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Epidemiology of select species of filarial nematodes in free-ranging moose (*Alces alces*) of North America

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To the Graduate Council:

I am submitting herewith a dissertation written by Caroline Mae Grunenwald entitled "Epidemiology of select species of filarial nematodes in free-ranging moose (*Alces alces*) of North America." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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Epidemiology of select species of filarial nematodes in free-ranging moose (*Alces alces*) of North America

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DEDICATION

“Science knows no country because knowledge belongs to humanity, and is the torch that illuminates the world.”

Louis Pasteur

This dissertation is dedicated to all the Torchbearers.

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ABSTRACT

North American moose (*Alces alces*) are a culturally and economically valued species. Recent population declines raise concern for the survivability of this natural resource. The Minnesota population has experienced the most dramatic decline, with a 60% loss in total numbers since 2006. Nematode parasites, particularly some species of filarids, are important pathogens of moose and could be contributing to morbidity and mortality. This study investigates the eco-epidemiology of two filarial parasites of moose: *Rumenfilaria andersoni* and *Elaeophora schneideri.* By surveying cervid species from six U.S. states, we discovered *R. andersoni* was present in moose from all sample locations with prevalence varying between 20-40%. This suggests *R. andersoni* is distributed throughout North America. We also observed *R. andersoni* in white-tailed deer (*Odocoileus virginianus*) but not elk (*Cervus canadensis*) or caribou (*Rangifer tardanus*). Sequence analysis of *R. andersoni* suggested the existence of two distinct clades. Molecular screening of Minnesota tabanid flies discovered 1.5% harbored *R. andersoni*, with only *Chrysops* spp. deerflies containing *R. andersoni* DNA. This is the first report of *R. andersoni* in moose and white-tailed deer herds within the contiguous U.S. and the first time horseflies have been implicated as the vector. Molecular surveys for parasitic nematodes in brain tissues from Minnesota moose that died from unknown causes revealed 63% produced 18S sequences closely aligning with *E. schneideri*, a neurological pathogen of moose previously unreported in Minnesota. Molecular screening of Minnesota tabanid flies revealed *E. schneideri* was present in the environment and transmission could occur locally. Prevalence ranged between 0-100% per trapping site, with *Chrysops* spp. and *Hybomitra* spp. horseflies implicated as vectors. This is the first report of *Chrysops* spp. serving as a carrier of *E. schneideri* and the first report of *E. schneideri* in Minnesota, suggesting *E. schneideri* is an emerging pathogen in the Minnesota herd. Together these data demonstrate the presence of multiple parasitic nematode species in vulnerable moose populations, yet it is still unclear what the implications are for herd health. Further research is warranted to determine if a link between nematode infections and declining moose populations exists.

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CHAPTER I
INTRODUCTION

INTRODUCTION TO THE NORTH AMERICAN MOOSE (*ALCES ALCES*)

General Life History and Ecology of North American Moose

As majestic as they are enormous, moose (*Alces alces*) of North America are the largest extant species of the cervid family (Artiodactyla: Cervidae), which includes various species of deer, elk (*Cervus canadensis*), and caribou (*Rangifer tardanus*). Moose are distributed throughout most of Canada and Alaska, as well as northern New England and New York, northern Minnesota, and Michigan's upper peninsula. In the western U.S., moose occupy regions in North Dakota, Montana, Wyoming, Colorado, Idaho, Utah, Washington, and Oregon (*1*) (Fig. 1[1]). In general, moose occupy a variety of habitats including boreal forest, mixed forest, large delta flood-plains, tundra and subalpine shrub, and stream valleys (*2*). As strict herbivores, moose consume a diet primarily composed of woody plants (i.e. browse) including twigs and stripped leaves from deciduous trees. Specifically, willow (*Salix* spp.) tends to be an important and preferred food source of moose (*3*).

Four separate subspecies of moose are recognized in North America: *A. alces shirasi (4), A. alces americana (5), A. alces, andersoni (6), and A. alces gigas (7),* with each occupying a distinct geographical range and exhibiting slight variations in size, appearance, and habitat preference. Adult moose weigh between at 380-710k and stand approximately 1.4-2.1 meters high, with males slightly larger than females (*8*). As is with other species of cervids, adult male moose are identified by their characteristic large set of palmate antlers. In general, moose are solitary animals and do not regularly congregate in herds like elk or deer; however adult females tend to remain with their calves for the first 9-10 months of life (*9*). Moose are also relatively long-lived, with an estimated lifespan of 15-20 years (*10*).

North American Moose are a Valued Species

Moose have long been recognized as a cultural icon and symbol of the wilderness. Seeking opportunities for viewing and encountering moose can compel humans to visit state or national parks and recreation areas harboring moose (*11*). In 1984 Bisset estimated the total gross value of all moose-related recreation to be $1.3 billion U.S. dollars ($1.6 billion Canadian dollars) (*12*). These revenues brought in through ecotourism benefit local businesses as well as state government agencies. Although it may be difficult to accurately estimate the non-consumptive value of moose, human activity motivated by a general desire to enjoy nature and its inhabitants, as well as to protect the natural ecosystem is certainly of intrinsic value.

[1] All figures are located in the Appendix

Perhaps more easily calculated is the economic value and impact associated with moose hunting activities. Total economic activity generated from moose hunting includes both direct (e.g. purchase of hunting license) and indirect expenditures (e.g. lodging, provisions, etc.). In 1982 Bisset estimated the total annual expenditures for moose hunting in North America to be $464 million Canadian dollars (approximately $357 million U.S. dollars) (*12*). Currently 11 U.S. states and 10 Canadian providences host an annual, regulated moose hunt. Although current economic impact estimates are not available, moose hunting is still viewed as an economically important activity to local governments and businesses.

Pathogens and Moose Population Decline

Over the last decade some areas of North America have experienced a decline in total moose population numbers (*13-15*). The cause of North American moose population declines likely vary with each moose herd and a variety of factors including a warming climate, predation, destruction of habitat, increased interspecies competition, and disease may contribute to morbidity, mortality, and poor recruitment rates. Although many disease agents have been documented in moose, only a few known pathogens are suspected to have the potential to significantly impact moose populations. These include winter ticks (*Dermacentor albipictus*), meningeal worm (*Parelaphostrongylus tenuis*), the arterial worm (*Elaeophora schneideri*), and possibly *Brucella abortus,* the causative agent of brucellosis (*16*).

Negative impacts of winter ticks and meningeal worm infections on moose are probably the most well known and well documented. Heavy winter tick infestations induce hair loss and subsequent clinical signs and behaviors (e.g. excessive grooming, hypothermia, etc.), acute anemia, and possibly death (*17, 18*). These ectoparasites have been implicated as factors in the decline of moose populations in Isle Royale (*19*), New Hampshire (*18*), Ontario (*20*), and Alberta (*21*). The meningeal worm is a common parasite of white-tailed deer and distributed throughout the northeastern regions of North America. Moose are considered to be aberrant hosts to *P. tenuis* and acquire the parasite when they share the same habitat as the deer definitive host. Sometimes referred to as “moose sickness”, infections with meningeal worm can result in severe neurological disease and death in moose and other ruminants (*22, 23*). Although *P. tenuis* likely contributes to some moose declines in eastern North America, there is not a clear consensus that *P. tenuis* is the most important factor driving these declines (*24-27*). Thus the true impact of *P. tenuis* on moose populations is still the subject of much debate and research. The impact of brucellosis and *E. schneideri* infections on moose populations are also not well understood. Brucellosis can cause fetal abortion in moose, affecting reproductive rates (*16*) and cases of morbidity and mortality due to *E. schneideri* infections in moose have been documented (*28, 29*). However, current data describing natural rates of morbidity and mortality for these pathogens in the moose host are lacking. Although previous documentation of this parasite in moose has been sparse, *Rumenfilaria andersoni,* a filarial parasitic nematode that resides within the lymphatic vessels of the rumen, has exhibited a significant surge in prevalence and an

expansion in range over the last decade in parts of Europe. Very little is known about *R. andersoni* in North American moose and if this parasite could contribute to moose morbidity and mortality. To better understand how these and other pathogens influence moose population dynamics, additional research is needed.

Rumenfilaria andersoni: A Cryptic, Lymphatic-Dwelling Filarid of Cervids

Discovery of Rumenfilaria andersoni

Rumenfilaria andersoni was first documented in 1982 by Lankester and Snider when they discovered filarial nematodes loosely coiled in the subserosal lymphatic vessels in the rumens of moose from Ontario, Canada (*30*). Since the initial description, only a handful of reports have been published documenting this parasite and thus many of the details surrounding the life cycle and epidemiology of *R. andersoni* are unknown or inferred based on other related species of filarial worms. Microfilariae morphologically consistent with *R. andersoni* have been reported in blood samples from moose of Alaska (*31*). More recently, Laaksonen, et. al. documented *R. andersoni* in multiple species, including wild and domestic reindeer (*Rangifer tardanus*)[2], moose, white-tailed deer, and roe deer (*Capreolus capreolus*) in Finland (*32, 33*). Combined, these data suggest cervids serve as the main reservoir hosts for *R. andersoni* and the nematode is likely widely distributed across North America and possibly Europe, however the true host and geographic distribution of this nematode has yet to be determined.

The prevalence of *R. andersoni* in cervid hosts, especially in North America, is not well known. Approximately 70% of Alaskan moose were reported to be infected with *R. andersoni* (*31*), whereas Lankester and Snider found *R. andersoni* in 6 of 41 (15%) moose examined from Ontario (*30*). Recent studies in Finland describe prevalence rates in moose ranging from 0-12% (*33*). Similarly, Laaksonen et. al. also reported 15-22% of white-tailed deer and 3% of roe deer were harboring *R. andersoni* in Finland (*33*). Finnish domesticated and wild reindeer demonstrated very high prevalence rates from 0-90% and 41-100%, respectively (*33*).

Rumenfilaria andersoni Morphology and Parasitic Life Cycle

Adult *R. andersoni* are white and thread-like with long, slender bodies and a thin, smooth cuticle (*30*). Anterior and posterior ends are tapered with the head bluntly rounded and the tail without protuberances. Adult males range from 55-62mm long by

[2] Currently reindeer and caribou are considered to be the same species (*Rangifer tardanus*), however in North America “reindeer” often refers to the semi-domesticated variety of *R. tardanus* and “caribou” is used to describe wild *R. tardanus*. In Europe, all *R. tardanus* (wild or domesticated) are referred to as “reindeer.” In this case, we chose to use the same terminology as the author. From this point forward “reindeer” will refer to *R. tardanus* of Europe and “caribou” will refer to wild *R. tardanus* of North America.

197-205μm wide with a pair of short, unequal spicules with rounded tips on the posterior end. Females are larger in size, ranging from 127-144mm long by 340-351μm wide. *Rumenfilaria andersoni* microfilariae are ensheathed with blunt anterior and tapered, rounded posterior ends. The microfilariae range in size from 140-165μm long by 4-5μm wide (*30*).

Many of the details surrounding the life cycle of *R. andersoni* have yet to be elucidated. Like other members of the Onchocercidae family *R. andersoni* is presumably transmitted by hematophagous arthropods, however the identity of the vector has yet to be discovered. Following sexual reproduction, adult females release microfilariae into the blood stream of the ungulate host where they are acquired by the arthropod vector via a blood meal. Once ingested by the vector, the microfilariae mature into an infectious larval form and are then deposited into a susceptible host by the biting arthropod. It is unknown what length of time is required for the microfilariae to mature into the infectious larval form within the vector, nor is it known how this developmental process occurs. Initial infection with *R. andersoni* is followed by the migration and maturation of the larval form into sexually mature adults within the ruminal lymphatic vessels of the cervid host. By monitoring the emergence of *R. andersoni* microfilariae in captive reindeer calves reared on pastures with *R. andersoni*-infected adult reindeer, Laaksonen et. al. estimated the prepatent period, the time from infection to the appearance of microfilariae in the circulatory system, to be approximately 5 months.

Rumenfilaria andersoni Infection Dynamics and Associated Pathology

Current knowledge of *R. andersoni* infection dynamics in the cervid host is based on data collected by Laaksonen, et. al. examining blood samples from Finnish cervids for the presence of *R. andersoni* microfilariae (*33*). Finnish reindeer exhibit an extremely high density of microfilariae in the blood stream averaging between 596-3752 microfilariae/mL, whereas white-tailed deer have a mean density of 10 microfilariae/mL and moose have a mean density of 308 microfilariae/mL. These data indicate *R. andersoni* microfilariae intensity varies among host species, suggesting moose and deer may be better adapted to *R. andersoni* and therefore possibly more resistant than reindeer. This hypothesis is also supported by the lower prevalence rates in Finnish moose and deer compared to the prevalence rates in reindeer. However, it is also possible *R. andersoni* may proliferate more readily in reindeer. It should be noted that the density of microfilariae does not directly correlate with total worm burdens. Although microfilariae density can correlate to the number of adult female worms present, it does not account for single sex infections or the number of adult male nematodes present. Furthermore, in other filarial worm species, namely *Brugia malayi,* a human parasite and the cause of lymphatic filariasis, and *Onchocerca volvulus*, which causes river blindness in humans, host acquired immunity can significantly influence microfilaraemia levels resulting in a decrease in microfilariae blood density despite increased worm burdens (*34, 35*).

Evidence of cyclic variations of microfilariae density and persistence of *R. andersoni* infections over time has also been documented in Finnish reindeer (*33*). Monitoring *Rumenfilaria andersoni* microfilariae density in eight naturally infected reindeer over 24 hours revealed intensity of microfilariae was the highest at night between 21:00-3:00, gradually subsiding to its lowest level at 12:00 noon (*33*). Many species of filarial nematodes, including the human pathogens *Wucheria bancrofti* which causes lymphatic filariasis, the eye worm *Loa loa,* and *B. malayi* exhibit similar circadian rhythms, often coinciding with the feeding behaviors of the vector (*36*). It is unknown if *R. andersoni* microfilariae behave in a similar manner in moose or other cervid host species. Moreover infected reindeer were shown to maintain microfilariae in their blood throughout a three-year sampling period (*33*), suggesting *R. andersoni* infections may persist for multiple seasons. It is unknown how long an individual nematode may survive in the cervid host or if persistence of microfilaraemia is due to subsequent re-infections.

The impact of *R. andersoni* on cervid health is unknown. M.W. Lankester and J.B. Snider did not record any pathological changes in *R. andersoni* infected moose of Ontario (*30*) and no case reports implicating *R. andersoni* in moose morbidity or mortality have been published. Poor body condition, dry hair coat, and stiffness were observed in Finnish reindeer with high *R. andersoni* microfilariae densities, however it is inconclusive whether *R. andersoni* was the causal factor (*33*). Lymphoedematous swelling of the vessel walls, fibrosis, and granulomatous inflammation have been observed in the subserosal lymphatic vessels harboring adult worms in Finnish reindeer (*32*). It is unknown if *R. andersoni* infections can result in clinical or sub-clinical disease in cervids or if infection can lead to an immunocompromised state, leaving the host susceptible to other pathogens and development of disease.

Emergence of Rumenfilaria andersoni in Finland

Recently *R. andersoni* has experienced a rapid geographic expansion in Finland, prompting much of the research discussed above. The discovery of *R. andersoni* in Finnish reindeer coincided with the outbreak of parasitic peritonitis caused by another filarial nematode *Setaria tundra (32, 37),* thus prompting concern for the spread of filarial parasites and the diseases associated. Comparing prevalence of *R. andersoni* microfilariae cervid blood samples taken in 1997, 2006, and 2006, Laaksonen, et. al. observed prevalence in adult reindeer of Finland increased from 8% in 1997 with only the southern most region of the Finnish study area (near the 65°N latitude) harboring *R. andersoni* to *R. andersoni* being detected near the 70°N latitude with prevalence rates between 42-83% in 2006. It has yet to be determined how this rapid expansion has impacted native and non-native cervid populations and the significance of these findings.

The rapid emergence of *R. andersoni* in Finland has also prompted researchers to speculate as to the origin of the Finnish *R. andersoni*. Laaksonen et. al. hypothesized *R. andersoni* was recently introduced to the area, possibly by the importation of the non-native white-tailed deer from North America (*33*). In 1935 the first and only introduction

of non-native white-tailed deer from North America, specifically the U.S. state of Minnesota occurred when five deer, one male and four females, were imported into southern Finland as a gift from Finnish immigrants to the homeland (*38*). It is certainly possible that *R. andersoni* was introduced to Finland via this translocation event, however evidence to confirm these suspicions is lacking. If indeed the hypothesis is correct, the introduction of *R. andersoni* and its extensive proliferation in the local native cervid populations, especially domestic and wild reindeer, serves as another cautionary tale demonstrating the potential consequences of spreading parasites into naïve populations via the anthropogenic movement of cervid hosts.

Disease Dynamics of *Elaeophora schneideri*

Introduction to Elaeophora schneideri

Elaeophora schneideri is a filarial nematode of the Onchocercidae family and the etiological agent of the disease elaeophorosis in ruminants, including moose. First described in domestic sheep (*Ovis aries*) from New Mexico in 1935 (*39*), E. schneideri has since been reported in a variety of domestic, wild, and exotic game species including moose, elk, Barbary sheep (*Ammotragus lervia*), bighorn sheep (*Ovis canadensis*), domestic goats (*Capra hircus*), sika deer (*Cervus nippon*), white-tailed deer (*Odocoileus virginianus*), mule deer (*Odocoileus hemionus*), and black-tailed deer (*Odocoileus hemionus columbianus*) (*29, 40-46*). The parasite has been documented throughout much of the western and southwestern U.S., including Nebraska, South Dakota, Montana, Wyoming, Colorado, Washington, Oregon, California, Utah, Arizona, New Mexico, and Texas (*28, 29, 43, 44, 47-54*). Mule deer and black-tailed deer are considered to be the most well adapted to *E. schneideri* and thus the definitive hosts for the parasite (*44, 46, 55*). Prevalence rates in mule deer vary, with historical reports ranging from 19-58% in Arizona, 4%-44% in California, 4-57% in Colorado, 60-100% in New Mexico, and 100% in Texas (*41, 56*). Prevalence rates in black-tailed deer are also considerably high, with 78% of black-tailed deer reported to be infected with *E. schneideri* in California (*44*).

Sporadic reports of *E. schneideri* in white-tailed deer in Arkansas and the Atlantic and Gulf costal plain of Florida, Georgia, and South Carolina have also been documented (*57, 58*). The reported prevalence rates of 2-10% are relatively low compared to mule deer in the western U.S. It has been suggested that the presence of *E. schneideri* in the Southeast is due to a recent introduction of the nematode, possibly by the translocation of animals from enzootic regions of the west to the Southeast (*57, 59*). However, it is also possible the parasite was endemic to the Southeast and the nearly complete extirpation of white-tailed deer in the early 1900's left only a few pockets of *E. schneideri* in the Southeast (*60*). *Elaeophora schneideri* has not been reported in the Midwest or northeastern U.S., suggesting the parasite is not endemic to these regions.

Morphological Description and Lifecycle of Elaeophora schneideri

Commonly referred to as “arterial worm”, adult *E. schneideri* nematodes are creamy white in color, sexually dimorphic, and considerably large in size with tapered extremities. Adult females measure between 60-120mm long by 0.6-0.9mm wide and males are 55-85mm long by 0.4-0.9mm wide with tightly coiled tails (*61*). The estimated life span of the adult nematode is three to four years (*62*). Within the definitive deer host adult *E. schneideri* nematodes reside within the cephalic arteries, particularly the carotids. *Elaeophora schneideri* worms tend to coil within the vessels, presumably as an adaption to the dynamic environment within the arterial system to prevent the nematodes from being dislodged (*46*). Following sexual reproduction, the females release microfilariae directly into the bloodstream where they are carried to the capillary beds of the dermis of the host’s head and face (*63*). Microfilariae are 239-279µm long by 11-15µm wide with a rounded anterior end and the posterior end tapering to a blunt point (*61*).

Transmission of *E. schneideri* occurs via the bite of infected tabanid horseflies (Diptera: Tabanidae). While taking a blood meal from an infected definitive host, the tabanid fly acquires the *E. schneideri* microfilariae (*56*). The microfilariae invade the fat body lining the abdomen of the vector where they molt into first-stage (L1) larvae (*56, 64*). Eventually the L1 larvae exit the fat body and continue their development into second (L2) and third stage (L3) larvae in the hemocoel. L3 larvae eventually migrate from the hemocoel to the head and mouthparts of the horsefly vector (*56*), where they are deposited into the subcutaneous tissues of the cervid host while the vector obtains a blood meal. The exact details describing the behavior and development of the L3 larvae into adult *E. schneideri* within the definitive host are not fully understood. Eventually the L3 larvae exit the subcutaneous injection site and enter the arterial system where they migrate to the leptomeningeal arteries and grow into immature adults (10-13mm in length) (*55, 64, 65*). At approximately 3-4 weeks post initial infection, immature adult *E. schneideri* migrate from the leptomeninges to the carotids where they mature into adults (*64*). The entire developmental process from initial infection with the L3 larvae to the maturation into sexually mature adult nematodes takes approximately 4.5 months (*65*).

