

**Epidemiology of *Toxoplasma gondii* and a survey of other parasites in
the West Indian manatee (*Trichechus manatus*)**

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Degree
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DEDICATION

To my loving and ever patient husband, Brian. Thank you for your unfailing support and showing as much dedication to this degree as me.

To my beautiful, smart, and spunky baby girl, Marisol Olivia Wyrosdick. Your passion for biology and reading inspires me to be a better scientist for the next generation of researcher.

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ABSTRACT

Toxoplasma gondii is a protozoan parasite of felids reported to cause morbidity and mortality in the two subspecies of the West Indian Manatee (*Trichechus manatus*): the Antillean manatee (*Trichechus manatus manatus*) in Puerto Rico and the Florida manatee (*Trichechus manatus latirostris*). Sera or plasma (n=343) were collected from live-capture *T. m. latirostris* through the US Geological Survey (USGS) manatee health assessment program and serosanguinous fluid (n=10) were collected from necropsies conducted by the Marine Mammal Pathobiology Laboratory (MMPL). Additionally, serum or serosanguinous fluid samples (n=5) were collected from rehabilitated or necropsied *T. m. manatus* at the Manatee Conservation Center (MCC) of Puerto Rico. Free-roaming cat (*Felis catus*) serum samples (n=25) from Puerto Rico were collected for *T. gondii* testing. All serum, plasma, and serosanguinous fluid samples (n=383) were screened using the Modified Agglutination Test (MAT) to determine *T. gondii* seroprevalence in manatee and cat. All manatee samples tested on the MAT were seronegative for *T. gondii* except for one Antillean manatee which was inconclusive. Nested PCR on the blood clot of this animal was negative. Seroprevalence of *T. gondii* in cat sera was 16%. Fecal samples (n=79) from free-roaming cats in Florida were collected to determine the shedding prevalence of *T. gondii* oocysts. No oocysts consistent with *T. gondii* morphology were detected in any felid samples. This is the first attempt to connect *T. gondii* genotypes in manatees to the hypothesized contamination source, feral cat populations. To determine manatee habitat contamination, 33 seagrass samples (*Thalassia testudinum*, *Syringodium filiforme*, and *Halodule wrightii*) from 19 sites on the main island of Puerto Rico were collected for concentration and PCR detection of *T. gondii* oocysts. No *T. gondii* oocysts were detected in any of the seagrass samples. Freshly defecated fecal samples (n=24) were also collected from *T. m. latirostris* (n=21) and *T. m. manatus* (n=3) for parasite surveillance. Parasite ova found included: 2 *Chiorchis* spp., *Pulmonicola cochleotrema*, *Moniligerum blairi*, *Nudacotyle undicola*, 2 *Eimeria* spp., *Heterocheilus tunicatus*, and several unidentified eggs, cysts, and larvae. To the author's knowledge, this is also the first report of *Eimeria* spp. in *T. m. manatus* from Puerto Rico.

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CHAPTER I
Literature Review

Wyrosdick, H. and J. Schaefer, *Toxoplasma gondii*: history and diagnostic test development, *Animal Health Research Reviews*: Cambridge University Press, 16, 02, p. 1-14, reproduced with permission.

Introduction to the West Indian Manatee

The Sirenians are one of four taxonomic orders in the unique group called the ‘subungulates’ which lack clavicles and hooves but have nails and hypsodont molariform teeth that are horizontally replaced. The Sirenia includes 3 species of manatees and the dugong. The West Indian Manatee is further divided into 2 subspecies the Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*; Reeves *et al.* 2002). The Florida manatee’s range is very restrictive to warm water sites in coastal areas of Florida in the winter; however, its range is much more varied in the summer, with some animals migrating as far north (Atlantic) as Rhode Island and as far west (Gulf of Mexico) as Texas (Hamilton & Puckett 2006). The Antillean manatee’s range is dramatically varied with fractured populations throughout the Caribbean, along the eastern coast of Central America, the northeastern coast of South America, and rarely venturing east toward the Bahamas (USFWS West Indian Manatee Report).

Both subspecies are listed as endangered; however, the Florida subspecies population is listed as stable, whereas the Antillean subspecies population is listed as decreasing. The majority of deaths of the Antillean manatee in Puerto Rico and the Florida manatee have been contributed to human interaction including collisions with watercraft, reduction of habitats and food source contamination from anthropogenic pollutants (Burgelt and Bonde 1983, Mignucci-Giannoni *et al.* 2000). Additionally, some Florida manatee deaths are attributed to cold water stress, indicating lack of sufficient warm water sites to sustain the rising manatee populations (Laist *et al.* 2013). Another component of manatee morbidity and mortality may be a result of infectious diseases and toxins such as papillomavirus, toxoplasmosis, and brevetoxicosis. Both manatee subspecies are protected from hunting but conservation efforts have primarily benefitted the Florida manatee to date (IUCN, “*Trichechus manatus*.”). Stronger conservation efforts focused toward the Antillean manatee and better understanding of infectious disease effects on the population in Puerto Rico is needed to stabilize this population. Sirenian parasites and theriothasonotic parasites (transmittable from domestic animals to wild animals) are also of concern when investigating mortalities in these subspecies (“*Georgis’ Parasitology for Veterinarians: Introduction, edition 9*”).

All reports of parasites in the West Indian manatees have been from adult parasites collected at necropsy with the exception of two studies that performed microscopic examination on the feces (Bando *et al.* 2014, Upton *et al.* 1989). Trematodes reported in both subspecies of the West Indian manatees include *Chiorchis fabaceus*, *C. groschafti*, *Moniligerum blairi*, *Nudacotyle undicola*, and *Pulmonicola cochleotrema* (previously *Cochleotrema cochleotrema*; Beck & Forrester 1988, Llavina *et al.* 2009, Mignucci-Giannoni 1999a, b). Only one nematode has ever been reported in any manatee species (*Heterocheilus tunicatus*). A single report describes one specimen of an immature stage of an Anoplocephalan tapeworm from a Florida manatee from Dade County and the authors suggest that this was an aberrant infection (Beck & Forrester 1988). Three *Eimeria* spp. have been reported, two in the Florida manatee (*E. manatus*, *E. nodulosa*) and one more *Eimeria* sp. (*E. trichechi*) in the Amazonian manatee. A

Cryptosporidium sp. was also reported in the Amazonian (*T. inunguis*) and Antillean manatees (Laison *et al.* 1983, Upton *et al.* 1989, Borges *et al.* 2009, 2011). No other endoparasites have been reported in manatees.

History

The history of *Toxoplasma gondii* discovery, identification of hosts, description of its life cycle, diagnostic strategies and treatment have been thoroughly reviewed with ongoing research efforts and clinical experience continuing to advance our understanding of this important pathogen (Dubey 2008, Dubey 2010, Ferguson 2009). One investigative team in the early 20th century noted the presence of protozoal organisms in their research gundy (*Ctenodactylus gundi*) in Tunisia and another in rabbits (*Oryctolagus cuniculus*) in Brazil; both initially attributed the observation to *Leishmania* sp., before the former group realized it was a previously undescribed species and named it *T. gondii* (Nicolle & Manceaux 1909, Splendore 1908). One year later, a clinical case of toxoplasmosis was described in the domestic dog (*Canis familiaris*) in Italy, an early insight into the distribution of this parasite across divergent animal species and geographic areas (Mello 1910). Tissue cyst structures were first noted in a group of rabbits and mice and described cytologically about 20 years after the organism's initial identification (Levaditi *et al.* 1928). Early investigators depended on microscopic work and staining techniques. The advent of serological tests and molecular diagnostic techniques led to a rapid increase in understanding of the parasite and its role in clinical disease.

Life Cycle

The demonstration of successful *T. gondii* transmission by congenital, carnivorous and fecal-oral routes stimulated research interest into the parasite's life strategy that allows survival in widely diverse hosts (Dubey *et al.* 2008a,b, Frenkel *et al.* 1970, Hutchison 1965, Weinman & Chandler 1954). *Toxoplasma gondii* has a complex life cycle that can only be completed in the feline definitive host (DH) (**Figure 1**). The most prolific and destructive stage is the tachyzoite, formerly called the trophozoite or endozoite. This is the active stage of the parasite and the most likely stage to cause clinical disease. The second life stage is the bradyzoite, which is the slow-replicating chronic stage that is most commonly within a tissue cyst. The oocyst is the life stage that results from sexual reproduction within the gut and is shed in the feces of the DH, the wild or domestic felid (Dabritz *et al.* 2007). In intermediate hosts (IH), sporozoites released from sporocysts penetrate through intestinal epithelial cells and migrate to the lamina propria, where numerous different types of cells are invaded, and two tachyzoites are produced by approximately 12 hours post infection. Following invasion of additional tissues with tachyzoites, tissue cysts have been noted to start forming by 6 days post infection and these likely persist for the life of the animal. The life cycle in felids can follow this same course, making felids uniquely both potential intermediate and definitive hosts. The latter, due to their hosting the sexual stages of *T. gondii*, in which gametogony is noted after intestinal invasion, leading to the production of sexual stages (female macrogamonts and male microgamonts) by 2 days after infection.

Following the formation of zygotes and oocyst walls, the host cell ruptures, discharging oocysts into the intestine (Dubey 2010). Both IH and DH life cycles require evasion of the host's immune system to proceed successfully, and the same white blood cells that respond to the

presence of the parasite are utilized to spread infection throughout the host (Denkers *et al.* 2012). Each cat has the potential to shed over 1 million *T. gondii* oocysts during the initial phase of the infection (Dabritz *et al.* 2007). This increases the contamination potential exponentially. The oocysts become infective when they sporulate in as little as 24 hours in the environment (www.cdc.gov/parasites/toxoplasmosis/biology). The prepatent period (PPP) between ingestion of infective stages and shedding of oocysts in domestic cats varies depending on the stage of the parasite initiating infection (Frenkel *et al.* 1970). The PPP after tissue cyst ingestion is considerably shorter (3-10 days) than after sporulated oocyst ingestion (18 days) in cats (Weiss & Kim 2007). The abbreviated PPP following tissue cyst ingestion in the cat with the similarly brief time from infection to tissue cyst production in the IH, contamination can spread quickly through the host/prey ecosystem, e.g. felids and rodents.

Human Toxoplasmosis

Though felids act as the only DH, birds and mammals, including humans, are known to serve as IH (Assadi-Rad *et al.* 1995). Toxoplasmosis was established as a disease of humans by the observation of a case of congenital transmission in 1939 and experimental transmission to animals from this case was demonstrated (Ferguson 2009, Wolf *et al.* 1939a). The recognition of the potential importance of toxoplasmosis in human health precipitated development of diagnostic tests to allow antemortem detection. Transmission to an IH occurs via ingestion of sporulated oocysts from felid fecal contamination, ingestion of tissue cysts from raw or undercooked infected meat, or congenital transmission (transplacentally). In a European study, contact with domestic cats was not identified as a risk factor for seropositivity in humans. Rather, eating undercooked meat, contact with soil and travel outside of Europe, the United States and Canada were significant. In all, 30-63% of infections were attributed to consumption of undercooked or cured meat and 6-17% to soil contact (Cook *et al.* 2000). The lack of a requirement for a DH in the parasite's propagation was noted by oral infection of pigs with mouse tissue, and of mice with pork, also establishing the potential risk to humans from meat consumption (Weinman & Chandler 1954). In the United States, disease transmission in adults typically occurs by ingestion of infected meats, ingestion of contaminated vegetables, or aspiration of aerosolized sporulated oocysts from cat litter boxes. In addition, there are an estimated 400-4,000 new cases of congenital toxoplasmosis annually in the United States (www.cdc.gov/parasites/toxoplasmosis, Lopez *et al.* 2000). The prevalence of infection in humans varies widely, with a seroprevalence of 38% previously noted in the United States and a range of 4-92% noted in other countries. The fecundity of oocyst production (an average of 10,000,000 oocysts shed per infected cat has been estimated) and their environmental durability, with survival times of greater than one year noted in both natural and experimental settings, contribute to the ubiquity of the infection (Dubey 2010). However, an estimate from the latest National Health and Nutrition Examination Survey (2009-2010) continues to recognize a trend towards decreasing rates of infection in the overall United States human population with a seroprevalence of 22.5% noted (Jones *et al.* 2014).

