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TOXOPLASMA GONDII ANTIBODY PREVALENCE AND TWO NEW GENOTYPES OF THE PARASITE IN ENDANGERED HAWAIIAN GEESE (NENE: *BRANTA SANDVICENSIS*)

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ABSTRACT: *Toxoplasma gondii* is a protozoan parasite transmitted by domestic cats (*Felis catus*) that has historically caused mortality in native Hawaiian birds. To estimate how widespread exposure to the parasite is in nene (Hawaiian Geese, *Branta sandvicensis*), we did a serologic survey for *T. gondii* antibody and genetically characterized parasite DNA from the tissues of dead birds that had confirmed infections by immunohistochemistry. Of 94 geese sampled, prevalence on the island of Kauai, Maui, and Molokai was 21% (n=42), 23% (n=31), and 48% (n=21), respectively. Two new *T. gondii* genotypes (ToxoDB #261 and #262) were identified by PCR-restriction fragment length polymorphism from four geese, and these appeared segregated geographically. Exposure to *T. gondii* in wild nene is widespread and, while the parasite is not a major cause of death, it could have sublethal or behavioral effects. How to translate such information to implement effective ways to manage feral cats in Hawaii poses challenges.

Key words: Birds, cats, genotype, island, PCR-RFLP genotyping, strain.

INTRODUCTION

Hawaii has the highest per-capita number of endangered birds in the US (Dobson et al. 1997) due to introduced predators, habitat loss, and disease. Because birds on island ecosystems evolved without the complement of pathogens and parasites found on the continents, introduced infectious agents may play a disproportionate role in morbidity and mortality. For example, the apicomplexan blood parasite *Plasmodium relictum* was introduced into Hawaii along with its mosquito vector and likely contributed to the extirpation of several species of native Hawaiian honeycreepers (Lapointe et al. 2012). Another apicomplexan parasite, *Toxoplasma gondii*, also probably played a role in decline of Hawaii's only extant native crow (the Hawaiian Crow or "'alala," *Corvus hawaiiensis*) and prompted the removal of all crows from the wild (Work et al. 2000). *Toxoplasma gondii* exists in island ecosystems in the presence of felids, where the parasite completes its sexual cycle (Wallace et al. 1972), and the only wild felids in the Hawaiian islands are nonnative feral cats (*Felis catus*) likely introduced from Europe (Hansen et al. 2007).

The Hawaiian Goose (or "nene," *Branta sandvicensis*) is the only remaining extant native goose in Hawaii and is listed as endangered (US Fish and Wildlife Service 2004). Trauma and starvation are the major causes of death in nene; however *T. gondii* accounts for ~4% of mortalities (11/300 birds examined) and is the most-commonly encountered infectious disease in nene (Work et al. 2015). This contrasts with wild geese in the US where *T. gondii* is rarely documented as a cause of death; we found only one report of fatal toxoplasmosis in the literature in two of 12 (16%) Magpie Geese (*Anseranas semipalmata*) in a Texas zoo (Dubey et al. 2001).

However, diseases in captive animals are not apt comparisons because such animals may have a greater likelihood of exposure to domestic cats than to wild animals (De Camps et al. 2008).

Necropsy surveys are useful to assess whether pathogens cause mortality in wildlife. However, not all infections with *T. gondii* lead to mortality, so assessing exposure to parasites through serologic surveys might provide a better understanding of the parasite's distribution in ecosystems. In contrast to Hawaiian Crows where *T. gondii* appears to be more lethal (Work et al. 2000), the relatively lower mortality induced by the parasite in nene (Work et al. 2015) provides the opportunity to query how widespread it might be in a native Hawaiian birds. We also have little information on the genetic diversity of the parasite in Hawaii. Accordingly, we evaluated apparently healthy nene for exposure to *T. gondii* and genotyped *T. gondii* in archived tissues from nene that died from toxoplasmosis.

