ECOLOGICAL INVESTIGATION OF A NEW HOST-PARASITE RELATIONSHIP: *PARELAPHOSTRONGYLUS ODOCOILEI* IN THINHORN SHEEP (*OVIS DALLI*)

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By

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ABSTRACT

Discovery of a new host-parasite relationship, *Parelaphostrongylus odocoilei* in Dall's sheep (*Ovis dalli dalli*) in the Canadian North, prompted the first investigation of the geographic distribution, pathogenesis, ecology and epidemiology of this parasite, as well as the related protostrongylid *Protostrongylus stilesi*, at Subarctic latitudes (60-65ºN). All protostrongylid parasites have an indirect life-cycle, where first-stage larvae are shed in the feces of a mammalian definitive host, penetrate the foot of a gastropod intermediate host, and develop to infective third-stage larvae.

Protostrongylid larvae were recovered from over 2000 fecal samples from thinhorn sheep (*Ovis dalli*) and other hosts for *P. odocoilei* and *P. stilesi* across northwestern North America (38-69 ºN). Through novel application of molecular techniques to identify morphologically indistinguishable first-stage larvae, new records for *P. odocoilei* were established at 20 locations. This provided insight into the historical origins and biogeography of this new host-parasite relationship, and greatly expanded the known geographic range of both protostrongylids.

Clinical effects, including a neurological syndrome, were described in five thinhorn sheep experimentally infected with *P. odocoilei*. Neural and respiratory pathology in these five sheep were compared with over 50 wild Dall's sheep from a population naturally infected with *P. odocoilei* and *P. stilesi*. In the end stages, diffuse verminous interstitial pneumonia associated with *P. odocoilei* led to respiratory failure, and may have acted as a predisposing factor for bacterial pneumonia, which caused sporadic mortalities in this wild population.

At Subarctic latitudes, seasonal patterns in host and parasite availability, including larval shedding by Dall's sheep and larval development in experimentally infected gastropods, suggested that lambs become infected with *P. odocoilei* in a narrow seasonal window in their first fall on winter range. In combination with laboratory experiments, a degree day model for

temperature-dependent larval development was developed, validated, and applied to describe and predict the effects of climate warming on protostrongylid parasites of thinhorn sheep in northern North America. In a future of climate warming, the narrow seasonal window for parasite development and transmission would be significantly extended, leading to amplification of populations of *P. odocoilei* and *P. stilesi* in endemic regions, and possibly range expansion of *P. odocoilei*. This may have consequences for the health of thinhorn sheep, as well as other wildlife that are important resources in the Canadian North.

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Dedication

To my family, especially my husband, Aaron Genest, for unflagging support and a life-saving sense of humor.

"Never about imposing a thesis, only about seeking truth."
Ken Alexander, The Walrus, May 2005

Written in memory of journalist Bill Cameron, but equally applicable to my grandfather, Bill Jenkins, and my friend, Bill Sarjeant, who told stories, many of which were true, and many of which should have been.

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BAL	Bronchoalveolar lavage
ca	Circa, approximately
cf	Compare with (similar in appearance to)
CNS	Central nervous system
CSF	Cerebrospinal fluid
CT	Computed tomography
D1-D6	Development trials
DD	Degree days, amount of warming above To
dpi	Days post infection
DRWED	Department of Resources, Wildlife, and Economic Development, Government of the NT (now Environment and Natural Resources)
DS	Dall's sheep (*Ovis dalli dalli*)
DSL	Dorsal-spined larvae
DSLPG	Dorsal-spined larvae per gram of feces
E1-E3	Emergence trials
Exp.	Experiment
IHC	Immunohistochemistry
IM	Intramuscular
ITS2	Second internal transcribed spacer
L1	First-stage larvae
L2	Second-stage larvae
L3	Third-stage larvae
LPG	Larvae per gram of feces
MRI	Magnetic resonance imaging
NT	Northwest Territories
PCR	Polymerase chain reaction
PPP	Pre-patent period
PrLPG	*Protostrongylus* spp. larvae per gram of feces
SS	Stone's sheep (*Ovis dalli stonei*)
To	Threshold temperature for larval development
TS4-7	Thinhorn sheep hybrids (*O. d. stonei* X *O. d. dalli*)
WCVM	Western College of Veterinary Medicine

CHAPTER 1

1 INTRODUCTION

1.1 Background and discovery

In 1998, as part of a survey for the newly discovered lungworm *Umingmakstrongylus pallikuukensis* of muskoxen (*Ovibos moschatus*), new host and geographic records were established for *Parelaphostrongylus odocoilei* in Dall's sheep (*Ovis dalli dalli*) in the Mackenzie Mountains, Northwest Territories (NT), Canada (Kutz et al., 2001c). This muscle-dwelling nematode is a member of the Subfamily Elaphostrongylinae (Superfamily Metastrongyloidea, Family Protostrongylidae), which is a monophyletic group comprised of two genera, *Parelaphostrongylus* (of North American, or Nearctic origin), and *Elaphostrongylus* (of Eurasian, or Palearctic, origin) (Carreno and Lankester, 1994; Lankester, 2001; Carreno and Nadler, 2003). Unlike other metastrongyloids, which are primarily "lungworms", adult elaphostrongylines dwell in the tissues of the mammalian host (central nervous system or musculature), with eggs and larvae passing through the lungs (Anderson, 2000). This group of pathogenic nematodes of wild and domestic ungulates is of veterinary and socioeconomic importance, and has been the subject of extensive research (Lankester, 2001).

The discovery of *P. odocoilei* in Dall's sheep, a new genus of host for this parasite, was serendipitous. Cervids are considered the basal hosts for the Elaphostrongylinae, with secondary colonization of caprines and lagomorphs by other protostrongylids in the Subfamilies Muellerinae and Protostrongylinae (Carreno and Hoberg, 1999; Carreno and Nadler, 2003). For the neurotropic species in the Elaphostrongylinae, domestic sheep and goats are considered

"atypical" hosts, in which infections rarely become patent and frequently result in severe neurological disease (Mayhew et al., 1976; Pybus et al., 1996; Handeland et al., 2000b; Handeland et al., 2001). The discovery of *P. odocoilei* in the Mackenzie Mountains was also surprising because it extended the known range of *P. odocoilei* 1000 km further north (Fig. 1.1). Combined with recent reports of two other protostrongylids in the Canadian North, the muellerine *U. pallikuukensis* in muskoxen (Hoberg et al., 1995), and the protostrongyline *Protostrongylus stilesi* in Dall's sheep and muskoxen (Kutz et al., 2001c; Hoberg et al., 2002), these discoveries highlighted the lack of knowledge of parasite biodiversity in northern ecosystems, even in wildlife important for subsistence and sport hunting in northern communities.

These discoveries also provided the basis for comparison between protostrongylids in the same host (*P. odocoilei* and *P. stilesi* in Dall's sheep), and between related protostrongylids (*P. odocoilei* and *U. pallikuukensis*) in different hosts in the North: the Elaphostrongylinae and the Muellerinae are sister groups (Carreno and Hoberg, 1999). Northern systems are attractive for study because they are "simple" compared to temperate and tropical systems, and northern wildlife have had minimal contact with domestic livestock and their parasites (Weider and Hobaek, 2000; Hoberg et al., 2001). As such, changes, such as colonization of new hosts by parasites, can be detected and interpreted more easily than in more complex systems (Hoberg et al., 2002). Northern environments are also "younger", with more recent evolutionary histories. The formative events in northern North America (such as vicariance due to glaciation, interspersed with periods of expansion in interglacial periods and with the formation of the Bering land bridge) are well characterized, facilitating interpretation of host and parasite biogeography (Hoberg et al., 1999; Weider and Hobaek, 2000). Finally, northern ecosystems are

"fragile", sensitive to disturbance, and serve as records of the effects of historical climate change and as sentinels for current and future climate change (Hoberg et al., 2001; Kutz et al., 2005; Hoberg, 2005).

The discovery of *P. odocoilei* at northern latitudes also prompted exploration of the ecological factors that determine the distribution of parasites and hosts, including life history adaptations to northern environments. All protostrongylid parasites have indirect life cycles, in which first-stage larvae (L1) are shed in the feces of the mammalian definitive host, invade a gastropod intermediate host, and develop to third-stage larvae (L3) infective for another definitive host (Fig. 1.2) (Anderson, 2000). As a result, transmission of protostrongylid parasites requires spatial and temporal overlap of definitive hosts, intermediate hosts, and parasite life stages in a suitable environment. These complex relationships favor an ecological approach to investigation, one that considers the influence of abiotic and biotic factors on parasite distribution and abundance. Knowledge of the ecosystem is essential to understanding the host-parasite relationship, and likewise, knowledge of parasite distribution and abundance provides information about the ecosystem (i.e. the presence of suitable hosts and climatic conditions).

The following reviews the state of the knowledge for elaphostrongylines and *P. odocoilei* at the time of discovery in Dall's sheep, and defines needs for further investigation.

1.2 Host range and geographic distribution

Of the elaphostrongylines, *P. odocoilei*, *P. andersoni*, and *P. tenuis* are endemic to North America, and *E. rangiferi* has been introduced with reindeer to the island of Newfoundland (Lankester and Fong, 1989). Prior to its discovery in Dall's sheep, *P. odocoilei* had been considered primarily a parasite of cervids in west-central North America (Fig. 1.1), including black-tailed deer (*Odocoileus hemionus columbianus*) from the central Coast mountain range of California (CA), USA (Hobmaier and Hobmaier, 1934), and from Vancouver Island, British

Columbia (BC) (Pybus et al., 1984); California mule deer (*O. h. californicus)* from the western Sierra Nevada mountains, CA (Brunetti, 1969); and mule deer (*O. h. hemionus*) from Jasper National Park, Alberta (AB) (Platt and Samuel, 1978a) and from the Okanagan Valley, BC (Lankester, 2001). *Parelaphostrongylus odocoilei* had also been confirmed in woodland caribou (*Rangifer tarandus caribou*) from west-central AB (Gray and Samuel, 1986). Experimentally, *P. odocoilei* established patent infections in moose, but did not establish well in white-tailed deer (Pybus and Samuel, 1980; Pybus and Samuel, 1984a). Domestic goats and sheep were refractory to infection (Pybus and Samuel, 1984c; S. Kutz, unpubl. data). Surprisingly, wild mountain goats (*Oreamnos americanus*) from AB, central BC, and Washington, USA (Pybus et al., 1984), and Dall's sheep in the NT were seemingly suitable hosts for this "cervid" parasite. The discovery of *P. odocoilei* in Dall's sheep in the NT raised the possibility that this parasite was established in other populations of Dall's sheep throughout North America, and possibly in Stone's sheep (*Ovis dalli stonei*), the other subspecies of thinhorn sheep (*Ovis dalli*).

All elaphostrongylines, and some other protostrongylid genera present in North America (*Muellerius*, *Umingmakstrongylus*, and *Varestrongylus*), produce morphologically similar dorsal-spined L1 (DSL) (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). As a consequence, and because concurrent infections are common, identification based on morphology of L1 shed in feces is rarely possible (Pybus and Shave, 1984; Gray et al., 1985b; Anderson, 2000). Definitive identification of protostrongylids has traditionally relied on morphometrics of tails of adult male nematodes recovered from tissues at necropsy. Largely because of such diagnostic challenges, knowledge of the geographic distribution and host range of most protostrongylids (including *P. odocoilei*) is likely incomplete. With the advent of molecular techniques in parasitology (Gasser et al., 1993; Anderson et al., 1998; Hoberg et al., 1999; Monis et al., 2002),

species-level identification of parasite life stages in feces of definitive hosts became possible, but has not been broadly applied to characterize the distribution of protostrongylid nematodes.

1.3 Development in definitive hosts

The route of migration and target organ within the definitive host varies among the elaphostrongylines, with the ancestral pattern (plesiomorphic condition) unknown, but thought to involve the CNS (Platt, 1978; Carreno and Lankester, 1994). Adults of *Elaphostrongylus* spp. (*E. rangiferi*, *E. cervi*, and *E. alces*) travel through some aspect of the CNS (epidural or subdural spaces) before establishing in the skeletal musculature (Hemmingsen et al., 1993; Anderson, 2000; Handeland and Gibbons, 2001). Adults of *P. tenuis*, the meningeal worm, migrate through the neural parenchyma and then into the subdural space and cranial venous sinuses, and do not invade the muscles (Anderson, 1963; Anderson, 1968). Adults of *P. odocoilei* and *P. andersoni* were considered to be strictly muscle-dwelling, with a pattern of migration through the central nervous system (CNS) having either been secondarily lost or never acquired (Platt, 1984; Anderson, 2000). For all elaphostrongylines, adult females deposit eggs in the vasculature, which become trapped in the capillary bed of the lungs, embryonate, and hatch into L1, which are coughed up, swallowed, and passed in feces (Anderson, 2000).

Pathology associated with adult nematodes in the definitive host varies depending on localization and host species. Neurotropic elaphostrongylines may cause neurological deficits and even mortality, especially in atypical hosts (Anderson, 1972; Pybus et al., 1996). In "typical" (basal or ancestral) hosts, neurotropic nematodes often cause little inflammatory reaction or damage to the neural parenchyma: for example, *P. tenuis* in white-tailed deer (Anderson, 1972), or *E. cervi* in red deer (Mason, 1995). Muscle-dwelling protostrongylids, such as *P. odocoilei* and *P. andersoni*, cause localized myositis and hemorrhage (Nettles and Prestwood, 1976; Pybus and Samuel, 1984b), but have not been linked to recognizable disease

syndromes and were not thought to undergo a neural migration (Platt and Samuel, 1978b; Pybus and Samuel, 1981; Anderson, 2000; Lankester, 2001). As a result, detailed examination of the CNS in the pre-patent period, necessary to demonstrate the CNS-muscle migration pattern of *E. rangiferi* (Hemmingsen et al., 1993), has not been accomplished for hosts infected with the "muscleworms", *P. odocoilei* and *P. andersoni*. As well, clinical monitoring of cervids experimentally infected with *P. odocoilei* and *P. andersoni* has not included specific tests of neurological function. Clinical signs (such as weakness and reluctance to move) have been dismissed as locomotor abnormalities associated with muscle pathology, and exercise intolerance associated with respiratory pathology (Nettles and Prestwood, 1976; Pybus and Samuel, 1984b).

In the lungs, eggs and larvae of elaphostrongylines are associated with granulomatous inflammation, and the resulting verminous interstitial pneumonia is often a primary lesion, even for the neurotropic elaphostrongylines (Nettles and Prestwood, 1976; Sutherland, 1976; Stockdale, 1976; Pybus and Samuel, 1981; Watson, 1983; Pybus and Samuel, 1984b; Handeland, 1994; Lankester, 2001). *Parelaphostrongylus odocoilei* has been linked to severe, even fatal, respiratory pathology in naturally-infected black-tailed deer (Hobmaier and Hobmaier, 1934; Brunetti, 1969) and experimentally infected mule deer (Platt and Samuel, 1978b; Pybus and Samuel, 1984b). Most recently, in Dall's sheep of the Mackenzie Mountains, eggs and larvae of *P. odocoilei* and all life stages of the lungworm *P. stilesi* caused concurrent pulmonary damage (Kutz et al., 2001c). *Protostrongylus stilesi* was once thought to cause fatal pneumonia in bighorn sheep (*Ovis canadensis*) (Forrester, 1971; Spraker et al., 1984), but is now relegated to the role of a predisposing factor in the multifactorial pneumonia complex that has decimated bighorn sheep populations in North America (Samson et al., 1987; Monello et al., 2001). The

role of protostrongylid parasites in the health of Dall's sheep populations, and the possibility of a fatal pneumonia complex similar to that of bighorn sheep, has not been investigated.

1.4 Development in intermediate hosts

All protostrongylids have an indirect life cycle, where larvae develop to infective L3 in a gastropod intermediate host (Fig. 1.2). In North America, terrestrial gastropods in Suborders Heterurethra (Family Succineidae) and Sigmurethra (Families Arionidae, Endodontidae, Haplotrematidae, Helicidae, Helminthoglyptidae, Limacidae, Polygyridae, and Zonitidae) are suitable intermediate hosts for many of the Elaphostrongylinae. In contrast, larvae of elaphostrongylines do not establish or develop in gastropods of the Suborder Orthurethra (Pupillidae and Valloniidae), which are hosts for *Protostrongylus* spp. (Hobmaier and Hobmaier, 1934; Pillmore, 1955; Lankester and Anderson, 1968; Platt, 1978; Skorping and Halvorsen, 1980; Samuel et al., 1985; Upshall et al., 1986; Whitlaw et al., 1996; Lankester and Peterson, 1996; Anderson, 2000). Gastropod species potentially important for transmission of *P. odocoilei* have been identified in a temperate region (Platt and Samuel, 1984; Samuel et al., 1985), but not in the Subarctic, where gastropod species in general have not been well described.

Determination of gastropod species suitable as intermediate hosts is difficult, because morphological identification of both gastropod and larvae requires considerable expertise. This is further complicated by 1) overlapping size ranges and intraspecific variation of L3 tail morphology (Ballantyne and Samuel, 1984; Kralka and Samuel, 1984b; Gray et al., 1985b; Lankester and Hauta, 1989); 2) multiple species of protostrongylids endemic to a region, often using the same gastropod species as intermediate hosts (Gray et al., 1985b; Robb and Samuel, 1990); and 3) low prevalence of protostrongylid infection in gastropods (generally <10%), even in areas heavily used by infected definitive hosts (Lankester and Peterson, 1996; Lankester, 2001). Molecular techniques hold great promise for identification of protostrongylid L3 from

gastropods (Gajadhar et al., 2000), but do not eliminate the need for large sample sizes because of low prevalence in gastropods. As an alternative to identification of L3 in naturally infected gastropods, experimental infection of native gastropods provides basic information about host suitability (larval establishment, development rate, and survival) and has implications for, but does not demonstrate a role in, natural transmission of protostrongylids (Lankester and Anderson, 1968; Urban, 1980; Platt and Samuel, 1984). Under natural conditions, transmission also depends on ecological factors, such as intermediate host distribution, abundance, microhabitat selection, and behavior (Urban, 1980; Halvorsen, 1986; McCoy and Nudds, 1997; Anderson, 2000).

In the laboratory, the polygyrid snail *Triodopsis multilineata* is commonly used as an intermediate host for *P. odocoilei* because it is easily maintained (Platt and Samuel, 1978b; Pybus and Samuel, 1984c; Gray et al., 1985a), but low levels of larval establishment suggest that this species is not a good host for *P. odocoilei* (Shostak and Samuel, 1984; Lankester, 2001). In contrast, *P. odocoilei* has high rates of larval establishment and rapid larval development in the slug *Deroceras laeve* (Kutz et al., 2001c). *Deroceras laeve* is easily maintained in the laboratory, and is a native gastropod species important in natural transmission of elaphostrongylines, including *P. odocoilei* (Lankester and Anderson, 1968; Samuel *et al.*, 1985; Ball *et al.*, 2001). As well, slugs do not undergo aestivation, which is common in wild-caught or otherwise stressed snails in captivity. Larval development may be retarded in aestivating or otherwise unsuitable intermediate hosts, confounding efforts to describe and compare development rates among different gastropod species (Lankester and Anderson, 1968; Solomon et al., 1996; Kutz et al., 2000; Kutz et al., 2001b).

Third-stage larvae of some species of protostrongylids develop in and emerge from the gastropod, where they may be ingested directly from the vegetation (Boev, 1975; Kralka and Samuel, 1984c; Cabaret and Pandey, 1986; Kutz et al., 2000). Emerged protostrongylid L3, like L3 of trichostrongyles (Saunders et al., 2001), may survive in the environment for weeks to months, and exhibit behaviors that increase probability of transmission, such as selectively ascending the main food plant of their hosts. Although most definitive hosts are thought to become infected by accidental ingestion of gastropods containing protostrongylid L3 (Anderson, 2000) (Fig 1.2), ingestion of emerged L3 may play a role in transmission of protostrongylids, especially in northern regions where gastropods are seasonally unavailable for much of the year (Kutz et al., 2000). Larvae of *P. odocoilei* emerged from experimentally infected *D. laeve* in the laboratory, the first report for any elaphostrongyline (Kutz et al., 2001c). The proportion and survival of emerged larvae has not been determined for *P. odocoilei* in the laboratory or in the field.

1.5 Ecology and epidemiology

Transmission of parasites of ungulates at Subarctic and Arctic latitudes is strongly influenced by the unique history and conditions (both abiotic and biotic) of these regions (Nielsen and Neiland, 1975; Halvorsen et al., 1999; Kutz, 2000; Irvine et al., 2000; Hoberg, 2005). Spatial and temporal patterns in transmission likely vary across the broad geographic distribution of parasites such as *P. odocoilei* and *P. stilesi*, which are distributed from approximately 35 to 65 ºN in North America. As well, seasonal and altitudinal migrations of definitive hosts strongly influence patterns of parasite transmission, and likely vary among different host species (Morgan et al., 2004; Hoberg, 2005). For example, hosts in grassland or woodland areas in temperate regions may become infected with protostrongylid parasites throughout the entire snow-free season (Slomke et al., 1995; Lankester and Peterson, 1996). For

hosts with a seasonal migration, such as mountain-dwelling ungulates like bighorn sheep and some populations of mule deer, infection is thought to occur primarily in fall and spring on the winter range (Samuel et al., 1985; Robb and Samuel, 1990). The epidemiology and ecology of protostrongylids in Dall's sheep, which inhabit Subarctic and Arctic regions and have seasonal migrations, have not been described.

Temporal patterns of parasite transmission and spatial patterns in the distribution of both parasites and hosts are strongly influenced by climate (Levine, 1963; Martin, 2001). Climate change has already influenced habitat, distribution, and phenology for animals across several taxonomic classes (Dunn and Winkler, 1999; Inouye et al., 2000; Lenart et al., 2002; Reale et al., 2003; Parmesan and Yohe, 2003). Climate change, especially of the magnitude projected at high latitudes and elevations (IPCC, 2001; McBean, 2005), will also affect parasites (Lindgren et al., 2000; Hoberg et al., 2001; Epstein, 2001; Harvell et al., 2002). Climate warming will accelerate rates of temperature-dependent development of parasitic larvae in the environment and in poikilothermic intermediate hosts, possibly causing shifts in parasite distribution and phenology (Dobson and Carper, 1992; Kovats et al., 2001; Dobson et al., 2003). Based on work on *U. pallikuukensis*, climate change has already altered the pattern of transmission for this northern protostrongylid, and may have contributed to a decline in an infected population of muskoxen (Kutz et al., 2002; Kutz et al., 2005). Similarly, outbreaks of clinical cerebrospinal elaphostrongylosis (due to *E. rangiferi*) in reindeer in northern Norway have been linked to warmer summer temperatures (Handeland and Slettbakk, 1994). It is likely that climate change will have similar effects on other northern protostrongylids, including *P. odocoilei* and *P. stilesi* in thinhorn sheep, whose distribution overlaps the two districts (i.e., North British Columbia

Mountains/Yukon and Mackenzie District) currently experiencing the strongest warming trends in Canada (http://www.msc-smc.ec.gc.ca/ccrm/bulletin/ accessed April 2005).

Predicting and quantifying the effects of climate change on protostrongylid parasites requires a mathematical model based on the relationship between temperature and rates of development of protostrongylid larvae in the poikilothermic intermediate host. This relationship can be described using a degree day model, which assumes that a fixed amount of heating accumulated above a critical threshold temperature is necessary for development (Campbell et al., 1974; Saunders et al., 2002; Harvell et al., 2002). Degree day models have been developed for several protostrongylids, including *E. rangiferi*, *P. stilesi*, and *U. pallikuukensis* (Halvorsen and Skorping, 1982; Samson and Holmes, 1985; Kutz et al., 2001b), but have only been validated in the field for *U. pallikuukensis* (Kutz *et al.*, 2002). Such models are powerful tools for comparing development of protostrongylid species, isolating temperature as a factor potentially limiting parasite distribution and abundance, and describing and predicting temporal and spatial patterns in larval development and transmission.

1.6 Rationale

Discovery of a new host-parasite relationship, *P. odocoilei* in Dall's sheep, launched an investigation that spanned several disciplines and involved a broad network of collaborators across North America. This comprehensive approach was in part driven by concerns among outfitters, wildlife managers, and veterinarians about the effects of *P. odocoilei* in thinhorn sheep, a valuable game species for First Nations, northern residents, and non-resident sport hunters (Bowyer and Leslie, 1992; Veitch et al., 1998; Crapo, 2000; Nadasdy, 2003). Based on what is known about *P. odocoilei* and related protostrongylids in other hosts, this parasite has the potential to cause respiratory disease in thinhorn sheep. Respiratory disease is one of the most important factors limiting establishment and maintenance of bighorn sheep populations in North

America, but has not been reported as a cause of mortality in thinhorn sheep. The reasons for this difference in susceptibility and/or virulence are unknown, and investigation of the role of *P. odocoilei* and other respiratory pathogens in the health of thinhorn sheep provided a valuable opportunity to isolate differences in predisposing factors.

This investigation was also motivated by theoretical interests of classical and ecological parasitology. Discovery of a broader host and geographic range for *P. odocoilei* raised many questions: What other wildlife species are suitable hosts for *P. odocoilei*? Where else is this parasite established in northwestern North America? When and how did this parasite establish in thinhorn sheep? What can parasite distribution and genetics tell us about the historical biogeography and genetic structure of hosts? What are the drivers and barriers for range expansion and host specificity of parasites?

Investigating the pathogenesis of *P. odocoilei* in thinhorn sheep provided the opportunity to describe the life cycle of this elaphostrongyline in a new host species, and begin to address some fundamental questions: Does parasite development (i.e., length of the pre-patent period, migration route, and patterns of larval shedding) differ among host species? How do patterns of development differ among the elaphostrongylines, and what does this tell us about the relationships within this group of nematodes? What are the subclinical and clinical effects of parasitism in wildlife populations? And, how do we monitor the effects of parasitism and disease in wildlife populations in remote, inaccessible regions of the Canadian North?

Finally, this discovery prompted investigation of the epidemiology (prevalence, intensity, and seasonal patterns of development and transmission) and ecology (relationships among definitive hosts, intermediate hosts, parasite life stages, and the environment) of protostrongylids *(P. odocoilei* and *P. stilesi)* in thinhorn sheep near the northern distributional limits for these

parasites. By comparing patterns in development and transmission of these protostrongylid parasites in northern regions to those in temperate regions, and to a related protostrongylid restricted to the North, we explored the following questions: How are parasites, especially protostrongylid parasites with complex life cycles, maintained at latitudes north of 60 ºN? Which aspects of life history, including parasite fecundity, life span, and alternative "infection strategies" such as larval emergence (as per Fenton and Hudson, 2002), are key to success in these regions? Which abiotic and biotic factors limit transmission? And, of great importance in the North, what are the predicted impacts of climate change on the epidemiology and ecology of parasite transmission, and ultimately on biodiversity and wildlife health?

1.7 Specific objectives

1) To review the state of the knowledge about *P. odocoilei* and related parasites in their respective hosts, and define areas where further investigation is needed (Chapter 1).

2) To more fully describe the host range and geographic distribution of *P. odocoilei* in northwestern North America, using novel application of molecular techniques (Chapter 2).

3) To describe development, pathogenesis, and pathology of *P. odocoilei* in naturally and experimentally infected thinhorn sheep, using local expertise and specialized monitoring techniques in veterinary and human medicine (Chapters 3 and 4).

4) To describe the ecology and epidemiology of protostrongylids in thinhorn sheep and gastropods in northern Canada, and to describe and predict the effects of climate warming by developing, validating, and applying a degree day model for parasite development in the laboratory (Chapter 5) and field (Chapter 6).

5) To explore larger questions in the field of ecological parasitology, using a multidisciplinary approach and drawing on molecular and traditional parasitology, veterinary and human medicine, and disease ecology and epidemiology (Chapter 7).

Figure 1.1: Proposed distribution of *Parelaphostrongylus odocoilei* in cervids and mountain goats from Lankester (2001) (shaded area), and initial discovery in Dall's sheep in 1998 (*).

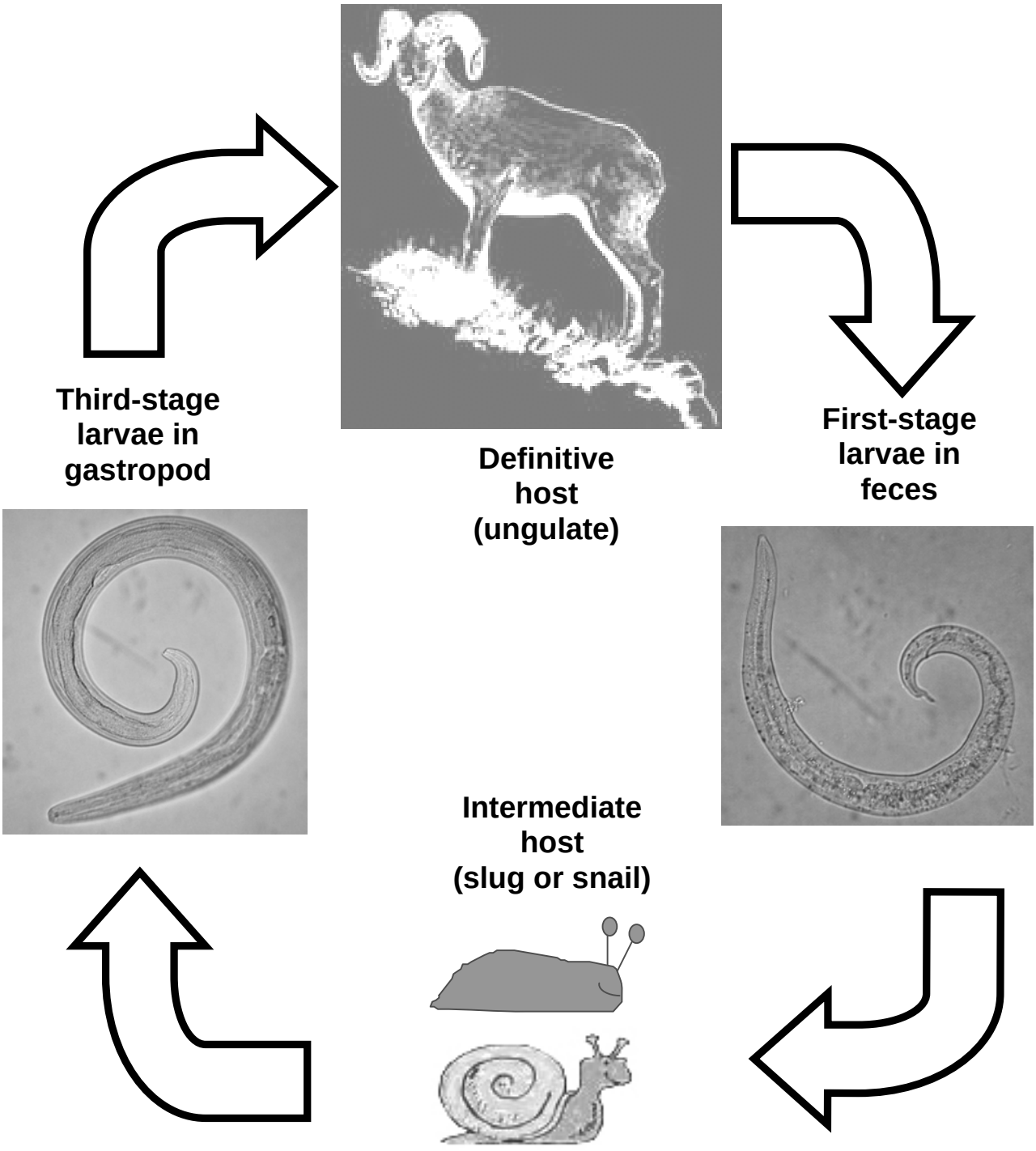

Figure 1.2: Life cycle of *Parelaphostrongylus odocoilei* and other protostrongylids.

Note that sheep can become infected by consuming gastropods containing infective third-stage larvae, or possibly larvae that have emerged from gastropods and are free on vegetation. Larvae not to scale. Image of sheep from Alaska Fish and Game, and slug image from S. Kutz.

CHAPTER 2

2 GEOGRAPHIC DISTRIBUTION AND MOLECULAR IDENTIFICATION

The manuscript[1] comprising this chapter describes how we applied molecular techniques to identify species of nematode larvae shed in feces of the definitive host. This allowed us to comprehensively describe the geographic distribution of *Parelaphostrongylus odocoilei*, an important protostrongylid parasite of wild ungulates in North America. In turn, this provided the foundation for describing factors limiting the geographic distribution of protostrongylid parasites (Chapter 6), and understanding the historical origins of these host-parasite assemblages (Chapter 7). I established an extensive network of collaborators, and coordinated the collection and analyses of over 2,000 fecal samples from wild ungulates at 29 locations in western North America. With guidance and assistance from co-authors and collaborators, I was involved in sample collection, larval recovery, DNA extraction, PCR, and sequencing. I was ultimately responsible for sample tracking and analyses of data, and, as corresponding author, researching and writing the publication with input from co-authors.

[1] Jenkins, E.J., G.D. Appleyard, E.P. Hoberg, B.M. Rosenthal, S.J. Kutz, A.M. Veitch, H.M. Schwantje, B.T. Elkin, and L. Polley. 2005. Geographic distribution of the muscle-dwelling nematode *Parelaphostrongylus odocoilei* in North America, using molecular identification of first-stage larvae. Journal of Parasitology 91: 574-584.
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2.1 Abstract

Molecular identification of dorsal-spined larvae (DSL) from fecal samples indicates that the protostrongylid parasite *Parelaphostrongylus odocoilei* occupies a broader geographic range in western North America than previously reported. We analyzed 2,124 fecal samples at 29 locations from thinhorn sheep (*Ovis dalli dalli* and *O. d. stonei*), bighorn sheep (*Ovis canadensis canadensis* and *O. c. californiana*), mountain goats (*Oreamnos americanus*), woodland caribou (*Rangifer tarandus caribou*), mule deer (*Odocoileus hemionus hemionus*), and black-tailed deer (*O. h. columbianus*). DSL were recovered from populations of thinhorn sheep south, but not north, of the Arctic Circle, and were not recovered from any of the bighorn sheep populations examined. In total, DSL were recovered from 20 locations in Alaska, Yukon Territory, Northwest Territories, British Columbia, Alberta, and California. DSL were identified as *P. odocoilei* by comparing sequences of the second internal transcribed spacer (ITS2) region of ribosomal RNA among 9 protostrongylid species validated by adult comparative morphology. ITS2 sequences were markedly different among *Parelaphostrongylus* and other protostrongylid genera. Fewer fixed differences served as diagnostic markers for the three species of *Parelaphostrongylus*. ITS2 sequences (n=60) of *P. odocoilei* were strongly conserved across its broad geographic range from California to Alaska. Polymorphism at 5 nucleotide positions was consistent with multiple copies of the ITS2 within individual specimens of *P. odocoilei*. This work combines extensive fecal surveys, comparative morphology, and molecular diagnostic techniques to comprehensively describe the host associations and geographic distribution of a parasitic helminth.

2.2 Introduction

Protostrongylid nematodes are important parasites associated with recognized disease syndromes and, in some instances, declines in wildlife populations (Lankester, 2001). All

protostrongylids have a similar life cycle, where first-stage larvae are shed in the feces of the mammalian definitive host, invade a gastropod intermediate host, and develop to third-stage larvae infective for another definitive host. Species representing five genera of protostrongylids (*Elaphostrongylus*, *Parelaphostrongylus*, *Muellerius*, *Umingmakstrongylus*, and *Varestrongylus*) are known to parasitize artiodactyls in North America, and all produce morphologically similar first-stage larvae with a characteristic "dorsal-spine" (DSL) (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). The other species of protostrongylids present in North America are members of the genera *Protostrongylus* and *Orthostrongylus*, parasitize artiodactyls and lagomorphs, and produce spike-tailed larvae clearly distinguishable from DSL but not from each other (Boev, 1975; Mason, 1995; Carreno and Hoberg, 1999). Length of first- and third-stage larvae has been used to differentiate protostrongylid species; however, measurements overlap among the various species (Pybus and Shave, 1984; Kralka and Samuel, 1984b; Gray et al., 1985b; Pybus et al., 1989). Consequently, identification of protostrongylid genera or species based on larval length or morphology is rarely possible.

The known geographic distributions of protostrongylid parasites in North America, especially the muscleworms *Parelaphostrongylus odocoilei* and *P. andersoni*, are based on isolated reports and therefore often appear disjunct (Platt and Samuel, 1978a; Pybus and Samuel, 1981; Lankester and Fong, 1989; Lankester, 2001). Efforts to more thoroughly define distributions have been constrained by the necessity to kill hosts for recovery and identification of adult male nematodes. Recovery of adult parasites is challenging and tedious, even in heavily-infected ungulates, and often unsuccessful in lightly-infected or atypical hosts (Pybus and Samuel, 1981; English et al., 1985; Lankester, 2001). Presumptive identification based on host and geographic locality is not advisable because sympatric hosts commonly share multiple

species of protostrongylid parasites, and mixed infections are possible (Carreno and Hoberg, 1999; Hoberg et al., 2002). For example, DSL from white-tailed deer at some locations could represent single or mixed infections of *P. tenuis*, *P. andersoni*, and/or *Varestrongylus alpenae*. Bioassay of unknown first-stage larvae in captive intermediate and definitive hosts, and subsequent recovery of adult nematodes, is a reliable method for identification (Pybus et al., 1984; Samuel et al., 1985; Gray and Samuel, 1986), but requires specialized animal care facilities, a supply of captive uninfected hosts, and significant amounts of time (pre-patent periods alone can be as long as 3 mo). For these reasons, bioassay is rarely logistically feasible, and ante-mortem identification of protostrongylids has remained a diagnostic challenge.

Molecular techniques, especially in combination with comparisons of the morphology of adult parasites, are now commonly applied to identification of nematodes (Divina et al., 2000; Blouin, 2002; Monis et al., 2002; Nadler, 2002). The internal transcribed spacers of ribosomal RNA genes (ITS) have been used to differentiate a variety of nematode species where morphological differences, particularly among larvae, eggs, and adult females, are subtle or non-existent (Gasser and Hoste, 1995; Powers et al., 1997; Anderson et al., 1998). Identification of protostrongylids has recently been attempted using banding patterns on PCR of the ITS2 region, and sequences for the ITS2 of 6 protostrongylid species are now available in GenBank (Gajadhar et al., 2000; Junnila, 2002).

Recently, based on comparative morphological studies of adult nematodes, *P. odocoilei* was identified for the first time in Dall's sheep, a new genus of host for this parasite (Kutz et al., 2001c). This finding in the Mackenzie Mountains, Northwest Territories (NT), Canada, also represented a new geographic record for *P. odocoilei*, approximately 1,000 km farther north than previously reported. This discovery highlighted the need to more comprehensively define the

host associations and geographic distribution of *P. odocoilei*. We validated ITS2 sequence for adult and larval protostrongylid specimens of known identity, and then obtained and compared ITS2 sequences to identify unknown DSL from feces of wild caprine and cervid hosts across western North America. This combination of morphological and molecular identification led to the first comprehensive description of the geographic distribution of a protostrongylid parasite, and illustrated the concept of an "epizootiological probe" (Hoberg et al., 2001).

2.3 Materials and Methods

2.3.1 Parasite source and recovery

We obtained reference specimens or previously published sequences representing 7 species of protostrongylid parasites that produce DSL and are present in North America, and two that could be introduced by animal translocation (Table 2.1). Adult nematodes were identified using comparative morphology and standard criteria for protostrongylids (Boev, 1975; Anderson, 1978; Carreno and Lankester, 1993). Male and female nematodes were cut into sections, and heads and tails were identified and deposited as validated physical vouchers in the US National Parasite Collection (USNPC) at the Animal Parasitic Diseases Laboratory of the Agricultural Research Service, USDA, Beltsville, Maryland. Remaining portions of the body were used for subsequent molecular analyses. First-stage larvae in feces from experimentally- or naturally-infected hosts from which adult parasites had been identified were also used as representatives of known species. Larvae were recovered from fecal samples using a beaker Baermann technique modified from Forrester and Lankester (1997) by placing a single layer of cheesecloth or single ply White Swan Quick-Wipes (Scott Paper Limited, Streetsville, Ontario, Canada) between the fecal pellets and a supporting mesh layer. Physical vouchers of larval parasites were deposited in the USNPC. Where possible, molecular data from larvae were validated against that from adult specimens of known identity.

Between 1995 and 2003, collaborators across northwestern North America collected a total of 2,124 fecal pellet groups from thinhorn sheep, bighorn sheep, mountain goats, and cervids (Table 2.2). Samples were collected from the ground or from captured animals, and frozen at –20 C. DSL and spike-tailed *Protostrongylus* spp. larvae were recovered using the modified beaker Baermann technique and counted in 3 aliquots of 0.05 ml of the Baermann sediment on a slide under a compound microscope. If very few or no larvae were detected using the aliquot technique, the entire sediment was examined in a gridded Petri dish or on a slide, and all the larvae counted. The proportion of samples positive for each type of larvae (prevalence) was calculated for each sampling location. DSL were hand-picked under a dissecting microscope from at least one individual host from each location, and immediately processed for molecular analyses or frozen in tap water at -75 ºC in 2 ml polypropylene vials (Wheaton Cryule® Millville, New Jersey).

2.3.2 Molecular analyses

DNA was obtained from pieces of adult nematodes, individual larvae, or pooled larvae (n ranged from 6 to 50) by heating to 90 ºC for 10 min in 10 ul of water and cooling on ice for 25 min. Extraction buffer (20 ul composed of 0.5 mg/ml proteinase K, 1X PCR Buffer, and 2.5% 2-mercaptoethanol) was added and the mixture incubated at 65 ºC for 2 hr followed by heating to 90 ºC for 10 min. The samples (2 ul per PCR reaction) were used immediately or stored frozen (-20 ºC).

A PCR modified from Gajadhar et al. (2000) was performed using the primers NC1 (5'-ACG TCT GGT TCA GGG TTG TT-3') and NC2 (5'-TTA GTT TCT TTT CCT CCG CT-3') (Ellis et al., 1986; Gasser et al., 1993). Each 50 ul PCR reaction contained 34 ul of water, 5 ul of 10x PCR buffer, 4 ul of 25 mmol $MgCl^{2+}$, 0.5 ul of 25mmol dNTP's, 2 ul (40 pmol) of each

primer, 0.25 ul (1 Unit) Taq DNA polymerase, and 2 ul of sample DNA, overlaid with one drop of mineral oil. Amplification conditions consisted of an initial 3 min denaturation at 94 ºC followed by 35 cycles of 94 ºC for 60 sec, 60 ºC for 60 sec, and 72 ºC for 60 sec. A final extension phase of 72 ºC for 10 min was followed by cooling to 4 ºC. Reagent-only (DNA not added) reactions were used as negative controls to detect potential contamination. Reactions were analysed by electrophoresis through a 2% agarose gel with ethidium bromide staining. Amplification products ranged from 472 to 590 base pairs (bp) and were visualized with an Alphaimager gel documentation camera (Alpha Innotech, San Leandro, California).

PCR products amplified from individual larvae and adult *P. odocoilei* were sequenced directly. In directly sequenced PCR products, overlapping traces were observed at several nucleotide positions. To identify the bases present at these positions, DNA was extracted from a single adult female *P. odocoilei* using DNAeasy columns according to the manufacturer's protocol (Qiagen Corp., Valencia, California), and used as template in PCR reactions. PCR products from this specimen of *P. odocoilei*, as well as specimens of other adult protostrongylids and all the pooled larvae, were cloned using the Topo TA cloning kit (Invitrogen, Burlington, Ontario, Canada), and sequenced using M13 forward and reverse primers in both directions. Sequence chromatograms were aligned using Sequencher 4.1 (GeneCodes Corp., Ann Arbor, Michigan) or Seqman and Megalign (DNA Star, Madison, Wisconsin) software, and overall percent similarities between sequences were recorded. Gapped-BLAST searches of the GenBank database were performed to access previously published sequences (Altschul et al., 1997). Sequences were manually examined for species-specific differences and polymorphic sites.

2.4 Results

Selected sequences were deposited in GenBank under accession numbers AY648379-AY648409, AY679527, and AY679528. Length and raw similarity of ITS2 sequences among the 9 species of protostrongylids are reported in Table 2.3. The three species of *Parelaphostrongylus* could be differentiated at 5 sites ranging from single base substitutions to a 4 base insert, and at two larger variable regions (Table 2.4). Single nucleotide polymorphisms were observed at alignment positions 93 (A or T), 302 (T or C), 334 (C or A), 341 (G or A), and 391 (G or A) in cloned ITS2 sequences of *P. odocoilei* (n=26). These corresponded to overlapping traces/ambiguous bases when PCR products were sequenced directly (n=34). In total, we obtained 60 sequences for ITS2 of *P. odocoilei*, 51 from larvae in feces of wild caprines and cervids, and 9 from specimens of known identity.

Based on ITS2 sequences, we identified DSL of *P. odocoilei* from at least one host in thinhorn sheep (Dall's and Stone's sheep) at 13 locations in British Columbia (BC), the Northwest Territories (NT), the Yukon Territory (YT), and Alaska (AK); in mountain goats at 4 sites in BC, NT, and AK; and in woodland caribou in NT (Table 2.2, Fig. 2.1). We found no evidence of other species of *Parelaphostrongylus* in wild sheep or mountain goats, but our techniques would not necessarily detect mixed infections, especially at low levels of larval shedding. Prevalence of DSL and *Protostrongylus* spp. larvae in feces of wild ungulates are reported in Table 2.2.

2.5 Discussion

Parelaphostrongylus odocoilei occupies a far greater geographic range in northwestern North America than has been previously reported. Prior to its discovery in Dall's sheep of the Mackenzie Mountains, NT (Kutz et al., 2001c), the presence of *P. odocoilei* had been confirmed by identification of adult parasites at only 6 locations in west-central North America (Table 2.2,

Fig. 2.1). Based on observations of dorsal-spined larvae (DSL) in feces of wild cervids and mountain goats, a more widespread distribution of *P. odocoilei* was suspected, but not confirmed by adult parasite recovery and identification (Pybus et al., 1984; Lankester, 2001). Efforts to characterize the distribution of *P. odocoilei* in North America have been hampered by unidentifiable, or mistakenly identified, DSL. For example, DSL from Stone's sheep in northern BC were tentatively identified as *Muellerius* sp. (Seip and Bunnell, 1985), but, based on the findings in the current study, were likely *P. odocoilei*. By integrating comparative morphology of adult parasites and molecular identification of first-stage larvae, we more thoroughly defined the geographic distribution of *P. odocoilei* in wild cervids and caprines from western North America.

