THE GEOGRAPHIC DISTRIBUTION AND GENETIC VARIATION OF *ECHINOCOCCUS MULTILOCULARIS* IN CANADA

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Saskatoon

By

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ABSTRACT

Echinococcus multilocularis, Leuckart, 1863, is a small cestode that averages 4mm in length. It utilizes carnivores and rodents as definitive and intermediate hosts, respectively, and is increasing in distribution and prevalence in people and animals across its circumpolar range. The inadvertent ingestion of infectious eggs by people results in alveolar hydatid disease. The larval stage grows throughout liver like a metastatic tumour and if left untreated has an extremely high fatality rate. While early diagnosis is difficult, due to a long latency period, it is paramount to patient survival.

Until recently, *E. multilocularis* received very little attention outside Europe, Asia and Alaska, primarily due to the number of human infections in those areas. The recent discovery of a European strain in British Columbia and a seemingly increased presence in Canadian urban-based wildlife, however, has raised alarms, as increased zoonotic potential may be associated with strains of Eurasian origin.

Identification of areas of potential risk for infection by *Echinococcus* species often involves a survey of wild animals. The recommended method for the extraction of adult cestodes is the sedimentation and scraping technique (SCT), which requires an hour of sedimentation per sample. A new method of extraction, the scraping, filtration and counting technique (SFCT), was developed to increase the efficiency and decrease the time associated with extraction of cestodes from the small intestines of carnivore hosts. A comparison of SFCT and SCT was performed. The SFCT took an average of 8.5 minutes less to quantify than the SCT, not including the one-hour sedimentation, which is a significant decrease ($p = 0.0001$, $\alpha = 0.05$). There was no significant difference in adult cestode count between the two methods ($p = 0.801$, $\alpha = 0.05$); however, SCT had a sensitivity of 91% and a negative predictive value of 97% when compared to SFCT. Therefore, SFCT is more sensitive and decreases time required for the extraction of adult cestodes from carnivore intestines.

SFCT was used to investigate the potential establishment of *E. multilocularis* in wild carnivores in Quesnel, BC, Canada as follow-up to the discovery of a European strain in a BC dog in 2009. SFCT was used to identify infection prevalence in 10 of 33 carnivores collected within 80 km of Quesnel. Analysis of four mitochondrial loci (*nad1*, *nad2*, *cob* and *cox1*) indicates that the same strain was infecting the domestic dog and the wild carnivores, implying

local transmission of this parasite. This study confirms the establishment of this parasite in local wildlife and is the first report of *E. multilocularis* in the Boreal forest habitat previously considered incapable of supporting sufficient rodent densities for life cycle requirements. This is also the first report of a European-type strain of *E. multilocularis* in North American wildlife.

This finding highlighted the lack of understanding of the current diversity and origins of *E. multilocularis* in North America, which has been hampered by early impressions of low genetic diversity within *E. multilocularis*. The currently documented haplotypes found in North America are based on findings from Alaska and the contiguous United States without sampling from the intervening region in northwestern Canada. In this study, analysis of the mitochondrial gene *nad1* revealed seven previously unrecognized haplotypes, undermining the reasoning that this gene does not hold significant variation. The newly identified haplotypes were found in BC, SK and Nunavut (NU). All haplotypes (B-H) displayed distinct geographic associations with the exception of haplotype A, which had a broad distribution within North America and was identical to sequences reported from Europe and Asia. This illustrates the need for large-scale geographic sampling when determining genetic polymorphisms, or mutations, and generates hypotheses about the origins and movements of this parasite and its hosts across the circumpolar north.

A second European strain was recovered from a coyote in Saskatoon, SK, which was remarkably different from published sequences. There were six nucleotide mutations between its closest European relation (E4) and seven differences between it and the European haplotype found in BC wildlife. In addition, seven samples resulted in six unique haplotypes were detected in SK deer mice. Four additional mouse samples were identical to the N2 North American strain, present in the northcentral United States.

Identification of areas of endemicity or newly established foci for this parasite may be a step towards mitigating the risk of human disease. In southern Saskatchewan, 8% of 516 adult and subadult deer mice collected in 2009, and 2% of 252 in 2010, had alveolar hydatid cysts confirmed using both morphological and molecular identification techniques. There was no difference in infection prevalence between male and female mice. A slightly higher prevalence in infected mice was found in native and hay field habitat types, compared to active croplands. Flintoft was an area of increased focus, with a site prevalence of 26%. Therefore, areas with

higher risk of transmission are present within agriculturally altered landscapes, which may have significance for both animal and human health as this parasite continues to emerge worldwide.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AB - Alberta, Canada

AE - Alveolar echinococcosis

AHD - Alveolar hydatid disease

BC – British Columbia, Canada

bp - Base pairs

CCWHC – Canadian Cooperative Wildlife Health Centre

Cob – cytochrome b – mitochondrial gene

Cox1 – Cytochrome c oxidase subunit 1 – mitochondrial gene

EtOH – Ethanol

GIT – gastrointestinal tract

MB - Manitoba, Canada

mtDNA - Mitochondrial DNA

NAD2 - NADH dehydrogenase subunit 1 and 2 - mitochondrial genes

NCR - North central region

NTZ - Northern tundra zone

NU – Nunavut, Canada

PCR - Polymerase chain reaction

RMNP - Riding Mountain National Park, Manitoba, Canada

SK - Saskatchewan, Canada

SLI - St. Lawrence Island, Alaska, USA

U of S - University of Saskatchewan

1. LITERATURE REVIEW

Phylum: Platyhelminthes
Class: Cestoda
Subclass: Eucestoda
Order: Cyclophyllidea
Family: Taeniidae
Genus: *Echinococcus*
Species: *E. multilocularis*

In North America, *Echinococcus multilocularis* and *Echinococcus granulosus* are the only two cestodes of zoonotic importance in the *Echinococcus* genera. *E. multilocularis* and *E. granulosus*, originally grouped together as *Echinococcus* Rudolphi, 1801 as a true tapeworm (Eucestoda) exhibiting all the classic morphological features that characterize this group. Originally described by Karl Georg Friedrich Rudolf Leuckart, a German zoologist, *E. multilocularis* was granted species status in 1863. Still, in the early 20th century confusion about morphological characteristics of this separate species made identification difficult. As an example, an alveolar form of hydatid disease thought to be conspecific with an infection type in Eurasia was found in an Alaskan rodent and was identified as a new species and named *E. sibericensis* (Rausch & Schiller, 1956). However, it was later recognized as *E. multilocularis* and subsequently, the name was changed (Palmer et al., 2011). What is now known as *E. multilocularis* has also been referred to as *E. alveolaris* (James & Boyd, 1937).

1.1 General Life Cycle

Echinococcus multilocularis is a taeniid cestode responsible for alveolar hydatid disease in people in the circumpolar North. Adults of this cestode are present in the small intestine of wild and domestic canids and felids with the majority of worms aggregating in the distal regions of ileum and jejunum (Eckert et al., 1983). Eggs passed in the feces are immediately infective for rodent intermediate hosts. Accidental ingestion may happen while the sticky eggs are affixed to fecal particles, small invertebrates, or grains. In the intermediate host, larval stages (metacestodes) develop into alveolar hydatid cysts, which generally originate in the liver but can fill the entire abdominal cavity. An aberrant host can be described as a non-traditional host that

may or may not participate in the continuation of the lifecycle. Humans and other mammals can serve as aberrant hosts, in which protoscolices may not develop but the germinal membrane continues to spread throughout the liver and metastasize to other organs. When the definitive host consumes the alveolar hydatid cyst in the rodent, the protoscolices evert, attach to the intestinal wall, and begin to produce proglottids. There is a prepatent period (time before egg production) of 35 days after ingestion of the hydatid cyst (Thompson & Eckert, 1983; Thompson, 1986; Conboy, 2009). The general life cycle is outlined below in Figure 1-1.

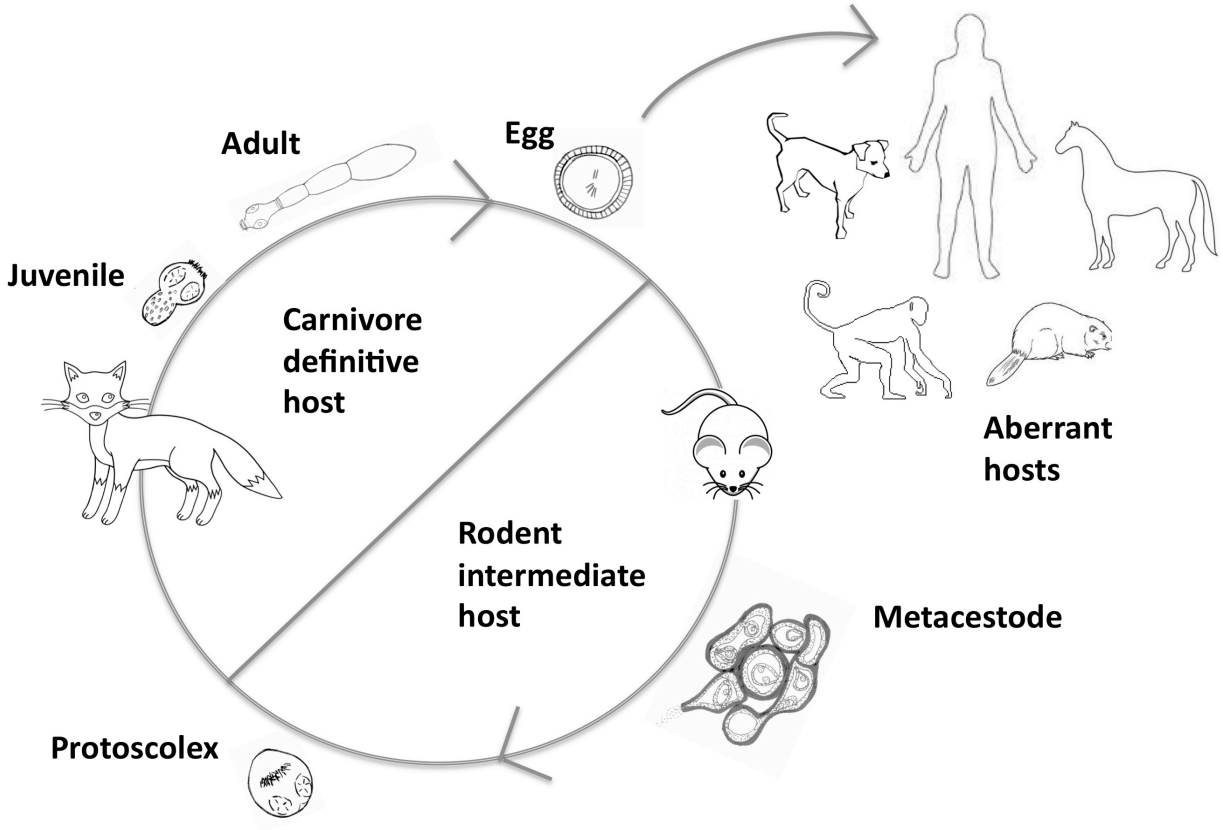

Figure 1-1 Diagrammatic representation of the general life cycle of *Echinococcus multilocularis* depicting the stages of cestode development in natural definitive and intermediate hosts and identifying numerous aberrant hosts. Definitive hosts may include, but are not limited to, foxes, coyotes, dogs and cats. Intermediate hosts include mice, lemmings and voles. Aberrant hosts may include, but are not limited to humans, dogs, monkeys, horses and beaver.

1.2 Biology of *Echinococcus multilocularis*

1.2.1 Adult Cestodes in Definitive Hosts

Identification of *Echinococcus multilocularis* by morphology is less difficult with the adults than the eggs. Defining characteristics used for identification include the placement of the genital pore and uterine formation. Although these characteristics are also shared by *E. oligarthus*, *E. multilocularis* only shares its northern distribution with *E. granulosus*, whose characteristic morphology is somewhat different, making identification less complex.

1.1.1.1 Adult Cestode Morphology

Echinococcus multilocularis adults have from 3 to 5 segments and are 1.2 to 4.5 mm in length (Webster & Cameron, 1967; Sreter et al., 2003). Like all tapeworms, the adults have no gut and therefore metabolic exchange takes place across the thin tegument or syncytial membrane (Thompson & McManus, 2002). The body is divided into three regions: the scolex (anterior), the neck and the strobila (posterior), which is sub-divided into proglottids or segmented reproductive units.

The scolex, located anteriorly, contains the specialized organ used to attach to the intestinal lining of the definitive host (Figure 1-2). The scolex has three main parts consisting of two rows of hooks, a rostellum and four suckers. The hooks, one large row and one small row, range from 24.9-34 μm and 20.4-30 μm, respectively (Thompson & McManus, 2002). The hooks are located on the anterior end of an extensible, beak-like rostellum. Posterior to the hooks and rostellum are four muscular suckers, which also aid in intestinal adherence.

The neck region is the site of proglottid germination. Maturing proglottids forming the strobila bud off from this point. The strobila, posterior to the neck region, consists of reproductive units called proglottids (segments) that vary from three to five in number. The segment proximal to the neck is the most immature proglottid and is followed distally by maturing and finally gravid (containing mature eggs) proglottids. The proglottids contain the reproductive units of the hermaphroditic tapeworm and follow a protandrous method of development with male organs maturing before female (Rawson, 1966). Within each proglottid are 16 to 35 testes (mean 18 to 26) and a sac-like uterus of varying maturity (Thompson &

McManus, 2002). The common reproductive ducts of *E. multilocularis* open in lateral genital pores, located in the anterior to middle region of each proglottid. *E. multilocularis* can be morphologically distinguished from *E. granulosus* using the genital pore placement and uterine structure (Figure 1-3).

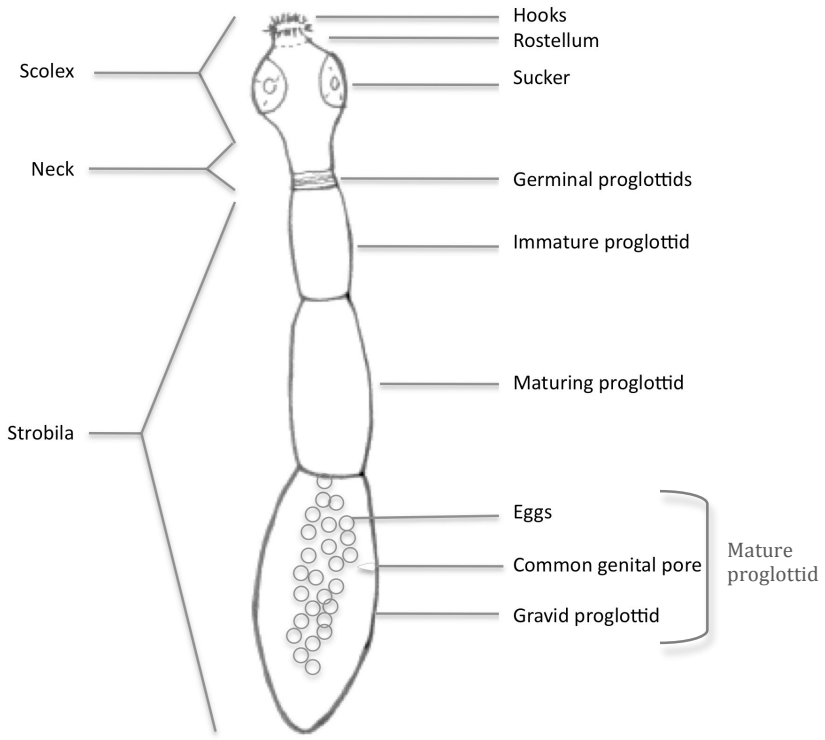

Figure 1-2 Diagrammatic representation of the adult *Echinococcus multilocularis* cestode outlining body divisions, organs of attachment, and the progression of body maturation.

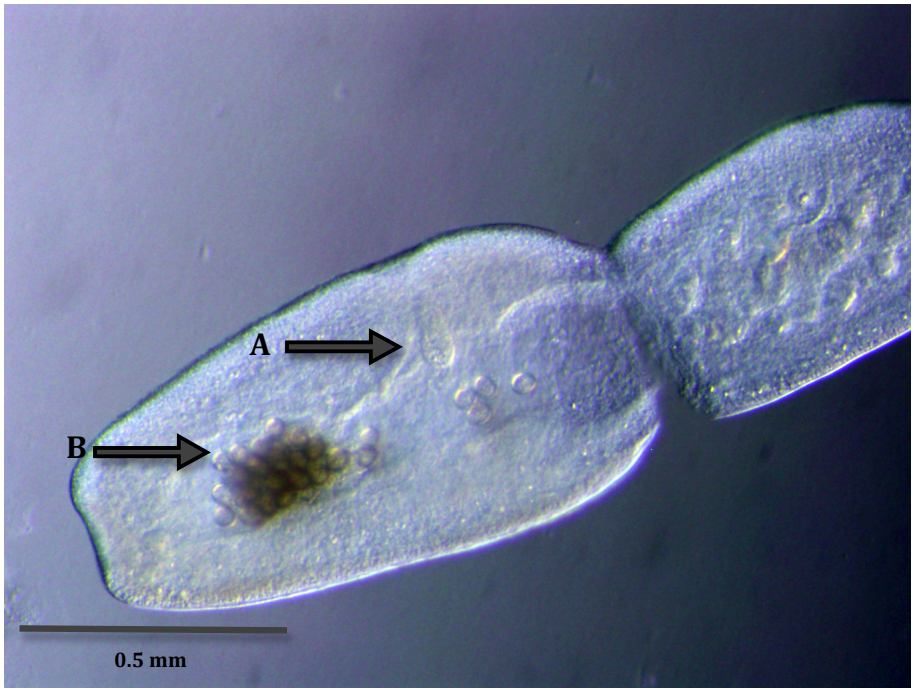

Figure 1-3 Photomicrograph displaying the mid to anterior placement of the genital pore (A) and the sac-like formation of the uterus complete with eggs (B).

1.1.1.2 Adult Growth

Within 6 hours of ingestion of fertile protoscolices (larvae) by the definitive host, approximately 87% begin transforming into juvenile worms in the GIT (gastrointestinal tract) by the process of evagination with complete transformation occurring within three days (Thompson, 1977). At about day 20, post-infection, the adult worms permanently adhere to their chosen locations between the villi of the small intestine (Figure 1-4) (Polley, 1978; Thompson, 1979). As adults, the apical suckers attach to the base of the villi and the rostellum extends into the crypt of Lieberkühn, where the hooks superficially attach to the mucosal epithelium (Thompson, 1979, 1986; Thompson & Eckert, 1983). *Echinococcus* is the only genus within Taeniidae to utilize the crypt of Lieberkühn, presumably because of its small size (Thompson, 1986). Proglottid differentiation is divided into two parts: germinal and somatic. Germinal differentiation refers to proglottisation by proximal growth and maturation, which is the formation and maturation of the reproductive units (Thompson, 1986). Somatic differentiation is responsible for size increase and the delineation, or strobilization, of each proglottid by an infolding of the tegument (Thompson, 1986).

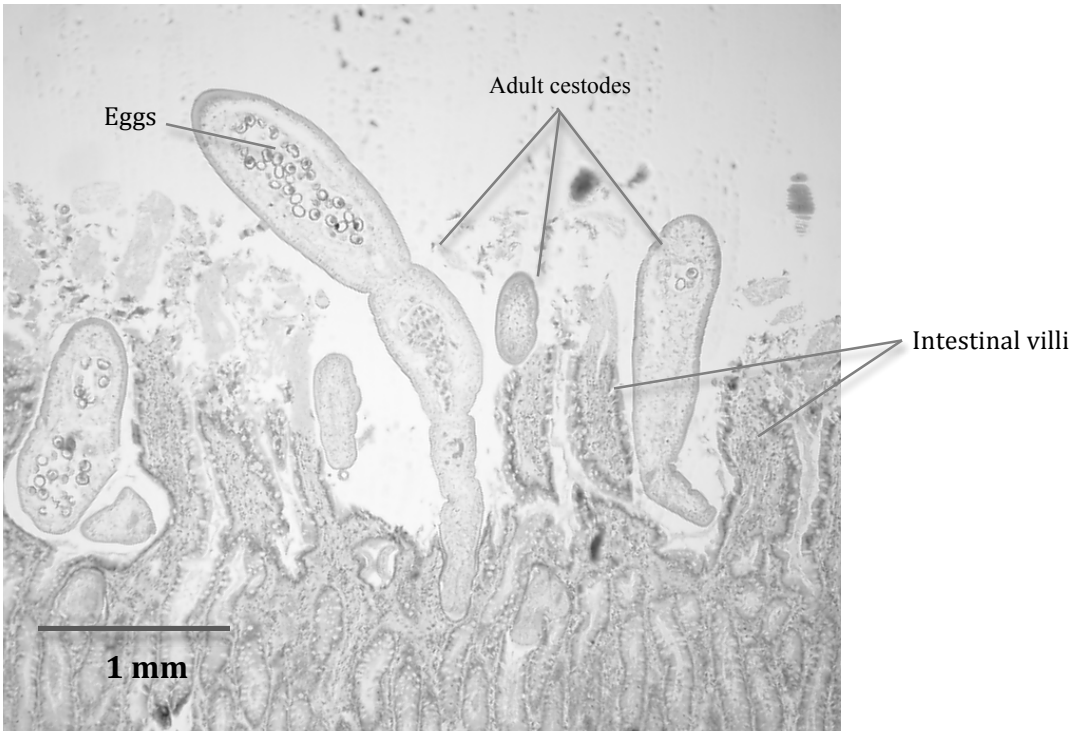

Figure 1-4 Photomicrograph/histology showing placement of adult *Echinococcus multilocularis* cestodes within the villi of the small intestine of a coyote found on the University of Saskatchewan campus.

1.1.1.3 Egg Production in Definitive Hosts

Egg production occurs between 26-35 days from the time of protoscolex ingestion by a definitive host (Thompson & Eckert, 1983; Thompson, 1986; Conboy, 2009). The number of eggs produced by each gravid segment has been a subject of debate with recordings of 100 to 1000 per proglottid (Hubbert et al., 1975; Thompson & Eckert, 1983; Conboy, 2009). In experimentally infected domestic dogs no more than 200 eggs were produced per proglottid (Thrompson & Eckert, 1982). Adult worms have reportedly lived for up to two years in the definitive host but shedding of eggs only occurred in the first one to four months of infection (Thompson, 1986; Conboy, 2009). The number of worms present limits the number of eggs, shed in the feces of definitive hosts; but foxes with high parasite intensity have been recorded to shed as many as 100,000 eggs per day (Eckert et al., 2002).

1.2.2 Eggs Shed by Definitve Hosts

The eggs of *E. multilocularis* are morphologically indistinguishable from other members of the family Taeniidae (*Echinococcus* and *Taenia* spp.). Identification of genera and species can only occur with molecular analysis.

1.2.2.1 Egg Morphology

The small spherical or ellipsoid eggs average 32-38 μm in diameter and consist of a hexacanth embryo (oncosphere), named for the presence of immature hooks in viable larvae, and several external envelopes (Figure 1-5; Wharton, 1983). The oncosphere is considered the first larval stage (Thompson & McManus, 2002). The inner layer of the taeniid egg gives rise to the oncospheral membrane and the embryophore simultaneously (Rybicka, 1972). The embryophore, or the outer shell, is made of small rod-shaped keratin blocks held together by a cement-like substance (Silverman, 1954). The blocks form a continuous shell, inwardly smooth and outwardly irregular in structure (Rybicka, 1972). It is the highly keratinized embryophore that gives the egg its yellow-brown colouration; the characteristic dark striated appearance of the eggshell and provides chemical and mechanical resistance (Silverman, 1954; Rybicka, 1972; Wharton, 1983). The composition of the eggs allows prolonged resistance to a variety of environmental conditions.

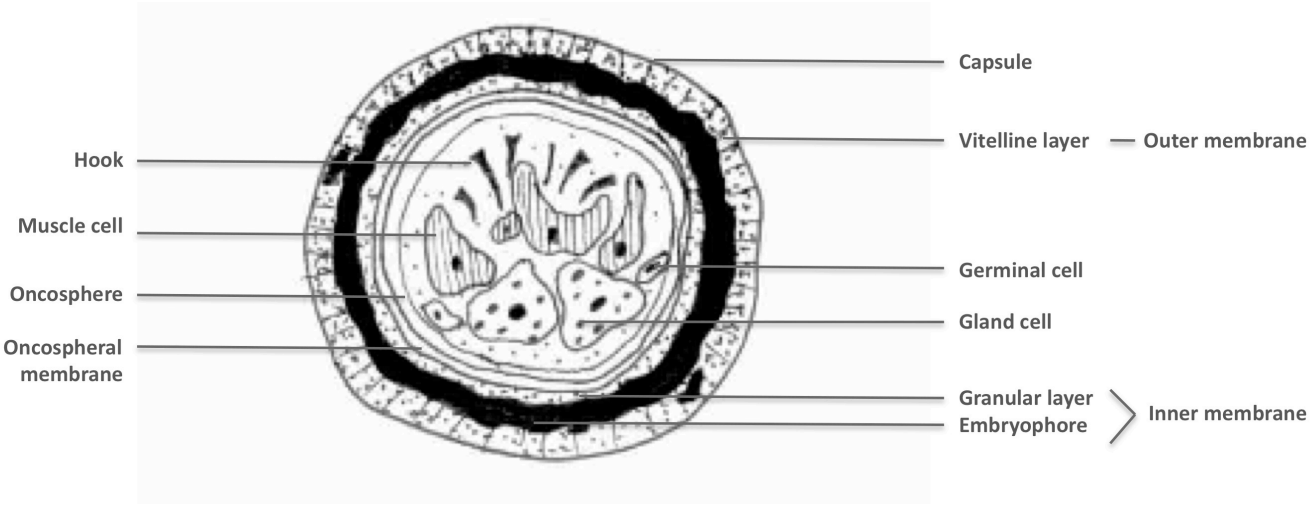

Figure 1-5 Diagrammatic representation of a taeniid egg depicting outer layers making up the "shell" and inner structures responsible for larval growth.

1.2.2.2 Hatching of Eggs Within the Intermediate Host

The eggs of *Echinococcus* are thought to be in various stages of development when expelled from the definitive host. While the vast majority of eggs are presumed to be fully embryonated at the time of expulsion from the definitive host, some eggs may require a short maturation period in the environment before becoming infective (Willis et al., 1981; Thompson, 1986). Eggs may endure for years in the environment under suitable conditions (Thomas et al., 1954). Hatching is a two-step process, involving the breakdown of the embryophoric blocks in the stomach and intestine of the intermediate host, and oncospheric activation (Thompson, 1986). Breakdown of the embryophoric blocks depends on proteolytic enzymes in the stomach but does not appear to rely on one particular enzyme (Thompson, 1986). Once block morphology has been compromised, altering membrane permeability, oncospheric activation is thought to take place with the introduction of bile salts (Thompson, 1986). However, eggs have been shown to hatch in extra-intestinal sites, such as the liver, when directly injected (Sakamoto et al., 1982). In this case, it is assumed that the interaction of neutrophils and macrophages with the eggs enables the creation of hydrolytic enzymes necessary for embryophore dissolution (Thompson, 1986).).

1.2.3 Larvae (or Metacestode stage) Within Intermediate Hosts

Once hatching is complete, the oncosphere penetrates the intestinal walls of the intermediate/aberrant host via the villi tips in the ileum and jejunum (Heath, 1971). Studies of *E. granulosus* show that oncospheres reach the lamina propria (connective tissue in the mucosa of gut) after breaching the epithelial border of the villi within two hours of hatching (Thompson, 1986). Gland secretions that cause degeneration of the host tissue at the attachment site aid in intestinal penetration by the juvenile cestodes (Heath, 1971). Coordinated hook and body movement further aids in penetration (Swiderski, 1983). Finally, the oncospheres are thought to migrate passively to the liver, via the lymphatic or venous system, where they begin the transformation to the metacestode, or second larval stage (Heath, 1971; Thompson, 1986).

1.2.3.1 Morphology and Growth of Larvae

Development of the metacestode stage of *Echinococcus multilocularis* is the most complex pattern of growth in the genera (Ohbayashi et al., 1971; Thompson, 1986). The

metacestode consists of multichambered microcysts in dense connective tissue surrounding vesicles (brood chambers), which are filled with a semi-solid viscous fluid, that eventually house the protoscolices (Figure 1-6; Figure 1-7) (Thompson & McManus, 2002). Microcysts are composed of a nucleated germinal layer, surrounded by an acellular laminated layer that takes on a honeycomb-like appearance. Localized microcyst expansion occurs endogenously by asexual budding resulting in a multilocular aggregation of cyst-like growths (Thompson & McManus, 2002).