The pathogenicity of toxoplasmosis varies depending on the age and species of host infected, the mode of infection, and the *T. gondii* genotype present (Assadi-Rad *et al.* 1995). Mortality due to toxoplasmosis in humans occurs in very few cases, most notably due to reactive encephalitis in immunocompromised patients and abortion in naïve mothers on first exposure

(Dabritz & Conrad 2010). However, morbidity due to toxoplasmosis can take many forms, related to the diverse tissue types that can be invaded in IHs. Clinical signs and pathology in adults range from no clinical signs to ocular disease, lymphadenopathy, tissue-specific lesions and localized edema, and death (Weiss & Kim 2007). It was linked to potentially fatal central nervous system disease in AIDS patients in the late 1980's (Luft *et al.* 1983, Tuazon 1989, Harden & Hair 1994). Congenital toxoplasmosis occurs when a mother is infected during pregnancy and commonly causes fetal death, though severity of disease and probability of congenital transfer depends on stage of pregnancy at time of maternal infection (Weiss & Kim 2007). Though incidence of congenital transmission increases as pregnancy progresses, the pathogenicity of the congenital disease tends to decrease in severity (Rorman *et al.* 2006, Lynfield & Guerina 1997, Remington & Desmonts 1990, Freeman *et al.* 2005). The most commonly observed clinical and pathological signs in infants range from no clinical disease to chorioretinitis, blindness, severe hydrocephaly, meningoencephalitis, encephalomyelitis, intracerebral calcification, mental retardation, abortion, and postpartum death (Frenkel and Friedlander 1951, Hogan 1951, Janku 1923, Remington *et al.* 2001, Sabin 1942, Torres 1927, Weiss & Kim 2007, Wolf *et al.* 1939a, 1939b). Methods to reduce human exposure include: proper cooking of meat and vegetables, hand hygiene after handling meat or soil contact, preventing predation by cats, providing litter boxes and controlling feral cat populations (Dabritz & Conrad 2010). However, the recognition of *T. gondii* genotypes in wildlife capable of infecting humans implies a sylvatic life cycle that can independently perpetuate the parasite's population (Dubey *et al.* 2008a, Wendte *et al.* 2011).

Infection Sources/Reservoirs and Aquatic Contamination

The search for IHs for *T. gondii* has been accomplished by numerous investigators with notable success (Dubey 2010). Estimated numbers of feral cat populations within the United States have reached into the tens of millions (Torrey and Yolken 2013). Current indication that any warm-blooded animal can serve as an IH for the organism and the identification of marine invertebrates as potential transport hosts significantly broadens the number of animals of potential interest (Lindsay *et al.* 2003a). Mortality in southern sea otters (*Enhydra lutris nereis*) attributed to toxoplasmosis demonstrates how pervasive environmental contamination with oocysts is in both terrestrial and marine environments (Cole *et al.* 2000). The ability of *T. gondii* to infect a wide host range and to have a broad geographic distribution (described from all continents) makes it one of the most common protozoal parasites in the world.

Despite the strictly terrestrial nature of the DH, marine mammal morbidity and mortality have been attributed to toxoplasmosis. The first major case in marine mammals occurred in sea otters off the coast of California (Miller *et al.* 2002). These infections were linked to coastal freshwater runoff of feces from feral cat populations (Cole *et al.* 2000, Miller *et al.* 2002) and Shapiro *et al.* determined that further dispersal of the oocysts into the marine environment was facilitated by aquatic polymers (biofilm) and invertebrates (2014). Lindsay *et al.* (2003) determined that not only could *T. gondii* oocysts sporulate in seawater within 3 days, they also remain viable in seawater for up to 6 months. The established feral cat colonies in the continental United States increase exponentially every year and estimated numbers of these populations within the United States have reached into the tens of millions (Torrey and Yolken 2013). Feral cat colonies exist across the island of Puerto Rico but population numbers are not

known. Current claims that the Trap, Neuter, Release program has cut the population in half have not been substantiated through scientific studies (unpublished data). Though the stray cat population on the main island of Puerto Rico has never been surveyed for *T. gondii*, Dubey *et al.* (2007) found virulent genotypes of *T. gondii* from feral cats on the neighboring Mona Island, PR.

The diversity of the marine mammal habitats and foraging habits make it challenging to determine how many are infected with *T. gondii* and to elucidate modes of infection for these animals. Coastal runoff may explain infection in the benthoneuritic zone but does not explain discovery of the disease in more pelagic marine mammals. A few studies have suggested the dispersal of *T. gondii* oocysts is possible to more pelagic fish-eating marine mammals via transport host or mechanical vector. Eastern oysters can filter and maintain viable *T.gondii* oocysts from the seawater within their tissues for up to 85 days (Lindsey *et al.* 2001, Lindsay *et al.* 2005). Migratory filter feeding fish (including sardines and anchovies) have been implicated in the dispersal of oocysts into the marine environment by serving as transport hosts or biotic vectors. In addition to the oocysts being maintained in the alimentary tract of the fish, 30% of fish retained oocysts that were infective to mice (Massie *et al.* 2010).

Marine Mammal Toxoplasmosis

Despite the ability to detect infection using antibody testing, understanding the pathogenicity and progression of toxoplasmosis in marine mammals is difficult. More than twenty seroprevalence studies have been conducted on more than fifty species of cetaceans, pinnipeds, marine otters, etc.; however, there are much fewer studies that describe the histological changes caused by toxoplasmosis and even fewer that can connect toxoplasmosis to clinical signs before death (Oksanen *et al.* 1998, Lambourn *et al.* 2001, Cabezon *et al.* 2004, Measures *et al.* 2004, Murata *et al.* 2004, Conrad *et al.* 2005, Omata *et al.* 2005, Aguirre *et al.* 2007, Forman *et al.* 2009, Pretti *et al.* 2010, Santos *et al.* 2011, Alvarado-Esquivel *et al.* 2012, Mathews *et al.* 2012, Sulzner *et al.* 2012, Delgado *et al.* 2013).

Cetaceans have been the most extensively studied for toxoplasmosis, with both serological surveys (**Table 1**) and clinical cases reported (**Table 2**). The most commonly infected tissues include brain, heart, lungs, adrenal glands and lymph nodes. The most common histological findings include encephalitis, gliosis, encephalomeningitis, arteritis and myocarditis. Lesion descriptions vary depending on the stage of *T. gondii* found. Tissue cysts are often associated with minimal tissue damage and occasionally lack evidence of inflammatory reactions from the host tissues (Cruickshank *et al.* 1990). Lesions are most commonly described as multifocal areas of necrosis, and associated with non-suppurative inflammation with intralésional *T.gondii* tachyzoites (Migaki *et al.* 1990, Cruickshank *et al.* 1990, Inskeep *et al.* 1990, Domingo *et al.* 1992, Mikaelian *et al.* 2000, Resendes *et al.* 2002, Bowater *et al.* 2003, Dubey *et al.* 2008, Di Guardo *et al.* 2010, Pretti *et al.* 2010, Mazzariol *et al.* 2012, Arbelo *et al.* 2013, Gonzales-Viera *et al.* 2013). Meningoencephalitis has been most commonly reported within the neuropil of the brain (Bowater *et al.* 2003, Di Guardo *et al.* 2010). Co-infections with *Morbillivirus* and *Sarcocystis* sp. have been reported and can result in death (Mazzariol *et al.* 2012, Miller *et al.* 2001).

Significantly fewer reports of toxoplasmosis exist for pinnipeds. Lesion descriptions in pinnipeds are similar to those described in cetaceans (Van Pelt & Dieterich 1973, Migaki *et al.* 1977, Holshuh *et al.* 1985, Dubey *et al.* 2003, Dubey 2004) but the tissues affected are less

consistent. Lesions in pinnipeds have been reported in the heart, brain, liver, lungs, spleen, and gastrointestinal tract (Ratcliff and Worth 1951, Migaki *et al.* 1977, Holshuh *et al.* 1985, Dubey *et al.* 2003, Dubey 2004) but unlike in cetaceans, only three cases report lesions in the brain of pinnipeds (Holshuh *et al.* 1985, Dubey 2004). Dubey *et al.* (2003) reported multiple tachyzoites replicating within the myocardial valves of a California sea lion.

The discovery of toxoplasmosis in marine otters off the coast of California by Cole *et al.* (2000), Krueder *et al.* (2003) and Miller *et al.* (2002, 2008a, b) spurred many studies on the mechanisms of infection from the entirely terrestrial feline DH. The clinical presentation of toxoplasmosis and descriptions of severe necrotic lesions in the brain on histology in marine otters closely resemble those seen in other marine mammals (Cole *et al.* 2000, Kreuder *et al.* 2003, Thomas *et al.* 2007, Miller *et al.* 2008b).

Toxoplasma in Manatees

Numerous reports of *T. gondii* infection in species of marine mammals have been described, but three reports exist for manatees and for the dugong (**Table 3**). Due to its predominantly herbivorous diet, the primary mode of transmission for the manatee is presumed to be accidental oocyst ingestion while feeding on aquatic vegetation (seagrasses), especially given the report of oocysts persistence in seawater (Frenkel 1990, Jacobs *et al.* 1960, Lindsay *et al.* 2003b). Coastal runoff is suspected to be the most significant source of seawater contamination (Miller *et al.* 2002). In Puerto Rico and coastal Florida sizeable populations of feral cat colonies may serve as point sources for contamination of marine waters with viable *T. gondii* oocysts. Therefore, a possible correlation between the prevalence of *T. gondii* infection in the manatees and the feral cat populations is likely.

Serosurvey studies have found a wide range of seroprevalence in manatees, from 0% in captive West Indian manatees in Mexico to as high as 63% in the Amazonian manatees (**Table 4**) (Alvarado-Esquivel *et al.* 2012, Bossart *et al.* 2012, Delgado *et al.* 2013, Dubey *et al.* 2003, Mathews *et al.* 2012, Smith *et al.* 2015, Sulzner *et al.* 2012). Seroprevalence is a good indicator of infections in wild populations but cannot be linked to clinical disease.

Death due to toxoplasmosis has been reported in six manatees and one dugong (Buergelt and Bonde 1983, Dubey *et al.* 2003, Ladds 2009, Owen *et al.* 2012, Bossart *et al.* 2012). Four of the Antillean manatees were from Puerto Rico and a subsequent serosurvey for *T. gondii* in this populations revealed a seroprevalence of 3% (Bossart *et al.* 2012). This is especially interesting given that the four manatees died with disseminated toxoplasmosis. Necropsy of the four animals revealed severe emaciation and empty gastrointestinal tracts with widespread inflammatory lesions, similar to those previously described for toxoplasmosis in other animals. Intralesional *T. gondii* tachyzoites were observed by histological, ultrastructural and immunohistochemical techniques in the gastrointestinal tract and heart. Lesions were moderate to severe in the heart, and generally less severe in the liver, lung and mesenteric lymph node (Bossart *et al.* 2012). Buergelt and Bonde (1983) described mild lesions consistent with *T. gondii* bradyzoites within tissue cysts in the brain of a Florida manatee; however, tachyzoites were not observed on histopathological examination. Finally, Dubey *et al.* (2003) found multifocal areas of myocardial necrosis from a single Antillean manatee from Caribbean waters. Non-suppurative myocarditis was evident as well as generalized encephalitis with tachyzoites consistent with *T. gondii* present in the lesions. Toxoplasmosis was reported as the likely cause

of the myocarditis in this case (Dubey *et al.* 2003). Ladds (2009) offers the first putative report of toxoplasmosis in the dugong but offers no description of pathologic changes. Owens *et al.* (2012) noted granulomas in the liver and mesenteric lymph nodes of the same animal but the brain was not examined due to hunting laws afforded to the aboriginal people which required that the researchers return the heads of the dugongs undamaged. Based on the above cases, the most prevalent clinical findings for toxoplasmosis in manatees are lymphadenopathy and myocarditis (Dubey *et al.* 2003, Bossart *et al.* 2012). Although congenital toxoplasmosis remains a concern, it has not been reported in manatees; however, there have been reports of vertical transmission in dolphins and otters and is a common mode of transmission in people (Resendes *et al.* 2002, Jardine & Dubey 2002, Lopez *et al.* 2000, Miller *et al.* 2008b). Thus, it is likely also possible in manatees.