Pathology in Moose and Other Atypical Hosts

Although *E. schneideri* infections are clinically silent in the definitive mule deer and black-tailed deer hosts, infection of atypical hosts can result in the development of clinical elaeophorosis. This disease is characterized by obstructed blood flow, endothelial damage, thrombosis, and infarction due to the presence of adult nematodes in the arteries and microfilariae in the carotid and cephalic arterial system (*29, 40, 59*). This disruption in arterial circulation can lead to blindness, ischemic necrosis of the brain, ears, muzzle, and other cephalic tissues; poor antler development, oral impactions, and death (*28, 29, 40, 41, 47, 66*). Hibler, et. al. suggests there are three critical periods of infection for atypical hosts in which dysfunctional behavior of *E. schneideri* nematodes leads to the development of disease (*64*). Within aberrant hosts like sheep and elk, larval stages fail to migrate to larger arteries as they grow and

instead remain in the smaller arteries of the leptomeninges for a longer period of time. Subsequently, these larvae and immature adults inhibit blood flow to the brain, causing ischemia and resulting in tissue damage, blindness, and death. The second critical period occurs when the immature adult *E. schneideri* attempts to traverse the cerebral rete to migrate to the carotid or maxillary arteries. Due to the large size of the immature adult (30-40mm), rupture of the arterial rete or capillaries can occur resulting in sudden death from cerebral hemorrhage (*64*). The final critical period occurs following the migration of the immature adult *E. schneideri* to the carotid arteries. Here, the nematodes continue to grow and if sufficient in numbers can result in interference of blood flow. This further damages tissues of the head, as well as leads to intimal proliferation of the carotid artery (*40, 64, 67*).

Severity and manifestations of elaeophorosis can differ between species of atypical hosts. Elaeophorosis in sheep typically results in a hypersensitivity reaction in response to the presence of microfilariae. This manifests as chronic dermatitis and leads to the production of skin lesions on the poll, face, nostrils, lips, legs, feet, and abdomen (*55, 62, 68*). Intimal thickening, verminous thrombosis, and proliferation of fibrous tissue in cephalic arteries, as well as sublingual oral food impactions have been reported in white-tailed deer infected with *E. schneideri* (*66, 69*). Elaeophorosis pathology in elk is much more severe, with animals developing ischemic damage to the brain, eyes, optic nerves, ears, muzzle, and other tissues of the head. Infected elk exhibit signs of neurological impairment, emaciation, abnormal or poor antler growth, cropping of the ears, and blindness; infections in elk are often fatal (*40*).

Elaeophorosis severity also varies in moose. Madden, et. al. reported an adult female moose from Colorado with poor body condition and cropped ear tips exhibiting ataxia and wandering in circles; after three days the animal expired (*47*). Necropsy revealed the presence of 40 adult *E. schneideri* nematodes in the carotid arteries and histological lesions on the carotid arteries and rete mirabile cerebri. Reports of moose exhibiting clear-eyed blindness, necrotic lesions of the brain, and cerebral hemorrhage have also been documented (*28, 29, 50*). Development of elaeophorosis does not seem to be dependent on parasite burden; low-level infections can still result in morbidity and mortality. For instance, in Washington a yearling moose exhibiting ataxia, poor coordination, anorexia, and blindness was euthanized and necropsied. Histological findings were consistent with elaeophorosis, yet only a single juvenile *E. schneideri* was recovered (*28*). Nevertheless, recent surveillance efforts to document the prevalence of *E. schneideri* in hunter-killed moose suggest infections do not always result in clinical disease. A study in Wyoming revealed 82 of 168 (48.8%) healthy moose carcasses examined contained *E. schneideri* nematodes (*50*). Histological examination of carotid arteries from 109 healthy moose of Colorado revealed 83% (n=91) had lesions consistent with *E. schneideri* infections (*67*), however *E. schneideri* nematodes were recovered from only 13% (n=14) of moose surveyed. These studies indicate moose may survive and potentially recover from *E. schneideri* infections.

Disease Ecology of Elaeophora schneideri

Like most parasites with multiple required hosts, the maintenance of *E. schneideri* in the environment is dependent on several variables. The density of reservoir hosts and susceptible atypical hosts can significantly impact the prevalence of *E. schneideri.* Observations of *E. schneideri* microfilariae in the dermal tissues of elk, moose, sheep, and white-tailed deer atypical hosts indicates these species can serve as patent hosts and therefore contribute to the lifecycle of this parasite (*29, 40, 62, 66*). Thus the parasite may be able to persist in geographic regions outside the range of the definitive black-tailed deer and mule deer hosts if there is a high enough density of competent mammalian hosts and vectors. This phenomenon has already been observed in some white-tailed deer populations of the Southeast (*57, 58*).

Abundance and behaviors of tabanid vectors can also significantly impact the prevalence of *E. schneideri* in the environment. Tabanid horseflies are a diverse taxonomic group with not all species appearing to be competent vectors. Surveys across *E. schneideri* endemic areas indicate *Hybomitra* spp., *Tabanus* spp., and *Silvius* spp. are the vectors responsible for transmission (*70-73*). Previous research investigating the ecology of *E. schneideri* infected horseflies in New Mexico indicated fly activity was dependent on temperature, humidity, precipitation, and wind conditions (*48, 63*). Tabanids were found only at higher elevations (above 6000 feet) and most active during mid day at temperatures between 32-38°C and humidity less than 10%. Overall abundance of horseflies was also reported to be much greater in dry rather than wet years (*59*). This suggests weather patterns could significantly influence tabanid population size, timing of fly emergence, and transmission rates. Furthermore, host selection and landing preferences may influence parasite prevalence. Many horsefly species exhibit feeding and landing preferences (*74*), thus those that prefer to land and feed upon the face or head of cervids are more likely to come in contact with *E. schneideri* microfilariae. If the abundance of these particular cervid-biting species is high, theoretically the prevalence of the parasite could also increase. Development of *E. schneideri* within the tabanid host may also influence parasite prevalence levels. The fly must survive long enough for the parasite to mature and to be transmitted to the next host. Within the tabanid it takes approximately 14 days for the L1 larvae to mature into the infectious L3 larvae (*56, 64*). The lifespan of most horseflies is between one to three weeks, but significant variation exists among and within species (*75*).

Impact of Elaeophorosis and Management Implications

The impact of *E. schneideri* on ruminant host populations is not understood. Reported infection rates in domestic sheep are relatively low (1%), likely due to the use of antihelminthics for routine parasite control (*46*). Therefore elaeophorosis in domestic sheep is probably a rare occurrence and it is unlikely that *E. schneideri* infections are economically important in these animals (*76*). However, wild sheep populations that are sympatric with mule deer may have a greater risk of infection and development of elaeophorosis. Pence, et. al. observed severe dermatitis caused by a hypersensitivity response to *E. schneideri* microfilariae reduced the number of trophy quality wild

Barbary sheep (*41*). Outbreaks of *E. schneideri* in wild sheep could potentially affect state and commercial revenues associated with hunting, however current data describing the prevalence of the parasite and incidence rate of clinical elaeophorosis in wild sheep populations is lacking.

Historical reports describing elaeophorosis outbreaks in elk of New Mexico suggests *E. schneideri* infections can impact calf recruitment. Significant loss of elk caves due to elaeophorosis was reported in the Gila National Forest in southwestern New Mexico following the introduction of elk in 1954-1956 (*48*). Here researchers reported a calf survival rate of only 15-20%, with *E. schneideri* considered to be the most important factor affecting survival. Since the 1950's, reports of elaeophorosis in elk are relatively rare. It unknown how prevalent the parasite or what the incidence rate of clinical elaeophorosis is in wild or captive elk herds. The impact of this parasite on elk herd health and population dynamics remains a mystery.

The effects of *E. schneideri* infections on moose are also unclear. High prevalence of *E. schneideri* in apparently healthy moose of Wyoming and Colorado suggests infections can be tolerated and are not necessarily debilitating to the moose host (*50, 67*). Yet, several cases of morbidity and mortality attributed to *E. schneideri* have been documented (*28, 29, 47, 50*). It is unknown if subclinical infections can have adverse affects on overall moose health, leading to reductions in reproductive rates or predisposing moose to be more susceptible to other diseases or predation. It is possible clinical and subclinical infections could be a contributing factor to moose population declines, but incidence rates of morbidity and mortality attributed to *E. schneideri* infections in moose populations have not been measured. Additional studies are required to address the question regarding the impact of *E. schneideri* on moose survival and recruitment.

Elaeophora schneideri has important management implications for wildlife agencies, commercial hunting operations, zoos, and domestic farmers. Due to the complex nature of the *E. schneideri* lifecycle and the potential for multiple ruminant and vector species to serve as competent hosts, the possibility of transporting *E. schneideri* to new locations via the translocation of cervids, especially mule deer and black-tailed deer, must be considered. Movement of atypical hosts such as elk, white-tailed deer, moose, and sheep from enzootic areas to non-endemic locations could also pose a risk for the introduction of elaeophorosis into naïve populations due to the ability of *E. schneideri* to produce patent infections in these host species. Furthermore the possible impact of elaeophorosis must be considered when introducing susceptible animals, including exotic species, into *E. schneideri* endemic locations.

CHAPTER II
PREVALENCE AND ECO-EPIDEMIOLOGY OF THE LYMPHATIC-DWELLING NEMATODE *RUMENFILARIA ANDERSONI* IN FREE RANGING MOOSE OF NORTH AMERICA

Abstract

Moose (*Alces alces*) have long been a culturally and economically valued species in Minnesota, however since 2006 the Minnesota moose population has decreased by 60%. The cause of the decline is currently unclear. Nematode parasites are important pathogens in moose, causing severe disease and mortality. *Rumenfilaria andersoni* is a filarial nematode of moose, however very little is known about the epidemiology of this parasite in free-ranging moose of North America. To investigate the prevalence and distribution of *R. andersoni*, blood samples were collected from live-caught and deceased animals. Blood was screened microscopically for the presence of microfilariae using a modified Knott's test and the microfilariae were identified based on morphological characteristics. A subset of Knott's-positive animals was subjected to PCR with nematode-specific primers targeting the first internal transcribed spacer region (ITS-1). *Rumenfilaria* microfilariae were present in 20.5% (N=352) of Minnesota moose, with only slight fluctuations seen over time. Minnesota white-tailed deer (*Odocoileus virginianus*) (5.6%, N=36) and moose from Alaska (40.1%, N=27), Montana (28.8%, N=73), Washington (31.3%, N=16), Maine (21.4%, N=14), and New Hampshire (25%, N=16) also harbored *R. andersoni*, suggesting this parasite is widely spread throughout North American moose herds and white-tailed deer can serve as a patent host. Sequence analysis of blood samples confirmed the identity of *R. andersoni* and suggested the existence of two distinct clades. In addition, tabanid flies were collected to molecularly screen for the presence of parasite DNA. Of the 618 horseflies molecularly screened using Nematoda-wide 18S primers, *R. andersoni* DNA was detected in 1.5% and associated with a single fly genus (*Chrysops* spp.), suggesting horseflies may serve as the vector for *R. andersoni* transmission. This is the first report of *R. andersoni* in moose and white-tailed deer herds within the contiguous US. At this time the implications for *R. andersoni* infection on moose health is unknown. Future research is warranted to determine the potential impact of *R. andersoni* on moose populations and to elucidate the details of *R. andersoni* ecology and transmission.

Introduction

Moose (*Alces alces*) have long been a culturally and economically valued species in North America, however some North American moose populations have exhibited a serious decline in total population numbers (*13, 14, 77*). Nowhere has this decline been more dramatic than Minnesota where the estimated number of moose has decreased by 60%, from 8,840 animals in 2006 to 3,450 in 2015 (*15*). To prevent the loss of this precious natural resource, a better understanding of the driving factors of mortality in free-ranging moose of Minnesota, as well as the herd's overall health is desperately needed.

Nematode parasites, particularly species of lungworms and filarids, are important pathogens in moose and known to cause morbidity and mortality in free-ranging populations (*23, 26, 47*). *Rumenfilaria andersoni* is a lymphatic-dwelling filarial nematode associated with moose and reindeer (*Rangifer tardanus*). Although very little is known about the life history of this specific organism, *R. andersoni* belongs to the Onchocercidae family, which is a group of filarial nematodes transmitted by

hematophagous arthropods. Adult filarids produce larval stages called microfilariae that reside in the circulatory system or skin of the definitive cervid host. When an arthropod intermediate host (vector) obtains a blood meal and ingests the microfilariae, the microfilariae will ensheath, penetrate the vector's gut wall, migrate to the haemocoel, and develop into an infectious larval stage. The lifecycle is complete when the infected vector deposits the infectious larvae into a definitive host during a subsequent blood meal.

Originally described in moose from Ontario (*30*) and more recently in Alaska (*78*) and Finland (*32, 33*), the exact geographical distribution and the identity of the insect vector of *R. andersoni* is unknown. Although the pathological impact of *R. andersoni* on cervid health remains unclear, recent studies documenting the rapid expansion of *R. andersoni* in Finnish reindeer describe infections with inflammation of ruminal lymphatic vessels and the development of high microfilaraemia within the bloodstream (*32, 33*), both of which would be predicted to have a negative impact on overall cervid health (*40, 79, 80*). This suggests *R. andersoni* infections may have important health implications for cervids, including moose.

The objective of this study was to investigate the eco-epidemiology of *R. andersoni* in Minnesota moose compared to other North American herds. To accomplish this, we explored the basic epidemiology of this parasite, including the identification of other patent host species, geographic distribution, prevalence of infection, host demographics, and the identification of potential vector species by collecting parasitological samples across spatial and temporal scales. We also performed the first genetic characterization and comparison of *R. andersoni* isolates, providing a basis for future population genetics studies. These data contributed to a greater understanding of the life history of *R. andersoni* and provide baseline epidemiological data for future reference and research.

Materials and Methods

Sampling of Hosts and Detection of Microfilariae

To estimate the prevalence of *R. andersoni* within the Minnesota moose population, 352 blood samples were collected from moose over a period of approximately four years from 2012-2015. Blood was collected from either hunter-killed animals, opportunistic mortalities, or live-captured research animals and placed into EDTA blood tubes. A limited number of blood samples were also obtained from live-captured Minnesota elk (*Cervus canadensis*) (N=14) and white-tailed deer (*Odocoileus virginianus*) (N=36). Additional blood samples were donated by various state wildlife agencies from live-captured animals, including 12 caribou and 27 moose from Alaska; 14 moose from Maine, 73 moose from Montana, 16 moose from New Hampshire; and 16 moose, 1 white-tailed deer, and 3 mule deer (*Odocoileus hemionus*) from Washington. Whenever possible, the age, sex, and geographic location of the animal was noted. Animals were classified as either calves (<1 year of age), yearlings (approximately 1 year old), or

adults (>1 year old). The blood samples (1ml/animal) were examined using a modified Knott's test and bright-light microscopy (*81*) for the presence of *R. andersoni* microfilariae (RMF). Microfilariae were identified based on comparative morphological features (*32*).

Prevalence Estimates and Statistical Analyses

Prevalence of *R. andersoni* within cervid populations was estimated for each cervid species based on the modified Knott's test results. Prevalence was defined as the percentage of RMF positive animals compared to the entire population. To determine if *R. andersoni* prevalence differed between geographical locations, a Pearson's chi-squared test and Fisher's exact test were utilized with Bonferroni correction ($p \leq 0.01$). The Minnesota moose population was further analyzed, comparing prevalence between age classes, gender, and sample year using the Pearson's chi-squared test with Bonferroni correction ($p \leq 0.01$). Statistical analyses were performed with SPSS software version 23.0 (IBM Corporation, Armonk, NY).

Molecular Confirmation of Microfilariae as R. andersoni

To confirm the identity of the RMF, as well as investigate intra-species genetic variation, 30 Knott's-positive blood samples containing microfilariae that were morphologically consistent with *R. andersoni* were selected for molecular analysis. DNA was extracted using the ZR Fecal DNA Kit (Zymogen, Irvine, CA) according to the manufacturer's instructions. RMF DNA was amplified using a previously described semi-nested PCR protocol targeting portions of the 18S and 5.8S rRNA genes flanking regions of the first internal transcribed spacer region (ITS-1) (*82*). The PCR products were separated by agarose gel electrophoresis and visualized under UV light. PCR amplicons around 600bp were excised using a clean razor blade and the PCR product purified using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). The ITS-1 PCR product was then cloned into a pGEM-T Easy vector (Promega, Madison, WI) and transformed into competent DH5α *Escherichia coli* cells (Invitrogen, Grand Island, NY) via a 40 second heat shock at 42°C. Transformed cells were cultivated in S.O.C. medium (Life Technologies, Grand Island, NY) for 1.5 hours at 30°C with 225rpms shaking. 100µl of the transformed cells were then plated on Luria broth (LB) agar plates containing 100µg/mL carbenicillin and ChromoMax IPTG/X-gal solution (Thermo Fisher Scientific, Waltham, MA). Cultures were incubated 24-48 hours at 30°C; single, white colonies were selected with a sterile toothpick and grown overnight in 5mL of LB with 100µg/mL carbenicillin. Cultures were centrifuged twice for 10 minutes each at 1500rpms and the supernatant removed. Plasmids were purified from the remaining cell pellet using the Qiagen Spin Miniprep Plasmid Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. To confirm the presence of the filarial ITS-1 PCR product insert, the plasmids were digested with EcoR1 restriction enzyme (Thermo Fisher Scientific, Waltham, MA) and examined via gel electrophoresis for multiple bands. Plasmids containing an insert approximately 600bp in size were sequenced at the University of Tennessee's Molecular Core Facility (Knoxville, TN).

Molecular Survey of Tabanid Flies

To identify potential hematophagous insect vectors of *R. andersoni*, 618 tabanid horse flies (Diptera: Tabanidae) were collected during the months of June-August of 2013 from 4 separate locations (Fig. 2)[3] using CO_2 traps and their genera identified based on morphological characters. Flies were preserved in 70% ethanol until ready for DNA extraction. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Flies were divided into groups of 10 and 5µl of DNA from each fly was pooled into the corresponding group's tube. To screen for the presence of *R. andersoni* within pooled fly DNA, nematode-specific primers targeting the 18S rRNA gene were used in a polymerase chain reaction as previously described (*83*). Individual flies with PCR-positive DNA pools were subjected to an additional PCR reaction using the Nem18S primers. All positive reactions were purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) following manufacturer's instructions and sequenced at the University of Tennessee's Molecular Core Facility (Knoxville, TN).

Phylogenetic Analysis of R. andersoni Sequences

All 18S and ITS-1 consensus sequence chromatograms were trimmed and edited by hand using Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI). Edited sequences were compared against the NCBI GenBank database, however very little genetic data exist for filarial nematodes known to infect cervids. Therefore, we also compared our genetic data with sequences obtained from adult reference nematodes (Table 1) that were identified morphologically and subjected to DNA extraction and PCR amplification in our laboratory as described above. Alignment and construction of neighbor-joining trees of ITS-1 and 18S filarial worm sequences was performed using MEGA 6.0 (*84*). All consensus sequences were deposited into GenBank.

Results

Prevalence of R. andersoni in Wild Cervids

During 2012-2015, RMF occurred in 20.5% (72 of 352) of Minnesota moose. We did not find any significant associations with the presence of RMF and either gender (Pearson's chi squared test; p=0.061) or age of the Minnesota moose (Pearson's chi squared & Fishers exact test; p=0.232). RMF were also detected in Minnesota white-tailed deer, with an overall prevalence at 5.6% (2 of 36), but no RMF were detected in the Minnesota elk samples (0 of 14) (Fig. 3A). Over the 4-year sampling period, RMF prevalence in the Minnesota moose varied slightly, ranging from 12.7% (8 of 55) in 2015 to 18.4% (36 of 160) in 2013, however no significant difference between sampling years was detected (Fishers exact test; p=0.607) (Fig. 3B). Outside of Minnesota, RMF were

[3] All figures and tables are located in the Appendix.

observed in all other moose populations we surveyed including Maine (21.4%; 3 of 14), New Hampshire (25.0%; 4 of 16), Montana (28.8%; 21 of 73), Washington (31.3%; 5 of 16), and Alaska (40.7%; 11 of 27) (Fig. 3C); no statistical difference was detected between the populations (Fishers exact test, p=0.013). Moreover, we failed to observe RMF in any of the Alaskan caribou blood samples or in either species of deer from Washington.

Genetic Characterization of RMF Isolates

To confirm the identity of the RMF observed in the Knott's tests and to investigate the intra-species genetic variation of *R. andersoni*, we successfully amplified, cloned, and sequenced partial filarial 18S rRNA, full ITS-1, and partial 5.8S rRNA sequences obtained from RMF-positive blood samples of 15 North American moose and one white-tailed deer (Table 2). Isolates varied in geographical origin, with 6 from Montana, 8 from Minnesota, and 2 from Maine. Attempts to amplify additional isolates were not successful. Sequences obtained from a morphologically confirmed *R. andersoni* adult nematode (RA-F3) isolated from a Finnish reindeer served as the standard. Overall, the RMF ITS-1 sequences were AT-rich, with multiple sections of repeats with variations in length between isolates. Phylogenetic analysis revealed all of the ITS-1 sequences obtained from the RMF-positive blood clustered with the RA-F3 standard, branching into two distinct *R. andersoni* lineages (Fig. 4). The Minnesota isolates had representatives clustering into both clades, which we simply denoted as Clade A and B. All of the Montana isolates and six Minnesota isolates clustered into Clade A, which also contained the RA-F3 standard. Clade B contained both Maine isolates and two Minnesota isolates. Isolates of Clades A and B had a mean difference of 0.038 (SE=0.007) base substitutions per base pair. Within clades, isolates had a mean difference of 0.015 (Clade A SE=0.003; Clade B SE=0.004) base substitutions per base pair for both Clades A and B.

