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NOTE

Disseminated toxoplasmosis *Toxoplasma gondii* in a wild Florida manatee *Trichechus manatus latirostris* and seroprevalence in two wild populations

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ABSTRACT: Marine mammals are important indicators for ecosystem health and serve as sentinel species for infectious agents including zoonoses. Histological examination of tissues from a stranded Florida manatee *Trichechus manatus latirostris* revealed protozoal cysts in the cerebrum and intrahistiocytic tachyzoites in the liver and caudal mesenteric lymph node. Disseminated *Toxoplasma gondii* infection was confirmed by immunohistochemistry and sequencing of the nuclear ribosomal internal transcribed spacer region of formalin-fixed tissues. The lack of baseline information on Florida manatees' exposure to this pathogen prompted a study into the seroprevalence of *T. gondii* in 2 separate geographic habitats in Florida, USA, during the winters from 2011–2014. Serum was collected during routine health assessments of 44 apparently healthy manatees from Crystal River (n = 26) on the west central coast of Florida and Brevard County (n = 18) on the east coast of Florida. Serum was screened for detection of *T. gondii* immunoglobulin G (IgG) antibodies via the modified agglutination test. Two animals from Crystal River from 2011 and 2012 (7.7%) and one animal from Brevard County from 2011 (5.6%) tested positive for *T. gondii* antibodies. Overall seroprevalence for *T. gondii* was low in the 2 sampled populations and may reflect a low seroprevalence or animal susceptibility. However, continued monitoring of this pathogen in aquatic ecosystems is warranted due to both possible anthropogenic sources and zoonotic potential.

KEY WORDS: Florida manatee · Disseminated toxoplasmosis · Modified agglutination test · Serology · *Toxoplasma gondii* · Zoonosis

INTRODUCTION

Toxoplasma gondii causes disease in a variety of mammals including marine mammals (Miller et al. 2002, Honnold et al. 2005, Dubey et al. 2008, 2009, Jones & Dubey 2010). Marine mammals are considered potential important markers for ecosystem health, since they can serve as sentinel species for infectious/zoonotic agents (Bossart 2011). *T. gondii* was linked to mortality events in southern sea otters *Enhydra lutris nereis* off the US Californian coast from 1998–2004 (Thomas & Cole 1996, Miller et al.

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2002, Dubey et al. 2003), and a study revealed a *T. gondii* seroprevalence of 52% in 305 freshly dead sea otters and 38% in 257 live sea otters (Conrad et al. 2005). Typically, *T. gondii* infects the definitive host (members of the family Felidae) when cats consume an intermediate host (i.e. bird or rodent) containing tissue cysts harboring bradyzoites. Unsporulated oocysts are then shed in the environment through feline feces, where they are able to sporulate over the course of 1–5 d. The sporulated oocysts are ingested by an intermediate host, resulting in tachyzoite replication in intermediate hosts leading to morbidity and potential mortality (Hill & Dubey 2002, Dubey 2008).

T. gondii is a waterborne pathogen with a worldwide distribution. While Lindsay & Dubey (2009) demonstrated that *T. gondii* oocysts can survive in seawater up to 24 mo, the epidemiology and pathophysiology of infection in marine mammals is not well understood. Studies along the California coast identified sandy bays with close proximity to urban areas and freshwater runoff as being associated with a higher risk of exposure (Conrad et al. 2005). Exactly how *T. gondii* is transmitted into the aquatic environment and the factors that dictate whether a marine mammal will develop clinical disease are areas of active research.

There are few reports of toxoplasmosis infection in manatees. Buergelt & Bonde (1983) reported toxoplasmic meningoencephalitis in a manatee originally observed in respiratory distress in the Indian River, St. Lucie County, Florida, USA. The animal died shortly after rescue plans were initiated, and diagnosis was determined upon histopathological and ultrastructural evaluation. More recently, Bossart et al. (2012) reported 4 cases of disseminated toxoplasmosis in stranded Antillean manatees *Trichechus manatus manatus* from Puerto Rico. The seroprevalence of *T. gondii* in free-ranging and captive Antillean manatees in Puerto Rico (Bossart et al. 2012) and Belize (Sulzner et al. 2012) is low at 3 and 7%, respectively, as compared to managed populations of Amazonian manatees *Trichechus inunguis* in Peru (Delgado et al. 2013) and Brazil (Mathews et al. 2012) at 63.2 and 39.2%, respectively.