Detection and Diagnosis

There are three types of *T. gondii* infections: acute, chronic, and congenital. Self-limiting acute infections are most commonly associated with clinical disease due to tachyzoite replication. This is followed by the chronic stage of infection containing the bradyzoite tissue cysts, which remains throughout the life of the animal. Due to the chronic stage persisting in the tissues, the infection can never be completely eliminated. Congenital infections are reported in many species of mammals. Congenital transmission rates depend on mammal species infected: rates in sheep are estimated at 66% of pregnancies, mice are estimated at 75%, whereas, congenital transmission in humans is as low as 19.8% (Hide *et al.* 2009).

Antibody Testing

Seroprevalence of *T. gondii* immunoglobulin IgG antibodies can be evaluated in the intermediate and DH populations using several techniques. The Sabin Feldman test is considered to be the gold standard utilized for *T. gondii* antibody testing in human medicine, but is no longer used in diagnostic testing due to the complication of the technique and zoonotic potential of the live tachyzoites to the technician running the test (personal communication). In medical diagnostics, a number of immunofluorescence (IFA) and enzyme-linked immunosorbent assays (ELISA) were developed but have limited utility in veterinary diagnostics due to their requirement for species-specific protein conjugate. Few ELISA and IFA tests for domestic animals are commercially available. Testing serum by modified agglutination test (MAT) for IgG titers to the parasite is the best method of screening for antibodies to *T. gondii* in exotic animals because it does not require an animal specific conjugate for testing. The development of the direct agglutination and later the modified agglutination tests, techniques that utilized formalin-killed rather than live tachyzoites and did not have a conjugate requirement was revolutionary in the diagnosis and investigation of toxoplasmosis in animals (Desmonts & Remington 1980, Dubey & Desmonts 1987). These techniques have been further modified to produce additional agglutination tests that utilize the same strategy: the indirect hemagglutination test using antigen coated sheep red blood cells and the latex agglutination test using antigen coated latex beads. Indirect hemagglutination test results were noted to agree with Sabin-Feldman dye test results in human patients, and remain positive for the life of the patient (Lunde 1973). In a study of experimental infection using cats that were monitored for up to 950 days after inoculation, the Sabin-Feldman dye test, modified agglutination test (MAT), indirect

hemagglutination test (IHA) and latex agglutination test (LAT) were compared and the MAT was found to be the most sensitive of the agglutination methods (Dubey & Thulliez 1989). The MAT and IHA were found to produce similar results in a study of dairy goats (Patton *et al.* 1990). The MAT also had good correlation with an ELISA test developed specifically for porcine testing (Gamble *et al.* 2005). Agglutination testing remains the most commonly used modality for the diagnosis of toxoplasmosis in animals, despite the loss of availability of some commercial kits. The MAT test is an IgG immunoglobulin antibody test that utilizes formalin-fixed, lab propagated *T. gondii* tachyzoites to produce a lattice appearance when added to antibody filled animal serum (Desmonts & Remington 1980, Dubey & Desmonts 1987).

In veterinary investigation of *T. gondii*, the production of recombinant antigen and advances of molecular techniques have expanded diagnostic options. Some species specific ELISAs have been developed and performed well when compared to reference serological techniques, notably for cats, sheep and pigs (Dabritz *et al.* 2007, Hill *et al.* 2006, Kimbita *et al.* 2001, Klun *et al.* 2007, Mainar-Jaime & Barberan 2007, Shaapan *et al.* 2008). Some cautions that must be noted in interpretation of these results include the potential of cross-reactivity in the presence of infections with other coccidia and the difficulty of determining true sensitivities and specificities without a reliable gold standard for comparison (Gardner *et al.* 2009, Silva *et al.* 2002).

Polymerase Chain Reaction and Histology

Tissues of deceased animals can also be tested for *T. gondii* by polymerase chain reaction (PCR) and immunohistochemical examination (IHC). PCR detects DNA of the parasite and can be difficult to use in detecting *T. gondii* in the tissues due to the sparse nature and small size of tissue cysts and the small sample size used for testing. Sucrose centrifugation flotation on feline feces can be used to detect *T. gondii* oocysts in the feral cat colonies close to waterways known to be habitats for manatees (Dryden 2005, Dabritz *et al.* 2007).

Bioassay and Genotyping by multiplex multilocus nested PCR-Restriction Fragment Length Polymorphism (Mn-PCR-RFLP) method employing 10 genetic markers (Su *et al.* 2010) can be useful to increase likelihood of parasite production and identification. Using multiplex PCR the markers are preamplified using external primers in a single reaction. These products are used as templates to amplify each individual marker by nested PCR. Those products are treated with restriction enzymes to reveal different alleles.

While serological detection is the primary mode of *T. gondii* diagnosis in humans and other animals, molecular techniques are increasingly being used, since the first identification of a gene target for polymerase chain reaction amplification (Burg *et al.* 1989). PCR is now commonly used to test amniotic fluid in pregnant women with suggestive serological results, and has been used for environmental detection of oocysts and characterization of *T. gondii* genotypes in vertebrate hosts (Schwab & McDevitt 2003, Su *et al.* 2006). The difficulty of acquiring the appropriate tissue sample for antemortem diagnosis makes PCR a modality of questionable sensitivity but potentially excellent specificity when used in diagnostic setting. While serology provides evidence of infection, a single sample does not allow the characterization of an acute versus a chronic infection, nor the severity of the infection. The direct detection of DNA by polymerase chain reaction is a promising method in that a positive result establishes the presence

of the organism in the tested tissue. However, the nature of the organism's complex life cycle (**Figure 1**) poses a challenge in translating a successful PCR assay into a useful clinical test.

There are two commonly used targets for the detection of genomic DNA by either traditional or real time PCR, the *BI* gene and AF146527 (529 base pair repeat element). The *BI* gene was initially detected as a PCR target with 35 repeats in the *T. gondii* genome by Burg *et al.* (1989), who determined a limit of detection of one organism in cell lysate and 10 organisms in a human leukocyte preparation by traditional PCR, with no evidence of detection of closely related pathogens. The *BI* gene has become a standard target for PCR detection in clinical samples and, significantly for veterinary diagnostics, does not occur in *Neospora caninum* (Chabbert *et al.* 2004, Schatzberg *et al.* 2003). More recently, a 529 base pair target (AF146527) that is repeated 200-300 fold in the *T. gondii* genome has been described and utilized as a target for PCR detection (Homan *et al.* 2000).

Samples commonly evaluated from suspected human cases include: amniotic fluid, placenta, brain, blood, cerebrospinal fluid, urine, vitreous, aqueous, bronchoalveolar lavage, pleural and peritoneal fluids (Remington *et al.* 2004). Veterinary diagnostic laboratories offer evaluation of blood, feces, CSF, brain and other tissues, with fecal examination used to distinguish *T. gondii* oocysts from morphologically similar *Hammondia hammondi* in feline feces (Schaes *et al.* 2008). Knowledge of the parasite's life cycle is critical to increase the utility of PCR testing, *e.g.* blood stages are transient and expected early in infection, and detection of the parasite in tissue relies on the presence of a tissue cyst in the aliquot being tested. Even with a good sampling strategy, molecular detection must be considered a method of unproven sensitivity but good specificity, where the predictive value of a positive result is much higher than that of a negative result (Remington *et al.* 2004). This makes molecular detection a reasonable adjunct to serological investigation.

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Appendix

Table 1: Published reports of *Toxoplasma gondii* seroprevalence studies in cetaceans.

LAT=Latex Agglutination Test, IHAT=Indirect Hemagglutination Test, MAT=Modified Agglutination Test, PCR=Polymerase Chain Reaction, ELISA=Enzyme-Linked Immunosorbent Assay, SFD=Sabin-Feldman Dye Test.

ANIMAL	SEROPREVALENCE	LOCATION	n	DIAGNOSTIC TEST	REFERENCE			
Cetaceans (6 species)	11.9%	Japan	59	LAT/IHAT	Murata <i>et al.</i> 2004			
Striped Dolphins (<i>Stenella coeruleoalba</i>)	100.0%	Tuscany, Italy	6	MAT/PCR	Pretti <i>et al.</i> 2010			
Bottlenose Dolphin (<i>Tursiops truncatus</i>)	88.0%		8					
Pacific Bottlenose Dolphin (<i>T. aduncus</i>)	13.8%	Solomon Islands	58	LAT/ELISA	Omata <i>et al.</i> 2005			
Amazon River Dolphin (<i>Inia geoffrensis</i>)	86.3%	Amazon, Brazil	95	MAT	Santos <i>et al.</i> 2011			
Striped Dolphin	11.1%	Spain	36	MAT	Cabezón <i>et al.</i> 2004			
Common Dolphins	50.0%		4					
Bottlenose Dolphins	57.0%		7					
Harbor Porpoise (<i>Phocoena phocoena</i>)	100.0%		1					
Risso's Dolphin (<i>Grampus griseus</i>)	0.0%		9					
Long-finned Pilot Whale (<i>Globicephala melas</i>)	0.0%		1					
Short-beaked Dolphin (<i>Dolphinus delphis</i>)	28.6%		British Water			21	SFD	Forman <i>et al.</i> 2009
Risso's Dolphin	0.0%					1		
Atlantic White-sided Dolphin (<i>Lagenorhynchus acutus</i>)	0.0%	1						
Humpback Whale (<i>Megaptera novaeangliae</i>)	100.0%	1						
Harbor Porpoise	1.4%	70						
Striped Dolphin	0.0%	5						
Cuvier's Beaked Whale (<i>Ziphius cavirostris</i>)	0.0%	1						
Bottlenose Dolphin	0.0%	1						
Atlantic Bottlenose Dolphin	87.3%	Mexico (Captive)		63	MAT	Alvarado-Esquivel <i>et al.</i> 2012		
Bottlenose Dolphin	97.8%	Florida & California		141	MAT	Dubey <i>et al.</i> 2003		

Table 2: Necropsy findings in published reports of clinical toxoplasmosis in cetaceans.
 Only animal with clinical signs of seizures and depression. **As reviewed by. +Similar report on same case.