Identification of Unknown Filarid in Moose

In addition to RMF, another morphologically distinct group of microfilariae was observed in 1.4% (5 of 352) of Minnesota moose. These microfilariae were characterized by blunt, rounded heads and long, thinly tapered tails, measuring between 285-315µm in length and 5-7.5µm in width (Fig. 5). Dual infection with RMF and the non-RMF microfilariae was observed in a single Minnesota moose. To identify this unknown filarid, we attempted to sequence a portion of the 18S rRNA gene using nematode-wide primers (*83*). Only one blood sample was successfully PCR amplified. A comparison of 796 base pairs from the unknown filarid and 18S sequences from our reference nematodes revealed the unknown filarid most closely resembled *Setaria yehi* (Spirurida: Onchocercidae) (Fig. 6), a filarial nematode commonly associated with white-tailed deer (*85, 86*). The 18S rRNA sequence was deposited into GenBank under the accession number KT878969.

Molecular Prevalence of R. andersoni in Tabanid Flies

Rumenfilaria andersoni was detected in 1.5% (9 of 618) of the tabanid flies tested, with only *Chrysops* spp. from the Grand Portage trapping site testing positive (Table 2). All other trapping sites and genera, including *Tabanus* spp. and *Hybomitra* spp., were PCR negative. All DNA extraction and PCR positive and negative controls were as expected. Sequence and phylogenetic analysis confirmed the identity of *R. andersoni* sequences isolated from the Minnesota horse flies (Fig. 7).

Discussion

Prior to the conclusion of our study, very little was known about the occurrence of *R. andersoni* in cervid hosts. Knowledge about the distribution of *R. andersoni* was limited to the original description of this nematode in moose of Ontario, Canada (*30*) and was only recently expanded to include Alaska (*31*) and Finland (*32*). Our survey of moose herds for *R. andersoni* suggests the geographical range of this filarial nematode is much more extensive than was previously appreciated. RMF were detected in all of the wild moose herds we sampled, including herds geographically isolated from one another, with an average prevalence of 28%±7.5SD (Fig.2C). This implies *R. andersoni* nematodes are widely distributed throughout the North American moose range and supports previous studies suggesting moose serve as the main reservoir host of *R. andersoni* (*30, 33*). Interestingly, Alaskan moose had a higher RMF prevalence (40% ($p=0.013$)) compared to other moose herds surveyed, however this is not entirely unexpected as previous reports for *R. andersoni* prevalence in Alaska have been as high as 70% (*31*). It is possible *R. andersoni* and/or its preferred vector may be more highly adapted to subarctic climates and may proliferate more easily in a colder environment. The recent rapid expansion of *R. andersoni* into the subarctic regions of Finland (*32, 33*) supports this hypothesis, but conclusive evidence is still lacking.

To assess the host range, surveys for RMF in other cervid species revealed *R. andersoni* is naturally harbored by white-tailed deer and deer can serve as a definitive host for the filarid (Fig. 3A). The presence of RMF in white-tailed deer has been documented once before by Laaksonen, et. al. in 2015 while investigating the emergence of *R. andersoni* in Finnish woodland reindeer (*33*). The authors observed 22% of the deer surveyed in Finland were RMF-positive, slightly higher than the 5.6% prevalence value observed in deer from Minnesota. As mentioned above, it is possible the parasite is better adapted to sub-arctic environments, thus explaining the lower parasite prevalence at the more southern latitude, however a larger sample size along a latitudinal gradient would be needed to properly address that particular hypothesis.

In addition to white-tailed deer, our survey included specimens from caribou, elk, and mule deer from various geographic locales. We were unable to find evidence of *R.*

andersoni infections within any of these species. This was a little surprising, as Laaksonen, et. al. found *R. andersoni* to be a common and abundant parasite in reindeer in Finland, with prevalence as high as 90% in some locations (*33*). Although the caribou and reindeer herds from Alaska and Finland differ by subspecies[4] and it is possible Alaskan caribou may be genetically more resistant to *R. andersoni* infection, we suspect the absence of *R. andersoni* in our Alaskan caribou specimens may be due to an insufficient sample size rather than a lack of a host-parasite relationship. However, further field and laboratory experiments are needed to determine what, if any, differences in host-parasite relationship exist between European and North American reindeer and caribou populations. Additionally the number of elk and mule deer surveyed may have been insufficient resulting in no RMF detected in the modified Knott's test; yet, it is also possible these species are not suitable hosts for the nematode. Nonetheless it would be irresponsible to come to a definitive conclusion without additional research.

It should be noted that our use of the modified Knott's test to estimate prevalence based on the visualization of microfilariae in blood samples could result in an underestimation of the true prevalence of *R. andersoni* in their mammalian hosts. Many species of filarids exhibit periodicity in which the density of microfilariae in the blood increases and subsequently decreases to coincide with the feeding behavior of hematophagous insects (*87-90*). For instance, *Wucheria bancrofi*, a parasitic nematode that causes human lymphatic filariasis exhibits nocturnal periodicity, presumably as an adaptation to the night-biting habits of certain mosquito species (*91*). Although it is unknown if *R. andersoni* exhibits this type of circadian rhythm in the North American moose host, while monitoring the density of RMF in captive reindeer Laaksonen, et. al. observed the number of microfilariae in the reindeer blood peaked at night between 21:00 and 3:00 and a subsequently declined tenfold by 12:00 noon (*33*). While the exact timing may vary from host to host, as well as be influenced by the global position (i.e. higher latitudes vs. lower latitudes) or seasonal conditions, this suggests *R. andersoni* likely exhibits some form of a circadian rhythm in cervid hosts. Additionally, the pre-patent period between initial infection of the host and the time it takes for the adult *R. andersoni* to produce microfilariae is estimated to be at least 5 months long (*33*). Timing of the sample collection could significantly influence the estimated prevalence values, potentially missing early-stage infections or the synchronous influx of microfilariae into general circulation coinciding with circadian rhythms. Furthermore, the modified Knott's test is unable to detect infections with nematodes of a single sex due to the female filarid being unable to mate and produce progeny. At this time the only alternative

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[4] *Rangifer tardanus granti*, commonly referred to as Grants caribou, is the predominant caribou subspecies found in Alaska. A small herd of woodland caribou, *Rangifer tardanus caribou*, is found in the south central region of Alaska. *Rangifer tardanus fennicus*, the Finnish forest reindeer, and *Rangifer tardanus tardanus,* the mountain reindeer, are the subspecies found in Finland.

method to estimate prevalence would be to grossly examine host carcasses for the presence of adult *R. andersoni*, presumably in the lymphatic vessels of the rumen. In addition to being a laborious and tedious process, the collection of fresh wildlife carcasses can be difficult to obtain. Development of sensitive and specific molecular or immunological assays would provide additional tools for a more accurate estimation of *R. andersoni* prevalence.

Despite the recent advance in knowledge regarding *R. andersoni* distribution and host range, the details describing the nematode's life outside the cervid host remain mostly a mystery. Just as other filarids are transmitted by arthropod vectors, presumably *R. andersoni* is as well; yet, prior to our study the identity of the vector was unknown. In Finland, Laaksonen, et. al. dissected and examined approximately 1000 *Aedes* spp. mosquitoes for the presence of filarial larvae, but only *Setaria tundra* larvae were observed despite the mosquitoes sharing a habitat with RMF-positive reindeer (*92*). This suggested another type of arthropod likely served as *R. andersoni's* vector. Horseflies of the family Tabanidae are distributed throughout most of North America and Europe (*93, 94*) and known to vector other filarial nematodes, including the moose pathogen *Elaeophora schneideri* (*56, 70, 95, 96*). By molecularly testing 618 tabanid horseflies, our study identified *Chrysops* spp. as a potential insect vector of *R. andersoni* (Fig. 6). *Chrysops* spp., commonly referred to as deerflies, are distributed worldwide with at least 80 different species found in North America (*94*). Due to the ubiquitous nature of *Chrysops* spp. flies, it is not surprising that we find *R. andersoni* in moose herds across the United States. It is possible other species of horseflies, or even other arthropods, carry *R. andersoni* and facilitate the parasite's lifecycle, but we were unable to find any evidence in this study.

Although other nematode parasites are known to contribute to moose morbidity and mortality, our study was unable to establish a clear association between prevalence of *R. andersoni* and declining populations of moose. No significant difference in RMF prevalence was observed between the Minnesota moose herd, which is exhibiting a severe population decline (*15*), and those of Montana and New Hampshire, which are also decreasing (*13, 14*), albeit less dramatically; Washington, which has steadily increased in numbers since 1922 (*97*); or Maine, which has seen an increase in the number of moose over the last decade (*98, 99*) (Fig. 3C). Thus prevalence of *R. andersoni* does not appear to have an obvious association with declining moose populations. At this time it is still unknown the impact, if any, *R. andersoni* infections may have on the health of the moose host. Laaksonen, et. al., observed macroscopic inflammatory changes within the ruminal lymphatic vessels of infected Finnish reindeer (*32*), however there have yet to be any reports of pathological changes associated with moose and surveying moose carcasses for such changes was beyond the scope of our study. However, given the high prevalence of RMF in moose and Finnish reindeer future studies on potential subclinical and clinical disease is warranted. It is logical to hypothesize infection with *R. andersoni* has a metabolic cost and it is possible heavy worm burdens or systemic microfilaraemia would result in adverse health effects,

potentially rendering the host more susceptible to other diseases or poor body condition, but future studies will be required to assess the validity of these hypotheses.

In addition to lacking complete comprehension of *R. andersoni's* basic biology and life history, there is an even larger deficit of knowledge regarding the population genetic structure of this parasite. To gain a better understanding of the diversity and genetic variation of *R. andersoni* nematodes, we compared ITS-1 sequences obtained from RMF-positive blood samples (Fig. 4). ITS-1 is a non-coding region of the genome located between the 18S rRNA and the 5.8S rRNA genes. We selected this genetic target for two main reasons. First, the ITS-1 is under less conservational pressure, resulting in higher variability between individuals (*100, 101*). Secondly, universal filarial primers for a nested PCR reaction were readily available (*82*), allowing us to increase our assay's sensitivity to ensure amplification of samples with low densities of microfilariae in the blood. Initial attempts to amplify microfilariae DNA using the Nematoda-wide 18S primers utilized in the tabanid fly screening had an extremely low success rate, possibly due to low parasite DNA levels in the blood or the presence of PCR inhibitors, prompting us to move to the nested PCR assay.

Based on our phylogenetic analysis *R. andersoni* isolates can be distinguished into two separate clades, with all Montana isolates associating with Clade A and all Maine isolates falling into Clade B, suggesting the two populations are genetically isolated. This conclusion is logical given the extensive geographical distance between the host species populations; it would be expected that isolates from Montana and isolates from Maine would be geographically isolated and thus promote genetic divergence from one another. Interestingly, Minnesota isolates have representatives in both clades. Possible reasons for the mixed clades in Minnesota include potential overlapping RMF populations or previous cervid translocation events, resulting in the presence of multiple paratypes circulating within Minnesota cervid populations. Previous morphological analyses of *R. andersoni* specimens revealed nematodes often differ by the number and arrangement of caudal papillae present, with parasites isolated from moose having 7-8 pairs and parasites isolated from reindeer having 8-10 pairs (*30, 32*). Combined, our ITS-1 data and the morphological descriptions suggest at least two separate *R. andersoni* populations exist, however a dual DNA-based and adult morphological study will be needed to identify if there is a distinct relationship between caudal papillae phenotype and corresponding phylogenetic assortment. Furthermore, studies comparing *R. andersoni* genotypes in the vector and cervid hosts would provide additional insight into *R. andersoni* population dynamics and may help to identify factors driving *R. andersoni* transmission and maintenance within the environment.

We also observed our *R. andersoni* reference nematode's (RA-F3) ITS-1 sequence clustered into Clade A with isolates from Minnesota and Montana (Fig. 4). This is significant as RA-F3 was originally isolated from a reindeer in Finland, which recently experienced a significant expansion of *R. andersoni* in wild and domestic reindeer herds (*33*). Researchers hypothesized the colonization of Finnish cervids with *R. andersoni*

resulted from the first and only introduction of non-native white-tailed deer from North America, specifically the U.S. state of Minnesota. Five deer, one male and four females, were imported into southern Finland in 1935 as a gift to the homeland from Finnish immigrants from northern Minnesota (*38*). If Finnish *R. andersoni* nematodes' ancestors originated from Minnesota, we would expect the Finnish ITS-1 sequences to be quite similar to those of North American specimens, especially Minnesota; conversely, we would expect significant genetic variation if the Finnish population had an extended history of geographic distribution and isolation in Fennoscandia. Our data demonstrate a lack of divergence between these isolates, supporting the theory and indicating a more recent, anthropogenically-driven introduction of the parasite occurred rather than an introduction coinciding with the geographic colonization by moose from Central Europe and Russia after the last glaciation, approximately 10,000 years ago (*102*).

In addition to the presence of RMF, our data revealed another filarid circulating within the Minnesota moose herd (Fig. 4, 6). Sequencing and phylogenetics confirmed the identity of this filarial nematode to be that of *S. yehi, a* common parasite of cervids in North America (Fig. 6). Adult *S. yehi* are most commonly found in the abdominal cavity and produce microfilariae that circulate within the bloodstream (*31, 86*). Various mosquito genera are considered to be the major vectors of the nematode (*93*). Although not much is known about the distribution of *S. yehi* in moose, previous reports documented *S. yehi*-infected moose in Alaska and Wisconsin (*103*), suggesting *S. yehi* is also likely widespread amongst North American moose herds. Although this parasite can cause mild fibrin formation on serosal surfaces in infected white-tailed deer and Dieterich and Luick reported chronic peritonitis in Alaskan reindeer infected with *S. yehi* (*104*), little if any clinical disease has been observed with *S. yehi* infections in moose. A similar filarid species, *S. tundra* has been shown to have negative impacts in European reindeer leading to peritonitis and poor hair coat [27], however, *S. tundra* has not been reported in North America. Further research is needed to determine if *S. yehi* infection may lead to subclinical or clinical disease, especially in moose.

Rumenfilaria andersoni nematodes are now recognized to be widespread throughout moose herds of North America. In addition to moose, white-tailed deer are also now accepted as natural, definitive hosts of this parasite. Moreover, this study reveals tabanid flies likely carry *R. andersoni*, with *Chrysops* spp. being implicated as the specific insect vector. Recognizing the geographic distribution and host range of this parasite is especially important for preventing the introduction of *R. andersoni* into naïve populations by translocation of animals by state conservation agencies or commercial hunting businesses. Our genetic comparison of *R. andersoni* isolates supports the hypothesis that the recent, rapid spread of *R. andersoni* in Finnish reindeer was due to the introduction of white-tailed deer from North America (*32, 33*) and further underscores the importance of a better awareness of *R. andersoni* biology. Although we were unable to connect levels of parasite prevalence with moose population declines, it is still unknown if *R. andersoni* infection can lead to clinical or subclinical disease.

Continued efforts to document this parasite in natural cervid hosts will help to provide clarity on this topic.

CHAPTER III
Emergence of the arterial worm *Elaeophora schneideri* in free-ranging moose of Minnesota

Abstract

Moose (*Alces alces*) are a culturally and economically valued species in Minnesota, however since 2006 total population numbers have decreased approximately 60%. Previous studies indicate some species of parasitic nematodes can cause significant morbidity and mortality in moose, resulting in population declines. Due to the recent, rapid decline we hypothesize an emerging parasitic pathogen may be significantly impacting moose survival. Brain tissues from 32 individual Minnesota moose that died of an unknown cause were histologically examined and molecularly tested for the presence of parasitic nematodes. Molecular testing of the brain tissue utilizing nematode-specific primers targeting the 18S rRNA gene revealed 20 (62.5%) individuals produced 18S sequences that most closely aligned with the arterial worm *Elaeophora schneideri,* a filarial parasite known to cause ischemic necrosis of the brain but previously unreported in Minnesota. *Setaria yehi*, a common filarial parasite of deer, was also detected via 18S sequencing in tissues from 5 (15.6%) moose. Additional molecular screening using *Parelaphostrongylus* spp. specific primers targeting the ITS-2 revealed 16 (50%) moose produced sequences 99% identical to *P. tenuis,* an endemic metastrongylid nematode associated with neurological disease in moose. Molecular screening of 618 wild-caught tabanid flies from four separate trapping sites revealed *E. schneideri* was indeed in the Minnesota environment and transmission could occur locally. Prevalence rates ranged between 0-100% per trapping site, with *Chrysops* spp. and *Hybomitra* spp. implicated as the vectors. This is the first report of *Chrysops* spp. serving as a carrier of *E. schneideri.* Furthermore, an unidentified filarial species most closely related to *Dipetalonmema* was detected in 3 (0.49%) flies. We were unable to identify the species and it is unknown the significance of these findings. Together, these data reveal multiple species of parasitic nematodes are circulating within the Minnesota moose population. Most notably, the moose parasitic pathogen *E. schneideri* is emerging in the Minnesota herd and could contribute to morbidity and mortality in Minnesota moose. Further research is needed to determine if a link between emerging nematode infections and the declining moose population exists.

Introduction

In addition to being cultural icons of the wilderness, moose (*Alces alces*) are economically valued by state and local governments due to revenues generated through ecotourism and hunting. Despite some regions boasting thriving moose populations, some areas are exhibiting a serious decline in total population numbers (*13, 14, 77*). Nowhere has this decrease been more dramatic, than that of Minnesota where the estimated number of moose has decreased by 60%, from 8,840 animals in 2006 to 3,450 in 2015 (*15*). Aerial surveys conducted in 2002-2007 of the Minnesota moose population indicated annual survival and reproductive rates of Minnesota moose were notably lower than anywhere else in the United States (*105*), raising significant concern for the future survival of the Minnesota population. To prevent the loss of this precious natural resource, researchers and wildlife agencies have strived to better understand the driving factors of mortality in free-ranging moose of Minnesota. A recent study examining carcasses of Minnesota moose that died from unknown causes or

were euthanized due to perceived signs of illness found 45% of these animals had lesions consistent with nematode neural migration within central nervous system (CNS) tissues (*26*). The authors presumed the pathogenic nematode species to be that of *Parelaphostrongylus tenuis* (Metastrongyloidea: Protostrongylidae) based on histological appearance.

Parelaphostrongylus tenuis is a common nematode parasite of white-tailed deer (*Odocoileus virginianus*) and distributed throughout the eastern United States, including Minnesota (*106*). Transmission of *P. tenuis* occurs when a host ingests an infected gastropod intermediate host while grazing on vegetation. The infectious L_3 *P. tenuis* larvae penetrate the gastrointestinal wall and migrate through the subdural space of the spine, eventually reaching the cranium. Adult worms most frequently reside within the veins or venous sinuses of the cranial meninges. Although infected white-tailed deer are generally asymptomatic, *P. tenuis* infection of an atypical host, including moose, elk, llamas, sheep, and goats, can result in severe neurological disease and mortality due to aberrant migration of the nematode through CNS tissues (*25, 107-111*).

It is well documented that *P. tenuis* is established within the Minnesota environment and is an important parasite in Minnesota cervids (*112-114*), however it is unknown if other nematode species may be contributing to CNS disease and mortality events in Minnesota moose. With the sudden decline in total moose numbers, we hypothesize a previously unrecognized pathogen may be circulating in the Minnesota herd. *Elaeophora schneideri* (Spirurida: Onchocercidae) is a filarial nematode of cervids and can be an important pathogen in wild and domestic ruminants. The parasite is harbored by mule deer (*Odocoileus hemionus*), black-tailed deer (*Odocoileus hemionus columbianus*), and less commonly white-tailed deer (*44, 46, 48, 115*). Transmission of *E. schneideri* occurs via the bite of a tabanid horsefly (Diptera: Tabanidae) (*56, 64*). Third stage larvae (3L) migrate from the fly mouthparts into the host circulatory system, where they eventually migrate to the carotid or leptomeningeal arteries and mature into adults (*55, 64, 65*). Microfilariae progeny generally reside within the smaller capillaries of the head and neck where they can be acquired by a feeding tabanid fly to complete the parasitic lifecycle. Mule deer and black-tailed deer are considered the definitive hosts of the parasite and are typically asymptomatic. However, within certain species of wild and domestic ruminant hosts, including moose, elk (*Cervus canadensis*), sheep (*Ovis* spp.), and goats (*Capra hircus*) infection leads to the development of elaeophorosis (*46*). Elaeophorosis is characterized by obstructed blood flow, endothelial damage, thrombosis, and infarction due to the presence of adult nematodes in the arteries and microfilariae in the carotid and cephalic arterial system (*29, 40, 59*). This disruption in arterial circulation can lead to blindness, ischemic necrosis of the brain, ears, muzzle, and other cephalic tissues; poor antler development, oral impactions, and death (*28, 29, 40, 41, 47, 66*).