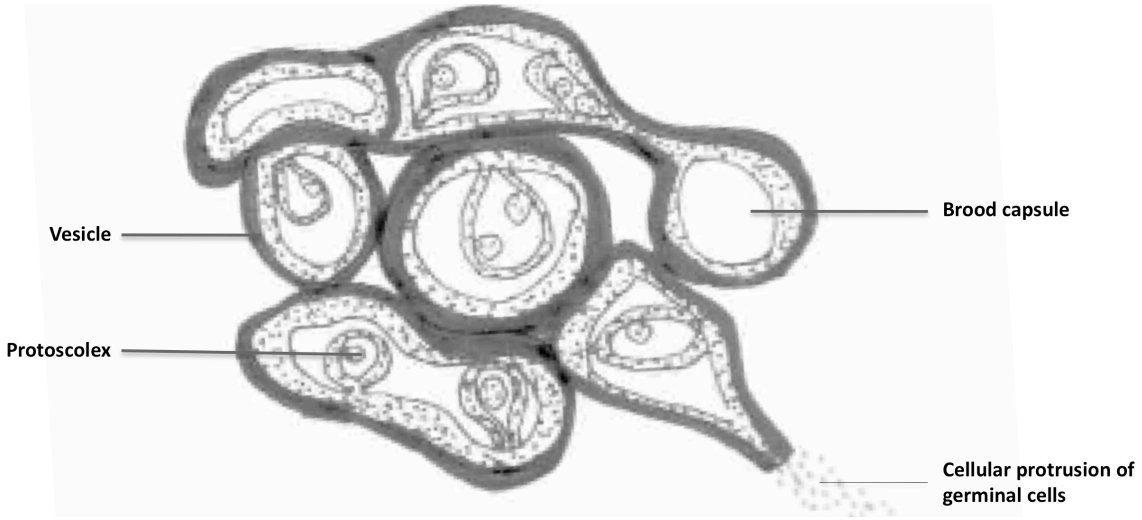

Figure 1-6 Diagrammatic representation of the multilocular metacestode stage of the *Echinococcus multilocularis* life cycle, also called an alveolar hydatid cyst

1.2.3.2 Alveolar Hydatid Growth Within Intermediate Hosts

Typically, there is an aggregation of cyst material in the liver of intermediate hosts. The infiltrative nature of growth replaces healthy liver tissue with cyst material. Although growth primarily begins in the liver, it is not restricted to this organ and as such is limited only by the life and breadth of its host (Thompson, 1986; Whitfield & Evans, 1983). Some experimentally infected rodents in laboratory studies have died from liver damage after 30 days while others, after 50 days, had growths whose weight exceeded the remainder of the animal (Rausch & Schiller, 1956). In many instances, and depending on the amount of growth, infiltration or

necrosis of the surrounding abdominal organs may occur. Alveolar hydatid disease is considered pathogenic in intermediate hosts as it contributes to parasite induced trophic transmission, as larval growth hampers host movement.

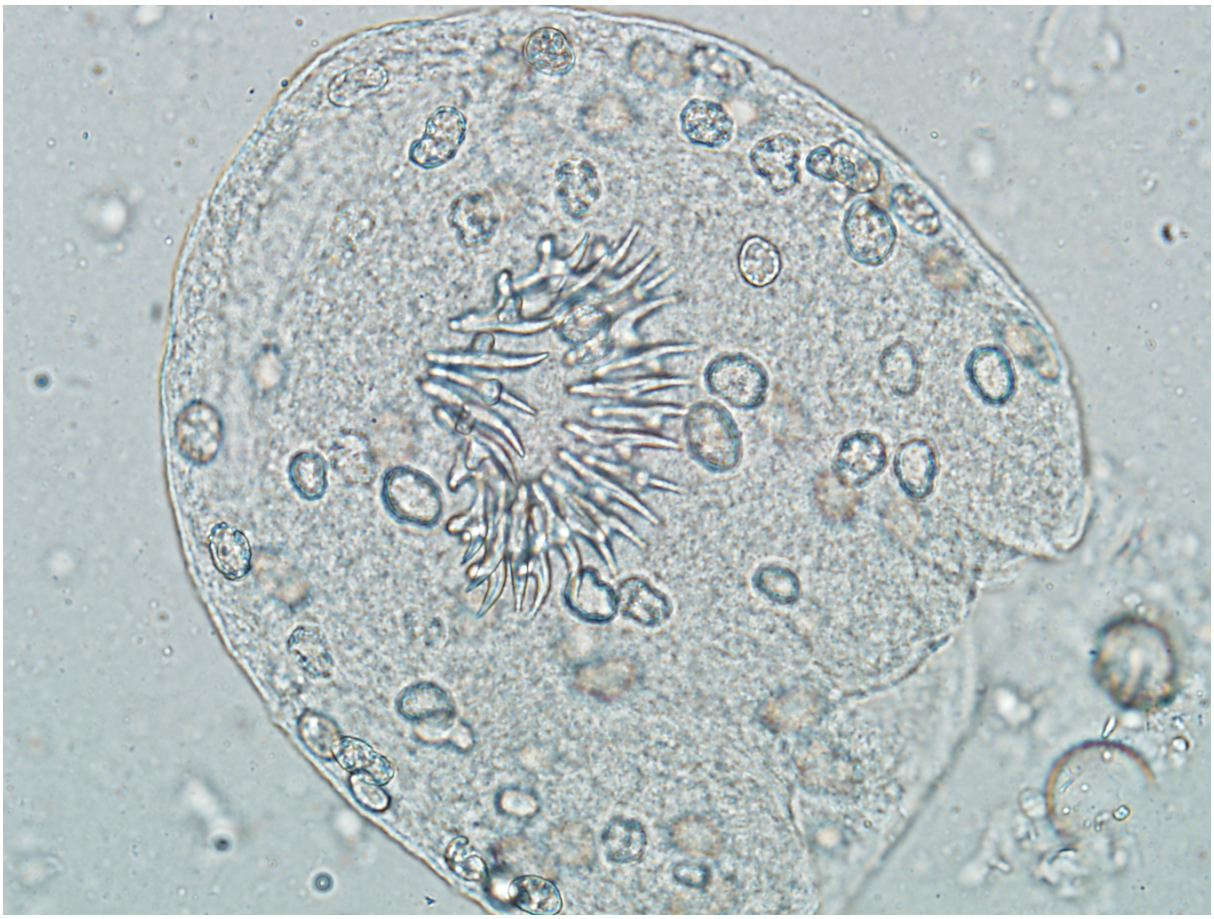

Figure 1-7 Photomicrograph of a protoscolex emphasizing the 2 rows of small and large hooks from an alveolar cyst in the liver of a Saskatchewan deer mouse.

1.2.3.3 Larval Growth Within Aberrant Hosts

Cyst growth in aberrant hosts occurs in much the same way as in traditional intermediate hosts. But, while extensive growth of parasitic material occurs, protoscolex development in aberrant hosts is usually stunted in the early stages of development (Rausch & Schiller, 1956). In some humans, larval development may be arrested in the early stages, although development into protoscolices of any form is rare (Wilson & Rausch, 1980). In most human cases, there is no development of protoscolices, but the proliferative stage (germinal cells and asexual budding) can still lead to progressive and extensive growth of parasitic material (Webster & Cameron,

1967; Wilson & Rausch, 1980; Rausch & Wilson, 1985). When humans serve as aberrant hosts, cysts generally originate in the liver and, in about one third of cases, metastasize throughout the body (Kern et al., 2003). The lack of protoscolex development in humans is likely due to the high degree of incompatibility between host and parasite. Most infections are thought to occur early in life, with a preclinical period lasting up to 25 years, allowing the parasitic material to enjoy a prolonged period of undisturbed development (Gemmell, 1968; Rausch & Wilson, 1985). Symptoms, if they present at all, are generally represented by mild abdominal pain and epigastric discomfort caused by a palpable abdominal mass or an enlarged liver (Wilson & Rausch, 1980). Other symptoms, though less common, include shortness of breath, ascites and jaundice (Wilson & Rausch, 1980). Because of the wide-ranging and non-specific nature of the symptoms, cases of alveolar hydatid disease are often misdiagnosed, delaying treatment (Wilson & Rausch, 1980). Misdiagnoses, or differential diagnoses, even in advanced stages, often include metastatic carcinoma and amebiasis (Leiby & Kritsky, 1972). As an example, advanced stages of alveolar hydatid disease were discovered in 67 patients in Switzerland during routine autopsy, of which only 18 had been correctly diagnosed prior to death (Webster & Cameron, 1967). Because of the severity of the disease and the prolonged period of parasitic growth, detection of alveolar hydatid disease in its early stages is essential to survival (Webster & Cameron, 1967). Case fatality rates have exceeded 90% within 10 years, unless treated early and aggressively (Kern et al., 2003). Even with treatment, which usually involves resection of the affected tissue, followed by life-long chemotherapy (e.g. with albendazole), the average survival rate is 19 years (Eckert et al., 2002; Rausch & Wilson, 1985). Though surgical resection of the affected tissue remains the treatment of choice, there is risk of cyst rupture during the procedure, which can lead to extensive secondary growth through the dissemination of protoscolices (hydatid sand), anaphylaxis, or acute eosinophilia (Smith & Rickman, 2001). Iatrogenic mortality caused by surgical resection is approximately 3% (Khuroo et al., 1997; Gilman & Lee, 2000).

1.3 Host and Geographic Distribution

There are two distinct populations of *Echinococcus multilocularis* in Canada and the United States: the northern tundra zone (NTZ) population in Arctic Canada and Alaska, and the north central region (NCR) population corresponding to the northern contiguous United States and three Canadian prairie provinces (Figure 1-8) (Nakao et al., 2009). Another potential region of *E. multilocularis* occupation has been recently been identified in British Columbia with the

detection of a European strain in a domestic dog, with no history of travel outside BC (Jenkins et al., 2012). This discovery may represent a new endemic focus for *E. multilocularis* in Canada and may be the result of translocation of another domestic animal or of fox populations in the surrounding area (Jenkins et al., 2011). Further research is needed to determine if this strain of *E. multilocularis* has established in British Columbia wildlife.

While the prevailing hypothesis for the existence of *E. multilocularis* in Arctic North America is attributed to the movement of Arctic fox *Vulpes lagopus* (formerly *Alopex lagopus*) across the Beringia Strait between 2 million and 10,000 years ago, the origins in the NCR have been the subject of much debate (Rausch, 1994; Nakao et al., 2009). One hypothesis stipulates that the translocation of red foxes from Europe in the early 1800s for hunting purposes may be responsible for the establishment of *E. multilocularis* in the NCR (Rausch, 1985). The occurrence of *E. multilocularis* in the NCR may be compounded by the natural southward movement of infected Arctic foxes in search of food during low lemming years, which was first noticed in the 1970s (Wrigley & Hatch, 1976). The second hypothesis pertains to the isolation of infected carnivores that predates the Last Glacial Maximum, approximately 26,500 to 19, 000 years ago (Nakao et al., 2009). The glaciation hypothesis suggests that infected fox populations from the Arctic used the NCR as a refugium during glacial periods. The molecular evidence supporting this argument uses a 10% divergence per million years and places divergence of the two North American strains at about 75, 000 years (Nakao et al., 2009). As there is no molecular clock for tapeworms, the 10% per million years divergence rate is based on the upper limits of the molecular clock of the parasite's intermediate host *Microtus oeconomus* (Nakao et al., 2009). It is presumed that the two haplotypes, N1 in the NTZ and N2 in the NCR, have remained disjunct, due to insufficient rodent densities in the boreal regions of North America (Rausch, 1967). However, it is possible that the prevalence of *Echinococcus* in this region is below the detection limit (Rausch, 1967).

1.3.1 NTZ (Northern Tundra Zone) Arctic Canada

Echinococcus multilocularis in the NTZ is thought to roughly follow the distribution of Arctic fox *Vulpes lagopus* (formerly *Alopex lagopus*), which in turn, roughly follows northern reaches of the North American tree line. This pattern of distribution in Canada is extrapolated from one report on the Arctic mainland and two in the high Arctic islands (Choquette et al.,

1962; Eaton & Secord, 1979). In Alaska, the distribution of *Echinococcus multilocularis* is also thought to correspond to the distribution of the Arctic fox but appears to be limited to coastal regions of the north Alaskan mainland and the Alaskan Archipelagos (Fay & Williamson, 1962). Basing the distribution of *Echinococcus multilocularis* solely on the distribution of Arctic fox may be questioned however by the occurrence of Arctic fox in Greenland, where to date no *Echinococcus multilocularis* has been found (Braestrup, 1941; Kapel & Nansen, 1996; Akuffo et al., 2003). It must also be noted that the red fox (*Vulpes vulpes*) is a suitable definitive host whose distribution is at times sympatric with its Arctic kin (Hersteinsson & Macdonald, 1992). The coyote (*Canis latrans*) is also a highly suitable host whose distribution range includes all of North America except the northeastern most islands (Sillero-Zubiri et al., 2004).

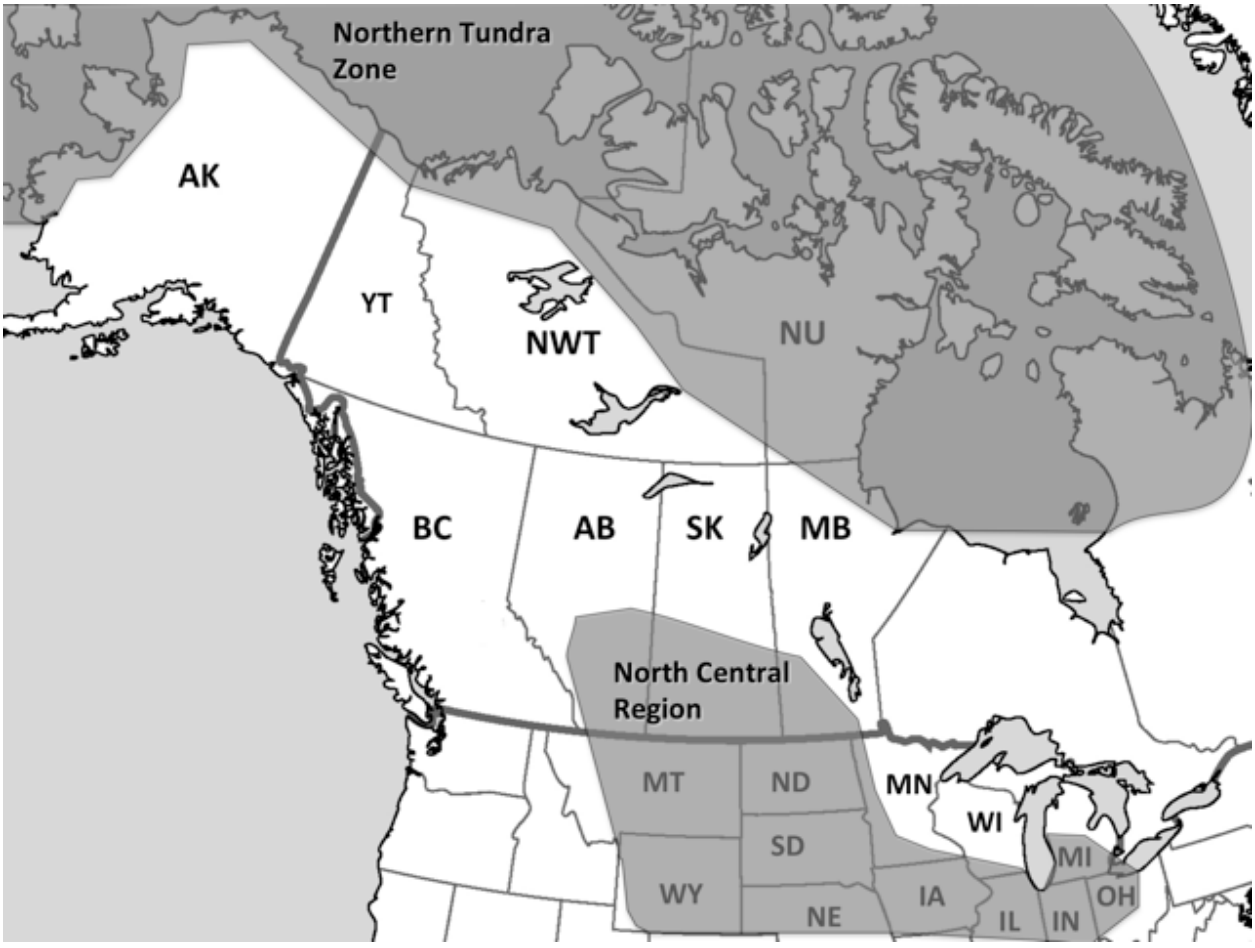

Figure 1-8 Map displaying the presumed distribution of *Echinococcus multilocularis* in North American with reference to the Arctic northern tundra zone (NTZ) and the north central region (NCR).

1.3.1.1 Definitive Hosts in the NTZ

The NTZ is loosely defined as any land north of 60° latitude. Definitive hosts (Table 1-1, Figure 2-1) in the North American Arctic include canid species including Arctic fox, red fox, coyote and domestic dogs (*Canis lupus familiaris*). Arctic foxes generally represent the most important host species in the North. However, where the home ranges of red foxes are parapatric or sympatric with Arctic foxes (Hersteinsson & Macdonald, 1992), the red fox may supplant Arctic fox as primary definitive host. Red foxes outcompete the Arctic fox for food sources and often usurp the den and resting areas of the Arctic fox (Rudzinski et al., 1982). In fact, Arctic foxes introduced into a region, where the red fox was already established, were soon eradicated (Evermann, 1914; Bower & Aller, 1917). In northern villages, free-roaming dogs may become the primary definitive host (Rausch & Wilson, 1985). This potentially exposes a greater number of humans to infective eggs via increased fecal contamination. Wolves (*Canis lupus*) may also serve as definitive hosts but are more likely to be infected by parasitic tapeworms of larger ungulate species, namely *E. granulosus* (Rausch, 2003). Although the domestic cat (*Felis catus familiaris*) and lynx (*Lynx canadensis*) may also serve as definitive hosts, though less suitable, as parasitic growth and egg production is reduced (Thompson, 1986). The lynx is largely restricted to the boreal forest (de Vos & Matel, 1952) and domestic cats are uncommon.

1.3.1.2 Intermediate Hosts in the NTZ

Intermediate hosts (Table 1-1, Figure 2-1) include arvicoline rodents such as lemmings and voles, and neotomine rodents like deer mice (Jones & Pybus, 2001). In Greenland, the only potential intermediate host is the Greenland (collared) lemming (*Dicrostonyx groenlandicus*); however, field and laboratory studies indicate that the collared lemming, despite being a close relative of the brown lemming (*Lemmus trimucronatus*), is not a suitable host (Ohbayashi et al., 1971; Rausch, 1995). Other Northern intermediate hosts include shrews of the family Soricidae and squirrels of the family Sciuridae.

1.3.1.3 Prevalence in Animals in the NTZ

In the Canadian North the prevalence of *Echinococcus multilocularis* in both definitive and intermediate hosts is thought to be relatively low (Table 1-1). On Banks Island in the Northwest Territories, only 1 of 50 Arctic foxes surveyed was infected with *E. multilocularis* (Eaton & Secord, 1979). To our knowledge, larval cysts have not been identified in rodent hosts

in the Canadian Arctic. The low prevalence in the Canadian North may be a reflection of limited sampling and challenges in diagnosis. However, analytical techniques (i.e. fecal flotation) may have opened up new opportunities for large-scale surveys. However, as eggs are morphologically indistinguishable between *Echinococcus* and *Taenia* genera, molecular analysis still needs to occur. On mainland Alaska, as in Canada, the prevalence of *E. multilocularis* is relatively low in definitive hosts. A prevalence of only 2% and 4%, was found in red and Arctic fox, respectively (Rausch, 1956) and has not been found in excess of 1% in rodent intermediate hosts despite many surveys (Holt et al., 2005). In contrast to the low prevalence on the mainland, St. Lawrence Island can by considered a hyperendemic region. The discrepancy between mainland and island infection rates may be due to the differences in stability and abundance of rodent populations. Island host populations tend to remain relatively stable from year to year, while populations on mainland Alaska experience dramatic fluctuations (Rausch & Fay, 2002). Even in years when the prevalence in definitive hosts was between 76 and 100%, the prevalence in island rodent intermediate hosts did not exceed 20% (Rausch, 1956). However, the prevalence in rodent hosts have at times, exceeded 50 and 80%, but no concurrent study on definitive and intermediate hosts was performed (Rausch et al., 1990). Variations in infection rate among definitive and intermediate host species serve to highlight the role that definitive hosts play in parasite bioaccumulation in the environment. In fact, Fay et al. (1962) noted that an infection rate of less than 10% in tundra voles (*Microtus oeconomus*) could yield almost 100% infection in Arctic foxes. In Alaska, the mean prevalence in Arctic fox was 52%, while in red foxes and dogs was 29% and 8%, respectively (Table 1-1). Among intermediate hosts, shrews (*Sorex jacksoni*) had the highest prevalence of infection, followed by tundra voles (*Microtus oeconomus*), Arctic ground squirrels (*Citellus undulatus*) and red-backed voles (*Clethrionymus rutilus*) (Table 1-1).

Before the implementation of an *Echinococcus* control program near Savoonga on St. Lawrence Island, Alaska, the prevalence of infection was approximately 53% in voles (Rausch et al., 1990b). The control program, consisting of monthly doses of praziquantel for local dogs, decreased the occurrence of this parasite in voles by approximately 83% (Rausch et al., 1990b). Holt et al. (2005) found a low prevalence (0.9%) in brown lemmings (*Lemmus trimucronatus*) after implementation the control program. To our knowledge, there is no control program currently in effect in the North, and *E. multilocularis* is considered a parasite of limited animal

health significance with the exception of infected rodents at an increased risk of predation due to large alveolar hydatid cysts (Castrodale, 2003).

1.3.1.4 Aberrant Hosts in the NTZ

An aberrant host may be described as a host that does not normally contribute to the continuation of the life cycle. Humans and other aberrant hosts may become infected with *Echinococcus multilocularis* by the accidental ingestion of eggs shed in carnivore feces. The sticky eggs may adhere to multiple surfaces in the environment including vegetation, fur and human skin and may even float in water (Laws, 1968; Hildreth et al., 2000). In humans, alveolar hydatid disease behaves like an invasive neoplasm, most often originating in the liver. The high fatality rate of human alveolar hydatid disease is likely due to late detection, as the symptoms associated with infection are broad and nondescript.

1.3.1.5 Prevalence in Humans in the NTZ

In North America, cases of human alveolar hydatid disease (AHD also known as alveolar echinococcosis) have occurred mainly in Alaska, with only two autochthonous cases in central North America (Manitoba, Canada and Minnesota, USA) (James & Boyd, 1937; Gamble et al., 1979). Within Alaska, autochthonous cases of human alveolar hydatid disease have been primarily focused in the Alaskan Archipelago with a few cases in the North Slope of the Alaskan mainland (Table 1-2, Figure 2-1) (Wilson et al., 1995).

Between 1947 and 1986, 54 cases of alveolar hydatid disease were reported to the Alaska State Section of Epidemiology (Castrodale, 2003). Historically, the rate of human infection coincided with the transition of the Alaskan Inuit from a nomadic to a sedentary way of life (Rausch, 2003). Domestic dogs, replacing the Arctic fox as definitive host in villages, have access to stable and abundant populations of infected intermediate hosts, which serves to perpetuate the life cycle of *E. multilocularis* and increase its prevalence in the immediate surroundings (Stehr-Green et al., 1988; Eckert & Deplazes, 2004). Domestic dogs are considered the most important source of transmission of this parasite to humans, primarily due to close physical proximity of egg-shedding animals to people in the community (Rausch, 1956; Wilson et al., 1995). Trappers and hunters may be at increased risk when handling infected foxes, although trapping foxes was not a significant risk factor in adults in a case-control study in Alaskan Inuit (Rausch, 1956; Stehr-Green et al., 1988). Around the world, the highest prevalence

of human infection with alveolar hydatid disease was seen primarily in rural and remote areas, where exposure was thought to occur through the accidental ingestion of eggs on local produce and vegetation (Hildreth et al., 2000). In some regions, consumption of surface (vs. well) water is also associated with increased risk of alveolar hydatid disease (Yang et al., 2006).

At its peak, seropositivity for alveolar hydatid disease on St. Lawrence Island reached 98/100,000 (Wilson & Rausch, 1980; Schantz et al., 1995). However, there are significant challenges in the sensitivity and specificity of immunological testing for exposure to *E. multilocularis*, including the possibility of cross-reaction with *E. granulosus* and related helminths. On the mainland, the historical presence of *E. multilocularis* in both human and animal hosts has been comparatively low (Rausch & Fay, 2002). Since successful implementation of education and control programs in 1986, there have been no reports of human *E. multilocularis* infection in Alaska (Castrodale, 2003). There is; however, the possibility of undiagnosed cases within Alaska and in other Arctic regions, as alveolar hydatid disease can mimic other conditions, including hepatic carcinoma (Webster & Cameron, 1967; Hildreth et al., 2000). This may be particularly true in Arctic Canada, where there is a low index of suspicion on the part of medical practitioners; hydatid disease is not notifiable at the national level; and many records do not differentiate between alveolar (*E. multilocularis*) and cystic (*E. granulosus*) hydatid disease (Gilbert et al., 2010).

North American strains, or at least the NCR (N2) strain, may have lower zoonotic potential than European or Asian strains of *E. multilocularis* (Jenkins et al. 2012) This suggests that at least one of the human cases reported in western Alaska was due to infection with Asian haplotypes (Nakao et al., 2009). The idea of lower zoonotic potential for North American strains is somewhat controversial, but probably stems from the more common occurrence of human and wildlife infections in Europe and Asia. With no information on effects on humans, strain differences of N1 and N2 are seen in experimentally infected gerbils (Bartel et al., 1992). In the gerbils, the N2 strain took longer to invade surrounding host tissue and develop protoscolices and, despite the increase in vesicle diameter, did not have the typical opaque coloration associated with hydatid fluid (Bartel et al., 1992).

While it is possible that the low prevalence of human infection in the rest of North America is due to a decreased opportunity for human exposure, this seems unlikely given that many residents of northern North America (especially Indigenous peoples) hunt, trap, consume

untreated surface water, and harvest wild foods. The transmission of cystic hydatid disease (*E. granulosus*) continues to occur in northern and Indigenous communities within the range of *E. multilocularis* in North America (Hildreth et al., 2000; Gilbert et al., 2010; Himsworth et al., 2010). This reinforces the idea that differences in parasite genetics may account for the lack of human cases of *E. multilocularis* observed in North America, despite relatively high prevalence in wild canids in the NCR.

1.3.2 NCR (North Central Region) Central North America

The North American strain N2, which is distinct from the N1 strain present on St Lawrence Island, Alaska, has been described in the north central region (NCR) of North America (Nakao et al., 2009). The NCR of North America corresponds to the southern portion of the three Canadian prairie-provinces and the north central contiguous American states. The NTZ and NCR are distinct populations due to the discontinuity of supportive vegetation for intermediate rodent hosts between Canada's northern and southern regions (Schantz et al., 1995). The lack of supportive vegetation in the boreal region has created an area of low rodent intermediate host density, resulting in separate northern and southern populations of *E. multilocularis* in North America (Schantz et al., 1995; Nakao et al., 2009).

1.3.2.1 Hosts in the NCR

Definitive hosts in the NCR include carnivore species that utilize rodents as a food source (Table 1-3, Figure 2-1). The main definitive hosts in the NCR are red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*) and potentially dogs (*Canis lupus familiaris*) and felids. While foxes and coyotes are the definitive hosts in the sylvatic life cycle, the domestic life cycle may involve domestic cats and dogs as definitive hosts (Wobeser, 1971; Leiby & Kritsky, 1972). While domestic cats have been naturally infected, there is little direct evidence of both host types (definitive and intermediate) being infected in the same location (Leiby & Kritsky, 1972). However, circumstantial evidence of domestic cat and intermediate host infections within a 32 km radius support the claim of a domestic life cycle (Leiby & Kritsky, 1972). This claim is further substantiated by successful experimental infections of domestic cats in which viable protoscolices have been produced (Vogel, 1957; Kapel et al., 2006). Although cats may serve as definitive hosts, parasitic growth is often stunted in size and egg production is diminished which may minimize zoonotic potential (Thompson, 1986; Kapel et al., 2006).

In prairie Canada, the main intermediate hosts are rodents of the sub-families Arvicolinae (voles) and Neotominae (mice). In the sylvatic life cycle, voles and mice are often the main food source of smaller carnivores like coyotes and foxes. The two main species of intermediate hosts in the NCR are the meadow vole, *Microtus pennsylvanicus*, often mistakenly called a field or meadow mouse, and the deer mouse, *Peromyscus maniculatus* (Table 1-3).