MATERIALS AND METHODS

Between 2011 and 2012, we captured 94 nene opportunistically by hand net at one site each on the island of Kauai (Kauai Lagoons golf course), the island of Molokai (Puu Hoku Ranch), and at four sites on the island of Maui (Olowalu Reservoir, Olowalu golf course, Lahaina Luna Aquaculture, and Haleakala National Park) under existing National Wildlife Health Center Institutional Animal Care and Use Committee (IACUC) protocols for capture and blood sampling of birds (IACUC ST090715). Birds were aged as described by Hunter (1995), physically examined to ensure absence of grossly visible lesions or behavioral abnormalities, and 3–5 mL of whole blood was collected from an ulnar vein using 5-mL syringes and 22-gauge needles. Blood was centrifuged, serum harvested, and stored at −70 C until shipping to the US Department of Agriculture, Agricultural Research Service, Animal Parasitic Diseases Laboratory in Beltsville, Maryland for serologic testing. Sera were tested with the modified agglutination test (MAT) as described by Dubey and Desmonts (1987) with a titer of >25 scored as positive. Sera (post- and preinfection) from an experimentally infected pig were used as controls. The MAT is highly specific for the detection of *T. gondii* antibodies in all hosts, including birds, and recently the MAT was validated using 2,066 free-range chickens from 19 countries by comparing results of serology and bioassays of chicken (*Gallus gallus domesticus*) tissues using domestic cats and laboratory mice (*Mus musculus*) (Dubey et al. 2016).

For genotyping, we selected archived frozen lung or liver from four nene that died with histologic lesions of *T. gondii*, all of which were confirmed by immunohistochemistry (Work et al. 2002, 2015). These organs were chosen based on histologic evidence of large numbers of tachyzoites. *Toxoplasma gondii* DNA was extracted from tissues of histologically positive geese using DNeasy® Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's instructions. The DNA quantification and quality were determined by NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delaware, USA). The multilocus PCR-restriction fragment length polymorphism (PCR-RFLP) typing of 10 genetic markers; SAG1, SAG2 (5′→3′ SAG2, and alt. SAG2), SAG3, BTUB, GRA6, c22-8, c29-2, L358, PK1, and Apico was done following Su et al. (2010). Appropriate positive (reference strains) and negative (H_2O) controls were included for all experiments. Positive reference strains included GT-1 #10 (Type I), PTG #1 (Type II), CTG #2 (Type III), MAS #17, TgCgCa1 #66, TgCtBr5 #19, TgCtBr64 #111, and TgRsCr1 #52 (Su et al. 2012).

Prevalence and 95% confidence intervals were calculated as described by Newcombe (1998). Effects of age, sex, and island of collection relative to antibody prevalence were evaluated with binomial regression. All statistics were done with R (Ihaka and Gentleman 1996) using an alpha level of 0.05.

RESULTS

Of 94 geese sampled, overall prevalence of *T. gondii* antibody was highest on Molokai (10/21, 48%) followed by Maui (7/31, 23%) and Kauai (9/42, 21%). For all islands, prevalence was higher in males than in females, and no immature birds were antibody positive (Table 1). Binomial regression failed to detect significant differences in prevalence between islands, ages, or sexes. Two strains of *T. gondii* were detected in liver or lung from four nene from Kauai collected in February, May, and December 2012 and January 2014 (Table 2). Three animals infected with strain #261 were found from 1 to 10 km apart on

TABLE 1. Antibody *to Toxoplasma gondii* in Hawaiian Geese ("nene," *Branta sandvicensis*) in Hawaii, USA. Sample size, number positive, and antibody prevalence against *T. gondii* with 95% confidence intervals (CI) in nene by island, sex, and age. Sex was not determined for 11 birds.

Row labels	*n*	Positive	Prevalence (%)	95% CI
Kauai	42	9	21	11–37
Maui	31	7	23	10–41
Molokai	21	10	48	26–70
Female	41	8	20	9–35
Male	42	15	36	22–52
Adult	74	26	35	25–47
Immature	20	0	0	0–20

North Kauai whereas strain #262 was found on South Kauai ~40 km away.

DISCUSSION

Prevalence of antibody to *T. gondii* in nene from the Hawaiian Islands was 21–48%. Although there was not a statistically significant difference among age groups, prevalence was higher in adults than in immature nene, where the sample size was small. In the Netherlands, adult Barnacle (*Branta leucopsis*) and Pink-footed Geese (*Anser brachyrhynchus*) had a higher prevalence of *T. gondii* antibody than did immatures (Sandstrom et al. 2013). Similar, age-related prevalence results were seen for domestic geese from China (Rong et al. 2014). However, in both the aforementioned studies, immature geese were antibody positive whereas none of the immature nene from Hawaii had evidence of exposure. Antibody prevalence in males was higher than in females, but this could be confounded by age because our sample size was not stratified by age and sex. In domestic geese from China, there was no difference in antibody prevalence between sexes (Rong et al. 2014).