ANIMAL	LOCATION	n	NECROPSY FINDINGS	REFERENCE
Tucuxi (<i>Sotalia fluviatilis</i>)	Brazil	1	Lymphadenitis	Bandoli &DeOliveira 1977
Atlantic Bottlenose Dolphin (<i>Tursiops truncatus</i>)	USA	1	T.gondii-like tachyzoites seen but unable to confirm diagnosis	Schulman <i>et al.</i> 1977; **Dubey <i>et al.</i> 2003
Spinner Dolphin (<i>Stenella longirostris</i>)	Hawaii	1	Bronchopneumonia, adrenalitis, encephalitis, COD: disseminated toxoplasmosis	Migaki <i>et al.</i> 1990
Atlantic Bottlenose Dolphin	Florida, USA	1	Hepatitis, adrenalitis, COD: toxoplasmosis	Cruickshank <i>et al.</i> 1990
Atlantic Bottlenose Dolphins (Dam & Calf)	Florida, USA	2	Dam: interstitial pneumonia, adrenalitis, cardiac myonecrosis, myocarditis, COD: toxoplasmosis Calf: encephalitis, COD: toxoplasmosis	Inskip <i>et al.</i> 1990
Striped Dolphin (<i>Stenella coeruleoalba</i>)	Spain	1	Lymphadenitis, encephalitis	Domingo <i>et al.</i> 1992
Atlantic Bottlenose Dolphins	Italy	2	Encephalitis, pneumonia	DiGuardo <i>et al.</i> 1995
Striped Dolphins		4	Coinfection with morbillivirus, encephalitis	
Risso's Dolphin (<i>Grampus griseus</i>)		1	T.gondii tachyzoites noted	
Striped Dolphin	Italy	1	Coinfections T.gondii, Listeria, and Brucella	Grattarola <i>et al.</i> 2016
Beluga Whale (<i>Delphinapterus leucas</i>)	Port-au-Persil, CA	1	Encephalitis, COD: disseminated toxoplasmosis	DeGuise <i>et al.</i> 1995; **Mikaelian <i>et al.</i> 2000
Beluga Whale	Canada	1	Encephalitis, lymphadenopathy, COD: disseminated toxoplasmosis	Mikaelian <i>et al.</i> 2000
Indo-Pacific Bottlenose Dolphin (Late Term Fetus) (<i>T. aduncus</i>)	Australia	1	Myocarditis, encephalitis, COD: toxoplasmosis	Jardine & Dubey 2002
Risso's Dolphins (Dam & Fetus)	Spain	2	Dam: lymphadenomegaly, splenomegaly, adrenomegaly and hemorrhage, ulcerative glossitis and stomatitis, laryngeal erosions, COD: disseminated toxoplasmosis	Resendes <i>et al.</i> 2002b
			Fetus: encephalomyelitis, pneumonia, necrotizing hepatitis, cholangiohepatitis, gastritis, adrenalitis, lytic necrosis of heart, lungs, and kidney, COD: toxoplasmosis	
Indo-Pacific Humpbacked Dolphins (<i>Sousa chinensis</i>)	Indo-Pacific	4	Tissue cysts in brain, tachyzoites in lungs, heart, liver, spleen, adrenal gland, pancreatitis, pneumonia, peritonitis, COD: toxoplasmosis	Bowater <i>et al.</i> 2003
Striped Dolphin	Costa Rica	1	Meningoencephalitis	Dubey <i>et al.</i> 2007
Bottlenose Dolphins	Unknown	3	Unreported, T.gondii infection confirmed via bioassay from heart tissue	Dubey <i>et al.</i> 2008c
Bottlenose Dolphins	Sea Aquarium, CA	2	Meningoencephalitis	Dubey <i>et al.</i> 2009
Striped Dolphins	Italy	8	Meningoencephalitis, toxoplasmosis confirmed COD (4)	DiGuardo <i>et al.</i> 2010
Striped Dolphins	Italy	3	Meningoencephalitis	DiGuardo <i>et al.</i> 2011
Mediterranean Fin Whale (<i>Balaenoptera physalus</i>)	Italy	1	Coinfection with morbillivirus, hepatomegaly, splenomegaly, lymphadenopathy, hydronephrosis	Mazzariol <i>et al.</i> 2012 +DiGuardo <i>et al.</i> 2013
Atlantic Spotted Dolphins (<i>Stenella frontalis</i>)	Canary Islands	2	Encephalitis	Arbelo <i>et al.</i> 2013
Guiana Dolphin (<i>Sotalia guianensis</i>)	Brazil	1	Pneumonia, artemitis, adrenalitis, hepatitis	Gonzales-Viera <i>et al.</i> 2013
Striped Dolphins	Tuscany, Italy	6	T.gondii infection confirmed by DNA Sequencing	Pretti <i>et al.</i> 2010
Bottlenose Dolphins		8		
Hector's Dolphins (<i>Cephalorhynchus hectori</i>)	Unknown	5	Disseminated necrotic lesions in lungs, lymph nodes, and adrenals, metritis, COD: toxoplasmosis	Roe <i>et al.</i> 2013
Maui's Dolphins (<i>C. hectori maui</i>)		2		

Table 3: Necropsy findings in published reports of clinical toxoplasmosis in Sirenia.

ANIMAL	LOCATION	n	NECROPSY FINDINGS	REFERENCE
DUGONG (<i>Dugong dugon</i>)	Australia	1	Granulomas in liver and mesenteric lymph nodes, COD: toxoplasmosis	Owen <i>et al.</i> 2012
FL MANATEE (<i>Trichechus manatus latirostris</i>)	Florida, USA	1	T.gondii-like tissue cysts in brain	Buergelt & Bonde 1983
ANTILLEAN MANATEE (<i>T. m. manatus</i>)	Caribbean	1	Myocarditis, myocardial necrosis	Dubey <i>et al.</i> 2003
ANTILLEAN MANATEES	Puerto Rico, USA	4	Emaciation, GI lesions, pneumonia, gastritis, colitis, hepatitis, lymphadenitis, enteritis, myocardial lesions, pericarditis, COD: toxoplasmosis	Bossart <i>et al.</i> 2012

Table 4: *Toxoplasma gondii* seroprevalence in Sirenia.

ANIMAL	LOCATION	n	SEROPREVALENCE	METHOD	REFERENCE
Antillean Manatee (<i>Trichechus manatus manatus</i>)	Belize	115	7%	IFAT/LAT	Sulzner <i>et al.</i> 2012
Amazonian Manatee (<i>T. inunguis</i>)	Peruvian Amazon	19	63.2%	MAT	Delgado <i>et al.</i> 2013
Amazonian Manatee	Rehab Facility, Brazil	74	39.2%	MAT	Mathews <i>et al.</i> 2012
West Indian Manatee (<i>T. manatus</i>)	Mexico (Captive)	3	0%	MAT	Alvarado-Esquivel <i>et al.</i> 2012
Antillean manatee	Puerto Rico, USA	30	3%	MAT	Bossart <i>et al.</i> 2012
Florida manatee (<i>T. m. latirostris</i>)	Florida, USA	384	0%	MAT	This study
Florida manatee	Florida, USA	44	6%	MAT	Smith <i>et al.</i> 2015
Antillean manatee	Brazil	55	10.9%	MAT	Attademo <i>et al.</i> 2016

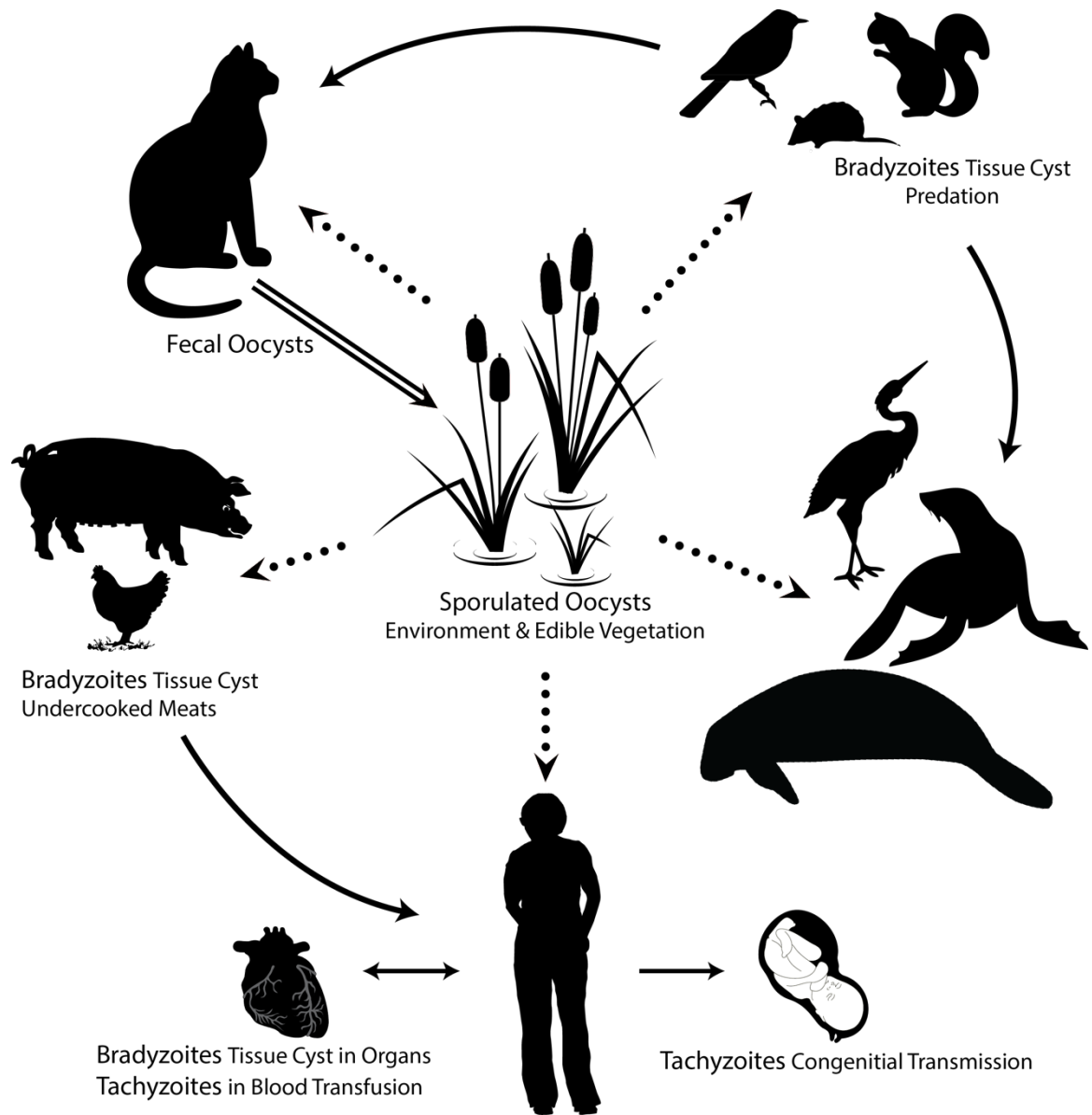


Figure 1. *Toxoplasma gondii* life cycle.
 Illustrated by Aly Chapman and Greg Schweiger.

CHAPTER II

Epidemiology of *Toxoplasma gondii* in the Florida manatee (*Trichechus manatus latirostris*) and Antillean manatee (*Trichechus manatus manatus*)

Co-researchers for this chapter included Dr. R. K. Bonde and the staff at the USGS; Dr. Martine de Wit and the staff at the MMPL (within the FWS); Dr. Mignucci-Giannoni, Carly Rivera and the MCC (Manatee Conservation Center) staff; Linda Lovering; Jessica Martinez; and Drs. Rick Gerhold, Debra Miller, and Chunlei Su. Co-researchers aided in securing financial support, sample collections, field work, sample analysis, or manuscript review.

Abstract

Toxoplasma gondii is a feline protozoan recently reported to cause mortality in Antillean manatees (*Trichechus manatus manatus*) in Puerto Rico. Toxoplasmosis is well documented in marine mammals causing morbidity and mortality with a wide range of clinical signs. Prior to 2015, only two reports of disseminated toxoplasmosis in 4 Antillean manatees and 1 Florida manatee (*Trichechus manatus latirostris*) were documented. Our study is the first attempt to determine an association between manatee toxoplasmosis and free-roaming cats. Given their herbivorous nature, accidental ingestion of oocysts from sea grass is presumed to be the primary mode of infection for manatees. Objective one of the study was to determine prevalence of *T. gondii* in the Florida manatees and the Antillean manatees in Puerto Rico. Objective two was to determine the prevalence of *T. gondii* in the free-roaming cats in Florida and Puerto Rico. Objective three was to investigate oocyst contamination of sea grass beds in Puerto Rico. Finally, objective four was to compare the *T. gondii* genotypes between the two populations. Sera or plasma (n=343) from live-captured Florida manatees and serosanguinous fluid (n=10) collected opportunistically at necropsy from Florida manatees were tested using the modified agglutination test (MAT) to determine prevalence of *T. gondii*. Similarly, sera (n=3) from live Antillean manatees and serosanguinous fluid (n=2) from dead Antillean manatees were tested for *T. gondii* antibodies using MAT. In addition, various tissues from one of the three necropsied Antillean manatees were collected in 10% buffered neutral formalin (BNF). No *T. gondii* antibodies (0%) were detected from any of the 358 manatee samples from either location; however, one dead Antillean manatee was inconclusive on MAT. Nested PCR on the blood clot from this animal was negative. These data suggest an even lower prevalence of *T. gondii* in the Florida and Antillean manatees than previously reported. Freshly defecated free-roaming cat feces (n=79) from Florida were tested for *T. gondii* oocysts by centrifugal flotation to determine parasite prevalence. No oocysts consistent with *T. gondii* were seen in any of the cat feces (0%). A seroprevalence of 16% resulted from MAT testing on sera from 25 free-roaming cats in Puerto Rico. Environmental sea grass samples (n=33) from areas surrounding the Puerto Rico's main island were tested for *T. gondii* using centrifugal flotation followed by PCR. All sea grass samples were negative for *T. gondii*. No genotypes were obtained for comparison. Although no contamination of seagrass was successfully determined, antibody detection in the cat samples suggest an epidemiological connection yet to be confirmed and support continued monitoring of both manatee populations for *T. gondii* infection. Furthermore, an intensive survey of *T. gondii* in free-roaming cats in proximity to the watershed regions is warranted.