1.3.2.2 Prevalence in Animals in the NCR

In the NCR, the primary definitive hosts are red foxes and coyotes (Table 1-3, Figure 2-1). The highest prevalence recorded for red foxes come from North and South Dakota with infection rates of 70% (n = 96) and 75% (n = 137) (Rausch & Richards, 1971; Hildreth et al., 2000). In these same areas, the highest prevalence in an intermediate host was recorded as 6% (n = 3335) in deer mice (Leiby et al., 1970). The discrepancy in host infection rate supports the theory that a low prevalence in intermediate hosts can maintain a very high prevalence in definitive hosts (Fay & Williamson, 1962). In the United States, it appears that red foxes are the primary definitive hosts with a higher overall prevalence (75%) than coyotes (44%, n = 9) (Hildreth et al., 2000). However, this may result from the number of red foxes and coyotes studied. In Canada, the highest infection rate in red foxes was found near Riding Mountain National Park (RMNP) in Manitoba (3 of 6 positive) (Samuel et al., 1978). Recently, 25% of 91 coyotes from the Edmonton and Calgary areas of Alberta were infected with *E. multilocularis* infection (Catalano et al., 2012). This is similar to findings of 23% of 43 coyotes in RMNP, Manitoba (Samuel et al., 1978). The results may indicate that coyotes are an important host species in the western Canadian prairie-provinces. The highest prevalence in intermediate hosts recorded was in periurban Edmonton, AB with rates of 22% (n = 283) in meadow voles and 28% (n = 216) in deer mice (Holmes et al., 1971). Edmonton and RMNP appear to be areas of endemic focus in Canada, which could be a reflection of increased study in each location. The mean prevalence for all definitive host species is 29% (range 1-75%) and 25% (6-35%) in red foxes and coyotes, respectively (Table 1-3). The mean prevalence in rodent species is 8% (range 0.9-28%) in meadow voles, 6% (0.4-15%) in deer mice, and 2% in house mice.

1.3.2.3 Aberrant Hosts in the NCR

Only two autochthonous cases of human alveolar hydatid disease have occurred in central North America (Manitoba, Canada and Minnesota, USA) (James & Boyd, 1937; Gamble et al.,

1979). In Canada, the patient diagnosed with AHD was originally from Iceland, but, as *E. multilocularis* does not occur in Iceland, infection was thought to be of Canadian origin (James & Boyd, 1937; Polley, 1978). Two additional cases were described in the Canadian NCR, one in Ontario and one in Alberta; however, the patients originated from Germany and the Ukraine, respectively, where *E. multilocularis* is endemic (Geddes Smith & Hanson, 1961; Sereda et al., 1961; Langer et al., 1984). Two foreign-acquired cases have also been identified in the United States and an in Canada, the origin of infection likely occurred in their countries of birth (Figure 2-1) (Weinberg, 1947; LaFond et al., 1963).

1.4 Characterization of Genetic Markers

It was originally thought that *E. multilocularis* was genetically uniform across its distribution in the northern hemisphere (Bowles & McManus, 1993; Okamoto et al., 1995). Findings that *E. multilocularis* is not genetically uniform could have significance for understanding pathogenicity, host specificity, and zoonotic potential (Bartel et al., 1992; Kapel & Nansen, 1996; Nakao et al., 2009).

1.4.1 *Nad1* Mitochondrial Gene

NADH dehydrogenase subunit 1 (*nad1*) is a mitochondrial gene that has been used for species level identification of *Echinococcus* (Bowles and McManus, 1993). Multiplex polymerase chain reaction (PCR) is used as a tool for the identification and differentiation of species of taeniid eggs, which are morphologically indistinguishable from one another. The primers are designed to amplify 395 base pairs (bp) of the *nad1* gene in *E. multilocularis*, a 117 bp region of (ribosomal RNA) *rrnS* gene in *E. granulosus,* and a 267 bp region of the *rrnS* gene in *Taenia* spp. (Trachsel, Deplazes, & Mathis, 2007). Differentiation between species can be visualized as distinct bands on an agarose gel. *Nad1* sequences of *E. multilocularis* distinguish two geographic genotypes, M1 (European) and M2 (China and North America) (Bowles & McManus, 1993; Okamoto et al., 1995; Nakao et al., 2009). Beyond identifying genotype and species, the usefulness of the *nad1* gene is limited, as differences at the genetic level were thought to be restricted to a few single nucleotide mutations, which does not constitute enough of a difference for detailed haplotype characterization (Nakao et al., 2009). By increasing the number and geographic distance of samples, more diversity may be found within this gene.

1.4.2 *NAD2*, *COB* AND *COX1* Mitochondrial Genes

Finer-scale determination of haplotypes can be accomplished by looking at the variation in multiple mitochondrial DNA (mtDNA) genes. Mitochondrial DNA may be best suited for population and species level comparison when little variance is seen in nuclear DNA, because it evolves more rapidly, has a high copy number, undergoes a non-Mendelian mode of maternal inheritance without recombination, and exhibits homoplasmy (mutations affect all mtDNA copies) in most individuals (Brown et al., 1979). The three genes that are primarily used for analysis in *E. multilocularis* are cytochrome b (*cob*), NADH dehydrogenase subunit 2 (*nad2*), and cytochrome c oxidase subunit 1 (*cox1*) (Nakao et al., 2009). The primers amplify 1068 bp (nucleotide base pairs) of the *cob* gene, 882 bp of *nad2,* and 1608 bp of *cox1*. Based on the amplification and sequence analysis of these three genes, a total of 18 haplotypes have been described (Nakao et al., 2009). The resulting nucleotide network suggests that *E. multilocularis* is more diverse than currently understood.

Characterization at multiple mitochondrial loci has demonstrated distinct North America, Asian, and European haplotypes of *E. multilocularis* (Nakao et al., 2009). On St. Lawrence Island, Alaska, there are three known strains of *E. multilocularis*, a North American N1 haplotype and two Asian strains, A2 and A4 (Nakao et al., 2009). A second North American haplotype (N2) was found in Indiana and South Dakota, within the United States portion of the NCR (Nakao et al., 2009). This implies that the identification of only two haplotypes in North America is an underestimation. The presence of the two Asian haplotypes in Alaska is likely due to the natural movement of Arctic foxes (*Vulpes lagopus*), which can travel extensive distances across the ice (Fay & Williamson, 1962; Dalen et al., 2005). This movement could lead to the eventual introduction or identification of additional European or Asian haplotypes of *E. multilocularis* in North America. In fact, a fifth haplotype was recently discovered in British Columbia, Canada and identified with those of Eurasian origin (Jenkins et al., 2012). This may have been a result of natural and/or anthropogenic translocation of wild and/or domestic animals or be an established but previously undetected occurrence (Jenkins et al., 2012).

1.5 Anthropogenic Drivers of Emergence

There are many possible drivers of parasite emergence (increasing in prevalence or distribution). Anthropogenic forces, such as climate and landscape changes at the human/wildlife

interface, and the translocation of infected animals, are among the top reasons for parasitic emergence in a human population. In North America, high prevalence of *E. multilocularis* has been observed in localized regions, such as in agricultural lands, where deer mice were more abundant (Holmes et al., 1971). The transformation of native grasslands for agricultural use may alter the landscape in a way that is favourable for rodent intermediate host habitation, which will attract carnivore definitive hosts, thereby increasing the prevalence of *E. multilocularis* (Samuel et al., 1978; Deplazes et al., 2004). Alternatively, an increase in rodent diversity, due to an increase in food sources, or a decrease in rodent density, due to the formation of new habitat sites, may counteract the effects of increasing carnivore populations by the 'dilution effect' (Bradley & Altizer, 2006). In the case of *E. multilocularis*, the dilution effect may decrease transmission of alveolar hydatid disease. If a small percent of infected rodents are in the same region as many uninfected rodents, then the odds of an infected rodent being caught are decreased. Transmission may also be decreased by lessening the odds of an infected rodent being ingested by a carnivore, as there are more rodents to choose from. On the other hand, a decrease in rodent density may help to insure that a higher percent of the population is infected, therefore increasing the chance of ingestion by a carnivore host.

Additional anthropogenic landscape changes, such as deforestation, may also lead to intermediate host range expansion in North America. While the dispersal of helminths is naturally occurring, ecosystem alteration is increasing the rate of dispersal of intermediate and definitive hosts, thereby increasing the distribution of *E. multilocularis* (Rausch, 1985). In Europe, for example, rodent and fox distributions are enhanced by deforestation and agricultural practices that create a favourable habitat for rodent species, to which foxes are attracted (Veit et al., 1995; Giraudoux, 2004; Romig et al., 2006). Increased fox abundance as a result of rabies vaccination programs in Europe has also played a role in the increased occurrence of *E. multilocularis* (Deplazes et al., 2004; Romig et al., 2006). It is possible that the recent identification of a European strain-type in British Columbia is the result of translocation of red foxes during the last century from France, an area of endemicity (Kamler & Ballard, 2002). It is also possible that this is a more recent introduction from wildlife or domestic animals brought into the area (Jenkins et al., 2012).

1.6 Climate Change Impacts

The survival of infective eggs of *Echinococcus multilocularis* in the environment is affected by both temperature and moisture. The eggs of *E. multilocularis* are susceptible to desiccation and warmer temperatures, which is thought to limit the parasite to the northern hemisphere (Mas-Coma et al., 2008). In the future, increased precipitation and enhanced snow melting in the north (Furgal & Prowse, 2008) may facilitate survival of the eggs of *E. multilocularis* in the summer, while warmer temperatures may decrease overwinter survival (Mas-Coma et al., 2008). Arctic-adapted strain(s) may see decreases in survival and occurrence at their southern limit, while the strain(s) present in the NCR may expand northward into the Arctic as a result of increased moisture in northern microhabitats.

Climate alterations, including global warming, will affect the structure and functioning of parasitic ecosystems (Parmesan & Yohe, 2003; Polley & Thompson, 2009). These effects will be especially felt in the Arctic where average temperatures have increased at almost twice the global average in the last 100 years (Kutz et al., 2009). One consequence of climate change in the north is the alteration of restrictive temperatures that limit survival and development times of parasites (Kutz et al., 2009). The current distribution of *E. multilocularis* is restricted to the northern hemisphere, where the impacts of climate change are already being felt. The distributional restriction may therefore make *E. multilocularis* highly susceptible to the effects of climate change (Mas-Coma et al., 2008; Jenkins et al., 2011). This parasite is emerging (increasing in distribution and prevalence) in wildlife and human hosts elsewhere in the circumpolar North and is colonizing new regions through anthropogenic and natural movements of wild and domestic hosts (Eckert et al., 2000; Jenkins et al., 2005; Schweiger et al., 2007; Davidson et al., 2012; Jenkins et al., 2012).

Alterations to the distribution of *E. multilocularis* may largely be mediated through changes in rodent intermediate host distribution and abundance. Climate change may open up previously unsuitable areas (such as the boreal forest) by clearing the way for early successional plant species, supporting higher densities of rodent intermediate hosts. At the same time, rising temperatures in the NCR may decrease rodent habitat suitability, causing a northward shift in geographic range, which could decrease the occurrence of *E. multilocularis* in their current locations. Climate change is predicted to increase overall precipitation in northwestern North America, which will lead to higher primary productivity and in turn, increased stability and

density of rodent populations, facilitating amplified transmission of *E. multilocularis* on the Arctic mainland. Climate change is also predicted to increase extreme weather events, which may in turn increase the frequency and amplitude of fluctuations in Arctic rodent populations. Fluctuating rodent numbers may decrease overall transmission of *E. multilocularis*, as seen on mainland Alaska, as prevalence may be density-dependent (Rausch & Schiller, 1951).

In North America, warming temperatures may also increase international shipping through the Northwest Passage, enabling further opportunities for translocation of infected rodents and dogs. The recent establishment of *E. multilocularis* on Svalbard in the Norwegian Arctic and in Sweden is most likely by the introduction of suitable rodent hosts from shipping, and infected domestic dogs (Henttonen et al., 2001; Osterman Lind et al., 2011). The transportation of infected domestic dogs into BC may also have been responsible for the infection a domestic dog in Quesnel, BC (Jenkins et al., 2012). Alternatively, the recent identification of the BC/European strain of *E. multilocularis* in central British Columbia (a previously non-endemic region) may have occurred as a result of importation of infected red foxes from Europe in the last century (Jenkins et al., 2012). If this parasite has established in local wildlife, it could also be a result of climate shifts and deforestation that created a favourable habitat for intermediate hosts, which, in turn, attracted definitive carnivore hosts.

In addition to altering the ecology of intermediate hosts, climate change may also affect the distribution and abundance of sylvatic definitive hosts in Alaska and Canada. In the early part of the 20th century in North America, red foxes moved north by successfully outcompeting Arctic fox for food and den sites, possibly as a result of climate alterations (Hersteinsson & Macdonald, 1992; Post et al., 2009). With encroachment of the red fox into Arctic fox territory, there may be an opportunity for mixing of Arctic and southern strains of *E. multilocularis*, which may differ in pathogenicity, zoonotic potential, and host-specificity. Further understanding of the genetic variability, distribution, and zoonotic potential of strains of *E. multilocularis* is needed in northern North America and elsewhere in the circumpolar North to assess risks posed by a future of climate and other anthropogenic changes.

Table 1-1 Prevalence of *Echinococcus multilocularis*, identified at necropsy, in naturally infected definitive and intermediate hosts in Alaska and northern Canada.

Host	Location	Prevalence [% (n)]	References
O. Carnivora			
Arctic fox (*Vulpes lagopus*)	St. Lawrence Island, AK	71 (7)	(Rausch & Schiller, 1956)
		67 (6)	(Thomas et al., 1954)
		40 (106)	(Rausch & Schiller, 1956)
		100 (40)	*ibid*
		75 (1527)	(Rausch, 1967)
		80 (1579)	(Rausch & Fay, 2002)
	St. George Island, AK	67 (5)	(Fay & Williamson, 1962)
		32 (28)	(Rausch, 1967)
	Nunivak Island, AK	73 (33)	*ibid*
	Seward Peninsula, AK	2 (11)	*ibid*
	Mainland, AK	4 (94)	(Rausch, 1956)
	Mainland, AK	9 (207)	(Rausch, 1967)
	Banks Island, NT	2 (50)	(Eaton & Secord, 1979)
	Sachs Harbour, NT	1 case	CCWHC (W1997.149.1)
	Eskimo Point (Arviat), Resolute Bay, Cornwallis Island, NU	NR [a]	(Choquette et al., 1962)
Dog (*Canis lupus familiaris*)	St. Lawrence Island, AK	6 (89)	(Rausch, 1960)
		5 (110)	(Rausch, 1967)
		13 (31)	(Rausch & Fay, 2002)
Red fox (*Vulpes vulpes*)	Nunivak Island and Point Barrow, AK	2 (100)	(Rausch, 1956)
	Nunivak Island and Brooks Range, AK	55 (11)	(Rausch, 1967)
O. Rodentia			
Brown lemming (*Lemmus trimucronatus)*	Mainland Alaska	0.9 (467)	(Holt et al., 2005)
Ground squirrel (*Citellus undulatus*)	St Lawrence Island, AK	17 (12)	(Thomas et al., 1954)
Red-back vole (*Clethrionymus rutilus*)	St Lawrence Island, AK	18 (22)	(Rausch & Schiller, 1951)
		12 (25)	*ibid*
		5 (22)	*ibid*
Tundra vole (*Microtus oeconomus*)	St Lawrence Island, AK	2 (385)	*ibid*
		8 (905)	*ibid*
		16 (200)	*ibid*
		10 (320)	*ibid*
		5 (528)	(Rausch et al., 1990a)
		28 (1115)	(Rausch et al., 1990b)
		63 (329)	(Rausch & Fay, 2002)
		17 (198)	(Thomas et al., 1954)

O. Soricomorpha			
Shrew	St Lawrence Island, AK	25 (4)	(Thomas et al., 1954)
(*Sorex jacksoni*)		23 (13)	(Rausch & Schiller, 1951)
a. NR = not recorded			

Table 1-2 Prevalence [% (n)] of alveolar hydatid disease in people in Alaska from west to east, then chronologically within a location.

Location	Prevalence [% (n)]	Method	References
St. Lawrence	33 (233)	Casoni skin test [a]	(Rausch & Schiller, 1956)
Island (SLI)	16 (233)	Casoni skin test [b]	*ibid*
	15 (233)	CF [c]	*ibid*
	8 (153)	CF	*ibid*
	24 (232)	SE [d]	*ibid*
Gambell, SLI	20 (126)	Skin test	(Rausch & Schiller, 1951)
	2 (372)	SE, x-ray, biopsy	(Wilson & Rausch, 1980)
Savoonga, SLI	28 (106)	Skin test	(Rausch & Schiller, 1951)
	2 (364)	SE, x-ray, biopsy	(Wilson & Rausch, 1980)
Little Diomede	1 (84)	SE, x-ray, biopsy	*ibid*
Wales	2 (131)	SE, x-ray, biopsy	*ibid*
Point Hope	2 (386)	SE, x-ray, biopsy	*ibid*
Noatak	0.7 (293)	SE, x-ray, biopsy	*ibid*
Kotzebue	0.2 (1696)	SE, x-ray, biopsy	*ibid*
Kiana	0.4 (278)	SE, x-ray, biopsy	*ibid*
NR [e]	42 cases	SE, x-ray	(Wilson et al., 1995)

[a] Alveolar hydatid antigen

[b] Cystic hydatid antigen

[c] Complement fixation

[d] SE = serology

[e] NR – not recorded

Table 1-3 Prevalence of *Echinococcus multilocularis* of naturally infected definitive and intermediate hosts in the North American north central region, identified at necropsy.

Hosts	Location	Prevalence [%(n)]	References
O. Carnivora			
Red fox	SK	1 case	(Hnatiuk, 1969)
(*Vulpes vulpes* formerly *Vulpes fulva)*	Riding Mountain National Park (RMNP), MB	50 (6)	(Samuel et al., 1978)
	Morris, MB	1 (75)	(Baron, 1970)
	North Dakota	14 (830)	(Leiby et al., 1970)
	North Dakota	70 (96)	(Rausch & Richards, 1971)
	North Dakota	49 (61)	*ibid*
	South Dakota	0.8 (222)	(Leiby et al., 1970)
	South Dakota	75 (137)	(Hildreth et al., 2000)
	Nebraska	16 (1153)	(Kritsky & Leiby, 1978)
	Nebraska	42 (64)	(Storandt et al., 2002)
	Minnesota	5 (277)	(Leiby et al., 1970)
	Iowa	0.5 (200)	*ibid*
	Indiana	23 (71)	(Storandt & Kazacos, 1993)
	Ohio	27 (22)	*ibid*
Coyote	Edmonton, AB	25 (91)	(Catalano et al., 2012)
(*Canis latrans*)	RMNP, MB	23 (43)	(Samuel et al., 1978)
	North Dakota	6 (111)	(Leiby et al., 1970)
	South Dakota	44 (9)	(Hildreth et al., 2000)
	Illinois	35 (17)	(Storandt & Kazacos, 1993)
	Indiana, US	19 (70)	*ibid*
Domestic cat	Saskatoon, SK	2 (131)	(Wobeser, 1971)
(*Felis catus*)	Pick City, ND	1 case	(Leiby & Kritsky, 1972)
	Vela, North Dakota	2 cases	*ibid*
O. Rodentia			
Meadow vole	Edmonton, AB	22 (283)	(Holmes et al., 1971)
(*Microtus pennsylvanicus*)	Saskatoon, SK	0.9 (114)	(Hnatiuk, 1966)
	North Dakota	3 (565)	(Leiby et al., 1970)
	Iowa	6 (36)	*ibid*
Deer mouse	Edmonton, AB	28 (216)	(Holmes et al., 1971)
(*Peromyscus maniculatus*)	Lethbridge, AB	1 (180)	(Chalmers & Barrett, 1974)
	Kyle, SK	1 case	CCWHC.000042281
	Stony mountain/Argyle, MB	15 (99)	(Leiby et al., 1970)
	Gunton, MB	4 (25)	(Lee, 1969)
	Montana	0.4 (436)	(Leiby et al., 1970)
	North Dakota	6 (3335)	*ibid*

	South Dakota	1 (234)	*ibid*
	Minnesota	2 (53)	*ibid*
	Iowa	0.7 (151)	*ibid*
House mouse (*Microtus musculus*)	North Dakota	2 (47)	(Leiby et al., 1970)
Bushy-tailed woodrat (*Neotoma cinerea*)	Wyoming	1 case	(Kritsky et al., 1977)
Muskrat (*Ondatra zibethicus*)	Eyebrow Lake, SK	2 cases	CCWHC (W1993-102.1 and 103.1)
	North Dakota	0 (12, 142)	(Rausch & Richards, 1971)

Table 1-4 People diagnosed with alveolar hydatid disease in the north central region of Canada and the United States.

Location	Prevalence	Method	References
Edmonton, AB	1	unspecified	(Geddes Smith & Hanson, 1961; Sereda et al., 1961)
Winnipeg, MB	1*	autopsy	(James & Boyd, 1937)
Toronto, ON	1[a]	unspecified	(Langer et al., 1984)
Milwaukee, WI	1	unspecified	(LaFond et al., 1963)
Minnesota, US	1*	bioassay	(Gamble et al., 1979)
New York, NY	1[a]	unspecified	(Weinberg, 1947)

* Autochthonous cases
a North American cases outside NCR

2. OBJECTIVES

The objectives of this study were to: 1) to identify the haplotypes of *E. multilocularis* present in Canada; 2) to understand the host and geographic distribution of *E. multilocularis* in northwestern Canada; and 3) provide baseline information about this parasite and identify potential regions of emergence with corresponding animal and public health significance.

2.1 Hypotheses

The two general hypotheses are that; 1) there is greater genetic diversity of *E. multilocularis* than previously thought in Canada and that 2) the geographic distribution of *E. multilocularis* in Canada is more widespread than previously thought.

In terms of genetic diversity, the hypothesis can be divided into numerous subhypothesis: 1) In British Columbia, where a domestic dog was infected with a European strain of *E. multilocularis*, wild animals in this region will harbour the European strian; 2) there will be evidence to support both theories of establishment in the NCR; and 3) there will be distinct zones of occurrence for different strains (haplotypes) in Canada.

The prevalence of *E. multilocularis* is thought to be higher in areas where deer mice are more abundant (Holmes et al., 1971), therefore a subhypothesis is that in southern Saskatchewan agricultural areas, prevalence will be higher in hay field and native prairie habitats (generally reclaimed pastureland) than in active crop lands.

Screening wild carnivores for the presence of adult cestodes is a time consuming and arduous task. The sedimentation and counting technique (SCT), is recommended by the World Health Organization for the quantification of cestodes from the definitive host's small intestines (Kamiya, 2008). The SCT is laborious and also includes a 1-hour sedimentation phase for each sample. Therefore, it is hypothesized that the addition of an intermediate filtration step will reduce labour and time associated with cestode extraction.

2.2 Importance of Study

That Canada has not been included in genetic examination leaves a large gap in world knowledge on the subject of *E. multilocularis*, on a geographic and genetic scale. As there have been few human cases, especially in the prairie regions of Canada and the United States, North

American haplotypes are considered less pathogenic than their Eurasian counterparts (Nakao et al., 2009; Jenkins et al., 2012). Although this is controversial, there have been more cases of infection in Eurasia than in North America (Nakao et al., 2009). This may be due to increased surveilance, better detection methods, increased human exposure or may in fact be due to genetic differences in the parasite. As pathogenic differences are a possibility, variations found in Canadaian isolates should be determined. Research into the occurrence of *E. multilocularis* in Canada will provide baseline distribution information, and be useful in tracking future changes of a parasite that is emerging/re-emerging world wide. Hot zones, including the potential establishment of a European haplotype in British Columbia, and preferred habitat sites of host species need to be identified to mitigate possible public health concerns.

2.3 Scope of Study

Samples will be collected from three different habitat types in southern Saskatchewan farmlands to provide information about habitat preference of intermediate hosts and information about genetic differences of *E. multilocularis* in areas with high rodent densities. Samples from this region will provide information on prevalence of *E. multilocularis* in three habitat subtypes: 1) active croplands; 2) hay fields and; 3) native prairie. Samples from Edmonton and Calgary in Alberta and Saskatoon in Saskatchewan will provide information about this parasite in urban settings. Samples from the area surrounding Quesnel, British Columbia will provide information about the potential establishment of a European haplotype in Canadian wildlife.

In the North, sample sites will include Karrak Lake and Bylot Island, Nunavut (Figure 2-1). Samples from the Northwest Territory will also be available; however, the majority of samples will be from wolves, which are more often definitive hosts for *E. granulosus*, not *E. multilocularis.*

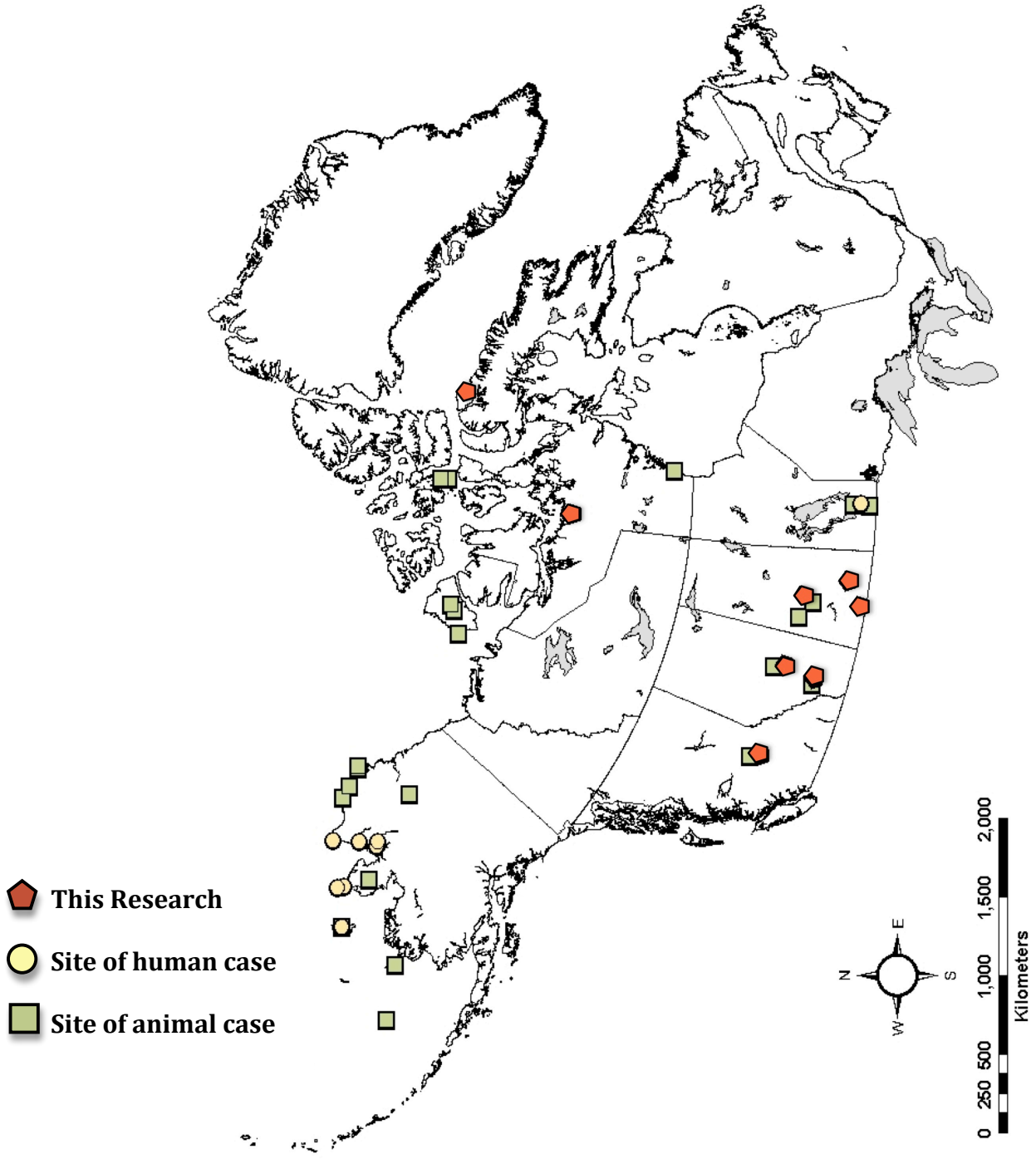

Figure 2-1 Map displaying the sites of current research (red polygons) along with the literature reports for positive animals (green squares) and human infections (yellow circles). The contiguous United States is not represented on this map.

3. AN IMPROVED METHOD FOR THE EXTRACTION AND QUANTIFICATION OF ADULT *ECHINOCOCCUS* FROM WILDLIFE DEFINITIVE HOSTS

This section has been submitted, in part, to the journal of Parasitology Research for publication (PR-2013-0096).

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Brent Wagner: University of Saskatchewan, Veterinary Microbiology. Method development support.

Brett Elkin: Government of Northwest Territories, Wildlife Veterinarian. Specimen supply.

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Emily Jenkins: University of Saskatchewan, Veterinary Microbiology. Supervisor, method development support.