Recently white-tailed deer exhibiting facial swellings consistent with oral food impactions have been observed in Minnesota (M. Carstensen, Minnesota Department of

Natural Resources, Personal Communication). Intriguingly, these oral impactions are similar to what have been previously described in *E. schneideri*-infected deer of South Carolina (*66*). These observations further raise suspicions that the parasite is present in the Minnesota cervid population. Historically *E. schneideri* was thought to be limited to the western half of North America and small pockets of the southeastern U.S. However with the increasing density and expanded range of white-tailed deer (*60*) and the human-mediated translocation of captive cervids potentially harboring the parasite, it is possible *E. schneideri* could be an emerging pathogen in the Minnesota moose population.

In the present study we investigated the potential for non-endemic parasitic nematodes, particularly *E. schneideri*, to be associated with CNS disease in Minnesota moose. To accomplish this, we molecularly tested Minnesota moose CNS tissue samples from the Wunschmann, et. al. study (*26*), as well as collected and screened Minnesota tabanid flies for the presence of filarial parasites. Our data reveal *E. schneideri* is indeed present in the Minnesota moose herd and horsefly vectors, suggesting the parasite could be contributing to morbidity in moose and transmission is occurring locally. This study serves as the first documentation of *E. schneideri* in Minnesota and the Midwest, as well as provides new insight into the causes of morbidity and mortality in the Minnesota moose population with implications for future herd management.

Methods and Materials

Moose Sample Collection and Tissue Histology

As previously described, carcasses from 62 Minnesota moose were necropsied and CNS tissues examined histologically for any pathological changes (*26*). Briefly, tissues were collected from carcasses of moose that died of an unknown cause, were euthanized by tribal or Department of Natural Resources personnel because of perceived signs of illness, or died from vehicular collisions. Sections of central nervous system (CNS) tissues were preserved in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for microscopic examination. Based on the histological examination of the CNS, moose were categorized as histologically positive or negative for a nematode CNS infection. The presence of migration tracts, larvae, morulae, or adult nematode cross-sections were considered to be histologically positive (HP) and no pathological changes in the CNS was categorized as histologically negative (HN). Based on these results, 35 animals (23 HP; 12 HN) were randomly selected for further molecular analysis.

Molecular Testing of Moose Tissues

To screen the preserved moose CNS tissues for the presence of pathogenic nematodes, two separate 10μm shavings were obtained from the formalin-fixed, paraffin-embedded tissue blocks for molecular analysis. Testing multiple shavings allowed us to decrease the likelihood of obtaining a false-negative result. Each shaving was subjected to DNA extraction according to manufacturer's instructions. A DNA

extraction control was included to detect contamination during the DNA extraction process. To screen for the presence of nematodes in the moose CNS tissues, the Nematoda-wide primers Nem18SF (5'-CGCGAATRGCTCATTACAACAGC-3') and Nem18SR (5'-GGGCGGTATCTGATCGCC-3') targeting the 18S rRNA gene were utilized. The PCR reaction was performed as previously described (*83*). PCR amplicons were separated via gel electrophoresis and visualized using UV light. All positive PCR products were purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) and sequenced at the Molecular Core Facility at the University of Tennessee (Knoxville, TN).

Additional molecular screening for the presence of *P. tenuis,* an endemic nematode known to infect moose in Minnesota, was performed to ensure this parasite was not missed during the 18S amplification. We utilized a nested polymerase chain reaction (PCR) designed to selectively amplify an 110bp portion of the second internal transcribed spacer region (ITS-2) of *Parelaphostrongylus* spp. (*116*). The following primer pairs were used in the nested PCR: primary primer pair PTP1 (5'-CCGTCGAATACATGTCATCC-3') and PTP2 (5'-TCGTCAAGACGATGATTCCC-3'); secondary primer pair PtIntITSF (5'-AGAATTACGACAATGGCAAC-3') and PtIntITSR (5'-ATGATACCCATTGATAATC-3') (*109, 111, 116*). Both the primary and secondary reactions were separated by electrophoresis and the PCR products visualized using UV light. Nuclease-free water and DNA from a *P. tenuis*-infected sika deer (*109*) served as the PCR negative and positive controls, respectively.

Tabanid Fly Collection and Molecular Screening

To survey for the presence of cervid nematode parasites in tabanid flies, 618 flies were collected June-August of 2013 from four separate locations as described in Chapter 2, page 16. Briefly, the flies were ensnared using CO_2 traps and their genera identified based on morphological characters. Flies were preserved in 70% ethanol until ready for DNA extraction. DNA was extracted using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) following manufacturer's instructions. Flies were divided into groups of 10 and 5µl of DNA from each fly was pooled into the corresponding group's microcentrifuge tube. To screen for nematode DNA within pooled fly DNA, a PCR reaction with the Nem18S primers described above were utilized. Individual flies with PCR-positive DNA pools were subjected to an additional PCR reaction using the same Nem18S primers. All positive reactions were purified using the Qiagen PCR Purification Kit (Qiagen, Valencia, CA) following manufacturer's instructions and sequenced at the University of Tennessee's Molecular Core Facility (Knoxville, TN).

Phylogenetic Analysis of Parasite Sequences

All 18S and ITS-2 consensus sequence chromatograms were trimmed and edited by hand using Sequencher 5.3 (Gene Codes Corporation, Ann Arbor, MI). Edited sequences were compared against the NCBI GenBank database. Due to the lack of published genetic data for parasitic nematodes known to infect cervids, we also compared our genetic data with sequences obtained from adult reference nematodes

(Table 3)[5] that we identified morphologically and subjected to DNA extraction and PCR amplification as described above. Alignment and construction of neighbor-joining trees of 18S nematode sequences were done using MEGA 6.0 (*84*). All unique consensus sequences were deposited into GenBank.

Parasite Prevalence Estimates and Statistical Analysis

To estimate the prevalence of infected fly vectors, we compared the percentage of infected flies for each nematode species that was identified through DNA sequencing and phylogenetics. Our analysis also included a comparison of parasite prevalence between trapping sites, as well as between different fly genera. To determine if the prevalence of the various parasitic nematode species significantly differed between location or if a particular fly genera was more commonly associated with a parasitic nematode species, a Pearson's Chi-Squared test and Fisher's Exact Test with Bonferroni correction were utilized ($p \leq 0.015$). Statistical analyses were performed with SPSS software version 23.0 (IBM Corporation, Armonk, NY).

Results

Detection of Multiple Nematode Species in Minnesota Moose CNS Tissues

Molecular analysis of all moose CNS tissue samples using Nematoda-wide 18S primers was carried out to detect if parasitic nematodes were present in the Minnesota moose that died from unknown causes. Nematode DNA was successfully amplified and sequenced from 24 individual moose. Additionally, 18S sequences were obtained from morphologically identified species of filarial nematodes known to infect cervids. When compared against GenBank, sequences most closely aligned with 18S sequences from nematode species of the Onchocercidae family, including *Loa loa* (Accession Number: DQ094173.1) and *Setaria digitata* (Accession Number: DQ094175.1) with a 98-99% identity. Despite the high percent identity, it is well known that neither *L. loa* nor *S. digitata* are endemic to North America, nor are they associated with cervids (*46, 120*). Due to the highly conserved nature of the 18S rRNA target, this suggests the sequences from the Minnesota moose tissues indeed belonged to filarial nematodes, however they are most likely not *L. loa* or *S. digitata*.

In light of the GenBank results, a phylogenetic comparison against 18S sequences from other known filarial parasites of cervids was conducted. The analysis revealed the presence of 3 distinct species of filarids in the moose CNS tissue samples (Fig. 8): *Setaria yehi*, *E. schneideri*, and *Rumenfilaria andersoni*. The arterial worm, *E. schneideri,* was detected in 20 individual moose, 11 (55%) of which had no pathological signs of CNS nematode infections and nine moose had either migration tracts or

[5] All tables and figures are located in the Appendix.

nematodes visible in the CNS tissues. In addition to *E. schneideri, S. yehi*, a common filarial parasite of white-tailed deer (*86*), was detected in one histologically-negative moose, three moose with migration tracts in the CNS, and one moose with an adult nematode visible in the CNS tissues. Two animals (MO-15, MO-21) were positive for *E. schneideri* and *S. yehi*. *Rumenfilaria andersoni,* a filarial parasite of moose, deer, and reindeer (*30, 33*), was detected in a single moose. See Table 5 for a detailed summary of molecular testing results and Table 4 for sequence accession numbers.

Due to the possibility that the 18S PCR assay may miss the presence of dual infections, particularly with the endemic metastrongylid parasite *P. tenuis*, we chose to further test the Minnesota moose CNS tissues using *Parelaphostrongylus* spp.-specific nested primers. Molecular screening revealed 16 of 35 (40.6%) moose were PCR-positive, with all ITS-2 sequences (150 base pairs) having a 99% Maximum Identity to other *P. tenuis* isolates in GenBank (Accession Numbers: GU122925.1, GU122924.1). All negative and positive PCR and DNA extraction controls were as expected. Of the 16 *P. tenuis*-positive moose, three *P. tenuis*-positive moose had visible migration tracts in the CNS tissues, 12 had nematodes (morulae, larvae, and/or adults) visualized, and one was histologically negative for signs of a nematode CNS infection (Table 5). Interestingly, two animals (MO-5, MO-31) were positive for both *E. schneideri* and *P. tenuis.* A dual infection of *P. tenuis* and *S. yehi* was detected in three animals (MO-8, MO-19, MO-27) and a single animal had a dual infection with *P. tenuis* and *R. andersoni.*

Identification and Prevalence of Filarial Nematodes in Minnesota Horseflies

In light of our sequence results from the moose CNS tissues, we sought to determine whether we could find *E. schneideri* within the Minnesota environment by screening the fly vector for the filarid. In total, 618 horseflies were acquired for molecular analysis from four separate locations. Within the fly collection, three distinct tabanid fly genera were identified, including *Hybomitra* spp., *Chrysops* sp., and *Tabanus* spp.; representatives from all three genera were collected at each of the trapping sites with the exception of the Schultz location, where only *Chrysops* spp. were obtained (Table 4). PCR screening and sequencing with nematode-specific 18S rRNA primers revealed 5.8% (36 of 618) of the horseflies tested were positive for *E. schneideri* (Fig. 9).

A comparison of all *E. schneideri* 18SrRNA sequences from tabanid and moose hosts revealed the presence of four distinct 18S haplotypes. Two 18S haplotypes were observed among the tabanid *E. schneideri* sequences, with 94% (34 of 36) representing a single haplotype we denoted as “ES-1”; the other haplotype had a single representative and was labeled as “ES-2” (Table 7). The sequence quality was quite poor for one isolate, CA-CH369, and was therefore not included in this analysis. Analysis of the 18S rRNA sequences from the moose CNS tissues revealed 90% (18 of 20) of the sequences were identical to the ES-1 haplotype found in the flies. Two additional unique haplotypes, each with a single representative (“ES-3”, “ES-4”; Table 4), were also observed.

Among the *E. schneideri*-positive flies, the majority belonged to the *Chrysops* genus (86%; 31 of 36), with *Hybomitra* spp. representing only 14% (5 of 36) of the positive flies. *Elaeophora schneideri* was not detected in any of the *Tabanus* spp. tested (0%; 0 of 10). Prevalence of *E. schneideri* across trapping sites varied greatly, exhibiting a wide range between 0% (0 of 10) at Grand Portage to 2.3% (10 of 433) at Carlos Avery, 9.3% (5 of 54) at Isabella, and 100% (21 of 21) at the Schultz location (Fig. 10). Statistical analyses revealed positive correlations between some fly genera and locations (Fishers Exact Test, p=0.001) indicating a lack of independence between the two variables (Table 4); therefore a three-way test was performed to determine if prevalence of *E. schneideri* significantly differed between location and fly genus. Our statistical analysis revealed the number of *E. schneideri* sequence positive *Chrysops* spp. flies were significantly higher than expected at the Schultz location but significantly lower than expected at the Carlos Avery site (Fishers Exact Test, p=0.001). For *E. schneideri*-positive *Hybomitra* spp., the inverse was true, with a significantly higher number of positive *Hybomitra* spp. flies discovered at the Carlos Avery site than expected, but significantly lower at the Schultz location (Fishers Exact Test, p=0.001).

In addition to *E. schneideri*, two other species of filarial worms were detected in the Minnesota horse flies. Previous analysis discovered the presence of *Rumenfilaria andersoni* in 1.5% (9 of 616) of the flies tested (Chapter 2, p. 17) and were included within the nematode 18S phylogenetic analysis (Fig. 9). Furthermore, additional unique filarial sequences were detected in three *Chrysops* spp. flies (0.5%; 3 of 618). All three isolates came from the Grand Portage location and phylogenetic analysis revealed they were most closely related to *R. andersoni* as compared to our other filarial reference sequences (Fig. 9). When compared against GenBank, the unknown filarid sequences most strongly aligned with 18S sequences from *Dipetalonema* spp. (Accession Number: DQ531723.1) and *Loa loa* (Accession Number: DQ094173.1) with 99% identities. Based on the sequence data, we were unable to definitely identify the genus or species of this unknown nematode(s).

Discussion

For the first time ever reported, this study reveals the arterial worm *E. schneideri*'s presence in the Midwest, specifically Minnesota, indicating *E. schneideri* could be an important emerging pathogen in the Minnesota herd. Historically, *E. schneideri* was thought to be limited to the western half of North America, presumably in conjunction with the geographic range of the well-adapted mule deer and black-tailed deer definitive hosts. In these hosts prevalence of *E. schneideri* is often high, sometimes reaching levels of 78-100% (*41, 56*). Although a few cases have been reported in white-tailed deer in Florida, Georgia, South Carolina, and Texas, the prevalence ranged from only 2-10% (*57, 115*), much less than what has been reported for mule and black-tailed deer. It has been suggested that *E. schneideri* was recently introduced into the southeastern United States, likely by the translocation of animals from endemic areas in the west to new habitats in the east (*46, 57, 59*). At this time, it is uncertain if the emergence of *E. schneideri* in Minnesota is due to a recent importation event(s) of the parasite or a

recent expansion of the parasite's geographic range. Alternatively, *E. schneideri* could be endemic to the Minnesota herd but remained undetected due to low prevalence levels. Future studies comparing various geographic isolates genetically are warranted to provide insight into the origin of *E. schneideri* of Minnesota.

The presence of *E. schneideri* has significant health implications for both wild and domestic ruminants of Minnesota. Although *E. schneideri* infections in definitive mule deer and black-tailed deer hosts are typically asymptomatic, within abnormal hosts, such as elk and moose, *E. schneideri* can cause significant damage by restricting blood flow in the carotids and other cephalic arteries (*28, 29, 40*). Reports of elaeophorosis in elk describe ischemic lesions in the brain, eyes, and optic nerves; necrosis of the muzzle and ears, antler deformities, blindness, and subsequent death (*40*). Infections in sheep result in severe skin lesions on the head, with the condition commonly referred to as "sorehead disease" (*41*); domestic kids are also extremely sensitive to this parasite, with the majority of infections resulting in brain lesions (*46*) . Specifically in moose, elaeophorosis lesions are associated with sloughed ear tips (*47*), blindness (*28, 29*), and neurological impairment (*50*). With the discovery of *E. schneideri* in Minnesota, awareness and consideration of elaeophorosis as a differential diagnosis in free-ranging and domestic ruminants displaying neurologic impairment or ischemic necrosis of the head and face should be implemented.

Intriguingly, we discovered *E. schneideri* in CNS tissues of moose that died from unknown causes or were euthanized due to illness (Fig. 8). Over half (66%) of the moose had lesions (i.e. migration tracts, the presence of nematode parasites) associated with nematode CNS infections (Table 1). Typically *P. tenuis* infection would be considered the most likely diagnosis, however we were unable to confirm the presence of *P. tenuis* in seven animals containing lesions consistent with nematode neural migration and a single animal with an adult nematode in the CNS. Instead, we obtained DNA sequences consistent with *E. schneideri* in all of the aforementioned animals, as well as in two molecularly confirmed *P. tenuis* positive animals (MO-5, MO-31) indicating the presence of a dual infection. As stated above, one animal had an adult nematode in the CNS that was PCR negative for *P. tenuis* but PCR positive for *Elaeophora*. However, this does not unequivocally indicate that the adult nematode observed is indeed *E. schneideri*. A dual infection of *P. tenuis* and *Elaeophora* may have occurred and only the *Elaeophora* DNA amplified with PCR, while the *P. tenuis* nematode did not. However, this seems unlikely given the use of nested PCR for *P. tenuis* detection. It would be useful for future histological studies on cervids to have immunohistochemical-specific antibodies for *P. tenuis* and *Elaeophora* to localize the nematodes in tissue sections. *Elaeophora schneideri* was also detected in 11 of the 12 histologically negative moose. The presence of the arterial worm in both histologically negative and positive animals signifies *E. schneideri* infections may not necessarily result in the development of clinical CNS disease. Moreover, we were unable to confirm the lesions that were observed were directly caused by *E. schneideri.* As mentioned above, neurological impairment due to elaeophorosis is most commonly caused by the

disruption of blood flow within the cranial vascular system by *E. schneideri* nematodes. It is reasonable to presume that the *E. schneideri* we detected in the Minnesota moose CNS tissues originated in the arteries, most likely the leptomeninges, of the cranium, and may have been dislodged during necropsy. Under these conditions, the parasites may not have caused clinical disease or overt gross or histological lesions. It is also possible *E. schneideri* nematodes exhibited aberrant migration through the CNS tissues, however this has not been previously reported in elaeophorosis cases. Experimental infection studies would be needed to determine the neurotropic nature and potential for CNS lesions, if any, is associated with elaeophorosis in moose.

The overall effect of *E. schneideri* infections on moose populations is still unknown. Previous studies surveying hunter-killed moose for *E. schneideri* implied elaeophorosis is relatively mild in these animals, with many exhibiting subclinical infections (*50, 67*), however both surveys took place in endemic regions (Colorado, Wyoming) and only examined seemingly healthy individuals. Due to a lack of host-parasite coevolution, it is possible there may be differences between the western *A. alces shirasi* moose subspecies versus *A. alces andersoni* of Minnesota and their ability to tolerate *E. schneideri* infections; potentially leading *A. alces andersoni* to be more prone to developing clinical disease. However, experimental infections would need to be performed to further investigate the potential health impacts of *E. schneideri* on *A. alces andersoni.* Furthermore, it would be worth investigating if *E. schneideri* infection could lead to a compromised immune state allowing greater susceptibility to other pathogens, predation, or result in decreased reproductive rates. We would predict co-infections with *E. schneideri* and *P. tenuis* or other nematodes would have a negative impact on overall moose health. In areas where moose populations are already impacted negatively by parasites including *P. tenuis*, liver flukes (*Fascioloides magna*), and winter ticks (*Dermacentor albipictus*), as is the case in Minnesota (*26*), the emergence of another nematode pathogen could further repress an already struggling population. Further analysis of the epidemiology of *E. schneideri* in Minnesota moose host is needed to address these hypotheses.

Our surveillance of tabanid flies for the presence of filarial nematodes further asserts the presence of *E. schneideri* in Minnesota and suggests the nematode is being transmitted and maintained in the environment. An overall prevalence of 5.8% in the Minnesota flies was relatively high compared to the 0.3% reported in South Carolina (*73*) and 0.8% in Montana (*72*), however prevalence rates as high as 20% have been reported in New Mexico (*56, 70*). Interestingly, we were able to sequence *E. schneideri* from *Chrysops* spp. and *Hybomitra* sp flies (Fig. 9); previous surveys implicated *Hybomitra* sp. as an intermediate host for *E. schneideri* (*70-72*), but this is the first time the parasite has been detected in *Chrysops* sp. flies. Although we failed to detect *E. schneideri* in our *Tabanus* sp. flies, we recognize our sample size for this particular genus was quite limited and thus it remains undetermined if *Tabanus* spp. contribute to the eco-epidemiology of *E. schneideri* in the Minnesota system.

A comparison of the *E. schneideri* 18S sequences isolated from the tabanid flies and moose CNS tissues revealed the presence of at least four unique 18S haplotypes, with most sequences corresponding to a single haplotype (Table 2, 5). Due to its conserved nature, relatively low levels of 18S sequence diversity between isolates are not unexpected, however our data suggest multiple *E. schneideri* populations may be present. Without a clear understanding of the origin of *E. schneideri* parasites in Minnesota, it is difficult to determine the significance of these data. The presence of multiple haplotypes could be due to overlapping of *E. schneideri* populations or the importation of new nematode populations via the anthropogenic translocation of cervids. Additionally, the 18S diversity observed could be attributed to variation between 18S copies within the genome. Future studies evaluating the population genetic structure of this parasite are warranted to evaluate the true diversity of *E. schneideri* parasites in Minnesota.