Introduction

Toxoplasma gondii is an intracellular obligatory protozoan of felids (Nicolle and Manceaux 1909, Splendore 1908). Though it typically causes no clinical disease in the definitive host (DH), it can cause disease in a wide range of severity and even mortality in many intermediate hosts (IHs; Weiss and Kim 2007). Disease severity depends on the species of IH infected, IH's age and immunity status, the *T. gondii* genotype and mode of infection (Assadi-Rad *et al.* 1995). Ingestion of sporulated oocysts from felid fecal contamination, predation (ingestion of tissue cysts), or congenital transmission are the likely modes of infection to IH.

Estimates for feral cat population in the U.S. range from 13 million in the winter to over 100 million in summer and estimates within Florida differ almost as dramatically from 2.8-5.3 million, depending on the source (Alley Cat Allies 2005, Clifton 2003, Levy and Crawford 2004). Feral cat colonies exist across the island of Puerto Rico as well but population numbers are not known. Current claims that the Trap, Neuter, Release (TNR) program has cut the population in half have not been substantiated through scientific studies (Gunther *et al.* 2011, Miller *et al.* 2014). Though the stray cat population on the main island of Puerto Rico has never been surveyed for *T. gondii*, Dubey *et al.* (2007) found virulent genotypes (Type I lineage) of *T. gondii* from feral cats on the neighboring Mona Island, PR. Contamination potential is high with the feral cat colony numbers increasing each year and the oocysts shedding potential of >1 million oocysts per cat per defecation (Dabritz *et al.* 2007, Torrey and Yolken 2013). Reports of *T. gondii* contaminated felid feces in freshwater runoff causing mortalities in sea otters in California (Cole *et al.* 2000, Miller *et al.* 2002), oocysts survival and even sporulation in seawater (Lindsay *et al.* 2003), and the herbivorous diet of the manatee suggests oocysts ingestion from felid fecal contamination as the likely mode of infection for manatees.

The Florida manatee (*T. m. latirostris*) and the Antillean manatee (*T. m. manatus*) are subspecies of the West Indian manatee (*T. manatus*). The Florida manatee is a temperature sensitive, marine mammal native to coastal Florida. Its winter range is regulated by water temperature, forcing them to seek warm water refuges such as freshwater springs or power plant outflows where the water temperature remains constant year round. Whereas, the Antillean manatee has a wide distribution across the tropical waters of the Caribbean so their movements are not hindered by water temperature. Due to hunting, fishery by-catch, watercraft collision, and disease (brevetoxicosis, toxoplasmosis, papillomavirus), both subspecies are now listed as endangered (IUCN 2008a, b). Conservation efforts have focused mostly on the Florida manatee which has produced a rebound in the population; however, the Antillean manatee populations are fractured making it more difficult to establish successful conservation methods (FWC 2016, Mignucci-Giannoni *et al.* 2000). The manatee's affinity for shallow, clear water bays and inlets make it a common target for the anthropogenic calamities listed above (Gerstein 2002). Both subspecies are herbivores, prefer to feed at 2 meters of water or less, and eat primarily seagrasses. *Syrigodium filiforme* (manatee grass), *Thalassia testudinum* (turtle grass), and *Halodule wrightii* (shoal grass) are the three sea grasses making up the majority of their diet (Lefebvre *et al.* 1999, Miller and Lugo 2009).

Two mortalities in the Florida manatee have been reported due to toxoplasmosis. Buergelt and Bonde (1983) reported *T. gondii* tissue cysts with mild lesions but no tachyzoites present in the brain of a Florida manatee. Most recently, Smith *et al.* (2015) reported *T. gondii* tissue cysts stained by Immunohistochemistry (IHC) and confirmed by PCR. The study also

reported a 6% sero-prevalence in the Florida manatee (n=44). Bossart *et al.* (2012) reported the deaths of four Antillean manatees due to toxoplasmosis within a single year. They also reported a *T. gondii* seroprevalence in the Antillean manatee of 3% (n=30).

Our study is the first attempt to link the *T. gondii* genotypes from the free-roaming cats to the genotypes found in clinically ill or dead manatees in two geographic locations. We also attempted to concentrate oocysts from the Antillean manatee's main food source (seagrass) in order to determine oocyst contamination of the marine environment.

Methods

Collections

Puerto Rico, USA

Antillean Manatees: Live Animals

Serum samples (n=3) were collected from live manatees at MCC during routine health examinations. These wild Antillean manatees were sick or injured and brought to the center for health assessment and rehabilitation. They have been in captivity for a few months to a few years depending on the severity of their debilitation. Logistical issues hindered health assessment captures from 2014 to present (2016) and are indefinitely halted; thus, we were unable to collect additional samples from live animals in this location.

Antillean Manatees: Dead Animals

Serosanguinous fluid samples (n=2) were collected from two stranded Antillean manatees at necropsy and formalin fixed heart, lung, diaphragm, and liver were collected from one of them. Fresh tissues were not collected due to advanced autolysis of the carcasses.

Free-roaming Felids

Attempts to collect fecal samples from free-roaming cats submitted to animal shelters throughout Puerto Rico were unsuccessful; however, we were able to obtain 25 serum samples in 2015 from veterinarians in coastal and inland municipalities of San Juan, Puerto Rico.

Seagrasses

Thirty-three 0.5L sealed plastic bags of seagrass were collected from the ocean floor at 17 sites (**Figure 3**). Most specimens had leaves and sheaths intact and some had sections of rhizome still attached. Three seagrass species were collected (one species per bag and approximately 2 bags per site) and included *Thalassia testudinum* (turtle grass), *Syringodium filiforme* (manatee grass), *Halodule wrightii* (shoal grass). *Halodule wrightii* is not wide spread in this region so it was only collected at 2 sites (Miller and Lugo 2009).

Florida, USA

Florida Manatees: Live Animals

Serum or plasma samples (n=343) obtained from live Florida manatees were tested to determine *T. gondii* IgG antibody titers using the MAT as previously described by Dubey and Desmonts (1987). Of these samples, 21 serum samples were collected as part of the health survey performed by Dr. Bob Bonde with the USGS. The remaining 322 samples consisted of archived plasma from previous USGS health assessments from various capture locations (**Figure 2**).

Florida Manatees: Dead Animals

Refrigerated heart, brain, and liver from 10 deceased manatees were collected during necropsies by Dr. Martine deWit and staff of the Marine Mammal Pathobiology Laboratory, Florida Fish and Wildlife Conservation Commission (MMPL, FWC). Pooled blood from heart tissue was centrifuged to obtain serosanguinous fluid for MAT testing. Fresh heart, brain, and liver tissues were kept to attempt propagation of the *T. gondii* for genotyping from seropositive manatees.

Free-roaming Felids

Four grams of freshly defecated (n=79) fecal samples were collected from free-roaming cats from Florida's northwest coast to test for *T. gondii* oocysts shedding via fecal centrifugal flotation with Sheather's sugar. Unfortunately, blood collection was not possible due to limited staffing.

Modified Agglutination Test

Assay

All sera, plasma, and serosanguinous fluid collected from manatees and cats were screened for *T. gondii* antibodies using the MAT. The procedure used incorporates all modifications (Desmonts and Remington 1980, Dubey and Desmonts 1987, Dubey *et al.* 1995) to the original agglutination test designed by Fulton and Turk (1959) maximizing the sensitivity and specificity of the test (Dubey *et al.* 1995). Briefly, this test is a kit (Biomerieux, Marcy-l'Etoile, France) that uses whole formalin-fixed tachyzoite antigen to detect antibodies to *T. gondii* in animal sera by antigen-antibody agglutination.

Interpretation

Negative test wells with no lattice of reacted tachyzoites show a tight pellet. In a positive reaction, the tachyzoites bind to the corresponding antibodies in the animal sera and form a matted appearance in the test well. Following the standard MAT procedures of the University of Tennessee, Diagnostic Veterinary Parasitology Laboratory (UTDVPL), an IgG titer greater than or equal to 32 is considered a positive reaction.

Seagrass Processing, Concentration method, and PCR

Rinsing and Concentration

Seagrass samples were blended with Tween 20 detergent and water, strained through cheesecloth, and underwent a series of rinsing, centrifugation, and decanting supernatant until only a small pellet remained. Concentration methods were as described by Gerhold *et al.* (2015). A small portion of the pellet was concentrated using centrifugal flotation with sugar as described by Dryden *et al.* (2005) for diagnosis of parasites to determine if oocysts were present.

PCR

The products of the rinsing and concentration methods underwent DNA extraction (Qiagen, Valencia, California) and PCR according to the procedure listed in Gerhold *et al.* (2015). Primers TOX4 and TOX5 that amplify a 529 basepair repeat in *T. gondii* were used for PCR (Homan *et al.* 2000). 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.

Sucrose Centrifugal Flotation

Centrifugal flotations with Sheather's sugar solution for fecal diagnosis of parasites were performed on all free-roaming cat fecal samples (Dryden *et al.* 2005). The procedure is similar to those described by Dryden *et al.* (2005) with addition of water step to remove excess debris and centrifugal speeds set at 1,500 rpm instead of 1,200 rpm.

Nested PCR

Tissues or whole blood from animals testing positive on the MAT, underwent nested PCR following the procedure illustrated by Su *et al.* (2010).

Results

Florida and Antillean Manatees

All Antillean and Florida manatee sera, plasma, and serosanguinous fluid samples (n=358) were negative 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. Unfortunately, no seropositive manatees were found, so bioassay and genotyping of the parasite from the manatees was not possible.

Florida and Puerto Rico Felids

No oocysts consistent with size and morphology of *T. gondii* were detected on fecal flotations of the free-roaming cat samples collected (n=79) from Florida; thus, bioassay and genotyping of the parasite from the cats were not possible. Seroprevalence of *T. gondii* in the cat samples from Puerto Rico was 16%, three of which had very high titers (IgG>1:2048).

Puerto Rico Seagrass

No *T. gondii* oocysts were recovered from the seagrass rinsing method followed by centrifugal flotation. On PCR, two weak bands were sequenced and found to be uncultured bacteria on BLAST analysis.

Discussion

Clinical toxoplasmosis has rarely been reported in manatees and the dugong (*Dugong dugon*; Bossart *et al.* 2012, Buergelt and Bonde 1983, Dubey *et al.* 2003, Owen *et al.* 2012, Smith *et al.* 2015). The two previously reported mortalities in the Florida manatees documented disseminated toxoplasmosis as the cause of death (Buergelt and Bonde 1983, Smith *et al.* 2015). Neither of these studies were able to obtain serum from the deceased animal for antibody testing. Our study highlights the need for future investigators to routinely collect serum during necropsies so that cut off titers can be substantiated when *T. gondii* infection is confirmed by other testing (*e.g.* on histology or PCR). Clinical disease has not been reported in the Amazonian manatee (*T. inunguis*) despite several reports of high seroprevalence in this species ranging from 39.2 – 63.2% seropositive animals (Delgado *et al.* 2013, Mathews *et al.* 2012). Bossart *et al.* (2012) reported disseminated toxoplasmosis in 4 Antillean manatees and included a

seroprevalence study of 3% (n=30). No studies could be found on toxoplasmosis in the West African manatee (*Trichechus senegalensis*) but disease research in this manatee species is lacking. Toxoplasmosis has been reported in many terrestrial mammals in Africa (including wild cats capable of shedding oocysts) thus there is no reason to believe this manatee species would not be infected with *T. gondii* (Penzhorn *et al.* 2002).