Abstract

Most methods available for extraction and quantification of adult *Echinococcus* species from the intestine of wild carnivores require chemical solutions, significant technician time, or specialized equipment. The scraping and counting technique (SCT), with sensitivity close to 100%, has been the standard protocol recommended by global regulatory bodies. A scraping, filtration and counting technique (SFCT) method performed on two 10% aliquots of total small intestinal contents maintained the sensitivity ($p = 0.801$, $\alpha = 0.05$) and increased the efficiency of recovery and quantification of adult cestodes of *E. granulosus* and *E. multilocularis* from intestines of 85 wild carnivores. The SCT had a sensitivity of 91% and negative predictive value of 97% relative to SFCT. The SFCT significantly decreased time ($p = 0.0001$, $\alpha = 0.05$) and effort needed to process each sample, because of reduced debris in the filtered solution. Positive samples took an average of 25 minutes (per 20% aliquot) to quantify with SFCT and 33.5 minutes (per 20% aliquot) with SCT, not including the 1-hour sedimentation time required by SCT. The SFCT is therefore an appealing option to determine if these important zoonotic parasites are newly established in a region or host species, and if the prevalence and intensity of infection are changing in a region over time. This is particularly important for *E. multilocularis*, which is increasing in prevalence and distribution worldwide.

3.1 Introduction

Members of the *Echinococcus* genera are the smallest cestodes in the Taeniidae family, measuring less than 7 mm in length (Eckert et al., 2002). Though all species are of zoonotic concern, *E. multilocularis* and *E. granulosus* may be most important on a global scale as a cause of human disease and socioeconomic loss. *E. granulosus*, present almost worldwide, is the causative agent of cystic hydatid disease and mainly affects the lungs and liver of intermediate (cervids) and aberrant hosts (humans) (Rausch, 2003). Cystic hydatid disease in intermediate hosts can cause a dramatic decline in health and productivity leading to widespread industry and economic loss (Parodi et al., 2002). The expenditures associated with human infection are also considerable, with high cost treatments, if surgical resection is an option, and a significant decrease in lifespan, if it is not (Parodi et al., 2002).

Infection with *E. multilocularis* may not hold the same potential for economic loss, as it cycles through rodents and carnivores, rather than cervids and carnivores, but the effects on

human health are profound. Alveolar hydatid disease, caused by *E. multilocularis*, has been described as one of the most insidious of human diseases and is considered near incurable with a fatality rate of over 90%, if untreated (Bresson-Hadni et al., 1991; Kern et al., 2003). Humans and other aberrant intermediate hosts become infected through the inadvertent ingestion of eggs, of either species of *Echinococcus*, from feces of wild and domestic carnivores. Large-scale surveillance of wild carnivore species for the presence of *E. multilocularis* is the recommended method to monitor the occurrence, prevalence and potential emergence of this parasite in wildlife reservoir hosts.

Of the many tests used to screen wild carnivores for *Echinococcus* infection, including fecal egg and antigen testing, microscopic examination of cestodes recovered from total intestinal contents at necropsy is unsurpassed for obtaining the best estimate of prevalence and intensity of infection at the individual and population levels (Duscher et al., 2005). Detection of *E. multilocularis* in definitive hosts by fecal examination alone may not be an accurate measure of infection as typically, egg production only occurs in the first 1- 4 months of infection (Duscher et al., 2005). Eggs are morphologically indistinguishable between the genera of *Taenia* and *Echinococcus* and, therefore, identification can only be accomplished with molecular analysis. Traditional analyses of intestinal contents can be time consuming and arduous, especially with large sample sizes used in screening surveys. The gold standard method of extraction and counting is currently the scraping and counting technique (SCT), which involves hours of sedimentation time prior to sifting through large volumes of debris-laden liquid, and may produce false negatives if intensity of infection is low (Thompson, 1986; Conboy, 2009). Many other processes involve the use of additives, such as saline, that may interfere with PCR amplification; require the creation of specialized equipment; or compromise morphological structure (Rausch et al., 1990a; Hofer et al., 2000; Davidson et al., 2009). Conservation of adult specimens is necessary for accurate morphological and molecular characterization. For these reasons, we compared the sensitivity, specificity, and efficiency of SCT with a proposed modification, the scraping, filtration and counting technique (SFCT).

3.2 Materials and methods

The small intestines of 85 wild canids (55 wolves - *Canis lupus*, 24 coyotes - *Canis latrans*, and 6 red foxes - *Vulpes vulpes*) were collected from trappers, hunters, and wildlife personnel in northwestern Canada (British Columbia and the Northwest Territories) for other

purposes. The small intestines were sealed by tying a string around the intestine at the anterior end of the duodenum and another just anterior to the caecum then excised. The intestines were frozen at -80°C for a minimum of seven days prior to processing in order to inactivate eggs potentially infective to people (Jacobs et al., 1994; Eckert, 2003; Duscher et al., 2005). Although this was not a double blind survey, a single technician performed all steps for both SCT and SFCT in an attempt to minimize bias and error.

After thawing, each intestine was divided into four to eight equal parts depending on species and length. The portions of intestine were opened longitudinally and placed in sealable glass jars (one to four) jars depending on length of the segment) with approximately 250 ml distilled H_2O (dH_2O), then shaken vigorously for one to two minutes (Eckert et al., 2002). The intestine portions were scraped twice between two fingers and discarded (Deplazes & Eckert, 1996; Eckert, 2003). The resulting liquid was strained through a large mesh sieve (1mm pore size, 20.3 cm diameter, U.S.A. standard test sieve no. 18, Fisher Scientific Company, Ottawa, Ontario) and gently washed with dH2O to remove large particles (Jacobs et al., 1994; Umhang et al., 2011). Filtrate containing the cestodes, from the jars, was homogeneously mixed and divided into two equal portions for analysis by the SCT and SFCT protocols, respectively.

One portion was set aside for the hour-long sedimentation required by the SCT protocol (Rausch et al., 1990a; Kamiya, 2008). The second portion was immediately filtered through a second sieve (150 µm pore size, 20.3 cm diameter, Canadian standard sieve series no. 100, W.S. Tyler, Fisher Scientific Company, Ottawa, Ontario). Retention of cestodes on the second sieve previously had been tested using 40 samples. Only one scolex (of approximately 2,500) in one sample was detected in the filtrate of this second filtration step. In the remaining 39 samples, including one containing approximately 300,000 cestodes, all scolices and segments were retained on the sieve.

After filtration, the sieves were rinsed with dH2O until clear, using a swirling motion, and gentle tapping on the underside of the sieve to encourage flow-through. When the contents retained on the sieve were clear of debris, the contents were rinsed with dH2O into a receptacle for quantification. The liquid resulting from the SFCT averaged 10-15 ml. Approximately 190 ml of water was added to this SFCT liquid for comparison with the SCT liquid, which averaged 200 ml. Intact cestodes and scolices of *Echinococcus* spp. were counted in two 10% aliquots (10% of 200 ml) for both methods. After use, sieves were soaked in Dri-Clean® enzyme cleaner

(Decon Laboratories, cat no. 04-355-75, Fisher Scientific, Ottawa, Ontario) according to manufacturers instructions, scrubbed on both sides, rinsed with bleach (10%), and then rinsed with dH2O between samples to avoid contamination of subsequent samples. After cleaning, no cestodes were detected on the sieves.

Statistical analyses of count and time, not including the hour-long sedimentation time for SCT, were performed on positive samples (n = 23). To compare methods, the sensitivity [true positives/(true positives + false negatives)] and negative predictive value [true negative/(true negative + false negative)] of SCT relative to SFCT for detection of *Echinococcus* was determined for all 85 samples (Table 3---1). A t-test for equality of means was used to determine if further analysis required parametric or non-parametric testing. A one-way ANOVA test ($\alpha = 0.05$) was used to analyze the difference in worm count between the two methods; time differences were analyzed using the non-parametric Wilcoxon Signed Ranks Test ($\alpha = 0.05$).

3.3 Results

In two samples, cestodes of *Echinococcus* spp. were detected by SFCT and not by SCT (sensitivity of SCT = 91% and NPV = 97% - Table 3-1). Therefore, apparent prevalence (% positive samples) using SFCT was 23/85 (27%), whereas apparent prevalence using SCT was only 21/85 (25%). The highest count came from a coyote with a projected worm burden of approximately 3,900 (in a 10% aliquot, there was a mean of 391 *Echinococcus* cestodes) according to SFCT. The same sample when quantified with SCT had an estimated worm burden closer to 4,100 (in a 10% aliquot, there was a mean of 416 *Echinococcus* cestodes). The total count of all positive samples was approximately 26,000 (mean count of 2603) with SFCT and 24,000 (mean of 2422) with SCT. The smallest number of *Echinococcus* cestodes detected in a 10% aliquot using either method was 2. There was one animal from which worms were detected in both aliquots using SFCT, but in only one aliquot using SCT.

There was no significant difference between counts between methods ($p = 0.801$, $\alpha = 0.05$). Mean counts for SFCT and SCT, for the positive samples, were 113 (range 2 to 428) and 105 (range 2 to 519), respectively. Counts for SFCT were higher than those for SCT in 15/23 samples (Table 3-2). Quantification with SFCT (mean 25 minutes, range 3 to 98) took significantly less time than with SCT (33.5 minutes, range 9 to 69) ($p = 0.0001$, $\alpha = 0.05$), which did not include the 1-hour sedimentation time required by SCT (Figure-3-1). Samples processed with SFCT were consistently clearer than those processed with SCT.

3.4 Discussion

The proposed SFCT proved to be a more efficient and sensitive way to detect and quantify adult *E. granulosus* and *E. multilocularis* in three naturally infected wildlife definitive hosts, relative to the currently accepted gold standard (SCT) (Kamiya, 2008). The enhanced efficiency and sensitivity of SFCT is likely reflective of a reduction in the amount of debris masking adult cestodes. It is unlikely that the enhanced sensitivity with SFCT could be due to worms from previous counts contaminating subsequent samples, as stringent sieve cleaning (previously tested for efficiency) was carried out between samples. Sample processing with SFCT also reduced the need for serial dilutions, making identification and quantification quicker and easier. SFCT could also lead to a reduction in false positive and negative tests, as unfiltered SCT sediment contains material, such as intestinal villi, that could be mistaken for cestodes or could potentially mask their presence. The reliability of both positive and negative diagnoses is therefore increased with filtration. In the instances where SCT had a higher count (n = 8/23, 35%), the chance of worms passing through the retention sieve (SFCT) and being lost to the count must be considered. However, previous testing indicated almost 100% retention on the sieve.

In many instances, screening wildlife for zoonotic diseases may be the first line of defence for mitigating disease occurrence in people (Davidson et al., 2009). However, large scale screening of wild carnivores for the presence of pathogenic parasites is a daunting and time consuming task. Use of the proposed SFCT method maintains, if not improves upon the sensitivity of SCT while significantly decreasing processing time per sample. With the addition of the 1-hour sedimentation time required by SCT the average time saved per sample is 68.5 minutes. In Sweden a total of 1,140 foxes were screened for *E. multilocularis* infection in 2011 (Osterman Lind et al., 2011). If necropsies were performed on all samples, application of this protocol would save saved over 1300 hours of processing time. The SFCT is therefore an appealing option to determine if these important zoonotic parasites are present in a region or host species, and if the prevalence and intensity of infection are changing over time. Maximizing sensitivity, specificity, and efficiency of surveillance tests is especially important when demonstrating country-level freedom status or to detect the emergence (increasing in abundance and distribution) of parasites such as *E. multilocularis*.

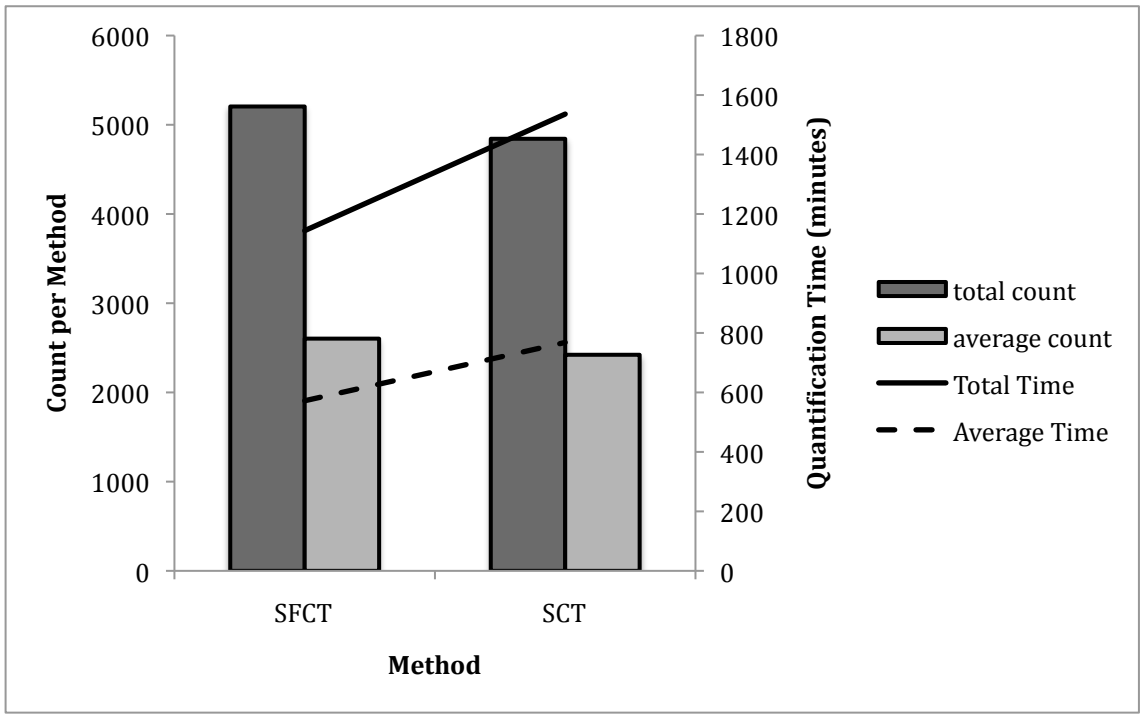

Figure-3-1 Sum and average of adult cestodes in a 20% aliquot of total canid intestinal contents, as well as total and average time for quantification, in the SCT and SFCT methods for 23 samples positive for *Echinococcus* infection. Time does not reflect the one-hour sedimentation time required by SCT.

Table 3-1 Values used for the calculation of sensitivity and negative predictive values of the SCT and the SFCT for 85 wild carnivore definite hosts for *Echinococcus* spp.

	Positive SFCT	Negative SFCT	
Positive SCT	21 (True Positive TP)	0 (False Positive FP)	21 Total positive SCT
Negative SCT	2 (False Negative FN)	62 (True Negative TN)	64 Total negative SCT
	23 Total positive SFCT	62 Total negative SFCT	Total = 85

Table 3-2 Number of adult cestodes detected by SCT and SFCT methods in intestinal samples from 23 carnivores, and time required for each method.

Method	Total	High	Mean	Low	Higher Total [a]
SFCT Count	***5205**	**428**	***113**	**2**	**15/23 (65%)**
SFCT Time	***1144**	**98**	***25**	**3**	**3/23 (13%)**
SCT Count	***4843**	**519**	***105**	**2**	**8/23 (35%)**
SCT Time	***1536**	**69**	***33.5**	**9**	**20/23 (87%)**

[a] SFCT produced a higher individual cestode count in 65% of the trials and an increased quantification time in only 13%. Cestode counts were higher in SCT in 35% of the trials. Quantification time was higher in 87% of the trials using SCT. The time values do NOT reflect the one-hour sedimentation required by SCT.

* Number detected in two 10% aliquots of each of 23 animals.

4. RODENT INTERMEDIATE HOSTS AND INFECTION PREVALENCE OF *ECHINOCOCCUS MULTILOCULARIS* IN SOUTHERN SASKATCHEWAN

4.1 Introduction

Rodent-borne zoonotic diseases are an emerging public health concern worldwide. Rodents in prairie areas of western Canada are known reservoirs of zoonoses, including alveolar hydatid disease (AHD) caused by the cestode *Echinococcus multilocularis*. The presence of this parasite in rodent populations poses a potential public health risk that includes occupational and peridomestic exposure of rural and urban residents. While rodents infected with *E. multilocularis* do not in themselves place humans at risk for exposure, they do pose a threat when consumed by domestic carnivores, which will, in turn, increase the risk of human exposure. Human AHD has an extremely high fatality rate when left untreated (Kern et al., 2003). Although human infections are, at present, rare in North America, the annual cost of treatment can exceed $300,000 US per patient (Sreter et al., 2003). A substantial cost of almost $6 million US per patient is assumed for the average 19-year survival with treatment of this disease (Rausch & Wilson, 1985; Eckert et al., 2002).

In Europe, distribution of *E. multilocularis* is increasing at an alarming rate, as is occurrence in animals and humans (Romig et al., 2006; Moro & Schantz, 2009). In Europe, there are direct links among prevalence of the parasite, local abundance of rodent intermediate hosts, and habitat type, including agriculturally disturbed and deforested areas (Viet et al., 1999). The threat of this disease in North America may also be increasing, as more occurrences of this parasite are being discovered in both wild and domestic animals in urban settings (Rausch & Wilson, 1985; Eckert et al., 2002; Catalano et al., 2012), as well as in previously non-endemic regions (Jenkins et al., 2012). In central North America, the primary intermediate hosts are deer mice, *Peromyscus maniculatus*, and meadow voles, *Microtus pennsylvanicus*, and the prevalence of *E. multilocularis* is thought to be higher where deer mice were more abundant (Holmes et al., 1971). Deer mice may also be hosts for other rodent-borne zoonoses, including Hantavirus pulmonary syndrome and tularaemia; therefore it is important to determine the habitat preferences of deer mice to determine risks to public health posed by these diseases with wildlife reservoirs. The objective of this study was to discern the habitat preferences and alveolar hydatid infection status of deer mice in rural settings in southern Saskatchewan. Therefore, we

determined links between infection status of rodent intermediate hosts among mice trapped at sites with three habitat types: active croplands, hay fields, and native prairie (community pastures).

4.2 Materials and Methods

The study area in southern Saskatchewan, Canada, is roughly bounded by the towns of Estevan (49°13' N, 102°58' W), Strasbourg (51°04' N, 104°57' W) and Moose Jaw (50°20' N, 105°33' W) in the moist-mixed grassland region and by the towns of Moose Jaw, Val Marie (49°22' N, 107°50 W) and Bengough (49°23'N, 105°08' W;) in the mixed grassland region (Figure 4-1). In summer 2009, rodents were trapped at 18 sites, each containing the three habitat types; active croplands, hay fields and native prairie (primarily community pastures). Each habitat type was within 2-10 km of the others. In summer 2010, trapping was repeated at one site (Val Marie) sampled in 2009; 3 additional sites sampled in 2010 included Lone Tree, Masefield, SK and Walker Ranch, Montana, USA, (n = 252) but did not include habitat type specific trapping. Sixteen mice from Cypress Hills were examined that were trapped in the summers 2009 and 2011.

4.2.1 Samples

Victor Professional Snap-Traps® (Woodstream Corporation, Lititz, PA, USA) were set simultaneously for three consecutive nights in each of the three habitat types to ensure consistent trapping conditions. Traps were checked and reset each morning. Traps were baited with a mix of peanut butter, birdseed and rolled oats and spaced approximately 10 m apart on 500 m transects. Within each habitat type, transects were placed approximately 100 m from the site edge to minimize edge effects. An equal number of traps were set in each habitat type. A total of 2596 individual rodents were captured during 25,126 trap nights (trap number x transects x active sets) in 2009. Deer mice were the most frequently captured species (90.4% of total captures) in all regions (sum total). The remaining 9.6% were voles (*Microtus* and *Lemmiscus* spp.) and other mice (*Zapus* and *Perognathus* spp). Trapping occurred courtesy of the Royal Saskatchewan Museum and University of Regina (University of Regina President's Committee on Animal Care Protocol #08-11).

A total of 784 adult and sub-adult deer mice, classified by size and weight, and frozen at -20 °C since 2009/2010, were sent to the University of Saskatchewan, Veterinary Microbiology

department for analysis. No juveniles were studied, as metacestode development only becomes detectable at week four to six post ingestion of the egg, by which time deer mice are reproductively mature and no longer considered juveniles (Blair, 1940; Thompson & McManus, 2002). All necropsies took place using level 2 bio-safety measures to guard against the airborne transmission of potential pathogens. The abdominal cavity was opened and the liver excised. The liver was visually inspected for the presence of larval cysts. If suggestive lesions were observed, the cysts were carefully cleared of surrounding host tissue. Large cysts were punctured, fluid collected and examined microscopically for the presence of protoscolices (Figure 1-7), as other conditions that could be confused with early alveolar hydatid cysts include infections with the nematode *Capillaria hepatica* and other taeniid cestode larval stages, granulomas, and autolysis. Fluid and tissue from microscopically confirmed alveolar hydatid cysts were placed in separate 1.5 ml microcentrifuge tubes and frozen at -20 °C until DNA extraction..

4.2.2 DNA Analysis

DNA extraction and analysis was performed to confirm visual identification of *Echinococcus* infection. Extraction of genomic DNA (gDNA) was performed using the DNeasy Blood and Tissue Kit® (Qiagen Incorporated, Toronto, ON, Canada). If available, extraction was preferentially performed on the hydatid fluid to reduce contamination with host tissue. Extraction followed manufacturer's guidelines with the addition of a second wash with buffer AW2. Genomic DNA was stored at -20 °C until PCR amplification.

Amplification of the mitochondrial DNA (mtDNA) gene NADH dehydrogenase subunit 1 (*nad1*) was accomplished using the simplex PCR (outlined in section 5.2.2) using published primers (Trachsel et al., 2007). Samples verified as *E. multilocularis* at *nad1* were sequenced at three additional mtDNA loci: NADH dehydrogenase subunit 2 (*nad2*), cytochrome b (*cob*) and cytochrome c oxidase subunit 1 (*cox1*). Because of the lack of amplification or decreased efficiency using published primers (Nakao et al., 2009), alternative primers were developed to amplify the additional mtDNA loci (Table 6-2). The developed primers amplified 623 base pairs (bp) of *nad2* (nt 104 – 727), 693 bp of *cob* (nt 209-902) and 966 bp of *cox1* (nt 363-1327), relative to the complete genome (accession no. NC_000928).

Four positive mice captured in 2010 and one positive mouse from 2009 did not have complete collection information, and were not included in the analysis. Sex, prevalence and

habitat comparisons were therefore based on 516 mice captured in 2009, which included 39 infected mice. Prevalence of infection was calculated as the percent infected per total number examined.

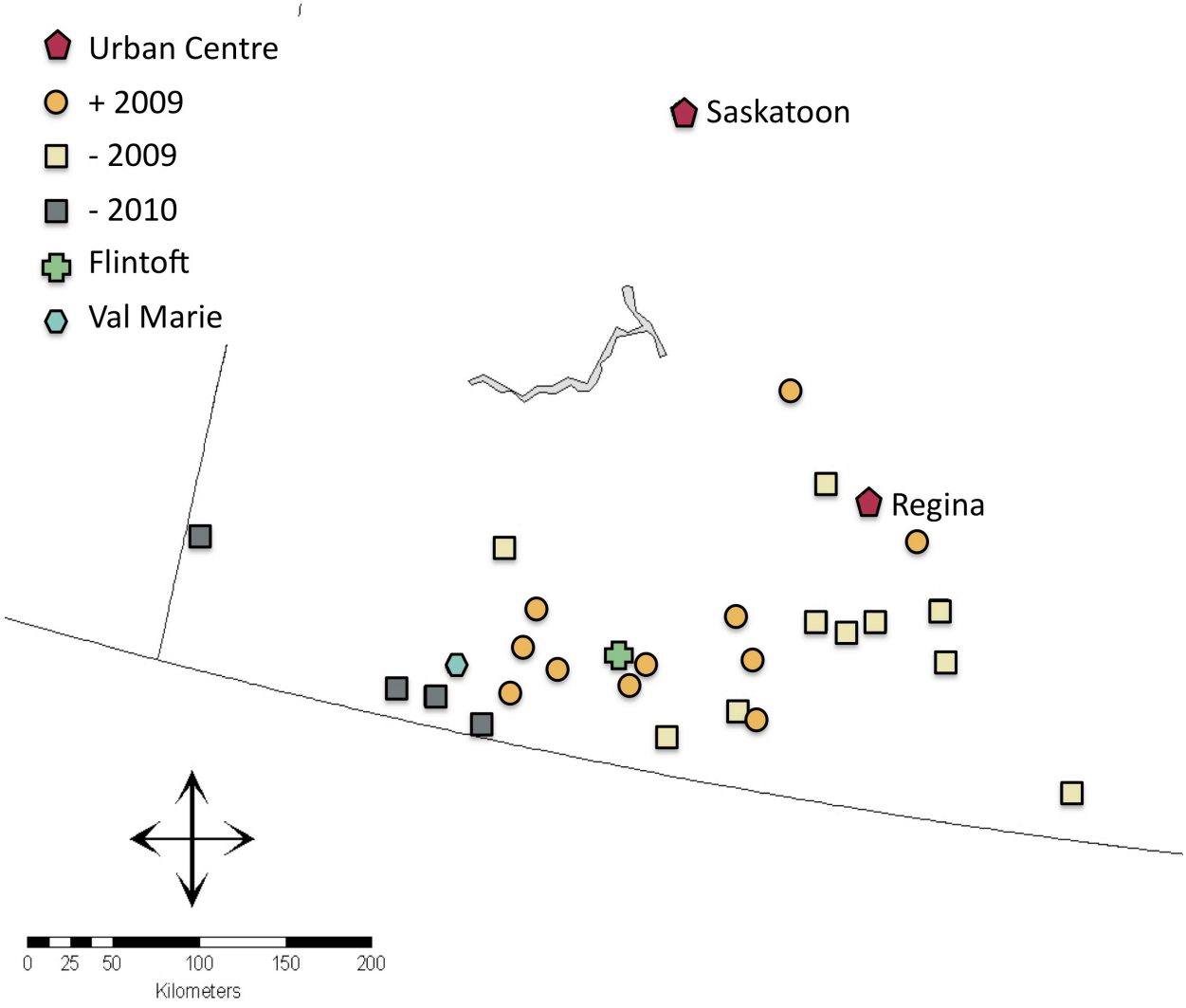

Figure 4-1 Study sites for collection of *Echinococcus multilocularis* infected deer mice across southern Saskatchewan relative to Saskatoon and Regina (red polygons). Markers represent positive and negative sites from 2009 and 2010. The highest infection prevalence was found in Flintoft (green cross); and Val Marie (blue hexagon) was positive in 2009 and 2010.

4.3 Results

Larval cysts of *Echinococcus multilocularis* were found in 39 of 516 (8%) mice captured in 2009 and 5 of 252 (2%) captured in 2010. None of 16 mice from Cypress Hills was positive.

The average prevalence of infection in the 2 years was 44 of 784 (6%). Of 516 mice captured in 2009, 55% (n = 284) were male and 43% (n = 220) were female. Fifty nine percent (23 of 39) of infected mice were male. However, both male and female mice had an overall infection prevalence between 7 and 8%, which was not statistically significant (Table 4-1).

Table 4-1 Prevalence of *E. multilocularis* in male and female deer mice captured in Saskatchewan in 2009.

Sex	Total Captured (n (%))	Total Positive	% Prevalence	% of Positive (n=39)
Female	220 (43)	16	7.3	41
Male	284 (55)	23	8.1	59
Unknown	12 (2)	0	0	0
Total	516	39		100

The estimated proportion of cyst to healthy liver tissue varied greatly between specimens. In some animals, the cysts encompassed less than 5% of the liver. In other cases, cyst material had replaced an estimated 80% of the liver, leaving very little unaffected liver tissue (Figure-4-2).

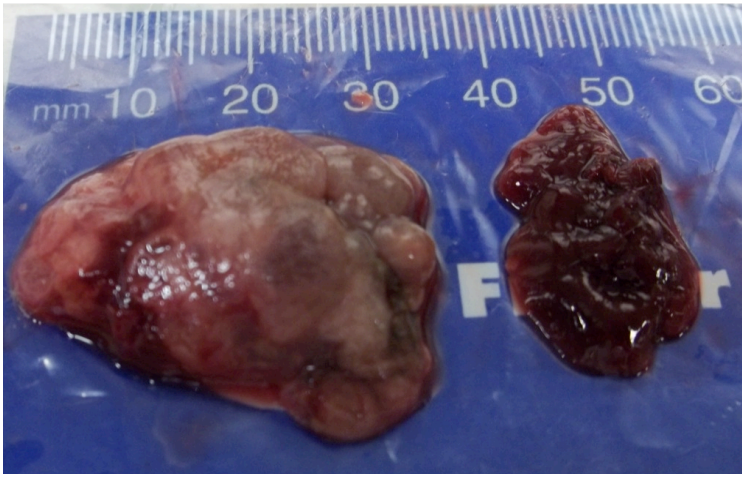

Figure-4-2 Photograph showing size of extracted cyst material (left) relative to unaffected liver tissue (right) from infected Saskatchewan deer mouse.