The Florida manatee is legally protected and is the focus of numerous conservation efforts, including state, federal, non-governmental organizations and educational institutions. Overall, little is known about the impact of infectious diseases on wild manatee populations. In the present study, we present a case of disseminated toxoplasmosis in a free-ranging Florida manatee, as well as a serologic survey of *T. gondii* in 2 wild Florida manatee populations.

MATERIALS AND METHODS

Postmortem examination, histopathology and immunohistochemistry

The carcass of a 247 cm, 268.5 kg, fresh, subadult female Florida manatee was recovered from Ormond Beach at Tomoka State Park, Volusia County, on 29 March 2012 and brought to the Florida Fish and Wildlife Conservation Commission (FWC) Marine Mammal Pathobiology Laboratory (MMPL) in Saint Petersburg, Florida. The carcass was placed in a cooler overnight, and necropsy was performed the following day. Tissue samples were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, sectioned at 5 µm and stained with hematoxylin and eosin for histological evaluation. No bacteriology or virology was pursued prior to tissue fixation.

Following histological evaluation and a suspicion of toxoplasmosis, immunohistochemistry (IHC) using a rabbit primary polyclonal antisera against *Toxoplasma gondii* was performed according to Miller et al. (2001). Formalin-fixed paraffin-embedded (FFPE) tissue sections of the cerebrum, liver and lymph node were processed for IHC alongside appropriate negative and positive controls at the California Animal Health and Food Safety Laboratory Systems (Davis, CA).

Polymerase chain reaction, Sanger sequencing and BLASTN analysis

The cerebrum and appropriate negative and positive controls were tested for *T. gondii* DNA by PCR at the University of Florida Wildlife and Aquatic Veterinary Disease Laboratory. DNA was extracted from a 50 µm thick section of formalin-fixed, paraffin-embedded cerebrum using a Maxwell16 automated extractor and FFPE Plus LEV DNA Purification Kit (Promega). Initial nested PCR was performed using 2 pairs of primers complementary to portions of the nuclear ribosomal internal transcribed spacer 1 (ITS1) sequences conserved among *Hammondia* spp., *Neospora caninum* and *T. gondii* as previously described (Silva et al. 2009). When this was initially unsuccessful, a semi-nested protocol targeting a shorter sequence, more likely to be successful with DNA damaged by formalin fixation, was designed. The first round used primers CT1 (5′-TGA ATC CCA AGC AAA ACA-3′) and CT2 (5′-GCG CGA GCC AAG ACA TCC AT-3′) and was amplified as previously described (Silva et al. 2009). The second round

used novel primer ToxHamNeoITS1F (5′-TTG CCT TCT TCA TGT TGG AT-3′) with CT2 and was amplified using conditions identical to the first round. After electrophoresis in 1% agarose gels, bands were extracted and purified using a QIAquick gel extraction kit (Qiagen). Purified DNA samples were submitted to the University of Florida's Interdisciplinary Center for Biotechnology Research (ICBR) for Sanger sequencing in both directions on an ABI 3130 DNA sequencer (Applied Biosystems). Sequences were aligned, edited and primer sequences removed using CLC Genomics Workbench 7.0.3 (www.clcbio.com). BLASTN searches of the edited sequence was performed (Altschul et al. 1997).

