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Toxoplasma gondii: the dynamics between free-roaming cats and wildlife

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To the Graduate Council:

I am submitting herewith a dissertation written by Tania J. Dawant entitled "Toxoplasma gondii: the dynamics between free-roaming cats and wildlife." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Comparative and Experimental Medicine.

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Toxoplasma gondii: the dynamics between free-roaming cats and wildlife

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ABSTRACT

Toxoplasma gondii is a single celled, apicomplexan parasite with a complicated life cycle. Felids are the definitive hosts, and shed oocysts in their feces, which are extremely hardy in the environment. Intermediate hosts maintain a life-long infection with the parasite encysted in tissues. Due to this phenomenon, being seropositive is indicative of infection. The transmission dynamics between cats and wildlife is not completely understood. With its zoonotic risk, it is important to understand how *T. gondii* is spreading between domestic and wild populations. We sought to deepen the understanding of the dynamics between free-roaming cat populations and select wildlife species. With the three R's of animal research (Reduce, replace, refine) in mind, we developed an isolation technique that directly inoculates cell culture without the need for animal subjects. In order to develop a standardized procedure, the authors developed a protocol for this technique. Experimental in vitro isolation of the parasite was successful in 3/5 (60%) ducks, 4/4 (100%) turkeys, 1/6 (17%) white-tailed deer, and 4/6 (67%) kangaroos using Vero or Human Foreskin Fibroblast (HFF) cells. Isolation was successful in 5 (100%) duck samples using mouse bioassay. Isolates were confirmed using multiloculus PCR-RFLP markers. To investigate the presence of the parasite in different populations, blood samples were tested using the Modified Agglutination Test (MAT). Free-roaming domestic cats in East Tennessee showed 56.4% (2,757/4,863) seropositive. Age was

determined as a significant (p=<0.0001) risk factor. Raptors from North and South Carolina (NC and SC), USA were tested and 18.6% (30/161) were seropositive. No risk factors analyzed, species, age, or county, were significant. Northern fur seals (NFS) and owned free-roaming cats on St. Paul Island, AK, USA were 21.1% (4/19) and 6.7% (2/30) seropositive respectively. Cat feces were collected from litter boxes when possible and fecal floatations in Sheather's sugar solution were done, none were positive for oocysts. We found that the parasite is present in all populations studied, including marine mammals. *Toxoplasma gondii* is emerging in many novel species and has unknown effects on naïve populations. Understanding these dynamics is important for mitigation of future infections in both humans and animals.

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Introduction

Background

Etiology and Clinical Signs

Toxoplasma gondii is an apicomplexan parasite that can infect any warm-blooded animal including humans and birds (Dubey, 2010). The only definitive hosts are felids, where the parasite completes sexual reproduction. A single oocyst is enough for an infective dose, and a cat can shed millions of oocysts per day for 1-2 weeks during acute infection (Dubey, 2005; Siński & Behnke, 2004). Although many hosts can be infected, toxoplasmosis is generally benign. Signs, if shown, are limited to flu-like symptoms associated with lymphadenopathy particularly those in the pre- and postauricular and cervical regions (Dubey, 2010). The infection then transitions to a chronic, subclinical state (Gross, 1996). Chronic toxoplasmosis has been suggested as a risk factor for schizophrenia and autism spectrum disorders in humans (Prandota, 2010; Strobl et al., 2012). Immunocompromised or naïve populations are at higher risk of disease. Clinical signs of disseminated toxoplasmosis include encephalitis, myocarditis, hepatitis, chorioretinitis, and/or pneumonia (Dubey, 2010). If a woman is exposed to the parasite for the first time while pregnant, this could result in congenital toxoplasmosis. This manifests as hydrocephaly, microphthalmia, retinochoroiditis, intracranial calcifications, abortion, or neonatal death (Tenter et al., 2000). Despite these severe clinical signs and broad reach of the parasite, it

is considered one of the neglected parasitic diseases in the United States due to insufficient research funding (CDC, 2020; Jones et al., 2014).

Life Cycle

Toxoplasma gondii has two main portions of its life cycle, the cycle within the definitive host and that within the intermediate hosts (Figure 1; all tables and figures are located at the end of each chapter). Starting with the definitive host, cats are infected by ingesting oocysts from the environment or infected tissue from their prey. If the parasite is ingested in oocyst form, the spores mature to bradyzoites. The parasite then invades intestinal epithelial cells and begins dividing asexually. The bradyzoites then differentiate into tachyzoites and gametocytes. Zygotes then mature into oocysts which are excreted in the cat's feces. These sporulate within three days in the environment, including sea water, and are then infective (Dubey et al., 2011; Lindsay et al., 2003). Intermediate hosts (mammals, birds, humans) are infected by ingesting oocysts in the environment or contaminated water or bradyzoites encysted in their prey. Once the intermediate host is infected, the bradyzoites invade the intestinal epithelial cells and begin to divide asexually. Bradyzoites then differentiate to tachyzoites and travel throughout the body via the blood stream for a brief period of parasitemia. Tachyzoites can invade many tissues within the body, with the typical locations being brain, liver, heart and muscle. Once the tachyzoites have reached their target cell, they differentiate back to bradyzoites and encyst within

that tissue where it remains within the host. These cysts remain dormant within the host for the duration of its life, and may reactivate if the host's immune system is compromised or the animal is stressed (Derouin et al., 1989).

Diagnosis and detection/isolation

There are currently several different diagnostic approaches, including:(1) tests that target anti-*Toxoplasma* antibodies; these include the modified agglutination test (MAT), immunofluorescent antibody test (IFAT), Sabin-Feldman dye test (DT), enzyme linked immunosorbent assay (ELISA), direct agglutination test (DAT), indirect hemagglutination test (IHA), and latex agglutination test (LAT). (2) Other diagnostics target the antigen or organism itself; these include polymerase chain reaction (PCR), microscopic identification with histology, immunohistochemistry, feline and mouse bioassay, cell culture, and fecal flotation (Wyrosdick & Schaefer, 2015). These diagnostic techniques are summarized in Table 1.

To optimally study the *T. gondii* lifecycle and to be able to carry out epidemiological, genetic, and population studies, it is important to be able to isolate the parasite. The gold standard has been cat and mouse bioassay to be able to isolate *T. gondii* from infected hosts (Garcia et al., 2006). Cat bioassay is used to harvest oocysts shed by the feline host fed the parasite in either oocyst, bradyzoite, or tachyzoite form. Mouse bioassay is used to isolate the parasite in the form of tachyzoites or bradyzoites from mice that are infected with the

parasite in oocyst, bradyzoite, or tachyzoite form. Both mice and cats are natural hosts for the parasite, making them sensitive to infection, though in different ways. Mice are more sensitive to oocysts, they need as few as 1 oocyst to become infected while infection was shown to be dose dependent in cats with the lowest infective dose being 100 oocysts (Dubey, 1996, 2006). On the other hand, cats are more sensitive to bradyzoites needing as few as one bradyzoite to shed oocysts while 100 bradyzoites were needed for inoculation to lead to infection in mice (Dubey, 2001, 2005). Cats have been the preferred model for isolating *T. gondii* due to the ease of infection, however, due to the high cost, high biosafety requirements for handling oocysts, and the limitations to using cats as lab animals, this method is not desirable.

As an alternative, mouse bioassay can be used to isolate the parasite from infected tissue. Two big advantages of this method is that it is lower cost and requires a lower biosafety level. The parasite is either in the form of tachyzoites or bradyzoites throughout the process. The immune system of the mouse can tolerate a certain level of bacterial and fungal contamination in a tissue, which is helpful for opportunistic sampling. The major disadvantage of this method is that mice require a relatively high concentration of bradyzoites as an infective dose as it is not the natural route of infection (Dubey, 2001, 2005). Though the cat bioassay is more sensitive, the majority of *T. gondii* isolates reported in the literature are from mouse bioassay.

These are the current typical options for the isolation of *T. gondii*, however, efforts have been made to bypass the use of animals and use cell culture directly.

Cell culture, bioassay, and gene amplification

Cell culture is not a new technique, many cell types have been shown to support the growth of parasites including embryonic heart, leg muscle, chicken lung and intestine, and mouse embryos among others (Cook & Jacobs, 1958). Cook and Jacobs (1958) used trypsinized monkey kidney cells that formed a continuous monolayer in 4-7 days. They infected these cells with RH strain tachyzoites harvested from mouse bioassay, and the cells showed complete degeneration on day five or six (Cook & Jacobs, 1958). Similar to viral isolation, *Toxoplasma* has been isolated from the blood of infected AIDS patients in several different studies. In 1985, an AIDS patient presenting with pneumonia was found to have a 1:16 titer for *T. gondii* antibodies using the Sabin-Feldman dye test, but no parasite was identified on brain biopsy or direct inoculation in mice. A blood culture was done on this patient, and tachyzoite plaques were seen after 5 days and mice inoculated with material from this culture developed toxoplasmosis (Hofflin, 1985). This study shows that cell culture of *T. gondii* could be a useful diagnostic tool similar to viral isolation. Serology can be insensitive in immunocompromised patients as it is a measure of an individual's immune response. Blood cultures, however, show what is actively happening at

that moment in time in a patient's body. Due to the high cost of mouse bioassay, labs do not keep a colony available for testing. In 1985, Shepp et al. performed a viral blood culture on a patient with AIDS and isolated *T. gondii* tachyzoites. All of these patients had evidence of previous exposure by serology prior to acute disease, showing that it may be possible for the parasite to reactivate in times of a lowered immune system (Shepp et al., 1985). Reactivation of a latent infection was also demonstrated in AIDS patients where *T. gondii* was isolated via culture of buffy coat, bronchioalveolar lavage, and lung biopsy (Derouin et al., 1989). Two days after inoculation, foci of parasitized cells were present. All patients were positive for IgG antibody titers, indicating a chronic infection (Derouin et al., 1989). Not only has cell culture been useful to demonstrate parasitemia, but it can also be helpful to diagnose ocular toxoplasmosis. Vitreous humor from patients with toxoplasmic retinochoroiditis was cultured on human fibroblast MRC-5 cells (MRC5, Bio-Mérieux, Lyon, France), and within an average of 12 days tachyzoites were present with a positive predictive value of 100% (Miller et al., 2000). Culture, as well as mouse bioassay, have been used to diagnose prenatal congenital toxoplasmosis via amniotic fluid. In mouse bioassay it took at least 45 days for mice to show tissue cysts in the brain, while on human fibroblast MRC-5 cells tissue culture showed positive results after 4 days of incubation (Derouin et al., 1988).

Different strains of *Toxoplasma* have different levels of virulence, which can affect the response time in mouse bioassay. Mice inoculated with different strains and life stages of *T. gondii* show different responses (Dubey et al., 2016). Those inoculated with the virulent RH strain developed acute toxoplasmosis and died within 9 days, while those inoculated with the low virulence H strain tachyzoites developed chronic toxoplasmosis. Mice inoculated with low-virulence bradyzoites developed chronic toxoplasmosis and those inoculated with avirulent C strain tachyzoites or bradyzoites developed chronic toxoplasmosis (Derouin et al., 1987). Though there was a difference between inoculation materials, the medium did not show a significant difference in sensitivity; however, all strains grew in a shorter time frame on cell culture. The RH strain grew within 6-9 hours and both the C and H strains had early multiplication by days 1 or 2 (Derouin et al., 1987). This study showed that cell culture may be a viable option for diagnosis in cases that are more time-sensitive and serology is inconclusive.

Mouse bioassay and cell culture are not the only techniques used to identify *T. gondii*. Gene amplification can also be used to determine if there is parasitic DNA present in a sample. Gene amplification, cell culture and mouse bioassay were compared to identify parasitemia in experimentally infected rabbits. In this study, mouse bioassay was shown to be the most sensitive technique followed by gene amplification and cell culture (Hitt & Filice, 1992). This study used the buffy coat because of a higher likelihood of detecting the

intracellular parasite versus extracellular tachyzoites to inoculate cell culture which may have been detrimental due to the toxic nature of the cells within this sample. This sample also requires parasitemia which is a stage of the disease that is short-lived. Though mouse bioassay was the most sensitive technique, it is not practical for many clinical labs due to the high financial and labor cost associated with maintaining a mouse colony (Hitt & Filice, 1992).

Storage conditions also have a significant effect on isolation techniques. The sensitivity of both cell culture and mouse bioassay are significantly affected. Tachyzoites stored at -20°C for 48 hours were still able to grow when inoculated on cell culture at a concentration of 10^4 while there was not a response in mice (James et al., 1996). This study showed that sensitivity changes were comparable in cell culture and mouse bioassay, however, cell culture gave results within 10 days which is still clinically relevant. It was also shown that PCR could detect the parasite at low numbers (10-10^3 tachyzoites) and even when the parasite was deteriorated (James et al., 1996). Though PCR is sensitive, it requires the parasite to be within the very small sample that is processed which may lead to the parasite being missed entirely if a larger organ is being sampled. These results show that all techniques are useful in different situations; however, cell culture offers a good alternative for laboratories that do not have the space or funding for PCR and/or mouse bioassay.

Cell culture has already been used successfully to isolate *T. gondii* from tissue samples. Fresh brain and heart muscle from one cat and the lung of another were inoculated onto cell culture in Finland resulting in the isolation of the parasite; a steady infection was established after the third passage on African green monkey kidney epithelial cells (Vero) and continued to be maintained by serial passage and then cryopreserved (Jokelainen et al., 2012). The Type X *Toxoplasma* strain that is infecting southern sea otters (*Enhydra lutris nereis*) was identified using cell culture. Fresh brain tissue was inoculated onto MA-104 (monkey kidney) cells, the parasite that was isolated was then cryopreserved and genotyped (Shapiro et al., 2019). This study provides a brief history of the direct cell culture technique and a defined protocol for better access and standardization across studies.