2.5.1 Molecular identification of dorsal-spined larvae

We obtained and compared sequences for the ITS2 region of ribosomal RNA genes of 9 protostrongylid species validated by comparative morphology of adult parasites, and used this database to identify unknown DSL as *P. odocoilei*. Identifications were based on large differences among representatives of *Parelaphostrongylus* and other genera, and smaller fixed differences among the three species of *Parelaphostrongylus*. Interestingly, consistent with observations by Junnila (2002), there were no fixed differences in the limited number of sequences available for *E. rangiferi* and *E. cervi*. In combination with controversy over the taxonomy of *Elaphostrongylus* spp. based on adult parasite morphology (Carreno and Lankester, 1993), this suggests that further molecular characterization at multiple loci is needed to define the relationship of these two species.

Taxon-level differences among the ITS2 sequences of the Protostrongylidae were roughly comparable to those reported for other nematode parasites, with raw similarities among genera of 30-60% as compared to 60-80% for gastro-intestinal trichostrongyles (Heise et al., 1999).

Similarity among species of *Parelaphostrongylus* was 90-98%, comparable to the 89-99% reported among gastro-intestinal trichostrongyles (Hoste et al., 1995; Newton et al., 1998; Heise et al., 1999). *Elaphostrongylus alces* had only 63-65% similarity with *E. rangiferi* and *E. cervi*, closer to the similarity (44-79%) among species of *Dictyocaulus* (Hoglund et al., 2003), and among genera of the Protostrongylidae. Caution must be exercised when comparing sequences outside of the Elaphostrongylinae, which were so divergent as to preclude reliable alignment. In addition, genetic "yard-sticks" for ITS2 sequence divergence have not been developed for nematodes (Anderson et al., 1998; Monis et al., 2002; Hoglund et al., 2003). Finally, genetic distances based on limited sequence data from a single locus cannot be used to infer phylogenetic relationships; decisions about species diversity and validity should be based on multiple loci and interpreted within a sound phylogenetic framework (Nadler, 2002).

Based on ITS2 sequences of a protostrongylid parasite across its known geographic range, we describe higher levels of intraspecific polymorphism within *P. odocoilei* than previously reported (Junnila, 2002), although comparable with that reported for gastro-intestinal nematodes (Heise et al., 1999), and for *Dictyocaulus eckerti* (Hoglund et al., 1999). Intraspecific polymorphism likely accounts for small differences in length of the ITS2 region of *P. odocoilei* within the current study (557-562 base pairs, Table 2.3), and between ITS2 sequences of protostrongylids in the current and previous studies (Junnila, 2002). The lengths reported by Gajadhar et al. (2000) were estimates using the relative positions of bands on a gel (Table 2.3). Intraspecific polymorphism within *P. odocoilei* was in part due to 5 single nucleotide polymorphisms (SNP) in cloned ITS2 sequences, which corresponded to overlapping traces when PCR products were sequenced directly. By sequencing clones of ITS2 from an individual adult female nematode, we confirmed the presence of two distinct copies of ITS2, which differed

at these 5 polymorphic sites, within the individual genome (GenBank accession numbers AY648401-AY648406). ITS2 "types" have been reported in other nematode species (Divina et al., 2000), and such heterogeneity among rDNA copies may persist within a lineage for more than a million generations (Coen et al., 1982). Only those differences that have become fixed between related lineages provide positive evidence for their differentiation (Williams et al., 1988; Rich et al., 1997; Santin-Duran et al., 2002).

We observed no fixed differences in sequence of ITS2 of *P. odocoilei* relative to host or geographic location, despite the broad distribution of this parasite from California to Alaska. The ITS2, while useful for species identification, appears to be unsuitable for detecting population genetic structure (Hoste et al., 1993; Anderson et al., 1998). In addition, the minimal level of genetic diversity of the ITS2 among the elaphostrongylines explains why only sequencing, and not differences in the mobility of PCR products of ITS2 through conventional agarose gels, could distinguish the 3, closely-related species of *Parelaphostrongylus* (Gasser and Hoste, 1995; Gajadhar et al., 2000). Sequencing is time- and labor-intensive, and mixed infections may not be detected unless large numbers of larvae are analyzed. If PCR product from samples of pooled larvae is sequenced directly instead of cloned, overlapping electrophoretograms may reveal the presence of mixed infections (Junnila, 2002; E. Jenkins, G. Appleyard, S. Kutz, unpubl. obs.). PCR in combination with species-specific primers, restriction fragment length polymorphism, or single-strand conformation polymorphism, would significantly increase the ability to identify species of protostrongylids, especially in mixed infections (Gasser and Monti, 1997; Monis et al., 2002). Finally, a mitochondrial locus such as cytochrome oxidase, which is less strongly conserved, is better suited for determining the

presence of population genetic structure or cryptic species, and to address phylogenetic hypotheses (Avise, 1994; Anderson et al., 1998; Hoberg et al., 1999; Blouin, 2002).

2.5.2 Epizootiology of protostrongylids

We describe the epizootiology, i.e., prevalence, host associations, and geographic distribution, of *P. odocoilei*, identified using molecular analyses of DSL, and of *Protostrongylus* spp., identified using larval morphology. Larvae of *Protostrongylus* spp. were recovered from fecal samples from all thinhorn sheep, bighorn sheep, and mountain goat populations examined. Shedding of *Protostrongylus* spp. larvae in feces is affected by season; therefore, despite variability in the data in Table 2.2, it is likely that close to 100% of thinhorn sheep, like bighorn sheep, are infected with *Protostrongylus* spp. (Forrester and Senger, 1964; Uhazy et al., 1973; Pybus and Shave, 1984). The larvae recovered were likely *P. stilesi*, or *P. rushi*, or both; larvae are morphologically indistinguishable and both species have been reported in these hosts (Uhazy et al., 1973; Samuel et al., 1977; Pybus et al., 1984; Kutz et al., 2001c). Using NC1 and NC2 primers, we could not amplify the ITS2 region of larvae of *Protostrongylus* spp., and therefore could not differentiate larvae of *P. stilesi* from *P. rushi* using molecular analyses.

DSL were present in 80-100% of samples from Dall's sheep and mule deer, but prevalence was more variable in samples from Stone's sheep (66-100%), mountain goats (41-100%), woodland caribou (28 and 43%), and black-tailed deer (25 and 56%). Logistical constraints generally entailed identification of DSL from only a single host at each location. Therefore, the prevalence of DSL, assumed to be *P. odocoilei*, should be interpreted in light of the possibility of infection with other protostrongylids such as *P. andersoni*, which has been reported in woodland caribou elsewhere in Canada (Lankester and Hauta, 1989; Lankester and Fong, 1989) and in experimentally-infected mule deer (Pybus and Samuel, 1984a). In addition, although we standardized methods used for larval recovery as much as possible, sample age and

storage were somewhat variable. Season of collection and host factors, such as age and sex, are known to influence larval shedding (Uhazy et al., 1973; Samuel et al., 1985; Festa-Bianchet, 1991; Peterson et al., 1996; Forrester and Lankester, 1997). Despite these sources of variability, the high prevalence of DSL in Dall's sheep and mule deer suggests that they are equally suitable hosts. Lower prevalence in Stone's sheep, mountain goats, black-tailed deer, and woodland caribou in both the current and previous studies suggests that they may be less suitable hosts, either inherently or because of differences in exposure due to behavior, habitat use, or host density.

DSL were not recovered from any of the three bighorn sheep populations examined in the current study. Indeed, DSL have only rarely been observed in fecal samples from bighorn sheep across North America, which are extensively monitored to determine the prevalence and intensity of *Protostrongylus* spp. larvae. DSL have been reported, but not identified, from bighorn sheep from East Kootenay and Premier Ridge, British Columbia, Canada; Banff, Alberta, Canada; Lower Rock Creek, Montana; and North Dakota (Hudson et al., 1972; Pybus and Shave, 1984; Aune et al., 1998). Identification based on recovery of adult parasites has only been accomplished in bighorn sheep in South Dakota, which proved to be infected with *Muellerius capillaris* (Pybus and Shave, 1984). It is likely that many populations of bighorn sheep have been exposed to *P. odocoilei* by sharing range, at least seasonally, with infected mule deer and mountain goats (e.g. sites 20 and 28, Table 2.2, Fig. 2.1). Currently, however, there is no evidence that bighorn sheep, unlike thinhorn sheep and mountain goats, are hosts for *P. odocoilei*. This is surprising in light of the broad host range of *P. odocoilei*, and the similarity of the endemic parasite fauna of thinhorn sheep, bighorn sheep, and mountain goats (Samuel et al.,

1977; Hoberg et al., 2001). The suitability of bighorn sheep as a host for *P. odocoilei* warrants further investigation.

DSL were not recovered from several populations of thinhorn sheep, despite repeated survey and adequate sample size, the latter based on high prevalence (80-100%) of DSL in infected thinhorn sheep populations elsewhere (Table 2.2, Fig. 2.1). In several instances, uninfected populations of thinhorn sheep were in close proximity to infected populations. Parasite transmission may not occur between these populations because mountain sheep exhibit high fidelity to their seasonal ranges (Geist, 1971; Valdez and Krausman, 1999). This philopatry is consistent with genetic structure suggesting isolation and limited opportunities for gene flow, and by extension parasite transmission, among thinhorn sheep populations in close proximity (Worley et al., 2004). Also, geographic barriers to sheep movement are known to exist between sheep in the Nahanni range, where DSL were not recovered, and those in the Mackenzie Mountains, where *P. odocoilei* is well established (N. Larter, pers. comm.). No geographic barriers are currently evident between an uninfected population of Stone's sheep at Williston Lake and nearby infected mountain goats and Stone's sheep; however, sheep are sparse at the southern end of the range and habitat-sharing may not occur among herds (Shackleton, 1999; J. Elliott, pers. comm.).

Samples from Dall's sheep north of the Arctic Circle (Baird, Brooks, British, and Richardson Mountains) were negative for DSL (Table 2.2, Fig. 2.1). *Muellerius* sp., which produces DSL, has been reported in Dall's sheep from the Brooks Range, Alaska (Dau, 1981); however, this record was based on findings at necropsy and it is possible that lung lesions typical of *Protostrongylus* sp. were mistakenly identified as those of *Muellerius* sp. *Parelaphostrongylus odocoilei* may be absent in the geographically isolated populations of

Dall's sheep north of the Arctic Circle because it has never been introduced, or because it could not establish in this high-latitude environment. The latter explanation is less likely, as favorable abiotic conditions and gastropod intermediate hosts suitable for transmission of some species of protostrongylids in the arctic regions may be inferred from the presence of *Protostrongylus* spp. in these populations of Dall's sheep, and of other protostrongylids in muskoxen and caribou (Hoberg et al., 1995; Lankester, 2001; Hoberg et al., 2002).

To explain the historical origins and current distribution of *P. odocoilei* in North America, phylogeographic and population genetic studies of both parasite and hosts are needed (Avise, 2000; Hoberg et al., 2003). Our finding that *P. odocoilei* is well-established in western North America is consistent with Platt's hypothesis that *P. odocoilei* developed with the ancestor of mule deer endemic to this region (Platt, 1984). Mule deer do not, however, currently share range with Dall's sheep, and have limited range overlap with Stone's sheep (Shackleton, 1999; Kutz et al., 2001c). The widespread distribution of *P. odocoilei* in thinhorn sheep cannot be explained by recent transmission of *P. odocoilei* from mule deer. Interestingly, Platt suggests that first-stage larvae of *P. odocoilei* are freeze-tolerant relative to those of *P. tenuis* (Platt, 1984; Shostak and Samuel, 1984), and this may account for the successful establishment of *P. odocoilei*, but not *P. tenuis*, in northern North America.

2.5.3 Molecular techniques in parasitology

Molecular identification, validated by adult parasite morphology, of unknown protostrongylid larvae in a broad-based survey illustrates the concept of "epizootiological probes", which have many applications in parasitological studies (Divina et al., 2000; Hoberg et al., 2001; Hoberg et al., 2003). The literature includes many reports of unidentifiable first-stage larvae of protostrongylid parasites in North American wildlife (Lankester et al., 1976; Pybus and Samuel, 1981; Pybus et al., 1984; Gray and Samuel, 1986). In addition to first-stage larvae,

third-stage larvae from gastropod intermediate hosts, adult female nematodes, and partial nematodes can now be identified. Applications in wildlife management and conservation biology include diagnosis of parasites in hosts that cannot be sacrificed for adult parasite recovery, such as endangered species or those that inhabit remote locations, or in risk assessments prior to translocation of either wild or game-ranched animals (Hoberg et al., 2001; Hoberg et al., 2003). The use of non-invasive molecular techniques for identifying protostrongylid parasites represents a breakthrough for this group of nematodes, which has hitherto proved to be diagnostically intractable. Further refinement of molecular techniques to allow detection of mixed infections and to minimize cost and time (i.e. for sequencing) are needed before they can be put to widespread use for identifying parasites of wild, game-ranched, and domestic animals, particularly in areas of natural range overlap and at the interface of managed and wild systems.

In addition to these practical diagnostic applications, epizootiological probes will finally allow comprehensive descriptions of the host associations and geographic distributions of parasites. While the current study has greatly increased the known geographic distribution of *P. odocoilei* in North America, sequence of first-stage larvae from mule deer in Montana matching that of *P. odocoilei* (Junnila, 2002) suggests that the full range of this parasite has yet to be described. As molecular identification of first-stage larvae becomes more feasible and more widely applied, *P. odocoilei* and other protostrongylids will likely prove to have broader and more overlapping distributions than previously suspected. For example, DSL from barren-ground caribou in northwestern Canada (YT and NT) were assumed to be *P. andersoni*, which has been reported in the Beverly herd in central NT and, based on unpublished reports, in caribou in Alaska (Lankester and Hauta, 1989; Lankester, 2001). Recent molecular characterization of

these DSL has not only confirmed the presence of *P. andersoni*, but also led to the discovery of a previously undescribed species of protostrongylid in barren-ground and woodland caribou at multiple locations in northwestern Canada (S. Kutz, G. Appleyard, and E. Jenkins, unpubl. obs.).

Knowledge of the species of parasite(s) present in a region is necessary to identify risks of parasite transmission and disease emergence under changing environmental conditions (Hoberg, 1997; Hoberg et al., 2003; Kutz et al., 2004b). Several protostrongylids are linked to neurological disease syndromes, and all have the potential to cause pulmonary pathology (Lankester, 2001). *Parelaphostrongylus odocoilei*, for example, can cause respiratory failure in experimentally and naturally-infected hosts (Brunetti, 1969; Platt and Samuel, 1978b; Pybus et al., 1984; Pybus and Samuel, 1984b). Stress due to environmental factors, in combination with various bacteria and protostrongylid lungworms, has been linked to pneumonia epizootics in bighorn sheep, and isolated cases of fatal pneumonia in wild Dall's sheep have been reported (Bunch et al., 1999; Jenkins et al., 2000). Outbreaks of clinical cerebrospinal elaphostrongylosis in reindeer in Norway (Handeland and Slettbakk, 1994), and accelerated development (from a 2- to a 1-yr cycle) of the muskox lungworm *U. pallikuukensis* in Nunavut, Canada (Kutz et al., 2004b), have been linked to warmer summer temperatures. In addition to unprecedented rates of warming, northern ecosystems are facing disturbance due to increasing oil, gas, and mineral exploration.

Shifts in host distributions, possibly in response to such habitat disruption, and the breakdown of isolation could lead to exchange of parasites such as *P. odocoilei*, and even disease outbreaks (Rausch, 1972; Hoberg, 1997; Hoberg et al., 2002). For example, muskox populations are expanding across the Arctic, and soon both subspecies of muskoxen in the Northwest Territories (*Ovibos moschatus wardi* and *O. m. moschatus*) may bridge the gap between thinhorn

sheep populations in the Mackenzie Mountains infected with *P. odocoilei*, and naïve populations in the Richardson Mountains. Although it is not known if muskoxen are suitable hosts for *P. odocoilei*, muskoxen and Dall's sheep share some species of gastro-intestinal parasites and the protostrongylid lungworm *Protostrongylus stilesi* (Hoberg et al., 2001; Hoberg et al., 2002; Kutz et al., 2004a).

Epizootiological investigations employing techniques from traditional parasitology combined with modern molecular analyses, such as the current study, are needed to characterize the parasite fauna of wildlife and their geographic distributions. Such knowledge is key to understanding how host-parasite assemblages originated, and to predicting how they may respond to environmental change, an increasingly recognized concern for wildlife management and conservation.

2.6 Acknowledgements

Many people (see Table 2.2) collected and shipped fecal samples and we gratefully acknowledge the considerable cost and effort involved, particularly in remote locations. Special thanks to Wayne Heimer and Randy Zarnke of the Alaska Department of Fish and Game, Fairbanks, AK, and Claire Jardine at the Western College of Veterinary Medicine (WCVM). In addition, we gratefully acknowledge Steve Nadler at University of California-Davis, as well as Robert M. Timm and Robert J. Keiffer from the University of California Hopland Research and Extension Center, for their invaluable assistance in obtaining samples from this region. Parasitology technicians and summer students at the WCVM who helped process fecal samples include Brent Wagner, Darren Harder, Jennifer Dodd, Jeremy Thomas, Farhad Ghandi, and Mathew Herperger. Expert PCR technical assistance was provided by Rebecca Cross, Anita Quon, and Anju Tumber at the WCVM, and Mayee Wong, Detiger Dunams, and Arthur Abrams at the USNPC. Many thanks to Alvin Gajadhar and Murray Lankester at the Centre for Animal

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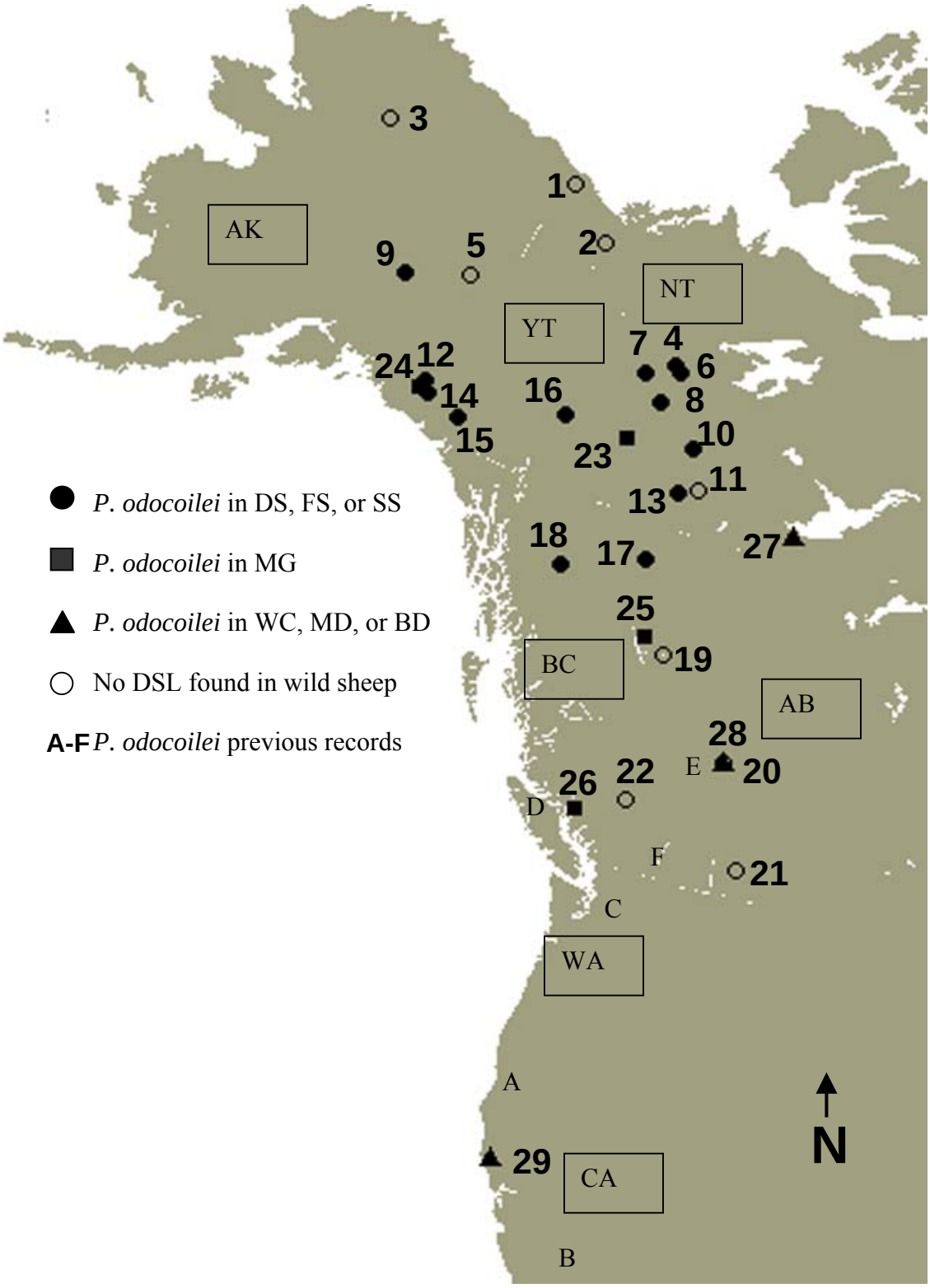

Figure 2.1: Known geographic distribution of *Parelaphostrongylus odocoilei* in North America. Abbreviations, numbers, and letters as for Table 2.2.

Table 2.1: Reference specimens and sequences of the ITS2 region of the ribosomal RNA gene of protostrongylid parasites.

Parasite	Stage	Host [a]	Origin	Accession number(s) [c]
P. odocoilei	1 adult [b]	DS	Mackenzie Mts., Northwest Territories (NT)	USNPC 94329-94334 AY648401-648406
P. odocoilei	4 adults [b] and L1 [e]	SS [d]	Mackenzie Mts., NT	USNPC 94891-94894 AY648380, AY648392-648393
P. odocoilei	sequence	MD	British Columbia (BC); Genbank	AF504031 & AF504037
P. andersoni	L1 [f]	BGC	Beverly herd, NT	USNPC 94890; AY648400
P. andersoni	sequence	BGC	NT; Genbank	AF504030 & AF504036
P. tenuis	sequence	WTD [d]	BC and Minnesota, USA; Genbank	AF504029 & AF504035
E. rangiferi	L1 [g]	WC	Gros Morne National Park, Newfoundland	AY648408
E. rangiferi	sequence	WC	Newfoundland; Genbank	AF504027 & AF504033
E. cervi	sequence	RD [d]	New Zealand; Genbank	AF504026 & AF504032
E. alces	sequence	MS [d]	Sweden; Genbank	AF504034
U. pallikuukensis	L1 [e]	MX [d]	Kugluktuk, Nunavut	USNPC 94884; AY648409
Muellerius capillaris	adult [b]	DmS	Guelph, Ontario	USNPC 94888-94889 AY679527-679528
Varestrongylus alpenae	adult [b]	WTD	Riding Mountain National Park, Manitoba	USNPC 94204; AY648407

[a] DS Dall's sheep (*Ovis d. dalli*); SS Stone's sheep (*O. d. stonei*); MD mule deer (*Odocoileus h. hemionus*); BGC barren-ground caribou (*Rangifer tarandus groenlandicus*); WTD white-tailed deer (*Odocoileus virginianus*); WC woodland caribou (*R. t. caribou*); RD red deer (*Cervus elaphus*); MS moose (*Alces alces*); MX muskoxen (*Ovibos moschatus*); DmS domestic sheep (*Ovis aries*)
[b] identification based on morphology and measurements
[c] United States National Parasite Collection Numbers (USNPC) and GenBank accession numbers (AY or AF)
[d] experimentally infected host
[e] identification of L1 (first-stage larvae) based on recovery and identification of adult parasites from experimentally infected host
[f] L1 from herd known to be infected with *P. andersoni* (Lankester and Hauta, 1989)
[g] L1 from herd known to be infected with *E. rangiferi* (Lankester and Fong, 1989)

Table 2.2: *Protostrongylus* spp. larvae (Proto) and dorsal-spined larvae (DSL) in fecal samples from wildlife hosts collected 1995-2003: number of samples (N), prevalence (%), and number of ITS2 sequences from unknown DSL.
Numbers 1-29 represent collections in the current study, while letters A-F represent previous studies where *P. odocoilei* was confirmed by adult parasite identification (Map column corresponds to Fig. 2.1).

Map	Host [a]	Locality [b]	Coordinates North; West	N	Proto (%)	DSL (%)	No. sequences	No. hosts	Collectors/source
1	DS	Ivvavik National Park, British Mts, NT	69°12'; 140°15'	6	67	0	-	-	J. Carey, I. McDonald
2	DS	Richardson Mts, NT	67°55'; 136°00'	259	81	0	-	-	J. Nagy
3	DS	Baird & Brooks Mts, AK	67°-68°; 150°-159° [c]	21	100	0	-	-	L. Adams, C. Kleckner
4	DS	Katherine Creek, Mackenzie Mts, NT	65°01'; 127°35'	650	84	96	15	3	A. Veitch, R. Popko, et al.
5	DS	Yukon-Charley R. Preserve, AK	65°; 143° [c]	19	95	0	-	-	J. Burch, J. Lawler
6	DS	Sheep Mt, Mackenzie Mts, NT	64°52'; 127°06'	22	100	100	1	1	R. Popko, K. Hickling
7	DS	Palmer Lk, Mackenzie Mts, NT	64°28'; 129°24'	36	56	92	1	1	A. Veitch, R. Popko
8	DS	Keele River, Mackenzie Mts, NT	64°19'; 126°48'	1	100	100	1	1	R. Popko
9	DS	Central Alaska Range, AK	63°56'; 147°28'	7	71	86	1	1	S. Arthur
10	DS	Trench Lk, Mackenzie Mts, NT	62°38'; 124°29'	8	75	100	1	1	K. Davidge, E. Jenkins
11	DS	Nahanni Range, Mackenzie Mts, NT	61°28'; 123°20'	31	90	0	-	-	K. Davidge, A. Gunn
12	DS	Rex Creek, Wrangel Mts, AK	61°19'; 142°31'	5	NA	40 [d]	3	2	E. Hoberg
13	DS	Tlogotscho Plateau, Mackenzie Mts, NT	61°07'; 124°32'	15	53	100	2	2	D. Tate
14	DS	Goat Creek, Chugach Mts, AK	60°60'; 142°02'	3	NA	100	4	2	E. Hoberg
15	DS	Kluane National Park, St Elias Mts, YT	60°45'; 139°30' [c]	9	100	100	2	1	J. Carey
16	FS	Faro, Anvil Mts, YT	62°15'; 133°15'	43	56	91	1	1	J. Adamczewski, J. Loehr
17	SS	Muskwa-Kechika, Rocky Mts, BC	58°45'; 125°10'	408	75	75	1	1	T. Ennis, H. Schwantje
18	SS	Spatsizi Plateau, Skeena Mts, BC	57°41'; 129°53'	4	50	75	3	2	L. Gawalko, H. Schwantje
19	SS	Williston Reservoir, Rocky Mts, BC	56°05'; 122°30' [c]	55	95	0	-	-	M. Wood
20	RBS	Cardinal R., Rocky Mts, AB	53°15'; 117°30'	10	100	0	-	-	Hinton Veterinary Clinic
21	RBS	Radium, Kootenay Mts, BC	49°56'; 115°35'	21	81	0	-	-	H. Schwantje
22	CBS	Fraser R., Lillooet Mts, BC	51°19'; 122°08'	412	93	0 [e]	1	1	P. Dielman, H. Schwantje
23	MG	Ramhead, Mackenzie Mts, NT	62°18'; 128°58'	22	50	41	1	1	A. Veitch, R. Popko
24	MG	Tana Lake, Chugach Mts, AK	61°00'; 142°45'	3	NA	67	NA	NA	E. Hoberg

25	MG	Ospika R., Rocky Mts, BC	56°30'; 123°55'	22	55	64	2	1	M. Wood
26	MG	South Coast Mts, mainland BC	50°31'; 124°39'	18	78	89	2	2	S. Taylor, H. Schwantje
27	WC	Hay River, NT	60°45'; 116°38'	7	NA	43	1	1	D. Johnson, B. Elkin
28	MD	Cardinal R., Rocky Mts, AB	53°15'; 117°30'	4	NA	100	5	2	W. Samuel
29	BD	Hopland, CA	38°58'; 123°07'	3	NA	100	3	3	R. Carreno et al.
A	BD	Coastal range, CA	41°00'; 123°02' [c]	100	NA	25	-	-	Hobmaier & Ibid, 1934
B	CMD	W. Sierra Nevada Mts, CA	36°34'; 118°41' [c]	59	NA	98	-	-	Brunetti, 1969
C	MG	Newhalem, WA	48°; 121°	1	100	100	-	-	Pybus et al., 1984
D	BD	Vancouver Island, BC	50°; 126°	16	NA	56	-	-	Pybus et al., 1984
E	MD	Jasper National Park & vicinity, AB	52°59'; 118°06'	496	NA	93	-	-	Samuel et al., 1985 [f]
E	MG	Jasper National Park & vicinity, AB	52°59'; 118°06'	1	100	100	-	-	Pybus et al., 1984
E	WC	Jasper National Park & vicinity, AB	52°59'; 118°06'	155	NA	28	-	-	Gray and Samuel, 1986
F	MD	Penticton, BC	49°30'; 119°34'	NA	NA	82	-	-	Lankester, 2001

[a] abbreviations as for Table 2.1; also FS Fannin sheep (*Ovis dalli stonei*); RBS Rocky Mountain bighorn sheep (*Ovis c. canadensis*); CBS California bighorn sheep (*Ovis canadensis californiana*); MG Mountain goat (*Oreamnos americanus*); BD Black-tailed deer (*Odocoileus h. columbianus*); CMD California mule deer (*Odocoileus h. californicus*)

[b] NT (Northwest Territories); AK (Alaska, USA); YT (Yukon Territory); BC (British Columbia); AB (Alberta); CA (California, USA); WA (Washington, USA)

[c] approximate map reference based on place name

[d] prevalence questionable, samples analyzed under field conditions

[e] one composite pellet group contained DSL, *Protostrongylus* spp. larvae, and eggs of gastro-intestinal parasites typical of bighorn sheep, but may have included feces from sympatric mule deer infected with *P. odocoilei*

[f] original identification by Platt and Samuel (1978a)

Table 2.3: Size (base pairs = bp) and similarity (%) of ITS2 region of protostrongylid species using the primers NC1 and NC2. Sequence pair distances from the Clustal V method (Megalign, DNA Star, Madison, Wisconsin).

Species	Size (bp) Gajadhar et al., 2000	Size (bp) Junnila, 2002	Size (bp) Current study	Po	Pa	Pt	Er	Ec	Ea	Up	Mc	Va
P. odocoilei (Po)	571	561	560[a]	-------	91-97	94-97	58-63	53-63	52-55	34-46	31-41	40
P. andersoni (Pa)	566	545	550		-------	97-98	51-62	52-55	50-56	35-49	33-41	38-39
P. tenuis (Pt)	576	554	NA			-------	60-66	53-54	45-53	47-49	38-43	39-42
E. rangiferi (Er)	597	585	590				-------	95	63	40	42	39
E. cervi (Ec)	597	585	NA					-------	65	36	40	38
E. alces (Ea)	581	575	NA						-------	41	35	39
U. pallikuukensis (Up)	445	445	472							--------	51	36
M. capillaris (Mc)	NA	NA	495								-------	30
V. alpenae (Va)	NA	NA	556									

[a] range 557-562

Table 2.4: Fixed differences among ITS2 sequences of the three species of *Parelaphostrongylus*.
Number of sequences (n), aligned using Seqman and Megalign (DNA Star, Madison, Wisconsin) software.

Alignment position	79	84	201	231	346	351	468
P. odocoilei (n=60)	A	A	G	GATG	A	GAAAA**G**AAAAAAAAG[a]	TTATTACTAGGT
P. andersoni (n=17)	G	G	A	----	A	GAAAAAAAAA	TTATTATTATGT or TTAT_____ GT
P. tenuis (n=3)	G	A	A	----	C	GAAAAAAAAAC	TTATTATTATGT

[a] most common variant; in a minority of sequences, bold G substituted by an A, and the number of A's was variable (range 8-14).

CHAPTER 3

3 LIFE CYCLE AND PATHOGENESIS

The manuscript[2] comprising this chapter describes how we completed the life cycle and described clinical effects in five thinhorn sheep experimentally infected with *P. odocoilei*. This included a neurological syndrome not previously described in any definitive host for *P. odocoilei*, which raised the possibility of a neural migration. This was later supported by histological examination of the brains of experimentally and naturally infected thinhorn sheep (Chapter 4). Knowledge of the life cycle was key to interpreting the patterns of larval shedding observed in wild Dall's sheep (Chapter 6). As well, these findings have implications for the health significance of this parasite, and the phylogeny of the elaphostrongylines (further discussed in Chapter 7). I designed a monitoring program for the experimentally infected sheep, and supervised or directly monitored sheep with assistance from technicians, clinicians, and diagnosticians. As a veterinarian, I was ultimately responsible for the health and welfare of the animals, including obtaining appropriate documentation from the University Animal Care Committee, and for obtaining anesthetic drugs on an Emergency Drug Release. I analyzed the data and, as corresponding author, researched and wrote the manuscript with input from co-authors.

[2] Jenkins, E.J., E.P. Hoberg, and L. Polley. Development and pathogenesis of *Parelaphostrongylus odocoilei* (Nematoda: Protostrongylidae) in experimentally infected thinhorn sheep (*Ovis dalli*). Journal of Wildlife Diseases, In press.
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3.1 Abstract

Recently, the protostrongylid nematode *Parelaphostrongylus odocoilei* has been reported in a new host species, thinhorn sheep (*Ovis dalli*). For the first time, we completed the life cycle of *P. odocoilei* in three Stone's sheep (*O. d. stonei*) and two thinhorn hybrids (*O. d. stonei* x *O. d. dalli*), each infected with 200 third-stage larvae from slugs (*Deroceras laeve*). The pre-patent period ranged from 68-74 days, and shedding of first-stage larvae (L1) peaked at >10,000 L1 per gram of feces between 90 and 110 days post infection. A total of 75, 27, and 14 adult *P. odocoilei* were recovered from skeletal muscles of three Stone's sheep. Starting in the pre-patent period, all infected sheep lost weight and developed peripheral eosinophilia. At two weeks prior to patency, two thinhorn hybrids developed neurological signs (hind end ataxia, loss of conscious proprioception, and hyperesthesia) that resolved at patency. Eosinophilic pleocytosis and antibody to *Parelaphostrongylus* spp. were detected in the cerebrospinal fluid of the affected sheep, suggesting that the migration route of the "muscleworm" *P. odocoilei* may involve the central nervous system. Twenty days after treatment with ivermectin, neurological signs recurred and larval shedding ceased in one infected thinhorn hybrid, while multiple treatments transiently suppressed but did not eliminate larval shedding in the other. During patency, two Stone's sheep with numerous eggs and larvae of *P. odocoilei* in the lungs died of respiratory failure following anesthesia or exertion. *Parelaphostrongylus odocoilei* has widespread geographic distribution, high prevalence, the possibility of neurological and respiratory disease, and resistance to treatment, and may constitute a significant emerging disease risk for thinhorn sheep.

3.2 Introduction

In 1998, the protostrongylid nematode *Parelaphostrongylus odocoilei* was discovered in a new host, Dall's sheep (*Ovis dalli dalli*) in the Mackenzie Mountains, Northwest Territories,

Canada (Kutz et al., 2001c). Subsequent investigations revealed that *P. odocoilei* was well-established in many populations of thinhorn sheep (*Ovis dalli*), including both Dall's sheep (*O. d. dalli*) and Stone's sheep (*O. d. stonei*), in north-western North America (Jenkins et al., 2005a). Thinhorn sheep were traditionally hunted by northern First Nations, and currently generate significant revenue for northern communities through their value as a trophy game species (Crapo, 2000; Nadasdy, 2003).

Parelaphostrongylus odocoilei is a member of the Elaphostrongylinae (Family Protostrongylidae), which is comprised of two genera, *Parelaphostrongylus* (of North American origin), and *Elaphostrongylus* (of Eurasian origin) (Lankester, 2001). All protostrongylids undergo a similar life cycle, where first-stage larvae (L1) are shed in the feces of the mammalian definitive host, invade a gastropod intermediate host, and develop to third-stage larvae (L3) infective for another definitive host. The route of migration and target organ within the definitive host, usually a cervid, varies among the elaphostrongylines. Adults of *P. odocoilei* and *P. andersoni* are considered to be strictly muscle-dwelling, with a pattern of migration through the central nervous system (CNS) having either been secondarily lost or never acquired (Platt, 1984; Anderson, 2000). Adults of *P. tenuis*, the meningeal worm, establish in the CNS and cranial venous sinuses, and do not invade the muscles. Adults of *Elaphostrongylus* spp. (*E. rangiferi*, *E. cervi*, and *E. alces*) travel through some aspect of the CNS before establishing in the skeletal musculature (Anderson, 2000; Lankester, 2001).

Elaphostrongylines with a neural migration may cause neurological disease in "typical" or "atypical" definitive hosts, and eggs and larvae of all elaphostrongyline species travel via the venous circulation to the lungs, causing granulomatous interstitial pneumonia (Lankester, 2001). All elaphostrongylines have the potential to cause disease in individual hosts, and may have

effects at the population level (Anderson, 1972; Lankester, 2001; Ball et al., 2001). Based on clinical respiratory disease and lung pathology in both naturally and experimentally infected hosts (Brunetti, 1969; Platt and Samuel, 1978b; Pybus et al., 1984; Pybus and Samuel, 1984b; Kutz et al., 2001c), *P. odocoilei* could be a significant pathogen in thinhorn sheep.

Using various gastropod intermediate hosts, the life cycle of *P. odocoilei* of cervid origin has been completed in mule deer (*Odocoileus h. hemionus*) (Platt and Samuel, 1978b; Pybus and Samuel, 1984a), black-tailed deer (*O. h. columbianus*) (Hobmaier and Hobmaier, 1934; Brunetti, 1969; Platt and Samuel, 1978b), and moose (*Alces alces*) (Platt and Samuel, 1978b). Attempts to infect white-tailed deer (*O. virginianus*) were only marginally successful, whereas domestic goats (*Capra hircus*) and domestic sheep (*Ovis aries*) seem to be refractory to infection (Platt and Samuel, 1978b; Pybus, 1983; Pybus and Samuel, 1984a; S. Kutz, unpublished data). For the first time, we complete the life cycle and describe the pathogenesis of *P. odocoilei* in experimentally infected thinhorn sheep. This work has implications for understanding life history patterns among the Elaphostrongylinae and the significance of a newly discovered host-parasite relationship, *P. odocoilei* in thinhorn sheep.

3.3 Materials and Methods

3.3.1 Sources of experimental animals and *P. odocoilei*

Three captive Stone's sheep (SS1, SS2, and SS3; Table 3.1) were acquired from a zoo in June 2000, and housed individually (SS1) or together (SS2 and SS3) in concrete pens measuring 2.3 x 4 m at the Western College of Veterinary Medicine (WCVM), Saskatoon, Saskatchewan, Canada. Four hand-raised thinhorn hybrids (*O. d. stonei* x *O. d. dalli*, TS4-TS7; Table 3.1), originally from a game ranch in Saskatchewan, were brought in from pasture in October 2003, and housed in pairs (TS4 with TS5, and TS6 with TS7) in concrete rooms measuring 3.4 x 4.5 m at WCVM. Animals had unlimited access to water, mineral blocks, and grass or alfalfa hay, and

were offered ruminant pellets daily (Landmark Feeds, Winnipeg, Manitoba), and browse as available. SS1, SS2, and SS3 were housed in natural lighting conditions, while TS4-TS7 had regulated photoperiods (12 hr light:12 hr dark). Housing and procedures were approved by the University of Saskatchewan Committee on Animal Care (protocol 2000-0040).

Upon arrival, eggs and oocysts of several species of gastrointestinal parasites were present in feces from all three Stone's sheep, and low numbers of *Protostrongylus* sp. larvae were present in pooled feces from SS2 and SS3. Sheep received benzimidazole anthelmintic and anticoccidial treatment at least 28 days prior to infection with *P. odocoilei*. At 17 months prior to infection, the thinhorn hybrids had been exposed to the muskox lungworm *Umingmakstrongylus pallikuukensis* (Kutz et al., 2004a), but showed no evidence of infection with this or any other parasite and were not treated. As part of a separate study, one control sheep (TS7) was experimentally infected with the gastro-intestinal nematode *Marshallagia marshalli* at 252 days into the *P. odocoilei* study.

For infection of SS1, first-stage larvae (L1) were obtained from four fecal samples collected in May 2000 from a Dall's sheep population known to be infected with *P. odocoilei*, and no other elaphostrongylines, in the Mackenzie Mountains, Northwest Territories (65º 01' N; 127º 35' W) (Kutz et al., 2001c). For the other sheep, L1 were obtained from feces of SS1. Slugs (*Deroceras laeve*) were infected with L1 and, after 18-35 days at room temperature, infective third-stage larvae (L3) harvested (Hoberg et al., 1995). Motile or tightly coiled L3 were hand-picked under a dissecting microscope and held in tap water at 4 ºC overnight. Sheep were fasted for 24 hours, anesthetized (protocol modified from Kutz et al., 2004), and infected with 200 L3 in 60 ml of tap water administered by dose syringe and gastric tube (9.5 mm outer diameter), which were immediately flushed with a minimum of 60 ml of water, then twice with

60 ml of air. Control animals (TS6 and TS7) received the same treatment, except no L3 were administered.

3.3.2 Parasitological monitoring

For SS1 in the pre-patent period (PPP), we used a funnel Baermann technique to examine twelve 20 g samples from feces collected over 24 hours every 4 days until 38 days post infection (dpi), then every 2 days (Kutz et al., 1999a). Otherwise, protostrongylid larvae were recovered from feces using a modified beaker Baermann sedimentation and counted using an aliquot or total count method (Forrester and Lankester, 1997; Jenkins et al., 2005a). Larvae per gram (LPG) of wet feces was calculated, and one to three 5 g samples from each fecal collection were air dried for at least two weeks, and LPG of dry feces calculated. From each pair of infected sheep housed together in the PPP, we examined 6-12 5 g samples from pooled feces collected over a 24 hr period every 2 days starting at 30-40 dpi. Once patency was established, SS2 and SS3 were separated for 24 hr, and TS4 and TS5 for 4-6 hr, every Monday and Thursday. Three 5 g samples were examined from feces collected from each sheep over the period of separation, or over a 24 hr period every Monday and Thursday for SS1. From TS6 and TS7, we examined six 5 g samples from pooled feces collected over a 24 hr period once a week from 43 to 380 dpi.

TS4 and TS5 were treated with 200 µg/kg subcutaneous ivermectin (10 mg/ml, Ivomec®, Merial, Baie d'Urfe, Quebec, Canada) at 180, 278, and 292 dpi. One uninfected control sheep (TS6) was treated with ivermectin at the same dose and time as infected sheep. Following treatment, we examined six 5 g samples from feces pooled from TS4 and TS5 collected over a 24 hr period every Monday and Thursday until 361 dpi. If larvae were detected, samples were collected from individual animals to identify the source.

The ITS-2 regions of rDNA of L1 shed in feces from SS1, TS4, and TS5 were sequenced (Jenkins et al., 2005a). Total worm counts were obtained for SS1, SS2, and SS3 (Kutz et al.,

2001c). Five or six adult male nematodes from each Stone's sheep were fixed in 70% ethanol and 5% glycerol, cleared with lactophenol, and identified using standard morphometric techniques, including measurements of spicules and gubernacula.

3.3.3 Clinical monitoring

Sheep were observed daily by staff of the Animal Care Unit, and at least twice a week by researchers. Animals with clinical signs were examined by researchers several times a day, and at least once per episode by large animal clinicians from the WCVM. Clinical pathology, including complete blood counts (CBC) and serum chemistry, was performed by Prairie Diagnostic Services, WCVM, Saskatoon, Saskatchewan, Canada. The three Stone's sheep (SS1-SS3) were anesthetized every 1-4 wk for monitoring changes in the lungs associated with *P. odocoilei* (reported elsewhere), at which times body weight and blood were also obtained. Starting at one wk prior to infection, TS4-TS7 were weighed every week. Starting at 6 wk prior to infection, blood for CBC was obtained every 1-2 mo from TS4-TS7 under manual restraint, with increased frequency during times of clinical illness (weekly) or following treatment (every two wk for six wk). Blood from TS4-TS7 for serum chemistry was obtained every 2-3 mo. TS4-TS7 were anesthetized for cerebrospinal fluid (CSF) taps at 61-64 dpi, for medical imaging at 140 dpi, and, for TS4 only, for magnetic resonance imaging of the spinal column at 223 dpi (anesthetic protocols modified from Kutz et al., 2004). After each procedure, TS4-TS7 were treated prophylactically with subcutaneous injections of 10 mg/kg tilmycosin (300 mg/ml, Micotil®, Provel, division of Eli-Lilly, Guelph, Ontario, Canada). Clinical pathology on CSF included differential white blood cell counts, total numbers of nucleated and red blood cells, and total protein. Serum and CSF from TS4-TS7 were analyzed using protein electrophoresis and assay for specific antibody for excretory-secretory products of *Parelaphostrongylus* spp. (Ogunremi et al., 1999).

3.4 Results

3.4.1 Parasitological observations

Prior to infection with *P. odocoilei*, dorsal-spined larvae were not recovered from the feces of any sheep, nor from the feces of uninfected control sheep at any time in the study. Dorsal-spined larvae were first detected in feces from infected sheep from 68-74 dpi (Table 3.1). For SS1, larval shedding increased gradually in the first few weeks, reached a peak at 106 dpi, and then stabilized at a mean value of 6117 LPG (wet weight) for a 2-month plateau period (Table 3.1, Fig. 3.1). During this period, based on average daily production of 320 g of feces (wet weight), SS1 shed approximately 2 million larvae per day (120 million larvae in total). For TS4 and TS5, larval shedding increased exponentially in the first few weeks, reaching a peak between 95 and 110 dpi, then gradually decreased (Fig. 3.1).