In addition to *E. schneideri*, we also detected the presence of other filarid species in the moose CNS tissues and tabanid flies. Sequences from unidentified species of filarial nematode(s) were amplified from three *Chrysops* spp. flies from the Grand Portage trapping site (Fig. 9). We were unable to determine the identity or the significance, if any, of these findings. Future studies examining the flies for infectious third stage larvae may allow these species to be differentiated based on morphological characters. Screening of the CNS tissue samples revealed *S. yehi* and *R. andersoni* were present in a select number of Minnesota moose (Fig. 8), all of which were PCR positive for *P. tenuis* as well (Table 2). A common parasite of white-tailed deer, *S. yehi* is found in the peritoneal cavity. No associations with disease in moose have been reported, although other *Setaria* species, namely *Setaria digitata* and *Setaria cervi* have documented neurotropism in cattle and deer, respectively (*46, 121*). *Rumenfilaria andersoni*, another filarial nematode, infects moose, caribou, and white-tailed deer (*30, 32, 33*). Adult *R. andersoni* reside within the lymphatic vessels of the rumen and microfilariae can be observed in the general circulatory system (*30, 32*). It is unknown if infections with *R. andersoni* can lead to clinical or subclinical disease; only macroscopic inflammatory changes within the ruminal vessels of infected reindeer have been described (*33*). Microfilariae identified as *S. yehi* and *R. andersoni* have been observed in blood samples from live-captured moose of Minnesota (Chapter 2, page X), thus *S. yehi* and *R. andersoni* DNA was probably introduced to the CNS tissues by contamination with microfilariae-containing blood during necropsy, rather than a direct CNS infection with these two species of filarids.

In conclusion, our study documents for the first time the presence of the pathogenic nematode *E. schneideri* in moose and tabanid fly populations of Minnesota, indicating the occurrence of local transmission and expanding the current known distribution of *E. schneideri.* A better understanding of the distribution of *E. schneideri* is essential to help prevent the spread of this parasite to other non-endemic locations through human-mediated translocation of infected cervids, as well as its potentially negative economic impact on domestic farmers (loss of livestock, cost of treatment, etc.) or state and local

governments (loss of hunting and ecotourism revenues). Furthermore we were able to enhance our understanding of *E. schneideri* eco-epidemiology by implicating another genus of tabanid flies as a newly discovered vector of *E. schneideri*. These data will help set the foundation for future research investigating *E. schneideri*, particularly with regards to elaeophorosis and the potential impact it may have on moose and other cervid populations.

CHAPTER IV
CONCLUSIONS

Filarial Worm Infections: Moose and Beyond

Summary of Research Findings and Implications for Moose Populations

Exploring the eco-epidemiology of filarial nematodes in North American moose revealed the presence of multiple filarid species circulating within these animals. We were able to demonstrate *Rumenfilaria andersoni*, a lymphatic-dwelling nematode, is distributed throughout moose herds of North America and possibly transmitted by *Chrysops* spp. flies. We were unable to find a correlation between prevalence rates of *R. andersoni* and declining populations, suggesting this parasite may be of little health consequence to the moose host. However, additional research is warranted to test this hypothesis and determine if *R. andersoni* can cause clinical or subclinical disease in moose or other cervid species. Our data establishes a baseline for future *R. andersoni* epidemiological studies and will allow researchers to gain a more comprehensive view on the dynamics of *R. andersoni* infections in moose over time and space.

Perhaps our most significant finding was the presence of *Elaeophora schneideri* in the CNS tissues of moose and tabanid flies of Minnesota. As a known pathogen of moose but previously unreported in eastern North America, we suspect *E. schneideri* is an emerging pathogen in Minnesota moose and a contributing factor to the recent population decline. It is still unclear if these findings represent a recent introduction of *E. schneideri* into Minnesota or conversely a recent increase in *E. schneideri* population levels to a detectable level. Moreover, a direct connection between *E. schneideri* infections and Minnesota moose morbidity and mortality has yet to be proven. What is clear is that the parasite is established within the Minnesota ecosystem and transmission is occurring locally. This has significant implications for the management of native moose, elk, and white-tailed deer populations, as well as commercial game farms and zoos. In addition to increasing morbidity and mortality in native wildlife, elaeophorosis could result in the loss of valuable or endangered exotic species in zoos or game farms and a decrease in trophy-quality game for commercial hunting operations. Future research is warranted to identify the factors driving *E. schneideri* transmission and maintenance in the environment, as well as incidence rates of clinical and subclinical disease in Minnesota cervids. Using a multi-faceted approach that incorporates the full host-parasite-vector ecosystem, researchers will be able to better understand *E. schneideri* disease ecology and assess its impact on the Minnesota moose population.

Future Research Directions

We still know very little about *R. andersoni* in the natural environment and thus future research examining *R. andersoni* ecology would result in significant advances in the understanding *R. andersoni* epidemiology. Dissection of *R. andersoni*-infected *Chrysops* spp. flies for the presence of infectious larvae will allow us to confirm that *Chrysops spp.* indeed serve as vectors. Furthermore, documentation of the larval stages within the vector would provide insight into the developmental process of the filarid and provide a more accurate timeline to the *R. andersoni* lifecycle. Additional

surveys of tabanid flies or other potential arthropod vectors would also allow us to identify which species are most important for transmission, as well as predict risk of infection. Furthermore, tracking yearly variations in prevalence of *R. andersoni* in the arthropod vectors may provide insight into which environmental factors significantly influence *R. andersoni* distribution and prevalence. Moreover, surveys of *R. andersoni* across latitudinal gradients, either in cervid hosts and/or vectors, would help to test the hypothesis that *R. andersoni* is specifically adapted to sub-arctic climates.

The health implications for *R. andersoni* have yet to be determined. Although experimental infections in moose may not be feasible, experimental infections in white-tailed deer would help to elucidate the developmental process and infection dynamics of *R. andersoni* in cervid hosts. Live animal infection studies would allow us to assess the immune response of the cervid host to *R. andersoni*, monitor closely for any development of clinical disease, and better understand the physiological impact *R. andersoni* has on the cervid host. Moreover, cervid infection studies could be used to test the competence of suspected *R. andersoni* arthropod vectors.

Knowledge regarding the recent emergence of *E. schneideri* in Minnesota is also lacking. Comparative genetic analysis of *E. schneideri* isolates from diverse geographical locations would help to identify the origin of the Minnesota *E. schneideri.* If *E. schneideri* is truly contributing to the Minnesota moose population decline, one of the first steps towards mitigating the problem is to identify the source of the pathogen. If the emergence of *E. schneideri* is a result of a recent importation event, we would expect the Minnesota isolates to be closely related to geographically distant isolates. Conversely, if *E. schneideri* emergence is simply due to an increase in prevalence of the parasite to detectable levels, we would expect some divergence between Minnesota isolates and isolates from other geographical regions.

Although our study was able to document the presence and prevalence of *E. schneideri* in the tabanid horseflies, we have yet to determine how prevalent the parasite is within the Minnesota moose and white-tailed deer population. In contrast to *R. andersoni*, previous studies indicate *E. schneideri* microfilariae inhabit the epidermal capillary beds of the head and face in the cervid host rather than within the general circulation (*40, 45, 55, 69*). Thus, the use of a Knott's test is insufficient for epidemiological studies. Development of a serological test to detect *E. schneideri*-specific antibodies in live hosts would allow us to assess the prevalence of *E. schneideri* in the Minnesota moose population, providing a better estimate of the geographic distribution and to identify risk factors correlated with infection (habitat, host demographics, host population density). Preliminary experiments indicate moose do produce IgG antibodies capable of recognizing *E. schneideri* proteins (Figures 11-13, Tables 8,9), thus implying there is potential for the development of a serological assay.

Connecting the emergence of *E. schneideri* in the Minnesota herd to moose population decline may be difficult. Comparing *E. schneideri* seroprevalence of Minnesota moose

prior to the start of the major decline (late 1990's to early 2000's) and over the course of the decline (2005-current) may provide insight, however this will require access to banked Minnesota moose sera collected over the last few decades. A comparison of seroprevalence from seemingly healthy live capture animals and diseased or deceased animals may also reveal a pattern, but recovering carcasses in sufficient time to collect samples before they are scavenged or decomposed can be challenging. Furthermore, development of an immunohistological probe would also help researchers to identify *E. schneideri* within host tissue samples. This would aid in the diagnosis of elaeophorosis, as well as the documentation of pathology associated with *E. schneideri* infection.

Parasite Hitchhikers and the Consequences of Translocating Wildlife

As demonstrated by our genetic analysis of *R. andersoni* isolates revealing a lack of divergence between Minnesotan and Finnish *R. andersoni,* human-mediated movement of cervid hosts presents an opportunity to introduce parasites into naïve populations. This translocation of parasite hitchhikers can lead to outbreaks and an increase in morbidity and mortality in native wildlife. For example, the translocation of North American elk into Italy in 1865 resulted in the introduction of the giant liver fluke (*Fascioloides magna*) into Europe (*122*). Since then, extensive fluke-related mortality of native European elk has occurred, with outbreaks continuing to surface (*123*) and the parasite spreading to new geographic regions (*124, 125*). Of course this phenomenon is not limited to nematode parasites; other pathogens, including viruses, ectoparasites, and bacterial agents can also be spread via translocation of host species (*126*).

Although protocols and governmental regulations exist to help prevent the spread of disease through translocation (*127*), cryptic parasitic species like *R. andersoni* may go undetected in wildlife. Furthermore, very few diagnostic tools exist to detect the presence of these parasitic nematodes in live animals, thus adding an additional layer of difficulty when determining the infection status of a wild animal. Increased knowledge and awareness of which parasite species are naturally circulating within cervid hosts will help wildlife management officials and state agencies make informed decisions regarding quarantine provisions, testing requirements, and restrictions for the translocation of these animals. Furthermore, a better understanding of nematode parasite ecology is essential for the implementation of control strategies to mitigate the impacts of parasitic infections on native and non-native hosts when parasites are incidentally introduced to the ecosystem.

Filarial Parasites and Global Climate Change

Due to the complex nature of the life cycle of filarial nematodes, changes in climate patterns can significantly influence pathogen transmission and interactions between wildlife hosts, vectors and even humans. Furthermore, climate patterns are increasingly recognized or predicted to be important factors in the emergence of parasitic diseases in humans and wildlife (*128-131*). Changes in precipitation, temperature, and humidity can have direct and indirect effects on the development and survivability of parasites, thus affecting their abundance in the environment. Additionally, abundance and feeding

behaviors of arthropod vectors are influenced by weather patterns. Therefore, long term changes, especially warming temperatures, could lead to significant alterations in the distribution and prevalence of filarial nematodes in wildlife populations. Emergence of filarids in previously unexposed host populations or a surge in parasite prevalence in endemic populations could lead to significant disease outbreaks (*130*).

It is uncertain how future global climate change will affect *R. andersoni* and *E. schneideri* distributions. The successive proliferation of *R. andersoni* into northern Finland exemplifies the potential for this parasite to spread given adequate abundance of hosts and vectors (*33*). Additionally, our documentation of *E. schneideri* in Minnesota indicates this nematode may have also recently increased its range. Continued surveillance and longitudinal epidemiological studies documenting the distribution of these parasites as well as their hosts will provide data from which researchers can assess the impact of environmental change on parasite prevalence and abundance, as well as disease incidence in North American cervids.

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APPENDIX

Chapter I

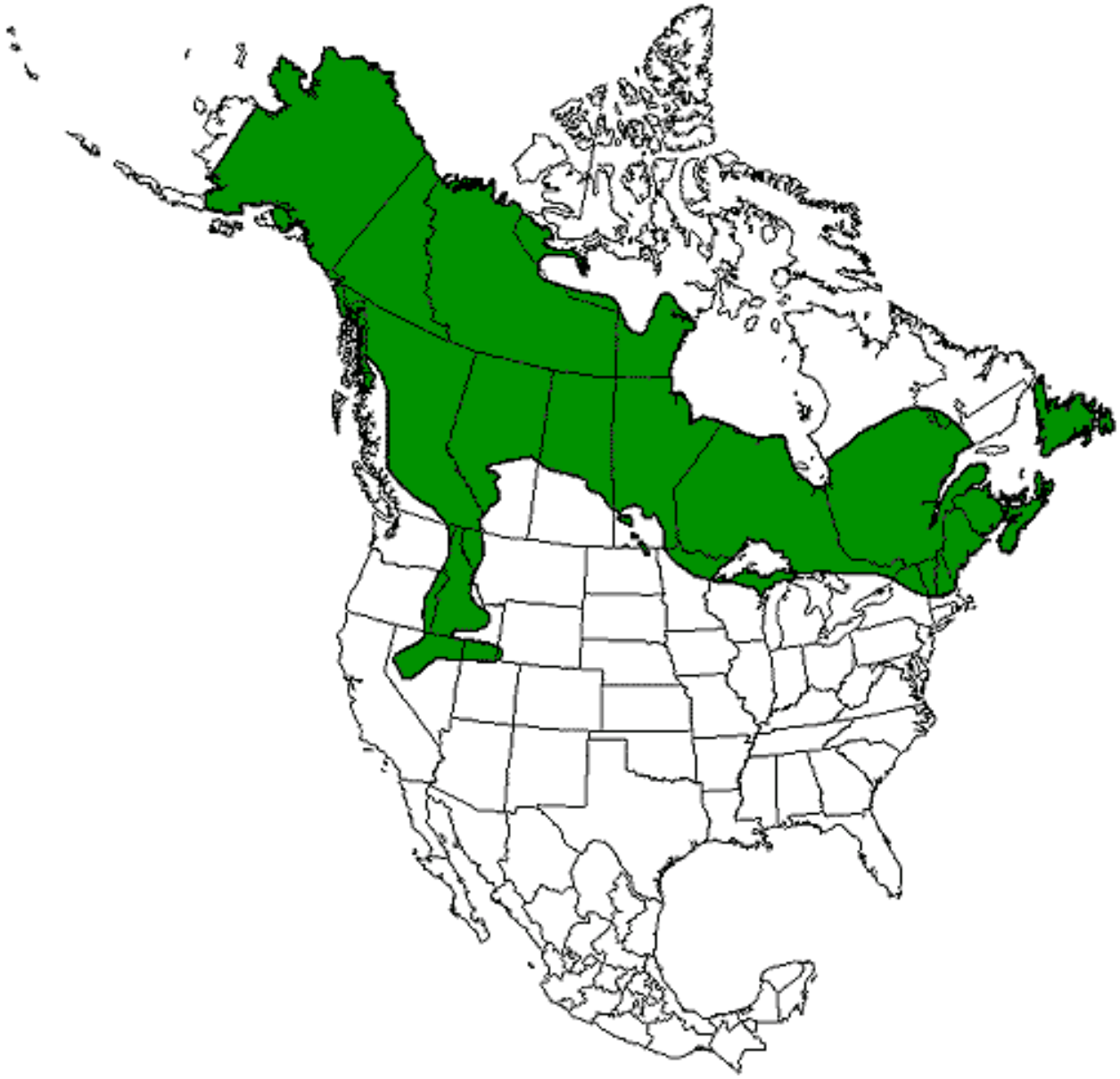

Figure 1. Distribution of moose (*Alces alces*) in North America. Image by Manitoba Wildlife Division (*132*).

Chapter II

Table 1. Reference nematodes utilized in molecular analysis. Adult nematodes were identified based on morphological characters. Geographic origin and host species refer to the place and host from which the nematode was isolated. Gene refers to the targeted gene sequence (18S rRNA or ITS-1) that was amplified.

Isolate	Species	Geographic Origin	Host Species	Gene	GenBank Accession No.
RA-F124	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	18S	KT878978
RA-F113	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	18S	KT878977
RA-F128	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	18S	KT878979
RA-F3	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	ITS-1	KT873731
ES-WY11	*Elaeophora schneideri*	Wyoming, USA	*Alces alces*	18S	KT020850
ES-WY50	*Elaeophora schneideri*	Wyoming, USA	*Alces alces*	ITS-1	KT873732
ES-CA1	*Elaeophora schneideri*	California, USA	*Rusa unicolor*	18S	KT020850
OC-AK1	*Onchocerca cervipidis*	Alaska, USA	*Alces alces*	18S	KT031393
SY-AK1	*Setaria yehi*	Alaska, USA	*Alces alces*	18S	KT878970
SY-GA3	*Setaria yehi*	Georgia, USA	*Odocoileus virginianus*	ITS-1; 18S	AY621478; KT878972

Table 2. *Rumenfilaria andersoni* sequences obtained from moose and tabanid flies.

Isolate	Host Species	Geographic Origin	Gene	GenBank Accession No.
RA-MT4	*Alces alces*	Montana, USA	ITS-1	KT873721
RA-MT8	*Alces alces*	Montana, USA	ITS-1	KT873724
RA-MT31	*Alces alces*	Montana, USA	ITS-1	KT873720
RA-MT43	*Alces alces*	Montana, USA	ITS-1	KT873722
RA-MT44	*Alces alces*	Montana, USA	ITS-1	KT873723
RA-MN1	*Alces alces*	Minnesota, USA	ITS-1	AF228560
RA-MN2	*Alces alces*	Minnesota, USA	ITS-1	KT873733
RA-MN3	*Alces alces*	Minnesota, USA	ITS-1	KT873727
RA-MN4	*Alces alces*	Minnesota, USA	ITS-1	KT873728
RA-MN5	*Alces alces*	Minnesota, USA	ITS-1	KT873729
RA-MN6	*Alces alces*	Minnesota, USA	ITS-1	KT873730
RA-MN7	*Alces alces*	Minnesota, USA	ITS-1	AF228561
RA-MN9	*Alces alces*	Minnesota, USA	ITS-1	KT873719
RA-ME1	*Alces alces*	Maine, USA	ITS-1	KT873726
RA-ME2	*Alces alces*	Maine, USA	ITS-1	KT873725
GP-CH546	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878984
GP-CH582	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878985
GP-CH545	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878983
GP-CH537	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878982
GP-CH527	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878988
GP-CH521	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878987
GP-CH502	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878981
GP-CH490	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878980
GP-CH481	*Chrysops spp.*	Minnesota, USA	18S rRNA	KT878986

Table 3. Reference nematodes utilized in 18S molecular analysis of moose CNS tissues and tabanid horseflies. Adult nematodes were identified based on morphological characters. Geographic origin and host species refer to the place and host from which the adult nematode was isolated.

Isolate	Species	Geographic Origin	Host Species	GenBank Accession No.
RA-F124	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	KT878978
RA-F113	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	KT878977
RA-F128	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	KT878979
RA-F1	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	KT885224
RA-F2	*Rumenfilaria andersoni*	Finland	*Rangifer tardanus*	KT885225
ES-WY11	*Elaeophora schneideri*	Wyoming, USA	*Alces alces*	KT020850
ES-WY1	*Elaeophora schneideri*	Wyoming, USA	*Alces alces*	KT878975
ES-WY2	*Elaeophora schneideri*	Wyoming, USA	*Alces alces*	KT878976
ES-GA1	*Elaeophora schneideri*	Georgia, USA	*Odocoileus virginianus*	KT878974
ES-CA1	*Elaeophora schneideri*	California, USA	*Rusa unicolor*	KT020850
OC-AK1	*Onchocerca cervipidis*	Alaska, USA	*Alces alces*	KT031393
SY-AK1	*Setaria yehi*	Alaska, USA	*Alces alces*	KT878970
SY-GA1	*Setaria yehi*	Georgia, USA	*Odocoileus virginianus*	KT878971
SY-GA2	*Setaria yehi*	Georgia, USA	*Odocoileus virginianus*	KT878973

Figure 2. Trapping locations of tabanid flies in northern Minnesota, USA. Each trapping site is labeled with their respective site name. Dark green shaded region marks the primary range of Minnesota moose. Light green shaded region represents the secondary range.