A focal point of infection in Saskatchewan deer mice appears to be the area surrounding Flintoft, with 26% of the 29 positive samples originating here. Infection prevalence was also

26% (10 of 38) for deer mice captured around Flintoft in 2009 (Figure 4-1; Table 4-2). Other sites with prevalence greater than or equal to 10% included Crane Valley, Glentworth, Mankota and Wood Mountain. Negative sites include Bienfait, Francis, Ironchild, Pambrun, and Rockglen. Val Marie was the only site to be sampled in both 2009 and 2010. In 2009, there was an infection prevalence of 5% (3 of 65 captured) in Val Marie. In 2010, infection prevalence was 2% (3 positive) even though more than twice the number of deer mice was captured that year (n = 153).

Table 4-2 Prevalence of *E. multilocularis* in deer mice at positive capture sites in Saskatchewan in 2009.

Site	Total Captured	Prevalence (n = (%))
12 Mile	40	2 (5)
Bengough	19	1 (5)
Crane Valley	11	2 (18)
Flintoft	38	10 (26)
Glentworth	51	6 (12)
Hazenmore	41	1 (2)
Kronau	1	1 (100)
Lipp	4	1 (25)
Mankota	45	5 (11)
McCord	46	2 (4)
Val Marie	65	3 (5)
Viceroy	22	1 (5)
Wood Mntn	41	4 (10)
Total	424	39

Of 136 deer mice captured in native prairie habitat, 9% were infected. A habitat specific prevalence of 8% (of 184 captured) and 7% (of 196) was found in hay fields and active cropland habitats, respectively. Native prairie account for 33.2% (n = 13) of infected deer mice in this study while active croplands account for 31% (n = 12). Hay field habitats accounted for 35.8% (n = 14) of positive infections (n = 39).

Infections identified visually were confirmed as *E. multilocularis* in 39 of 40 positive samples by sequencing of the *nad1* gene. One sample was characterized as 99% identical to

Taenia polyacantha across 486 nucleotides. *Taenia polyacantha*, found in one sample is a larger cestode that also utilizes rodent species as its intermediate host (Fujita et al., 1991). Genetic characterization of the SK isolates is discussed further in section 6.

4.4 Discussion

Sites with increased prevalence of *E. multilocularis* in rodent intermediate hosts were identified, which may indicate sites of increased risk for human infection in southern Saskatchewan. One such area is the agricultural zone surrounding Flintoft, SK, with an infection prevalence of 26%. This is higher than that reported in deer mice in endemic regions of central North America (mean 4%, range 0.4-15%) (Table 1-3). The exception was an area in central Alberta that once had a prevalence of 28% (Holmes et al., 1971). Spatial differences in parasite prevalence likely reflect variation in rodent density and diversity.

An overall prevalence of 6% in the current study is comparable with that reported for alveolar hydatid cysts in deer mice elsewhere in North America (Table 1-3). There was interannual variation in prevalence (Val Marie) noted at one site sampled in 2009 (5%) and 2010 (2%), which likely reflects interannual variation in deer mouse abundance seen in previous years (Wobeser et al., 2007).

There was no significant sexual difference in prevalence. Both male and female deer mice had an infection prevalence of approximately 8% in 2009, despite more males being captured in that year. There were 284 males captured and only 220 females. This may be a reflection of a true male sampling bias or a reflection of actual population sex dynamics. Males were infected more often than females but this was not significant.

There were 116 male mice captured in croplands and 101 caught in hay field sites as compared to the 67 caught in native prairie sites. Although there was also no significant difference in habitat type prevalence, a slightly higher number of mice were infected in native and hay field habitats. Although all habitat types have been altered at one time or another (native is likely reclaimed pasture land), active croplands undergo seasonal disruption, such as tilling, which may explain the lower occurrence of infection. The seasonal disruptions of active cropland may therefore decrease the abundance and stability of food sources and rodent populations, and/or decrease use by infected definitive hosts (such as coyotes, foxes, and domestic dogs and cats). Further sampling in the three habitat types should be considered to verify these results and

identify agricultural regions and practices that favour parasite transmission and may pose a threat to human health.

Despite the fact that *E. multilocularis* is obviously well established in wildlife in southern SK, there is no evidence of human infection. The low incidence of human infection with AHD in Canada may be directly related to low human densities and decreased risk of exposure at the human/wildlife interface. In Europe, where 67 cases were reported in 2010 alone (EFSA and ECDC, 2012), human populations may have higher exposure because of the increased density and distribution of human populations in relation to that of wildlife. Humans in Canada may have less chance of being infected by *E. multilocularis* than their European counterparts because of decreased exposure to infective eggs. However, it is also possible that strains of North American origin have less zoonotic potential than Eurasian strains (Jenkins et al., 2012). This reinforces the need for further characterization of strains of *E. multilocularis*, particularly in endemic foci such as those identified in the current study.

5. ESTABLISHMENT OF A EUROPEAN STRAIN OF *ECHINOCOCCUS MULTILOCULARIS* IN CANADIAN WILDLIFE

This section has been submitted, in part, to the journal of Emerging Infectious Diseases for publication (EID-12-1738).

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Janet E. Hill, University of Saskatchewan, Veterinary Microbiology. Haplotype analysis support.

Helen Schwantje: Government of British Columbia, Wildlife Veterinarian. Specimen supply.

Stefano Liccioli: University of Calgary. Adult cestode lysis support.

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Abstract

In 2009, a haplotype most closely related to European strains of the cestode *Echinococcus multilocularis* was detected in a dog from the Quesnel region of British Columbia, Canada. We now report the establishment of this European haplotype in seven coyotes (*Canis latrans*) trapped within 40 km of Quesnel, BC. In addition, three coyotes and one red fox (*Vulpes vulpes*) harboured adult cestodes morphologically compatible with that of *Echinococcus* (overall prevalence 33% in 33 carnivores). None of 156 potential intermediate hosts, including 131 representatives of two highly suitable rodent species, *Peromyscus maniculatus* and *Microtus pennsylvanicus*, trapped from a region 120-210 km south of Quesnel were infected. This report confirms the establishment and local transmission of an introduced strain of *E. multilocularis* (the causative agent of human alveolar hydatid disease), in forested region of North America where this cestode had not been previously detected, with significance for public and animal health.

5.1 Introduction

Recently, a dog (*Canis lupus familiaris*) native to Quesnel, British Columbia (BC), Canada was diagnosed with an alveolar hydatid cyst, the larval stage of the cestode *E. multilocularis* (Peregrine et al., 2010; Jenkins et al., 2012). Molecular analysis identified this strain as European in origin (Jenkins et al., 2012). There was no history of travel outside of BC for the dog, suggesting that the parasite was locally acquired; however, BC was not considered an endemic region, with the nearest report of this parasite in North America 600 km to the east in Alberta, Canada. How this dog came in contact with eggs of European origin remained unanswered (Jenkins et al., 2012). Furthermore, the dog was serving as an aberrant intermediate host (harboring the larval or metacestode stage) rather than the typical carnivore definitive host with adult cestodes in the intestines. The normal intermediate hosts for this parasite are rodents, which develop alveolar hydatid cysts upon ingesting eggs shed in the feces of an infected carnivore; the life cycle is completed when a carnivore consumes a cyst in an intermediate host. If this dog was exposed locally, it implies that the infectious eggs of the parasite were shed in the feces of carnivore, and that people were potentially at risk from the same source.

The identification of a European cestode strain in Canada is a potential public health concern as European strains are thought to have more zoonotic potential than their North American counterparts (Nakao et al., 2009). In 2010 alone, 67 human cases were reported in the

European Union (EFSA and ECDC, 2012), while only 2 authochthonous cases have ever been reported in Canada and the lower 48 US states (James & Boyd, 1937; Gamble et al., 1979). This may be a reflection of the pathogenic differences between the haplotypes, as intraspecific variation has already proved responsible for differences in larval development and host response (Bartel et al., 1992). People affected by this parasite often experience a prolonged preclinical period of 10 to 25 years, during which larval growth causes extensive, and often irreparable liver damage (Rausch & Wilson, 1985). Without early and extensive surgical resection of the affected liver tissue and long term cestostatic drug therapy, life expectancy is reduced to 10 years post diagnosis for more than 90% of patients (Kern et al., 2003). Even with aggressive surgical resection, the average post-surgery survival time is only 19 years (Rausch & Wilson, 1985). *E. multilocularis* is emerging (increasing in prevalence and range) across its circumpolar distribution as a result of anthropogenic and environmental alterations (Romig et al., 2006). This is reflected in the range expansion of infected red foxes in Europe, which has increased from 4 to 21 affected countries since the 1980's (Eckert et al., 2002). Human cases of alveolar hydatid disease have also increased. In Switzerland, for example, the occurrence of human infections has more than doubled in 12 years (Davidson et al., 2012). Determining if *E. multilocularis* was present in wildlife in the Quesnel region, and therefore the possibility of local transmission with concomitant risks of human exposure, was of the utmost importance.

5.2 Materials and Methods

In the summer of 2010, 156 rodents and small mammals were captured by snap-trap between the communities of Williams Lake (52°07'46" N; 122°08'18" W) and 100 Mile House (51°38'30"N; 121°17'50" W), 120 to 210 km south of Quesnel, BC (Government of British Columbia permit no. 78470.25) (Figure 5-1). Species trapped include 72 deer mice (*Peromyscus maniculatus*), 59 meadow voles (*Microtus pennsylvanicus*), 16 North American jumping mice (*Zapus hudsonius*), 7 shrews (*Sorex* spp.), 1 red squirrel (*Tamiasciurus hudsonicus*), and 1 weasel (*Mustela frenata altifrontalis*). All of these species are considered common in the area (Nagorsen, 2002). Intact carcasses were kept cool, shipped to the Zoonotic Parasitology Research Unit (ZPRU), at the University of Saskatchewan in Saskatoon, Saskatchewan, Canada, and frozen at - 20 °C. Carcasses were thawed and abdominal contents were visually inspected for the presence of alveolar hydatid cysts of *E. multilocularis* by trained personnel.

In winter 2011-2012, wild canids (27 coyotes, *Canis latrans*, and 6 red fox, *Vulpes vulpes*) were trapped within 80 km of Quesnel, BC, Canada (52°58'42" N; 122°29'35" W), as part of a legal fur harvest by BC licensed trappers (Figure 5-1). The intestinal tracts were removed intact and frozen at -20 °C until transport to the ZPRU. The samples were frozen at -80 °C for a minimum of seven days to inactivate any eggs of *E. multilocularis* before processing (Eckert et al., 2002). Adult cestodes were recovered from the intestines using a modified scraping and counting technique (Eckert et al., 2002; Eckert, 2003, Gesy et al. unpub. data). Adult cestodes were lysed using previously described techniques (Catalano et al., 2012). Three representative adult cestodes from each of the seven coyotes (n = 21) that harboured fully intact cestodes were prepared for molecular characterization.

5.2.1 Molecular Characterization

Adult cestode lysate was used as template in a simplex PCR amplification of a 395 bp region of the mitochondrial gene encoding NADH dehydrogenase subunit 1 (*nad1*) using the *E. multilocularis* specific primers Cest1 and Cest2 (Trachsel et al., 2007). PCR was performed with 23 µL using: 1X PCR Buffer (20 mM Tris-HCl (pH 8.4), 50 mM KCl), 200 µM dNTP, 4 mM $MgCl_2$, 200 µM forward and reverse primer (10 mM), 1.5 U Taq (AccuStartTM, Quanta Biosciences), dH_2O and 2 µL adult lysate for a total of 25 µL (Catalano et al., 2012). PCR was performed in duplicate using neat and dilute lysate (diluted 1:20 in sterile water) using previously described primers and thermocycling parameters (Trachsel et al., 2007). Samples displaying the expected 395 bp amplicon were further characterized at 3 mitochondrial loci (NADH dehydrogenase subunit 2 (*nad2*), cytochrome b (*cob*) and cytochrome oxidase subunit 1 (*cox1*)) as previously described using *E. multilocularis* specific primers and a PCR reaction mix of 25 µL (Nakao et al., 2009). All amplicons were purified and sequenced using the amplification primers.

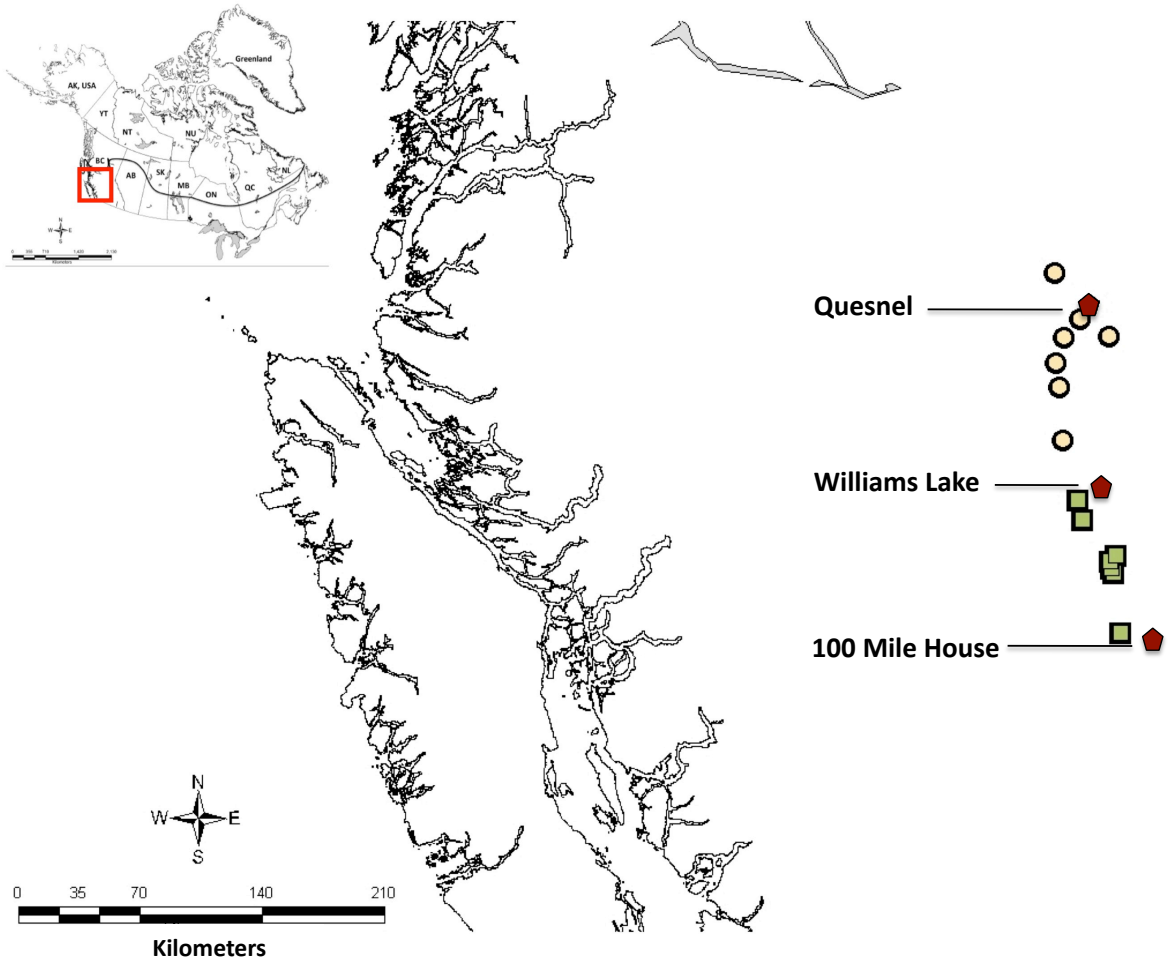


Figure 5-1 Sites of specimen acquisition in BC. Quesnel, Williams Lake and 100 Mile House are represented by burgundy polygons, carnivores by yellow circles and small mammals by green squares.

5.3 Results

Adults of *Echinococcus* spp. (based on morphology) were found in 10 of 27 (37%) coyotes and in 1 of 6 (17%) red fox, for an overall prevalence of 33% in 33 carnivores. The mean number of *Echinococcus* adult cestodes in the two 10% aliquots was 488 (range 2-4,145 cestodes per animal), extrapolating to a total mean intensity of 4,880 per coyote (range 20-41,450). No alveolar hydatid cysts were found in any of the 156 rodents and small mammals surveyed from south of Quesnel, BC.

Four *E. multilocularis* positive samples were detected by PCR amplification of neat worm lysates not detected in the 1:20 dilute samples. One positive was detected in the 1:20 dilute PCR that was not recognized by neat amplification. This highlights the benefit of PCR

amplification using dual lysate concentrations. Tissue lysis was successful for 19 of the 21 cestodes studied. Sequence of a 395 bp region (nt 158 – 553) of the *nad1* gene of 19 adult cestodes from 7 coyotes was 99%–100% identical to the partial *E. multilocularis* sequence representing the European M1 genotype (Genbank Accession AJ237639). Complete sequences for all 4 loci (*nad1, nad2*, *cob*, and *cox1)* were available from 6 cestodes from 4 coyotes, and were 100% identical to the sequence from the BC dog (accession nos. JF751034, JF751033, JF751035, and JF751036) (Jenkins et al., 2012). Sequence analysis also confirmed the presence of an additional nucleotide difference at position 663 of the *nad2* gene unique to the BC haplotype (Jenkins et al., 2012). In a mitochondrial haplotype network of *E. multilocularis* based on published sequences for *nad2*, *cob*, and *cox1*, the sequences from the coyotes in BC (and the original sequence from the dog) grouped with the European haplotypes (Figure 6-3).

5.4 Discussion

Our results confirm the presence of a European strain (haplotype) of *E. multilocularis* in wildlife in a forested region of North America where it was not previously undetected. The haplotype is identical to that previously identified in the domestic dog in Quesnel, BC (Jenkins et al., 2012), and suggests local tranmission of this parasite. Although this strain was only identified in carnivore definitive hosts in the immediate area, and not in rodent intermediate hosts 120-210 km to the south, distribution is not necessarily limited to the area surrounding Quesnel. It is not uncommon for rodent intermediate hosts to have a prevalence of infection of 0-10%, even in regions where 100% of carnivores are infected (Fay & Williamson, 1962).

Coyotes may be the primary host for *E. multilocularis* in North America, as compared to fox species elsewhere in the circumpolar North (Samuel et al., 1978). In this study 33% of carnivores studied were *Echinococcus* positive with a mean intensity of more than 4800 adult cestodes. The prevalence in coyotes was 37% (10/27) while only one of six red fox (17%) was infected. A recent study in Alberta, Canada found a prevalence of 25% in coyotes (Catalano et al., 2012). In addition, the mean intensity of infection in the current study (with an extrapolated intensity of 4800 adult cestodes) was higher than that reported in the recent Alberta study (1400 adult cestodes) (Catalano et al., 2012). The infection rate in the United States reflects a difference in host species infection rate with a recent study citing 29% (2/7) prevalence in coyotes (Storandt & Kazacos, 2012).

While resident coyotes may only have a mean annual home range of approximately 11 km^2, transient coyotes can range more than 100 km^2 and disperse up to 300 km from their natal den sites (Gese et al., 1988; Harrison, 1992). Given the extensive range of transient coyotes and the limited scope of the current study, there may well be a risk for human infection outside the immediate surroundings of Quesnel. This is particularly alarming in light of growing evidence to support the establishment of infected coyote populations in urban areas in western Canada (Catalano et al., 2012; Liccioli et al., 2012). This may increase the risk of alveolar hydatid disease for humans and dogs that ingest infective eggs in contaminated environments. The presence of wildlife definitive hosts shedding eggs in urban areas will increase the prevalence of infection in rodents. These bridging hosts serve to increase the risk of human infection when consumed by domestic dogs and cats with access to the outdoors.

The haplotype present in wildlife in BC is distinct from North American strains of *E. multilocularis* established in Indiana, South Dakota, and St. Lawrence Island, Alaska (Nakao et al., 2009), as well as from haplotypes present in rodents and coyotes in Saskatchewan (unpublished data). Sequence results indicate a close relation to the haplotypes present in the core endemic region for this parasite in Europe (Austria, Belgium, France, and Germany) (Nakao et al., 2009; Jenkins et al., 2012). It is not known how long this haplotype has been established in BC, although a complex history of red fox introductions as well as lack of requirement for anthelmintic treatment of imported domestic dogs offer appealing hypotheses for its recent introduction. In the last century, red fox of European origin (United Kingdom, France, and Scandinavia) were introduced in the Pacific coastal states and in the eastern United States, from which they subsequently moved north and west across North America (Kamler & Ballard, 2002). As well, foxes of unknown origin may have escaped from fur farms throughout southern BC (McTaggert Cowan & Guiguet, 1965). To date, the BC haplotype of *E. multilocularis* has not been detected east of the Rocky Mountains (Catalano et al., 2012); however further research is needed to determine the full extent of the distribution, diversity, prevalence, and public health significance of this pathogenic parasite in the rest of North America.

6. GEOGRAPHIC DISTRIBUTION AND GENETIC CHARACTERIZATION OF *ECHINOCOCCUS MULTILOCULARIS* IN CANADA

6.1 Introduction

Previous investigation into the genetics of *Echinococcus multilocularis* involved short mitochondrial (mt) gene sequences. Short sequences included 366 nucleotide (nt) sites from the mitochondrial (mt) gene cytochrome c oxidase subunit 1 (*cox1*) and 471 nt sites from the NADH dehydrogenase subunit 1 (*nad1*) (Nakao et al., 2009). From this, a total of four single polymorphisms, or mutations, were found, placing all specimens into one of two geographic genotypes, M1 (Europe) or M2 (China, Alaska and North America) (Okamoto et al., 1995). The classification of strains into the two genotypes was subsequently reinforced by the amplification of the nuclear DNA genes 18S rRNA and homeobox (Haag et al., 1997; Rinder et al., 1997). The findings, associated with amplification of the two partial mtDNA genes (*cox1* and *nad1*) and the nuclear genes (18S rRNA and homeobox), implied that *E. multilocularis* was primarily uniform across its Holarctic distribution. More recently; however, the amplification of larger gene sequences has resulted in division of the species into 18 distinct haplotypes (Nakao et al. 2009).

The 18 *E. multilocularis* haplotypes currently recognized are grouped into four clades representing Europe, Asia, Inner Mongolia, and North America (Nakao et al., 2009). In Europe, there are five recognized variations with three occuring in France, one in Austria (E1) and one in Slovakia (E5). Six of the Asian haplotypes are found in China (A5 – A10), two in Japan (A3 – A4) and two in Kazakstan (A1 – A2). An additional strain, O1, found in Inner Mongolia, appears to be quite unique in this haplotyping scheme. Two Asian haplotypes, A2 and A4 associated with Kazakstan and Japan, respectively, have been described in western Alaska, along with the Arctic North American haplotype (N1). Finally, a second North American haplotype (N2) was identified in central North America. The division of specimens into distinct clades reflects the clonal nature of larval and adult reproduction through division and self-fertilization, causing distinct geographic variation (Nakao et al., 2009).

Although mitochondrial haplotying holds great promise for understandind the biogeography of *E. multilocularis*, the number of sequences available and sampling effort, especially for North America, are quite limited. The Arctic North American haplotype, N1, was recovered from 11 voles (*Microtus* spp.) from St Lawrence Island, Alaska and the central North

American haplotype N2 strain from one red fox (*Vulpes vulpes*) in Indiana and one laboratory strain in South Dakota (Catalano et al., 2012). Mitochondrial haplotypes of Canadian or US samples between these two locations have not been described, with the exception of a European haplotype recovered from a dog in central BC (Jenkins et al., 2012). Therefore, more sequences of *E. multilocularis* from naturally infected wildlife hosts are needed to address a potential underestimation of genetic variation of this important parasite in North America, especially when examining only short nucleotide sequences (Nakao et al., 2009)

In an effort to compare Canadian samples to published sequences, this research combines short and long sequence amplification and follows the approach of comparing nucleotide sequences associated with the mtDNA genes NADH dehydrogenase subunits 1 and 2 (*nad1* and *2*), cytochrome b (*cob*) and *cox1* for haplotype determination. Nucleotide sequence lengths vary and include 395 sites from *nad1*, 882 sites from *nad2*, 1068 from *cob* and 1608 sites from *cox1*using published primer sets (Trachsel et al., 2007; Nakao et al., 2009). Use of published primers did not produce the expected amplicons for the mitochondrial genes, *nad2*, *cob* and *cox1*, when used on the Saskatchewan deer mice. In response, primers were developed for *nad2*, *cob* and *cox1* amplification from Saskatchewan deer mice in lengths of 623, 693 and 899 nucleotides, respectively. I sought to better characterize the geographic distribution and genetic differences of *E. multilocularis* in Canada, by surveying naturally infected wildlife definitive and intermediate hosts and generating sequence data at four mitochondrial genes, in order to explore hypotheseses about the origins (further discussed in section seven), distribution and significance of this important wildlife-reservoired parasite of animal and public health significance.

6.2 Materials and Methods

Adult cestodes were recovered from carnivore definitive hosts including 19 coyotes (*Canis latrans*) from British Columbia (BC), Alberta (AB) and Sasktchewan (SK) and 1 red fox (*Vulpes vulpes*) from British Columbia. The animals were trapped as part of a fur harvest (BC) or pest control (AB) or submitted to the Canadian Cooperative Wildlife Health Centre for routine diagnostic pathology (SK) (Catalano et al., 2012; Gesy et al., in prep) (Table 6-1). Safety precautions, as outlined in section 3.2 were followed for each sample. Cestodes were recovered from intestinal tracts of carnivores from Saskatchewan and British Columbia using the scraping filtration and counting technique (SFCT) developed in-house (Gesy et al., in prep) (section 3.2). Adult cestodes were kept in 95-100% ethanol (EtOH) until ready for DNA extraction. Adult

cestodes from 5 coyotes collected in Alberta, Canada were fixed in 100% ethanol and shipped to the Zoonotic Parasite Research Unit (ZPRU) at the University of Saskatchewan.

A total of 965 rodents including 7 species (Table 6-1) were examined for the presence of larval cestodes under level 2 bio-safety conditions. The 783 SK deer mice, courtesy the Royal Saskatchewan Museum in Regina, SK, were captured in 2009 and 2010 as part of a small mammal diversity study. Rodents from BC and NU were captured in summer, 2010 and 2011 (permit WL 10-65638 and N2008N0028) for this project. Fluid drawn from suspicious lesions was examined under the microscope for the presence of protoscolices (Figure 1-7). The remaining tissue (macerated in 100% EtOH) and fluid were placed microcentrifuge tubes and frozen at -20 °C until DNA extraction.

Arctic fox fecal samples (n = 404) were examined for the presence of taeniid eggs using a modified fecal flotation technique on 1g samples following freezing at -80°C to inactivate the infectious eggs (Salb et al., 2008). Of the 404 Arctic fox fecal samples, 278 were collected in 2000-2003 as part of dietary survey at Karrak Lake, NU, by Environment Canada and held frozen at -20°C since that time. The additional samples from Karrak Lake and Bylot Island, NU, collected in 2010 and 2011 for this study, were frozen at -20°C prrior to shipment to ZPRU. Taeniid eggs seen on microscope cover slips, were quantified then washed with distilled H_2O into glass petri dishes to remove any residual sugar solution. The eggs were placed in a 1.5ml Eppendorf tubes using a Pasteur pipet and washed 3 times in dH_2O to remove any residual sugar solution (Sheather's solution used in flotation). Samples were subsequently frozen at -20 °C until DNA extraction.

Table 6-1 Specimens used for the geographic and genetic survey of *Echinococcus multilocularis* in Canada.