Following the first treatment with ivermectin, larval counts in TS4 and TS5 were maintained at 1,700-4,800 LPG of dry feces for a week, then fell to 0 at 20 days after treatment. In TS5, but not TS4, intermittent, low-level (< 10 LPG) shedding resumed at 30 days after the first treatment and continued until 20 days after a second treatment. Larvae shedding from TS5 resumed 69 days after the third and final treatment.

Sequences from L1 from SS1, TS4, and TS5 were identical to each other and to that of *P. odocoilei* (GenBank accession numbers AY648380, 648392, and 648393). Seventy-five adult nematodes were recovered from skeletal muscles of SS1 (38% recovery of infective dose of 200 L3), 27 from SS2 (13.5%), and 14 from SS3 (7%). Measurements of 17 adult male nematodes from SS1-SS3 were within the range reported for *P. odocoilei* (Platt and Samuel, 1978a; Kutz et al., 2001c). At least three voucher specimens from each Stone's sheep were deposited at the United States National Parasite Collection, Beltsville, Maryland, USA (accession numbers 94891-94894, 95314, and 95315).

3.4.2 Clinical observations

Weight loss in SS1, SS2, and SS3 began at ~28 dpi and continued until death, at which point sheep had lost, respectively, 16%, 7%, and 13% of body weight at time of infection. Weight loss was observed initially in all thinhorn hybrids upon confinement, but weights of control sheep (TS6 and TS7) stabilized at about 30 dpi, while infected sheep (TS4 and TS5) continued to lose weight until 85-99 dpi (Fig. 3.2), losing a maximum of 23% and 16% of body weight at time of infection. The infected thinhorn hybrids reached their lowest weights shortly after patency, and subsequently gained weight, corresponding with decreasing larval counts (Fig. 3.3). All thinhorn hybrids began to gain weight after 260 dpi (Fig. 3.2).

At 60-70 dpi, relative to their own baselines, SS1 and SS3 developed mild eosinophilia ($< 1.2 \times 10^9$/L) which continued to increase until death. At 60 dpi, relative to control sheep, TS4 and TS5 developed mild eosinophilia which became a sustained leukocytosis due to an absolute eosinophilia ($>1.2 \times 10^9$/L) (Fig. 3.4). Increasing eosinophil counts correlated with spontaneous declines in larval shedding in TS4 and TS5 prior to treatment with ivermectin (Fig. 3.5). Fifteen days after the first treatment, counts peaked at 7.5 and 6.2×10^9 eosinophils/L (54% and 48% of total white blood cells) and then fell, but rose again in TS5, which continued to shed larvae after the first treatment. After the second and third treatments, eosinophil counts in both TS4 and TS5 returned to baseline values (Fig. 3.4). Mildly elevated eosinophil counts in a control sheep (TS7) after 280 dpi probably reflect experimental infection with a gastrointestinal parasite. No abnormalities were observed in serum chemistry panels from any sheep.

At 79 dpi for SS1, all three Stone's sheep developed transient bloody diarrhea due to bovine corona virus identical to that isolated from similarly affected calves housed nearby (E. Jenkins and K. West, unpubl. data). At 173 dpi (101 days after patency), following exertion, SS1 died with fluid draining from the lungs, and hemorrhagic foam in the airways. At 92 dpi (19

days after patency), following anesthesia the previous day, SS3 died with rumen fluid draining from the nose and mouth. Granulomatous inflammation, hemorrhage, and edema associated with high intensities of eggs and larvae of *P. odocoilei* were observed on histopathology of the lungs of both SS1 and SS3, although this pattern was somewhat obscured in SS3 by aspiration of rumen contents, possibly agonally. At 84 dpi (16 days after patency), SS2 presented staggering, with what appeared to be a left hind lameness, and later that day had a prolonged recovery from anesthesia, was shaky and unable to rise without difficulty, and was unusually aggressive. The leukogram (neutrophilia with left shift and toxic change) suggested inflammation consistent with severe, chronic, bacterial pneumonia, which was confirmed on post-mortem following euthanasia at 87 dpi.

TS5 (infected) and TS6 (control) developed signs consistent with a mild bacterial pneumonia shortly after anesthesia at 0 dpi, but these resolved within 2-3 weeks following antibiotic treatment. Otherwise, no clinical abnormalities were observed in TS4-TS7 until 54 dpi, when the two infected sheep (TS4 and TS5) developed depression and incoordination, had difficulty getting up and lying down, lay with hind, and sometimes fore, limbs in full extension, and assuming a "stacked up" posture (Fig. 3.6). If made to move, the animals frequently slipped and fell, and spontaneously crossed hind limbs when turning or trying to lie down. Both animals ground their teeth and TS4 rubbed her head frequently. Treatment with an anti-inflammatory analgesic (1 mg/kg IM flunixin meglumine, 50 mg/ml, Cronyxin™, Bioniche Animal Health Inc., Belleville, Ontario, Canada) at 55 and 67 dpi did not alleviate clinical signs. Examination by large animal clinicians, including specific tests of neurological function (sensation, reflexes, placing reactions, gait) at 60 and 67 dpi established that signs were consistent with posterior ataxia, loss of conscious proprioception, increased extensor tone, exaggerated reflexes, and

hyperesthesia. At 61 dpi, eosinophilic pleocytosis, hemorrhage, and increased total protein were present in CSF from infected (TS4 and TS5), but not control (TS6 and TS7) sheep (Table 3.2). There was an equivocal increase in the beta-globulin protein fraction in the CSF of TS4 and TS5, but not TS7 (not available for TS6), and no abnormalities were observed in serum protein profiles of any of the four sheep. Antibodies for *Parelaphostrongylus* spp. were detected in the cerebrospinal fluid and serum of infected, but not control, sheep. Neurological signs stabilized at patency and subsequently disappeared until a mild recurrence in TS4, 20 days following the first treatment with ivermectin. At this time, this sheep also developed signs consistent with neurological bladder dysfunction, with frequent ($\leq$ 1-2 min) posturing to urinate, spastically producing only a small amount of urine each time. The urinary abnormalities resolved within a few days, but this sheep dragged the dorsal surface of the right hind foot slightly until the end of the monitoring period. Onset of shedding of the winter coat, which began in April 2004 for the two control sheep (TS6 and TS7), was delayed until June-July 2004 for the two infected sheep (TS4 and TS5). Uninfected control sheep showed no clinical abnormalities at any time.

3.5 Discussion

3.5.1 Life cycle and clinical effects

This is the first time that the life cycle of *P. odocoilei* has been completed in experimentally infected slugs (*D. laeve*) and thinhorn sheep (*Ovis dalli*). Sheep were each infected with 200 L3, a "moderate" experimental inoculum based on doses of 14-1000 L3 of *P. odocoilei* administered to mule deer as a single exposure (Platt and Samuel, 1978b; Pybus and Samuel, 1984a; Pybus and Samuel, 1984b). Recovery rate of adult *P. odocoilei* (38%) from a Stone's sheep at necropsy on 173 dpi was comparable to the mean recovery rate of 45+/- 8% reported in mule deer experimentally infected with 300 L3 of *P. odocoilei* (Pybus and Samuel, 1984a). Nematode recovery rates were lower in two Stone's sheep examined earlier in patency,

which may have been due to the small size of muscle hemorrhages associated with adult nematodes early in infection (Pybus and Samuel, 1984b; Lankester and Hauta, 1989). Using the same recovery techniques, infection intensities in the experimentally infected Stone's sheep (14, 27, and 75 adult worms per carcass) were similar to the range of 16-56 worms per carcass in seven naturally infected Dall's sheep in the Mackenzie Mountains, NT (E. Jenkins, S. Kutz, and A. Veitch, unpubl. data).

The pre-patent period in five thinhorn sheep (68-74 d) was longer than that reported for mule deer infected with up to 334 L3 of *P. odocoilei* (45-62 d), but similar to that reported in black-tailed deer and moose infected with 50-750 L3 of *P. odocoilei* (58-72 d) (Platt and Samuel, 1978b; Pybus and Samuel, 1984a). The pattern of larval shedding in thinhorn sheep was similar to that observed in mule deer infected with *P. odocoilei*, with larval counts increasing for the first 3-4 weeks, peaking at 20-40 days after patency, then reaching a plateau or gradually declining over a period of weeks to months (Platt and Samuel, 1978b; Pybus and Samuel, 1984a). Differences in patterns and magnitude of larval shedding among sheep in the current study likely reflect individual variation in susceptibility or immunity. Stressors such as fasting or anesthesia were associated with transient peaks in larval shedding (Fig. 3.1); stress-related immunosuppression has been linked to increased shedding of larvae of *E. rangiferi* (Gaudernack et al., 1984).

Stone's sheep infected with 200 L3 of *P. odocoilei* developed relative eosinophilia and weight loss, similar to the mild eosinophilia and overall loss of condition observed in mule deer fawns experimentally infected with 300 L3 of *P. odocoilei* (Pybus and Samuel, 1984b). Absolute eosinophilia in the two thinhorn hybrids was associated with a spontaneous decrease in larval counts, as well as treatment-induced inflammatory destruction of parasites. Marked

weight loss in the two thinhorn hybrids that developed neurological signs could in part reflect muscle atrophy due to inactivity, while delayed shedding of the winter coat was likely a nonspecific indicator of poor health (Woolf and Kradel, 1973). Emaciation has been reported in other definitive hosts with naturally-acquired infections of *P. odocoilei* (Brunetti, 1969; Pybus et al., 1984). Following treatment, infected sheep reached and even exceeded weights at time of infection, possibly in part due to seasonal effects, as control sheep also gained weight at this time (Fig. 3.2).

Timing of the deaths of two experimentally infected Stone's sheep (SS1 and SS3) and pathology associated with eggs and larvae of *P. odocoilei* in the lungs suggest that infection was at least a contributing factor. Fatal respiratory failure following exertion has been observed in a naturally infected Dall's yearling with similar pulmonary lesions associated with *P. odocoilei* (S. Kutz, T. Bollinger, E. Jenkins, unpubl. data). Two mule deer experimentally infected with *P. odocoilei* developed clinical signs suggestive of gastroenteritis shortly after patency, and had severe interstitial pneumonia on post-mortem (Platt and Samuel, 1978b). Pybus and Samuel (1984b) found that all experimentally infected mule deer developed signs of respiratory distress at 20-25 days post-patency, with one animal dying at 49 days post-patency "with much hemorrhagic foam…discharged from the mouth and nares", similar to that observed in SS1.

Aspiration and stress associated with multiple anesthetics likely caused the bacterial pneumonia that led to euthanasia of one older Stone's ewe (SS2). Mild bacterial pneumonia in two of four thinhorn hybrids following a routine anesthetic, and transmission of bovine corona virus from nearby calves to the three Stone's sheep (the first report of this pathogen in thinhorn sheep), reinforce the need for strict biosecurity for wildlife species kept in captivity in a veterinary college.

3.5.2 Neurological syndrome

This is the first report of a neurological syndrome associated with *P. odocoilei* in any host species. Loss of conscious proprioception and increased extensor tone in the hind limbs are associated with lesions in the superficial dorso-lateral funiculi of the spinal cord between T3 and L3, while hyperesthesia is associated with lesions of dorsal roots (DeLahunta, 1983). Neurogenic bladder dysfunction (possibly reflex dyssynergia or detrussor hyper-reflexia) of one thinhorn sheep following treatment can be linked to partial disruption of the long spinocerebellar tracts (superficial lateral funiculi) (Oliver et al., 1997), possibly due to inflammation associated with dying nematodes. The Stone's ewe that was euthanized due to bacterial pneumonia first presented with unilateral lameness and staggering, which may have been ataxia or weakness; without a neurological examination, the two cannot easily be differentiated. The clinical signs that we observed were similar to those described in both typical and atypical hosts infected with *E. rangiferi* (which localizes in the leptomeninges and subarachnoid space), or with *P. tenuis* (which invades the grey matter of the dorsal horns before migrating to the subdural space and venous sinuses) (Handeland, 1994; Pybus et al., 1996; Anderson, 2000; Lankester, 2001). Clinical signs due to cerebrospinal parelaphostrongylosis and elaphostrongylosis are nonspecific, often multifocal, and difficult to localize (Anderson, 1968).

Clinical signs, as well as eosinophilic inflammation and hemorrhage in cerebrospinal fluid, would be considered diagnostic for cerebrospinal nematodiasis in domestic animals (Mayhew et al., 1976; DeLahunta, 1983). These findings in the thinhorn hybrids, combined with detection of parasite-specific antibody in the CSF (Dew et al., 1992), strongly suggest that *P. odocoilei* was present in the CNS and the cause of inflammation in the subarachnoid space, leptomeninges, and superficial dorsal regions of the spinal cord and nerve roots. Onset of clinical signs at 20 d prior to patency and cessation at patency are consistent with transient

migration of immature *P. odocoilei* through the central nervous system (CNS), similar to the route demonstrated for *E. rangiferi* (Hemmingsen et al., 1993) (Fig. 3.7). The recurrence of clinical signs and mild residual gait abnormalities in one animal following treatment suggests that, like *E. rangiferi*, some nematodes have prolonged residence in the CNS (Hemmingsen et al., 1993). Magnetic resonance imaging (MRI) of the spinal cord at this time did not reveal nematodes or lesions; MRI at the onset of clinical signs prior to patency might have been revealing (Wood et al., 1991), but was delayed until after treatment because animals would likely not have survived the necessary extended anesthesia.

Definitive neurological signs developed in only two of the five experimentally infected sheep. In addition to variation in host factors such as genetics and age, these two sheep had been previously exposed to *U. pallikuukensis*, which did not establish, but may have primed the immune system against protostrongylids. The spontaneous decline in larval shedding accompanied by marked eosinophilia in these two sheep suggest a strong immune response against *P. odocoilei*. Increased severity of inflammation associated with neural migration may have caused a normally subclinical process to become clinically evident. Alternatively, a stronger immune response in these two animals may have driven *P. odocoilei* into the "immunological harbour" of the CNS (Hemmingsen et al., 1993), implying that neural migration of *P. odocoilei* may not normally occur.

A neural migration for *P. odocoilei* and *P. andersoni* has, however, been suggested previously, based on the migration pattern of related parasites (Anderson, 1968), and because adults, eggs, and L1 of both "muscleworm" species have been found in the epidural tissues and vertebral canal in experimentally infected deer (Pybus, 1983; Pybus and Samuel, 1984a). In early patency two white-tailed deer experimentally infected with 5000 L3 of *P. andersoni*, and in

the PPP one mule deer infected with 300 L3 of *P. andersoni*, developed unilateral or bilateral posterior lameness and weakness, as well as postural and gait abnormalities that could, in retrospect, be neurological (Nettles and Prestwood, 1976; Pybus, 1983). Serial post mortem examinations of the CNS of experimentally infected hosts are needed to confirm the migration route of these "muscleworms"; to our knowledge, such examinations have not been accomplished for *P. odocoilei* or *P. andersoni* in the pre-patent period in any definitive host. No adult *P. odocoilei* were found upon gross examination of the vertebral canal or neural parenchyma of the three experimentally infected Stone's sheep examined after patency, nor from naturally infected Dall's sheep (E. Jenkins, S. Kutz, and A. Veitch, unpubl. data); however, detection may depend on the stage of infection, as well as techniques used for examination (Pybus and Samuel, 1984a). Further experimental work is needed to determine if neurological signs occur in thinhorn sheep infected with *P. odocoilei* at doses and methods of exposure comparable to naturally-acquired infections.

If detailed and timely examinations of the CNS of both experimentally and naturally infected hosts confirm the hypothesis that the "muscleworms" (*P. andersoni* and *P. odocoilei*) are neurotropic, the life-history patterns and phylogeny of the elaphostrongylines may need to be revisited (Platt, 1984; Carreno and Lankester, 1994). Migration through the CNS followed by establishment in skeletal muscles may be the ancestral pattern, occurring in all species of *Elaphostrongylus*, and possibly in *P. odocoilei* and *P. andersoni*. The CNS-only migration of *P. tenuis* may represent attenuation or loss of the ancestral pattern. If the "muscleworms" (*P. andersoni* and *P. odocoilei*) are neurotropic, this supports the alternative phylogeny outlined by Platt (1984), where *P. odocoilei* is the sister of *P. tenuis* and *P. andersoni*. Further experimental

work and molecular characterization are needed to fully resolve the phylogeny of the elaphostrongylines.

3.5.3 Management significance

Treatment for protostrongylid parasites in bighorn sheep has been a common, if controversial, management intervention (Schmidt et al., 1979; Jones and Worley, 1997; Miller et al., 2000), and there has been significant interest in the feasibility of such treatment in thinhorn sheep. Following parenteral ivermectin, neurological signs recurred and larval shedding ceased in one thinhorn hybrid infected with *P. odocoilei*. In the other thinhorn hybrid, which remained clinically normal following treatment, larval shedding was transiently suppressed but not eliminated, as reported in cervids infected with *P. andersoni*, *P. tenuis*, and *Elaphostrongylus cervi* (Kocan, 1985; Watson, 1987; Samuel and Gray, 1988). An extended monitoring period (at least 69 days in the current study) was necessary to detect resumption of larval shedding. Multiple treatments with oral fenbendazole did not eliminate shedding of *Protostrongylus* spp. larvae in at least one naturally infected Stone's sheep, or in a thinhorn hybrid experimentally infected with *Protostrongylus stilesi* (E. Jenkins and J. Skific, unpubl. data). For these reasons, anthelmintic treatment may not be effective or realistic for translocations or health management of thinhorn sheep infected with protostrongylid parasites.

Mixed infections with both *P. odocoilei* and *P. stilesi* are common in Dall's and Stone's sheep in the Subarctic (Jenkins et al., 2005a), and additive, or even synergistic, pulmonary pathology is likely (Petney and Andrews, 1998; Kutz et al., 2001c). Bighorn sheep (*O. canadensis*) undergo all-age die-offs due to respiratory disease (which may involve pre-existing lung damage by protostrongylid lungworms), and thinhorn sheep are also susceptible to fatal pneumonia (Foreyt et al., 1996; Jenkins et al., 2000). Thinhorn sheep inhabit a high-latitude, high-altitude environment, and are subject to predation by wolves. Parasites that cause

respiratory compromise at high infection intensities (such as *P. stilesi* in bighorn sheep, and *Echinococcus granulosus* in moose) may increase the susceptibility of ungulates to such chase-predators (Uhazy et al., 1973; Messier et al., 1989). As well, if *P. odocoilei* causes even minor, transient neurological signs in naturally infected thinhorn sheep, it could increase susceptibility to predation and falls in hazardous terrain. It would be interesting to compare health, fecundity, and causes of mortality in Dall's sheep populations infected with both *P. odocoilei* and *P. stilesi* (e.g. in the Mackenzie Mountains, NT) with nearby populations where only *P. stilesi* is present (e.g. in the Nahanni or Richardson Mountains, NT) (Hoberg et al., 2002; Jenkins et al., 2005a).

Dall's sheep in the Mackenzie Mountains will soon share range with muskoxen (*Ovibos m. moschatus*) infected with *U. pallikuukensis* (Kutz et al., 2004a). Although *U. pallikuukensis* does not establish in Dall's sheep, it may "prime" a stronger immune response against migrating *P. odocoilei*, resulting in clinical disease. While it is not known if muskoxen can be infected with *P. odocoilei*, muskoxen in the Richardson Mountains are naturally infected with *P. stilesi* from Dall's sheep, and elsewhere are susceptible to cerebrospinal elaphostrongylosis (Holt et al., 1990; Hoberg et al., 2002). Historical precedent for colonization of new host species by protostrongylid parasites (i.e., *P. stilesi* in muskoxen, and *P. odocoilei* in thinhorn sheep) and the potential health consequences for naïve hosts, warrant continued surveillance by wildlife managers.

The widespread geographic distribution, high prevalence, resistance to treatment, and the possibility of respiratory and neurological disease indicate that *P. odocoilei* may constitute a significant emerging disease risk for thinhorn sheep. Because of its wide host range and the possibility of a neural migration, *P. odocoilei* should be considered a differential diagnosis for dorsal-spined larvae in feces and cerebrospinal nematodiasis (often identified only on

histopathology) in a number of ungulate hosts, including mule and black-tailed deer, caribou, moose, mountain goat, bighorn and thinhorn sheep. Definitive diagnosis and differentiation from closely-related elaphostrongylines require comparative morphology of adult nematodes, or identification of larvae using validated molecular techniques.

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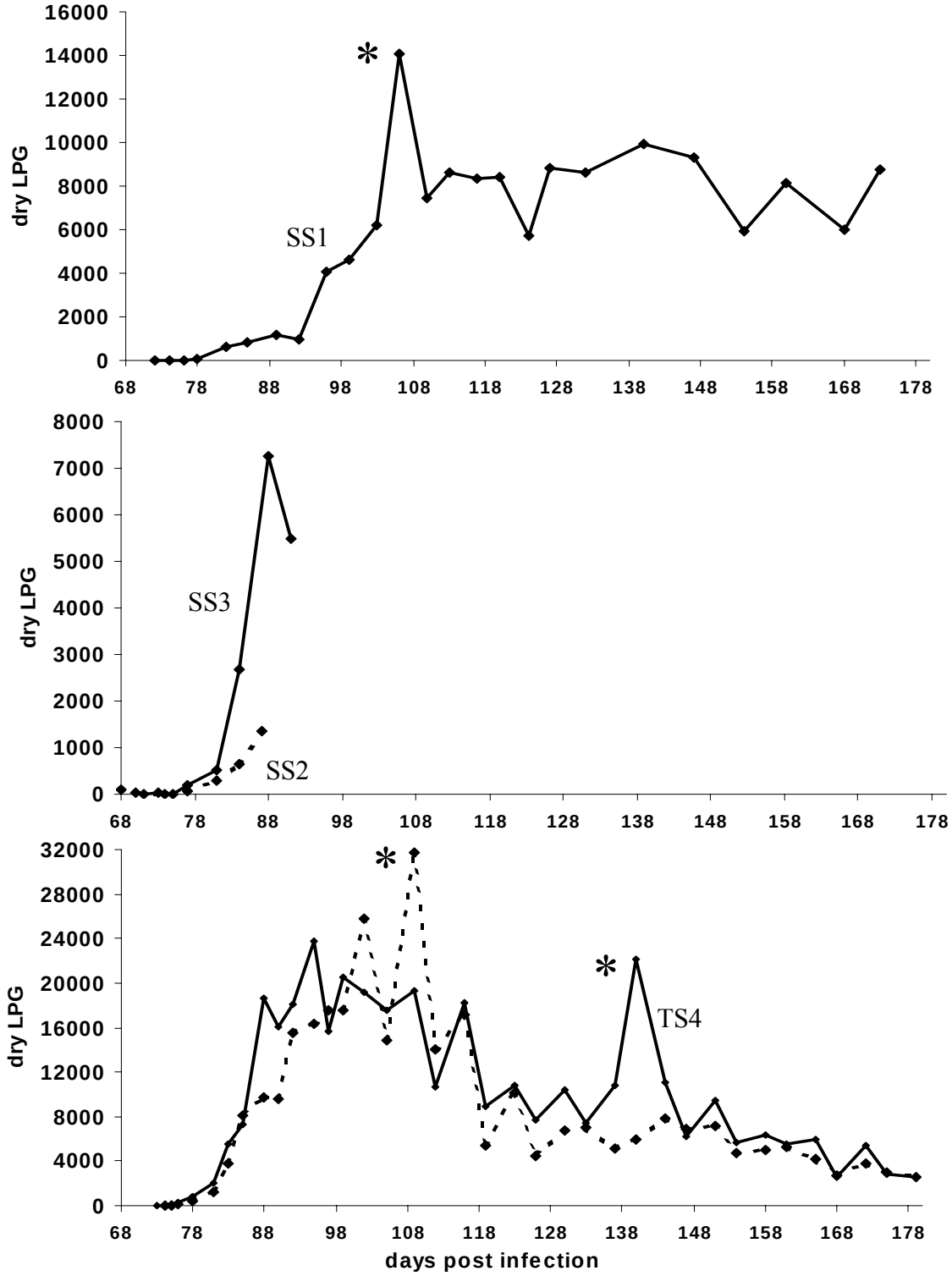

Figure 3.1: Patterns of larval shedding (in larvae per gram of dry feces) for thinhorn sheep experimentally infected with *P. odocoilei*.

Note different scales on y-axes. *Transient peaks following anesthesia and a monitoring procedure at 106 dpi (SS1), transfer to an unfamiliar room for fecal collection at 109 dpi (TS5), and fasting for a procedure at 140 dpi, which visibly caused stress (TS4).

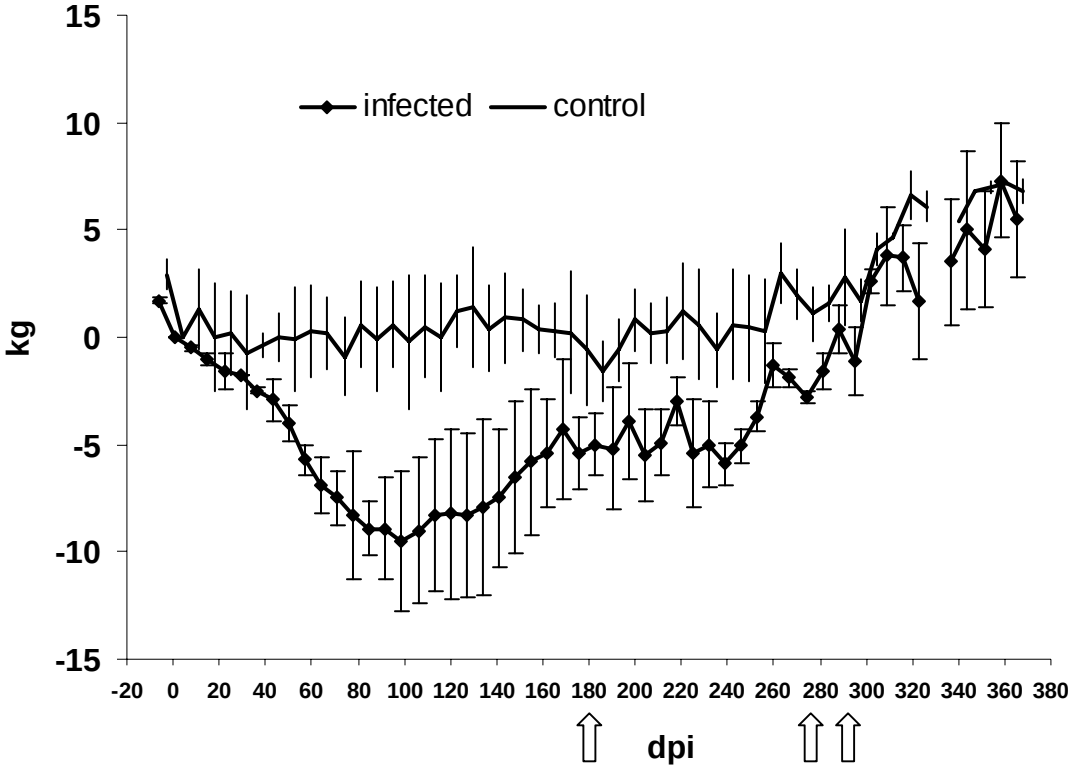

Figure 3.2: Weekly mean changes in weight relative to beginning of monitoring of infected (TS4 and TS5) and control thinhorn sheep (TS6 and TS7).

Dpi = days post infection, arrows indicate treatment with ivermectin, and error bars are +/- one S.D.

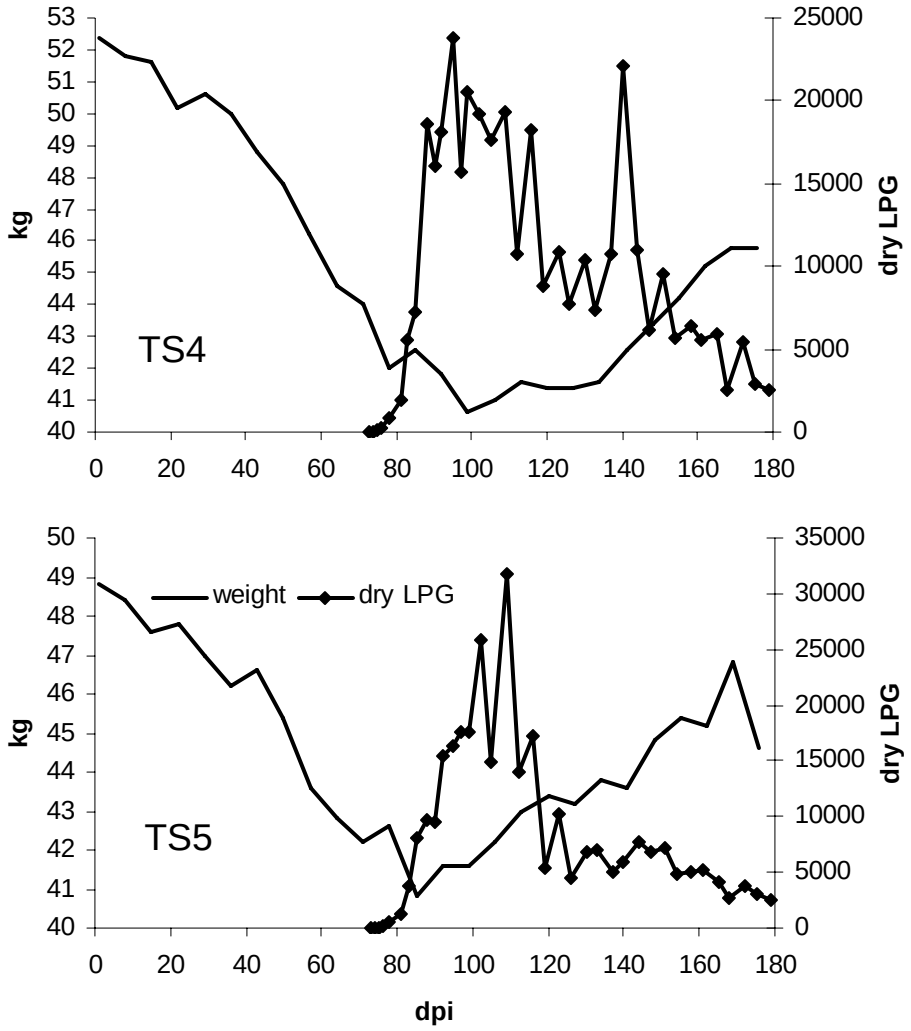

Figure 3.3: Weight (kg) and larvae per gram of dry feces (dry LPG) for infected thinhorn sheep (TS4 and TS5) prior to treatment.

Dpi = days post infection.

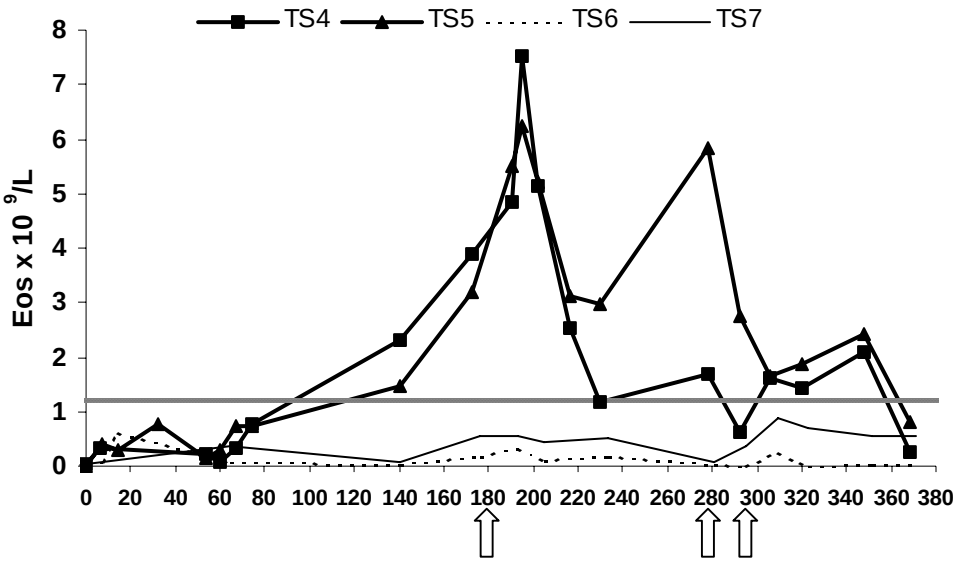

Figure 3.4: Peripheral eosinophil counts (Eos x 10^9/L) for infected (TS4 and TS5) and control (TS6 and TS7) thinhorn sheep.

Dpi = days post infection, arrows indicate treatment with ivermectin, and the hatched line corresponds to 1.2 x 10^9 eosinophils/L.

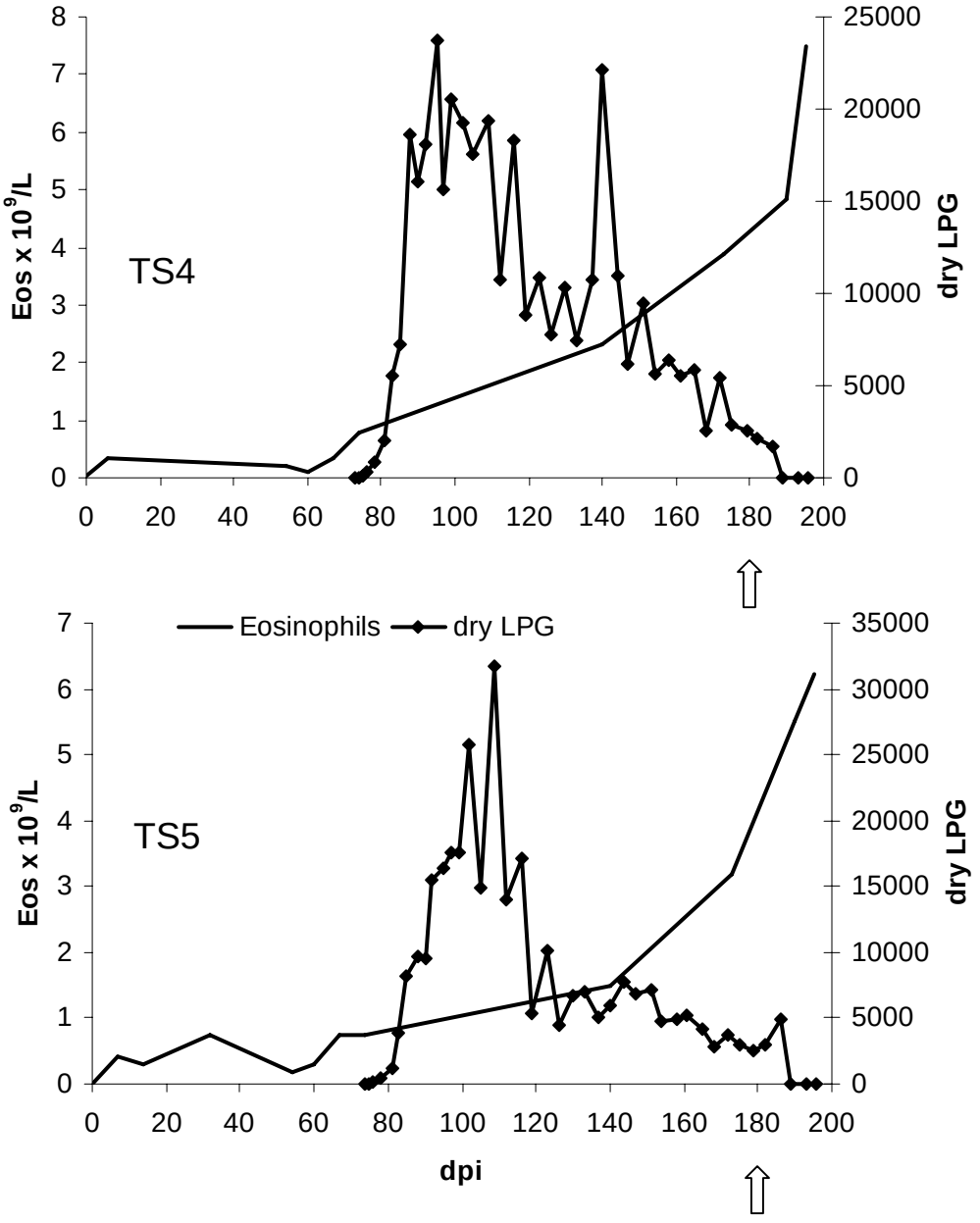

Figure 3.5: Peripheral eosinophil counts (Eos x 10^9/L) and larvae per gram of dry feces (dry LPG) for infected thinhorn sheep (TS4 and TS5).

Following the first treatment with ivermectin at 180 dpi (arrow), larval counts were not 0, as suggested by the scale of these graphs, until 200 dpi.

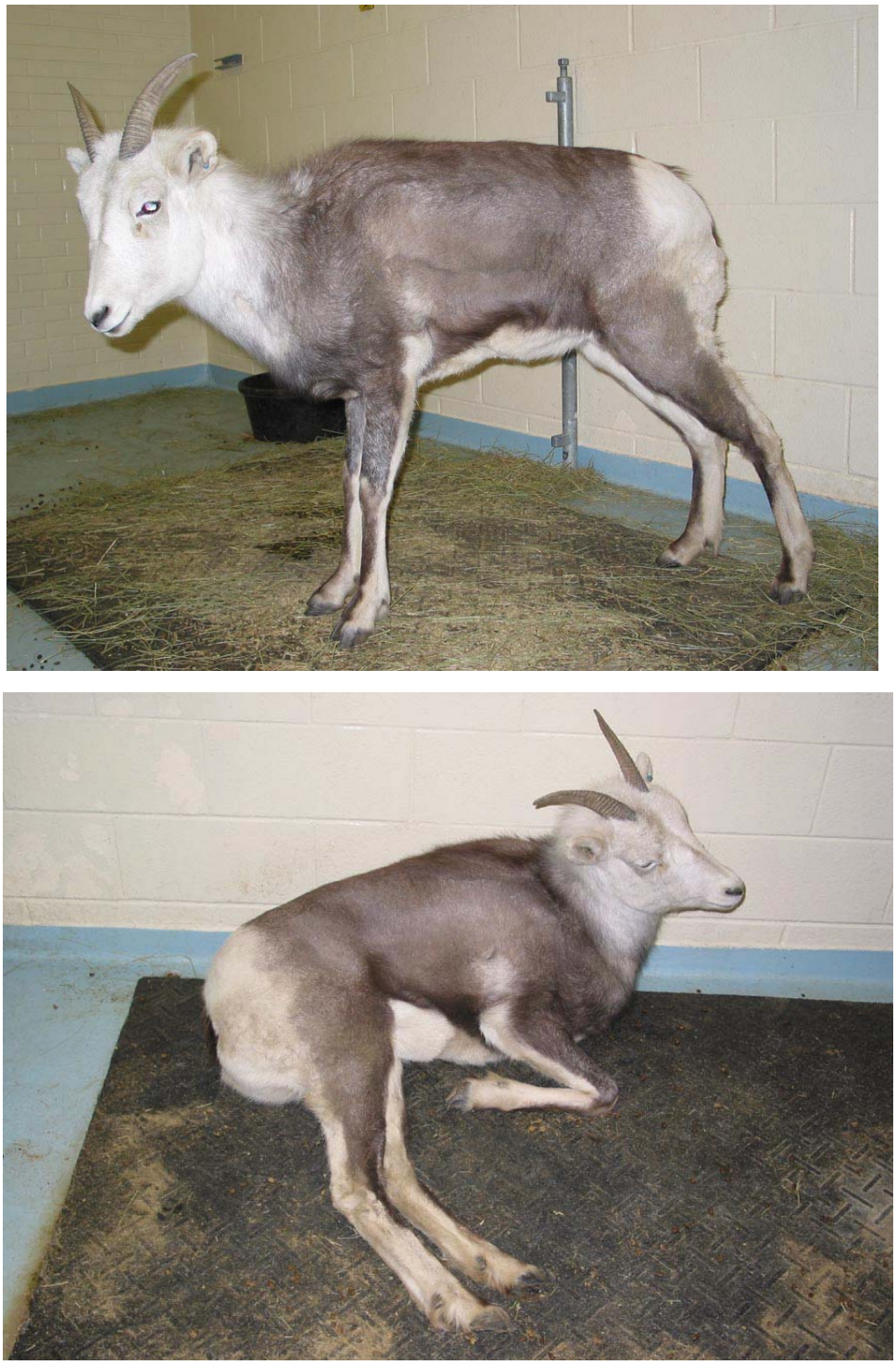

Figure 3.6: Abnormal posture of a thinhorn sheep (TS4) at 55 days following infection with *P. odocoilei*, 18 days prior to patency.

Note that the majority of weight is carried on the forelegs when standing, and the abnormally extended hind limbs, both standing and in sternal recumbency.

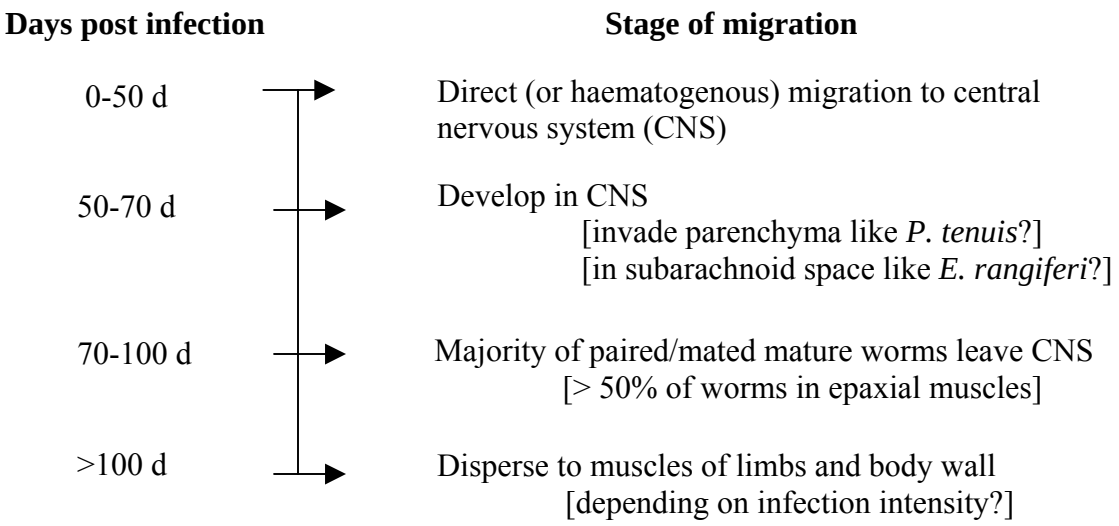

Figure 3.7: Proposed migration route for *P. odocoilei* in thinhorn sheep.

Based on the current study, findings in experimentally infected mule deer (Pybus and Samuel, 1984a), and the migration route of *E. rangiferi* in reindeer (Hemmingsen et al., 1993).

Table 3.1: Thinhorn sheep experimentally infected with 200 infective larvae of *Parelaphostrongylus odocoilei*.

ID	Species	Age (y)	Sex	Date of infection or placebo	PPP[a]	Peak LPG[b]	Dpi[c] peak	Dpi at end[e]
SS1	*Ovis dalli stonei*	2	M	08/02/00	72	14,071	106	173
SS2	*O. d. stonei*	16	F	03/14/02	68	NA[d]	NA	87
SS3	*O. d. stonei*	15	F	03/07/02	73	NA	NA	92
TS4	*O. dalli* hybrid	2	F	12/04/03	73	23,750	95	180
TS5	*O. dalli* hybrid	2	F	12/04/03	74	31,697	109	180
TS6	*O. dalli* hybrid	2	F	12/01/03	-	-	-	183
TS7	*O. dalli* hybrid	2	F	12/01/03	-	-	-	183

[a] pre-patent period
[b] larvae per gram of dry feces
[c] days post infection
[d] not available as animals died shortly after patency
[e] death (SS1 and SS3), euthanasia (SS2), or first treatment with ivermectin (TS4-TS7)

Table 3.2: Cerebrospinal fluid cytology and chemistry for thinhorn sheep experimentally infected with *Parelaphostrongylus odocoilei* (TS4 and TS5) and uninfected control animals (TS6 and TS7).

	TS4	TS5	TS6	TS7
Eosinophils (%)	67.5	70	0	NA[a]
Mononuclears (%)	31	28.5	100	NA
Neutrophils (%)	1	1.5	0	NA
plasma cells (%)	0.5	0	0	NA
Nucleated cells x 10^6/L	105	149	3	1
RBC x 10^6/L	1951	818	272	27
Total protein (g/L)	2.35	2.23	0.25	0.26

[a] not available due to low numbers of nucleated cells, which is normal in CSF

CHAPTER 4

4 LOCALIZATION AND PATHOLOGY

In this chapter[3], we describe and compare parasite localization in thinhorn sheep experimentally and naturally infected with *P. odocoilei*, and found post mortem evidence to support the hypothesis, suggested in Chapter 3, that this parasite undergoes a neural migration. Using a semi-quantitative scoring system for density and size of lesions associated with eggs and larvae of protostrongylids in histological sections of the lungs, we demonstrated seasonal patterns consistent with our understanding of the epidemiology of *P. odocoilei* and *P. stilesi* in Dall's sheep in the Mackenzie Mountains (Chapter 6). Diffuse verminous interstitial pneumonia associated with *P. odocoilei*, as well as chronic and acute bacterial pneumonia, were sporadic causes of mortality in this population of Dall's sheep. Along with co-authors and collaborators, I performed ante mortem monitoring of the respiratory system and post mortem examination of the 3 Stone's sheep experimentally infected in Chapter 3. As well, I designed, assembled, and analyzed sample collection kits for targeted sampling of hunter-killed Dall's sheep, and participated in necropsies, parasite recovery, and interpretation of pathology. I analyzed the data, and as corresponding author, wrote the manuscript with input from co-authors.

