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The transmission ecology of Echinococcus multilocularis in a North American urban landscape

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The transmission ecology of *Echinococcus multilocularis* in a North American urban landscape

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Abstract

The cestode *Echinococcus multilocularis* is a trophically-transmitted parasite that is typically maintained in a sylvatic cycle involving wild canid definitive hosts and small mammal intermediate hosts. As etiological agent of Alveolar Echinococcosis in humans, the cestode is currently the third most impacting food-borne parasite globally. Understanding *E. multilocularis* transmission ecology, combined with reliable diagnostic and surveillance of parasite infection in wild hosts, is crucial for disease prevention and risk management.

In Calgary, Canada, *E. multilocularis* was found to infect 29.5% (n = 61) of road-killed urban coyotes (*Canis latrans*) collected in 2009-2010. For the detection of *E. multilocularis* eggs in coyote feces, sugar fecal flotation showed lower sensitivity than the $ZnCl_2$ centrifugation and sedimentation protocol (0.46 *vs*. 0.75). The latter technique is therefore recommended for screening of canid hosts.

This study provided the first evidence of an urban sylvatic cycle of *E. multilocularis* in North America. Parasite prevalence in coyote feces was shown to vary temporally (10.5-43.5%) and spatially (5.3-61.5%) across five city parks, and infection of small mammal intermediate hosts was documented in three competent species (prevalence ranging between 0.7 and 1.4%). Genotyping coyote feces (n = 142) at 4-6 microsatellite loci allowed to correct for repeated sampling of individuals, and to estimate an overall parasite prevalence of 25.0%. Importantly, individual patterns of infection (i.e., parasite egg intensity in feces) allowed to observe temporal variations in parasite transmission that were not detected when examining fecal samples of unknown identity. By looking at the recovery of parasite eggs in genotyped feces over time, a re-infection rate of 57% (n =7) was estimated for coyotes in an area of parasite hyper-endemicity.

Winter was a crucial period for *E. multilocularis* transmission, as the encounter rate of coyote with the parasite (number of infected hosts ingested) was higher than any other season (95% CI: 1.0-22.4). Voles (*Microtus pennsylvanicus* and *Myodes gapperi*) were consumed by coyotes proportionally more than their availability, and likely played a key role for the maintenance of the urban sylvatic life-cycle of *E. multilocularis* in this urban landscape.

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Chapter 1

INTRODUCTION

It is today well accepted that the impact of parasites on hosts goes well beyond individual health, and that it extends to include a much broader influence on the ecological interactions within populations and their role in shaping ecological communities (Anderson & May 1979; May & Anderson 1979; Hudson 2002; Collinge & Ray 2006). Due to a natural intimate association, parasites evolve around the ecology (i.e., movements, habitat requirements, behaviour, population biology, trophic interactions) of their hosts (e.g., Poulin 2011), which ultimately drives and shapes the dynamics of parasite transmission - thus determining what is referred to as the ecology of parasite transmission (e.g., Emerson *et al.* 2000; Cattadori *et al.* 2006; Giraudoux *et al.* 2006).

Changes in host communities (e.g., introduction or removal of host species; Tompkins, White & Boots 2003), biodiversity (Ostfeld, Keesing & Logiudice 2003), host ecology and/or trophic interactions (Ostfeld & Holt 2004; Levi *et al.* 2012) may thus have dramatic effects on parasite transmission. This is particularly evident for micro (i.e., bacteria, viruses) and macro (i.e., helminths) parasites that are characterized by complex life cycles (e.g. trophically-transmitted parasites: most trematodes and cestodes, many nematodes; Lafferty 1999) or that are transmitted by arthropod vectors (e.g. *Borrelia burgorferi*; Ostfeld, Keesing & Logiudice 2003; Levi *et al.* 2012), although host trophic ecology was recently shown to play a role in the transmission of direct life-cycle parasites (Strona 2014).

By modifying natural environments, urbanization (growth in the area and number of people inhabiting cities; Shochat *et al.* 2006) can affect ecosystems in different ways, including:

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i) loss (but in few instances also increase) of biodiversity (i.e., mammals, birds, reptiles, amphibians, plants; McKinney 2008); ii) alteration of abundance and feeding behavior of wild species as a consequence of altered seasonal and yearly fluctuations of resources (e.g. Faeth *et al.* 2005); and iii) increased resource competition among wild species following enhanced habitat productivity (e.g. Shochat *et al.* 2006), which may cause species that are highly efficient in exploiting anthropogenic food resources to outcompete others and dominate the community (e.g. *Lepidodactylus lugubris* and *Hemidactylus frenatus*; Petren & Case 1996). For this reason, in the last decade interest and concerns have risen towards understanding the impact of urbanization on infectious diseases (e.g. Skelly *et al.* 2006) and more in general, their emergence as a consequence of human alteration to ecosystems (Williams *et al.* 2002). Through its impact on ecological communities and landscape characteristics, urbanization indeed has the potential to affect host-parasite interactions and disease dynamics, which may pose risk to the health of wildlife populations and consequently humans (Bradley & Altizer 2006). Indeed, zoonoses (diseases transmitted from animals to humans) represent the majority (60%) of emerging infectious diseases of humans, of which 72% are estimated to originate in wildlife (Jones *et al.* 2008). For instance, overall loss of predators and non-competent host species in urban fragmented landscapes resulted in higher prevalence of *B. burgdorferi* in white-footed deer mouse (*Peromyscus leucopus*), consequently leading to higher infection rates of ticks and higher risk of transmission of Lyme disease to humans (LoGiudice *et al.* 2003). Alternatively, anthropogenic food resources available in urban habitats were observed to induce higher population densities and contact rates in racoons (*Procyon lotor*), thus resulting in enhanced transmission and higher prevalence of *Baylisascaris procyonis* (Wright & Gompper 2005), a nematode of zoonotic relevance.

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Understanding the effect of urbanization on the transmission ecology of wildlife parasites becomes therefore particularly relevant under a public health perspective. Furthermore, ecological processes and patterns that shape parasite transmission, such as dilution effect (reduction in transmission of a disease agent due to the presence of non-competent host species; e.g. Schmidt & Ostfeld 2001), predator-prey functional response (relationship between the density of preys and the consumption of preys by a predator; e.g. Hegglin *et al.* 2007) and heterogeneity of parasite infection (variability in the distribution of parasite infection in the host population; e.g., Hudson 2002; Altizer *et al.* 2006), are still relatively unexplored in urban habitats.

By providing habitat for wildlife species that positively respond to anthropogenic resources and tolerate habitat fragmentation and human presence (e.g., medium-sized carnivores, omnivores), urban landscapes offer the conditions to study the circulation of zoonotic parasites at the interface of wildlife, domestic animals and humans. In these specific settings, it is possible to investigate driving processes in disease ecology and parasite transmission while obtaining crucial information for public health risk management.

The cestode *Echinococcus multilocularis* is the causative agent of Alveolar Echinococcosis (AE) in humans, which is among the most serious parasitic zoonoses of the northern hemisphere (case fatality rate > 90% when untreated; Craig, Rogan & Allan 1996). According to a 2014 report of the World Health Organization (WHO) and the UN's Food and Agricolture Organization (FAO), *E. multilocularis* represents the third most impacting food-borne parasite globally (FAO/WHO 2014). Typically, humans acquire infection by accidentally ingesting parasite eggs through contaminated food (e.g., berries and vegetables), soil, or contact with infected dogs (Eckert *et al.* 2001). The disease is estimated to affect more than 18,000

people each year on a global scale, with over 90% of the cases recorded in China (Torgerson *et al.* 2010), but with increasing rates in Europe since the '90s (e.g., from 0.10 to 0.26 in Switzerland; Moro & Schantz 2009). In North America, one area of high endemicity was reported in the 50's and in following years in St. Lawrence Island, Alaska (Rausch & Schiller 1954; Rausch & Schiller 1956; Rausch, Wilson & Schantz 1990). Outside of this historic (and now estinguished) hotspot, only three human cases have been so far recorded for Canada and USA (Klein & Massolo 2014), of which the most recent was documented in 2013 in Edmonton, Canada (Massolo *et al.* 2014) and the other two occurred rispectively in Manitoba, Canada (James & Boyd 1937), and Minnesota, USA (Gamble *et al.* 1979). Effectiveness of medical treatment for AE has significantly improved in the last decades, and reduction of life expectancy dropped from 18-20 years in the '70s to 2-4 years in 2005 (Hegglin & Deplazes 2013). However, AE is still a severe disease that requires long-term (or lifelong) and highly expensive medical treatment. This is aggravated by the high incidence of the disease in areas of the world where public health care is low and many infected people might not even receive proper diagnosis (Torgerson *et al.* 2010; Hegglin & Deplazes 2013), as well as the long latency (5-15 years) in human patients which futher complicates epidemiological studies (Craig, Rogan & Allan 1996).

Adults of *E. multilocularis* typically parasitize the small intestine of wild canids, such as foxes (*Vulpes* spp.) and coyotes (*Canis latrans*), although domestic dogs are competent definitive hosts that can be locally very important (Eckert *et al.* 2001). Eggs of the parasite are shed in the environment through the feces of definitive host, for which a pre-patency period of 32-33 days (Rausch, Wilson & Schantz 1990) and a patency of 90-110 days post-infection (varying among definitive host species; Kapel *et al.* 2006) are recorded. However, no experimental infection of coyotes have been performed to assess the exact dynamics of egg excretion in this host. Adult

worms are 1.2-4.5 mm long and have four to five segments, of which the antepenultimate in the strobila is characteristically mature (Eckert *et al.* 2001). Morphological diagnostic features for the identification of the worms are the position of the genital pore (anterior to the middle of both gravid and mature segments) and the sac-like shape of the uterus (Jones & Pybus 2008). Eggs of *E. multilocularis* are ovoid (30-40 µm diameter) and characterized by a highly resistant keratinized embryophore which confers the dark striated appearance (Eckert *et al.* 2001). As eggs of *Echinococcus* and *Taenia* cannot be morphologically distinguished, molecular tests are needed to identify parasite eggs at the species level (Trachsel, Deplazes & Mathis 2007).

Like other taeniid species (e.g. *Taenia crassiceps*), *E. multilocularis* is a dixenous parasite that relies on the predator-prey relationship to complete its life cycle. For *E. multilocularis* this is predominantly sylvatic, and more than 40 species of rodents and lagomorphs (mainly pikas) have been recorded to date as intermediate hosts (Giraudoux *et al.* 2003; Vuitton *et al.* 2003). In natural intermediate hosts, the larval (i.e. metacestode) stage of the parasite develops a multivesicular and infiltrating structure that grows rapidly and produces protoscolices (i.e. infectious stages) in 2-4 months (Eckert *et al.* 2001). Initial infection is typically in the liver (Jones & Pybus 2008), although larval mass extends to other organs of the abdominal cavity (i.e., spleen, pancreas, gastrointestinal tract) and reproductive organs as the structure grows (see this research - Liccioli *et al.* 2013).

Epizootiology of *E. multilocularis* varies across the parasite's range, in relation to the local intermediate and definitive host species. In China, red fox, Tibetan fox (*Vulpes ferrilata*) and the corsac fox (*Vulpes corsac*) are the typical wild definitive hosts, although locally (e.g., Gansu, Ningxia) domestic dogs may act as the main reservoir (Giraudoux *et al.* 2013a; Giraudoux *et al.* 2013b). Although exhaustive data are not available to fully characterize the

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community of small mammal intermediate hosts in China, several focal species have been here identified, namely *Ellobius tancrei*, *Ochotona curzoniae*, *Lasiopodomys brandtii* and *Eospalax fontanierii* (Giraudoux *et al.* 2013a). In Europe, the parasite is maintained by the red fox and the two most abundant intermediate hosts, the common vole (*Microtus arvalis*) and the European water vole (*Arvicola terrestris*). In Japan (i.e. Hokkaido Island), red foxes and raccoon dogs (*Nyctereutes procyonoides*) are the two most relevant definitive hosts (Yimam *et al.* 2002), while the main intermediate hosts are *Myodes* species (*M. rufucanus bedfordiae*, *M. rutilus mikado*, *M. rex*; Takahashi & Nakata 1995; Saitoh & Takahashi 1998; Eckert *et al.* 2001). In North America, *E. multilocularis* has two main areas of distribution: the Northern Tundra Zone (NTZ) of Alaska and Canada, and the North Central Region (NCR), which includes four Provinces of Canada (Alberta, Saskatchewan, Manitoba and British Columbia) and 13 contiguous States of the USA (Davidson *et al.* 2012; Gesy *et al.* 2013). Throughout the NTZ, the parasite circulates mainly among arctic fox (*Vulpes lagopus*) and its arvicoline rodent prey, such as the northern vole (*Microtus oeconomus*), the brown lemming (*Lemmus sibiricus*), and to a lesser extent, the northern red-backed vole (*Myodes rutilus*) (Eckert *et al.* 2001). In the NCR, definitive hosts are mainly represented by red foxes and coyotes, while the intermediate host species reported to date include deer mouse (*Peromyscus maniculatus*), meadow vole (*Microtus pennsylvanicus*), house mouse (*Mus musculus*), bushy tailed woodrat (*Neotoma cinerea*) (Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Kritsky, Leiby & Miller 1977) and the southern red-back vole *Myodes gapperi* (this research - Liccioli *et al.* 2013).

A relatively new phenomenon documented for *E. multilocularis* is the completion of its life-cyle within urban settings, as reported for example in cities of Europe (Hofer *et al.* 1999; Deplazes *et al.* 2004) and Japan (Tsukada *et al.* 2000; Yimam *et al.* 2002). Such observation is

coincident with growing urban populations of canid hosts (e.g. red fox and coyote, as well as domestic dogs), and can be a potential threat to public health, given the close proximity of the parasite to humans (Deplazes *et al.* 2004). As *E. multilocularis* mainly circulates among wild host species - with the exception of synanthropic (i.e., ecologically associated with humans) cycles maintained by domestic dogs in few areas of China (Giraudoux *et al.* 2006) - AE is not considered an eradicable disease (Ito, Romig & Takahashi 2003). Understanding the transmission ecology of the parasite in urban habitats is therefore crucial for assessing the risk of zoonotic transmission.

The PhD research herein presented investigated how the ecology of wildlife populations in a predator-prey system influences the maintenance of a trophically-transmitted parasite in an urban landscape, using the coyote, their prey (i.e. small mammals) and *Echinococcus multilocularis* in Calgary, Alberta, Canada, as a study system. By integrating information acquired on the ecology of the wild definitive and intermediate hosts and of the parasite, the overarching objective of the research was to assess processes and patterns that shape *E. multilocularis* transmission within urban environments, which can consequently inform disease risk prevention and management. Furthermore, by relying on trophic transmission and the predator-prey relationship between definitive and intermediate hosts, *E. multilocularis* represents an excellent model species to investigate the role played in parasite transmission by ecological processes such as dilution effect, predator functional response and feeding behavior.

Chapter overview

In the second chapter, I present a preliminary survey on the gastrointestinal parasites of urban coyotes (i.e., coyotes inhabiting and/or breeding within urban settings) in Calgary through

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analysis of carcasses and fecal samples collected in the metropolitan area in 2009-2010. By documenting - for the first time in North America - the infection of urban coyotes with *Echinococcus multilocularis*, this study opened questions on the role played by this carnivore in the transmission and maintenance of the parasite in the urban landscape.

Although necropsies and post-mortem examination traditionally represent the gold standard in the investigation of gastro-intestinal parasites in wildlife (Wobeser 2007), analysis of fecal samples is a valid alternative in urban habitats, where carcass collection cannot be planned according to a specific study design. However, parasitological studies based on fecal samples need to know the reliability of the techniques adopted, in order to correctly interpret the results and consider alterative diagnostic tests if necessary.

In the <u>third chapter,</u> I therefore assessed the sensitivity of the double centrifugation sugar fecal flotation and kappa agreement between fecal flotation and postmortem examination of intestines for the detection of helminths in coyotes, with particular attention to *E. multilocularis*, the main focus of this thesis. As the results showed that a more sensitive diagnostic technique was needed to reliably assess *E. multilocularis* infection in coyote feces, an adaptation of the $ZnCl_2$ centrifugation and sedimentation protocol described by Davidson *et al*. (2009) was consequently developed and tested. This modified technique was subsequently adopted for all the parasite testing.

Such technique is introduced in the <u>fourth chapter</u>, in which I investigated the presence of an urban sylvatic life-cycle of *E. multilocularis* in the city of Calgary by assessing spatial and temporal patterns of *E. multilocularis* infection in coyotes and intermediate hosts.

Regardless the sensitivity of fecal diagnostic techniques, estimates from fecal prevalence are still potentially biased whenever the identity of the sampled animal is unknown, as unplanned

repeated testing of unknown individuals can occur (Marathe *et al.* 2002). An integrated approach combining fecal genotyping with parasitological analysis has been recently introduced as a tool to prevent bias in estimates of parasite prevalence based on fecal samples alone (e.g. Zhang *et al.* 2011), but it has never been applied in the context of *E. multilocularis* research. Fecal genotyping is of particular importance in social definitive hosts and for those using territorial fecal marking, a behaviour that is exhibited by all canid species (Bekoff & Andrews 1978; Macdonald 1979; Mech & Boitani 2003). Moreover, identifying individual hosts potentially allows estimation of additional relevant epidemiological parameters, such as host population size (Creel *et al.* 2003), its temporal fluctuations (Prugh *et al.* 2005), and repeated individual-based measurements of parasite infection or diet (Prugh, Arthur & Ritland 2008), which represent key information to understanding the epidemiology and zoonotic transmission of *E. multilocularis* (Eckert & Deplazes 1999). The <u>fifth chapter</u> is dedicated to the evaluation of fecal genotyping as an epidemiological tool to assess parasite prevalence and patterns of individual infection in coyotes. Although *E. multilocularis* in urban coyotes was used as a study system, the results are to be interpreted considering the broader application of such an approach in wildlife disease ecology and parasitology.

In the <u>sixth chapter</u>, I investigated the feeding ecology of urban coyotes in relation to the transmission of *E. multilocularis*. In particular, I assessed seasonal variations of parasite infection in coyotes (presented in the fourth chapter) in relation to their diet and the estimated encounter rate with *E. multilocularis*. I also determined the role of different intermediate host species in the maintenance of the parasite's life-cycle given their relative abundance and the coyotes' selective consumption of small mammal species.

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In the <u>seventh chapter</u>, I finally integrated my contribution with the existing literature in a critical review on *E. multilocularis* transmission ecology in urban habitats. By expanding from the specific context of my research and reviewing the broader literature, the last section of the thesis aimed to broaden the concept of *E. multilocularis* "urbanization", as previously presented by Deplazes *et al*. (2004), and to describe the key changes in host community and host-parasite interactions affecting *E. multilocularis* transmission in urban settings.

Chapter contributions

Chapter 2: SL and AM designed the study. SL conducted field data collection and parasitological analyses of coyote feces, assisted in coyote post-mortem examination, ran statistical analyses, and wrote the manuscript. SC was responsible for parasitological analysis of coyote intestines and identification of adult parasite specimens, with the assistance of GV, ML and SL (*Taeni*a species). CF and PD conducted post-mortem examination of coyotes. MH analyzed coyote feces for *Giardia* spp. and assisted in lab work. SK provided support and guidance in laboratory activities. SK and KR contributed to study design. AM, SK and KR edited the manuscript.

Chapter 3: SL designed the study, conducted parasitological analysis of coyote feces, assisted in coyote post-mortem examination, ran statistical analysis, and wrote the manuscript. SC was responsible for parasitological analysis of coyote intestines and identification of adult parasite specimens, with the assistance of GV, ML and SL (*Taenia* species). CF and PD conducted post-mortem examination of coyotes. SK provided support and guidance in laboratory activities. AM, SK and KR edited the manuscript.

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Chapter 4: SL and AM designed the study, analyzed the data and wrote the manuscript. SL was responsible for field collection and laboratory analysis. SK and KR contributed to study design, provided laboratory support and edited the manuscript.

Chapter 5: SL and AM designed the study. SL was responsible for field collection of coyote feces, extraction, amplification and genotyping of coyote fecal DNA, data analysis, and writing of the manuscript. FC was responsible for extraction, amplification and genotyping of coyote tissue DNA. SR and CG provided laboratory support and supervision. AM, SK, SR and KR edited the manuscript.

Chapter 6: SL and AM designed the study. SL was responsible for field collection of coyote feces. CB analyzed fecal samples for qualitative assessment of coyote diet. SL and CB collected data to estimate consumption of small mammal biomass. SL analyzed the data and wrote the manuscript. AM and KR edited the manuscript.

Chapter 7: SL and AM ideated the review. SL wrote the manuscript under revision and constant discussion with AM. PG and PD edited the manuscript and provided data for Table 2 (Zurich and Nangxia).

Appendix

Manuscripts included in the appendix section represent additional contributions to the advance of knowledge on *E. multilocularis*. These include: i) a first-authored case report describing a new species of intermediate host for *E. multilocularis*, originated from the broader investigation on the parasite transmission ecology in the urban landscapes of Calgary (presented in chapter four); ii) two scientific papers that were co-authored with collaborators at the University of Calgary and University of Saskatoon; iii) a methodological study to identify factors

affecting the sensitivity of *E. multilocularis* detection at PCR. My contribution to these manuscripts is as follows. Appendix I: organization and coordination of carcass collection, coyote post-mortem examination, editing of the manuscript; Appendix II: study design, field collection, post-mortem and molecular analysis, writing of the manuscript; Appendix III: molecular analysis, editing of the manuscript; Appendix IV: collection and parasitological analysis of fecal samples, editing of the manuscript.

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Chapter 2

GASTROINTESTINAL PARASITES OF COYOTES (*CANIS LATRANS*) IN THE

METROPOLITAN AREA OF CALGARY, CANADA

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Abstract

Disease ecology is a relevant but relatively unexplored subject of urban coyote (*Canis latrans* Say, 1823) research. In fact, this carnivore may play a role in the circulation of parasites that can have implications on human and domestic dog health, but can also be affected by pathogens transmitted from domestic reservoirs. To investigate the gastrointestinal parasites of urban coyotes in Calgary, Alberta, Canada, we analyzed 61 carcasses and 247 fecal samples collected within the metropolitan area, including city parks, in 2009-2010. We found nine parasite taxa: *Toxascaris leonina* (Linstow, 1902), *Uncinaria stenocephala* (Railliet, 1884), *Ancylostoma caninum* (Ercolani, 1859), *Pterygodermatites affinis* (Jägerskiold, 1904), *Trichuris vulpis* (Froelich, 1789), *Echinococcus multilocularis* (Leuckart, 1863), *Taenia crassiceps* (Zeder, 1800), *Giardia* Kunstler, 1882, *Cystoisospora* Frenkel, 1977. Factors related to coyote ecology, habitat characteristics and dog management likely influence the community of coyote parasites in an urban environment, and need to be taken into account to assess the actual role of this carnivore in the maintenance of parasites in the city landscape. Further research is needed to assess the current risk for transmission of potentially zoonotic parasites (e.g., *E. multilocularis*, *T. crassiceps*, *Giardia* sp.) among coyotes, dogs and humans.

Introduction

Coyote (*Canis latrans* Say, 1823) populations have significantly expanded their range throughout North America in the last few decades (Laliberte & Ripple 2004). The species has become increasingly common also within metropolitan areas, showing great capacity for ecological adaptation (Grinder & Krausman 2001a; Gehrt, Anchor & White 2009).

To date, coyote research in urban environments has focused on spatial behavior (Grinder & Krausman 2001a; Gehrt, Anchor & White 2009), feeding ecology (Morey, Gese & Gehrt 2007) and management of human-coyote conflicts (Baker & Timm 1998), whereas only a few studies have investigated aspects related to their parasitology and disease ecology (Grinder & Krausman 2001b). The lack of research on parasitism in urban coyotes is surprising, since the species may be host to several gastrointestinal (GI) parasites that can be shared with domestic dogs, including the causative agents of important zoonoses (e.g., *Echinococcus* Rudolphi, 1801, *Giardia duodenalis* (Lambl, 1859), *Toxocara canis* (Werner, 1782)). At the same time, large populations of domestic animals (i.e., dogs and cats) in urban settings can facilitate the transmission of pathogens from domestic reservoirs to wildlife populations (Thompson, Kutz & Smith 2009), and this might affect urban coyotes as well.

From a public health perspective, monitoring the presence of zoonotic parasites in urban wildlife and domestic animals should be a priority. In fact, the rapid expansion of metropolitan areas, their large domestic dog populations, and the presence of wild species particularly adapted to the urban environment, could potentially increase the transmission risk at the interface between wildlife, domestic animals, and humans.

The objective of this study was to survey the GI parasites of coyotes in the metropolitan area of Calgary, as a first step towards the assessment of the transmission risk for GI parasites among wildlife, dogs and humans within an urban setting. We expected urban coyotes to harbor parasites that were previously recorded for the species in prairie ecosystems of North America (Seesee, Sterner & Worley 1983; Henke, Pence & Bryant 2002), including potentially zoonotic parasites.

Materials and Methods

Study area

The study occurred in the Census Metropolitan Area (CMA) of Calgary (51°5'N, 114°5'W), a region with a population of 1,230,248 (Statistics Canada, 2009[1]) that extends over 5,107 km^2 in the grasslands of southern Alberta (Fig 1A), Canada. Within Calgary city limits, the elevation ranges from 1,060 m.a.s.l. in the two river valleys (Bow and Elbow) to 1,240 m.a.s.l. in the surrounding hills. Several other creeks and water bodies are present within the city limits and provide a large amount of riparian habitat (Foley 2006), often encompassed in city parks, natural areas and golf courses, while the city is mainly surrounded by agricultural land.

Postmortem examination of carcasses

Between October 2009 and March 2010, 61 hunted and road-killed coyotes were collected in the Calgary CMA. Carcasses were frozen at -20 °C until postmortem examination,

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[1] http://www.statcan.gc.ca/pub/91-214-x/2008000/t021-eng.htm

performed at the Faculty of Veterinary Medicine, University of Calgary. During necropsy, coyotes were sexed, aged according to tooth eruption and wear, distinguishing between juveniles (<1 years old) and adults. Necropsied coyotes included 31 males (50.8%) and 30 females (49.2%), with 16 juveniles (9 males, 7 females), 43 adults (21 males, 22 females), and two individuals of undetermined age (1 male, 1 female).

Intestinal tracts were collected at postmortem examination, refrozen at -80 °C for 72 hours to inactivate eggs of *Echinococcus* spp. (Veit *et al.* 1995), and then stored at -20 °C. Small and large intestines were examined separately for the presence of helminths using scraping, washing and sieving (Eckert *et al.* 2001) through a 1x1 mm sieve. Nematodes were preserved in 70% ethanol and later cleared in lactophenol and identified under the microscope at 100-400x magnification (Anderson *et al.* 2009).

Taenia L., 1758 specimens were identified to species level through measurement of large and small hooks (Jones & Pybus 2008) and blade: handle ratios (Hoberg, Ebinger & Render 1999) for each scolex. When scolices were absent or in conditions that were inadequate for morphological identification based on hooks, representative proglottids were stained with Semichon's acetic carmine, dehydrated in ethanol, cleared in xylene, mounted in Canada balsam and analyzed for internal morphology, according to Jones and Pybus (2008).

Echinococcus specimens were identified to species level based on morphology (Jones & Pybus 2008) and confirmed using species-specific PCR (Trachsel, Deplazes & Mathis 2007) on DNA extracted from representative worms as reported in Liccioli *et al.* (in press).
For all helminth species, representative specimens were deposited with the University of Alberta Parasite Collection, Alberta, Canada (accession numbers: UAPC11573 to 11581).

Collection and analysis of fecal samples

From May to September 2010 coyote feces were collected in 9 sites in urban Calgary. These included the following city and Provincial Parks, natural areas, and golf courses: Elk Lodge (EL), Fish Creek East (FCE), Fish Creek West (FCW), Inglewood (IN), Nose Hill (NHP), Southland Lowlands (SL), Stanley Park (SP), Weaselhead (WSH) and Willow Park (WP) (Figure 1B).

Study sites represented significant habitat patches for coyotes in the city landscape, and included both areas where dog access was allowed ($N = 6$) and areas of no dog access ($N = 3$) (Figure 1B). Definition of 'no dog access' sites was based on official designation, and was confirmed by direct observation.

We collected feces along standardized trails and paths (4.2 ± 2.8 km) that were known as routes used by coyotes, based on observation of presence indices (i.e., tracks, feces). Trails and paths were covered on foot or bike with a maximum interval of ten days between visits. Coyote feces were determined by size, shape, content and texture (Halfpenny 1986), and aged according to their external appearance as follows: 48 hours old (class 1), 3-5 days old (class 2) and 5-10 days old (class 3). An additional collection trial with an interval of 48 hours was performed in two sites (FWP and WP) to calibrate the operator ability to properly identify fresh feces. Feces were collected in plastic bags (Ziploc®) and frozen at -80 °C for 72 hours to inactivate *Echinococcus* spp. eggs (Veit *et al.* 1995), and subsequently stored at -20 °C until examination.

Samples classified as not older than 3-5 days (classes 1 and 2) were analyzed for parasites within four months from the collection date. To account for potential heterogeneity in distribution of parasite eggs, oocysts and cysts, feces were sub-sampled in the following manner.

First, 2 grams of fecal material were taken from three different regions (two peripheral and one central) of the sample. The total fecal material sampled (6 grams) was then homogenized with addition of water (11.4 ± 2.7 mL). The resultant slurry was mixed thoroughly and then equally divided in three aliquots, each representing 2 grams of the original fecal material plus the water required for its homogenization. Two of these subsamples were analyzed modifying the Wisconsin double centrifugation technique (Cox & Todd 1962) as follows: we used Sheather's sucrose floating solution (S.G. = 1.26) and centrifugation at 2,000 rpm for 5 minutes, and placed 18x18 mm cover slips on top of each test tube during the second centrifugation.

All helminth eggs and coccidian oocysts observed were identified at 100-400x (Foreyt & Foreyt 2001; Hendrix 2006). Parasite intensity was quantified until 500 eggs/oocysts per slide, above which they were no longer counted, using such value as approximation for higher intensities. Parasite eggs/oocyst counts in the two replicates of each fecal sample were averaged.

Taenia spp. and *Echinococcus* spp. eggs are morphologically undistinguishable (Foreyt & Foreyt 2001), and were initially classified as Taeniidae. Further molecular identification of Taeniidae eggs was performed using the protocol developed and described by Trachsel *et al.* (2007), with modifications as reported in Catalano *et al.* (in press.). Briefly, DNA lysate was prepared from 10 µL of solution containing parasite eggs, 50 µL of lysis buffer [500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 10mM DTT, and 4.5% Tween 20] and 4 µL of Proteinase K. A simplex PCR was performed to distinguish the eggs of Taeniidae species based on amplicon size as follows: *Taenia* sp. (267 bp), *E. multilocularis* (Leuckart, 1863)(395 bp) and *E. granulosus* (Batsch, 1786)(117 bp) (Trachsel, Deplazes & Mathis 2007). Fecal prevalence of *Giardia* Kunstler, 1882 and *Cryptosporidium* Tyzzer, 1910 was assessed using immuno-fluorescence detection with Cyst-a-Glo™ Comprehensive Kit (Waterborne, Inc., New Orleans,

Louisiana, USA). In detail, a total of 2 grams of feces were taken from three different sections (two peripheral and one central) of the sample, homogenized with 12 mL of water and filtered into test tubes through a double layer of cheesecloth. Test tubes were first centrifuged at 2,000 rpm for 5 minutes. The majority of the supernatant was then discarded, and the sediment and approximately 1 mL of supernatant were transferred into a microcentrifuge tube and homogenized through vortexing.

Once mixed, 20 µL of this solution was pipetted on a glass slide, allowed to dry, and 20 µL of Cyst-a-Glo™ reagent was then added. Slides were incubated in the dark for 45 minutes in a humid chamber at room temperature (21 °C). The slides were then air-dried in the dark, the kit mounting medium was added and the slide examined using a fluorescent microscope (Olympus BX-51). A slide with 10 µL of the kit solution containing *Giardia* cysts and *Cryptosporidium* oocysts was prepared at the same time as a positive control. We recorded the number of positive samples (presence/absence of cysts and oocysts).

Statistical analysis

Differences in prevalence of adult parasites relative to coyote sex and age were analyzed with Fischer's Chi-squared test. Adult parasite intensity (Bush *et al.* 1997) in coyote carcasses was estimated using median values and group differences were compared using the Mann-Whitney U test. For coyote carcasses, Simpson's index was used to quantify the diversity of parasite species (Bush *et al.* 1997), with value ranging between zero (maximum diversity) and one (no diversity). To calculate Simpson's index, parasite specimens that were not identified at the species level were pooled in the same genus (e.g., *Taenia* sp.).

For all species, prevalence of parasite eggs did not differ between fecal samples of class

1 and 2 (Fisher's Chi-squared test, $P > 0.05$), and fecal prevalence was therefore calculated

pooling these classes. Difference in fecal parasite prevalence between sites with and without dog

access, as well as difference in helminth prevalence between carcasses and feces, were tested

with Fisher's Chi-squared test.

Statistical analysis was run on the SPSS 17.0 package (IBM Corporation, Armonk, New

York, USA).

Results

GI parasite profiles from carcasses

Parasites collected from coyote carcasses (Table 1) included four nematode species,

namely *Toxascaris leonina* (Linstow, 1902), *Uncinaria stenocephala* (Railliet, 1884),

Pterygodermatites affinis (Jägerskiold, 1904), *Ancylostoma caninum* (Ercolani, 1859), and at

least two cestodes, *Echinococcus multilocularis* and *Taenia crassiceps* (Zeder, 1800). For 11 of

13 coyotes infected with *Taenia* sp., the cestodes were in very poor condition (likely as

consequence of freezing treatment) and there were no hooks on the scolices, and morphology of

proglottids was inconclusive.

Simpson's index was 0.79. Information on sex and age composition of coyotes infected

with gastrointestinal parasites is reported in Table 1.

For *T. leonina*, prevalence was significantly higher in juveniles than in adults (100%

and 34.4%, respectively; $X^2 = 5.03$, $df = 1$, $P = 0.026$). Similarly, juveniles had significantly

higher prevalence for *E. multilocularis* (43.8% and 25.6%, respectively; $X^2 = 4.89$, $df = 1$, $P = 0.035$). With respect to the overall parasitism (presence of at least one parasite), as well as all the

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other parasite species, no differences ($P > 0.05$) in parasite prevalence or intensity were observed according to age class or gender.

GI parasite profiles from fecal samples

A total of 247 fecal samples (class 1, $N = 114$; class 2, $N = 133$) were collected and analyzed for parasites. Of these, 61.1% were found positive for parasite eggs, cysts or oocysts.

We identified two nematode species (*Toxascaris leonina*, *Trichuris vulpis* (Froelich, 1789), two protozoan genera (*Giardia*, *Cystoisospora* Frenkel, 1977) and Taeniidae cestodes (Table 1). Taeniidae eggs were molecularly identified in five of 15 samples, confirming the presence of *Echinococcus multilocularis* (4/5) and *Taenia* sp. (1/5).

Cryptosporidium sp. was not detected in coyote feces.

Eighteen of 106 samples positive to *Cystoisospora* contained oocysts of different sizes (length: 30-36 µM and 17-24 µM), suggesting the presence of at least two different species. These measurements are consistent respectively with *Cystoisospora canis* Neméseri, 1959 and *Cystoisospora ohioensis* Dubey, 1975 (Taylor, Coop & Wall 2007).

Giardia sp. was detected in 19.8% of the feces, with significantly higher prevalence in sites with dog access than in sites without dogs (23.2% and 10.6%, respectively; $X^2 = 4.83$, $df = 1$, $P = 0.031$). Fecal prevalence of *Cystoisospora* sp. was significantly higher in sites with no dog access than in sites with dogs (59.7% and 35.7%, respectively; $X^2 = 7.90$, $df = 1$, $P = 0.006$). For all the other parasites, as well as for overall parasite prevalence, no differences were detected between parks with different dog management.

Parasite prevalence in carcasses was significantly higher than in feces for *T. leonina* ($X^2 = 85.68$, $df = 1$, $P < 0.001$), Taeniidae ($X^2 = 72.86$, $df = 1$, $P < 0.001$), *U. stenocephala* ($X^2 =$

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77.40, *df* = 1, *P*< 0.001) and *P. affinis* (X^2 = 8.15, *df* = 1, *P*< 0.001) (Table 1), whereas no difference (*P*> 0.05) were detected for the other helminths.

Discussion

Occurrence and prevalence of parasite species we reported for coyotes in the Calgary CMA were similar to what was previously observed for the species in northern prairie ecosystems (Seesee, Sterner & Worley 1983), but differed from studies carried out in other biogeographic regions of Canada (Holmes & Podesta 1968; Bridger, Baggs & Finney-Crawley 2009) and USA (Gompper *et al.* 2003).

Toxascaris leonina was the most prevalent intestinal parasite of coyotes, consistent with previous reports of prevalence of up to 80-100% (Pence 1979; Seesee, Sterner & Worley 1983; Wirsing *et al.* 2007; Thompson *et al.* 2009). The dominance of one species within the helminth community is supported also by the high value (0.79) of the Simpson's index. *Toxascaris leonina* was more prevalent in juveniles, as previously reported for coyotes (Franson *et al.* 1978). Prevalence of this nematode was also higher in carcasses (collected in the winter) than in feces (collected during the summer). In a study on the sensitivity of fecal examination that used the analysis of intestines as gold standard (Liccioli *et al.*, in press), we reported high fecal detectability for *T. leonina* (0.84), suggesting that the summer fecal prevalence we estimated was representative of intestinal presence. Additionally, results of a pilot study conducted on a limited number of feces that were collected in the same sites in winter 2011 (*N* = 28, fecal prevalence = 75.0%) support the hypothesis of higher winter prevalence. Causes of seasonal differences might be related to seasonal variations in exposure, either through environmental contamination or predation on paratenic hosts (i.e., rodents).

