The occurrence and ecology of *Toxoplasma gondii* in a terrestrial arctic food web

Submitted to the College of Graduate Studies and Research of the University of Saskatchewan in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Veterinary Microbiology at the University of Saskatchewan.

By

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ABSTRACT

The occurrence and ecology of the apicomplexan parasite *Toxoplasma gondii* in arctic wildlife is not well understood. Transmission cycles, especially in terrestrial systems, are enigmatic because environmentally resistant oocysts, shed by felid definitive hosts, might be less responsible for transmission in the Arctic than in more southern latitudes. *Toxoplasma gondii* can also be transmitted through the food web by carnivory, and by transmission from mother to fetus during gestation, and these routes are thought to play a large role in the ecology of *T. gondii* in the Arctic. In this thesis, I examine *T. gondii* in a well-described part of the food web at Karrak Lake, Nunavut, in the central Canadian Arctic and through experimental infections of domestic waterfowl. In the field over 3 years, I sampled generalist carnovires (arctic foxes), migratory herbivores (Ross's Geese and Lesser Snow Geese), and resident herbivores (lemmings). Using an occupancy modeling approach that accounted for imperfect detection, I compared commonly used serological assays to estimate prevalence of *T. gondii* antibodies in sera from arctic foxes and eluted blood on filter paper from Ross's geese and Lesser Snow Geese and compared commonly used serological assays. I also used a naïve estimator to determine prevalence of *T. gondii* antibodies in sera from Ross's Geese and Lesser Snow Geese, and blood on filter paper from lemmings. I detected antibodies against *T. gondii* in sera from arctic foxes (47-60%, depending on age category), Ross's Geese (32%) and Lesser Snow Geese (28%). I also detected antibodies in blood on filter paper from Ross's Geese (39% seropositive) and Lesser Snow Geese (36% seropositive) but not in lemmings. These findings suggest that light geese might introduce *T. gondii* to the Karrak Lake ecosystem with the annual spring migration and that oocyst transmission might not occur in the

terrestrial system, because the parasite was not detected in resident rodents. For the *in vivo* experimental infections, we used a novel application of a multi-scale occupancy framework to determine within-host detection probability of *T. gondii* in experimentally inoculated domestic geese and then used those results to guide tissue sampling in wild Ross's Geese and Lesser Snow Geese. In the experimental inoculation trial, the heart and brain had the highest detection probability for *T. gondii* through a real-time PCR with melt-curve analysis. *Toxoplasma gondii* DNA was not detected in tissues from wild geese, suggesting that the parasite was either not present, or methodological difficulties prevented its detection. The research presented in this thesis forms the groundwork for further *T. gondii* studies in this region.

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LIST OF COMMONLY-USED ABBREVIATIONS

AICc: corrected Akaike's Information Criterion
CI: confidence interval
DAT: direct agglutination test
DNA: deoxyribonucleic acid
ELISA: enzyme-linked immunosorbent assay
FITC: fluorescein isothiocyanate
IFA: immunofluorescent assay
IFAT: indirect fluorescent antibody test
IgG: immunoglobulin G
LCI: lower confidence interval
MAT: modified agglutination test
PBS: phosphate-buffered saline
PCR: polymerase chain reaction
PCR-MCA: polymerase chain reaction with melt curve analysis
ROGO: Ross's Goose
SE: standard error
SNGO: Snow Goose
S/P ratio: sample to positive ratio
UCI: upper confidence interval

CHAPTER 1. Introduction and Literature Review

The occurrence and ecology of *Toxoplasma gondii* and other parasites in the Arctic is an area of increasing interest as researchers, public health officials, and arctic residents continue to understand how the health of people and wildlife in the north is uniquely intertwined, both through scientific study and through traditional ecological knowledge. As the world changes rapidly due climate change and other anthropogenic pressures such as deforestation and resource extraction, there is a need to monitor trends and shifts in parasite distribution and hosts (Davidson et al., 2011). For remote regions such as the arctic tundra, baseline data against which comparisons can be made are rare and much needed.

The major objective of this PhD thesis was to characterize endoparasite occurrence and ecology within major food web components in an arctic tundra ecosystem in the central Canadian Arctic, focused primarily on the ubiquitous apicomplexan, *Toxoplasma gondii*. This zoonotic parasite is a concern in the Arctic, where the cultural practice of eating raw meat from wild sources occurs routinely. The most severe manifestations of toxoplasmosis include neurological disease, miscarriages, and ophthalmic disease. *Toxoplasma gondii* can use many transmission strategies to infect hosts, including fecal-oral transmission of oocysts from cats to many species of warm-blooded vertebrate hosts (e.g. birds and mammals), the transmission of intracellular tachyzoites from mother to fetus, or the transmission of tissue cysts (containing bradyzoites) from intermediate host to a consumer species (Dubey, 2009).

In this series of studies, we focused on arctic foxes, as common predators, Ross's Geese and Lesser Snow Geese, as seasonally abundant migratory herbivores, and annually variable populations of lemmings (*Dicrostonyx groenlandicus* and *Lemmus trimucronatus*), as resident herbivores. The results of this thesis are being used to guide future arctic research and serve as a baseline for the occurrence of *T. gondii* and other endoparasites in this region.

1.1 Description of the field site and wildlife species studied

The fieldwork was conducted within the nesting colony of Lesser Snow Geese and Ross's Geese surrounding Karrak Lake, Nunavut (67° 14' N, 100° 15' W) in the Queen Maud Gulf Migratory Bird Sanctuary in the central Canadian Arctic, approximately 60 km south of the Arctic Ocean. The nearest human community (Cambridge Bay, Nunavut) is approximately 300 kilometers and the site is only accessible by small aircraft, or snowmobile in the winter-spring. The landscape within the Karrak Lake ecosystem is rolling tundra, characterized by rocky outcrops and surrounding areas of lowland wetlands, sedge meadows and shallow ponds (Ryder, 1972; Didiuk and Ferguson, 2005; Alisauskas et al., 2006).

Karrak Lake is in the Arctic Tundra climate region, which in 2012 and 2011 experienced the 2nd and 3rd warmest summers, respectively, on record since 1948. During the study period, annual regional temperatures were 1.0°C (2013), 2.4°C (2012) and 2.0°C (2011) above average. Overall, from 1948-2013, the Arctic Tundra region of Canda had an annual warming trend of 2.1°C, behind only the immediately adjacent Mackenzie District (2.6°C) and the North British Columbia Mountains (2.2°C) in northwestern Canada (data available upon request from http://www.ec.gc.ca/adsc-

cmda/default.asp?lang=EN&n=8C7AB86B-1). Generally, the western Canadian Arctic is experiencing more rapid warming than the eastern Arctic (Furgal and Prowse, 2008).

The Karrak Lake ecosystem supports high arctic fox abundance and breeding density that is about 2-4 times higher than outside the goose colony (Samelius et al., 2011). Arctic foxes at Karrak Lake are known to prey on or scavenge ptarmigan, geese, bird eggs, lemmings, caribou, muskoxen, and arctic hare (Bantle and Alisauskas, 1998; Samelius et al., 2007). Reproductive output of arctic foxes is highly correlated with small mammal (i.e. lemmings and voles) abundance (Samelius et al., 2011). Grey wolves (*Canis lupus*), wolverines (*Gulo gulo*), and grizzly bears (*Ursus arctos*) are sporadic but common at Karrak Lake, while red foxes are infrequent visitors to the area with only one sighting in 12 years of ongoing arctic fox study (G. Samelius, unpublished data).

The light goose colony at Karrak Lake is one of the largest in the Arctic, with more than 750,000 Ross's and 450,000 Lesser Snow Geese nesting in a region spanning over 200 square kilometers of contiguous nesting habitat in 2012 (Alisauskas et al., 2012). Ross's Geese are smaller than Lesser Snow Geese, with a smaller bill suited for grazing in pastures and short tundra grasses (Alisauskas et al., 1988; Jonsson et al., 2013). The larger Lesser Snow Geese grub in the soil for roots and tubers. Geese typically arrive on the breeding grounds by the end of May.

Small mammal abundance at Karrak Lake fluctuates considerably among years (Samelius et al., 2007; 2011) with collared (*Dicrostonyx groenlandicus*) and brown lemming (*Lemmus sibricus*) abundance at a record-high of 5.7 lemming-captures per 100 trap-nights in 2011 followed by a low of 0 lemming captures per 100 trap-nights in 2012 (R. Alisauskas, unpublished data). Lemmings are active year-round with a winter diet of

twigs and bark, and a summer diet of grasses and sedges (Nowak, 1999). Lemmings have small home ranges; the average for a collared lemming is 2.03 ha for males and 0.16 ha for females, while brown lemming males have an average of 1.0 ha and females 0.5 ha (Nowak, 1999).

1.2 Trophically transmitted protozoan and helminth parasites in fox-rodent-goose food web components.

The trophic interactions among arctic foxes (*Vulpes lagopus*), lemmings (*Dicrostonyx* sp. and *Lemmus* sp.), and arctic-nesting geese in the Karrak Lake, Nunavut ecosystem have been well documented (Samelius et al., 2007). Some studies have examined the helminth parasites of Ross's Geese and Lesser Snow Geese (Forbes et al., 1999; Mellor and Rockwell, 2006; Shutler et al., 2012), but few have focused on trophically transmitted protozoan parasites in geese, and none document the occurrence of endoparasites within the fox and lemming populations at Karrak Lake.

Arctic foxes are known to serve as intermediate hosts of *T. gondii.* In wild arctic foxes from Svalbard, Norway, Sorensen et al. (2005) described acute clinical toxoplasmosis and Prestrud et al. (2007) reported widespread seroprevalence (43%) of *T. gondii*. In North America, there were no previously (prior to Elmore et al., 2014) reports of *T. gondii* isolation from Lesser Snow Geese or Ross's Geese, but in arctic Scandinavia (Russia and Svalbard), Barnacle Geese (*Branta leucopsis*) and Pink-Footed Geese (*Anser brachyrhynchus*) have been seropositive for the parasite (Prestrud et al., 2007; Sandström et al., 2013). Also, related rodents in the subfamily Arvicolinae are known intermediate hosts for *T. gondii*, suggesting that lemmings might also be capable of hosting the

parasite, given an opportunity for transmission (Fuehrer et al., 2010); however, sibling voles from Svalbard were not exposed to *T. gondii* (Prestrud et al. 2007).

The most consistently described helminth parasite of arctic foxes is the ascarid, *Toxascaris leonina* (Eaton and Secord, 1979; Skirnisson et al., 1993; Aguirre et al., 2000). This parasite is also common in subarctic and temperate regions, but an explanation for its persistence in extreme arctic environments could be its ability to use paratenic hosts to accomplish its life cycle. Arctic rodents, such as lemmings and voles, might fulfill this purpose in an arctic food web, harboring the parasite safely when environmental conditions are too harsh for *T. leonina* ova to persist in the environment. Other helminths that have been reported to transmit from arctic rodents to arctic foxes include the cestodes *Echinococcus multilocularis*, *Mesocestoides* sp., and *Taenia* sp. (Rausch, 1956, Eaton and Secord, 1979; Stien et al., 2010).

Apart from *T. gondii*, other trophically transmitted protozoan parasites that might be found in the ecosystem include *Sarcocystis* sp., which could be transmitted from either rodents or waterfowl, depending on the species; presumably, arctic foxes could be the definitive host for many different types of *Sarcocystis. Cystoisospora* sp. is a common canid intestinal coccidian that could also use a rodent paratenic host for trophic transmission. Also, *Neospora caninum,* a coccidian closely related to *T. gondii,* uses a canid definitive host but requires an intermediate host for transmission, such as rodents, ungulates, or birds. The role of arctic foxes as definitive hosts for *N. caninum* is currently unclear.

1.3 *Toxoplasma gondii* as a parasite of concern in the arctic

The zoonotic parasite, *Toxoplasma gondii* has a worldwide distribution and a cosmopolitan suite of hosts. Oocyst-derived infections are the result of environmental contamination by felids, the definitive hosts of *T. gondii* (Dubey et al., 1970). In arctic tundra regions, felids are rare to absent and, while the complete transmission routes in such regions have yet to be fully elucidated, trophic routes and transmission from mother to offspring (vertical transmission) are likely to be important (McDonald et al., 1990; Messier et al., 2009; Jenkins et al., 2013). Alternatively, it is possible that oocysts could travel through freshwater drainage en route to the Arctic coasts (Simon et al., 2013a, Simon et al., 2013b). As the Arctic continues to experience increased warming, the home ranges of definitive hosts for *T. gondii* (*Lynx canadensis*) are predicted to expand northward and survival of feral or domestic cats will likely increase (Davidson et al., 2011). This could result in more oocyst-derived infections in both wildlife and people through a contaminated environment (Davidson et al., 2011).

Toxoplasmosis, while often subclinical, can be a very serious zoonosis. When people become infected, the parasite can cause significant ocular, neurological, or reproductive disease, especially in immunocompromised individuals (Dubey, 2009). Also, if a pregnant woman becomes infected, the parasite can cross the placenta to the fetus, leading to congenital toxoplasmosis. Due to the close relationship between people and wildlife in northern Canada, and the cultural propensity for eating raw meat and organs of harvested wildlife, Inuit and other residents in the Arctic might be at a higher risk of toxoplasmosis. Following the discovery of *T. gondii* in pregnant women from Nunavik, Quebec (McDonald et al., 1990), Messier et al. (2009) reported a very high

seroprevalence rate in this region (60% on average). Major risk factors for *T. gondii* seropositivity in both studies included handling wild animal carcasses and ingesting raw meat, where people could be exposed to *T. gondii* tissue cysts in wildlife acting as intermediate hosts.

The stability of country food sources (harvested wildlife) is vital for food security in the Arctic. Our study site is in Nunavut, which has the highest reported rate of food insecurity (69%) of any indigenous population in any developed country (Rosol et al., 2011). Healthy and sustainable populations of wildlife, especially caribou and marine mammals, are needed to ensure affordable and nutritionally valuable protein sources for people living in the North. For example, barren-ground caribou populations across the Canadian Arctic are already experiencing declines. Additive pressure from parasites, especially those like *T. gondii*, which cause reproductive failure or congenitally affected offspring, could negatively affect remaining animals, thus contributing to the declines (Elmore et al., 2012; Jenkins et al., 2013). Wild birds also represent a significant source of energy for Northern residents. In 2004, birds, mostly geese, accounted for 16% of the traditional food consumed among the Inuit of Nunavik (Blanchet et al., 2008)

1.4 Occupancy modeling approaches

Occupancy-modeling approaches are analogous to mark-recapture analyses from wildlife biology and were originally used to estimate the occurrence of cryptic or rare species within habitat patches where they may be detected imperfectly (MacKenzie et al., 2006). These approaches are useful in wildlife disease ecology because they acknowledge that detection is imperfect and account for this uncertainty in final parameter estimates of disease/pathogen prevalence (McClintock et al., 2010, Lachish et al., 2012). Under a

typical occupancy framework, multiple randomly selected 'sites' are surveyed on multiple occasions within a time frame where the occupancy state (species present or species absent) does not change. These repeated survey occasions at each site enables estimation of two parameters: occupancy (ψ), defined as the probability that a site is occupied by the species of interest, and detection probability (p), the probability that the species is detected during a given occasion, given that the site is occupied (MacKenzie et al., 2006).

Some extensions exist to the basic occupancy model. Traditional static occupancy models, in a diagnostic context, insulate prevalence estimates against false-negatives but these models assume that false-positive results do not occur. Yet, in serological assays, and especially with samples from wildlife species, there is a risk of cross-reactivity with unknown non-target antibodies, which could lead to ambiguous test results. Results from some serological assays are subject to observer experience and opinion, which might cause ambiguous test results to be misclassified, leading to false-positive results. A multi-state occupancy approach (Nichols et al. 2007; Miller et al. 2011) can be used to account for both false-positive and false-negative observational errors.

In a multi-scale occupancy model, occupancy and detection probabilities can be estimated at multiple spatial scales. With this approach, there are estimates of occupancy on both large and small scales (Nichols et al., 2008). For example, the larger scale measurement could estimate the probability that a park or wildlife reserve is occupied by a species of interest, while the smaller estimate could describe the probability that a species of interest was available for sampling at a specific habitat type within the park or reserve. Then, detection probabilities could be estimated for each specific habitat type. In

a wildlife disease context, we could use this type of model to estimate the probability that an animal is occupied by a pathogen of interest, and, given that an animal is occupied, that the pathogen is available to be detected at each smaller site (i.e. different organs).

Lastly, we could measure the detection probability of the pathogen for each smaller site, given that both the animal and the organ are occupied by the pathogen of interest. Thus, occupancy modeling offers not only a method of accounting for uncertainty of pathogen distribution in a wildlife host or population, but also for measurement error when using imperfect diagnostic tests to detect pathogens in wildlife. This uncertainty in diagnostic testing is a serious problem. Because very few assays intended for veterinary or human use are actually validated for wildlife species, no measurements of sensitivity or specificity exist. Although traditional occupancy models have been used to generate prevalence estimates in several cases, in this thesis we apply multi-state and multi-scale occupancy models to wildlife disease estimates for the first time.

OBJECTIVES

- To determine if *T. gondii* occurs within the primary food web at Karrak Lake and if so, at what prevalences in the wildlife (arctic foxes, light geese, and lemmings) involved?

- What are potential trophic pathways of *T. gondii* transmission to arctic foxes in the Karrak Lake ecosystem?

- Utilize occupancy models to estimate the prevalence and detection probability of *T. gondii* in tissues and *T. gondii* antibodies in serum samples from geese, arctic foxes, and lemmings in the Karrak Lake ecosystem

CHAPTER 2 - Endoparasites in the feces of arctic foxes in a terrestrial Arctic ecosystem in Canada

Copyright

Citation

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Author Contributions

SAE and GS collected fecal samples. SAE, LFL, GS, and EJJ contributed to study design. RTA and AAG provided field and laboratory mentoring, support, and logistics. SAE and GS drafted the manuscript and figures. All authors read and approved the final manuscript.

Chapter 2 Transition Statement

The objective of this study was to examine feces from a common carnivore in the Karrak Lake region to determine what parasites were present in the ecosystem. This helped to understand what trophically transmitted parasites were in the system and what trophic relationships might be relevant to the transmission of *Toxoplasma gondii*. Although arctic foxes are unlikely definitive hosts, this also addressed the possibility that they shed *T. gondii* in their feces. This study also identified other coccidian species that infected foxes and also might be responsible for antibody cross-reactions during serological testing for *T. gondii.* Some of the text presented in this thesis chapter is different from the published manuscript.

Abstract

The parasites of arctic foxes in the central Canadian Arctic have not been well described. Today, the Canadian Arctic is experiencing dramatic environmental change, which is predicted to cause shifts in parasite and wildlife species distributions, and trophic interactions, requiring that baselines be established to monitor future alterations. This study used conventional, immunological, and molecular fecal analysis techniques to survey the current gastrointestinal endoparasite fauna present in arctic foxes in central Nunavut, Canada. Ninety-five arctic fox fecal samples were collected from the terrestrial Karrak Lake ecosystem within the Queen Maud Gulf Migratory Bird Sanctuary. Samples were examined by fecal flotation to detect helminths and protozoa, immunofluorescent assay (IFA) to detect *Cryptosporidium* and *Giardia*, and PCR with melt-curve analysis (PCR-MCA) to detect coccidia. Positive PCR-MCA products were sequenced and analyzed phylogenetically. Arctic foxes from Karrak Lake were routinely shedding eggs from *Toxascaris leonina* (63%). Taeniid, Capillarid, and hookworm eggs. *Sarcocystis* sp. sporocysts, and *Eimeria* sp., and *Cystoisospora* sp. oocysts were present at a lower prevalence on fecal flotation. *Cryptosporidium* sp. and *Giardia* sp. were detected by IFA. Through molecular techniques and phylogenetic analysis, we identified two distinct lineages of *Sarcocystis* sp. present in arctic foxes, which probably derived from cervid and avian intermediate hosts. Additionally, we detected previously undescribed genotypes of *Cystoisospora*, and, in one sample, either *Hammondia* sp. or *Neospora caninum*. Our survey of gastrointestinal endoparasites in arctic foxes from the central Canadian Arctic provides a unique record against which future comparisons can be made.

2.1 Introduction

Arctic foxes (*Vulpes lagopus*) have a circumpolar distribution and are common throughout the North American and Russian Arctic. Few reports of parasites in North American arctic fox populations currently exist, although the parasite fauna of the endangered Fennoscandian arctic fox has been well documented in recent years (Meijer et al, 2011). As anthropogenic environmental change in the Arctic biome continues to accelerate, parasite communities are also expected to change (Kutz et al., 2009). Already, the Canadian Arctic Tundra climate region has experienced an observed warming trend of 2.1°C in annual temperature since observations began in 1948 (Environment Canada Annual Regional Temperature Departures, Climate Trends and Variation Bulletin 2011, http://www.ec.gc.ca/adsc-cmda/default.asp?lang=en&n=B49D9F0B-1, accessed Dec. 14, 2012).

Potential shifts in the distribution and abundance of parasites and hosts threaten our current understanding of trophic interactions in the Arctic, including the host-parasite relationship (Ims and Fuglei, 2005). Parasites are also known to play a part in destabilizing some host populations (Anderson and May, 1978), and, when combined with anthropogenic pressures, parasitism could have an additive negative effect on arctic fox populations. Also, red fox (*Vulpes vulpes*) populations continue to expand northward, creating opportunity for interspecific competition and transmission of parasites from southern canid populations (Hersteinsson and McDonald, 1992).