Samples	Location	Collection Year	Number Tested	Positive	Cestodes analyzed [a]
Definitive hosts - intestinal tracts (positive denotes adult Echinococcus cestodes detected)					
Coyote (Canis latrans)	Quesnel, BC	2011-2012 (Gesy et al., in prep)	27	10	21
	Edmonton, AB	2009-10 (Catalano et al., 2012)	5	5	5
	Unknown, SK	2010	4	3	3
	Saskatoon, SK	2012	1	1	3
Red fox (*Vulpes vulpes*)	Quesnel, BC	2011-2012 (Gesy et al., in prep)	6	1	0
Sub-Total			43	20	32
Definitive hosts - feces (positive denotes taeniid-type eggs detected)					
Samples	**Location**	**Collection Year**	**Number**	**Positive**	***Echinococcus multilocularis* identified**
Arctic fox (*Vulpes lagopus*)	Karrak Lake, NU	2000-2003	278	3	3
	Karrak Lake, NU	2011	76	14	0
	Bylot Island, NU	2010	50	11	0
Sub-Total			404	28	3
Intermediate hosts - (positive denotes detection of alveolar hydatid cysts and protoscolices)					
Samples	**Location**	**Collection Year**	**Number**	**Positive**	***Echinococcus multilocularis* identified**
Brown lemming (*Lemmus trimucronatus*)	Karrak Lake, NU	2011	37	0	0
Collared lemming (*Dicrostonyx groenlandicus*)	Karrak Lake, NU	2011	72	0	0
Deer Mice (*Peromyscus maniculatus*)	100 Mile House, BC	2010 (Gesy et al., in prep)	72	0	0

	Southwest, SK	**2009/2010**	**700**	**39**	**39**
Meadow voles (*Microtus pennsylvanicus*)	**100 Mile House, BC**	**2010 (Gesy et al., in prep)**	**59**	**0**	**0**
North American jumping mice (*Zapus hudsonius*)	**100 Mile House, BC**	**2010 (Gesy et al., in prep)**	**16**	**0**	**0**
Red Squirrel (*Tamiasciurus hudsonicus*)	**100 Mile House, BC**	**2010 (Gesy et al., in prep)**	**1**	**0**	**0**
Shrews (*Sorex* spp.)	**100 Mile House, BC**	**2010 (Gesy et al., in prep)**	**7**	**0**	**0**
Tundra red-backed voles (*Clethrionomys rutilus*)	**Karrak Lake, NU**	**2011**	**8**	**0**	**0**
Weasel (*Mustela frenata altifrontalis*)	**100 Mile House, BC**	**2010 (Gesy et al., in prep)**	**1**	**0**	**0**
Sub-Total			**973**	**39**	**39**
Total			**1423**	**87**	**74**

a. Adults: Amplification was attempted on 3 intact cestodes from each host. 21 cestodes were analyzed from 7/10 coyotes from BC and 3 cestodes from 1/3 positive SK coyotes (unknown location). Only 5 individual cestodes were sent from AB. Note: not all samples were used in sequence analysis.

6.2.1 DNA Extraction

DNA was extracted from three representative intact adult cestodes from each carnivore host. Fully intact worms were soaked in *Echinococcus* lysis buffer to rehydrate and rid worms of excess ethanol (Catalano et al., 2012). After 10 minutes, each cestode was placed in an individual 0.2 ml PCR tube with 50µl lysis buffer and 1µl Proteinase K (Qiagen, Toronto, ON, Canada), and placed in a thermocycler for 98 minutes at 60°C to lyse the cestodes, then 94°C for 15 minutes to denature the Proteinase K (Catalano et al., 2012). DNA was not extracted from six BC and SK coyote samples and one red fox sample, as they did not contain intact adult cestodes. The five coyote samples from AB were only amplified at the *nad1* locus due to lysate degradation.

DNA was extracted from samples of 50 eggs from a single fecal sample from a carnivore host using the FastDNA kit (MP Biomedicals, Solon, Ohio, USA), as previously described (Da Silva et al., 1999) and substituting binding matrix A for binding matrix F (Kirk, 2012). DNA was extracted from hydatid fluid/tissue from hydatid fluid/tissue from deer mice using the DNeasy Blood and Tissue Kit® (Qiagen, Toronto, ON, Canada), as per manufacturers instructions, with an additional wash with buffer AW2, included in the kit. The additional wash improved PCR amplification results.

6.2.2 Gene Amplification/PCR

For adult cestodes, extracted DNA was amplified using *Echinococcus multilocularis*-specific primers for a 395 bp region of the *nad1* mitochondrial gene (Table 6-2) (Trachsel et al., 2007). For eggs, as mixed infections were possible, extracted DNA was amplified using a mixture of primers in a multiplex PCR for genus and species level identification (Trachsel et al., 2007). The resulting PCR products from egg samples were cloned to increase DNA yield and purify the results using pGEM®-T Easy vector (Promega Corporation, Madison, WI, USA). Samples with the expected product size of 395 bp were sequenced and underwent further analysis using published primers for three mitochondrial genes, *cob, cox1,* and *nad2* ((Nakao et al., 2009); Table 6-2). Published primers for *cob, nad2* and *cox1* did not produce PCR products when used with Saskatchewan rodent samples, likely due to mutations in the annealing regions. Therefore, modified primers, specifically designed for the N2 haplotype, were used (Table 6-2). That published primers would not work for amplification suggests that mutations have occurred in the primer annealing regions for these haplotypes. PCR products were purified using the QIAquick PCR Purification Kit® (Qiagen, Toronto, ON, Canada), and then sequenced using PCR primers.

The resulting sequences were checked for sequence accuracy by protein translation (eBIOX: www.ebioinformatics.org/**ebiox**/) prior to haplotype determination. Subsequent sequences were aligned using the sequence alignment software, Clustal W (www.ebi.ac.uk/Tools/msa/**clustalw**2/), shortened to reflect differential loci and transformed from the 'aln' format into a 'nexus' file format using Alignment Transformation EnviRonment (ALTER: http://sing.ei.uvigo.es/ALTER/). The nexus formatted single nucleotide polymorphisms (SNPS) were subsequently transferred into the Java computer program based

TCS cladogram estimation program (TCS v1.21) to produce each haplotype network. Haplotype analyses were performed three times; once using only *nad1* sequences (Trachsel et al., 2007); once using truncated sequences of *cob*, *nad2* and *cox1* resulting from the use of developed primers; once using elongated sequences of *cob*, *nad2* and *cox1* resulting from the use of published primer sets (Nakao et al., 2009). Analysis of the *cob*, *nad2* and *cox1* genes was performed in tandem, rather than individually, for short and long sequences, as aggregate analysis produced clearer definition between the haplotypes.

Table 6-2 Primers used for the amplification of mitochondrial genes of E. multilocularis including published primer sets from Nakao et al., 2009[b] and Trachsel et al., 2007, used for simplex PCR[d] of adult cestodes and alveolar hydatid cyst fluid and multiplex PCR[c] on egg samples where mixed infections were possible. In-house developed primers[a] were used for the amplification of SK alveolar hydatid fluid samples.

Gene	Primer Set[1]	Amplification Protocol	Amplicon Size	Reference
NADH dehydrogenase subunit 2 (*nad2*)[a]	F: GGGTTTTTTTGGAGTTGTG R: AAGGCATAGAYACAGGAGTCA	95°C for 3 min; (94°C for 30 sec, 54°C for 30 sec, 72°C for 45 sec) X 40; 72°C for 5 min	623 bp (nt 104 - 727)	In-house development
Cytochrome B (*cob*)[a]	F: TGCGTTATTGGCATATGGTAG R: GTGCCACCCTCAGTTGGTACT		693 bp (nt 209 - 902)	
Cytochrome C oxidase subunit 1 (*cox1*)[a]	F: TGGGTGCTGGGTGTTGGTTGG R: TACACACACGACGMGGAAC	94°C for 3 min; (94°C for 30 sec, 55°C for 30 sec, 72°C for 1 min) X 40; 72°C for 5 min	966 bp (nt 362 - 1327)	
NADH dehydrogenase subunit 2 (*nad2*)[b]	F: GCGTTGATTCATTGATACATTGT R: TAGTAAAGCTCAAACCGAGTTCT	94°C for 3min; (94°C for 30 sec, 55°C for 30 sec, 72°C: 45 sec *nad2*/*cob* and 1min for *cox1*) X35, 72°C for 5 min	882 bp	Nakao et al., 2009
Cytochrome B (*cob*)[b]	F: GTTTAAACTGGTAGATTGTGGTTC R: CTCCACAGTAGAAATCACCATCA		1068 bp	
Cytochrome C oxidase subunit 1 (*cox1*)[b]	F: GACTTTCTCTTTGGTTGGTGTAAG R: AACCTAAACAACCAACTTCACAG		1608 bp	
Echinococcus multilocularis nad1[c,d]	F: TGCTGATTTGTTAAAGTTAGTGATC R: CATAAATCAATGGAAACAACAACAAG	94°C for 15 min; (94°C for 30 sec, 58°C for 90 sec, 72°C for 10 sec) X40, 72°C for 5 min	395 bp (nt 155 - 550)	Trachsel et al., 2007
*E. granulosus rrn*S[c]	F: GTTTTTGTGTGTTACATTAATAAGGGTG R: GCGGTGTGTACMTGAGCTAAAC		117 bp	
Taenia spp. *rrn*S[c]	F: YGAYTCTTTTTAGGGGAAGGTGTG R: GCGGTGTGTACMTGAGCTAAAC		267 bp	
[1] F: Forward, R: Reverse				

6.3 Results

As a result of protein translation verification only 28 study samples were used in the haplotype analysis of *nad1*. Study samples were grouped into eight haplotype categories (A – H).

Samples included 11 coyotes from BC and SK, 3 Arctic fox samples, and 14 deer mice from SK. Only 11 SK deer mice passed the protein accuracy screening at all three additional loci (*nad2*, *cob* and *cox1*), and subsequently, were used in haplotype analysis. Adult cestodes included in the analysis of the additional mtDNA loci include three cestodes from one SK coyote and 19 cestodes from seven BC coyotes. In total, 32 samples were used in haplotype analysis.

6.3.1 Nad1

Analysis of 28 sequences from this study and 9 published *nad1* sequences resulted in the identification of 10 unique haplotypes based on mutations found at 9 different loci, or particular location in the gene (Figure 6-1, Table 6-3). Mixed haplotypes were found in two BC coyotes (haplotypes A, B and E).

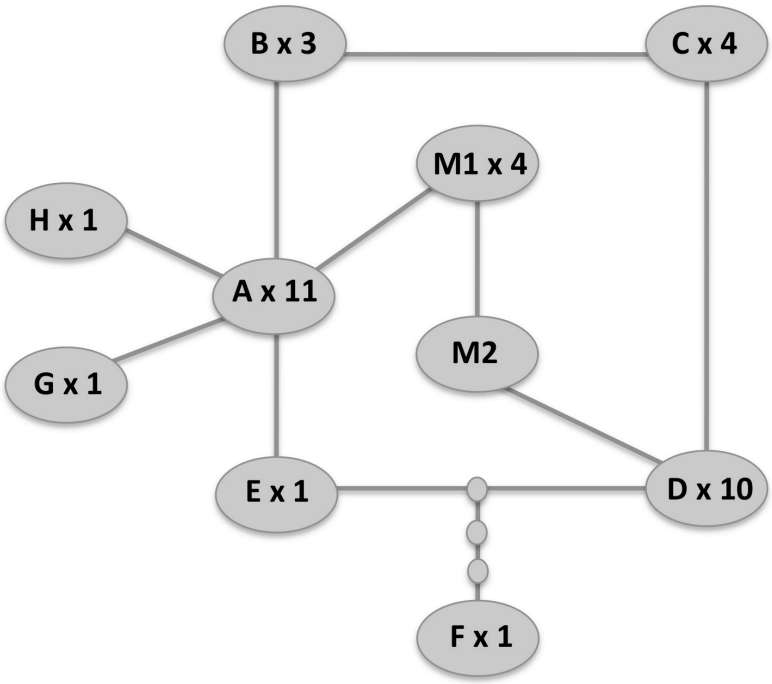


Figure 6-1 Mitochondrial haplotype network of *Echinococcus multilocularis* based on statistical parsimony of the *nad1* gene demonstrating 8 new North American haplotypes in addition to the European (M1) and Asian/North American (M2) genotypes. Large circles denote haplotypes found in this study and small circles denote hypothetical haplotypes. Letters denote haplotype names while numbers indicate the number of sequences of that haplotype in the current study (Table 6-3).

Table 6-3 Chart showing the haplotype designations for 28 Canadian *Echinococcus multilocularis* samples and 9 published *nad1* sequences. Divisions are based on differences found at 9 nucleotide sites. Breakdown by sample, accession number and province is also shown. Table corresponds to Figure 6-1.

Nucleotide Position													
Haplotype	n [a] =	170	233	252	367	461	462	464	465	470	Sample	n [b] =	Sample ID / GenBank Accession No.
A [c]	11	A	T	T	C	A	A	T	T	T	BC coyote	6	84A, 94B, 97A, 102A, 107A, 107B
											BC dog	1	JF751034
											Poland (human)	2	JX266825 and JX266826
											China	1	AY389984
											Nunavut Arctic fox	1	CWS 1-90
B [c]	3	T	T	T	C	A	A	T	T	T	BC coyote	3	94C, 94A, 102C
C [c]	4	T	T	T	T	A	A	T	T	T	SK deer mouse	4	20, 679, 1014, 1019
D [c]	10	A	T	T	T	A	A	T	T	T	SK deer mouse	10	G13, F23, 17-12, 77, 281, 613, 899, 1005, 1006 and 1085
E [c]	1	A	T	T	C	A	A	G	T	T	BC coyote	1	103A
F [c]	1	A	T	T	T	G	C	G	A	T	South SK coyote	1	SK 9
G [c]	1	A	T	T	C	A	A	T	T	C	Nunavut fox	1	CWS 45
H [c]	1	A	T	A	C	A	A	T	T	T	Nunavut fox	1	CWS 39
M1	4	A	C	T	C	A	A	T	T	T	M1 genotype	1	AJ237639
											China (larvae)	1	EU704122
											Complete Genome	1	AB018440
											Iran (red fox)	1	AB621793
M2	1	A	C	T	T	A	A	T	T	T	M2 genotype	1	AJ237640

[a] Number of identical sequences identified as haplotype

[b] Number from each region and host/GenBank sequence

[c] Designation of new haplotype

6.3.2 *Nad2*, *Cob* and *Cox1*

When truncated sequences (due to the modified primers) were used in the haplotype analysis, four Saskatchewan deer mouse isolates grouped with the North American N2 strain as expected. The remaining seven deer mouse samples resulted in six new haplotypes, N2 through N8 (Figure 6-2). Each new North American haplotype contained a single sample, with the exception of N3, which contained two samples.

Two sequences from coyotes in BC and SK grouped with European haplotypes. However, the use of shorter sequences resulted in and underestimation of differences between the existing Eurasian haplotypes and those found in the BC and SK coyotes.

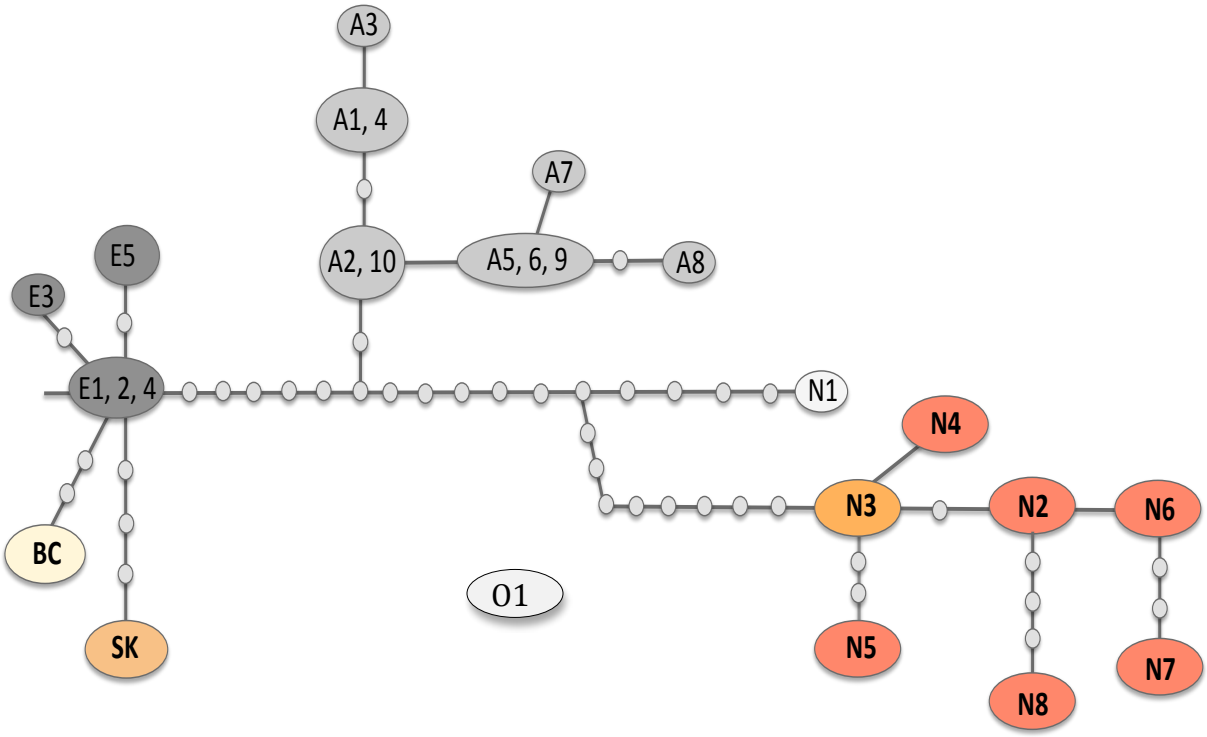


Figure 6-2 Haplotype network using truncated sequences resulting from the use of in-house primers based on statistical parsimony of the *cob, nad2* and *cox1* mitochondrial genes of *Echinococcus multilocularis*. See Figure 6-1 for symbol descriptions. BC represents uniform sequences from 19 individual cestodes of 7 coyotes. SK represents uniform sequences from 3 individual cestodes from 1 coyote. N3 to N8 represent new haplotypes from 7 SK deer mice. The remaining 4 mouse samples were identical to the N2 published sequence (Nakao et al., 2009). Figure based on (Nakao et al., 2009).

By eliminating the SK mice from haplotype analysis, and using larger gene fragments a more detailed picture of the genetic makeup of the European strains (SK and BC) found in Canada (Figure 6-3). Haplotype 'BC' is the same as that found previously in a dog in a newly endemic region of BC in 2009 (Jenkins et al., 2012) and is most closely related to the European E4 haplotype. The haplotype found in the coyote from Saskatoon (SK) contains six nucleotide mutations that separate it from its closest relation, also E4 (Figure 6-3).

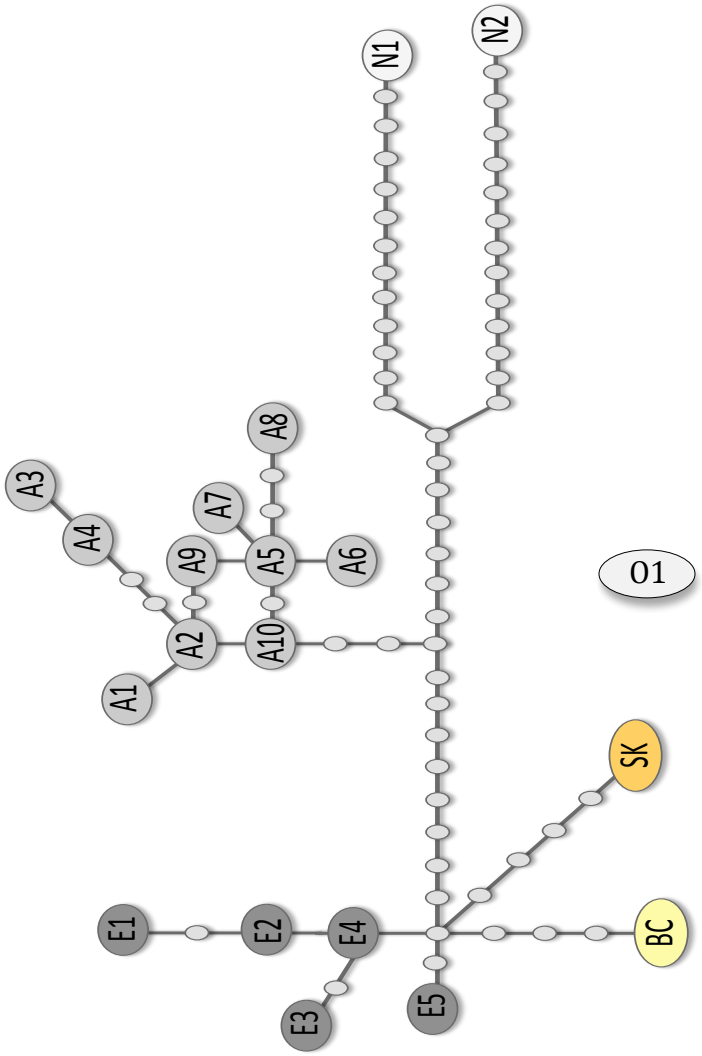


Figure 6-3 Haplotype network based on statistical parsimony using long sequences of the *cob*, *nad2* and *cox1* genes of *Echinococcus multilocularis*. See Figure 6-1 for symbol descriptions. BC and SK represent the uniform sequences from individual worms from those coyotes, respectively. Figure based on (Nakao et al., 2009).

Table 6-4 Nucleotide sequences used in the creation of haplotype networks for *Echinococcus multilocularis* isolates found in Canadian samples. Short sequences were used for Figure 6-2 and long sequences were used for Figure 6-3. Nucleotide positions for each mutational change are shown along the top for each respective gene.

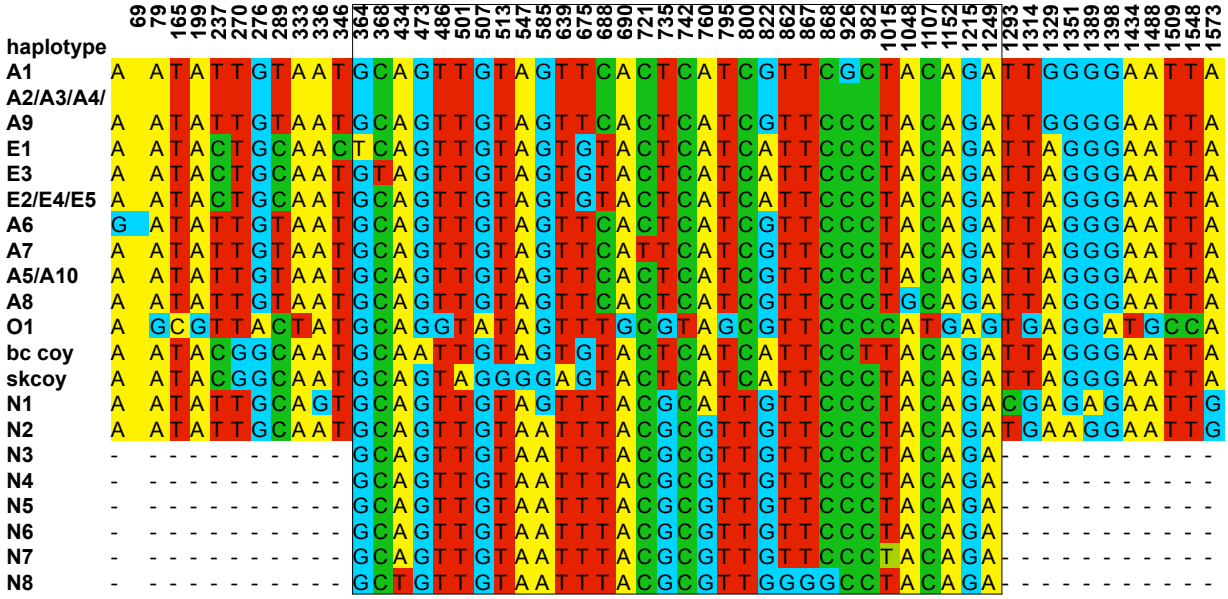
COB

haplotype	72	81	133	138	141	165	241	306	332	369	380	408	438	451	453	481	486	543	606	610	663	666	688	729	733	756	760	804	812	825	843	853	960	984	990	1006	1038	1062
O1	C	A	C	C	A	T	T	C	A	T	A	C	G	A	T	G	G	A	A	C	A	T	T	A	A	T	C	T	C	C	T	C	A	T	A	A	G	A
E3	T	G	T	T	A	C	C	T	A	C	A	C	G	G	C	T	G	A	A	A	A	C	C	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
E1/E2/E4	T	G	T	T	A	C	C	T	A	C	A	C	G	G	C	G	G	A	A	A	A	C	C	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
E5	T	A	T	T	A	C	C	T	A	C	A	C	G	G	C	G	G	A	G	A	A	C	C	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
A1/A2/A4/ A5/A6/A7/ A9/A10	T	A	T	T	A	T	C	T	A	T	A	C	G	G	C	G	G	A	A	A	A	C	T	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
A8	T	A	T	T	A	T	T	T	A	T	A	C	G	G	C	G	G	A	A	A	A	C	T	A	A	T	C	C	C	T	G	T	G	C	A	G	G	A
A3	T	A	T	T	A	T	C	T	A	T	G	C	G	G	C	G	G	A	A	A	A	C	T	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
BC	T	A	T	T	A	C	C	T	A	C	A	C	G	G	C	G	G	A	A	A	G	C	C	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
SK	T	A	T	T	A	C	C	T	A	C	A	C	G	G	C	G	G	A	A	A	A	C	C	A	A	T	C	C	C	T	G	T	G	T	A	G	G	A
N1	C	A	T	T	G	T	C	T	G	T	A	T	A	G	C	G	G	G	A	A	A	C	T	A	A	C	C	C	C	T	G	T	A	T	A	G	G	G
N2	C	A	T	T	A	T	C	T	A	T	A	T	G	G	T	G	T	A	A	A	A	C	T	G	G	T	T	C	C	T	G	T	A	T	G	G	A	A
N3	C	A	T	T	A	T	C	T	A	T	A	T	G	G	T	G	G	A	A	A	A	C	T	G	G	C	T	C	C	T	G	T	A	T	G	G	A	A
N4	-	-	-	-	-	-	C	T	A	T	A	T	G	G	T	G	G	A	A	A	A	C	T	G	G	C	T	C	T	T	G	T	-	-	-	-	-	-
N5	-	-	-	-	-	-	C	T	A	T	A	T	G	G	T	G	G	A	A	A	A	C	T	G	G	C	T	C	C	T	G	T	-	-	-	-	-	-
N6	-	-	-	-	-	-	C	T	A	T	A	T	G	G	T	G	T	A	A	A	A	C	T	G	G	T	T	C	C	T	G	T	-	-	-	-	-	-
N7	-	-	-	-	-	-	C	T	A	T	A	T	G	G	T	G	T	A	A	A	A	C	T	G	G	T	T	C	C	T	G	T	-	-	-	-	-	-
N8	-	-	-	-	-	-	C	T	A	T	A	T	G	G	T	G	T	A	A	A	A	C	T	G	G	T	T	C	C	T	G	T	-	-	-	-	-	-

NAD2

haplotype	16	42	72	123	157	183	198	234	237	240	246	252	282	338	348	393	423	471	486	507	566	624	631	633	649	656	675	679	690	696	716	717	732	786	793	796	827
A3/A4	A	G	G	T	G	T	C	A	C	T	A	A	G	T	C	G	T	T	A	A	A	T	A	T	G	C	C	T	T	T	T	A	A	G	G	A	T
A1/A2/A10	G	G	G	T	G	T	C	A	C	T	A	A	G	G	C	G	T	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
A5/A6/A7/ A8/A9	G	G	G	T	G	T	C	A	C	T	A	A	G	G	C	G	T	T	A	A	A	T	A	T	G	T	C	T	T	T	T	G	A	G	G	A	A
E5	G	T	G	C	G	T	C	A	C	T	A	T	G	G	C	G	C	T	G	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
E1/E2	G	T	A	C	G	T	C	A	C	T	A	T	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
E3/E4	G	T	G	C	G	T	C	A	C	T	A	T	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
O1	G	G	G	C	G	C	T	A	T	C	G	A	A	G	T	G	C	C	A	G	G	C	G	T	T	C	T	T	T	T	T	G	G	G	T	A	T
BC COY	G	T	G	C	G	T	C	A	C	T	A	T	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
SK COY	G	T	G	C	G	T	C	A	C	T	A	T	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	A	T
N1	G	G	G	C	G	T	C	A	C	T	G	A	G	G	C	A	A	T	A	A	A	T	A	G	G	C	C	T	T	T	T	G	A	A	G	A	T
N2	G	G	G	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	G	G	G	T
N3	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	-	-	-	-
N4	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	-	-	-	-
N5	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	A	A	T	A	-	-	-	-
N6	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	A	T	T	G	A	-	-	-	-
N7	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	A	A	T	A	T	A	-	-	-	-
N8	-	-	-	C	T	T	C	G	C	T	G	A	G	G	C	G	C	T	A	A	A	T	A	T	G	C	C	T	T	T	T	G	A	-	-	-	-

COX1



A map showing the distribution of the new haplotypes is displayed below (Figure 6-4). Based on *nad1*, haplotypes C, D and F were restricted to southern Saskatchewan, while G and H were found only in Nunavut. Haplotypes B and E were present in coyotes from British Columbia. Haplotype A occurs both in Nunavut Arctic fox and in the BC coyotes. The SK and BC haplotypes were not found outside of Saskatoon, SK, and Quesnel, BC, respectively. The seven haplotypes resulting from the aggregate (*nad2*, *cob,* and *cox1*) analysis of *E. multilocularis* represent 10 southern SK locations from 11 deer mice.