The occurrence of Taeniidae in urban coyotes suggests that these multi-host helminths might complete their life cycle within the urban landscape. With respect to *E. multilocularis*, its detection in coyotes inside the city limits and in dog parks is important from a public health perspective (Catalano *et al.*, in press). Species of rodents that were found in Calgary CMA (Perrigo & Bronson 1985; Smith 1993) and that are known to be susceptible to *E. multilocularis* (Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971) include deer mice (*Peromyscus maniculatus* (Wagner, 1845)), meadow vole (*Microtus pennsylvanicus* (Ord, 1815)), and house mice (*Mus musculus* L., 1758). However, no evidence of their role in an urban cycle has been provided yet. Higher prevalence of *E. multilocularis* in juveniles can be relevant under an epidemiological perspective, considering that animals of this age class are the most likely to disperse and colonize new territories (Bekoff 1977).

Taenia crassiceps is rare in coyotes (Seesee, Sterner & Worley 1983), and although recently reported in this host in Saskatchewan (Wirsing *et al.* 2007), ours is the first report of it in coyotes in Alberta. It circulates through small to medium size rodents, and is common in both red fox (*Vulpes vulpes* L., 1758) and arctic fox (*Alopex lagopus* L., 1758) (Jones & Pybus 2008). This parasite has zoonotic potential, particularly when domestic dogs are the definitive hosts (Hoberg, Ebinger & Render 1999), and its presence in wild canids in urban settings should be further investigated.

The hookworms *U. stenocephala* and *A. caninum* are often reported in coyotes, and the prevalence we observed is consistent with previous studies (Conder & Loveless 1978; Wirsing *et al.* 2007). Eggs of these parasites were not detected in coyote feces, likely as a consequence of the deep freezing treatment (Liccioli *et al.*, in press). These hookworms are common parasites of dogs (Unruh *et al.* 1973; Salb *et al.* 2008), but severe clinical signs are reported only for

infections with *A. caninum* (Epe 2009), which can occasionally infect humans (Prociv & Croese 1996). Given the high prevalence of *U. stenocephala* observed in carcasses, the role of coyotes in contaminating city parks with infective stages may be relevant for dog health and for the maintenance of this parasite in the city environment. In contrast, *A. caninum* is rarely reported in wild canids in northern regions (Holmes & Podesta 1968), and southern Canada is currently considered the limit of its distribution (Zajac & Conboy 2011). The role of domestic dogs in the circulation of *A. caninum* among wild canids cannot be excluded (Holmes & Podesta 1968) and should be further investigated.

Trichuris vulpis has been previously reported for coyotes (Van Den Bussche, Kennedy & Wilhelm 1987), red foxes (Erickson 1944) and wolves (*Canis lupus* L., 1758) (Byman *et al.* 1977) in North America. The low prevalence of *T. vulpis*, detected only in feces in two collection sites (FCW and WSH) in the SW of the City (Figure 1), could be locally related to reservoir hosts such as foxes (Foreyt & Foreyt 2001) or environmental conditions (soil and vegetation, Schmidt & Roberts 1989). However, these sites were characterized by the largest sample sizes (FCW, $N = 68$, prevalence = 2.9%; WSH, $N = 49$, prevalence = 22.4 %), suggesting that adequate sampling may be the primary issue for the detection of this parasite.

Prevalence of *Giardia* sp. is comparable with what was previously reported in coyotes in Alberta (Thompson *et al.* 2009) and New York (Gompper *et al.* 2003). Lower prevalence of *Giardia* in sites with no dog access, coupled with high prevalence in dogs in Calgary (up to 25%, Smith *et al.*, unpubl.[2]), suggests that domestic dogs may play an important role in the

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[2] A. F. Smith, C.A.D. Semeniuk, S.J. Kutz, A. Massolo. A risk analysis for gastrointestinal parasitism in Calgary park-going dogs: Implications for public health and city park management.

maintenance of this parasite in city parks. Nonetheless, further research and genotyping of the parasite (Thompson, Palmer & O'Handley 2008) are needed to evaluate transmission between these two host species. Although *Cryptosporidium* was not detected with the method of immuno-fluorescence, preliminary results of an ongoing study based on immunomagnetic separation of oocysts and molecular analysis of DNA extracts from the same feces are currently indicating the presence of the parasite, therefore suggesting a low sensitivity of the protocol adopted in the current study.

Cystoisospora sp. was commonly found in coyote feces. The species of this coccidian that are most frequently reported in coyotes are *C. canis* and *C. ohioensis* (Arther & Post 1977; Gompper *et al.* 2003). These species are also commonly reported in dogs (Mitchell *et al.* 2007), and although these hosts could be involved in their circulation in the environment, lower fecal prevalence was found in sites with dog access. Identification of parasite species, as well as information on definitive host (coyote *vs.* dog) susceptibility and the possible role of paratenic hosts are needed to investigate the transmission of this parasite between the two hosts.

Pterygotermatites affinis (syn. *P. cahirensis*, Gibbs 1957) was previously found in coyotes (Pence 1979; Seesee, Sterner & Worley 1983) and other carnivores (Rausch, Maser & Hoberg 1983; Tiekotter 1985) in USA, but to our knowledge ours is the first report of this species in Canada. This parasite is transmitted by insect (Orthoptera Latreille, 1793) intermediate hosts (Luong & Hudson 2012), that can seasonally (i.e., summer) represent an important part of coyote diet (Andelt *et al.* 1987). *Pterygotermatites affinis* has no zoonotic relevance and isassociated with clinical signs in the definitive host only in cases of high intensity infections (Bowman 2002), not documented in the present study.

Toxocara canis was not observed in fecal or postmortem examinations, but this does not rule out its presence. In fact, given the typical age distribution of the parasite (Barutzki & Schaper 2003; Gates & Nolan 2009), higher prevalence would be expected for pups (< 6 months), and these were not adequately represented in our sample. *Toxocara canis* has been reported at low prevalence in other parasitological surveys of coyotes in western Canada (Holmes & Podesta 1968; Samuel, Ramalingam & Carbyn 1978; Wirsing *et al.* 2007; Thompson *et al.* 2009, Watts 2011), emphasizing the need of larger sample size for proper estimates.

Similarly, we did not record *Alaria* Schrank, 1788, although it is quite widespread in coyotes (Samuel, Ramalingam & Carbyn 1978; Pence 1979; Seesee, Sterner & Worley 1983). This trematode has been previously reported in Calgary at low fecal prevalence (1.6%, Watts 2011). Although the specific gravity of the floating solution we used was not ideal for the recovery of trematode eggs (recommended S.G. = 1.30-1.35, Taylor, Coop & Wall 2007), we did not detect this parasite in intestinal tracts either. The complex life cycle of this trematode, which requires a minimum of two intermediate hosts (acquatic snails, tadpoles and frogs, Pearson 1956), might be rarely completed within and in proximity to ecologically fragmented habitats available in urban Calgary, and could be the cause of its low prevalence.

Our results showed that coyotes are host to several gastrointestinal parasites, including potentially relevant zoonoses, and may have a role for their circulation within the urban settings. Coyote ecology (i.e., diet, spatial behavior), habitat characteristics and dog management likely influence the community of coyote parasites in urban environments, and should be taken into account to assess the actual role of this carnivore in the maintenance of parasite species in the city landscape. Moreover, further research is needed to evaluate whether there is any risk for

transmission of zoonotic parasites (e.g., *E. multilocularis, T. crassiceps*, *Giardia* sp.) among coyotes, dogs and humans.

Acknowledgments

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List of tables

Table 1: Percent prevalence and intensity (median and range, when applicable, in parentheses) of adult gastrointestinal parasites in carcasses of coyotes (*Canis latrans*) collected in themetropolitan area of Calgary, Alberta, Canada (October 2009 – March 2010), and of parasite eggs, oocysts, or cysts in coyote feces collected in Calgary city parks (May 2010 –September 2010).

List of figures

Figure 1: Maps of A) the CMA of Calgary (Geography Division, Statistics Canada, 2006) where 61 coyote carcasses were collected between October 2009 and March 2010; B) the study sites in urban Calgary where 247 coyote feces were collected between May and September 2010. Collection sites in Calgary are respectively: 1) Nose Hill Park; 2) Elk Lodge[*]; 3)Inglewood[*]; 4) Stanley Park; 5) Weaselhead; 6) Southland Lowlands; 7) Willow Park[*]; 8) Fish Creek West; 9) Fish Creek East.

[*] sites where dog access was not allowed

Table 1

Parasite species	Adults			Juveniles			Total in all intestines (N = 61)	Total in feces (N = 247)
	Males (N = 21)	Females (N = 22)	Total (N = 43*)	Males (N = 9)	Females (N = 7)	Total (N = 16*)		
Nematodes								
Toxascaris leonina	76.2 (34; 3-150)	72.7 (14; 1-77)	74.4 (17.5; 1-150)	100 (44; 6-90)	100 (50; 1-146)	100 (45.5; 1-146)	80.3 (29.5; 1-150)	19.0 (49.1; 0.2-253.8)
Uncinaria stenocephala	23.8 (2; 1-5)	27.3 (2; 1-49)	25.6 (2; 1-49)	33.3 (12; 3-24)	57.1 (2; 1-5)	43.7 (3; 1-24)	29.5 (2; 1-49)	-
Pterygodermatites affinis	-	4.5 (2)	2.3 (2)	11.1 (3)	-	6.2 (3)	3.3 (1.5; 2-3)	-
Ancylostoma caninum	4.8 (4)	-	2.3 (4)	-	-	-	1.6 (4)	-
Trichuris vulpis	-	-	-	-	-	-	-	4.4 (3.0; 0.5-250.4)
Cestodes								
Taenia crassiceps	4.8 (9)	-	2.3 (9)	-	14.3 (4)	6.2 (4)	3.3 (6.5; 4-9)	na
Taenia sp.	14.3 (7; 3-16)	18.2 (3; 1-91)	16.3 (4; 1-91)	22.2 (3; 1-5)	28.6 (4.5; 2-7)	25 (3.5; 1-7)	18.0 (4; 1-91)	na
Echinococcus multilocularis	33.3 (3; 1-240)	18.2 (32; 14-151)	25.6 (18.5; 1-540)	55.5 (9; 1-84)	28.6 (59; 9-103)	43.7 (15.5; 1-103)	29.5 (18; 1-240)	na
Taeniidae	-	-	-	-	-	-	-	6.1 (8.1; 0.5-252.4)
Protozoans								
Cystoisospora sp.	-	-	-	-	-	-	-	42.9 (36.5; 0.2-253.8)
Giardia sp.	-	-	-	-	-	-	-	19.8 (na)

Note: Parasite intensity in feces (last column) is the median number of eggs per gram (epg) of feces. na indicates information was not available.

* For two animals (one female and one male), information on the estimated age is not available.

Figure 1

A) Calgary CMA

B) City of Calgary

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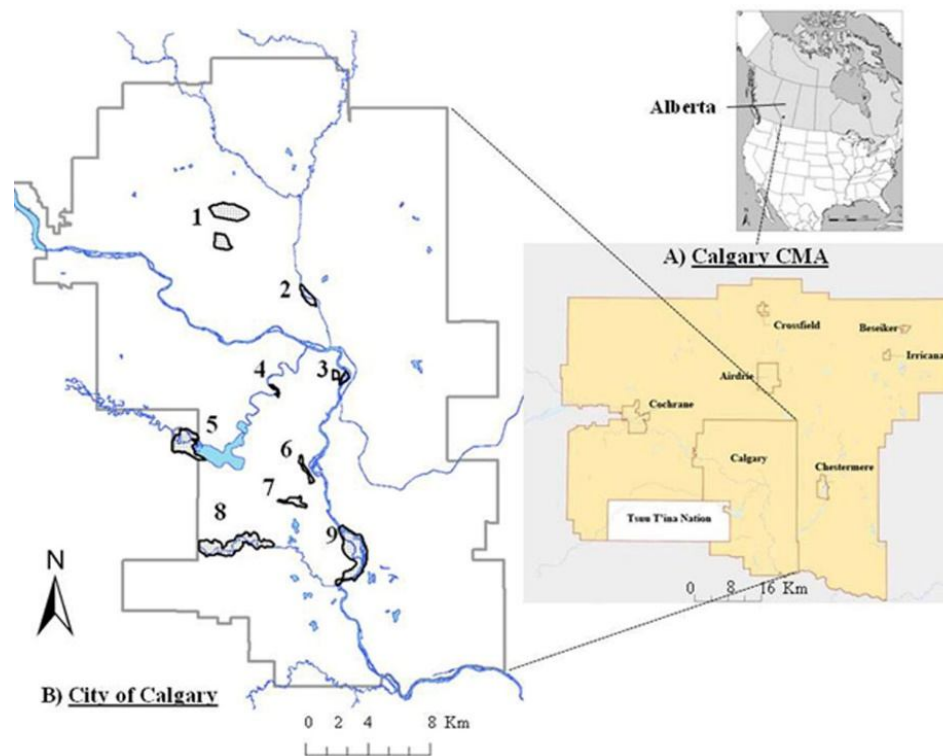
Chapter 3

SENSITIVITY OF DOUBLE CENTRIFUGATION SUGAR FECAL FLOTATION FOR DETECTING INTESTINAL HELMINTHS IN COYOTES (*CANIS LATRANS*)

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Abstract

Fecal analysis is commonly used to estimate prevalence and intensity of intestinal helminths in wild carnivores, but few studies have assessed the reliability of fecal flotation compared to analysis of intestinal tracts. We investigated sensitivity of the double centrifugation sugar fecal flotation and kappa agreement between fecal flotation and postmortem examination of intestines for helminths of coyotes (*Canis latrans*). We analyzed 57 coyote carcasses that were collected between October 2010 and March 2011 in the metropolitan area of Calgary and Edmonton, Alberta, Canada. Before analyses, intestines and feces were frozen at -80 C for 72 hrs to inactivate *Echinococcus* eggs, protecting operators from potential exposure. Five species of helminths were found by postmortem examination, including *Toxascaris leonina*, *Uncinaria stenocephala*, *Ancylostoma caninum*, *Taenia* sp. and *Echinococcus multilocularis*. Sensitivity of fecal flotation was high (0.84) for detection of *T. leonina*, but low for *Taenia* sp. (0.27), *E. multilocularis* (0.46) and *U. stenocephala* (0.00). Good kappa agreement between techniques was observed only for *T. leonina* (0.64), for which we detected also a significant correlation between adult female parasite intensity and fecal egg counts (R_s=0.53, P=0.01). Differences in sensitivity may be related to parasite characteristics that affect recovery of eggs on flotation. Fecal parasitological analyses are highly applicable to study the disease ecology of urban carnivores, and often provide important information on environmental contamination and potential ofzoonotic risks. However, fecal-based parasitological surveys should first assess the sensitivity of used techniques to understand their biases and limitations.

Introduction

Parasitological surveys of wild carnivores are often based on necropsies and direct examination of dead or culled animals (Wobeser 1994). Fecal analysis for parasites is an alternative (Watve & Sukumar 1995; Kloch, Bednarska & Bajer 2005; Miller *et al.* 2009; Stronen *et al.* 2011), even though this technique has some limitations, including variable sensitivity depending on parasite species (Dryden *et al.* 2005), inability to identify eggs morphologically beyond family or genus level (Taylor, Coop & Wall 2007), or sampling temporal mismatches, i.e. samples might be collected during the prepatent period (Snyder & Fitzgerald 1987) or outside egg excretion peaks, when the number of eggs excreted is markedly reduced (i.e., Kapel *et al.* 2006). Moreover, in areas where potential zoonoses are suspected, routine precautions such as deep freezing should be applied to fecal samples in order to protect researchers from potential exposure (Veit *et al.* 1995; Hildreth, Blunt & Oaks 2004). This procedure can additionally interfere with the detection of parasites, depending on morphological characteristics of eggs (Lucker 1941).

Despite these limitations, fecal examinations may be informative for urban wildlife (e.g., Jacobson, Kazacos & Montague 1982), endangered species (e.g., Sleeman *et al.* 2000) or small scale investigations, where animal carcasses are rarely available, or their sampling cannot be planned according to specific study designs. There are, however, only a few examples (e.g., Page, Gehrt & Robinson 2005; Sexsmith *et al.* 2009) where the reliability of fecal flotation has been assessed in relation to examination of intestinal tracts.

As part of a larger research program on coyote disease ecology in urban Calgary, mainly relying on the collection and the analysis of fecal samples, this study aimed to estimate the

reliability of fecal flotation for detection of intestinal helminths of coyotes (*Canis latrans*), and to highlight what factors might influence the detection of parasites in feces.

Material and Methods

Between October 2009 and March 2010, 57 hunted and road-killed coyotes were collected in the metropolitan area of Calgary (51°5'N, 114°5'W; *n*=50) and Edmonton (53°33'N, 113°30'W; *n*=7), AB, Canada. Carcasses were frozen at -20 C until post mortem examination, performed at the Faculty of Veterinary Medicine, University of Calgary.

Intestinal tracts were collected, refrozen at -80 C for 72 hrs to inactivate eggs of *Echinococcus* spp. (Veit *et al.* 1995), and then stored at -20 C until examination. Intestines were thawed and approximately 20.8±5.0 grams of feces were collected from the distal colon and refrozen at -20 C until analysis. Small and large intestines were examined separately for helminths using scraping, washing and sieving (Eckert *et al.* 2001) through a 1x1 mm strainer. Nematodes were preserved in 70% ethanol and later cleared in lactophenol and identified under 100-400x microscope (Anderson *et al.* 2009).

Taenia specimens were identified examining hook size (Jones & Pybus 2008) and relative measure of blade and handle (Hoberg, Ebinger & Render 1999) for each intact scolex. If scolices did not allow for species identification, taeniids were classified only to the genus level. *Echinococcus* specimens were identified morphologically and then molecularly confirmed using species-specific PCR on DNA extracts from worm tissues, following a modification of the methodologies described by Trachsel *et al.*(2007). In our study, simplex-PCR was used to

distinguish among *E. multilocularis*, *E. granulosus*, *Taenia* sp., since it provided more consistent results than those obtained from multiplex PCR.

For fecal analysis, 2 grams of feces were taken from two peripheral and one central region of the fecal sample and homogenized with water (9±1.99 ml). The total volume was equally divided by weight into three subsamples, each representing 2 grams of the original fecal material (plus the water required to homogenize the slurry): two of these subsamples were analyzed using Wisconsin double centrifugation technique and Sheather's sucrose floating solution (S.G.=1.26). Slides were examined at 100-400x and all helminth eggs identified (Foreyt & Foreyt 2001; Hendrix 2006) and counted. *Taenia* sp. and *Echinococcus* sp. eggs are morphologically undistinguishable and were only classified as Taeniidae (Foreyt & Foreyt 2001). Parasite eggs per gram of feces (epg) in the two replicates of each fecal sample were averaged.

Sensitivity of fecal flotation was estimated using postmortem recovery of adult parasites as the gold standard, and calculated as the ratio between the number of true positives correctly detected and the total number of true positives (true detected positives + false negatives).

Kappa coefficient was used to estimate agreement between presence of adult parasites recovered at postmortem examination and detection of parasite eggs by fecal flotation. Kappa values between 0.4 and 0.75 were considered representing fair to good agreement, whereas values below 0.4 were interpreted as poor agreement (Basu & Basu 1995).

Differences in estimated prevalence between fecal flotation and postmortem examination, and in sensitivity between parasite species, were tested with Fisher's Chi-squared test. Differences in parasite intensity (*sensu* Bush *et al.* 1997) between carcasses with positive and

negative fecal samples were assessed with Mann-Whitney U test. Correlation between adult parasite intensity and parasite epg in feces was measured with Spearman's correlation coefficient (R_s). Sensitivity and Kappa analysis were implemented in Win Episcope 2.0 (Thrusfield *et al.* 2001). Statistical analysis was run on the SPSS 17.0 package (IBM, Inc., Armonk, New York, USA).

Results

Five species of helminths were found by postmortem examination, including three nematodes (*Toxascaris leonina*, *Uncinaria stenocephala*, *Ancylostoma caninum*) and two cestodes (*Taenia crassiceps*, *Echinococcus multilocularis*). However, species level identification of *Taenia* could be only performed for specimens from two of 11 intestines. Therefore, in our results we refer only to the genus *Taenia* (*Taenia* sp.).

Only *T. leonina* and Taeniidae eggs were found on fecal flotation. For *U. stenocephala*, prevalence obtained from intestinal tracts was significantly higher (*P*<0.001) than that estimated through fecal examination (Table 1). For the other parasite species, prevalence estimates from postmortem examination were higher, but not significantly.

Sensitivity of fecal flotation differed among parasite species (Table 2), with high sensitivity for *T. leonina* (0.84) and low for Taeniidae (0.36). Based on examination of the intestines, only one coyote was co-infected with *Taenia* and *Echinococcus*: this animal was removed to allow a separate analysis of the sensitivity for *Taenia* sp. (0.27) and *E. multilocularis* (0.46). There was no significant difference between the two taxa (*P*=0.25).

Agreement between fecal flotation and postmortem examination was moderate (kappa=0.64) for *T. leonina*, and poor (kappa<0.4) for all the other parasites. For *T. leonina*, egg detection was associated with adult female parasite intensity (R_s=0.53, P=0.01; Figure 1A and 1B). *Toxascaris leonina* adult females, adult males and total parasite number were significantly higher (all P≤0.001) in coyotes with *T. leonina* positive feces than in those with negative feces.

There was no association between the intensity of parasites in the carcasses and their detectability in feces for either *Taenia* sp. or *E. multilocularis* (P=0.15 and P=0.77, respectively) and no significant correlation between adult parasite intensity and fecal epg (*Taenia*, R_s=0.48, P=0.13; *Echinococcus*, R_s=-0.22, P=0.48).

Discussion

Our results show that double centrifugation sugar fecal flotation underestimates true prevalence of intestinal helminths in coyotes that went under deep-freezing (-80 C) treatmentand two freeze-thaw cycles, and that the magnitude of this relationship is not consistent across parasite species.

Fecal flotation was very sensitive (≥0.89) for detecting presence of *T. leonina* at parasite intensities greater than 25 adult female worms (Figure 1A). We observed higher sensitivity for fecal detection of *T. leonina* than was reported in a previous study on coyotes (0.84 *vs*. 0.64) with similar infection intensities (45.0 *vs*. 43.3, Foreyt & Foreyt 1982). Our higher sensitivity might be related to the use of double centrifugation (Dryden *et al.* 2005), not mentioned in Foreyt and Foreyt (1982). High sensitivity of fecal flotation has also been reported for another

ascarid nematode, *Baylisascaris procyonis* (Sexsmith *et al.* 2009). Ascarids are highly fecund (Richards & Lewis 2001) and produce eggs that are resistant to a variety of environmental and chemical extremes (Parsons 1987) and these characteristics may contribute to good egg recovery despite multiple freeze-thaw cycles.

Fecal flotation was highly variable and unsatisfactory for estimating the prevalence of *Taenia* sp. and *E. multilocularis*. Low sensitivity for Taeniidae may be related to how eggs of these parasites are shed. They are released in proglottids (Jones and Pybus, 2001) and not homogeneously distributed in feces and could, therefore, be missed in the subsamples of feces examined (Gillespie, Greiner & Chapman 2005). Additionally, proglottid release may be highly irregular, with up to five days without shedding (Gregory 1976); low parasite intensity could possibly accentuate this problem. On the other hand, Taeniidae eggs are fairly robust and were reported to be resistant to freezing at -3 C (Lucker 1960), and this should facilitate their detection, even after long term storage and multiple freeze-thaw cycles. Our sensitivity for detection of *Taenia* sp. eggs was higher (0.27 *vs.* 0.13) than that observed previously for coyotes (Foreyt & Foreyt 1982), despite lower infection intensities in our study (10.1 *vs.* 22.0). Subsampling three discrete regions of the fecal samples and the use of the double centrifugation may have increased the sensitivity of our test.

We did not detect any *U. stenocephala* eggs in the feces, despite a prevalence of 28.1% on post mortem examination. *Uncinaria* eggs are recovered quite easily through sugar fecal flotation (Gompper *et al.* 2003; Salb *et al.* 2008) and Foreyt and Foreyt (1982) found a sensitivity of 0.57 for feces collected from coyote carcasses that went through a single freeze-thaw cycle. Strongyles eggs are much more fragile than ascarid or Taeniidae eggs and the initial

deep freeze to inactivate *Echinococcus* eggs, followed by two freeze-thaw cycles likely destroyed many of the *Uncinaria* eggs (Lucker 1941). Additionally, the mean infection intensity in our study was much lower than the intensity found by Foreyt and Foreyt (4.6 *vs*. 82.6), and no adult female parasites were found in 25% of our positive cases (*n*=16). Sensitivity of fecal flotation for this parasite should be tested on a broader range of infection intensities and tolerance of strongyles to freezing should be further investigated to identify storage conditions conducive to egg recovery (Wetzel & Weigl 1994).

Collection and analysis of feces are of value in parasitological assessment of urban carnivores. Fecal parasitological examination allows for larger sample sizes and detection of not only helminths but also protozoan parasites (e.g., *Giardia* sp., *Cryptosporidium* sp., *Cystoisospora* sp.) that are not detectable on gross postmortem examination. Combined with molecular techniques, fecal analyses can identify parasites at the species level, offering a tool to investigate the transmission of zoonotic parasites such as *Echinococcus* (Taylor, Coop & Wall 2007), *Giardia* (Ballweber *et al.* 2010) and *Cryptosporidium* (Hunter & Thompson 2005). Also, for some parasites such as the Taeniidae, copro-antigen enzyme-linked immunosorbent assays (ELISA) tests are available (Torgerson & Deplazes 2009), and have reasonable sensitivity (up to 0.80, Deplazes *et al.* 1999), although the specificity of these tests decreases at low parasite intensity (Allan & Craig 2006). However, in parasitological investigations based on fecal analysis, researchers should to take into account the limitations of the technique.

Furthermore, deep-freezing has likely affected the overall detectability of parasite eggs in feces. Nonetheless, in areas where *Echinococcus* is suspected, freezing at -80 C should be a routine precaution applied to field samples (e.g., Veit *et al.*, 1995; Hildreth *et al.*, 2004) for the

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safety of operators. This represents therefore a reasonable "post-field" condition that had to be included for the interpretation of similar surveys. Other factors, such as field conditions (e.g., weather, environmental exposure before collection) and specific gravity of the floating solution, might also affect the sensitivity of fecal flotation, and should be considered when dealing with fecal samples.

Our results stress the importance of assessing the sensitivity of the fecal analysis technique to properly interpret results of fecal surveys of wildlife populations.

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List of tables

Table 1: Comparison of prevalence and median intensity of helminths in intestines (adult parasites) and feces (epg, eggs per gram) of 57 coyotes collected from October 2009 to March 2010 in Calgary and Edmonton, Canada; *n* represents the number of carcasses infected with each parasite species. Pearson's Chi square test results for difference in parasite prevalence (GI tracts *vs*. feces) are reported.

Table 2: Sensitivity of Wisconsin double centrifuge flotation and agreement coefficient with the postmortem examination for helminths found in 57 coyotes collected in Calgary and Edmonton, Canada, from October 2009 to March 2010. For *Taenia* sp., *T. crassiceps* and *E.multilocularis* only monoinfections were considered.

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Figure 1A: Frequency (grey bars) distribution of *Toxascaris leonina* adult female infection intensity and corresponding sensitivity (solid line) of fecal flotation in coyotes (*n*=57) collected in Calgary and Edmonton, Canada, from October 2009 to March 2010.

Figure 1B: Correlation between adult female intensity and fecal epg (R_s=0.53, *P*=0.01) for *Toxascaris leonina* (*n*=44) in infected coyotes collected in Calgary and Edmonton, Canada, from October 2009 to March 2010.

Table 1

		Prevalence (%)			Intensity (range)	
Parasite species	*n*	Intestines	Feces	*P value*	Intestines	Feces (epg)
Toxascaris leonina	45	78.9	68.4	0.29	27 (2-150)	5.3 (0.25-263.6)
Taeniidae	25	43.9	28.1	0.12	6 (1-240)	17.6 (0.5-252.5)
Taenia sp.[a]	12	21.1	n/a	n/a	4.5 (1-61)	n/a
T. crassiceps	2	3.5	n/a	n/a	6.5 (4-9)	n/a
E. multilocularis	14	24.6	n/a	n/a	16.5 (1-240)	n/a
Uncinaria stenocephala	16	28.1	0.0	<0.001	2.5 (1-24)	n/a
Ancylostoma caninum	1	1.8	0.0	1	4 (4)	n/a

[a] Include specimens identified as *T. crassiceps*, that are presented separately below.

Table 2

Parasite species	*n*	Sensitivity (95% C.I.)	*Kappa* coefficient (95% C.I.)
Toxascaris leonina	45	0.84 (0.74-0.95)	0.64 (0.42-0.87)
Taeniidae	25	0.36 (0.17-0.55)	0.15 (-0.14-0.43)
Taenia sp.[a]	11	0.27 (0.1-0.54)	0.06 (-0.25-0.37)
T. crassiceps	1	0	n/a
E. multilocularis	13	0.46 (0.19-0.73)	0.24 (-0.06-0.55)
Uncinaria stenocephala	16	0	n/a
Ancylostoma caninum	1	0	n/a

[a] Include specimens identified as *T. crassiceps*, that are presented separately below.

Figure 1A

Toxascaris leonina adult female intensity class
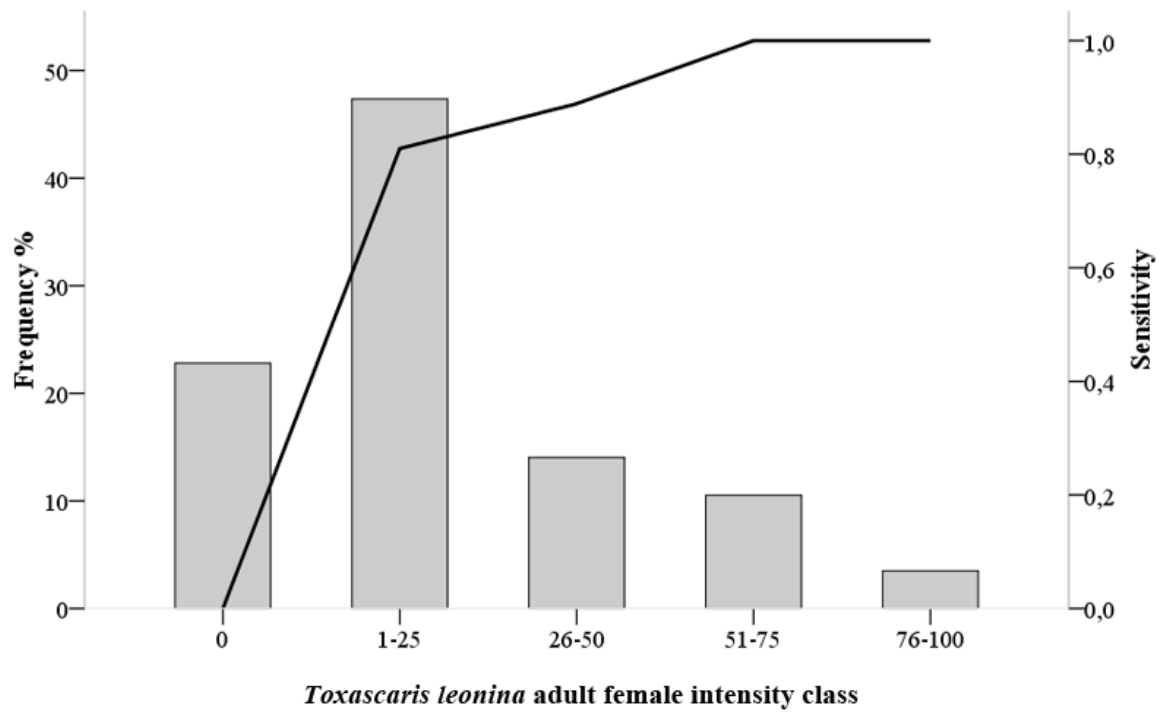
Figure 1B
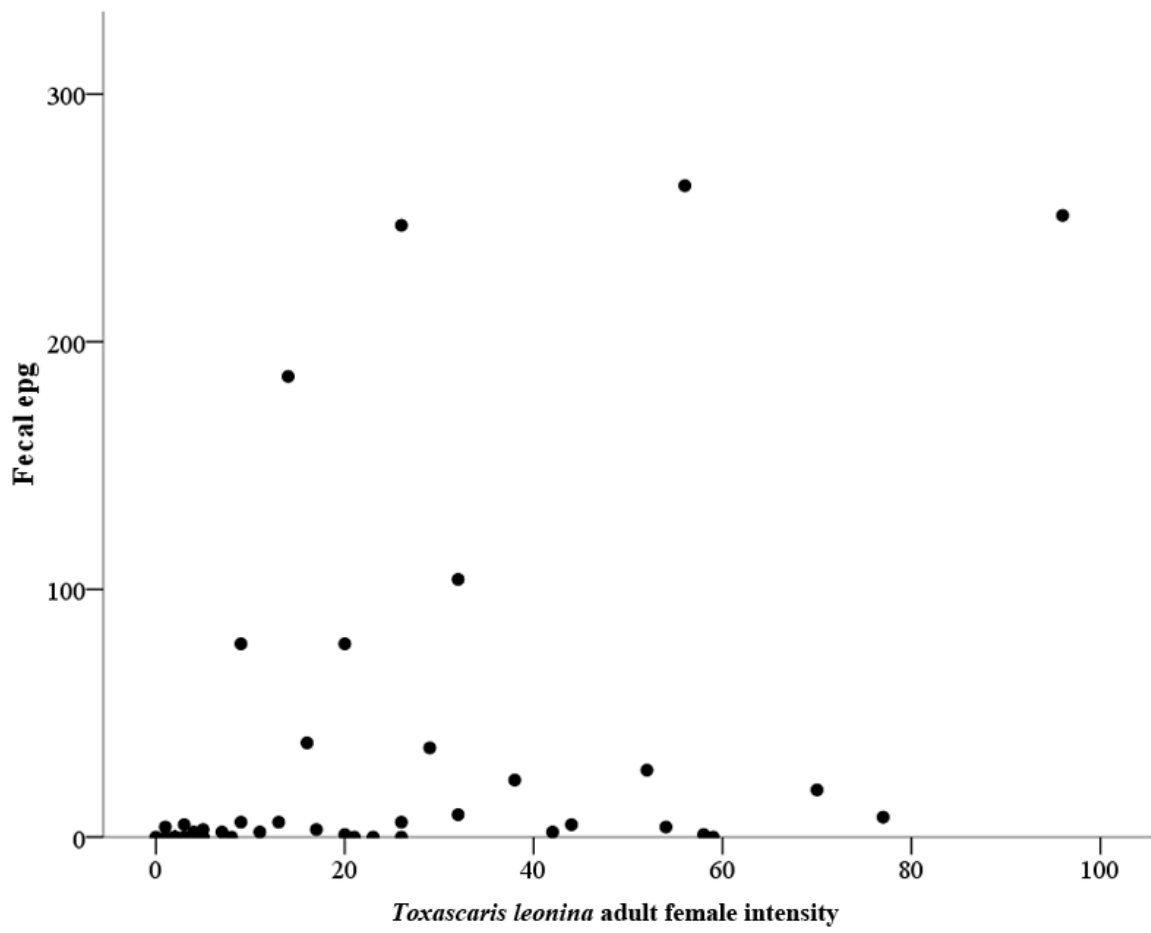
Chapter 4

SPATIAL HETEROGENEITY AND TEMPORAL VARIATIONS IN *ECHINOCOCCUS MULTILOCULARIS* INFECTIONS IN WILD HOSTS IN A NORTH AMERICAN URBAN SETTING

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Abstract

Echinococcus multilocularis, the causative agent of human alveolar echinococcosis, has the potential to circulate in urban areas where wild host populations and humans coexist. The spatial and temporal distribution of infection in wild hosts locally affects the risk of transmission to humans. We investigated the spatial and temporal patterns of *E. multilocularis* infection in coyotes and rodent intermediate hosts within the city of Calgary, Canada, and the association between spatial variations in coyote infection and the relative composition of small mammal assemblages. Infection by *E. multilocularis* was examined in small mammals and coyote feces collected monthly in five city parks from June 2012 to June 2013. Coyote feces were analyzed using a $ZnCl_2$ centrifugation and sedimentation protocol. Infection in intermediate hosts was assessed through lethal trapping and post-mortem analysis. Parasite eggs and metacestodes were morphologically identified and molecularly confirmed through species-specific PCR assays. Of 982 small mammals captured, infection was detected in 2/305 (0.66%) deer mice (*Peromyscus maniculatus*), 2/267 (0.75%) meadow voles (*Microtus pennsylvanicus*), and 1/71 (1.41%) southern red backed voles (*Myodes gapperi*). Overall fecal prevalence in coyotes was 21.42% (*n*=385) and varied across sites, ranging from 5.34% to 61.48%. Differences in coyote fecal prevalence across sites were consistent with local variations in the relative abundance of intermediate hosts within the small mammal assemblages. Infections peaked in intermediate hosts during autumn (0.68%) and winter (3.33%), and in coyotes during spring (43.47%). Peaks of infections in coyote feces up to 83.8% in autumn were detected in a hyper-endemic area. To the best of our knowledge, our findings represent the first evidence of a sylvatic life-cycle of *E.*

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multilocularis in a North American urban setting, and provide new insights into the complexity of the parasite transmission ecology.

Introduction

Echinococcus multilocularis is considered a globally emerging pathogen (Davidson *et al.* 2012). This cestode is the causative agent of human alveolar echinococcosis (HAE), which is among the most serious parasitic zoonoses of the northern hemisphere (Craig, Rogan & Allan 1996). As *E. multilocularis* mainly circulates among definitive and intermediate wild host species, HAE is not considered an eradicable disease (Ito, Romig & Takahashi 2003); understanding the transmission ecology of the parasite is therefore crucial for disease prevention.

The geographic distribution of the parasite, as well as its prevalence in wild hosts, seems to be increasing as a direct or indirect consequence of human activities (Giraudoux *et al.* 2003; Davidson *et al.* 2012). During the last decade, coincident withgrowing urban populations of red foxes (*Vulpes vulpes*), *E. multilocularis* has been documented to circulate within numerous cities of Europe (Hofer *et al.* 1999; Deplazes *et al.* 2004) and Japan (Tsukada *et al.* 2000; Yimam *et al.* 2002). Given the close proximity to humans, such a phenomenon can potentially represent a public health emergency. This is well exemplified by the densely inhabited grasslands of the Tibetan plateau and China, where intense parasite transmission results in high incidences of HAE (Tiaoying *et al.* 2005; Giraudoux *et al.* 2006).