Serologic survey

Sera were tested for *T. gondii* antibodies from a total of 44 free-ranging Florida manatees during health assessments in 2 different winter habitats, Crystal River (n = 26) and Brevard County (n = 18) from 2011–2014. The habitats include a complex of artesian springs on Florida's west central coast (Crystal River) and a region along Florida's east central coast, where manatees rely on one industrial warm-water effluent and a network of secondary warm-water sites (Brevard County). Abundance estimates for 2011 (west coast) and 2012 (east coast) (Martin et al. 2015) included a mean number of 610 manatees (95% CI: 360–950) for the northwest region and 2480 (95% CI: 1880–3180) for Brevard County (H. Edwards pers. comm.). All manatees appeared clinically healthy based on physical examination and fat measurements. Complete blood count (CBC) and chemistry were mostly unremarkable based on previously established reference intervals for hematology and chemistry analytes in Florida manatees (Harvey et al. 2007, 2009). Prior to testing, samples collected from years 2011, 2012, and January 2013 were kept frozen at −80°C. Samples collected in December 2013 and January 2014 were shipped on ice following collection at the health assessment site. Detection of *T. gondii* antibodies was performed by the modified agglutination test (MAT) as previously described (Dubey et al. 1995). A titer was considered positive for *T. gondii* at or above 1:32, as established by the University of Tennessee's College of Veterinary Medicine Diagnostic Laboratory (Knoxville, TN). Statistical analysis was performed using JMP 11 (SAS). The wild manatee sampling was conducted under Federal Fish and Wildlife Permit MA067116-2 and University of Florida's IACUC (permit 201308161).

RESULTS

Postmortem examination, histopathology and immunohistochemistry

On gross examination, the carcass was fresh. Small uterus diameter and absence of uterine scars suggested the manatee was nulliparous. There was depleted, serous abdominal fat, and a moderate amount of omental, and mesenteric fats. The abdominal cavity contained an abundant amount of red-tinged fluid. The caudal mesenteric lymph nodes were severely enlarged, with the largest measured at $9 \times 7 \times 3.5$ cm. On cut surface, the lymph node was marbled tan to pink. Axillary, anorectal and transverse septal (T-septal) lymph nodes were also enlarged. The spleen was enlarged, lobulated ($7.4 \times 4.5 \times 1.5$ cm) and was mottled pink to dark purple on serosal surface, and red to dark red on cut surfaces. There was a moderate amount of serous epicardial fat. The heart valves and chambers appeared unremarkable. Both hemidiaphragms were intact, and the lungs were mottled pink and purple on serosal surfaces. On cut surfaces, the lung parenchyma was dark plum in color and exuded a small amount of blood. The primary and secondary airways contained a small amount of red-tinged fluid, and the mucosa was dark pink. Evaluation of the brain revealed congested meninges, but no other lesions were reported. The liver was grossly enlarged, firm and the margins were rounded. The serosal surfaces were mottled dark pink to purple. Cut surfaces were friable, red and exuded dark red fluid. The stomach contained 0.5 l of brown fluid mixed with a small amount of light green vegetation, and the mucosa was green to grey in color with a few small, ulcerative lesions. The cardiac gland was hemorrhagic and extremely bloody. The remainder of the gastrointestinal tract was relatively empty of contents, and there were multiple ulcerative lesions noted in the mucosa of the duodenum. The mucosa of the proximal one-third of the small intestine was thickened and black in color, and the remainder of the proximal small intestine mucosa was dark grey in color. There were scattered, irregularly shaped yellow lesions throughout the jejunum and ileum. The colon wall was thickened with mucosal sloughing present. Both kidneys were dark purple on serosal surfaces. On cut surfaces, the cortices were dark pink and the medullae were tan to plum in color. There was a dark red line of congestion at the corticomedullary boundaries.

Histological examination confirmed all tissues were well preserved. Evaluation of the cerebrum revealed