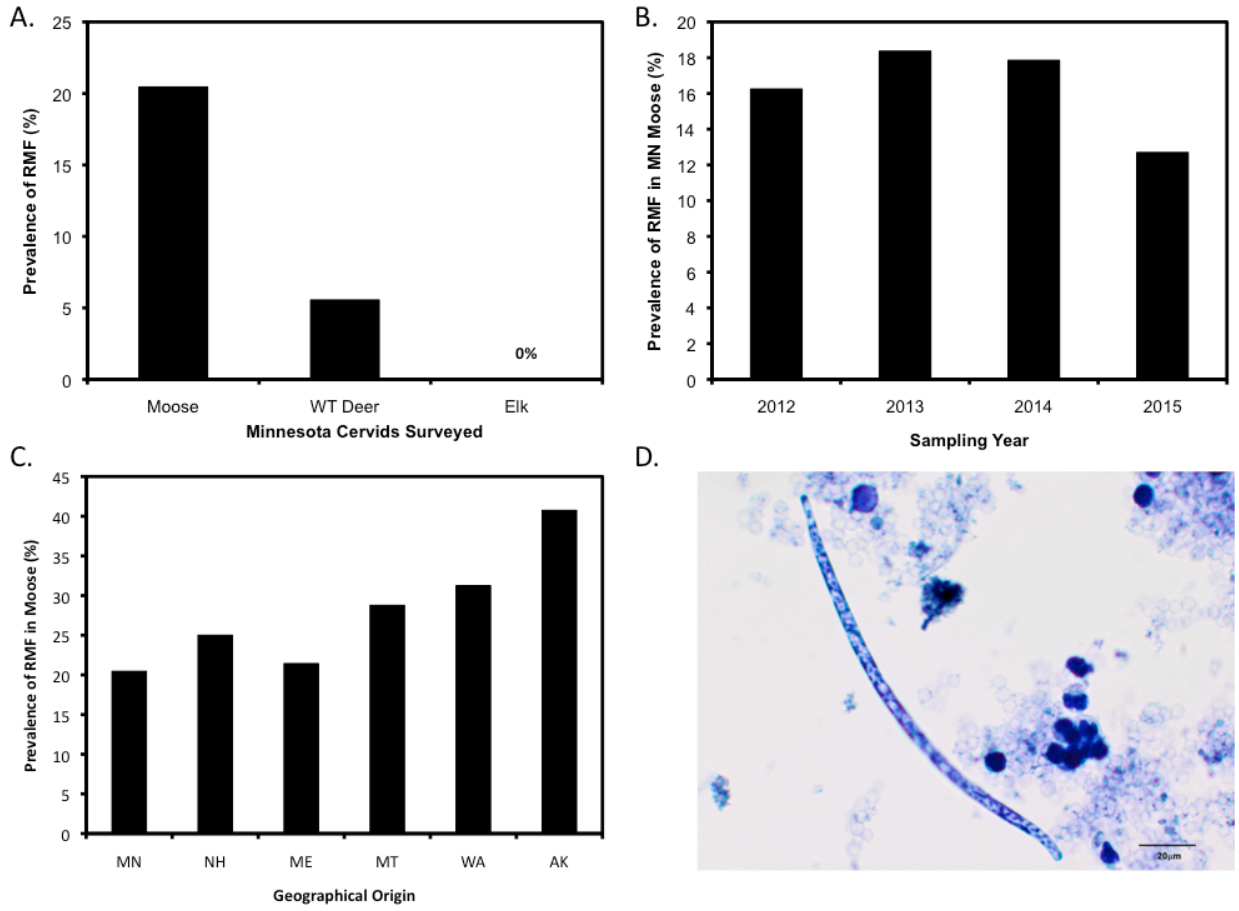

Figure 3. Prevalence of *R. andersoni* microfilariae (RMF) in free-ranging cervids. Prevalence is defined as the percentage of RMF-positive isolates compared to the entire population using a modified Knott's test. A) Prevalence of RMF in three species of Minnesota cervids. (Moose N=352; Elk N=14; White-tailed (WT) Deer N=36). Blood samples were collected over 4 year period for moose and 2 year period for elk and deer. Fishers exact test; p=0.013 B) Comparison of RMF prevalence in Minnesota moose over time. (2012 N=80, 2013 N=196, 2014 N=84, 2015 N=63) Fishers exact test; p=0.607. C) Comparison of RMF prevalence in moose from multiple U.S. states. (Total samples examined: MN N=352; NH N=16; ME N=14; MT N=73; WA N=16; AK N=27). Fishers exact test; p=0.013. D) Image of RMF from Minnesota moose blood. Sample was stained with methylene blue and visualized with a bright light microscope at 20X.

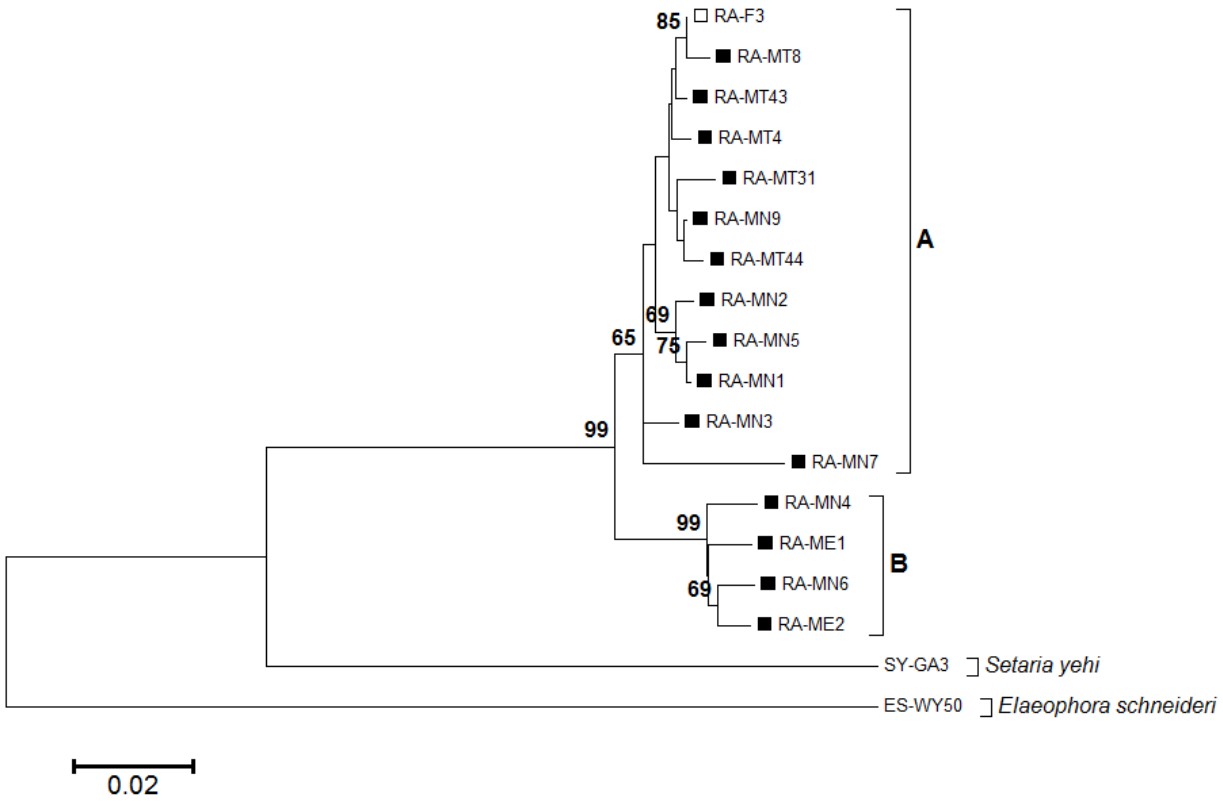

Figure 4. Phylogenetic analysis of ITS-1 sequences obtained from RMF-positive blood samples from cervid hosts. Sequences of 609 base pairs were aligned using ClustalW and the evolutionary history was inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Kimura 2-parameter method. The tree is drawn to scale. Bootstrap values (x1000) greater than 50% are shown above the branches. RMF isolates are marked with solid boxes; ITS-1 Clades A and B are labeled. RA-F3 (*Rumenfilaria andersoni*; open box), *Setaria yehi*, and *Elaeophora schneideri* serve as standards. GenBank accession numbers for all isolates are located in Table 1 and 2.

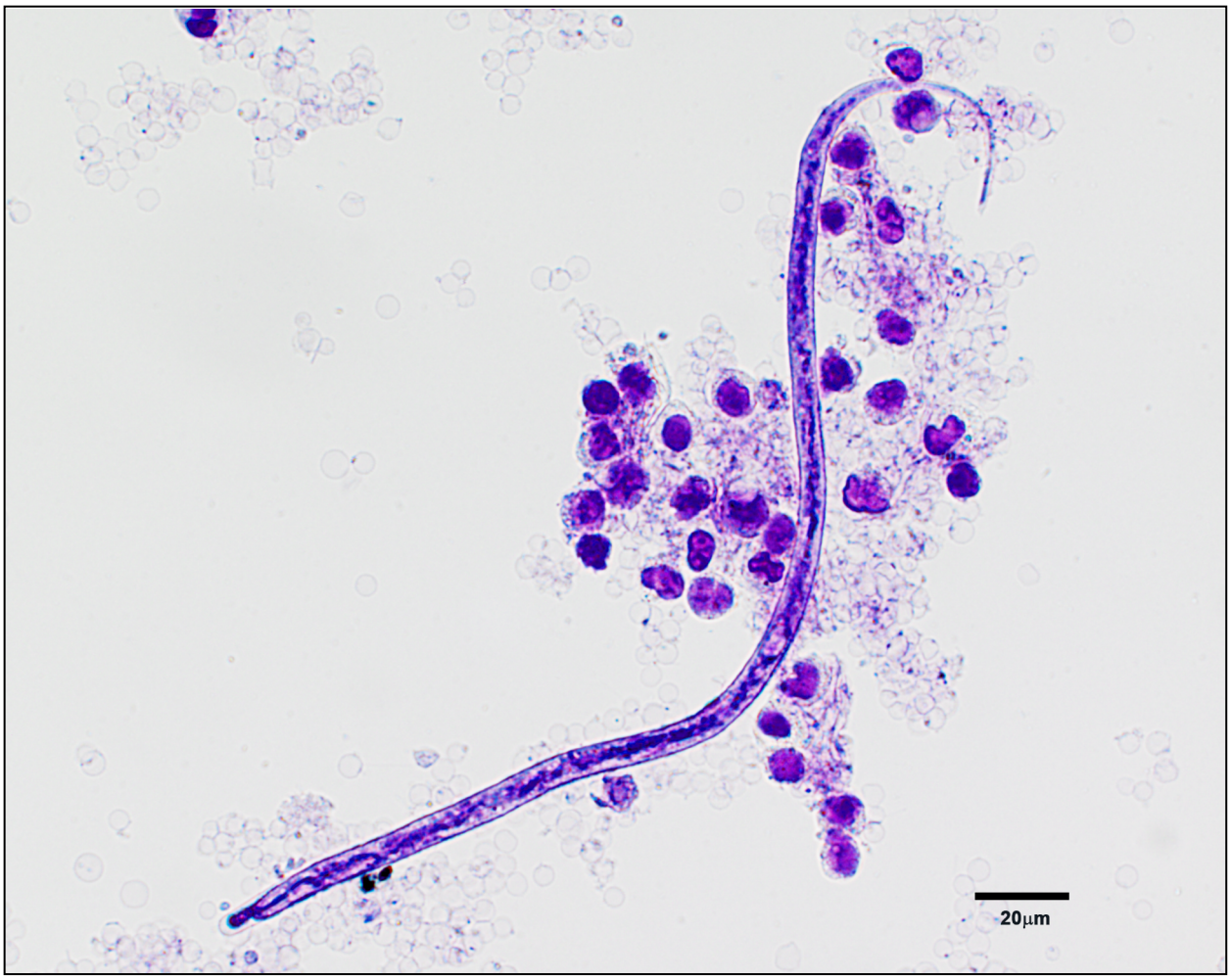

Figure 5. Unidentified microfilariae observed in blood from Minnesota moose. Sample was stained with methylene blue and image taken under a bright light microscope at 20X.

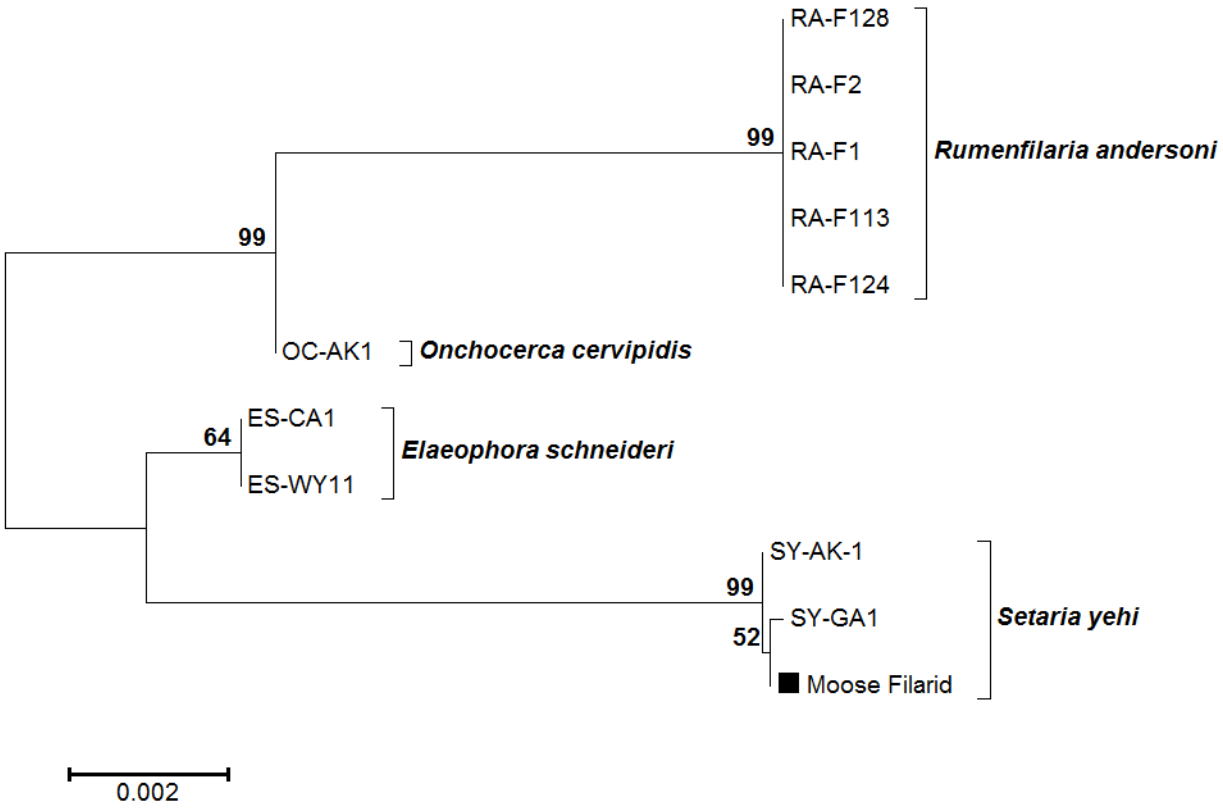

Figure 6. Genetic characterization of unidentified filarid in Minnesota moose blood. 18S rRNA sequences (796 base pairs) from unknown filarid (black box) was compared against known filarial parasites of ungulates (ES: *E. schneideri*; SY: *S. yehi*; RA: *R. andersoni*; OC: *O. cervipidis*). The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances were computed using the Kimura 2-parameter method. The tree is drawn to scale. Bootstrap values (x1000) are shown above the branches. GenBank accession numbers for all isolates are located in Table 1 and 2.

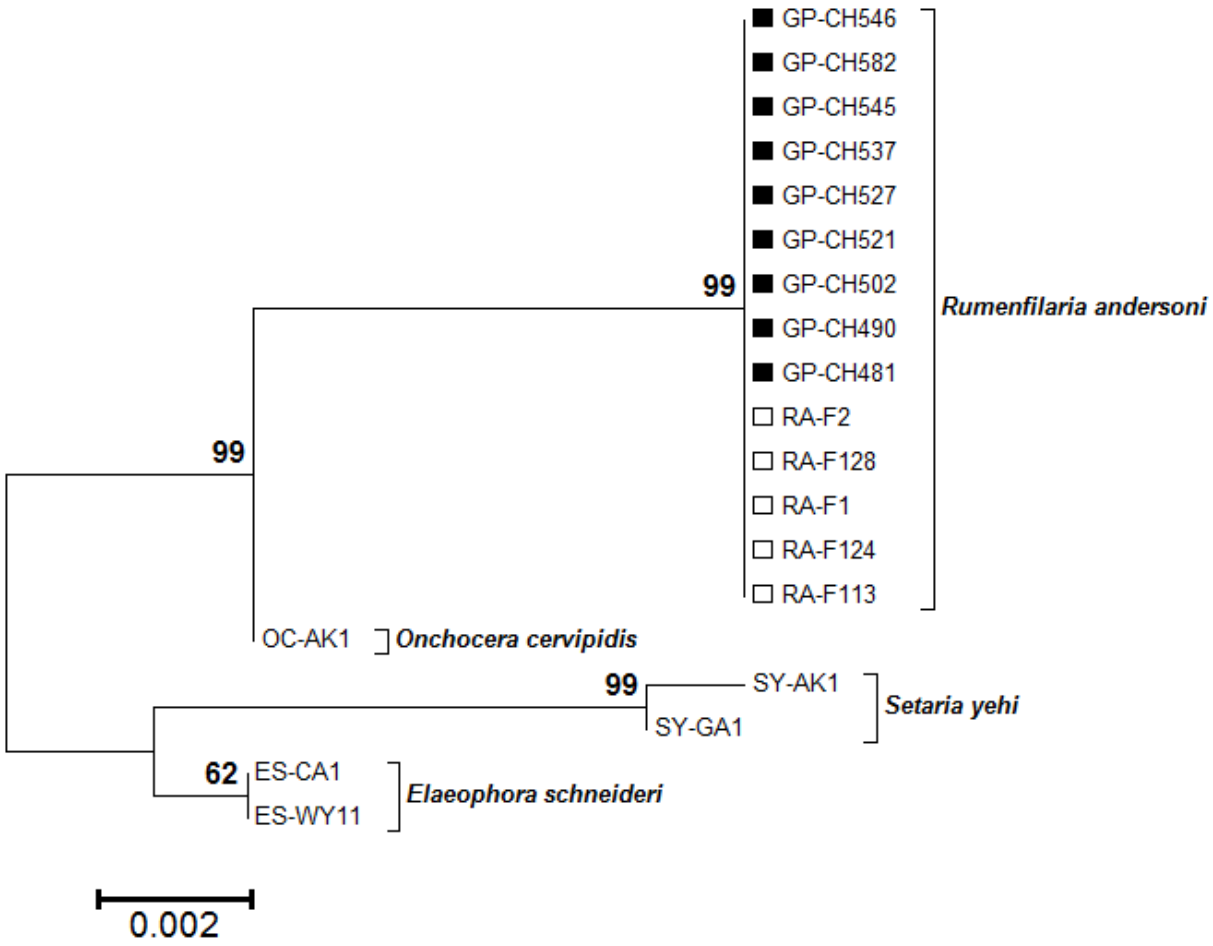

Figure 7. Phylogenetic tree of nematode 18S rRNA sequences isolated from Minnesota tabanid horseflies. Sequences of 795 base pairs were aligned and the evolutionary history inferred using the Neighbor-Joining method. Evolutionary distances were computed using the Kimura 2-parameter method. The tree is drawn to scale. Bootstrap values (x1000) are shown above the branches. Solid boxes indicated fly isolates and open boxes indicate R. andersoni (RA) reference nematodes. GenBank accession numbers for all isolates are located in Table 1 and 2.

Chapter III

Table 4. Filarial 18S rRNA sequences obtained from Minnesota moose CNS tissues. *Elaeophora schneideri* isolates with identical 18S sequences are assigned to the same Haplotype number. A representative from each haplotype was deposited into GenBank.

Moose ID	Nematode Species	*E. schneideri* Haplotype	GenBank Accession No.
MO-2	*Elaeophora schneideri*	ES-1	KT885226
MO-3	*Elaeophora schneideri*	ES-1	KT885226
MO-5	*Elaeophora schneideri*	ES-1	KT885226
MO-6	*Elaeophora schneideri*	ES-1	KT885226
MO-9	*Elaeophora schneideri*	ES-1	KT885226
MO-11	*Elaeophora schneideri*	ES-1	KT885226
MO-12	*Elaeophora schneideri*	ES-1	KT885226
MO-14	*Elaeophora schneideri*	ES-1	KT885226
MO-16	*Elaeophora schneideri*	ES-1	KT885226
MO-17	*Elaeophora schneideri*	ES-1	KT885226
MO-20	*Elaeophora schneideri*	ES-1	KT885226
MO-22	*Elaeophora schneideri*	ES-1	KT885226
MO-23	*Elaeophora schneideri*	**ES-3**	KT907504
MO-24	*Elaeophora schneideri*	ES-1	KT885226
MO-25	*Elaeophora schneideri*	ES-1	KT885226
MO-28	*Elaeophora schneideri*	**ES-4**	KT907505
MO-29	*Elaeophora schneideri*	ES-1	KT885226
MO-31	*Elaeophora schneideri*	ES-1	KT885226
MO-15	*Elaeophora schneideri;* *Setaria yehi*	ES-1	KT885226
MO-21	*Elaeophora schneideri;* *Setaria yehi*	ES-1	KT885226; KT934942
MO-8	*Setaria yehi*	NA	KT907506
MO-19	*Setaria yehi*	NA	KT907507
MO-27	*Setaria yehi*	NA	KT907508
MO-4	*Rumenfilaria andersoni*	NA	KT907509

Table 5. Summary of histology and sequence results for Minnesota moose CNS tissues. 35 moose were included in the study. Negative histology results refer to animals with no pathological changes in the CNS tissues consistent with a nematode infection. Total number of moose refers to the total number of animals that were sequence positive for each parasite species.