Early parasitological studies of arctic foxes in North America were primarily focused on detection of *E. multilocularis*, a cestode of public health importance in western Alaska and elsewhere in the circumpolar North. These studies led to the

detection of *Toxascaris leonina, Taenia crassiceps, Echinococcus multilocularis,* and coccidia resembling *Eimeria* sp. (Rausch, R.L., 1956; Choquette et al., 1962; Eaton and Secord, 1979). Otherwise, little is known about the prevalence, distribution, and diversity of arctic fox parasites in the Canadian Arctic.

Fecal-based studies are non-invasive and logistically feasible in remote environments. Results from fecal studies are increasingly meaningful as molecular tools improve to distinguish between morphologically similar parasite species and to answer questions about the genotypes or subspecies present. In a Northern context, molecular parasitology provides information about the zoonotic potential of parasites in wildlife and the environment. For example, molecular diagnostic tools are used to identify zoonotic genotypes or canid-specific genotypes of *Giardia* (Thompson et al., 2009). Knowing which genotypes are present in the ecosystem is necessary to help northern residents develop safe food and water guidelines.

The objective of this study was to survey and describe helminth and protozoal gastrointestinal endoparasites from feces of arctic foxes at Karrak Lake, Nunavut, Canada. Although trophic interactions in this ecosystem are well described (Samelius et al., 2007), this was the first parasitological study on this population. This study provides the first regional record of arctic fox endoparasites in the central Arctic of North America based on conventional, immunological, and molecular analysis of parasites present in feces. Additionally, we used a real-time PCR with melt-curve analysis method to detect and distinguish different genera and species of coccidians (PCR-MCA; Lalonde and Gajadar, 2011). The results of this study serve as important baseline information against which we can evaluate changes in parasite distribution and prevalence from predicted

environmental change, and to better understand the wildlife and human health significance of parasites in terrestrial Arctic ecosystems.

2.2 Methods

2.2.1 Study Area

The fieldwork was conducted within the nesting colony of Lesser Snow Geese and Ross's Geese surrounding Karrak Lake, Nunavut (67° 14' N, 100° 15' W) in the Queen Maud Gulf Migratory Bird Sanctuary in the central Canadian Arctic (Figure 2.1). The nearest human community is approximately 300 kilometers and the site is only accessible by small aircraft. Karrak Lake is in the Arctic Tundra climate region, which in 2012 and 2011 experienced the 2nd and 3rd warmest summers on record since 1948. During this period, temperatures were 2.3°C (2012) and 2.1°C above average (Ranked summer regional temperatures table, http://www.ec.gc.ca/adsc-cmda/default.asp?lang=En&n=30EDCA67-1, accessed Dec 14, 2012).

The Karrak Lake ecosystem supports high arctic fox abundance and breeding density (Samelius et al., 2011). Red foxes are infrequent summer visitors to the area as only one sighting was reported in 12 summers of ongoing arctic fox study (G. Samelius, unpublished data). The landscape within the Karrak Lake ecosystem is rolling tundra, characterized by rocky outcrops and surrounding areas of lowland wetlands, sedge meadows and shallow ponds (Ryder, 1972; Didiuk and Ferguson, 2005; Alisauskas et al., 2006). Arctic foxes at Karrak Lake are known to prey on or scavenge ptarmigan, geese, bird eggs, lemmings, caribou, muskoxen, and arctic hare (Bantle and Alisauskas, 1998; Samelius et al., 2007).

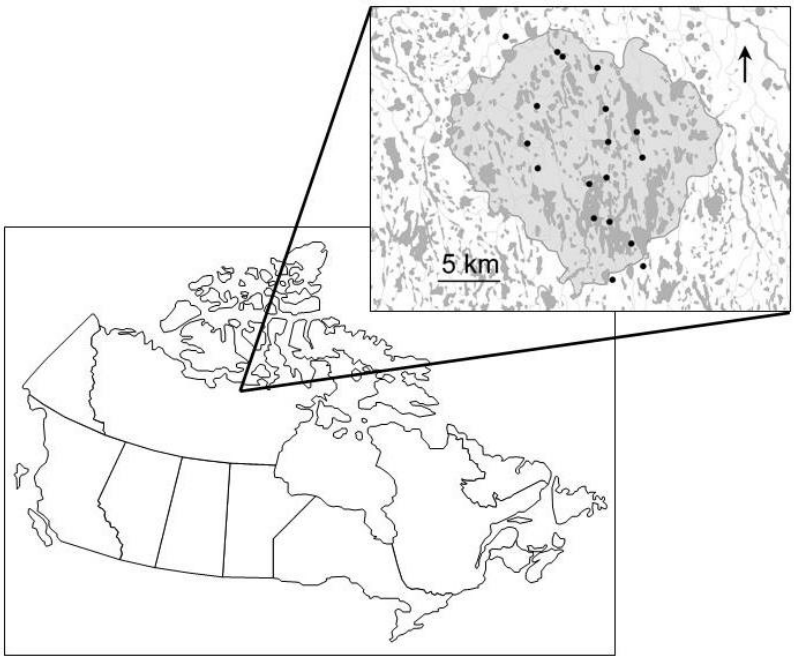

FIGURE 2-1. KARRAK LAKE GOOSE COLONY WITHIN THE QUEEN MAUD GULF BIRD SANCTUARY, NUNAVUT. INSET MAP: SAMPLE COLLECTION SITES WITHIN THE GOOSE COLONY.

2.2.2 Sample Collection

During mid-May to mid-June in 2011 and 2012, 95 fecal samples were collected from snow cover surrounding known arctic fox den sites distributed throughout the Karrak Lake goose colony (Figure 2-1). Feces were also recovered from arctic foxes captured during an ongoing population dynamics study, either from the trap or the ground after the fox was released. For each fecal sample, the location and date of collection were recorded. Fecal samples were stored in individual plastic bags at approximately 1-6°C until shipment to the University of Saskatchewan. In the laboratory, fecal samples were held at -80°C for 7 days to kill ova of *Echinococcus* sp., then at -20°C for 1-3 weeks until analysis and permanently thereafter. The fecal samples in this study were from at least 30 individual foxes, based on capture data from these two years (R. Alisauskas and G. Samelius, unpublished data). However this is likely a low estimate due to the presence of unsampled transient foxes passing through the sampling area, and resident foxes that went uncaptured during this study.

2.2.3 Fecal Flotation

A modified double centrifugation Sheather's sucrose flotation technique was performed on a known quantity (1-3 grams) of feces from each sample. All parasite eggs, cysts, and oocysts, observed by light microscopy, were counted and the number per gram recorded. Parasite identifications were based on morphology and morphometrics (Foreyt, 2001).

2.2.4 Immunofluorescent Assay (IFA)

To concentrate the ova, cysts, and oocysts in the feces in preparation for the immunofluorescent assay and molecular analysis, a known quantity (0.5-3 grams) of feces from each sample was mixed with 10 mL phosphate-buffered 0.9% saline (PBS). This liquid was filtered through two layers of cheesecloth into a 15 mL centrifuge tube and centrifuged for 10 minutes. The resulting fecal pellet was resuspended in 6 mL PBS and then centrifuged as above (Seifker, et al. 2002). Finally, the washed fecal pellet was resuspended in 1 mL PBS and the fecal suspension was refrigerated at 4°C until further analysis.

Cysts of *Giardia* sp. and oocysts of *Cryptosporidium* sp. were counted in 15 ul of fecal suspension prepared with a commercially available test kit specific for these protozoans (Cyst-a-glo; Waterborne, Inc.) according to manufacturer instructions. The following formula was used to estimate the number of oocysts or cysts in each gram of feces: ((1000 * number cysts / cysts counted) / # μL on IFA slide) / grams feces used).

2.2.5 Molecular Techniques

Genomic DNA was extracted from 300 μL of fecal suspension (Da Silva et al., 1999) using the Fast-DNA kit (MP Biomedicals) with Lysis Matrix E beads (MP Biomedicals), followed by the PCR Purification kit (Qiagen). Coccidian species in each fecal sample were detected using a universal coccidia primer cocktail designed to amplify a ~315-bp region of 18S rDNA in a real-time quantitative PCR assay and differentiated using melt-curve analysis (Lalonde and Gajadhar, 2011). The PCR product melting temperature (T_m) is based on the nucleotide sequence composition, length, and G-C content, so genetically distinct members of the same genus or species can be

differentiated by the melt curve shape and T_m and identified by comparison to in-run controls and/or sequencing (Lalonde and Gajadhar, 2011). All PCR analyses were performed using the CFX96 Real-Time PCR detection system (Bio-Rad Laboratories) as described previously (Lalonde and Gajadhar, 2011) except 1X Evagreen Supermix (Bio-Rad Laboratories) was used in the final reaction mix. Each PCR reaction plate included two negative control wells (water), a standard curve consisting of plasmid DNA from *Eimeria bovis* (10^5 to 10^1 oocysts), and wells containing DNA from *Toxoplasma gondii* (genomic, ATCC, Virginia, USA), *Cystoisospora* sp. (plasmid, in house), *Neospora caninum* (genomic, ATCC, Virginia, USA), *Sarcocystis cruzi* (genomic, in house), and *Eimeria bovis* (plasmid, in house) as positive controls.

Melt curves from positive samples were visually compared to the controls for preliminary identification, and amplified DNA from positive reactions were sequenced by Macrogen (Seoul, South Korea) for confirmation using original primers. Forward and reverse sequences were assembled with PreGap4 and Gap4 (Staden Package) and consensus sequences were compared with reference sequences in GenBank™ using the nucleotide Basic Local Alignment Search Tool (BLASTN). Multiple alignments of reference and sample sequences were performed with CLUSTAL X. Neighbor-joining phylogenetic trees and branch reliability bootstrap values (100 iterations) were constructed with the program PHYLIP 3.69 (Chaban and Hill, 2012).

2.3 Results

We detected eight morphologically different types of gastrointestinal endoparasites by fecal flotation in 95 fecal samples from arctic foxes in this study. Eggs of *Toxascaris leonina* were most common (63% prevalence), with a median shedding

intensity of 33 (range 1-800) eggs per gram of feces. Taeniid, anoplocephalid, capillarid, and hookworm eggs and *Sarcocystis* sp., *Eimeria* sp., and *Cystoisospora*-like oocysts were observed at lower prevalence and shedding intensity (Table 2-1). On immunofluorescent antibody assay, *Giardia* and *Cryptosporidium* were detected in 16 and 8% of fecal samples, respectively (Table 2-1). The median shedding intensity of *Giardia* was 162 (range 2-12,080) cysts per gram feces, while the median shedding intensity of *Cryptosporidium* was 77 (range 18-146) oocysts per gram feces.

The PCR-MCA analysis, subsequent sequencing, and phylogenetic analysis confirmed at least two distinct species of *Sarcocystis* (in 16/95 samples), two species of *Cystoisospora* (5/95), at least two species of *Eimeria* (8/95), and one sample with either *Hammondia* sp. or *Neospora* sp. (1/95). Test agreement between PCR and fecal flotation was low (0-25%) for these coccidians (Table 2). When unknown samples were visualized together, distinct melt peaks for different types of coccidia were clear (Figure 2-2).

TABLE 2-1. PREVALENCE OF GASTROINTESTINAL PARASITES FOUND IN ARCTIC FOX FECES AND THE FREQUENCY OF AGREEMENT AMONG DETECTION METHODS.

Parasite	**Overall Prevalence n=95**	**Prevalence Detected by Flotation**	**Prevalence Detected by PCR-MCA**	**Prevalence Detected by IFA**	**Test Agreement[b]**
Toxascaris leonina	60 (63%)	60 (63%)	-	-	-
Taeniid	14 (15%)	14 (15%)	-	-	-
Anoplocephalid	20 (21%)	20 (21%)	-	-	-
Capillarid	1 (1%)	1 (1%)	-	-	-
Hookworm	2 (2%)	2 (2%)	-	-	-
Sarcocystis sp.	16 (16%)	3 (3%)	14 (15%)	-	1/16 (6.3%)
Cystoisospora sp.	8 (8%)	5 (5%)	5 (5%)	-	2/8 (25%)
Eimeria sp.	14 (15%)	6 (6%)	9 (9%)	-	1/14 (7.1%)
Neospora/Hammondia-like	1 (1%)	0	1 (1%)	-	0/1 (0.0%)
Cryptosporidium sp.	9 (9%)	0	0	9 (9%)	0
Giardia sp.	15 (16%)	0	-	15 (16%)	-

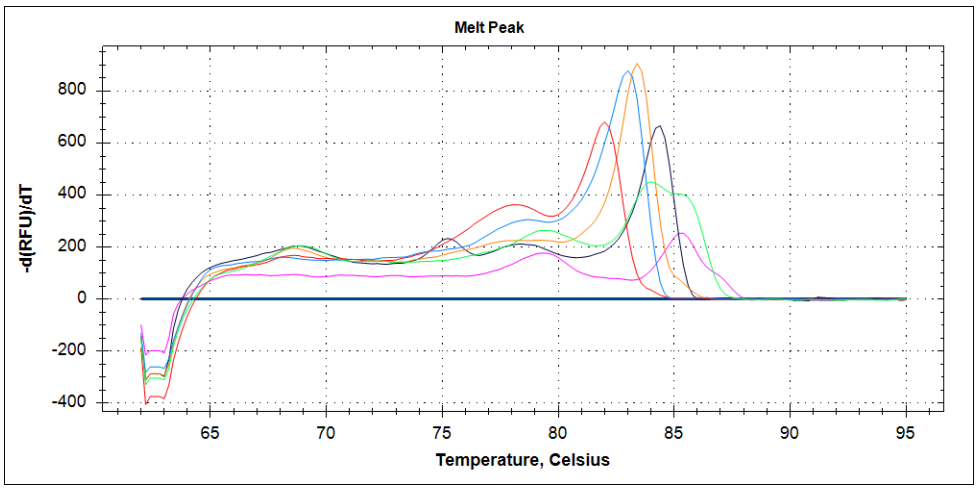

FIGURE 2-2. MELT CURVES FOR UNKNOWN SAMPLES. EACH PEAK SHOWS THE MELTING TEMPERATURE FOR A DIFFERENT COCCIDIAN SPECIES. RED: *SARCOCYSTIS* (CERVID), BLUE: *NEOSPORA/HAMMONDIA,* ORANGE: *SARCOCYSTIS* (AVIAN), BLACK: *CYSTOISOSPORA*, GREEN: *EIMERIA* SP., PINK: *EIMERIA SP.* THE HORIZONTAL AXIS INDICATES MELTING TEMPERATURE (ºC) AND THE VERTICAL AXIS [–D(RFU)/DT] IS RELATED TO THE AMOUNT OF DNA PRESENT.

Additionally, individual melt curves were visualized against the positive control for each parasite (Figure 2-3), highlighting similarities and differences between the unknowns and positive controls. Phylogenetic analysis of nucleotide sequences showed at least two different lineages of *Sarcocystis*. One grouping included species of *Sarcocystis* that use cervid intermediate hosts (Lineage 2; Figure 4; Dahlgren et al., 2007) and the other with species of *Sarcocystis* that use avian intermediate hosts (Lineage 1; Figure 2-4; Kutkiené et al., 2012). Samples that grouped in Lineage 1 (Figure 2-4) were 99-100% similar to *Sarcocystis albifronsi* (GenBank Accession no: EU502868) and *Sarcocystis anasi* (GenBank Accession no: EU553477), The second *Sarcocystis* lineage (Lineage 2; Figure 2-4) were only 77-78% similar to *S. anasi* and *S. albifronsi* at the target region but 91-93% similar to *Sarcocystis capreolicanis* and 100% similar to *Sarcocystis tarandivulpes*. When melt curves of a representative sample from each lineage of *Sarcocystis* were compared, there were two distinct curves, one that was similar to the *Sarcocystis cruzi* positive control (Lineage 2; T_m=82.0°C) and one that was unique (Lineage 1;T_m=83.4°C) (Figure 3c). Likewise, there were two separate melt curves for *Eimeria* sp. (Figure 2-3b), which differed from the *E. bovis* control, suggesting that at least two different species of *Eimeria* were present.

Sequences from *Cystoisospora*-like oocysts in the current study were 96% (GenBank Accession nos: KC262748 and KC262749) or 98% (GenBank Accession nos: KC262746 and KC262747) similar to *C. ohioensis* (GenBank Acession no: GU292305) at the target region of the 18S gene (Figure 2-5). A multiple sequence alignment of the target locus showed that the reference sequences for *Hammondia* spp. (GenBank Accession: GQ984222) and *Neospora caninum* (GQ899206; U16159) were

indistinguishable from each other and the unknown sample from the arctic fox was 99% similar to the reference sequences.

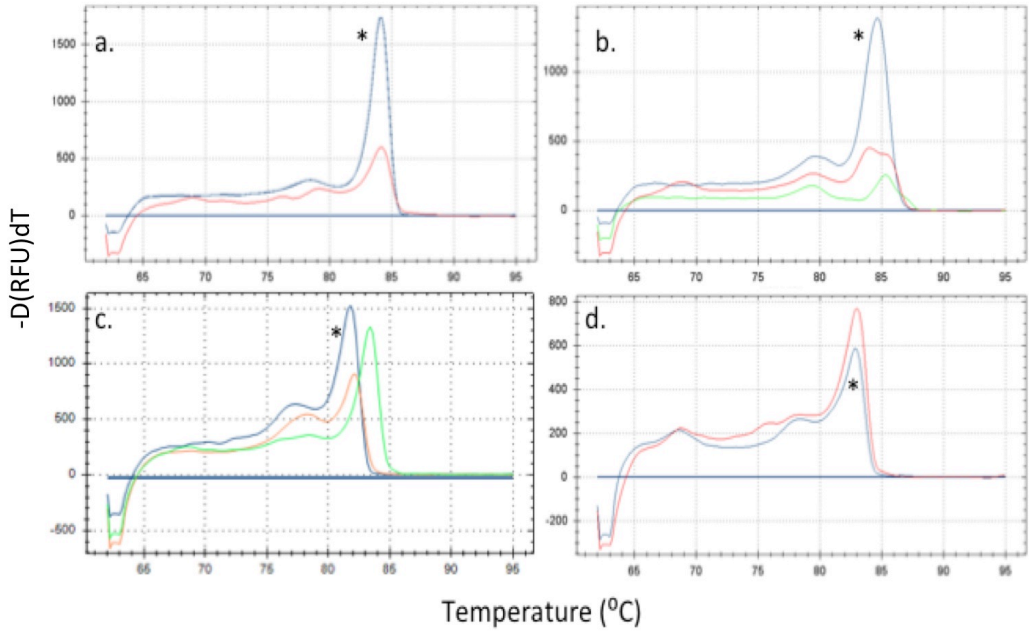

FIGURE 2-3. INDIVIDUAL MELT CURVES FOR UNKNOWN SAMPLES SHOWN WITH MELT CURVE FOR RESPECTIVE CONTROL DNA (*). THE COLORED LINES INDICATE THE MELT CURVE FOR DIFFERENT GENOTYPES. A. *CYSTOISOSPORA* SP. B. *EIMERIA* SPP. C. *SARCOCYSTIS* SPP. D. *HAMMONDIA/NEOSPORA* SP. THE HORIZONTAL AXIS INDICATES MELTING TEMPERATURE (ºC) AND THE VERTICAL AXIS [–D(RFU)/DT] IS RELATED TO THE AMOUNT OF DNA PRESENT

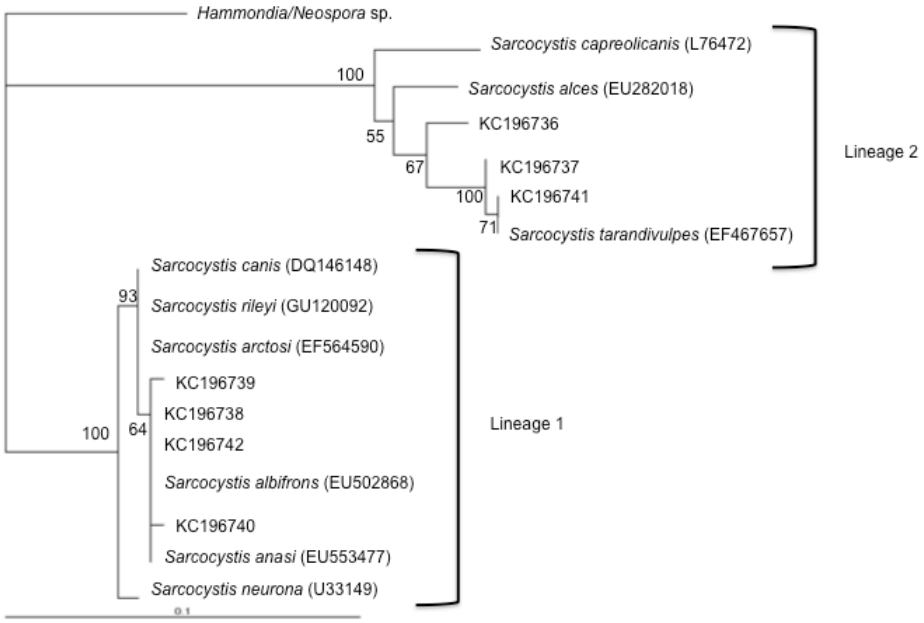

FIGURE 2-4. PHYLOGENETIC TREE SHOWING RELATIONSHIP OF *SARCOCYSTIS* SPP. DETECTED IN THIS STUDY WITH EXISTING REFERENCE SEQUENCE DATA IN GENBANK.

