZINB)
lrtest(DSL_abund_Wetland_ZIP, DSL_abund_Wetland_ZINB)

Anova(DSL_abund_Wetland_ZIP, type = "II", test = "Chisq")
nagelkerke(DSL_abund_Wetland_ZIP)

#Gastropod host density
DSL_abund_host_dens_ZIP <- zeroinfl(DSL_abund ~ host_dens + offset(log(pellets_baer)) |
        host_dens + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_host_dens_ZIP)

DSL_abund_host_dens_ZINB <- zeroinfl(DSL_abund ~ host_dens + offset(log(pellets_baer)) |
         host_dens + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)
summary(DSL_abund_host_dens_ZINB)

lrtest(DSL_abund_host_dens_ZIP, DSL_abund_host_dens_ZINB)


Anova(DSL_abund_host_dens_ZIP, type = "II", test = "Chisq")
```

```
nagelkerke(DSL_abund_host_dens_ZIP)

#WTD fecal pellet density
DSL_abund_Fecal_dens_ZIP <- zeroinfl(DSL_abund ~ Fecal_dens + offset(log(pellets_baer)) |
        Fecal_dens + offset(log(pellets_baer)), dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_Fecal_dens_ZIP)

DSL_abund_Fecal_dens_ZINB <- zeroinfl(DSL_abund ~ Fecal_dens + offset(log(pellets_baer)) |
        Fecal_dens + offset(log(pellets_baer)), dist = "negbin", link = "logit", data = All_data_DSL)
summary(DSL_abund_Fecal_dens_ZINB)
lrtest(DSL_abund_Fecal_dens_ZIP, DSL_abund_Fecal_dens_ZINB) #Zip

Anova(DSL_abund_Fecal_dens_ZIP, type = "II", test = "Chisq")
nagelkerke(DSL_abund_Fecal_dens_ZIP)

#Models with significant factors from univariate models
DSL_abund_f2 <- formula(DSL_abund ~  Mixedwood_D + Coniferous_D + Grass_D +
offset(log(pellets_baer)) |
        Mixedwood_D + Coniferous_D + Grass_D +  offset(log(pellets_baer)), data = All_data_DSL)

DSL_abund_Zip2 <- zeroinfl(DSL_abund_f2, dist = "poisson", link = "logit", data = All_data_DSL)
summary(DSL_abund_Zip2)

test <- lm(DSL_abund ~ Coniferous_D + Mixedwood_D+ Developed_D + offset(log(pellets_baer)), data =
All_data_DSL)
vif(test)

Anova(DSL_abund_Zip2, type = "II", test = "Chisq")
nagelkerke(DSL_abund_Zip2)
```

```
######## RDA: host density and habitat (adjusted for snails)
library(readxl)
library(tidyverse)
library(vegan)

T_host_dens_all <- read_excel("Moose_Project_Data.xlsx", sheet = "T_hosts_dens_all")
T_host_dens_all<- column_to_rownames(T_host_dens_all, var = "Location")

T_habitat_all <- read_excel("Moose_Project_Data.xlsx", sheet = "All_habitat")
T_habitat_all<- column_to_rownames(T_habitat_all, var = "Location")

T_host_dens_all_log <- decostand(T_host_dens_all, method = "log")

status_groups = (c("green3", "green3","green3","green3","green3","green3","red","red", "red", "red",
"red", "red", "dark green", "dark green","dark green","dark green","dark green","dark green","dark
red","dark red", "dark red", "dark red", "dark red", "dark red" ))

year_groups = (c(pch = 16, pch = 16, pch = 16, pch = 16, pch = 16, pch = 16, pch = 16, pch = 16, pch = 16,
pch = 16, pch = 16, pch = 16,pch = 15, pch = 15, pch = 15, pch = 15, pch = 15, pch = 15, pch = 15, pch =
15, pch = 15, pch = 15, pch = 15, pch = 15 ))

Snail_dens_all.rda <- rda(T_host_dens_all_log ~., T_habitat_all)
summary(Snail_dens_all.rda) #RDA1: 11.9%, RDA2 #6.9%, Total 18.7%


plot(Snail_dens_all.rda, scaling =0)
        plot(Snail_dens_all.rda, type = "n")
        points(Snail_dens_all.rda, col = status_groups, pch = year_groups, cex = 1.5)
        text(Snail_dens_all.rda, display = "bp")
```

```
        points(Snail_dens_all.rda, display = "species", scaling = 1)

anova(Snail_dens_all.rda)

######## Column plots ##################

library(ggplot2)

library(readxl)


All_data <- read_excel("Moose_Project_Data.xlsx", sheet = "All_data")

All_data$GHA <-as.factor(All_data$GHA)