In North America, *E. multilocularis* was historically reported in the Northern Tundra Zone of Alaska and Canada (Eckert, Conraths & Tackmann 2000). Since the 1960s, the parasite has been reported in 13 US states and four Canadian provinces (Alberta (AB), Saskatchewan (SK), Manitoba (MB), British Columbia (BC)) (Eckert *et al.* 2001; Jenkins *et al.* 2012), thus defining a second area of distribution, the North Central Region (NCR). In the NCR, definitive hosts are mainly represented by red foxes and coyotes (*Canis latrans*), whereas intermediate host

species listed to date include deer mouse (*Peromyscus maniculatus*), meadow vole (*Microtus pennsylvanicus*), southern red-backed vole (*Myodes gapperi*), house mouse (*Mus musculus*) and bushy-tailed woodrat (*Neotoma cinerea*) (Hnatiuk 1966; Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Kritsky, Leiby & Miller 1977; Liccioli *et al.* 2013). Recently, *E. multilocularis* was reported in coyotes within metropolitan areas of Alberta, Canada (Catalano *et al.* 2012), but no information was yet available regarding the transmission ecology of the parasite in this environment.

Recent evidence indicated that *E. multilocularis* transmission is spatially clustered, both at a regional (Viel *et al.* 1999; Said-Ali *et al.* 2013; Tolnai, Szell & Sreter 2013) and local spatial scale (Giraudoux *et al.* 2007). Although over large regions the main intermediate host species have been clearly identified (e.g., *Arvicola sherman* and *Microtus arvalis* in Europe, Deplazes *et al.* 2004), understanding *E. multilocularis* transmission dynamics still needs to be approached by looking at the entire complexity and composition of small mammal assemblages (Giraudoux *et al.* 2003). Unfortunately, to date research has often focused only on intermediate host population density as the main parameter regulating patterns of infection (Saitoh & Takahashi 1998; Hegglin *et al.* 2007; Raoul *et al.* 2010).

Previous research also suggested that infections by *E. multilocularis* follow seasonal patterns, with higher prevalence recorded during winter for both definitive (Hofer *et al.* 2000) and intermediate hosts (Burlet, Deplazes & Hegglin 2011). However, only a few studies have described the temporal patterns of infection throughout the year for both definitive and intermediate hosts within the same area (Stieger *et al.* 2002), and certainly no information is available for North American urban landscapes.

Herein, we aimed to: i) investigate spatiotemporal patterns of *E. multilocularis* infections in coyotes and rodent intermediate hosts in an urban landscape; and ii) assess the association between spatial variations in coyote infection and the relative abundance of intermediate host species within the small mammal assemblages.

Given the low overall prevalence (<1%) observed for intermediate hosts (Giraudoux *et al.* 2003) and the trophic linkage between intermediate and definitive hosts, we expected infection in coyotes to respond to spatial and temporal variations of prevalence in intermediate hosts. We also expected *E. multilocularis* fecal prevalence in coyotes to be higher in areas where the relative abundance of intermediate host species within the small mammal assemblage is higher, and to observe a time lag (3-4 months) between the peak of infection in rodents and coyotes, consistent with the prepatent period in the canid host (Eckert *et al.* 2001; Jones & Pybus 2008).

Materials and methods

Study area and sample collection

The study occurred in five urban parks and natural areas of the city of Calgary (51°5′N, 114°5′W), AB, Canada: Nose Hill Park (NHP; 1,127.9 hectares (ha)), Bowmont (BM; 63.5 ha), Weaselhead (WSH; 208.7 ha), Southland Lowlands (SL; 15.0 ha) and Fish Creek Provincial Park (FCPP; 3,400.0 ha) (Fig. 1A).

From June 2012 to June 2013, in each area coyote feces were collected along standardized trails and paths (Liccioli *et al.* 2012a), as well as opportunistically in areas known

to be used by the animals. Feces were identified as from coyotes and aged as described in (Liccioli *et al.* 2012a).

In the same parks, small mammals were trapped, mostly using Woodstream© Museum Special Traps (7 x14cm) baited with a mixture of oatmeal and peanut butter. To reduce the risk of trap misfire, at specific points with low vegetation and in proximity of human trails, snap traps were replaced by Longworth© small mammal live traps (14 x 6.5 x 8.5cm), always representing ≤ 10% of the total number of traps set.

Small mammal capture sites constituted rectangular grids of 200 traps, set at regular intervals (7-10 m, depending on the shape and size of the site). Traps were checked and re-set every morning for three days (Millar, Xia & Norrie 1991), for a maximum total of 600 trap-nights per capture session (not controlling for misfires). Capture sites were selected in order to be representative of the main habitat types available in each park (e.g., grassland, shrubs, forest). Each park had three different capture sites, with the exception of SL which had only two due to its small area. Even in the smallest park (SL), the shortest distance between capture sites was >300 m (and up to 1 km in larger parks), bigger than the average dispersal distance recorded for *Peromyscus* and vole species (King 1968; Boonstra *et al.* 1987; Andreassen & Ims 2001). Every month we sampled all the parks, rotating among different capture sites (Fig. 1B).

For each capture day, we recorded the number of traps that were active throughout the night (Village & Myhill 1990), considering as misfired any trap found sprung, missing, with no bait, or not triggered. Small mammals that were caught alive (in live traps or not killed in snap-traps) were immediately euthanized through cervical dislocation by trained personnel.

The animal use protocol was approved by the Animal Care Committee of the University of Calgary (AC12-0037), Canada.

Echinococcus multilocularis in intermediate hosts

Small mammals were necropsied by trained personnel under level 2 conditions in a biosafety cabinet, in order to protect operators from potential Hantavirus exposure. Animals that could not be necropsied on the day of collection were frozen at -20°C until analysis. At postmortem examination, small mammals were classified as adults or juveniles, based on combined information on body weight, body length and gonad development (Henttonen *et al.* 2001). Animals were morphologically inspected for *E. multilocularis* alveolar hydatid cysts (Liccioli *et al.* 2013). Any suspected lesion or mass in the abdomen was collected, stored in 95% ethanol and tested molecularly. Extraction of DNA was performed using the QiagenDNeasy Blood & Tissue kit, with a final elute of 300 µL (Liccioli *et al.* 2013). Parasite identity was then confirmed through species-specific PCR as described by (Catalano *et al.* 2012).

Small mammal availability and assemblage composition

The relative availability of small mammals was assumed to be reflected in their different capture rate (Calhoun 1956; Woodman *et al.* 1996). For each trapping session, the effective capture rate of small mammals was calculated by dividing the number of animals caught by the number of active traps and multiplying it by 100 (%) (Village & Myhill 1990). For this estimate, capture nights during which snowfall resulted in complete coverage of the traps were excluded from the analysis.

For each season and park, the proportionof intermediate hosts (IH) out of the total of small mammals captured was calculated as IHs/(IHs + NIHs), where NIHs are individuals of species not previously reported as intermediate hosts (Hnatiuk 1966; Lee 1969; Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Liccioli *et al.* 2013). As older animals are more likely to have been infected by *E. multilocularis* (Burlet, Deplazes & Hegglin 2011), we also calculated the proportion of adult animals out of the total number of intermediate hosts captured as adults/(adults + juveniles).

Echinococcus multilocularis in coyotes

Immediately after collection, coyote feces were frozen at -80°C for 72 h to inactivate *E. multilocularis* eggs and protect operators from exposure (Veit *et al.* 1995), and then stored at -20°C until analysis. Feces were processed using the $ZnCl_2$ centrifugation and sedimentation protocol (Davidson *et al.* 2009), with the modifications herein described. We analyzed a total of 2 g of fecal material, taken from three different areas of the feces to maximize the chance of sampling *E. multilocularis* proglottids (Liccioli *et al.* 2012b). After the addition of $ZnCl_2$ (specific gravity = 1.45) and centrifugation of samples as described in (Davidson *et al.* 2009), sequential sieving of supernatant was performed using nylon filters of 44 μm and 21 μm mounted on an inverted plastic cup used as a pedestal after removal of the bottom. Sieved supernatant was allowed to sediment in distilled water at 4°C overnight, and further concentrated into a final volume of 500 μL. After proper homogenization of the total sediment, a maximum of three aliquots of 100 μL were microscopically analyzed and taeniid eggs identified and counted at 100X-400X magnification (Foreyt & Foreyt 2001). Microscopic analysis was stopped after the

first positive aliquot was examined. Infection intensity with Taeniidae (eggs per gram of feces) was calculated using the estimate from the aliquots (*n* eggs/volume analyzed) and relating it to the total sediment volume (500 μL) and to the original weight of examined feces (in g) as the following: ((*n* eggs / aliquot volume)x(total volume/aliquot volume)/(g of feces analyzed)).

To estimate the sensitivity of our fecal analysis protocol, we applied the same methodology to feces collected from the intestinal tract of 16 coyotes, for which postmortem recovery of adult parasites indicated infection with *E. multilocularis*, but no other taeniid species (Liccioli *et al.* 2012b). Parasitological examination of coyote intestines was a modification of the procedure described in (Catalano *et al.* 2012). We added two more sieves to the overall process using mesh sizes of 1000 μm, 500 μm and 212 μm. After homogenization, 25% of the material collected by each filter was then analyzed for adult *E. multilocularis*; in cases of low infection (<100 worms) levels, the whole volumes were analyzed.

DNA was extracted from fecal samples positive for taeniid eggs using the pathogen detection protocol of the E.Z.N.A.® Stool DNA Kit (Omega bio-tek, USA), with a final elution of 150 μL. One μL of extracted DNA was then amplified in a simplex PCR containing 6.25 μL of Accustart™ II PCR Supermix (Quanta BioSciences Inc., USA), 4.75 μL of distilled water, and 0.25 μL of species-specific primers (Trachsel, Deplazes & Mathis 2007; Catalano *et al.* 2012), using the following protocol: 94°C for 1 min, 40 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final 10 min extension at 72°C. Amplicons of expected 395 bp, 117 bp and 267 bp were used to identify infection with *E. multilocularis, Echinococcus granulosus* and *Taenia* spp., respectively (Trachsel, Deplazes & Mathis 2007).

We estimated *E. multilocularis* fecal prevalence for each site and each season by correcting the fecal prevalence of taeniid eggs by the proportion of samples molecularly confirmed to be positive for *E. multilocularis*, as calculated by the formula $P_{Em} = P_T$ x (N_{Em}/Tot_{MolID}), where P_{Em} is the fecal prevalence of *E. multilocularis*; P_T is the fecal prevalence of Taeniidae; N_{Em} is the number of samples molecularly identified as positive for *E. multilocularis*; Tot_{MolID} is the total number of samples for which molecular confirmation was obtained.

Statistical analysis

For each park, *E. multilocularis* prevalence confidence intervals (CIs) in intermediate hosts and coyote feceswere estimated using Sterne 95% CI,when at least one positive case was detected (Reiczigel, Foldi & Ozsvari 2010), or the Median Unbiased Estimate (MUE), when no positive cases were found (Hirji, Tsiatis & Mehta 1989).

For both coyote and intermediate hosts, differences in occurrence of *E. multilocularis* among seasons and sites were tested with Pearson's Chi-square permutation test.Variations in the proportion of intermediate hosts captured among sites, as well as the proportion of adult intermediate hosts among seasons, were tested using the Pearson's Chi-square test. Differences in the availability of intermediate hosts among sites and seasons, as well as differences in taeniid eggs per gram of feces of definitive hosts, were tested by a Kruskal-Wallis test for *k* independent samples, or by a Friedman test for paired samples as appropriate (i.e. comparisons across seasons). For multiple comparisons, we used a Mann-Whitney test for independent samples or the Wilcoxon test for paired ones and applied the Bonferroni correction to the type one error threshold (Curtin & Schulz 1998). The probability levels were computed using a complete

randomisation method (permutation or exact test; P_{Exact}) or by a Monte Carlo simulation based on a 10,000 sampled tables ($P_{MonteCarlo}$) when computation was not possible (Mehta & Patel 1996; Good 2000).

Means ± S.E.M. are reported throughout the text. All the analyses were run on SPSS version 20.0 (IBM Corporation, USA) and EpiTools (http://epitools.ausvet.com.au/). $P \leq 0.05$ was considered significant.

Results

Echinococcus multilocularis in intermediate hosts

Over 24,821 trap-nights (5,379 misfires on a total of 30,200 night-traps), 982 small mammals were captured and necropsied (3.95% capture rate), detecting *E. multilocularis* infection in three rodent species: 0.66% (2/305) in deer mouse (*Peromyscus maniculatus*), 0.75% (2/267) in meadow vole (*Microtus pennsylvanicus*) and 1.41% (1/71) in southern red-backed vole (*Myodes gapperi*). Of five positive cases, only one (20%) contained *E. multilocularis* protoscolices (*M. gapperi*) (Table 1).

The estimated parasite prevalence in intermediate hosts showed a positive trend from summer (0.46%) to autumn (0.68%), reaching its peak during winter (3.33%) and its minimum during spring (0%) (Fig.2A). Among study areas, the highest prevalence was estimated for BM (2.13%), followed by NHP (1.22%) and SL (1.06%), while no positive cases were found in WSH or FCPP (Fig. 3A). Due to the low frequency of positive cases, we could not detect any significant difference among sites (X^2_{Exact}=6.075; df=4; P=0.144) and seasons (X^2_{Exact}=4.161; df=3; P=0.258).

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Small mammal availability and assemblage composition

The mean capture rate of intermediate hosts varied significantly across seasons (Friedman test, X^2= 12.600; df=3; *P*=0.001), with a peak during summer (4.25 ± 1.17) and the minimum values during winter (1.03 ± 0.21) and spring (0.92 ± 0.19), although no significant difference was detected between seasons once the type one error threshold was modified according to the Bonferroni method to account for multiple comparisons (Wilcoxon paired test, $P_{(0.05;\ 6)}$ >0.0083).

Conversely, among intermediate hosts, the proportion of adults significantly differed across seasons (Friedman test, X^2= 11.809; df= 3; *P* = 0.002) overall, with a maximum in winter (0.99±0.01) and spring (0.95±0.02), and a decrement in summer (0.77±0.04) and autumn (0.76±0.08), but no significant difference was detected between seasons once the type one error threshold was modified according to the Bonferroni method to account for multiple comparisons (Wilcoxon paired test, $P_{(0.05;\ 6)}$ >0.0083).

For all the species, no juveniles were captured between November 2012 and April 2013. For deer mouse and meadow vole, the most common IHs, the peak in the capture rate (%) of juveniles was recorded in September 2012 (deer mouse=2.45±1.35; meadow vole=4.27±2.53).

Mean overall capture rate of intermediate hosts did not differ among parks (X^2=3.447; df=4; *P*=0.486), but the proportion of intermediate hosts (IHs/(IHs + NIHs)) overall varied significantly (X^2=110.671; df=4; *P*<0.001), ranging from a maximum of 0.77 (±0.07) in BM to a minimum of 0.36 (±0.07) in SL. Differences across sites were more pronounced during autumn/winter (X^2=77.950; df=4; *P* <0.001), when the proportion of IHs was highest in BM (0.82 ± 0.07) and lowest in SL (0.31± 0.07) (Fig. 3B). During this time interval the proportion of

intermediate hosts in SL was significantly lower than NHP (X^2=48.280; df=1; P <0.001), BM (X^2= 32.410; df=1; P <0.001) and WSH (X^2= 51.997; df=1; P <0.001). Among NIHs, the most common species was represented by shrews (*Sorex* sp.), which are a prey species of coyotes in the study area, occurring in around 7.5% of feces submitted to diet analysis (Liccioli *et al.*, unpublished data).

Echinococcus multilocularis in coyotes

Sensitivity of the $ZnCl_2$ centrifugation and sedimentation used for the detection of *E. multilocularis* eggs in coyote feces was estimated at 75% (12/16).

A total of 385 coyote fecal samples were collected, of which 95 (24.68%) were positive for taeniid eggs. Molecular identification of parasites was performed for 53 of 95 samples, for a total identification success rate of 55.79%. The identified samples were distributed as follows: 40 (75.47%) amplified only for *E. multilocularis*, six (11.32%) for both *E. multilocularis* and *Taenia* spp., and seven (13.21%) for *Taenia* spp. only. No infections with *E. granulosus* were detected.

The overall estimated fecal prevalence of *E. multilocularis* in coyotes was 21.42%, with significant variations over time (X^2= 35.654; df=3; $P_{MonteCarlo}$<0.001). Summer fecal prevalence (10.52%) increased during autumn (21.8%) and winter (19.4%), and reached a peak during spring (43.47%). Fecal prevalence in spring was significantly higher than in summer (X^2= 28.164; df=1; $P_{MonteCarlo}$<0.001), autumn (X^2= 13.264; df=1; $P_{MonteCarlo}$<0.001) and winter (X^2= 7.352; df=1; $P_{MonteCarlo}$= 0.005) (Fig. 2B).

A similar significant pattern was detected for BM and NHP (respectively, X^2= 13.011; df = 3; $P_{MonteCarlo}$= 0.005, and X^2= 19.682; df=3; $P_{MonteCarlo}$<0.001), but not for the other parks ($P_{MonteCarlo}$> 0.05).

Parasite prevalence in coyote feces differed significantly across study areas (X^2= 171.857; df=4; P <0.001); corrected *E. multilocularis* fecal prevalence in BM (63.07%) was significantly higher than in all the other parks (NHP= 17.28%, X^2= 38.897, df=1, P <0.001; FCPP= 6.23%, X^2= 91.252, df=1, P <0.001; WSH=6.22%, X^2= 82.697, df=1, P <0.001; SL=5.42%, X^2= 71.372, df=1, P <0.001) (Fig.3A). No significant difference was detected among the other study areas. As sample size and fecal prevalence differed among parks, we also analyzed seasonal prevalence separately for each study area. For three parks (NHP, SL, FCPP) higher fecal prevalence was observed in spring, whereas for WSH higher fecal prevalence occurred in winter. An exception was represented by BM, which represented a focus of infection with high fecal prevalence constantly detected from autumn to spring. In this park, a higher intensity of taeniid eggs in coyote feces was estimated in winter, although due to the small sample size we could not detect any significant difference among seasons (Kruskal-Wallis test, X^2= 3.386, df=3, $P_{MonteCarlo}$=0.332). Overall, we could not detect any significant difference in the amount of taeniid eggs per gram of feces across seasons (Friedman test, X^2= 1.408, df=3, P_{Exact}=0.742) (Fig.4A), but clear differences across parks were detected (Kruskal-Wallis test, X^2= 25.268, df=4, $P_{MonteCarlo}$<0.001), with intensity of taeniid egg infection in Bowmont being significantly higher than that in FCPP, NHP and SL (Mann-Whitney test with Bonferroni corrections for multiple comparisons) (Fig.4B).

Discussion

Our data support the existence of a sylvatic life-cycle of *E. multilocularis* in urban Calgary, and we detected temporal variations in the infection of wild hosts. Furthermore, our data highlighted a clear spatial heterogeneity in infection in definitive hosts, likely as the result of the interplay of prevalence in intermediate hosts and their relative abundance in the small mammal prey assemblage.

To our knowledge, this is the first study documenting the existence of a sylvatic life-cycle of the parasite in an urban setting of North America. The circulation of *E. multilocularis* within urban parks and recreational areas has been investigated in Europe (Hofer *et al.* 1999; Deplazes *et al.* 2004) and Japan (Yimam *et al.* 2002). In Europe, increased prevalence and range expansion have been reported for *E. multilocularis* during the last decade (Davidson *et al.* 2012), and the parallel increase in the number of human cases (Moro & Schantz 2009) emphasizes the relevance of this potential zoonosis, especially where the parasite circulates in close proximity to humans. As recreational areas in Calgary are heavily used by people and dogs, it is first of all a public health priority to increase general awareness of the subject, in order to minimize the risk of zoonotic transmission. Other rodent species, not sampled in our study, could also have a significant role in maintaining the parasite in the environment; in particular, the muskrat (*Ondatra zibethicus*) appears to be a competent intermediate host (Baumeister *et al.* 1997; Umhang *et al.* 2013) that needs to be considered in future studies. Further research is certainly required to fill the knowledge gap on the circulation of *E. multilocularis* within North America urban settings.

The overall estimated parasite fecal prevalence in coyotes (21.42%) confirms the sylvatic cycle of the parasite can persist even with low (<2%) prevalence in intermediate hosts (Eckert *et al.* 2001). Furthermore, the fecal prevalence in coyotes is consistent with what we observed by postmortem analysis (Liccioli *et al.* 2012a), possibly reflecting an improved detection of the parasite in feces compared with the sugar flotation technique previously adopted (Liccioli *et al.* 2012b); we thus recommend the use of $ZnCl_2$ centrifugation and sedimentation for large screening of canid hosts.

Seasonal variations in the age structure of rodent populations are considered an important factor influencing *E. multilocularis* prevalence in intermediate hosts, as lifetime exposure to parasite infection in adult animals is greater than in younger individuals (Reperant *et al.* 2009; Burlet, Deplazes & Hegglin 2011). Despite the lack of power of our statistical analysis - a common challenge when dealing with the low prevalence observed in intermediate hosts (Giraudoux *et al.* 2003) - our results seem to be consistent with these findings. At our study location, reproductive activity of intermediate hosts was highly seasonal, with a peak in the number of juveniles observed at the end of the summer. Throughout this season, juvenile intermediate hosts are therefore likely to reduce the encounter rate of definitive hosts with the parasite. Starting from autumn, such 'dilution' would progressively decrease, as intermediate host populations mostly consist of adult animals. Additionally, better survival of parasite eggs in cold weather (Veit *et al.* 1995; Burlet, Deplazes & Hegglin 2011) can further explain the higher rate of infection observed for intermediate hosts in our study during winter. As alveolar cysts of *E. multilocularis* develop within 2 weeks after infection (Thompson & Lymbery 1995), seasonal

prevalence in intermediate hosts should reflect exposure during the same time interval (Henttonen *et al.* 2001).

Temporal variations in *E. multilocularis* fecal prevalence in coyotes apparently responded to fluctuations in the infection rate recorded in intermediate hosts. It seems reasonable to infer that autumn-winter are crucial seasons for the infection of definitive hosts; the time lag observed between the peak of prevalence in intermediate hosts and infection in coyotes was consistent with the time required for protoscolices to develop in the rodent liver (2-3 months after infection; Thompson & Lymbery 1995) and the patency period observed in the canid host (30-90 days post-infection; Eckert *et al.* 2001; Kapel *et al.* 2006; Jones & Pybus 2008). In a focus of infection (BM), a lack of a temporal pattern in the definitive host fecal prevalence could be caused by a higher re-infection rate (Tackmann *et al.* 2001); however, the trend observed for the intensity of taeniid eggs in coyote feces seems consistent with temporal variations recorded for the other sites.

As rodent intermediate host populations can dramatically fluctuate following pluriannual cycles (Tkadlec & Zejda 1998), which may differ among species (Giraudoux *et al.* 2007), there is high potential for complex interactions between variations in the prey assemblage composition and seasonal patterns of infection. Unfortunately, our temporal series of data is still limited in its ability to properly understand the role of different species in the maintenance of the parasite. Moreover, small mammal capture rates may reflect more environmental conditions or behavioral differences across species (Gentry, Golley & McGinnis 1966; Wiener & Smith 1972), rather than actual animal abundances; our estimates of intermediate host abundance, therefore, need to be confirmed with further field experiments testing different trapping methods.

We detected a clear spatial heterogeneity in *E. multilocularis* infection, with a highly endemic focus of infection in a park (BM), and lower fecal prevalence observed elsewhere. As the functional response of a generalist carnivore host to prey availability can alter the dynamics of trophically transmitted parasites (Hegglin *et al.* 2007; Raoul *et al.* 2010), density of intermediate host populations is commonly assumed to be the main variable shaping *E. multilocularis* transmission patterns (Saitoh & Takahashi 1998; Giraudoux *et al.* 2003; Raoul *et al.* 2010). However, our data support the hypothesis that the relative composition of prey assemblages - rather than the abundance of single species - is a key feature in the transmission ecology of the cestode. Among our study sites, the only exception to the relationship between definitive host and intermediate host prevalence is represented by SL (Fig. 3A), where detectable prevalence of *E. multilocularis* in intermediate hosts is not reflected in coyote feces. Although for this study area the capture rate of intermediate hosts per se did not differ from other parks, when we examined the whole assemblage of small mammals we recorded the lowest proportion of intermediate hosts. This was particularly evident in autumn/winter, which our data suggested to be the most important season for the infection of coyote hosts. Similarly, for BM and NHP the highest proportion of intermediate hosts is associated with high fecal parasite prevalence in definitive hosts. As morphological detection of taeniid infection is likely to miss immature liver lesions (Al-Sabi *et al.* 2013), morphologically undetectable larval stages might have not been tested molecularly and the parasite prevalence in intermediate hosts that we reported could consequently be an underestimation. Although further research is needed to confirm these results and address these limitations, to our knowledge, this is one of the first studies in which the

relevance of prey assemblage composition in the transmission ecology of *E. multilocularis* is supported by data.

The relative abundance of competent intermediate hosts of *E. multilocularis* is thus possibly shaped at two scales: i) temporally, by the dynamics of small mammal populations, which influence the relative abundance of juvenile and adult animals; and ii) spatially, by habitat characteristics determining the composition of small mammal assemblages. As the definitive host infection rate can be reduced by non-competent intermediate hosts through the 'dilution effect'(Keesing, Holt & Ostfeld 2006; Randolph & Dobson 2012), these factors could interact and determine the pattern of infection observed in coyotes.

A potential for biases that need to be acknowledged arises from the large variability in the area of the study sites, as well as from possible repeated sampling of the same individual coyotes. Consequently, it is now necessary: i) to investigate diet and parasitic infection of individual coyotes and confirm seasonal and spatial patterns; ii) to conduct long-term monitoring of rodent intermediate hosts and assess more robust estimates of population density (e.g. capture-mark-recapture methods), and apply more sensitive techniques to detect infection in IHs; and iii) to investigatethe definitive host functional response to variations in prey abundance.

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Figure 1: Study sites for *Echinococcus multilocularis* detection in wild hosts in urban Calgary, Alberta, Canada: (A) Nose Hill Park (NHP; 1,128.0 hectares (ha)), Bowmont (BM; 63.5 ha), Weaselhead (WSH; 208.7 ha), Southland Lowlands (SL; 15.0 ha) and Fish Creek Provincial Park (FCPP; 3,400.0 ha).(B) A representative flow chart of rodent capture site rotation within a park (June 2012 to June 2013): 1 (June, September, December, March), 2 (July, October, January, April), 3 (August, November, February, May), where the distance between capture sites was always >300m.

Figure 2: Estimated seasonal prevalence of *Echinococcus multilocularis* in intermediate (A; rodents) and definitive (B; coyote feces) hosts in five sites in urban Calgary, Alberta, Canada, in 2012-2013.Means, 95% confidence intervals (CIs) and Pearson's Chi square results are displayed. Sample sizes are indicated below the x-axis.IHS, intermediate hosts.***$P_<$0.001.

Figure 3:(A) Estimated prevalence of *Echinococcus multilocularis* in coyote faeces and rodent intermediate hosts (IHs) in five sites in urban Calgary, Alberta, Canada, 2012-2013. Means, 95% confidence intervals (CIs) and Pearson's Chi square results are displayed. Sample sizes (coyote; IHs) are indicated below the bars. (B) Proportion of *E. multilocularis* IHs of the total small mammal assemblage (including not intermediate host species, NIH) collected in five sites in urban Calgary, Alberta, Canada, between September 2012 and February 2013. Pearson's Chi square results are displayed. Boxes representinter-quartile distances; thick line, median;

whiskers, extreme values. Circles and asterisks represent outliers (thatextend for more than 1.5 times the interquartile distance) and extreme outliers (i.e. extend more than three times the inter-quartiledistance), respectively. Numbers along the x axis represent the total number of cases. Details of location abbreviations are provided in the Fig. 1 legend.***$P < 0.001$.

Fig. 4.Temporal (A) and spatial (B) variation of taeniid egg infection intensity in coyote faeces (epg, eggs per gram of faeces) collected in five sites in urban Calgary, Alberta, Canada, in 2012-2013. Boxes represent inter-quartile distances; thick line, median; whiskers, extreme values. Circles and asterisks represent outliers (that extend for more than 1.5 times the interquartile distance) and extreme outliers (i.e. extend more than three times the inter-quartile distance), respectively. Numbers along the x axis represent the total number of cases. Details of location abbreviations are provided in the Fig. 1 legend. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Table 1

Species	n	Lesions	Taenia spp. % (pos.)	E. multilocularis % (pos./inf.)
Deer mouse (*Peromyscus maniculatus*)[a]	305	34	3.93 (12)	0.66 (2/0)
Meadow vole (*Microtus pennsylvanicus*)[a]	267	29	5.24 (14[1])	0.75 (2[1]/0)
Shrews (*Sorex* sp.)	296	2	0	0
Southern red-backed vole (*Myodes gapperi*)[a]	71	5	4.23 (3)	1.41 (1/1)
Western jumping mouse (*Zapus princeps*)	32	5	0	0
Thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*)	6	2	0	0
Least chipmunk (*Tamias minimus*)	4	0	0	0
Northern pocket gopher (*Thomomys talpoides*)	3	0	0	0
House mouse (*Mus musculus*)[a]	2	0	0	0

[a] Previously documented intermediate hostspecies for *E. multilocularis* in North America (Hnatiuk 1966; Lee 1969; Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Liccioli *et al.* 2013).

[1] The number of *Taenia* spp. and *Echinococcus multilocularis* co-infections.

Figure 1

Figure 2

A

% Prevalence IHs

| Summer | Autumn | Winter | Spring |
| n=215 | n=294 | n=60 | n=76 |

B

% Prevalence Coyote

***

| Summer | Autumn | Winter | Spring |
| n=134 | n=142 | n=36 | n=73 |

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Figure 3

A

B
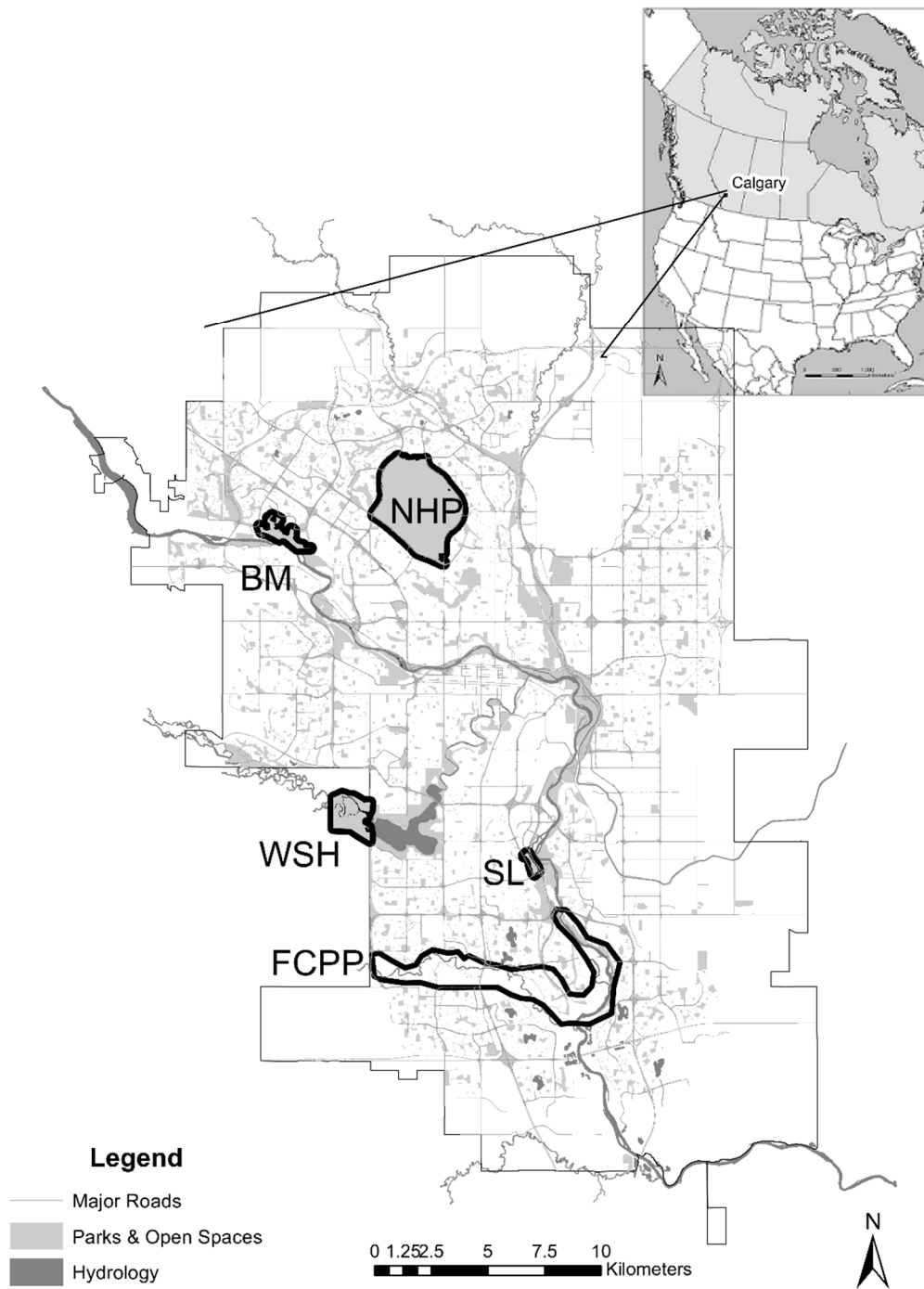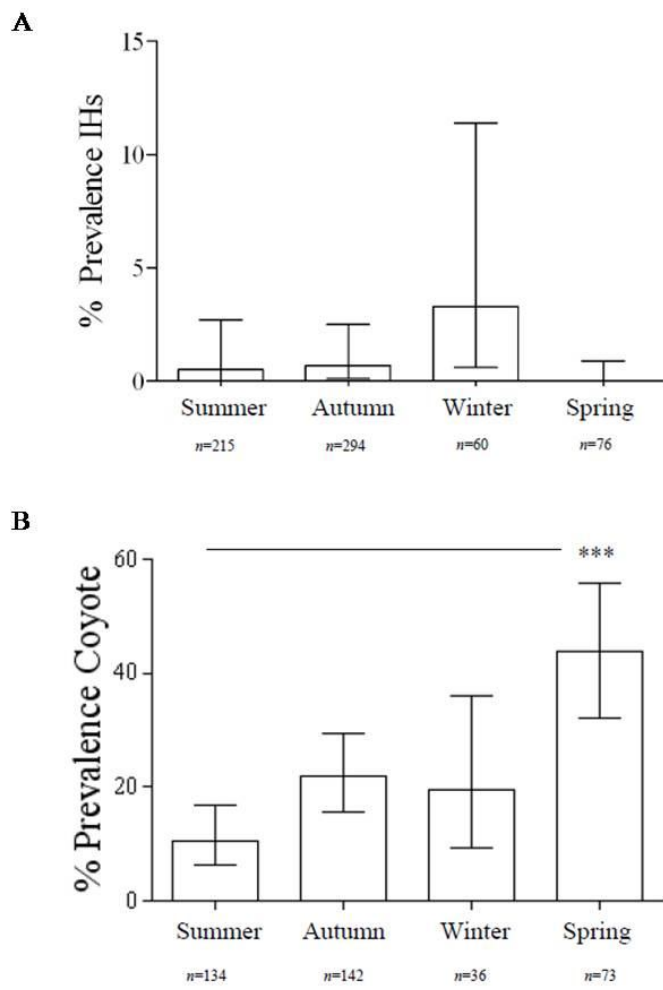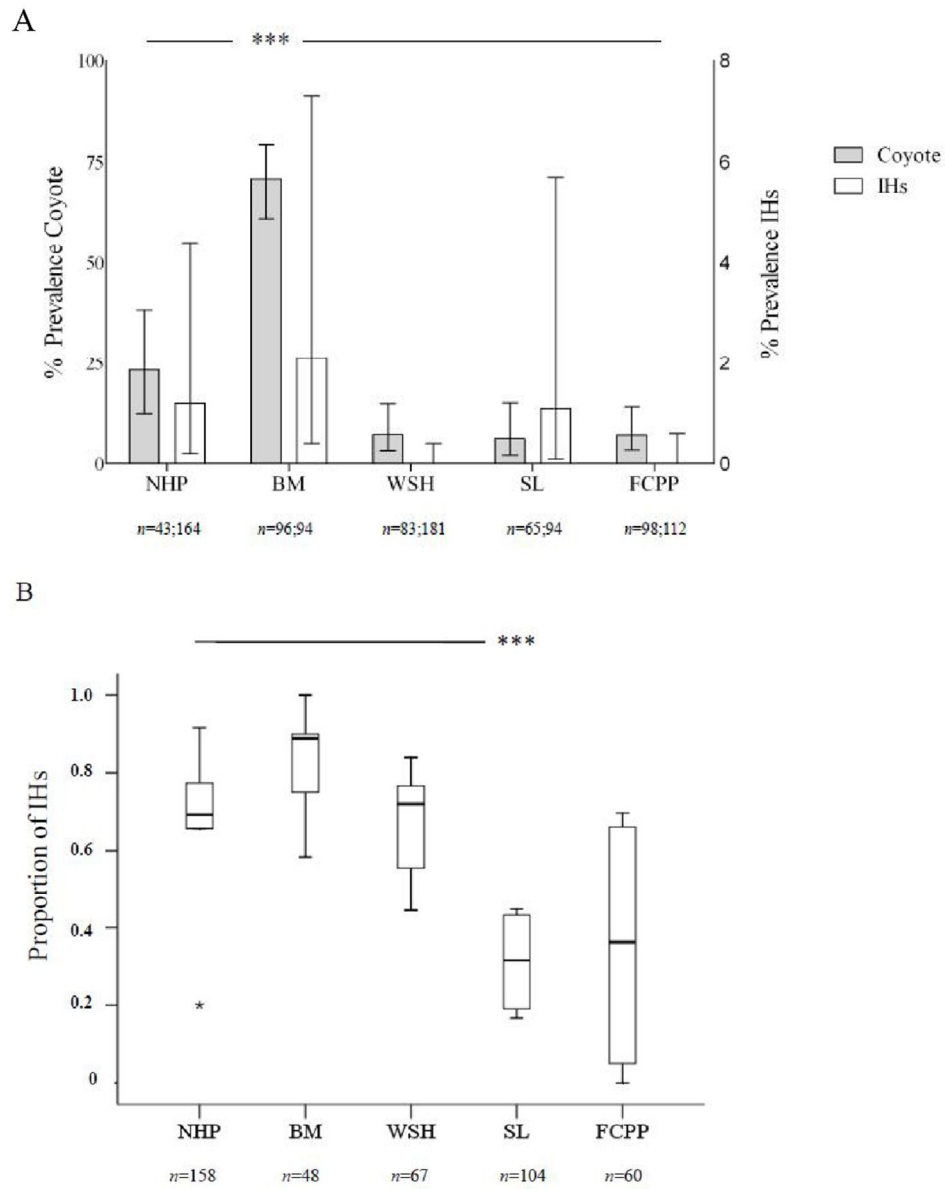
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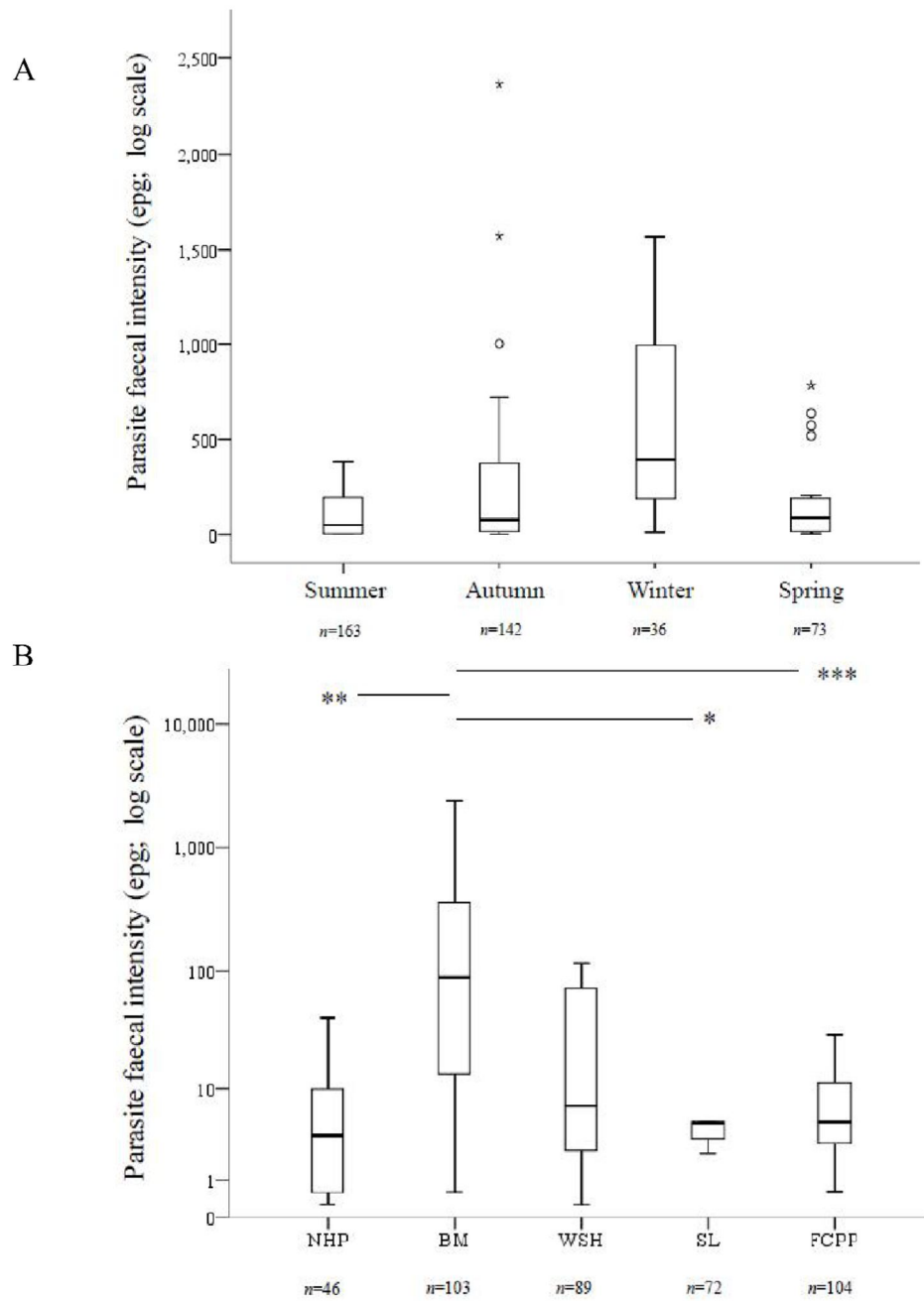
Chapter 5

ASSESSING INDIVIDUAL PATTERNS OF *ECHINOCOCCUS MULTILOCULARIS* INFECTION IN URBAN COYOTES: NON-INVASIVE GENETIC SAMPLING AS EPIDEMIOLOGICAL TOOL

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Abstract

In epidemiological studies of wildlife parasites, faecal genotyping has been introduced to prevent bias in estimates of parasite prevalence from faecal samples collected in the field. Such an approach could be particularly relevant in the study of *Echinococcus multilocularis* transmissionin urban settings, where estimates of prevalence and patterns of infection in wild canid hosts are key parameters used in zoonotic risk assessment and management. However, no previous study has evaluated the reliability of *E. multilocularis* faecal prevalence, and individual patterns of infection in definitive hosts remain poorly understood.