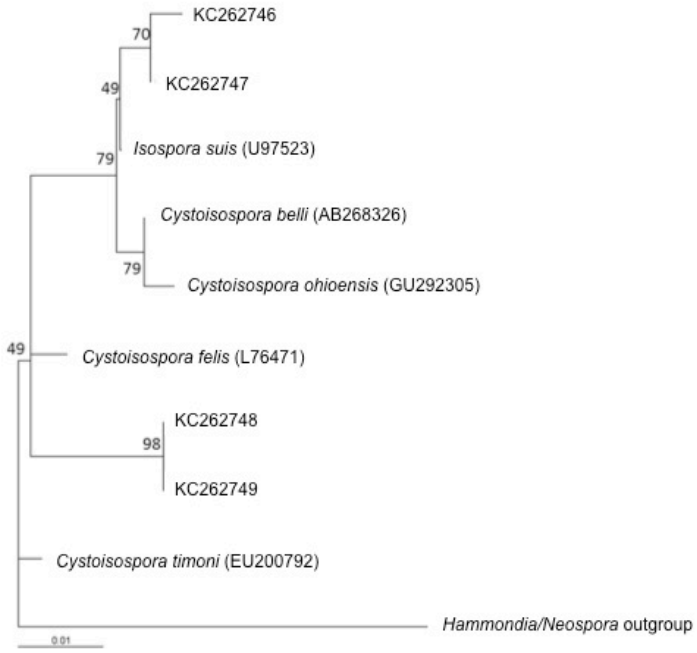

FIGURE 2-5. PHYLOGENETIC TREE SHOWING RELATIONSHIP OF *CYSTOISOSPORA* SPP. DETECTED IN THIS STUDY WITH EXISTING REFERENCE SEQUENCE DATA IN GENBANK.

2.4 Discussion

These results show that arctic foxes in the Karrak Lake ecosystem host a number of potentially undescribed coccidian genotypes or species and are routinely infected with parasites common to wild canids. As in other studies of arctic foxes, *Toxascaris leonina* was the most abundant helminth, and in general is the most prevalent ascarid nematode in Arctic carnivores (Eaton and Secord, 1979; Kapel and Nansen, 1996; Aguirre et al., 2000, Meijer et al., 2011; Rausch and Fay, 2011). Notably absent were *Toxocara canis* ascarids, which are common in canids in temperate regions but have not been detected above the Arctic Circle in North America, likely due to the low freeze-tolerance of the parasite ova (O'Lorcain, 1995; Jenkins et al., 2011). The hookworm eggs observed by fecal flotation were significantly collapsed due to freezing at -80°C. These were most likely *Uncinaria* sp., which has been reported occasionally in arctic foxes in Europe (Aguirre et al., 2000; Meijer et al., 2011), on St. Lawrence Island, Alaska, USA, (Rausch et al., 1990), and in domestic dogs from the Northwest Territories, Canada (Salb et al., 2008). The eggs of *Ancylostoma caninum* have similar morphology, however this parasite has not been reported in arctic canids.

We did not detect any trematodes and acanthocephalans; however, these would not necessarily have been detected with our methods. Sedimentation is a more reliable assay than flotation for detection of these species and we did not have sufficient sample quantity for both tests. The absence of these parasites, which are commonly associated with marine environments, is consistent with the non-marine diet of arctic foxes at Karrak Lake, consisting mostly of birds and small mammals (Samelius et al., 2007; Meijer et al., 2011). Arctic fox in coastal regions of Greenland have a more diverse diet and parasite

fauna including trematodes (*Echinoparyphium* sp., *Plagiorchis elegans, Cryptocotyle concavum*), cestodes (*Diphyllobothrium dendriticum, Mesocestoides lineatus*), the nematode (*Strongyloides stercoralis*) and the acanthocephalan *Polymorphus* sp. (Rausch, 1983; Kapel and Nansen; 1996).

Taeniid eggs were present in 15% of samples at low intensity on fecal flotation. However, eggs might not have been detected in either early infections due to the prepatent period or late infections in which eggs are no longer being shed (Duscher et al., 2005). On Banks Island, Northwest Territories, Canada, Eaton and Secord (1979) recovered adult *T. crassiceps* (78%) and *E. multilocularis* (2%) from the intestinal tracts of arctic foxes. Further molecular characterization to determine if the eggs in the current study were *E. multilocularis* or *T. crassiceps* was not successful; however *E. multilocularis* has been detected at low prevalence in the Karrak Lake fox population (Gesy et al., 2014).

We detected protozoa by fecal flotation, immunological, and molecular methods. The qPCR-MCA assay was especially useful in detecting protozoan species that produce small oocysts and sporocysts, which might have been missed on flotation. One exception was *Cryptosporidium* sp., which was detected by IFA, but not by qPCR-MCA. Melt-curve analysis combined with phylogenetic analysis of sequences led to detection of multiple species of *Sarcocystis*, *Cystoisospora*, and *Eimeria* present in feces of arctic foxes. These results support at least two separate lineages of *Sarcocystis*. Samples that grouped in Lineage 1 were genetically similar to *Sarcocystis albifronsi* (GenBank Accession no: EU502868) and *Sarcocystis anasi* (GenBank Accession no: EU553477), which Kutkiené et al. (2012) isolated from tissue of greater white-fronted geese (*Anser*

albifrons) and mallard ducks (*Anas platyrhynchos*), respectively. Samples in Lineage 2; were related to *Sarcocystis* spp. previously detected in cervids (Dahlgren et al., 2007). Arctic foxes are definitive hosts for both *S. albifronsi* and *Sarcocystis capreolicanis* (Gjerde, 2012; Kutkiené et al., 2012), and at Karrak Lake, foxes feed on both geese and cervids (Samelius et al., 2007).

Oocysts morphologically consistent with *Cystoisospora ohioensis* were detected in 8% of samples. *Cystoisospora ohioensis* oocysts are also morphologically similar to other canid coccidia, including *Cystoisospora burrowsi* and *Cystoisospora neorivolta*, and molecular diagnostic tools are necessary for species determination (Samarasinghe et al., 2008). *Cystoisospora* spp. detected by PCR-MCA (Figure 2-3a) could be an undescribed parasite infecting the fox, or could also be a dietary artifact from an incompletely digested prey item, such as a rodent. Additional molecular work is needed to further identify the *Cystoisospora* present in arctic foxes and their prey species. Infection with *Cystoisospora* spp. can cause diarrhea, anorexia, and weight loss in young and immunocompromised canids (Mitchell et al. 2007), which might impact fox health and survival when combined with other pathogen infections or environmental stressors.

DNA from either *Neospora caninum* or *Hammondia* spp. was detected in one sample in the current study. Neither *N. caninum* nor *Hammondia* spp. oocysts have been reported in North American arctic foxes, but *N. caninum* antibodies have been detected in a fox from Karrak Lake (S. Elmore, unpublished data). Other species of foxes can become infected with *N. caninum* and are probable definitive hosts (Hurková and Modry, 2006; Wapenaar et al., 2006); multiple fox species are known definitive hosts for *Hammondia* spp. (Gjerde and Dahlgren, 2011). The melting temperature of the positive

sample matched the *N. caninum* positive control, however we did not have *Hammondia* sp. positive control for comparison, so diagnosis could not be made based on qPCR-MCA. To distinguish the two species, *Hammondia* and *Neospora*-specific PCR assays targeting different loci would be necessary.

Giardia sp. and *Cryptosporidium* sp. were present in arctic foxes from Karrak Lake, a previously undescribed host species and region. Zoonotic genotypes of *Giardia* appear to be established in arctic wildlife, while *Cryptosporidium* is thought to be uncommon, or at least not commonly detected, at Arctic latitudes (Jenkins et al. 2013). Kutz et al. (2008) detected *Giardia duodenalis* Assemblage A in muskoxen from Banks Island, Northwest Territories, Canada, while Olson et al. (1997), reported the presence of *Giardia* cysts in ringed seals (*Phoca hispida*) from Holman, Northwest Territories, Canada. The few reports of *Cryptosporidium* in northern canids are limited to domestic dogs from British Columbia and Saskatchewan (Himsworth et al., 2010; Bryan et al., 2011, Schurer et al., 2012), but Siefker et al. (2002) detected a novel genotype of *Cryptosporidium* in Alaskan caribou and Dixon et al. (2008) found the parasite in the intestinal contents of ringed seals. The results of the current study suggest that IFA remains a highly sensitive and specific method of detection of these protozoan parasites compared to fecal flotation. There was no test agreement between IFA and PCR-MCA for detection of *Cryptosporidium* oocysts. This could be due to the low oocyst concentration, the small amount of sample used for the qPCR-MCA as compared to the IFA, or inefficient DNA extraction from the robust oocysts.

Agreement between the three tests used: fecal flotation, IFA, and PCR-MCA results, was low, but the combination of tests increased coccidia detection. The presence

of PCR inhibitors, low oocyst counts, and inefficient DNA isolation technique are potential reasons for the failure of PCR-MCA to detect fecal flotation positive samples. Conversely, a low oocyst quantity and loss of structural integrity after freeze/thaw cycles might lead to a positive PCR result and lack of detection on fecal flotation. PCR also offers the potential to capture free DNA in feces after parasite egg rupture.

Parasites that are not known to infect canids (*Eimeria* spp., Anoplocephalidae-like) were detected in fox feces in the current study. This reflects the limitations of fecal surveys in wild carnivore hosts and the need to interpret results with a broad understanding of parasite life cycles and trophic interactions. It is possible that these oocysts and eggs were present in rodent or bird prey and passed through the fox gastrointestinal tract with other undigested prey tissue (Chu et al., 2004; Stronen et al., 2011). This is supported by the PCR-MCA and subsequent phylogenetic analysis, which demonstrated that the *Eimeria* spp. present in arctic fox feces were most closely related to species such as *E. myoxi* that infect rodents. Gastrointestinal scraping or histopathology would be necessary to determine if the *Eimeria* spp. were truly parasitizing the foxes. Skirnisson et al. (1993) detected either *Eimeria* sp. or *Isospora* sp. in the microvilli of the small intestine of arctic foxes.

The anoplocephalid-like tapeworm eggs, morphologically distinct from eggs from tapeworms for which foxes are known definitive hosts such as *Mesocestoides* sp. and taeniids, were most likely artifacts in rodent prey species. Lemmings (*Dicrostonyx* and *Lemmus* spp.) host multiple species of *Paranoplocephala* (Haukisalmi et al., 2001; Haukisalmi et al., 2006), *Anoplocephaloides* spp., and *Hymenolepis* spp. (Hymenolepididae; Haukisalmi and Henttonen, 2001). Molecular techniques, in addition

to parasitological studies of rodent carcasses, are needed to further characterize these tapeworm eggs in foxes from Karrak Lake.

This study broadened our understanding of parasites of arctic foxes from the central Canadian Arctic and serves as a record for comparison to future parasite work in this rapidly changing region. A more complete parasitological study of arctic foxes at Karrak Lake would require gastrointestinal tracts from trapped or euthanized animals, which is logistically challenging in this remote environment. Overall, the PCR-MCA assay using a universal primer set that targets multiple coccidia species was a useful surveillance method that improved prevalence estimates and also allowed for DNA sequence analysis to determine identity and relationships of coccidian parasites. Combining traditional fecal flotation techniques with immunological and molecular assays offers more powerful methods to characterize the parasite fauna of remote and endangered wildlife species in non-invasive, fecal-based surveys, and also offer insights into trophic relationships of wild carnivores and their prey species.

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CHAPTER 3 - Estimating *Toxoplasma gondii* exposure in arctic foxes while navigating the imperfect world of wildlife serology

Citation

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Author Contributions

SAE and GS captured the foxes and collected the blood samples. SAE, GS, BA, KPH, LLB and EJJ contributed to study design. RTA and AAG provided field and laboratory mentoring, supplies, and logistics. SAE and GS drafted the manuscript, tables, and figures with feedback from other coauthors. All authors read and approved the final manuscript.

Chapter 3 Transition Statement

This chapter was the first step in demonstrating that Toxoplasma gondii was likely to be present in this terrestrial Arctic ecosystem. This study was also an application of occupancy modeling to help reduce bias in seroprevalence estimates and determine what serological assays performed the best while accounting for uncertainty in detection.

Abstract

The protozoan parasite *Toxoplasma gondii* is ubiquitous in birds and mammals worldwide and the full suite of hosts and transmission routes of *Toxoplasma gondii* is not completely understood. In arctic regions, *T. gondii* occurrence in people and wildlife can be high, despite apparently limited opportunities for transmission of oocysts shed by felid definitive hosts. Arctic foxes are under increasing anthropogenic and ecological pressure, leading to historic population declines in parts of the Arctic. Our understanding of *T. gondii* occurrence in arctic foxes is limited to only a few regions, but *T. gondii*-associated mortality can occur. Therefore, we investigated the exposure of arctic foxes (*Vulpes lagopus*) to *T. gondii* in the Karrak Lake goose colony, Queen Maud Gulf Migratory Bird Sanctuary, Nunavut, Canada. Following an occupancy modeling framework, we performed replicated tests of the same serum samples and compared the detection of *T. gondii* antibodies in arctic foxes measured by direct agglutination test (DAT), an indirect fluorescent assay (IFAT), and an indirect enzyme-linked immunosorbent assay (ELISA), and estimated probability of detecting *T. gondii* antibody for each of the tests as a metric of test performance. Occupancy estimates for *T. gondii* antibodies in arctic foxes under this framework were between 0.582 and 0.689. Detection probability was highest for IFAT (0.704) and lower for DAT (0.599) and ELISA (0.437), indicating that the test of choice for antibody detection in arctic foxes is the IFAT. This study documents a new geographical record of *T. gondii* exposure in arctic foxes and demonstrates an emerging application of ecological modeling techniques to account for imperfect diagnostic tests in wildlife species.

3.1 Introduction

Few parasites are known to infect as many vertebrate species as *Toxoplasma gondii*. This protozoan is ubiquitous in birds and mammals worldwide (Dubey, 2009). Felids are the only known definitive hosts of the parasite and are responsible for environmental contamination by passing oocysts in their feces (Dubey et al., 1970). Because felids are rare visitors to the arctic tundra and it is difficult to work in this remote ecosystem, the complete transmission routes and the full suite of hosts of *T. gondii* in the Arctic are not known. The low density of felids above the Arctic Circle reduces the opportunities for locally produced oocyst transmission, although oocysts from domestic and wild felids (e.g. lynx) might travel from subarctic (boreal and temperate) regions through watersheds that drain into the Arctic Ocean (Simon et al., 2013). It is probable that arctic carnivores can also become exposed to *T. gondii* through carnivory and transmission from mother to fetus during gestation.

Arctic foxes (*Vulpes lagopus*) are circumpolar canids with generalist diets (Audet et al., 2002). Although currently common throughout northern Canada, rapid climactic and environmental change likely contribute to an increasing number of stressors on the Canadian population, such as increasing interspecific competition due to red fox (*Vulpes vulpes*) range expansion (Fuglei and Ims, 2008). Elsewhere, the presence of red foxes (*Vulpes vulpes*) restricted the southern limit of arctic foxes in Norway (Hersteinsson and Macdonald, 1992), and competition between the two species negatively affected arctic fox occurrence and abundance during recolonization efforts in Fennoscandia (Hamel et al., 2013). Red foxes and other migrants from subarctic or temperate regions might introduce novel pathogens to an arctic ecosystem, which could affect the health of naïve

arctic foxes. While not an immediate conservation concern, it is important to acquire baseline data on arctic fox populations (and their parasites) in northern Canada.

Arctic foxes are known intermediate hosts of *T. gondii* and the parasite can cause mortality, such as reported in the Svalbard, Norway population (Sorensen et al., 2005; Prestrud et al., 2008), in which 43% of 594 arctic foxes examined were seropositive (Prestrud et al., 2007). In North America, *T. gondii* detection in arctic foxes is rare, most likely due to lack of surveillance and the high cost of obtaining samples. Dubey et al. (2011b) contributed the only published report of *T. gondii* isolation from 5 of 14 arctic foxes from Alaska. However, antibodies have been detected in other canid species from the Northwest Territories and Alaska (Zarnke, et al. 2000; Salb, et al. 2008; Stieve, et al. 2010), indicating that northern canids are routinely exposed to *T. gondii*.

Most of the studies that examined *T. gondii* in wildlife were done with serological assays (Elmore et al., 2012; Jenkins et al., 2013); these are common techniques used as alternatives to direct demonstration of *T. gondii* infection in tissues by PCR or histopathology This is likely due to the difficulty of isolating the parasite *ante mortem* and the relative ease of accessing blood samples in live animals. These assays are rarely formally validated due to the inaccessibility of known positive and negative control samples for many wildlife species (Elmore et al., 2012; Peel et al., 2013) and also to the lack of monetary incentives for developing veterinary diagnostic tests for non-food and non-companion animal species. As a result, assay sensitivity and specificity are rarely available for the species of interest, creating uncertainty in interpreting the test results (Elmore et al. 2014), and leading to results being falsely categorized using traditional naïve prevalence estimators. Increasingly, this problem is being addressed by analyzing

wildlife disease data within an occupancy modeling framework (e.g. Lachish et al., 2012, Elmore et al., 2014).

Occupancy modeling approaches are analogous to mark-recapture analyses from wildlife biology and were initially used to estimate the occurrence of cryptic or rare species within habitat patches where they may be detected imperfectly (MacKenzie et al., 2006). These approaches are useful in wildlife disease ecology because they acknowledge that uncertainty exists when studying wild populations and when using imperfect diagnostic tests (McClintock et al., 2010, Lachish et al., 2012). Under a typical occupancy framework, multiple randomly selected 'sites' are surveyed on multiple occasions within a time frame where the occupancy state (species present or species absent) is assumed not to change. These repeated survey occasions at each site enables estimation of two parameters: occupancy (ψ), defined as the probability that a site is occupied by the species of interest (equal to prevalence in a wildlife disease context), and detection probability (p), the probability that the species is detected during a given occasion, given the site is occupied (MacKenzie et al., 2006). In our application, diagnostic samples are 'sites' (i.e., serum samples from individual foxes), the "species of interest" are antibodies against *T. gondii*, and the repeated survey occasions are multiple test repetitions performed on each sample for each assay. The reinterpreted parameter definitions in this study are 1) ψ_i = the probability that serum sample *i* is positive for *T. gondii* antibodies and 2) p_{it} = the probability of detecting *T. gondii* antibodies in sample *i,* from assay *t.*

Adding to the uncertainty of *T. gondii* serology is the absence of a "gold standard" diagnostic test. The direct agglutination test (DAT; equivalent to the modified

agglutination test, MAT) is a commonly used serological test for wildlife exposure to *T. gondii* because it is flexible for use in multiple species (Prestrud et al., 2007). The DAT has not been formally validated for wildlife and performance can vary among different species (Macrí et al., 2008). Indirect fluorescent antibody tests (IFAT) are also used with wildlife sera (Miller et al., 2002; Dabritz et al., 2008), but their use has been limited to animals for which a taxon-specific secondary antibody has been produced. Both assays have subjective endpoint criteria based on visual inspection, which suggests the potential exists for misclassification and biased reporting of seroprevalence. Commercially available enzyme-linked immunosorbent assays (ELISA) can also be used to determine *T. gondii* seroprevalence, but these assays are also limited by requiring a taxon-specific secondary antibody. To address this problem, Al-Adhami et al. (2014) developed an indirect ELISA using a protein A/G conjugate that can be used in multiple mammal species, although the problem of obtaining known positive and negative samples to determine cut-off values still exists.

In this study, our objectives were to use occupancy modeling approaches to: 1) estimate the seroprevalence of *T. gondii* in arctic foxes from Karrak Lake, Nunavut, Canada and 2) determine which serological assay gives the best detection probability for *T. gondii*. Due to previous descriptions of *T. gondii* exposure in prey items [geese (*Chen* sp.*,* caribou (*Rangifer tarandus*), and musk ox (*Ovibos moschatus*)] that are commonly scavenged or hunted by foxes within our study area (Kutz et al., 2000, Kutz et al., 2001, Elmore et al., 2014), we hypothesized that arctic foxes in the Karrak Lake ecosystem would demonstrate exposure to *T. gondii*. Because the IFAT has been demonstrated to have a higher detection probability than DAT in a previous study on geese (Elmore et al.,

2014) and the protein A/G ELISA had high agreement with the IFAT and DAT (Al-Adhami and Gajadhar, 2014), we also hypothesized that either of these tests would have a higher detection probability than would the DAT.

3.2 Methods

3.2.1 Study area

The field portion of this study took place within the Karrak Lake region (67º 14' N, 100º 15' W) of the Queen Maud Gulf Bird Sanctuary in the central Canadian Arctic (Figure 3-1). This area is a large goose colony where over one million Ross's Geese (*Chen rossii*) and Lesser Snow Geese (*Chen caerulescens*) nest annually (Alisauskas et al. 2012). The landscape is characterized by rolling tundra with rock outcrops, sedge meadows, marshes, and freshwater ponds (Ryder, 1972).