Moose ID	**Histology Results**	***P. tenuis* (+/-)**	***E. schneideri* (+/-)**	***S. yehi* (+/-)**	***R. andersoni* (+/-)**
MO-32	Migration tracts present	+	-	-	-
MO-19	Migration tracts present	+	-	+	-
MO-27	Migration tracts present	+	-	+	-
MO-6	Migration tracts present	-	+	-	-
MO-2	Migration tracts present	-	+	-	-
MO-17	Migration tracts present	-	+	-	-
MO-3	Migration tracts present	-	+	-	-
MO-25	Migration tracts present	-	+	-	-
MO-15	Migration tracts present	-	+	+	-
MO-10	Migration tracts present	-	-	-	-
MO-18	Morulae present	+	-	-	-
MO-7	Morulae present	+	-	-	-
MO-1	Larvae present	+	-	-	-
MO-13	Larvae present	+	-	-	-
MO-34	Adult, larvae present	+	-	-	-
MO-4	Adult, morulae present	+	-	-	+
MO-5	Adult, morulae present	+	+	-	-
MO-35	Adult present	+	-	-	-
MO-26	Adult present	+	-	-	-
MO-33	Adult present	+	-	-	-
MO-30	Adult present	+	-	-	-
MO-31	Adult present	+	+	-	-
MO-21	Adult present	-	+	+	-
MO-8	Negative	+	-	+	-
MO-9	Negative	-	+	-	-
MO-11	Negative	-	+	-	-
MO-12	Negative	-	+	-	-
MO-14	Negative	-	+	-	-
MO-16	Negative	-	+	-	-
MO-20	Negative	-	+	-	-
MO-22	Negative	-	+	-	-
MO-23	Negative	-	+	-	-
MO-24	Negative	-	+	-	-
MO-29	Negative	-	+	-	-
MO-28	Negative	-	+	-	-
Total Number of Moose:		16	20	5	1

Table 6. Fly counts per genus and trapping location used in the molecular survey for filarial nematodes. Trapping locations correspond to the locations detailed in Figure 9.

	Trapping Location				
Fly Genus	Carlos Avery	Grand Portage	Isabella	Schultz	Total No. Flies
Chrysops spp.	364*	103*	18	21*	506
Hybomitra spp.	62	6	34*	0	102
Tabanus spp.	7	1	2	0	10
Total No. Flies	433	110	54	21	618

*Indicates a significant positive association between the number of flies (per genus) and the trapping location (Fisher's Exact Test; p=0.001).

Table 7. Filarial 18S rRNA sequences obtained from Minnesota tabanid horseflies. *Elaeophora schneideri* isolates with identical 18S sequences are assigned the same Haplotype number. Sequences with ambiguous characters are labeled with ND and were not included in the haplotype analysis.

Fly ID	Trapping Location	Fly Genus	Filarid Species	*E. schneideri* Haplotype	GenBank Accession No.
SH-CH396	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH397	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH398	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH399	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH400	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH401	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH402	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH403	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH404	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH405	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH406	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH410	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH408	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH409	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH412	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH414	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH415	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH407	Shultz	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
SH-CH413	Shultz	*Chrysops*	*Elaeophora schneideri*	**ES-2**	KT885227
CA-CH334	Carlos Avery	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
CA-CH367	Carlos Avery	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
CA-CH368	Carlos Avery	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
CA-CH369	Carlos Avery	*Chrysops*	*Elaeophora schneideri*	**ND**	KT885228
CA-CH371	Carlos Avery	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
CA-HY372	Carlos Avery	*Hybomitra*	*Elaeophora schneideri*	ES-1	KT885226
CA-HY380	Carlos Avery	*Hybomitra*	*Elaeophora schneideri*	ES-1	KT885226
CA-HY382	Carlos Avery	*Hybomitra*	*Elaeophora schneideri*	ES-1	KT885226
CA-HY386	Carlos Avery	*Hybomitra*	*Elaeophora schneideri*	ES-1	KT885226
CA-HY383	Carlos Avery	*Hybomitra*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH419	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH422	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH416	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH417	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH420	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH421	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
IS-CH411	Isabella	*Chrysops*	*Elaeophora schneideri*	ES-1	KT885226
GP-CH556	Grand Portage	*Chrysops*	Unknown Filarid spp.	NA	KT907501
GP-CH571	Grand Portage	*Chrysops*	Unknown Filarid spp.	NA	KT907502
GP-CH555	Grand Portage	*Chrysops*	Unknown Filarid spp.	NA	KT907503

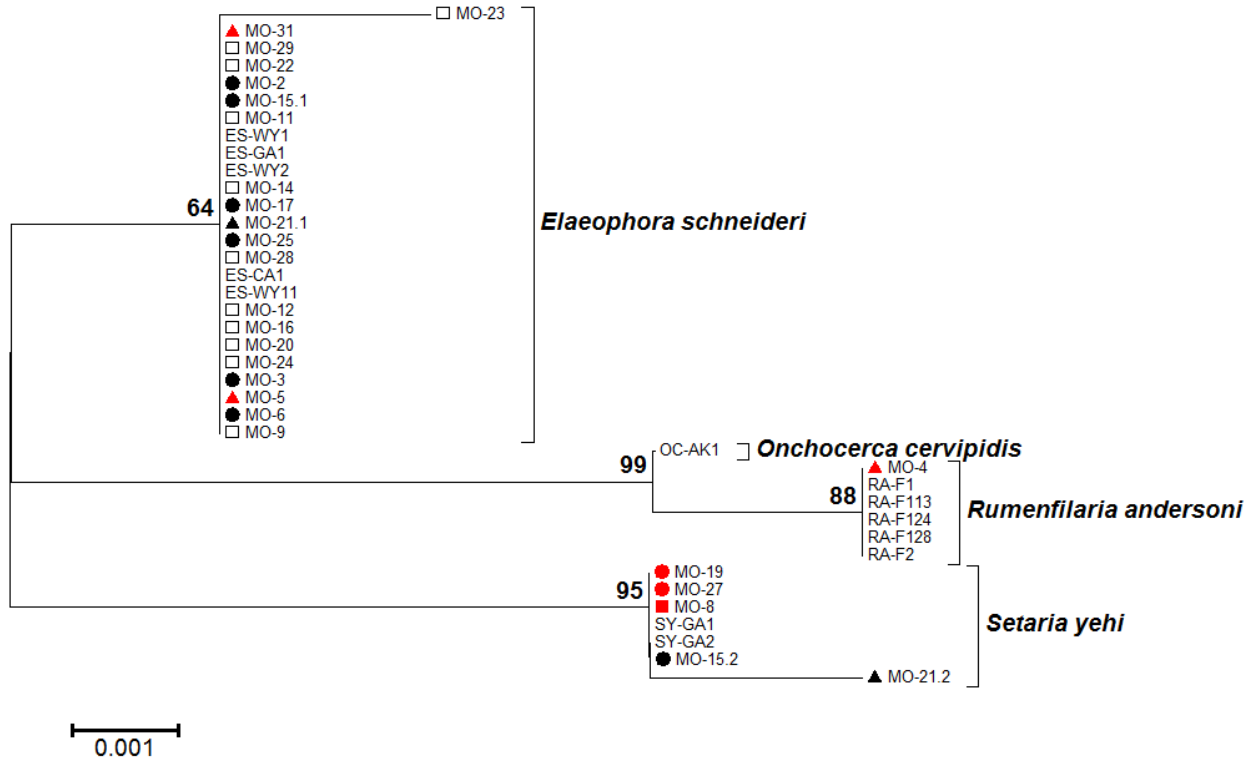

Figure 8. Phylogenetic analysis of partial nematode18S sequences (508bp) obtained from formalin-fixed paraffin-embedded CNS tissues of Minnesota moose. Tree was constructed using the Neighbor-Joining method and the evolutionary distances computed using the Kimura 2-parameter method. Bootstrap values (x1000) ≥50% are shown above the branches. Tree is drawn to scale. Isolate labels and markers correspond to the moose ID numbers and histology results, respectively, in Table 1 (squares = negative; circles = migration tracts; triangles = nematodes visualized). Red markers indicate the animal was also PCR positive for *P. tenuis.* Reference nematodes labeled as ES (*E. schneideri*), OC (*O. cervipidis*), RA (*R. andersoni*), and SY (*S. yehi*). *Elaeophora* reference nematodes originated from either moose from Wyoming (WY), white-tailed deer from Georgia (GA), or sambar deer from California (CA).

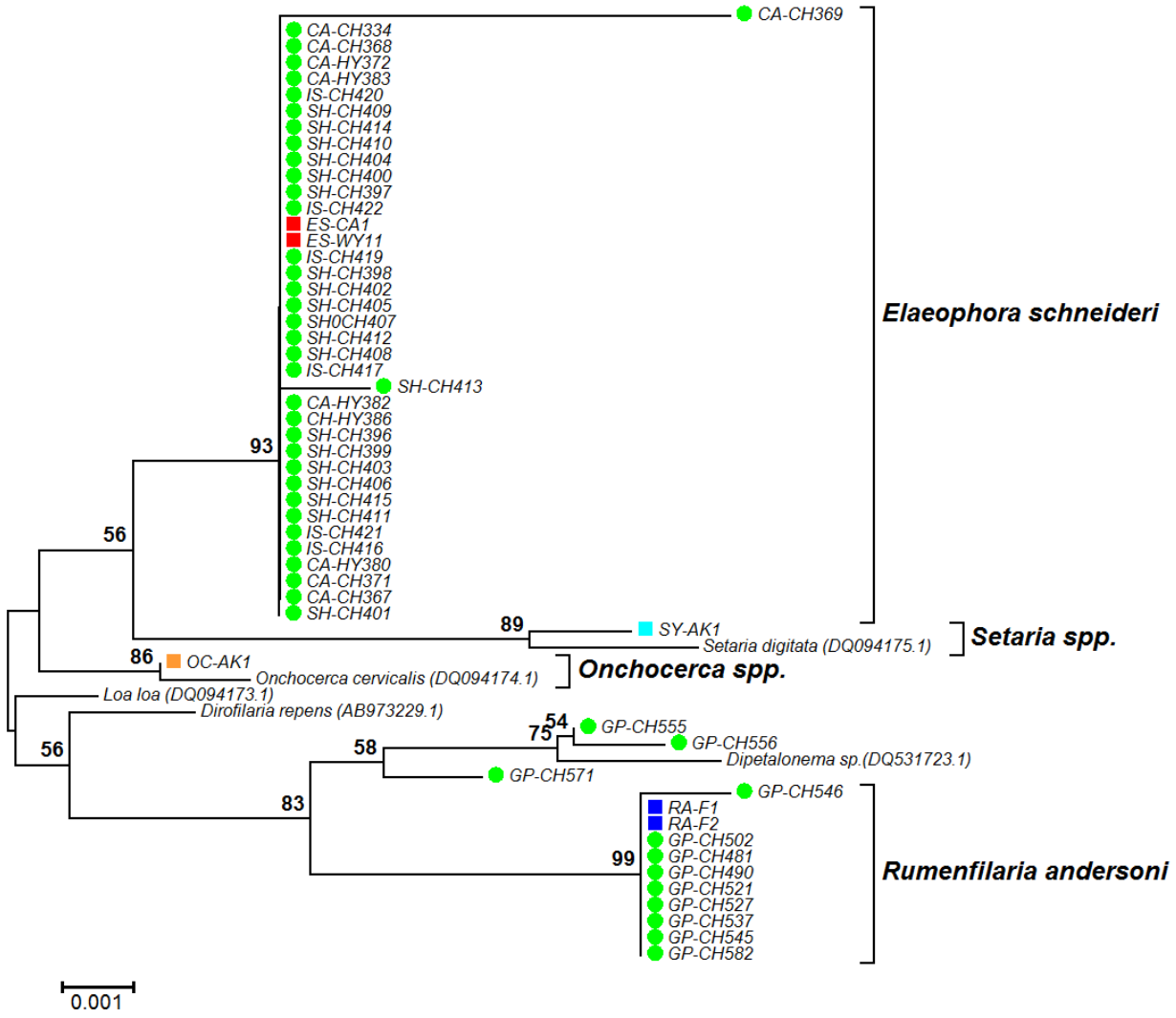

Figure 9. 18S rRNA sequencing and phylogenetic analysis reveals the presence of multiple filarial nematodes in Minnesota tabanid horseflies. 59 nematode 18S rRNA sequences (795 base pairs) were used in the analysis. The evolutionary history was inferred using the Neighbor-Joining method and evolutionary distances computed using the Kimura 2-parameter method. Tree is drawn to scale. Bootstrap values (x1000) greater than 50% are shown above the branches. Green circles indicate fly isolates, boxes indicate reference nematodes: *E. schneideri* (red); O*. cervipidis* (orange); *S. yehi* (light blue); *R. andersoni* (dark blue). Other reference nematodes obtained from GenBank are labeled with their respective GenBank Accession Number.

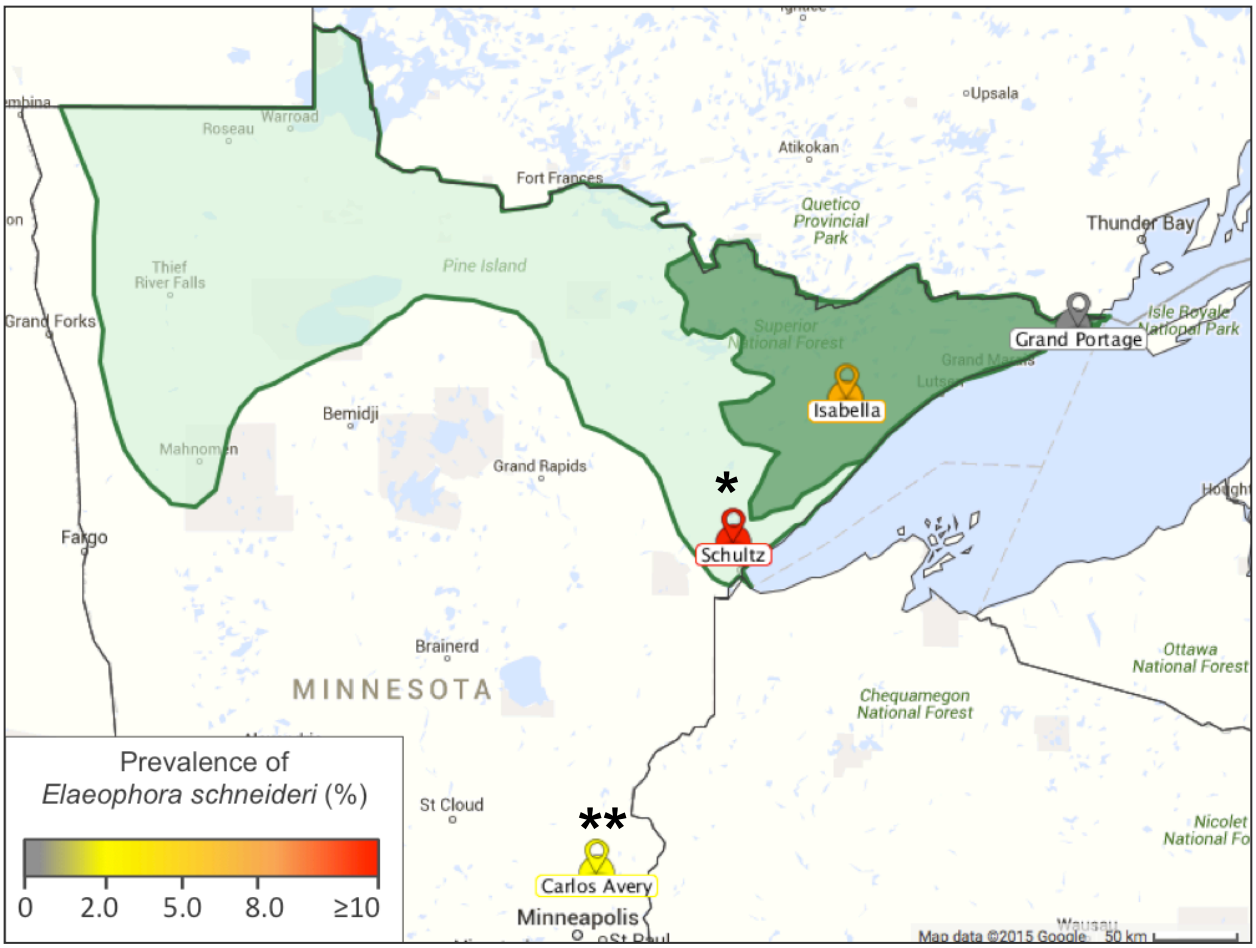

Figure 10. Prevalence of *Elaeophora schneideri* in tabanid horseflies differs between Minnesota trapping sites. 618 tabanid flies were trapped and screened for the presence of *E. schneideri*. Each trapping location marker is color-coded based on the prevalence (% infected). Green shaded region indicates moose range: dark green=primary moose range; light green=secondary moose range. Range data adapted from Minnesota DNR.
*Indicates site with significantly higher prevalence of infected *Chrysops* spp. (p=0.001).
**Indicates site with significantly higher prevalence of infected *Hybomitra* spp. (p=0.001).

Investigation of Immunogenic *Elaeophora schneideri* Proteins

Experiment Summary

A serological test to detect *Elaeophora schneideri*-specific antibodies in live hosts is an essential tool to assess the prevalence of the parasite in moose or other cervid populations. Measuring the seroprevalence of *E. schneideri* would allow us to better estimate the geographic distribution of the nematode and to identify risk factors correlated with infection (e.g. habitat type, host demographics, host and/or vector density, climate and weather patterns). Furthermore this type of assay may also aid in clinical settings, providing a non-invasive diagnostic tool. The main objective of this study was to produce a working enzyme-linked immunosorbent assay for the detection and measurement *E. schneideri*-specific antibodies in cervid serum.

To identify *E. schneideri* protein antigen candidates for the development of this assay, we obtained a fresh adult nematode extracted from the carotid artery of an adult male moose in Wyoming. The nematode was washed in phosphate buffered saline and stored on dry ice. The whole, frozen nematode was submitted to Applied Biomics Inc. (Hayward, CA) for protein extraction, 2D poly-acrylamide gel electrophoresis, and western blotting. Dual 2D poly-acrylamide gels were run with the *E. schneideri* extracted protein and fluorescently stained to compare protein profiles (Fig. 11A, D). Once it was confirmed that the protein profiles were congruent, both gels were subjected to western blotting with either *Elaeophora*-negative serum obtained from Alaskan moose (Fig. 11B, C) or with *Elaeophora*-positive serum obtained from an infected Wyoming moose (Fig. 11E, F). Infection with *E. schneideri* was confirmed by morphologically identifying adult nematodes extracted from the Wyoming moose. Fluorescently labeled goat-anti deer IgG (Alpha Diagnostic Intl. Inc., San Antonio, TX) served as the secondary antibody. Results from the 2D western blotting indicated multiple protein spots specifically reacted with the *E. schneideri*-positive moose serum, implying *E. schneideri* infected moose produce IgG antibodies capable of recognizing *E. schneideri* proteins (Fig. 11C, F).

When the 2D western blots were more closely compared, we found 69 specific protein spots that only reacted with the *E. schneideri*-positive moose serum and showed no reactivity with the *Elaeophora*-negative moose serum (Fig. 12). Fifteen of these *Elaeophora*-specific immunogenic protein spots were selected, purified, and subjected to MALDI-TOF (using MS spectra) and MALDI-TOF/TOF (using MS/MS spectra) analysis. To identify the protein spots, the MS and MS/MS data were analyzed using GPS Explorer software with the MASCOT search engine to identify the most closely matching proteins in the NCBI protein sequence database. Results from the NCBI query revealed 10 of the 12 protein spots most closely aligned with proteins of other filarial nematodes including *Brugia pahangi*, a tropical filarid of domestic cats and wildlife, and *Brugia malayi,* a tropical filarial parasite of humans that causes lymphatic filariasis (Table 8). Various protein families were represented, including heat shock proteins, elongation factors, actins, tubulins, SPARCs (secreted protein, acidic and cysteine-rich), a pyrophosphatase, an aldo-keto reductase and oxidoreductase, an enolase, an adenylate kinase, and a PAN-domain protein.

Due to the lack of available *E. schneideri* genome or proteome data, we were unable to pursue the production of complete *Elaeophora* homologs of the proteins identified in the MS, MS/MS analysis. Instead, we examined each of the peptide sequences obtained from the MALDI-TOF/TOF analysis for their potential to serve as antigens in the immunoassay. To assess the

antigenic potential of the peptide sequences, the peptides were analyzed using the Kolaskar and Tongaonkar Antigenicity Scale (*117*), Bepipred Linear Epitope Prediction (*118*), and the Parker Hydrophobicity Scale (*119*). Peptides with epitopes predicted by Kolaskar and Tongaonkar (K&T) and/or Bepipred, a Parker hydrophobicity score (Parker Score) of ≤ 4.0 were then compared against the NCBI protein sequence database and ranked as either "non-conserved", "partially conserved" or "conserved". Peptides were considered non-conserved if they only closely aligned with proteins from filarial worms; peptides were categorized as non-conserved if they closely aligned with proteins from various nematode species, both filarid and non-filarids. Peptides were ranked as conserved if they closely aligned with proteins from multiple taxonomic groups. Based on the analysis, 12 non-conserved peptides were selected and synthesized (GenScript, Piscataway, NJ) (Table 9).