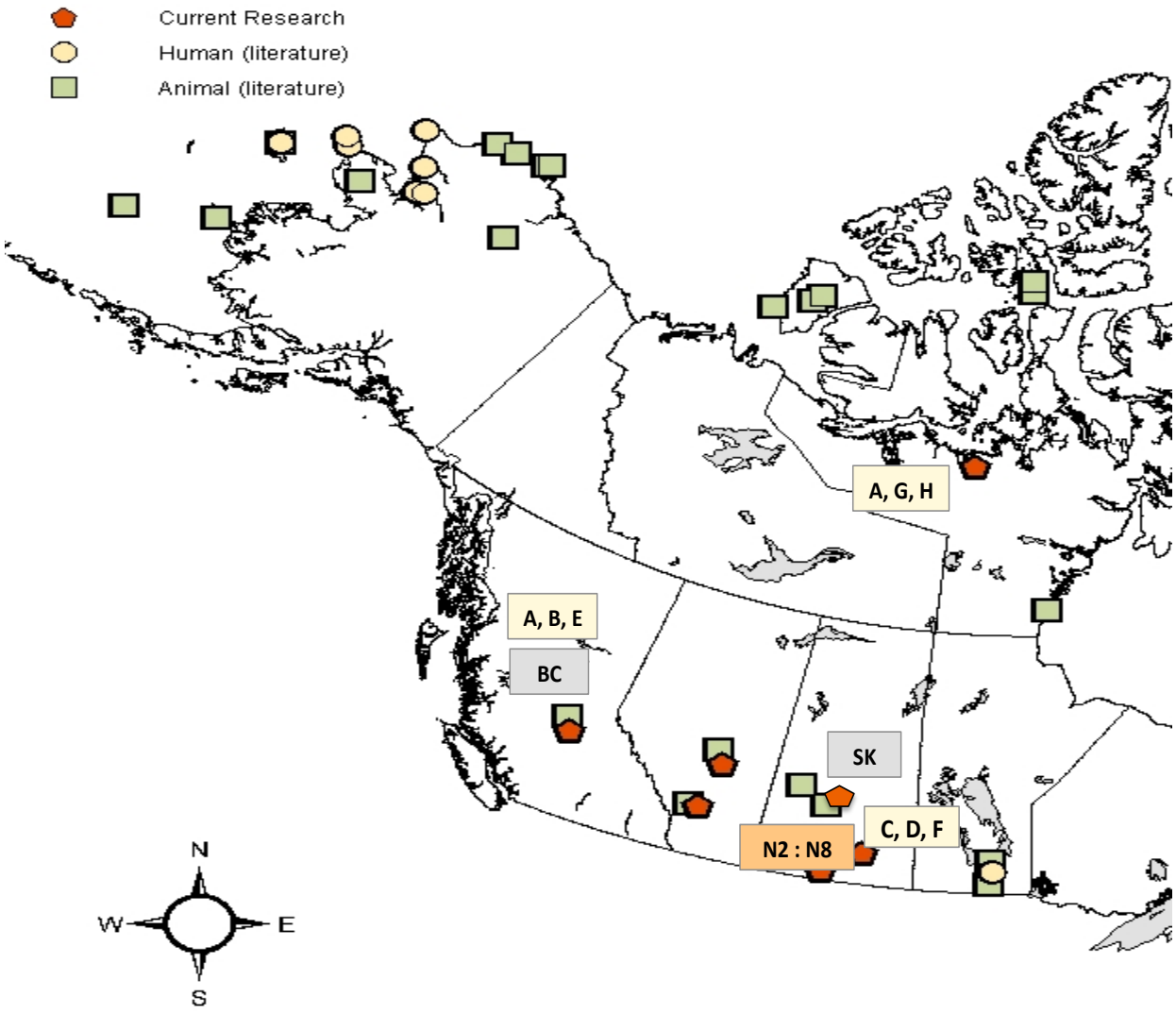


Figure 6-4 Locations of identified haplotypes of *Echinococcus multilocularis* in Canada. Yellow boxes represent haplotypes based on the *nad1* gene while grey boxes identify haplotypes based on the analysis of long sequences using 3 mitochondrial genes *nad2*, *cob* and *cox1*. Short sequence haplotypes, N2 to N8, are shown in orange. Haplotype 'C' represents isolates from coyotes and 'D and F' are from mice.

6.4 Discussion

Judging by the 8 new haplotypes that resulted from analysis of *nad1* it is clear that there is more diversity within this gene, and within *Echinococcus multilocularis,* than previously thought. This speaks to the limits of a small sample size when determining genetic diversity. Indeed, the original designation of only two genotypic groups, M1 (European) and M2 (Asia and North America), likely represented an underestimation of genetic diversity due to a limited

number and range of samples (Okamoto et al., 1995; Nakao et al., 2009). It is important to note that the differences between *E. multilocularis* haplotypes are often only single nucleotide mutations, as compared to much greater diversity observed within the closely related *E. granulosus* species complex. The biogeographical significance of this genetic variation may become clearer with increasing availability of *nad1* sequences in GenBank and comparison of samples from a broader geographic range.

We report unique zones of occurrence for all Canadian *nad1* haplotypes of *E. multilocularis* with the exception of haplotype 'A'. Haplotype 'A' was identified in 6 of 13 British Columbia wildlife samples and in one sample isolated from Arctic foxes in Nunavut. The occurrence of this haplotype in the two regions may mean that the European strain found in central BC is also found in Arctic Canada. However, caution must be taken when interpreting data based on a single mitochondrial locus. Molecular characterization at multiple mitochondrial and nuclear loci of *E. multilocularis* from across its broad range in the North American Arctic is needed to explore hypotheses about the origins and relationships of *E. multilocularis*, such as introduction of Asian strains with natural movements of Arctic fox across the Bering strait, anthropogenic introduction of European strains from the importation of European foxes for hunting purposes into North America (Rausch, 1985) and/or genetic exchange between strains of *E. multilocularis* established in Arctic and prairie regions of North America through southward incursions of naturally infected Arctic fox (Wrigley & Hatch, 1976).

We encountered many challenges in molecular characterization of taeniid eggs relative to alveolar hydatid cyst material and adult cestodes. Fecal samples had been stored for a decade under varying conditions and contained relatively few eggs. Previous studies called for the use of a large number of eggs for adequate sequencing of the *cox1* gene (Cabrera et al., 2002). Gene amplification of the three additional mitochondrial loci in eggs was attempted on several occasions but results were inadequate for sequence analysis.

This study sheds light on the possible origins of *E. multilocularis* in a newly endemic region of central BC. The strain(s) present in BC were distinct from those present in southern SK (related to the N2 haplotype present in South Dakota and Indiana), supporting the hypothesis that this was a relatively recent introduction and not a range expansion from the NCR. Partial sequence of the *nad1* gene from an alveolar hydatid cyst in a dog from Quesnel, BC, was identical to that present in 10 of 13 coyotes from within 80 km of Quesnel (denoted as haplotype

'A' in the current study), suggesting that the parasite was locally acquired. One additional coyote sample had a single mutation at nucleotide 464 and another sample had two changes at nucleotide 458 and 459. Changes in these single nucleotides suggest prolonged establishment in BC wildlife as mutations often take prolonged periods of time to develop. However, establishment and isolation probably did not occur as far back as the last ice age, as there would likely be more sequence divergence than currently present. This finding may support either the hypothesis of establishment in the last 200 years resulting from the translocation of red foxes from Europe for hunting purposes (Rausch, 1985) or the anthropogenic movement of companion animals.

Sequences from coyotes in BC and Saskatoon, SK, clustered with European haplotypes. There were six nucleotide differences between the Saskatoon sample and the next closest European haplotype (E4; Figure 6-3). The genetic distance between the SK coyote sample and the current haplotype scheme suggests that this strain has, like the BC parasites, been present in Canada for quite awhile. Given the high degree of genetic variability one can deduce that this finding supports the hypothesis of establishment prior to, or during the last ice age, and not as a result of fox relocation in the last century. If it were a recent introduction, one would expect a higher degree of similarity between the SK coyote and its European kin. At the very least, the SK coyote haplotype appears to have been present longer than the BC haplotype, as more mutational changes have occurred.

This study demonstrated that isolates of *E. multilocularis* in southern SK were sufficiently distinct from previously described European, Asian, and the N1 North American haplotypes, that we had to develop in-house primer sets for *nad2*, *cob* and *cox1*. These generated shorter sequences limited to a small genetic region containing very less variation; however, the analysis of 11 deer mice resulted in 7 North American haplotypes (N2 – N8), based on the aggregate analysis of 3 genes. These haplotypes represent samples from 10 locations in southern Saskatchewan with no clear distinct zones of occurrence.

Based on nad1 analysis, two haplotypes, 'C' and 'D', were distinguished by a nucleotide alteration at position 170, though the majority of samples grouped with haplotype 'C'. Both haplotypes were close to the recognized N2 strain established in South Dakota and Indiana (NCR), giving credence to the hypothesis of divergence from strain(s) present on St Lawrence Island, Alaska, sometime in the last 75,000 years and not as a result of recent introduction with

incursions of Arctic fox in the 1960s. The third *nad1* variation found in adult cestodes in a coyote from Saskatchewan is haplotype 'F'. This was distinct from the European-type haplotype in a coyote collected within the urban region of Saskatoon, and from the haplotypes present in deer mice in southern Saskatchewan. This suggests a complex mosaic of European type haplotypes possibly introduced thousands of years ago overlaid on a well-established prairie population of *E. multilocularis*. Further sampling is needed to substantiate this hypothesis.

In summary, this study served to identify some of the genetic diversity of *E. multilocularis* found in Canada. As hypothesized, the genetic diversity is greater than previously thought. However, a larger-scale survey of carnivores and rodents is needed to develop a better understanding of the distribution and diversity of this important parasite in Canada and in North America. An understanding of the basic distribution of the different haplotypes is paramount because of the presumed differences in pathogenicity. Only with a complete understanding of the diversity in this species can we begin to understand the impact those differences may have on human and animal health.

7. GENERAL DISCUSSION

One major outcome of this research is the creation of a new protocol for the extraction of adult cestodes of *Echinococcus* species from carnivore intestinal tracts (Chapter 3), originally conceived in order to decrease the associated processing time. This protocol was used in the extraction of adults from intestinal tracts from animals from British Columbia in order to determine if *E. multilocularis* had established in local wildlife (Chapter 5). The previously recommended gold standard for extraction (sedimentation and counting technique (SCT) was time consuming and laborious (Kamiya, 2008). The new scraping, filtration and counting technique (SFCT) not only significantly decreased processing time, but it also was a better predictor of negative samples (i.e. we could believe our negative findings when using the SFCT, which was particularly important for screening samples from BC, a potentially newly endemic geographic region). Although there was no significant difference in counts between the two methods, the SFCT improved method sensitivity, with SCT having 91% sensitivity relative to SFCT. Using SFCT in this project allowed recovery of cestodes from carnivores across a wide geographic range in Canada, necessary for determining genetic diversity of *E. multilocularis* (Chapter 6).

As part of efforts to obtain material for genetic characterization, this project involved sampling a large number of rodents from endemic regions of SK (Chapter 4). This in turn enabled determination of spatial, sex, and habitat patterns in prevalence of infection with *E. multilocularis* in the most important intermediate host in the NCR. Identification of areas of endemic, or newly established, foci for this parasite may be the first step towards mitigating human disease. In southern Saskatchewan the prevalence of 6% in 783 deer mice collected in 2009 and 2010, is comparable to that reported elsewhere in the NCR, and much higher than that reported in rodents in the mainland NTZ. The highest prevalence was in mice near Flintoft, SK, with a site prevalence of 26%. Flintoft can therefore be considered an area of focus in Saskatchewan. There was no difference found between male and female mice, both with an infection prevalence of 8%. Prevalence was slightly higher in native and hay field habitat types than in active croplands, possibly due to periodic disturbances such as tilling, of the latter.

Another significant outcome is the first concentrated effort to characterize the genetic diversity of *E. multilocularis* in Canada, accomplished through amplification and sequence analysis of mitochondrial genes *nad1, nad2, cob,* and *cox1*. It was expected that the results of the

nad1 analysis would indicate uniformity with the M2 genotype previously reported in North America (Okamoto et al., 1995; Nakao et al., 2009). However, analysis of 28 samples identified 8 new haplotypes, none of which exactly matched either M1 (European) or M2 (North American and Asian) sequences. This was unexpected as the *nad1* gene was thought to hold too little variation to be useful in distinguishing haplotypes linked to geographic distribution (Ohbayashi et al., 1971; Bowles & McManus, 1993). With the discovery of eight new *nad1*-based haplotypes, perhaps this gene is more diverse in Canada than elsewhere.

More likely, though, the small sample sizes and limited geographic range in previous studies were too narrow to detect more than the four distinct nucleotide polymorphisms previously identified (Okamoto et al., 1995). In Europe, the smaller land mass means human and animal populations are geographically closer than in Canada, providing more opportunity for parasite gene flow and multiple infections in host species, perhaps accounting for greater observed genetic homogeneity within European isolates of *E. multilocularis*. Increased geographic isolation and distance in Canada could account for an increase in the number of haplotypes. This idea is reinforced by the clear separation of most new haplotypes into distinct geographic zones. In southern Saskatchewan, for example, larval cysts of *E. multilocularis* in deer mice sampled were in haplotype groups, D and E (Figure 6-1), which were distinct from the M2 (North American) haplotype. Haplotype F, also different from the M2 haplotype, detected in a southern SK coyote was not found in the deer mice sampled. This suggests that this coyote of unknown origin is not from the same region as the sampled mice. It is probable that the coyote harbours a strain found in mice from the region it inhabited and was too far away from the rodents sampled in this study. This reflects the need for large-scale sampling when determining haplotype diversity.

In Arctic Canada, we detected three haplotypes (A, G and H) of *E. multilocularis* in 3 fecal samples collected from Arctic fox at Karrak Lake, NU. Only one isolate of *E. multilocularis* from the mainland North American Arctic has ever been characterized previously (Kirk, 2012); most "North American" Arctic samples originated from St Lawrence Island in the Bering Strait, and likely include Asian haplotypes (Nakao et al., 2009). Variants, G and H, were only found in *E. multilocularis* at Karrak Lake, NU (i.e. were not detected in any other areas sampled), but, without additional samples from the northern region, it is difficult to say with certainty that they are confined to this area. Since Arctic foxes travel long distances to fulfil

dietary requirements and to locate den sites, often resulting in an exchange of foxes across the circumpolar North (Samelius et al., 2004), it is possible that Alaskan and even Asian genotypes may be present in the Canadian Arctic.

In BC, haplotypes B and E appear to be limited to the area surrounding Quesnel, but, without further samples, this is conjecture. The variant identified as haplotype A is unique in this study as the same variations in the *nad1* gene were found in both BC and NU isolates. This could indicate that the occurrence in BC is the result of the natural movement of infected foxes crossing the frozen tundra to interact with southern carnivore populations. It could also be the result of early translocation of infected red foxes from Europe establishing in the area and moving northward to interact with the Arctic fox population. Since haplotype A has also been reported in Poland and China, this may represent a Holarctic strain of *E. multilocularis.*

Further characterization of genetic diversity in Canada utilized aggregate analysis of three additional mtDNA loci, *cob*, *cox1* and *nad2*. Using published primer sets (Nakao et al., 2009), 2 different haplotypes of *E. multilocularis* were found in coyotes in Quesnel, BC and Saskatoon, SK. Results indicate that the European strain (BCc1) originally detected in the BC dog (Jenkins et al., 2012) is also present in coyotes in this region (Chapter 5). The identification of a nucleotide change at position 663 of the *nad2* gene indicates that the establishment of *E. multilocularis* in this region did not happen recently. To account for alterations in the *nad1* and *nad2* genes, a period of isolation was needed. Mitochondrial mutations occur more rapidly than in nuclear DNA: however, a short time with relative isolation from the parental group is still needed for the change to occur. The European-type haplotype found in SK (Saskatoon) was vastly different than the other European variant and that present in BC. The six nucleotide differences found between the established European haplotypes and the SK coyote haplotype indicates prolonged period of isolation from the parental group. The SK haplotype was not found elsewhere in Canada. However, only one sample from the Saskatoon region was analyzed. If this coyote was a local resident, local rodent and carnivore species might also harbor this variant. It is possible, however, that this coyote travelled from elsewhere in Canada. This again serves to highlight the need for additional testing of host species (especially those with large home ranges and dispersal distances) from a large geographic region.

Published primers normally used to amplify the three additional mitochondrial genes did not work for the Saskatchewan rodent samples. This could be indicative of sequence alterations

in the primer annealing regions. This supports findings that *E. multilocularis* present in central North America (the proposed N2 strain) is genetically distinct from other strains of *E. multilocularis* characterized to date, including the N1 North American strain established on St Lawrence Island, Alaska (Nakao et al., 2009). Additional primer sets (Table 6-2) were developed to amplify partial sequences of *cob*, *cox1* and *nad2,* resulting in characterization of eight haplotypes in deer mice in Saskatchewan. Though four of the shorter amplicons matched the published N2 sequence (Nakao et al., 2009), seven mouse samples did not. Nucleotide changes, found in all three genes, further separate the southern SK isolates from the presumed parent group, N1, with the exception of N3, which serves as a link between the two groups (Table 6-4). The new haplotype positions, relative to N2, is indicative of prolonged isolation of the NCR mouse isolates from the N1 haplotype present in the Alaskan Arctic, and does not support a more recent colonization event.

The new genotypic variations identified in this study indicate greater genetic diversity in *E. multilocularis* in North America than previously thought. Because diversity may still have been grossly underestimated, further investigation is required. Limited sample sizes from vastly different regions will not be reflective of the genetic alterations brought on by the highly variable and changing Canadian environment. It is likely that further variation could be identified on an individual cestode level as procreation is primarily by self-fertilization and clonal division (Nakao et al., 2009). It could, therefore, be assumed that, even within a relatively small region (i.e. the size of the European Union), a tremendous amount of diversity could be found. Moreover, it is important to note that mitochondrial genes of *E. multilocularis*, once thought to hold little genetic diversity, could hold a greater amount of variation than previously thought and should be re-examined on a larger geographic scale.

This study also addressed the question of how *E. multilocularis* originally came to inhabit central North America. Three prevailing hypotheses are: 1) infected Arctic foxes brought *E. multilocularis* down into the Prairie provinces during population irruptions in the 1960's; 2) the translocation of infected red foxes from Europe for hunting purposes in the 19th century brought *E. multilocularis* to the area and 3) prior to, or during, the last ice age, foxes already infected with the N1 strain used the NCR as a refugium and as a consequence infected local wildlife (Rausch, 1985; Nakao et al., 2009). Subsequent isolation of the refugees from the parent group (N1) after the ice sheet retreated resulted in divergence of the N2 strain. Our findings suggest

that the first hypothesis is supported, as isolate from the BC and NU were closely related, as would be consistent with a recent range expansion or natural wildlife translocation. If the second hypothesis was correct, Canadian isolates should be highly similar to presently identified European haplotypes. If the third hypothesis were correct, we would find that the NCR and NTZ sequences were quite distinct, resulting from a prolonged period of isolation. The results of this study indicate that all three hypotheses may be correct.

The first and second hypotheses may explain the presence of the European-type strain in the BC coyotes. According to haplotype analysis, there was very little genetic difference between the coyote isolates and European varieties. If establishment occurred earlier than a few hundred years ago, we would likely see more divergence in these regions than what was detected. This negates the possibility of a pre-ice age establishment for *E. multilocularis* in this region. As stated in section 6, the occurrence of this strain in BC may be the result of the natural movement of foxes and coyotes, which may have very large home ranges. This possibility is highlighted by the identification of the same *nad1* haplotype (A) being found in BC as well as Karrak Lake, NU. Foxes, ranging far south of their normal distribution in the Arctic and interacting with wildlife, could account for the occurrence in BC. Therefore, there is some support for genetic interchange between Arctic and southern populations of *E. multilocularis* (hypothesis 1). However, without further investigation into the NTZ haplotypes, an exact point of origin is difficult to determine.

Based on the concatenation of the mitochondrial genes, evidence for the third hypothesis includes the verification and identification of distinct haplotypes of *E. multilocularis* in southern SK, closely related to the N2 haplotype in South Dakota and Indiana (Figure 6-2). Mutations in the three genes, *cob*, *cox1* and *nad2,* serve to both separate and link N1 to N2. The number of mutations suggests a prolonged isolation of the N1 from the N2 groups, and that mutations in these genes are continuing to occur, creating more genetic differentiation. The substantial number of differences between these samples and other isolates imply a very long period of separation from the parent strain. This idea is reinforced by the exclusion of specific *nad1* haplotypes from other parts of the country. This portion of the study therefore finds in favour of the pre-ice age hypothesis of establishment for the N2 and related strains in the NCR.

We found another haplotype in Saskatoon, SK that is of European origin. The degree of separation between this new haplotype and its European kin, as based on the aggregate analysis

of three mitochondrial genes, suggest that this particular strain has been isolated in Canadian wildlife for many years. The sequences associated with this novel haplotype include six nucleotide polymorphisms that separate it from European haplotype, E4. The SK isolate is not the same haplotype that was recently found in BC wildlife. There are seven mutations that separate this new haplotype (SK) from the BC coyote samples, indicating a closer relationship to the native European haplotypes than BC. The existence of this strain in SK is enigmatic. It is possible that foxes infected with European strains have entered the eastern Canadian Arctic in the past and have become isolated in the NCR during a period of North American glaciation.

This, again, highlights the need for further sampling in the eastern Canadian Arctic. While this study confirmed the previous distribution of *E. multilocularis* in the NCR, it did little to verify the distribution in the North. We did not find evidence to support the existence *of E. multilocularis* in fecal samples from 50 Arctic foxes on Bylot Island, NU; however, this was a very small sample size and the parasite could have very low prevalence. *E. multilocularis* has not been found in either of the two fox species in Greenland, likely because there are no suitable rodent intermediate hosts (Braestrup, 1941; Kapel & Nansen, 1996; Akuffo, 2003).

Considering the number of nucleotide differences found between isolates, and the resulting haplotype placement in relation to other haplotypes, using four mitochondrial genes, it is unlikely that a full picture of the genetic diversity in Canada has been captured. This is probably the consequence of limited specimen and geographic sampling. It may also be a reflection of the limited scope of the gene loci chosen for characterization. The genetic makeup of *E. multilocularis* is obviously not as clear-cut as previously thought, and the mitochondrial loci typically selected in variation analysis could be insufficient to accurately measure its true diversity. Examination of multiple nuclear and mitochondrial genes, or even full genome sequencing, is needed to fully characterize genetic variation across the circumpolar distribution of *E. multilocularis*, and the significance of this variation for understanding the origins, translocation history, and zoonotic significance of this important pathogen.

This research highlights the need for large sample sizes when determining genetic variation. Coordinated efforts with other research groups may increase sample yield with each group taking the desired samples from both rodents and carnivores. An increase in the number of carnivore samples processed can be aided by use of the new scraping, filtration and counting technique (SFCT), with drastically reduced processing time. This method is not only comparable

to existing protocols, but ensures the retention of whole body structures needed for molecular analysis. The necessity of analysis of single cestodes stems from the need for sample purity, as mixed infections can exist within a host, especially carnivores consuming multiple intermediate hosts. There may be other haplotypes present or there may be a co-infection with *E. granulosus*. Only whole worms should be used in sequence analysis for the determination of strain-type used in the potential differentiation of pathogenicity.

Genetic differences resulting varying degrees of zoonotic potential are at this point, only suspect, but should be further investigated. In Europe, the annual incidence of human infection is 1:10 million as compared to US statistics citing 1:2.5-3 billion (Davidson et al., 2012). European and Asian countries may be more densely populated, but given the current distribution and abundance of *E. multilocularis* in Canada's prairies, there must be ample opportunity for human exposure. This is especially true, as infected wild canids have been increasingly found in urban centres (Catalano et al., 2012; Liccioli et al., 2012). Recently, *E. multilocularis* was ranked third on the list of important food borne parasites (FAO/WHO, 2012). This calls for enhanced surveillance in wild and domestic carnivores, based on recovery of adult cestodes or improved methods of detection, to determine if this cestode is emerging in Canada as it is elsewhere in the world.

8. REFERENCES

Akuffo, H. (2003). Parasites of the colder climates. London, England and New York, USA. Taylor and Francis.

Baron, B. W. (1970). The occurrence of *Echinococcus multilocularis* Leuckart, 1863 and of other helminths in the red fox, *Vulpes vulpes*, in southern Manitoba. *Canadian Journal of Zoology*, *48*, 1132.

Bartel, M. H., Seesee, F. M., & Worley, D. E. (1992). Comparison of Montana and Alaska isolates of *Echinococcus multilocularis* in gerbils with observations on the cyst growth, hook characteristics and host response. *Journal of Parasitology*, *78*, 529–532.

Blair, W. F. (1940). A study of prairie deer-mouse populations in southern Michigan. *The American Midland Naturalist*, *24*, 273–305.

Bower, W. T., & Aller, H. D. (1917). Alaska fisheries and fur industries in 1916. Bureau of Fisheries Doc. (USA) - Document No. 838. Washington, DC: Government Printing Office.

Bowles, J., & McManus, D. P. (1993). NADH dehydrogenase 1 gene sequences compared for species and strains of the genus *Echinococcus*. *International Journal for Parasitology*, *23*, 969–972.

Bradley, C. A., & Altizer, S. (2006). Urbanization and the ecology of wildlife diseases. *Trends in Ecology and Evolution*, *22*, 95–102.

Braestrup, F. W. (1941). A study on the Arctic fox in Greenland. *Medd Grønl*, *131*, 1–101.

Bresson-Hadni, S., Miguet, J. P., & Mantion, G. (1991). Orthotopic liver transplantation for incurable alveolar echinococcosis of the liver: report of 17 cases. *Hepatology*, *13*, 1061.

Brown, W. M., George, J. M., & Wilson, A. C. (1979). Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the United States of America*, *76*, 1967–1971.

Cabrera, M., Canova, S., Rosenzvit, M., & Guarnera, E. (2002). Identification of *Echinococcus granulosus* eggs. *Diagnostic Microbiology and Infectious Disease*, *44*, 29–34.

Castrodale, L. (2003). Echinococcosis in Alaska. State of Alaska Epidemiology Bulletin. Government of Alaska, US - Bulletin no. 2.

Catalano, S., Lejeune, M., Liccioli, S., Verocai, G. G., Gesy, K. M., Jenkins, E. J., Kutz, S. J., et al. (2012). *Echinococcus multilocularis* in urban coyotes, Alberta, Canada. *Emerging Infectious Diseases*, *18*, 1625–1628.

Chalmers, G. ., & Barrett, M. W. (1974). *Echinococcus multilocularis* Leuckart, 1863 in rodents in southern Alberta. *Canadian Journal of Zoology*, *52*, 1091.

Choquette, P. E., MacPherson, A. H., & Cousineau, J. G. (1962). Note on the occurrence of *Echinococcus multilocularis* Leuckart, 1863 in the Arctic fox in Canada. *Canadian Journal of Zoology*, *40*, 1167.

Conboy, G. (2009). Cestodes of dogs and cats in North America. *Veterinary Clinics of North America: Small Animal Practice*, *39*, 1075–1090.

Da Silva, A. J., Bornay-Llinares, F. J., Moura, I. N. S., Slemenda, S. B., Tuttle, J. L., & Pieniazek, N. J. (1999). Fast and reliable extraction of protozoan parasite DNA from fecal specimens. *Molecular Diagnosis*, *4*, 57–64.

Dalen, L., Fuglei, E. V. A., Hersteinsson, P., Kapel, C. M. O., Roth, J. D., Samelius, G., Tannerfeldt, M., et al. (2005). Population history and genetic structure of a circumpolar species: The Arctic fox. *Biological Journal of the Linnean Society*, *84*, 79–89.

Davidson, R. K., Oines, O., & Madslien, K. (2009). *Echinococcus multilocularis* - adaptation of a worm eggs isolation procedure coupled with multiplex PCR assay to carry out large-scale screening of red foxes (*Vulpes vulpes*) in Norway. *Parasitology Research*, *104*, 509–514.

Davidson, R. K., Romig, T., Jenkins, E. J., Tryland, M., & Robertson, L. J. (2012). The impact of globalization on the distribution of *Echinococcus multilocularis*. *Trends in Parasitology*, *28*, 239–247.

Deplazes, P., & Eckert, J. (1996). Diagnosis of *Echinococcus multilocularis* infection in final hosts. *Applied Parasitology*, *37*, 247–252.

Deplazes, P., Hegglin, D., Gloor, S., & Romig, T. (2004). Wilderness in the city: the urbanization of *Echinococcus multilocularis*. *Trends in Parasitology*, *20*, 77–84.

de Vos, A., & Matel, S. E. (1952). The status of lynx in Canada, 1920-1952. *Journal of Forestry*, *50*, 742–745.

Duscher, G., Prosl, H., & Joachim, A. (2005). Scraping or shaking—a comparison of methods for the quantitative determination of *Echinococcus multilocularis* in fox intestines. *Parasitology Research*, *95*, 40–42.

Eaton, R. D. P., & Secord, D. C. (1979). Some Intestinal Parasites of Arctic Fox, Banks Island, N. W. T. *Canadian Journal of Comparative Medicine*, *43*, 229–230.