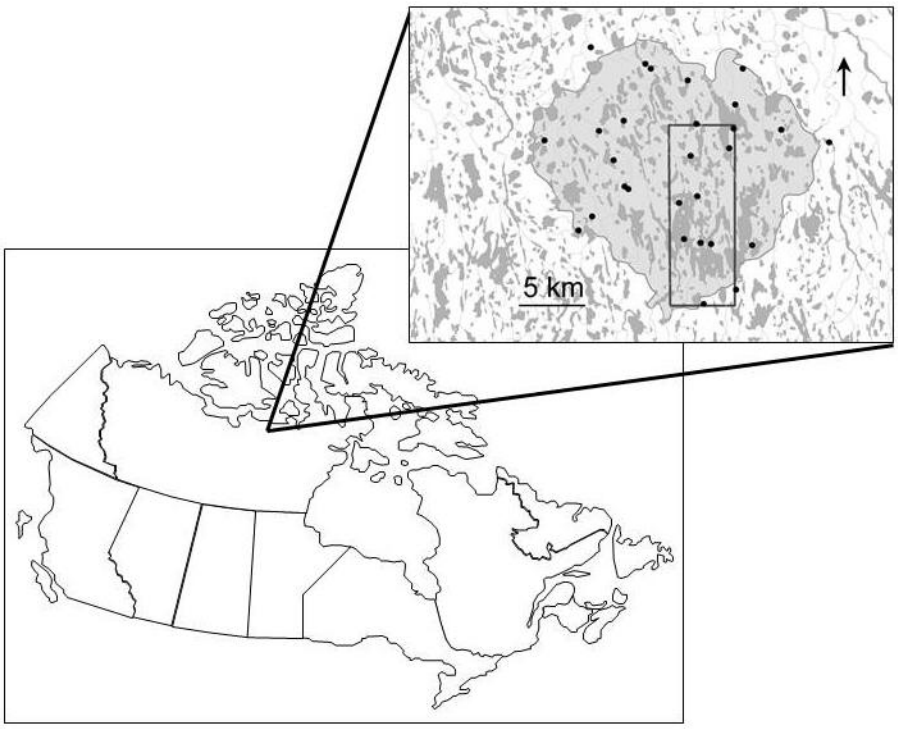

FIGURE 3-1. LOCATION OF THE KARRAK LAKE GOOSE COLONY IN NUNAVUT, CANADA. INSET: DOTS REPRESENT KNOWN ARCTIC FOX DENS AND RECTANGLE REPRESENTS TRAPPING AREA WITHIN THE COLONY (ELMORE ET AL., 2013).

3.2.2 Field sample collection

From mid-May to mid-June 2011-2013, we live-captured 39 different adult arctic foxes. Foxes were sedated with 15 mg Telazol® (tiletimine/zolazepam) following Samelius et al. (2003). We placed plastic ear tags in both ears for future identification, estimated sex and age (based on tooth-wear measurements), and collected up to 3 mL of blood by cephalic venipuncture. Following blood clot formation, we centrifuged the blood at 8000 rpm for 10 minutes to separate the serum from the cellular portion and stored the sera at -20ºC. Foxes caught multiple times over the course of the 3 year study were sedated and had blood drawn once per year.

We estimated age through tooth wear classifications as "not worn, slightly worn, and worn" (see Landon et al. 1998 for similar categories). Tooth wear of known-age foxes (n=7 foxes marked as kits) and of foxes recaptured in more than one year (n=38 recaptures of 26 foxes) showed the following: 1) 5 of 5 known yearling foxes had no tooth wear but one of 24 foxes known to be at least 2 years old was included in the "not worn" category, 2) 27 of 33 foxes known to be at least 2-4 years old had "slightly worn" teeth, and 3) that 4 of 5 foxes that were known to be at least 5 years old had "worn" teeth (G. Samelius, unpublished data). In accordance with these data, we structured our age categories as: 1 year (n=19), 2-4 years (n=14), and >5 years (n=6).

3.2.3 Laboratory analysis

Following an occupancy modeling framework, we tested each serum sample (n=46) from each fox using three separate replicates each of the direct agglutination test (DAT), indirect fluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA), resulting in a detection/non-detection sequence for each serum sample

consisting of 9 replicates for which we observed and recorded antibody states (antibodies detected = 1; no antibodies detected = 0). Equivocal results were treated as missing data (recorded as "."), and were excluded from the analysis.

Direct agglutination tests were performed on serum using a commercially available kit (DAT; ToxoScreen-DA, Biomerieux, Marcy l'Etoile, France) according to manufacturer instructions. Each serum sample was tested at 1:40 dilution on three discrete replications, or "site visits" (Jokelainen et al. 2010; Elmore et al., 2014). Test-wells indicating agglutination covering 50-100% of the well were recorded as "*T. gondii* antibodies clearly detected (0). Following manufacturer's instructions, test-wells with a solid dot or small ring in the center were recorded as "no antibodies detected" (0). Wells with mild agglutination covering less than half of the test well were recorded as equivocal.

Indirect fluorescent antibody tests (IFAT) were performed using anti-canine fluorescein isothiocyanate (FITC; rabbit origin) conjugate and antigen-coated slides from VMRD (Pullman, WA, USA). The staining procedure followed manufacturer's (VMRD) instructions. We scanned the slides by fluorescent microscopy at 40x magnification. Sample wells with unbroken staining surrounding the entire tachyzoite were recorded as "*T. gondii* antibodies clearly detected"(1). Sample wells with little or no staining, or where tachyzoites in the well demonstrated discontinuous staining, were recorded as "no antibodies detected" (0). Sample wells with dim fluorescence and both intact and discontinuous staining were recorded as equivocal.

We analyzed all samples by indirect ELISA with protein A/G conjugate at a 1:200 dilution as previously described (Al-Adhami et al. 2014). Due to lack of species-specific

positive and negative control samples to estimate the cut-off value by traditional methods, we calculated an intrinsic cut-off value for each ELISA plate (Jacobson 1997; Desquesnes et al., 2009; Pruvot et al., 2013). Suspect negative samples were identified by cluster analysis using a k-means procedure using Proc CLUSTER implemented in SAS v9.3 (SAS Institute Inc. 2011). Positive and negative control sera from experimentally infected pigs were used to calculate sample to positive ratio (S/P) values for all optical density (OD) results. Final cut-off values were determined by calculating 2 x standard deviation (upper bound) of the mean S/P value for the suspect negative sera (Crowther, 2012).

3.2.4 Data Analysis

We analyzed detection histories for *T. gondii* antibodies using the single-season occupancy model in Program MARK to estimate seroprevalence within the arctic fox population at Karrak Lake (White and Burnham, 1999). When multiple blood samples were available per fox, we only included the serology results from the first year; serology results from subsequent years were not included in the occupancy. We developed an *a priori* candidate model set (Table 1) to analyze the potential effects of sex and age class on the probability of seropositivity (ψ) and the effect of serological assay type on detection probability (p). We performed model selection using the small sample bias-corrected Akaike Information Criterion (AICc; Burnham and Anderson 2002). We ranked models by calculating the differences in AICc (ΔAIC) between the highest-ranked model and remaining models; model weights (w_i) were then calculated for each model. Similar weights for multiple models indicated some model selection uncertainty, so we

used model averaging to calculate parameter estimates (Anderson, 2008). Unconditional variances were used to construct 95% confidence intervals.

3.3 Results

We analyzed 46 serum samples from 39 foxes by DAT, IFAT, and ELISA for *T. gondii* antibodies. For the 6 foxes tested in multiple years, one fox was seronegative throughout the study (3 subsequent years). Four foxes were tested for two consecutive years; two of these were seropositive in both years and two tested seronegative in the first year and seropositive in the second year.

Age of foxes was an important determinant of seropositivity (ψ) in the top-ranked model, which carried an Akaike weight of 0.48 (Table 3-1). Serological assay type ("test") influenced the probability of detecting *T. gondii* antibodies, given that a fox was seropositive (p; Table 3-1); this effect was represented in all models carrying Akaike weights >0.10. There was little evidence of sex-specific differences in seroprevalence (Table 3-1). As hypothesized, young foxes were least likely to be seropositive for *T. gondii* (Table 3-2). The middle age group (2-4 years) had the highest seroprevalence estimates, while the seroprevalence for the highest age group was slightly lower (Table 3-2). A comparison of detection probabilities for the three diagnostic tests showed that detection probability was higher for the IFAT [$\hat{p} = 0.704$ (95% CI = 0.569, 0.811)] than for both the DAT and the ELISA [$\hat{p} = 0.599$ (95% CI = 0.478, 0.70), and 0.437 (95% CI = 0.306, 0.577), respectively; Table 3-2].

TABLE 3-1. CANDIDATE MODEL SET AND MODEL SELECTION RESULTS OF THE OCCUPANCY ANALYSIS TO DETERMINE SEROPREVALENCE, DETECTION PROBABILITY OF *T. GONDII* ANTIBODIES IN ARCTIC FOXES FROM KARRAK LAKE, NUNAVUT, CANADA.

Model	No. Parameters	-2log likelihood	AICc	ΔAICc	AICc Weights
psi(age) p(test)	6	299.94	314.57	0.00	0.48
psi(.) p(test)	4	306.17	315.34	0.78	0.33
psi(sex) p(test)	5	305.61	317.43	2.86	0.12
psi(age) p(.)	4	310.81	319.98	5.42	0.03
psi(sex*age) p(test)	9	297.16	321.37	6.80	0.02
psi(.) p(.)	2	317.03	321.37	6.80	0.02
psi(sex) p(.)	3	316.47	323.16	8.59	0.01
psi(sex*age) p(.)	7	308.02	325.64	11.07	0.00

TABLE 3-2. MODEL-AVERAGED ESTIMATES OF *T. GONDII* SEROPREVALENCE AND DETECTION PROBABILITY FOR ARCTIC FOXES FROM KARRAK LAKE, NUNAVUT, CANADA.

Parameter	Estimate	SE	LCI	UCI
$\psi_{\text{Young Males}}$	0.467	0.147	0.216	0.736
$\psi_{\text{ Young Females}}$	0.452	0.138	0.217	0.710
$\psi_{\text{ Mid-Aged Males}}$	0.689	0.145	0.370	0.893
$\psi_{\text{ Mid-Aged Females}}$	0.676	0.159	0.335	0.896
$\psi_{\text{ Older Males}}$	0.628	0.161	0.304	0.867
$\psi_{\text{ Older Females}}$	0.599	0.183	0.252	0.869
p_{DAT}	0.599	0.060	0.478	0.708
p_{IFAT}	0.704	0.063	0.569	0.811
p_{ELISA}	0.437	0.071	0.306	0.577

3.4 Discussion

This study documents a new geographical record of *T. gondii* exposure in arctic foxes and shows that arctic foxes within the Karrak Lake ecosystem in Nunavut are routinely exposed to *T. gondii*. While *T. gondii* is known to circulate in marine environments in the North (Simon et al., 2011; Tryland et al., 2014), our finding of *T. gondii* antibodies in terrestrial predators suggests that the parasite also circulates in terrestrial Arctic ecosystems.

At Karrak Lake, arctic foxes are probably exposed to *T. gondii* by trophic transmission (e.g. consumption of tissue cysts in other intermediate hosts), but transmission from mother to offspring might also occur during gestation (Dubey, 2009). Oocyst transmission of *T. gondii* to arctic foxes in our study system is less likely, due to the distance from lynx habitat (subarctic boreal ecosystems) and human communities where cats might be present. Alternatively, oocysts might enter terrestrial systems by traveling through watersheds from more southern latitudes, followed by ingestion by wildlife (Simon et al., 2013a). Arctic foxes in this population are known to consume collared lemmings (*Dicrostonyx groenlandicus*), birds (including adult geese), and cached goose eggs; however, foxes also scavenge larger prey such as caribou and muskoxen (Samelius et al., 2007). All of these animals have the potential to serve as intermediate hosts of *T. gondii,* and antibodies have been reported in geese sympatric with the foxes in this study, caribou, and muskoxen in the Canadian Arctic (Kutz et al., 2000, 2001; Elmore et al., 2014). Also, geese and caribou conduct long-distance migrations between subarctic and temperate regions (where they might encounter *T. gondii* oocysts) and the Arctic.

Observing *T. gondii* antibodies in a predator such as arctic foxes might also serve as an indicator for potential human exposure and transmission of *T. gondii* in wildlife from this region. For example, caribou, musk oxen, and geese are important foods for both subsistence hunters and foxes (Samelius et al., 2007), and exposure to *T. gondii* in these species has been documented. We stress the importance of better understanding the distribution and transmission of *T. gondii* in arctic wildlife and the significance for wildlife and human health in northern Canada. In the recent Inuit Health Survey, Inuit populations in Nunavut have a higher seroprevalence of *T. gondii* (32%) than those in the west (Inuvialuit Settlement Region, 7.5%), although still only half of that observed in the Nunavik region east of Hudsons Bay (~60%) (Messier et al., 2009; Goyette et al., 2014). While the Inuit Health Survey revealed that the consumption of seafood and marine mammals was a higher risk factor for human exposure to *T. gondii* than birds and ungulates, our study serves as a reminder that this parasite circulates in terrestrial arctic ecosystems as well. Our current understanding of *T. gondii* in arctic foxes does not imply direct transmission from foxes to humans, except possibly in cases of carcass handling or, rarely, consumption of foxes in some traditional communities, but rather reflects the ubiquity of the parasite within a terrestrial food web.

In our study, middle-aged foxes were most likely to be seropositive, and older foxes had a slightly lower probability of seropositivity. This was unexpected; we expected the oldest age class to have the highest seroprevalence due to the cumulative increased risk of exposure throughout the lifetime of a fox and previous observations (Mitchell et al., 1999). It is probable that the low overall sample size in this study, the relatively low number of known-aged foxes, and potential misclassifications of age based

on tooth wear might have influenced these results by underestimating the seroprevalence in the highest age class. Another explanation of the lower seroprevalence in older foxes than middle-aged foxes is that the presence of *T. gondii* antibodies wanes with time if animals are not continually exposed to the parasite. It is generally assumed that antibodies against *T. gondii* persist throughout the life of an animal (Osteegh et al., 2011). It is also possible that seropositive foxes die before they reach the older age class. Disseminated toxoplasmosis can influence survival in arctic foxes (Sörensen et al., 2005), but the effects of chronic *T. gondii* infections on wildlife populations are largely unknown.

By taking blood samples from recaptured animals in subsequent years, we might begin to understand the dynamics of antibody persistence and colonization in arctic foxes, possibly using a robust design occupancy approach (Eads et al., 2014) if enough recapture data could be collected in a long-term study. It would also be beneficial to understand the movements of arctic foxes at Karrak Lake throughout the year to estimate where and when they become exposed to *T. gondii.* It is unknown whether Karrak Lake foxes maintain territories or emigrate during the winter, although there is a tendency to stay in an area once settled (Samelius et al., 2007). However, even foxes known to hold successful breeding dens in consecutive years will disperse during periods of low prey abundance, such as during a lemming population lowpoint (G. Samelius, unpublished data). Because Karrak Lake is only approximately 60 km from the Arctic Ocean, it is possible that foxes arriving in the ecosystem could have been exposed in a marine environment, although in a previous study, the stable isotope signatures of arctic fox blood did not reveal marine signatures at significant levels and were instead heavily

represented by collared lemmings (*Dicrostonyx groenlandicus*) and goose eggs (*Chen* sp.) (Samelius et al., 2007).

Detection probability of *T. gondii* antibodies varied among the three serological assays examined. The IFAT had the highest detection probability for antibodies ($\hat{p} = 0.704$), outcompeting the DAT and ELISA ($\hat{p} = 0.599$ and 0.437, respectively). This is in accordance with a previous study that demonstrated higher sensitivity in the IFAT over DAT in canine serum samples (Macrí et al., 2008). The low detection probability from the ELISA might be due to our conservative determination of a positive cut-off value, in the absence of known control samples. This suggests that the ELISA will underestimate seroprevalence unless a more rigorous method of cut-off value determination can be used with known positive and negative control samples. When the appropriate controls were available, this ELISA was very effective and showed excellent agreement with the MAT, western blot, and an ELISA-IgG kit (Al-Adhami et al., 2014). Our work demonstrates the utility of an occupancy framework to evaluate the performance of diagnostic assays not validated in wildlife species.

In summary, this study documents a new geographical record of *T. gondii* exposure in arctic foxes and demonstrates an emerging application of ecological modeling techniques to account for the use of imperfect diagnostic tests in wildlife species. The logistic and financial challenges to pursuing arctic fieldwork also further highlights the value of this data set. Future research bridging the occurrence of *T. gondii* with potential routes of exposure will be important to increase our understanding of *T. gondii* ecology in the Arctic.

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CHAPTER 4. *Toxoplasma gondii* exposure in arctic-nesting geese: a multi-state occupancy framework and comparison of serological assays

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Citation

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Author Contributions

SAE collected samples. SAE, KPH, LLB, and EJJ designed the study. SAE, KPH, LLB, and JM analyzed data. RTA and AAG provided mentorship, logistics and supplies. SAE drafted the manuscript, tables and figures with feedback from coauthors. All authors read and approved the final manuscript.

Chapter 4 Transition Statement

In the chapters 2 and 3, I demonstrated that foxes were exposed to *T. gondii* in the Karrak Lake ecosystem, most likely as intermediate hosts. The next step was to determine how they might be exposed to T. gondii. In this chapter we demonstrate that migratory geese are likely vectors for *T. gondii* into terrestrial Arctic ecosystems in Canada.

Abstract

The zoonotic parasite, *Toxoplasma gondii,* has a worldwide distribution and a cosmopolitan suite of hosts. In arctic tundra regions, the definitive felid hosts are rare to absent and, while the complete transmission routes in such regions have yet to be fully elucidated, trophic and vertical routes are likely to be important. Wild birds are common intermediate hosts of *T. gondii,* and in the central Canadian arctic, geese are probable vectors of the parasite from temperate latitudes to the arctic regions. Our objective was to estimate seroprevalence of *T. gondii* in Ross's and Lesser Snow Geese from the Karrak Lake ecosystem in Nunavut, Canada. After harvesting geese by shotgun, we collected blood on filter paper strips and tested the eluate for *T. gondii* antibodies by indirect fluorescent antibody test (IFAT) and direct agglutination test (DAT). We estimated seroprevalence using a multi-state occupancy model, which reduced bias by accounting for imperfect detection, and compared these estimates to a naïve estimator. Ross's Geese had a 0.39 probability of seropositivity, while for Lesser Snow Geese the probability was positive for *T. gondii* antibodies was 0.36. IFAT had a higher antibody detection probability than DAT, but IFAT also had a higher probability of yielding ambiguous or unclassifiable results. The results of this study indicate that Ross's Geese and Lesser Snow Geese migrating to the Karrak Lake region of Nunavut are routinely exposed to *T. gondii* at some point in their lives and that they are likely intermediate hosts of the parasite. Also, we were able to enhance our estimation of *T. gondii* seroprevalence by using an occupancy approach that accounted for both false-negative and false-positive detections and by using multiple diagnostic tests in the absence of a gold standard serological assay for wild geese.

4.1 Introduction

The zoonotic parasite, *Toxoplasma gondii* has a worldwide distribution and a cosmopolitan suite of hosts; evidence of exposure was even recently detected in pinnipeds from Antarctica (Jensen et al., 2012). Oocyst-derived infections are the result of environmental contamination by felids, the definitive hosts of *T. gondii* (Dubey et al., 1970). In arctic tundra regions, felids are rare to absent and, while the complete transmission routes in such regions have yet to be fully elucidated, trophic routes and transmission from mother to offspring (vertical transmission) are likely to be important (McDonald et al., 1990, Messier et al., 2009).

Wild birds are common intermediate hosts of *T. gondii* (Dubey et al., 2002). The most common infective forms of *T. gondii* for herbivorous birds, such as geese, are sporulated oocysts, which can be found in contaminated water bodies or soil (Dubey, 2009) to which these birds may be exposed. When high densities of waterfowl congregate in a contaminated environment, oral transmission is likely to occur. If the birds become intermediate hosts of the parasite, they will eventually develop cysts in their organs and musculature. The population-level significance of infection in wild birds is unclear, but avian mortality has been reported in heavily infected birds (Dubey et al., 2001, Work et al., 2002). Arctic-nesting geese are probable vectors of the parasite from temperate latitudes to the arctic region of Svalbard (Prestrud et al., 2007) and likely along other migratory routes as well.

In North America, Ross's Geese (*Chen rossii*) and Lesser Snow Geese (*Chen caerulescens*) are two common arctic-nesting geese that overwinter in the southern United States and migrate through the midcontinent of North America to breeding areas

in arctic Canada and Alaska (Alisauskas et al., 2011). Thus, these two populations of arctic-nesting geese are sympatric with both domestic and wild felids for at least 8 months of the year (September to April), but are thought to be largely allopatric to felids for about 4 months (May to August), when in the Arctic. Felid and goose ranges may overlap in the southern portions of breeding range where about 10% of subarctic geese nest at known colonies near Hudson or James Bay, but 90% nest well above treeline, such as near Queen Maud Gulf, Southampton Island and Baffin Island (Alisauskas et al., 2011); these regions are thought to be largely or completely absent of felid populations. These geese are considered overabundant (Leafloor et al., 2012) because of demonstrated impacts on arctic vegetation (Abraham et al., 2012) from overgrazing (Alisauskas et al., 2012). Such high goose densities across an expanding range represent an increasing potential for seasonal *T. gondii* introduction to wildlife predators in ecosystems of both arctic and temperate latitudes. However, no estimates exist for the seroprevalence of *T. gondii* in these goose populations. Potential predators of geese in the Karrak Lake ecosystem include arctic foxes (*Alopex lagopus*), wolverines (*Gulo gulo*), wolves (*Canis lupus*), and barren-ground grizzly bears (*Ursus arctos horribilis*), and it is possible that infected geese could transmit *T. gondii* to these animals (Bantle and Alisauskas, 1998; Wiebe et al., 2009).