All_data$GHA <- factor(All_data$GHA,levels = c("13", "18", "27", "22"))

All_data_pellets$GHA <- factor(All_data_pellets$GHA,levels = c("13", "18", "27", "22"))

All_data$Status <- as.factor(All_data$Status)

All_data$Year <- as.factor(All_data$Year)

All_data$Trip <- as.factor(All_data$Trip)


#Host density

p_bars <-ggplot(All_data, aes(fill = GHA, x = Year , y = host_dens)) + labs( y = "Gastropod host density", x
= "Trip") +   geom_col() +  facet_grid(~ Trip,

        scales = "free_x", # Let the x axis vary across facets.

        space = "free_x",  # Let the width of facets vary and force all bars to have the same width.

        switch = "x") +     # Move the facet labels to the bottom

 theme(strip.placement = "outside",                   # Place facet labels outside x axis labels.

      strip.background = element_rect(fill = "white"),  # Make facet label background white.

      panel.grid.major = element_blank(), panel.grid.minor = element_blank(),

      panel.background = element_blank(), axis.line = element_line(colour = "black"),
```

```
      text = element_text(size=20))  # Remove x and y axis titles.
p_bars + geom_bar(position = "stack", stat = "identity") + scale_fill_manual(values=c("Dark Red", "Red",
"Dark Green", "Green3"))

#Host diversity
p_bars <-ggplot(All_data, aes(fill = GHA, x = Year , y = host_spp)) + labs( y = "Gastropod host species
richness", x = "Trip")+  geom_col() + facet_grid(~ Trip,
          scales = "free_x", # Let the x axis vary across facets.
          space = "free_x",  # Let the width of facets vary and force all bars to have the same width.
          switch = "x") +     # Move the facet labels to the bottom
  theme(strip.placement = "outside",                   # Place facet labels outside x axis labels.
      strip.background = element_rect(fill = "white"),  # Make facet label background white.
      panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
      panel.background = element_blank(), axis.line = element_line(colour = "black"),
      text = element_text(size=20))  # Remove x and y axis titles.
p_bars + geom_bar(position = "stack", stat = "identity") + scale_fill_manual(values=c("Dark Red", "Red",
"Dark Green", "Green3"))

#Fecal pellet density
p_bars <-ggplot(All_data, aes(fill = GHA, x = Year , y = Fecal_dens)) + labs( y = "WTD fecal pellet density",
x = "Trip")+  geom_col() +  facet_grid(~ Trip,
          scales = "free_x", # Let the x axis vary across facets.
          space = "free_x",  # Let the width of facets vary and force all bars to have the same width.
          switch = "x") +     # Move the facet labels to the bottom
```

```
 theme(strip.placement = "outside",                  # Place facet labels outside x axis labels.
       strip.background = element_rect(fill = "white"),  # Make facet label background white.
       panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
       panel.background = element_blank(), axis.line = element_line(colour = "black"),
       text = element_text(size=20))  # Remove x and y axis titles.
p_bars + geom_bar(position = "stack", stat = "identity") + scale_fill_manual(values=c("Dark Red", "Red",
"Dark Green", "Green3"))


All_data_DSL <- read_excel("Moose_Project_Data.xlsx", sheet = "All_data_DSL")
All_data_DSL <- na.omit(All_data_DSL) #transects with no fecal pellets collected
All_data_DSL$GHA <-as.factor(All_data_DSL$GHA)
All_data_DSL$GHA <- factor(All_data_DSL$GHA,levels = c("13", "18", "27", "22"))
All_data_DSL$Status <- as.factor(All_data_DSL$Status)
All_data_DSL$Year <- as.factor(All_data_DSL$Year)
All_data_DSL$Trip <- as.factor(All_data_DSL$Trip)


p_bars <-ggplot(All_data_DSL, aes(fill = GHA, x = Year , y = Prevalence)) + labs( y = "DSL prevalence", x =
"Trip")+ geom_col() +  facet_grid(~ Trip,
           scales = "free_x", # Let the x axis vary across facets.
           space = "free_x",  # Let the width of facets vary and force all bars to have the same width.
           switch = "x") +     # Move the facet labels to the bottom
 theme(strip.placement = "outside",                  # Place facet labels outside x axis labels.
       strip.background = element_rect(fill = "white"),  # Make facet label background white.
       panel.grid.major = element_blank(), panel.grid.minor = element_blank(),
```

```
    panel.background = element_blank(), axis.line = element_line(colour = "black"), # Remove x and y
axis titles.
    text = element_text(size=20))
p_bars + geom_bar(position = "stack", stat = "identity") + scale_fill_manual(values=c("Dark Red", "Red",
"Dark Green", "Green3"))
```