We evaluated faecal genotyping as an epidemiological tool, using *E. multilocularis* in urban coyotes (*Canis latrans*) as our study system. Combining parasitological analysis and multilocus individual genotyping of coyote faeces, we compared faecal parasite prevalence with the prevalence obtained from genotyped faecal samples. Furthermore, we assessed patterns of individual infection, such as re-infection rates and phenology of parasite egg excretion. Of 425 feces collected in five urban sites, we genotyped 142 samples (33.4%) corresponding to 60 unique individual coyotes. Number of genotyped samples per coyote ranged between 1 and 10 (mean = 2.3). Genotypes were obtained at 4-6 microsatellite loci and had a mean reliability of 0.9975. Faecal prevalence of *E. multilocularis* in genotyped coyotes was 25.0%, and similar to results previously obtained from non-genotyped faeces. Faecal genotyping allowed estimating a re-infection rate of individual coyotes of 57.1%, and to observe temporal patterns of parasite infection that were not detected using non-genotyped faeces.

If compared to independent data obtained through coyote post-mortem examination, our results suggest that reliable estimates of overall parasite prevalence in definitive host populations

can be efficiently obtained through well-designed field collection and traditional faecal parasitological analysis. However, faecal genotyping allows assessing the dynamics of individual infections, which could otherwise only be estimated by using invasive techniques. Combining faecal genotyping with parasitology has a great potential in assessing zoonotic risk transmission in urban areas, as well as advancing the field of wildlife ecology, disease ecology and conservation.

Introduction

   Growing research efforts have been directed towards the ecology of wildlife parasites in recent decades, as a consequence of their recognized role in shaping individual fitness (Hudson 2002), host population dynamics (Grenfell & Dobson 1995), communities and ecosystems (Collinge & Ray 2006), and because of their public health relevance as zoonoses (Thompson 2013). However, epidemiological studies require reliable estimates of parasite prevalence and transmission parameters (e.g., exposure, rate of encounter, patency) (Heinzmann & Torgerson 2008; Altizer *et al.* 2013), which are particularly difficult to obtain from wildlife populations (Wobeser 2007).

   For gastro-intestinal parasites, gold standard methodologies are generally based on post-mortem examination (Wobeser 2007). However, faecal analyses can be a valid alternative for rare and elusive host species (Carbonell & Rodriguez 1998) or in urban environments (Jacobson, Kazacos & Montague 1982), where invasive sampling techniques are often not a feasible option. Yet, estimates from faecal prevalence are potentially biased whenever the identity of the sampled animal is unknown, as repeated testing of a small group of individuals can occur (Marathe *et al.* 2002). An integrated approach combining faecal genotyping with parasitological analysis has been recently introduced as a tool to prevent bias in estimates of parasite prevalence based on faecal samples alone (e.g. Zhang *et al.* 2011). Identifying individual hosts potentially allows estimation of additional relevant epidemiological parameters, such as host population size (Creel *et al.* 2003), its temporal fluctuations (Prugh *et al.* 2005), and repeated individual-based measurements of parasite infection or diet (Prugh, Arthur & Ritland 2008). Although highly promising, such an approach may suffer from low genotyping success (Taberlet & Luikart 1999),

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which can reduce sample sizes and consequently the detection probability of parasites at low prevalence of infection. Costs associated with reliable genotyping can be also very high, so its utility has to be properly weighed against the needs of the research (Cullingham *et al.* 2010).

A unique study system to test the integrated use of faecal genotyping is the transmission of *Echinococcus multilocularis* in urban settings, where estimates of prevalence in wild hosts and spatio-temporal patterns of infection are key for zoonotic risk assessment and management. This trophically-transmitted parasite mainly infects wild canids (foxes *Vulpes* spp., coyotes *Canis latrans* and wolves *Canis lupus*) as definitive hosts and more than 40 species of small mammals (rodents and pikas) as intermediate hosts (Eckert *et al.* 2001). Despite its predominantly sylvatic life-cycle, *E. multilocularis* can circulate in urban habitats (Yimam *et al.* 2002; Deplazes *et al.* 2004; Liccioli *et al.* 2014), where it rises public health concern as aetiological agent of Alveolar Echinococcosis (AE), a potentially fatal disease in humans (Craig, Rogan & Allan 1996). Assessing the infection of *E. multilocularis* in definitive hosts from faeces is a valuable option, as diagnostic tests based on parasite-specific coproantigens (cELISA assays) (Deplazes *et al.* 1999) or identification of parasite DNA isolated from eggs (Mathis *et al.* 1996; Davidson *et al.* 2009) have been successfully implemented. However, no previous studies have properly evaluated the reliability of faecal prevalence, or accounted for potential re-sampling of individuals. Furthermore, estimates of re-infection rates and temporal patterns of infection are also required to understand the parasite epidemiology, assess the risk of zoonotic transmission (Eckert & Deplazes 1999), and evaluate management solutions (Hegglin & Deplazes 2008). Such parameters are not easy to obtain, since wild canids are generally nocturnal, elusive and wide ranging. Although radiotelemetry studies can assist in estimating definitive host densities

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and acquiring individual-based information (Deplazes *et al.* 2004), wildlife captures may be problematic in urban and recreational areas that are frequently used by people and domestic animals. As such, individual patterns of infection with *E. multilocularis* in definitive hosts remain poorly understood.

In this manuscript, we evaluated faecal genotyping as an epidemiological tool, using *Echinococcus multilocularis* in urban coyotes (*Canis latrans*) as study system. In particular, we aimed to assess key parameters of the epidemiology of *E. multilocularis* in a North American urban landscape, the city of Calgary. Our specific objectives were: 1) to assess potential biases in estimates of parasite prevalence in coyotes, comparing fecal prevalence estimates to the prevalence estimates obtained from individually genotyped faecal samples; and 2) to assess the dynamics of individual infections, re-infection rates and phenology of parasite egg excretion.

Herein, we provide evidence that a faecal sampling protocol and traditional faecal parasitological analysis can lead to accurate estimates of parasite prevalence in definitive hosts without the high costs associated with non-invasive genetic sampling. Additionally, we show that faecal genotyping can be a powerful tool to assess key epidemiological parameters, and in particular the dynamics of individual infections, which could otherwise only be estimated by using invasive techniques.

Material and Methods

Study area and sampling

The study was done in the City of Calgary (51°5′N, 114°5′W), Alberta, Canada. Between May 2012 and July 2013, 425 coyote faeces were collected in the following urban parks and

natural areas: Nose Hill Park (NHP; 1129.7 hectares (ha)), Bowmont (BM; 63.5 ha), Weaselhead (WSH; 208.7 ha), Southland lowlands (SL; 15.0 ha) and the west end of Fish Creek Provincial Park (FCPP; 3400.0 ha) (Fig. 1; for details on the study area, see Liccioli *et al.* 2012a). Faecal samples were collected along standardized trails and paths, as well as opportunistically in areas used by coyotes, on a 10-days sampling interval (Liccioli *et al.* 2012a; Liccioli *et al.* 2014). Faeces were aged according to the estimated time of deposition (class 1, <48 hrs; class 2, 3-5 days; class 3, 5-10 days; Liccioli *et al.* 2012a), and analyzed for *E. multilocularis* as described in Liccioli *et al.* (2014). Briefly, we applied a modification of the $ZnCl_2$ centrifugation and sedimentation protocol described by Davidson *et al.* (2009)*,* followed by molecular identification of *E. multilocularis* eggs through species-specific amplification of the NADH dehydrogenase subunit 1 (*nad1*) (Trachsel, Deplazes & Mathis 2007).

For each faecal sample, a subsample destined for molecular analysis was stored in 95% ethanol (1:10 ratio between faeces and alcohol) at -20ºC to minimize DNA degradation (Santini *et al.* 2007). Additionally, samples of skeletal muscle were taken from coyote carcasses (*n*=71) collected in 2012-2013 within Calgary city limits (Fig. 1). Coyote muscle was also preserved in 95% ethanol at -20ºC until analysis.

Coyote genotyping

Genomic DNA was extracted from coyote faeces using the pathogen detection protocol of the E.Z.N.A.® Stool DNA Kit (Omega bio-tek, USA), with a final elution of 150 µL. Faeces were genotyped at six unlinked tetranucleotide microsatellite markers (*FH2096*, *FH2001*, *FH2010*, *FH2054*, *FH2079*, *FH2380*) (Sacks, Brown & Ernest 2004; Prugh *et al.* 2005)

following the multi-tube approach (Taberlet *et al.* 1996). The six loci were selected among other available markers (Sacks, Brown & Ernest 2004), considering their allelic variability and amplification success, to maximize genotyping effectiveness. DNA amplification was carried out in 12.5 μL reactions containing 6.25 μL of Accustart™ II PCR Supermix (Quanta BioSciences Inc., USA), 4.75 μL of distilled water, 2.5 ρmol of forward and reverse primers and 1 μL of DNA template with the following PCR protocol: 94°C for 1 min, 40 cycles of 94°C for 30 s, 58°C (64°C for *FH2380*) for 30 s, 72°C for 30 s, and a final 10 min extension at 72°C. To minimize risk of cross-contamination, extraction and amplification of faecal DNA were conducted in separate dedicated rooms (Waits & Paetkau 2005). Furthermore, PCR replicates included negative quality controls (i.e., reagents with no DNA). DNA quality was initially screened by four independent amplifications at three loci (*FH2096*, *FH2001*, *FH2010*), after which only samples with PCR success >50% were further amplified and genotyped at the remaining loci (Caniglia *et al.* 2012). Samples that could not be reliably genotyped after ten independent amplifications were discarded.

Following PCR amplification, 1 μL of amplicon was diluted in a 10 μL solution of Hi-Di™ Formamide with GeneScan 500LIZ (Applied Biosystems, ABI) added as internal size standard. Electrophoresis of the PCR products was then conducted in an ABI 3500xL automated sequencer to separate alleles according to size (in base pairs), with allele sizes scored against the size standard using the software GENEMAPPER 4.0 (ABI). Reliability of faecal genotypes was determined with a maximum likelihood approach that assesses allelic dropout and minimize genotyping errors, using the software RELIOTYPE (Miller, Joyce & Waits 2002). Unreliable genotypes were further amplified until they met the reliability threshold (>0.95), following

Miller *et al.* (2002). We regrouped genotypes differing at one or two alleles with the software

GIMLET 1.3.3 (Valière 2002) and further checked for scoring inconsistencies. Consensus

genotypes were constructed accepting heterozygotes when both alleles were present in at least

two independent replicates, and homozygotes when the same allele was confirmed in at least

four replicates (Caniglia *et al.* 2012). GIMLET was also used to calculate rates of allelic dropout

(ADO) and false allele (FA), following Broquet & Petit (2004).

To calculate the probability of identity (P_{ID}), we used allele frequencies obtained by

genotyping coyote carcasses at the same six microsatellite loci. DNA was extracted from tissue

samples with the Qiagen DNeasy Blood & Tissue kit, with a final elute of 300 μL. P_{ID} for

populations where individuals randomly mate (P_{IDrand}) (Paetkau & Strobeck 1994), P_{ID} with

sample size correction ($P_{IDunbias}$)(Kendall & Stuart 1977) and P_{ID} for related individuals (P_{IDsibs})

(Taberlet & Luikart 1999) were computed following the equations provided by Waits *et al.*

(2001) using the software GIMLET.

Species verification

DNA extracted from faecal samples was molecularly confirmed as of coyote origin by

amplifying a fragment of the cytochrome *b* region of the mitochondrial DNA (Adams, Kelly &

Waits 2003). We modified the protocol described in Prugh & Ritland (2005) as follows: ScatID

primers (Adams, Kelly & Waits 2003) were used to amplify 1 μL of faecal DNA in a 12.5 μL

PCR reaction at the conditions outlined for nuclear markers and with an annealing temperature of

52°C. Subsequently, 10 μL of PCR product where mixed with 3.0 μL of distilled water, 0.5 μL

of Taq$^{\alpha}$ I restriction enzyme (Thermo Fisher Scientific, Canada) and 1.5 μL of corresponding

Taq$^\alpha$ I buffer (with BSA), and digested in Bio-Rad thermocyclers with the following protocol: 65°C for 90 min, 80°C for 15 min. Digested PCR products were visualized on 2% agarose gel electrophoresis. Samples showing the diagnostic 100 bp digested fragment were confirmed as of coyote origin (Prugh & Ritland 2005).

Coyote population size

   To estimate the total coyote population size in the five study areas, we first used multilocus genotypes as capture-recapture records, following the approach described in Caniglia *et al*. (2012). We checked for heterogeneity in detection probability of individual genotypes, to take into account potential bias that could cause underestimate of population size (Cubaynes *et al.* 2010). Assumptions of homogeneous capture probability could be violated due to coyote social behavior (Bekoff & Wells 1986) and territoriality (Gese 2001), as previously reported for wolves (*Canis lupus*) (Cubaynes *et al.* 2010; Caniglia *et al.* 2012). We divided the sampling period into 4-months intervals, according to the main biological seasons (breeding, pup-rearing, dispersal) described for the species (Gehrt, Anchor & White 2009). We used the software U-CARE 2.2 (Choquet *et al.* 2009) to evaluate and exclude the potential effect of transience and trap-dependence (Pradel 2005). Subsequently, we estimated coyote population size using the faecal genotype rarefaction curve (Kohn *et al.* 1999) calculated through the equations of Eggert (Eggert, Eggert & Woodruff 2003) and Chessel (Valière 2002). Compared to the equation proposed by Kohn *et al.*(1999), the selected equations are reported to perform better and result in lower variance among replicates (Valière 2002; Eggert, Eggert & Woodruff 2003). We ran the rarefaction curve analysis in the software GIMLET, estimating mean population size (±SD) with

1,000 iterations to take into account possible effects of the order in which samples were analyzed (Kohn *et al.* 1999). Finally, we estimated the minimum number of residents in each area, considering only genotypes that were captured in the same park for more than one season (Grinder & Krausman 2001).

True parasite prevalence and re-infection rate

We referred to true parasite prevalence as the percentage of individuals (unique multilocus coyote genotypes) that tested positive to *E. multilocularis* at least once during the entire study period. We calculated the true parasite prevalence as $P_{Em} = N_{Em}/ \text{Tot}_N$, where P_{Em} is the prevalence of *E. multilocularis*; N_{Em} is the number of unique genotypes associated with faeces molecularly confirmed to be positive for *E. multilocularis* eggs (Liccioli *et al.* 2014); Tot_N is the total number of unique coyote genotypes identified. We estimated the true parasite prevalence separately for each site and for the whole sampled population. Confidence intervals (CI) were estimated using Sterne 95% CI, when at least one positive case was detected (Reiczigel, Foldi & Ozsvari 2010), or the Median Unbiased Estimate (MUE), when no positive cases were found (Hirji, Tsiatis & Mehta 1989).

Based on the population size estimated in our study sites, we calculated the number of faecal samples required to obtain true *E. multilocularis* prevalence (at 95% confidence level and 5% precision), taking into account the genotyping success rate and the variability in capture probability across individuals. We calculated the sampling effort as

$$N_f = (N_i /G_{sr}) \times \sum_{i=1}^{k} i \times freq_i,$$

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where N_f is the number of faecal samples required; N_i is the number of different individuals to be tested, given the estimated parasite prevalence, as calculated with the utility available in Epitools (http://epitools.ausvet.com.au/); G_{sr} is the overall genotyping success rate; $freq_i$ is the frequency of individuals whose genotype was captured i times.

We assumed the patterns of *E. multilocularis* eggs excretion in coyotes to be similar to those described for red foxes (*Vulpes vulpes*) and domestic dogs (Kapel *et al.* 2006), with a patency of maximum 71 days (Kapel *et al.* 2006) unless a re-infection had occurred. We calculated the rate of re-infection as $R_r = N_r / Tot_{pos}$, where R_r is the re-infection rate; N_r is the number of coyotes (identified by their unique multilocus genotype) for which *E. multilocularis* eggs were detected in faeces collected more than 81 days apart (thus accounting for a 10-days buffer between faecal deposition and collection); Tot_{pos} is the total number of coyotes that tested positive to *E. multilocularis* at least once during the study period, and for which recapture of the multilocus genotype occurred. Given the sensitivity (0.75) of the diagnostic technique adopted (Liccioli *et al.* 2014), we compensated for potential errors (false negatives) in assessing the dynamics of individual infections by repeating the parasitological analysis for negative samples of animals that tested positive at previous or subsequent faecal collections.

Statistical analysis

Differences in genotyping success of faeces according to age of deposition or season were tested with a Pearson's Chi-square test. For multiple comparisons, we applied the Bonferroni correction to the type one error threshold (Curtin & Schulz 1998). Fisher's exact Chi-square test was used to compare faecal and true parasite prevalence in each park and within the

overall study area. Statistical analyses were run using SPSS 20.0 (IBM Corporation, Armonk, New York, USA).

Results

Genetic analyses

Of 425 faecal samples that were initially screened for DNA quality at three loci, 135 (31.7%) were discarded and not further amplified at the other loci. Of the remaining 286 faeces, we were able to obtain reliable genotypes at ≥ 4 loci for 142 (49.7%) samples, for an overall genotyping success of 33.4%. After screening with the mitochondrial DNA test, all the genotyped samples were confirmed to be from coyote origin (data not shown). Genotyping success did not differ significantly according to the estimated age of faecal deposition (X^2=5.629; df =2; P=0.131), ranging from a maximum of 40% and 37.2% (class 1 and 2, respectively) to a minimum of 26.7% (class 3). However, genotyping success varied across seasons (X^2= 25.941; df = 2; $P_<$0.001): faeces collected during the dispersal season (September-December) had higher genotyping success than those collected during the pup-rearing (May-August) (49.3% and 23.5%, respectively; X^2= 25.964; df = 1; $P_<$0.001), whereas no significant differences were observed among the other classes, once the type I error threshold was modified according to Bonferroni method to account for multiple comparisons (Pearson Chi-square test, $P_{(0.05;\ 3)\ >}$0.016; genotyping success in breeding season = 35.1%). All six loci were polymorphic, with 47 alleles in total and an average of 7.8 alleles per locus (range = 5–11). Considering all six loci, the probability of identity varied from a minimum of 0.0000014 ($P_{ID\textit{rand}}$) to a maximum of 0.004837 for siblings ($P_{ID\textit{sibs}}$). As probability of identity obtained at 4 microsatellite loci varied between

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0.0000299 (P_{IDrand}) and 0.01986 (P_{IDsibs}), we included samples genotyped at 4-6 loci in our analysis (Prugh *et al.* 2005). Of 142 genotypes, 121 (85.2%) were obtained at 6 loci, 18 (12.7%) at 5 loci, 3 (2.1%) at 4 loci.

Mean (±SD) per-locus per-replicate ADO rate was 0.107 (± 0.115), while the equivalent mean FA rate was 0.038 (± 0.079). Mean genotype reliability was 0.9975 (± 0.0055). From a total of 142 non-invasive samples we were able to identify 60 unique multilocus genotypes. The number of faeces identified per individual ranged between one and 10, with a mean of 2.31 (± 2.05). We did not detect significance in capture heterogeneity according to test for transience (Test 3.SR, X^2= 0.4434, df=2, *P*=0.801) and trap-dependence (Test 2.Ct, X^2=2.0337, df=2, *P*=0.154). In the five study sites, total coyote population size ranged from a minimum of 51 (95% CI: 50.9-51.3) to a maximum of 59 (95% CI: 58.1-58.9) individuals according to Chessel's and Eggert's equations (Fig. 2).

True parasite prevalence and re-infection rate

We estimated an overall prevalence of *E. multilocularis* in coyotes of 25.0% (95% CI: 15.6 - 37.4%), ranging widely across sites. No statistical differences were observed between faecal and true prevalence, although visible discrepancies were recorded for NHP, SL and FCPP (Tab. 1). With an estimated parasite prevalence of 25% and a population of 51-59 individuals, between 44 and 49 individuals need to be tested in order to estimate the prevalence of *E. multilocularis* with a 95% confidence level.

In BM, seven of 17 individual genotypes met our criteria to detect variations in their infection status through time. For four of these animals (genotype ID: 22, 40, 46, 51), shedding

of *E. multilocularis* eggs was recorded for longer than a single patency period (Fig.3A), resulting

in an estimated rate of re-infection of 57.1%. Furthermore, three individuals (genotype ID: 22,

34, 46) were repeatedly (3-6 times) sampled at close time intervals, allowing the observation of

temporal patterns of parasite egg excretion. Over a 20-days period, egg excretion increased from

an average minimum of 10.9 ± 5.1 epg to an average peak of 965 ± 703.1 epg. From a population

standpoint, the trend described for the genotyped individuals indicated an increment in shedding

of parasite eggs from August to November, with a subsequent plateau that lasted until spring

(Fig. 3A). Interestingly, no similar pattern was detected using data from non-genotyped faeces

(Fig. 3B).

Discussion

Our results suggest that a well-designed faecal sampling protocol can lead to accurate

estimates of parasite prevalence in the wild host population without the high costs associated

with non-invasive genetic sampling. However, the great potential of faecal genotyping is

expressed by the opportunity of assessing key epidemiological parameters, and in particular the

dynamics of individual infections, which could otherwise only be estimated by using invasive

techniques (i.e., capture/recapture, direct observation of tagged individuals).

Estimates of E. multilocularis prevalence

Using an integrated approach of faecal genotyping and faecal parasitology, our study

provided an accurate estimate of *E. multilocularis* prevalence in the definitive host in an urban

setting. In previous work, we reported an overall parasite faecal prevalence of 21.3% (Liccioli *et*

al. 2014), but we could not rule out the potential bias caused by repeated sampling of individuals (Marathe *et al.* 2002). Very few coproscopy-based wildlife parasitological studies have addressed such limitations (Zhang *et al.* 2011), and to the best of our knowledge ours is the first one to evaluate the problem in the context of *E. multilocularis* research. This is particularly relevant considering the behavior of the wild definitive hosts: foxes, coyotes and wolves exhibit social dominance and territorial faecal marking (Bekoff & Andrews 1978; Macdonald 1979; Mech & Boitani 2003), which can potentially increase the risk of such a sampling bias. An overall prevalence of 25% is consistent with the previous estimates obtained for the Calgary Metropolitan Area (20.5-29.5 %; Catalano *et al.* 2012; Liccioli *et al.* 2012a) through scraping and sieving of intestines (Eckert *et al.* 2001) collected at post-mortem analysis of coyote carcasses. Interestingly, our results suggest that faeces can give a reliable estimate of parasite prevalence in a population without the need of individual genotyping, provided that samples are collected over large areas (e.g., large portions of city parks and natural areas) and during multiple seasons. As a downside, the relatively low genotyping success of faecal DNA can eventually lead to underestimates of infection rates in sites characterized by low (<10%) faecal prevalence, or for which collection of samples is more difficult. In our study, this is exemplified by SL and FCPP (low prevalence), and NHP (low number of samples collected) (Tab. 1). In facts, our integrated approach requires a relatively large sample size, depending on parasite prevalence (which determines the number of individuals to be tested) and genotyping success rate (which influences the number of faeces that need to be collected from each animal) (Tab. 2). Cost effectiveness of non-invasive genotyping could be optimized by collecting faecal samples during autumn and winter, for which genotyping success was reported to be highest, likely due to

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favorable environmental conditions for the preservation of DNA (e.g., cold temperatures and low humidity) (Santini *et al.* 2007). However, reliable estimates of parasite prevalence still require a solid understanding of parasite transmission ecology: in the case of *E. multilocularis*, those seasons may be strategic since they are also the ones during which infection of coyotes is more likely to occur (Liccioli *et al.* 2014). Yet, faecal genotyping can significantly inflate the operational costs (in our study, up to $ 90-100/successfully genotyped sample), and its use needs to be carefully evaluated. When the estimation of parasite prevalence in the host population is the only desired outcome of the study, faecal prevalence obtained through a well-designed sampling protocol may be the most effective solution, possibly integrated with post-mortem examination of carcasses opportunistically collected.

Epidemiological parameters

Combining faecal genotyping with faecal parasitology allowed us to assess key parameters for the understanding of *E. multilocularis* epidemiology in an urban setting. There are currently no alternatives to monitor temporal variations of individual infection without direct observation or capture of the animals, and therefore no feasible alternatives for wild carnivores such as coyotes. Although local foci of high *E. multilocularis* endemicity were previously reported (Hofer *et al.* 2000; Liccioli *et al.* 2014), the re-infection rate of wild definitive hosts has never been estimated. According to our data, more than 50% of the coyotes in an area of high prevalence are infected multiple times throughout the year, thus supporting the hypothesis that the lack of temporal patterns observed in that site (Liccioli *et al.* 2014) is due to a high re-infection rate (Tackmann *et al.* 2001).

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Furthermore, individual patterns of infection have the potential to inform on temporal variations (i.e. seasonality) of parasite transmission that could be otherwise not detected when examining faecal samples of unknown identity (Fig. 3). Individually genotyped faeces allow also to account for individual heterogeneity of parasite infection and to identify super-spreaders. By shaping parasite-host interactions, parasite aggregation (i.e. overdispersal) is highly relevant in epidemiological studies (Hudson 2002), but cannot be investigated without invasive techniques (i.e., post-mortem analysis, repeated animal capture) unless non-invasive genetic sampling is integrated in faecal parasitological studies.

Finally, the temporal variations in individual infection can provide insights into the dynamics of parasite egg excretion. Such data are normally obtained through infection trials with captive animals (Kapel *et al.* 2006), but are rare for wild populations. For *E. multilocularis*, no previous information is available on the dynamics of egg excretion in coyote hosts. Although preliminary, our data suggest an exponential increase of egg excretion in a 20-days period, possibly resembling more the dynamics observed in wild canid hosts than that documented for domestic dogs (Kapel *et al.* 2006).

Management implications

Our results suggests that prevalence of *E. multilocularis* in definitive hosts can be reliably and efficiently achieved through well-designed studies based on the collection and parasitological analysis of faecal samples, without the need of correcting for sampling bias through expensive non-invasive genetic sampling. Importantly, faecal collection can be planned according to site and season, thus allowing for the detection of spatial heterogeneity and

temporal variations in parasite infection, as well as the evaluation of the subsequent risk of zoonotic transmission (Liccioli *et al.* 2014).

However, mark-recapture of individual faecal genotypes is a powerful tool to estimate abundance and recruitment rate of species (e.g. carnivores) that are difficult to observe and capture (Prugh *et al.* 2005), and to obtain repeated individual-based measures of parasite infection. With respect to *E. multilocularis*, the combination of host population parameters with information on parasite prevalence is fundamental to assess the risk of zoonotic transmission (Eckert & Deplazes 1999) and plan baiting programs (Hegglin & Deplazes 2008), currently considered the most effective control measure of this zoonotic parasite (Tsukada *et al.* 2002; Hegglin, Ward & Deplazes 2003). Additionally, the actual efficiency of anthelmintic baiting can be properly assessed through faecal genotyping and monitoring of individual infection through time (i.e., before and after intervention). Interestingly, faecal genotyping allows to identify resident animals (Caniglia *et al.* 2014), whose infection status is likely to be the most relevant for environmental contamination and human exposure to the parasite.

By allowing assessment of individual patterns of infection, faecal genotyping can greatly contribute to the understanding of *E. multilocularis* transmission ecology, as well as the management of this zoonotic parasite in urban areas. The broad adoption of such integrated approach in other parasitological studies has the potential to significantly advance the field of wildlife disease ecology, conservation and management.

Data Accessibility

DRYAD archives the following microsatellite data: Reliotype input and summary, Gimlet input and output for genotype identification, consensus and population size.

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List of tables

Table 1: Number of faeces collected (genotyped), faecal prevalence (95% Confidence Intervals), number of unique multilocus genotypes, mean number of genotype recapture events, minimum number of residents (individuals whose genotype was captured in the area for more than one season), and true *Echinococcus multilocularis* prevalence (95% Confidence Intervals) estimated for coyotes in five urban sites in Calgary, AB, Canada, between May 2012 and August 2013. Fisher's Chi-square exact tests (*P* values displayed) were used to compare faecal and true parasite prevalence.

Table 2: Number of faecal samples required to estimate true *Echinococcus multilocularis* prevalence in the definitive host with a 95% confidence and 5% precision, according to the heterogeneity in capture probability encountered in this study (range 1-10, mean=2.31). Sample size is calculated for a population of 60 individuals, in relation to variable genotyping success rate and parasite prevalence.

List of figures

Figure 1: Geographic distribution of genotyped coyote faeces (grey circles, *n*=142) and tissues (black stars, *n*=71) collected in Calgary, AB, Canada, between May 2012 and July 2013. City parks and natural areas sampled include Nose Hill Park (NHP), Bowmont (BM), Weaselhead (WSH), Southland Lowlands (SL) and the west end of Fish Creek Provincial Park (FCPP).

Figure 2: Coyote population size according to the genotype rarefaction curve. Green line and blue line refer to the equations of Chessel and Eggert, respectively. Black line represents the mean asymptote over 1,000 iterations.

Figure 3: Patterns of *Echinococcus multilocularis* eggs excretion recorded for individual coyotes (*n*=14) (A) and non-genotyped coyote faeces (B) sampled at 10-days intervals (represented as I, II and III for each month) between May 2012 and July 2013 in Calgary, AB. Values on the Y-axis (log scale) indicate the number of eggs per gram of faeces. In graph A, intensity is reported as zero for animals that tested negative before or after shedding of parasite eggs was detected. Data labels indicate unique multilocus genotype ID of individual coyotes. For individuals that were sampled multiple times, lines connect data points. In graph B, interpolation line is displayed.