Most evidence for the occurrence of *T. gondii* in wildlife is obtained through serological tests, which, while providing limited information on current infection status, can be useful tools in determining exposure within a population. Filter paper blood collection is a technique that is increasingly used for post-mortem antibody detection in wildlife (Jakubek et al., 2012, Aston et al., 2014). The technique is especially useful in

remote areas where sera cannot be refrigerated or frozen, and is commonly used in wild waterfowl (Maksimov et al., 2011). The direct agglutination test (DAT; equivalent to modified agglutination test (MAT)), is a widely used serological test for wildlife exposure to *T. gondii* because it is flexible for use in multiple species and can also be used with eluate from blood stored on filter paper (Jakubek et al., 2012). Although often described as sensitive and specific in wildlife serological applications (Hollings et al. 2013), the DAT has not been formally validated for wildlife and performance can vary among different species (Macrí et al., 2008). Indirect fluorescent antibody tests (IFAT) are also used with wildlife sera (Miller et al., 2002; Dabritz et al., 2008), but their use has been limited to animals for which a taxon-specific secondary antibody has been produced. The use of IFAT with eluate from blood-soaked filter paper is not often reported in *T. gondii* diagnostics, but is commonly used for other types of antibody detection in waterfowl (Maksimov et al., 2011). Both assays have subjective cut-off values based on visual inspection, which suggests the potential exists for misclassification and biased reporting of seroprevalence. In a comparison between IFAT and MAT, Macrí et al (2008) reported 97.8% sensitivity in cat serum by MAT (with IFAT as the comparative test), but only 73.4% sensitivity in dog serum by MAT. In this case, the IFAT was considered the "gold standard" for comparison by the MAT.

The risk of *T. gondii* transmission from geese to other wildlife populations and people emphasizes the need for reliable parameter estimates from serosurvey data (McClintock et al., 2010). Observation error due to non-detection is not commonly accounted for in prevalence estimates from wildlife disease studies, although the increasing use of occupancy modeling approaches shows more attention to this concern

(e.g., McClintock et al., 2010, Gómez-Diaz et al., 2010, Lachish et al., 2011, Eads et al., 2013). Occupancy approaches are analogous to mark-recapture analyses from wildlife biology and were originally used to estimate the probability of occurrence of cryptic or rare species within habitats where they may be detected imperfectly (MacKenzie et al., 2006). These approaches are useful in wildlife disease ecology because they acknowledge that detection is imperfect and account for this uncertainty in parameter estimates of disease prevalence (McClintock et al., 2010, Lachish et al., 2011). Because most wildlife serological assays are not formally validated and thus have no information on test sensitivity and specificity, occupancy approaches provide a method for quantifying some of the uncertainty in the diagnostic system.

Under a typical occupancy framework, multiple randomly selected 'sites' are repeatedly surveyed within a time frame where the occupancy state (species present or species absent) does not change. Surveys, or replicates, can be conducted at multiple times or at the same time by multiple independent observers or different detection methods. These replicates at each site enables estimation of two parameters: occupancy, defined as the probability that a site is occupied by the species of interest, and detection probability, the probability that the species is detected during a given survey (replicate), given the site is occupied (MacKenzie et al., 2006). In our application, diagnostic samples are 'sites' (i.e., an eluate produced from blood-soaked filter paper taken from each goose) and the species of interest are antibodies against *Toxoplasma gondii*, and the replicates are repeated assays (DAT or IFAT) performed on each sample.

In more complex occupancy models, such as those handling multiple occupied states or multiple scales, additional parameters can be estimated. Traditional static occupancy

models, in a diagnostic context, insulate prevalence estimates against false-negatives but these models assume that false-positive results do not occur. Yet, in all serological assays, and especially with samples from wildlife species, there is a risk of cross-reactivity with unknown non-target antibodies, which could lead to ambiguous test results. Results from the IFAT and DAT are subject to observer experience and opinion, which might cause ambiguous test results to be misclassified, leading to false-positive results. In this paper we utilized a generic multi-state occupancy approach (Nichols et al. 2007) and interpret model parameters in a disease ecology context. A similar approach was also proposed by Miller et al. (2011) and both approaches account for both false-positive and false-negative observational errors.

We hypothesized that Ross's and Lesser Snow Geese are routinely exposed to *T. gondii* because they overwinter in and migrate to areas where *T. gondii* oocysts are likely to be present in the environment. Our main objectives in this study were to: 1) estimate seroprevalence in hunted Ross's and Lesser Snow Geese using a general static multi-state occupancy approach to account for both false-positive and false-negative observational errors, and 2) compare seroprevalence, estimated with occupancy models, to naïve estimates of seroprevalence that assume detection probability is complete and diagnosis is error-free. An additional objective was to evaluate whether species and/or sex had an effect on the probability of seropositivity in a given individual, suggesting apparent differences exist between the species in foraging behavior (Jonsson et al., 2013) or that androgens might suppress immune function, thus leading to increased parasite susceptibility in males (Owen-Ashley et al., 2004). We propose that using different serological assays with multiple replicates and modeling techniques that account for

imperfect detection in wildlife samples will reduce bias in estimates of *T. gondii* seroprevalence.

4.2 Methods

4.2.1 Field Sample Collection

Ross's Geese and Lesser Snow Geese were sampled from respective populations by shooting from late May to early June each year, 2011-2013, as part of ongoing studies at Karrak Lake on these species. In both years, geese were collected over a period of 2 weeks and it is unlikely that collected individuals were related. During field necropsies, we collected serosanguineous fluid from the thoracic cavity of each goose on Nobuto filter paper strips (Advanetec MFS, Inc, Dublin, CA, USA). The strips were dried at ambient temperature overnight, placed into individual envelopes, and frozen at -20°C until further analysis at the University of Saskatchewan. All field activities were conducted in accordance with The University of Saskatchewan Animal Care and Use Committee (Protocol 20100159), the Canadian Wildlife Service (Permits NUN-SCI-11-02; NUN-MBS-11-03), and the Government of Nunavut (Permits 2011-019, 2012-021, 2013-017).

4.2.2 Filter Paper Elution

To facilitate antibody elution from the filter paper strips, we followed the method used by Curry et al. (2011). Briefly, two filter paper strips from each goose were cut into pieces and placed in a microcentrifuge tube. Then, 800 µL Dulbecco's Phosphate Buffered Saline with antibiotic was added to each tube and left to elute in a refrigerator overnight (approximately 16 hours). To eliminate debris from dried blood clots on the

filter paper, we filtered all eluate samples through a 19-gauge filter needle (Becton Dickenson Canada, Inc., Mississauga, ON) and placed into a new microcentrifuge tube. Eluate samples were stored at -20°C until further analysis.

4.2.3 Serological analysis

Following a static occupancy-modeling framework, we tested each eluate sample from each goose using three separate replicates of the direct agglutination test (DAT) and two additional replicates using IFAT, resulting in a detection/non-detection sequence for each eluate sample consisting of 5 replicates for which observed antibody states were recorded.

Direct agglutination tests were performed on filter paper eluate using commercially available kits (ToxoScreen-DA, Biomerieux, Marcy l'Etoile, France) according to manufacturer instructions. Each eluate sample was tested in duplicate wells at 1:40 dilution on three discrete occasions. Test wells indicating agglutination covering 50-100% of the well were recorded as "*T. gondii* antibodies clearly detected". Following manufacturer's instructions, test wells with a solid dot or small ring in the center were recorded as "no antibodies detected", and wells with mild agglutination covering less than half of the test well were recorded as "ambiguous or unclassifiable".

Indirect fluorescent antibody tests (IFAT) were performed using anti-duck fluorescein isothiocyanate (FITC; rabbit origin) conjugate from Nordic Laboratories (Copenhagen, Denmark) and antigen-coated slides from VMRD (Pullman, WA, USA). The IFAT was optimized using *T. gondii*-positive and negative control filter paper samples from experimentally infected and unexposed control Pekin ducks (Appendix), concluding that the assay best performed at both a sample dilution and conjugate dilution

of 1:20. The staining procedure followed manufacturer's (VMRD) instructions. We scanned the slides by fluorescent microscopy at 40x magnification. Sample wells with unbroken staining surrounding the entire tachyzoite were recorded as "*T. gondii* antibodies clearly detected". Sample wells with little or no staining, or where tachyzoites in the well demonstrated discontinuous staining were recorded as "no antibodies detected"; sample wells with dim fluorescence and both intact and discontinuous staining were recorded as "ambiguous or unclassifiable".

4.2.4 Data Analysis

We calculated naïve seroprevalence by dividing the number of geese that were clearly *T. gondii* seropositive on at least one replicate, of either assay, by the total number of samples tested.

To utilize the general multi-state occupancy model (Nichols et al. 2007) we first describe three true states; each eluate sample must be in one of these mutually exclusive states: i) Eluate sample contains no antibodies that may lead to non-negative assay results (True state = 0), ii) Eluate sample contains non-target antibodies or other material that may lead to an ambiguous or unclassifiable assay result (True state = 1), and iii) Eluate sample contains targeted *T. gondii* antibodies (True state = 2). The multi-state occupancy model contains two conditional occupancy parameters, ψ^1 and ψ^2, that are used to estimate the probability that a given eulate sample is in each of the three true states (see Table 4-1). We utilized our observed detection histories for antibodies results for each replicate assay with the static multiple-state occupancy model implemented in Program MARK (White and Burnham, 1999) to estimate the two occupancy parameters and derive seroprevalence for our sampled goose populations ($\psi^1 \times \psi^2$; Table 1). In addition, the multi-state

occupancy model contains two detection probability parameters (p^1 and p^2; see Table 4-1), and a correct classification parameter (δ; see Table 4-1). Here, p^1 represents the probability of an ambiguous or unclassifiable assay result even though the eluate does not contain *T. gondii* antibodies. We assume that an eluate without *T. gondii* antibodies cannot produce an unambiguous positive result (i.e., the "*T. gondii* antibodies clearly detected" result as defined above). Notice that an eluate that contains *T. gondii* antibodies can produce an assay with any of the three possible observations: *T. gondii* antibodies are clearly detected (probability = $p^2 \times \delta$), ambiguous or unclassifiable results (probability = $p^2 \times (1-\delta)$), or non-detection results (probability = $(1-p^2)$).

TABLE 4-1. PARAMETER DEFINITIONS FOR THE MULTIPLE-STATE OCCUPANCY MODEL.

Parameter	Definition
ψ^1_i	Probability that an eluate sample *i* might give a non-negative result.
ψ^2_i	Probability that an eluate sample *i* is occupied by *T. gondii* antibodies, given a non-negative result is possible.
$\psi^1_i * \psi^2_i$	Unconditional probability that sample *i* contains *T. gondii* antibodies.
p^1_{it}	Probability of a non-negative result from assay *t*, given the eluate sample *I* is in the unclassifiable state (state 1).
p^2_{it}	Probability of a non-negative result from assay *t*, given *T. gondii* antibodies are in eluate sample *i* (i.e., the sample is in state 2).
δ_{it}	Probability that a non-negative result from assay *t* is correctly classified as *T. gondii* seropositive, given that the eluate sample *i* is seropositive.

Using our multi-state occupancy model, we developed an *a priori* candidate model set (Table 4-2) to examine the effects of sex and species on the probability of *T. gondii* seropositivity, and the effect of serological method (DAT vs IFAT) on both detection probabilities (p^1 and p^2) and the correct classification probability (δ). We performed model selection using small sample bias-corrected Akaike Information Criterion values (AICc; Burnham and Anderson, 2002). We ranked candidate models by calculating the differences in AICc (ΔAICc) between the highest-ranked model and the other models, and then calculated model weights (w_i) for each model; higher-ranked models carry the most weight and thus best explain the data (Anderson, 2008). Because most of the weight was distributed between the two highest ranked models, we addressed model selection uncertainty by performing model averaging to obtain final parameter estimates (Anderson, 2008).

4.3 Results

We analyzed blood filter paper eluent by DAT and IFAT for *T. gondii* antibodies in 121 Lesser Snow Geese and 123 Ross's Geese from Karrak Lake Nunavut. We detected *T. gondii* antibodies in samples from both species (Figure 4-1). Our occupancy-based model-averaged estimates of seroprevalence in geese were higher than those calculated using a naïve approach (Figure 4-1). The occupancy seroprevalence estimate for Ross's Geese was 0.39 (95% CI = 0.27, 0.51) while the naïve estimate was 0.26. In Lesser Snow Geese, results were similar, with an occupancy estimate of 0.36 (95% CI = 0.25-0.49) and a naïve estimate of 0.25. Naïve seroprevalence estimates were either below or equivalent to the lower bound of the model-based confidence intervals. Our

results indicate that naïve values using only a single assay would have been well below estimated seroprevalence estimates for both species.

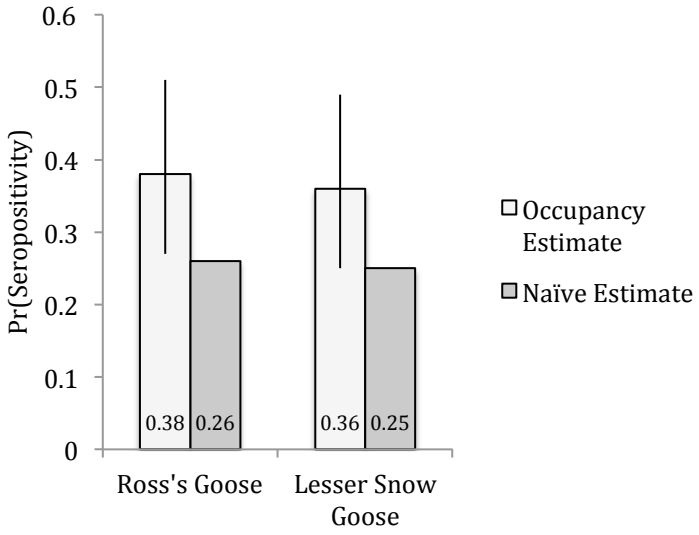

FIGURE 4-1. COMPARISON OF SEROPREVALENCE ESTIMATES FOR ROSS'S GEESE AND LESSER SNOW GEESE GENERATED BY NAÏVE AND OCCUPANCY ESTIMATORS.

TABLE 4-2. CANDIDATE MODEL SET AND MODEL SELECTION RESULTS OF MULTI-STATE OCCUPANCY ANALYSIS TO DETERMINE SEROPREVALENCE, DETECTION PROBABILITY, AND CLASSIFICATION PROBABILITY OF *T. GONDII* ANTIBODIES IN WILD GEESE FROM KARRAK LAKE, NUNAVUT, CANADA.

Model	No. Parameters	-2 Log Likelihood	AICc	Δ AICc	AICc Weight
ψ^1(species) ψ^2(species) p^1(test) p^2 (test) δ(.)	9	1180.73	1199.50	0.00	0.62
ψ^1(species) ψ^2(species) p^1(test) p^2 (test) δ(test)	10	1179.77	1200.71	1.21	0.34
ψ^1(.) ψ^2(.) p^1(test) p^2(test) δ(.)	7	1191.83	1206.30	6.81	0.02
ψ^1(.) ψ^2(.) p^1(test) p^2(test) δ(test)	8	1190.72	1207.33	7.83	0.01
ψ^1(sex) ψ^2(.) p^1(test) p^2(test) δ(.)	9	1189.85	1208.61	9.11	0.01
ψ^1(sex) ψ^2(.) p^1(test) p^2(test) δ(test)	10	1188.46	1209.40	9.90	0
ψ^1(species) ψ^2(species) p^1(.) p^2(.) δ(.)	7	1275.35	1289.82	90.32	0
ψ^1(species) ψ^2(species) p^1(.) p^2(.) δ(test)	8	1275.18	1291.79	92.29	0
ψ^1(.) ψ^2(.) p^1(.) p^2(.) δ(.)	5	1285.49	1295.74	96.24	0
ψ^1(.) ψ^2(.) p^1(.) p^2(.) δ(test)	6	1285.35	1297.70	98.20	0
ψ^1(sex) ψ^2(sex) p^1(.) p^2(.) δ(.)	7	1284.50	1298.98	99.48	0

TABLE 4-3. MODEL-AVERAGED PARAMETER ESTIMATES OF SEROPREVALENCE, DETECTION PROBABILITY, AND CLASSIFICATION.

Parameter	Estimate	SE	95% LCI	95% UCI
$\psi^1_{\text{Female SNGO}}*\psi^2_{\text{Female SNGO}}$	0.36	0.06	0.25	0.49
$\psi^1_{\text{Male SNGO}}*\psi^2_{\text{Male SNGO}}$	0.36	0.06	0.25	0.49
$\psi^1_{\text{Female ROGO}}*\psi^2_{\text{Female ROGO}}$	0.39	0.06	0.27	0.51
$\psi^1_{\text{Male ROGO}}*\psi^2_{\text{Male ROGO}}$	0.39	0.06	0.27	0.51
$\psi^1_{\text{Female SNGO}}$	0.99	*	*	*
$\psi^1_{\text{Male SNGO}}$	0.99	*	*	*
$\psi^1_{\text{Female ROGO}}$	0.55	0.15	0.28	0.80
$\psi^1_{\text{Male ROGO}}$	0.55	0.15	0.28	0.80
$\psi^2_{\text{Female SNGO}}$	0.36	0.08	0.23	0.53
$\psi^2_{\text{Male SNGO}}$	0.36	0.08	0.23	0.52
$\psi^2_{\text{Female ROGO}}$	0.71	0.17	0.32	0.92
$\psi^2_{\text{Male ROGO}}$	0.71	0.17	0.32	0.93
(p^1_{DAT})	0.01	*	*	*
(p^1_{IFAT})	0.17	0.04	0.10	0.27
(p^2_{DAT})	0.21	0.03	0.16	0.28
(p^2_{IFAT})	0.54	0.05	0.44	0.64
(δ_{DAT})	0.55	0.06	0.43	0.67
(δ_{IFAT})	0.58	0.06	0.47	0.68

*Parameter estimated at boundary of parameter space. Standard error cannot be estimated.

4.4 Discussion

Our study suggests that both populations of Ross's and Lesser Snow Geese sampled from the central Canadian Arctic were exposed to *T. gondii* at some point in their lives, supporting the hypothesis that waterfowl can be a source of *T. gondii* introduction in the terrestrial Canadian Arctic. Although *T. gondii* antibodies have been detected in other species of wild goose (Prestrud et al., 2007, Murao et al., 2008, Sandström et al., 2013), to our knowledge this study is the first to document seropositive Ross's and Lesser Snow Geese. *T. gondii* exposure in Ross's and Lesser Snow Geese probably occurs on wintering grounds in the southern United States and along migratory flyways, where they feed in agricultural fields at numerous stopover points in North America (Alisauskas et al., 1988, Alisauskas and Ankney, 1992). We did not test blood filter paper samples from juvenile geese that had not yet migrated south, thus we cannot rule out possible exposure to *T. gondii* on the nesting grounds at Karrak Lake, Nunavut. However, Sandström et al. (2013) did not detect any antibodies in juvenile geese from Arctic brood-rearing locations on Svalbard, suggesting that these animals were exposed solely at temperate latitudes. *T. gondii* antibodies were detected in adult geese, however, indicating that geese are exposed after migrating from the brooding ground. A survey of hatch-year geese on the brooding grounds in the Queen Maud Gulf Bird Sanctuary would help determine whether geese are exposed to *T. gondii* while in the Canadian central arctic.

Few studies report the seroprevalence of *T. gondii* in wild geese from North America. Dubey et al. (2014) reported the detection of *T. gondii* antibodies in 2 of 2 Canada Geese (*Branta canadensis*) from Pennsylvania, USA. The occurrence of *T. gondii*

in other species sympatric with Ross's and Lesser Snow Goose wintering grounds is unknown. In Europe, Prestrud et al. (2007) reported a 7% sample seroprevalence in Barnacle Geese (*Branta leucopsis*) from Svalbard. Sandström et al. (2013) reported seroprevalence rates of 6.5% in Pink-Footed Geese (*Anser brachyrhynchus*) from Svalbard, and 25% in migratory Barnacle Geese on wintering grounds in the Netherlands. Neither of these studies accounted for potential false-positive or false-negative errors. Sex of goose hosts did not influence seroprevalence, but we found support for species-specific differences in model parameters (Table 2). Our estimates suggest than nearly all samples collected from Snow Geese contain antibodies or other material that may lead to non-negative assay results ($\hat{\psi}^{1}_{SNGO}$ = 0.99), while only 55% of samples from Ross's Geese has these antibodies. Given that antibodies or similar material existed in a sample, Ross's Geese were much more likely to contain *T. gondii* antibodies ($\hat{\psi}^{2}_{ROGO}$ = 0.71) than Snow Geese ($\hat{\psi}^{2}_{SNGO}$ = 0.36).

Ross's Geese might be more likely to be exposed to *T. gondii* oocysts than Lesser Snow Geese due to differences in feeding ecology. Ross's Geese have a smaller bill that is better suited for grazing in pastures and short tundra grasses, whereas the larger Snow Goose is known to grub in the soil for roots and tubers (Alisauskas et al., 1988, Jonsson et al., 2013). Presence of *T. gondii* oocysts might be more likely on shoots of vegetation than on below-ground portions of plants or in the soil, suggesting that differences in feeding mechanism could explain the differences in parameter and seroprevalence estimates we observed.

Lesser Snow Geese and Ross's Geese are commonly hunted waterfowl species throughout central North America, along the Pacific Flyway, and in their arctic summer

habitat. The seroprevalences (36 and 39%, respectively) of *T. gondii* in these species demonstrate the potential for geese to transmit infection to predator animals, hunters and people who process the carcasses of hunted geese. However, current food preparation practices in the central Canadian Arctic might already be protective; goose meat is commonly boiled, following a specific carcass-handling procedure (D. Stern, personal communication). In other areas of the Canadian North, goose meat is smoked and barbecued (Ohmagari and Berkes, 1997). Thoroughly boiling and otherwise cooking *T. gondii*-infected meat at 60° C or higher will kill tissue cysts (Dubey, 2009). The viability of cysts after drying is unclear and probably variable; however Lundén and Uggla (1992) did not recover infective *T. gondii* from mutton that was cured, smoked, or frozen. Also, cats fed sausage, igunaq (fermented muscle), and nikku (dried muscle) from experimentally infected seals (*Halichoerus grypus*) did not shed oocysts after exposure (Forbes et al., 2009). Sanitary measures during processing and preparing meat, and thorough cooking of meat (if culturally acceptable) before consumption would help reduce transmission of *T. gondii* to people (Kapperud et al., 1996).