Epidemiology

Free-roaming cats

Though *T. gondii* generally does not cause significant disease in felids that are infected, it does have the potential to cause significant disease. Cats diagnosed with acute toxoplasmosis show signs of lung infections, neuropathy, hepatitis, pancreatitis, cardiovascular and ophthalmic changes (Bresciani et al., 2016). The parasite can also be passed transplacentally or via milk from a queen to her offspring and can manifest as uveitis, lethargy, depression, ascites,

encephalitis, hypothermia and sudden death (Bresciani et al., 2016). Felids can get infected by consuming contaminated vegetation, produce, and/or water or by consuming raw or undercooked meat containing tissue cysts with bradyzoites, which is a problem with the increased popularity of raw food diets (Hussain et al., 2017; van Bree et al., 2018). When a cat is acutely infected, they can shed millions of oocysts in the environment over a one to two week period (Siński & Behnke, 2004).

Free-roaming domestic cats (*Felis catus*), both owned outdoor cats and feral cats, present an increased risk for wildlife exposure to *T. gondii*, as they share a habitat with wildlife species that are susceptible to the disease (Conrad et al., 2021). Infections are emerging in areas that should not have exposure in the natural life-cycle of the parasite. Marine environments are becoming contaminated due to growing feline populations, urbanization, and increased contamination of waterways. In California, outbreaks in sea otters were associated with heavy rainfall events that produced washout that was contaminated with oocysts (Miller et al., 2002). The same pattern was seen in a study looking at deaths due to toxoplasmosis in the Hawai'ian monk seal (*Monachus schauinslandi*) on the Hawai'ian main islands (Robinson et al., 2023). This infection route is becoming more and more important with the trends in rainfall associated with climate change. Extreme weather events in the USA have increased by 40% since the 1950s, which increases the amount of runoff that is

coming from terrestrial environments where felids are present (Kunkel et al., 2013). With more intense rainfall the sediment at the bottoms of rivers and lakes is getting disrupted which may contain oocysts. These oocysts are extremely hardy in the environment, surviving for up to 54 months in cold, fresh water and at least 24 months in seawater (Dubey, 1998, 2004; Lindsay et al., 2003; Lindsay & Dubey, 2009). Not only do the oocysts survive for a long period in aquatic conditions, but they are only 10-20 µm in size, making them able to travel long distances in the water column as well as evade some filtration systems (Bowman, 2013; Jones & Dubey, 2010).

Some studies have looked at the prevalence of *T. gondii* in cats throughout the United States. Using the modified agglutination test (MAT), Smith et al. (1992) reported the prevalence of cats on swine farms in Iowa, USA as 42% (n=74). In 1995, Dubey et al. reported a prevalence of 76% (n=295) in swine farm cats in Illinois using the MAT. Another study looking at cats on swine farms in Iowa in 1998 reported a prevalence of 80% (n=20) (Hill et al., 1998). DeFeo et al. (2002) tested cats from veterinary clinics and shelters in Rhode Island reporting a prevalence of 42% (n=200) using the MAT. Also in 2002, Dubey et al. reported a prevalence of 48% (n=275) in rural Ohio. A large scale, retrospective study of clinically ill cats throughout the united states reported a prevalence of 29% (n=12628) (Vollaire et al., 2005). Owned cats in Pennsylvania, USA had a prevalence of 20% (n=210) in 2008 (Dubey et al.,

2009). In a study investigating free-roaming cats in Ohio, the cats had a prevalence of 51.5% (n=200) (Ballash et al., 2015). Two studies in Virginia looked at the feces of domestic cats to determine the presence of oocysts being shed. Lilly and Wortham (2013) used PCR on owned and stray domestic cats showing 6% positive for *T. gondii* DNA by PCR. Taetzsch et al. (2018) did fecal flotations on 275 cats finding oocysts in 2% of these samples, the prevalence using the MAT, however, was 22.4%. A prevalence of 56.3% (n=20) was reported in wild and domestic cats in Minnesota (Verma et al., 2016). A few other studies tested banked samples from domestic cats to look at general pathogen exposure. In northern Florida, Luria et al. (2004) showed a prevalence of 8.9% (n=553) using the commercially available enzyme-linked immunosorbent assay (ELISA). Banked samples from feral cats on the Channel Islands, California, USA were tested showing 34% (n=92) positive for the parasite by indirect fluorescent antibody test (IFAT) (Clifford et al., 2006). Palerme et al. (2019) showed a prevalence of 30% in free-roaming cats sampled between 2015-2016 in Iowa. There have not been any published prevalence estimates of *T. gondii* in domestic cats in Tennessee and the results reported in this study will help fill this gap.

Raptors

As previously mentioned, *T. gondii* can infect most warm-blooded animals including birds. Existing studies have investigated toxoplasmosis in various species of birds, but not much is known about the disease in birds of prey. There

have been a limited number of studies looking directly at *Toxoplasma* in these species. Williams et al. (2006) reported a prevalence of 46% in 50 tawny owls (*Strix aluco*), but these were owls that were roadside casualties and were specifically chosen due to ocular lesions thus may be a biased pool since *T. gondii* is associated with chorioretinitis in birds (Williams et al., 2001). On the other hand, Keenan et al. (2020) evaluated ocular lesions in wild barred owls *(Strix varia*) and great horned owls *(Bubo virginianus*) admitted to the Auburn University Southeastern Raptor Center (SRC), and found that they were not associated with *T. gondii*. This demonstrates that there is a knowledge gap about the pathophysiology of the parasite in avian species. Fatal toxoplasmosis has also be identified in the Bald Eagle (*Haeliaeetus leucocephalus*) and disseminated toxoplasmosis was found in an osprey (*Pandion haliaetus*) (Szabo et al., 2004; Wang et al., 2022). The prevalence of the parasite in birds of prey is also understudied. In 1993, Lindsay et al. looked at the prevalence of *T. gondii* in raptors from Alabama, USA and reported a prevalence of 26.7% (n=101) using the mouse bioassay method explained previously. Though the Keenan (2022) study looking at ocular lesions in owls found no significant difference between positive serology and affected eyes, the prevalence in affected birds was 47% (n=15) and 20% (n=5) in normal birds. Dubey et al. (2010) isolated the parasite from a roughed-legged hawk, a barn owl, two Swainson's hawks, an American kestrel, a ferruginous hawk, and a red-tailed hawk giving an 11.6% (n=60)

success rate. Using the commercial latex agglutination test, samples collected from California condors, golden eagles, and turkey vultures at various sites in California, USA showed 3% (n=92), 15% (n=26), and 11% (n=66) positive respectively (Straub et al., 2015). Love et al. (2016) looked at 14 different species of raptors in the southeastern United States and reported a total prevalence of 34.5% (n=281) with individual species varying from 0-100%. Another study looked at carnivorous birds in the Eastern United States and found a prevalence of 20.6% (n=155) (Ammar et al., 2021). There are no studies reporting the prevalence of *T. gondii* in birds of prey in western North and South Carolina showing a gap the present study can close.

Northern Fur Seals (Callorhinus ursinus) on St. Paul Island, Alaska, USA

Toxoplasma gondii has been emerging in new environments including in marine environments; notably, it has been associated with severe disease and die offs in marine mammals (Barbieri et al., 2016; Kreuder et al., 2003; Miller et al., 2023). The parasite has been identified in many marine species including those in the dolphin (*Tursiops, Sotalia, Cephalorhynchus, Lagenorhynchus* and *Stenella* spp.), dugong (*Dugong dugong*), manatee (*Trichechus* spp.), otter (*Enhydra lutris* spp.), porpoise (*Phocoena* spp.), sea lion (*Zalophus, Eumetopias* and *Phocaractos* spp.), seal (*Neomonachus, Mirounga, Halichoerus, Pagophilus, Phoca, Cystophora, Pusa* and *Arctocephalus* spp.), walrus (*Balaenoptera edeni* and *Odobenus rosmarus*), and whale (*Delphinapterus, Balaenoptera, Orcinus,*

kogia, and Physeter spp.) families (Dubey et al., 2020) It has been elucidated in existing literature that *T. gondii* has differing effects on species. In sea otters (*Enhydra lutris nereis*) it can cause severe subcutaneous and peritoneal steatitis and intralesional parasites were found in the pancreas, cardiac muscle, lungs, lymph nodes, adrenal glands, uterus, duodenum, skeletal muscle and meningeal tissue (Miller et al., 2023). In Hawai'ian monk seals (*Monachus schaunislandi)* it was identified in the lymph nodes, spleen, adrenal glands, diaphragm, heart, and brain associated with necrosis and intracellular and extracellular tachyzoites (Barbieri et al., 2016; Honnold et al., 2005). Few studies have assessed the prevalence of *T. gondii* in fur seals (*Arctocephalinae*). Jensen et al. (2012) looked at different pinnipeds in Antarctica and reported a prevalence of 57% at a cut-off titer of 1:40, but 0% when further diluted; thus all samples for the Antarctic fur seal (*Arctocephalus gazella*) were considered negative. Blood from 125 adult female Australian fur seals (*Arctocephalus pusillus doriferus*) was tested by Lynch et al. (2011) and showed a 0% prevalence. Using PCR Gibson et al. (2011) reported evidence of *T. gondii* DNA in tissue from Guadalupe fur seals (*Arctocephalus townsendi*). Clinical toxoplasmosis characterized by myocarditis, meningoencephalitis, ocular lesions, pituitary lesions, and tissue cysts has been documented in Hawaiian monk seals (*Neomonachus schauinslandi*), New Zealand fur seals (*Arctocephalus forsteri)*, and South American fur seals (*Arctocephalus australis) (Barbieri et al., 2016; Donahoe et al., 2014; Reisfeld et*

al., 2019). Other pinnipeds that have been shown to have antibodies against *T. gondii* include Atlantic harbor seals (*Phoca vitulina vitulina*), harbor seals (*phoca vitulina*), bearded seals (*Erignathus barbatus)*, Caspian seals (*Pusa capsica)*, crabeater seals (*Lobodon carcinophaga*), grey seals (*Halichoerus grypus*), ringed seals *(Pusa hispida*), southern elephant seals (*Mirounga leonina*), Weddell seals (*Leptonnychotes weddellii*), and monk seals (Barbieri et al., 2016; Dubey et al., 2020; Honnold et al., 2005). To my knowledge, there has been a single report of toxoplasmosis in a northern fur seal (*Callorhinus ursinus*), and population studies or an up to date prevalence are not currently available (Holshuh et al., 1985). The present study will add to the current body of knowledge by reporting the prevalence from a sample of northern fur seals (*Callorhinus ursinus*) from St. Paul Island, Alaska, USA.

The parasite has been shown to be present in Alaska, USA in other wildlife species. A retrospective study of samples collected from 1976-1996 found antibodies in black bears (*Ursus* americanus; 43%, n=143), wolves (*Canis lupus;* 9%, n=125), Dall sheep (*Ovis dalli*; 7%, n=319), caribou (*Rangifer tarandus;* 6%, n=241), moose (*Alces alces*; 1%, n=240), and bison (*Bison bison*; 1%, n=241) throughout Alaska (Zarnke et al., 2000). A later, similar study was done looking at wolf (collected 1996-2008; 17.8%, n=324*)*, caribou (1996-2008; 0.4%, n=453), moose (2001-2005; 0.0%, n=201), black-tailed deer (*Odocoileus hemionus*; 1980-2000; 0.0%, n=55), fox (*Vulpes vulpes*; 1985-2006; 12.5%, n=9),

and coyote (*Canis latrans*; 2005; 0.0%, n=12) samples from Alaska, USA and Yukon, Canada. The prevalence was 17.8%, 0.4%, 0.0%, 0.0%, 12.5%, and 0.0% respectively (Stieve et al., 2010).

One question that remains unanswered is *how* the northern fur seals on St. Paul Island, Alaska, USA are getting infected with the parasite. As mentioned above, marine environment contamination has been associated with the presence of domestic cats (owned and feral) and freshwater runoff contaminated with oocysts (Miller et al., 2002; Robinson et al., 2023). For this reason, the present study assessed the prevalence of *Toxoplasma gondii* antibodies in the owned cat population of St. Paul Island as well, to determine if this is a potential source of infection for the northern fur seal.

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Appendix

Tables and Figure

Table 1 Summary of diagnostic techniques for *Toxoplasma gondii* infection.