[3] E.J. Jenkins, A.M. Veitch, S.J. Kutz, T. Bollinger, M. Chirino-Trejo, B.T. Elkin, K. West, E.P. Hoberg, and L. Polley. Respiratory and neural pathology in thinhorn sheep (*Ovis dalli*) infected with protostrongylid parasites.
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4.1 Abstract

To describe the role of protostrongylid parasites (*Parelaphostrongylus odocoilei* and *Protostrongylus stilesi*) and other respiratory pathogens in the health of thinhorn sheep (*Ovis dalli*), we investigated parasite localization and associated pathology in over 50 naturally-infected Dall's sheep (*O. d. dalli*) from the Mackenzie Mountains, Northwest Territories, as well as in three Stone's sheep (*Ovis dalli stonei*) experimentally infected with *P. odocoilei*. Histological lesions in the brain and distribution of *P. odocoilei* in the muscles of experimentally and naturally infected sheep were consistent with a previously hypothesized 'central nervous system to muscle' migration pattern for *P. odocoilei*. In the lungs, size of granulomas associated with eggs of *P. odocoilei* and density of protostrongylid eggs and larvae in the cranial lung were significantly correlated with intensity of larvae of *P. odocoilei* in feces, and varied with season of collection. Due to marked seasonal effects on larval production and focal distribution of lesions in the lungs, prevalence of *P. stilesi* based on examination of lungs did not correlate with that based on presence of larvae of *P. stilesi* in feces. Diffuse, interstitial pneumonia due to *P. odocoilei* led to fatal pulmonary hemorrhage and edema following exertion in one experimentally infected Stone's sheep and one naturally infected Dall's sheep. Protostrongylid parasites, especially *P. odocoilei*, may be predisposing factors in the development of acute or chronic bacterial pneumonia (associated with *Arcanobacterium pyogenes*, *Pasteurella* sp., and *Mannheimia* sp.), which caused sporadic mortalities in this population of Dall's sheep. There was no evidence of respiratory viruses or bacterial strains characteristically associated with domestic animals, nor of an epizootic pattern of mortality due to respiratory disease in Dall's sheep of the Mackenzie Mountains.

4.2 Introduction

In 1998, the protostrongylids *Parelaphostrongylus odocoilei* and *Protostrongylus stilesi* were reported in Dall's sheep (*Ovis d. dalli*) in the Mackenzie Mountains, Northwest Territories (Kutz et al., 2001c). Subsequent widespread survey of Dall's sheep within the Mackenzie Mountains and elsewhere in Subarctic Canada revealed a prevalence of infection with these parasites approaching 100% (Jenkins et al., 2005a). In 1999, the first cases of fatal bacterial pneumonia were observed in Dall's sheep in the Mackenzie Mountains (Jenkins et al., 2000). This wildlife population (14,000 – 26,000 Dall's sheep in a 140,000 km^2 area) is a valuable resource for subsistence harvest and trophy hunting (Veitch et al., 1998). Despite this importance, little is known about disease-related mortalities in this or any other population of thinhorn sheep (Dall's and Stone's sheep, *O. d. stonei*) (Nielsen and Neiland, 1975; Hoefs and Cowan, 1979; Simmons et al., 1984; Demarchi and Hartwig, 2004).

In contrast, much is known about a multifactorial pneumonia complex in bighorn sheep (*Ovis canadensis*) that causes outbreaks with high rates of mortality throughout North America (Forrester, 1971). The role of protostrongylid lungworms (e.g., *P. stilesi*) in this pneumonia complex is controversial, but has lately been relegated to that of a predisposing factor (Spraker et al., 1984; Samson et al., 1987; Monello et al., 2001). Adults of the protostrongylid "muscleworm" *P. odocoilei* produce eggs which travel through the vasculature to the lungs, where they may cause respiratory disease in their hosts, including cervids and mountain goats (Brunetti, 1969; Platt and Samuel, 1978b; Pybus et al., 1984; Pybus and Samuel, 1984b). In Dall's sheep in the Mackenzie Mountains, *P. odocoilei* and *P. stilesi* cause additive or even synergistic pulmonary damage (Petney and Andrews, 1998; Kutz et al., 2001c). To isolate the effects of *P. odocoilei*, we experimentally infected and monitored thinhorn sheep. In the process, we described the first neurological syndrome associated with *P. odocoilei* in any definitive host,

and hypothesized a neural migration for this "muscleworm", based on clinical appearance and ante-mortem monitoring of captive thinhorn sheep (Jenkins et al., 2005b).

In the current study, we describe parasite localization and respiratory pathology associated with protostrongylids in three captive Stone's sheep experimentally infected with *P. odocoilei*, and compare to that in over 50 naturally infected Dall's sheep from the Mackenzie Mountains, NT. This is the first investigation of the effects and seasonal patterns of prevalence and intensity of protostrongylid parasites in the lungs of thinhorn sheep, and complements investigation of seasonal patterns of larval shedding in Dall's sheep in the Mackenzie Mountains (Jenkins et al., 2005d). In addition, we investigated disease-related causes of mortality in this population of Dall's sheep, including targeted sampling for pathogens that have been implicated in respiratory disease in domestic, bighorn, and captive Dall's sheep (Parks et al., 1972; Clark et al., 1985; Spraker and Collins, 1986; Black et al., 1988; Martin, 1996; Aune et al., 1998; Van Campen et al., 2003; Garde et al., 2005), or have been identified in free ranging Dall's sheep in Alaska based on serological evidence (Foreyt et al., 1983; Zarnke, 2000). To our knowledge, there are no investigations of population health in wild Dall's sheep in Canada available for comparison.

4.3 Materials and Methods

4.3.1 Experimentally infected Stone's sheep

Three captive Stone's sheep (SS1, SS2, and SS3) were each infected with 200 third-stage larvae of *P. odocoilei* originating from first-stage larvae (L1) shed by Dall's sheep in the Mackenzie Mountains (Jenkins et al., 2005b). Prior to infection with *P. odocoilei*, low numbers of larvae of *P. stilesi* were present in pooled feces from SS2 and SS3. In the pre-infection period, each sheep had bronchoscopy and bronchoalveolar lavage (BAL) twice, in which sheep were chemically immobilized, the trachea and larger bronchi were visually examined with a

fibreoptic endoscope, and 50 ml of sterile saline were inserted rapidly into the lower lung via a plastic tube and immediately aspirated a total of three times (adapted from Begin et al., 1981; Silflow and Foreyt, 1988; Meyer et al., 1998). Following infection, SS1 had BAL a total of 7 times at 2-4 week intervals from 28-161 days post infection (dpi), while SS2 and SS3 had BAL on 35, 63, and, for SS3 only, 91 dpi.

Examination of BAL fluid included determination of total protein levels, a differential count of 100-200 nucleated cells stained with Diff-Quick® (Dade Behring, Newark, Delaware), and, in SS2 and SS3, the proportion of 100 macrophages that stained with Perl's Prussian blue for hemosiderin (hemosiderophages). For SS1, BAL fluid was examined at 25X for L1. For SS2 and SS3, BAL fluid was centrifuged at 1200 RPM at 4°C for 12 minutes and sediment resuspended in 2% formalin, centrifuged again, and sediment resuspended in a drop of methylene blue and examined at 40-100X for the presence of eggs and L1 (modified Knott's test).

For SS1, thoracic radiographs were obtained at each BAL procedure. Images of transverse sections (7 mm, and also 1 mm at 126 and 173 dpi) through the entire lung field were obtained using a Hi Speed Computed Tomography/i® helical scanner (GE Medical Systems, Milwaukee, Wisconsin, at the Royal University Hospital, Saskatoon, Saskatchewan) at 37 days prior to infection, at 70 and 126 dpi, and on excised, inflated lungs at necropsy at 173 dpi (Cadore et al., 1994; Kutz et al., 1999b; Kutz et al., 2004a).

A routine necropsy was performed on each sheep, including gross examination of the brain, meninges, spinal cord, and spinal canal. As well, all of the skeletal muscles were examined for hemorrhages from which adult *P. odocoilei* were recovered (as per Kutz et al., 2001c). Fecal samples were collected, and sections taken for histology from all lung lobes,

brain, and for SS3 only, spinal cord. Lung sections from all three sheep were cultured for bacteria and, for SS2 and SS3, examined using immunohistochemistry for respiratory viruses, including respiratory syncytial virus (RSV), parainfluenza III (PI3), and infectious bovine rhinotracheitis (IBR) (Haines and Chelack, 1991).

4.3.2 Dall's sheep from the Mackenzie Mountains

We sent sample collection kits to the 8 outfitters who guide non-resident hunters (NH) in the Mackenzie Mountains (Fig. 4.1), and received opportunistic submissions of lungs (entire) and feces from Dall's sheep taken by resident hunters (RH) (Table 4.1). From NH and RH-killed sheep, when possible, 2x2 cm samples were collected from a site in the dorsal region of the left cranial lobe, and a caudo-dorsal site near the apex of the left diaphragmatic lobe. From NH killed rams, fecal samples, chest fluid/whole blood, and throat swabs (Cary-Blair or Amies transport media, BBL CultureSwab®, Becton-Dickinson, Sparks, Maryland, USA) were also collected. All samples were frozen within 48 hours of collection, and shipped to the Western College of Veterinary Medicine (WCVM), Saskatoon, Saskatchewan.

In November 2001, three ewes and one young ram (Fall Collected, FC 23-26, Tables 4.1 and 4.2) were randomly selected from the Katherine Creek area in the Mackenzie Mountains (65°01' N; 127°35' W) and shot. Samples were collected as for NH-killed rams, but processed immediately, including direct inoculation of swabs onto agar plates for culture, and separation of sera from whole blood. Airway washes were examined for adult lungworms (as per Oakley, 1980). As well, adult *P. odocoilei* were recovered from the left half of each carcass (Kutz et al., 2001c); in cervids, adult *P. odocoilei* were distributed evenly on the left and right sides (Pybus, 1983). The brain, meninges, spinal cord, and spinal canal were examined grossly, and sections of the brain were fixed for histology. We also obtained fixed lung tissue and data not previously reported from 6 adult ewes from the Mackenzie Mountains collected in October 1998 (FC 1-3)

and April 1999 (FC 5-7) (Kutz et al., 2001c). These "straggler" ewes, slower than others in the herd, were selected to maximize chances of parasite recovery.

Reports of 1-3, coughing, dyspneic, and lethargic rams with nasal discharge were received in each of Sept 99, Jul 01, and Sept 02. In two of these instances, one affected ram was shot and we received lung samples (Sick, SK13 and SK18, Table 4.3). Lastly, entire carcasses of Dall's sheep (Spring Collected, SC15, FC21, FC27, and all Mortalities, MO; Tables 4.1-4.3) that were found in the Mackenzie Mountains were kept cool, and shipped within a few days to WCVM for examination. At necropsy, samples were collected for histological and microbiological examination in order to recover pathogens and to determine cause of death. Estimates of body condition included calculating the fat percentage of bone marrow (Mech and Delgiudice, 1985). The brain and meninges were examined grossly and fixed for histology.

4.3.3 Sample processing

When the entire lungs were available, the parenchyma was examined grossly for lesions and airways were cut open and examined for adult lungworms. Samples for histology were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin by Prairie Diagnostic Services (PDS), Saskatoon, SK. Cranial and caudal lung sections were examined at 100X, and the presence of protostrongylid eggs, larvae, and adult nematodes recorded. Eggs of *P. odocoilei* were clearly distinguishable from freshly-deposited eggs of *P. stilesi*, although developing stages and larvae could not be differentiated unless the tails of the larvae were visible (Kutz et al., 2001c). Protostrongylid life stages (eggs and larvae) in ten, arbitrarily chosen, 100X fields in the cranial lung section were counted as an estimate of density. The dimensions (length and width) of the largest granuloma surrounding eggs of *P. odocoilei* observed in each section was measured using a calibrated slide micrometer (Pybus and Samuel, 1984b). When reading histology slides, the observer was blinded from the results of Baermann

analyses of fecal samples for protostrongylid larvae (Forrester and Lankester, 1997). For all Dall's sheep, the prevalence of infection with each protostrongylid was determined separately for lung and fecal samples, and the two methods were combined to obtain the overall prevalence. For Dall's sheep without bacterial pneumonia, density and size of granulomas in the cranial lung were compared with intensity of L1 of *P. odocoilei* in feces using Pearson's correlation coefficient (two-tailed, significant at the 0.01 level) (Statistical Package for the Social Sciences 12.0, SPSS Inc., Chicago, Illinois).

Bacteria isolated from throat swabs and lung samples were identified to genus, and when possible, to species level, using standard techniques by PDS and the Bacteriology Research Laboratory, WCVM. Sera from the four sheep collected in November 2001 were tested for antibodies to *Mannheimia haemolytica* (serotypes A1 and A2) and *Pasteurella trehalosi* (T10) in direct agglutination assays, as well as a leukotoxin neutralizing assay, by the Department of Pathobiology, Ontario Veterinary College, Guelph, Ontario, Canada. Sera from these four animals as well as chest fluid from seven sheep killed by hunters in 2001 were tested for antibodies to respiratory viruses (RSV, PI3, IBR), bovine viral diarrhea (BVD), maedi-visna virus, and *Mycobacterium paratuberculosis* by PDS. Histology sections from the lungs of seven sheep with bacterial pneumonia were stained using immunohistochemistry for antigens of *M. haemolytica* and respiratory viruses (IBR, PI3, BRSV), as well as *Mycoplasma* spp. (n=5), *Mycobacteria* spp. (n=2), *Haemophilus somnus* (n=2), and BVD (n=2) (Table 4.3).

4.4 Results

4.4.1 Experimentally infected Stone's sheep

In SS1, there were no abnormal findings on bronchoscopy, or on thoracic radiography. In all sheep, the majority of cells recovered from BAL were macrophages (70-99%) and small mononuclear cells/lymphocytes (0-29%), with few neutrophils (generally < 10%), eosinophils

(generally ≤ 2.5%), and hemosiderophages (≤2 of 100 macrophages). In BAL fluid from SS1, increased numbers of hemosiderophages were observed starting at 56 dpi, larvae of *P. odocoilei* were detected at 77 dpi, and total protein increased starting at 105 dpi. A diffuse reticulonodular infiltrate was first observed on CT of the lungs of SS1 at 126 dpi, and this sheep died of respiratory failure following exertion at 173 dpi (101 days after patency). At necropsy, nodules on CT of excised lungs corresponded with granulomas that were grossly visible on cut section and consisted of lymphocytes, plasma cells, and macrophages surrounding 3-13 eggs and larvae of *P. odocoilei* on histological examination (Fig. 4.2). In addition to generalized, granulomatous, interstitial pneumonia, there was diffuse pulmonary edema and hemorrhage (acute and chronic), with petechial and ecchymotic hemorrhages grossly visible on the pleural surface.

In BAL fluid from SS2, 27 protostrongylid eggs, 40 x 80 um in size, were detected at 35 dpi and one egg was present at 63 dpi. SS2 was euthanized at 87 dpi (19 days after patency), after developing bacterial pneumonia and unilateral hind limb "lameness", recumbency, and behavioural changes which may in retrospect have been neurological in origin. Chronic, locally extensive, fibrinopurulent bronchopneumonia and pleuritis were confined to the ventral portions of the cranial lobes and the right middle lung lobe, from which *Pasteurella* sp. and *Corynebacterium* sp. were isolated. There was little inflammatory response surrounding eggs of *P. odocoilei* in the lungs (Table 4.2). On histological examination of the cerebellum, there were discrete, multiple foci of eggs and developing larvae of *P. odocoilei* associated with encephalomalacia and, at one site, hemorrhage (Fig. 4.3). There was lymphoplasmacytic cuffing of blood vessels nearby and in the choroid plexus, as well as plasma cells and hemosiderophages in the meninges of the medulla.

In BAL fluid from SS3, *Protostrongylus* spp. larvae were observed prior to, but not after, infection with *P. odocoilei*. One protostrongylid egg was recovered from BAL fluid at 63 dpi, and larvae of *P. odocoilei* and increased numbers of hemosiderophages were present in BAL fluid at 91 dpi. SS3 died at 92 dpi (19 days after patency) of unknown causes, possibly as a complication of anesthesia and old age. There were high densities of eggs and larvae of *P. odocoilei* in the lungs, surrounded by granulomas smaller than those observed in SS1 (Table 4.2). There was no evidence of infection with respiratory viruses in the lungs of SS2 or SS3.

In the muscles of SS1-SS3, adult *P. odocoilei* were associated with inflammatory infiltrates and chronic hemorrhage in fascial planes. Tails of female nematodes were frequently found inside blood vessels. In SS1, adult worms were evenly distributed throughout the body, in SS2 the majority were in the axial and trunk muscles, and in SS3 the majority were in the hindquarters (Table 4.2).

4.4.2 "Healthy" Dall's sheep

Lung samples from a total of 47 Dall's sheep with no evidence of active bacterial pneumonia were examined (Table 4.1). For animals in which the whole lungs were available (n=20), petechial and ecchymotic hemorrhages were observed on the visceral pleura throughout the lung, and firm, pale, consolidated lesions typical of *P. stilesi* were grossly visible on the dorsum and apices of the diaphragmatic lobes of many sheep (Fig. 4.4). Chronic fibrous adhesions between the lung and chest wall were reported or observed in a few sheep. No nematodes, such as *P. rushi* or *Dictyocaulus* spp., were observed in the airways of any sheep.

Eggs of *P. odocoilei* were frequently observed within alveolar septa near small capillaries (and occasionally within capillaries), and were surrounded by granulomatous inflammation, with the occasional eosinophil and multinucleated giant cell. Density and size of granulomas in the cranial lung were positively and significantly correlated with intensity of larvae of *P. odocoilei*

in feces (Pearson's coefficient 0.729 for density, 0.630 for granuloma size, n=47, p<0.001). Density, granuloma size, and larval counts were higher in sheep collected in spring than those collected in summer and fall (Table 4.1). In SC straggler sheep (SC5-7), but not FC straggler sheep (FC1-3), granulomas coalesced and surrounded terminal and respiratory bronchioles, leading to impairment of entire alveolar units (bronchiolitis obliterans) (Fig. 4.5). One yearling (SC15) died of respiratory failure following exertion, with gross (Fig. 4.4a) and histological (Fig. 4.5c) lesions similar to those observed in SS1.

Protostrongylid larvae (both *P. odocoilei* and *P. stilesi*) were observed in the lumen of airways (in one instance, embedded in the epithelium), in alveolar spaces, and in the alveolar interstitium of both cranial and caudal lobes. Lesions associated with adults of *P. stilesi* that were actively producing eggs were locally severe, including lymphoplasmacytic, granulomatous inflammation and fibrosis, and were frequently (but not always) limited to the caudal lobes. Adult *P. stilesi* without associated eggs or developing larvae were observed, especially in summer and fall collected sheep, and generally elicited little inflammatory reaction. The prevalence of *P. stilesi* in the lungs was greater than that based on feces in sheep collected in summer and fall, whereas for *P. odocoilei*, prevalence based on lungs and feces were similar in sheep collected in all seasons (Table 4.1).

The majority of adult *P. odocoilei* were in the appendicular musculature of sheep collected in spring (81% of adults) and fall (66%) (Table 4.2). No nematodes were observed in the CNS on gross examination, but in SC15, focal eosinophilic and lymphoplasmacytic meningitis, as well as hemosiderophages, surrounded a blood vessel deep in the cerebral cortex (Fig. 4.3c).

Arcanobacterium pyogenes was isolated from throat swabs plated immediately following collection from two healthy sheep. Other bacteria isolated were consistent with normal fauna of the upper respiratory and gastrointestinal tract (*Streptococcus* spp., usually alpha or non-hemolytic, *E. coli*, *Staphylococcus* sp., *Enterobacter* sp., *Enterococcus* sp., and *Lactobacillus* sp.). There were low titres (1:12 to 1:108) of antibody to a pestivirus related to BVD in 3 of 11 samples from 'healthy' sheep. Otherwise, there was no serological evidence of exposure to bacterial or viral pathogens of domestic livestock.

4.4.3 Sick and dead Dall's sheep

We examined samples from two sick sheep that were shot (SK), and nine carcasses of sheep found dead (MO), with the diagnoses and pathogens reported in Table 4.3. Older ewes were frequently lactating and in poor body condition. Most submissions were from the northern half of the Mackenzie Mountains in the NT, with one case from the southern half and one from the Yukon Territory (Fig. 4.1). In the northernmost zone in September 1999, three adult sheep were found dead in close proximity, with no visible signs of predation or trauma, but necropsies were not performed.

In sheep with bacterial bronchopneumonia, the cranial and middle lung lobes, and occasionally the cranial portion of the diaphragmatic lobes, were consolidated, with purulent foci ranging from 1 mm to several cm in diameter (Fig. 4.6). Fibrinous and fibrinopurulent pleuritis, as well as fibrous adhesions, were also observed. The caudal portions of the diaphragmatic lobes were less affected by the bronchopneumonia, but parenchymal lesions associated with *P. stilesi* were frequently observed. On histological examination, normal lung architecture was obliterated by focal necrosis and abscesses. Protostrongylid eggs and larvae in various stages of decomposition could occasionally be seen on the periphery of "microabscesses", and larvae were observed in the midst of necrotic/bacterial debris in airways (Fig. 4.6). In these sheep, both *P.*

odocoilei and *P. stilesi* had higher prevalence based on lung histology than on detection of larvae in feces (Table 4.1).

4.5 Discussion

4.5.1 Localization of *P. odocoilei* in CNS and muscles

Contrary to current hypotheses about migration patterns among species of *Parelaphostrongylus* (Platt and Samuel, 1978b; Anderson, 2000; Lankester, 2001), *P. odocoilei* may have a neural migration in thinhorn sheep (Jenkins et al., 2005b). Histological lesions (hemorrhage, malacia, and inflammation) in the brains of experimentally and naturally-infected sheep were similar to those reported for *P. tenuis* and *E. rangiferi*, which establish in or migrate through the CNS (Anderson, 1965; Lankester and Northcott, 1979; Handeland and Norberg, 1992; Lankester, 2001). Based on clinical signs in the pre-patent period in experimentally infected thinhorn sheep (Jenkins et al., 2005b), immature adult *P. odocoilei* may migrate through the CNS during the pre-patent period, as observed for species of *Elaphostrongylus* (Roneus and Nordkvist, 1962; Handeland and Norberg, 1992; Hemmingsen et al., 1993). As eggs were present in neural parenchyma, some nematodes may remain during the reproductive phase, although we cannot rule out the possibility that eggs of *P. odocoilei* in the brain of a captive Stone's sheep (SS2) may have been of hematogenous origin. Migration of adult *P. odocoilei* from the CNS into the surrounding axial and trunk muscles may be followed by dissemination throughout the musculature of thinhorn sheep, consistent with observations in deer (Pybus and Samuel, 1984a). Adult *P. odocoilei* have been observed in connective tissue beneath the spine in black-tailed deer (Hobmaier and Hobmaier, 1934), and in the epidural space and in association with nervous tissue in muscles of mule deer killed in early patency (Pybus, 1983).

Two yearlings (FC24 and FC27) and an 11 month old lamb (SC15) were naturally infected with *P. odocoilei* (Table 4.2). Young of the year likely become infected in late summer

and early fall of their first year, based on the epidemiology of *P. odocoilei* in this population of Dall's sheep and in mule deer (Samuel et al., 1985). If our hypothesis about the timing of the neural migration is correct, examination of young of the year in September-October may demonstrate the presence of adults or developing fourth-stage larvae in the CNS. Examination of the CNS of adult animals in October-November did not reveal evidence of *P. odocoilei*; however, nematodes may only be present in the CNS of young of the year, as observed for *E. alces* (Handeland and Gibbons, 2001) and *E. cervi* (Handeland et al., 2000a). Dall's sheep infected as lambs may be subsequently immune to infection, as suggested for *P. andersoni* and *P. tenuis* (Prestwood and Nettles, 1977; Slomke et al., 1995; Lankester, 2001), with the long-life span of adult *P. odocoilei* accounting for its high prevalence in sheep.

4.5.2 Protostrongylids in lungs of thinhorn sheep

Pathogenicity of *P. odocoilei* in the lungs of thinhorn sheep primarily depended on density of eggs and larvae, and chronicity and magnitude of the inflammatory reaction. Some lung pathology may occur in the pre-patent period, as protostrongylid eggs were detected in BAL fluid of two captive Stone's sheep before larvae were present in feces. While these could have been eggs of *Protostrongylus* spp., the dimensions were consistent with those of *P. odocoilei* (Hobmaier and Hobmaier, 1934). Following patency in experimentally infected thinhorn sheep, as for mule deer (Pybus and Samuel, 1984b), granuloma size increased with time since infection. In naturally infected sheep, seasonal patterns in larval shedding correlated with granuloma size and density in the cranial lung (Table 4.1).

Using staging adapted from interstitial lung disease of humans (Crystal et al., 1981), stage 1 would be considered mild alveolitis and cuffing of capillaries associated with eggs and larvae of *P. odocoilei* (i.e. in SS2 killed in early patency). In stage 2, granulomas form and distort alveolar septa, but remain discrete, as in most sheep collected in summer and fall (Fig.

4.5). In stage 3, coalescing granulomas cause diffuse distortion of the lung architecture and may compromise vascular walls, terminal respiratory bronchioles and associated alveolar units (Fig. 4.5). Stage 3 pathology was observed in "straggler" sheep collected in spring, the season of peak larval shedding in this population (Jenkins et al., 2005d), and was comparable to that observed in experimentally infected Stone's sheep (Table 4.2). It is likely that increased blood pressure during exertion led to edema and fatal hemorrhage in one experimentally (SS1) and one naturally infected sheep (SC15), similar to stress failure of the capillary walls observed in exercise-induced pulmonary hemorrhage in racehorses (Donaldson, 1991; West et al., 1993). Based on observations of interstitial lung disease in people and domestic sheep (Crystal et al., 1981; Collie et al., 1993), hypoxemia due to reduced lung volumes, compliance, and gas exchange may compromise respiratory function of Dall's sheep, especially at high altitudes and when escaping predation.

On CT, eggs and larvae of *P. odocoilei* were distributed diffusely throughout the lung (probably due to hematogenous arrival), and therefore even 2x2 cm samples were likely representative of the changes throughout the lung and amenable to a semi-quantitative approach (Hobmaier and Hobmaier, 1934; Crystal et al., 1981; Pybus and Samuel, 1984b; Cadore et al., 1994). In contrast, lesions associated with *P. stilesi* were focal and frequently limited to the caudodorsal tips of the diaphragmatic lung lobes, and are probably best assessed using a surface-area scoring system (Forrester and Senger, 1964; Uhazy et al., 1973), or perhaps computed tomography (Kutz et al., 1999b). Prevalence and intensity of *Protostrongylus* spp. larvae in feces were unreliable indicators of prevalence and pathology associated with *P. stilesi* in the lungs, especially in summer and fall, when we observed adult nematodes in the lungs of sheep which were not shedding *Protostrongylus* spp. larvae in feces. In summer, there was a marked

drop in prevalence and intensity of shedding of larvae of *P. stilesi* in this population of Dall's sheep (Jenkins et al., 2005d), possibly due to seasonal decreases in parasite fecundity or immune-mediated destruction of eggs and larvae (Gaudernack et al., 1984). Infections with *P. stilesi* were present in Dall's lambs as young as 2 mo old (Table 4.3), suggesting that transplacental transmission, similar to that reported for *P. stilesi* in bighorn sheep (Hibler et al., 1974), may also occur in thinhorn sheep.

Protostrongylus rushi, the airway dwelling lungworm of bighorn sheep and mountain goats, does not appear to be prevalent in Dall's sheep in the NT, although it has been reported in the Yukon Territory (Kutz et al., 2001c). It is possible that maintenance of this parasite in thinhorn sheep requires sympatry with mountain goats, which are present in only a few isolated pockets in the Mackenzie Mountains (Veitch et al., 2002). Alternatively, other historical (such as founder effects) or ecological conditions may have prevented establishment of *P. rushi* in Dall's sheep in the NT.

4.5.3 Pneumonia in thinhorn sheep

Granulomatous interstitial pneumonia associated with *P. odocoilei* and *P. stilesi* (verminous pneumonia) was present in almost all Dall's sheep examined from the Mackenzie Mountains. Diffuse, severe respiratory pathology associated with *P. odocoilei* was a direct, if infrequent, cause of mortality in thinhorn sheep, similar to naturally infected black-tailed deer with "overwhelming parasitemia" (Brunetti, 1969) and "indurations" of the lungs (Hobmaier and Hobmaier, 1934), and to experimentally infected mule deer (Pybus and Samuel, 1984b). The interstitial pneumonia associated with *P. odocoilei* appears similar to that reported for other elaphostrongylines and metastrongyles (Nettles and Prestwood, 1976; Stockdale, 1976; Pybus and Samuel, 1984b; Handeland, 1994), except that peak larval shedding ($\geq$ 15,000 LPG) in mule deer and thinhorn sheep experimentally infected with *P. odocoilei* was much higher than for

other elaphostrongylines, regardless of infective dose or Baermann technique (Platt and Samuel, 1978b; Pybus and Samuel, 1984a; Lankester, 2001; Jenkins et al., 2005b).

While the "role of any nematode in the pneumonia complex of bighorn sheep is poorly understood" (Demartini and Davies, 1977), as for humans with interstitial lung disease (Crystal et al., 1981), diffuse interstitial pneumonia caused by protostrongylids (especially *P. odocoilei*) may predispose sheep to bacterial pneumonia. Lesions associated with *P. stilesi* were generally limited to the caudo-dorsal lobes, where they may compromise the only remaining functional lung tissue in sheep with concurrent lesions of bacterial bronchopneumonia (which typically have a cranio-ventral distribution). In Dall's sheep with active bacterial pneumonia, detection of protostrongylid infection based on larval shedding in feces was unreliable, probably due to inflammatory destruction and mechanical trapping of larvae in the airways similar to that described for bighorn sheep (Spraker et al., 1984; Onderka and Wishart, 1984; Schwantje, 1986). If relying on fecal larval counts, managers may underestimate the prevalence of protostrongylids in sheep with concomitant bacterial pneumonia, which further complicates interpretation of the role of these parasites as predisposing factors.

Bacterial pneumonia caused clinical illness and mortality in 9 Dall's sheep examined from the Mackenzie Mountains between 1999 and 2002. Chronic, fibrinopurulent bronchopneumonia was associated with opportunistic bacterial invaders (*A. pyogenes* and *Mannheimia* spp.), and frequently also with lactation, emaciation, and dental disease (for details see Jenkins et al., 2000). The emaciated condition of these animals may be a predisposing factor, or a consequence of the disease process, as reported in free ranging bighorn sheep and captive thinhorn sheep (Spraker and Hibler, 1982; Black et al., 1988). Other animals died acutely in good body condition with fibrino-necrotizing bronchopneumonia, also associated with *A.*

pyogenes and *Mannheimia* spp. Differences in virulence of bacterial strains or host susceptibility may account for the two types of bacterial pneumonia (chronic and acute). In four cases, groups of 2-3 sick and dead sheep, often rams, were observed, suggesting direct transmission or a common predisposing factor. Increased scrutiny by hunters may be responsible for this gender bias; however, older rams experienced greater losses in one pneumonia epizootic in bighorn sheep (Onderka and Wishart, 1984).

Bacterial species and lung pathology in Dall's sheep (and one captive Stone's sheep) were similar to those observed in outbreaks of pneumonia in bighorn sheep (Spraker et al., 1984; Schwantje, 1986; Aune et al., 1998). *Arcanobacterium pyogenes* was isolated from the pharynx of healthy sheep as well as from pneumonic and septicemic Dall's sheep in the Mackenzie Mountains, and is commonly isolated from mandibular osteomyelitis (lumpy jaw) in Dall's sheep (Glaze et al., 1982; Bowyer and Leslie, 1992). The strains of *Mannheimia* sp. present in Dall's sheep in the NT (based on serology and immunohistochemistry) and in Alaska (Jaworski et al., 1998; Kelley et al., 2005) differed from strains described in bighorn and domestic sheep. We did not recover any *Pasteurella/Mannheimia* spp. from pharyngeal swabs, even those plated immediately on blood agar; for hunter-killed sheep, poor recovery may have been due to the choice of transport media, freezing, and long periods between collection and culture (Ward et al., 1997). Collection and freezing of tonsillar biopsies of Dall's sheep (Wild and Miller, 1991; Foreyt and Lagerquist, 1994), as well as strain typing of *Pasteurella/Mannheimia* spp., are recommended for future investigations (Jaworski et al., 1998; Miller, 2001; Weiser et al., 2003). Although we found minimal evidence of exposure to the respiratory viruses common in bighorn and domestic sheep, tests designed for pathogens of domestic animals would probably not detect other viruses unique to Dall's sheep.

4.5.4 Management significance

The effects of disease on wildlife populations are difficult to demonstrate, and range from obvious mortality to subtle, subclinical effects, which include reduced growth rate, fecundity, and body condition (for review see Morgan et al., 2005). *Parelaphostrongylus odocoilei*, a newly-recognized pathogen of thinhorn sheep, has been linked to poor body condition and respiratory failure in mountain goat, mule deer, and thinhorn sheep (Pybus et al., 1984; Pybus and Samuel, 1984b; Jenkins et al., 2005b), and caused neurological disease and pathology in experimentally infected thinhorn sheep. Therefore, *P. odocoilei* has the potential to cause population level effects, and may contribute to mortality, especially of young of the year in their first winter.

The role of protostrongylids in the multifactorial pneumonia complex (involving verminous, bacterial, and sometimes viral etiologies) of bighorn sheep has been the subject of intense discussion and controversy. The current study highlights the importance of distinguishing the health significance of verminous interstitial pneumonia (likely present in almost all wild sheep, healthy or otherwise, and fatal only in the end stages) from that of bacterial bronchopneumonia (generally associated with clinical illness and often mortality). The diffuse interstitial pneumonia caused by *P. odocoilei* probably leads to greater respiratory compromise than the focal interstitial pneumonia caused by *P. stilesi*, and may play a predisposing role in the development of bacterial pneumonia. In sheep with concomitant verminous and bacterial pneumonia, shedding of protostrongylid larvae in feces was reduced or eliminated, suggesting that fecal larval counts are unreliable indicators of infection status in these animals.

Thinhorn sheep occupy most of their historic range at high densities and abundance (Heimer et al., 1992; Worley et al., 2004), and no disease-related die-offs have been reported

(Hoefs and Cowan, 1979; Dau, 1981; Bowyer et al., 2000). As thinhorn sheep have not been extensively translocated (Heimer and Taylor, 1996; Hatter and Blower, 1996; Veitch, 1996) and have had minimal opportunities for contact with domestic livestock, the pathogen fauna of thinhorn sheep may reflect the endemic pathogen fauna of wild sheep prior to contact with domestic animals (Uhazy et al., 1971; Nielsen and Neiland, 1975). Domestic livestock harbor parasites, viruses, and strains of bacteria to which thinhorn sheep are naïve, but likely susceptible (Nielsen and Neiland, 1975; Foreyt et al., 1996; Zarnke, 2000; Kelley et al., 2005; Garde et al., 2005). Thinhorn sheep are also likely susceptible to the same ecological factors (such as habitat fragmentation and degradation) that have hampered conservation efforts for bighorn sheep. Therefore, efforts to avoid translocation, contact with domestic animals, and anthropogenic stressors are a sound basis for proactive management for thinhorn sheep in North America, and may forestall the breakdown of protective ecological barriers.

4.6 Acknowledgements

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Outfitters, guides, resident, and non-resident hunters in the remote Mackenzie Mountains, as well as wildlife officers, technicians, and pilots, were involved in surveillance, storage, and shipping of carcasses and samples. Without their cooperation and "eyes on the land", this project could not have been accomplished. Processing was a joint effort between members of the

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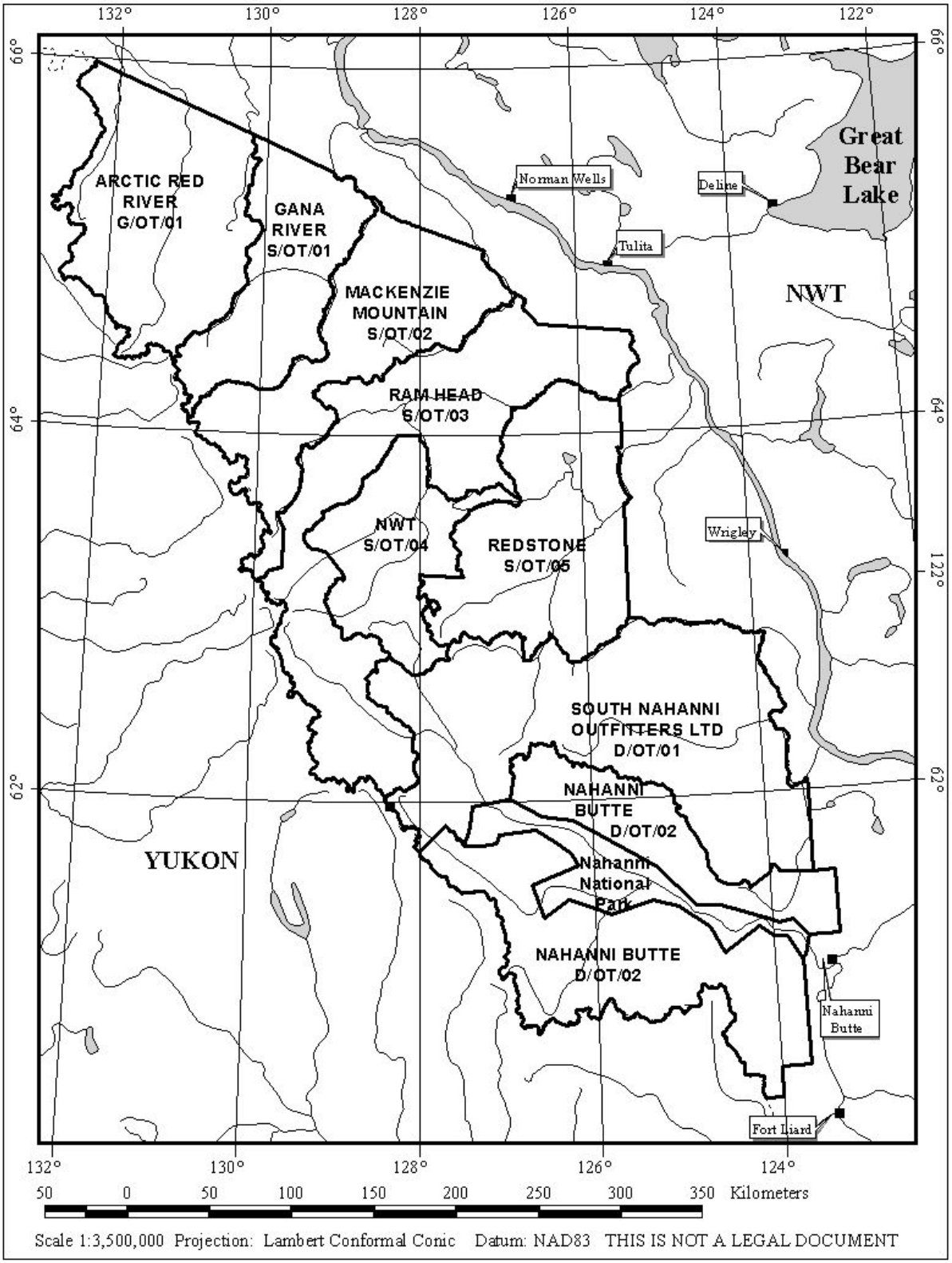
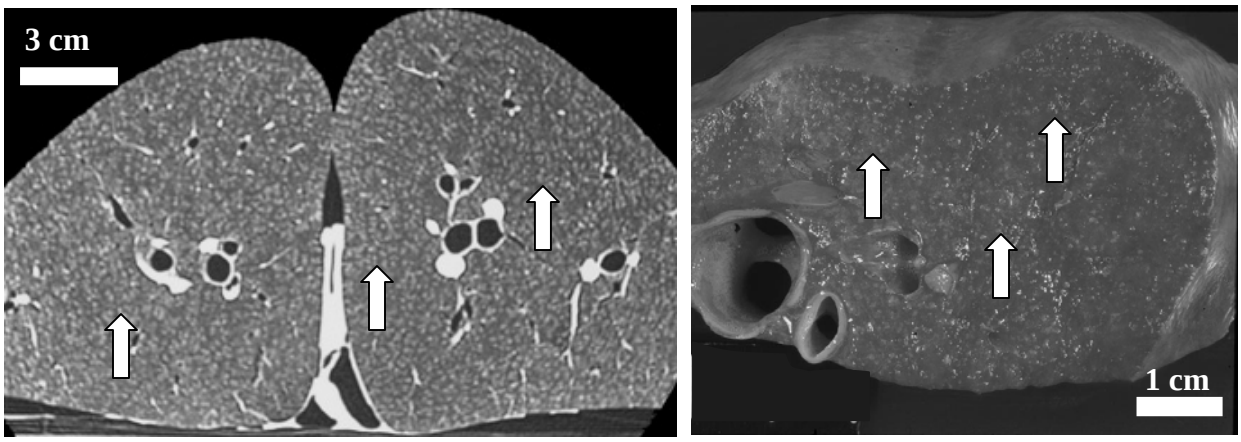
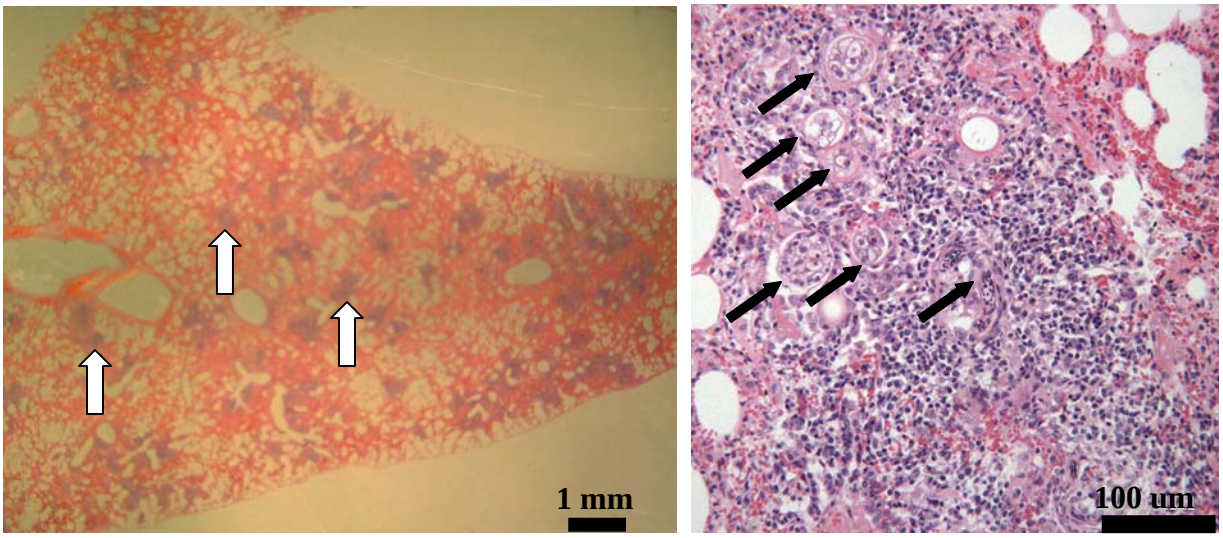


Figure 4.1: Outfitting zones and Nahanni National Park in the Mackenzie Mountains, Northwest Territories, Canada.
Courtesy of the Sahtu GIS project and A. Veitch.



a) b)

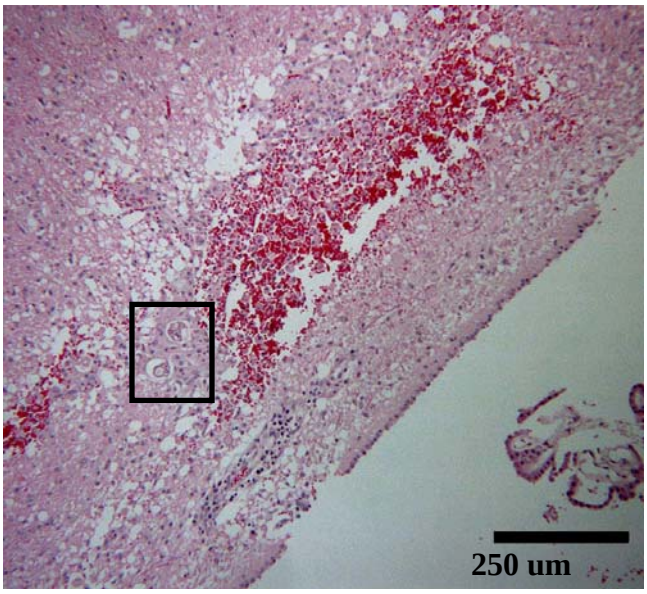


c) d)

Figure 4.2: Granulomas (white arrows) associated with eggs and larvae of *P. odocoilei* (black arrows) in the lungs of an experimentally infected thinhorn sheep (SS1).

a) computed tomography, 1 mm section through entire lungs; b) cut section at necropsy; c) histological sections at 7X; and d) 200X.

a)



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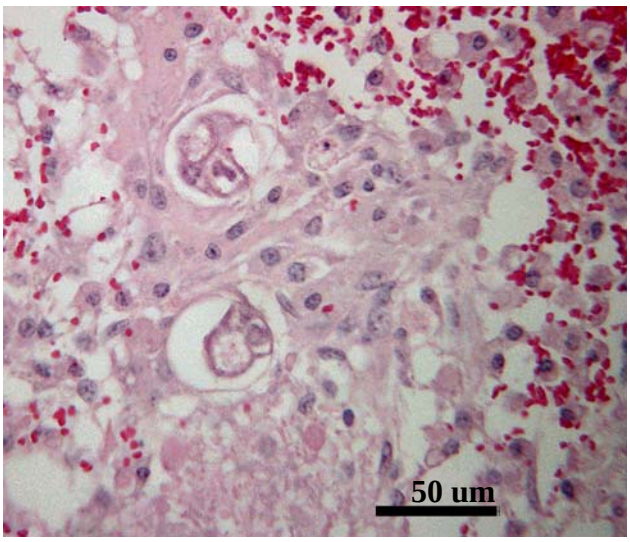

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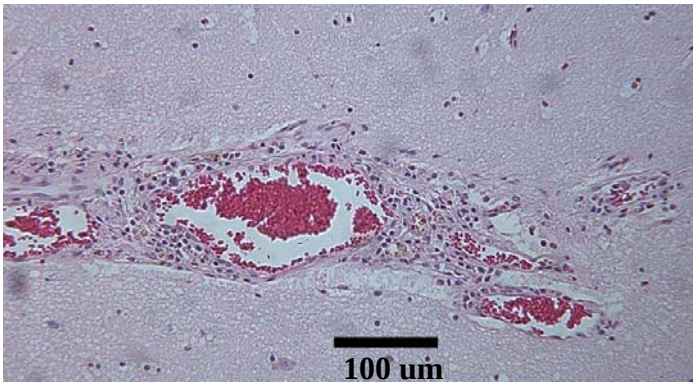
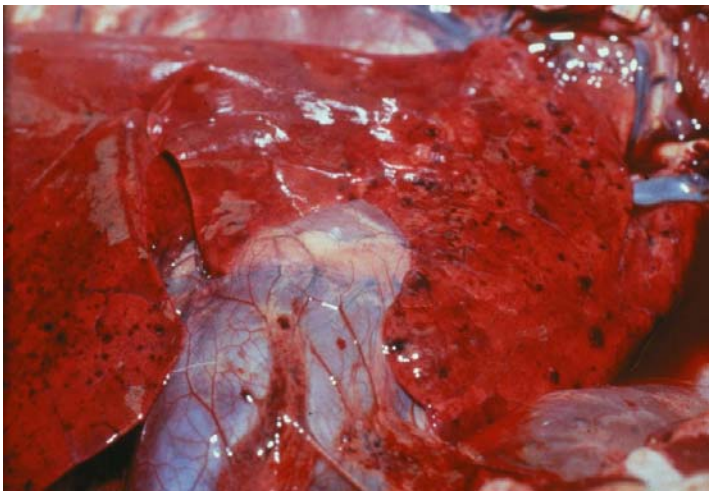