We compared seroprevalence estimates using occupancy-based and naïve estimation methods. The occupancy approach demonstrated a higher estimated seroprevalence in both goose species than the naïve estimators. From the difference between types of estimates, it is clear that failure to account for detection probability results in an underestimate of seroprevalence, and thus could result in an underestimation of infection status in wild geese.

When comparing the two assays used in this study, the IFAT test seemed to outperform the DAT when used on wild goose filter paper eluate samples, because it

showed both a higher probability of *T. gondii* antibody detection and a higher probability of correct classification of serology conditional on the antibody being present. The higher detection probability of IFAT reflects more consistency in the test across the multiple replicates than in the DAT. However, IFAT also resulted in a higher probability of unclassifiable or ambiguous serological results when only not-target antibodies are present (True state = 1), which could lead to bias in serological studies if false-positive errors are not considered in the analysis. The high occurrence of unclassifiable or ambiguous results might reflect inaccurate optimization of the IFAT assay, in which case more replicates of known positive and negative animals might improve assay validity. Filter paper eluent is variable in nature and differences will exist in sample handling between filter paper from experimentally infected versus free-ranging animals. These factors, plus the differences between laboratory samples and field-collected samples might have contributed to uncertainty in the IFAT results. The low false-positive (p^1) and true-positive (p^2) detection probabilities estimated with the DAT indicates that a simple occupancy-modeling framework (MacKenzie et al. 2006) is ideal for this assay, as repeated sampling should provide a more precise estimate of true detections or non-detections. Study designs that rely only on one DAT repetition are more likely to underestimate the seroprevalence, and thus lead to biased inference about occurrence of *T. gondii* infection within the study population.

Both DAT and IFAT performed similarly in correctly classifying positive results, which indicates that if *T. gondii* antibodies exist and are detected, the result is categorized correctly 55-58% of the time. Given the different types of uncertainty introduced by each test, we recommend parallel testing with both DAT and IFAT assays to improve the

sensitivity of seroprevalence estimates in waterfowl. Although DAT demonstrated a much lower antibody detection probability than IFAT, it also had a much lower probability for unclassifiable results, suggesting that the DAT results provide less ambiguous estimates of *T. gondii* seroprevalence, provided that one accounts for non-detection (false negative errors). However, the tradeoff is that, because DAT appears to detect antibodies in fewer seropositive samples, these estimates are likely to be biased if DAT is used alone without accounting for non-detection. Moreover, lower detection probability associated with any method would reduce the precision of the estimate of seroprevalence, the parameter of main interest.

Both assays used in this study demonstrated strengths and weaknesses in detection ability. The IFAT and DAT diagnostic tests used in this study have positive/negative cut-off characteristics that are inherently subjective, thereby increasing the risk of violating traditional occupancy modeling assumptions of no observational error. With a multi-state occupancy approach, we were able to estimate the seroprevalence of *T. gondii* in wild geese while accounting for both false positive and false negative results. Also, we were able to gather information about the uncertainty of both assays through the use of equivocal test results, which might ordinarily be discarded during a traditional serological analysis.

Sample collection in remote and arctic wildlife systems is both logistically and physically challenging. Reducing bias in seroprevalence estimates through thoughtful study design and rigorous data analysis, such as with repeated tests and an occupancy analysis approach, can increase the quality of serosurveys to match the effort required. Wildlife researchers might reduce uncertainty in future serosurveys if repeat testing is

performed, if the data analysis accounts for imperfect detection, and if multiple assays are used. Multiple analytical models can be used to accommodate this type of data to provide unbiased estimates of seroprevalence (Nichols et al. 2007, Miller et al. 2011) and are readily available in various, free programs (e.g., programs MARK and Presence).

The potential for *T. gondii* to impact wildlife health and reproduction and the risk of transmission from geese to people and predators such as arctic canids and subarctic felids emphasizes the need for robust estimates of parasite prevalence. Because serosurveys only indicate exposure and not true infection, future work could focus on testing tissues of arctic-nesting geese to determine in which organ the parasite can be most easily detected and molecular characterization of arctic isolates of *T. gondii.* Such research will provide information about how the parasite is introduced and maintained in terrestrial Arctic ecosystems.

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CHAPTER 5. Using A Multi-Scale Occupancy Approach To Determine *Toxoplasma Gondii* Detection Probability In Goose Tissues

Citation

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Author Contributions

SAE collected samples. SAE, KPH, LLB, and EJJ designed the study. SAE, KPH, and LLB analyzed data. CS performed the PCR-RFLP genotyping. RTA and AAG provided mentorship, logistics and supplies. SAE drafted the manuscript, tables and figures with feedback from other coauthors. All authors read and approved the final manuscript.

Chapter 5 Transition Statement

In chapter 4, I demonstrated that wild Arctic nesting geese were exposed to *T. gondii*. The next step was to demonstrate that the organism was present in tissues of wild Arctic nesting geese. However, there is very little information about tissue predilection sites of *T. gondii* in geese. Therefore, I used a multi-scale occupancy model to determine what tissue types maximize detection probability following molecular detection methods in experimentally inoculated geese. I used this information to guide tissue selection in wild geese.

Abstract

Although it is increasingly recognized that birds are an important host for the ubiquitous coccidian parasite *Toxoplasma gondii*, there is little experimental evidence to determine which tissues are best to test for *T. gondii* detection in waterfowl and other birds. To determine what organ had the highest detection probability, we orally inoculated 28 domestic geese (*Anser anser domesticus*) and collected the brain, heart, lungs, liver, spleen, and kidneys during necropsy at 7 weeks post-inoculation. We randomly selected three portions of each tissue, which were tested for *T. gondii* DNA by real-time PCR with melt-curve analysis (PCR-MCA) and confirmed by restricted fragment length polymorphism (RFLP). We applied a multi-scale occupancy framework, where each goose was considered to be a "habitat" and each organ was considered a "site" within that habitat. Each "site visit" was replicated 3 times by randomized tissue testing. Model selection results indicated that all tissues were equally likely to be PCR positive, but probability of detection of *T. gondii* was highest in the brain (0.689, 95% CI = 0.486, 0.839) and the heart (0.809, 95% CI = 0.693, 0.888), similar to findings in mammals. Geese within the study had a *T. gondii* prevalence of 0.885, 95% CI = (0.672, 9.966), highlighting the system uncertainty even when animals were experimentally inoculated. Based on these results, we sought to to detect *T. gondii* DNA in the brains and hearts of wild Ross's Geese (*Chen rossii*, n=50) and Lesser Snow Geese (*Chen caerulescens*, n=50) from the Karrak Lake populations in the Queen Maud Gulf Migratory Bird Sanctuary, Nunavut. Preliminary screening by PCR-MCA indicated 60 samples from 25 individual geese were positive for *T. gondii*, based on comparison to a positive control in melt-curve analysis. However, only DNA of *Sarcocystis* sp. was detected by RFLP. It is

possible that the birds were coinfected with *T. gondii* and *Sarcocystis* spp. and that *Sarcocystis* outcompeted *T. gondii* DNA in the RFLP analysis. Further work using larger sample sizes and more specific molecular characterization is needed to determine the infection status and genetic diversity of *T. gondii* present in arctic-nesting geese, which may bring the parasite from the southern overwintering grounds into the Arctic, following the spring migration.

5.1 Introduction

Knowing what tissue is best for testing can make the difference between detection and non-detection of a pathogen or disease process (McClintock et al., 2010). In most settings, where space, time, and financial resources are limited, testing all possible samples is infeasible. Tissue selection during post-mortem analysis is not always straightforward, however, because reports regarding pathogen tissue predilection sites can be conflicting and detection in a specific tissue type might be sensitive to laboratory methodology or pathogen/host species. Tissue coccidians, such as *Toxoplasma gondii*, can be difficult to detect, and more information on tissue predilection sites and detection probability is needed to improve diagnostics, especially in wildlife and food animal species.

Toxoplasma gondii is ubiquitous in many vertebrate species, including humans (Dubey, 2009). Hosts can become infected by three different life stages: oocysts, tissue cysts, or tachyzoites, and transmission occurs through both direct and indirect routes. Aside from the felid definitive hosts, infected vertebrates serve as intermediate hosts, developing tissue cysts containing bradyzoites in their soft tissues (Dubey, 2009). Previous studies in mammals provide comparisons of detection frequency in tissues, suggesting that *T. gondii* tissue cysts are consistently found in the brain and heart of infected mammals (Jurankova et al., 2014), although these sites are often the only ones tested (Krijger et al., 2014; Feitosa et al., 2014). Despite this assertion, other reports provide conflicting evidence of tissue tropism, and findings might also differ by host species, parasite genotype, or inoculation route (Zöller et al., 2013; Bangoura et al., 2013; Jurankova et al., 2013; 2014). Occurrence of *T. gondii* in wild or domestic birds is not

reported as often as in mammals, but numerous avian species worldwide are recognized as intermediate hosts (Dubey, 2002).

Another difficulty surrounding tissue diagnostics for cyst-forming coccidian parasites (e.g. *T. gondii*) is the size of the organ in relation to the amount of tissue screened in the laboratory analysis. Because it is not usually feasible or possible to test an entire organ, uncertainty exists when an infectious organism (e.g. *T. gondii* cyst) is not detected. While some DNA extraction methods effectively extract DNA from up to a gram of tissue, most commercial kits require much less (e.g. 25µg for the Qiagen Blood and Tissue Kit), possibly decreasing the chances that the tissue sample will contain the parasite; *T. gondii* might be unevenly distributed in the organ and processing less tissue dilutes the opportunity for detection.

Occupancy modeling approaches are traditionally used to estimate the probability of occurrence of wildlife species within a habitat, and are especially useful for rare or cryptic species (McKenzie et al., 2006). Occupancy approaches also allow for the estimation of detection probability, or, the probability that a species of interest will be detected, given that an area is occupied by that species. These methods are increasingly used in wildlife disease ecology because pathogens can be difficult to detect or unevenly distributed within a host, and imperfect detection methods can add to uncertainty in prevalence estimates (e.g. McClintock et al., 2010; Lachish et. al. 2011; Eads et al., 2014; Elmore et al., 2014). To address this concern of imperfect detection, we used a multi-scale occupancy approach (Nichols et al., 2008) to determine if organs vary in their likelihood of containing *T. gondii,* and if so, determining what organ(s) have the highest detection probability using molecular methods. To accomplish this, we used in an

experimental goose model from which detection probability results could be extrapolated into a wild goose population.

Our first objective was to determine probability of detection of DNA of T. gondii in tissues from experimentally inoculated domestic geese. We hypothesized that *T. gondii* would be most likely detected in the brain of experimentally inoculated birds and that each organ would have a different probability of *T. gondii* occupancy because previous research has demonstrated varying detection of *T. gondii* in mammals and birds (Bangoura et al., 2013; Jurankova et al., 2013; 2014). The results from the first objective helped to guide our second objective, which was to collect and test tissue samples from wild geese for *T. gondii.* We hypothesized that wild geese from Karrak Lake would be infected with *T. gondii*, based on previous serological data (Elmore et al. 2014). We also hypothesized that sex of goose would not influence occupancy probability due to results from a previous serological study (Elmore et al., 2014). Identifying *T. gondii* in wild geese would strengthen the findings from a previous study based only on serological data (Elmore et al., 2014), supporting the idea that migratory birds bring the parasite from temperate regions to arctic ecosystems. Also, it would confirm that wild geese are a potential source of *T. gondii* for wildlife predators, such as arctic foxes, because it would show that the parasite DNA is present in the viscera, and that ingestion of *T. gondii* by predators could perpetuate its life cycle in the Arctic.

5.2 Methods

5.2.1 Experimental infection of domestic geese with *T. gondii*

We obtained domestic goose goslings (*Anser anser domesticus*) on the day of hatch from a local hatchery in Saskatoon, Saskatchewan, Canada. To avoid

contamination with coccidian oocysts in the environment, goslings were kept from contacting the ground until released in a biosecurity level 1 room in the Animal Care Unit at the University of Saskatchewan Western College of Veterinary Medicine. Goslings were offered water and chick starter feed (without antibiotics) *ad libitum* and maintained under a 12h light/12h dark cycle. At 7 weeks of age, we inoculated 26 of the geese with 500 sporulated *T. gondii* oocysts in 1 mL 0.9% saline (Genotype Type III; originally isolated from swine) by oral gavage with an 8 Fr. red rubber feeding tube into the distal esophagus (Bartova et al., 2004). Two negative control geese were given 1.0 mL 0.9% saline. To prevent contamination with undigested oocysts from the feces of experimentally infected geese, the control geese were isolated by a plastic barrier for 36 hours, then allowed to commingle with the rest of the geese. We obtained blood samples from all geese prior to inoculation and at weekly intervals thereafter. At 49 days post-inoculation, we anesthetized all geese with isoflurane and euthanized with an intravenous injection of T-61 (0.5mL/kg). Upon necropsy, we collected the entire liver, spleen, brain, heart, kidneys, and lungs and stored them at -20ºC until further analysis. The experimental infection was performed in compliance with the University of Saskatchewan Committee on Animal Care and Supply (Certificate of Approval 20120050).

5.2.2 Serological methods

To determine whether experimentally inoculated geese were exposed to *T. gondii*, we optimized and applied an indirect ELISA using a goat-anti-duck horseradish peroxidase IgG conjugate (KPL, Inc., Gaithersburg, Maryland, USA). The assay conditions followed a previously described procedure (Al-Adhami et al., 2014), except

that we used a conjugate dilution of 1:2500 and a serum dilution of 1:12.5. Known *T. gondii* negative Pekin duck sera (see Appendix) were used to determine cut-off values for the ELISA by measuring 3 x standard deviation of the average negative optical density (OD) values.

5.2.3 Goose tissue processing and PCR-MCA analysis

We systematically divided the heart, lungs and kidneys into 9 pieces of approximately equal size, and the liver into 14 approximately equal pieces, from which we randomly selected three pieces from each organ for subsequent processing. The entire spleen and brain were divided into three approximately equal pieces, all of which were processed. We assigned a number to each tissue piece and used a random number generator to select three pieces from each organ's "set" of tissue pieces for sugsequent prodessing.

We immersed each tissue piece in liquid nitrogen to flash freeze it and then pulverized the tissue using the blunt side of a meat tenderizer. Following manufacturer's instructions, we extracted genomic DNA from 100 mg of tissue homogenate from each piece of tissue (Easy-DNA™ kit, Invitrogen™, Life Technologies, Burlington, ON). We then analyzed genomic DNA by real-time PCR with melt-curve analysis (PCR-MCA; Lalonde and Gajadhar, 2011), and used a primer cocktail to amplify a 329 bp fragment of the 18S region; PCR reaction mixture and thermocycler conditions were as described by Lalonde and Gajadhar (2011). We ran duplicate wells of DNA samples from each tissue piece. DNA samples with a melt peak of 83.0-83.2°C were considered to be *T. gondii*-positive. To confirm the presence of *T. gondii*, positive tissue sections and genomic DNA from one experimentally inoculated goose were sent to collaborators at Bureau of

Microbial Hazards, Food Directorate, Health Canada, Ottawa, for confirmation and genotyping (Howe et al., 1997).

5.2.4 Wild goose sample collection, processing, and PCR-MCA analysis

Ross's Geese (*Chen rossii*) and Lesser Snow Geese (*Chen caerulescens*) carcasses were sampled from Karrak Lake, Nunavut, Canada in late May to early June in 2011-2012 as previously described (Elmore et al., 2014). Using results from the experimental infection as a guide, we collected the brain and heart from 100 geese (n=50 for each species) harvested by shotgun for ongoing biological and ecological research. We processed and analyzed the tissue samples using real time PCR-MCA as described in Section 2.2 (Lalonde and Gajadhar, 2011), except that we performed confirmatory PCR using the primers TOX4 and TOX5, which amplify a 529 bp repeating region within the *T. gondii* genome (Homan et al. 2000). The master mix consisted of 2 ul template DNA, 5 ul 10X PCR Buffer, 2.5 ul MgCl (50mM), 20 mM each primer, 1 ul dNTP (10mM), and 0.5 ul taq polymerase (Invitrogen). All samples positive by both methods were sent to the University of Tennessee for confirmation of *T. gondii* presence and genotyping to determine what *T. gondii* genotypes circulate in the Karrak Lake ecosystem (Su et al. 2010). All field activities were conducted in accordance with The University of Saskatchewan Animal Care and Use Committee (Protocol 20090126), the Canadian Wildlife Service (Permits NUN-SCI-11-02; NUN-MBS-11-03), and the Government of Nunavut (Permits 2011-019, 2012-021, 2013-017).

5.2.5 Data analysis

Following a multi-scale occupancy framework (Nichols et al., 2008), we considered each experimentally inoculated goose to represent a "habitat" or "primary

site" and each organ to represent a "secondary site" within each goose (Figure 5-1). Three randomly chosen tissue pieces from each organ served as the replicate surveys that allowed us to estimate detection probability, or the probability that *T. gondii* DNA was detected via PCR-MCA from a similar sized piece of tissue obtained from an organ in which the goose was experimentally inoculated. Locations for the 3 pieces of tissue from each organ ("site surveys") were selected randomly as previously described (Section 2.2). We constructed an encounter history for each survey using all PCR-MCA runs where detections of *T. gondii* DNA were represented with a "1" and non-detections with a "0" under the definitions of "*T. gondii* positive" and *T. gondii* negative" as described in Section 5.2.3. All DNA samples from individual tissue pieces were run in duplicate wells on the PCR plate, however, results were recorded so that a detection in either well was considered "*T. gondii* positive".

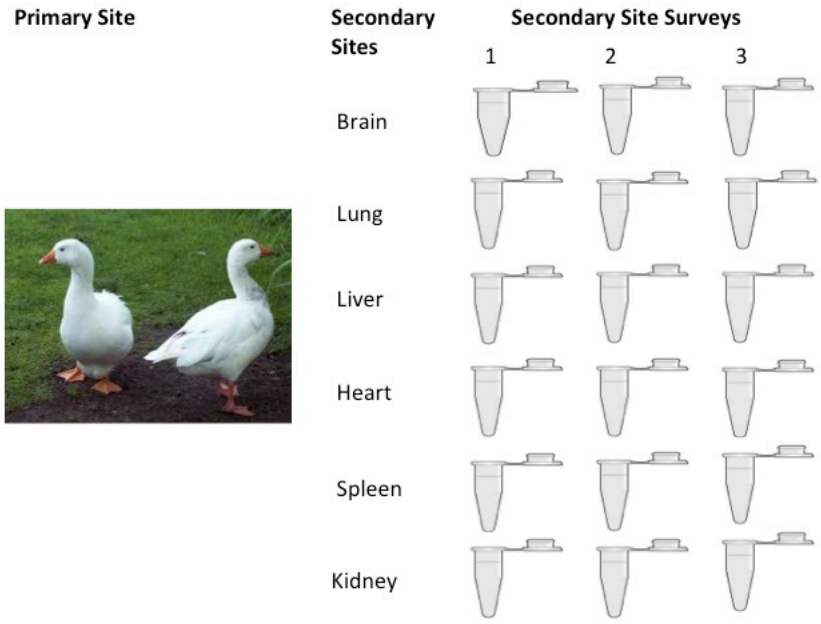

FIGURE 5-1. MULTI-SCALE OCCUPANCY FRAMEWORK FOR ANALYZING THE OCCURRENCE AND DETECTION PROBABILITY OF *TOXOPLASMA GONDII* IN THE TISSUES OF EXPERIMENTALLY INOCULATED DOMESTIC GEESE.

We analyzed the encounter histories using the multi-scale occupancy model in Program MARK (White and Burnham, 1999), to estimate the following parameters: 1) ψ_i = probability that goose *i* is infected with *T. gondii*; 2) θ_{ij} = the probability that *T. gondii* is present in an organ *j*, given that goose *i* is infected with *T. gondii*, and 3) p_{ij} = probability of detecting *T. gondii* DNA, given the goose *i* is infected with *T. gondii,* and the parasite is present in the organ *j* (Table 1). Given that a goose is the primary site (n=26 exposed goslings), we considered K=6 secondary sites (organs) and L=3 surveys at each secondary site.

We developed an *a priori* candidate model set (Table 5-2) to determine the effect of organ type on the probability of 1) an organ being occupied by *T. gondii* DNA, given the goose is infected (θ), and 2) detecting *T. gondii* DNA (p) within an organ, given the organ was occupied by the parasite and the goose was infected. We performed model selection using small sample bias-corrected Akaike Information Criterion values (AICc; Burnham and Anderson, 2002) and ranked models by calculating the differences in AICc (ΔAICc) between the highest-ranked model and the other models. We calculated the model weights (w_i) for each model; higher-ranked models carried the most weight, thus best explaining the data (Anderson, 2008). Model selection uncertainty was minimal, so we used estimates from the best model with no need to perform model averaging.