Our study shows a lower prevalence of *T. gondii* in the Florida manatee than previously reported (Smith *et al.* 2015). Out of 44 animals tested, Smith *et al.* (2015) found a 6% seroprevalence; whereas, seroprevalence from our study was 0% out of 353 animals tested. Our study also demonstrated a lower seroprevalence of 0% (n=5) in the Antillean manatee as compared to the report of 3% (n=30) by Bossart *et al.* (2012). The MAT is considered the gold standard in *T. gondii* testing due to its high sensitivity and specificity without the need for a host species specific conjugate (Dubey and Thulliez 1989). Although this test is well established for most mammal and bird intermediate host, a true understanding of positive titer cutoffs in exotic animals is lacking; as such, a limitation of studying toxoplasmosis in manatees (or any endangered, exotic animal) is the inability to set accurate positive and negative titer cutoffs. Further research, however unlikely, would require experimental infection of manatees with various *T. gondii* genotypes to determine the respective cutoff values. Many marine mammals tested have shown very high titers but most species of manatee reportedly positive for *T. gondii* antibodies have had very low positive titers at 1:25 or 1:32 (Bossart *et al.* 2012; Dubey *et al.* 2003, 2009; Lambourn *et al.* 2001; Mathews *et al.* 2012; Measures *et al.* 2004; Mikaelian *et al.* 2000). The closest living relative to the manatee is the elephant; however, multiple elephant titers have been reported as high as 1:4096 so these animals do not fit the pattern of very low positives (Dangolla *et al.* 2006, Riemann *et al.* 1975). Another explanation for the low seroprevalence could be a lack of sero-conversion (antibody titers never develop) in the manatee species similar to what is seen in wild rodents (DeFeo *et al.* 2002, Dubey and Frenkel 1998). Dubey and Frenkel (1998) reviewed the literature on *T. gondii* in wild rodents and reported that some species could die from toxoplasmosis before mounting an immune response. This is also true for host species that are highly susceptible to toxoplasmosis such as in macropods (Patton *et al.* 1986, Portas 2010). In contrast, manatees likely seroconvert given the high seroprevalence in some species without signs of clinical disease. A possible difference in genotype virulence could also result in different rates of sero-conversion. Biologists have suggested that manatees have “immunity” to toxoplasmosis and other pathogens because of the lack of clinical disease; however, in the case of *T. gondii*, tissue cysts are never cleared from the body so the animal would still develop an antibody reaction detectable on the MAT (Bonde *et al.* 2004). Although our study produced no seropositive wild manatees, captive animals are at a higher risk of *T. gondii* infection.

Toxoplasma gondii infections are higher in captive animals with free-roaming cats in close proximity to holding tanks or secluded wild animals living with high free-roaming cat populations, such as the Amazonian manatee whose migration patterns are more reliant on the wet and dry seasons of the Amazon Basin (Arraut *et al.* 2010, Attademo *et al.* 2016, Buergelt and Bonde 1983, Mathews *et al.* 2012). Transmission to various exotic species in zoos and aquaria from improperly controlled free-roaming cat populations or from captive exotic cats is well documented (Basso *et al.* 2007, de Camps *et al.* 2008, Hermosilla *et al.* 2010, Ippen *et al.* 1981, Patton *et al.* 1986).

Despite the low percentage (<1%) of cats shedding *T. gondii* at any given time, a single cat can shed tens of millions of oocysts during initial infection (Dabritz *et al.* 2007, Dubey *et al.* 2009, Weiss and Kim 2007). Keeping this in mind, the lack of *T. gondii* oocysts found in the felid feces is not surprising. A larger survey is recommended to verify our findings. If we set the shedding prevalence at 1%, then binomial distribution sets the recommended sample size at 161 cats for a power of 80.2%. Although estimates of feral cat colonies exist for some geographical locations, no one truly knows the density of cat populations within the United States and it grows exponentially (Clifton 2003, Levy and Crawford 2004, Torrey and Yolken 2013). The largest feral cat colonies in Puerto Rico are located in the city of Old San Juan. Here cats are free to roam the city and are even fed by local shop keepers and volunteers from the Save the Gato Foundation. The estimated feral cat numbers for Old San Juan range from 250 (Save the Gato Foundation) to 415 (NSF 2016), but no other estimates exist for the rest of the island. The 16% *T. gondii* seroprevalence reported in our study in cats (n=25) is lower than the estimates of 30-40% worldwide and inconsistent with the higher seroprevalences reported in more humid tropical climates (Elmore *et al.* 2010). Our sample size is very low when compared to the overall feral cat populations; therefore, further testing of free-roaming cat populations on the main island of Puerto Rico is warranted. Also, given that antibodies are not yet detectable in cats actively shedding oocysts, even the smaller seroprevalence of *T. gondii* in this cat population is suggestive of oocysts contamination; however, the extent of contamination cannot be determined from seroprevalence alone (Pena *et al.* 2006). Fecal examinations on the free-roaming cats and environmental sampling are useful for estimating the extent of oocyst contamination or for providing supportive data on transmission (Dumètre and Dardé 2003, Isaac-Renton *et al.* 1998, Shapiro *et al.* 2010).

Oocysts reportedly remain viable in sea water for as long as 6 months and at cooler temperatures oocysts remain viable for up to 24 months (Lindsay *et al.* 2003, Lindsay and Dubey 2009). This suggests that the cooler winter water temperatures of Florida could extend the viability of the oocysts. Environmental sampling might be a viable option for detecting oocyst contamination or for providing supportive data on transmission. Molecular and concentration techniques that overcome issues such as dilution of oocysts in water and difficulty in removing oocysts from vegetation, are available and enhance the sensitivity of environmental sampling (Dumètre and Dardé 2003, Isaac-Renton *et al.* 1998, Shapiro *et al.* 2010). Kniel *et al.* (2002) reported the ability of *T. gondii* oocysts to “stick” to berries and is implicated as a common mode of transmission of this parasite to people, along with raw meat consumption. Loss of seagrass and wetland habitats allows significantly more oocysts to disseminate into the aquatic environment, providing validity to the hypothesis that oocysts are attracted to vegetation (Shapiro *et al.* 2010, VanWormer *et al.* 2016). VanWormer *et al.* (2016) found that when coastal development and precipitation occur together oocyst runoff delivery to the ocean increased by 175%! Biofilm, invertebrate movement, and filter feeding fish and bivalves have also been implicated as facilitators for *T. gondii* distribution in the ocean (Massie *et al.* 2010, Shapiro *et al.* 2014).

Avenues for future investigations on this topic are multifaceted. Future research should also investigate other marine life, such as bi-valves, to explore other avenues for transmission or environmental persistence of *T. gondii*. For example, Lindsay *et al.* (2001, 2004) and Massie *et al.* (2010) investigated oocysts concentration in filter feeding invertebrates and fish, respectively,

to determine their potential role as transport hosts. Considering the mode of infection for marine mammals is suspected to be oocyst ingestion, it would make sense to investigate their major food sources. Exploring the potential modes of aquatic contamination is vital for impeding further infections to marine mammals. Additionally, future research could focus on more extensive cat surveillance and environmental testing to determine the true contamination status of *T. gondii* and continue the investigation into marine mammal transmission of toxoplasmosis. Ultimately, given the limitations of testing, fresh carcasses of seropositive manatees would be required to confirm serological results. Both bioassay and genotyping should remain components of future studies if we are to draw clear conclusions regarding the source and transmission routes of *T. gondii* to marine mammals.

A challenge for any serological study in wildlife is the interpretation of results as tests are only validated in domestic species. This challenge can be overcome partially by increasing sample sizes, which could be done by impressing upon field biologists and other researchers the importance of serum collection. Although many samples would be collected from injured or dead animals, these values can be compared to those from health assessments in similar species, and thus provide valuable test interpretations. Knowing how to interpret test results in marine mammals is key to any study that investigates health issues in these species.

In conclusion, our 0% seroprevalence results in the manatees may be explained by four possible scenarios, listed in order of decreasing likelihood. First, *T. gondii* infection in these manatee populations is rare. Second, the animals are vulnerable to toxoplasmosis and they succumb to the disease before antibody detection is possible. Third, the sensitivity of the MAT test is unknown for manatees. Lastly, manatees are immune to *T. gondii* infections. Without experimental infection, it is impossible to determine which one or more of these scenarios accurately describes the disease status in these manatee populations. Two approaches to enhance our understanding of this host-parasite relationship are to take advantage of future outbreaks and find confirmed toxoplasmosis through opportunistic sampling from manatees.

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Appendix

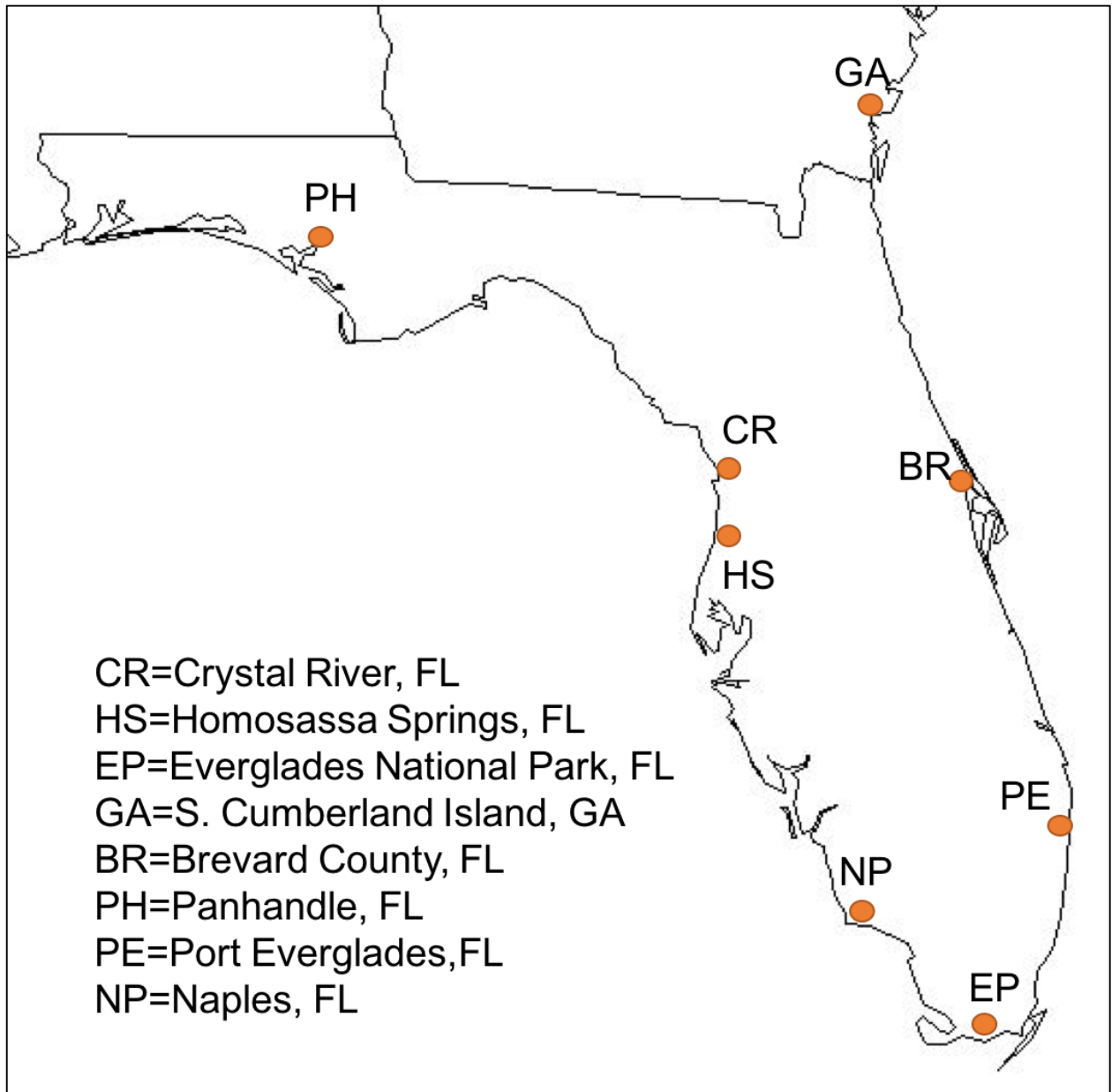


Figure 2. Florida Manatee (*T. m. latirostris*) collection locations for USGS Health Assessments in Florida and Georgia.

