

NOTE

# Investigating seagrass in *Toxoplasma gondii* transmission in Florida (*Trichechus manatus latirostris*) and Antillean (*T. m. manatus*) manatees

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**ABSTRACT:** *Toxoplasma gondii* is a feline protozoan reported to cause morbidity and mortality in manatees and other marine mammals. Given the herbivorous nature of manatees, ingestion of oocysts from contaminated water or seagrass is presumed to be their primary mode of infection. The objectives of this study were to investigate oocyst contamination of seagrass beds in Puerto Rico and determine the seroprevalence of *T. gondii* in Antillean (*Trichechus manatus manatus*) and Florida (*T. m. latirostris*) manatees. Sera or plasma from Antillean (n = 5) and Florida (n = 351) manatees were tested for *T. gondii* antibodies using the modified agglutination test. No *T. gondii* DNA was detected via PCR in seagrass samples (n = 33) collected from Puerto Rico. Seroprevalence was 0%, suggesting a lower prevalence of *T. gondii* in these manatee populations than previously reported. This was the first study to investigate the potential oocyst contamination of the manatee diet, and similar studies are important for understanding the epidemiology of *T. gondii* in herbivorous marine mammals.

**KEY WORDS:** *Toxoplasma* · Toxoplasmosis · Sirenia · Oocyst

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## INTRODUCTION

*Toxoplasma gondii* is an intracellular obligatory protozoan of felids (Splendore 1908, Nicolle & Mancaux 1909). Although it typically causes no clinical disease in the feline definitive host, it causes a wide range of disease severity and even mortality in many intermediate hosts (Bowman 2013). Disease severity depends on the species, age, and immunity status of the infected intermediate host, the *T. gondii* geno-

type, and the mode of infection (Assadi-Rad et al. 1995). Ingestion of sporulated oocysts from cat fecal contamination, predation (ingestion of tissue cysts), or congenital transmission are the only modes of infection for the intermediate and definitive hosts.

Although estimates for feral cat populations within Florida range from 2.8 to 5.3 million (Levy & Crawford 2004), population estimates for feral cats across the island of Puerto Rico are not known. With the oocyst shedding potential of >1 million oocysts per

cat per defecation, contamination potential increases as the feral cat colony numbers increase (Dabritz et al. 2007). An association between contaminated water sources and sea otter mortalities (Miller et al. 2002) suggests that oocyst viability in the marine environment, including seagrass beds serving as primary food sources for manatees, may be an exposure risk for several manatee populations.

The West Indian manatee *Trichechus manatus* has 2 Endangered subspecies, the Antillean (*T. m. manatus*) and Florida (*T. m. latirostris*) manatees. Manatee grass *Syringodium filiforme*, turtle grass *Thalassia testudinum*, and shoal grass *Halodule wrightii* make up the majority of the diet of both subspecies (Mignucci-Giannoni & Beck 1998, Lefebvre et al. 1999). The first documented toxoplasmosis mortality in Florida manatees revealed tissue cysts with mild lesions but no tachyzoites present in the brain (Buergelt & Bonde 1983). Most recently, Smith et al. (2016) reported disseminated toxoplasmosis with *T. gondii* tissue cysts and tachyzoites stained by immunohistochemistry and confirmed by PCR in a Florida manatee. That study also reported a 6% seroprevalence in Florida manatees (n = 44). Bossart et al. (2012) reported the deaths of 4 Antillean manatees in Puerto Rico due to toxoplasmosis within a single year, as well as a *T. gondii* seroprevalence in Antillean manatees of 3% (n = 30).

Our study is the first attempt to concentrate oocysts from the Antillean manatee's main food source (seagrasses) to determine oocyst presence in the marine

environment and includes a *T. gondii* seroprevalence survey on Florida manatees and Puerto Rico populations of the Antillean manatee.