Table 1

Site	N faeces	Faecal prevalence % (CI)[a]	N genotypes	N recaptures (range)	N residents	True Prevalence % (CI)	P
NHP	55 (27)	23.3 (12.4-38.3)	15[1]	1.80 (1-6)	2	6.7 (0.3-30.2)	0.199
BM	112 (43)	70.8 (61.0-79.3)	17	2.53 (1-7)	8	76.5 (51.1-91.5)	0.182
WSH	83 (24)	7.2 (3.2-14.9)	13	1.5 (1-6)	5	7.7 (0.4-34.2)	0.593
SL	70 (22)	6.2 (2.1-15.1)	5	4.4 (1-10)	4	0.0 (0-15.9)	0.806
FCPP	105 (26)	7.2 (3.4-14.1)	11[1]	2.27 (1-5)	4	0.0 (0-6.1)	0.520
TOT	425 (142)	21.3 (20.5-29.2)	60	2.31 (1-10)	23	25.0 (15.6-37.4)	0.310

[a] (Liccioli *et al.* 2014)

[1] Number of genotypes captured in more than one site

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Table 2

	Parasite Prevalence						
Genotyping rate	**0.01**	**0.05**	**0.1**	**0.2**	**0.3**	**0.4**	**0.5**
0.1	306	777	990	1131	1178	1202	1225
0.2	153	389	495	565	589	601	613
0.3	102	259	330	377	393	401	408
0.4	77	194	247	283	295	300	306
0.5	61	155	198	226	236	240	245
0.6	51	130	165	188	196	200	204
0.7	44	111	141	162	168	172	175
0.8	38	97	124	141	147	150	153
0.9	34	86	110	126	131	134	136

Figure 1
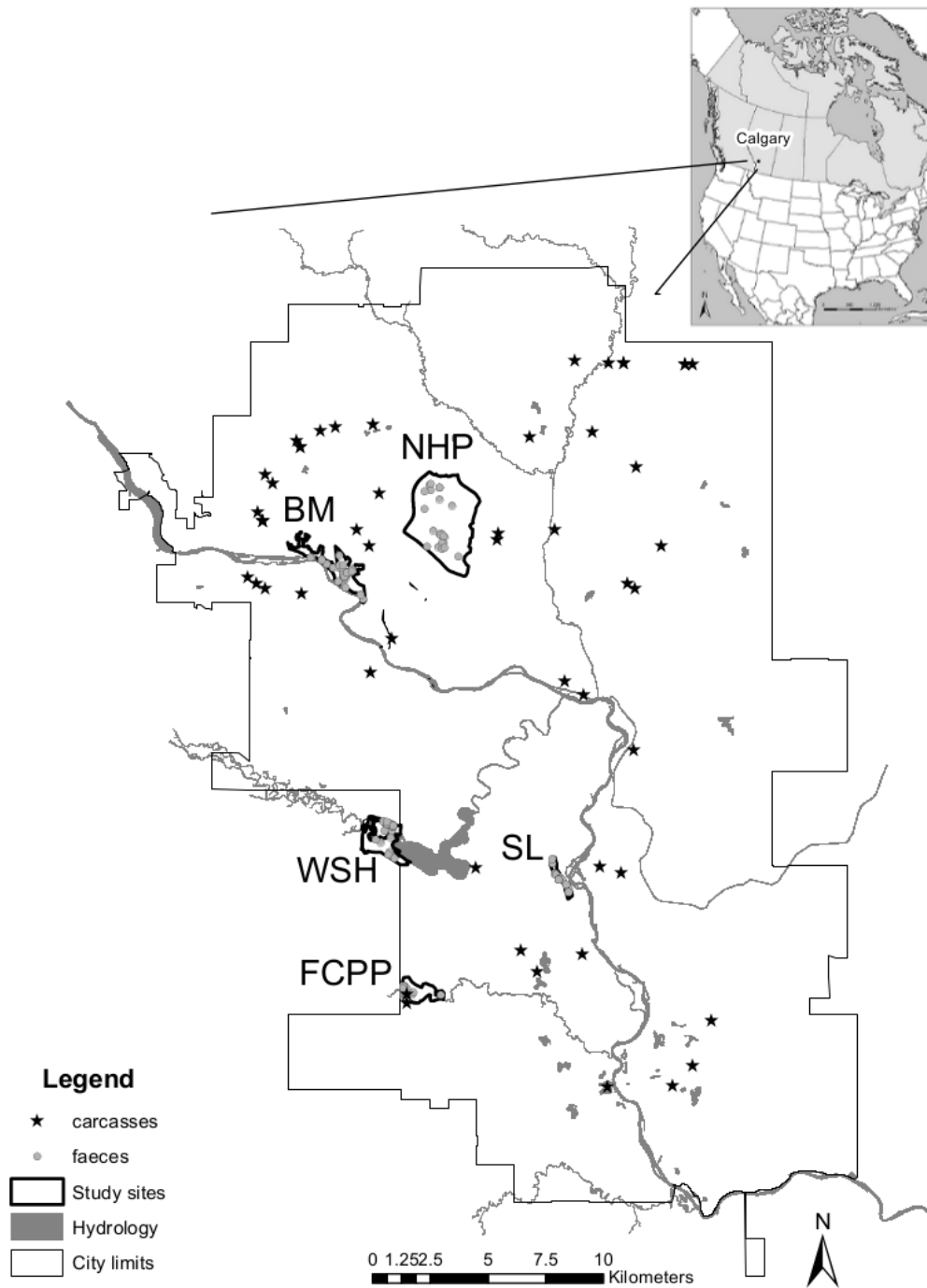
Figure 2
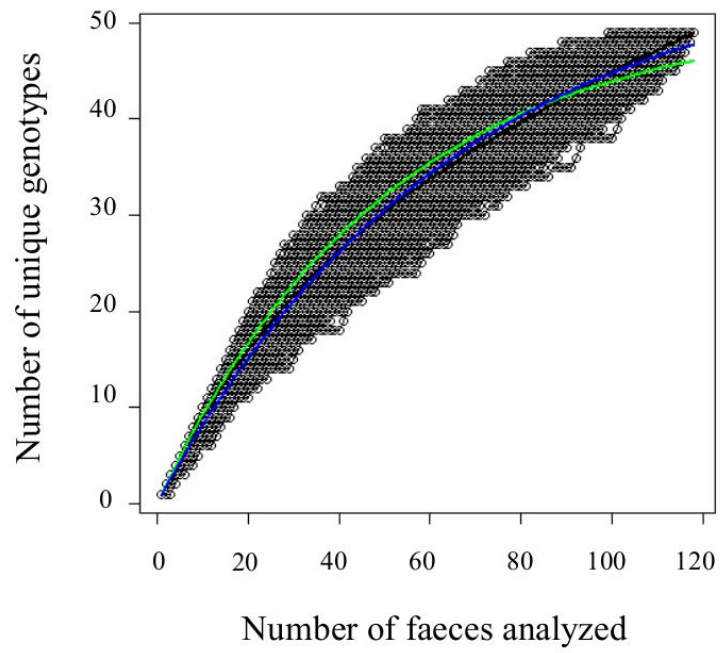
Figure 3
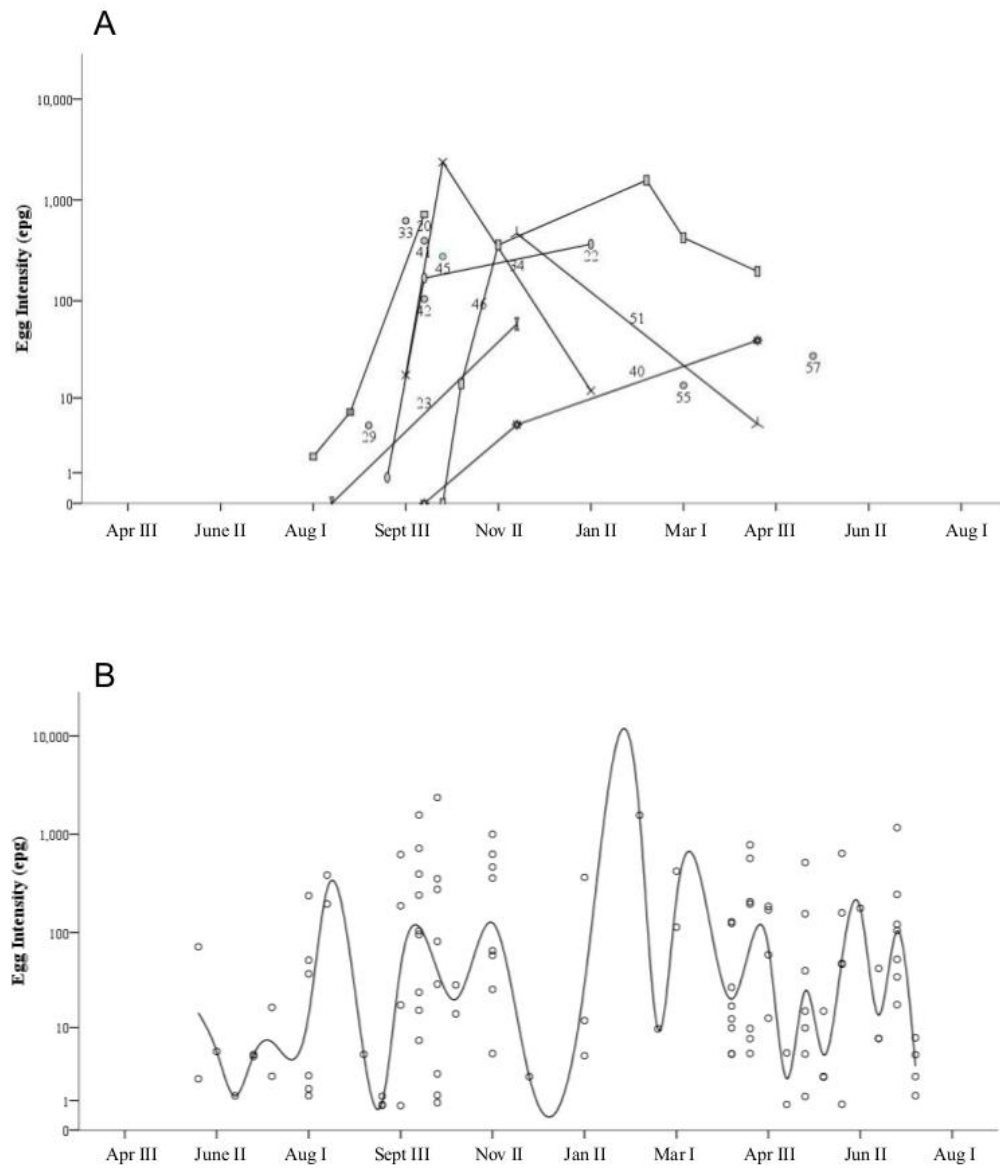
Chapter 6

FEEDING ECOLOGY INFORMS PARASITE EPIDEMIOLOGY: PREY SELECTION MODULATES ENCOUNTER RATE WITH *ECHINOCOCCUS MULTILOCULARIS* IN URBAN COYOTES

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Abstract

The diet of wild species is rarely analyzed to inform on the ecology of trophically-transmitted parasites. Here, we investigated the role of urban coyote feeding ecology in the transmission of *Echinococcus multilocularis*, the causative agent of Alveolar Echinococcosis in humans. As coyotes can play a main role in the maintenance of this zoonotic parasite within North American urban settings, such study can ultimately aid disease risk management. Between June 2012 and June 2013, we collected 251 coyote feces and conducted trapping of small mammals (n = 971) in five parks in the city of Calgary, Alberta, Canada. We investigated *E. multilocularis* epidemiology by assessing seasonal variations of coyote diet and the selective consumption of different rodent intermediate host species. Furthermore, accounting for small mammal digestibility and coyote defecation rates we estimated the number of small mammal preys ingested by coyote and consequently, coyote encounter rates with the parasite. Dominant food items included small mammals, fruit and vegetation, although hare and deer were seasonally relevant. The lowest frequency of occurrence per scat of small mammals was recorded in winter (39.4 %), when consumption of deer was highest (36.4 %). However, highest encounter rates (number of infected hosts predated/season) with *E. multilocularis* (95% CI: 1.0-22.4), combined with the lack of predation on non-competent small mammal species, suggest that winter is the critical season for transmission and control of this parasite. Within the small mammal assemblage, voles (*Microtus pennsylvanicus* and *Myodes gapperi*) were the preferred preys of urban coyotes and likely played a key role for the maintenance of the urban sylvatic life-cycle of *E. multilocularis* in Calgary.

Introduction

The feeding ecology of wild species is traditionally investigated to assess predator-prey relations and dietary preferences (e.g., Farrell, Roman & Sunquist 2000; Bagchi, Mishra & Bhatnagar 2004; Hayward *et al.* 2006), habitat requirements (e.g., Loucks *et al.* 2003), causes of decline (e.g., Ramakrishnan, Coss & Pelkey 1999) and human-related conflicts (e.g., Bagchi & Mishra 2006), overall aiming to identify conservation policies and inform management strategies. Much less frequently, animal feeding ecology is analyzed under a disease ecology perspective, considering its implications for the transmission of those parasites that depend on the predator-prey relationship between definitive and intermediate hosts (Lafferty 1999).

Among trophically-transmitted parasites, *Echinococcus multilocularis* offers an interesting and complex system that may shed light on important ecological and epidemiological processes. This parasitic cestode is widely distributed in the Northern Hemisphere (Eckert *et al.* 2001) and mainly infects wild canids such as foxes (*Vulpes* spp.) and coyotes (*Canis latrans*) as definitive hosts, and more than 40 species of small mammals (mainly Arvicolidae, Cricetidae and Muridae;Vuitton *et al.* 2003) as intermediate hosts (Eckert *et al.* 2001). Importantly, the parasite is the causative agent of Alveolar Echinococcosis in humans, currently considered among the most serious zoonotic diseases of the northern hemisphere (case fatality rate >90% if untreated; Craig, Rogan & Allan 1996). Despite a primarily sylvatic life-cycle, *E. multilocularis* can circulate and be maintained within urban habitats (Tsukada *et al.* 2000; Deplazes *et al.* 2004; Liccioli *et al.* 2014): here, given the high risk of zoonotic transmission, understanding parasite ecology becomes crucial for disease prevention and risk management.

In the Northern-central region of North America (13 US states and the four Canadian western provinces (Eckert *et al.* 2001; Gesy *et al.* 2013)), meadow voles (*Microtus pennsylvanicus*) and deer mice (*Peromyscus maniculatus*) are traditionally considered the most important intermediate hosts (Eckert *et al.* 2001). However, parasite prevalence in small mammals alone cannot illuminate *E. multilocularis* transmission unless combined with information on dietary preferences of the definitive host (Giraudoux *et al.* 2003).

Several studies have explored coyote feeding ecology in natural (Thurber *et al.* 1992; Gese & Grothe 1995; Gese, Ruff & Crabtree 1996), suburban (Grigione *et al.* 2011) and urban areas (Quinn 1997; Morey, Gese & Gehrt 2007). Although the relevance of small mammals in coyote diet has been widely documented (Litvaitis & Shaw 1980; Gese, Ruff & Crabtree 1996; Quinn 1997; Morey, Gese & Gehrt 2007; Grigione *et al.* 2011), no research has ever specifically explored the feeding ecology of this opportunistic predator in relation to the transmission of *E. multilocularis*. Such a study is particularly relevant in North American urban habitats, where coyotes can act as the main definitive host and be responsible for the maintenance of the urban sylvatic life-cycle of this parasite (Liccioli *et al.* 2014). To date, studies of coyote diet in urban habitats mainly aimed to investigate the usage of human-associated food sources and inform management of coyote-human conflict (Morey, Gese & Gehrt 2007; Lukasik & Alexander 2012), whereas aspects of disease ecology are still unexplored.

Herein, we investigated the role of definitive host feeding ecology on *E. multilocularis* transmission in urban habitats, using coyotes as the focal species. Specifically, we aimed to i) assess seasonal variations of *E. multilocularis* infection in coyotes in relation to their diet; ii) determine the impact of coyote selective consumption of small mammal preys on *E.*

multilocularis transmission; and iii) evaluate how these processes affect parasite transmission through changes in encounter rate of coyotes with *E. multilocularis*.

Material and Methods

Ethic statement

Small mammals were captured in the field with lethal traps (Woodstream© Museum Special Traps). If necessary, animals were euthanized through cervical dislocation by trained operators. The animal use protocol was approved by the Animal Care Committee of the Faculty of Veterinary Medicine, University of Calgary (protocol number: AC12-0037). Field permit was granted by the City of Calgary and Alberta Government (Provincial Parks).

Study area and sample collection

The study was conducted in the following five parks and natural areas of the City of Calgary (51°5′N, 114°5′W), Alberta, Canada: Nose Hill Park (NHP), Bowmont (BM), Weaselhead Natural Environment Areas (WSH), Southland Lowlands (SL) and Fish Creek Provincial Park (FCPP) (for details and map, see Liccioli *et al.* 2014). Between June 2012 and June 2013, coyote feces were collected on a ten-day sampling schedule following methodologies and protocols previously described (Liccioli *et al.* 2012a; Liccioli *et al.* 2014). After collection, feces were weighed and stored at -80º C for 72 hrs to inactivate *Echinococcus* spp. eggs (Veit *et al.* 1995) and protect operators from potential exposure. Samples were then stored at -20º C until analysis.

In the same study sites, the relative abundance of small mammals was estimated through lethal trapping sessions conducted within a broader investigation on the prevalence of *E. multilocularis* in intermediate hosts (Liccioli *et al.* 2014). Small mammals were captured monthly with Woodstream© Museum Special traps deployed along 1-2 ha rectangular grids and with a sampling effort of 200 traps per three nights (for details, see Liccioli *et al.* 2014). Relative abundance of the species was assumed to be reflected in their effective capture rate (Village & Myhill 1990), controlling for the number of misfires (number of animal captured divided by the number of active traps, multiplied by 100%), with the exception of the northern pocket gopher (*Thomomys talpoides*). For this species, capture rate was not considered a reliable indicator of relative availability in the study area, given its fossorial activity (Engeman, Campbell & Evans 1993) and the type of traps used, and was consequently removed from the analysis of small mammal abundance. Small mammal species that were captured only occasionally (i.e., <10 occurrences) and were not encountered in coyote diet, were also removed from the analysis.

Coyote diet analysis

After collection, a subsample (4-6 g) of feces was removed and saved for parasitological and molecular analysis (Liccioli *et al.* 2014). The remaining portion of the fecal sample was then weighed, hand-washed, and macroscopic fragments isolated using a 500 µm mesh sieve (Reynolds & Aebischer 1991) and let to dry at room temperature.

Coyote diet was analyzed following the point-frame method (Chamrad & Box 1964): undigested macro-components were mixed and evenly spread on a glass tray (18x28cm, 15x20cm or 11x16.5cm, depending on the amount of material) with an underlying grid of 50

points equally spaced to allow for systematic sampling (Ciucci *et al.* 1996). Food items were identified by three trained operators, whose reliability and concordance were previously tested. In particular, operators were evaluated on their ability to correctly identify small mammals and *E. multilocularis* intermediate host species through a blind test (Ciucci *et al.* 1996) based on 11 hair samples belonging to the following 8 species: meadow vole, southern red-backed vole (*Myodes gapperi*), house mouse (*Mus musculus*), muskrat (*Ondatra zibethicus*), deer mouse, shrews (*Sorex* spp.), northern pocket gopher and western jumping mouse (*Zapus princeps*). Prey remains were microscopically identified using mammalian hair and skull keys (Adorjan & Kolenosky 1969; Jones & Manning 1992; Moore *et al.* 1997). An archive of locally collected mammalian hair, as well as the zoological collection of the Department of Biological Sciences at the University of Calgary, were used as additional supporting reference for the identification of hairs, bones and skulls. Presence of fruit and vegetation was assessed through the recovery of seeds, berries and leaves (Lukasik & Alexander 2012), although plants were not identified taxonomically. Similarly, occurrence of reptile, bird and invertebrate remains was recorded on the basis of diagnostic items (i.e., scales, feathers, exoskeleton), but taxa within each category were not classified. Deer (*Odocoileus* spp.) and hares (*Lepus* spp.) were identified at the genus level. Ground squirrels (genus *Urocitellus* and *Ictidomys)* and tree squirrels (genus *Sciurus*) were identified at the species level but grouped together as Sciuridae.

Overall coyote diet was quantified using the frequency of occurrence per scat (i.e., percentage of scats containing a given food item) (Klare, Kamler & Macdonald 2011), as well as the frequency of occurrence per item (i.e., percentage of the number of occurrences of a given food item of the total number of occurrences of all food items), which estimates the relative

importance of each food item in the animal diet (Loveridge & Macdonald 2003). Items accounting for less than 2% of the scat volume, as estimated by the proportion of grid points occupied by each food item (Ciucci *et al.* 1996), were excluded from the analysis (Prugh 2005).

Small mammal consumption and encounter rate with E. multilocularis

Samples containing remains of small mammals were further analyzed to estimate the number of individual preys associated with each coyote scat. First, for each fecal sample, hairs of small mammal species were meticulously separated by hand upon morphological identification, and their total amount weighed at four decimal precision with an Ohaus PA214 scale. Mass of prey hairs in the sample analyzed was extrapolated to the total fecal mass. Undigested prey hair mass was then converted to ingested biomass according to an existing predictive model of prey (house mouse *Mus musculus*) consumed by coyotes (Kelly & Garton 1997). Specifically, we applied the average digestibility recorded along sets of feeding trials differing in the number of preys fed per meal (i.e., 10-50) and meal composition (i.e., with or without ground meat filler) (Kelly & Garton 1997). For the purpose of this study, the indigestibility coefficient reported for house mouse hair (0.0252) was considered representative of vole and mouse-size preys (i.e., genus *Microtus*, *Myodes*, *Peromyscus*, *Zapus*, *Sorex*, and *Thomomys talpoides*) (Kelly 1991). Finally, the number of preys per coyote scat was estimated dividing the total biomass ingested by the mean mass recorded for each small mammal species in our study area (Liccioli *et al.* 2014).

For each species of small mammal occurring in the diet of coyote, the total number of preys ingested per coyote per season was then estimated as

$$N_{\frac{Prey}{season}} = NPrey_{feces} * r_{dej} * d$$

where $NPrey_{feces}$ is the mean number of ingested small mammal preys estimated per coyote feces; r_{dej} is the coyote mean daily dejection rate (0.79 scats/day) observed in feeding tests of coyotes, and reported as independent of consumed biomass (Monroy-Vilchis & Frieven 2006); d is the number of days in each three-months season (summer: June-August; fall: September-November; winter: December-February; spring: March-May). For each season, we calculated the proportion (0 to 1) of intermediate hosts within the total number of ingested small mammals using the formula

$$[IHs/(IHs + NIHs)]$$

where IHs and NIHs are, respectively, the number of intermediate and non-intermediate hosts ingested per individual coyote (Liccioli *et al.* 2014).

Finally, seasonal encounter rate (mean, 95% CI) of coyotes with *E. multilocularis* was estimated as

$$N_{Encounters} = \sum_{1}^{k} N\,IHPrey_i * p_{IHi}$$

where $N\,IHPrey_i$ is the total number of ingested preys of the i intermediate host species, and p_{IHi} is the parasite prevalence (mean, 95% CI) in the i intermediate hostspecies as observed in the study area during the same time period (Liccioli *et al.* 2014).

Statistical analysis

Differences in frequency of occurrence per scat of food items across seasons were tested by Fisher's exact Chi-square test. Variations in the capture rate of different small mammal species were tested by Kruskal-Wallis test for k independent samples. For each species,

differences of relative abundance across seasons were tested by Friedman test for paired samples. For pairwise comparisons, we used Mann-Whitney U-tests for independent samples or the Wilcoxon test for paired ones and applied the Bonferroni correction to the type I error threshold (Curtin & Schulz 1998). Following ln-transformation and normalization of data, seasonal variations in the relative abundance of small mammals were tested with a one-way ANOVA and Fisher's least significant difference (LSD) Post-hoc test. Seasonal variations in the proportion of intermediate hosts within the total number of ingested small mammals were tested using the Pearson's Chi-square test. To test for selective consumption of small mammal species by coyotes, we used Fisher's exact Chi-square (Byers, Steinhorst & Krausman 1984) calculating, for each species, the proportion of individual preys ingested by coyotes (observed) and comparing it to the proportion of animals captured in the field (expected). Ivlev's electivity index (Ivlev 1961) was calculated to measure the degree of coyote selection (positive or negative) for small mammal species. Means and Standard Errors (± SEM) are reported throughout the text, unless otherwise specified. All the analyses were run on SPSS version 20.0 (IBM Corporation, USA).

Results

Coyote diet

A total of 251 coyote fecal samples were collected in the five sites (NHP, n = 37; BM, n = 71; WSH, n = 51; SL, n = 38; FCPP, n = 54) and submitted to diet analysis. Among vertebrates, small mammals had the highest frequency of occurrence per scat overall (57.1%) and in each season, followed by hares (20.3%), deer (17.5%), Sciuridae (15.1%) and birds (14.3%), whereas muskrat, porcupine, cat, red fox and domestic dog were detected in less than

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5% of the feces (Table 1). Remains of cattle and skunk were identified in one single occasion (<0.5%; data not shown). Small mammals, hares and deer represented the key preys of coyotes, overall accounting for 30% of the total food items (range: 23.6%, summer - 44.7%, winter) (Table 1). Frequency of occurrence per scat of small mammals varied across seasons ($X^2 = 8.290$; df = 3; p = 0.039), with a maximum in summer (65.1%) and a minimum in winter (39.4%), although no differences among seasons were detected once the type I error threshold was modified according to the Bonferroni method to account for multiple comparisons (Fisher's exact Chi-square test, $p_{(0.05; 6)} > 0.0083$). An opposite trend was observed for deer, for which the frequency of occurrence per scat also varied across seasons ($X^2_{Exact} = 16.224$; df = 3; p = 0.001), reaching its peak in winter (36.4%) and the minimum in spring (4.7%) (winter *vs.* spring, $X^2_{Exact} = 16.540$; df = 1; all the other comparisons, $p_{(0.05; 6)} > 0.0083$). Occurrence of hare showed a similar seasonal trend ($X^2_{Exact} = 11.263$; df = 3; p = 0.009), with the lowest frequency observed in summer (11.6 %) and the highest in spring (32.8%) (summer *vs.* spring, $X^2_{Exact} = 10.044$; df = 1; p = 0.002; all the other comparisons, $p_{(0.05; 6)} > 0.0083$).

Vegetation (25.6%) and fruit (16.7%) were relevant components of coyote diet in terms of frequency of occurrence per item, although vegetation was often present only in small amounts (46.5% of the cases with ≤10% volume).

Small mammal assemblage and relative abundance

A total of 971 small mammals were captured over 30,200 trap-nights, for an overall capture rate of 5.61% when accounting for trap misfires. Small mammal species included meadow vole (n = 267), southern red-backed vole (n = 71), deer mouse (n = 305), western

jumping mouse (n = 32) and shrews (n = 296). Relative abundance (effective capture rate, in %) of small mammals varied across species (Kruskal-Wallis test, X^2 = 70.583; df = 3; p<0.001), and was maximal for deer mouse (1.53±0.29; median = 1.03; range = 0 - 10.7), shrews (1.37±0.26; median = 0.72; range = 0 – 9.7) and meadow vole (1.27±0.37; median = 0.28; range = 0 – 14.4), and minimal for southern red-backed vole (0.61±0.38; median = 0; range = 0-16.7) and western jumping mouse (0.24±0.12; median = 0; range = 0-5.0). No statistical difference (Mann-Whitney test with Bonferroni corrections for multiple comparisons, $p_{(0.05; 10)}$ >0.005) was observed between the relative abundance of meadow vole, deer mouse and shrews, as well as between southern red-backed vole and western jumping mouse (all the other comparisons, p<0.001). Overall, small mammal relative abundance varied across seasons ($F_{3, 43}$ = 5.226; p = 0.004), with a maximum during summer (6.84±1.47) and fall (8.39±3.12) and a minimum during winter (2.13±0.33) and spring (1.49±0.29) (Fisher's LSD Post-hoc: summer *vs.* winter, p = 0.026; summer *vs.* spring, p = 0.001; fall *vs.* spring, p=0.004; all the other comparisons not significant).

Small mammal consumption and encounter rate with E. multilocularis

According to the frequency of occurrence per scat, consumption of meadow vole (33.9%) was significantly higher than any other species of small mammals (southern red-backed vole = 8.0%, X^2_{Exact} = 50.880, df = 1, p<0.001; deer mouse =5.6%, X^2_{Exact} = 63.428, df = 1, p<0.001; northern pocket gopher = 10.8%, X^2_{Exact} =38.661, df=1, p<0.001; western jumping mouse = 3.6%, X^2_{Exact} =75.604, df=1, p<0.001; shrews = 2.8%, X^2_{Exact} = 80.969, df = 1, p< 0.001) (Fig.1). Meadow vole ranked as the most consumed small mammal also according to the mean number of animals ingested per coyote feces (0.90±0.13; median = 0, range = 0-12.44), which was

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significantly higher than all the other species (southern red-backed vole = 0.39± 0.14; median = 0, range = 0 – 25.39; U = 23597.5, df = 1, p<0.001; deer mouse = 0.17 ± 0.07; median = 0, range = 0 – 16.54; U = 22700.5, df = 1, p<0.001; northern pocket gopher = 0.15 ± 0.04; median = 0, range = 0 – 3.78; U = 23960.5, df = 1, p<0.001; western jumping mouse = 0.08±0.03; median = 0, range = 0 – 5.84; U = 22097.0, df = 1, p<0.001; shrews = 0.02±0.02; median = 0, range = 0-4.60; U = 21484.000, df =1, p<0.001).

Compared to their relative availability, vole species were overall consumed significantly more than expected (meadow vole, X^2_{Exact} = 65.288, df = 1, p<0.001; southern red-backed vole, X^2_{Exact} = 71.301, df = 1, p<0.001), whereas deer mouse (X^2_{Exact} = 82.390, df = 1, p<0.001) and shrews (X^2_{Exact} = 161.512, df = 1, p<0.001) were consumed significantly less than expected (Table 2). Positive selection of voles (*M. pennsylvaniscus* and *M. gapperi*) was exhibited in every season with the exception of the fall, during which both species were consumed in proportion of their availability. Deer mouse was preyed less than expected in summer (X^2_{Exact} = 64.099, df = 1, p<0.001), winter (X^2_{Exact} = 11.745, df = 1, p<0.001) and spring (X^2_{Exact} = 47.685, df = 1, p<0.001), but consumed significantly more than expected during fall (X^2_{Exact} = 5.527, df = 1, p = 0.023). Shrews were negatively selected during every season (summer, X^2_{Exact} = 27.748, df = 1, p<0.001; fall, X^2_{Exact} = 36.515, df = 1, p<0.001; winter, X^2_{Exact} = 25.686, df = 1, p<0.001; spring, X^2_{Exact} = 75.892, df = 1, p<0.001), whereas western jumping mouse was consumed according to its availability with the exception of a significantly higher consumption during fall (X^2_{Exact} = 32.897, df = 1, p<0.001) (Table 2).

Based on the defecation rate applied in this study, we estimated a mean of 116.7±24.2 small mammals consumed per individual coyote per 3-month season, ranging from a maximum

of 178.7 during spring to a minimum of 70.6 in winter. Most of the predated small mammals were competent species for *E. multilocularis*, resulting in a mean of 101.7±19.8 intermediate hosts ingested per season, and ranging from a maximum of 157.3 in spring to a minimum of 70.6 in winter (Table 3). The proportion of intermediate hosts predated varied across seasons (X^2_{Exact} = 23.646, df = 3, p<0.001), with that in winter (1.0) being significantly higher than values observed in summer (0.79; X^2= 17.767, df =1, p<0.001), fall (0.87; X^2= 9.649, df =1, p = 0.003) and spring (0.88; X^2 = 9.148, df =1, p = 0.001). Meadow voles accounted for the majority of ingested intermediate hosts, with a mean of 60.6±21.9 individuals preyed by coyote per season, and ranging from a minimum of 24.4 in fall to a maximum of 118.8 in spring. Southern red-backed vole was the second most recurrent prey species (mean=29.6±5.9), followed by deer mouse (11.5±9.3), northern pocket gopher (9.1±4.4), western jumping mouse (4.8±2.0) and shrew (1.1±2.2) (Fig. 2B). Given the prevalence of *E. multilocularis* in intermediate hosts in the study area, the encounter rate with the parasite was highest in winter, with an estimate mean of 1.05 (95% CI: 0.99-22.44) infected intermediate hosts ingested per coyote, and lowest in spring (mean = 0; 95% CI: 0-3.38; Table 3).

Discussion

Our results suggest that small mammals, fruit and vegetation constituted the bulk of urban coyote diet in the study area, although hare and deer were seasonally very relevant. Within the small mammal assemblage, voles were the preferred preys of coyotes and likely played an important role in the transmission of *E. multilocularis* in urban Calgary during our study. Despite a relatively lower consumption of small mammals in winter, the encounter rate of coyote with

the parasite was at a maximum during this season, when the number of infected intermediate hosts ingested was estimated to be highest.

Seasonal variations in coyote diet and encounter with E. multilocularis

In urban Calgary, small mammals were the most frequent prey found in coyote feces in all seasons. For most of the year (summer-winter), their frequency of occurrence in diet reflected their relative availability, as previously documented in prairies (Litvaitis & Shaw 1980) and boreal forests (Nellis & Keith 1976). However, this trend was not confirmed for spring, during which we observed a high occurrence of small mammals in coyote diet despite a low capture rate in the field. Due to abundant snow cover, trapping success of small mammals in winter and spring is likely influenced not only by their relative abundance, but also by their activity in the subnivean space (Pruitt 1984; Schwingel & Norment 2010). A reduction of the snow cover depth and hardness during spring could expose small mammals to higher risk of predation by coyotes (Gese, Ruff & Crabtree 1996) without necessarily increasing their activity above the snowpack and the success of snap-trapping sessions, thus explaining the discrepancy observed.

Interestingly, lower occurrence of small mammals during winter was concurrent with higher consumption of deer. In this season, deer (juveniles in particular) are more exposed to the risk of coyote predation (Lingle 2002), as snow cover and low forage abundance/quality can hinder animal movements and negatively affect their body condition (Nelson & Mech 1986; Deelen *et al.* 1997; DelGiudice *et al.* 2002). An increased access of coyotes to alternative (and abundant) food sources could thus concur to explain the decrement in the consumption of small mammals observed in winter. The interdependence between the consumption of small mammals

and the availability of deer (i.e., prey switch) has been previously suggested for coyotes (Bowen 1981; Hamlin *et al.* 1984; Lingle 2002), and could affect the transmission ecology of *E. multilocularis* in areas where this canid is the parasite's main definitive host. Nonetheless, despite a relatively lower number of small mammals predated, encounter rate of coyotes with *E. multilocularis* was highest in winter, given the peak in parasite prevalence reported for intermediate hosts in this season (Liccioli *et al.* 2014). Importantly, composition of local small mammal assemblages can further shape spatial patterns of infection with *E. multilocularis* in definitive hosts (Liccioli *et al.* 2014), and our data suggest that such a dilution effect (*sensu* Keesing, Holt & Ostfeld 2006) may be significant in summer but absent in winter, when only competent intermediate host species are consumed (Fig. 2B). These observations are consistent with the higher parasite prevalence documented in coyote feces during spring (Liccioli *et al.* 2014) (Fig. 2A), once the patency period in the canid host (30-90 days post-infection, Kapel *et al.* 2006) is taken into account, further emphasizing the relevance of winter for *E. multilocularis* transmission (Burlet, Deplazes & Hegglin 2011; Liccioli *et al.* 2014).

Our estimates of parasite encounter rate, however, are conservative and need to be interpreted as the minimum number of intermediate hosts ingested per season. Although likely not applicable in urban habitats, higher predation rates on small mammals (and higher defecation rates) were reported for coyotes in Yellowstone National Park (Gese, Ruff & Crabtree 1996). Further research, possibly combining field observation with feeding trials, would allow refining correcting factors for small mammal digestibility and coyote defecation rates. Similarly, larger sample size, multiannual data series and indices of availability for other prey species (i.e., deer,

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hare) would aid in a better understanding of seasonal variations in coyote diet and parasite encounter rates.

Coyote diet and E. multilocularis transmission ecology

Among small mammals, meadow vole and southern red-backed vole were the preferred preys of coyotes in the study area. Considering their competence for the parasite (Eckert *et al.* 2001; Liccioli *et al.* 2013), they are likely playing a key role in the transmission of *E. multilocularis* in Calgary. In particular the meadow vole, given its high abundance, is likely very important for the maintenance of the sylvatic life-cycle of *E. multilocularis* in this urban landscape. On the other hand, the southern red-backed vole could be locally highly relevant in relation to suitable wood patches (Bondrup-Nielsen 1987), that are in Calgary less abundant and more heterogeneously distributed than grasslands. This species is likely very important in winter, when its contribution to coyote diet and parasite transmission are higher. Although the deer mouse is a locally abundant competent host (Eckert *et al.* 2001), its under-representation in coyote diet would suggest that it is unlikely to be a key species for the infection of coyotes in urban Calgary.

Microtine multi-annual population fluctuations are considered major drivers of *E. multilocularis* transmission intensity (Giraudoux *et al.* 2003). In particular, the relevance of *Microtus* species (i.e. *M. arvalis*) for the maintenance of the parasite life-cycle has been recently demonstrated also at large scales (Guerra *et al.* 2014) where red foxes are the main (and often only) wild definitive host (Deplazes *et al.* 2004; Hegglin *et al.* 2007), although several other intermediate host species (i.e., *Myodes* sp., *Ellobius* sp., *Ochotona* sp., *Eospalax* sp., *Cricetulus*

sp.) can be locally important, depending on ecosystem characteristics (Saitoh & Takahashi 1998; Giraudoux *et al.* 2013). While our data emphasize the importance of considering the whole small mammal assemblage (Liccioli *et al.* 2014), as the relevance of intermediate hosts can vary depending on asynchronous population fluctuations of different species (Giraudoux *et al.* 2003; Giraudoux *et al.* 2013), it is necessary to further investigate the interaction between availability of suitable habitats (i.e. ROMPA; Lidicker 2000) and population dynamics of meadow voles in urban habitats, as well as their effect on *E. multilocularis* transmission. Integrated with existing knowledge on parasite ecology and epidemiology, such information could help prevent and manage potential disease outbreaks (Viel *et al.* 1999).

Finally, our study suggests that coyotes may predate upon red foxes, although occasionally. Such information was not reported in earlier investigations on coyote diet in our study area (Lukasik & Alexander 2012), possibly also due to its rare occurrence. The suppression of red fox populations by coyotes has been documented in North America (Harrison, Bissonette & Sherburne 1989; Levi & Wilmers 2012), and it has been hypothesized as the mechanism explaining the low abundance of foxes in urban Calgary (Catalano *et al.* 2012). By shaping the composition of the definitive host community and the density of highly susceptible host species such as foxes, the existence of such a direct competition might have consequences on the transmission dynamics of *E. multilocularis*. Further research is needed to quantify interference competition between coyote and red fox, and assess the relative role of these species (as well as of domestic dogs) in the circulation of *E. multilocularis* in Calgary urban and rural habitats.

Conclusions

In this study we estimated, for the first time, the seasonal encounter rate of a canid host with *E. multilocularis* infected intermediate hosts, thus offering a quantitative framework for further epidemiological studies. Our data shows that winter is the most important season for parasite transmission, due to higher encounter rates when coyotes predate upon susceptible intermediate hosts, and the lack of dilution offered by non-competent small mammal species; eventual control strategies (i.e., coyote deworming with anthelmintic baits) should be planned with this in mind (Burlet, Deplazes & Hegglin 2011). Furthermore, we provided evidence that within the small mammal assemblage, the meadow vole *Microtus pennsylvanicus* is likely playing a key role in the maintenance of the urban sylvatic life-cycle of *E. multilocularis* in Calgary, along with the southern red-backed vole (*Myodes gapperi*) which may be locally very important. Long-term studies of population dynamics of these species, in response to changes in habitat availability and land use, should be integrated in future research on *E. multilocularis* transmission in urban habitats to inform disease risk management.

