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Toxoplasma gondii prevalence in carnivorous wild birds in the eastern United States

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ABSTRACT

Toxoplasma gondii is an important zoonotic protozoan parasite that can infect all warm-blooded animals including mammals and birds. Raptors can be intermediate hosts for *T. gondii* and the infection may be dependent on their feeding habits. In this study, we investigated the seroprevalence of *T. gondii* in ten raptor species from Florida, Pennsylvania, and Tennessee followed by a parasite bioassay on select seropositive samples. From a total of 155 raptors, we detected *T. gondii* antibodies using a modified agglutination test (cutoff 1:25) in 32 (20.6%) birds. The *T. gondii* seroprevalence was 44.8% in Falconiformes (13/29), 75% in Strigiformes (15/20), and 3.8% in Ciconiiformes (4/106). All Ciconiiformes samples (hearts and sera) were collected from Pennsylvania during nuisance wildlife removal projects and all birds were apparently healthy. Falconiform and Strigiform samples were collected from an exotics clinic in Tennessee and a rehabilitation center in Florida. All sampled birds were dead or euthanatized due to failure of rehabilitation or treatment. There was no statistically significant difference in *T. gondii* seroprevalence between Tennessee and Florida in the tested raptors. There was also no statistically significant difference in *T. gondii* exposure between males and females or adults and subadults. Mice bioassay attempts using fresh brain and/or heart tissue were performed on four seropositive birds. We isolated viable *T. gondii* tachyzoites from one red-shouldered hawk (*Buteo lineatus*) and genotyped the isolate using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of ten genetic markers. The isolated strain was designated as TgHawkFL1, which is ToxoDB PCR-RFLP genotype #28. Further research is needed to investigate the prevalence of *T. gondii* in raptors in the United States to obtain a better understanding of the life cycle, wildlife population impacts, and transmission dynamics of the parasite.

1. Introduction

Toxoplasma gondii is a protozoan parasite capable of infecting all mammals and birds (Dubey, 2010). The parasite is distributed worldwide and can cause severe clinical disease especially in immunocompromised humans and unborn fetuses (Tenter et al., 2000). *Toxoplasma gondii* oocysts, which are produced by the feline definitive host, are widely distributed in the environment including aquatic habitats (Adamska, 2018; Aramini et al., 1999) and soil (Wang et al., 2014; Frenkel et al., 1975). Investigating the level of environmental contamination with *T. gondii* oocysts is crucial to understanding its life cycle and transmission dynamics.

An indirect approach for determining *T. gondii* distribution in the environment is accomplished through detecting the parasite prevalence in intermediate hosts. Rodents and birds play an important role as intermediate hosts in the *T. gondii* life cycle because they are the main source of infection for several feline definitive hosts (Love et al., 2016; Gilot-Fromont et al., 2012). Rodents and birds also serve as a food source for predatory birds, such as raptors. For example, one common kestrel (*Falco tinnunculus*) pair can ingest approximately 520 small vertebrates in a breeding season (Geng et al., 2009). We hypothesized that raptors are intermediate hosts of *T. gondii* and susceptibility to infection varies

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among species. In this study, we aimed to estimate the seroprevalence of *T. gondii* in various raptor species and attempted to isolate the parasite from select seropositive birds to add to the knowledge on exposure and parasite diversity within raptors.

2. Materials and methods

Throughout 2016, 2017, and 2018, a total of 155 raptor carcasses or tissue samples representing ten species (Table 1) were opportunistically collected from the University of Tennessee, College of Veterinary Medicine Exotics Clinic (Knoxville, Tennessee, USA); Busch Gardens Rehabilitation Center (Tampa, Florida, USA); and Lancaster, Lebanon, Perry and Philadelphia Counties in Pennsylvania. Data were collected on birds' clinical history, age, sex, and state of origin. Bird samples collected from Pennsylvania were sampled during nuisance wildlife removal projects and all birds appeared outwardly healthy when dispatched and sampled. Birds from Tennessee and Florida died or were euthanized at veterinary clinics or rehabilitation centers due to causes other than toxoplasmosis. Most of these birds were suffering from various severe bone fractures with failure of fixation and/or infection leading to death or euthanasia. Few birds were shot, paralyzed, or blind and died or were euthanized (Supplementary Table 1).