## MATERIALS AND METHODS

### Collections

#### Puerto Rico seagrasses

Seagrass samples (n = 33) were collected by hand from the ocean floor at 17 sites (Fig. 1) where manatees are known to forage, and placed in 0.5 l sealed plastic bags with seawater. Three seagrass species were collected (1 species bag<sup>-1</sup>, 2 bags site<sup>-1</sup>) and included turtle grass, manatee grass, and shoal grass. Shoal grass is not widespread in this region (Lefebvre et al. 1999), so it was only collected at 2 sites. Seagrass was refrigerated (4°C) at the Puerto Rico Manatee Conservation Center (PRMCC) until it was shipped on ice to the University of Tennessee College of Veterinary Medicine (UTCVM) for processing and PCR.

#### Puerto Rico Antillean manatees

Serum samples (n = 3) from Antillean manatees at the PRMCC were collected during routine health examinations (Bonde et al. 2012). Serum samples (n = 2) from stranded Antillean manatees were collected

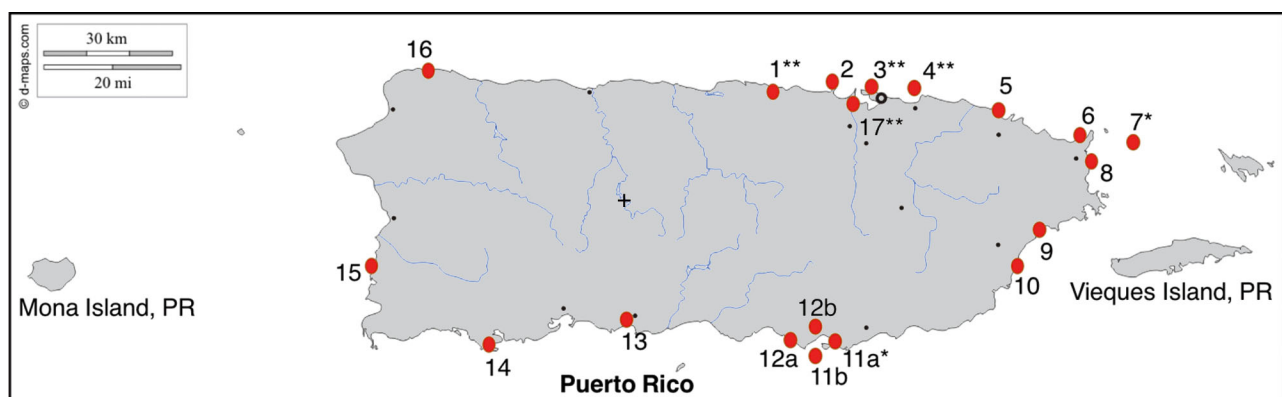


Fig. 1. Collection sites for 3 species of seagrass around the main island of Puerto Rico (+: 18.22° N, 66.59° W) in 2015 for *Toxoplasma gondii* testing. Manatee grass *Syringodium filiforme* and turtle grass *Thalassia testudinum* were collected from all locations, but shoal grass *Halodule wrightii* was only collected at locations marked with a single asterisk (\*). 1 = Villa Pesquera, Dorado; 2 = Punta Salinas, Toa Baja; 3 = Escambrón, San Juan; 4 = Isla Verde, Carolina; 5 = Bahia Beach Resort, Rio Grande; 6 = Palm Vista Seven Seas, Fajardo; 7 = Palomino Island, Fajardo; 8 = Fajardo (Marina); 9 = El Corcho, Naguabo; 10 = Playa de Humacao (Humacao Beach); 11a = Jobos Bay's Dock, Guayama; 11b = Jobos Bay's Boca del Infierno, Guayama; 12a = Las Mareas, Salinas; 12b = Central Aguirre, Salinas; 13 = La Guancha, Ponce; 14 = Guánica; 15 = Joyuda, Cabo Rojo; 16 = Isabela; 17 = Cataño. \*\*Denotes locations where seagrass collections and manatee toxoplasmosis mortalities have been documented (Bossart et al. 2012). Map source: D-maps.com ([http://d-maps.com/carte.php?&num\\_car=23081&lang=en](http://d-maps.com/carte.php?&num_car=23081&lang=en))

during necropsy, and formalin-fixed heart, lung, diaphragm, and liver were collected from 1 of these animals. Serum samples were refrigerated (4°C) and shipped on ice to the UTCVM. Once received, samples were frozen (−80°C) until testing.