Figure 4.3: Neural lesions associated with *P. odocoilei* in experimentally (SS2) and naturally (SC15) infected thinhorn sheep.

a) Hemorrhage and encephalomalacia in cerebellum of SS2 b) Inset, eggs of *P. odocoilei*. c) Lymphocytes, plasma cells, eosinophils, and haemosiderophages surround a blood vessel in the cerebral cortex (SC15).

a)



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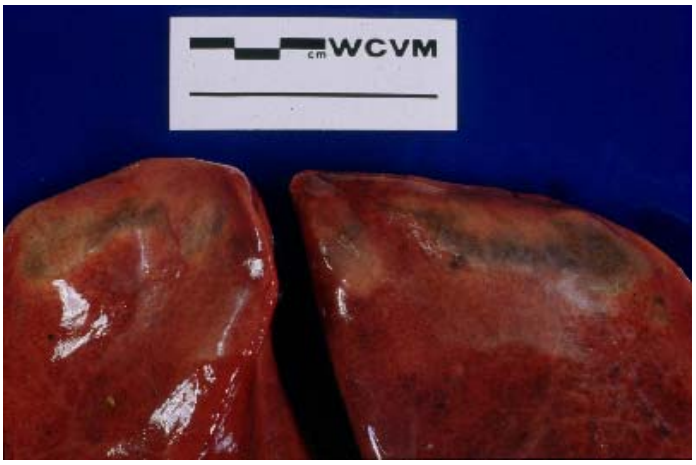

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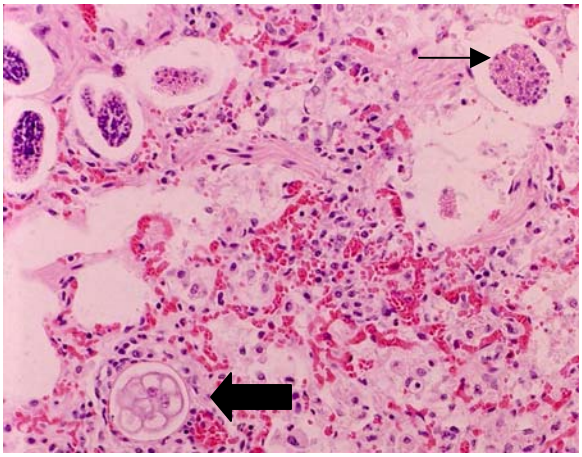
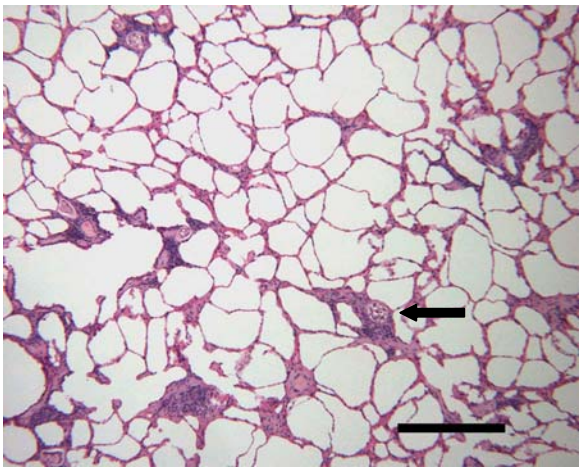


Figure 4.4: Pulmonary lesions associated with protostrongylid parasites in Dall's sheep from the Mackenzie Mountains.

a) Right lung *in situ*, pleural hemorrhages associated with *P. odocoilei* (SC15), b) Ventral view of apices of diaphragmatic lobes with parenchymal lesions associated with *P. stilesi* (FC21), and c) eggs of *P. stilesi* (thin arrow) and *P. odocoilei* (thick arrow) at 200X on histological examination (SC15)

a)



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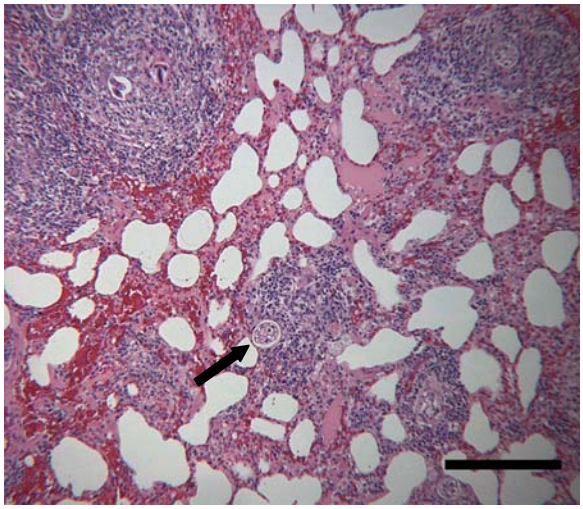

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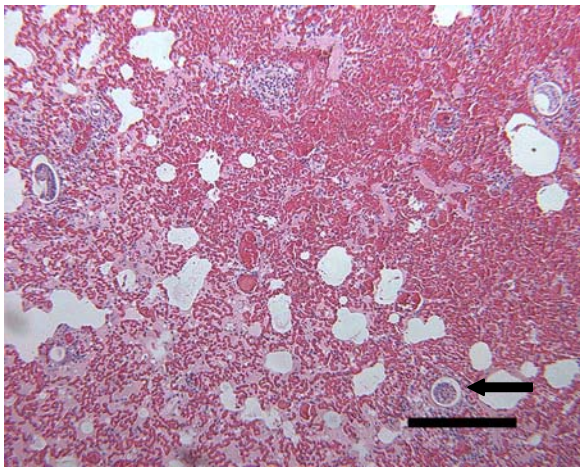
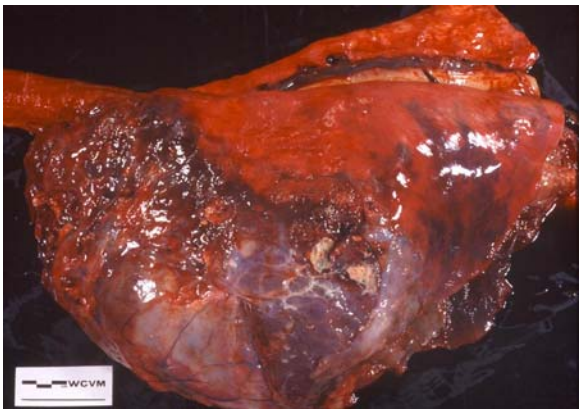


Figure 4.5: Stages of pulmonary pathology associated with *P. odocoilei* in Dall's sheep of the Mackenzie Mountains.

a) Stage 1-2 (NH-killed ram) b) Stage 3 (spring straggler SC7) c) End-stage, diffuse hemorrhage and oedema (SC15). Magnification 100X, bars = 250 um, arrows = eggs

a)



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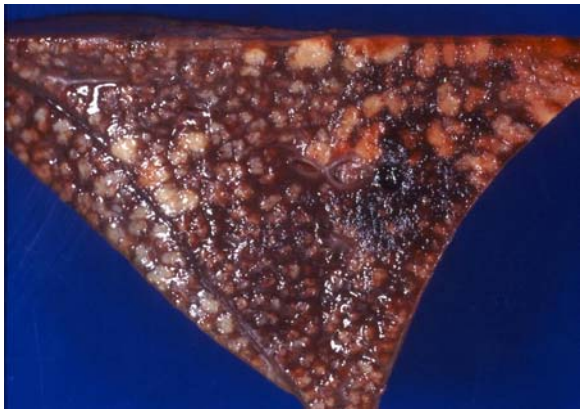

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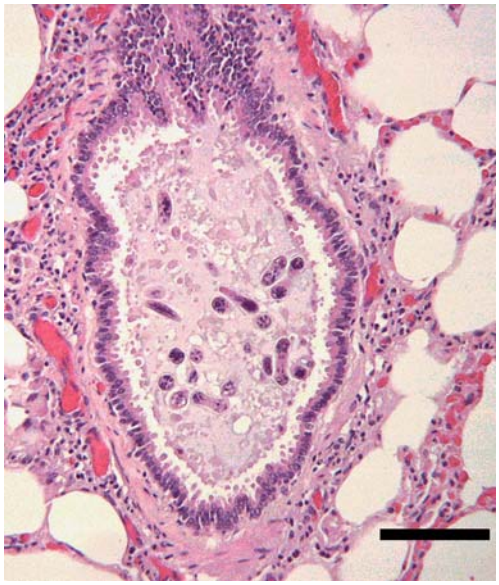

Figure 4.6: Chronic, fibrinopurulent bronchopneumonia in Dall's sheep from the Mackenzie Mountains.

a) Left lung, lateral view, MO9, b) Cranial lobe, cut section, MO16, and c) larvae trapped in airways in a histological section from lung of MO8, bar = 100 um

Table 4.1: Prevalence (%) and intensity (density and LPG) of *Parelaphostrongylus odocoilei* and *Protostrongylus stilesi* in lung and feces of Dall's sheep from the Mackenzie Mountains, NT, 1998-2002.

				P. odocoilei (%)			*P. stilesi* (%)			Density in	Granuloma	Fecal	Fecal
Collection	n	Sex	Age (y)	lungs	fecal	overall	lungs	fecal	overall	cranial lung[c]	size in lung[d]	DSLPG	PrLPG
NH[a] killed Jul-Aug, 00-02	27	M	10.1 (7.5-12.5)	93	96	100	93	63	96	8 (0-50)	114 (49-330)	109 (2-748)	155 (5-1075)
RH[b] killed Aug-Oct, 99-02	7	6M/1F	5.7 (3.5-9.5)	100	100	100	86	57	86	11 (2-40)	158 (110-286)	182 (44-387)	51 (22-123)
Fall Collected (FC) Aug-Nov, 98-02	9	1M/8F	4.9 (1.3-11.5)	100	89	100	89	67	89	14 (2-50)	182 (121-340)	557 (157-1301)	194 (15-458)
Spring Collected (SC) April, 99-00	4	F	3.5 (0.9-7)	100	100	100	100	100	100	52 (27-60)	1077 (588-2000)	2614 (187-5993)	1044 (8-2800)
Mortalities (MO) Jun-Aug, 99-02	9	3M/6F	6.8 (0.2-10)	56	11	56	89	67	100	8 (0-30)	NA	1.2	66.3 (1.2-300)

[a] non-resident hunter
[b] resident hunter
[c] number of protostrongylid eggs and larvae in 10 fields at 100X
[d] length x width x 100 um^2 of largest granuloma surrounding egg(s) of *P. odocoilei*

Table 4.2: *Parelaphostrongylus odocoilei* in lung, feces, and muscles of experimentally infected Stone's sheep (SS1-3), and naturally infected Dall's sheep collected in spring (SC) and fall (FC) from the Mackenzie Mountains, NT, 1998-2002.

ID	Collection	Age (y)	Sex	Density in cranial lung[a]	Granuloma size in lung[b]	Fecal DSLPG	Muscle No. worms	Percent Axial	(%) worms recovered Trunk	Hindquarters	Forelimbs	Nematode M:F ratio
SS1	173 dpi	2	M	27	1400	3508	75	37	13	33	16	0.8
SS2	87 dpi	16	F	27	88	1187	27	33	33	11	22	0.8
SS3	92 dpi	15	F	60	399	4499	14	14	21	64	0	1
SC15	Apr	0.9	F	27	588	187	----	----	----	----	----	----
SC5	Apr	2.5	F	60	840	5993	17	12	0	53	35	0.4
SC6	Apr	adult	F	60	880	1983	30	17	0	40	43	1.3
SC7	Apr	7	F	60	2000	2293	48	13	15	33	40	1.7
FC27	Aug	1.3	F	2	121	0	----	----	----	----	----	----
FC21	Sep	5	F	8	196	484	----	----	----	----	----	----
FC1	Oct	adult	F	9	140	896	6 [c]	17	0	17	67	0.7
FC2	Oct	adult	F	6	168	198	3 [c]	67	0	0	33	1
FC3	Oct	adult	F	8	150	408	0 [c]	----	----	----	----	----
FC23	Nov	5.5	F	7	224	157	11 [d]	18	18	36	27	1.3
FC24	Nov	1.5	M	40	340	794	18 [d]	22	17	39	22	2
FC25	Nov	11.5	F	27	160	1301	28 [d]		29	21	50	1.7
FC26	Nov	4.5	F	8	143	220	6 [d]	17		50	33	3

[a] number of protostrongylid eggs and larvae in 10 fields at 100X
[b] length x width x 100 um^2 of largest granuloma surrounding egg(s) of *P. odocoilei*
[c] right hind leg not examined, first attempt at recovery
[d] only left side of carcass examined

Table 4.3: Pathology and microbiology in sick (SK) and dead (MO) Dall's sheep from the Mackenzie Mountains, NT, 1999-2002.

ID	Age (y)	Sex	Condition (Marrow fat)	Month	Diagnosis	Culture results	IHC	Parasite status [f]
MO8	10	F	thin (45%)	Jun-99	Chronic, fibrinopurulent bronchopneumonia	*A. pyogenes* [a,b]	Neg [c,d]	Po & Ps
MO9	8	F	thin (51%)	Jul-99	Chronic, fibrinopurulent bronchopneumonia	*Mannheimia* sp.[a]	Neg [c]	Ps
					Dental disease, lumpy jaw	*A. pyogenes* [a,b]	[d]*Mycobacterium +ve*	
MO16	6	F	thin (6%)	Jun-00	Chronic, fibrinopurulent bronchopneumonia	*A. pyogenes* [a,b]	Neg [c,e]	Po & Ps
MO17	7	F	thin (14%)	Jul-00	Chronic, fibrinopurulent bronchopneumonia	*A. pyogenes* [a,b]	Neg [c,e]	Po & Ps
MO19	7	M	good (88%)	Aug-00	Acute, fibrino-necrotizing, bronchopneumonia	*A. pyogenes* [a,b]	Neg [c,e]	Po & Ps
MO20	9	M	good (89%)	Aug-00	Acute, fibrino-necrotizing, bronchopneumonia	*A. pyogenes* [a,b]	Neg [c,e]	Po & Ps
MO22	0.2	F	good	Jul-01	Acute, fibrinous pleuropneumonia	*P. multocida* [a,b]	NA	Ps
MO18	9	F	thin (33%)	Aug-00	Septicemia, dental and renal disease	*A. pyogenes* [a,b]	Neg [c,e]	Ps
MO28	0.25	M	moderate	Aug-02	Suppurative meningoencephalitis	*E. coli* (brain) [b]	NA	Ps
SK13	11.5	M	thin	Sep-99	Cough, dyspneic, depressed, purulent abscess	*A. pyogenes* [a]	NA	Po
SK18	NA	M	NA	Jul-01	Cough, dyspneic, depressed	*Mannheimia* sp.[a]	NA	Po & Ps

[a] lung
[b] septicemia
[c] immunohistochemistry (IHC) on lung for PI3, BRSV, IBR, and *Mannheimia haemolytica*
[d] IHC for BVD, *Mycobacterium* spp., *Haemophilus somnus*
[e] IHC for *Mycoplasma bovis*
[f] *Parelaphostrongylus odocoilei* (Po) and *Protostrongylus stilesi* (Ps)

CHAPTER 5

5 LARVAL BIONOMICS IN GASTROPODS

In this chapter[4], we investigate bionomics of larvae of *P. odocoilei* in gastropod intermediate hosts. In this case, bionomics ($\simeq$ ecology) is the study of factors influencing the survival and development of parasitic larvae in the gastropod intermediate host (see Rose, 1957a; Levine, 1963; and Beresford-Jones, 1966). Through experimental infection of gastropods collected in the Mackenzie Mountains, we determined gastropod species native to the Subarctic that are suitable intermediate hosts for *P. odocoilei*. In the laboratory, we described the proportion and survival of infective larvae that emerged from two important gastropod intermediate hosts, the slug *Deroceras laeve* and the snail *Catinella* sp. Finally, we developed a degree day model for *P. odocoilei* in *D. laeve* that can be used to predict development rates over a range of temperatures. This work in the laboratory provided the foundation for describing the epidemiology and factors limiting the distribution of this parasite in the Subarctic (Chapter 6). I did the experimental work, analyzed the data and, as corresponding author, researched and wrote the manuscript with input from co-authors. Note that the Appendix on larval morphometrics is based on experiments described in this chapter.

[4] Jenkins, E.J., S.J. Kutz, E.P. Hoberg, and L. Polley. Bionomics of larvae of *Parelaphostrongylus odocoilei* (Nematoda: Protostrongylidae) in experimentally-infected gastropod intermediate hosts. Journal of Parasitology, In press.
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5.1 Abstract

Parelaphostrongylus odocoilei is a protostrongylid parasite that has recently been recognized at a number of locations in Subarctic, but not Arctic, North America. We investigated factors that may determine the distribution of *P. odocoilei*, including suitable gastropod intermediate hosts, temperature requirements for larval development in gastropods, and larval emergence facilitating overwinter transmission. We collected and experimentally infected gastropods from a site in the Subarctic where *P. odocoilei* is at the northern limit of its distribution. *Deroceras laeve*, *Catinella* sp., and *Euconulus* cf *fulvus*, but not members of the Pupillidae, were suitable intermediate hosts. We described bionomics of larvae of *P. odocoilei* in *D. laeve* and *Catinella* sp. Infective larvae emerged from all slugs (*D. laeve*) and 60% of *Catinella* sp. snails, and emergence from *D. laeve* was intensity-dependent. Emerged infective larvae survived up to 6 mo under conditions approximating that of the subnivean environment. In *D. laeve*, there was a direct relationship between temperature and development rate of larvae of *P. odocoilei*. Larvae of *P. odocoilei* did not develop to infective stage below the theoretical threshold (8.5 ºC), and required a minimum of 163 degree days to complete development. These developmental parameters can be incorporated into a model to predict larval development in the field. Knowledge of the factors influencing larval bionomics provides the foundation for predicting temporal and spatial patterns of parasite distribution, abundance, and transmission.

5.2 Introduction

Parelaphostrongylus odocoilei, a protostrongylid muscleworm, has traditionally been considered a parasite of temperate and coastal regions in western North America. Recently, however, it was discovered in populations of thinhorn sheep (*Ovis dalli*), mountain goats (*Oreamnos americanus*), and woodland caribou (*Rangifer tarandus caribou*) in Subarctic, but not Arctic, North America (Kutz et al., 2001c; Jenkins et al., 2005a). Similar to other

protostrongylids, *P. odocoilei* has an indirect life-cycle, where first-stage larvae (L1) shed in the feces of a mammalian definitive host penetrate the foot of a gastropod intermediate host, develop to second-stage larvae (L2), and then to infective third-stage larvae (L3). Native gastropod species naturally infected with larvae morphologically indistinguishable from those of *P. odocoilei* have been described in a temperate region, in Jasper, Alberta, Canada (52ºN; 118ºW) (Samuel et al., 1985). Prevalence of infection was less than 5%, necessitating the capture of more than 10,000 gastropods to determine suitable intermediate host species. Most logistically feasible sampling techniques underestimate gastropod abundance and the prevalence of protostrongylid infection (McCoy and Nudds, 1997; Hawkins et al., 1998). As well, species of protostrongylid larvae cannot reliably be identified on the basis of morphology alone. Therefore, to determine gastropod species potentially important for natural transmission, experimental infection of native gastropods is an effective and attractive alternative (Platt and Samuel, 1984).

Definitive hosts may become infected by ingesting gastropods containing L3, or L3 that have emerged from gastropods. Reports of larval emergence vary among different species of protostrongylids and gastropod hosts, and the significance of this phenomenon is controversial. Other than a single report of larval emergence of *P. odocoilei* of thinhorn sheep origin (Kutz et al., 2001c), emergence has not been reported for any elaphostrongyline, nor considered an important transmission route for most protostrongylids (Anderson, 2000). In contrast, recent work on a protostrongylid lungworm of muskoxen, *Umingmakstrongylus pallikuukensis* (Kutz et al., 2000), and older, less-accessible studies in the Russian literature (summarized by Boev, 1975), indicate that emergence is important for transmission of some protostrongylids, especially in northern regions where gastropods are seasonally unavailable for up to 8 mo of the year.

Further work is needed to determine the significance of larval emergence for *P. odocoilei*, including survival of emerged larvae under simulated natural conditions.

Temperature is probably the most important abiotic factor affecting rates of development of parasites with free-living developmental stages, or larval stages in poikilothermic intermediate hosts, such as gastropods (Halvorsen and Skorping, 1982; Shaw et al., 1989). Degree day models, which describe the amount of heating above a critical threshold temperature, are widely used to determine conditions allowing parasite development and can be incorporated into deterministic, predictive models for rates of parasite development under different climatic conditions (Campbell et al., 1974; Saunders et al., 2002; Harvell et al., 2002). Early workers began to examine the effects of weather and climate on parasite transmission (Levine, 1963), but "this became a neglected and low prestige area over the last two decades as our ability to study the…more molecularly glamorous aspects of the parasite life-cycle increased" (Smith et al., 1995). Perhaps as a result, there are few empirical studies available for protostrongylids (Halvorsen and Skorping, 1982; Samson and Holmes, 1985; Kutz et al., 2001b), and the effects of temperature on larval development have not been investigated for *P. odocoilei*.

Many biotic and abiotic factors, including species of gastropod, infection intensity, and temperature, influence larval bionomics, including development, emergence, and survival of larval stages (Gerichter, 1948; Halvorsen and Skorping, 1982; Skorping, 1984; Kutz et al., 2000). In turn, factors affecting larval bionomics drive temporal and spatial patterns of transmission for protostrongylid parasites. In order to explore the determinants for the distribution of *P. odocoilei* in North America, therefore, our objectives were: 1) to identify gastropod intermediate hosts native to the Subarctic; 2) to determine the significance of larval emergence, including survival of emerged larvae; 3) to determine the effects of temperature on

development of larvae; and 4) to develop a degree day model that can be used to predict larval development under different climatic conditions.

5.3 Materials and Methods

5.3.1 Infection of gastropods with *P. odocoilei*

First-stage larvae (L1) of *P. odocoilei* were obtained from feces of captive Stone's sheep (*O. d. stonei*) experimentally infected with *P. odocoilei* from naturally infected Dall's sheep (*O. d. dalli*) in the Mackenzie Mountains, Northwest Territories, Canada (65º01'N; 127º35'W). Adult parasites and L1 from the captive sheep were confirmed as *P. odocoilei* based on morphological and molecular data . Feces were held at –20 ºC for 5-33 mo before L1 were recovered using a modified beaker Baermann technique (Forrester and Lankester, 1997). Only motile L1 were used to calculate infection doses. Gastropods were fasted for 24 hr, housed 6 to a medium petri dish (9 cm in diameter), and exposed to L1 suspended in a small amount of water at room temperature (Hoberg et al., 1995). Gastropods that moved away from the L1 suspension were returned to the suspension every 15-20 min for 3 hr. To synchronize infection in development trials, gastropods were rinsed with water at the end of the infection period to remove L1 that had not penetrated the gastropod.

5.3.2 Native intermediate hosts

Native gastropods were collected in July and August 2002 in the Mackenzie Mountains (64º28'N; 129º37'W) and snails identified on the basis of shell morphology (Pilsbry, 1946; Pilsbry, 1948; Burch, 1962). Twelve specimens of *Catinella* sp., 6 specimens of *Euconulus* cf *fulvus* (possibly *E. fulvus alaskensis*), and 5 specimens of the Pupillidae (*Vertigo modesta* and/or *V. alpestris oughtoni*, and *Columella edentula* and/or *C. alticola*) were each exposed to 500 L1 of *P. odocoilei* and group-housed at 20 ºC (9-28 ºC) in large plastic petri dishes (15 cm in diameter) containing native vegetation and dried leaf litter. Equal numbers of uninfected control

snails of each species were treated in a similar fashion, except they were not exposed to L1. As part of a separate experiment, 10 slugs (*Deroceras laeve*) were also collected from the same site, each exposed to 250 L1 of *P. odocoilei*, and housed under the same conditions. At 20 days post infection (dpi), groups of 5-6 snails (including mortalities) were digested in a pepsin and hydrochloric acid solution for 1.5-3 hr to recover larvae (Hoberg et al., 1995). All larvae were examined and quantified at 100-400X, and larval stages determined using morphological criteria established for *U. pallikuukensis* (Kutz et al., 2001b). The L1, L2, and early L3 (dead and live early L3, which had poor motility and survival in digest fluid) were considered pre-infective larvae, while intermediate L3 (partially ensheathed in the L2 cuticle) and late L3 (ex-sheathed) were considered infective larvae (as defined by Gerichter, 1948; Rose, 1957b; Platt, 1978).

5.3.3 Larval emergence and survival

Three larval emergence trials (E1-E3) were established from September 2001 to September 2003 (Table 5.1). *Deroceras laeve* were obtained from protostrongylid-free laboratory colonies and *Catinella* sp. were collected from the Mackenzie Mountains in August 2003. Gastropods were each exposed to 1000 (E1) or 500 (E2 and E3) L1, and housed at room temperature (20-21 ºC). In E1 from 2-14 dpi, 6 gastropods were group-housed in each of 3 large plastic petri dishes (15 cm in diameter), which were examined for emerged larvae at 14 dpi. In E1 after 14 dpi, and in E2 and E3 after 4 dpi, gastropods were housed individually in small glass petri dishes (6 cm in diameter) containing 1-2 ml of tap water and maintained as per Kutz et al. (2000). Dishes were examined under a dissecting microscope for emerged larvae every 2 days from 14-60 dpi in E1, every 4 days from 6-18 dpi in E2 and E3, and every 2 days from 18-84 dpi in E2 and E3. For examination, gastropods were moved to new dishes, and tops and bottoms of vacated dishes were half-filled with tap water and examined immediately, or dishes were refrigerated for up to 4 days prior to examination. In E1 only, at each transfer the feet of slugs

were examined under a dissecting microscope for lesions associated with larvae. As positive controls, 3 gastropods were digested at 26 dpi (E1) or 21 dpi (E2 and E3). At the end of each emergence trial, all gastropods were individually digested, and larvae counted and classified as above.

The cumulative percentage of L3 that emerged was calculated by dividing the number of L3 that emerged from an individual gastropod up to and including a given dpi by the sum of the number of L3 that emerged and the number of L3 recovered from digest of that gastropod at the end of the trial. The group mean cumulative percentage included only those gastropods experiencing emergence by the end of the trial. Data from 22 slugs in E1 and E3 were used for regression analysis of intensity of infection (L3/slug) on the percentage of infective L3 emerging from each slug by 60 dpi.

Pre-infective larvae that emerged from slugs in E1 were held in 10 ml of tap water in a small glass petri dish at room temperature and monitored for survival for up to 7 days. Infective L3 that emerged from slugs in E3 were refrigerated in 10 ml of tap water in a small glass petri dish until the end of the experiment. From these, a total of 60 motile or tightly-coiled L3 were hand-picked and held in darkness at 1.6 ºC (range -3.4 to 9.8 ºC) in 10 ml of tap water in 25 ml Erlenmeyer flasks with a foam cork, 25 larvae/flask (one with 10 emerged L3/flask). The number of dead (visibly dead or missing and presumed dead), and live (tightly coiled or motile) L3 were recorded at 3, 6, and 12 mo.

5.3.4 Larval development and temperature

Six larval development trials (D1-D6) were established from November 2002 to March 2003 (Table 5.2). *Deroceras laeve* were obtained from laboratory colonies, each exposed to 250 L1 of *P. odocoilei*, and group-housed in containers filled with moist soil and vermiculite at the temperatures indicated in Table 5.2 (Kutz et al., 2001b). Temperatures were recorded every 30-

60 min using HOBO XT external or H08 internal temperature sensors (Onset Computer Corporation, Pocasset, Massachusetts). Three slugs were collected every 7 days (D1, D2, and D3) or 2 days (D4, D5, and D6) for the monitoring periods indicated in Table 5.2. Collected slugs were weighed, and individually digested immediately or after refrigeration for up to 4 days. Larvae were counted and classified to developmental stage as described above.

Development rate was defined as the inverse of the first day post infection (dpi) that infective L3 were observed. The theoretical threshold temperature (To) and the thermal constant (DD, degree days, or the minimum amount of heating above threshold necessary for development of infective L3) were calculated from the intercept and slope of the line generated by a regression analysis of temperature on larval development rates for D2 to D6 [development rate = (b x temperature) - a, To = -a/b, DD = 1/b] (Campbell et al., 1974; Samson and Holmes, 1985; Kutz et al., 2001b). The number of days at a given temperature necessary to obtain infective larvae was predicted using [dpi = DD/(Temperature – To)].

5.4 Results

5.4.1 Native intermediate hosts

All specimens of *E.* cf *fulvus*, 75% of *Catinella* sp., and 40% of the Pupillidae were alive at 20 dpi. There were no differences in survival between infected and uninfected snails. At digest at 20 dpi, 93% (26/28) of larvae recovered from 6 *E.* cf *fulvus*, and 11% (18/171) of larvae recovered from 12 *Catinella* sp., were infective L3. For comparison, at 19 dpi, 76% (295/386) of larvae were infective L3 from 9 experimentally-infected native *D. laeve*. Only 3 dead pre-infective larvae were recovered from 5 pupillid snails. Nine infective L3 were recovered from 5 uninfected control *Catinella* sp. snails.

5.4.2 Larval emergence and survival

Small numbers of pre-infective larvae (36 in E1, 5 in E2, and 2 in E3) emerged from ~20% of gastropods, and the first infective L3 emerged between 22 and 26 dpi in all trials. Emergence of infective larvae occurred from 100% of *D. laeve*, but only 60% of *Catinella* sp. snails, and the mean percentage of infective L3 emerging from *D. laeve* was greater (Table 5.1). The percentage of infective larvae emerging from *D. laeve* correlated with infection intensity ($F = 6.136$, $p = 0.022$) (Fig. 5.1). The mean number of larval-associated foot lesions visible in *D. laeve* in E1 increased from 14 to 44 dpi, then decreased steadily until the end of the trial at 60 dpi (Fig. 5.2).

Both pre-infective and infective larvae emerged in high numbers from sick *D. laeve* and *Catinella* sp., often in the days immediately prior to death. In E1, 11 pre-infective larvae emerged in the 2 days prior to death of a slug at 16 dpi, and 11 pre-infective larvae and 46% of infective L3 emerged from a slug that was sick for the duration of the monitoring period. In E2, 2 L3 emerged the day before death of a *Catinella* sp. snail at 72 dpi. *Catinella* sp. snails aestivated and fasted occasionally.

Pre-infective larvae that emerged did not survive for longer than a few days, nor did they undergo further development. At 3, 6, and 12 mo, 83, 65, and 0% of emerged infective L3 were alive.

5.4.3 Larval development and temperature

Development rates of larvae of *P. odocoilei* in *D. laeve* had a direct relationship with temperature, which could be described by a linear model ($y = 0.0061x - 0.0522$, $R^2 = 0.979$, $F = 139.75$, $P = 0.001$) (Fig. 5.3). An exponential model also fit the observed data ($y = 0.0038e^{0.1399x}$, $R^2 = 0.981$, $F = 154.8$, $P = 0.001$), while a power model had the best fit ($y = 0.00008 \times ^{2.244}$, $R^2 = 0.992$, $F = 356.7$, $P = 0.000$). In D2 (9.8 ºC), development occurred more

rapidly than predicted by the linear model and was less synchronous, with infective L3 not present in all 3 slugs from each collection until 91 dpi (Table 5.2). In D1 at 7.8 ºC (range 7.4 to 10.2 ºC), development to L2, but not to L3, occurred by 91 dpi (Fig. 5.4). After 91 dpi, when slugs were housed at 20.6 C, the first infective larvae were observed at 14 days (105 dpi on the D1 timeline), similar to D5 at 20 ºC (Table 5.2, Fig. 5.4). The To was 8.5 C, and the thermal constant was 163 DD.

5.5 Discussion

Bionomics of larvae of *P. odocoilei* in intermediate hosts potentially important in transmission at Subarctic latitudes have not been previously investigated. We explored factors that may determine the distribution of *P. odocoilei*, including suitable gastropod intermediate hosts, larval emergence (which creates the potential for overwinter transmission), and temperature requirements for larval development in gastropods. By comparing our findings to those for *P. odocoilei* in temperate and coastal regions, and to protostrongylid parasites limited to Arctic latitudes, such as *U. pallikuukensis*, we gain insight into parasite life histories, as well as abiotic and biotic constraints on parasite development and transmission.

5.5.1 Intermediate host suitability

Based on experimental infections, several species of gastropods native to the Mackenzie Mountains in Subarctic North America are suitable intermediate hosts for *P. odocoilei*, including *D. laeve*, *Catinella* sp., and *E.* cf *fulvus*. This is the first published report that *Catinella* sp., or indeed any member of the Family Succineidae (suborder Heterurethra), can serve as a suitable intermediate host for *P. odocoilei*, although succineid snails are suitable hosts for other protostrongylids (Lankester and Anderson, 1968; Kutz et al., 2000). Following exposure to similar numbers of L1, more larvae established in *Catinella* sp. than *E.* cf *fulvus* and *D. laeve*, but larvae required more time to develop to infective stage. This may reflect delayed

development due to aestivation of *Catinella* sp. snails, as almost all larvae eventually reached infective stage in E2 (Table 5.1) (Lankester and Anderson, 1968; Solomon et al., 1996; Kutz et al., 2000; Kutz et al., 2001b) (but see Gerichter, 1948). Interestingly, infective L3 morphologically indistinguishable from *P. odocoilei* were recovered from uninfected control specimens of *Catinella* sp. collected in the Mackenzie Mountains, which may represent contamination or, possibly, natural infection of one or more snails with a protostrongylid native to this region. If the latter, this likely had negligible effects on our results from experimentally infected snails, as prevalence of naturally infected gastropods is low (less than 5% in Samuel et al., 1985), and mean intensity of protostrongylid larvae is much lower in natural infections (3 larvae/gastropod in Lankester and Anderson, 1968) as compared to experimental infections (53 larvae/*Catinella* sp. snail in E2 in the current study).

Both *D. laeve* and *E. fulvus* are considered important intermediate hosts for *P. odocoilei* in temperate regions, based on abundance and prevalence of larvae in naturally-infected gastropods (Samuel et al., 1985). In addition, the snails *Vitrina limpida*, *Zonitoides* spp., and *Discus* spp. in temperate regions are potentially important in transmission (Platt and Samuel, 1984; Samuel et al., 1985). Members of the Pupillidae appear to be refractory to infection with *P. odocoilei*, and had poor survival in captivity, as observed in subsequent trials with larger numbers of snails (n = 48, unpubl. obs.) and elsewhere (Samuel et al., 1985; Gray et al., 1985a). These findings suggest that *D. laeve* and *Catinella* sp. are the most important intermediate hosts for *P. odocoilei* in the Subarctic, with *E.* cf *fulvus* possibly playing a lesser role, based on lower larval establishment. Both *D. laeve* and *Catinella* sp. are hydrophilic gastropods, abundant in sedges, grasses, and willow stands, while *E. fulvus* is more xerotolerant and can be found in a wider variety of habitats in temperate regions and in the Mackenzie Mountains (Boag and

Wishart, 1982; unpubl. obs.). Thinhorn sheep primarily encounter habitat suitable for gastropods on their winter range, and possibly at heavily-used, naturally-occurring mineral licks and water sources on summer range (Hoefs and Cowan, 1979; Simmons, 1982). Such habitat patches may serve as foci of infection (Lankester and Anderson, 1968; Anderson, 1972).

North of the Arctic circle, larvae of *P. odocoilei* were not present in fecal samples from thinhorn sheep, barren-ground caribou, or muskoxen, although other protostrongylid species are present in and frequently shared among these hosts (Hoberg et al., 2002; Jenkins et al., 2005a; unpubl. obs.). The slug *D. laeve* has been reported from Alaska and the Arctic Islands, and *D. laeve*, *Catinella* sp. and *E. fulvus* have been recovered from the coast of the Arctic mainland at 68ºN (Pilsbry, 1948; Kutz, 2000). Both *D. laeve* and *E. fulvus* have Holarctic distributions (Burch, 1962). Therefore, at least some of the gastropod species important for transmission of *P. odocoilei* in Subarctic and temperate regions are present in the Arctic. The apparent absence of *P. odocoilei* from these regions suggests that either this parasite has not been introduced into the historically isolated Dall's sheep populations in the Arctic, or other factors may currently be limiting its distribution, such as abiotic conditions unfavorable for survival of first-stage larvae or development of larvae in gastropods.

5.5.2 Role of emergence in transmission

Emergence of infective larvae from intermediate hosts can be viewed as 'bet-hedging', where a parasite produces offspring that differ in infection strategy (Fenton and Hudson, 2002). Infective larvae of *P. odocoilei* emerged from healthy specimens of *D. laeve* and *Catinella* sp., both important intermediate hosts for *P. odocoilei* in the Subarctic. Emergence occurred less commonly from *Catinella* sp. despite higher larval establishment rates, perhaps because of delayed larval development and aestivation (Kutz et al., 2000). Larvae emerged in disproportionately large numbers from sick or dying gastropods (similar to observations by Rose,

1957b; Kutz et al., 2000), suggesting that protostrongylid larvae are sensitive to changes in the physical milieu or immunity of the host. Marked host reactions were associated with larvae in the feet of *D. laeve*, with lesions similar in appearance to those reported for *P. odocoilei* in *Triodopsis multilineata* (Platt, 1978), and for *Elaphostrongylus rangiferi* in *Arianta arbustorum* (Skorping, 1984). Visibility of lesions initially increased, possibly due to increasing host reaction surrounding larvae, then decreased, correlating with emergence of a greater percentage of larvae and resolution of lesions (Fig. 5.2).

Emerged infective larvae of *P. odocoilei* survived for 6 mo in darkness in water at near-freezing temperatures (at times below freezing) under conditions simulating the subnivean environment, where there is little direct sunlight, 100% relative humidity, and temperatures are stable at or just below 0 ºC despite much colder air temperatures (Marchand, 1996; Forrester and Lankester, 1998). Survival of emerged infective larvae has been observed under various environmental conditions for other protostrongylids (Rose, 1957b; Boev, 1975; Cabaret and Pandey, 1986; Kutz et al., 2000). Emergence of infective larvae may therefore be an important aspect of transmission of some protostrongylids, especially in northern regions where gastropods are seasonally unavailable for much of the year (Kutz et al., 2000). Gastropods become inactive when temperatures fall below 0 ºC and when snow cover becomes continuous, although some activity may occur in the subnivean space in early winter and late spring (Aitchison, 1979). Larval emergence may facilitate overwinter transmission of free-living L3 of *P. odocoilei* to ungulates foraging beneath the snowpack, as suggested for *U. pallikuukensis* in the Canadian Arctic (Kutz et al., 2000), and for free-living L3 of gastrointestinal nematodes in the Eurasian Arctic (Halvorsen et al., 1999).

Pre-infective larvae of *P. odocoilei* that emerged did not survive or continue development, and likely have no significance for transmission. Emergence of pre-infective larvae has not been previously reported for any protostrongylid species, nor has it been considered a mechanism of host resistance (Cabaret, 1979; Kralka and Samuel, 1984c; Kutz et al., 2000). Emergence of pre-infective larvae of *P. odocoilei*, which occurred from both healthy and sick gastropods, likely reflects "spill-over" at high infection intensities, or expulsion by the intermediate host. This may also occur with some infective larvae at high intensities, as the percentage of infective larvae of *P. odocoilei* that emerged from *D. laeve* was correlated with intensity of infection. While mean infection intensities were artificially high in experimentally infected gastropods, 295 larvae of *P. odocoilei* have been reported in a naturally infected slug (Platt, 1978). Interestingly, infective L3 were significantly smaller in *D. laeve* in E1 at high infection intensity [5], suggesting that larval growth may be intensity-dependent, as observed for larvae of other protostrongylid species (Boev, 1975; Skorping, 1984).

Even at high infection intensities, the percentage of infective larvae that emerged from *D. laeve* never exceeded 50%, similar to *Protostrongylus boughtoni* and *P. stilesi* (Monson and Post, 1972; Kralka and Samuel, 1984c). In contrast, 100% of L3 of *U. pallikuukensis* emerged from some slugs regardless of infection intensity (Kutz et al., 2000). This may reflect specific adaptations to different environmental constraints; *P. odocoilei* is present at latitudes ranging from 36º to 65º N (Jenkins et al., 2005a), whereas *U. pallikuukensis* is present at latitudes ranging from 65º to 69º N (Kutz, 2000). The possibility of latitudinal differences in the proportion of larvae that emerge within and among species of protostrongylids warrants further investigation. Where overwinter transmission is not limiting, i.e. in warm temperate or tropical

[5] See Appendix.

regions, few or no larvae may emerge, which may in part account for the controversy over the significance of this phenomenon.

5.5.3 Temperature, development, and the degree day model

Heating above the threshold temperature (To) may be most important for development of *P. odocoilei* from L2 to L3. Development to infective L3 did not occur below To (8.5 C), but larvae did develop from L1 to L2. When warmed above To, L2 developed to L3 at a rate similar to that predicted if the larvae had started as L1, as observed for other protostrongylids (Gerichter, 1948). At or below To, development from L1 to L2 occurred for *U. pallikuukensis* (Kutz, 2000), but not *E. rangiferi* (Halvorsen and Skorping, 1982). It is possible that stochastic variation in temperature may trigger development from L1 to L2 when temperatures briefly reach or exceed a critical threshold (Saunders et al., 2002). Alternatively, the threshold temperature for development from L1 to L2 may be lower than that for L2 to L3.

The threshold temperature of protostrongylids from temperate and northern regions, including *P. odocoilei*, *E. rangiferi*, *U. pallikuukensis*, and *Protostrongylus* spp., appears to be strongly conserved, ranging from 8-10 ºC (Samson and Holmes, 1985; Kutz et al., 2001b). In contrast, To of *Muellerius capillaris*, a protostrongylid parasite of more temperate climates, is 4.2 ºC (Rose, 1957b; Kutz et al., 2001b). The paradoxically higher To in protostrongylids in temperate and northern climates may be an adaptation to strong seasonal patterns of transmission, delaying onset of development until temperatures are consistently warmer and development to infective stage occurs rapidly (Schjetlein and Skorping, 1995). Slowly developing larval stages may place increased demands on their gastropod hosts for a longer period of time, or may elicit stronger host reactions, decreasing survival of both gastropods and larvae.

Using the degree day (DD) concept, or the amount of heating above threshold, larvae of *P. odocoilei* needed 163 DD to develop to infective stage, similar to *U. pallikuukensis* and *M. capillaris*. Higher values have been reported for *Protostrongylus* spp. (305) and *E. rangiferi* (250) (Samson and Holmes, 1985; Kutz et al., 2001b). For *Protostrongylus* spp., Samson and Holmes (1985) used a more conservative endpoint (>50% of gastropods with infective larvae) that minimized the effects on the model of asynchronous development at low temperatures. Further investigation is needed to determine the influence of intermediate host species, phylogenetic relationships, and environment, as well as methods used by investigators, on developmental parameters of protostrongylid larvae.

Developmental parameters of *P. odocoilei* may vary across its broad host range and geographic distribution extending from California to Alaska. Using To and DD of *P. odocoilei* of Subarctic origin, larvae would need 17 days at 18 ºC to reach infective stage in *D. laeve*, an intermediate host native to both temperate and Subarctic regions. At this temperature, larvae of *P. odocoilei* from mule deer in temperate regions (Jasper, Alberta, Canada) needed a minimum of 22 days to develop to infective L3 in *T. multilineata* (Platt, 1978), whereas larvae of *P. odocoilei* from black-tailed deer in milder coastal regions (Vancouver Island, British Columbia, Canada) needed 35-38 days in various gastropods, including *T. multilineata*, *Deroceras reticulatum* (previously *Agriolimax agrestis*) and *Helix aspersa* (Hobmaier and Hobmaier, 1934; Shostak and Samuel, 1984). Establishment and development rates of larvae of *P. odocoilei* may be sub-optimal in these gastropod species, which are not native intermediate hosts for *P. odocoilei* (Platt and Samuel, 1984; Shostak and Samuel, 1984; Kutz et al., 2001c). Nonetheless, the possibility of a continuum of developmental capacity of *P. odocoilei* across its geographic and host range warrants further exploration. For example, the pre-patent period of *P. odocoilei*

in thinhorn sheep was approximately 20 days longer than that of *P. odocoilei* in mule deer given similar doses of infective L3 (Jenkins et al., 2005b). Genetic differences at the COX II locus have been observed in mitochondrial DNA of *P. odocoilei* from different locations, but were not restricted latitudinally (unpubl. obs.).

Our work suggests that a degree day model (which assumes a linear relationship) may adequately predict development rates of larvae of *P. odocoilei*. However, an exponential or power relationship cannot be excluded, and larval development at cooler temperatures may be faster than predicted by the linear model (Saunders et al., 2002); it is not known whether the amount of development that occurs at or below threshold temperature is biologically significant. For this reason, and in light of the possibility of biological differences in *P. odocoilei* across its broad geographic range, the degree day model needs to be validated using observations of larval development in the field before it can be broadly applied (Kutz et al., 2002). Nonetheless, it is instructive to compare temperatures across the range of *P. odocoilei* to determine if climate currently determines the northern limits of its distribution in North America. At most sites in temperate Canada where *P. odocoilei* is established, daily average temperatures exceed To (8.5 ºC) for 5-7 mo (May-September in temperate regions, or April-October in the coastal regions). In the western Canadian Subarctic and Arctic, daily average temperatures exceed To for only 3 mo (June-August), and in the Arctic, never exceed 14 ºC (versus 17 ºC in the Subarctic) (http://www.climate.weatheroffice.ec.gc.ca/climate_normals/index_e.html, accessed April 2005). Therefore, *P. odocoilei* may be restricted from northward range expansion by temperature-dependent limitations on development of larvae in intermediate hosts.