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Table 1

	Summer (n = 86)		Fall (n = 69)		Winter (n = 33)		Spring (n = 63)		Overall (n = 251)	
	Scat	Occur	Scat	Occur	Scat	Occur	Scat	Occur	Scat	Occur
Vertebrates										
Small mammals	65.1	16.3	50.7	17.2	39.4	17.1	62.5	23.0	57.1	18.0
Hare (*Lepus* spp.)	11.6	2.9	17.4	5.9	27.3	11.8	32.8	12.1	20.6	6.5
Deer (*Odocoileus spp.*)	17.4	4.4	20.3	6.9	36.4	15.8	4.7	1.7	17.5	5.5
Birds	27.9	7.0	11.6	3.9	9.1	3.9	1.6	0.6	14.3	4.5
Sciuridae	30.2	7.6	8.7	2.9	6.1	2.6	6.2	2.3	15.1	4.8
Muskrat	11.6	2.9	-	-	-	-	6.2	2.3	5.6	1.8
Porcupine	4.6	1.2	4.3	1.5	12.1	5.3	1.6	2.6	4.8	1.5
Cat	3.5	0.9	2.9	1.0	-	-	-	-	2.0	0.6
Red fox	4.7	1.2	-	-	-	-	1.6	0.6	2.0	0.6
Dog	1.2	0.3	-	-	3.0	1.3	-	-	0.8	0.3
Invertebrates	5.8	1.5	14.5	4.7	3.0	1.3	3.1	1.1	7.1	2.3
Vegetation	97.7	24.4	82.6	27.9	27.3	11.8	84.4	31.0	81.0	25.6
Fruit	57.0	14.2	37.7	21.1	42.4	18.4	42.2	15.5	52.8	16.7
Anthropogenic	18.6	4.7	7.2	2.5	15.1	6.6	1.6	0.6	10.7	3.4

Table 2

	Summer		Fall		Winter		Spring		Overall	
Species	Ivlev	p	Ivlev	p	Ivlev	p	Ivlev	p	Ivlev	p
Microtus pennsylvanicus	**0.31**	< 0.001	0.07	0.686	**0.30**	0.043	**0.27**	0.001	**0.28**	< 0.001
Myodes gapperi	**0.59**	< 0.001	0.18	0.258	**0.97**	< 0.001	**1.00**[b]	< 0.001	**0.53**	< 0.001
Peromyscus maniculatus	**-0.99**	< 0.001	**0.42**	0.023	**-1.00**	< 0.001	**-0.82**	< 0.001	**-0.54**	< 0.001
Zapus princeps	-0.19	0.298	**0.95**	< 0.001	- [a]	-	1.00[b]	0.559	0.14	0.296
Sorex sp.	**-0.73**	< 0.001	**-1.00**	< 0.001	**-1.00**	< 0.001	**-1.00**	< 0.001	**-0.93**	< 0.001

[a] species not captured in the field and not found in feces

[b] species not captured in the field, but found in feces

Table 3

Season	Summer	Fall	Winter	Spring	Total
Small mammals ingested per feces	1.8	1.2	1.0	2.5	1.6
IHs ingested per feces[a]	1.4	1.1	1.0	2.2	1.4
N small mammals ingested [b]	130.0	87.4	70.6	178.7	466.7
N IHs ingested [b]	102.5	76.4	70.6	157.3	406.8
N infected IHs ingested (95% CI) [c]	0.002 (0.002-1.397)	0.509 (0.496-1.175)	1.052 (0.994-22.443)	0 (0-3.383)	1.563 (1.492-28.398)

[a] Small mammals reported as susceptible hosts for *E. multilocularis* in North America (Hnatiuk 1966; Lee 1969; Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Liccioli *et al.* 2013).

[b] Daily defecation rate of coyotes (0.79 scats/day) (Monroy-Vilchis & Frieven 2006).

[c] Seasonal prevalence (95% CI) of *E. multilocularis* in intermediate hosts in the study area (Liccioli *et al.* 2014).
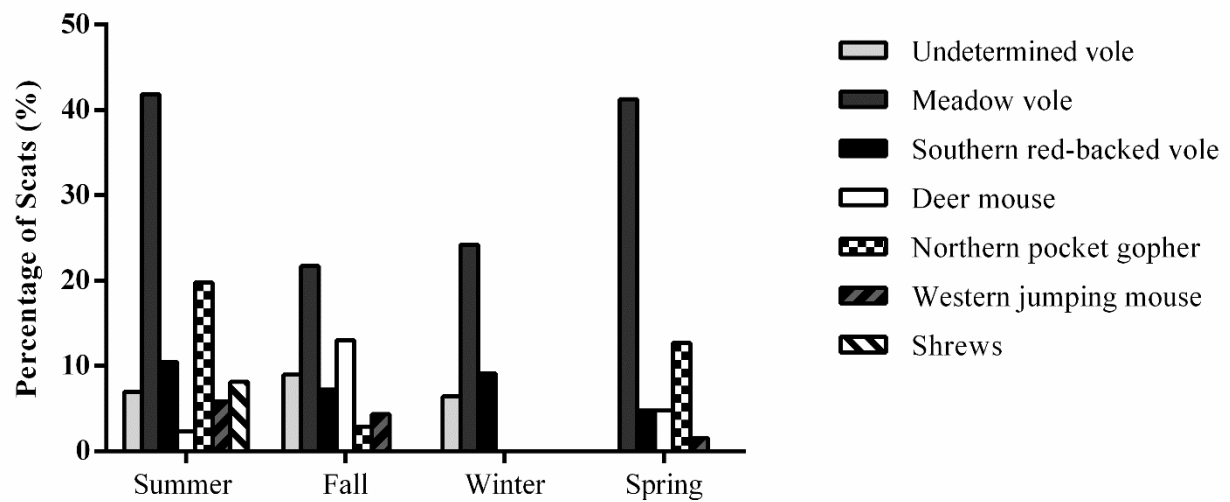
Figure 1

Figure 2

A

B

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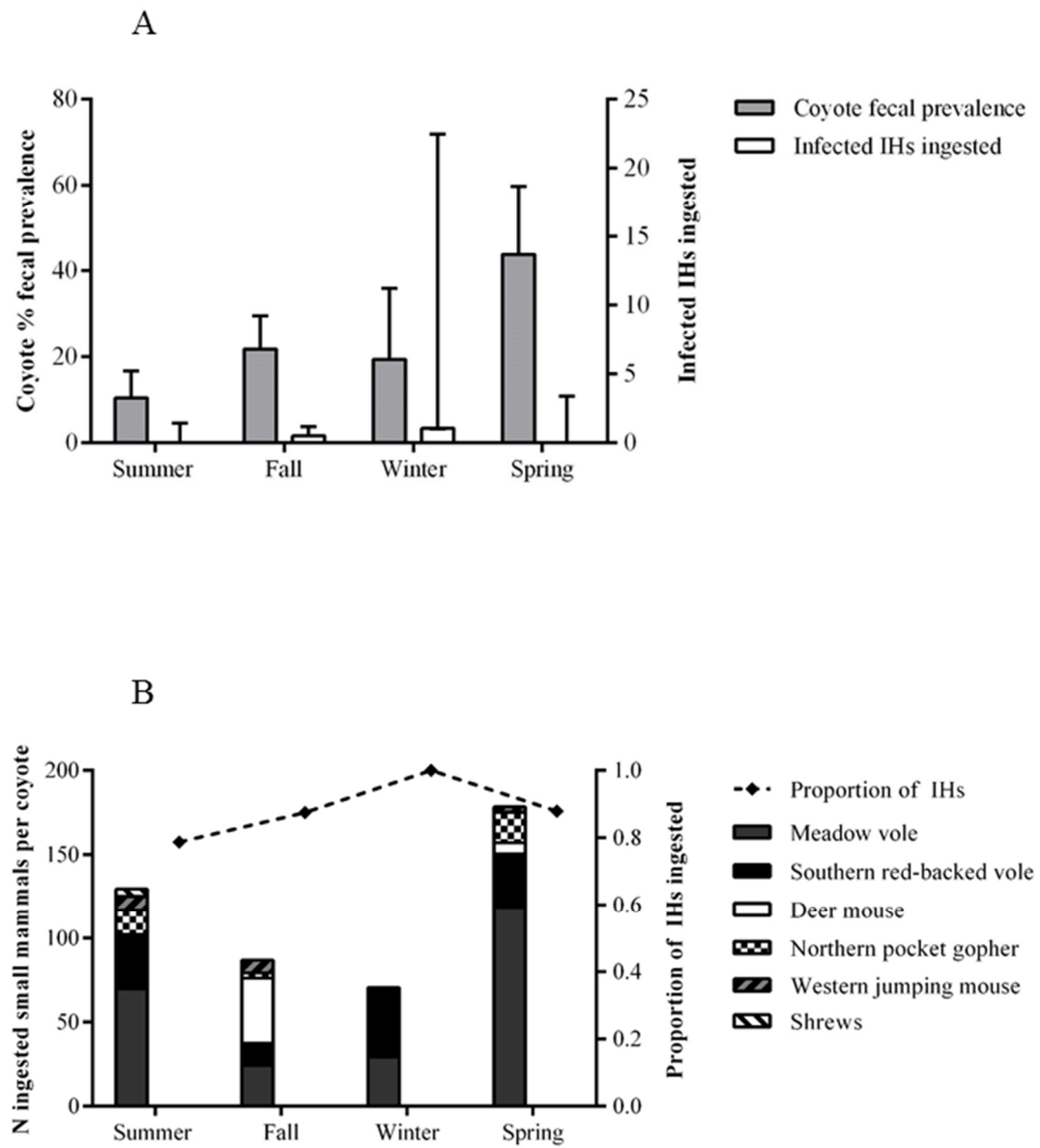
Chapter 7

WILDERNESS IN THE CITY REVISITED: ALTERATIONS OF PREDATOR AND PREY COMMUNITIES SHAPE URBAN CYCLES OF *ECHINOCOCCUS MULTILOCULARIS*

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Abstract

The urbanization of *Echinococcus multilocularis*, the causative agent of the Alveolar Echinococcosis, is a public health concern worldwide. Herein, we propose to consider "urban" habitats under a broad ecological perspective, and discuss the effects of human settlings on host communities and the process of parasite urbanization. We argue that interactions between landscape features (i.e., landscape composition and configuration) and host communities can shape the heterogeneity of transmission gradients observed within and across different urban settings.

Due to unique ecological characteristics and public health management priorities, we envisage urban landscapes as a model system to further increase our understanding of host – parasite interactions shaping the circulation of *E. multilocularis*.

Introduction

Urbanization has broad effects on ecosystem biodiversity (McKinney 2002), ecological mechanisms (Faeth *et al.* 2005; Shochat *et al.* 2006), and disease dynamics (Bradley & Altizer 2006). Exploring its effects on host-pathogen interactions becomes particularly interesting for trophically-transmitted parasites, given the complex life-cycles often involving intermediate and definitive host species with very different ecology (Lafferty 1999).

The cestode *Echinococcus multilocularis* is the causative agent of Alveolar Echinococcosis (AE) in humans, which is among the most relevant emergent parasitic zoonoses for the Northern hemisphere (case fatality rate > 90% when untreated; Craig, Rogan & Allan 1996). According to a 2014 report of the World Health Organization (WHO) and the UN's Food and Agriculture Organization (FAO), *E. multilocularis* is the third most impacting food-borne parasite globally (FAO/WHO 2014). Several interacting factors are possibly responsible for the parasite's global emergence (Davidson *et al.* 2012), including: the increase of definitive host populations (Romig, Craig & Pawlowski 2002; Deplazes *et al.* 2004); landscape changes outside cities (e.g. deforestation or grassland extension) that promote high densities of intermediate host populations in endemic areas (Giraudoux *et al.* 2003; Graham, Danson & Craig 2005; Romig, Thoma & Weible 2006); the involvement of domestic animals into the parasite cycle or transmission to humans (Rausch, Fay & Williamson 1990; Craig *et al.* 2000; Hegglin & Deplazes 2013); the sprawl of cities into carnivore natural habitats (Catalano *et al.* 2012), along with growing trends in planning green spaces within urban landscapes (Romig, Dinkel & Mackenstedt 2006).

The circulation of *E. multilocularis* in urban settings not only changed our perception of the risk of zoonotic transmission (Deplazes *et al.* 2004), but also generates new questions on the evolution of a

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complex ecological system. Yet, ecological mechanisms underlying the parasite's urbanization are still poorly understood. Seminal contribution to the understanding of *E. multilocularis* urban transmission comes from research done in European cities (Deplazes *et al.* 2004; Hegglin *et al.* 2007; Robardet *et al.* 2008), but there is certainly the need to extend the concept of 'urban' to include other landscapes human settlements (Table 1), where ecological processes (Liccioli *et al.* 2014) and control strategies (Hegglin & Deplazes 2013; Takahashi *et al.* 2013) may be significantly different.

Herein, we aim to i) revisit and broaden the concept of urban landscapes in relation to the urbanization of *E. multilocularis*; and ii) describe how the urbanization process affects key changes in intermediate and definitive host communities, their interactions, and consequently *E. multilocularis* transmission within and in proximity to urban settings.

Defining '*urban*': a broader perspective

The term 'urban' derives from the latin noun *urbs*, which means 'city', generally referred to the settlings delimited by the defensive walls. This perhaps explains why there is no clear agreement on what distinguishes a city from a town or a village, thus suggesting to consider a wide range of human aggregations and landscapes when referring to urban settings. Overall, a system is herein defined 'urban' depending on the density of human settlements (e.g., few isolated houses are not an "*urbs*") and the relative impact of such aggregations on parasite transmission (e.g., few dogs would not significantly contribute to alter or maintain the circulation of the parasite).

Analysing the transmission of *E. multilocularis*, it becomes evident the broad variety of urban settings in which wild hosts, domestic animals and humans may interact. These can include i) small settlements and villages surrounded by farmland, such as the ones found in highly endemic areas of

western China (e.g., Tibetan plateau) (Giraudoux *et al.* 2006; Giraudoux *et al.* 2013a) or Germany (Bavaria) (Janko *et al.* 2011); ii) rural towns and surrounding districts in Japan (e.g. Abashiri, Hokkaido Island; Saitoh & Takahashi 1998) or Iran (Cheneran county; Beiromvand *et al.* 2013); iii) relatively low density residential areas (1,000-3,000 inhabitant/km^2) of medium-large cities in North America (Liccioli *et al.* 2014), where green spaces and parks represent a fairly connected significant portion of the urban landscape (>10%); iv) low density cities of Japan (Tsukada *et al.* 2000) and high density cities (>4,000 inhabitants/km^2) of Europe (e.g., Switzerland and France) where green parks are limited in extension and scarcely connected (Deplazes *et al.* 2004; Robardet *et al.* 2008).

Although research on *E. multilocularis* traditionally referred to the urbanization gradient as the one existing from the city periphery to the inner urban core (Deplazes *et al.* 2004; Reperant *et al.* 2007), such a large variety of urban settings introduces other elements of heterogeneity, which ultimately create additional gradients (Fig. 1). Human aggregations differ in size and population density, thus resulting in a broad variety of landscapes and ecosystems, where the ecological processes shaping parasite transmission can be highly diverse. Furthermore, habitat connectivity in urban landscapes affects permeability to wildlife movements within urban green areas (e.g. Adkins & Stott 1998), and between city settings and surrounding habitats (Beier & Noss 1998). Similarly, urban landscape composition and its heterogeneity might influence the diversity of the host community, with important consequences on the dynamics and resilience of parasite transmission (Giraudoux *et al.* 2007). Along with a degree of urbanization, a gradient of 'urban density' (e.g. high density *vs.* low density residential areas) and/or 'green area' connectivity needs therefore to be taken into account, and its consequences on *E. multilocularis* transmission further explored (Fig. 1).

Urbanization and host communities

In the last decade, the 'urbanization' of *E. multilocularis* has been considered as a process primarily driven by changes in the wild definitive host ecology, which consequently allowed the parasite to circulate and be maintained within urban habitats (Deplazes *et al.* 2004). However, the urbanization of the parasite can potentially result also from any alteration of the hosts' community (both intermediate and definitive) induced by the presence of human settlings. Domestic dogs, for example, are not only a source of human AE (Rausch, Wilson & Schantz 1990; Craig *et al.* 2000; Tiaoying *et al.* 2005; Hegglin & Deplazes 2013), but can locally act as the main definitive host in a semi-domestic cycle of *E. multilocularis*, as documented in China and Alaska. Here, domestic canids play a significant - and occasionally, primary - role for the maintenance of *E. multilocularis*, as supported by high parasite prevalence (13-33%; Budke *et al.* 2005) and re-infection rates (15.5-57%; Moss *et al.* 2013) observed in dogs, their major contribution to environmental contamination (Vaniscotte *et al.* 2011), and the reduction of intermediate host infection following dog deworming (Rausch, Wilson & Schantz 1990). In such systems, human aggregations are actively influencing and altering the parasite transmission by bringing a competent and abundant definitive host in the environment, without affecting necessarily the ecology of the wild definitive hosts (e.g. *Vulpes* spp.). Similarly, urbanization has the potential to enhance parasite transmission through the alteration of the intermediate host community, for example by providing variety of suitable habitat patches for the main intermediate hosts, such as *Microtus* sp. and *Myodes* sp. (Dickman & Doncaster 1987) and *Arvicola* sp. (Hofer *et al.* 2000), as well as for more synanthropic competent species such as the house mouse (*Mus musculus*; Leiby, Carney & Woods 1970) (Rowe 1975). When referring to the urbanization of *E. multilocularis*, we therefore stress the need of considering it as a multifaceted process, derived by the adaptation of

wildlife populations to urban environments ("synurbization"; Andrzejewski *et al.* 1978) and/or changes in the environment (and in this case, host communities) caused by urban development ("urbanization").

Intermediate host communities along urban gradients

In European landscapes, the density of the main intermediate host species (water vole *Arvicola terrestris* and common vole *Microtus arvalis*) is reported to increase from the urban cores to surrounding rural habitats (Deplazes *et al.* 2004; Hegglin *et al.* 2007; Reperant *et al.* 2007; Robardet *et al.* 2008). Due to the definitive host (red fox *Vulpes vulpes*) functional response to variations in prey availability (Hegglin *et al.* 2007), higher *E. multilocularis* prevalence and environmental contamination were observed along this urbanization gradient (Stieger *et al.* 2002; Lewis *et al.* 2014), causing recreational areas located at city peripheries to be hotspots for zoonotic transmission risk and public health surveillance (Deplazes *et al.* 2004). However, such a transmission gradient may not adequately describe the circulation of *E. multilocularis* in other urban and peri-urban landscapes.

Although several variables may interact to determinewhether a system is able to sustain *E. multilocularis* transmission, the proportion of landscape occupied by optimal habitat (ROMPA; Lidicker 2000) for intermediate hosts is considered one of the key factors, as it influences small mammal population dynamics, definitive hosts (i.e., foxes) density and intensity of parasite transmission (Giraudoux *et al.* 2003).

Habitat diversity influences small mammal assemblages throughout the geographic range of *E. multilocularis* by shaping both the overall abundance of intermediate hosts and their relative abundance compared to not susceptible small mammal species. The presence of not susceptible species is likely decreasing the rate of transmission diluting the risk of encounter of the predator with infectious hosts

(Liccioli *et al.* 2014). Thus, whereas the proportion of grassland has been the key parameter driving the parasite transmission dynamics in Western Europe until the '90s (at least before the substantial increase of red fox population; Deplazes *et al.* 2004), given its association with the main local intermediate hosts (Giraudoux *et al.* 2003), other habitat types (and intermediate host species) are determinant elsewhere. For example, in the Southern Gansu plateau (China) the population dynamics of *Microtus limnophilus* and *Cricetulus longicaudatus* are strongly influenced by the availability of shrubs and scrublands (Giraudoux *et al.* 2006), whereas in Hokkaido (Japan) the parasite transmission is determined by the presence of woodlands (Tsukada *et al.* 2000), the optimal habitat for main local intermediate host species (*Myodes* spp.; Tsukada *et al.* 2002), as well as red foxes (Tsukada *et al.* 2000).

Variations in the circulation of *E. multilocularis* along an urbanization gradient thus reflect the spatial arrangement of particular habitat types in the urban matrix, as well as their suitability for intermediate and definitive hosts. Whereas the presence of woodlands at the urban fringe of Sapporo (Hokkaido, Japan) might sustain the life-cycle of *E. multilocularis* at the city periphery and not in its inner core - although further research is needed to confirm such hypothesis, (Tsukada *et al.* 2000) - large grassland and forest patches within metropolitan areas of Canada support parasite transmission within the city limits (Liccioli *et al.* 2014). Factors affecting parasite circulation and infection pressure thus vary with the characteristics of the landscape beyond the traditional idea of a gradient from city centres to the surroundings (Fig. 3).

Investigating how landscape composition and configuration influence the dynamics of infectious diseases, landscape ecology and spatial epidemiology have recognized potential to help assessing zoonotic risk (Ostfeld, Glass & Keesing 2005). However, their actual integration in urban

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planning (i.e. spatial arrangement of habitats and outbreaks of parasites in reservoir species) to manage disease incidence still has to be actualized, possibly making *E. multilocularis* in urban habitats an excellent, although challenging, study system.

Urban carnivore community

Although most of our knowledge on the transmission ecology of *E. multilocularis* in urban landscapes is based on the role of the red fox (e.g. Deplazes *et al.* 2004), the parasite is not the exclusive of this single urban-adapted carnivore host. Other species of wild carnivores are associated with the circulation of *E. multilocularis* in urban settings, such as raccoon dogs (*Nyctereutes procyonoides*) in Japan (Yimam *et al.* 2002), the Tibetan fox (*Vulpes ferrilata*) on the Tibetan plateau (Vaniscotte *et al.* 2011) and coyotes in Canada (Catalano *et al.* 2012). Carnivores species are reported to differ not only in terms of susceptibility to *E. multilocularis* infection and parasite reproductive potential (i.e. number of eggs released; Kapel *et al.* 2006), but also in feeding behaviour (and thus encounter rate with the parasite), especially when living in sympatry (Kitchen, Gese & Schauster 1999; Neale & Sacks 2001). Along ecological gradients (e.g. rural to urban), the relative abundance of different species in the definitive host community can vary (Oehler & Litvaitis 1996), according to habitat suitability and interspecific competition (Levi & Wilmers 2012). Ultimately, competition can result in complete exclusion of one species, as documented for coyotes and sympatric foxes (Fedriani *et al.* 2000; Kamler *et al.* 2003; Karki, Gese & Klavetter 2007; Thompson & Gese 2007). Intra-guild competition therefore has the potential to influence *E. multilocularis* transmission: depending on relative composition of the definitive host community, as well as on susceptibility and biological

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potential for the parasite of the different species, parasite circulation could be either attenuated or amplified.

Further complicating the relationship between intra-guild competition and parasite transmission is the role of domestic dogs, for which ecological rules (i.e. interspecific competition, carrying capacity) do not necessarily apply. Interestingly, even if the prevalence of *E. multilocularis* in dogs is usually reported to be low (<1 %) (Deplazes *et al.* 2004), their contribution to the parasite cycle could be relevant given their large populations (Vaniscotte *et al.* 2011). By reinforcing and amplifying the wildlife cycle, their role on parasite transmission can be therefore highly significant (Rausch, Wilson & Schantz 1990; Vaniscotte *et al.* 2011; Hegglin & Deplazes 2013), especially in urban areas where dog populations reach high numbers (e.g. >100,000 dogs in metropolitan areas of North America or densely inhabited cities of Europe) and in China where hundreds of dogs can be present in each village of the eastern border of the Tibetan plateau and the mountain hills of the loess plateaus (Gansu, Ningxia) (Giraudoux *et al.* 2013b).

Given the particular ecological characteristics of urban settings (i.e. habitat heterogeneity and fragmentation, presence of dogs), these may be an ideal study system to investigate the effects of change in definitive host community composition on *E. multilocularis* transmission.

Concluding remarks

Intense research effort highly improved our comprehension of the complex *E. multilocularis* system in the last decade. Nonetheless, the great ecological heterogeneity observed within and among urban landscapes requires to carefully interpret and compare patterns observed in specific environments. Urbanization affects the ecological mechanisms and processes that regulate the

transmission dynamics of *E. multilocularis*, altering host communities both through changes in landscape composition and configuration (i.e. habitat types and connectivity), as well as directly modifying the definitive host community (i.e. with domestic dogs; Fig. 4). Priority areas for further research are represented by the study of predator functional response to variations in abundance and composition of small mammal assemblages within urban mosaics, the role of landscape configuration as well as the effect of intra-guild competition on definitive host community composition and parasite transmission.

Urban landscapes therefore represent not only a research priority under a health risk management standpoint, but also an excellent opportunity to increase our understanding of this complex host-parasite system.

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Figure 4:   Diagrammatic representation of the effects of urbanization on *Echinococcus multilocularis* host community and life cycle dynamics, through alteration of landscape and through direct modification of the definitive host community (dogs).

Table 1

Ref	Urban setting	Location	Object	Definitive hosts	%Prev. intestines	%Prev. feces	Intermediate hosts (%Prev., n)
1	high-density city	Zurich (CHE)	Prevalence	*Vulpes vulpes*	36		*Arvicola terrestris* (14%, 135)
2	high-density city	Zurich (CHE)	Prevalence	*Vulpes vulpes*		25.8[a]	*Arvicola terrestris* (9.1%, 889)
2	"	"					*Myodes glareolus* (2.4%, 83)
3	high-density city	Zurich (CHE)	Control	*Vulpes vulpes*		n/a[a]	*Arvicola terrestris* (6.8%, 1229)[3]
4	high-density city	Geneva (CHE)	Prevalence	*Vulpes vulpes*	31		
5	high-density city	Zurich (CHE)	Ecology	*Vulpes vulpes*	16.5		*Arvicola terrestris* (n/a)
6	high-density city	Geneva (CHE)	Prevalence	*Vulpes vulpes*	48.8		
7	high-density city	Nancy (France)	Prevalence	*Vulpes vulpes*	4		
8	"	"	Epidemiology	"			
9	low-density city	Sapporo city (JPN)	Prevalence	*Vulpes vulpes*		21.3[a]	*Myodes rufocanus* (0%, 3)
10	low-density city	Sapporo city (JPN)	Prevalence	*Vulpes vulpes*		16-49[a,b]	
11	low-density city	Calgary (CAN)	Prevalence	*Canis latrans*	25.6	≤6.1[b]	
12	low-density city	Calgary, Edmonton (CAN)	Prevalence	*Canis latrans*	25.3		
13	low-density city	Calgary (CAN)	Ecology	*Canis latrans*		21.4[b]	*Peromyscus maniculatus*(0.66%, 310)
13	low-density city	Calgary (CAN)	Ecology				*Microtus pennsylvanicus* (0.75%, 267)
13	low-density city	Calgary (CAN)	Ecology				*Myodes gapperi* (1.41%, 71)
14	rural town	Koshimizu (JPN)	Control	*Vulpes vulpes*		38.7-13.3[b]	
15	rural town	Abashiri, Nemuro, Kushiro (JPN)	Prevalence	*Vulpes vulpes*	4		*Myodes rufocanus* (n/a)
16	rural town	Nemuro City (JPN)	Control	*Vulpes vulpes*	49.4[c]		
17	rural town	Otaru, Koshimizu, Nemuro (JPN)	Control	*Vulpes vulpes*	not rep	not rep.	
17	rural town	Otaru, Koshimizu, Nemuro (JPN)	Control	*Canis lupus familiaris*		0.4[a,b]	
18	rural town	Otaru city (JPN)	Prevalence	*Vulpes vulpes*	56.7		
18	rural town	Otaru city (JPN)	Prevalence	*Nyctereutes procyonoides*	23.1		
19	rural town	Chenaran county (IRN)	Prevalence	*Vulpes vulpes*	10-22.9[27-30]		*Microtus transcaspicus* (29.6%, 54)
19	"	"	"	*Canis aureus*	16 [30]		*Ochtona rufescens* (75%, 4)

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Table 1 – continued

20	rural village	Oberammergau and Starnberg (GER)	Prevalence/Ecology	*Vulpes vulpes*	41.9-45.5	26.1[b]	
21	rural village	Zang county (CHN)	Epidemiology	*Canis lupus familiaris*	10		
22	rural village	Tuanji, Shiqu county (CHN)	Ecology				*Microtus limnophilus* (14.7%, 34)
23	rural village	Shiqu county (CHN)	Ecology	*Canis lupus familiaris*	13-33		*Cricetulus kamensis* (5.3%, 19)
24	rural village	Savoonga, St. Lawrence Island (USA)	Control				*Microtus oeconomus* (22-35%, n/a)[3]
25	rural village	Shiqu county (CHN)	Ecology	*Canis lupus familiaris*		23[b]	
25	"	"	"	*Vulpes vulpes*		15[b]	
26	rural village	Tuanji, Shiqu county (CHN)	Ecology				*Microtus* cf. *irene* (25%, 12)
26	"	"	"	*Canis lupus familaris*	12.1-25		*Ochotona curzoniae* (6.7%, 75)
26	"	"	"	*Vulpes ferrilata*	44.4		*Lepus oiostolus* (7.1%, 14)

References as follows: 1 (Hofer *et al.* 2000); 2 (Stieger *et al.* 2002); 3 (Hegglin, Ward & Deplazes 2003b); 4 (Fischer *et al.* 2005); 5 (Hegglin *et al.* 2007); 6 (Reperant *et al.* 2007); 7 (Reperant *et al.* 2009); 8 (Robardet *et al.* 2011); 9 (Tsukada *et al.* 2000); 10 (Lagapa *et al.* 2009); 11 (Liccioli *et al.* 2012a); 12 (Catalano *et al.* 2012); 13 (Liccioli *et al.* 2014); 14 (Morishima *et al.* 1999); 15 (Saitoh & Takahashi 1998); 16 (Takahashi *et al.* 2013); 17 (Nonaka, Kamiya & Oku 2006); 18 (Yimam *et al.* 2002); 19 (Beiromvand *et al.* 2013); 20 (Janko *et al.* 2011); 21(Craig *et al.* 1992); 22(Raoul *et al.* 2006); 23(Budke *et al.* 2005); 24 (Raush *et al.* 1990) 25(Vaniscotte *et al.* 2011); 26(Qiu *et al.* 1999); 27(Mobedi & Sadighian 1971) 28(Mobedi *et al.* 1973); 29(Zariffard & Massoud 1998); 30(Eckert *et al.* 2001).

Table 2

			Zurich	Sapporo City	Calgary	Abashiri	Ningxia [ɗ]
Urban characteristics							
Size			92 km²	1,121 km²	704.5 km²	470.9 km²	9.855 km²
Population (year)			403,000 (2014)	1, 118, 096 (2013)	1,096,833 (2011)	38, 966 (2011)	460,000 (2003)
Mean density/km² (range)			4,350 (12-20,050)	1,710	1,329 (566-18,937)	85.6	115 (27-118,107)
E. multilocularis							
Definitive hosts	Dogs	Population	106,000	unknown [a]	120,000	unknown [a]	100,000-400,000 [12]
		Density	115/ km²	-	170.3/km²	-	25.1-100.4/km²
		Prevalence	0.3% [1]	unknown [a]	0.46% (feces, n=218) [§]	unknown [a]	unknown [b]
	Fox	Population	~1000 [2]	unknown	unknown	unknown	-
		Density	11/km² [2]	unknown	unknown	unknown	- [‡]
		Prevalence	17-63% [3]	16-49% (feces) [5-6]	0% (intestines; n=1)	20–42 % [11]	-
	Coyote	Population	-	-	777-899 [†]	-	-
		Density	-	-	3.4-3.9/km² [†]	-	-
		Prevalence	-	-	20-29% [8-10]	-	-
Intermediate hosts			*A. terrestris*: 9.1% [4]	0% [7]	*P. maniculatus*: 0.66% [10]	unknown	*E. fontanieri*: 0.3%
			M. arvalis: 4.9% [*]		*M. pennsylvanicus*: 0.75% [10]		*S. alashanicus*: 0.6%
			M. glareolus: 2.4% [4]		*M. gapperi*: 1.41% [10]		
Zoonotic risk parameters							
AE prevalence			unknown	unknown	0 (unreported)	unknown	2% (0-8%) [12]
AE incidence (per 100,000)			0.25-0.5 [**]	unknown [a]	-	unknown [a]	unknown

Table 2 - continued

References as follows: [1](Deplazes *et al.* 1999a); [2](Gloor 2002); [3](Hegglin *et al.* 2007); [4](Schwarzenbach *et al.* 2004) ;[5](Tsukada *et al.* 2002b);[6](Lagapa *et al.* 2009);[7](Tsukada *et al.* 2000);[8](Catalano *et al.* 2012);[9](Liccioli *et al.* 2012a); [10](Liccioli *et al.* 2014); [11](Saitoh & Takahashi 1998); [12](Yang *et al.* 2012).

* Hegglin, pers comm;

** Deplazes, pers comm;

§ Massolo *et al.*, unpubl;

† Liccioli *et al.*, unpubl.;

ʄ Small mammal communities include several other possible intermediate host species (*C. longicaudatus*, *T. triton*, *M. unguiculatus*, *O. daurica*, *O. huangensis*, *M. musculus*), and the real role in parasite transmission of those incidentally found naturally infected as the others is virtually unknown (Giraudoux *et al.* 2013a);

‡ Extinct in 2000s (Giraudoux *et al.* 2006);

a Values for the whole Hokkaido Island as follows: dog population=248,149 (Nonaka, Kamiya & Oku 2006); *E. multilocularis* prevalence in dogs= 0.4-1% (Nonaka, Kamiya & Oku 2006); AE incidence = 5–19/year (Tsukada *et al.* 2000; Tsukada *et al.* 2002b);

b Veterinary surveillance data on dog population are missing since 1991-1992 (Yang *et al.* 2012)
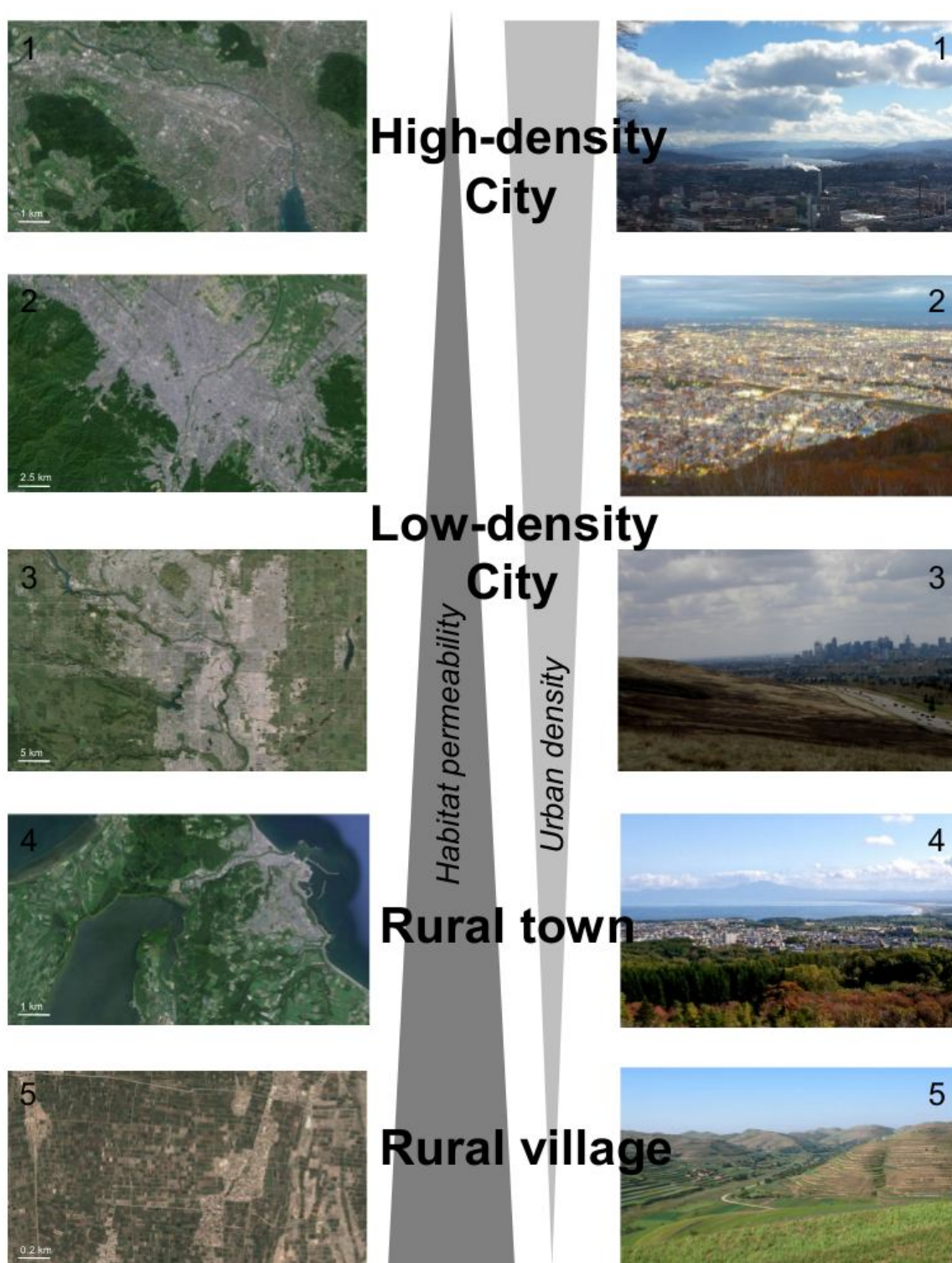
Figure 1

High-density
City

Low-density
City

Rural town

Rural village

Habitat permeability
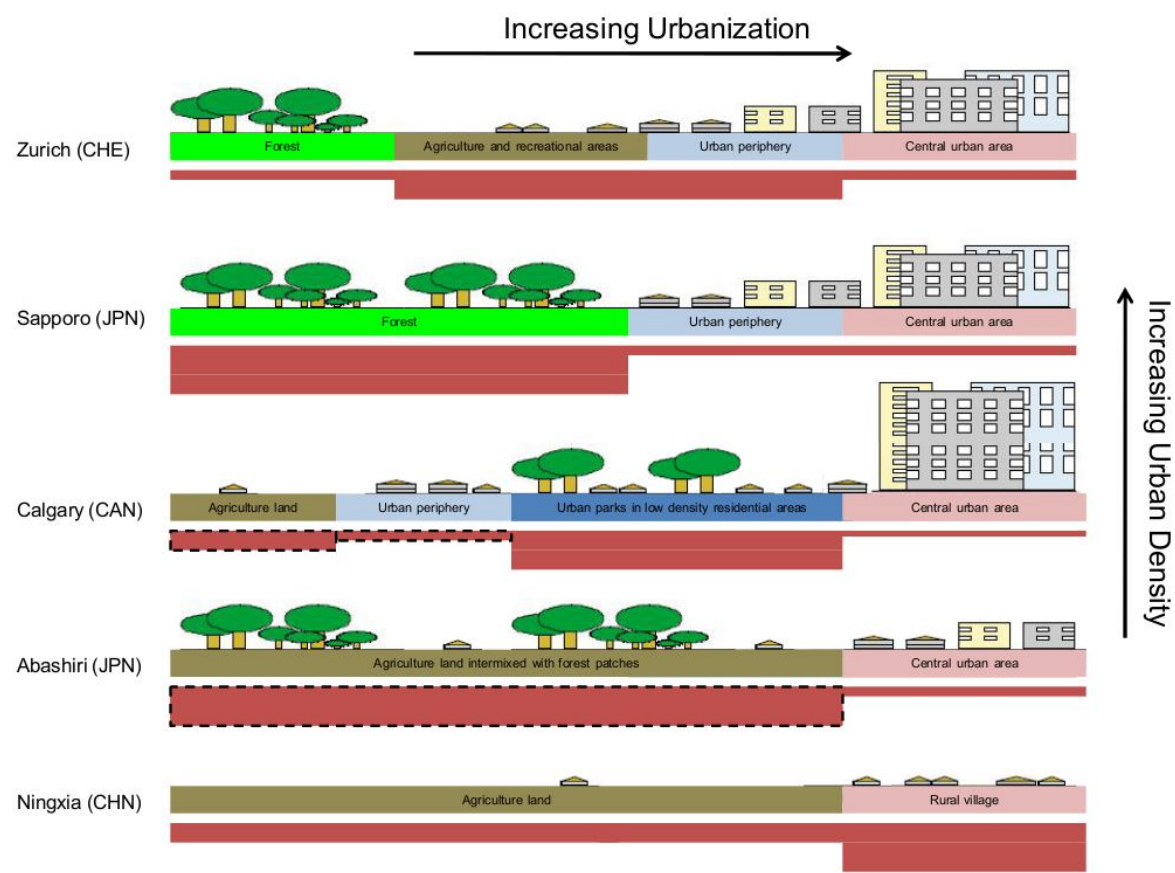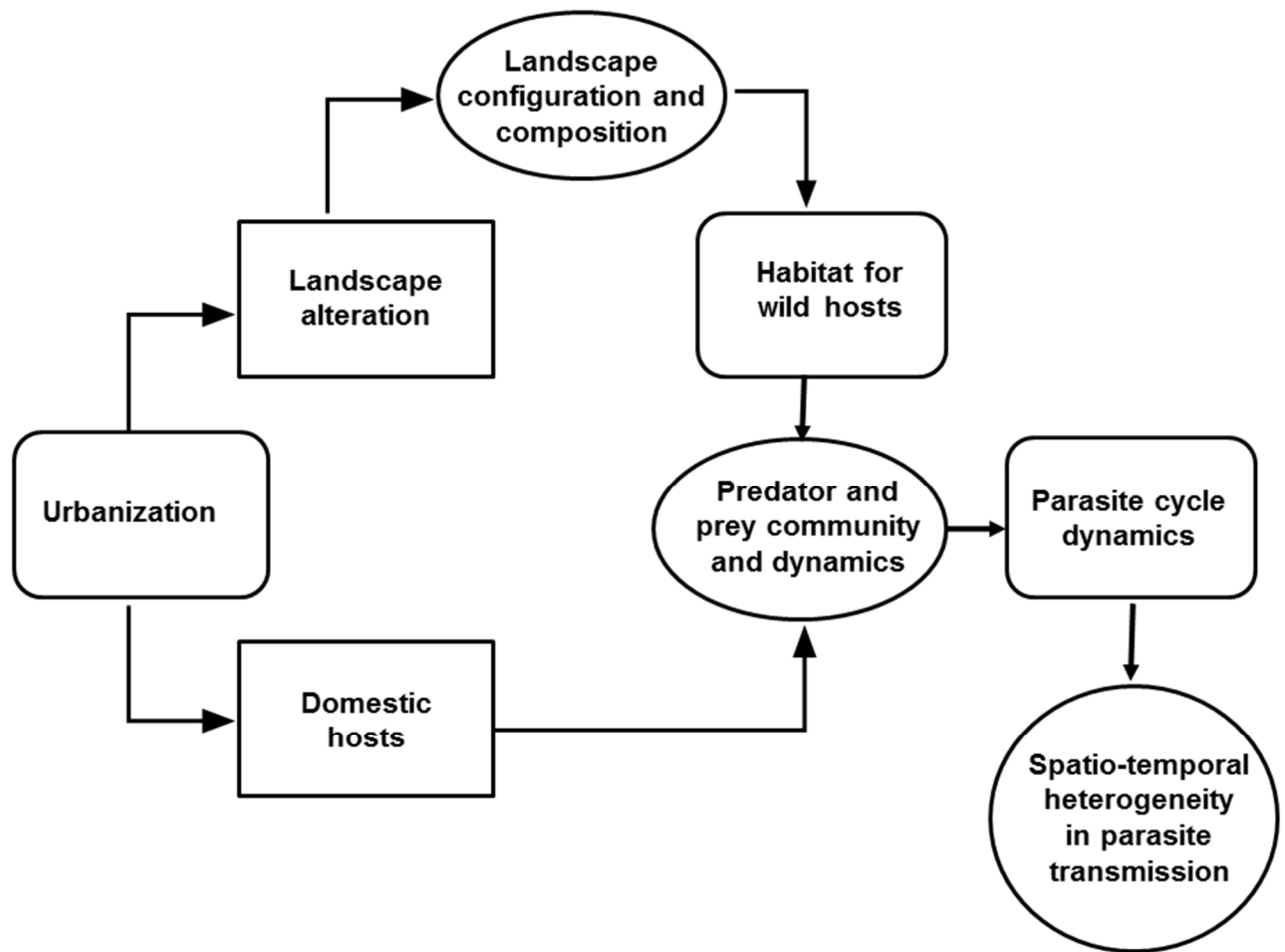
Urban density

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Figure 2

Figure 3

Figure 4

Chapter 8

CONCLUSIONS

The research presented in this dissertation was initiated to investigate the disease ecology of urban coyotes, with a specific focus on their gastrointestinal parasites. The early detection of *Echinococcus multilocularis* in coyotes inside Calgary city limits and in dog parks (Catalano *et al.* 2012; Liccioli *et al.* 2012a) has been extremely relevant from a public health perspective, but more information was needed to understand the transmission ecology of the parasite in this environment. In particular, it was unknown whether natural habitats available in urban Calgary could sustain and maintain a sylvatic life-cycle of *E. multilocularis*, or whether definitive hosts could only acquire the infection in surrounding rural areas. Furthermore, processes and patterns of parasite transmission in North American urban landscapes were still unexplored, and certainly further research was required to assess the risk of zoonotic transmission and to inform disease prevention and management.