Eckert, J. (2003). Predictive values and quality control of techniques for the diagnosis of *Echinococcus multilocularis* in definitive hosts. *Acta Tropica*, *85*, 157–163.

Eckert, J., Conraths, F. ., & Tackmann, K. (2000). Echinococcosis: an emerging or re-emerging zoonosis? *International Journal for Parasitology*, *30*, 1283–1294.

Eckert, J., & Deplazes, P. (2004). Biological, epidemiological, and clinical aspects of Echinococcosis, a zoonosis of increasing concern. *Clinical Microbiology Review*, *17*, 107–135.

Eckert, J., Gemmell, M. A., Meslin, F. X., & Pawlowski (Eds.). (2002). *WHO/OIE manual on Echinococcosis in humans and animals: a public health concern of global concern*. World Organization for Animal Health (OIE), Paris, France.

Eckert, J., Thompson, R. C. A., & Mehlorn, H. (1983). Proliferation and metastases formation of larval *Echinococcus multilocularis*. *Zeitschrift Fur Parasitenkunde-Parasitology Research*, *69*, 737–748.

European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) (2012). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2010. *European Food Safety Authority*, *10*, 2597.

Evermann, B. W. (1914). Alaska fisheries and fur industries in 1913. Bureau of Fisheries Doc. (USA) - Document No. 797. Washington, DC: Government Printing Office.

FAO/WHO. (2012). *Multicriteria-based ranking for risk management of foodborne parasites*. FAO, Rome, Italy.

Fay, F. H., & Williamson, F. S. L. (1962). Studies on the helminth fauna of Alaska. XXXIX. *Echinococcus multilocularis* Leuckart, 1862, and other helminths of foxes on the Pribilof Islands. *Canadian Journal of Zoology*, *40*, 767–772.

Fujita, O., Oku, Y., Okamoto, M., Sato, H., Ooi, H. K., Kamiya, M., & Rausch, R. L. (1991). Early development of larval *Taenia polyacantha* in experimental intermediate hosts. *Journal of the Helminothological Society of Washington*, *58*, 100–109.

Furgal, C., & Prowse, T. D. (2008). *Northern Canada*. *From Impacts to Adaptation: Canada in a Changing Climate* (pp. 57–118). Ottawa, ON: Government of Canada.

Gamble, W. G., Segal, M., Schantz, P. M., & Rausch, R. L. (1979). Alveolar hydatid disease in Minnesota: First human case acquired in the contiguous United States. *The Journal of the American Medical Association*, *241*, 904–907.

Geddes Smith, E. M., & Hanson, S. (1961). Alveolar echinococcosis: a case report with a discussion of the ecology of the disease. *American Journal of Clinical Pathology*, *35*, 160–165.

Gemmell, M. A. (1968). Safe handling of infected definitive hosts and eggs of *Echinococcus* spp. *Bulletin of the World Health Organization*, *39*, 122.

Gese, E. M., Rongstad, O. J., & Mytton, W. R. (1988). Home range and habitat use of coyotes in southeastern Colorado. *The Journal of Wildlife Management*, *52*, 640–646.

Gilbert, N. L., Oluwayemisi, K. D., Libman, M. D., Muchaal, P. K., & Odgen, N. H. (2010). Hospitalization for trichinellosis and echinococcosis in the Canadian Arctic, 2001 - 2005: the tip of the iceberg? *Canadian Journal of Public Health*, *101*, 337–340.

Gilman, R. H., & Lee, B. H. (2000). Alveolar hydatid disease. In *Hunter's Tropical Medicine and Emerging Infectious Disease* (8th ed.). Philadelphia, PA, USA: WB Saunders.

Giraudoux, P. (2004). Interactions between landscape changes and host communities can regulate *Echinococcus multilocularis* transmission. *Parasitology*, *127*, 121–131.

Haag, K. L., Zaha, A., Araujo, A. M. & Gottstein, B. (1997). Reduced genetic variability within coding and non-coding regions of the *Echinococcus multilocularis* genome. *Parasitology*, *115*, 521-529.

Harrison, D. J. (1992). Dispersal characteristics of juvenile coyotes in Maine. *Journal of Wildlife Management*, *56*, 128–138.

Heath, D. D. (1971). The migration of oncospheres of *Taenia pisiformis*, *T. serialis* and *Echinococcus granulosus* within the intermediate host. *International Journal for Parasitology*, *1*, 145–152.

Henttonen, H., Fuglei, E., Gower, C. N., Haukisalmi, V., Ims, R. A., Niemimaa, J., & Yoccoz, N. G. (2001). *Echinococcus multilocularis* on Svalbard: introduction of an intermediate host has enabled the local life-cycle. *Parasitology*, *123*, 547–552.

Hersteinsson, P., & Macdonald, D. W. (1992). Interspecific competition and the geographical distribution of red and arctic foxes *Vulpes vulpes* and *Alopex lagopus*. *Oikos*, *64*, 505–515.

Hildreth, M. B., Sriram, S., Gottstein, B., & Schantz, P. M. (2000). Failure to identify alveolar echinococcosis in trappers from South Dakota in spite of high prevalence of *Echinococcus multilocularis* in wild canids. *Journal of Parasitology*, *86*, 75–77.

Himsworth, C. G., Jenkins, E. J., Hill, J. E., Nsungu, M., Thompson, R. C. A., Covacin, C., Ash, A., et al. (2010). Emergence of sylvatic *Echinococcus granulosus* as a parasitic zoonosis of public health concern in an Indigenous community in Canada. *American Journal of Tropical Medicine and Hygiene*, *82*, 643–645.

Hnatiuk, J. M. (1966). First occurrence of *Echinococcus multilocularis* Leuckart, 1863 in *Microtus pennsylvanicus* in Saskatchewan. *Canadian Journal of Zoology*, *44*, 493.

Hnatiuk, J. M. . (1969). Occurrence of *Echinococcus multilocularis* Leuckart, 1863 in *Vulpes fulva* in Saskatchewan. *Canadian Journal of Zoology*, *47*, 264.

Hofer, S., Gloor, S., Muller, U., Mathis, A., Hegglin, D., & Deplazes, P. (2000). High prevalence of *Echinococcus multilocularis* in urban red foxes (*Vulpes vulpes*) and voles (*Arvicola terrestris*) in the city of Zurich, Switzerland. *Parasitology*, *120*, 135–142.

Holmes, J. C., Mahrt, J. L., & Samuel, W. M. (1971). The occurrence of *Echinococcus multilocularis* Leuckart, 1863 in Alberta. *Canadian Journal of Zoology*, *29*, 575–576.

Holt, D. W., Hanns, C., O'Hara, T., Burek, K., & Frantz, R. (2005). New distribution records of *Echinococcus multilocularis* in the brown lemming from Barrow, Alaska, USA. *Journal of Wildlife Diseases*, *41*, 257–259.

Hubbert, W. T., Hull, T. G., McCulloch, W. F. and Schnurrenberger (Eds.) (1975). Taeniidae. In *Diseases Transmitted from Animals to Man*. CC Thomas Publisher Limited. Springfield, IL, USA.

Jacobs, D. E., Arakawa, A., Courtney, C. H., Gemmell, M. A., McCall, J. W., Myers, G. H., & Vanparijs, O. (1994). World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) guidelines for evaluating the efficacy of anthelmintics for dogs and cats. *Veterinary Parasitology*, *52*, 179–202.

James, E., & Boyd, W. (1937). *Echinococcus alveolaris*. *The Canadian Medical Association Journal*, *36*, 354–356.

Jenkins, D. J., Romig, T., & Thrompson, R. C. A. (2005). Emergence/re-emergence of *Echinococcus* spp.-a global update. *International Journal for Parasitology*, *35*, 1205–1219.

Jenkins, E. J., Hill, J. E., Somers, C., Gesy, K., & Barnes, B. (2012). Detection of European strain of *Echinococcus multilocularis* in North America [letter]. *Emerging Infectious Diseases*, 18, 1010-1012.

Jenkins, E. J., Schurer, J. M., & Gesy, K. M. (2011). Old problems on a new playing field: Helminth zoonoses transmitted among dogs, wildlife, and people in a changing northern climate. *Veterinary Parasitology*, *182*, 54–69.

Jones, A., & Pybus, M. J. (2001). Taeniasis and Echinococcosis. *Parasitic diseases of wild mammals* (2nd ed.). Ames, Iowa: Iowa State University Press/Ames.

Kamiya, M. (2008). Echinococcosis/hydatidosis. WHO/OIE manual of diagnostic tests and vaccines for terrestrial animals. *Office International Des Epizooties*, Paris, France.

Kamler, J. F., & Ballard, W. B. (2002). A review of native and nonnative red foxes in North America. *Wildlife Society Bulletin*, *30*, 370–379.

Kapel, C. M. O., & Nansen, P. (1996). Gastrointestinal helminths of Arctic foxes (*Alopex lagopus*) from different bioclimatological regions in Greenland. *American Society of Parasitologists*, *82*, 17–24.

Kapel, C. M. O., Torgerson, P. R., Thompson, R. C. A., & Deplazes, P. (2006). Reproductive potential of *Echinococccus multilocularis* in experimentally infected foxes, dogs, raccoon dogs and cats. *International Journal for Parasitology*, *36*, 79–86.

Kern, P., Bardonnet, K., Renner, E., Auer, H., Pawlowski, Z., Ammann, R. W., Vuitton, D. A., et al. (2003). European Echinococcosis registry: human alveolar echinococcosis Europe, 1982-2000. *Emerging Infectious Diseases*, *9*, 343–349.

Khuroo, M., Wani, N., Javid, G., Khan, B. A., Yattoo, G. N., Shah, A. H., & Jeelani, S. G. (1997). Percutaneous drainage compared with surgery for hepatic hydatid cysts. *New England Journal of Medicine*, *337*, 881–887.

Kirk, C. (2012). Molecular ecology of *Echinococcus* and Taeniinae in Arctic fox (*Vulpes lagopus*) of Northern Alaska: characterization of the nad1 gene fragment. M. Sc. Thesis. University of Colorado, Colorado, USA.

Kritsky, D. C., & Leiby, P. D. (1978). Studies on sylvatic echinococcosis. V. Factors influencing prevalence of *Echinococcus multilocularis* Leuckart 1863, in red foxes from North Dakota, 1965-1972. *Journal of Parasitology*, *64*, 625–634.

Kritsky, D. C., Leiby, P. D., & Miller, G. E. (1977). The natural occurrence of *Echinococcus multilocularis* in the bushy-tailed woodrat, *Neotoma cinerea rupicola*, in Wyoming. *American Journal of Tropical Medicine and Hygiene*, *26*, 1046–1047.

Kutz, S. J., Jenkins, E. J., Veitch, A. M., Ducrocq, J., Polley, L., Elkin, B., & Lair, S. (2009). The Arctic as a model for anticipating, preventing and mitigating climate change impacts on host-parasite interactions. *Veterinary Parasitology*, *163*, 217–228.

LaFond, D. J., Thatcher, D. S., & Handeyside, R. G. (1963). Alveolar hydatid disease. *The Journal of the American Medical Association*, *186*, 35–37a.

Langer, J. C., Rose, D. B., Keystone, J. S., Taylor, B. R., & Langer, B. (1984). Diagnosis and management of hydatid disease of the liver. A 15-year North American experience. *Annals of Surgery*, 199, 412-417.

Laws, G. F. (1968). Physical factors influencing survival of taeniid eggs. *Experimental Parasitology*, *22*, 227–239.

Lee, C.-F. (1969). Larval *Echinococcus multilocularis* Leuckart, 1863 in the southern Interlake area in Manitoba. *Canadian Journal of Zoology*, *47*, 733–734.

Leiby, P. D., Carney, W. P., & Woods, C. E. (1970). Studies on sylvatic echinococcosis. III. Host occurrence and geographic distribution of *Echinococccus multilocularis* in the North Central United States. *The Journal of Parasitology*, *56*, 1141–1150.

Leiby, P. D., & Kritsky, D. C. (1972). *Echinococcus multilocularis*: A possible domestic life cycle in central North America and its public health implications. *The Journal of Parasitology*, *58*, 1213–1215.

Liccioli, S., Catalano, S., Kutz, S. J., Lejeune, M., Verocai, G. G., Duignan, P. J., Fuentealba, C., et al. (2012). Gastrointestinal parasites of coyotes (*Canis latrans*) in the metropolitan area of Calgary, Alberta, Canada. *Canadian Journal of Zoology*, *90*, 1023–1030.

Mas-Coma, S., Valero, M., & Bargues, M. D. (2008). Effects of climate change on animal and zoonotic helminthiases. *Revue Scientifique et Technique de L Office International des Epizooites*, *27*, 443–452.

McTaggert Cowan, I. & Guiguet, C. J. (1965). *The mammals of British Columbia*. Victoria, BC, Canada: British Columbia Provincial Museum. Queen's Press, Victoria, BC.

Moro, P., & Schantz, P. M. (2009). Echinococcosis: a review. *International Journal of Emerging Infectious Diseases*, *13*, 1618–1619.

Nagorsen, D.W. (2002). An identification manual to the small mammals of British Columbia. Minitstry of Sustainable Resource Management; Ministry of Water, Land and Air Protection; Royal British Columbia Museum. Accessed (September, 2012) from http://www.env.gov.bc.ca/wld/idkey_intropg.htm

Nakao, M., Xiao, N., Okamoto, M., Yanagida, T., Sako, Y., & Ito, A. (2009). Geographic pattern of genetic variation in the fox tapeworm *Echinococcus multilocularis*. *Parasitology International*, *58*, 384–389.

Ohbayashi, M., Rausch, R. L., & Fay, F. H. (1971). On the ecology and distribution of *Echinococcus* spp. (Cestoda: Taeniidae), and characteristics of their development in the intermediate host. II. Comparative studies on the development of the larval *E. multilocularis* Leuckart, 1863, in the intermediate host. *Japanese Journal of Veterinary Research*, *19*, 1–53.

Okamoto, M., Bessho, Y., Kamiya, M., Kurosawa, T., & Horii, T. (1995). Phylogenetic relationships within *Taenia taeniaeformis* variants and other taeniid cestodes inferred from the nucleotide sequence of the cytochrome c oxidase subunit 1 gene. *Parasitology Research*, *81*, 451–458.

Osterman Lind, E., Juremalm, M., Christensson, D., Widgren, S., Hallgren, G., Ågren, E. O., Uhlhorn, H., et al. (2011). First detection of *Echinococcus multilocularis* in Sweden, February to March 2011. *Eurosurveillance: Europe's Journal on Infectious Disease Epidemiology, Prevention and Control*, *16*, 1–4.

Palmer, S. R., Soulsby, L., Torgerson, P. R. & Brown, D. W. G. (Eds.) (2011). Cystic echinococcosis. In *Oxford Textbook of Zoonoses: Biology, Clinical Practice, and Public Health Control* (2nd ed., pp. 630–666). Oxford University Press, USA.

Parmesan, C., & Yohe, G. (2003). A globally coherent fingerprint of climate change impacts across natural systems. *Nature*, *421*, 37–42.

Parodi, P., Mantovani, A., & Seimenis, A. (2002). Public health education and training in control programmes. *WHO/OIE manual on Echinococcosis in humans and animals: a public health concern of global concern*. World Organization for Animal Health (OIE), Paris, France.

Peregrine, A. S., Jenkins, E. J., & Barnes, B. (2010). Hepatic alveolar echinococcosis in a dog in British Columbia, Canada. *Proceedings of the 55th Annual Meeting of the American Association of Veterinary Parasitologists* (Vol. 2010, pp. 72–73). Atlanta, Georgia, USA.

Polley, L. (1978). Visceral larva migrans and alveolar hydatid disease. Dangers real or imagined. *Veterinary Clinics of North America*, *8*, 353–376.

Polley, L., & Thompson, R. C. A. (2009). Parasite zoonoses and climate change: molecular tools for tracking shifting boundaries. *Trends in Parasitology*, *25*, 285–291.

Post, E., Forchhammer, M. C., Bret-Harte, M. S., Callaghan, T. V., Christensen, T. R., Elberling, B., Fox, A. D., et al. (2009). Ecological dynamics across the Arctic associated with recent climate change. *Science*, *325*, 1355–1358.

Rausch, R. L. (1956). Studies on the helminth fauna of Alaska. XXX. The occurrence of *Echinococcus multilocularis* Leuckart, 1863, on the mainland of Alaska. *The American Journal of Tropical Medicine and Hygiene*, *5*, 1086–1092.

Rausch, R. L. (1960). Recent studies on hydatid disease in Alaska. *Parassitologia*, *2*, 391–398.

Rausch, R. L. (1967). On the ecology and distribution of *Echinococcus* spp. (cestoda: taeniidae), and characteristics of their development in the intermediate host. *Annales de Parasitologie*, *42*, 19–63.

Rausch, R. L. (1985). Parasitology: retrospect and prospect. *The Journal of Parasitology*, *71*, 139–151.

Rausch, R. L. (1994). Transberingian dispersal of cestodes in mammals. *International Journal for Parasitology*, *24*, 1203–1212.

Rausch, R. L. (1995). Life cycle patterns and geographic distribution of *Echinococcus* species. *Echinococcus and Hydatid Disease* (1st ed.). Oxfordshire, UK: CAB International.

Rausch, R. L. (2003). Cystic Echinococcosis in the Arctic and Sub-Arctic. Harold W. Manter Laboratory of Parasitology. Accessed (October, 2012) from http://digitalcommons.unl.edu/parasitologyfacpubs/515

Rausch, R. L., & Fay, F. H. (2002). Epidemiology of Alveolar Echinococcosis with reference to St Lawrence Island, Bering Sea. In P. Craig & Z. Pawlowski (Eds.), *Cestode Zoonoses: Echinococcosis and Cysticercosis* (pp. 309–324). Seattle, Washington, USA: IOS Press.

Rausch, R. L., Fay, F. H., & Williamson, F. S. L. (1990a). The ecology of *Echinococcus multilocularis* (Cestoda: Taeniidae) on St. Lawrence island, Alaska. II helminth populations in the definitive host. *Annales de Parasitologie Humaine et Comparee*, *65*, 131–140.

Rausch, R. L., & Richards, S. H. (1971). Observations on parasite-host relationships of *Echinococcus multilocularis* Leuckart, 1863, in North Dakota. *Canadian Journal of Zoology*, *49*, 1317–1330.

Rausch, R. L., & Schiller, E. L. (1951). Hydatid disease (Echinococcosis) in Alaska and the importance of rodent intermediate hosts. *Science*, *113*, 57–58.

Rausch, R. L., & Schiller, E. L. (1956). Studies on the helminth fauna of Alaska XXV. The ecology and public health significance of *Echinococcus sibiricensis* Rausch & Schiller, 1954, on St. Lawrence Island. *Parasitology*, *46*, 395–419.

Rausch, R. L., & Wilson, J. F. (1985). The current status of alveolar hydatid disease in northern regions. *Circumpolar Health*, *84*, 245–247.

Rausch, R. L., Wilson, J. F., & Schantz, P. M. (1990b). A programme to reduce the risk of infection by *Echinococcus multilocularis*: the use of praziquantel to control the cestode in a village in the hyperendemic region of Alaska. *Annals of Tropical Medicine and Parasitology*, *84*, 239–250.

Rausch, R. L. (2003). Cystic echinococcosis in the Arctic and sub-Arctic. Harold W. Manter Laboratory of Parasitology. Accessed (October, 2012) from http://digitalcommons.unl.edu/parasitologyfacpubs/515

Rawson, D. (1966). Germ cell migrations in a cestode. *Nature*, *209*, 315–316.

Rinder, H., Rausch, R. L., Takahashi, K., Kopp, H., Tomschke, A. & Loscher, T. (1997). Limited range of genetic variation in *Echinococcus multilocularis*. *Journal of Parasitology, 83*, 1045-1050.

Romig, T., Thoma, D., & Weible, A.-K. (2006). *Echinococcus multilocularis* - a zoonosis of anthropogenic environments? *Journal of Helminthology*, *80*, 207–212.

Rudzinski, D. R., Graves, H. B., Sargeant, A. B., & Storm, G. L. (1982). Behavioral interactions of penned red and arctic foxes. *Journal of Wildlife Management*, *46*, 877–884.

Rybicka, K. (1972). Ultrastructure of embryonic envelopes and their differentiation in *Hymenolepis diminuta* (cestoda). *The Journal of Parasitology*, *58*, 849–863.

Sakamoto, T., Kono, I., & Yasuda, N. (1982). Experimental infection of mice by intrahepatic inoculation of oncospheres and eggs of *Echinococcus multilocularis*. *Memoirs of the Faculty of Agriculture - Kagoshima University*, *18*, 141–147.

Salb, A. L., Barkema, H. W., Elkin, B. T., Thompson, R. C. A., Whiteside, D. P., Black, S. R., Dubey, J. P., et al. (2008). Dogs as sources and sentinels of parasites in humans and wildlife, Northern Canada. *Emerging Infectious Diseases*, *14*, 60–63.

Samelius, G., Alisauskas, R. T., Kellett, D. K., & Moore, F. (2004). Foraging behaviours and population dynamics of Arctic foxes. *Arctic*, 57, 441-450.

Samuel, W. M., Ramalingam, S., & Carbyn, L. N. (1978). Helminths in coyotes (*Canis latrans* Say), wolves (*Canis lupus* L.) and red foxes (*Vulpes vulpes* L.) of southwestern Manitoba. *Can*, *56*, 2614–2617.

Schantz, P. M., Chai, J., Craig, P. S., Eckert, J., Jenkins, D. J., Macpherson, C. N. L., & Thakur, A. (Eds.) (1995). Epidemiology and control of hydatid disease. *Echinococcus and Hydatid Disease*. Wallingford, UK: CAB International.

Schweiger, A., Ammann, R. W., Candinas, D., Clavien, P., Eckert, J., Gottstein, B., Halkic, N., et al. (2007). Human alveolar echinococcosis after fox population increase, Switzerland. *Emerging Infectious Diseases*, *13*, 878–882.

Sereda, M. M., Sherman, L., & Smith, E. M. G. (1961). Alveolar Echinococcosis. *Canadian Medical Association Journal*, *84*, 1138–1139.

Sillero-Zubiri, C., Hoffmann, M., & Macdonald, D. W. (Eds.). (2004). *Canids, foxes, wolves, jackals and dogs. Status survey and conservation action plan.* (1st ed.). Gland, Switzerland and Cambridge, UK: IUCN: The World Conservation Union.

Silverman, P. H. (1954). Studies on the biology of some tapeworms of the genus *Taenia*. *Annals of Tropical Medicine and Parasitology*, *48*, 207–215.

Smith, D. M., & Rickman, L. S. (2001). Echinococcosis: A drop of water-review on human disease, diagnosis, and management. *Infectious Diseases in Clinical Practice*, *10*, 355–359.

Sreter, T., Szell, Z., Egyed, Z., & Varga, I. (2003). *Echinococcus multilocularis*: an emerging pathogen in Hungary and central eastern Europe? *Emerging Infectious Diseases*, *9*, 384–386.

Stehr-Green, J. K., Stehr-Green, P. A., Schantz, P. M., Wilson, J. F., & Lanier, A. (1988). Risk factors for infection with *Echinococcus multilocularis* in Alaska. *American Journal of Tropical Medicine and Hygiene*, *38*, 380–385.

Storandt, S. T., & Kazacos, K. R. (1993). *Echinococcus multilocularis* identified in Indiana, Ohio, and East-central Illinois. *The Journal of Parasitology*, *79*, 301–305.

Storandt, S. T., & Kazacos, K. R. (2012). *Echinococcus multilocularis* identified in Michigan with additional records from Ohio. *The Journal of Parasitology*, *98*, 891–893.

Storandt, S. T., Virchow, D. R., Dryden, M. W., Hygnstrom, S. E., & Kazacos, K. R. (2002). Distribution and prevalence of *Echinococcus multilocularis* in wild predators in Nebraska, Kansas, and Wyoming. *The Journal of Parasitology*, *88*, 420–422.

Swiderski, Z. (1983). *Echinococcus granulosus*: hook-muscle systems and cellular organization of infective oncospheres. *International Journal for Parasitology*, *13*, 289–299.

Thomas, L. J., Babero, B. B., Gallicchio, V., & Lacey, R. J. (1954). Echinococcosis on St. Lawrence Island, Alaska. *Science*, *120*, 1102–1103.

Thompson, R. C. A. (1977). Growth, segmentation and maturation of the British horse and sheep strains of *Echinococcus granulosus* in dogs. *International Journal for Parasitology*, *7*, 281–285.

Thompson, R. C. A. (1979). Biology and speciation of *Echinococcus granulosus*. *Australian Veterinary Journal*, *55*, 93–98.

Thompson, R. C. A. (Ed.) (1986). Biology and systematics of *Echinococcus*. *The biology of Echinococcus and hydatid disease* (1st ed.). HarperCollins Publishers Limited, Scarborough, ON, Canada.

Thompson, R. C. A., & Eckert, J. (1982). The production of eggs by *Echinococcus multilocularis* in the laboratory following in vivo and in vitro development. *Zeitschrift Fur Parasitenkunde-Parasitology Research*, *68*, 227–234.

Thompson, R. C. A., & Eckert, J. (1983). Observations on *Echinococcus multilocularis* in the definitive host. *Parasitology Research*, *69*, 335–345.

Thompson, R. C. A., & McManus, D. P. (2002). Aetiology: parasites and life-cycles. *WHO/OIE manual on Echinococcosis in humans and animals: a public health concern of global concern*. World Organization for Animal Health (OIE), Paris, France.

Trachsel, D., Deplazes, P., & Mathis, A. (2007). Identification of taeniid eggs in the faeces from carnivores based on multiplex PCR using targets in mitochondrial DNA. *Parasitology*, *134*, 911–920.

Umhang, G., Woronoff-Rhen, N., Combes, B., & Boue, F. (2011). Segmental sedimentation and counting technique (SSCT): An adaptable method for qualitative diagnosis of *Echinococcus multilocularis* in fox intestines. *Experimental Parasitology*, *128*, 57–60.

Veit, P., Bilger, B., Schad, V., Schafer, J., Frank, W., & Lucius, R. (1995). Influence of environmental factors on the infectivity of *Echinococcus multilocularis* eggs. *Parasitology*, *110*, 79–86.

Viet, J. F., Giraudoux, P., Abrial, V., & Bresson-Hadni, S. (1999). Water vole (*Arvicola terrestris* Scherman) density as risk factor for human alveolar echinococcosis. *The American Journal of Tropical Medicine and Hygiene*, *61*, 559–565.

Vogel, H. (1957). *Echinococcus multilocularis* in South Germany. I. The tapeworm stage of strains from humans and animals. *Zeitschrift fuer Tropenmedizin und Parasitologia*, *8*, 404–405.

Webster, G., & Cameron, T. W. M. (1967). Epidemiology and diagnosis of *Echinococcus* in Canada. *Canadian Medical Association Journal*, *96*, 600–607.

Weinberg, T. (1947). *Echinococcus alveolaris* infection of the human liver. *Journal of Mount Sinai Hospital*, *13*, 331–335.

Wharton, D. A. (1983). The production and functional morphology of helminth egg-shells. *Parasitology*, *86*, 85–97.

Whitfield, P. J., & Evans, N. A. (1983). Pathogenesis and asexual multiplication among parasitic platyhelminths. *Parasitology*, *86*, 121–160.

Willis, J., Herbert, I. V., & Elliot, T. C. (1981). Some factors affecting the survival and transmission of *Taenia multiceps* eggs. Proceedings of the 9th International Conference of Wortd Association for the Advancement of Veterinary Parasitology, Budapest, 126.

Wilson, J. F., & Rausch, R. L. (1980). Alveolar hydatid disease: A review of clinical features of 33 indigenous cases of *Echinococcus multilocularis* infection in Alaskan Eskimos. *American Journal of Tropical Medicine and Hygiene*, *29*, 1340–1355.

Wilson, J. F., Rausch, R. L., & Wilson, F. R. (1995). Alveolar hydatid disease: Review of the surgical experience in 42 cases of active disease among Alaskan Eskimos. *Annals of Surgery*, *221*, 315–323.

Wobeser, G. (1971). The occurrence of *Echinococcus multilocularis* (Leuckart, 1863) in cats near Saskatoon, Saskatchewan. *The Canadian Veterinary Journal*, *12*, 65–68.

Wobeser, G., Ngeleka, M., Appleyard, G., Bryden, L., & Mulvey, M. R. (2007). Tularemia in deer mice (*Peromyscus maniculatus*) during a population irruption in Saskatchewan, Canada. *Journal of Wildlife Diseases*, *43*, 23–31.

Wrigley, R. E., & Hatch, D. R. M. (1976). Arctic fox migrations in Manitoba. *Arctic*, *29*, 147–158.

Yang, Y. R., Sun, T., Li, Z., Zhang, J., Teng, J., Lui, X., Liu, R., et al. (2006). Community surveys and risk factor analysis of human alveolar and cystic echinococcosis in Ningxia Hui autonomous region, China. Bulletin of the World Health Organization 84, 714-721.