5.3 Results

5.3.1 Results of experimental inoculation

Of the 26 domestic geese, 25 geese displayed positive serological results following inoculation with *T. gondii*. We included PCR-MCA results from all inoculated geese in the final data analysis, including two geese that were seropositive but for which all PCR-MCA tissue results were negative for *T. gondii,* and one goose that was both seronegative and negative on tissue PCR-MCA. The two negative control geese were negative on both serology and tissue PCR-MCA; we excluded PCR-MCA results from these animals in the occupancy analysis because they never received oocysts in their inoculum.

We analyzed encounter histories from PCR-MCA results with a multi-scale occupancy model to determine which organ (among heart, brain, lungs, liver, kidneys, and spleen) had the highest detection probability, when occupancy probability is close to 1, following known exposure. Model selection results indicated that, in the best model of the experimental goose data, goose occupancy probability (ψ) was constant among individuals and organ occupancy probability (θ) was similar, while detection probability (p) varied by tissue type (AICc weight=0.92; Table 5-2).

The probability that a goose became infected with *T. gondii* (ψ) after exposure was 0.849 (95% CI = (0.672, 0.966); Table 5-2) and was not influenced by the sex of the goose (Table 5-1). We estimated the probability of an individual organ being occupied by *T. gondii* in an infected goose (θ), was 0.841 (95% CI = (0.655, 0.937); Table 5-2); this value was not influenced by tissue type in the best model. The detection probability of *T. gondii* DNA, given the presence of the parasite in an organ, varied by tissue type (Figure

5-2). We estimated detection probability to be the highest in the heart (p=0.809, 95% CI = 0.693, 0.888) and the brain (0.689, 95% CI = 0.643, 0.946), and lower in the lung, liver, kidney and spleen (Figure 5-2; Table 5-2).

TABLE 5-1. CANDIDATE MODELS AND MODEL SELECTION RESULTS USING A MULTI-SCALE OCCUPANCY MODEL TO ESTIMATE *T. GONDII* OCCUPANCY AND DETECTION PROBABILITY IN SPECIFIC TISSUE TYPES IN EXPERIMENTALLY INNOCULATED DOMESTIC GEESE (N=26).

Model	No. Parameters	-2log likelihood	AICc	Δ AICc	AICc Weights
psi(.) theta(.) p(tissuetype)	8	296.452	321.453	0.000	0.924
psi(sex) theta(.) p(tissuetype)	9	296.445	326.445	4.992	0.076
psi(sex) theta(tissuetype) p(tissuetype)	14	283.174	353.174	31.721	0.000
psi(.) theta(.) p(tissuetype)	7	338.102	358.690	37.238	0.000
psi(.) theta(.) p(.)	3	385.998	393.141	71.688	0.000
psi(sex) theta(.) p(.)	4	385.990	395.990	74.538	0.000
psi(.) theta(tissuetype) p(tissuetype)	13	283.181	401.363	79.910	0.000

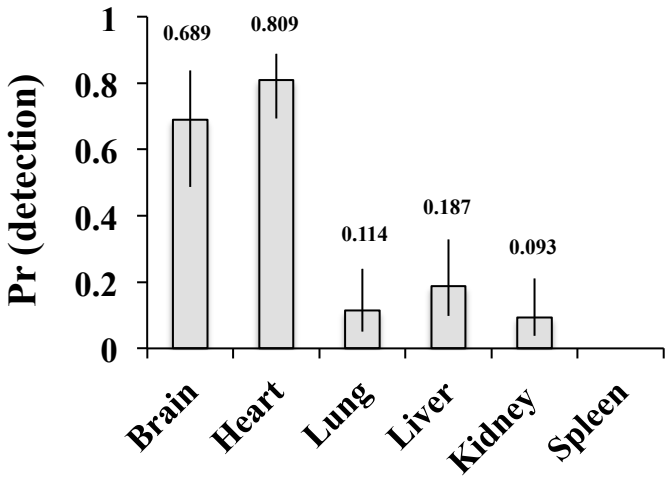

FIGURE 5-2. ESTIMATES FOR DETECTION PROBABILITY OF *T. GONDII* IN TISSUES OF EXPERIMENTALLY INNOCULATED DOMESTIC GEESE (N=26) USING A MULTI-SCALE OCCUPANCY MODEL.

TABLE 5-2. PARAMETER ESTIMATES FOR HOST OCCUPANCY, TISSUE OCCUPANCY, AND TISSUE-SPECIFIC DETECTION PROBABILITY OF *T. GONDII* IN EXPERIMENTALLY INOCULATED DOMESTIC GEESE USING A MULTI-SCALE OCCUPANCY MODEL.

Parameter	Estimate	SE	95% LCI	95% UCI
ψ	0.849	0.074	0.643	0.946
θ	0.841	0.070	0.655	0.937
$p_{(brain)}$	0.689	0.093	0.486	0.839
$p_{(heart)}$	0.809	0.050	0.693	0.888
$p_{(lungs)}$	0.114	0.046	0.050	0.240
$p_{(liver)}$	0.187	0.058	0.098	0.328
$p_{(kidney)}$	0.093	0.041	0.038	0.210
$p_{(spleen)}$	0	0	0	0

5.3.2 Results of wild goose tissue testing

Of the 100 wild geese tested, 17 brain piece DNA samples and 34 heart piece DNA samples from 33 individual geese were positive for *T. gondii* based on PCR-MCA (melt temperature was 82.8-83.4°C). Twenty-five of these suspect-positive samples also demonstrated positive reactions with the TOX4/TOX5 conventional PCR, and were sent for genotyping by RFLP. Twenty DNA samples were unable to be resolved using the RFLP procedure, but twenty-one DNA samples indicated the presence of an unknown coccidian, likely a *Sarcocystis* species for which positive control samples were not available for a comparable banding pattern. Four DNA samples were consistent with the RFLP banding pattern of *S. neurona. T. gondii* was not confirmed in any of the 51 previously suspect-positive DNA samples.

5.4 Discussion

In this study, we determined what organs best maximized the detection probability of *T. gondii* DNA using an occupancy modeling framework in an experimental goose model. We then applied similar techniques to 100 wild geese from Karrak Lake, Nunavut. Our study suggests that domestic geese are a good experimental host for *T. gondii*, that *T. gondii* is better or more frequently detected in some organs (brain and heart) than others, and that wild geese might be a vector for both *T. gondii* and *S. neurona* in the Canadian Arctic. Furthermore, our results demonstrate that occupancy modeling is a useful approach to addressing uncertainty in wildlife disease surveillance and diagnostics.

The probability that *T. gondii* DNA could be detected in an experimentally inoculated goose, given that a goose was orally inoculated with the parasite, was 0.849.

This result reflects imperfect detection of *T. gondii*, despite experimental inoculation, and emphasizes the utility of an occupancy modeling approach, where a goose was revisited multiple times in an effort to maximize the detection of the parasite. The imperfect detection also suggests that *T. gondii* is not evenly distributed among or within the tissues of a goose following exposure, which further complicates diagnostics and suggests the need for more research on where the parasite is likely to cluster within an organ, for more focused tissue sampling.

Surprisingly, the probability of an individual organ being positive for *T. gondii,* given that the goose is infected, was constant across all organs as reflected by the top-ranked model (0.841), although the raw data indicated a much higher occurrence in the brain and the heart than the other organs. This lack of resolution is probably related to our limited sample size and model failure due to the resulting overparameterization.

The heart and the brain of experimentally inoculated geese demonstrated the highest detection probabilities for DNA of *T. gondii*, suggesting that these organs are the best to test for for surveillance using our PCR-MCA detection system. Although our results do not necessarily demonstrate true tissue predilection sites, they are consistent with previous research on tissue tropism of *T. gondii* (. However, reports in the literature might vary based on species or inoculation route. In mammals, Jurankova et al. (2014) reported the brain as a predilection site for *T. gondii* in orally inoculated pigs by magnetic capture and real-time PCR. This is in contrast to a previous report of a higher parasite tissue burden in the lungs of orally inoculated goats, which suggests that host species may influence tissue predilection and/or detection probability (Jurankova et al., 2013). In birds, Zöller et al. (2013) intravenously inoculated domestic turkeys (*Meleagris*

gallopavo) with *T. gondii* tachyzoites and thoroughly examined carcasses by PCR; the parasite was most frequently detected in the liver, followed by breast muscle and heart. However, in a related study where turkeys were inoculated orally with oocysts, the brain and thigh muscle were most likely to host the parasite (Bangoura et al., 2013). Cong et al. (2014) tested brain, heart, and lung tissue of naturally infected house sparrows (*Passer domesticus*) and found the parasite most frequently in the brain. Also, Dubey and Jones (2008) tested 2094 retail chicken breasts and found that none were infected with viable tissue cysts of *T. gondii* using mouse bioassay; however, they speculated that meat processing might have inactivated the tissue cysts, arguing for the utility of molecular-based approaches in conjunction with traditional parasitological techniques such as *in vivo* bioassay to demonstrate parasite viability.

We extended the results of our experimental inoculation into a remote field setting where we selected the brains and hearts of wild Ross's and Lesser Snow Geese for collection and testing. Although we detected *T. gondii*-like melt curves and amplification products by real time PCR-MCA and conventional PCR of the 529 bp repeating sequence, further confirmation and genotyping revealed the presence of *Sarcocystis* spp. instead of *T. gondii*. It is possible that the geese were infected by multiple coccidian parasites and that abundant *Sarcocystis* DNA amplification masked any *T. gondii* DNA present during the genotyping procedure. Ross's Geese and Lesser Snow Geese, while not tissue-positive for *T. gondii* in this study, had *T. gondii* seroprevalences of 39% (Ross's Geese, n=123) and 36% (Lesser Snow Geese, n=121) (Elmore et al., 2014). Increasing the sample size in this study could potentially increase the probability of detecting *T. gondii* DNA. Also, the use of additional methods, such as the magnetic

capture PCR, bioassay, immunohistochemistry, and histopathology, could improve detection probability by providing multiple points of evidence and confirmation of *T. gondii* presence.

Waterfowl-associated *Sarcocystis* species are present in the food web at Karrak Lake. In a previous study (Chapter 2), DNA of *Sarcocystis albifrons* and *Sarcocystis anasi* were detected in arctic fox feces from Karrak Lake (Elmore et al., 2013), and waterfowl are the intermediate hosts of these parasites (Kutkiene et al., 2012). We found only one previous report of *Sarcocystis* infections in Ross's and Lesser Snow Geese; *Sarcocystis lankester* was reported in skeletal and cardiac muscle of geese that died during an avian cholera outbreak in Saskatchewan, Canada (Wobeser et al., 1981). Further investigation is necessary to determine the identity of the *Sarcocystis* in light geese at Karrak Lake, and how they are transmitted in this food web.

Only 7 of the 33 wild geese that tested positive for *T. gondii* by tissue PCR-MCA were seropositive in another study (Elmore et al., 2014), suggesting that our serological methods have a high frequency of false negatives. Tissue positive but seronegative deer mice experimentally innoculated with *T. gondii* have been reported, suggesting that this is biologically possible and raising concerns about studies relying solely on seroprevalence data, which might underestimate parasite prevalence (Rejmanek et al., 2010). Following an occupancy modeling framework for prevalence estimation could help to address some of this uncertainty. Alternatively, our PCR method could be amplifying DNA from organisms with a similar melting temperature as *T. gondii,* resulting in false positive PCR results; sequence data would be necessary for definitive diagnosis. PCR-MCA uses a universal primer cocktail that is known to amplify many coccidia species, although the

83°C melting peaks were consistent with the *T. gondii* positive control DNA. It is possible that the *Sarcocystis* detected by the RFLP analysis has a similar melting peak. However, we also detected *T. gondii* based on primers for a 529 bp repeating element generally considered to be *T. gondii*-specific (Homan et al., 2000). These results indicate a need to revisit the specificity of the TOX4/TOX5 primer pair, especially because they were found to weakly amplify a similar repeating element sequence in *Hammondia hammondi*, suggesting that they are not *T. gondii*-specific (Schares et al., 2008). Such considerations are quite important for use in wildlife, where multiple infections of closely related coccidia species (i.e. *Neospora* sp., *T. gondii*, *Hammondia* sp., and *Sarcocystis* sp.) are likely. Caution must be exercised when interpreting results of diagnostic techniques not validated in wildlife, about which very little is known regarding coinfection with other tissue coccidian parasites. Also, sequencing and genotyping of *T. gondii* from wild arctic-nesting geese was unsuccessful in this study, but would provide a great deal more information for the sources of *T. gondii* in the Canadian Arctic.

Because *T. gondii* is a zoonotic agent, detection of the parasite in tissues of animals destined for human consumption is a concern for food safety and public health (Dubey et al., 2011c; Vitale et al., 2014). The experimental approach used in this study could be applied to other species to optimize tissue selection for diagnostic testing of hunted wildlife or retail meats for *T. gondii*. This study represents a novel application of a multi-scale occupancy model to determine differential detection probability in organs.

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CHAPTER 6. Evidence For *Toxoplasma Gondii* In Migratory Vs. Non-Migratory Herbivores In A Terrestrial Arctic Ecosystem

Citation

Elmore SA, Samelius G, Fernando C, Alisauskas RT, Jenkins EJ. Evidence for *Toxoplasma gondii* in migratory vs. non-migratory herbivores in a terrestrial arctic ecosystem. In Prep.

Author Contributions

SAE collected samples. SAE, CF, and EJJ designed the study. SAE and CF performed labwork and reviewed laboratory results and SAE analyzed data. RTA and GS provided mentorship, logistics and supplies. SAE and GS drafted the manuscript, and figure with feedback from other coauthors. All authors read and approved the final manuscript.

Chapter 6 Transition Statement

In the previous chapters, I explored the occurrence of *T. gondii* in arctic foxes and arctic-nesting geese. In this chapter, I will examine another key prey item, lemmings, for *T. gondii*, using both serological and molecular techniques. Also, I include data from a large serosurvey of Ross's and Lesser Snow Geese, live-captured by helicopter-assisted drives.

Abstract

It is currently unclear how *T. gondii* persists in arctic tundra ecosystems in the absence of felid definitive hosts. To further investigate transmission of *T. gondii* in a terrestrial arctic food web, we collected samples from migratory herbivores, Ross's Geese (*Chen rossi*) and Lesser Snow Geese (*Chen caerulescens*) during routine banding operations in August 2012, and samples from resident herbivores, brown lemmings (*Lemmus trimucronatus*) and collared lemmings (*Dicrostonyx groenlandicus)* trapped in June-July 2011 at Karrak Lake, Nunavut. We hypothesized that geese would demonstrate widespread serological exposure to *T. gondii* because they migrate from agricultural areas where oocysts are likely to be present in the environment, and that resident herbivores (lemmings) would not demonstrate exposure to or presence of *T. gondii* DNA. Using an indirect ELISA, antibodies were detected in 76 of 234 (32.4%) serum samples from Ross's Geese and 66 of 233 (28.3%) serum samples from Lesser Snow Geese collected in August 2012. Using the Toxo-Screen DA kit (Biomerieux, France), we did not detect *T. gondii* antibodies in filter paper eluate tested at 1:40 dilution from thoracic fluid samples collected on filter paper from 84 lemmings. We did not detect *T. gondii* DNA in brain tissue from these lemmings using a *T. gondii*-specific PCR assay for the B1 gene. However, initial screening using a less specific PCR assay amplifying a 529 base pair repeating sequence in genomic DNA detected 16 suspect positives (likely *Neospora caninum* or *Hammondia* sp.). This research demonstrates a continued need for both sensitive and specific molecular and serological techniques to detect *T. gondii* in wildlife. Our findings suggest that lemmings in a terrestrial arctic ecosystem are not exposed to, or infected with, this parasite, further suggesting that oocysts are not introduced into

terrestrial arctic ecosystems via freshwater runoff from temperate regions. However, lemmings might serve as intermediate hosts for related parasites, *Neospora caninum* and *Hammondia* sp., for which sympatric arctic foxes are a likely definitive host. This study confirmed that live adult arctic-nesting geese are exposed to *T. gondii* and therefore migratory herbivorous hosts are important sources of *T. gondii* infection for arctic predators in terrestrial arctic ecosystems.

6.1 Introduction

The apicomplexan parasite, *Toxoplasma gondii*, occurs throughout the Arctic; however, the absence of felid definitive hosts in arctic tundra regions reinforces the need to examine other potential routes of *T. gondii* introduction to the region because oocyst transmission is less likely to occur than in more southern regions (Elmore et al., 2012, Jenkins et al., 2013). *Toxoplasma gondii* is a protozoan parasite of concern in the North because it is potentially transmissible from animals to people through cultural practices of eating raw or undercooked food from wild sources, and can cause significant morbidity in infected individuals (Dubey, 2009; Goyette et al., 2014). Wildlife populations might also be negatively affected by *T. gondii* infection (Shapiro et al., 2012).

Due to low densities/absence of wild or domestic felid definitive hosts, environmental transmission of oocysts is unlikely in terrestrial tundra ecosystems, although oocysts may travel through freshwater drainage from temperate and subarctic regions (Simon et al., 2013a). Several studies have implicated migratory birds as vectors of *T. gondii* to arctic tundra ecosystems (Prestrud et al., 2007; Sandstrom et al., 2013, Elmore et al., 2014). Geese infected by oocysts on wintering grounds or along their migratory pathway might transmit the parasite on return to the breeding grounds if eaten by predators or scavengers. Following introduction, the parasite could be maintained in arctic wildlife by trophic and vertical transmission (Elmore et al., 2012; Jenkins et al., 2013).

Arctic foxes in Canada and Norway showed seropositivity for antibodies of *T. gondii* ranging (Prestrud et al., 2007; Elmore et al., Chapter 3); however, it is currently unknown how exposure occurs, or the prey items responsible. Common prey and scavenged wildlife in a tundra ecosystem include caribou (*Rangifer tarandus*), muskoxen

(*Ovibos moschatus*), and wild birds, including migratory waterfowl (Anseriformes), all of which have demonstrated evidence of *T. gondii* exposure (Kutz et al., 2000; 2001; Elmore et al., 2014). The mainstay dietary items of arctic foxes, however, are rodents such as brown and collared lemmings (*Dicrostonyx groenlandicus* and *Lemmus trimucronatus*) (Bantle and Alisauskas, 1998; Samelius et al. 2007). *Toxoplasma gondii* infection and exposure has not been reported in lemmings, but outside of the Arctic, arvicoline rodents, such as voles (*Microtus* sp.), can be intermediate hosts of both *T. gondii* and the closely related parasite, *Neospora caninum* (Fuehrer et al., 2010).

In this study, we evaluated the exposure to *T. gondii* in common migratory (geese) and resident (lemmings) herbivores to determine potential sources of exposure for arctic foxes and other predators in an arctic tundra ecosystem. We tested serum samples from Ross's Geese and Lesser Snow Geese in the Queen Maud Gulf Migratory Bird Sanctuary for antibodies against *T. gondii*, to measure exposure within the population and to confirm and compare to previous findings in this goose population using blood collected on filter paper (Elmore et al., 2014). We hypothesized that geese will be seropositive for the parasite because they migrate from habitats where they are likely to be exposed to oocysts of *T. gondii*. We hypothesize that lemmings, however, will be negative because they are resident herbivores with assumed limited contact with *T. gondii* oocysts. Because it is possible for experimentally infected rodents to be seronegative, but tissue-positive for *T. gondii* (Rejmanek et al., 2010), we performed molecular analysis on the brains of seronegative lemmings to support the serological findings.

6.2 Methods

6.2.1 Goose blood collection

During August 2012, we collected 2-3 mL of blood from the medial tarsal vein of Lesser Snow Geese (*n*=233) and Ross's Geese (*n*=234) that were live-captured for annual banding procedures in the Queen Maud Gulf Migratory Bird Sanctuary, Nunavut, Canada. Blood was placed into serum separator tubes and temporarily maintained on ice packs in the field until it could be transferred to the field camp where it was centrifuged (3000 rpm for 10 minutes), then sera frozen at -20°C.

6.2.2 Lemming collection and necropsy

As part of long-term population monitoring studies, we lethally trapped lemmings with Victor Mouse Snap Traps M325 (Perry et al., 1996); traps were baited with a peanut butter and oat mixture. Two line transects (150 meters) were established and two traps were placed every 15 meters (Samelius et al., 2009). The two transects of twenty traps were set daily for 10 days, achieving a total of 400 trap-nights. We stored lemming carcasses (*n*=83) at -20°C and performed necropsies in the Zoonotic Parasite Research Unit at the University of Saskatchewan. We collected serosanguineous fluid from the thoracic cavity onto Nobuto filter paper strips (Advantec MFS, Inc, Dublin, CA, USA). The filter paper strips were allowed to dry at ambient temperature (~21°C) and frozen at -20°C. We also collected the entire brain, which was refrozen at -20°C.

6.2.3 Permits

We performed all field activities with the approval of federal and territorial governments, and the University of Saskatchewan Animal Research Ethics Committee (Canadian

Wildlife Service: NUN-SCI-11-02, NUN-MBS-11-03, Government of Nunavut: WL2011-017, WL2012-021, and the University of Saskatchewan UCACS: 2010-0159, 2011-0005, 2011-0030).

6.2.4 Serological analysis

We analyzed the serum samples from geese with an in-house indirect ELISA (Appendix; Al-Adhami et al. 2014) using a rabbit anti-duck horseradish peroxidase IgG conjugate (KPL, Inc., Gaithersburg, Maryland, USA). This test enables the processing of many samples at once and is less expensive than other methods (such as the DAT). We analyzed samples using a serum dilution of 1:12.5 and a conjugate dilution of 1:2500. Positive and negative controls from experimentally inoculated ducks were included on every plate (Appendix). We analyzed the lemming filter paper strips by direct agglutination test (DAT; Biomerieux, Canada) at a dilution of 1:40, following manufacturer's instructions. Elution of the antibodies from the filter paper was performed as previously described (Curry et al., 2011; Elmore et al., 2014). We used the DAT because it is described to work well for blood filter paper eluate (Jakubek et al., 2012).