#### Florida manatees

Serum and plasma samples (n = 341) from wild Florida manatees were collected by the US Geological Survey (USGS) during manatee health assessments at various capture locations (Fig. 2). Serum samples (n = 10) from dead Florida manatees were collected during necropsies by the Florida Fish and Wildlife Conservation Commission, Marine Mammal Pathobiology Laboratory. All serum and plasma samples were transported frozen on dry ice to the UTCVM and stored (−80°C) until testing.

#### Seagrass processing, concentration method, and PCR

Seagrass samples blended with Tween 20 detergent and water were strained through cheesecloth,

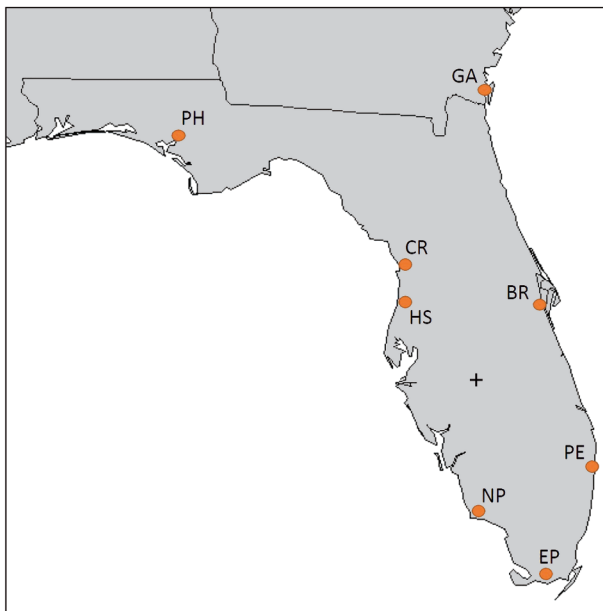


Fig. 2. Florida manatee *Trichechus manatus latirostris* US Geological Survey (USGS) health assessment locations in Florida (FL; +: 27.66° N, 81.52° W) and Georgia (GA), USA. CR = Crystal River, FL; HS = Homosassa Springs, FL; EP = Everglades National Park, FL; GA = South Cumberland Island, GA; BR = Brevard County, FL; PH = Panhandle, FL; PE = Port Everglades, FL; NP = Naples, FL. Figure provided by USGS

and underwent a series of rinsing, centrifuging, and decanting supernatant until a small pellet of sediment remained similar to that described by Gerhold et al. (2015). An aliquot of the pellet was concentrated using centrifugal flotation with sucrose solution as described by Bowman (2013) to determine if oocysts were present.

The rinsed, concentrated products underwent DNA extraction using the ZR Fecal DNA Prep Kit (Zymo Research). Primers TOX4 and TOX5 that amplify a 529 bp high-copy-repeat in *Toxoplasma gondii* were used for the PCR procedure described by Homan et al. (2000). DNA extracted from *T. gondii* oocysts that were obtained from fresh cat feces was used as a positive control, and water was used as a negative control. PCR products were separated on a 1% agarose gel with ethidium bromide, and amplified DNA was visualized using UV light. Target PCR products were excised, purified, and submitted to the University of Tennessee's sequencing laboratory. Resultant sequences were aligned in Sequencer, and consensus sequences were subjected to a BLAST analysis in GenBank.