encephalitis characterized by mild, heterophilic and histiocytic infiltrates, astrocytic hyperplasia, microgliosis, and multifocal intralesional protozoal cysts (Fig. 1a). Cysts were approximately 60 × 50 µm in diameter with a 0.5 µm cyst wall, and 2 to 3 µm long bradyzoites. There was myelin sheath swelling. Lymph nodes (multiple mandibular, cranial/caudal mesenteric) and spleen were characterized by lymphoid necrosis and depletion. The liver had patchy hepatocellular necrosis characterized by cytoplasmic pallor, fibrin exudation, and mild inflammatory infiltrate composed of macrophages and heterophils, as well as diffuse congestion (Fig. 1c). Changes in the caudal mesenteric lymph node were dramatic, as there was marked lymphoid necrosis and depletion (Fig. 1e) with expansion of the lymph node fibrin, edema, and multiple heterophilic and histiocytic foci with associated cellular debris and few 2 to 3 µm oval to crescent-shaped tachyzoites. Tachyzoites were elongate with tapering and rounded ends. The left and right cardiac ventricles had multifocal interstitial edema with associated macrophages, lymphocytes, and plasma cells. There was serous atrophy of epicardial adipose. Other histopathologic findings included mild multifocal lymphoplasmacytic gastroduodenitis, as well as submucosal edema and mild to moderate multifocal perivasculitis and vasculitis.

There was mild to moderate multifocal, transmural, fibrinous necrotizing enteritis with submucosal edema and diffuse congestion noted in the proximal and distal small intestine. There was mild to moderate, multifocal lymphoplasmacytic interstitial nephritis, along with moderate congestion and mild to moderate diffuse lymphoplasmacytic and histiocytic pyelitis. There was proliferative, diffuse, and moderate to marked necrosupperative epidermitis with intraepidermal micro-abscesses. Positive immunohistochemical staining of *Toxoplasma gondii* cysts was observed in the brain, and tachyzoites were observed in the liver and caudal mesenteric lymph node (Fig. 1). Neural cysts were typically adjacent to necrotic foci.

Polymerase chain reaction, Sanger sequencing and BLASTN analysis

The initial PCR protocol did not result in a product. The modified shorter protocol resulted in a product that was 269 bp after primers were edited out. A BLASTN search found 100% sequence identity to *T. gondii* strain RH (GenBank accession no. X75429) and 99–100% homology with 63 other *T. gondii* strains, 95–96% sequence identity with 6 available homologous *Hammondia hammondi* sequences, and 81–82% sequence identity with available *Neospora caninum* strains. The *T. gondii* sequence was deposited into GenBank under accession no. KM527503.

Serologic survey

Two animals from Crystal River from 2011 and 2012 had titers of 1:32 in the *T. gondii* MAT assay (2/26; 7.7%), and one animal from Brevard County from 2011 (1/18; 5.6%) tested positive for *T. gondii* antibodies at 1:32, yielding 6.8% seropositivity (confidence interval 1.4–18.66%). All seropositive animals from both locations were males with unremarkable blood work and good body condition. Although all seropositive manatees were males, the occurrence rate was not significantly different between females and males ($p = 0.27$; Fishers exact test). The animal from Brevard County was described as having severe skin lesions consistent with cold stress (Bossart et al. 2003). Two of the seropositive animals (from Crystal River and Brevard County) were >275 cm, therefore estimated at adult age, and one seropositive animal (from Crystal River) was 273 cm, which classifies as late subadult (O'Shea & Langtimm 1995).

DISCUSSION

Reports of *Toxoplasma gondii* infection in manatee species have increased in recent years (Bossart et al. 2012, Mathews et al. 2012, Sulzner et al. 2012, Delgado et al. 2013). Bossart et al. (2012) reported 4 cases of disseminated toxoplasmosis in Antillean manatees *Trichechus manatus manatus* from Puerto Rico based on histological, ultrastructural, and immunohistochemical evidence. The affected animals exhibited histological findings consistent with *T. gondii* infection including widespread inflammation with intralesional tachyzoites in the heart, gastrointestinal tract, liver, lung, and mesenteric lymph node. Similarly, our case demonstrated inflammation in the brain, as well as in other organ systems including the liver, heart, lung, lymph nodes and spleen. However, when compared to findings in terrestrial species and other marine mammals such as pinnipeds, cetaceans, and sea otters, inflammation present in the manatee brain was not as severe (Daily 2001, Thomas et al. 2007, Carlson-Bremer et al. 2015, Herder et al. 2015). The decreased inflammation noted in the cerebrum may be a result of sampling, since inflammatory lesions and protozoal cysts were