It is essential to develop models based on empirical observations in the laboratory and validate them in the field before applying such models to determine the effects of climate, and by

extension climate change, on parasite distribution, abundance, and transmission (Hoffmann and Blows, 1994; Harvell et al., 2002; Kutz et al., 2005). Laboratory based investigations, such as the current study, are key to understanding the biology and ecology of host-parasite systems, interpreting epidemiological patterns, and predicting shifts in the host-parasite equilibrium.

5.6 Acknowledgements

Darren Harder and Farhad Ghandi assisted with monitoring and maintenance of gastropods used in emergence trials. Sherry Hannon provided assistance with statistical analyses, and Arne Skorping provided helpful comments on the manuscript. Emily Jenkins' salary was provided by an Interprovincial Graduate Student Fellowship from the Western College of Veterinary Medicine, Saskatoon, Saskatchewan, Canada.

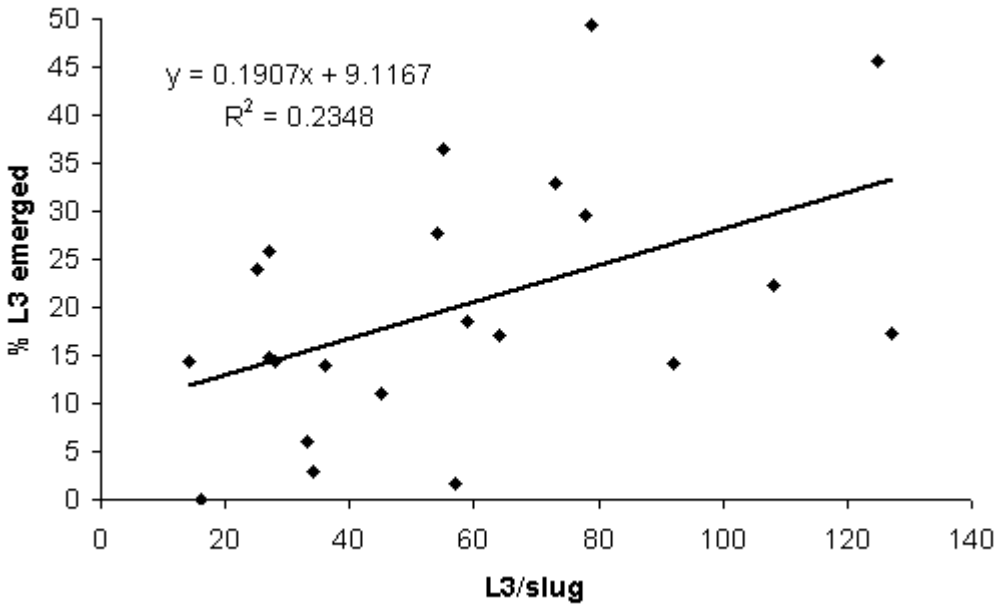

Figure 5.1: Intensity-dependence of emergence of infective larvae (L3) of *Parelaphostrongylus odocoilei* from *Deroceras laeve* in E1 and E3.

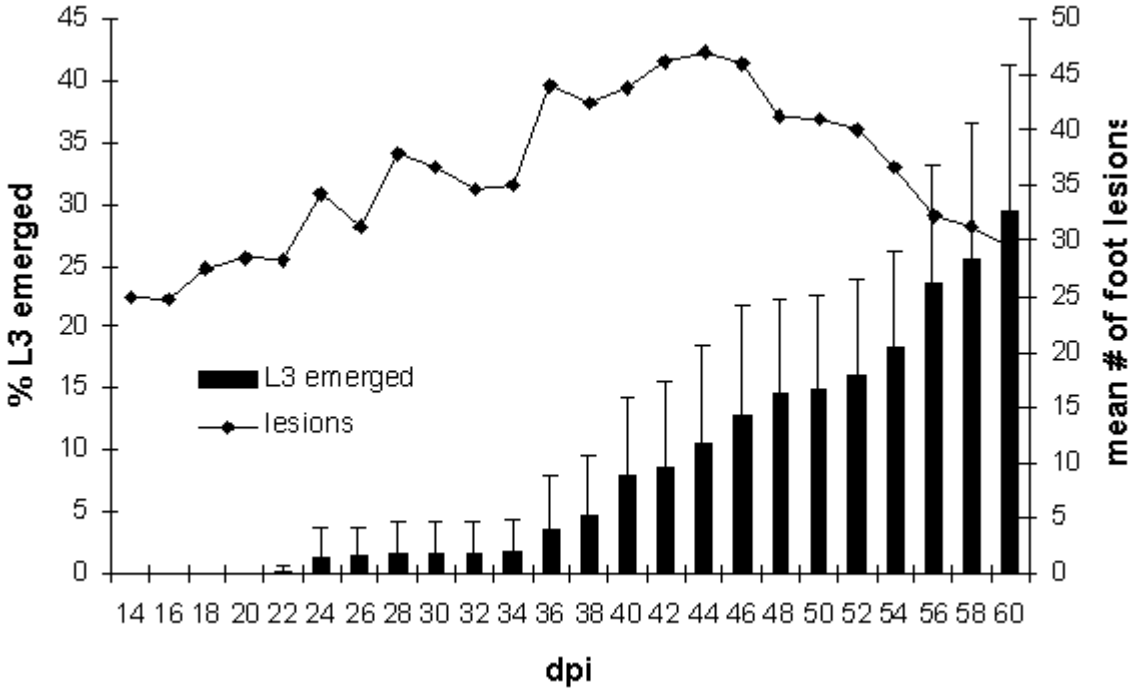

Figure 5.2: Mean cumulative percentage of infective larvae (L3) emerging from *Deroceras laeve* in E1, and mean number of foot lesions per slug.

Error bars = 1 S.D.

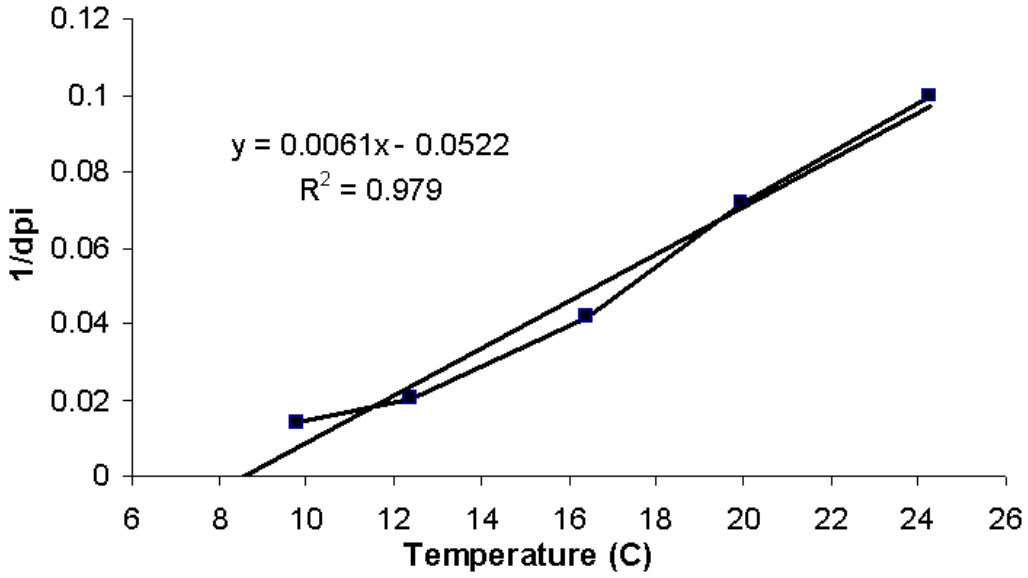

Figure 5.3: Relationship between temperature (ºC) and rate of development of larvae of *Parelaphostrongylus odocoilei* in *Deroceras laeve*.

(1/dpi = the inverse of the day post infection that the first infective larvae was observed). Note that although the linear model fits the data well, the relationship may actually be exponential.

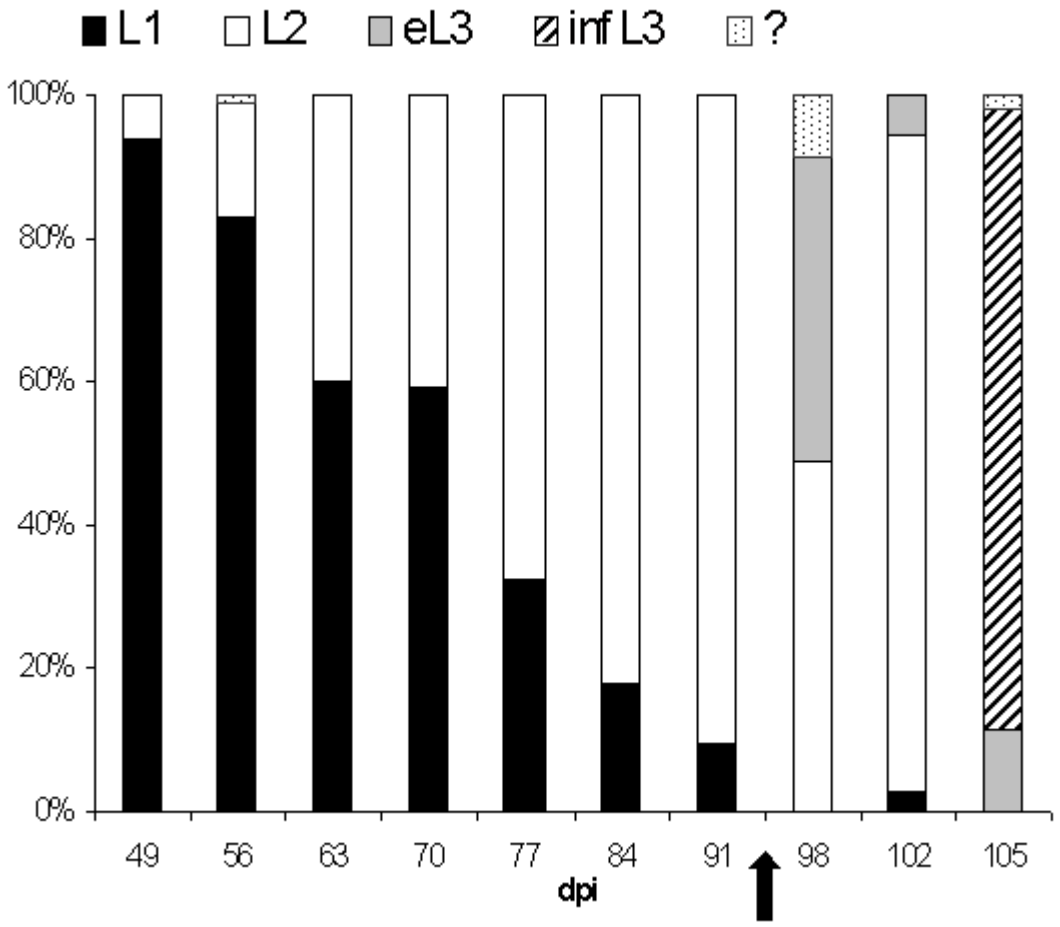

Figure 5.4: Distribution (as a percentage of total number of larvae) of larval stages of *Parelaphostrongylus odocoilei* recovered from *Deroceras laeve* digested on progressive days post infection (dpi) in D1.

After 91 dpi, temperature increased from 7.8 to 20.6 ºC (arrow). L1 = first-stage larvae, L2 = second-stage larvae, eL3 = early third-stage larvae, inf L3 = infective larvae, and ? = unidentifiable larvae.

Table 5.1: Emergence of infective third-stage larvae of *Parelaphostrongylus odocoilei* from *Deroceras laeve* and *Catinella* sp.

Trial	Species (number)	L1 dose	Mean L3[a] /gastropod (range)	Mean %L3 emerging [b] (range)	% gast.[c]	% L3/L[d] positive controls	% L3/L[d] at end of trial
E1	*D. laeve* (10)	1000	85 (54-127)	29 (14-49)	100	99	99
E2	*Catinella* (10)	500	53 (34-72)	4 (1-9)	60	34	97
E3	*D. laeve* (12)	500	34 (14-64)	21 (6-33)	100	84	100

Note that E2 and E3 ran concurrently.

[a] total number of L3, including emerged and recovered at digest
[b] L3 emerging as a percentage of total number of L3
[c] percentage of gastropods experiencing emergence by the end of the trial
[d] percentage of larvae (L) that were infective L3 at digest

Table 5.2: Effects of temperature (range 7.8-24.3 ºC) on development of larvae of *Parelaphostrongylus odocoilei* in *Deroceras laeve* each exposed to 250 first-stage larvae.

Trial	Mean temperature +/- S.D. (ºC)	Monitoring period (dpi[a])	Number of slugs	Mean slug weight +/- S.D. (mg)	Mean larvae/slug at digest (range)	Dpi first L3[b] (no. slugs)
D1	7.8 +/- 0.2	49-91	25	91 +/- 41	38 (14-79)	Not observed
	20.6 +/- 0.4	91-105[c]				105 (1/1)
D2	9.8 +/- 0.3	49-91	21	150 +/- 53	15 (7-29)	70 (1/3) 91 (3/3)
D3	12.4 +/- 0.2	28-56	15	123 +/- 34	12 (4-28)	49 (3/3)
D4	16.4 +/- 0.4	6-26[d]	26	95 +/- 31	20 (8-40)	24 (1/1)
D5	20.0 +/- 0.4	4-18	24	90 +/- 35	25 (5-44)	14 (2/3) 16 (3/3)
D6	24.3 +/- 0.8	3-14[e]	18	89 +/- 41	21 (10-39)	10 (1/3) 12 (3/3)

[a] days post infection
[b] infective third-stage larvae as defined in text
[c] at each of 98, 102, and 105 dpi, 1-2 slugs collected, including mortalities
[d] at each of 20, 22, 23, 24, and 26 dpi, one slug collected, including mortalities
[e] slugs were collected at 3 dpi, then every 2 days starting at 6 dpi

CHAPTER 6

6 EPIDEMIOLOGY AND CLIMATE CHANGE

In this chapter[6], we describe historical, present, and future patterns in development and transmission of *P. odocoilei* and the related protostrongylid *P. stilesi* in Dall's sheep in northern Canada. Through the collection (by collaborators in the Northwest Territories) and analyses (largely by me) of 650 fecal samples, we determined seasonal patterns in prevalence and intensity of shedding of L1 from Dall's sheep in the Mackenzie Mountains. By monitoring larval development in experimentally infected gastropods under field conditions, I determined when L3 would be available to infect sheep. These observations were also used to validate the degree day model developed in Chapter 5. I applied the degree day model to historical data sets of hourly temperatures recorded over the last 9-50 years for 4 locations in northern Canada, in order to describe historical patterns and factors limiting the geographic distribution of protostrongylid parasites. I combined temperature increases from a climate change scenario with the degree day model to predict the effects of climate warming on parasite phenology. I analyzed the data and as corresponding author, researched and wrote the manuscript with input from co-authors.

[6] Jenkins, E.J., A.M. Veitch, S.J. Kutz, E.P. Hoberg, and L. Polley. Climate change and the epidemiology of protostrongylid nematodes in northern ecosystems: *Parelaphostrongylus odocoilei* and *Protostrongylus stilesi* in Dall's sheep (*Ovis d. dalli*). Parasitology, In press. Used with kind permission from editor C. Arme and Parasitology.

6.1 Abstract

We describe the epidemiology of the protostrongylid parasites *Parelaphostrongylus odocoilei* and *Protostrongylus stilesi* in Dall's sheep (*Ovis dalli dalli*) from the Mackenzie Mountains, Northwest Territories, Canada (65º N; 128º W). Peak numbers of first-stage larvae of both parasites were shed by Dall's sheep on their winter range from March-May. In larval development experiments in the Mackenzie Mountains, peak numbers of infective third-stage larvae of *P. odocoilei* were available in gastropod intermediate hosts in August-September. For both protostrongylids, the majority of transmission likely occurs on the winter range, with infection of gastropods when they emerge from hibernation in spring, and infection of Dall's sheep upon their return in fall. We validated a degree day model for temperature-dependent development of larval *P. odocoilei* in gastropods, and applied degree day models to describe and predict spatial and temporal patterns in development of *P. odocoilei* and *P. stilesi* in northern North America. Temperature-dependent larval development may currently limit northward range expansion of *P. odocoilei* into naïve populations of Dall's sheep in the Arctic, but climate warming may soon eliminate such constraints. In Subarctic regions where both *P. odocoilei* and *P. stilesi* are endemic, the length of the parasite "growing season" (when temperatures were above the threshold for larval development) and amount of warming available for parasite development has increased over the last 50 years. Further climate warming and extension of the seasonal window for transmission may lead to amplification of parasite populations and disease outbreaks in host populations.

6.2 Introduction

New host and geographic records have recently been established for the protostrongylid parasites *Parelaphostrongylus odocoilei* (muscleworm) and *Protostrongylus stilesi* (lungworm) in wild thinhorn sheep (Dall's sheep, *Ovis d. dalli*, and Stone's sheep, *O. d. stonei*) in Subarctic

and Arctic North America (Kutz et al., 2001c; Hoberg et al., 2002; Jenkins et al., 2005a). Like all protostrongylid nematodes, these parasites have indirect life cycles, where first-stage larvae (L1) are shed in the feces of the mammalian definitive host, invade a gastropod intermediate host, and develop to infective third-stage larvae (L3). The life cycle of *P. odocoilei* has been described in experimentally infected thinhorn sheep (Jenkins et al., 2005b), but the epidemiology (including seasonal patterns of larval development, transmission, prevalence, and intensity) of protostrongylid parasites in populations of wild thinhorn sheep has not been investigated. Seasonal aspects of transmission of *P. odocoilei* in mule deer (*Odocoileus h. hemionus*) and *P. stilesi* in bighorn sheep (*Ovis c. canadensis*) at temperate latitudes in North America have been characterized (Samuel et al., 1985; Robb and Samuel, 1990).

Development of protostrongylid larvae within the poikilothermic intermediate host is temperature-dependent and can be predicted using degree day models, which assume that a fixed amount of heating accumulated above a critical threshold temperature is necessary for development (Saunders et al., 2002; Harvell et al., 2002). A degree day model has been developed and validated in the field for the protostrongylid *Umingmakstrongylus pallikuukensis*, a lungworm of muskoxen (*Ovibos moschatus*) (Kutz et al., 2002), whereas similar models for *P. stilesi* and *P. odocoilei* have been developed but not yet validated (Samson and Holmes, 1985; Jenkins et al., 2005c). Such models are powerful tools for comparing development of protostrongylid species across latitudinal gradients, isolating factors that are potentially limiting parasite distribution, and determining the potential for range expansion. A degree day model for *U. pallikuukensis* has also been used to describe historical patterns of parasite development and to predict responses of protostrongylid-host systems to climate change (Kutz et al., 2005). Climate change, especially of the magnitude projected in northern regions, will profoundly

influence ecosystems, including the ecological relationships between hosts and parasites (Harvell et al., 2002; Dobson et al., 2003). The distribution of thinhorn sheep in Canada (see Bowyer et al., 2000) corresponds to the 2 districts (i.e., North British Columbia Mountains/Yukon and Mackenzie District) currently experiencing the strongest warming trends in Canada (http://www.msc-smc.ec.gc.ca/ccrm/bulletin/, accessed April 2005).

Our objectives were: to describe the epidemiology of *P. odocoilei* and *P. stilesi* in Dall's sheep in the Mackenzie Mountains, Northwest Territories (NT), Canada; to validate a degree day model for development of larvae of *P. odocoilei* in an important gastropod intermediate host; and to apply degree day models to describe and predict trends in development of *P. odocoilei* and *P. stilesi* throughout the geographic range of Dall's sheep in northern North America, and in a future of climate warming.

6.3 Materials and Methods

6.3.1 Patterns of larval shedding

Every 1-3 mo. from March 2000 to April 2003, we collected approximately 30 separate fecal piles that appeared to have been deposited within the previous 48 h by Dall's sheep in the Katherine Creek study area, Mackenzie Mountains, NT (65°01' N; 127°35' W; elevation ca. 610-1100 m) (Fig. 6.1). Fecal pellets were sealed in plastic bags and frozen for 2-31 wk before recovery of L1 from 5 g samples using a modified beaker Baermann technique (Forrester and Lankester, 1997; Jenkins et al., 2005a). Samples with high numbers of free-living nematodes, insect larvae, or dead L1 were assumed to have been on the ground for longer than 48 h and results were not used in analyses. The prevalence (percent of samples positive) and mean intensity (mean number of L1 per gram of wet feces, or LPG) were calculated separately for *P. odocoilei* and *P. stilesi*.

6.3.2 Development of *P. odocoilei* in *D. laeve*

Using methods adapted from Kutz et al. (2002), in 2002 and 2003 we determined the rates of development for larvae of *P. odocoilei* in experimentally infected native slugs (*Deroceras laeve*) at the Palmer Lake study area, Mackenzie Mountains, NT (64°28' N; 129°37' W, elevation 1100 m) (Fig. 6.1). Slugs were collected from a wet sedge meadow separated by a packed trail from the 20 x 40 m study site, which was enclosed by a battery-powered electric fence to deter animals (e.g., grizzly bears, *Ursus arctos horribilis*, and woodland caribou, *Rangifer tarandus caribou*). The study site was a wet-mesic sedge meadow covered by 5-15% standing water depending on the time of year, and the vegetation was composed primarily of sedge and moss, with mats of mountain aven (*Dryas* sp.) and some willow (*Salix* spp.), dwarf birch (*Betula* sp.), and white spruce seedlings (*Picea glauca*).

Slugs were collected 1-3 times each day for 7-14 d prior to the start of each experiment (Table 6.1), except for Exp. 4, when slugs were collected for 26 days in May 2003 because the ground was still frozen, and traps were occasionally frozen or obscured by fresh snow. Slugs were measured at full extension and grouped into 1 of 3 size classes: small, up to 9 mm; medium, 10-14 mm; and large, greater than or equal to 15 mm (modified from Samuel et al., 1985). Slugs were housed in buckets containing native vegetation at mean temperatures of 6-12 ºC.

First-stage larvae of *P. odocoilei* (originally from the Mackenzie Mountains) were recovered from feces of experimentally infected Stone's sheep (Jenkins et al., 2005b). Groups of 12 slugs were exposed to 3000 motile L1 for 3 h (Hoberg et al., 1995), except for uninfected control slugs that were otherwise treated in a similar fashion. According to the size ratios at collection in each experiment, infected and uninfected slugs were housed in groups of 10 in separate enclosures randomly placed within the study site. Each enclosure consisted of a turf plug (approximate height 16 cm) inside a 5 L high density polyethylene bucket (21 cm top

diameter, 18 cm base diameter, height 19 cm; Ropak Packaging, Fullerton, California, USA) with drainage holes. Each bucket also contained a cut-down aluminum tomato cage (composed of 2 wire circles connected by 3 vertical rods, 24 cm top diameter, 18 cm base diameter, height 28 cm), and the entire enclosure was lined with white nylon/polyester netting (Fig. 6.2). To expel wild slugs, turf plugs were flooded for at least 24 h prior to addition of experimental slugs.

Starting at 14 days post-infection (dpi), every 7 d in 2002, and every 14 d in 2003, infected slugs were recovered by visual examination and sequential flooding for 3 d of 3 randomly-selected enclosures from each experiment (Table 6.1). In 2002, starting at 21 dpi, uninfected control slugs were recovered from 3 enclosures from each experiment every 14 d, and in 2003, starting at 28 dpi, uninfected slugs were collected from control enclosures every 28 d. In May-June 2003, overwintered enclosures (3 with infected slugs and 3 with uninfected slugs) from each experiment established in 2002 were collected at 308 dpi, and 3 overwintered enclosures with infected slugs from each experiment were collected on June 16 (Exp. 1, 336 dpi), July 7 (Exp. 2, 350 dpi), and July 21 (Exp. 3, 357 dpi) (Table 6.1).

Slugs recovered from enclosures were held at approximately 6 ºC for up to 7 d before individual digest in a pepsin-hydrochloric acid solution to recover, count, and classify stages of larvae (Hoberg et al., 1995; Jenkins et al., 2005c). In 2003, the seasonal abundance of infective L3 was calculated for slugs from experiments 4-7 by multiplying the mean number of infective L3/slug by the number of slugs recovered in each collection. In 2003, vegetation from enclosures containing infected slugs was examined for emerged infective L3 on July 28 (Exp. 4), August 11 (Exp. 4-6), and August 25 (Exp. 4-7) (Kutz et al., 2000). Any L3 recovered from uninfected control slugs were identified using molecular techniques (Jenkins et al., 2005a).

To ensure that larvae were viable, 10 infected and 10 uninfected slugs from each experiment (according to the size ratios at collection) were housed indoors at the warmest ambient temperatures possible under field conditions (Exp. 1-3 and 6-9, average 19.5 ºC, range 9-28 ºC; Exp. 4 and 5, average 15.5 ºC, range 3-24 ºC). These slugs were examined for survival and digested at 19 dpi in 2002, and 24 dpi in 2003, except for Exp. 4 where, because of cooler temperatures, slugs were examined for survival at 24 dpi and digested at 36 dpi.

6.3.3 Validation of model

Temperatures 2 cm below and at the soil surface were recorded inside and outside 3 enclosures containing no slugs placed randomly within the study area (Fig. 6.2). Temperatures were recorded every 1 h using external probes from HOBO XT monitors (Onset Computer Corporation, Pocasset, Massachusetts) (15 July to 25 August 2002), every 4 h using HOBO XT monitors (25 August 2002 to 7 May 2003), or every 1 h using HOBO H08 Pro monitors (7 May to 8 September 2003). The means for each temperature measurement for the 3 enclosures were used for further calculations, except for surface temperatures inside enclosures over the winter of 2002-2003, from which data were available from only 2 enclosures. Air temperatures 1.5 m above ground level were recorded every half h (15 July to 25 August 2002) or every 1 h (25 August 2002 to 8 September 2003) by a HOBO weather station in the study area.

To predict when infective L3 should have been present in experiments in 2002 and 2003, we used recorded temperatures (soil, surface, and air), the laboratory-derived threshold temperature (To: 8.5 ºC) and thermal constant (minimum amount of heating necessary for development of infective larvae, 163 degree days, or DD) (Jenkins et al., 2005c), and a degree day model for protostrongylid larval development incorporating slug avoidance of microhabitat temperatures exceeding 21 ºC (Dainton, 1989; Kutz et al., 2002). Temperatures above 21 ºC were converted to 21 ºC, the threshold temperature subtracted from all temperatures, and positive

values summed to determine the amount of heating above threshold on a daily basis. These were converted to degree days (DD) by dividing by 48 for temperatures recorded every half h, 24 for temperatures recorded every 1 h, or 6 for temperatures recorded every 4 h. For each experiment, we calculated cumulative DD based on soil, surface, and air temperatures, and compared dates of predicted (based on the thermal constant) versus observed development of the first infective L3.

6.3.4 Application of model

We obtained hourly air temperature data from Environment Canada weather stations at 4 locations in Subarctic and Arctic Canada, starting in the year that consecutive hourly temperature data were first recorded at each location (Fig. 6.1). These included the following: Ivvavik National Park, Yukon Territory (69º09' N; 140º09' W; elevation 244 m; 1996-2004); Inuvik, NT (68º18' N; 133º29' W; 68 m; 1961-2004); Norman Wells, NT (65º17' N; 126º48' W; 169 m; 1955-2004); and Fort Simpson, NT (61º52' N; 121º21' W; 132 m; 1955-1963; and 61º45' N; 121º14' W; 74 m; 1963-2004). We used temperature data between 1 April and 31 October for the 3 northernmost locations, and between 1 March and 30 November for Fort Simpson. As per (2005), at each location we used degree day models to describe historical trends and variation for theoretical development of larvae of *P. odocoilei* and *P. stilesi*, for the latter using laboratory-derived parameters (To: 7.8 ºC, 305 DD) from Samson and Holmes (1985). To adjust temperatures recorded at the northernmost location (Ivvavik), we used a crude correction factor of –1 ºC per 200 m gain in elevation (http://www.atmosphere.mpg.de/enid/16h.html, accessed Apr 2005), and an altitude of 1000 m for Dall's sheep winter range, which assumed that animals winter at altitudes closer to the valley bottoms than the high elevation summer range (http://www.taiga.net/wmac/consandmanagementplan_volume1/mlandscape.html, accessed Aug 2005).

We used surface temperatures recorded outside enclosures at Palmer Lake in 2003 as the baseline for climate change projections. The last date before 5 consecutive days with average surface temperatures below 0 ºC was assumed to be the last day for gastropod activity in each year (as per Kutz et al., 2005). From a climate change scenario (Canadian Centre for Climate Modeling and Analysis Global Coupled Model 2, A21 Special Report on Emission Scenarios, Economic Regional Focus Simulation 1) yielding mid-range values for the grid box incorporating the study site, we projected mean temperature increases of 1.2 ºC by 2020, 3.3 ºC by 2050, and 4.9 ºC by 2080 (http://www.cics.uvic.ca/scenarios/, accessed Feb. 2005). By adding these values to the observed temperatures in 2003, and using degree day models for development of larvae of *P. odocoilei* and *P. stilesi*, we predicted future trends in development of protostrongylids in the Mackenzie Mountains.

6.4 Results

6.4.1 Patterns of larval shedding

Larvae of *P. odocoilei* were present in 96% (84-100%), and *P. stilesi* in 82% (18-100%), of 650 fecal samples collected from March 2000 to April 2003 (9-47 samples per collection). The mean intensities of L1 of *P. odocoilei* and *P. stilesi* over all collections were, respectively, 568 LPG (141-1350 LPG per collection) and 348 LPG (32-1075 LPG per collection). The prevalence of L1 of *P. stilesi* fell in August of each year, and rose again each fall (Fig. 6.3). Larval intensity for both parasites peaked in the spring of each year, decreased in summer, and gradually increased throughout fall and winter (Fig. 6.3).

6.4.2 Development of *P. odocoilei* in *D. laeve*

Preinfective, but not infective, larvae developed under field conditions in Exp. 1-3 in 2002, and were recovered from live and dead slugs examined in 2003 after overwintering. In 2003, infective L3 were first observed on 28 July from Exp. 4-6, on 8 September from Exp. 7,

and were not recovered from Exp. 8 or 9 (for corresponding dpi, see Table 6.1). Abundance of infective L3 pooled from all experiments was highest in August and September 2003 (Fig. 6.4). Emerged protostrongylid larvae were not recovered from any enclosures. One L3 was recovered from each of 2 uninfected control slugs collected in late July from Exp. 6, which were identified as *P. odocoilei* and an unnamed protostrongylid species endemic in caribou. One unidentified protostrongylid L3 was recovered from an uninfected control slug collected in late August from Exp. 8.

Recovery of uninfected versus infected slugs from enclosures in the field did not differ consistently or substantially for any experiments, but decreased with the passage of time in all experiments. In enclosures from Exp. 1-3 that overwintered, slug recoveries were low, and dead slugs were frequently observed. Slug recoveries were also low in experiments 4, 5, 6, and 9, even at 14 and 28 dpi (Table 6.1). Moribund or dead slugs were often observed in collections in May and June 2003, especially those in the medium size class. Ratios of size classes of slugs were similar for experiments starting in July 2002 (Exp. 1-3) and 2003 (Exp. 6-8) (Table 6.1). In 2003, ratios were approximately the same for all 3 size classes in May (Exp. 4), the relative proportion of medium slugs dropped to its lowest in early June (Exp. 5), and the largest relative proportion of small slugs (60%) was observed in early August (Exp. 9). Eggs were observed in collection dishes and field enclosures from late May until early September in 2003, most noticeably in June and July. Many newly hatched slugs were observed on traps in late July and early August, and were recovered from field enclosures from mid-July to September 2003.

Survival of both uninfected and infected slugs housed indoors ranged from 80-100% for Exp. 1-3 and 7-9, and 40-80% for Exp. 4-6. Most (74-95%) larvae recovered from indoor

control slugs were infective L3, except in Exp. 5, in which 82% of larvae were early L3. Mean infection intensity in indoor controls ranged from 11-43 larvae/slug for the 9 experiments.

6.4.3 Validation of model

Based on all sources of temperature data (soil, surface, and air), larvae were not predicted to develop to infective stage for experiments starting in 2002 until May 19, 2003 (Exp. 1, 308 dpi), June 9, 2003 (Exp. 2, 322 dpi), and June 23, 2003 (Exp. 3, 329 dpi) (Fig. 6.5). For experiments starting in 2003, both air and surface temperatures adequately predicted when infective larvae were first observed, although air temperatures predicted somewhat slower development (Fig. 6.6). Degree days based on soil temperatures did not accumulate rapidly enough to explain observed rates of development.

In the summer of 2003, among the three enclosures where hourly soil and surface temperatures were monitored, the mean standard deviation for soil temperature was approximately 0.75 ºC (range 0-4.6 ºC), while surface temperatures were more variable, with a mean standard deviation of approximately 1.25 ºC (range 0.01-8.8 ºC). In general, surface temperatures in summer were higher than either soil or air temperatures, except in July 2003, when air and surface temperatures were comparable. In winter, soil temperatures were slightly higher than surface temperatures, and both were less variable and much warmer than air temperatures (Fig. 6.7). Surface and soil temperatures were marginally lower inside versus outside enclosures, except in summer, when soil temperatures inside enclosures were slighter warmer. Summer temperatures at Palmer Lake were, on average, 5 ºC cooler than at Norman Wells.

6.4.4 Application of model

For *P. odocoilei*, the length of the "growing season", when temperatures were above the threshold for larval development, and maximum number of cumulative degree days had an

inverse relationship with latitude (Table 6.2). As well, moving from north to south, larvae were predicted to develop to infective stage earlier in the year and could begin development later in the year and still reach infective stage. At Inuvik, Norman Wells (Fig. 6.8), and Fort Simpson, the maximum number of cumulative degree days and the length of the growing season for development has increased over the last 4-5 decades. Despite annual variability, this relationship was statistically significant for cumulative degree days at all three locations (Inuvik $R^2 = 0.14$, $F = 6.8$, $p = 0.013$; Norman Wells $R^2 = 0.15$, $F = 8.7$, $p = 0.005$; Fort Simpson $R^2 = 0.18$, $F = 10.3$, $p = 0.002$), but not growing season.

Using recorded air temperatures, larvae of both *P. odocoilei* and *P. stilesi* could have developed within 1 season at all locations in every year, although the thermal constant for *P. stilesi* was only narrowly exceeded at the 2 Arctic locations (Ivvavik and Inuvik). The two high-elevation data sets (Ivvavik corrected for elevation, and Palmer Lake) yielded approximately half the DD accumulated using uncorrected or nearby low elevation data sets (Norman Wells for Palmer Lake) (Table 6.2). Using elevation corrected data from Ivvavik, L3 of *P. odocoilei* would not have developed within a single summer in 5 of 9 years (1996-2004), and L3 of *P. stilesi* would not have developed within a single summer in any year.

Under a climate warming scenario, the maximum number of cumulative degree days at Palmer Lake increased through the 2020, 2050, and 2080 time slices for both *P. odocoilei* and *P. stilesi* (Table 6.3). As warming progressed, the transmission period lengthened (due to infective larvae becoming available earlier in the year and gastropod activity extending later in the year), and larvae could begin development later in the year and still reach infective stage. In all time slices, infective L3 of *P. stilesi* would be available approximately 3 weeks later in the year than

P. odocoilei, and the last date that larvae of *P. stilesi* could begin development and reach infective stage larvae occurred approximately 3 weeks earlier in the year (Table 6.3).

6.5 Discussion

6.5.1 Epidemiology of protostrongylid parasites

Parasites. In the Mackenzie Mountains, large numbers of L1 of *P. odocoilei* and *P. stilesi* were deposited by Dall's sheep over winter, with peaks in March-May. Using the mean intensity of larval shedding for November 2000-April 2001 (~600 LPG for each protostrongylid), and an average daily fecal production of ~300 grams (Jenkins et al., 2005b), each sheep would deposit approximately 30 million L1 of each protostrongylid species on the winter range. Based on laboratory investigation of the effects of temperature and humidity on survival of L1 of *P. odocoilei*, and laboratory and field trials with L1 of a related protostrongylid (*P. tenuis*) as well as *P. stilesi* (Forrester and Senger, 1963; Uhazy et al., 1973; Shostak and Samuel, 1984; Forrester and Lankester, 1998), a proportion (20-60%) of these L1 would survive overwinter in feces and be available to infect gastropods at snowmelt. Subsequently, gastropod infection may continue throughout summer, although L1 survival decreases with freeze-thaw cycles, increasing temperature, and exposure to sunlight (Rose, 1957a; Shostak and Samuel, 1984; Lorentzen and Halvorsen, 1986). Larval shedding by sheep in the Mackenzie Mountains declined precipitously over the summer, especially for *P. stilesi*. Although a summer decline in prevalence and intensity of *Protostrongylus* spp. has been reported in some bighorn sheep populations (Uhazy et al., 1973; Yde et al., 1988), the marked decline that we observed is unusual, and could be due to seasonal factors in combination with an interspecific interaction between parasites (Pybus et al., 1990; Ball et al., 2001; Lello et al., 2004).

In slugs infected June 2-30, 2003 (Exp. 4, 5, and 6), infective larvae of *P. odocoilei* were first present in all 3 experiments on July 28, 2003. As well, 2 uninfected control slugs collected

at this time contained L3 of *P. odocoilei* and an unnamed protostrongylid endemic to caribou in the Mackenzie Mountains, suggesting that L3 are available in naturally infected gastropods by late July. Long periods of minimal development at cooler temperatures are likely interspersed with rapid bursts of development at warmer temperatures, leading to "pulses" of infective larvae. In slugs infected in mid-July 2003 (Exp. 7), but not in those infected in mid-July 2002 (Exp. 1), infective larvae developed in early September. The summer of 2003 was approximately 0.5 ºC warmer than the summer of 2002, a normal year for this region of the Mackenzie District (http://www.msc-smc.ec.gc.ca/ccrm/bulletin/archive_e.cfm, accessed April 2005) (Fig. 6.9). Therefore, even small differences in overall temperature between years led to different seasonal patterns in larval development and availability.

Infective larvae had high abundance in experimentally infected *D. laeve* in August-September 2003, similar to the pattern observed in gastropods naturally infected with *P. odocoilei* and *P. stilesi* in temperate regions (Samuel et al., 1985; Robb and Samuel, 1990), as well as *U. pallikuukensis* in Arctic Canada (Kutz et al., 2002). Infective larvae in gastropods would remain available to sheep until late fall/early winter, when gastropods enter hibernation. Transmission after this point is theoretically possible, because a small proportion of infective larvae of both *P. odocoilei* and *P. stilesi* emerged from experimentally infected gastropods in the laboratory, and L3 of *P. odocoilei* survived in the environment for months under conditions simulating the subnivean environment (Monson and Post, 1972; Jenkins et al., 2005c). Although we did not observe emergence of larvae of *P. odocoilei* in the field, this may reflect poor slug survival and methods used for examination. Emergence may play a lesser role in transmission of *P. odocoilei* in the Subarctic than for *U. pallikuukensis* at higher latitudes, where emergence significantly extends both the spatial and temporal windows of transmission (Kutz et al., 2000).

Further investigation of the role of larval emergence and the possibility of overwinter transmission for *P. odocoilei* and other protostrongylids is indicated, as overwinter transmission of L3 of gastrointestinal nematodes occurs even in the High Arctic (Halvorsen et al., 1999).

Gastropods. The slug *Deroceras laeve* is important in transmission of elaphostrongylines in Canada (Lankester and Anderson, 1968; Samuel et al., 1985; Ball et al., 2001), and, along with the snails *Catinella* sp. and *Euconulus fulvus*, is probably an important intermediate host for *P. odocoilei* in the Mackenzie Mountains (Jenkins et al., 2005c). Based on temperature data and observations in the Mackenzie Mountains, slugs were active from May-September, as compared to April-November in temperate regions (Aitchison, 1979; Rollo and Shibata, 1991; Lankester and Peterson, 1996). In the current study, slugs of all size classes survived overwinter, with mortality of older individuals in May-June and recruitment primarily in July-August, as observed in temperate areas (Lankester and Anderson, 1968; Samuel et al., 1985). This pattern, and the appearance of moribund slugs consistent with senescence and shrinkage (Rollo and Shibata, 1991), likely account for low slug recoveries from field and laboratory enclosures for Exp. 4, 5, and 6. In Exp. 9, as 90-100% of indoor control slugs were recovered, low recoveries from field enclosures may have been due to the escape of small slugs (which constituted 60% of slugs in this experiment). Few slugs were recovered from overwintered enclosures (as per Kutz et al., 2002), which may reflect natural mortality in combination with colder soil and surface temperatures inside enclosures, probably due to decreased snow cover and limited options for microhabitat selection (South, 1989).

We observed a large cohort of slugs hatching in late summer/early fall, as has been reported in temperate areas (Lankester and Anderson, 1968; Boag and Wishart, 1982). This cohort would be immediately available to L1 the following spring and is likely the most

important source of infective larvae of *P. odocoilei* later in the summer, because young of the year slugs would not be available until July. This is consistent with the observation that larger, older slugs (versus young of the year) had higher prevalence of infective larvae of *P. odocoilei* in an endemic region at temperate latitudes (Samuel et al., 1985). The late-summer cohort may survive a second winter and die the following spring (i.e., a lifespan of ~1.5 years). This differs from the conventional view that *D. laeve* is an annual species (Lankester and Anderson, 1968; Boag and Wishart, 1982; Rollo and Shibata, 1991), although longer life spans for *D. laeve* and terrestrial snails have been described (Getz, 1959; Robb and Samuel, 1990).

Dall's sheep. In April-May, Dall's sheep leave their low elevation winter range (subalpine shrubs and boreal meadows), following the melting snow to higher elevations, where lambing occurs in late May (Hoefs and Cowan, 1979; Simmons, 1982). In June-early August, sheep primarily feed on alpine tundra, where suitable gastropod habitat is thought to be scarce (Hoefs and Cowan, 1979; Boag and Wishart, 1982), although heavily-used, naturally-occurring mineral licks and water sources may provide suitable gastropod habitat and thereby serve as foci of protostrongylid transmission. In late August, nursery groups of sheep (lambs, ewes, and yearlings) begin to return to the low elevation winter range, habitat more suitable for hydrophilic terrestrial gastropods like *D. laeve* and *Catinella* sp. (Hoefs and Cowan, 1979; Simmons, 1982). Upon their return, lambs likely become infected by consuming infective L3 in gastropods. Therefore, the observed increase in intensity of L1 shedding in November in all 3 years of the study may be due to the exponential rise in larval counts following the 70 day pre-patent period in newly infected lambs (Jenkins et al., 2005b), in combination with increased larval shedding by adult males under rutting stress (Halvorsen et al., 1985). Intensity of larval shedding continued to rise throughout winter, which may in part reflect immunological compromise due to

nutritional stress, and/or increased parasite fecundity due to seasonal triggers (Halvorsen et al., 1985; Slomke et al., 1995; Grenfell et al., 1995).

Based on seasonal overlap of protostrongylid larvae, gastropods, and Dall's sheep in the Mackenzie Mountains, the majority of Dall's sheep likely become infected in fall on their winter range, as suggested for *P. odocoilei* in mule deer and *Protostrongylus* spp. in bighorn sheep in temperate areas (Samuel et al., 1985; Robb and Samuel, 1990). Transmission in spring is also possible, most likely from infective larvae of *P. odocoilei* that have overwintered in gastropods. In the current study, preinfective larvae of *P. odocoilei* that overwintered in slugs were predicted to develop to L3 in late May and June, by which time sheep have left the winter range, nor did we observe development to L3 in overwintered slugs in Exp. 1 in mid-June (336 dpi). This, in combination with overwinter mortality of slugs, suggests that such larvae contribute only minimally to transmission of *P. odocoilei*. In contrast, overwinter survival and resumption of development of larvae of *U. pallikuukensis* in *D. laeve* are important for maintenance of this Arctic parasite in years when L3 do not develop in a single summer (Kutz et al., 2002).

6.5.2 Historical and future trends

Model validation. Using a degree day model, we successfully predicted rates of development of larval *P. odocoilei* in the summers of 2002 and 2003. Infective L3 were present in 1 enclosure in Exp. 6 somewhat earlier than anticipated, possibly due to heterogeneity of microhabitat within the study area, especially for soil surface temperatures. Despite concerns about a non-linear relationship at lower temperatures and the effects of stochasticity (Saunders et al., 2002; Jenkins et al., 2005c), degree day models have been validated for larval development of *U. pallikuukensis* (Kutz et al., 2002) and now *P. odocoilei*, which represent different sub-families (Muellerinae and Elaphostrongylinae) in different hosts (muskoxen and Dall's sheep) and habitats (Arctic tundra and Subarctic alpine). Such models, validated under field conditions,

may have broad applicability to other protostrongylids, and perhaps other host-parasite systems (Kutz et al., 2005).

We used air temperatures from weather stations located nearest to Dall's sheep habitat to describe trends in protostrongylid development over the last 50 years, and to compare parasite development on a latitudinal gradient. These stations were located at lower elevations than are typically used by Dall's sheep, but long-term data sets from high elevations are unavailable for northern Canada. This overestimated the cumulative degree days; for example, summer temperatures were ~5 ºC cooler, and approximately half the number of degree days were accumulated, at Palmer Lake in the Mackenzie Mountains than at Norman Wells in the Mackenzie River valley (Table 6.2, Fig. 6.1). Likewise, summer temperatures recorded at Ivvavik, when adjusted for elevation, were 4 ºC cooler, and the number of degree days decreased by a half.

Air temperatures, while predictive at Palmer Lake in 2003, generally produce conservative estimates of development rates (Kutz et al., 2002). Therefore, we used surface temperatures recorded at Palmer Lake in 2003 as the baseline for climate change projections. This was probably a "warm start" scenario, based on the 1955-2004 trend line for DD accumulated at Norman Wells (Fig. 6.8) and Environment Canada data, which indicated that average temperatures in the summer of 2003 were 0.4 ºC above the baseline since 1948 in the Mackenzie District (http://www.msc-smc.ec.gc.ca/ccrm/bulletin/archive_e.cfm, accessed April 2005).