In order to obtain accurate estimates of *E. multilocularis* prevalence in coyotes, it was necessary to first evaluate the reliability of the diagnostic technique adopted at the beginning of my research. The results of the test indicated the low (0.46) sensitivity of the sugar fecal flotation (Liccioli *et al.* 2012b), thus highlighting the risk of underestimating parasite fecal prevalence and the need for more reliable diagnostic techniques. Higher sensitivity (0.75; Liccioli *et al.* 2014) was obtained adapting the $ZnCl_2$ centrifugation and sedimentation protocol described by Davidson *et al.* (2009). This technique was consequently adopted in my research, and it is recommended for large screening of *Echinococcus* spp. in canid hosts. However, the success of molecular identification of parasite eggs experienced in my study was only moderate (55.8%). A

freeze-thaw protocol to be conducted prior to parasite DNA extraction has been shown to increase the sensitivity of *E. multilocularis* detection at PCR (Klein, Liccioli & Massolo 2014), and will allow future studies to further enhance the overall effectiveness of the fecal diagnostic test.

Based on a solid diagnostic, my research provided the first evidence of a sylvatic life-cycle of *E. multilocularis* in anurban landscape in North America (Liccioli *et al.* 2014); however, more information on the spatial behavior of urban coyotes will be needed in the future to better evaluate the role on parasite maintenance in urban settings played by coyote errands in the rural settings surrounding the city and by small mammal densities (and assemblage composition) in those habitats. Given their relatively high abundance (Catalano *et al.* 2012), coyotes are likely the main definitive host of the parasite in urban Calgary. Red foxes and dogs are recognized, respectively, as the parasite's main definitive hosts in Europe and Japan (Deplazes *et al.* 2004; Takahashi *et al.* 2013), and in certain areas of China (Moss *et al.* 2013). Assessing the role of coyotes and other potential definitive hosts in a multi-definitive host community, such as the one existing in Calgary, is fundamental to properly understand *E. multilocularis* ecology: further research is therefore needed to identify the role of these species in the urban life-cycle of the parasite in the study area. In particular, prevalence of *E. multilocularis* in coyotes in Calgary appeared to be generally lower than that recorded for urban foxes in cities of central Europe (i.e., 39.3 - 49.4%, Zurich; 35.3 - 51.2%, Geneva; Deplazes *et al.* 2004), although comparisons may be uninformative given the different sampling methods involved (i.e. hunted foxes *vs.* road-killed coyotes). If differences in parasite prevalence are to be considered as representative, they could be related for example to an overall higher suitability (i.e. worm establishment and reproductive

potential) of the fox host, although preliminary data currently do not support this hypothesis. In fact, intensities of parasite infection recorded for coyotes in our study area were comparable to those documented in foxes (median: 3,052 worm/coyote; range: 4-258,880; Massolo *et al.*, unpublished). Alternatively, densities of intermediate hosts could be a factor contributing to explain differences in parasite transmission intensity across different urban landscapes, although again inconsistencies in methods for estimating small mammal abundance (i.e., trapping *vs.* presence index data) may not allow such comparisons.

In Calgary urban settings, three main species of small mammals were identified as intermediate hosts for *E. multilocularis*. These included the deer mouse (*Peromyscus maniculatus*) and the meadow vole (*Microtus pennsylvanicus*), traditionally considered as the parasite's main intermediate hosts in North America (Eckert *et al.* 2001), but also the southern red-backed vole (*Myodes gapperi*), a competent host for *E. multilocularis* that was documented for the first time through my research (Liccioli *et al.* 2013).

The estimated parasite fecal prevalence in coyotes (21.4%) confirmed that the sylvatic cycle of the parasite can persist even with low prevalence in intermediate hosts (in this study, 0.66 - 1.41%), similar to what reported in studies in Europe, China and Japan (Saitoh & Takahashi 1998; Eckert *et al.* 2001; Giraudoux *et al.* 2003). Future research needs to consider other rodent species that were not sampled, such as the muskrat (*Ondatra zibethicus*), a competent intermediate host (Baumeister *et al.* 1997; Umhang *et al.* 2013) that was seasonally encountered in coyote diet (Liccioli *et al., submitted*). Additionally, future studies will benefit from adopting molecular techniques to detect immature liver lesions and morphologically undetectable larval stages that may otherwise result in underestimates of parasite prevalence in

intermediate hosts (e.g. Al-Sabi *et al.* 2013). This becomes particularly important given the very low parasite prevalence in intermediate hosts, which severely limits statistical comparisons across seasons and sites (Giraudoux *et al.* 2003).

Overall, spatial and temporal heterogeneity of parasite infection was observed in both definitive and intermediate hosts. In details, coyote fecal parasite prevalence varied widely across the sites I investigated, ranging from 5.3% to 61.5%, while infections peaked during spring (43.5%). Furthermore, prevalence in intermediate hosts increased from a low level in summer (0.46%) to a peak in winter (3.33%). The density of intermediate host populations is traditionally assumed to be the main variable driving *E. multilocularis* transmission (e.g., Saitoh & Takahashi 1998; Giraudoux *et al.* 2003; Raoul *et al.* 2010). However, this study suggested that local variations in the small mammal assemblage composition (i.e., proportion of intermediate host species) - rather than the abundance of intermediate hosts *per se* - and the dynamics of small mammal populations may shape the infection patterns observed in coyotes. These factors possibly interacted, reducing definitive host infection ratesthrough variation in the relative abundance of non-competent intermediate hosts, according to the "dilution effect" hypothesis (Keesing, Holt & Ostfeld 2006; Randolph & Dobson 2012). However, long-term studies are required to increase sample size and address the limitations caused by the low number of infections documented in intermediate hosts.

Integrating fecal parasitology analysis and fecal genotyping of individual coyotes, the study provided a more accurate estimate of *E. multilocularis* prevalence in an urban setting. After removing potential bias caused by re-sampling of individuals, the prevalence of 25.0% (95% CI: 15.6-37.4%) confirmed the results previously obtained with non-genotyped feces, thus

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suggesting that fecal prevalence estimates can be reliable and informative if the study design involves large sample collection across different areas and seasons. Importantly, fecal genotyping allowed to estimate a re-infection rate of 57.1% for coyotes in an area of high parasite prevalence (i.e., hyper-endemic area), supporting the hypothesis that the lack of temporal patterns observed for definitive hosts in that site (Liccioli *et al.* 2014) was likely caused by frequent exposure to the parasite (Tackmann *et al.* 2001). Individual genotyping of feces also shed light on temporal variations of parasite transmission (i.e., increment in shedding of parasite eggs during the fall) that were not detected when examining coyote fecal samples of unknown identity.

By analyzing the feeding ecology of urban coyotes with a disease ecology approach, the study estimated, for the first time, the encounter rate of the definitive host with *E. multilocularis*. Despite a relatively lower number of small mammals consumed in winter, this season was crucial for parasite transmission, due to higher parasite prevalence in intermediate hosts (Liccioli *et al.* 2014) and temporal variations in the consumption of competent small mammal species. Given its relative high abundance and the positive selection exhibited by coyotes (i.e., consumption higher than expected given the availability), the meadow vole (*Microtus pennsylvanicus*) may beplaying a key role in the maintenance of an urban sylvatic life-cycle of *E. multilocularis* in Calgary. These data support the relevance of microtine rodents in the parasite ecology and epidemiology, as recently demonstrated for wide areas in Europe (i.e., *Microtus arvalis*; Guerra *et al.* 2014). However, the southern red-backed vole (*Myodes gapperi*) could also be locally relevant depending on the abundance of suitable wood patches for this intermediate host. Widely distributed in forests and plains of North America, this species is present also in

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proximity to and within urban settings (Smith 1993), and is likely very important for parasite transmission in winter, when its contribution to coyote diet and the prevalence of infection are higher. Similarly, other intermediate hosts could become relevant depending on their population fluctuations.

This research also obtained important information from public health management and disease prevention perspectives. First of all, my results emphasized the value of surveillance studies aimed at detecting areas of high infection pressure (i.e. hyper-endemicity) and consequently, higher risk of zoonotic transmission. From a management standpoint, field studies based on the collection and analysis of fecal samples are invaluable, as they provide information on local environmental contamination (Deplazes *et al.* 2004). Foci of parasite transmission represent the areas where intervention strategies (e.g., anthelmintic baiting of definitive hosts) could be attempted or prioritized.

Second, this study identified fall and winter as the seasons during which infection of definitive hosts is more likely to occur. This was not only documented by assessing temporal patterns of fecal parasite prevalence in coyotes (Liccioli *et al.* 2014), but was also confirmed by investigating the histories of infection for individual hosts. Although rarely available in the research context of *E. multilocularis* urban transmission, information on individual patterns of infection is fundamental to assess the risk of zoonotic transmission (Eckert & Deplazes 1999) and plan baiting programs (Hegglin & Deplazes 2008), which should ideally take place just before and/or during winter.

Finally, my results suggest that future studies will have to investigate the population dynamics of meadow vole (*Microtus pennsylvanicus*), the preferred prey species of coyotes

within the small mammal assemblage. Along with other species of microtine rodents, meadow voles are likely to experience multi-annual fluctuations and cycles, which could have dramatic effects on the intensity of *E. multilocularis* transmission (Giraudoux *et al.* 2003) and consequently be used to predict higher risk of alveolar echinococcosis infection in humans (Viel *et al.* 1999; Giraudoux *et al.* 2013).

Although the present thesis has significantly contributed to the advancement of knowledge on this fascinating subject, further research is needed to fill knowledge gaps and fully understand the ecology of *E. multilocularis* in urban settings in North America. In particular, future research efforts should focus on: i) predator functional response to variations in small mammal assemblages; ii) intermediate and definitive host susceptibility to parasite infection; iii) the role of definitive host community composition and intra-guild competition; and iv) the role of rural areas in the maintenance of the urban life cycle of the parasite..

Overall, this thesis emphasized how studies of wildlife parasite transmission require to consider and fully comprehend the ecology of the wild populations involved. Patterns of parasite transmission observed in certain landscapes may be completely different in other environments, characterized by different landscape configuration and connectivity, as well as different host communities, with their unique ecology and population dynamics. The need of shifting from descriptive parasitological studies to a holistic comprehension of the ecology of host-parasite systems is broadly recognized (e.g., Hudson 2002; Collinge & Ray 2006). However, new approaches adopted in this thesis (e.g., fecal genotyping and the study of host feeding ecology to estimate epidemiological parameters) have the potential to be applied in other systems and further improve our understanding of wildlife disease ecology. Such approaches could be

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combined and integrated with additional tools offered by advances in laboratory techniques, such as stable isotopes and/or forensic genetics to investigate the foraging ecology of wild species (Farrell, Roman & Sunquist 2000; Hopkins & Ferguson 2012), which could become highly promising especially when operational costs will decrease.

Additionally, this research further stressed the feasibility of non-invasively assessing patterns of infection in individual hosts to understand their role on parasite transmission at the population level. As opposed to post-mortem examination and intestinal analysis, which provide a single snapshot of the history of the animal, fecal samples offer the opportunity of monitoring infection status of individuals through time. Combined with fecal genotyping, fecal parasitology has therefore the potential of illuminating patterns and processes in wildlife disease epidemiology. At the same time, it allows to obtain relevant information for zoonotic risk management, for example through the identification of super-spreaders, or by testing the effectiveness of control strategies (i.e. assessing individual infection before and after intervention). Finally, fecal genotyping also offers the opportunity of correcting for bias of parasite prevalence estimated through fecal samples caused by repeated sampling of individuals (e.g., Marathe *et al.* 2002). Although such problem was not significant in the study system herein investigated, it has to be evaluated and taken into account in any fecal parasitological study in which the identity of the animal sampled is unknown.

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Appendix I

ECHINOCOCCUS MULTILOCULARIS **IN URBAN COYOTES, ALBERTA, CANADA**

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Abstract

Echinococcus multilocularis is an important zoonotic parasite maintained in wild canids. We detected *E. multilocularis* by post mortem examination in 23 of 91 coyotes (*Canis latrans*) from the metropolitan areas of Calgary and Edmonton, Alberta, Canada. This parasite is emerging/re-emerging as a public health concern throughout the northern hemisphere, partly due to increased urbanization of wild canids.

Introduction

Echinococcus multilocularis is the causative agent of alveolar echinococcosis in humans. This disease is a serious problem because it requires costly long-term therapy, has high case-fatality rate, and is increasing in incidence in Europe (Moro & Schantz 2009). This parasitic cestode has a predominantly wild animal cycle involving foxes (*Vulpes* spp.) and other wild canids, including coyotes (*Canis latrans*), as definitive hosts. However, it can also establish an anthropogenic life cycle in which dogs and cats are the final hosts. Rodents are the primary intermediate hosts in which the alveolar/multivesicular hydatid cysts grow and are often fatal. Humans are aberrant intermediate hosts for *E. multilocularis* (Eckert & Deplazes 2004).

In North America, *E. multilocularis* was believed to be restricted to the northern tundra zone of Alaska, USA, and Canada until it was reported in red foxes (*Vulpes vulpes*) from North Dakota, USA (Leiby, Carney & Woods 1970). This parasite has now been reported in the southern half of 3 provinces in Canada (Manitoba, Saskatchewan, and Alberta) and in 13

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contiguous states in the United States (Moro & Schantz 2009). Foxes are the traditional definitive hosts for *E. multilocularis* worldwide. However, in North America, coyotes may be prominent hosts, particularly when they are more abundant than foxes. *Echinococcus multilocularis* was reported in 7 (4.1%) of 171 coyotes in the north-central United States in the late 1960s (Leiby, Carney & Woods 1970), and subsequently prevalences ranging from 19.0% to 35.0% have been reported in coyotes in the central United States (Kazacos 2003). In Canada, *E. multilocularis* was detected in 10 (23.0%) of 43 coyotes in Riding Mountain National Park, Manitoba (Samuel, Ramalingam & Carbyn 1978). In Alberta, 1 case was recorded from the aspen parkland in 1973 (Samuel, Ramalingam & Carbyn 1978) but it was not found in coyotes from forested regions and southern prairies (Holmes & Podesta 1968; Thompson *et al.* 2009). Nonetheless, *E. multilocularis* is generally considered enzootic to central and southern Alberta on the basis of its prevalence in rodent intermediate hosts. During the 1970s, sixty-three (22.3%) of 283 deer mice (*Peromyscus maniculatus*) trapped in periurban areas of Edmonton were positive for alveolar hydatid cysts (Holmes, Mahrt & Samuel 1971), and *E. multilocularis* was also detected in 2 deer mice collected <1.8 km from Lethbridge in southern Alberta (Chalmers & Barrett 1974). Because mice and voles (family Cricetidae, including *Peromyscus* spp.) have been reported as main prey (70.1%) of coyotes in Calgary (Lukasic & Alexander 2008), and coyotes are common in urban areas of Calgary and Edmonton, we suspected a role for this carnivore in the maintenance of the wild animal cycle of *E. multilocularis* in such urban settings. Thus, we aimed to ascertain the frequency of *E. multilocularis* in coyotes from metropolitan areas in Alberta, Canada.

The Study

Ninety-one hunted or road-killed coyotes were collected during October 2009–July 2011. Most (*n*=83) of the carcasses were from the Calgary census metropolitan area (CMA) (Figure 1). The remainder (*n*=8) were opportunistically collected from the Edmonton CMA. Of those from the Calgary CMA, the exact location of collection was known for 60 animals: 27 were from Calgary and 33 were from the rural fringe, including 2 near Strathmore. Of the carcasses from the Edmonton CMA, 7 were from Edmonton and 1 was from a periurban site. Sex and age of 90 of the coyotes were recorded. Before necropsy, all carcasses were stored at −20°C. Gastrointestinal tracts collected at necropsy were refrozen at −80°C for 3–5 days to inactivate *Echinococcus* spp. eggs. Once thawed and dissected, intestinal contents were washed, cleared of debris, and passed through a sieve (500 μm pores), and the material in the sieve was examined for *Echinococcus* spp. Adult tapeworms were counted and identified as *E. multilocularis* on the basis of morphologic features (Fig. 2). To confirm morphologic identification, PCR was performed by using species-specific primers (Trachsel, Deplazes & Mathis 2007). Briefly, a representative adult worm from each positive animal was lysed in 50 μL of DNA extraction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.3, 15 mmol/L $MgCl_2$, 10 mol/L dithiothreitol, and 4.5% Tween 20) containing 2 μL of proteinase K. This lysate was further diluted (1:20 in double-distilled water), and 2 μL was used for PCR. Amplicons of an expected 395 bp confirmed infection with *E. multilocularis*.

E. multilocularis was identified in 23 (25.3%) of 91 coyotes by using morphologic and molecular identification. Among positive animals, 18 (20.5%) of 83 were from the Calgary CMA and 5 (62.5%) of 8 were from the Edmonton CMA. In the Calgary CMA, 4 (14.8%) of 27

positive animals were found in the city and 9 (27.3%) of 33 were found in the rural fringe (Fig. 1). Five (21.73%) of 23 coyotes for which the location was not recorded were also positive. *E. multilocularis* intensity (number of cestodes per host) ranged from 1 to 1,400 (median 20.5). The frequency of infection was significantly higher in male coyotes (n = 44, 34.19%) than in female coyotes (n = 46, 15.2%; $\chi^2 = 4.337$, df = 1, $P_{exact} = 0.05$) (Table). No difference was detected between 43 juvenile coyotes and 47 adult coyotes (Table).

Conclusions

We demonstrated that *E. multilocularis* is common in coyotes of metropolitan areas of Calgary and Edmonton, Alberta, Canada, including their urban cores. This finding might indicate an emerging phenomenon similar to that observed in Europe with infiltration of urban centers by *E. multilocularis* caused by an increase in red foxes in cities such as Copenhagen, Geneva, and Zurich (Eckert & Deplazes 2004). In Alberta, the urban landscape or by city sprawl invading the natural habitats of coyotes. Our data suggest that *E. multilocularis* is enzootic in coyotes in Alberta and that perpetuation of the wild animal cycle of *E. multilocularis* within cities and surroundings and potential infection of domestic dogs may pose a zoonotic risk, as documented on Saint Lawrence Island, Alaska, and in China (Eckert & Deplazes 2004; Deplazes *et al.* 2011).

With a considerable increase in domestic dog population of Calgary (32.1% increase since 2001, a total of 122,325 dogs in 2010; Animal and Bylaw Services Survey 2010, ww.calgary.ca/CSPS/ABS/Pages/home.aspx) and substantial human population growth (32.9% increase in Calgary since 1999; Statistics Canada, 2009, www.statcan.gc.ca/start-debut-eng.html), awareness is needed of potential transmission risks associated with changing city

landscapes and *E. multilocularis* in the urban environment. In Canada, only 1 autochthonous human case of alveolar echinococcosis has been reported in Manitoba (James & Boyd 1937). However, imported cases have been described. In Alberta, there are no known reports of alveolar echinococcosis. This finding may be caused by the long incubation time required for clinical manifestation in humans (Deplazes *et al.* 2011) or a strain of *E. multilocularis* with a low zoonotic potential. Although there is little evidence of human risk from the strain of *E. multilocularis* in central North America (Hildreth *et al.* 2000), a human case caused by this strain has been confirmed (Yamasaki *et al.* 2008). Our finding of *E. multilocularis* in coyotes in urban regions in Alberta suggests that surveillance for this parasite should be increased in North America. Although removal of this parasite from domestic dogs and cats is effective, eradication from free-ranging definitive hosts may be unfeasible (Eckert & Deplazes 2004; Deplazes *et al.* 2011). Interventions other than improving public awareness about prevention and transmission risk are probably unnecessary, and public health messages should target veterinarians and dog owners because domestic dogs probably represent the main infection route for humans in North America (Eckert & Deplazes 2004; Deplazes *et al.* 2011). Genetic characterization of *E. multilocularis* and spatially explicit transmission models should also be developed to better assess risks of this emerging zoonosis in North America and worldwide.

Acknowledgments

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List of tables

Table 1. Analysis of the occurrence of *E. multilocularis* in coyotes carcasses, based on sex and age, collected from Calgary (n=83) and Edmonton (n=8) census metropolitan areas in Alberta, Canada from October 2009 to July 2011.

Table 1

SEX*		Total	Positive	Negative	%	X^2	df	p_{exact}
Occurrence¶	Males	44	15	29	34.09			
	Females	46	7	39	15.22	**4.337**	1	**0.05**
		Min	Max	Median	IQ distance	z		p_{exact}
Parasite intensity§	Males	1	1400	9	83			
	Females	9	822	59	137	-1.406		0.19
AGE*		Total	Positive	Negative	%	X^2	df	p_{exact}
Occurrence¶	Juveniles	43	14	29	33.33			
	Adults	47	8	38	17.02	1.661	1	0.226
		Min	Max	Median	IQ distance	z		p_{exact}
Parasite intensity§	Juveniles	1	151	9	71			
	Adults	1	1400	32	520	-0.737		0.518

*Sex and age of one of the coyote were not recorded.

¶The frequency of occurrence of in males *vs*. females and juveniles *vs*. adult was analyzed using Chi-square test.

§The parasite intensity (number of parasites per host) among sex and age classes was compared using Mann-Whitney tests for independent samples.

The probability of distribution was estimated using permutation approach (p_{exact}).

The values in bold (i.e. X^2 =4.337 and p_{exact} = 0.05) indicate significant difference. The higher prevalence in male coyotes may suggest a different role of the two genders in parasite dispersion.

List of figures

Figure 1: Maps of the Calgary and Edmonton census metropolitan areas (CMA) in Alberta from where 91 coyote carcasses have been collected from 2009 to 2011. The reference maps (2006) were obtained from the Geography Division, Statistics Canada. Urban core area indicated by red shade whereas, the surrounding rural fringe is indicated by white shade. The total number of coyote carcasses collected from each area (n) and the number positive for *E. multilocularis* (p) are reported along with the percentage (%) of coyotes positive for this cestode.

Figure 2: Morphological confirmation of *Echinococcus multilocularis* specimens from coyote carcasses collected from 2009 to 2011 in Alberta, Canada. Differential Interference Contrast-Microscopic (Olympus BX53) image of a representative *E. multilocularis* isolated from coyotes; its length measured using Olympus BX53 software.

Figure 1

Edmonton-CMA (n= 8); p= 5; %= 62.5

Urban (n= 8)
p= 5
%= 62.5

ALBERTA
Edmonton
Calgary

Calgary-CMA (n= 83); p= 18; %= 20.5

Rural Fringes (n=33)
p=9
%= 27.27

Urban (n= 27)
p= 4
%= 14.81

Location N/A (n= 23); p= 5; %= 21.73
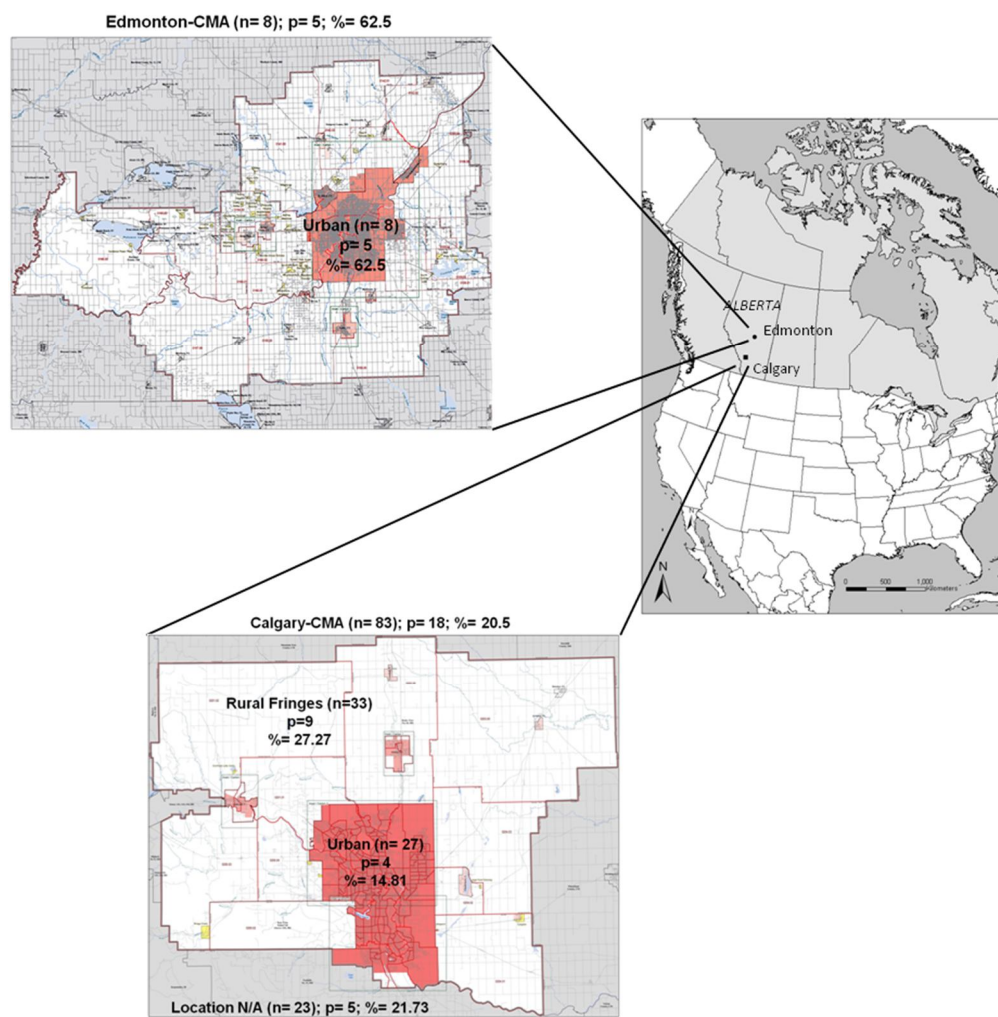
Figure 2

Appendix II

A NEW INTERMEDIATE HOST FOR *ECHINOCOCCUS MULTILOCULARIS:* THE SOUTHERN RED-BACKED VOLE (*MYODES GAPPERI*) IN URBAN LANDSCAPE IN CALGARY, CANADA

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Abstract

Human Alveolar Echinococcosis (HAE) is a potentially fatal parasitic disease caused by *Echinococcus multilocularis*, a cestode characterized by a sylvatic life-cycle involving several species of rodents and lagomorphs as intermediate hosts and canids as definitive hosts. Despite the wide distribution of the parasite in North America, the number of competent intermediate host species identified to date is still relatively small, and mainly includes the northern vole (*Microtus oeconomus*), brown lemming (*Lemmus sibiricus*), northern red-backed vole (*Myodes rutilus*), deer mouse (*Peromyscus maniculatus*) and meadow vole (*Microtus pennsylvanicus*).

By monitoring infections in rodents in the city of Calgary (Alberta, Canada), we have detected a case of severe alveolar echinococcosis in a southern red-backed vole (*Myodes gapperi*), a species never reported before as an intermediate host for this parasite. Observation of protoscolices in the intra-abdominal multilocular cysts indicates that *M. gapperi* could act as a competent intermediate host for the transmission of *E. multilocularis*.

Since *M. gapperi* can be found in close proximity to, and within metropolitan areas, this species could play a role in the establishment and maintenance of the sylvatic life-cycle of *E. multilocularis* in urban landscapes, where the potential for zoonotic transmission is higher. The new intermediate host reported needs to be taken into account in future surveys and transmission models for this parasite.

Introduction

Echinococcus multilocularis is a dixenous cestode (Family: Taeniidae) parasite, characterized by a predominantly sylvatic life-cycle involving wild canids, such as foxes (*Vulpes* spp.) and coyotes (*Canis latrans*), as definitive hosts (Eckert *et al.* 2001). More than 40 species of rodents and lagomorphs (mainly pikas) have been recorded to date as intermediate hosts, although this list is likely far from being definitive (Vuitton *et al.* 2003).

Echinococcus multilocularis is the causative agent of Human Alveolar Echinococcosis (HAE), currently considered one of the most lethal parasitic disease worldwide with a case fatality rate >90% if untreated (Craig, Rogan & Allan 1996). In the Northern Hemisphere, an increase in the parasite geographic range and its prevalence seem to suggest a possible emergence of this zoonosis, although the simultaneous increase of surveillance efforts would call for prudence when interpreting trends (Davidson *et al.* 2012).

In North America, *E. multilocularis* has two main areas of distribution: the Northern Tundra Zone (NTZ) of Alaska and Canada, and the North Central Region (NCR), which includes three Provinces of Canada (Alberta, Saskatchewan and Manitoba) and 13 contiguous States of the USA (Davidson *et al.* 2012). Given the wide geographic distribution of the parasite, the intermediate host species involved in its life-cycle can vary significantly. Throughout the NTZ, the parasite circulates mainly among arctic fox (*Vulpes lagopus*) and its arvicoline rodent prey, such as the northern vole (*Microtus oeconomus*), the brown lemming (*Lemmus sibiricus*), and to a lesser extent, the northern red-backed vole (*Myodes rutilus*) (Eckert *et al.* 2001). In the NCR, the intermediate host species reported to date include deer mouse (*Peromyscus maniculatus*), meadow vole (*Microtus pennsylvanicus*), house mouse (*Mus musculus*) and bushy tailed woodrat

(*Neotoma cinerea*) (Leiby, Carney & Woods 1970; Holmes, Mahrt & Samuel 1971; Kritsky, Leiby & Miller 1977).

As the variation in intermediate host consumption by definitive hosts in response to prey availability (i.e. functional response) is considered an important factor for the transmission ecology of *E. multilocularis* (Raoul *et al.* 2010), the addition of other species to the pool of competent intermediate hosts can be highly significant from an epidemiological standpoint. The aim of this paper is to document a case of *E. multilocularis* infection in a species of the genus *Myodes* (Cricetidae) previously unreported as intermediate hosts for *E. multilocularis* in North America.

Case report

In November 2012, an adult female southern red-backed vole (*M. gapperi*) was trapped with a Museum Special Trap (Woodstream Corp., Lititz, PA) in Bowmont Park Natural Area, Calgary, AB, Canada. Post-mortem examination of the carcass was performed immediately after the field collection.

At necropsy there was a firm multinodular to confluent off-white mass occupying the cranial half of the abdominal cavity and obliterating over 80% of the liver, pancreas and spleen. The nodules were often umbilicated, and on cut section had homogenous soft white foci with minimal free fluid interspersed with scirrhous strands reminiscent of a carcinoma. At its caudal margin, the mass infiltrated the gastrointestinal tract and reproductive organs. Protoscolices (Fig. 1) were identified microscopically in an impression smear of fluid from the mass (Jones & Pybus 2008).

On histology, the masses from the liver, pancreas and spleen were consistent with multilocular cysts described for *E. multilocularis* (Gardiner & Poynton 1999). In brief, the host tissue architecture was replaced by numerous variably sized cystic spaces lined by a thin (10 to 20μm), strongly eosinophilic bladder wall and a thick (up to 100 μm) laminated fibrous outer wall. The vesicular spaces were densely filled with ovoid protoscolices (100 to 150 μm diameter) often aggregated in brood capsules, interspersed with numerous basophilic calcareous corpuscles (Fig. 2). The protoscolices had circular arrays of well-defined bifurcated curved hooks (approx. 25 to 30 μm in length). The hepatic and pancreatic parenchyma was reduced to remnants between nodules and at the periphery of the mass, and had a mild to moderate mononuclear infiltration. Most of the spleen was replaced by cystic structures with only approximately 20% at the distal tip unaffected. Extraction of DNA from the liver cyst was performed using the Qiagen DNeasy Blood and Tissue kit, with a final elute of 300 μL. 2 μL of extracted DNA were then amplified with species-specific PCR following methodologies described by Catalano *et al.* (2012). The specific identity of the parasite was confirmed by amplicons of the expected size (395 bp). The 395 bp amplicon was then purified and sequenced using the amplification primers. The sequence confirmed to be 100% identical to the corresponding region of the *E. multilocularis* mitochondrial gene encoding NADH dehydrogenase subunit 1 (*nad1*).

Discussion

To our knowledge, this is the first report of *E. multilocularis* in southern red-backed vole. This rodent is widely distributed in forests and plains of North America, being found also in proximity to and within urban settings (Smith 1993). Species of the genus *Myodes* have been

previously documented to act as intermediate host for *E. multilocularis*: the northern red-backed vole (*M. rutilus*) in North America, the bank vole (*M. glareolus*) in Europe, and the red-backed voles in Japan (*M. rufucanus bedfordiae*, *M. rutilus mikado*, *M. rex*) (Eckert *et al.* 2001).