With the exception of the two vulture species, where only serum and heart tissue samples were obtained, raptor carcasses were grossly examined for any lesions. Heart blood, heart tissues, and brain tissues were collected and stored at 4 °C until the modified agglutination test (MAT) was conducted. The MAT was performed on all raptor serum samples as previously described (Desmonts and Remington, 1980). Sera were two-fold diluted starting at 1:25 followed by addition of formalin-fixed whole tachyzoites. Known negative and positive serum samples were included in each plate as controls. The plate was incubated overnight at 37 °C. Titers equal to or higher than 1:25 were considered positive. The mice bioassay was performed using procedures approved by the Institutional Animal Care and Use Committee at the University of Tennessee (ID: # 1419). The bioassay was performed using tissue samples from four seropositive birds including two eastern screech owls (*Megascops asio*) with antibody titers of 1:100 and two red-shouldered hawks (*Buteo lineatus*) with antibody titers of 1:400 and ≥ 1:3200. Brain and heart tissues were pooled and processed for the mice bioassay as previously described (Dubey, 1998). Briefly, tissues were cut into small pieces with clean scissors and ground with 0.85% NaCl, followed by pepsin digestion at 37 °C for about 50 min. The homogenate was filtered through gauze and centrifuged. The pellet was resuspended in phosphate buffered saline and gentamicin (10 μg/ml, ThermoFisher Scientific, Massachusetts, United States) was added. Each mouse was injected with 1 ml of the digested tissue supernatant intraperitoneally. Two mice were inoculated with each pooled tissue sample. Dexamethasone (15 μg/ml, Sigma-Aldrich, Missouri, United States) was administrated in drinking water to suppress the immune system and facilitate the parasite infection in mice (Djurkovic and Milenkovic, 2001). Mice were euthanized when they displayed clinical signs of *T. gondii* infection such as ruffled fur, reduced movement, and humped back or at four weeks post-infection. Peritoneal lavage of clinically ill mice was collected, seeded on human forehead fibroblast (HFF) cell culture, and incubated at 37 °C. Tachyzoites from mice peritoneal fluid were propagated using cell culture, then purified and genotyped by multiplex multilocus nested PCR-restriction fragment length polymorphism (PCR-RFLP) using ten different genetic markers as previously described (Su et al., 2010). Mice that did not manifest clinical signs at 28 days post-infection were bled via saphenous vein and tested via MAT to determine if they were seropositive or negative for *T. gondii* antibodies.

Statistics were performed using SPSS statistical package (IBM SPSS statistics 25). The chi-squared test was performed to detect if a difference in exposure to the parasite between avian order, age, and sex categories was evident. The chi-squared test was also performed to determine the *T. gondii* seroprevalence difference between Strigiformes and Falconiformes, and an odds ratio was calculated. Because only vulture samples were collected from Pennsylvania, we only examined *T. gondii* exposure via chi-squared tests between Tennessee and Florida. P-values <0.05 were considered significant.

3. Results

A total of 155 samples were collected from three different states (Table 1). Overall, 32 (20.6%) birds were seropositive (MAT ≥ 1:25) for *T. gondii* infection. Strigiformes had a seroprevalence of 75.0% (15/20) which was significantly ($p = 0.04$) higher than Falconiformes (44.8%, 13/29) and Ciconiiformes (3.8%, 4/106). Ciconiiformes had a

Table 1
Demographic data and counts of various Carnivorous bird species tested for *Toxoplasma gondii* using Modified agglutination test.

Order[a]	Host	Total number tested	Birds from Pennsylvania	Birds from Tennessee	Birds from Florida	M/F[b] (Unknown)	Adult/subadult (Unknown)
Falconiformes	Osprey *Pandion haliaetus*	2	0	1	1	2/0	1/1
	Cooper's hawk *Accipiter cooperii*	**5**	**0**	**3**	**2**	**2/3**	**1/2 (2)**
	Red-shouldered hawk *Buteo lineatus*	**18**	**0**	**3**	**15**	**13/5**	**7/5 (6)**
	Red-tailed hawk *Buteo jamaicensis*	**3**	**0**	**2**	**1**	**2/1**	**0/1 (2)**
	Sharp-shinned Hawk *Accipiter striatus*	**1**	**0**	**0**	**1**	**1/0**	**1/0**
Strigiformes	**Barred owl** *Strix varia*	**9**	**0**	**3**	**6**	**1/8**	**6/1 (2)**
	Great-horned owl *Bubo virginianus*	**3**	**0**	**1**	**2**	**3/0**	**3/0**
	Eastern screech owl *Megascops asio*	**8**	**0**	**1**	**7**	**5/2 (1)**	**2/1 (5)**
Ciconiiformes	**Black vulture** *Coragyps atratus*	**104**	**104**	**0**	**0**	**58/46**	**0/0 (104)**
	Turkey vulture *Cathartes aura*	**2**	**2**	**0**	**0**	**2/0**	**0/0 (2)**
Total		**155**	**106**	**14**	**35**	**89/65 (1)**	**21/11 (123)**