Diagnostic method	Sample used	Target	Advantages	Disadvantages	Species	Reference
Modified agglutination test (MAT)	Serum, plasma, or body juices	Antibody	Not species specific, antemortem, macroscopic	Requires whole tachyzoite, no commercial kit available	All mammal and bird species	(Dubey et al., 1995)
Direct agglutination test (DAT)	Serum	Antibody	Antemortem, not species specific, macroscopic, commercial kit available in France	Requires whole tachyzoite, no commercial kit available in the US	All mammals and birds	(Fulton & Voller, 1964)
Indirect hemagglutination test (IHAT)	Serum	Antibody	Non-species-specific, antemortem, commercial kits available	Less sensitive and specific than MAT	All mammals and birds	(Dubey & Thulliez, 1989; Lunde, 1973)
Latex agglutination test (LAT)	Serum	Antibody	Non-species-specific, antemortem, commercial kits available	Less sensitive and specific than MAT	All mammals and birds	(Dubey & Thulliez, 1989)
Indirect fluorescent antibody test (IFAT)	Serum	Antibody	Commercial kits available	Requires species specific conjugate	Requires conjugate for specific species	(Muranishi et al., 2004)
Immunohistochemistry (IHC)	Formalin fixed tissue	Antigen	Identifies antigen directly in a sample	Must have cysts in the sample	All mammals and birds	(Casartelli-Alves et al., 2014)
Sabin-Feldman dye test (DT)	Serum	Antibody	Antemortem	Requires live tachyzoites	All mammals and birds	(Sabin & Feldman, 1948)

Table 1 continued

Diagnostic method	Sample used	Target	Advantages	Disadvantages	Species	Reference
Enzyme linked immunosorbent assay (ELISA)	Serum, meat juice, milk	Antibody	Commercial kits available	Requires species specific conjugate	Species-specific and non-species-specific conjugates available	(Liyanage et al., 2021)
Polymerase chain reaction (PCR)	Fresh or fixed tissue	Antigen	Can confirm presence in tissues and/or blood, high specificity, allows for genetic analysis	Requires tissue cyst to be present in sample, high cost, specialty equipment needed, low sensitivity	All mammals and birds	(Burg et al., 1989; Noral et al., 2009; Singh, 1997)
Histological identification	Fixed tissue	Antigen	Visual confirmation of tissue cysts and associated pathology,	Needs PCR or IHC to confirm, requires histologic training, postmortem	All mammals and birds	(Dubey, 2010)
Cat bioassay	Fresh tissue	Antigen	Sensitive, oocysts can be collected and genetic material can be collected	High cost, laborious, requires live animals, biohazard of oocysts being handled	All mammals and birds	(Dubey, 2010)
Mouse bioassay	Fresh tissue	Antigen	Sensitive, tachyzoites can be collected and genetic material can be collected	High cost, laborious, requires live animals, biohazard of infectious tachyzoites	All mammals and birds	(Dubey, 2010)

Table 1 continued

Diagnostic method	Sample used	Target	Advantages	Disadvantages	Species	Reference
Direct cell culture	Fresh tissue, whole blood, or other bodily fluids	Antigen	Can be done on tissue up to 2 weeks old, does not require animal inoculation	Requires cell lines to be maintained,	All mammals and birds	(Dawant et al., 2023)
Fecal floatation	Feces	Oocysts	Easy to perform, passive sample collection, low cost	Transient shedding leads to low sensitivity, biohazard of infectious oocysts being handled	Felid hosts	(Dubey, 2010)

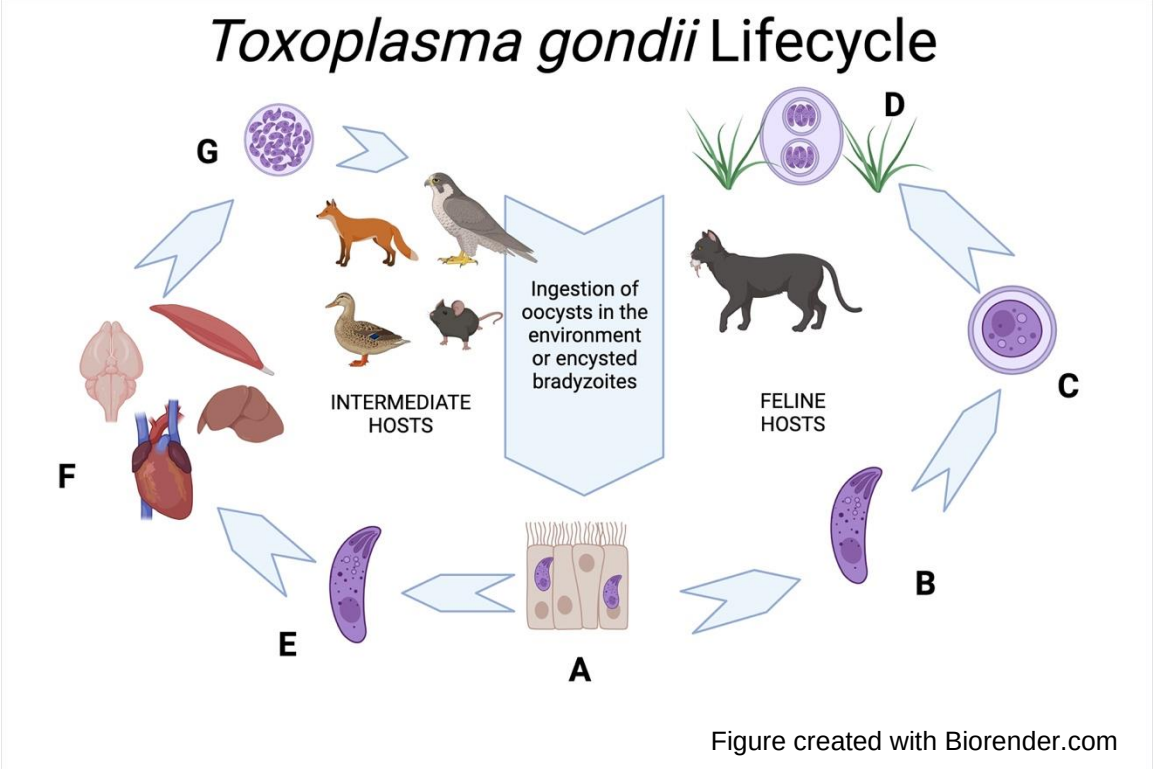

Figure 1 *Toxoplasma gondii* life cycle.

A) After infective oocysts or tissue infected with bradyzoites is consumed, the parasite invades intestinal epithelial cells and begin dividing. B) The bradyzoites in the epithelial cells differentiate into tachyzoites and gametocytes in the definitive hosts of the Felidae family. C) zygotes form and mature into oocysts within the host. D) Oocysts are shed in the feces and sporulate in the environment within three days and become infective. E) In an intermediate host, after the infected tissue or oocysts is consumed, the bradyzoites differentiate into tachyzoites only. F) These tachyzoites travel through the blood stream and invade cells of any tissue and multiply. G) Tachyzoites differentiate to bradyzoites and form cysts within tissues of the intermediate host. Figure created with Biorender.com

Chapter I:
Isolation of *Toxoplasma gondii* in cell culture: an alternative to bioassay

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Abstract

Toxoplasma gondii is a zoonotic, protozoan parasite that can infect most warm blood animals, leading to severe disease and death in susceptible hosts. Currently the standard for isolation of this parasite are cat and mouse bioassay. These are both labor intensive and costly procedures that involve using live animal subjects. This study aims to summarize the key literature and present a standard protocol for the isolation of *T. gondii* directly on cell culture without the use of mice or cats. The authors conducted four experiments to isolate *T. gondii* in vitro using Vero or Human Foreskin Fibroblast (HFF) cells. Samples from 5 wild ducks, 4 wild turkeys, 6 white-tailed deer, and 6 kangaroos were used to test the protocol. Isolates were successfully collected from 3 (60%) ducks, 4 (100%) turkeys, 1 (17%) white-tailed deer, and 4 (67%) kangaroos. All 5 (100%) duck samples resulted in successful isolation in mouse bioassay. These isolates were then confirmed using multiloculus PCR-RFLP markers. We show that it is practical to isolate *T. gondii* directly on cell culture providing a simpler, ethically more acceptable, and less time-sensitive protocol. In this paper we propose a procedure that may be applied and further optimized for isolation of *T. gondii*.

Introduction

Toxoplasma gondii is an apicomplexan protozoan parasite that can infect numerous warm-blooded animals, including humans, other mammals, and birds (Dubey, 2010). Although it can infect a wide variety of host species, only members of the Felidae family can shed oocysts following sexual replication. The parasite is one of the leading causes of foodborne illness in the United States, and *T. gondii* infection is considered one of the five neglected parasitic infections by the Centers for Disease Control and Prevention (CDC) due to insufficient research funding available (CDC, 2020; Jones et al., 2014). Infection can occur through a multitude of routes including ingestion of sporulated oocysts in water or food that is contaminated by feces of infected and shedding cats, or undercooked meat containing bradyzoites, the slow-replicating forms of the parasite (Benenson et al., 1982).

Toxoplasma gondii can readily grow in cell cultures (Chang and Gabrielson, 1984). This is a major advantage, enabling e.g. production of antigens for diagnostics, studying the biology of these organisms using cutting-edge methodologies, and cryopreservation of strains. For epidemiology, genetics, and population studies, it is often useful to isolate *T. gondii* from infected hosts. Bioassay, using cats and mice, has been the standard for the isolation of *T. gondii* from infected hosts for the past several decades (Dubey, 2010; Garcia et al., 2006), and has been an essential tool to study the biology of *T. gondii* in

general. Both cats and mice are natural hosts and susceptible to *T. gondii* infection, and can clear bacterial and fungal contamination in source animal tissues. Due to high sensitivity to bradyzoite infection and the ability to ingest a large number of animal tissues, bioassay using cats has been the gold standard for isolating *T. gondii* strains. However, due to the high cost, strict biosafety requirements to handle oocysts shed in the feces of infected cats, and ethical considerations and limitations of using cats as laboratory animals, isolation of *T. gondii* by bioassay in cats has been used in a limited number of research laboratories. For these reasons, mice have been the standard choice for bioassay. The key advantages of mouse bioassay are lower cost and lower biosafety requirements since the parasites are not shed by infected mice. Most *T. gondii* isolates reported in the currently available literature have been isolated using bioassay in mice. However, successful application of cell-culture-based methods to isolate *T. gondii* from biological samples has been reported in the literature, but not widely applied. In this article, we discuss isolation of *T. gondii* in cell culture, describe a set of experiments we carried out, and provide future perspectives.

Isolation of *T. gondii* in vitro by cell culture reported in literature

Isolation of T. gondii from human patients with acute toxoplasmosis

Several studies have reported the isolation of *T. gondii* parasites from blood or tissue samples from AIDS patients with acute toxoplasmosis directly to cell cultures. For example, Hofflin et al. inoculated a minced blood clot from an AIDS patient to an L cell culture (fibroblast cell line from mouse) and isolated a *T. gondii* strain five days after inoculation (Hofflin and Remington, 1985). Derouin et al. inoculated bronchoalveolar-lavage fluid or lung biopsy specimens from 4 AIDS patients with pulmonary toxoplasmosis to human lung embryonic fibroblast (MRC5) cell cultures and successfully isolated *T. gondii* from all 4 patients (Derouin et al., 1989). In a later report, the same lead author reported the isolation of *T. gondii* from 43 of 908 HIV-infected patients in cell culture (Derouin and Garin, 1992). Asensi et al. inoculated white blood cells of 71 HIV-positive patients to human embryonic lung fibroblast cell culture, *T. gondii* was isolated from the blood of 4 of 11 patients with AIDS who had cerebral toxoplasmosis and 6 of 60 HIV-seropositive patients with fever but no demonstrated organic infection with the parasite (Asensi et al., 1993). Contini et al., inoculated white-blood cells from 14 AIDS patients with toxoplasmic encephalitis to an MRC5 cell culture, and detected *T. gondii* parasites in 11 patients (Contini et al., 1995).

Besides the AIDS-related acute toxoplasmosis, *T. gondii* has been isolated from other forms of the disease. Shepp et al. inoculated white-blood cells from 3

organ transplant patients who developed acute toxoplasmosis to fibroblast cell culture and observed *T. gondii* in all 3 samples. These patients were initially chronically infected with *T. gondii*, and intensive immunosuppressive therapy after organ transplantation likely caused the reactivation of the dormant parasites leading to parasitemia (Shepp et al., 1985). Derouin et al. inoculated 9 amniotic fluid samples from cases of congenital toxoplasmosis to MRC5 cell culture and isolated 4 *T. gondii* strains (Derouin et al., 1988). Miller et al. isolated 5 *T. gondii* strains from the vitreous fluids of 5 patients with toxoplasmic retinochoroiditis (Miller et al., 2000). Taken together, these studies suggest that isolating *T. gondii* in cell culture from human acute toxoplasmosis appears effective.

Isolation of T. gondii from animals with acute toxoplasmosis and chronic infection

Isolation of *T. gondii* from clinical toxoplasmosis in animal hosts and chronically infected animals has also been reported in the literature. In one study in Finland, from 6 cats that had been diagnosed with generalized toxoplasmosis post-mortem, fresh tissues (brain, heart muscle, or lung) from 2 cats were inoculated to Vero cell cultures and *T. gondii* were isolated from both cats (Jokelainen et al., 2012). In another study in Portugal, brain homogenates from 76 animals (20 pigeons and 56 cats) seropositive for *T. gondii* infection but seemingly without clinical toxoplasmosis, were inoculated into Vero cell cultures, resulting in the isolation of the parasites from 13 of the 20 pigeons and 15 of the

56 cats. Inoculation of muscle homogenates (heart and limbs) prepared by acid-peptic digestion from a subset of 15 cats resulted in the isolation of the parasites from 10 of these cats. These results indicated an excellent success rate in isolating *T. gondii* using cell culture (Waap et al., 2012). Cell culture was also used to isolate *T. gondii* in sea otters from California. Brain tissues from stranded sea otter carcasses recovered less than three days since death were inoculated onto MA-104 (monkey kidney) cells and monitored for growth. The authors reported 135 isolates obtained but it is not clear how many samples were initially tested (Shapiro et al., 2019). In summary, the isolation of *T. gondii* from samples from animals by cell culture appears to be an effective approach.