Spatial patterns. We validated the degree day model for development of larvae of *P. odocoilei* in gastropods at the northern limits of its range (the northern Mackenzie Mountains in Subarctic Canada), and applied the degree day model to determine if larvae of *P. odocoilei* could

develop within 1 summer at the northern limits of the distribution of Dall's sheep in Canada (the British Mountains, Ivvavik National Park in Arctic Canada) (Fig. 6.1). At Ivvavik, using the degree day model and temperatures corrected for elevation, development to infective larvae could have occurred within 1 summer in only 4 of the last 9 years. Under current temperature conditions at high elevation at Arctic latitudes, larvae of *P. odocoilei* would therefore have to survive overwinter in slugs and resume development the following summer (a multi-year cycle); however, it is not clear that *P. odocoilei* can undergo a multi-year cycle in *D. laeve*, based on poor slug survival and failure of larvae to resume development after overwintering in the current study. If this, in combination with geographic barriers (isolation by distance), currently excludes *P. odocoilei* from Arctic latitudes, further climate warming may soon release this constraint on range expansion.

Under conditions suitable for larval development, *P. odocoilei* would likely establish at Arctic latitudes, as L1 are resistant to dessication and freezing (Shostak and Samuel, 1984), and gastropod species suitable as intermediate hosts for *P. odocoilei* are present in Alaska and on the Arctic coast (Pilsbry, 1948; Dau, 1981; Hoberg et al., 2002). If introduced, either through translocation or natural movements of definitive hosts (such as cervids), warming could allow *P. odocoilei* to establish in naïve populations of Dall's sheep in the Arctic. Such range expansions have been associated with increased prevalence and severity of clinical disease associated with elaphostrongylines, and even epizootics and population declines (Ball et al., 2001).

While *P. odocoilei* is absent in Dall's sheep populations in the Arctic ranges (Richardson, British, Brooks, and Baird), *P. stilesi* is present in all wild sheep populations surveyed in Subarctic and Arctic North America (Jenkins et al., 2005a). Based on a degree day model (using parameters from Samson and Holmes, 1985), larvae of *P. stilesi*, with its higher DD

requirements, would not have developed to infective stage in 1 summer at Ivvavik, the northernmost location, in any of the last 9 years. This suggests that *P. stilesi* must undergo a multi-year cycle at Arctic latitudes, as described for another Arctic parasite, *U. pallikuukensis* (Kutz et al., 2002). Alternatively, transplacental transmission (involving hypobiosis of L3 in ewes, possibly unique to this species of protostrongylid) may allow maintenance of *P. stilesi* in Dall's sheep at these latitudes (Hibler et al., 1974). The degree day model for *P. stilesi* should be validated in the field using a native intermediate host before it is broadly applied; the parameters derived by Samson and Holmes (1985) for *P. stilesi* were based on development in a snail species that may not be a natural intermediate host, potentially accounting for slower development and the higher thermal constant. A longer developmental period for *P. stilesi* may also reflect evolutionary constraints. *Protostrongylus* spp. are thought to have evolved with caprines in Eurasia, and later became distributed at high latitudes in North America.

Through application of a degree day model, we have examined temperature as a potentially limiting factor for parasite distribution and identified a wildlife population at risk. This provides the basis for wildlife managers to avoid translocation of thinhorn sheep, a common practice for bighorn sheep in North America, and eliminates the need for experimental translocation of parasites to determine if establishment is possible in non-endemic regions (Zarnke et al., 1990; Hoffmann and Blows, 1994).

Temporal trends. Based on our findings and a previous study on *U. pallikuukensis* (Kutz et al., 2005), climate warming has already increased the length of the growing season for protostrongylid development and the amount of heating available for larval development in Subarctic and Arctic Canada. Transmission of protostrongylids to Dall's sheep in fall likely occurs in a narrow window between mid-August, when sheep return to winter range, and mid-

September, when gastropods go into hibernation. Climate warming will cause L3 to be available earlier in the year, but this may have little significance if sheep do not return to the winter range before mid-August. Climate warming may have its most significant effects by extending gastropod activity earlier and later in the season, and by increasing the absolute numbers of infective larvae available to infect sheep. As well, at high elevations at Arctic latitudes, climate warming may increase the number of years when development within 1 summer is possible, facilitating a shift from a multi- to a 1-year cycle, similar to that described for *U. pallikuukensis* (Kutz et al., 2005).

Climate warming could lead to amplification of parasite populations and increased transmission to sheep, possibly resulting in disease outbreaks in endemic regions (Hoberg et al., 2001; Kutz et al., 2004b). Outbreaks of clinical cerebrospinal elaphostrongylosis (caused by *E. rangiferi*) in reindeer in northern Norway occurred in years when summer temperatures were 1.5 ºC above normal (Handeland and Slettbakk, 1994). Recent findings suggest that, in addition to muscular and respiratory pathology, *P. odocoilei* also has the potential to cause neurological disease in thinhorn sheep (Jenkins et al., 2005b). Based on its higher DD requirements and distribution at Arctic latitudes, climate warming could have disproportionately greater effects on transmission of *P. stilesi* as compared to *P. odocoilei*. *Protostrongylus stilesi* is a ubiquitous lungworm of bighorn and thinhorn sheep, and in thinhorn sheep, may cause additive or even synergistic pulmonary pathology in conjunction with eggs and larvae of *P. odocoilei* (Uhazy et al., 1973; Kutz et al., 2001c). Healthy Dall's sheep populations (total 14,000 – 26,000 animals) in the Mackenzie Mountains (140,000 km^2 area) are valuable resources for subsistence harvest and trophy hunting (Veitch et al., 1998).

Although our investigation focused on the positive effects of warming on larval development, warming may also increase mortality of free-living L1 (Levine, 1963), which could in part compensate for amplification of L3 numbers. However, L1 may be produced in sufficient numbers that increased mortality would have little effect on larval abundance in gastropods. Based on our field trials, there was no evidence that infected slugs experienced higher mortality than uninfected controls, suggesting that increased host mortality would not compensate for increased L3 availability.

In addition to temperature, humidity would also influence survival of L1 and the distribution and abundance of gastropods. It was excluded from our model for larval development within gastropods because it was assumed that slugs would buffer larvae from extremes of desiccation through microhabitat selection, behavior, and obligate maintenance of water homeostasis (Rollo and Shibata, 1991). Precipitation was the limiting factor for transmission of *Fasciola* sp., another gastropod-transmitted parasite, in temperate England (Ollerenshaw and Smith, 1969), and is important in the Mackenzie Mountains, which receive a desert-like 25-30 cm of rainfall each year, primarily in July and August (Simmons, 1982). However, all climate scenarios project wetter conditions for this region (http://www.cics.uvic.ca/scenarios/, accessed Feb. 2005), which favors protostrongylid transmission by increasing gastropod abundance and contact rates with L1 (Uhazy et al., 1973; Forrester and Littell, 1976).

6.5.3 Conclusions

The high prevalence of protostrongylid infections in Dall's sheep in Subarctic and Arctic North America bears testament to the success of these parasites at northern latitudes. This may in part reflect abundance and environmental resistance of free-living stages, seasonal concentration of hosts and parasites on winter range, and mobile intermediate hosts capable of

microhabitat selection (Inglis, 1965; Dobson et al., 2003). Under a regime of climate warming, these parasites may undergo amplification in endemic regions, and range expansion into naïve host populations. Without targeted surveillance programs, such signs may go undetected, especially in remote northern regions. Predictive models are key to determining vulnerabilities of wildlife due to climate warming, and to monitoring and proactively managing wildlife diseases (Kutz et al., 2004b). Unfortunately, few empirically-derived and field-validated models are available for the broad range of pathogens that will be affected by climate change. As well, such models must be complemented by sound knowledge of the biology and temporal and spatial patterns of availability of both pathogens and hosts (Morgan et al., 2004). Such data are critically lacking for northern wildlife, where survey and inventory of pathogen biodiversity (Hoberg et al., 2003), as well as baseline data on biologically relevant parameters recorded in appropriate microhabitat types (Danks, 1992), are still desperately needed.

6.6 Acknowledgements

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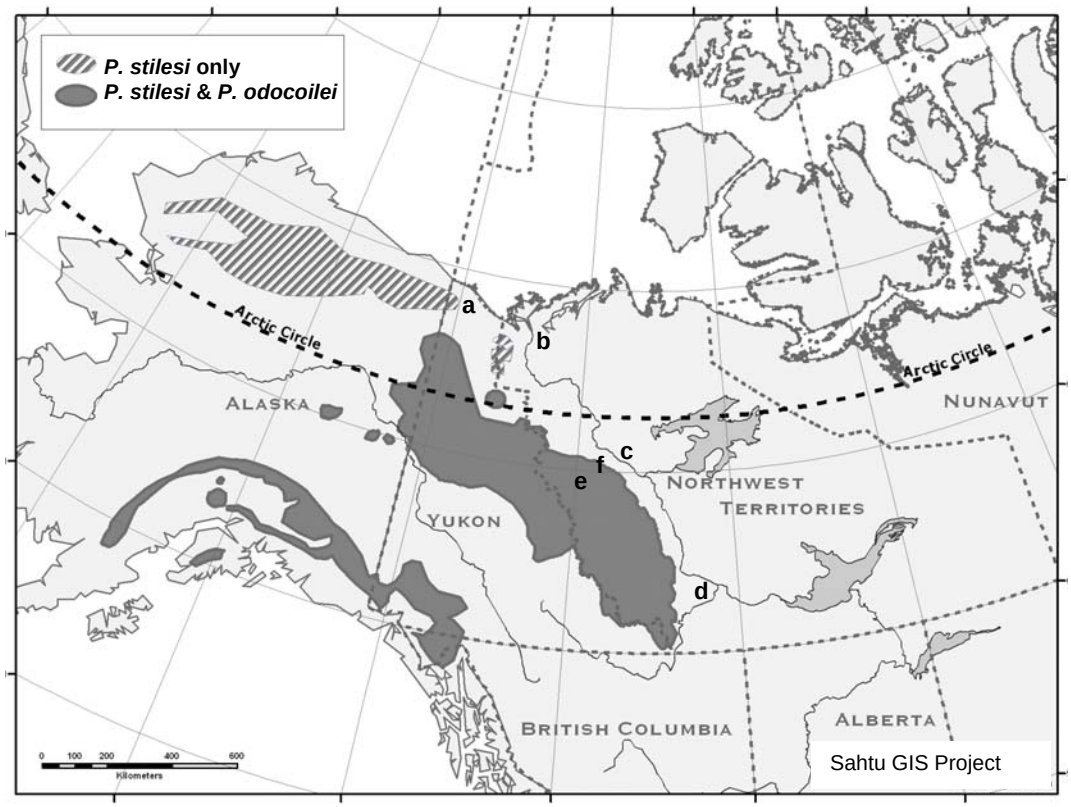


Figure 6.1: Distribution of protostrongylid parasites in Dall's sheep (extrapolated from Worley et al., 2004; Jenkins et al., 2005a) and locations of weather stations and study areas in northern Canada.

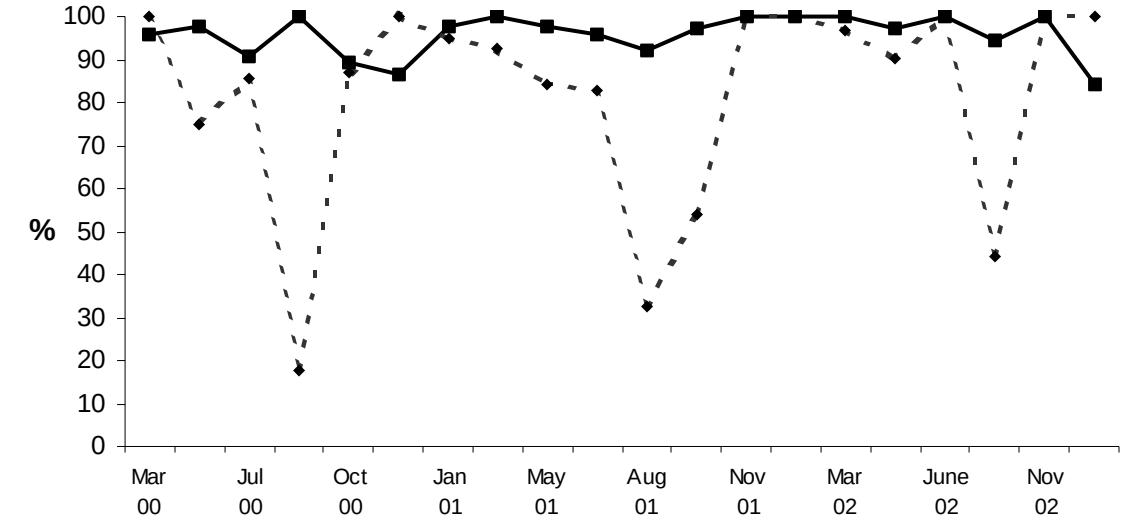
a) Ivvavik National Park, Yukon Territory (YT) (to the immediate left are the British Mountains, continuous with the Brooks and Baird Mountains in Alaska, USA); b) Inuvik, Northwest Territories (NT) (to the left are the Richardson Mountains, NT and YT); c) Norman Wells, NT; d) Fort Simpson, NT; e) Palmer Lake study area, Mackenzie Mountains, NT; f) Katherine Creek study area, Mackenzie Mountains, NT.

a)

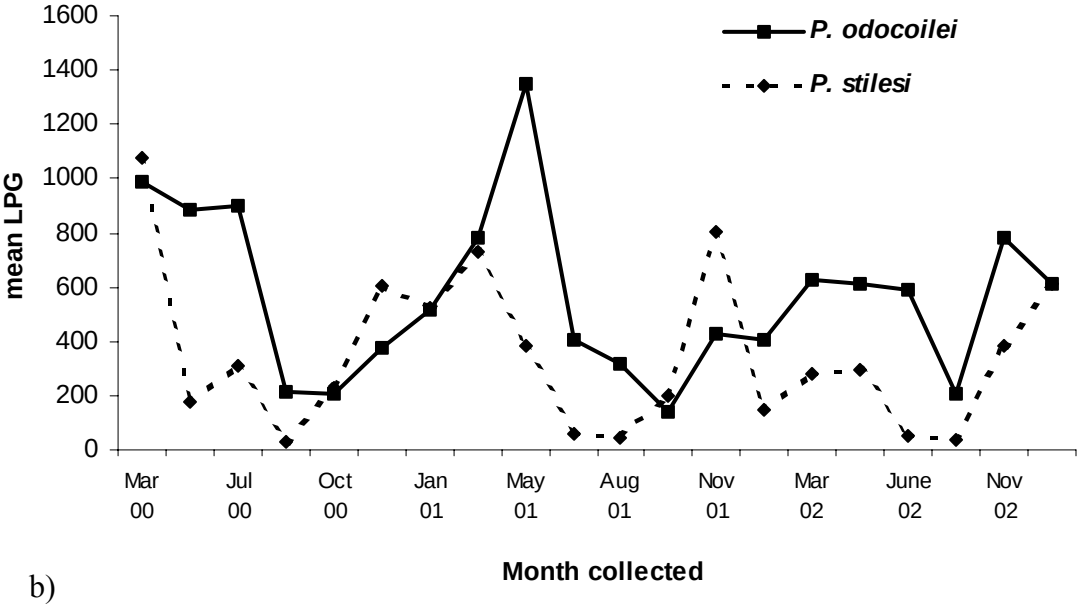
b)

Figure 6.2: Enclosures used in field development trials at Palmer Lake, Mackenzie Mountains, NT, 2002 and 2003.

a) Enclosures containing slugs (*Deroceras laeve*). b) Soil and surface temperatures are measured by HOBO monitors inside and outside an enclosure containing no slugs. Sensor wires are covered with metal tape to prevent damage by rodents.



a)



b)

Figure 6.3: a) Prevalence (%) and b) mean intensity (larvae per gram of feces, or LPG) of first-stage larvae of *P. odocoilei* and *P. stilesi* shed by Dall's sheep in the Katherine Creek study area, March 2000-April 2003.

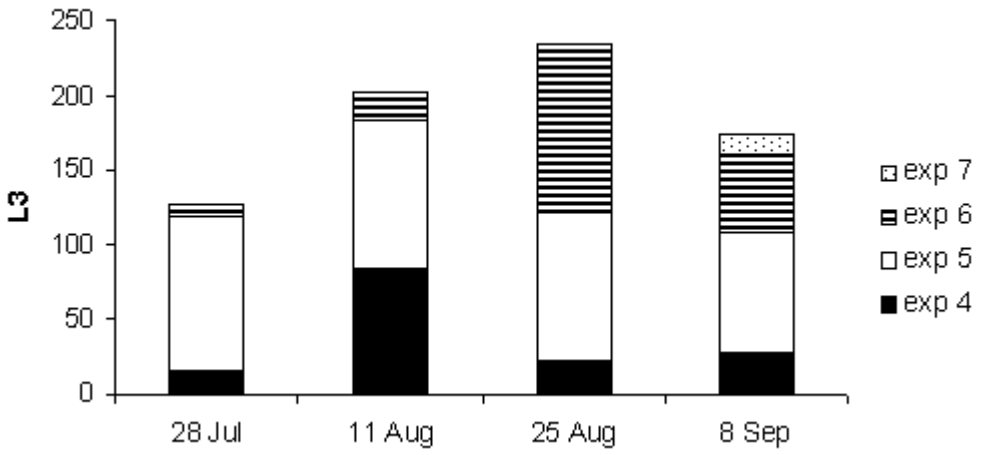


Figure 6.4: Seasonal abundance (number of slugs x mean number of L3/slug) of infective larvae (L3) of *P. odocoilei* in the slug *D. laeve* for Exp. 4-7 in 2003.

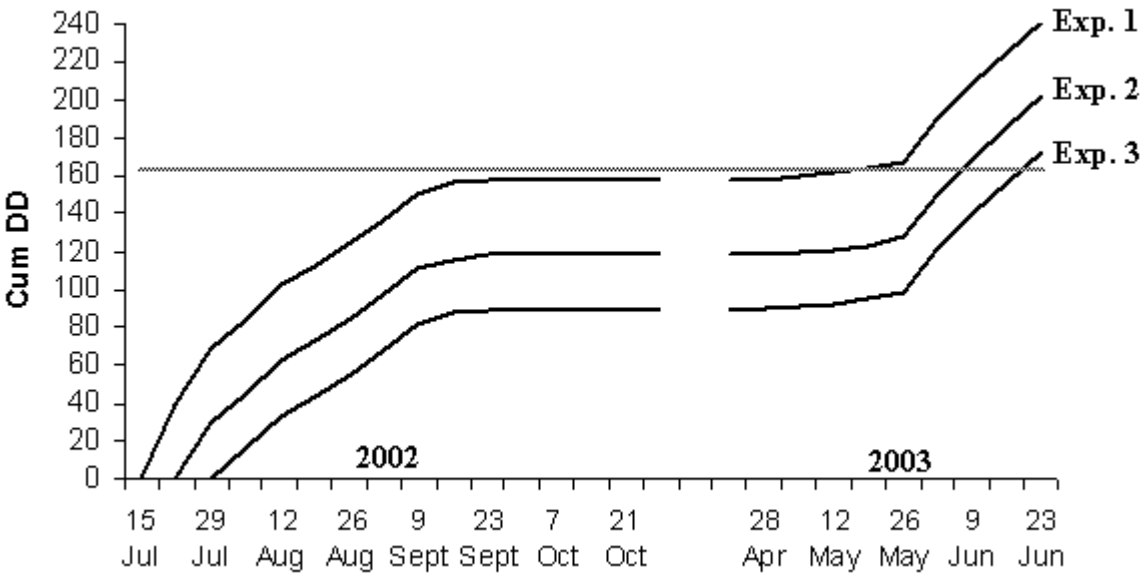


Figure 6.5: Predicted cumulative degree days (Cum DD) for larvae of *P. odocoilei* in experiments starting in 2002 using hourly surface temperatures inside enclosures at Palmer Lake.

Thermal constant (hatched line) = 163 DD.

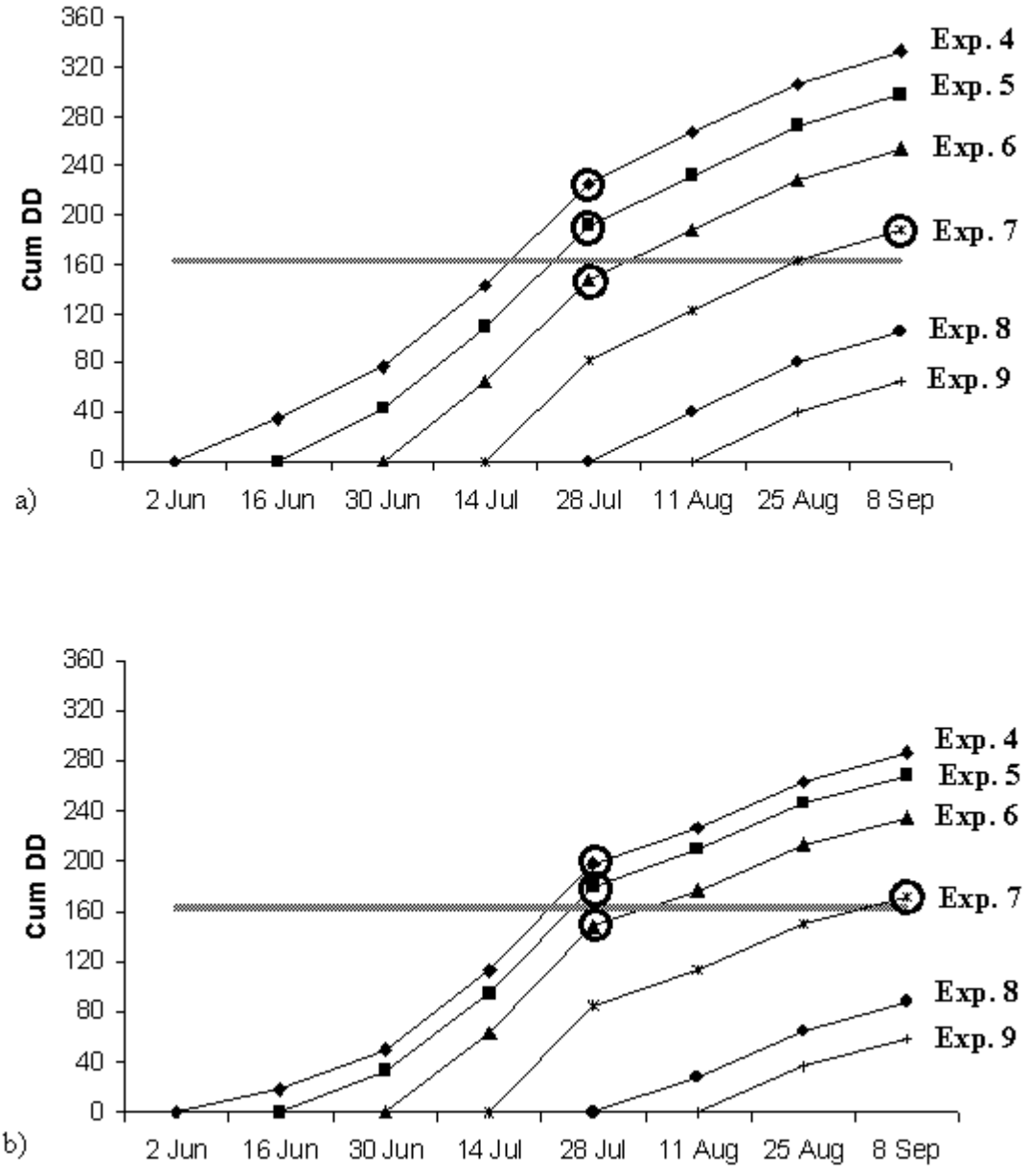


Figure 6.6: Predicted cumulative degree days (Cum DD) for larvae of *P. odocoilei* in experiments starting in 2003 at Palmer Lake using a) hourly surface temperatures inside enclosures and b) hourly air temperatures.

Circled markers indicate the first dates that infective larvae were observed. Thermal constant (hatched line) = 163 DD.

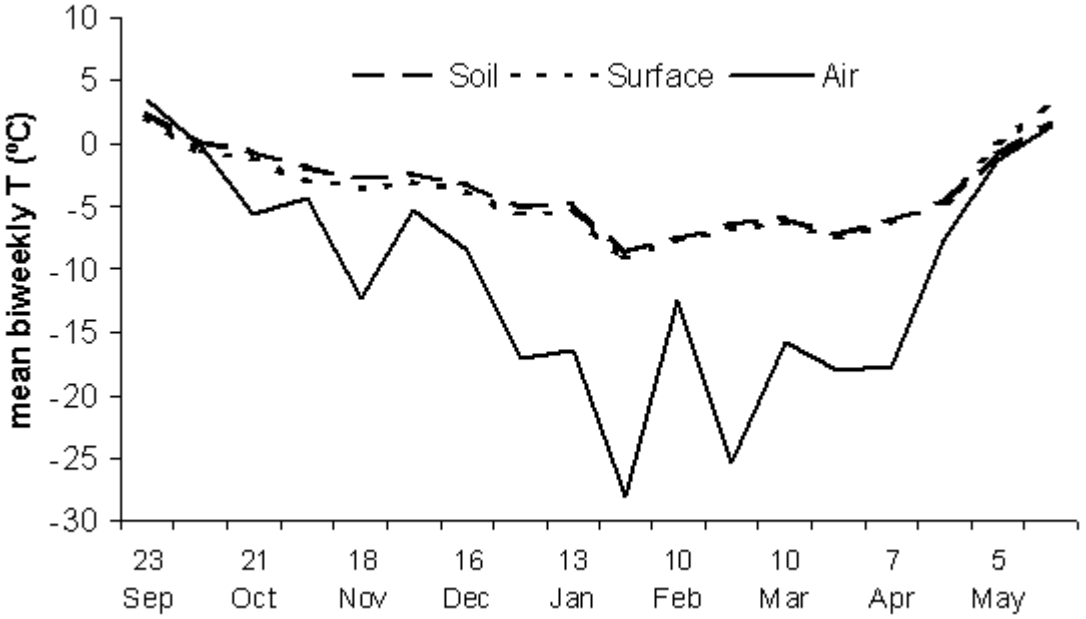


Figure 6.7: Mean biweekly air, surface, and soil temperatures (T, ºC) outside enclosures at Palmer Lake from September 2002 to May 2003.

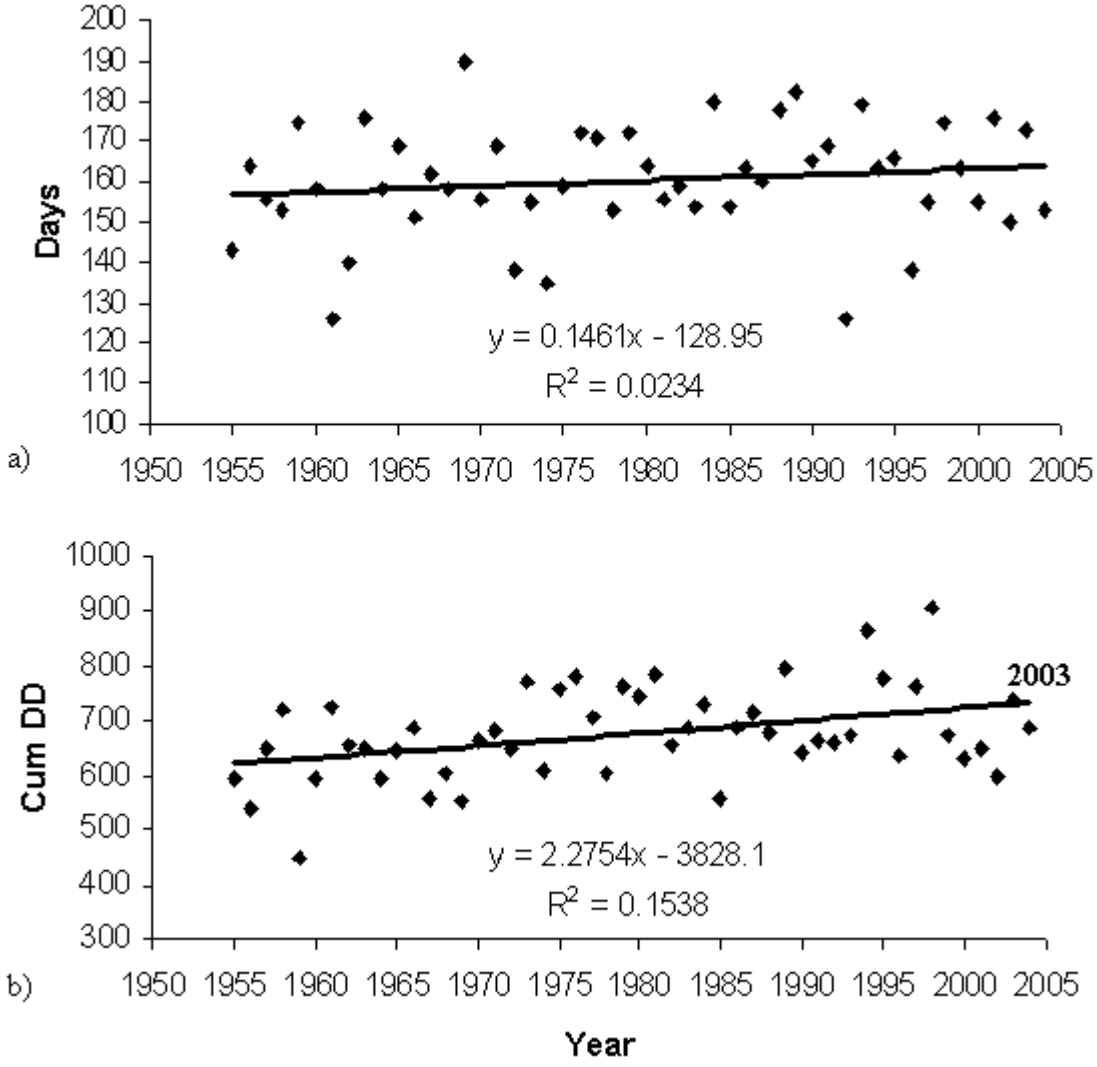


Figure 6.8: Historical trend and interannual variability for development of *P. odocoilei* using hourly air temperatures recorded in Norman Wells, NT, 1955-2004.

a) length of parasite growing season (linear regression, relationship not significant)
b) maximum number of cumulative degree days (linear regression, F=8.723, p=0.005)

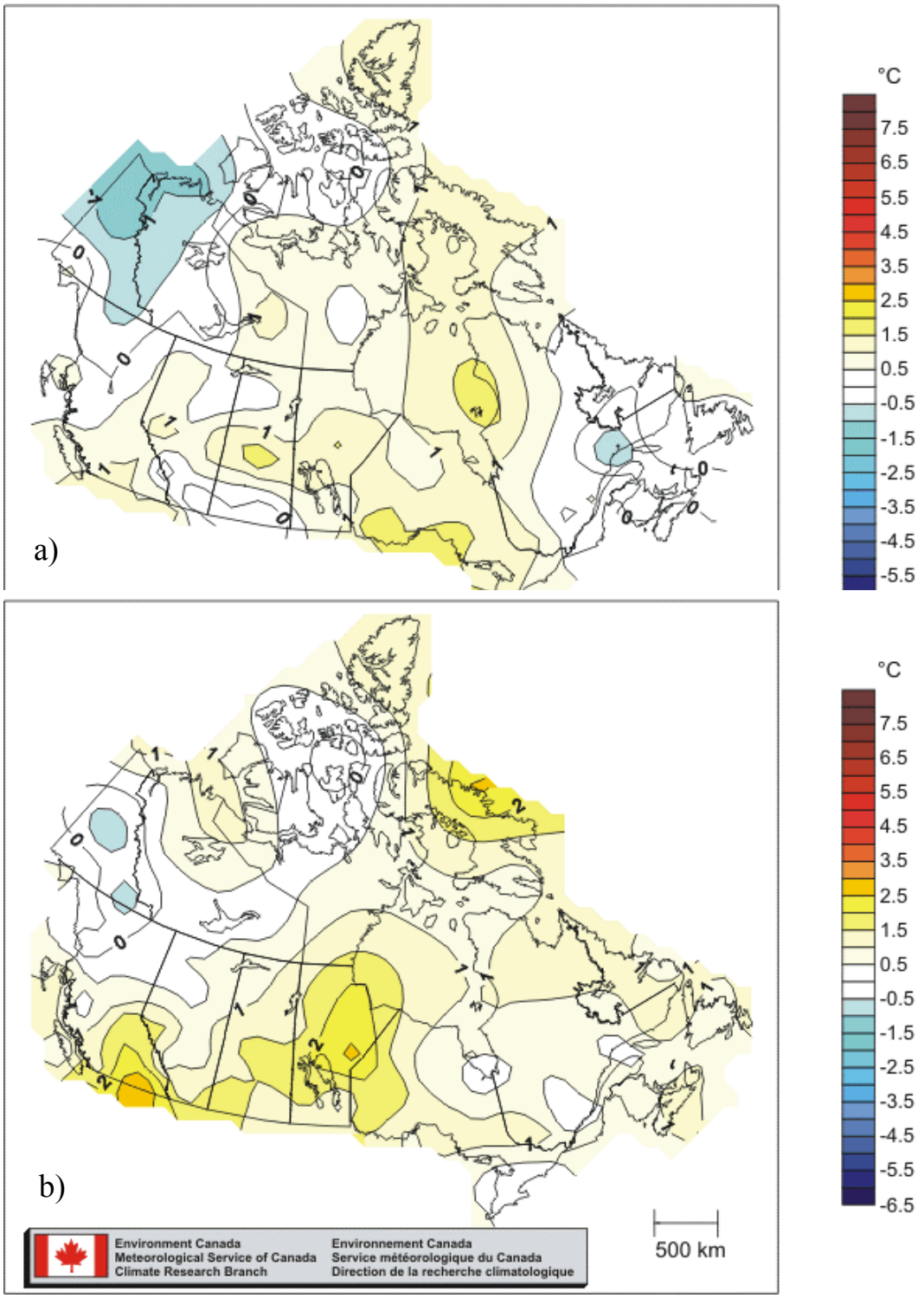


Figure 6.9: Departure of summer (Jun-Aug) temperatures from baseline since 1948 for a) 2002 and b) 2003.
Approximate location of field site at Palmer Lake marked with **X**. From Environment Canada, http://www.msc-smc.ec.gc.ca/ccrm/bulletin/archive_e.cfm

Table 6.1: Development trials with larvae of *Parelaphostrongylus odocoilei* in slugs (*Deroceras laeve*) at Palmer Lake, 2002-2003.

	Size ratios of slugs (%)				Number of enclosures with infective larvae/number with infected slugs (% slug recovery) at DPI[a]										
Exp.	Small	Med.	Large	Start date	14	21	28	35	42	56	70	84	98	308	336-357
1	10	50	40	15-Jul-02	0/3 (60)	0/3 (73)	0/3 (40)	0/3 (63)	0/3 (53)					0/0 (0)	0/2 (10)
2	10	40	50	22-Jul-02	0/3 (80)	0/3 (47)	0/3 (73)	0/3 (73)						0/2 (27)	0/0 (0)
3	10	40	50	29-Jul-02	0/3 (67)	0/3 (63)	0/3 (70)							0/1 (3)	0/0 (0)
4	30	30	40	2-Jun-03	0/3 (57)		0/3 (43)		0/3 (33)	3/3 (13)[b]	3/3 (23)	2/2 (7)	1/1 (7)		
5	40	10	50	16-Jun-03	0/3 (50)		0/3 (37)		2/2 (13)[b]	1/1 (17)	3/3 (17)	3/3 (13)			
6	20	40	40	30-Jun-03	0/3 (37)		1/3 (40)[b]		2/2 (10)	3/3 (13)	1/1 (7)				
7	10	40	50	14-Jul-03	0/3 (70)		0/3 (33)		0/3 (37)	2/3 (33)[b]					
8	20	30	50	28-Jul-03	0/3 (73)		0/3 (77)		0/3 (60)						
9	60	20	20	11-Aug-03	0/3 (47)		0/3 (40)								

[a] Days post infection
[b] Infective L3 observed for the first time on July 28 for Exp. 4, 5, and 6, and Sept. 8 for Exp. 7

Table 6.2: Historical patterns of theoretical development based on recorded air temperatures at locations in Subarctic Canada where *P. odocoilei* is present (Fort Simpson, Norman Wells, and Palmer Lake) and Arctic locations where it is absent (Inuvik and Ivvavik).

	Start of season[a] range	End of season[a] range	Growing season[b] mean (range)	Date of first L3[c] range	Last date for L3[d] range	Cumulative DD mean (range)
Ivvavik (corrected)	May 10 to Jun 3	Aug 28 to Oct 3	114 (86-133)	Jul 17 to Aug 10	Jun 9 to Jun 30	166 (117-249)
Ivvavik (uncorrected)	Apr 18 to May 31	Sep 6 to Oct 4	133 (102-162)	Jun 28 to Jul 19	Jul 2 to Jul 21	355 (283-510)
Inuvik	Apr 16 to May 30	Sep 5 to Oct 14	137 (110-166)	Jun 20 to Jul 22	Jun 30 to Aug 8	442 (300-674)
Palmer Lake 2003	Apr 24	Oct 5	164	Jul 19	Jul 18	320
Norman Wells						
	Apr 1 to May 15	Sep 10 to Oct 15	160 (126-190)	Jun 4 to Jul 4	Jul 17 to Aug 18	676 (450-906)
Fort Simpson	Mar 3 to Apr 30	Sep 22 to Nov 28	193 (155-236)	May 24 to Jun 29	Jul 23 to Aug 21	767 (540-996)

[a] First and last dates that temperatures were above threshold (8.5 ºC) and degree days (DD) were accumulated
[b] Number of days between the first and last dates that DD were accumulated in each year
[c] First date that infective third-stage larvae (L3) could have developed within each year
[d] Last date that first-stage larvae could begin development in gastropods and reach infective L3 stage before winter

Table 6.3: Present and future patterns of development for *P. odocoilei* and *P. stilesi* at Palmer Lake, based on surface temperatures recorded in 2003 and projected warming using a mid-range climate change scenario.

	Start of season[a]	End of season[a]	Growing season[b]	Date of first L3[c]	Last date gastropods[d]	Transmission period[e]	Last date for L3[f]	Cumulative DD
P. odocoilei								
2003	Apr 27	Oct 7	163	Jul 2	Sep 12	72	Jul 22	434
2020	Apr 27	Oct 9	165	Jun 26	Oct 12	108	Jul 26	508
2050	Apr 27	Oct 9	165	Jun 17	Oct 13	118	Aug 6	648
2080	Apr 26	Oct 13	170	Jun 11	Nov 16	158	Aug 12	763
P. stilesi								
2003	Apr 27	Oct 7	163	Jul 23	Sep 12	51	Jul 1	484
2020	Apr 27	Oct 9	165	Jul 16	Oct 12	88	Jul 9	564
2050	Apr 26	Oct 9	166	Jul 6	Oct 13	99	Jul 19	713
2080	Apr 26	Oct 13	170	Jun 30	Nov 16	139	Jul 26	836

[a] First and last dates that temperatures were above threshold (8.5 ºC) and degree days (DD) were accumulated
[b] Number of days between the first and last dates that DD were accumulated in each year
[c] First date that infective third-stage larvae (L3) could have developed within each year
[d] Last date before 5 consecutive days with average surface temperatures below 0 ºC, assumed to be last day for gastropods
[e] Number of days between the date that L3 were first available and the last date of gastropod activity
[f] Last date that first-stage larvae could begin development in gastropods and reach infective L3 stage before winter

CHAPTER 7

7 CONCLUSION

This investigation of *Parelaphostrongylus odocoilei* in thinhorn sheep (*Ovis dalli*) explored aspects of the host-parasite relationship from an ecological perspective, incorporating the influence of host and environment on parasite distribution, development, transmission, and abundance. In the gastropod intermediate host, establishment, growth, development, and the proportion of larvae of *P. odocoilei* which emerged and survived were influenced by abiotic (temperature) as well as biotic factors (species, physiological state, intensity of infection), exemplifying the ecological relationship between parasites and the microhabitat of the host (Crofton, 1971b). The geographic distribution of *P. odocoilei* may be limited by temperature-dependent larval development, and with climate warming, *P. odocoilei* may undergo northward range expansion and colonize new populations of hosts. In thinhorn sheep, *P. odocoilei* had high prevalence and intensity, and caused respiratory and neurological disease, with potentially significant consequences for wildlife health.

In addition to this specific knowledge, we gained broader insights into host-parasite systems, especially those in northern environments. This includes biologically relevant information that can be used to generate phylogenetic hypotheses for the Elaphostrongylinae (section 7.1). As well, we explored determinants and patterns of host specificity and parasite biogeography (section 7.2). We applied molecular techniques to differentiate closely related, morphologically similar parasite species, and to describe the distribution of a parasite and its significance for host biogeography (section 7.3). We identified factors important in success

(establishment and maintenance) of parasites with complex life cycles, especially in northern environments (section 7.4). We developed, validated, and applied a deterministic degree day model to predict specific outcomes, including amplification and range expansion, for host-parasite systems under climate warming (section 7.5). Finally, we developed an investigative approach, including targeted surveillance of wild populations and experimental infection of captive animals, which can be applied to investigate the health significance of any pathogen of wildlife (section 7.6).

7.1 Phylogenetics of the Elaphostrongylinae

Although not the focus of this investigation, this work has significant implications for current understanding of the phylogeny and evolution of the elaphostrongylines, *Parelaphostrongylus* and its sister group, *Elaphostrongylus* (Carreno and Hoberg, 1999). The possibility of a neural migration for *P. odocoilei* challenges the currently accepted phylogeny, which is based in part on migration patterns, as well as parasite morphology and pre-patent period. The current hypothesis places *P. tenuis* basal to the muscleworm lineage, *P. andersoni* and *P. odocoilei* (Platt, 1984; Carreno and Lankester, 1994) (Fig. 7.1a). If the "muscleworms" undergo the CNS-muscle migration pattern observed in *Elaphostrongylus* spp., this pattern may be symplesiomorphic (a shared ancestral character). In this case, the CNS-only migration of *P. tenuis* would be viewed as attenuated, having lost the last portion of the ancestral pattern - migration to the muscles (Platt, 1984; Carreno and Lankester, 1994).

These observations suggest that alternative phylogenetic hypotheses, previously rejected on the assumption that the muscleworm lineage does not undergo a neural migration, should now be reconsidered. Alternative phylogenetic hypotheses include that proposed by Platt (1984), which places *P. odocoilei* as basal to *P. tenuis* and *P. andersoni,* and another alternative detailed in Fig. 7.1b, which places *P. andersoni* basal to *P. tenuis* and *P. odocoilei*. The latter

incorporates a prominent distinguishing feature, the crura of the gubernaculum, while discounting unreliable characters such as shape and size of the dorsal ray, which is polymorphic in *Parelaphostrongylus* spp. (Platt and Samuel, 1978a). In Fig. 7.1b, a basal position for *P. andersoni*, compatible with a more plesiomorphic (ancestral or primitive) condition, corresponds with observations of broad host range (white-tailed deer, woodland and barren-ground caribou, and, experimentally, mule deer and moose) and distribution across North America (Pybus and Samuel, 1984a; Lankester, 2001). If, as is currently accepted, *P. andersoni* evolved in *Odocoileus*, *P. tenuis* and *P. andersoni* either underwent sympatric speciation in white-tailed deer, or allopatric speciation followed by cross-infection of white-tailed deer (secondary colonization) (Carreno and Lankester, 1994).

There is some evidence to support a sister group relationship for *P. odocoilei* and *P. tenuis* (Fig. 7.1b), which are so similar that the initial discovery of *P. tenuis* in the brain of white-tailed deer in Ontario was originally described as *P. odocoilei* (Anderson, 1956). *Parelaphostrongylus odocoilei* and *P. tenuis* may have diverged when ancestral *Odocoileus* speciated into the western, mountain-adapted mule deer, and eastern, woodland-adapted white-tailed deer. Both parasites are specialized for their respective basal hosts; *P. odocoilei* does not establish well in white-tailed deer (Pybus and Samuel, 1984a), while *P. tenuis* causes severe clinical disease in experimentally infected mule deer (Anderson et al., 1966; Tyler et al., 1980). Both parasites are currently geographically isolated, with *P. odocoilei* in the west and *P. tenuis* in the east (Lankester, 2001).

If further experimental work supports a neural migration for *P. odocoilei* and perhaps *P. andersoni*, phylogenetic hypotheses based on migration patterns in the definitive host should be reexamined. New hypotheses should be based on all types of relevant information (total

evidence analyses), including biological (such as migration patterns and host specificity), morphological, and molecular characters, carefully selected for the right balance of interspecific and intraspecific polymorphism. Although ITS sequences have been proposed for phylogenetic analyses of phenotypically similar protostrongylid species (Carreno and Hoberg, 1999), we found that sequences at the ITS2 locus were not sufficiently divergent to be useful for phylogenetic analyses of the closely-related *Parelaphostrongylus* spp. Likewise, Carreno and Nadler (2003) found that sequences of the rRNA and COX I loci had poor resolution and were unsuited to fine-scale comparisons among the Metastrongyloidea. Sequences at other, multiple loci, will likely be necessary to define phylogenetic relationships for this closely-related group of nematodes (Carreno and Hoberg, 1999; Nadler, 2002).

7.2 Determinants of host specificity and parasite distribution

Parasites display a great deal of variability in host specificity, which is likely constrained by phylogeny, as well as historical, biogeographical, physiological, and ecological determinants. *Parelaphostrongylus odocoilei* has a broad host range that follows ecological, as well as phylogenetic, determinants (Inglis, 1965; Platt, 1978; Pybus and Samuel, 1984c; Hoberg et al., 2002). All known, natural definitive hosts for *P. odocoilei* (mule deer, *Odocoileus h. hemionus*; black-tailed deer, *O. h. columbianus*; woodland caribou, *Rangifer tarandus caribou*; mountain goats, *Oreamnos americanus*; Stone's sheep, *Ovis dalli stonei*; and Dall's sheep, *O. d. dalli*) are mountain-adapted cervids and bovids. Mountain goats and thinhorn sheep (Caprinae) are phylogenetically distant from the other hosts (Cervidae), but based on high prevalence and broad distribution in the Subarctic, thinhorn sheep appear to be at least as suitable a host for *P. odocoilei* as the "preferred" host, mule deer (Platt and Samuel, 1978b).