[a] Classification of the bird species into orders is adapted from (Alsop, 2006).
[b] M/F is male to female counts.

seroprevalence of 3.8% (4/106) which was significantly lower than other tested bird orders. An odds ratio was calculated for Strigiformes and Falconiformes, indicating that Strigiformes have 3.7 times the odds of being seropositive compared to Falconiformes (OR = 3.69). Barred owls (*Strix varia*) had the highest seroprevalence of 77.8% (7/9), while turkey vultures (*Cathartes aura,* N = 2), ospreys (*Pandion haliaetus*, N = 2), and a sharp-shinned hawk (*Accipiter striatus,* N = 1) were all seronegative. (Table 2). Barred owls, eastern screech owls, and red-shouldered hawks had significantly higher *T. gondii* seroprevalence than other species, while black vultures (*Coragyps atratus*) had a significantly lower seroprevalence ($p < 0.001$). There was no statistically significant difference between males (18/89) and females (13/65) with both having a *T. gondii* seroprevalence of 20% ($p = 1.0$). We had only 32 birds with known age data. After running a chi-squared test, there was no statistically significant difference between adults (14/21, 66.7%) and subadults (6/11, 54.5%) in exposure to *T. gondii* ($p = 0.7$).

All the vulture samples were collected from Pennsylvania, and those samples had a seroprevalence of 3.8% (4/106). The four birds that were *T. gondii* seropositive were all black vultures. The *T. gondii* seroprevalence in raptors was 57% (8/14) in Tennessee and 57.1% (20/35) in Florida. There was no statistical difference in seroprevalence between raptors from the two states ($p = 1.0$).

The mice bioassay was performed using tissue samples from four seropositive birds including two eastern screech owls and two red-shouldered hawks. *Toxoplasma gondii* was isolated from one red-shouldered hawk sample with an antibody titer >1:3200. Genotyping revealed ToxoDB PCR-RFLP genotype #28. This bird was euthanized, necropsied 21 days later, and a mouse bioassay was performed six days after necropsy. The mice were asymptomatic two weeks post-infection. Twenty-six days after injecting mice with bird tissues, one mouse displayed clinical signs. The mouse was euthanized, and the peritoneal lavage was seeded on cell culture to maintain the *T. gondii* strain. In a second red-shouldered hawk sample (MAT = 1:400), mice bioassay trials were terminated on the second day post-injection due to a severe reaction in the mice and signs such as exudation of the eye, swelling, lethargy and ruffled fur. The *T. gondii* could therefore not be genotyped. Although two screech owls tested positive by MAT (1:100), inoculation of mice with their tissues yielded no *T. gondii* infections.

4. Discussion

Raptors serve as an intermediate host in the complex *T. gondii* life cycle (Atkinson et al., 2008). They can be infected via ingestion of small mammals and birds that harbor tissue cysts (Lindsay et al., 1993). This is similar to the mode of infection for felines, which acquire the infection through ingestion of *T. gondii* tissue cysts in raw meat provided by owners to domestic cats or in small animals tissues predated on by wild cats. Felines are the only host producing the resistant *T. gondii* oocyst in the environment (Dubey, 2010). For this reason, birds of prey may reflect the environmental contamination with *T. gondii* and they can be used to examine *T. gondii* epidemiology and transmission (Dubey et al., 2010).