A way to reduce and replace bioassays

From previous studies, it is clear that direct isolation of *T. gondii* in cell culture is feasible, particularly in cases of acute toxoplasmosis in humans and animals in which parasite load in tissues is expected to be high. However, publication bias are possible, and information regarding the relative efficiency of the cell-culture-based isolation method versus bioassay in mice is limited. A study comparing the sensitivity of isolating *T. gondii* by mouse bioassay and cell culture showed that the former is more sensitive than the latter (Hitt and Filice, 1992). A recent study indicated that the cell culture method is promising, but further optimization is needed before it can replace or reduce the number of mouse bioassays needed (Opsteegh et al., 2020). The reported results of the isolation of *T. gondii* directly

in cell cultures that are available in the literature are promising but scattered, and the methods used were often not described in detail. Considering the advantages and disadvantages of bioassay methods, and the three R's of a humane laboratory animal experimental technique: replacement, reduction, and refinement, more efforts should be made to adapt the cell-culture-based isolation method in future studies. Here we report results from a set of tests in isolating *T. gondii* in cell culture and propose a procedure that can be applied and tested for further optimization.

Isolating *T. gondii* by cell culture in this study

We conducted four batches of studies to isolate *T. gondii* from samples from animals collected as convenience sampling between 2019 and 2021.

In literature, the cutoff titer value for seropositivity by modified direct agglutination test (MAT) is often set at 1:5 for birds, and 1:25 for mammals. A review on isolation of *T. gondii* from chickens showed that the higher the MAT titers, the higher success rate of isolating *T. gondii* from chickens by bioassay in mice (Dubey et al., 2016). In this review article, from 2066 chicken serum samples collected from 19 countries worldwide and tested by MAT test, and 1041 samples had titers ≥ 1:5 and considered positive. Heart tissues from these positive chickens were homogenized and inoculated to mice, 522 *T. gondii* isolates were obtained. The success rates for isolation were 15% (16/105) for titer 1:5, 11% (9/79) for 1:10, 43% (42/98) for 1:20, 33% (43/132) for 1:40, 54% (86/159) for

1:80, 75% (136/182) for 1:160, 62% (115/186) for 1:320, 72% (57/79) for 1:640, and 86% (18/21) for titer ≥ 1:1280. Majority of these isolates (79%, 412/522) were obtained from chickens with MAT titers ≥ 1:80. The association of high MAT titers with high success rates of isolation suggests that the titers may reflect the parasite load in chicken heart tissues.

In another study, *T. gondii* isolates were obtained by bioassay from 19 seropositive mammals (MAT ≥ 1:32) including 13 white-tailed deer, 3 feral hogs, 2 coyotes and 1 mink. The rates of obtaining *T. gondii* isolates in mouse bioassay were 0% for titer 1:32, , 15% for 1:128, 12.5% for 1:512, 20% for 1:2048, 66.7% for 1:4096 and 62.5% for titer ≥ 1:8192. There was a significant correlation between MAT titers and the success rates of bioassay (GLM linear regression coefficient r = 0.88, P = 0.021) (Gerhold et al., 2017). Overall, this study is in agreement with the chicken studies above. This result indicates that success rate is higher at MAT titer ≥ 1:4096 for these animals.

With the outcome from isolation of *T. gondii* in chickens and other mammals in above mentioned studies as a general guideline, we carried out our experiments accordingly. In our MAT tests for birds, serum samples were diluted 1:5, 1:10, 1:20, 1:40, 1:80, 1:160 and 1:640, whereas for mammals the samples were diluted 1:25, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1600 and 1:3200. The animal samples were tested by MAT test and tissue samples from animals with

MAT titers towards the higher end were homogenized and inoculated to cell culture. This was to maximize the success rate for *T. gondii* isolation.

Cell culture procedure

All studies were carried out in a Biosafety Level-2 (BSL-2) laboratory at the University of Tennessee, Knoxville. Detailed protocol for cell culture is described in section 5. In brief, serum samples or exudates from fresh heart tissues of animals were collected and tested for anti-*T. gondii* antibodies by the modified agglutination test (MAT). Positive reactions at dilutions equal or greater than 1:25 were considered positive. Briefly, approximately one gram of fresh heart tissue from seropositive animals was processed for cell culture. To reduce the risk of contamination, each sample was cleaned by trimming off the exterior layer of tissue in a biosafety cabinet. The trimmed tissue was then rinsed with 70% ethanol followed by PBS (pH 7.4). The tissue was then homogenized, resuspended in PBS with antibiotics penicillin/streptomycin (pen/strep), and digested with trypsin to release *T. gondii* parasites from tissue cysts. The tissue preparation was then inoculated to human foreskin fibroblast (HFF) and/or African green monkey kidney epithelial (Vero) cell culture. The medium, containing pen/strep with or without a fungicide was changed every day for 3 days after the initial inoculation to remove cellular debris and contaminants. The cell cultures were observed daily to monitor for the presence or absence of parasite growth. The medium was changed every 2 to 3 days.

Bioassay in mice

The CD-1 outbred mice were used for one experiment following the protocol (IACUC #1419) approved by the Institution Animal Care and Use Committee at the University of Tennessee. Bioassay was performed by the previously reported protocol (Chunlei Su and Dubey, 2020). Briefly, one gram of animal heart tissue was homogenized in 10 ml PBS with pen/strep, centrifuged to remove the supernatant, the pellet was resuspended in 5 ml of PBS with pen/strep, mixed with 2 ml of 0.25% trypsin and digested at 37°C for 15 min. The digested content was spun down, the pellet resuspended in 5ml PBS with pen/strep, and 0.5 to 1 ml of processed heart tissue was inoculated into two outbred CD-1 mice per sample by intraperitoneal injection. Mice were observed daily for up to 14 days. At the end of the time, or earlier if a mouse started to show clinical signs of *T. gondii* infection (rough fur or lethargy), the mice were euthanized. A total of 5 ml of PBS with pen/strep was injected into the peritoneal cavity and peritoneal lavage fluid was collected. Between 0.25 and 0.5 ml of the fluid was inoculated to two 25cm^2 HFF cell flasks to further expand the parasites and for cryopreservation.

Experiments

Experiment 1. Wild ducks chronically infected with T. gondii.

Exudates from heart tissues of 51 ducks from Pennsylvania, sampled in 2019, were tested by MAT. Among these 51 samples, 9 had MAT titer < 1:5, 6 had 1:5,

9 had 1:10, 3 had 1:20, 3 had 1:40, 10 had 1:80, 6 had 1:160, 0 had 1:320 and 5 had ≥ 1:640. The 5 samples from ducks with titers ≥ 1:640 were used for parasite isolation by bioassay in mice. Four of the 5 samples were also inoculated directly to cell culture (Table 1).

To isolate *T. gondii* by mouse bioassay, trypsin-digested heart tissues were inoculated in 2 mice by intraperitoneal injection. A total of 10 mice were used for this experiment. These mice were euthanized on days 7 and 13 post-inoculation due to clinical signs of the infection. Parasites were collected from peritoneal lavage and mixed with pen/strep and inoculated to HFF cells. Altogether 5 isolates were obtained from the 5 samples (Table 1).

To isolate *T. gondii* from duck samples by direct cell culture, 0.2-0.5 ml of trypsin-digested heart tissues were added to three HFF cell monolayers. Pen/strep were added to the culture medium to prevent bacteria contamination. From the five duck heart tissue samples, PA-duck-127 had rotted and was not used. PA-duck-149 was slightly degraded and led to fungus-like contamination in cell culture and was discarded. Three isolates were obtained from the three fresh duck heart tissues. It took up to 50 days to observe the parasites in HFF cultures.

The five isolates were genotyped using 10 genetic markers following the previously published method (Su et al., 2010). Three ToxoDB PCR-RFLP genotypes were identified, including genotype #1 (also know as Type II) for PA-duck-125, PA-duck-127 and PA-duck-149; genotype #8 for PA-duck-135; and

genotype #216 for PA-duck-132 (Shwab et al., 2014). In summary, tissue samples from five wild ducks with MAT titers ≥ 1:640 were inoculated to mice and 5 isolates were obtained, accounting for 100% success rate by bioassay. Four of the 5 tissue samples, except one partially rotted, were inoculated to cell cultures, one of them was contaminated by fungus and discarded, the rest 3 were positive for *T. gondii* isolation, resulting in a 60% success rate by cell culture. These results showed that isolating *T. gondii* by cell culture is feasible, but control of fungal contamination is essential.

Experiment 2. Kangaroos with acute toxoplasmosis.

Six kangaroos from a zoologic park in the US died of acute toxoplasmosis between September 2019 and January 2020 (Table 2). Serum samples were tested by MAT test, all had titers ≥ 1:3200. Fresh heart tissues were collected and homogenized, digested by trypsin, and directly inoculated to cell culture in two HFF cell monolayers with pen/strep. Two cell cultures were contaminated by fungus-like organisms were discarded. Four isolates were obtained, accounting for a success rate of 67% (4/6). It took up to 63 days to see the parasites in the HFF cell culture (Table 2). Genotyping at the 10 genetic loci revealed ToxoDB PCR-RFLP genotype #7. Results suggested that the mortality in these zoo animals was likely due to an outbreak of toxoplasmosis. In summary, from 6 kangaroos with acute toxoplasmosis, one *T. gondii* isolate was obtained from each of 4 kangaroos, except 2 samples failed due to fungal-like contamination.

This results suggest that isolation of live *T. gondii* by cell culture is feasible, but again, contamination of fungi is critical.

Experiment 3. White-tailed deer with chronic infection of T. gondii.

A total of 24 heart samples were collected from white-tailed deer during the hunting season of 2020 from South Carolina, USA. Serum samples or heart exudates were used for the MAT test. Among these 24 samples, 10 had MAT titer < 1:25, 1 had 1:25, 2 had 1:50, 0 had 1:100, 2 had 1:200, 1 had 1:400, 2 had 1:800, 1 had 1:1600 and 5 had ≥ 1:3200. Trypsin-digested heart tissues from 6 white-tailed deer with MAT titers ≥ 1:1600 were directly inoculated to HFF cell culture. Cell culture medium was supplemented with pen/strep/Fungin. One isolate (ScWtd9) was obtained (Table 3). Genotyping at the 10 genetic loci revealed ToxoDB PCR-RFLP genotype #5, a common genotype from wild animals in North America (Shwab et al., 2014). In this experiment, only 1 isolate was obtained from 6 white-tailed deer, the success rate is 17%, which is low. However, none of the cell cultures was discarded due to fungal contamination, even though 2 of these cell cultures (SCWtd22 and SCWtd24, Table 3) were initially contaminated, but controlled by adding fungicide to the medium. This result suggests that fungicide and be an effective way to alleviate fungal contamination in direct cell culture. The low isolation rate may be due to low parasite load in white-tailed deer, or uneven distribution of the tissue cysts in the tissues.

Experiment 4. Wild turkeys with chronic infection of T. gondii.

In April 2021, 46 wild turkey heart samples were collected from Tennessee. Heart exudates were tested for antibodies to *T. gondii* infection. Twenty-eight samples had MAT titers <1:5, 6 had 1:5, 7 had 1:10, 1 had 1:20, 1 had 1:80, and 3 had ≥ 1:640. Heart tissues from the 4 samples with higher MAT titers (≥ 1:80) were homogenized, digested by trypsin, and inoculated to 2 flasks of Vero cells and 1 flask of HFF cells. Cell culture medium was supplemented with pen/strep/Fungin. Four isolates were obtained (Table 4).

A total of 5 isolates were obtained from 4 turkey samples, including one of the turkeys had two isolates. Three ToxoDB PCR-RFLP genotypes were identified, including genotype #5 for WITU2111, WITU2113 and WITU21-59, genotype #10 for WITU21-59, and genotype #36 for WITU21-12. Two genotypes were identified for WITU21-59, indicating mixed infection. However, we could not rule out the potential contamination of the WITU21-59 sample. Both HFF and Vero cells successfully propagated *T. gondii* directly from animal tissue. The process takes longer with HFF cells. In this experiment, the success rate of isolating *T. gondii* is 100% and no fungal contamination occurred. This may due to the effectiveness of adding fungicide in the cell culture medium.

Taken together, the outcome from our four experiments indicated that, isolating *T. gondii* from wild ducks and wild turkey with chronic infection, and kangaroos with acute toxoplasmosis in cell culture was effective as long as

fungal contamination can be controlled. Since we choose to use animals samples with high end MAT titers for the experiment, the high success rates may be biased. However, the results do show the promise of direct cell culture as the alternative to bioassay for *T. gondii* isolation in the future.

Perspective

Previous reports and the current studies showed that the isolation of *T. gondii* parasites directly in cell culture is a viable approach. This method would reduce the need for laboratory mice for isolating the parasite, which not only reduces the use of animals, one of the three R's of animal use in research, but it also significantly reduces cost and labor. The three R's of animal use in research are Reduce, Refine and Replace (Russell and Burch, 1959). The present technique should provide an alternative to isolate *T. gondii,* evolving parasite isolation to an in vitro process. This technique has the potential to facilitate the field of *T. gondii* research, making it possible for a wider variety of researchers who do not have the funds or ability to house laboratory mice to perform *T. gondii* isolation. There is also a significant decrease in labor with the present method. Rather than having to check a mouse colony twice daily, the cell culture flasks only need to be checked daily with media changes as nutrients are depleted and the media becomes acidic. Once the parasites have been established in the cell culture it can be maintained indefinitely or cryopreserved.

A variety of cell lines may be used to isolate *T. gondii*. However, some differences were observed when we used the HFF and Vero cells. Notably, Vero cells were more robust and grew faster. After splitting a flask of Vero cells, confluent monolayers were noted after one day of incubation at 37°C at 5% CO_2 , which is ready for inoculation of the parasites. HFF cells took 7-14 days before the monolayers become confluent for use. However, due to HFF cells propagating more slowly than Vero cells, HFF cells could be kept in an incubator at 37°C at 5% CO_2 for longer durations with less effort. Vero cells could be easily grown and maintained from a stock that had been frozen in either liquid nitrogen or at -80°C (Ammerman et al., 2008). This was helpful for opportunistic convenience sampling where the schedule was unknown and being able to quickly grow confluent cells was necessary. The choice of which cell line to use is dependent on the capability of the laboratory to maintain the cell lines. Our study clearly showed that Vero cells are more robust in propagating *T. gondii* than that of HFF cells (Table 4), it provided a much faster isolation of the parasites. The cell culture protocol for *T. gondii* isolation did not change with the different cell lines once confluent monolayers were developed.