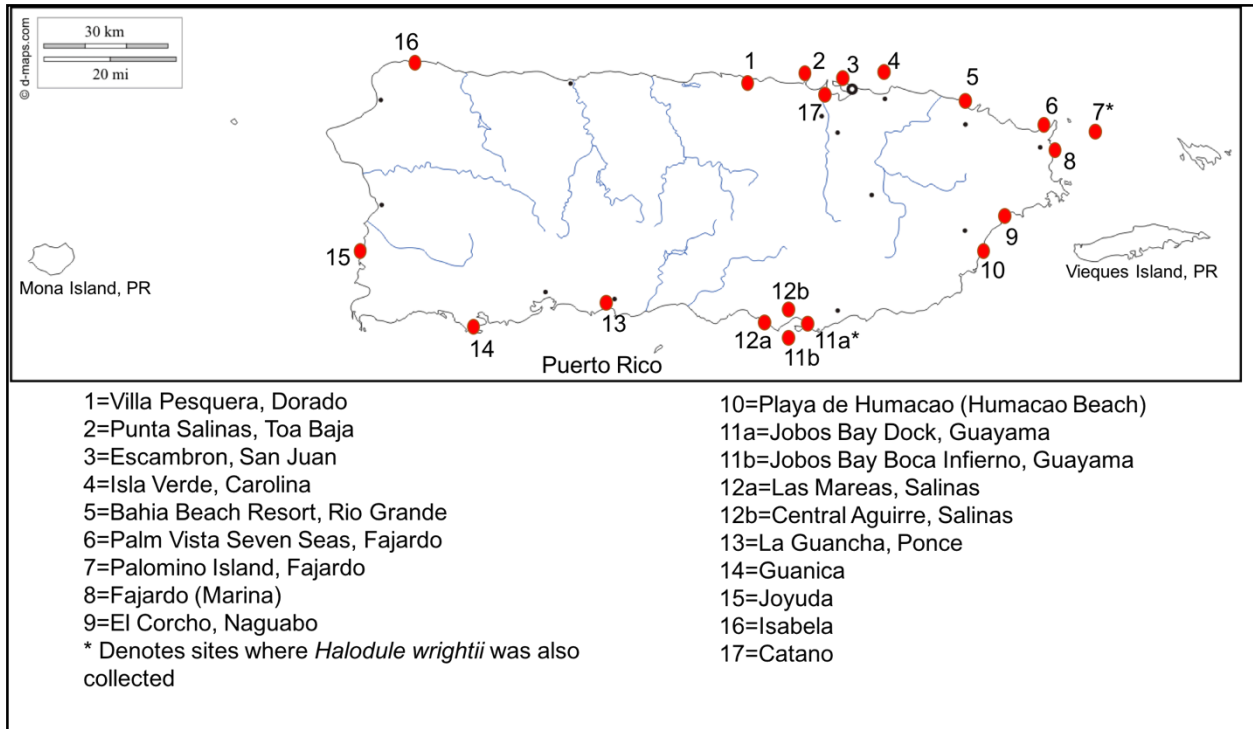


Figure 3. Seagrass Collection Map, Puerto Rico in 2015.

Syrigodium filiforme (manatee grass), *Thalassia testudinum* (turtlegrass), and *Halodule wrightii* (shoalweed) samples collected for *Toxoplasma gondii* testing. *Halodule wrightii* (*) is not as abundant in Puerto Rico as other seagrasses so this species was only collected at 2 sites.

CHAPTER III
Parasites of the West Indian manatee (*Trichechus manatus*)

Co-researchers for this chapter included Dr. R. K. Bonde and the staff at the USGS; Dr. Martine de Wit and the staff at the MMPL (within the FWS); Dr. Mignucci-Giannoni, Carly Rivera and the MCC (Manatee Conservation Center) staff; and Drs. Rick Gerhold, Debra Miller, and Chunlei Su. Co-researchers aided in securing financial support, sample collections, or manuscript review.

Abstract

The West Indian manatee (*T. manatus*) is divided into two subspecies the Antillean manatee (*T. m. manatus*) and the Florida manatee (*T. m. latirostrus*) and both are listed as endangered. Although necropsy is a valuable tool in diagnosing parasite infections, a need for better techniques in antemortem animals is important. With few exceptions, parasite diagnosis in manatees has been based primarily from necropsy findings. Fecal samples collected from Antillean manatees (n=3) from Puerto Rico and Florida manatees (n=10) collected during necropsies were evaluated by centrifugal flotation and ethyl acetate sedimentation to compare the parasites from each of the populations. An additional 11 fecal samples from live-captured Florida manatees were also tested. The most commonly found parasite eggs in both populations included the trematodes *Pulmonicola cochleotrema* (Family: Opisthotrematidae Poche, 1926) and *Nudacotyle undicola* (Family: Nudacotylidae Barker, 1916), the coccidian oocysts of *Eimeria* spp., and the ascarid *Heterocheilus tunicatus*. The trematode *Chiorchis fabaceus* was abundant in the Florida samples, but no eggs of this species were seen on fecal examination from the Puerto Rico populations. *Chiorchis groschafti* was found in both populations of manatees but at a higher prevalence in the Antillean manatee (100%) compared to the Florida manatee (33%). The trematode *Moniligerum blairi* was found in both populations but was more common in the Florida manatee (42%) than the Antillean manatee (33%). To our knowledge, this is the first report of both *Eimeria manatus* and *Eimeria nodulosa* in the Antillean manatee from Puerto Rico. This study reports prevalence of manatee parasites from two subspecies populations in different geographical locations.

Introduction

The West Indian manatee has two recognized subspecies the Florida manatee and the Antillean manatee; however, arguments for three subspecies have been proposed based on mitochondrial DNA studies of the different West Indian manatee populations (Garcia-Rodriguez *et al.* 1998). Although gene flow seems to be restrictive and suggests little or no intermingling of the separate populations, most populations seem to share the same parasites. Although most cases of parasitism in marine mammals are considered non-pathogenic, there are some parasites that can be fatal to their host. In general, host species, age, and immune status can affect the virulence of parasites; furthermore, factors such as the parasite species, infection intensity, and parasite metabolism can also complicate pathogenicity. It is especially important to understand all aspects of mortality due to parasitism in these animals given their endangered status (IUCN 2008a, b). Parasites of terrestrial mammals are typically transmitted via fecal contamination or predation. Given that manatees are not carnivorous, accidental ingestion of fecal material containing parasite infective stages is suspected to be the mode of transmission for most manatee endoparasites. Additionally, the bipolar filaments found on multiple manatee trematode eggs suggest that the eggs could be motile after released into the water column.

Chiorchis fabaceus was the first intestinal trematode reported in manatees in 1838 (Diesing; Price 1932); however, the closely related *Chiorchis groschafti* was not recognized until 1989 (Coy Otero). Other trematodes reported in manatees include intestinal trematodes *Moniligerum blairi* and *Nudacotyle undicola*, and the lung/nares trematode *Pulmonicola cochleotrema* (formerly *Cochleotrema cochleotrema*; Dailey *et al.* 1988, Forrester *et al.* 1980, Reynolds 1980, Travassos and Vogelsang 1931). The manatee ascarid *Heterocheilus tunicatus* was first described in the Antillean manatee by Khalil and Vogelsang (1932). More recent studies of manatee parasites have turned more to antemortum diagnosis such as fecal flotation procedures. Coccidian species are easily missed on necropsy but are detected well by centrifugal flotation of fresh feces. Bando *et al.* (2014) was the first comprehensive study on parasites of the Florida manatee and one of only two studies that looked at fecal examination for diagnosis of manatee parasites (Upton *et al.* 1989). Upton *et al.* (1989) examined the feces of the Florida manatee and discovered two new coccidian species *Eimeria manatus* and *Eimeria nodulosa*. Borges *et al.* (2011) first described *Cryptosporidium* spp. from the feces of Antillean and Amazonian manatees from Brazil. Given the difficulties with studying aquatic organisms, the majority of these parasite life cycles are unknown and most of what is known has been derived from knowledge of terrestrial parasite life cycles. The focus of this project was to compare and contrast the parasites present in the two distinct subspecies of manatees by fecal examination. Also parasite prevalence in these two populations will be reported. To the author's knowledge, this is the first report of *Eimeria* spp. oocysts in the Antillean manatee from Puerto Rico.

Methods

Collections

Live manatees

Fecal samples were collected from 11 manatees during routine health assessments conducted by the USGS in Crystal River, Florida. During these assessments, manatees were

capture for examination on the beach and freshly defecated feces was collected at this time (Merson *et al.* 2014).

Dead manatees

Fecal samples were collected from the gastrointestinal tract of 10 Florida manatees at necropsy by the staff at the MMPL and convenience samples of adult parasites were also recovered from two of these manatees. Similarly, fecal samples were collected from 3 Antillean manatees at necropsy directly removed from the gastrointestinal tract by the staff at the MCC but no adult parasites were recovered.

Testing

Centrifugal Flotation with Sucrose

Procedures for centrifugal flotation followed from Dryden *et al.* (2005) for fecal diagnosis of parasites were performed on all manatee fecal samples.

Ethyl Acetate Sedimentation

Fecal examination by sedimentation was done following the SOP guidelines from the UT CVM Diagnostic Parasitology Laboratory handbook and similar to the procedure described by Elkins *et al.* (1986) with the substitution of formalin for water in the final ethyl acetate centrifugation. This substitution kills eggs, oocysts, or larvae present making them easier to examine. Ethyl acetate sedimentation is the best diagnostic method for the recovery of heavy trematode eggs.

Parasite Ova Identification

All parasite evidence on fecal examinations were identified using the dichotomous key found in Bando *et al.* (2014). *Chiorchis fabaceus* and *C. groschafti* are distinguishable based on egg size (**Figure 4**). *Pulmonicola cochleotrema* eggs were distinguished from eggs of *M. blairi* and *N. undicola* by the appreciable difference in the base widths of the bipolar filaments (**Figure 5A, B**). *Nudacotyle undicola* (**Figure 5C, D**) and *M. blairi* (**Figure 5E, F**) are distinguishable by capsule lengths. A miracidium could also be appreciated in a few of the *M. blairi* eggs (**Figure 5F**). Eggs identified as *H. tunicatus* were easily identified by their dark brown color and large size with a thick shell and internal morula (**Figure 6**). *Eimeria* spp. were easily distinguishable by size and presence or absence of characteristic nodules on the shell surface of *E. nodulosa* (**Figure 7**).

Statistical Analyses

Analysis of variance (ANOVA) was used to examine the significance of age class (calf, juvenile, adult) on the number of parasites detected. Chi square and Independent t-test were used to evaluate the significance between infection of each parasite and the age class and sex of the manatees. An alpha level of 0.05 was used for all statistical analysis.