A high prevalence in Molokai might be explained by the anecdotal observations of a consistently conspicuous presence of feral cats in that area compared to other sampling sites. The complete lack of immature birds with antibody suggests nene become exposed to the parasite more often with time, or perhaps a dietary shift occurs from immature to adult, thereby exposing adults preferentially. Given that nene are mainly herbivores (Black et al. 1994), it is likely that adults are exposed by eating transport hosts such as insects (Wallace 1973) or by oocysts contaminating water, soil, or vegetation (Frenkel et al. 1975).

The two genotypes of *T. gondii* found in organs of nene that died from toxoplasmosis did not fit those previously documented from Hawaiian Crows (Dubey et al. 2011) or mouflon sheep (*Ovis ammon*) from Oahu (Verma et al. 2015) nor did they fit into the three major clades Types I, II, and III (Howe and Sibley 1995). Interestingly, neither of the two genotypes fell into described haplogroups or clades documented on all continents (excepting Antarctica) including Europe (Shwab et al. 2014), from whence cats in Hawaii presumably originated, at least on the island of Hawaii, the only place where genetics of feral cats have been examined

TABLE 2. Genotyping of *Toxoplasma gondii* strains from tissues of endangered Hawaiian Geese ("nene," *Branta sandvicensis*) from Hawaii, USA, by PCR-restriction fragment length polymorphism (PCR-RFLP). No data available for apicoplast DNA.

	ToxoDB PCR-RFLP genotype	SAG1	(5′→3′) SAG2	Alt. SAG2	SAG3	BTUB	GRA6	c22-8	c29-2	L358	PK1
21729-2 Liver	#261 (new)	I	I	I	III	I	I	II	III	III	I
21774-1 Lung	#261	I	I	I	III	I	I	II	III	III	I
25022-2 Liver	#261	I	I	I	III	I	I	II	III	III	I
21850-2 Liver	#262 (new)	I	III	III	III	I	III	II	III	III	u-2

(Hansen et al. 2007). These two genotypes have not been reported from America or any other regions based on our literature search. We designated these as ToxoDB PCR-RFLP genotype #261 and #262. Three geese that died from a new atypical strain #261 suggest a common source of infection because new strains having different biologic traits and virulence are generated in feline guts by genetic recombination (Grigg and Sundar 2009). Thus, *T. gondii* in Hawaiian nene appears to have diverged genetically from its source populations in Europe. Feral cats in Hawaii have also diverged genetically from their source population in Europe; however, there is high gene flow between cat populations on the islands (Hansen et al. 2007), and this might contribute to genetic diversity of *T. gondii*. The geographic segregation of parasite genotypes from dead nene merits further investigation. Future researchers might want to confirm this pattern and ask what role feral cats play relative to birds or to the role of multiple introductions of the parasite in contributing to the geographic structure for *T. gondii*.

The relatively high rate of exposure to *T. gondii* in apparently normal wild nene contrasts with the relatively lower prevalence of parasite-induced mortalities (4%) (Work et al. 2015), thus suggesting that the parasite may not have severe population impacts in nene (McCallum and Dobson 1995). This contrasts with the higher impact the parasite had on native Hawaiian Crows (Work et al. 2000). We caution, however, that a low prevalence of clinical disease does not necessarily equate to minimal population impacts. For example, a survey of exposure to *T. gondii* in native mammals (pandemelons, *Thylogale billardierii*) from Tasmania revealed a higher prevalence of infection in road-killed versus culled animals (Hollings et al. 2013), suggesting that infection with *T. gondii* may make them more susceptible to trauma. Given that trauma is a major cause of death in nene (Work et al. 2015), this hypothesis may be worth exploring, but obtaining a control group (culled animals) would make such a study problematic, particularly for endangered species. Another approach might be to compare *T. gondii* infection or exposure rate in animals dying from trauma versus all other causes of death. The correlation of *T. gondii* infections with trauma has also been seen in humans, where those exposed to *T. gondii* are more susceptible to workplace accidents and have higher suicide rates than the general population (Flegr 2013).

Exposure and mortality of native geese (this study) and crows (Work et al. 2000) to *T. gondii* on four Hawaiian islands indicate widespread contamination of habitat with oocysts, the main source of infection for intermediate hosts such as birds (Dubey 2002). Indeed, the parasite has been documented in endangered Hawaiian monk seals, *Neomonachus schauinslandi* (Honnold et al. 2005), suggesting that contamination extends into the marine environment. In addition to contaminating ecosystems with *T. gondii*, the adverse impact of cats through direct predation on endemic wild birds in island ecosystems has been documented (Medina et al. 2014). Further work is needed to find ways to address the multiple pressures caused by feral cats on nene and other Hawaiian wildlife.

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