With every technique, there are benefits and limitations. While mouse bioassay is more tolerant to contamination in the source tissue samples and tachyzoites are ready to harvest between 7 to 12 days after inoculation in mice, directly inoculated cell culture flasks are more likely to be contaminated and may

take up to eight weeks to propagate the parasites depending on the strain and the cells used. From our experiments from duck and kangaroo samples, contamination was an important obstacle for in vitro isolation of *T. gondii* (Table 1, Table 2). Therefore, it is very important to reduce the risk of contamination. This is why the sample must be trimmed and cleaned before inoculation and it is important to change media daily for the first few days, with fresh media supplemented with antimicrobials and antifungals. If the contamination is prevented, then cell culture can be maintained following previously established in vitro *T. gondii* maintenance protocols (Khan and Grigg, 2017).

Previous bioassays had shown a significant correlation between MAT titers and the success rates of bioassay tissues (Dubey et al., 2016; Gerhold et al., 2017), this implies the higher the MAT titers, the higher the parasite burdens in tissue samples. We expect this will also apply to the isolation of the parasites by cell culture. The relatively high success rates in isolating *T. gondii* from animal tissues in this study is likely biased to the selection of high MAT titer samples (Tables 1, 2, 4). It is worth pointing out that, our experiment from samples of 6 white-tailed deer only resulted in one viable isolate even though the MAT titers were relatively high (Table 3). This may due to uneven distribution of *T. gondii* tissue cysts in the heart tissues and a relatively small amount of tissues used for inoculation.

Using cell culture would allow for expanding the exploration of the disease ecology and epidemiology of *T. gondii* which was previously not cost-effective. Epidemiologic studies of wildlife can also be advanced with this technique. Frequently, samples from wildlife populations are from convenience sampling, and being able to receive samples and have cell culture flasks ready in the laboratory allows for a much quicker turnaround for these types of studies. The timeline is significantly decreased with the ability to inoculate a flask within two days of receiving the sample. While serology is being run on the sample using MAT, the investigator can prepare flasks of Vero cells that will quickly develop a monolayer. Being able to isolate *T. gondii* from a wider range of hosts, in particular wildlife, could elucidate the transmission dynamics from an expanded One Health point of view. Overall, this technique has the potential to push *T. gondii* research to the next level by making the process of isolation simple, more cost-effective, ethically more acceptable, and less time-sensitive.

While this technique has many benefits there are also some limitations. Namely, it has been shown that with multistrain co-infections, mouse and cat bioassay and cell culture have resulted in a single strain being isolated (Villena et al., 2004). This may bias the outcome of epidemiological study of the parasite. More research needs to be done develop tools that are able to isolate different strains of *T. gondii* from mixed infections. Experiments looking at the PCR results from inoculum compared to the isolated parasite from the same sample as well

as looking at different cell types to optimize the success of isolation should be considered.

Recommended protocol for direct isolation of *T. gondii* in cell culture

Materials and reagents:

PBS pH 7.4, Ca^{2+} free

Penicillin/streptomycin (pen/strep) antibiotics (penicillin 10,000 U/ml + streptomycin 10,000 µg/ml stock, 100x)

IKA ULTRA-TURRAX disperser or similar tissue homogenizer

15-ml IKA DT tubes pre-treated with 70% ethanol for 1 hour to sterilize

0.25% Trypsin-EDTA (Gibco, cat. no. 25200-056)

Fungin 10mg/ml (1000x) (InvivoGen, #ant-fn-1)

Vero and human foreskin fibroblast (HFF) cell culture (multiple 25cm^2 flasks). For detailed information, see the previously published protocol (Khan and Grigg, 2017).

PBS with pen/strep/Fungin (40 ml PBS +1 ml antibiotics + 40 µl Fungin). Need 20 ml for each sample

D5 medium (DMEM with 5% fetal bovine serum (FBS)

D5/pen/strep/Fungin (40 ml D5 + 1 ml Pen/Strep + 40 µl Fungin)

Procedure:

1. Collect fresh samples: Obtain heart tissues and serum samples (heart exudates will work) from animals. Note: Avoid contamination, use aseptic technique. From animals, heart tissues are preferred, but other tissues will work too.

   Human samples such as the placenta can be processed the same way. Blood, amniotic fluids, or similar samples can be inoculated to cell culture directly as in Step 7 below.
2. Determine MAT titers of serum samples. Select seropositive samples (MAT > 1:25; the higher the titers the better).
3. Tissue homogenization. For heart tissue, remove the surface layer to minimize bacterial contamination. Take 1-3 grams of fresh heart tissues. Rinse the tissue in 70% ethanol and PBS successively to reduce surface bacteria. Cut into small pieces. Place into a 15-ml IKA DT tube (pre-treated with 70% ethanol and drained), and add 10 ml of PBS. Homogenize by IKA ULTRA-TURRAX disperser (25 sec at 1000rpm, two to four times or until tissues are homogenized).
4. Remove blood and other soluble components: transfer into a sterile 50-ml centrifuge tube. Spin at 1500 rpm for 5 min. Discard supernatant. Add 5 ml PBS with pen/strep/Fungin, and resuspend the pellets. For samples collected from acute toxoplasmosis, there are a large amount of *T. gondii* tachyzoites in tissues, so inoculating homogenized tissues to cell culture is

sufficient. In this case, take two flasks of HFF and two flasks of Vero cells, remove the medium, and add 5 ml D5/pen/strep/Fungin medium to each flask. Inoculate 0.2 ml to 0.5 ml of homogenized tissue. Avoid inoculating large chunks of tissues. Follow the Step 8 below.

5. Trypsinization of tissue to release parasites: For samples from chronically infected animals or human placenta, it is necessary to digest the tissues to release *T. gondii* bradyzoites. Add 2 ml of trypsin to the homogenized tissue from Step 4, and resuspend. Incubate at 37°C for 15 to 30 min in a water bath.
6. Remove trypsin: centrifuge at ~ 1000x g for 5 min. Remove supernatant using pipets (Note: the pellet does not stick to the centrifuge tube well after trypsinization, so do not pour supernatant off). Resuspend in 5 ml PBS with pen/strep/Fungin.
7. Inoculate to cell culture: for each sample, take two to five flasks of HFF and two to five flasks of Vero cells, remove the medium, and add 5 ml D5/pen/strep/Fungin. Inoculate 0.2 ml to 0.5 ml of digested heart tissue in each flask. Avoid inoculating large chunks of undigested tissues. Note: It is a good practice to inoculate different amounts of digested tissues to each flask, which allows for identifying optimal doses to be used in future experiments.

8. Changing the medium to remove tissue debris: For the first three days after inoculating the tissue samples, replace the medium with 5 ml D5/pen/strep/Fungin daily, this is to remove tissue debris and wash away potential bacterial or fungal contamination in the cell culture.
9. Expand the parasites in cell culture. Observe daily to monitor for parasite growth in cell culture. Change the medium every two to three days or when the medium is acidic (slightly yellowish).

   Note: If tissues were collected from acute toxoplasmosis, it is expected to see tachyzoites in cell culture within two weeks. If tissues were collected from chronic infection with *T. gondii*, it may take 8 weeks to see tachyzoites in cell culture. Vero cells have a high level of metabolism, the culture medium needs to be changed every 2 to 3 days. Vero cell culture can be maintained for up to 12 days and then the cells age and start to die. To continue the culture, the infected Vero cells must be scraped, and homogenized by repeatedly passing through a 22-gauge needle with a syringe to break up cells, then 0.5 ml suspension to be passed to one or more new flasks of fresh Vero monolayers. HFF cells can be maintained in the same flask for 8 weeks or longer with a change of medium every one or two weeks. The success rate in isolating *T. gondii* is high (similar to bioassay) for cases of acute toxoplasmosis, but lower for chronic infections.

10. Cryopreserve the parasites (Khan and Grigg, 2017). Extract DNA and perform genotyping to identify the parasite isolates (e.g., Chunlei Su and Dubey, 2020; Joeres et al., 2023).

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Chapter II:
Survey and risk factor analysis of *Toxoplasma gondii* in free-roaming cats in eastern Tennessee

Abstract

Toxoplasma gondii is considered the most successful parasite in the world due its far reaches both geographically and host species diversity. It has numerous transmission routes including oral ingestion, in-utero transmission, and in the case of acute infections, blood transfusions. This apicomplexan parasite is an important pathogen for both human and animal health. It can infect and cause disease in any warm blooded animal from mammals, including humans, to birds. Animals in the Felidae family are the definitive hosts and can infect the environment by shedding oocysts in their feces. Once these oocysts are sporulated in the environment, they remain infective for years. Tennessee's placement on the edges of both the Atlantic and Mississippi flyways and along the Mississippi river watershed makes it an important location for pathogen dispersion. Large scale studies are lacking on free-roaming domestic cats in Tennessee to determine their impact on *Toxoplasma* shedding and pathogen ecology. We determined the presence of *T. gondii* in the Eastern Tennessee free-roaming cat population and analyzed risk factors for infection. Serum samples from 5,206 free roaming domestic cats were obtained from the Feral Fixin' sample bank at the University of Tennessee College of Veterinary Medicine (UTCVM) and tested using the Modified Agglutination Test (MAT) with a cut-off titer of 1:25. Of these, 4,863 were considered for statistical analysis due to poor sample quality (hemolysis or lack of demographic data). Overall, 2,747 (56.4%)

cats were positive for antibodies against *T. gondii*. Age was determined to be a significant risk factor (p=<0.0001) with cats >1 year of age being more likely to be infected. Sex, county, or colony size were not shown to be statistically significant risk factors. Free-roaming cats in East Tennessee are an important part of the sylvatic life cycle for *Toxoplasma gondii*. More studies are needed, however, to determine transmission dynamics and risk factors.

Introduction

Toxoplasma gondii is an apicomplexan parasite that can infect nearly all warm-blooded vertebrates, including humans, making it the most successful parasite in the world (Dubey, 2010). The only definitive hosts, where the parasite can complete sexual reproduction, are members of the Felidae family, including both wild and domestic cats. Typically, as a definitive host, cats do not show signs of disease, but in some cases, infection may lead to clinical disease. Clinical signs and lesions in adult cats diagnosed with toxoplasmosis include pneumonia, neuropathy, hepatitis, pancreatitis, and cardiovascular and ophthalmic abnormalities (Bresciani et al., 2016). Kittens can be infected transplacentally or via the milk of an infected queen and can present with uveitis, lethargy, depression, ascites, encephalitis, hypothermia and sudden death (Bresciani et al., 2016). Though some cats show clinical signs, most immunocompetent hosts are asymptomatic and shed oocysts in the environment. A single cat can shed millions of oocysts a day for a period of 1-2 weeks during acute infection (Siński & Behnke, 2004). Individuals can be infected by consuming uncooked or undercooked meat that is contaminated with cysts containing bradyzoites, or by consuming produce or water that is contaminated with oocysts (Fredebaugh et al., 2011; Hussain et al., 2017). Due to the presence of tissue cysts in commercial meat products, cats that eat a diet that contains raw meat are more likely to be infected with the parasite (Jokelainen et al., 2012).

Not only is toxoplasmosis important in our feline patients, but it can cause significant disease in humans as well. *Toxoplasma gondii* is one of the most common foodborne diseases in the world (Tenter et al., 2000). In 1999, Mead et al. estimated that 50% of human exposures to *T. gondii* are foodborne, and these cases of toxoplasmosis account for approximately 20% of all deaths due to foodborne pathogens in the United States. In immunocompetent people, toxoplasmosis is generally benign and self-limiting with flu-like symptoms that will transition into a long-term chronic state that largely remains subclinical (Dubey, 2010; Gross, 1996). It has, however, been associated with schizophrenia and autism spectrum disorders (Prandota, 2010; Strobl et al., 2012). It can also cause congenital disorders if a woman is infected for the first time during pregnancy (Dubey, 2010). Prenatal infection can lead to signs including low grade fever, malaise, fatigue, lymphadenopathy, hepatitis and chorioretinitis. (Aguirre et al., 2019; Ambroise-Thomas & Petersen, 2000). Determining the prevalence of *T. gondii* and understanding transmission is important to be able to properly prevent future infections. This study determined the proportion positive for antibodies against *T. gondii* in free roaming cats in eastern Tennessee over multiple years and assesses its association with a number of potential risk factors such as age, sex, and county.

Materials and methods

Sample collection

Aliquots of 5,206 serum samples were taken from samples that were banked at the University of Tennessee College of Veterinary Medicine (UTCVM), Knoxville, Tennessee, USA. Of these, 4,863 were used in this study, the others were excluded due to poor sample quality (hemolysis and/or excessive debris) or lack of demographic data. These samples were obtained for the Feral Fixin' program at the UTCVM that does spays and neuters of feral cats from East Tennessee (IACUC #1488-0621). The samples were collected from 2010-2019 from 29 different counties (Table 2).