#### Modified agglutination test (MAT) and nested PCR

All sera and plasma collected from manatees were tested for *T. gondii* immunoglobulin G (IgG) antibodies using the MAT test kit (Biomerieux). The procedure incorporated modifications to maximize sensitivity and specificity (Dubey & Desmonts 1987, Dubey et al. 1995) by using whole formalin-fixed tachyzoite antigen to detect antibodies, and each plate included a goat-derived positive and negative control from the kit. Following the standard MAT interpretations established at the UTCVM, Diagnostic Parasitology Laboratory, an IgG titer  $\geq 1:32$  is the lowest detectable positive titer. Confidence in interpretation increases with higher titers due to the subjectivity of the visual determination.

A whole blood clot (n = 1) from 1 Antillean manatee testing inconclusive on the MAT underwent nested PCR following the procedure of Su et al. (2010).

## RESULTS

### Puerto Rico seagrasses

No *Toxoplasma gondii* oocysts were recovered from seagrass samples. Sequences from 2 PCR bands were identified as bacterial by BLAST analysis.

### Florida and Antillean manatees

None of the Antillean and Florida manatee sera and plasma samples (0%; n = 356) was positive for *T. gondii* antibodies using the MAT. One necropsied Antillean manatee was inconclusive on MAT; however, histological examination of this animal's tissues showed no evidence of *T. gondii* cysts or tachyzoites, and nested PCR on the blood clot from this animal was negative.

## DISCUSSION

### Puerto Rico seagrasses

Determining the significance of our findings is difficult given the limited sample size and lack of published reports on *Toxoplasma gondii* oocyst contamination of seagrasses. However, we do know that freshwater runoff, coastal development, biofilm, invertebrate movement, filter-feeding fish, and bivalves have been implicated as facilitators for *T. gondii* distribution in the ocean and as modes of transmission to marine mammals (Lindsay et al. 2001, Shapiro et al. 2014, VanWormer et al. 2016). Oocyst accumulations within filter-feeding bivalves and aquatic snails affiliated with biofilm in brown kelp forests have been directly connected to toxoplasmosis-related mortalities in sea otters (Miller et al. 2002, Mazzillo et al. 2013). Given these connections, investigations of bivalves and aquatic snails in manatee environments is recommended.

### Florida and Antillean manatees

Our study indicates a lower seroprevalence (0%) of *T. gondii* in both subspecies than previous reports of 3% (n = 30) in Antillean manatees (Bossart et al. 2012) and 6% (n = 44) in Florida manatees (Smith et al. 2016). Possible explanations for the variation in seroprevalence reports in these manatee subspecies include: our sample size is too low, titers are below MAT-detectable limits, or the behavior of wild manatees reduces their exposure risk to the infective *T. gondii* oocysts and the cats that shed them as compared to captive or range-dependent animals.

The MAT is considered the gold standard in *T. gondii* testing due to its high sensitivity (82.9%) and specificity (90.29%) in pigs without the need for a host species-specific conjugate (Dubey et al. 1995). Although the MAT has been used extensively in

many terrestrial and marine species, it has not been validated in these species and does not determine the presence or absence of disease. Given previous reports of low titers (1:25 and 1:32) in both subspecies of the West Indian manatee (Bossart et al. 2012, Smith et al. 2016) and in the Amazonian manatee (Delgado et al. 2013), it is possible that our study titers were below the detection limit of 1:32. The high seroprevalence in Amazonian manatees often without signs of clinical disease suggests that *T. gondii* infections in immunocompetent manatees are likely subclinical (Delgado et al. 2013). *T. gondii* infection in the Antillean and Florida manatee populations is probably rare and suggests a low risk of parasite infection to sirenia. This risk is likely higher in areas of wetland loss or situations where a point source contamination (e.g. high feral cat numbers associated with natural watershed areas) can be identified, or in captive animals.

Serology, bioassay, and genotyping should be components of future studies if we are to draw clear conclusions regarding the source, transmission routes, and infection status of *T. gondii* to marine mammals. This was the first study to investigate the potential oocyst contamination of the manatee diet. Similar studies are important for understanding the epidemiology of *T. gondii* in herbivorous marine mammals.

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