Synthesized peptides were subjected to immunoblotting to assess their reactivity with *Elaeophora*-positive serum. Synthesized peptides (1µl of 1mg/mL) were spot-inoculated onto nitrocellulose membranes. Whole protein lysate (1µl of 500µg/mL) of two individual *E. schneideri* worms (ESWY 100, ESWY 93) served as positive protein controls. *Elaeophora*-negative moose serum (ES-) and *Elaeophora*-positive moose serum (ES+) served as positive controls to ensure proper binding of the secondary antibody (HRP-chicken anti-deer IgG, 50µg/mL, Gallus Immunotech Inc., Cary, NC). PBS was also spot-inoculated and served as a negative control. Three immunoblots with were performed with serum from three *E. schneideri*-infected moose (WY-MO2, WY-MO3, WY-MO5). Blots were blocked with 5% non-fat milk in TBS-T and developed using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA). Results from the immunoblot revealed all positive and negative controls were as expected, indicating the moose serum did contain *E. schneideri* IgG antibodies. Disappointingly, all 12 peptides failed to bind any of the *Elaeophora*-positive moose serum samples (Fig. 13).

Although our experiments did not yet identify an immunogenic peptide candidate for the development of an ELISA to detect *E. schneideri* seroprevalence, these data are promising. The 2D western blot experiments demonstrate moose indeed produce specific IgG antibodies capable of recognizing *E. schneideri* proteins and thus indicating significant potential for the development of a serological test. Due to financial and time constraints, this study only examined 12 peptides, a very small representative snippet of the MS, MS/MS data. Future experiments focusing on testing additional peptides or combinations of peptides for reactivity with *E. schneideri*-positive moose serum will provide a more comprehensive analysis and increase the likelihood of identifying a candidate for serological testing. Alternatively, comparing our data against a sequenced *E. schneideri* genome or proteome could lead to identification of *E. schneideri* protein homologs that could be purified and used as antigens for future assay development.

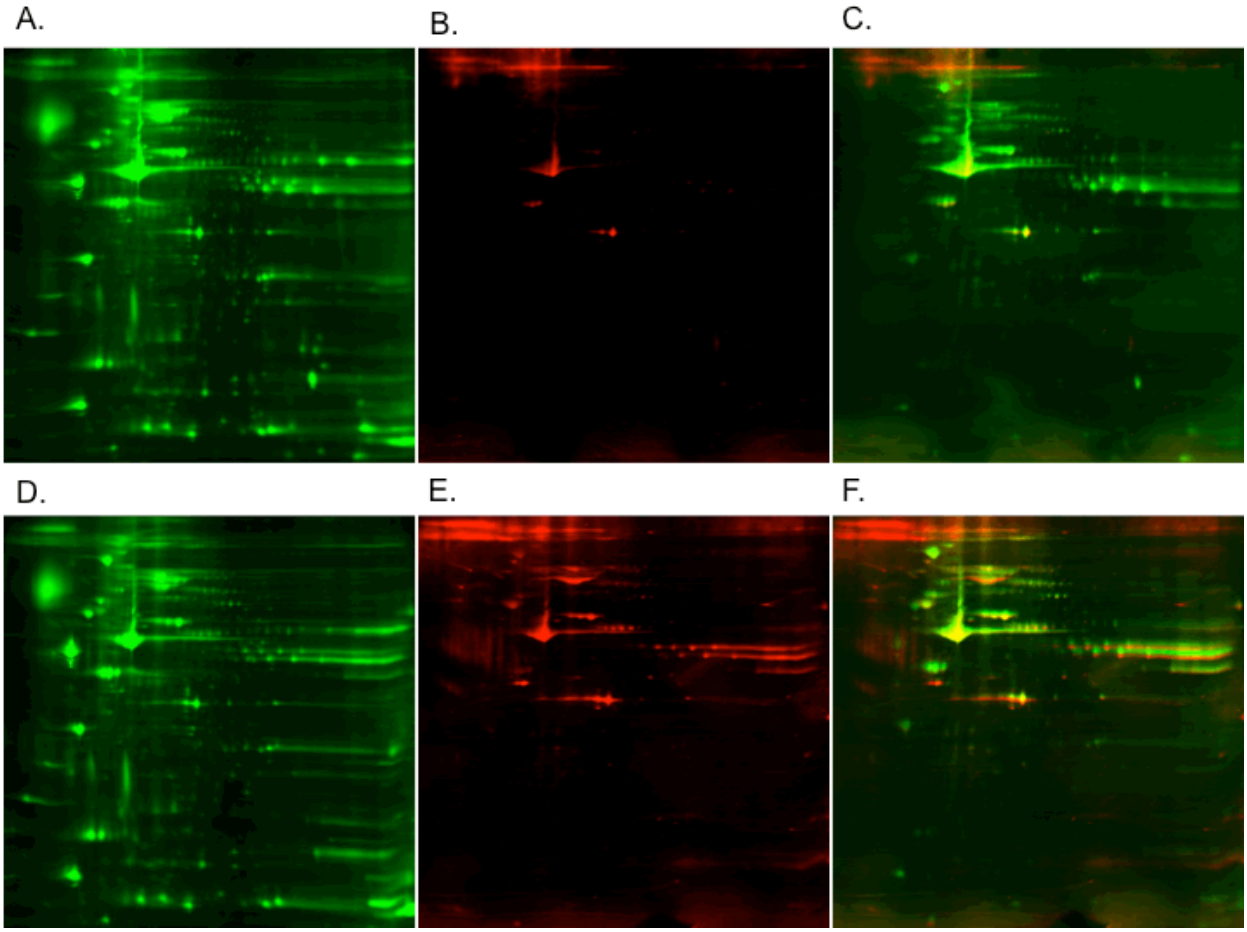

Figure 11. Identification of immunogenic *Elaeophora schneideri* proteins using 2D western blotting. A serological test to detect *E. schneideri*-specific antibodies in live hosts is an essential tool to assess the prevalence of *E. schneideri* in moose populations. To identify *E. schneideri* protein candidates for the development of this assay, we obtained a fresh adult nematode extracted from the carotid artery of an adult male moose in Wyoming. The nematode was washed in phosphate buffered saline and stored on dry ice. The whole, frozen nematode was submitted to Applied Biomics Inc. (Hayward, CA) for protein extraction, 2D poly-acrylamide gel electrophoresis, and western blotting. A,D). Dual 2D gels were run with the *E. schneideri* extracted protein. B) Western blot of 2D gel with *Elaeophora*-negative serum obtained from Alaskan moose. C) Combined image of A and B. E) Western blot with *Elaeophora*-positive serum obtained from infected Wyoming moose. F) Combined image of D and E. Fluorescently-labeled goat-anti deer IgG (Alpha Diagnostic Intl. Inc., San Antonio, TX) served as the secondary antibody.

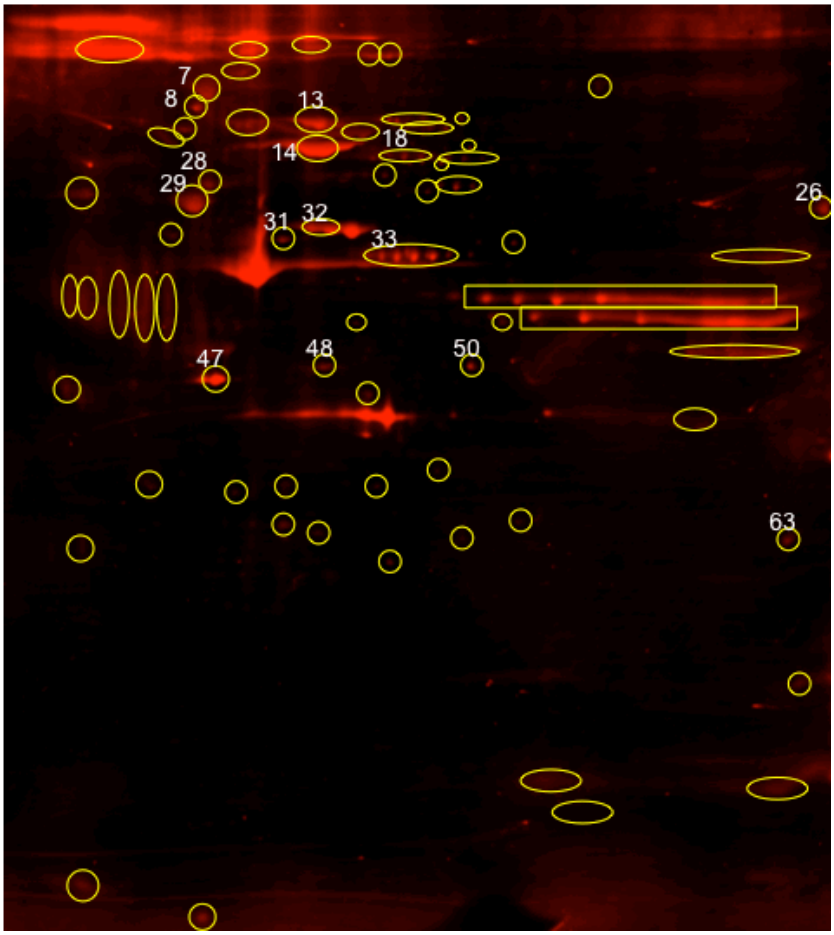

Figure 12. Identification of protein spots from 2D western blot that specifically reacted with the *E. schneideri*-positive moose serum. Protein spots are indicated by circles. Numbered spots were selected and purified for MALDI-TOF (using MS spectra) and MALDI-TOF/TOF (using MS/MS spectra) analysis.

Table 8. Results of the MALDI-TOF (using MS spectra) and MALDI-TOF/TOF (using MS/MS spectra) analysis of immunogenic *Elaeophora schneideri* proteins. Proteins were visualized using 2D western blot analysis (Fig. 11-12) and subjected to mass spectra analysis for sequence identification. MS and MS/MS data was analyzed using GPS Explorer software with the MASCOT search engine to identify the most closely matching proteins in the NCBI protein sequence database. Spot number corresponds to Fig. 12.

Spot number	Top Ranked Protein Name [Species]	NCBI Accession No.	Protein MW (kDa)	Protein PI	Peptide Count
7	Heat shock protein 90 [*Brugia pahangi*]	gi|3096951	82,525	5.0	28
8	PAN domain containing protein [*Brugia malayi*]	gi|170587418	81,228	4.9	24
13	Heat shock 70 kDa protein [*Brugia malayi*]	gi|170590876	73,833	5.7	25
14	Full=Intermediate filament protein B; Short=IF-B [*Ascaris suum*]	gi|124235	67,653	5.7	28
18	SPARC precursor [*Brugia malayi]*	gi|170586030	31,844	5.2	10
26	Elongation factor 1-alpha [*Brugia malayi*]	gi|170584161	56,035	9.2	14
28	Tubulin alpha chain - mouse [*Brugia malayi*]	gi|170588855	49,815	5.0	23
29	Beta-tubulin, identical [*Brugia malayi*]	gi|170583513	50,159	4.7	21
31	Actin 1 [*Brugia malayi*]	gi|170581695	41,777	5.3	15
32	Enolase [*Brugia malayi*]	gi|170582775	47,483	6.0	16
33	Actin 2 [*Brugia malayi*]	gi|170581703	41,778	5.3	11
47	Inorganic pyrophosphatase [*Brugia malayi*]	gi|170579482	38,880	5.9	6
48	SPARC precursor [*Brugia malayi*]	gi|170586030	31,844	5.2	10
50	Oxidoreductase, aldo/keto reductase family protein [*Brugia malayi*]	gi|170590818	34,897	7.1	6
63	Adenylate kinase isoenzyme 1 [*Brugia malayi*]	gi|170578003	44,494	9.1	9

Table 9. Peptides selected for synthesis and immunoblot testing. To assess the antigenic potential of the peptide sequences obtained from the MALDI-TOF/TOF analysis, the peptides were analyzed using the Kolaskar and Tongaonkar Antigenicity Scale (*117*), Bepipred Linear Epitope Prediction (*118*), and the Parker Hydrophobicity Scale (*119*). Peptides with epitopes predicted by Kolaskar and Tongaonkar (K&T) and/or Bepipred, a Parker hydrophobicity score (Parker Score) of ≤ 4.0 were then compared against the NCBI protein sequence database and ranked as either "non-conserved", "partially conserved" or "conserved". Peptides were considered non-conserved if they only closely aligned with proteins from filarial worms; peptides were categorized as non-conserved if they closely aligned with proteins from various nematode species, both filarid and non-filarids. Peptides were ranked as conserved if they closely aligned with proteins from multiple taxonomic groups. Based on the analysis, these 12 non-conserved peptides were selected and synthesized (GenScript, Piscataway, NJ). ES Pep. ID refers to the individual identification number assigned to the synthesized peptide. Spot number corresponds to Fig. 12.

ES Pep. ID	Top Ranked Protein Name [Species]	Peptide	K&T Antigenic Sequence	BepiPred Sequence	Parker Score	Spot Number
ES-P1	PAN domain containing protein [*Brugia malayi*]	LSGTPVVVSTAGHDLTCAQYCR	TPVVVSTAG	LSG_VVV_T_H	1.925	8
ES-P2	PAN domain containing protein [*Brugia malayi*]	NNIEPTTGAQRVCASFNFDGR	AQRVCASF	NNIEPTTGAQ	2.524	8
ES-P3	Heat shock protein 90 [Brugia pahangi]	FYTSASSEEMTSLKDYVSR	None	TSASSEEMT	3.336	7
ES-P4	PAN domain containing protein [*Brugia malayi*]	SVNYNYDTYLCELSTEDRR	DTYLCELS	NY_TEDRR	2.237	8
ES-P5	Heat shock protein 70 [*Brugia malayi*]	TSQTFTTYSDNQPGVLIQCVYEGER	QPGVLIQCV	TSQTFTTYSDNQP_ER	2.017	13
ES-P6	Tubulin alpha chain - mouse [*Brugia malayi*]	QSQIDDSFSTFFSETGTGR	None	QSQIDD_SETGTGR	2.434	28
ES-P7	PAN domain containing protein [*Brugia malayi*]	VGGEGDAVPFTIIENGCPR	None	VGGEGDAV_CPR	1.024	8

Table 9 Continued.

ES Pep. ID	Top Ranked Protein Name [Species]	Peptide	K&T Antigenic Sequence	BepiPred Sequence	Parker Score	Spot Number
ES-P8	Enolase [*Wuchereria bancrofti*]	QIALPLPAMNVIN GGSHAGNK	LPLPAMN	NGGSHAG NK	1.004	32
ES-P9	Heat shock protein 90 [*Brugia pahangi*]	YQALTEPAELET GKELYIK	None	ALTEPAEL ETG	2.454	7
ES-P10	Beta-tubulin [*Dirofilaria immitis*]	GGGFGQLFRPDN FVFGQSGAGNN WAK	FGQLFRP DNFVFGQ	GGG_F_S GAGNNWA K	1.433	29
ES-P11	Cytoplasmic intermediate filament protein [*Brugia malayi*]	INQWQHAIDDAQ SELEMLR	None	HAIDDAQS	2.557	14
ES-P12	SPARC precursor [*Brugia malayi*]	ELHNLEWEELIAE AESDDEKK	WEELIAE	AEAESDD EKK	2.394	18

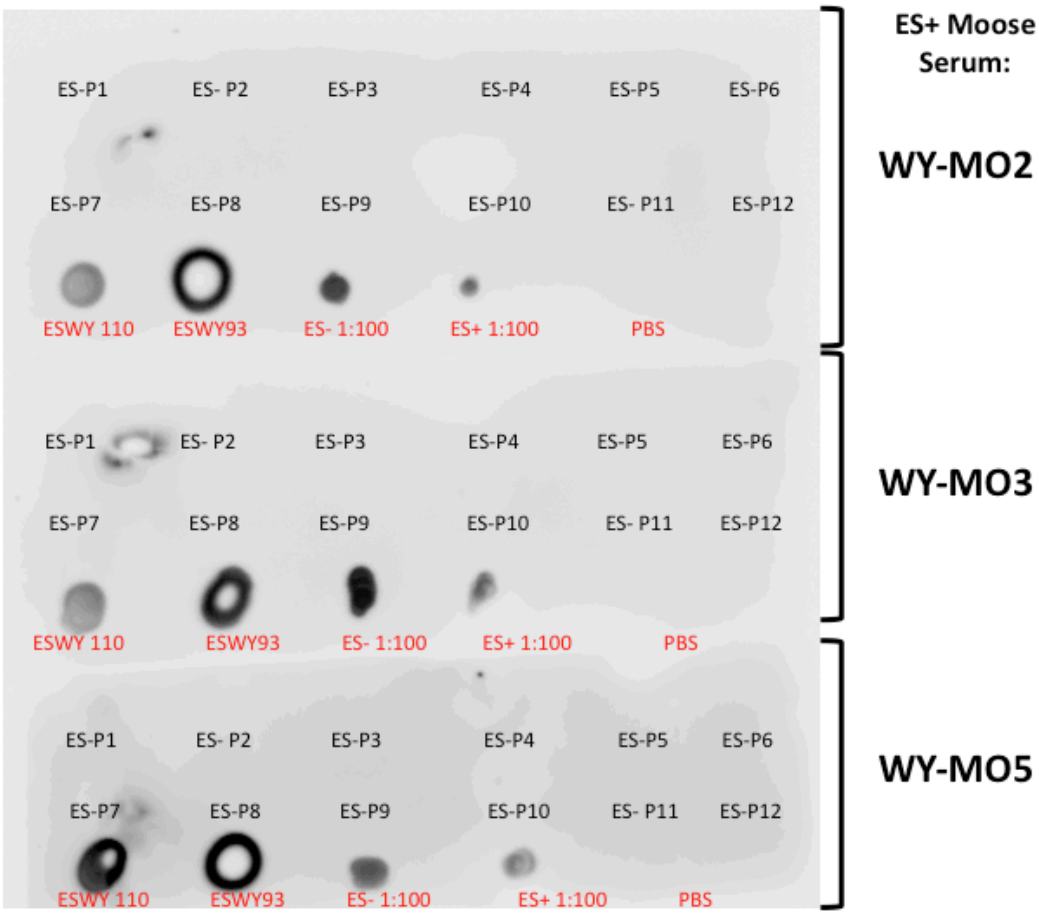

Figure 13. Immunoblot of *Elaeophora schneideri* peptides with serum from three *E. schneideri*-infected moose (WY-MO2, WY-MO3, WY-MO5). Peptides (1µl of 1mg/mL) were spot-inoculated onto a nitrocellulose membrane. Whole protein lysate (1µl of 500µg/mL) of two individual *E. schneideri* worms (ESWY 100, ESWY 93) served as positive protein controls (in red). *Elaeophora*-negative moose serum (ES-) and *Elaeophora*-positive moose serum (ES+) served as positive controls to ensure proper binding of the secondary antibody (HRP-chicken anti-deer IgG, 50µg/mL, Gallus Immunotech Inc., Cary, NC). PBS served as negative control. Blots were blocked with 5% non-fat milk in TBS-T and developed using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific, Waltham, MA). All peptides failed to bind the *Elaeophora*-positive moose serum.

VITA

Caroline Grunenwald was raised where the corn meets the deep woods in the small farming community of Merrill, Wisconsin. From 2004-2008 she attended the University of Dubuque in Dubuque, Iowa where she served as a student representative on the NCAA Student Athlete Advisory Committee, UD's Student Government Association, and as a member of the Alpha Chi National Honor Society. During her time at UD, she earned Iowa Intercollegiate Athletic Conference All-Conference and Academic All-Conference honors in cross country and track and field. Undergraduate research investigating the molecular control of anthocyanin production in orchids was conducted under the careful guidance of Dr. Rasika Mudilage-Jayawickrama. In recognition of her research efforts and academic achievement, Ms. Grunenwald was awarded the Robert M. Miller Excellence in Biology Award from the Department of Natural and Applied Sciences at UD. In May of 2008 she graduated from the University of Dubuque Summa Cum Laude, receiving her Bachelors of Science degree in Biology and Environmental Science (double major). From 2008-2011, Ms. Grunenwald attended Idaho State University where she was mentored by Dr. Gene Scalarone. During her time at ISU she studied the antigenic properties of the human fungal pathogen *Blastomyces dermatitidis*. In 2010, by the urging of Dr. Scalarone, Ms. Grunenwald served as a summer research intern studying the disease ecology of tick-borne relapsing fever in the laboratory of Dr. Tom Schwann at the NIH National Institute of Allergy and Infectious Disease's Rock Mountain Laboratories in Hamilton, Montana. In 2011, she received her Masters of Science degree in Microbiology from ISU and then went on to pursue a PhD at the University of Tennessee. Since arriving at UT, Ms. Grunenwald has received multiple awards including the David C. White Memorial Travel Award, the Graduate Teaching Assistant Award for Outstanding Teaching, the John C. New, Jr., Award for Excellence in Public Health or Epidemiological Research, and the Graduate Student Award of Merit for Excellence in Agricultural Research. Ms. Grunenwald was also fortunate to be awarded a research grant from Montana Fish, Wildlife, and Parks, as well as the Microbiology Across Campuses Educational and Research Venture Grant. Currently she plans to continue her career in scientific research with a focus on host-microbial interactions, microbial genomics and evolution, and disease ecology.