Serology

The Modified Agglutination Test (MAT) was used to determine the presence of antibodies in the serum samples. The antigen solution used was made in lab of senior author (C. Su) in the Department of Microbiology at the University of Tennessee, Knoxville, Tennessee, USA. Briefly, the serum samples were serially diluted from a concentration of 1:25 up to 1:3200. A solution with a concentration of $2x10^8$ whole formalin-fixed tachyzoites per mL was mixed with the diluted samples; Evan's blue dye was used to aid with contrast and visualization. A positive control of a known titer (here 1:400) and a negative

control were included on every plate run. Samples with a titer >=1:25 were considered positive.

Statistics

The association of sex, age and colony size on infection status of *T. gondii* were evaluated using Chi-square tests. Statistical significance was identified at $p<0.05$. All analyses were conducted in JMP pro for Windows 64x (SAS Institute Inc., Cary, NC, USA). An odds-ratio was done for age as this was the only risk factor that was found to be significant with the chi-squared analysis.

Results

Of the 4,863 tested samples across 29 counties in Tennessee (Figure 1a), 2,634 (54.2%) were female and 2,229 (45.8%) were male; 1,879 (39.1%) were less than one year of age and 2,929 (60.9%) were greater than one year of age. Overall, 2,747 (56.5%) cats were positive for antibodies against *T. gondii*. Of the tested females, 1,491 (56.6%) were seropositive and 1,256 (56.4%) of male cats were seropositive. Sex was determined not the be a significant risk factor ($p=0.86$; Figure 3). Cats <1 year of age had a significantly lower likelihood of being positive ($p= <0.0001$; Figure 2) with 895 (47.6%) testing positive, while those >1 year of age were 1.8 ($p=<0.001$) times more likely to be infected with 1,811 (61.8%) testing positive. Colony size was not shown to be a significant risk

factor for exposure to *T. gondii* (p=0.736). Sample distribution and prevalence are illustrated in Figure 1.

Discussion

This study showed an overall prevalence of 54.2%, which was higher than expected. In a meta-analysis of *T. gondii* infections in domestic felids by Hatam-Nahavandi et al. (2021) the average prevalence was 31.6% in North America and globally was 37.5% and globally. Though this needs to be assessed with caution due to different testing procedures between studies, there are some factors that would be interesting to examine further. Tennessee having a higher than average percent positive than the rest of North America could be due to environmental characteristics. According to the Tennessee Climate Office (TCO), Tennessee has two main climate types: Humid subtropical and Oceanic/Highland. These are both temperate zones that get an average of 55 inches up to 90+ inches of rain a year with hot summers and mild winters (*Tennessee Climatology*) It is known that *T. gondii* prefers humid, tropical environments meaning Tennessee offers an environment that is amenable to the maintenance of the parasite's lifecycle (Meerburg & Kijlstra, 2009).

There could be an aspect of population density with cats, but there is no published data on the relative number of free-roaming cats in Tennessee in comparison to other states (Rowan et al., 2019). The results in this study, however, did not show an association between colony size and infection with *T.*

gondii so the effect of the presence of more cats remains unknown. There was limited data on comorbidities such as Feline Leukemia Virus (FeLV) and Feline Immunodeficiency Virus (FIV) to determine if there is an association with changes in immunity due to these viruses, which may be warranted in future studies on *T. gondii* in free-roaming cats.

There was no association between sex and infection with *T. gondii*, thus sex was not shown to be a risk factor in this study. Age, however, was found to be significant. This is not unexpected because the longer an individual lives, the more opportunity it has to be exposed through the environment or prey. There could also be an aspect of maternal antibodies being present in samples from very young kittens (Remington et al., 2004). Future studies with standardized sample collection would be helpful to monitor the disease in the free-roaming cat population.

Understanding the risk factors leading to a high prevalence of *T. gondii* in Tennessee is important for our wildlife populations as well as human and feline health. Tennessee lies on the border between the Mississippi and Atlantic flyways, meaning that pathogens that migratory birds are exposed to in Tennessee may be carried anywhere along the Atlantic coast and the Gulf of Mexico and up to Alaska and Northeastern Canada (Elmore et al., 2014; Elmore et al., 2015). Furthermore, many of the waterways in Tennessee shed their water into the Gulf of Mexico and the Atlantic Ocean. The Mississippi River watershed,

to which the water bodies in Tennessee contribute, includes all or part of 31 different states and 2 Canadian provinces, and covers about 40% of the contiguous 48 states (*Watersheds*, 2021). Sediment from all along this watershed is carried down to the Gulf of Mexico. This sediment contains contaminants, such as *T. gondii* oocysts, which are carried throughout the waterway (Miller et al., 2002; Robinson et al., 2023). *Toxoplasma gondii* has been emerging in aquatic environments causing illness and death in certain marine mammals (Dubey et al., 2020). These events have been associated with increased runoff after heavy rain events in areas where domestic cats are present (Barbieri et al., 2016; Miller et al., 2002; Robinson et al., 2023). Future studies are needed to understand where these pathogens are entering the waterways in order to mitigate future infections. This study showed that cats in East Tennessee have a higher than average prevalence and more studies should be done to understand the impacts of this on the spread of *T. gondii.*

Cats may be a good candidate for a sentinel species in the area. Looking at human cases in the same counties would help determine if programs such as Feral Fixin', where free-roaming cats are being handled, could be useful disease sentinel programs as well as providing critical veterinary care. If a correlation is seen between the prevalence in the free-roaming cat population and the human population, this could help guide mitigation strategies to prevent future infections for both humans and animals.

Human impacts could also have an effect on the presence of *T. gondii* in the environment. It is known that large run-off events after storms are linked to infection with *T. gondii* in marine mammals, thus looking at rain and erosion patterns would be helpful to determine high risk areas for contamination of waterways (Robinson, 2023). Future studies looking at differences between urban and rural areas, socioeconomic status, and access to care for both human and veterinary medicine would be helpful to determine mitigation strategies for exposure in humans and animals.

Conclusion

Tennessee free-roaming cats have a higher prevalence of *T. gondii* than the published national average for domestic cats. More studies are needed to assess risk factors associated with this higher prevalence and the role Tennessee free-roaming cats play in the spread of the parasite.

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Appendix

Tables and Figures

Table 2 Distribution of titers of antibodies against *Toxoplasma gondii* in 4683 cats in eastern Tennessee. Samples that have a titer <1:25 are considered negative, all other titers are considered positive.

Titer	Number
<1:25	2116
1:25	277
1:50	27
1:100	340
1:200	170
1:400	135
1:800	128
1:1600	104
>1:3200	1160

Table 3. Percent positive of antibodies against *Toxoplasma gondii* by the modified agglutination test in free-roaming cats in East Tennessee, USA by sex.

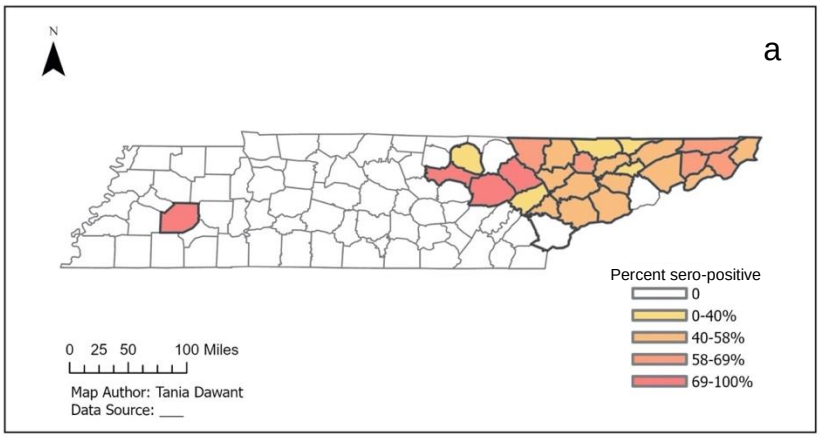
Sex	Positive (%)	Negative (%)	Total
Female	1450 (56.93)	1097 (43.07)	2547
Male	1222 (56.39)	945 (43.61)	2167

Table 4 Prevalence of antibodies against *Toxoplasma gondii* in free-roaming cats by age.

Age	Negative (%)	Positive (%)	Total
<1 year	953 (52.02)	879 (47.98)	1832
>1 year	1075 (38.00)	1754 (62.00)	2829

Table 5 Prevalence of antibodies against *Toxoplasma gondii* by the modified agglutination test in free-roaming cats in Tennessee, USA by county.

County	Negative	Positive	Total
Anderson	252 (42.1%)	346 (57.9%)	598
Blount	51 (54.3%)	43 (45.7%)	94
Campbell	31 (52.5%)	28 (47.5%)	59
Carter	199 (37.9%)	326 (62.1%)	525
Claiborne	7 (63.6%)	4 (36.4%)	11
Crab Orchard	0 (0%)	1 (100%)	1
Cumberland	13 (22.0%)	46 (78.0%)	59
Grainger	62 (47.3%)	69 (52.7%)	131
Greene	142 (45.4%)	171 (54.6%)	313
Hamblen	125 (64.1%)	70 (35.9%)	195
Hancock	3 (60.0%)	2 (40.0%)	5
Hawkins	33 (42.3%)	45 (57.7%)	78
Jefferson	121 (50.0%)	121 (50.0%)	242
Johnson	3 (50.0%)	3 (50.0%)	6
Knox	504 (42.6%)	608 (57.4%)	1184
Loudon	9 (45.0%)	11 (55.0%)	20
Madison	2 (22.2%)	7 (77.8%)	9
Monroe	1 (100.0%)	0 (0.0%)	1
Morgan	2 (11.8%)	15 (88.2%)	17
Overton	9 (60.0%)	6 (40.0%)	15
Putnam	0 (0.0%)	7 (100.0%)	7
Roane	45 (66.2%)	23 (33.8%)	68
Scott	11 (32.4%)	23 (67.7%)	34
Sevier	151 (48.1%)	163 (51.9%)	314
Sullivan	69 (34.2%)	133 (65.8%)	202
Unicoi	156 (44.6%)	194 (55.4%)	350
Union	9 (31.0%)	20 (69.0%)	29
Washington	89 (36.0%)	158 (64.0%)	247
Wise	1 (100.0%)	0 (0.0%)	1
Total	2100 (43.6%)	2715 (56.4%)	4815



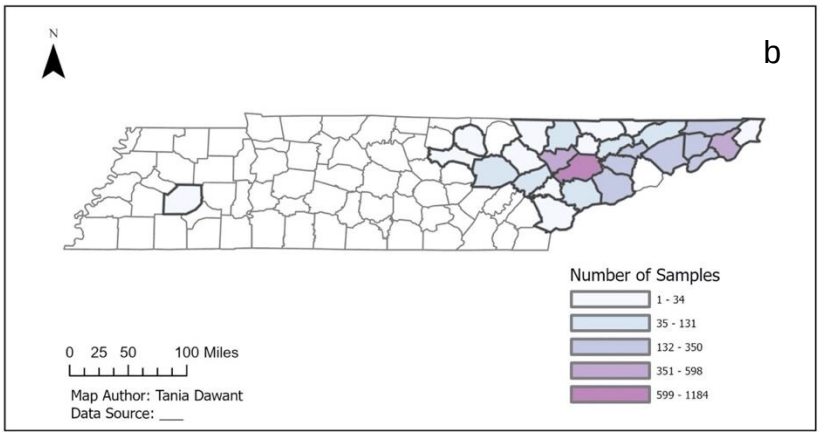


Figure 2 (a) Prevalence of antibodies against *Toxoplasma gondii* in free-roaming cats in east Tennessee, USA. (b) Geographical distribution of samples collected and tested in free-roaming cats in eastern Tennessee, USA.

Chapter III:
Investigation of *Toxoplasma gondii* in raptors in North and South Carolina, USA

Abstract

Toxoplasma gondii is a single celled parasite that can infect any warm-blooded animal from birds to humans. Its lifecycle is terrestrial biologically, however, it has been emerging in aquatic and marine environments and infecting naïve populations. Although birds have been implicated as being a part of the sylvatic life cycle for this parasite, their role in the epidemiology of the pathogen is not fully understood. This study aimed to determine the presence of antibodies against *T. gondii* in raptors from North and South Carolina, USA to understand the potential impacts of raptors on the disease ecology. Raptor carcasses were collected from the Carolina Raptor Center (CRC), Huntersville, North Carolina, USA. Blood samples from 161 raptors were tested using the Modified Agglutination Test (MAT) with a cut-off titer of 1:25 to determine the presence of antibodies against *T. gondii*. Statistical analysis was done to determine risk factors including age, species, and county. Twelve species were represented in this study: 6 Broad-winged Hawks (*Buteo platypterus)*; 19 Cooper's Hawks (*Accipiter cooperii)*; 1 Mississippi Kite (*Ictinia mississippiensis)*; 37 Red-shouldered Hawks (*Buteo lineatus)*; 20 Red-tailed Hawks (*Buteo jamaicensis)*; 2 Sharp-shinned Hawks (*Accipiter striatus)*; 9 Black Vultures (*Coragyps atratus)*; 5 Turkey Vulture (*Cathartes aura)*; 4 Osprey (*Pandion haliaetus)*; 43 Barred Owls (*Strix varia)*; 4 Eastern Screech Owls (*Megascops asio)*; and 11 Great Horned Owls *(Bubo virginianus)*. The overall prevalence was 18.6% (30/161). No risk

factors that were analyzed were determined to be significant for the risk of infection with *T. gondii.* This study shows that *T. gondii* is widely present in carnivorous birds. More studies are needed, however, to determine risk factors for infection and to understand the pathogenesis of the parasite in avian species.