Raptors are considered largely resistant to toxoplasmosis (Dubey et al., 1992; Lindsay et al., 1991). To date, one clinical case of toxoplasmosis was reported in a bald eagle and confirmed by immunohistochemistry (Szabo et al., 2004). Raptors are both carnivorous and scavengers and frequently ingest infected prey and carrion, which requires unique biological defense mechanisms. This concept is supported by the results obtained from testing vultures in present and in a previous study (Lindsay et al., 1993). Vultures have an acidic stomach environment and a unique intestinal microbiota that protect them from common pathogens and bacterial toxins present in degraded carcasses (Roggenbuck et al., 2014; Waite and Taylor, 2015). Their intestines are dominated by *Clostridium* spp. and *Fusobacteria* spp. and have a lower bacterial diversity than their facial skin (Roggenbuck et al., 2014). This suggests that most of the bacteria ingested with their decayed food do not survive their intestinal environment (Waite and Taylor, 2015). No data is available on the effect of the vultures' gastrointestinal tract environment on *T. gondii*, and future research is recommended.

Since toxoplasmosis is largely a food-borne disease (Mead et al., 1999), feeding habits play an important role in controlling the level of exposure to the parasite in various bird species. Although ingestion of the tissue cysts is the most common mode of infection in raptors, these birds may also be infected through direct ingestion of the oocysts (Dubey, 2002; Lindsay et al., 1993) present in contaminated water (Adamska, 2018), soil (Wang et al., 2014; Frenkel et al., 1975) or insects (Frenkel et al., 1975; Wallace, 1973). Two ospreys (*Pandion haliaetus*), which ingest mainly fish (Poole, 2019; Häkkinen, 1978), tested negative for *T. gondii* antibodies in the present study. Our results are consistent

Table 2
Toxoplasma gondii antibody titers and seroprevalence in wild bird species as tested by modified agglutination test.

Order[a]	Host	*N*	MAT titers									Seroprevalence % (95% CI[b])
			<1:25	1:25	1:50	1:100	1:200	1:400	1:800	1:1600	>1:3200	
Falconiformes	**Osprey** *Pandion haliaetus*	**2**	2	0	0	0	0	0	0	0	0	**0.0 (0.00–80.21)**
	Cooper's hawk *Accipiter cooperii*	**5**	3	0	2	0	0	0	0	0	0	**40.00 (7.26–82.96)**
	Red-shouldered hawk *Buteo lineatus*	**18**	8	2	4	1	1	1	0	0	1	**55.6 (31.35–77.60)**
	Red-tailed hawk *Buteo jamaicensis*	**3**	2	0	0	1	0	0	0	0	0	**33.3 (1.77–87.47)**
	Sharp-shinned hawk *Accipiter striatus*	**1**	1	0	0	0	0	0	0	0	0	**0.0 (0.00–94.54)**
Strigiformes	**Barred owl** *Strix varia*	**9**	2	2	4	1	0	0	0	0	0	**77.8 (40.19–96.05)**
	Great-horned owl *Bubo virginianus*	**3**	1	0	2	0	0	0	0	0	0	**66.7 (12.53–98.23)**
	Eastern screech owl *Megascops asio*	**8**	2	2	2	2	0	0	0	0	0	**75.0 (35.58–95.55)**
Ciconiiformes	**Black vulture** *Coragyps atratus*	**104**	100	2	2	0	0	0	0	0	0	**3.8 (1.24–10.12)**
	Turkey vulture *Cathartes aura*	**2**	2	0	0	0	0	0	0	0	0	**0.0 (0.00–80.21)**
Total		**155**										**20.6 (14.74–28.04)**

[a] Classification of the bird species into orders is adapted from (Alsop, 2006).
[b] CI is confidence intervals.

with Lindsay et al. (1993) who did not isolate the parasite by mice bioassay from four ospreys and Love et al. (2016) who tested one osprey by MAT that was negative. Owls and hawks have more diverse feeding habits and are more likely to ingest small mammals than ospreys. They also had a higher *T. gondii* seroprevalence in the present study and previous reports (Love et al., 2016; Dubey et al., 2010; Lindsay et al., 1993). Owl gizzards examined on necropsy in the present study often contained balls of fur and sometimes bones suggestive of small rodents. These birds were also collected from an exotics clinic or a rehabilitation center, where they were most likely introduced to small rodents as their food. The stomach from one seropositive red-shouldered hawk was full of undigested insect pieces, which is not surprising, as insects have been proven to serve as mechanical vectors for *T. gondii* (Wallace, 1973).