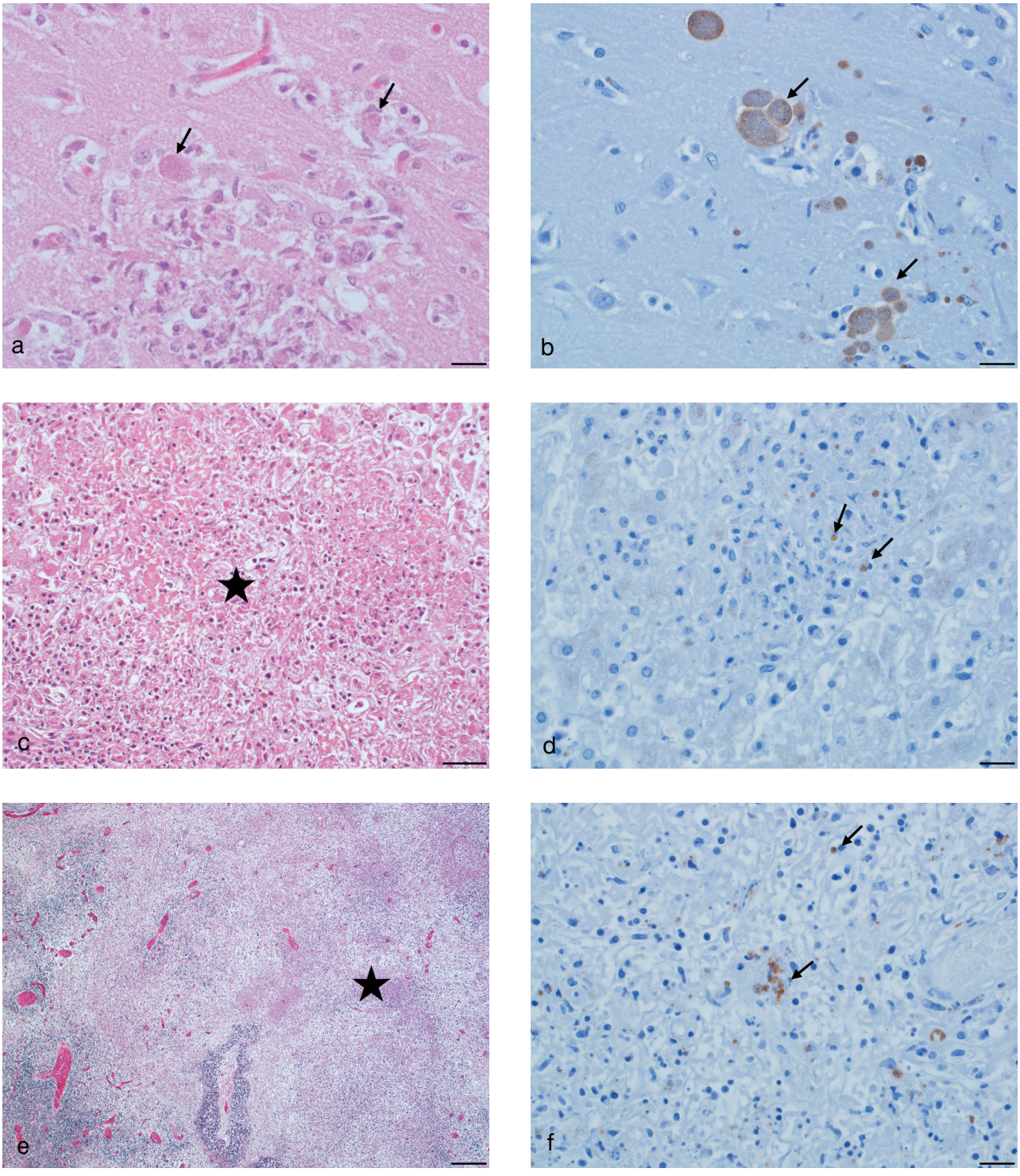

Fig. 1. *Trichechus manatus latirostris*. *Toxoplasma gondii* cysts present within the brain and tachyzoites within the liver, and lymph node. (a) Cerebrum, hematoxylin and eosin (H&E) stain. Within the brain there is disruption of the parenchyma by astrocytes, macrophages, and fewer lymphocytes. Protozoal cysts at the periphery of the inflammatory cell infiltrate (arrows). Scale bar = 20 µm. (b) Cerebrum, *T. gondii* immunohistochemistry (IHC). Prototozal cysts are stained (dark brown) within and at the periphery of inflammatory cell infiltrates (arrows). Scale bar = 20 µm. (c) Liver, H&E stain. There is hepatic necrosis (star). Viable hepatocytes are at the periphery. Scale bar = 50 µm. (d) Liver, *T. gondii* IHC. Positive staining of tachyzoites (arrows). Scale bar = 20 µm. (e) Caudal mesenteric lymph node, H&E stain. Lymph node is depleted and there are multifocal necrotic foci (star). Scale bar = 200 µm. (f) Mesenteric lymph node, *T. gondii* IHC. Positive staining of tachyzoites (arrows). Scale bar = 20 µm

noted in other tissues, such as the caudal mesenteric lymph nodes, indicating dissemination (Fig. 1). Although the use of formalin-fixed, paraffin-embedded tissues presented an initial challenge likely due to DNA degradation, a novel semi-nested PCR protocol was developed targeting a region of the *T. gondii* nuclear ribosomal ITS region. Thus, our study is the first to confirm the DNA sequence of *T. gondii* within the tissue of a sirenian.

In this investigation, a low seroprevalence of *T. gondii* was detected in both the Crystal River (n = 26, 7.7%) and Brevard County (n = 18, 5.6%) Florida manatee populations from 2011–2014. Recent studies have reported a similar low seroprevalence of *T. gondii* in free-ranging and captive Antillean manatees in Puerto Rico (n = 30, 3%) and Belize (n = 112, 7%) (Bossart et al. 2012, Sulzner et al. 2012, respectively). In contrast, a higher seroprevalence of *T. gondii* has been reported in free-living Amazonian manatees *Trichechus inunguis* taken into captivity in Brazil (n = 74, 39.2%) and Peru (n = 19, 63.2%) (Mathews et al. 2012, Delgado et al. 2013, respectively). Further research is