The development of morphologically complete protoscolices in *M. gapperi* indicates that this species could act as a competent intermediate host for *E. multilocularis*. Its presence in urban landscapes could therefore be potentially important for the establishment and maintenance of urban sylvatic life-cycles of the parasite. Preliminary data from our laboratory suggest that the meadow vole (*M. pennsylvanicus*) and deer mouse (*P. maniculatus*) may also act as intermediate hosts for the parasite in the Calgary area (Liccioli *et al.*, unpubl.). The prevalence of *E. multilocularis* in urban coyotes in Calgary has been estimated between 20% and 30% (Catalano *et al.* 2012; Liccioli *et al.* 2012a), but although voles and mice represent the main component of coyote diet in the study area (Lukasic & Alexander 2008), the frequency of occurrence in the coyote diet of the different species has not yet been quantified. Although red foxes (*Vulpes vulpes*) could also play a role in the urban cycle of the parasite, coyotes may be the main definitive host, given their higher abundance (Catalano *et al.* 2012).

Future parasitological surveys and transmission models investigating *E. multilocularis* will need then to consider *M. gapperi* among the susceptible intermediate host species. Moreover, as the transmission of *E. multilocularis* relies on predation of definitive hosts upon competent intermediate rodent species, it is now a priority: A) to identify other intermediate hosts in the prey assemblage; B) to estimate their occurrence in the definitive hosts diet; and finally C) to estimate the relative prevalence of infections in each of these species. Only by having reliable estimates of these parameters it will be possible to estimate the transmission

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dynamics of *E. multilocularis* in a multi-host system such as the one represented by the urban landscape of the city of Calgary.

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Figure 1: Microscope view (100X) of *Echinococcus multilocularis* protoscolex found in the cyst liquid extracted from the liver of a southern red-backed vole (*Myodes gapperi*) trapped in Calgary, AB, Canada.

Figure 2: Liver. Single cyst showing the thick fibrous outer wall, thin inner hyaline wall, densely packed protoscolices with hooks (in one), large laminated calcareous corpuscles in the cyst cavity and numerous small pale calcareous corpuscles within the protoscolices. Haematoxylin and Eosin stain, scale as shown.

Figure 1

Figure 2
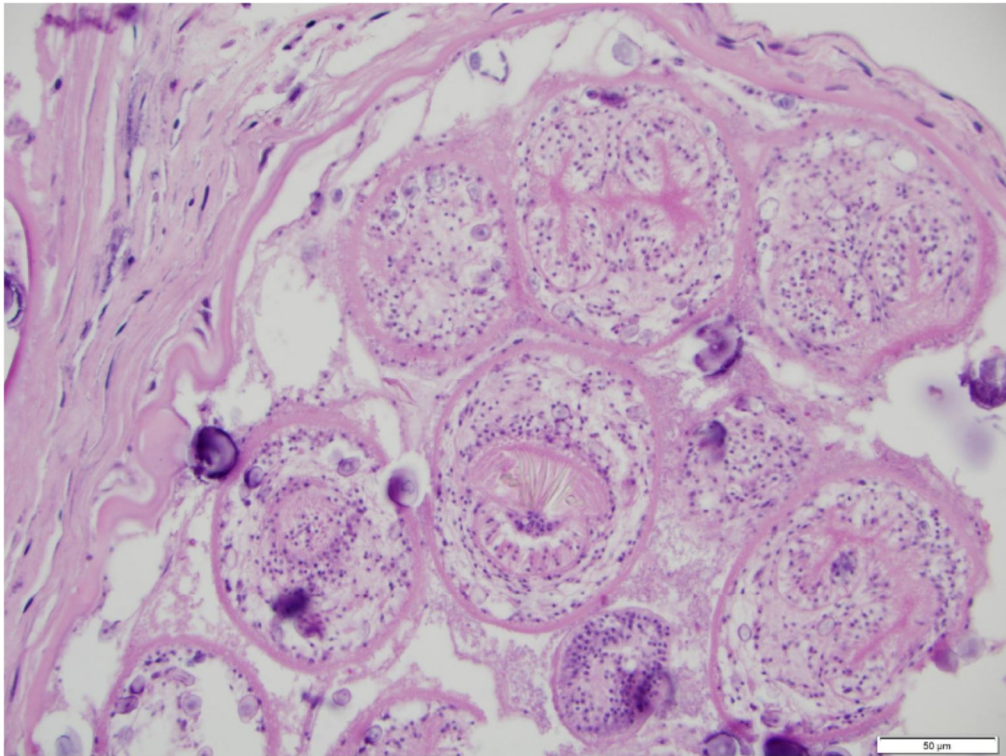
Appendix III

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

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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 7 coyotes (*Canis latrans*) trapped within 40 km of Quesnel, BC. In addition, 3 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 *Echinococcus 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.

Introduction

Recently, a dog (*Canis lupus familiaris*) native to Quesnel, British Columbia (BC), Canada was diagnosed with alveolar hydatid disease, the larval stage of the cestode *Echinococcus multilocularis* (Peregrine, Jenkins & Barnes 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 was 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 disease 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 infective eggs of the parasite were shed in the feces of wild carnivores, and that people were potentially at risk from the same source.

The identification of a European strain of *E. multilocularis* in Canada is a potential public health concern as European strains are suspected to have an increaed zoonotic potential than their North American counterparts (Nakao *et al.* 2009). In 2010 alone, 67 human cases were reported in the European Union (EFSA 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, Seese & Worley 1992). People affected by this parasite often experience a prolonged preclinical period of 10 to 25 years during which larval growth may cause 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). *Echinococcus multilocularis* is emerging (increasing in prevalence and range) across its circumpolar distribution as a result of anthropogenic and environmental alterations (Romig, Thoma & Weible 2006). This is reflected in the range expansion of infected red foxes in Europe, which changed from 4 to 21 affected countries since the 1980's (WHO 2001). In Switzerland, for example, the occurrence of human infections has more than doubled in 12 years (Schweiger *et al.* 2007). 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 considered to be of the utmost importance.

Materials and Methods

In the summer of 2010, rodents and small mammals (n = 156) were captured by Victor® Mouse Snap Traps (#M325: The Woodstream Corporation, PA, USA) 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 (permit no. 78470.25) (Figure 1). Species trapped

included 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. 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 a level 2 biosecurity hood.

In the winter of 2011-2012, wild canids (27 coyotes, *Canis latrans*, and 6 red foxes, *Vulpes vulpes*) were trapped within 40 km of Quesnel, BC, Canada (52°58'42" N; 122°29'35" W), as part of a legal fur harvest by trappers licensed in BC (Figure 1). The intestinal tracts were removed intact and frozen at -20 °C until transport to the ZPRU. Samples were frozen at -80 °C for a minimum of 7 days to inactivate any eggs of *E. multilocularis* before processing (WHO, 2001). Adult cestodes were recovered from the intestines using the scraping filtration and counting technique (SFCT) (Gesy *et al.* 2013), a modified sedimentation and counting technique (WHO 2001; Eckert 2003) in two 10% aliquots (20% of total intestinal contents). Three representative *Echinococcus* adults from each of the 7 coyotes that harbored fully intact cestodes were prepared for molecular characterization. Adult cestodes were lysed using previously described techniques (Catalano *et al*. 2012).

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, Deplazes & Mathis 2007). PCR was performed with 23 μL containing: 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), 0.3 U Taq (AccuStart™, Quanta Biosciences), dH$_2$O 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 thermocycling parameters (Trachsel, Deplazes & Mathis 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.

Results

Echinococcus adults were morphologically identified and differentiated based on the shape of the uterus and the placement of the genital pore. *Echinococcus multilocularis* has a sac-like uterus and a mid to anterior placement of the genital pore, as opposed to the fluted uterus and mid to posterior placement of the genital pore, found in *E. granulosus*. 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 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 per animal). 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 by the 95% dilute samples. Additionally, one positive was detected in the 95% dilute PCR that was not recognized by neat amplification. This highlights the benefit of PCR amplification using dual lysate concentrations. Sequence of a 395 bp (nt 158-553) region 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). 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 haplotype network of *E. multilocularis* based on published sequences for mitochondrial genes*nad2*, *cob*, and *cox1*, the sequences from the coyotes in BC (and the original sequence from the dog) grouped with the European haplotypes (Figure 2).

Discussion

Our results confirm the presence of a European-type strain (haplotype) of *E. multilocularis* in wildlife in a region of northwestern North America where it was not previously reported. In addition, this region is a forested habitat where this parasite was not thought to be

able to establish due to low density of rodent intermediate host populations (Schantz *et al.*1995). The haplotype is identical to that previously identified in the alveolar hydatid cysts found in a domestic dog in this region (Jenkins *et al.* 2012) and suggests that this dog acquired the parasite from local wildlife and not from a recently introduced domestic dog or cat. 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 wild canids are infected (Fay & Williamson 1962). In this study 33% of carnivores examined were *Echinococcus*-positive, with a mean intensity of more than 4,800 adult cestodes. The infection prevalence in coyotes was 37% (10/27), whereas in red foxes it was 17% (1/6). A recent study in Alberta, Canada provided evidence of infection with adult *Echinococcus* in 25% of urban-associated coyotes with an average infection intensity of 1,400 adult cestodes/host (Catalano *et al*. 2012). Therefore, the prevalence and mean intensity of infection in coyotes in the current study was higher than that reported in the recent Alberta study, suggesting that the parasite is well established in central BC.

Coyotes may be the primary host for *E. multilocularis* in central North America, as compared with fox species elsewhere in the circumpolar North (Samuel *et al.*1978). As in Canada, the infection rate in the USA reflects a difference in host species infection rate with a recent study citing 29% (2/7) in coyotes (Storandt & Kazacos 2012). While resident coyotes may only have a mean annual home range of approximately 11 km^2, transient coyotes can roam more than 100 km^2 and have dispersed up to 300 km from their natal range sites (Gese *et al.*1988; Harrison 1992). Given the extensive range sizes 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, BC. This is particularly alarming in light of growing evidence to support the establishment of infected coyote populations in urban areas of North America (Catalano *et al*. 2012; Liccioli *et al.* 2012). This may increase the risk of alveolar hydatid disease for humans and other animals (including, rarely, domestic dogs) who ingest infective eggs in contaminated environments. As well, the presence of wildlife definitive hosts shedding eggs in urban areas will also increase the prevalence of infection in intermediate rodent hosts. These bridging hosts serve to increase the risk of human infection when they are consumed by domestic dogs and cats with access to the outdoors. The haplotype present in wildlife in the Quesnel area of BC is distinct from North American strains of *E. multilocularis* established in Indiana, South Dakota and Alaska (Nakao *et al.*2009), as well as from the haplotypes present in coyotes in Alberta (Catalano *et al.*2012) and rodents 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 introduction. In the last century, red fox of European origin (UK, France and Scandinavia) were introduced in the Pacific coastal USA and in the eastern USA, 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 in southern BC (McTaggert Cowan & Guiguet 1956). To date the BC haplotype of *E. multilocularis* has not been detected east of the Rocky Mountains (Catalano *et al.* 2012; Gesy *et al.* unpublished data);

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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.

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Figure 1: Newly endemic area in the Quesnel region of British Columbia (BC), as well as US states and Canadian provinces/territories endemic for *Echinococcus multilocularis* in western North America (based on WHO/OIE manual). In the Northern Tundra zone: Alaska (AK), Yukon Territory (YT), Northwest Territories (NWT) and Nunavut (NU). In the North Central Region: Alberta (AB), Saskatchewan (SK), Manitoba (MB), Montana (MT), Wyoming (WY), North Dakota (ND), South Dakota (SD), Nebraska (NE), Minnesota (MN), Iowa (IA), Wisconsin (WI), Illinois (IL), Michigan (MI), Indiana (IN) and Ohio (OH).

Figure 2: Haplotype network of *Echinococcus multilocularis* based on statistical parsimony, showing the position of the sequences from a dog and coyotes from BC in the European cluster. Network is based on the mitochondrial genes nad2,cob and cox1. Haplotypes are named according to Nakao *et al.* (2009). Labelled ovals indicate previously published sequences representing haplotypes and small, unlabeled circles indicate hypothetical haplotypes as represented by a single nucleotide change from adjacent sequences. The large oval, labelled BC, indicates the position of the new found haplotype in relation to Nakao defined haplotypes. The network was constructed from the alignment of cox1, cob andnad2 PCR product sequences.
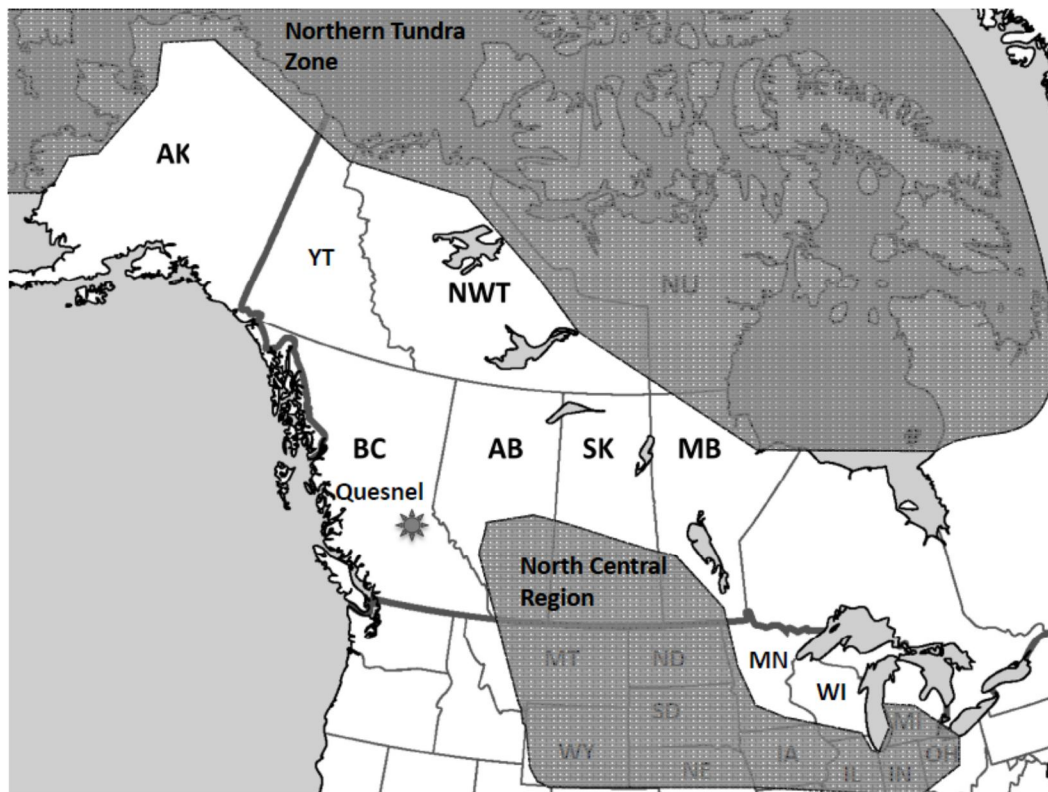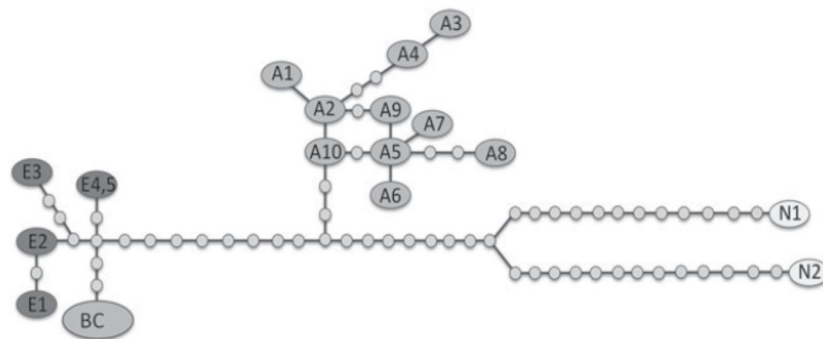
Figure 1

Figure 2

Appendix IV

EGG INTENSITY AND FREEZE-THAWING OF FECAL SAMPLES AFFECT SENSITIVITY OF *ECHINOCOCCUS MULTILOCULARIS* DETECTION BY PCR

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Abstract

Echinococcus multilocularis is one of the most relevant zoonotic parasites with about 18,000 human cases per year. Its detection in wild host is crucial for disease prevention. The present study aimed to determine factors affecting the sensitivity of *E. multilocularis* detection by PCR using DNA extracted from fecal samples of coyotes (*Canis latrans*). Fecal samples were screened for the presence of Taeniidae eggs through centrifugation and sedimentation. DNA was extracted from fecal samples with and without prior freeze-thawing of the sample and then subjected to PCR targeting a mitochondrial gene (nad1) and a multi-loci microsatellite marker (EmsB). The presence of PCR inhibitors was determined through internal amplification control. Subjecting the sample to repeated freeze-thaw cycles significantly increased the sensitivity of the PCR by 20%. Likewise, egg intensity had a significant effect on PCR, an effect which was more pronounced for samples not subjected to freeze-thawing. Two or more eggs per gram of feces significantly increased the odds for a positive PCR outcome. The presence of PCR inhibitors had no effect on PCR in samples subjected to freeze-thaw cycles, whereas in samples not subjected to freeze-thaw cycles the presence of PCR inhibitors was associated with a 0.78 lower odds ratio of positive PCR outcome. Targeting a nuclear versus a mitochondrial gene did not have a significant effect on the sensitivity of PCR. We recommend that freeze thawing samples prior to DNA extraction to become a standard procedure for *E. multilocularis* detection in canid feces.

Introduction

Echinococcus multilocularis is a parasitic tapeworm (family: Taeniidae) whose life-cycle includes canids as definitive hosts and small mammals (rodents and pikas) as intermediate hosts (Eckert *et al.* 2001). Parasite eggs are shed through feces of the definitive host; if ingested, they can lead to the formation of alveolar cysts in the liver and other organs of the intermediate host. Accidental ingestion of *E. multilocularis* eggs by humans (an aberrant host) can lead to human alveolar echinococcosis, the most serious helminthic zoonosis of the northern hemisphere, with a mortality rate > 90% if left untreated (Craig, Rogan & Allan 1996). Upon fecal flotation, *Echinococcus* spp. eggs cannot be morphologically distinguished from those of other Taeniidae (Jones & Pybus 2008) and primers targeting mitochondrial genes are commonly used to differentiate the different species via PCR analysis (Trachsel, Deplazes & Mathis 2007). The use of PCR to detect the presence of pathogens using DNA extracted from fecal samples is however commonly impaired by the presence of PCR inhibitors, such as complex polysaccharides possibly stemming from vegetables in taken as part of the diet (Monteiro *et al.* 1997). Several strategies can be taken to reduce the impact of PCR inhibitors, such as choosing a DNA isolation kit that uses a technology designed to reduce inhibitory substances from the sample (Waits & Paetkau 2005), using a polymerase that can overcome common PCR inhibitors or through the addition of amplification facilitators (e.g. bovine serum albumin, BSA) to the PCR reaction mixture (Al-Soud & Radstrom 2001).

The abundance of DNA is another key element affecting PCR success. This is often determined by two components: the abundance of the parasite itself and the ability to have access to its DNA.

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Ecological studies assessing the temporal and spatial factors affecting the prevalence of *E. multilocularis* in wild canids often rely on the analysis of fecal samples (e.g., Nonaka *et al.* 1998; Raoul *et al.* 2010). In order to provide reliable estimates of parasite prevalence and understand *E. multilocularis* transmission ecology, it becomes crucial to address limitations related to PCR testing of fecal samples as diagnostic tool.

The objective of the present study was to determine factors affecting the sensitivity of *E. multilocularis* detection by PCR using DNA extracted from fecal samples of the definitive host. Specifically, we aimed to test whether the sensitivity of *E. multilocularis* detection by PCR was affected by (1) subjecting the fecal samples to repeated freeze-thaw cycles prior to DNA extraction, (2) the egg intensity, (3) the presence of PCR inhibitors and/or (4) amplifying a nuclear gene versus a mitochondrial gene.

Materials and methods

Study design

A sub-sample of 100 coyote fecal samples was selected from 450 samples collected in 5 city parks in Calgary, Alberta, between May 2012 and August 2013. Fecal samples were initially screened for the presence of Taeniidae eggs by a modification of the centrifugation and sedimentation protocol (Davidson *et al.* 2009), as described in Liccioli *et al.* (2014). Egg intensity was recorded as number of eggs per gram (epg) of fecal material. Following microscopic analysis, Taeniidae eggs were further characterized through PCR analysis as described below.

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The following samples were included in the experiment: i) 30 samples that were positive for Taeniidae eggs and positive on PCR for *E. multilocularis* using DNA extracted directly from the stool sample without prior freeze-thaw cycles; ii) 30 samples that were positive for Taeniidae eggs, but negative on PCR for *E. multilocularis* using DNA extracted directly from the stool sample without prior freeze-thaw cycles; and iii) 40 samples that did not contain any Taeniidae eggs upon centrifugation and sedimentation and were not tested by PCR previously.

DNA extraction

DNA was extracted from fecal samples using the E.Z.N.A. Stool DNA kit (Omega bio-tek, Norcross, GA). DNA extraction from each sample was carried out twice; one time directly from the stool sample (no freeze-thaw (FT) group), the second after repeated freeze-thaw cycles (FT group). Freeze-thaw cycles were carried out as follows: 200 mg of fecal sample was suspended in extraction buffer and thoroughly mixed. The sample was then placed on dry ice for one minute followed by thawing the sample through incubation at 65°C for 1 min. Each sample was subjected to five freeze-thaw cycles. DNA extraction then proceeded according to the manufacturer's instructions, with a final elution volume of 150µL. Following extraction, DNA was stored at -20ºC until further use.

Assessment of the presence of PCR inhibitors

Preparation of the internal amplification control

The presence of PCR inhibitors was assessed through the use of an internal amplification control as described by Deer *et al.* (2010) with modifications. The 198-bp nucleotide sequence of

the internal amplification control (IAC) was cloned into the pIDT-Smart vector (Ampicillin resistance; Integrated DNA Technologies, Toronto, Ontario) and chemically transformed into TOP10 *Escherichia coli* cells following the manufacturer's instructions (Life Technologies, Burlington, Ontario). DNA was isolated from an overnight culture of the transformed *Escherichia coli* cells using the QIAprep Spin Miniprep kit (Qiagen, Toronto, Ontario).

Real-time PCR

Real-time PCR was carried out in 20 µl reactions using Taqman Fast Advanced master mix (Life Technologies), containing 400 nml l^{-1} IAC primers [IAC-forward (5'-CTAACCTTCGTGATGAGCAATCG-3') and IAC-reverse (5'-GATCAGCTACGTGAGGTCCTAC-3')], 200 nml l^{-1} IAC probe (5'-VIC-AGCTAGTCGATGCACTCCAGTCCTCCT-MGBNFQ-3') and 200 copies of the IAC plasmid. PCR reaction were carried out using the StepOnePlus™ Real-Time PCR System (Life Technologies) and the following conditions: 95°C for 1 min, 40 cycles of 95°C for 30 s and 59°C for 30 s. Five µl of DNA extract was added and resulting cycle threshold (Ct) values were compared to Ct values obtained for samples without added DNA extract. PCR inhibitors were considered present when the Ct value of the sample with DNA extract added was more than 1.5 cycles greater than the sample containing no DNA extract. A difference in Ct values between 1.5 and 3 was considered moderate inhibition, a difference in between 3 and 6 was considered intermediate inhibition, and a difference greater than 6 was considered strong inhibition.

Detection of *E. multilocularis* via PCR

PCR amplifying a mitochondrial gene

Detection of *E. multilocularis* DNA amplifying a mitochondrial gene was carried out using primers targeting nad1(Trachsel, Deplazes & Mathis 2007). PCR was carried out in 12.5 µl reactions using AccuStart II PCR SuperMix (Quanta BioSciences, Gaithersburg, MD) containing 0.2 µM forward and reverse primer, respectively, and 1 µl of DNA extract. PCR products were resolved on 1.5% agarose gels containing 0.5µg/mL ethidium bromide. The expected size of the resultant PCR product is 395 base pairs. Water was included as negative control. In addition, all samples were subjected to PCR using primers designed for *Taenia* sp. (Trachsel, Deplazes & Mathis 2007).

PCR amplifying a nuclear gene

Detection of *E. multilocularis* DNA amplifying a nuclear gene was carried out using primers targeting the tandem repeated multi-loci microsatellite EmsB (Bart *et al.* 2006). PCR was carried out in 12.5 µl reactions using AccuStart II PCR SuperMix (Quanta BioSciences) containing 0.2 µM forward and reverse primer, respectively and 1 µl of DNA extract. PCR products were resolved on 1.5% agarose gels containing 0.5µg/mL ethidium bromide. The expected size of the resultant PCR product is 209 - 241 base pairs. Water was included as negative control.

Statistical analyses

The frequency of positive and negative PCR outcomes in the two groups (i.e. no FT group and FT group) or between the outcomes using different primers (nad1 *vs.* EmsB), was

compared through a Pearson Chi-squared test. The effect of egg intensity and the presence of PCR inhibitors on the outcome of PCR in the two groups were preliminarily estimated using a Mann-Whitney test for two independent samples. For further analysis, intensity of eggs was divided into three classes: i) below 2 epg, ii) between 2 and 10 epg, and iii) above 10 epg. The effects of inhibition and egg intensity on FT and no FT samples were modelled by a logistic regression with a backward selection process using Likelihood Ratio as selection criterion. Model fit was estimated by proportion of correct classifications, Hosmer and Lemeshow test, and Cox & Snell R Square as well as the Nagelkerke R Square, while effects of the different categories of egg intensity and inhibition were compared using odds ratios (Hosmer, Lemeshow & Sturdivant 2013).

The probability levels for the Chi-square and the Mann-Whitney tests have been computed using a complete randomisation method (permutation or exact test; P_{Exact}) or by a Monte Carlo simulation based on a 10,000 sampled tables ($P_{MonteCarlo}$) when computation was not possible (Mehta & Patel 1996; Good 2000).

Means are reported with their standard error (SEM) throughout the text. All statistical analyses were performed using SPSS 20.0 (IBM Inc., Chicago, IL, USA).

Results

Relationship between the DNA extraction method and the detection of *E. multilocularis*

PCR analysis using primer specific for the mitochondrial gene nad1and DNA extracted following repeated freeze-cycles of the samples resulted in 42 out of 60 (70.0%) samples positive for Taeniidae eggs displaying a 395 base pair product. Subjecting the fecal samples to

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freeze-thaw cycles increased the sensitivity of the PCR (X^2= 25.714, df = 1, P_{Exact}< 0.001). PCR analysis using primers targeting the nuclear gene EmsB and DNA extracted following repeated freeze-cycles of the samples resulted in 43 out of 60 (71.7%) samples displaying a 209 - 241 base pair PCR product. Two samples, that were negative for *E. multilocularis* DNA, yielded a PCR product using primers targeting *Taenia* spp. Those samples were removed from further analysis.

The number of *E. multilocularis* positive PCR outcomes did not differ between primers targeting nad1 or EmsB (X^2_{Exact}=0.860, df=1, p=0.769), even though the overall sensitivity for *E. multilocularis* slightly increased from 0.72 (nad1, 42/58) to 0.74 (EmsB, 43/58). None of the samples negative for Taeniidae eggs displayed a PCR product.

Effect of egg intensity on sensitivity of PCR

Egg intensity in the 60 fecal samples positive for Taeniidae eggs ranged from 0.33 to 473 epg (mean: 41.4±11.29; median: 5.5). In both experimental groups, egg intensity of negative PCR cases was significantly lower than that of PCR positive cases (No FT group: 15.87 *vs.* 66.88 epg, z = -2.092, P_{Exact}= 0.036; FT group: 6.34 *vs.* 56.39 epg, z = -3.311, P_{Exact}< 0.001; Figure 1). Egg intensity had a greater effect on detection of *E. multilocularis* via PCR in samples in the no FT group than in the FT group (Table 1). In the no FT group, 2 – 10 epg were associated with a 5-fold higher odds ratio, and greater than 10 epg were associated with an 18-fold higher odds ratio of detecting *E. multilocularis* via PCR than samples containing less than 2 epg (Table 1). In the FT group, 2 – 10 epg were associated with a 5.4-fold higher odds ratio, and greater than 10

epg were associated with a 9.4-fold higher odds ratio of detecting *E. multilocularis* via PCR than samples containing less than 2 epg (Table 1).

In samples containing less than 10 epg, the sensitivity between groups did not differ significantly (Figure 2). In samples with less than 2 epg, PCR sensitivity was 0.357 (5/14) for both sample types ($X^2 < 0.001$, df = 1, $P_{Exact} = 1.000$), whereas for samples containing $2 - 10$ epg, PCR sensitivity was 0.5 (10/20) for no FT samples and 0.75 (15/20) for FT samples ($X^2 = 2.667$, df = 1, $P_{Exact} = 0.191$). On the other hand, for samples containing more than 10 epg PCR sensitivity was 0.6 and 0.88 for no FT and FT samples respectively ($X^2 = 6.011$, df = 1, $P_{Exact} = 0.022$; Figure 2). Overall, the positive output was increasing significantly with the egg intensity classes for the FT group ($X^2 = 10.609$, df=2, $P_{Exact} = 0.005$), but not for the no FT group ($X^2 = 1.625$, df=2, $P_{Exact} = 0.467$; Figure 2), although the proportion of positive cases increased in the high intensity group for the no FT group as well (5 positive cases out of 14 in egg intensity class 1, 10 out of 20 in class 2, 15 out of 26 in class 3).

Relationship between the presence of PCR inhibitors and the detection of *E. multilocularis*

PCR inhibition had no effect on PCR outcome in the FT group (i.e. did not enter into the logistic model), whereas PCR inhibition was associated with odds ratio significantly below 1 (indicating a negative effect on detection) for a positive PCR outcome in the no FT group (Table 1).

Models performance

Interestingly, despite having a comparable performance (R square values and Hosmer and Lemeshow tests; Table 2), the models for FT and no FT outcomes had different sensitivity and specificity in their ability to predict the PCR outcome (Table 2), being the first model very sensitive (0.878) but poorly specific (0.50), whereas the model for the no FT group was very specific (0.895), but poorly sensitive (0.50; Table 2).

Discussion

The current study was designed to determine factors affecting the sensitivity of PCR-based diagnosis of *E. multilocularis* in the definitive host. Specifically we aimed to test whether (1) subjecting the fecal samples to repeated freeze-thaw cycles prior to DNA extraction, (2) egg intensity, (3) the presence of PCR inhibitors, and/or (4) amplifying a nuclear gene versus a mitochondrial gene via PCR affect the sensitivity of *E. multilocularis* detection by PCR using DNA extracted from coyote fecal samples. Subjecting fecal samples to repeated freeze-thaw cycles resulted in a significant increase in PCR sensitivity. Egg intensity had a significant effect on PCR. The presence of PCR inhibitors had no effect on PCR in samples subjected to freeze-thaw cycles, whereas in samples not subjected to freeze-thaw cycles the presence of PCR inhibitors was associated with a 0.78 lower odds ratio of positive PCR outcome. Targeting a nuclear versus a mitochondrial gene did not have a significant effect on the sensitivity of PCR. Subjecting the sample to repeated freeze-thaw cycles in the present study significantly increased the sensitivity of the PCR by 20% (42/60 *vs.* 30/60 positive samples). The use of repeated freeze-thaw cycles to isolate DNA from *E. multilocularis* has been described (Beiromvand *et al.* 2011), but the effect of the latter on the sensitivity of PCR to detect *E. multilocularis* has not been

assessed before. Freeze-thawing mechanically disrupts the chitinous egg shell that Taeniidae eggs are surrounded by and liberates the DNA. Our results show that repeated freeze-thawing is a suitable method for liberating DNA without the need to add chemicals that can potentially impair down-stream PCR analysis as has been described by other authors (Ruecker *et al.* 2005).

Likewise, the intensity of eggs present upon fecal flotation affected the sensitivity of PCR to detect *E. multilocularis*. This effect was more pronounced for samples not subjected to freeze-thaw cycles than the ones subjected to freeze-thaw cycles. Samples with 2 to 10 eggs/gram feces had 5 times the odds of being PCR positive than samples containing less than 2 egg/gram feces (irrespective of DNA extraction method, i.e. FT or no FT). This indicates that the likelihood of sampling an egg when less than 2 eggs/gram are present is reduced when obtaining a 200 mg sample for DNA extraction. There was a tendency for samples (2 to 10 eggs/gram feces) subjected to freeze-thaw cycles to have a higher PCR sensitivity than samples not subjected to freeze-thaw cycles, albeit not significant (0.75 *vs.* 0.5; $P_{Exact} = 0.191$). Presence of ten and more eggs/gram feces significantly increased PCR sensitivity for both sample types, although more so for samples not subjected to freeze thaw cycles (odds ratio of 9 *vs.* 18). Although no previous study has correlated egg intensity and PCR sensitivity, our results are consistent with indication of a positive association of PCR sensitivity and the number of gravid worms (Dinkel *et al.* 1998) or the amount of fecal material sampled (Karamon 2014). Dinkel and co-workers correlated the number of *E. multilocularis* specimen present upon necropsy and their maturity stage with the sensitivity of a PCR targeting a mitochondrial rRNA gene on DNA extracted from fox fecal samples. PCR sensitivity was 100% for cases containing more than 1,000 gravid worms, and 70% for cases with less than 10 non-gravid worms (Dinkel *et al.* 1998). Karamon (2014) found

the highest number of PCR positive results when using 1g of faces for DNA extraction than 200 mg or less. This indicates that extracting DNA from a larger amount of fecal matter increases the possibility to sample eggs.

Inhibition of PCR is of particular concern when using DNA extracted from fecal samples for down-stream PCR analysis; PCR inhibitors reduce the amplification efficiency of PCR and can lead to false negative results (Monteiro *et al.* 1997; Oikarinen *et al.* 2009). We therefore assessed the presence of PCR inhibitors and determined the extent of PCR inhibition in the current study and correlated it to the outcome of PCR. Interestingly, results differed between the two different samples types. PCR inhibition in samples not subjected to freeze-thaw cycles was associated with a 0.76 lower odds of positive PCR outcome, whereas PCR inhibition had no significant effect on PCR outcome in samples subjected to repeated freeze thaw cycles. This suggests that in samples not subjected to repeated freeze-thawing the lower amounts of DNA present permits an inhibitory effect of PCR inhibitors, whereas in samples subjected to freeze-thawing the higher amounts of DNA present overcomes the effects of PCR inhibitors.

In the course of large scale ecological studies, DNA is often extracted directly from fecal samples and subjected to PCR analysis. The fact that the DNA extraction methods and egg intensity were the influential factors on detection of *E. multilocularis*, can cause a bias in the presentation of the data as cases with less than 2 eggs/gram have a lower odds of being detected or when performing PCR on DNA directly extracted from the stool sample.

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Table 1: Logistic regression models of PCR outcome for *Echinococcus multilocularis* infections in coyote feces from the City of Calgary (Alberta, Canada) using infection intensity classes (epg, class 1, below 2; class 2, from 2 to 10 epg; class 3, above 10) and inhibition levels (no inhibition, moderate, intermediate and high) using two different DNA extraction protocols: Freeze-thaw (FT) and without freeze-thaw cycles (noFT). In tables are reported the standardized coefficients (B) and their SE, the Wald statistics and the level of significance, and the odds-ratios and their 95% confidence intervals.

Table 2: Performance metrics for the logistic regression models of PCR outcome for *Echinococcus multilocularis* infections in coyote feces from the City of Calgary (Alberta, Canada) using infection intensity classes (epg, class 1, below 2; class 2, from 2 to 10 epg; class 3, above 10) and inhibition levels (no inhibition, moderate, intermediate and high) using two different DNA extraction protocols: Freeze-thaw (FT) and without freeze-thaw cycles (noFT). In tables are reported the percentage of correct classifications, the Hosmer and Lemeshow test results, and Cox & Snell and the Nagelkerke *R* Squares.

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Figure 1: Effect of egg intensity on PCR. Intensity of Taenidae eggs and PCR analysis outcome from coyote fecal samples collected in Calgary (Alberta, Canada) from May 2012 to August 2013. Egg intensity ranged from 0.33 to 473 eggs per gram (epg; 41.4 ± 11.29; median: 5.5) and was significantly different in PCR negative and positive cases in both experimental groups: samples submitted and not to freeze and thaw cycle (no FT *vs*. FT). Above panel, Freeze-Thaw group: 6.34 vs. 56.39 epg, $P_{Exact} = 0.001$. Below panel, No FT group: 15.87 *vs*. 66.88 epg, $P_{Exact} = 0.039$.

Figure 2: Effect of egg intensity on PCR sensitivity for Taenidae eggs in coyote fecal samples collected in Calgary (Alberta, Canada) from May 2012 to August 2013. In samples containing less than 2 epg, PCR sensitivity was 38% for both sample types, for samples containing 2 – 10 epg, PCR sensitivity was 50% for no FT samples and 75% for FT samples. For samples containing more than 10 eggs PCR sensitivity was 60% and 88% for no FT and FT samples respectively.

Table 1

	Variables	B	S.E.	Wald	df	Sig.	Odds-ratio	95% C.I. for odds-ratio	
								Lower	Upper
FT	Intensity (epg in classes)			8.980	2	.011			
	Intermediate (2 to 10)	1.686	.760	4.922	1	.027	5.400	1.217	23.956
	High intensity (>10)	2.246	.780	8.287	1	.004	9.450	2.048	43.606
	Constant	-.588	.558	1.111	1	.292	.556		
noFT	Intensity (epg in classes)			4.930	2	.085			
	Intermediate (2 to 10)	1.599	1.258	1.616	1	.204	4.949	.420	58.272
	High (>10)	2.938	1.344	4.777	1	.029	18.876	1.354	263.056
	Inhibition level			3.580	2	.167			
	Moderate	-2.529	1.336	3.580	1	.058	.080	.006	1.095
	Intermediate	-20.577	27191.192	.000	1	.999	.000	0.000	
	Constant	-1.628	1.093	2.221	1	.136	.196		

Table 2

			Predicted		Correct
			0	1	%
Observed	FT	0	9	9	50.0
		1	5	36	87.8
	Overall %				76.3
	noFT	0	17	2	89.5
		1	7	7	50.0
	Overall %				72.7

	-2 Log likelihood	Cox & Snell R Square	Nagelkerke R Square
FT	62.726	.154	.217
noFT	33.501	.294	.395

Hosmer and Lemeshow Test

	Chi-square	df	Sig.
FT	.000	1	1.000
noFT	.111	4	.999

Figure 1

Figure 2