None of the birds sampled in the present study had clinical signs suggestive of toxoplasmosis. However, raptors with subclinical disease due to *T. gondii* might be predisposed to trauma, the reason some of the sampled birds were admitted to a rehabilitation clinic. Toxoplasmosis has been related to increased risk of having a car crash and changes in personality profiles in humans (Gohardehi et al., 2018; Kocazeybek et al., 2009). Further research is needed to determine possible correlations between *T. gondii* subclinical disease and trauma in raptors.

Although *T. gondii* infection in raptors has been reported all over the world (Gazzonis et al., 2018; Gennari et al., 2017; Aubert et al., 2008; Dubey, 2002), few studies have been performed in the United States, and most of these studies consisted of opportunistic samples similar to our study (Gerhold et al., 2017; Love et al., 2016; Yu et al., 2013; Dubey et al., 2010; Lindsay et al., 1993). The fact that most of these studies, including ours, are opportunistic exposes to bias and results should be interpreted with caution. Studies with larger sample sizes and representative samples of birds from various locations, health conditions, and feeding habits are required to better understand the disease epidemiology and ecology. Nevertheless, prevalence of toxoplasmosis in birds is likely underestimated (Lindsay et al., 1991). This is due to the absence of an effective, sensitive, and specific serological test in birds (Frenkel, 1981). We used MAT with a cutoff of 1:25 to detect *T. gondii* antibodies in the serum of sampled raptors. A direct agglutination test using formalin-fixed tachyzoites was proven useful in red-tailed hawks with 1:20 as a cutoff point (Lindsay et al., 1991). MAT has been validated for use in chickens (Dubey et al., 2016) and thus is thought to be a specific and sensitive test for detecting *T. gondii* antibodies in birds (Dubey, 2002).

Mice bioassay, cell culture, and immunohistochemistry are considered the gold standard tests for diagnosis of infections with *T. gondii* in mammals and birds (Liu et al., 2015; Atkinson et al., 2008). However, limitations on the amount of tissues used for mice inoculation make it difficult to confirm the negative result of a bioassay and the mice bioassay may also cause selection of *T. gondii* strains in cases of coinfections with multiple *T. gondii* strains (Fernández-Escobar et al., 2020; Lindsay et al., 1993). Utilizing heart and brain tissues aids in increasing the success of the mice bioassay because these tissues are most frequently infected by the parasite (Sarkari et al., 2014; Dubey et al., 1993). Also, samples from mammals or birds with higher MAT titers have greater success when used in mice bioassay trials (Gerhold et al., 2017). In our study, we were not able to isolate the parasite from the two eastern screech owls, both of which had MAT titers of 1:100. We were able to isolate the parasite from a red-shouldered hawk by mouse inoculation of heart and brain tissues of the seropositive bird (MAT titer $\geq$1:3200). Even though the hawk was euthanized four weeks before inoculating the mice, we tried the mouse bioassay on this bird because it had a higher MAT titer compared to other birds tested. The hawk was refrigerated in a sealed bag and shipped after euthanasia. The heart and brain were kept at 4 °C in a sealed ziplock following necropsy and the mouse inoculation was performed a week later. Refrigerating the whole unopened carcass may have increased the parasite persistence in tissue but this needs to be researched further.

Mice inoculated for bioassay with *T. gondii* infected tissues should be kept for six to eight weeks prior to termination (Hill and Dubey, 2002). The mouse utilized for the bioassay of the seropositive red-shouldered hawk displayed clinical signs four weeks post-inoculation. We do not know if this is due to the duration of bird tissue processing before the inoculation or factors related to the mouse immunity or *T. gondii* strain.

The isolated strain from the red-shouldered hawk was designated as TgHawkFL1, and it is ToxoDB PCR-RFLP genotype #28. This genotype was previously isolated from a cat brain in Mexico (Rico-Torres et al., 2015). *Toxoplasma gondii* type I (ToxoDB genotype#10) was previously isolated from a red-shouldered hawk in Alabama (Yu et al., 2013). An additional eight isolates were obtained from red-shouldered hawks from Alabama; however, no genotyping data are available for these isolates (Lindsay et al., 1993). In conclusion, infection with *T. gondii* is common in various raptor species depending on their preferred diet and we isolated one *T. gondii* strain (ToxoDB genotype #28) from a red-shouldered hawk from Florida.

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Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.04.010.

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