needed to determine whether the reported differences in seroprevalence between Florida/Antillean manatee and the Amazonian manatee are due to differences in prevalence of *T. gondii* infection, differences in testing methodologies, or the possible presence of other serologically cross-reacting organisms in South America. These studies, except for Sulzner et al. (2012), used a MAT to detect *T. gondii* antibodies, and all positive titers were at the low cut-off of 1:25. In pigs, the MAT reportedly has a sensitivity of 82.9% and specificity of 90.29% (Dubey et al. 1995). As non-Boreoeutherian mammals, this may differ in manatees. The MAT is not validated in the manatee, and validation by experimental infections in manatees is not feasible given their endangered status.

Caution should be exercised when utilizing serologic assays of infectious agents in understudied species whose pathogens are not yet well surveyed. The presence of unknown closely related organisms that may cross-react is a possibility as observed in cetacean species (Nollens et al. 2008). There is antigenic similarity between *T. gondii* and *Hammondia hammondi* (Riahi et al. 1998), as well as between *T. gondii* and *Neospora caninum* (Zhang et al. 2011). As coccidial diseases of manatees to date have limited investigation, the presence of unknown antigenically related Toxoplasmatinae in manatees is possible.

Although reports of *T. gondii* infection in manatees appear to be increasing, this may be due to an increase in serologic testing and may not reflect an increase in disease. The threat it poses to the health of sirenian populations in Florida and elsewhere is unclear. Manatees serve as important sentinels for both aquatic ecosystem health as 'keystone species' in nearshore habitats and may even serve as sentinels of public health by accumulating important zoonotic protozoa (e.g. *T. gondii* in southern and northern sea otters) (Waltzek et al. 2012). Further research is needed to determine if zoonotic parasite flow from terrestrial species (e.g. *T. gondii* oocysts from domestic cats) into coastal waterways poses a significant risk to sirenian populations.

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