Introduction

Toxoplasma gondii is an apicomplexan parasite that can infect nearly all warm blooded animals including birds and humans (Dubey, 2010). *Toxoplasma gondii* has a complicated life cycle that requires a definitive host and intermediate hosts. The definitive hosts are animals in the Felidae family, while all other animals that are infected can act as intermediate hosts. Infected intermediate hosts, such as birds of prey, develop a lifelong infection with parasites encysted in their tissues (Derouin et al., 1989). Due to this phenomenon, seroprevalence of *T. gondii* is equivalent to the prevalence of active infections, though they may be acute or chronic. These tissue cysts contain bradyzoites, which are the slow replicating stage of the parasite.

Birds in general have been shown to be important for the maintenance of the parasite in the environment. Migratory birds in particular are important because these animals develop tissue cysts containing bradyzoites, which are infective, and can carry the parasite to wherever they fly (Elmore et al., 2015). Birds of prey can be good indicators of the infection in other intermediate hosts, which are lower on the food chain, and thus the presence of the parasite in the environment. The diet of a raptor, small rodents and smaller birds, paired with bioaccumulation, makes this group of animals a good potential candidate for a sentinel species. If a raptor is infected with *T. gondii*, this means it was exposed either by consuming prey that has tissue cysts or through environmental

contamination. Humans are also susceptible to the parasite and can exhibit serious disease leading to death (Derouin et al., 1989; Dubey, 2010). To mitigate for future infections in humans, it is important to understand the presence of the parasite in the environment and to examine the various circulating genotypes. This study estimated the prevalence of *T. gondii* in a total of 161 carnivorous birds of 12 different species from 40 counties in North and South Carolina, United States.

Materials and methods

Sample collection

Due to the Highly Pathogenic Avian Influenza (HPAI) outbreak in the United States, the Carolina Raptor Center (CRC), Huntersville, North Carolina, USA was not opening any carcasses within their hospital from January 2022 to present. Instead, carcasses were collected and frozen in 4 batches of about 50 birds of varying species and retrieved by our team. The carcasses were left to thaw at the University of Tennessee College of Veterinary Medicine (UTCVM), Knoxville, Tennessee, USA and necropsies were done on the birds. Samples collected included skeletal muscle, liver, heart, lung, spleen, kidney, gonads, feces, blood, and tail and wing feathers. For the purpose of this study only blood was needed and the rest of the samples were stored in a -80° C freezer for future projects.

Serology

Antibodies against *T. gondii* were detected using the Modified Agglutination Test (MAT) as described previously by Dubey and Desmonts (1987). The antigen solution used was made in the lab of Dr. Chunlei Su at the University of Tennessee Knoxville, Department of Microbiology, Knoxville, Tennessee, USA. The serum samples were diluted from 1:25 up to 1:3200. Samples that had titers >= 1:25 were considered positive.

Statistics

Statistics were done using RStudio (Version 1.4.1717). A chi-squared test was done to determine if there was a difference in proportion of sero-positive samples due to species, age, and county. P-values <0.05 were considered to be significant.

Results

A total of 161 birds were tested, consisting of 12 different species: six broad-winged hawks (*Buteo platypterus*), 19 Cooper's hawks (*Accipiter cooperii*), one Mississippi kite (*Ictinia mississippiensis*), 37 red-shouldered hawks (*Buteo lineatus*), 20 red-tailed hawks (*Buteo jamaicensis*), two sharp-shinned hawks (*Accipiter striatus*), nine black vultures (*Coragyps atratus*), five turkey vultures (*Cathartes aura*), four osprey (*Pandion haliaetus*), 43 barred owls (*Strix varia*), four eastern screech owls (*Megascops asio*), and 11 great horned owls (*Bubo*

virginianus) (Table 1). Forty different counties throughout North Carolina and part of South Carolina were represented in this study (Table 3). Age groups were separated by after hatch year (AHY; n=13), after second year (ASY; n=30), after third year (ATY; n=17), hatchling (L; n=6), hatch year (HY; n=73), second year (SY; n=19), third year (TY; n=2), and there was one unknown aged sample (Table 2). No Family showed to be more likely to be infected (p = 0.59). The overall prevalence was 18.6% (30/161). A map showing the sample distribution and positive cases is shown in Figure 1.

There were no statistically significant risk factors identified in this study. Age did not show a significant association (p=0.71), nor did species (p=0.29) or county (p=0.09).

Discussion

The overall proportion sero-positive found in this study (18.6%) was slightly lower than other published prevalence estimates in carnivorous birds in the southeastern United States. Lindsay et al. (1993) looked at 101 raptors of 5 different species from Alabama and were able to isolate the parasite from 27 (26.7%) of these birds by mouse bioassay. Love et al. (2016) showed a prevalence of 34.5% by the MAT in 281 wild raptor serum samples from 14 different species, many of which were also represented in the present study. Williams et al. (2006) found 46% (23/50) of tawny owls (*Strix varia*) to be seropositive for *T. gondii*, though these birds were chosen specifically because

they had ocular lesions which may have biased this sample group. The variability in these results indicates that further studies need to be done to understand the epidemiology of *T. gondii* in avian species. The current understanding of the behavior of *T. gondii* in birds of prey is limited. There are few studies on the disease process in raptors. Lindsay et al. (1991) fed tissue infected with *T. gondii* cysts to red-tailed hawks and Dubey et al. (1992) fed infected tissue to owls and neither determined a pattern of tissue tropism. In 1997, Mikaelian et al. diagnosed clinical toxoplasmosis in a barred owl from Canada showing tachyzoites within lesions in the liver. More studies are needed to understand *Toxoplasma gondii* in avian species.

There were no statistically significant risk factors identified in this study, however, this should be interpreted with caution. These samples were all opportunistically collected due to the inability of an individual to be released into the wild. This is a weakness of the study that may lead to a biased sample group. These birds were brought to the CRC due to injury or illness which could mean there are comorbidities that may be over represented in this sample group making true risk factors difficult to assess. A more standardized study with live caught birds could help with a more thorough assessment of risk factors.

The percent sero-positive seen in this study for the black vultures (33.3%) was unexpectedly high. In Brazil, Gennari et al. (2017) showed a proportion positive of 13% (16/121) in black vultures using the MAT with a 1:5 cutoff titer.

Ammar et al. (2021) showed a proportion positive of 12% (1/8) using the MAT with a 1:25 cutoff titer. Due to the opportunistic manner of sample collection, these results could overestimate the true prevalence in the wild population. There have been limited large scale studies reporting the presence *T. gondii* in wild birds of prey. It is important to have a baseline percent positive at different time points to be able to track the incidence of the disease in a population. This study shows that *T. gondii* is present in our avian predators and that they participate in the sylvatic lifecycle of the parasite. A more robust study is needed to determine the full picture of how birds participate in the sylvatic cycle of the parasite. This study was limited to two states, and a larger geospatial study would be needed to determine other risk factors. Future work is needed to determine risk factors for the wild population with a more targeted approach.

Conclusion

Toxoplasma gondii is emerging in more and more environments and it is becoming increasingly more important to understand the infection dynamics of this parasite (Dubey, 2004; Dubey et al., 2020). This study shows that *T. gondii* is present in our carnivorous bird population in a wide variety of species. More studies need to be done across a wider geographic scope and in more controlled settings to be able to properly assess risk factors in this population and what it means for zoonotic infection risk.

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Appendix

Tables and Figures

Table 6 Distribution of titers of antibiodies against *Toxoplasma gondii* in 163 raptors from North and South Carolina, USA

Titer	Number
<1:25	131
1:25	6
1:50	5
1:100	9
1:200	5
1:400	4
1:800	1
1:1600	0
>1:3200	0

Table 7 Prevalence of antibodies against *Toxoplasma gondii* in 12 different raptor species opportunistically collected from North and South Carolina, USA.

Species	total	positive	prevalence
Accipitridae			
Broad-winged Hawk (*Buteo platypterus)*	6	0	0.0%
Cooper's Hawk (*Accipiter cooperii)*	19	4	21.1%
Mississippi Kite (*Ictinia mississippiensis)*	1	0	0.0%
Red-shouldered Hawk (*Buteo lineatus)*	37	11	29.7%
Red-tailed Hawk (*Buteo jamaicensis)*	20	1	5.0%
Sharp-shinned Hawk (*Accipiter striatus)*	2	0	0.0%
Cathartidae			
Black Vulture (*Coragyps atratus)*	9	3	33.3%
Turkey Vulture (*Cathartes aura)*	5	1	20.0%
Pandionidae			
Osprey (*Pandion haliaetus)*	4	0	0.0%
Strigidae			
Barred Owl (*Strix varia)*	43	6	14.0%

Table 7 Continued

Eastern Screech Owl (*Megascops asio)*	4	2	50.00%
Great Horned Owl *(Bubo virginianus)*	11	2	18.2%
Total	161	30	18.6%

Table 8 Prevalence of antibodies against *Toxoplasma gondii* in raptors from 29 counties in North and South Carolina, USA.

County	total	positive	prevalence
Alexander	1	0	0.0%
Anderson	1	0	0.0%
Avery	2	2	100.0%
Buncombe	3	0	0.0%
Burke	2	1	50.0%
Cabarrus	11	3	27.3%
Cartret	1	0	0.0%
Catawba	6	4	66.7%
Cherokee	1	0	0.0%
Chester	2	1	50.0%
Cleveland	2	0	0.0%
Davidson	2	1	50.0%
Durham	1	0	0.0%
Forsyth	2	0	0.0%
Gaston	9	2	22.2%
Greenville	1	0	0.0%
Guilford	4	0	0.0%
Haywood	3	0	0.0%
Henderson	1	1	100.0%
Iredell	11	0	0.0%
Jackson	1	0	0.0%
Lancaster	2	0	0.0%
Lincoln	3	0	0.0%
McDowell	1	1	100.0%
Mecklenburg	25	4	16.0%
Montgomery	1	0	0.0%
Moore	1	1	100.0%
Pickens	1	0	0.0%
Polk	2	1	50.0%

Table 8 Continued

Randolph	1	0	0.0%
Richland	1	0	0.0%
Richmond	1	0	0.0%
Rowan	11	1	9.1%
Rutherford	1	0	0.0%
Scotland	1	0	0.0%
Stanly	2	1	50.0%
Union	10	1	10.0%
Wake	4	0	0.0%
Yardkin	1	0	0.0%
York	11	1	9.1%
Total	147	26	17.7%

Table 8 continued

County	**total**	**positive**	**prevalence**

Table 9 Prevalence of antibodies against *Toxoplasma gondii* in raptor species by age group opportunistically collected from North and South Carolina, USA. Legend: After hatch year (AHY), after second year (ASY), after third year (ATY), hatch year (HY), hatchling (L), second year (SY), third year (TY), unknown (UNK).

Age Group	total	positive	prevalence
AHY	13	3	23.1%
ASY	30	7	23.3%
ATY	17	2	11.8%
HY	73	11	15.1%
L	6	1	16.7%
SY	19	6	31.6%
TY	2	0	0.0%
UNK	1	0	0.0%
Total	161	30	18.6%

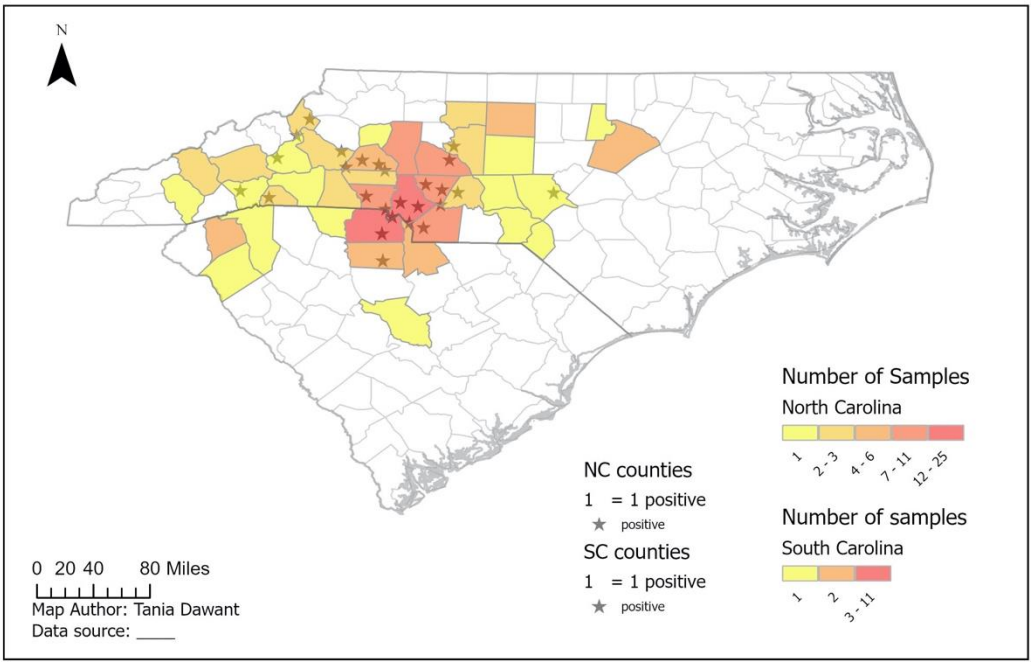

Figure 3 Positive cases for antibodies against *Toxoplasma gondii* in North and South Carolina, USA.

Sample distribution of samples collected from raptors in North and South Carolina. Positive cases for antibodies against *Toxoplasma gondii* using the Modified Agglutination Test (MAT) are represented by a star.

Chapter IV:
Investivation of the proportion positive of antibodies against *Toxoplasma gondii* in owned domestic cats (*Felis catus*) and northern fur seals (*Callorhinus ursinus)* on St. Paul Island, Alaska, USA.