In the Arctic (north of ~65 ºN), however, *P. odocoilei* does not appear to be established in Dall's sheep, barren-ground caribou (*R. t. groenlandicus*), or muskoxen (*Ovibos moschatus*)

(Appleyard et al., 2005). Populations of Dall's sheep in the Arctic have likely been separated from those in Subarctic North America (60-65ºN) since the last glaciation at the end of the Wisconsinan (10,000 years before present, ybp) (Sage and Wolff, 1986) (Fig. 7.2). This suggests that *P. odocoilei* colonized thinhorn sheep within the last 10,000 years, after the Subarctic and Arctic populations of Dall's sheep became isolated from each other. This colonization event may have occurred in the early Holocene, when new host communities formed on newly exposed or altered habitat (Guthrie, 1968; Kurten and Anderson, 1980; Hoberg et al., 2003; Hoberg, 2005), accompanied by rapid warming and climatic instability (McBean, 2005). The proposed timeline is further supported by the apparent absence of *P. odocoilei* in bighorn sheep (*Ovis canadensis*), ancestors of which moved south in the Sangamonian interglacial (120,000 to 75,000 ybp) and became isolated from the ancestors of thinhorn sheep by invasion of the Cordilleran and Laurentide ice sheets in the Wisconsinan (75,000 to 10,000 ybp) (Kurten and Anderson, 1980; Hoberg, 2005) (Fig. 7.2). Historical and contemporary ecological conditions do not appear to have driven a host switch for *P. odocoilei* from mountain goats and mule deer to bighorn sheep, which share range at many locations (Blood, 1963; Kistner et al., 1977; Aune et al., 1998).

A history of recent colonization is an important determinant of the current geographic distribution of *P. odocoilei* in thinhorn sheep. A much older host-parasite association likely accounts for the widespread distribution of the protostrongylid lungworm *Protostrongylus stilesi* in all thinhorn and bighorn sheep populations across North America (and possibly the closely-related Snow sheep in Siberia - see Neiland, 1976). *Protostrongylus* spp. lungworms and *Ovis* are Eurasian in origin, and likely entered North America in the Illinoian, 600-300 000 years before present (Pillmore, 1955; Kurten and Anderson, 1980; Hoberg, 2005) (Fig. 7.2).

Gastropod intermediate hosts for *Protostrongylus* spp. (Families Pupillidae and Vallonidae, Suborder Orthurethra) are also Eurasian in origin, and may have entered North America prior to or in combination with wild *Ovis*, which may themselves have mediated the introduction of these gastropods (Pillmore, 1955; Platt, 1978). In contrast, cervids colonized North America in the Pliocene, approximately 2-3 million years before present, about the time that *Ovis* was evolving in Eurasia in the Villafranchian (Kurten and Anderson, 1980). Ancestral *Parelaphostrongylus* would have been established in North American cervids and gastropods (Suborders Heterurethra and Sigmurethra) long before *Ovis* entered the Nearctic.

Parasite distributions in conjunction with host genetics can reveal patterns in historical host biogeography. For example, the distribution of *P. odocoilei*, present in the Subarctic but not Arctic Dall's sheep populations, is consistent with genetic evidence for isolation-by-distance of populations of Dall's sheep in the Arctic and those in the Subarctic, and between sheep populations in the Nahanni region (negative for *P. odocoilei*) and the rest of the Mackenzie Mountains (Worley et al., 2004), where close to 100% of Dall's sheep are infected with *P. odocoilei*. The geographic distribution of protostrongylids and other parasites in northern ungulates is the product of a series of historical and geographic events, including co-evolution, colonization, and host switching (a mosaic in the sense of Hoberg et al., 1999; Hoberg et al., 2001; Hoberg, 2005). Comparing distributions of sympatric parasites with different phylogenetic origins, histories, and host associations – for example, *Teladorsagia* and *Nematodirus* spp. (thought to have originated with the Caprinae), versus *Parelaphostrongylus* spp. (thought to have originated with the Cervidae) (Hoberg et al., 1999; Hoberg, 2005) would reveal historical patterns in host movement, as well as future potential for host switching.

7.3 Molecular techniques in parasitology

Host specificity and geographic distribution for many helminth parasites of wildlife are incompletely known, in part because of the difficulties in recovering and identifying adult parasites, and challenges of identifying morphologically similar juvenile stages (Hoberg et al., 2001; Criscione et al., 2005). Molecular techniques, validated with adult parasite morphology, were key to describing a far greater geographic range for *P. odocoilei* in north-western North America than had previously been demonstrated. This was the first time such an approach has been applied across the geographic distribution of a protostrongylid parasite. Despite this significant advance in molecular diagnostic techniques for this group of nematodes, challenges remain, including false positives based on ingestion and passage of small numbers of first-stage larvae in feces from sympatric definitive hosts (Duffy et al., 1999), long latent periods where no larvae are shed (Duffy et al., 2002), and specificity of serological diagnosis in fecal-negative hosts (Ogunremi et al., 2002).

In addition to diagnostic identification, molecular techniques have other applications in parasitology, including describing epidemiology and phylogeography of host-parasite relationships, and detecting cryptic species and population genetic structure (Avise, 1994; Hoberg et al., 1999; Paterson and Viney, 2000; Criscione et al., 2005). The population genetic structure of parasites reflects patterns of host gene flow (Blouin et al., 1995; Criscione et al., 2005), and can be used to gain insight into historical distribution and isolation of hosts. For example, both *P. odocoilei* and *P. stilesi* are established in many Dall's sheep populations in the Subarctic. The primary hosts for *P. stilesi* are thinhorn and bighorn sheep, which are restricted to mountain ranges by strong habitat preference, and to small seasonal ranges by marked philopatry (Geist, 1971; Worley et al., 2004). In contrast, potential hosts for *P. odocoilei* include several cervid species that are not restricted to mountainous habitat (such as caribou and moose)

and could serve as mediators of parasite transmission and gene flow between otherwise isolated populations of Dall's sheep, including those currently negative for *P. odocoilei*. As a result, the population genetic structure of *P. stilesi* is predicted to match that of sheep populations (i.e. highly structured), whereas *P. odocoilei* is predicted to have greater genetic homogeneity. For *P. odocoilei*, sequencing at a mitochondrial loci has confirmed high levels of homogeneity across its range, with some genetic structure following geographic lines (E. Hoberg, B. Rosenthal, E. Jenkins, unpubl. obs.), supporting a history of recent colonization and sustained gene flow among different ungulate hosts. Microsatellites hold great promise for describing fine scale genetic differences for *P. odocoilei* and other parasites (Blouin, 2002; Mortenson et al., 2005).

7.4 Ecology and epidemiology

Parasite transmission in northern ecosystems faces unique constraints and challenges, which include limited seasonal availability of intermediate and definitive hosts, and short "growing seasons" for development of parasites and poikilothermic intermediate hosts. For protostrongylids in thinhorn sheep, this is further complicated by the seasonal migration pattern of the definitive host, resulting in an extremely narrow window available for parasite transmission. This investigation of 2 protostrongylid parasites near their northern distributional limits has led to greater understanding of aspects of life history important in the epidemiology and distribution of parasites at northern latitudes (Table 7.1). Many of the factors in the left hand column in Table 7.1 represent life history characteristics that enhance success at northern latitudes, such as long life span and patency in the definitive host, providing temporal buffering during periods of unfavorable abiotic conditions. The high prevalence and broad distribution of *P. odocoilei* and *P. stilesi* in northwestern North America, and local establishment of "Arctic-adapted" parasites such as *U. pallikuukensis*, are testament to the success of these parasites, and may in part reflect advantages of the complex protostrongylid life cycle. Compared to parasites

with direct life cycles, indirect life cycles may be favored in extreme environments because intermediate hosts buffer developing larvae from harsh conditions, as well as increase chances of parasite transmission through greater mobility (Inglis, 1965; Dobson and Carper, 1992; Anderson, 2000). The ability to use multiple species of gastropods as intermediate hosts, and possibly emergence of larvae from gastropods, may increase chances of parasite transmission and larval survival overall (Gerichter, 1948; Fenton and Hudson, 2002).

Transmission in such host parasite systems involves a bewildering array of interacting factors, including distribution, density, and spatial and temporal patterns in recruitment of definitive and intermediate hosts, larval development and availability in intermediate hosts, and prevalence and intensity of shedding of L1 by definitive hosts (Kralka and Samuel, 1990). This complexity explains why models for indirectly-transmitted parasites are seldom available (Smith et al., 1995). Some of the factors in Table 7.1 could be used to develop such a model for *P. odocoilei*, and to define the basic reproductive number (Ro), a measure of parasite fitness. For macroparasites, Ro is defined as the number of female worms that are established from one female worm in a population of fully susceptible hosts, when there are no density-dependent constraints (Hudson et al., 2001). When Ro is greater than or equal to 1, colonizing parasites can invade, and currently established parasites will persist. Where Ro is less than 1, parasites will be unable to establish, and be eliminated. Although Table 7.1 addresses parasite "success" from a single-species perspective, this likely fails to capture the complexity of these multiple host, multiple parasite systems (Petney and Andrews, 1998; Morgan et al., 2004; Lello et al., 2004). In the Mackenzie Mountains, several species of protostrongylids are present in the same definitive hosts (i.e., *P. odocoilei* and *P. stilesi* in Dall's sheep) and intermediate hosts (i.e. larvae of *P. odocoilei* and an unknown protostrongylid in the slug *D. laeve*). Ecological or

immunological interactions are likely, but not well-described or understood (Pybus et al., 1990; Reguera-Feo et al., 1996; Ball et al., 2001). A multiple host system raises the possibility of apparent competition among different definitive hosts (Gilbert et al., 2001), which for *P. odocoilei* in the Mackenzie Mountains potentially include mountain goat, woodland caribou, moose, and Dall's sheep (Simmons et al., 1984).

There are still many unknowns in Table 7.1. Based on this investigation of *P. odocoilei* in Dall's sheep in the Subarctic, infection of definitive hosts likely occurs in fall. Further investigation is needed to determine if transmission can occur at other times of year (for example, in spring with L3 that have overwintered in gastropods), and if emerged larvae survive and remain infective under field conditions, and therefore play a role in transmission. As well, the role of other intermediate hosts, such as the snails *Catinella* and *Euconulus* spp., and the possibility of transmission on the summer range, perhaps at water sources and mineral licks, have not been investigated. Finally, transplacental transmission has been documented for *P. stilesi*, but has not been investigated for any of the elaphostrongylines. Further experimental observations in both laboratory and field environments are needed for protostrongylids from different latitudes and in different hosts and habitats (e.g., alpine, boreal, grassland, tundra).

7.5 Effects of climate change on parasites

Temperature-dependent larval development in intermediate hosts is an important factor determining the distribution and abundance of protostrongylids. To quantify this, we developed and validated a degree day model for larval development of *P. odocoilei* in the slug *D. laeve*, making this the second protostrongylid parasite for which this has been achieved (Kutz et al., 2002) and confirming that such models have broad applicability to other parasites for which temperature-dependent development is critical. Although this model is deterministic and likely oversimplified (Crofton, 1971a), it generated useful predictions of larval availability. It was,

therefore, sufficiently complex to include important biological variation, but simple enough to exclude that which was unnecessary to explain observations (Morgan et al., 2004).

Based on the degree day model and projected rates of climate warming, development of *P. odocoilei* will, like many parasites with both indirect and direct life cycles, continue to accelerate as climate warms (Dobson and Carper, 1992; Patz et al., 1996; Harvell et al., 2002). Climate change will influence all aspects of the life cycle of protostrongylids, and may have negative consequences for transmission; for example, increased mortality of free-living L1, and possibly decoupling of parasite and host phenology (Table 7.1). The general consensus, however, is that both protostrongylid parasites and gastropods will do well in warmer, wetter conditions, as predicted for the Mackenzie Mountains and other regions in the Subarctic where *P. odocoilei* is established (http://www.cics.uvic.ca/scenarios/, accessed Feb. 2005).

Climate change and other anthropogenic disturbances have and will continue to influence parasite distribution, phenology, and pathogenicity (Hoberg et al., 2002; Wasel et al., 2003; Kutz et al., 2005). This has led to recognition of the following critical steps (adapted from Dobson and Carper, 1992; Kovats et al., 2001; Harvell et al., 2002): 1) collect baseline data on distribution, prevalence, and effects of parasite/disease on wildlife; 2) isolate the effects of climatic factors, such as temperature, on transmission of parasite/disease, which requires experimental manipulation in the laboratory and field; 3) provide regional evidence of climate change based on monitoring, preferably over a long time period at multiple sites; 4) forecast amplification of parasite populations in endemic regions, range expansion, and emergence of disease; and 5) detect epidemiological and health consequences of climate change. For *P. odocoilei,* the first four objectives have now been accomplished, providing the means to detect changes associated with climate warming, and to forecast specific outcomes of climate warming,

including significant extension of transmission season, amplification in endemic regions, and range expansion. Demonstrating the epidemiological and health consequences of climate change (step 5), such as the link between warmer summers and outbreaks of clinical disease caused by *E. rangiferi* in semi-domesticated reindeer (Handeland and Slettbakk, 1994), will be difficult in the remote Canadian North, and requires the cooperation of multiple agencies and an interdisciplinary approach (Hoberg, 1997; Paterson and Viney, 2000; Kutz et al., 2001a; Hoberg et al., 2003).

"Determining how long-term climatic changes will affect…different parasites and pathogens at first seems a daunting task that almost defies quantification" (Dobson and Carper, 1992). In contrast with human health, the potential effects of climate change on wildlife health have not been well recognized (Klein, 2005), probably because established baselines and extensive monitoring are rarely available for diseases of wildlife, especially those that are not zoonotic. Establishing baselines requires long term monitoring, accurate taxonomic identification, and specimen banking, all of which are challenges faced by workers in the field of wildlife disease (Hoberg, 1997; Mills and Childs, 1998; Brooks and Hoberg, 2000; Hoberg et al., 2003).

7.6 Wildlife health and management

The approach used in this investigation of a newly discovered host-parasite relationship has relevance for exploring any new or newly recognized disease in wildlife hosts, especially in remote, undeveloped regions which pose unique logistical challenges. Remote sample collection and surveillance, which drew on the innovation and expertise of wildlife biologists, conservation officers, hunters and outfitters, combined with targeted collections, provided baseline information on the pathogens present and the role of disease in an important wildlife population. We also drew on the combined resources of veterinary and human medicine to describe and

isolate the effects of a specific pathogen in experimentally infected hosts. This investigation was limited by small sample sizes, the inability to examine large numbers of hosts (and difficulties in recovery of adult parasites from tissues), and the inability to mark known individuals and follow outcome of parasite infection in different subpopulations (Gulland, 1992; Mills and Childs, 1998). Nonetheless, we gained valuable insights into the role of this host-parasite relationship in wildlife health.

Regardless of whether *P. odocoilei* merely retains the ability to go through the CNS, or is neurotropic in the strict sense of the term (as defined by Anderson, 1968), this parasite can cause neurological disease in thinhorn sheep. This may in part reflect increased pathogenicity and/or a stronger immune response in a recently colonized host, as observed for *Elaphostrongylus* spp. and *P. tenuis* in domestic caprines (Handeland, 1994; Anderson, 2000). The possibility of neurological disease, in addition to respiratory pathology observed in both experimentally and naturally infected thinhorn sheep, suggest that *P. odocoilei* is a newly recognized threat to the health of thinhorn sheep. Based on the seasonal timing of infection and peak larval shedding, *P. odocoilei* probably contributes to mortality of young of the year in the first winter and spring of life, which may be masked by or concomitant with other causes of mortality, including severe weather, predation, and poor nutrition.

Although helminths are rarely considered to be the cause of emerging diseases of any importance (Daszak et al., 2000), they demonstrably regulate some ungulate populations (Gulland, 1992; Albon et al., 2002). We do not know if *P. odocoilei* regulates wildlife host populations, and determining population level effects through experimental manipulation of free-ranging populations is hindered by logistical difficulties as well as resistance of this parasite to anthelmintic treatment. Interestingly, some populations of Stone's sheep in northern British

Columbia are undergoing a decline from unknown causes, and *P. odocoilei* has recently been identified in at least one population in the Muskwa-Kechika region (Jenkins and Schwantje, 2004; Demarchi and Hartwig, 2004). For other protostrongylids, circumstantial evidence suggests that *U. pallikuukensis* may have contributed to a population decline in muskoxen in Canada (Kutz et al., 2004b), and *E. rangifer*i has been identified as the probable cause of a similar decline in a recently infected population of caribou in Newfoundland (Ball et al., 2001).

In most thinhorn sheep populations, little is known about disease or its interaction with other sources of mortality, such as predation. This in part reflects the remote nature of thinhorn sheep habitat in the alpine regions of the Subarctic and Arctic, largely unpopulated by people apart from the summer hunting season. Unsurprisingly, more is known about the effects of parasites in free-ranging ungulates in relatively accessible regions, often on islands where ranges are strongly delimited and there are few competitors or predators (Gulland and Fox, 1992; Halvorsen et al., 1999; Ball et al., 2001). Continued surveillance for specific pathogens and general monitoring of population health will give greater understanding of what pathogens and diseases are significant in thinhorn sheep, or are likely to emerge, either through introduction into naïve populations, or amplification in endemic regions due to changes in ecological or climatic factors (Hoberg, 1997).

Because of its broad host range and high prevalence (approaching 100% in some cervid and thinhorn sheep populations), *P. odocoilei* may pose a threat to other wildlife species, including endangered populations of woodland caribou. Generalist parasites pose greater risks to wildlife health than those with narrow host specificity (Morgan et al., 2004), and parasites with high prevalence are less likely to be lost in introductions and invasions (Torchin et al., 2003; Hoberg, 2005). *Parelaphostrongylus odocoilei* has great potential for range expansion, unlike

some protostrongylids which may be geographically limited due to narrow host specificity, such as *U. pallikuukensis* (Kutz et al., 2001a; Kutz et al., 2004a). Range expansion could occur through either anthropogenic or natural movements of ungulate hosts, perhaps in response to the breakdown of ecological borders due to climate warming (Hoberg, 1997). Such expansion could have complex ecosystem level effects, including exclusion of more susceptible host species (apparent competition), as has been suggested for the closely related, neurotropic *P. tenuis* (Anderson, 1972; Leighton, 2003).

Cases of fatal verminous and bacterial pneumonia in Dall's sheep have been described, but likely reflect an endemic, sporadic pattern of wildlife mortality, which depends on the balance between host immunity and pathogen virulence (Leopold, 1939). This is in contrast to the epidemic pattern of mortality due to bacterial pneumonia in bighorn sheep. Along with habitat loss, pathogen introduction from domestic animals (either through "spill-over" from established populations, or anthropogenic translocation) pose the greatest threats to survival of the fragmented, remnant populations of bighorn sheep in North America. The plight of these populations best approximates situation 2d in McCallum and Dobson (2002), where no level of habitat connectivity can save a species which has limited abilities to disperse (due to philopatry and limited available habitat) away from the pathogen reservoir (domestic livestock). In such cases, strict quarantine and controlled movement of disease-free individuals are the "only possible conservation strategies", bearing in mind that strains and species of pathogens originally from domestic livestock now circulate in many populations of bighorn sheep, and therefore mixing of bighorn populations is also a risk factor (Miller et al., 1991). In theory, reduction below a threshold host population size can decrease transmission and has been suggested as a management solution for pneumonia in bighorn sheep; in practice, population reduction is rarely

successful for disease control, and threshold population size varies depending on the relative virulence and transmission potential of circulating pathogens (Wobeser, 2002).

As defined by Wobeser (2002) and Leighton (2003), proactive management is the best management strategy for thinhorn sheep. Although currently isolated, thinhorn sheep are likely vulnerable to pathogens transmitted by direct contact with domestic livestock (Garde et al., 2005), as well as many parasites that can be transmitted to wild sheep through shared pools of infective larvae on range (Morgan et al., 2004). Currently, the gastrointestinal parasite fauna of Dall's sheep probably reflects the endemic parasite fauna of wild *Ovis* (Nielsen and Neiland, 1975; Hoberg et al., 2001), uncontaminated by "pathogen pollution" from domestic livestock (Daszak et al., 2000). Apart from the obvious health consequences of invasion of parasites of domestic livestock, conserving the unique host-parasite assemblages of the endemic Arctic fauna is key to maintaining biodiversity (Daszak et al., 2000).

7.7 Future directions

This investigation of a newly discovered host-parasite relationship has led to greater understanding of the health and parasite status of thinhorn sheep, and the need for proactive management. This in turn led to a risk assessment for pathogen transmission between Dall's sheep and domestic livestock in the Northwest Territories (Garde et al., 2005). Complementary to investigation of *P. odocoilei* in thinhorn sheep, we also completed the life cycle of *P. stilesi* in thinhorn sheep for the first time, and determined gastropod intermediate hosts potentially important in transmission of *P. stilesi* in the Subarctic (Skific, 2003). As well, application of molecular techniques developed in this investigation led to a new geographic record for *P. odocoilei* in Oregon (Mortenson et al., 2005) and discovery of an unknown protostrongylid L1 in caribou and L3 in slugs in northern Canada (Appleyard et al., 2005). This work, along with other recent investigations by the Research Group for Arctic Parasitology (Hoberg et al., 1995;

Hoberg et al., 1999; Hoberg et al., 2002), has increased our knowledge of parasite biodiversity in the remote Canadian North, while at the same time highlighted the need for more baseline data on parasites and other pathogens of wildlife in this region. This in turn has led to preliminary investigations of the gastrointestinal parasite fauna of Dall's sheep in the NT (Kutz et al., 2006), as well as the parasite fauna of Stone's sheep in BC (Jenkins and Schwantje, 2004; Demarchi and Hartwig, 2004), and mountain goats in the NT and BC (Jenkins et al., 2004). This work complements and confirms pioneering research on the effects of climate change on northern host-parasite systems and wildlife health (Kutz et al., 2004b; Kutz et al., 2005; Bradley et al., 2005). Finally, this thesis is testament to the efforts of biologists, veterinarians, parasitologists, researchers, outfitters, hunters, and other wildlife stakeholders across North America, who have laid the foundation for future investigation and cross-disciplinary collaboration.

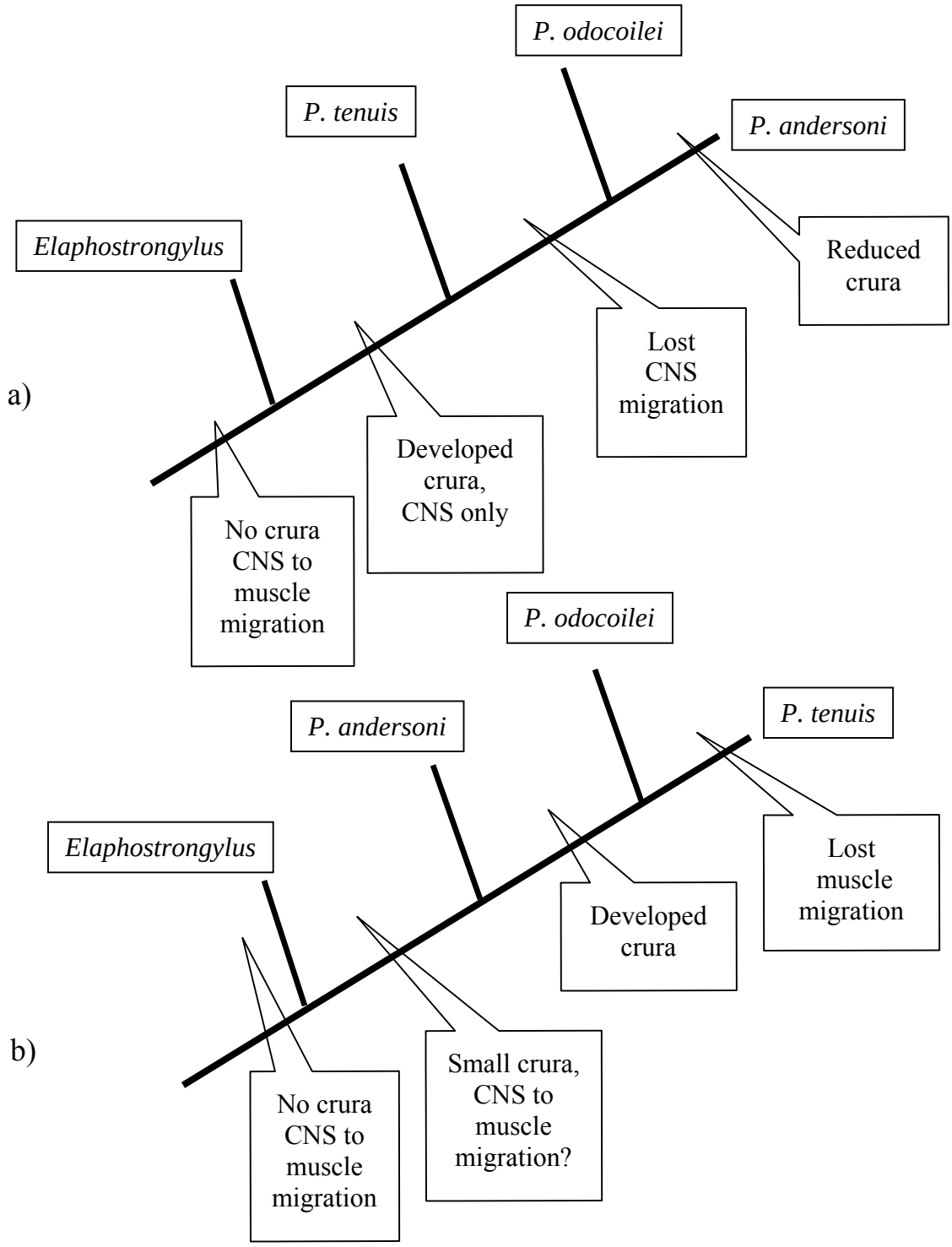


Figure 7.1: Phylogenetic relationships of the elaphostrongylines.
a) Currently accepted hypothesis (Platt, 1984; Carreno and Lankester, 1994) based on various morphological characters and the assumption that *P. odocoilei* and *P. andersoni* do not undergo a neural migration. b) Proposed hypothesis, based on a distinctive morphological character (crura of the gubernaculum) and new evidence that *P. odocoilei*, and possibly *P. andersoni*, may undergo a neural migration.

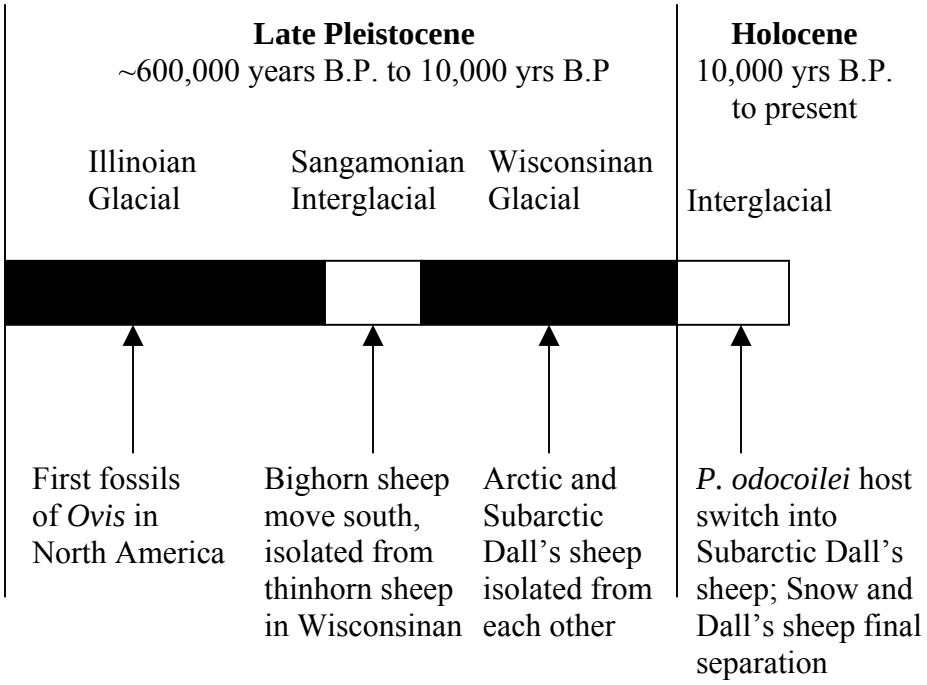


Figure 7.2: Time line showing movements of *Ovis* during the late Pleistocene, and timing of host-switch.

Ovis is thought to have evolved in Eurasia, with pachycerine sheep (bighorn and thinhorn sheep) possibly evolving in Beringia (Kurten and Anderson, 1980).

Table 7.1: Factors that may influence the success of *P. odocoilei* and protostrongylid parasites in general in a single parasite system

	Success	Limitations
L1	High prevalence and intensity in feces	Seasonal effects, spatial heterogeneity
	Environmentally resistant	CC may increase mortality
	Peak availability coupled to phenology and recruitment of IH	CC may decouple
	Can infect multiple species of IH	History, phylogeny, sympatry
L3	High IH and DH density on winter range, shared habitat preference	Low prevalence in IH and narrow seasonal window of transmission *
	Development within 1 year at all temperate and Subarctic locations	Not capable of 2-year cycle necessary in cold years at Arctic latitudes? *
	Larval emergence allows transmission if gastropods unavailable	Larvae may not emerge or survive in significant numbers for *P. odocoilei*
	Peak availability coupled to phenology and recruitment of DH	CC may decouple
	Infection of multiple species of DH	History, phylogeny, sympatry
	Manipulate behavior of IH to enhance transmission?	May not occur for *Parelaphostrongylus* spp. (McCoy and Nudds, 2000); adverse effects in heavily infected IH? (Skorping, 1985)
Adults	High prevalence and intensity in DH	Intensity-dependent effects and immunity in DH
	Long life expectancy	Life span of host
	Long patent period	Life span of host
	Little pathology in muscles	Severe lung pathology may kill DH
	Use CNS as immunological harbor?	CNS disease may kill DH in PPP
	Transplacental transmission, as for *P. stilesi* (Hibler et al., 1974)?	Probably does not occur for *P. odocoilei*

Note: abbreviations as defined previously; also IH = intermediate host, DH = definitive host, CC = climate change, * = limitation that may be removed by climate change

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APPENDIX: LARVAL MORPHOMETRICS AND GROWTH

Introduction

While the morphology and morphometrics of first-stage larvae (L1) and third-stage larvae (L3) of *Parelaphostrongylus odocoilei* of both cervid and thinhorn sheep origin have been described (Hobmaier and Hobmaier, 1934; Ballantyne and Samuel, 1984; Kutz et al., 2001c), the continuum of larval growth from L1 to L3 has only been described for *P. odocoilei* of cervid origin (Platt, 1978). Therefore, our objective was to describe morphometrics and patterns of growth of larval stages of *P. odocoilei* of thinhorn sheep origin in the slug *Deroceras laeve*. As well, we investigated whether gastropod species (*D. laeve* or *Catinella* sp. snails from the Mackenzie Mountains, NT), infection intensity, and method of recovery of L3 (spontaneous emergence or recovery by digest of gastropods) influenced larval morphometrics and growth. Larvae from gastropods in development (D4) and emergence trials (E1, E2, and E3) in Chapter 5 were used in the following analyses. Please refer to this chapter, and Tables 5.1 and 5.2, for detailed methods.

Materials and Methods

In trial D4, slugs (*D. laeve*) were held at 16.4 ºC and digested every other day from 6-26 days post infection (dpi). At each digest day, 10 larvae were heat-killed in a droplet of water on a slide and measured at 400X. For each morphometric character, descriptive statistics were calculated for the predominant larval stage at each digest day, and for pooled larvae of all stages, where L1 = first-stage larvae, L1-2 = developing first-stage larvae, L2 = second-stage larvae, eL3 = early third-stage larvae, l eL3 = live early L3, and iL3 = infective L3.

All gastropods for emergence trials were maintained at room temperature and emerged larvae collected every other day until 60 dpi (E1) or 84 dpi (E2 and E3). At 21 dpi for E2 and E3, 3 control gastropods were digested, and at the end of all emergence trials, remaining gastropods were digested and larvae recovered. In E1, 10 emerged infective L3 and 10 infective L3 from slugs digested at 60 dpi were refrigerated for 3-4 d prior to measurement. In each of E2 (*Catinella* sp. snails) and E3 (*D. laeve*), 10 emerged L3, 10 infective L3 from gastropods digested at 21 dpi, and 10 infective L3 from gastropods digested at 84 dpi were refrigerated for 1-3 mo prior to heat-killing and measurement as described above.

Measurements of infective L3 from *D. laeve* were compared using one-way ANOVA and included six comparison groups: D4 (n=12 L3 from digests at 24-26 dpi); E1 (n=10 emerged L3, n=10 L3 from digests at 60 dpi); and E3 (n=10 emerged L3, n=10 L3 from digests at 21 dpi, n=10 L3 from digests at 84 dpi). For E2 and E3, measurements of infective L3 were compared using univariate ANOVA for two factors, intermediate host species (*Catinella* sp. or *D. laeve*) and recovery method (emerged L3, L3 from digests at 21 dpi, or L3 from digests at 84 dpi). For both analyses of variance, if the p-value from the F-test was less than 0.05 and homoscedasticity was confirmed, a post-hoc Bonferroni multiple comparison was used. All statistical analyses were performed using SPSS 12.0 (Statistical Package for the Social Sciences, SPSS Inc., Chicago, Illinois).

Results

Larval growth was measured by changes in each of 10 characters (Fig. A1). Although 10 larvae were examined on each digest day, only the data for the predominant larval stage are presented. As well, not all characters were visible in all larvae, so the number of measurements for each character ranged from 5-10, and the genital primordium (GP) was generally not visible in L2. Initially, only body width (BW) increased substantially from L1 to L1-L2 (Table A1). The greatest change in characters, and the highest amount of variability in measurements, occurred in the L2 (Fig. A1). As larvae developed, most characters, including body length (BL) and BW, increased until the early L3, except the length of the esophagus proportional to body length (%E), which decreased from 40-50% in L1 and L1-2 to approximately 30% in infective L3 (Table A1, Fig. A1). After the early L3 stage at 22 dpi, only width of the base of the esophagus (EW), GP length (GPL), and GP width (GPW) continued to increase. Distance from anus to tail tip (TL), from head to GP (GPD), and from head to the base of the esophagus (ED), as well as BL and BW, decreased slightly between dead early L3 at 22 dpi and live early L3 at 24 dpi (Fig. A1).

Morphometrics of L1, L1-2, L2, early L3, and infective L3 of *P. odocoilei* are presented in Table A1. Values for L1 and infective L3 from Kutz et al. (2001) were included, as the larvae were similar to the current study both in origin (L1 from Dall's sheep in the Mackenzie Mountains, and L3 from the same colony of *D. laeve*) and method of fixation (heat-killed on slide). Values for infective L3, both emerged and from digests, were pooled for each of E1, E2, and E3 (Table A1). With one exception, there were no significant differences between L3 that emerged or L3 from gastropods digested in E1, E2 or E3, although variances of measurements of emerged L3 were generally greater. Among infective L3 from E2 and E3, mean BW of emerged L3 (37.0 +/- 2.1 um) and L3 from digests at 84 dpi (37.4 +/- 1.6 um) were significantly greater than that of L3 from digests at 21 dpi (35.6 +/- 1.2 um). In Table A1, values for body width were pooled within each trial despite this difference, as the magnitude was very small.

Means of ED, BW, EW, and %E were significantly greater in L3 from *D. laeve* in E3 than L3 from *Catinella* sp. in E2 (Table A1). Among infective L3 from *D. laeve* (D4, E1, and E3), L3 from digests in E3 and D4 were significantly larger than L3 from digests in E1 (Table A2). In E3, %E of emerged L3, and EW of L3 from digests at 84 dpi were significantly larger than L3 from digests in D4, which were primarily intermediate L3.

Discussion

Patterns in larval growth of *P. odocoilei* of thinhorn sheep origin in *D. laeve* were consistent with other members of the Elaphostrongylinae, including *P. odocoilei* of cervid origin (Platt, 1978) and *E. rangiferi* (Halvorsen and Skorping, 1982; Skorping, 1984), as well as a member of the Protostrongylinae, *Protostrongylus boughtoni* (Kralka and Samuel, 1984a). For these two subfamilies, the most dramatic changes in growth and development of all characters occur in the L2, in contrast with the Muellerinae, in which the majority of growth occurs in the L1-2 and late L2/early L3 (Gerichter, 1948; Beresford-Jones, 1966; Kutz et al., 2001b). Growth of L3 of *P. odocoilei* was limited to increases in length and width of the esophagus and the genital primordium, as well as body width; similarly, esophagus width, length of the genital primordium, and body width continued to increase from intermediate to late L3 of *U. pallikuukensis* (Kutz et al., 2001b).

This is the first description of morphometrics of developing larval stages of *P. odocoilei* of thinhorn sheep origin. Such data are necessary, but seldom available, for comparative studies of protostrongylid larvae and species identification based on larval morphology. Investigation is further limited by species-specific visibility of some characters; for example, the excretory pore was clearly visible, but the nerve ring was indistinct, in all larval stages of *P. odocoilei*, and the genital primordium was generally not visible in the L2 stage. In contrast, the nerve ring and genital primordium were often visible in larvae of *U. pallikuukensis* and *E. alces*, while the excretory pore was apparent in larvae of *E. cervi* but not *E. alces* (Lankester et al., 1998; Kutz et al., 2001b). In addition, measurements are affected by artifacts of fixation; for example, heat-killing, as compared to dying in digest fluid, may have caused artificial decreases in several characters of live early L3 of *P. odocoilei* of both thinhorn sheep and cervid origin (Platt, 1978).

Method of recovery of infective L3 (digested or emerged) did not influence larval measurements. In contrast, intermediate host species (and possibly physiological state, as *Catinella* sp. snails were observed aestivating) may influence larval growth, as infective L3 from *Catinella* sp. were smaller than those in the slug *D. laeve* (which does not undergo aestivation). As well, infective L3 were significantly smaller in *D. laeve* in E1, which had the highest infection intensity of all trials (Tables 5.1 and 5.2), suggesting that larval growth may be density-dependent, or more appropriately, intensity-dependent (Margolis et al., 1982), as has been observed for larvae of other protostrongylid species (*E. rangiferi* and *Protostrongylus pulmonalis*) (Boev, 1975; Skorping, 1984). All measurements of late L3 in Kutz et al. (2001c) were slightly greater than those of infective L3 in the current study, which may reflect differences in infection intensity, observer technique, or length of storage of L3 prior to examination. As a result of these limitations, molecular techniques are increasingly being applied to identification of protostrongylid larvae (Jenkins et al., 2005a), including L3 in gastropods.

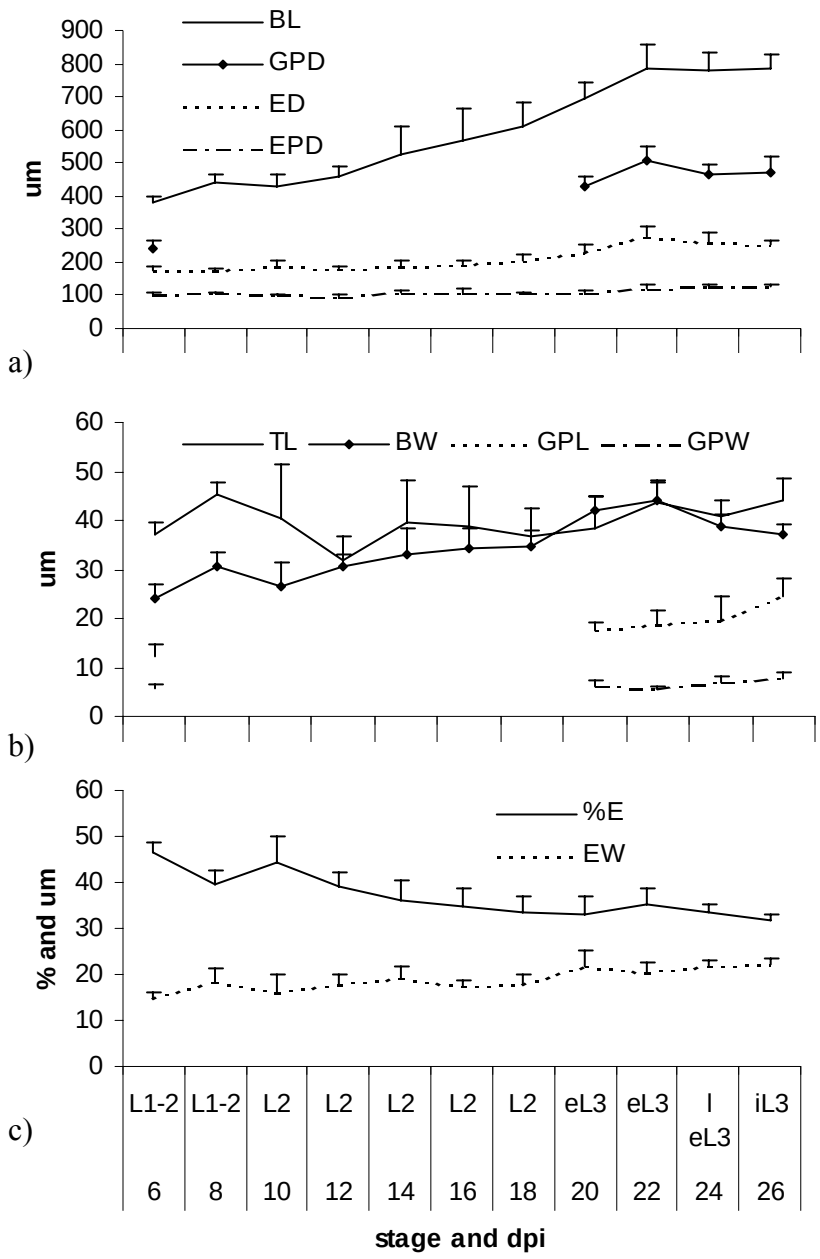

Figure A1: Patterns of growth of larval stages of *P. odocoilei* (as defined in text) at progressive days post infection (dpi).

a) Body length (BL), and distances from head to: genital primordium (GPD), base of esophagus (ED), and excretory pore (EPD). b) Anus to tail tip (TL), body width at base of esophagus (BW), genital primordium length (GPL) and width (GPW). c) %E and width of base of esophagus (EW). All measurements are in um except %E, and error bars = 1 S.D.

Table A1: Morphometric characters (in um except %E) of larval stages of *P. odocoilei* in development (D4) and emergence (E1, E2, and E3) trials, and from Kutz et al. (2001c). Data presented as mean ± standard deviation, range (minimum-maximum), and no. of larvae (n).

		D4	D4	D4	D4	E1	E2	E3	
	L1[ab]	L1-2	L2	eL3	iL3	iL3	iL3	iL3	iL3[b]
BL[a]	387 ± 30	408 ± 37	509 ± 97	746 ± 70	789 ± 42	739 ± 36	773 ± 54	782 ± 48	813 ± 42
	334-428	340-482	332-745	619-872	703-829	692-843	681-890	671-902	740-922
	n=21	n=19	n=48	n=28	n=12	n=20	n=30	n=30	n=24
BW	18 ± 2	27 ± 4	32 ± 5	41 ± 4	37 ± 2	37± 2	36 ± 2[c]	37 ± 2[c]	42 ± 4
	15-20	18-35	21-44	35-50	35-41	35-44	33-41	34-41	36-49
	n=21	n=19	n=48	n=27	n=12	n=20	n=30	n=30	n=24
EPD	105 ± 8	105 ± 4	103 ± 9	118 ± 12	127 ± 9	120 ± 7	128 ± 8	128 ± 8	132 ± 11
	92-117	97-112	76-129	100-150	115-141	106-133	114-142	112-147	119-162
	n=21	n=19	n=48	n=28	n=12	n=20	n=30	n=30	n=24
ED	181 ± 14	174 ± 11	190 ± 17	251 ± 32	253 ± 14	245 ± 17	252 ± 13[c]	261 ± 17[c]	266 ± 14
	160-206	144-199	159-244	188-326	226-279	221-284	231-282	236-305	235-293
	n=21	n=19	n=48	n=28	n=12	n=20	n=30	n=30	n=24
EW		16 ± 3	18 ± 3	21 ± 3	22 ± 1	22 ± 2	23 ± 2[c]	24 ± 1[c]	
		13-24	12-24	18-29	21-24	20-28	20-25	20-25	
		n=19	n=48	n=27	n=12	n=20	n=30	n=30	
%E	47 ± 2	43 ± 5	38 ± 6	34 ± 3	32 ± 1	33 ± 1	33 ± 1[c]	33 ± 2[c]	33 ± 2
	44-50	33-50	28-55	27-42	30-35	31-35	30-35	30-37	30-33
	n=21	n=19	n=48	n=28	n=12	n=20	n=30	n=30	n=24
GPD	256 ± 20	241 ± 27		461 ± 46	477 ± 42	454 ± 27	476 ± 36	487 ± 30	495 ± 37
	219-285	168-263		391-547	368-509	415-518	414-541	424-551	435-596
	n=21	n=10		n=21	n=12	n=20	n=30	n=30	n=24
GPL		12 ± 2		18 ± 3	25 ± 4	24 ± 2	22 ± 3	23 ± 2	
		10-15		15-26	18-29	20-28	18-28	18-27	
		n=9		n=19	n=12	n=20	n=30	n=30	
GPW		6 ± 1		6 ± 1	8 ± 1	7 ± 1	8 ± 1	8 ± 1	
		5-8		4-9	6-9	5-10	5-10	5-10	
		n=9		n=19	n=12	n=20	n=30	n=30	
TL	39 ± 4	40 ± 5	37 ± 8	41 ± 5	43 ± 5	40 ± 4	45 ± 4	44 ± 4	48 ± 4
	33-46	32-49	22-68	28-51	38-50	33-45	37-53	36-52	43-55
	n=21	n=19	n=48	n=28	n=12	n=20	n=30	n=30	n=24

[a] larval stages and characters as defined in text
[b] from Kutz et al. (2001c)
[c] significantly different ($p<0.05$) between pooled means for E2 and E3

Table A2: Means of measurements for morphological characters of infective L3 recovered from digests of *D. laeve* in D4, E1, and E3.

	D4 digested 26 dpi	E1 digested 60 dpi	E3 digested 84 dpi	Significant differences[b]
BL[a]	788.6	731.5	796.8	E3 & E1 D4 & E1
GPD	476.8	451.2	496.6	E3 & E1
ED	253.3	238.7	262.4	E3 & E1
EPD	127.2	119.5	130.6	E3 & E1
TL	43.3	40.4	46.0	E3 & E1
EW	22.2	21.8	24.4	E3 & E1

[a] as defined in text
[b] $p<0.05$ using one-way ANOVA

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