6.2.5 DNA Extraction and PCR analysis

We extracted genomic DNA from 25 mg of brain tissue from each lemming with the DNeasy® Blood and Tissue Kit (Qiagen, Toronto, Canada). All DNA extraction runs included negative extraction controls to ensure a clean procedure. Then, we analyzed the lemming DNA by polymerase chain reaction (PCR) with the TOX4/TOX5 primer set as described by Homan et al. (2000). All PCR products were visualized with ultraviolet light on a 1% agarose gel. DNA samples with a band at 529 bp were considered suspect-positive for a *T. gondii*. Because the TOX4/TOX5 primers are not specific for *T. gondii*

and also amplify *Neospora caninum* (but not *Hammondia* sp. or *Sarcocystis cruzi*; S. Elmore, unpublished data), we also tested the samples with primers targeting the B1 gene (B22/B23; Alfonso et al., 2013), which amplified an approximately 1.3 kb DNA fragment. The PCR reagent mixtures for both assays were as follows (Quanta Biosciences, Inc., Gaithersburg, MD, USA): 35 µL molecular grade water, 5µL 10x Buffer, 2.5 µL MgCl (50 mM), 2 µL of both forward and reverse primers (10 mM), 1 µL dNTP (10 mM), and taq polymerase 0.5 µL (5 units/uL); thermocycler settings were as previously described (Homan et al., 2000; Alfonso et al., 2013). All PCR runs included positive and negative controls.

6.3 Results

On indirect ELISA, 76 of 234 (32.4%) Ross's Geese were seropositive for *T. gondii,* while 66 of 233 (28.3%) Lesser Snow Geese were seropositive.

Using DAT, we did not detect *T. gondii* antibodies in any of the blood filter paper strip eluates from 84 lemmings. We analyzed genomic DNA from 84 lemmings with the TOX4/TOX5 primers and detected bands consistent with a size of 529 bp in 16 of 84 samples, compatible with either *T. gondii* or *N. caninum*. DNA of *T. gondii* was not detected in these 16 brain samples using *T. gondii*-specific primers targeting the B1 gene (B22/B23; Alfonso et al., 2009).

6.4 Discussion

Our findings further support that arctic-nesting geese are a potential prey source of exposure for arctic foxes and probable vector for the introduction of *T. gondii* from temperate regions to the Arctic. The seroprevalences of *T. gondii* antibodies in Ross's

Geese (32.4%) and Lesser Snow Geese (28.3%) in this study are comparable with naïve estimates from analysis of blood filter paper eluate from geese in the same study region (Ross's Geese 26% and Lesser Snow Geese 25%; Elmore et al. 2014). Sandström et al. (2013) reported a similar seroprevalence in barnacle geese (25%) migrating from the Netherlands to Svalbard, Norway and northwestern Russia, although estimates from other species of arctic-nesting geese were much lower. Although most reports of *T. gondii* in geese are serological surveys, we assume that wild geese are capable of serving as intermediate hosts of the parasite (i.e. harboring viable tissue cysts in their organs and muscle). Several reports exist of *T. gondii* infection in domestic geese (Dubey et al., 2007; Maksimov et al., 2011; Rong et al., 2014, Chapter 5), and disseminated toxoplasmosis in magpie geese (*Anseranas semipalmata*) has been previously described (Dubey et al., 2001).

We did not detect evidence of *T. gondii* in either blood filter paper eluate or brain tissue from brown or collared lemmings. This supports the hypothesis that environmental transmission of oocysts of this parasite is rare, or does not occur, in arctic tundra ecosystems. As herbivores, (Bergman and Krebs, 1993), lemmings would not be a part of any existing trophic transmission cycle, although the possibility of transmission by cannibalism or scavenging cannot be ruled out (Hofmannová et al., 2014). We could not find any published reports of *T. gondii*-seropositive lemmings, and Prestrud et al. (2007) did not detect *T. gondii* antibodies in sibling voles (*Microtus rossiaemeridionalis*) from Svalbard, thus concluding that oocyst transmission was not likely. It is also possible that lemmings are not suitable intermediate hosts for *T. gondii*, although natural infections have been reported in other rodents in the subfamily Arvicolinae (Fuehrer et al., 2010).

Also, false negative results are always a possibility if the antibody levels are below the assay's detection threshold. Our molecular findings, in combination with previous work on arctic foxes at this study site (Elmore et al., 2013), suggest that the related coccidian parasites *Neospora caninum* and/or *Hammondia* sp. cycle between lemmings and arctic foxes at this location. More molecular work with a *N. caninum*-specific primer set will be necessary to confirm this finding, and the role of arctic foxes as the definitive host of *Neospora caninum.* Wolves are known definitive hosts for the parasite (Dubey et al., 2011a), and are present in the Karrak Lake ecosystem.

The transmission cycle of *T. gondii* in terrestrial arctic ecosystems is still not well understood, but this study supports the hypothesis that the majority of transmission is trophic from migratory sources, and not through environmental routes by oocysts shed in felid feces. Lemmings, as resident herbivores, did not demonstrate *T. gondii* infection or exposure, while migratory herbivores (geese) were seropositive. This study also demonstrated the need for validating the specificity of published primers for closely related coccidian species as part of the PCR optimization process, especially in wildlife where mixed infections are likely to occur. Future research on *T. gondii* in terrestrial arctic ecosystems could include parasite isolation by molecular techniques and bioassay from goose tissues, other prey or scavenged animals such as muskoxen or caribou, and hatch-year Ross's Geese and Lesser Snow Geese, to determine if geese are being exposed on the tundra.

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CHAPTER 7. Discussion and Conclusion

7.1 Wildlife in the central Canadian arctic are exposed to *Toxoplasma gondii*

The transmission of *T. gondii* is enigmatic in terrestrial Arctic ecosystems. Due to the low density of felids in the region, transmission by oocysts might not occur as frequently as in more southern latitudes, so other transmission mechanisms, such as vertical and trophic transmission, must be considered.

Karrak Lake, Nunavut supports a very large goose colony, consisting mainly of Lesser Snow Geese and Ross's Geese. The trophic dynamics between arctic foxes, geese, and small mammals (lemmings) in this ecosystem have been well described (Samelius et al., 2007), which made this site ideal to study the potential for transmission of *T. gondii* from migratory prey (geese) and resident prey (lemmings) to a common predator (arctic fox). We collected blood samples from arctic foxes (Chapter 3), Ross's Geese and Lesser Snow Geese (Chapters 4 and 6), and lemmings (Chapter 6). Antibodies against *T. gondii* were detected in arctic foxes and geese, but not in lemmings. These findings can be added to a handful of other serological studies on *T. gondii* in arctic wildlife in Canada (Kutz et al., 2000; Kutz et al., 2001; Reichard et al., 2008; Simon et al., 2011).

7.1.1 Arctic foxes in the Karrak Lake ecosystem are seropositive for *T. gondii*

Very few serosurveys for *T. gondii* have focused on arctic foxes, probably due to the remote locations in which they are found. In Chapter 3, I reported the first finding of *T. gondii* antibodies in arctic foxes from the central Canadian Arctic and provide a comparison of results using cumulative findings from three serological assays.

In North America, only one other existing report gives evidence of *T. gondii* in arctic fox tissues (Dubey et al., 2011b). The foxes in our study were live-trapped as part of an ongoing population dynamics project, but it would also be beneficial to obtain carcasses from fur trappers in the region and investigate the occurrence and genotype of *T. gondii* in arctic fox tissues. We also investigated the possibility that arctic foxes might shed *T. gondii* oocysts in their feces as yet-unknown definitive hosts, but *T. gondii* oocysts were not detected by multiple methods of fecal analysis (Chapter 2).

As both predators and scavengers, arctic foxes are dietary generalists and, not unlike humans, access most of the common food sources on the tundra (Samelius et al., 2007). Because the two species share a top food chain position, arctic foxes exposure can serve as a proxy for potential human *T. gondii* exposure in a terrestrial arctic food web. Although there are competing hypotheses for the potential sources of *T. gondii* in the Arctic, we have taken steps forward in identifying what wildlife species at Karrak Lake are exposed to the parasite, thus contributing to our overall knowledge of *T. gondii* occurrence and ecology for the region.

7.1.2 Ross's Geese and Lesser Snow Geese in the Karrak Lake ecosystem are seropositive for *Toxoplasma gondii*

In Chapter 3, I reported the first known detection of *T. gondii* antibodies in Lesser Snow Geese and Ross's Geese (Elmore et al., 2014). *Toxoplasma gondii* occurs in other wild waterfowl species worldwide (Dubey et al., 2004; Murao et al., 2008; Manciati et al., 2013, Sandström et al., 2013), and disseminated infections can cause mortality in birds (Dubey et al., 2001; Work et al., 2002); however the general effects of chronic infection on wildlife populations are unknown. Ross's Geese and Lesser Snow Geese are

probably exposed to *T. gondii* in the wintering grounds in the southern United States or along their migratory pathways, thus introducing the parasite into arctic food webs upon their springtime return to the breeding grounds in the Arctic. This mechanism of introduction and transmission was originally proposed to explain the high seroprevalence of *T. gondii* in arctic foxes on Svalbard, Norway (Prestrud et al., 2007), and probably occurs in the Karrak Lake ecosystem as well.

7.1.3 Lemmings in the Karrak Lake ecosystem are not seropositive or PCR positive for *Toxoplasma gondii*

In Chapter 6, we examine lemmings, an integral component of the food web at Karrak Lake. As a major food source for arctic foxes, and other predators in the region, lemmings represent a source of trophic transmission of *T. gondii*. However, our serology results suggest that the lemming population at Karrak Lake is not exposed to *T. gondii* at detectable antibody levels, suggesting that lemmings are not a part of the *T. gondii* transmission cycle within the ecosystem. This is consistent with a study on Svalbard, Norway in which antibodies were not detected in sibling voles (*Microtus rossiaemeridionalis*) and the authors concluded that oocysts are not likely to be an important transmission mechanism in that ecosystem (Prestrud et al., 2007). In general, lemmings are described to "swim readily" (Nowak, 1999), therefore, exposure to oocyst-contaminated water drainages and thus exposure to *T. gondii* would seem likely, were oocysts of the parasite in the environment. This research supports the hypothesis that arctic foxes and other carnivores in the Karrak Lake ecosystem are primarily exposed to *T. gondii* through migratory prey that bring the parasite into the Arctic from subarctic and temperate regions.

7.2 Prioritizing testing of the brain and heart in geese will lead to higher detection probability than other organs

Due to a lack of information about *T. gondii* tissue predilection and detection probability in wild geese, we designed an experiment to determine what tissues were best to test in order to maximize detection probability of *T. gondii* (Chapter 5). First, to determine the optimal *T. gondii* oocyst dose for geese to become infected but not clinically ill, we conducted a pilot study in domestic ducks (Appendix) because goslings were not available during that time of year. Ducks that received 100 oocysts and 1000 oocysts were consistently and strongly seropositive (S. Elmore, unpublished data), so we chose to infect all geese with 500 sporulated *T. gondii* oocysts. We determined that testing the brain and the heart resulted in the highest detection probability in domestic geese, and used these results to guide our wild goose tissue sampling. We tested the brains and hearts of 50 Ross's Geese and 50 Lesser Snow Geese in an effort to detect *T. gondii*; however, we did not find any positive animals, possibly due to methodological considerations.

This is the first study in which a multi-scale occupancy approach (Nichols et al., 2008) has been used to determine tissue detection probability, although a basic occupancy model was recently used to determine tissue prevalence of hematozoa in northern pintails from California (Ramey et al. 2013). This approach is an alternative to prior studies that compare *T. gondii* presence in tissues within frequency tables (Dubey et al., 1997; Dabritz et al., 2007; Bangoura et al., 2013); assigning probability values provide easy comparisons among the organ types. Also, this approach accounts for the imperfect detection that occurs with all diagnostic tests. This portion of the study was

limited by sample size in both the experimental inoculation and the wild bird portion. More data in the experimental inoculation might have allowed for increased resolution of the organ occupancy estimate (θ), or the probability that the organ was occupied by *T. gondii* DNA, given that the goose itself was infected.

7.3 Embrace the uncertainty: living with imperfect detection and multiple detection methods

Applying imperfect diagnostic tests, or tests developed and validated for domestic animals or people, is a recurring theme in wildlife disease surveillance and research. In this thesis, no single diagnostic test for molecular or immunological evidence of *T. gondii* consistently gave convincing definitive results of detection. For this reason, I used an occupancy modeling approach that accounted for imperfect detection both within the study animal populations and within the biological samples themselves. Also, I used multiple diagnostic tests to reduce bias and generate the "best possible" seroprevalence estimates for *T. gondii* in a previously untested wildlife species (*Chen* spp.; Elmore et al., 2014).

In wildlife, uncertainty in interpreting results from serological test arises from lack of gold standards, test validation, and the high probability of cross-reaction with unknown or non-target antibodies (Table 7-1). I used an ELISA that was tested for specificity, using known positive sera that contained antibodies against closely related organisms (i.e. *Neospora caninum, Sarcocystis* sp.; Al-Adhami et al., 2014). This method allowed accurate determination of standard cut-off values for the goose serum analysis, because we had the appropriate positive and negative control sera (Chapter 6). It was of

less use in the arctic fox serum analysis (Chapter 3), due to the absence of known positive and negative controls. As a result, we used intrinsic cut-off values, which are relative to the sample population only, and this likely reduced detection probability. This does not reflect a fault of the assay, but rather highlights the difficulty in using diagnostic tests for wildlife. The high probability for unclassifiable results of the goose blood filter paper eluate testing in Chapter 4 indicates that a specificity issue exists with the IFAT, at least with that type of sample medium. Alternatively, the lower detection probability achieved with the DAT in both the foxes and the geese suggests that sensitivity is a concern with that assay. The commercial test kits for DAT were also expensive (in comparison to the other assays) and also were not always readily available through our suppliers. By using results of both DAT and IFAT assays in the goose filter paper study and the arctic fox serology study, we were able to utilize the best aspects of each test for our seroprevalence estimates.

The molecular diagnostics portion of this study highlighted difficulties with assay specificity and sensitivity (Chapters 5 and 6). The PCR-MCA was a useful screening assay for the detection of *T. gondii* in experimentally inoculated geese (Chapter 5), but we had difficulty confirming the PCR-MCA results for the wild geese, both by PCR-RFLP, as well as by conventional PCR and sequence analysis (see Table 7-2). Assay specificity was a problem when we tried to confirm the PCR-MCA results with the Homan et al. (2000) primer set, and we could not get quality DNA sequence from PCR products of either assay.

Although we did not detect *T. gondii* DNA in the wild geese using multiple molecular methods, some reasons might include: the low amount of tissue tested (100mg

per tissue sample), the selective amplification of other closely related species (i.e. *Sarcocystis* sp., *Neospora caninum*, *Hammondia* sp.) using a universal coccidian primer cocktail, the genuine absence of *T. gondii*, or that we randomly selected portions of the tissue samples in which *T. gondii* was absent, although the goose might actually have been infected. If these geese were really not infected with *T. gondii*, the specificity of both the IFAT and the DAT need to be further investigated for use in wildlife.

TABLE 7-1. SUMMARY OF SEROLOGICAL METHODS USED FOR *TOXOPLASMA GONDII* ANTIBODY DETECTION.

Assay Name	**Target Antigen**	**Comments**	**Reference**
Direct Agglutination TEST (DAT/MAT)	*T. gondii* surface protein	Can be used with many different species; sensitivity might vary among species[1]; Commercial kit expensive and not always available	Dubey and Desmonts, 1987
Indirect fluorescent antibody test (IFAT)	*T. gondii* tachyzoite surface protein via IgY FITC conjugate	Sensitive but might have a higher risk for false positives[1]	Maksmiov et al., 2011 Elmore et al., 2014
Protein A/G Enzyme-linked immunosorbent assay (ELISA)	*T. gondii* crude surface protein via Protein A/G conjugate	Good for large numbers of serum samples, can use with multiple mammalian species.[1]	Al-Adhami and Gajadhar, 2014 Elmore et al., Chapter 3 (this thesis)
IgY Enzyme-linked immunosorbent assay (ELISA)	*T. gondii* crude surface protein via anti-duck IgY	Good for large numbers of serum samples. Can use for multiple waterfowl species.[1]	Elmore et al., Chapter 6, Appendix A (this thesis) Al-Adhami and Gajadhar, 2014

[1]For optimal results, all of these assays require appropriate reference sera from the taxon being tested to determine precise negative cut-off values.

TABLE 7-2. SUMMARY OF PCR METHODS USED FOR *TOXOPLASMA GONDII* DETECTION.

Assay Type	**Target Region**	**Comments**	**Reference**
Real-time PCR with melt-curve analysis	18S	Good screening PCR, overlapping chromatograms with Sanger sequencing when multiple coccidian infections present	Lalonde and Gajadhar, 2011
Conventional PCR	529 bp repeating element	Not *T. gondii*-specific, also amplifies *N. caninum* and possibly *Hammondia* sp.	Homan et al., 2001
Conventional PCR	B1	Might not be as sensitive as other assays.	Alfonso et al., 2009

7.4 Conclusion

The study of *T. gondii* ecology in wildlife is rife with uncertainty, a characteristic of the field that must be acknowledged. The occurrence and transmission dynamics of the parasite within arctic ecological communities, populations, and hosts remains unclear, but in this thesis, I laid the groundwork for a further understanding of these characteristics in a terrestrial arctic food web in the central Canadian Arctic.

In this thesis, I also compared commonly used diagnostic methods in order to find the optimal assays for the system. I found that the use of multiple diagnostic tests might be more favorable than just one, although this is both labour and reagent intensive and is not always feasible for wildlife surveillance. Diagnostic tests, even when validated, are always imperfect, but for most wildlife species, the uncertainty in diagnostic assays for *T. gondii* remains unquantified. In this thesis, I have accounted for some of the system uncertainty by following an occupancy modeling framework, using both a traditional model (Chapter 3) and extensions of the original model (Chapters 4 and 5).

The groundwork presented here will enable ongoing and future research in the Karrak Lake ecosystem. Future studies could include an examination of vertical transmission of *T. gondii* in both foxes and geese from the region, the isolation and characterization of *T. gondii* from Ross's, Geese, Lesser Snow Geese, and arctic foxes, and an investigation on the host competency of lemmings for *T. gondii* and other coccidian parasites. Also critical is the ongoing monitoring of these wildlife species for changes in *T. gondii* dynamics in the region, which might indicate a shift in occurrence and persistence of the parasite in the Karrak Lake ecosystem and the central Canadian Arctic.

APPENDIX – Optimization of IgY ELISA in domestic ducks

We conducted a pilot experimental inoculation of *T. gondii* in domestic ducks to produce sera that could be used to optimize an ELISA for *T. gondii* serodetection. Our other goal was to determine what oocyst dose would result in chronic infection, but not clinical disease, in these birds.

Fourteen Embden-cross domestic ducks, received on the day after hatch, were randomly assigned into one of five inoculation groups: 10 oocysts, 50 oocysts, 100 oocysts, 1000 oocysts, and negative control (Bartova et al., 2004). Pre-inoculation sera were collected from all the ducklings when they were two weeks old. At 5 weeks old, the ducklings were inoculated with 10, 50, 100, or 1000 sporulated *T. gondii* oocysts in 1 mL of 0.9% saline. Negative control ducklings received 1 mL of saline without oocysts. Negative control ducks were housed in the same room as the infected ducks with a barrier placed between them for 3 days post-inoculation, when oocysts might be passed in feces. The ducklings were monitored for 28 days post-inoculation (dpi) and blood was collected weekly to monitor the serological responses of the birds. On 14 dpi, one duck from each group was euthanized for tissue examination (analyses are still outstanding). By 7 dpi, all birds in the 1000-oocyst group were seropositive by both the direct agglutination test (DAT) and the indirect fluorescent antibody test (IFAT). By 14 dpi, 2/3 ducks in the 100-oocyst group was seropositive. These seroresponses indicated that the optimal infectious dose was between 100 and 1000 oocysts, and therefore we used 500 oocysts for the upcoming goose inoculation (Chapter 5).

All ducklings were seronegative prior to inoculation by the DAT and indirect fluorescent assay (IFAT); these sera were used as "known negative" samples for the ELISA optimization. No *T. gondii* antibodies were detected in the negative control ducks using either assay throughout the study, except for a "weak positive" result in one duck on 28 dpi. This could be a false positive, or perhaps contamination occurred across the barrier between the negative control ducks and the inoculated ducks, despite biosafety control measures.

Following established ELISA procedures and conditions (Al-Adhami and Gajadhar, 2014), we performed a checkerboard titration with goat anti-duck horseradish peroxidase IgY conjugate (KPL, Inc, Gaithersburg, Maryland, USA) at dilutions 1:1250, 1:2500, 1:5000, and 1:10000, and positive control sera from experimentally inoculated ducks (Crowther. 2009) at dilutions of 1:12.5, 1:25, and 1:50. The optimal serum dilution was 1:12.5 and the optimal conjugate dilution was 1:2500.

We determined the negative cut-off value for the assay by performing the ELISA on the pre-inoculation blood samples from the ducklings, under the newly established conditions. The negative cut-off value of 0.277 (2 x MeanODvalue) was chosen for this assay, which more conservatively assigns positive results than the standard method (MeanODvalue + 3 x standard deviation; 0.237) (Crowther, 2009). These results were used to guide the analysis from naturally and experimentally infected geese in Chapters 5 and 6.

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