Abstract

Toxoplasma gondii is a single celled parasite that can infect any warm blooded animal from birds, terrestrial mammals, including humans, and more recently in marine mammals. It has recently been emerging in naïve populations such as the California sea otter (*Enhydra lutris nereis*) and the Hawai'ian monk seal (*Neomonachus schauinslandi*) and has caused mortality events in these populations. Northern fur seal (*Callorhinus ursinus;* NFS) populations in the Priblof Islands, Alaska, USA have been steadily declining since 1998 for unknown reasons. Cats, as definitive hosts, can contaminate the environment with infective oocysts. The Pribilof Islands, including St. Paul Island, in Alaska, USA have critical NFS rookeries. Free-roaming domestic cats inhabiting St. Paul Island may be a *T. gondii* source for the NFS. Cats and seals were sampled from St. Paul and tested for antibodies against *T. gondii* to determine whether the cat population on the island may be a risk factor for fur seal infection with *T. gondii*. Blood samples from NFS were collected by the National Oceanic and Atmospheric Administration (NOAA) as part of routine monitoring. Blood samples from cats were collected as part of the Cats of the Land and Sea Veterinary Clinic. Aliquots of these samples were sent to the University of Tennessee College of Veterinary Medicine (UTCVM) for testing. Serum samples were tested using the Modified Agglutination Test (MAT) with a 1:25 cut-off titer. Fecal samples were collected opportunistically from litter boxes of owned cats. Fecal

floatations using Sheather's sugar solution were done to determine the presence of oocysts. Nineteen NFS and 30 cats were tested. Of the NFS, four (21.1%) were seropositive, and 2 (6.7%) cats were seropositive. Both positive cats were from multi-cat households with no other positive housemates. All fecal samples were negative for the presence of oocysts. This study confirms that *T. gondii* is present in both the NFS and owned cat population on St. Paul Island. This study cannot, however, confirm that the cats are the source of infection for the NFS. Further studies are needed to understand the implication of the presence of the parasite and the role it plays, if any, in the decline of the NFS population.

Introduction

The population of northern fur seals (*Callorhinus ursinus*) on St. Paul Island, Alaska, USA has been declining steadily since 1998 even with the cessation of harvest pressure on adult females (Towell et al., 2006). The cause for this decline has yet to be identified but various wildlife diseases have not been completely investigated. In this study, we investigated the presence of *Toxoplasma gondii* in the northern fur seal and local owned cat populations on St. Paul Island, Alaska, USA. The goal was to assess the potential risk of domestic feline transmission of *T. gondii* to susceptible marine mammals. *Toxoplasma gondii* is a cyst-forming, apicomplexan parasite that can infect any warm-blooded animal including cats, humans, and marine mammals (Dubey, 2010; Dubey et al., 2020). Though it can infect any warm-blooded animal, it can only complete its life cycle and go through sexual reproduction in felid hosts (Dubey, 2010). Infected cats can then shed millions of oocysts per day over 1-2 weeks during acute infection (Siński & Behnke, 2004).

Biologically, *T. gondii* has a terrestrial lifecycle but it has been emerging in aquatic environments and infecting a wide variety of marine mammals including California sea otters (*Enhydra lutris nereis*), Hawaiian monk seals (*Neomonachus schauinslandi*), and beluga whales (*Delphinapterus leucas*) (Dubey, 2004; Dubey et al., 2020; Iqbal et al., 2018; Kreuder et al., 2003; Miller et al., 2004). *Toxoplasma gondii* has caused significant disease in Hawai'ian

monk seals and has been associated with the runoff after heavy rain events in areas with a free-roaming cat population (Barbieri et al., 2016; Honnold et al., 2005; Robinson et al., 2023). Additionally, Holshuh et al. (1985) reported a case of lethal toxoplasmosis in a northern fur seal listing sand that had been contaminated with cat feces on beaches nearby as one of the possible sources of infection.

This study aimed to determine if the parasite was present in the animals on St. Paul Island, Alaska by determining the percent positive of antibodies against *T. gondii* in a selection of the local owned cat and northern fur seal populations using the rookeries on the island. In this study, we used the Modified Agglutination Test (MAT) to determine antibody titers in the seal and cat samples. In the case of *T. gondii*, as a cyst-forming parasite, the presence of antibodies not only indicates exposure but confirms current infection with the parasite due to the parasite's permanent encystment in host tissue (Derouin et al., 1989).

Materials and Methods

Sample collection

Northern fur seal samples were collected by the National Oceanic and Atmospheric Administration (NOAA) as part of their routine monitoring of the population on the Pribilof Islands in October of 2023. Aliquots of sera from these

samples sent to the University of Tennessee College of Veterinary Medicine (UTCVM) where they were tested for the presence of antibodies against *T. gondii*.

Cat samples were collected when possible as part of a locally organized mobile veterinary clinic in October of 2023. Again, aliquots of sera were sent to the UTCVM for testing. Additionally, feces were opportunistically collected from litter boxes of owned cats when available in order to determine the presence of infective oocysts shed in their feces.

Serology

The Modified Agglutination Test (MAT) was used to detect IgG antibodies to *T. gondii* in serum. Briefly, samples were serially diluted from 1:25 to 1:3200 and mixed with an antigen solution containing whole tachyzoites of the RH strain fixed in formalin at a concentration of $2x10^8$ tachyzoites/mL. Evans blue dye was used to enhance the contrast and visualization; 2-alpha-mercaptoethanol was added to the solution to break non-specific IgM antibodies. Samples that showed a titer of >=1:25 were considered positive. A positive control with a known titer was included within every plate for quality control as well as a negative control of Fetal Bovine Serum.

Fecal floatation

Sheather's sugar solution was made using the standard protocol at the University of Tennessee College of Veterinary Medicine (UTCVM). A hydrometer was not available to ensure a specific gravity of 1.275, which could be a source of error in identifying shedding cats. Fecal samples were collected from the litter boxes of the cats that were seen by the locally run mobile veterinary clinic. These samples were then mixed with the sugar solution for a final volume of approximately 10ml and run through a two-ply cheesecloth. The resulting sludge was then poured into a 15 ml conical tube and spun for five minutes at 1500 RPM. The tubes were then removed from the centrifuge and filled with sugar solution until a meniscus was formed. A cover slip was placed on each sample and left to sit for 10 minutes. These coverslips were transferred to a microscope slide and examined under a microscope at 10x magnification.

Results

Nineteen fur seal samples and 30 feline samples were tested using the MAT. Of the 19 fur seals, 4 (21.1%) were seropositive for *T. gondii* (Table 1). The titers were 1:25 (1), 1:100 (2), and 1:200 (1). These were all adult female seals collected the same year. For the cats, 2/30 (6.7%) were positive for *T. gondii* antibodies using the MAT. The titers were both 1:50. These were both from multi-

cat households with no other housemates testing positive on MAT. All cats were negative for oocysts in the feces by fecal floatation using a sugar solution.

Discussion

Given the undermined population decline of the northern fur seal and previous reports of *T. gondii*- associated mortalities in Hawai'ian monk seals we decided to test northern fur seals for *T. gondii* antibodies (Barbieri et al., 2016). The monk seal mortality events were associated with runoff after heavy rain events on the Hawai'ian main islands that contain free-roaming cats (Robinson et al., 2023). Infections in southern sea otters (*Enhydra lutrus nereis*) off the coast of California have also been associated with large runoff events (Miller et al., 2002). Similarly, St. Paul Island, Alaska has a large owned indoor/outdoor cat population with limited access to veterinary care. Scavenging of bradyzoite infected animal tissues, including seals, by the St. Paul cats, can lead to shedding of a large number of oocysts during acute infection (Dubey, 2006; Siński & Behnke, 2004).

These oocysts are extremely hardy in the environment and are very difficult to remove once they are present (Dubey, 1998, 2004; Wainwright et al., 2010). During acute infection cats shed millions of oocysts a day for a period of 1-2 weeks, meaning a single cat has the potential to heavily contaminate an environment very quickly (Siński & Behnke, 2004). These oocysts can survive for years in the environment, both on land and in water (Dubey, 1998; Lindsay et al.,

2003; Yilmaz & Hopkins, 1972). As such, even though cat populations may not be actively shedding oocysts, they should not be dismissed as being important in the spread and epidemiology of the parasite. This is further supported given the two seropositive cats were identified, indicating that they previously shed the parasites. It is unknown, however, if these cats were seropositive before arriving on the island.

Due to the opportunistic nature of sample acquisition, there is a bias in the study population. The cats sampled were those that were either fully indoor, or able to be handled. The feral population was not sampled due to inability to capture these animals. This could be a source of sampling bias where feral and outdoor cats are underrepresented. Outdoor and feral cats are presumably hunting and scavenging more, increasing their risk of exposure to the parasite (Dabritz & Conrad, 2010). More studies need to be done to investigate the route of infection for both the local cat population and the northern fur seals. Future studies are needed to determine whether the cat population on the island is the source of infection for the seals. Genotyping the strain that is present in the seal tissue would help to answer this question. Landrau-Giovannetti et al. (2022) determined the genotype of *T. gondii* that was present in Hawai'ian spinner dolphins (*Stenella longirostris*) that had previously been detected in wild pigs (*Sus scrofa*) in O'ahu, bobcats (*Lynx rufus*) in Mississippi, USA, and chickens (*Gallus gallus*) in Costa Rica and Brazil. Similarly, knowing where the strain

present in the seals has been identified could help target future studies. Genotyping can also help determine if the seals are being infected with the same or different strains, and if so if they are of different virulence (Qian et al., 2012).

The local human population relies on subsistence harvesting of the fur seals managed by the National Oceanic and Atmospheric Administration (NOAA) and the tribal governments of St. Paul and St. George Islands. The meat is commonly consumed raw, which may lead to human infection (Administration, 2024). This is a potential risk for human infection with *T. gondii* and monitoring the fur seal population for *Toxoplasma* is important to understand the eco-epidemiology and zoonotic risk.

Conclusion

This study sets a good baseline for the presence of *Toxoplasma gondii* on St. Paul Island. Future work is needed to determine the impact the parasite plays on the population as well as the transmission dynamics. A future study is planned to look back at banked serum from the fur seals to look at the percent positive in different years to help determine infection patterns and trends.

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Appendix

Tables and Figures

Table 10 Distribution of titers of antibodies against *Toxoplasma gondii* in 30 owned cats on St. Paul island, Alaska, USA. Titers <1:25 were considered negative, all other titers were considered negative.

Titer	Number
<1:25	28
1:50	2

Table 11 Distribution of titers of antibodies against *Toxoplasma gondii* in 19 Northern Fur Seals on St. Paul island, Alaska, USA. Titers <1:25 were considered negative, all other titers were considered negative.

Titer	Number
<1:25	15
1:25	1
1:100	2
1:200	1

Table 12 Prevalence of antibodies against *Toxoplasma gondii* in domestic cats and northern fur seals on St. Paul Island.

Species	Prevalence (positives)	Total number of samples
Northern Fur Seal (*Callorhinus ursinus*)	21.1% (4)	19
Domestic cat (*Felis catus*)	6.7% (2/30)	30

Conclusions and Recommendations

Free-roaming cats are heavily implicated in the sylvatic lifecycle of *Toxoplasma gondii*

Toxoplasma gondii has extremely broad reaches and has detrimental effects on populations from marine mammals to humans. It is increasingly important to understand the transmission dynamics of this parasite and the effect that free-roaming cats have on the spread of the disease. As this study shows, free-roaming and feral cats are an important part of the sylvatic life cycle for *T. gondii*.

To properly understand the transmission dynamics, we must first understand the extent of the presence of the parasite. This study shows that the geographic range and host diversity of *Toxoplasma* is extensive. We confirmed it to be present in 3 distinctly different populations, both in terms of species and location. As this parasite emerges in naïve species, it is critical to track where it is going, how it is being spread, and the effect it has on these populations.

This study also demonstrates two good candidates for sentinel populations. The diet of many raptors consists of small rodents, which are the typical intermediate host for *T. gondii*. These animals are easily infected with oocysts, and then develop tissue cysts. If a raptor is seropositive for the parasite, this indicates that its prey was likely infected. This, in turn, indicates that the environment is contaminated. Raptors come into wildlife rehabilitation centers

regularly for various reasons. A sentinel protocol could be developed in partnership with these organizations to collect blood and test the birds for *T. gondii*.

Free-roaming cats are also a good candidate for a sentinel program. There are organizations across the US that participate in the Trap, Neuter, Release (TNR) program. Blood is often collected from these cats for disease testing, which provides the opportunity to have an extremely wide spread study across the United States. These free-roaming cats cross the boundaries between wildlife and domestic animals, as well as the human population. Future studies aiming to determine geographical and socioeconomic risk factors for this parasite would be extremely beneficial. *Toxoplasma gondii* is a parasite of utmost importance for human and animal health, and further understanding is needed to properly develop mitigation strategies for future infections.

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Vita

Tania Dawant was born in Nashville, Tennessee. She attended Vanderbilt University for her undergraduate studies getting a Bachelor of Science degree in Engineering Sciences and Biological Sciences with a Minor in Engineering Management. She then went on to attend the University of Tennessee College of Veterinary Medicine for her Doctorate of Veterinary Medicine (DVM). She enrolled in the dual degree DVM/Ph.D. program after her second year following a summer research opportunity with the Center of Excellence summer research program. Her research interests include disease transmission between domestic and wild animals, the interaction and disease transmission between wild animals and humans, and the One Health approach to environmental, animal, and human health. After graduation, she will be continuing on to work in veterinary practice in Denver, Colorado to strengthen her clinical skills. She hopes to continue on to do more wildlife field work as a veterinarian. She is extremely grateful for the support from the Gerhold Molecular Parasitology Lab, her family and friends and looks forward to the next chapter.