Results

All manatee fecal samples from both populations except a single sample contained the coccidian *Eimeria manatus* (95%). This is the first report of oocysts consistent in size and morphology to *E. manatus* and *E. nodulosa* (**Figure 7**) from Antillean manatees in Puerto Rico. Several coccidian oocysts inconsistent with size and morphology of reported *Eimeria* spp. in manatees were also observed in the Florida and Antillean manatee populations. Sizes of these oocysts ranged from 6µm to as large as 20µm. Trematodes *P. cochleotrema*, *N. undicola*, and *C.*

fabaceus were found in 62% of the Florida manatee fecal samples. The trematode *C. groschafti* was more predominant in the Antillean manatees (100%) than in the Florida manatees (33%); whereas, *C. fabaceus* was only found in the Florida manatee (62%). *Heterocheilus tunicatus* was found in over half of the manatee samples tested from each population. *Moniligerum blairi* was detected the least in 42% of the Florida manatee samples but was only detected in one of the Antillean manatee samples. All animal samples had at least two parasites detected. Of the Florida manatee samples in which *M. blairi* was detected, 89% of those were from dead animals and only 11% were in live animal feces. *Pulmonicola cochleotrema*, *N. undicola*, *C. groschafti*, and *E. manatus* were found in all 3 Antillean manatee fecal samples (100%). *Heterocheilus tunicatus* was found in two (67%) and *M. blairi* and *E. nodulosa* were found in one (33.3%) of the Antillean manatee samples (**Table 5**).

ANOVA showed no significant difference in numbers of parasites between age classes ($P=0.098$). Chi-square test showed that adult manatees (71.4%) had significantly higher infections with *C. groschafti* than juveniles (10%) and calves (25%; $P=0.028$). Chi-square test also showed that adult manatees (100%) had significantly higher infections with *H. tunicatus* than juveniles (40%) and calves (0%; $P=0.03$). No other infections showed significant differences between infection percentage and age class, sex, or status (dead/alive).

Discussion

Most parasite diagnosis in manatees takes place at necropsy and few reports have ever looked at the feces of the manatees for antemortem diagnosis (Bando *et al.* 2014, Borges *et al.* 2011, Lainson *et al.* 1983, Upton *et al.* 1989). Our study reports higher ova prevalence in the Florida manatees of *C. fabaceus*, *P. cochleotrema*, *M. blairi*, *N. undicola*, *H. tunicatus*, and *E. manatus* than previously reported by Bando *et al.* (2014); however, our study reports the same prevalence of *C. groschafti* (**Table 5**). Unfortunately, our small sample size and the lack of previous publications on parasite prevalence in the Antillean manatees from Puerto Rico make it impossible to derive many conclusions for the parasite results from these animals in this study. Colón-Llavina *et al.* (2009) corrected previous reports of *C. fabaceus* in the Antillean manatee by Mignucci-Giannoni *et al.* (1999a, b) to *C. groschafti* after a reclassification by Mora-Pinto (2000). Mora-Pinto (2000) described two distinct *Chiorchis* spp. from each manatee subspecies; however, it appears that although *C. fabaceus* has never been described from the Antillean manatee (post correction), both manatee subspecies do share *C. groschafti* (Bando *et al.* 2014). Our results are consistent with the corrected reports. The high prevalence of both *Eimeria* spp. is significant given what we know about the transmission of its terrestrial counterparts. *Eimeria* spp. in cattle, for instance, is best transmitted when animals are overcrowded and housing conditions are poor. Understanding the transmission of the *Eimeria* spp. in these aquatic herbivores requires knowledge of aggregation behavior (e.g. breeding masses and warm water sites). The widespread prevalence of the *Eimeria* spp. suggests that oocyst contamination is high in aggregation areas, such as warm water sites, to maximize transmission during these brief periods of accumulation.

Similarly, there are several possible explanations as to how these two presumably divided manatee populations could share parasite species. Rostral deflection and dental adaptations have provided unique insight into manatee evolution (Domning 1982, Domning and Hayek 1986). Significant differences in adaptations to temperatures and feeding behaviors suggest that

temperature, water depth, and the strength of water currents effectively segregate the two West Indian manatee subspecies (Domning and Hayek 1986), so perhaps the connection is evolutionary. Although these divisions in genetic variation suggest complete segregation it is likely that the two subspecies cross-migrate or fill niches previously utilized by other manatee subspecies. A parasite's ability to utilize these host habitats and ecosystems to await the next host could also facilitate the transmission of the parasites to seemingly unconnected host populations.

The trematode *C. groschafti* has been exclusively found in the Antillean manatee. This exclusivity suggests the lack of the proper snail or mollusc intermediate host in the Florida manatee habitat to perpetuate the trematode's life cycle. However, it is a bit puzzling that the subspecies share the closely related trematode *C. fabaceus* which would presumably utilize the same species of snail or mollusc intermediate host as *C. groschafti*.

Many organisms found on the fecal flotations from the Florida manatees were inconsistent with any descriptions previously reported (**Figures 8-13**). A single egg consistent with the descriptions of *C. fabaceus* except for the size (**Figure 4B**) was found in one of the samples. The typical trematode eggs reported in manatees have two bipolar filaments, one on each end of the capsule (*P. cochleotrema*, *N. undicola*, *M. blairi*); however, many trematode eggs were found with more than one filament per capsule end (**Figure 8B, D- I**), splits in one filament (**Figure 8A, E**), or with many smaller hair-like projections attached at one end surrounding the filament (**Figure 8C, D**). It is unknown if these are atypical mutations of known trematode eggs, stages of known species more developed than typically seen from fresh feces, or new species of trematode eggs not yet described. Unfortunately, since adult trematodes were not collected from the specific animals passing these unique eggs, new parasite species cannot be confirmed without further testing and more sampling. Two atypical ascarid egg characteristics are described as having a visible cap similar to that covering the micropyle of some coccidian species (**Figure 9A-D, F-I**) and appreciable larval development within the egg which was observed more often as the samples aged (**Figure 9A-E, G**). Many unidentifiable eggs were seen on fecal examinations including ascarid-like eggs (**Figure 10A & B**), trematode-type eggs (**Figure 10D, F-I**), hookworm-like eggs (**Figure 10C & D**), and an unidentified larvated egg (**Figure 10E**). A significant number of unidentified cysts were also detected on fecal examination and are suspected to be amoebae or ciliates (**Figure 11A-D**). Cysts collapsed in sugar solution (**Figure 11D**) but cysts that were not collapsed had 3-5 nuclei clearly visible inside the cyst (**Figures 11E & F**).

Three unidentified larvae were seen on fecal examination (**Figure 12**) with similar lengths and two had similar anterior and posterior morphology (**Figure 12A & B**). All larvae measured within the 400-500 μ m range. It is unknown if these are larvae or adult worms nor is it possible to determine if they are free-living or parasitic.

Little is known of parasites in manatees and their effect on the animal's overall health. Our results are consistent with other parasite reports in the two separate populations; however, this is the first report of *E. manatus* and *E. nodulosa* in the Antillean manatees from Puerto Rico. Research on aquatic parasite life cycles is vital to understanding the transmission of these organisms between subspecies. Further investigations should look at parasite pathogenicity in these animals. Given the large number of ova and larvae that were not identified and the very

few studies of manatee parasites from fecal examination, there is a great need for further research in this field.

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Appendix

Table 5: Parasite Prevalence in the Florida and Antillean manatees

PARASITE	FLORIDA N=21	ANTILLEAN N=3	TOTALS N=24	Prevalence Reported by Bando <i>et al.</i> 2014
TREMATODA				
<i>Pulmonicola cochleotrema</i>	62% (13)	100% (3)	67% (16)	14%
<i>Moniligerum blairi</i>	43% (9)	33.3% (1)	42% (10)	19%
<i>Nudacotyle undicola</i>	62% (13)	100% (3)	67% (16)	29%
<i>Chiorchis fabaceus</i>	62% (13)	0%	54% (13)	43%
<i>Chiorchis groschafti</i>	33% (7)	100% (3)	42% (10)	33%
NEMATODA				
<i>Heterocheilus tunicatus</i>	52% (11)	67% (2)	54% (13)	38%
PROTOZOA				
<i>Eimeria manatus</i>	95% (20)	100% (3)	96% (23)	83%
<i>Eimeria nodulosa</i>	38% (8)	33.3% (1)	37.5% (9)	50%

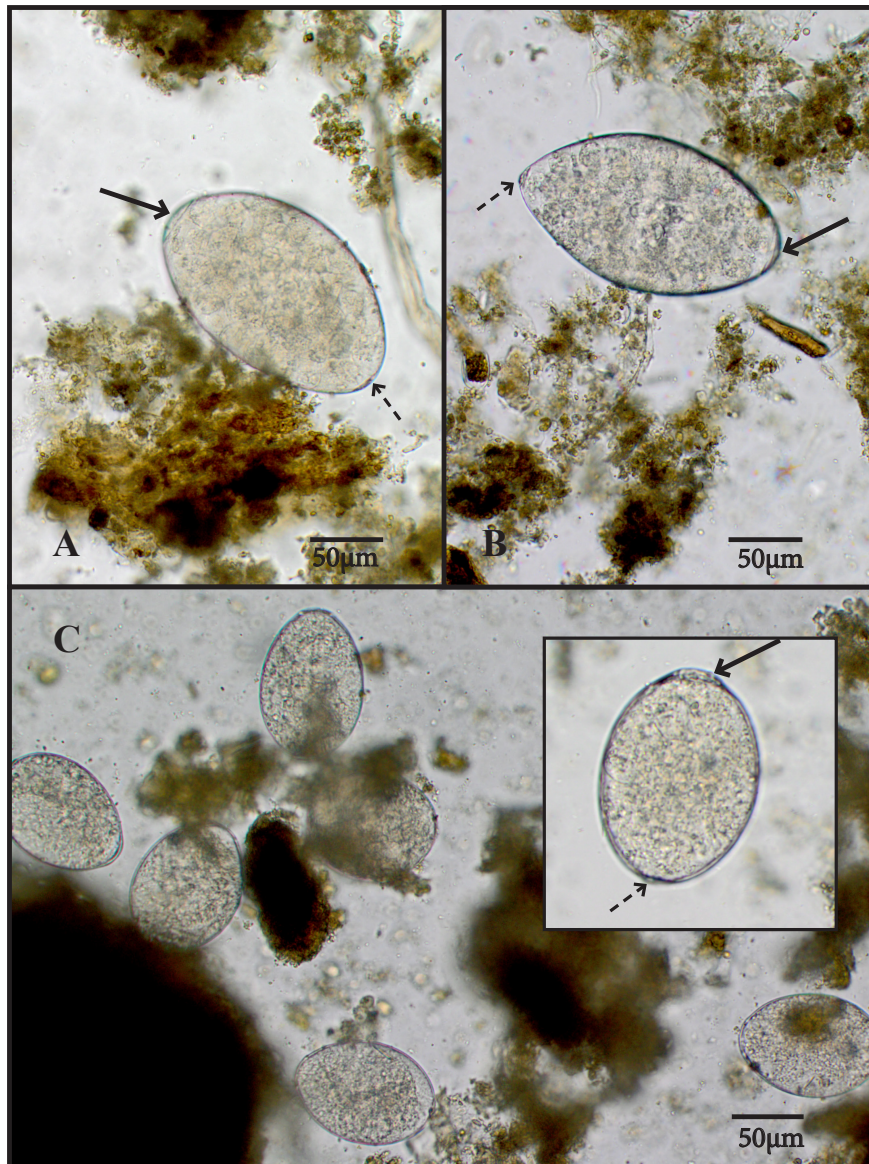


Figure 4. Trematode parasite eggs of the Florida manatee (*Trichechus manatus latirostris*). **A=** *Chiorchis fabaceus* egg (168 x 114 μ m) from a live male adult. **B=** Unusually large *Chiorchis fabaceus* egg (186 x 115 μ m) from a live male juvenile. **C=** *Chiorchis groschafti* eggs (110 x 78 μ m) from a dead female juvenile (**Inset:** two opercula visible on higher magnification from a live male adult). Large opercula (solid arrow) and opercular knobs (dotted arrow) noted.

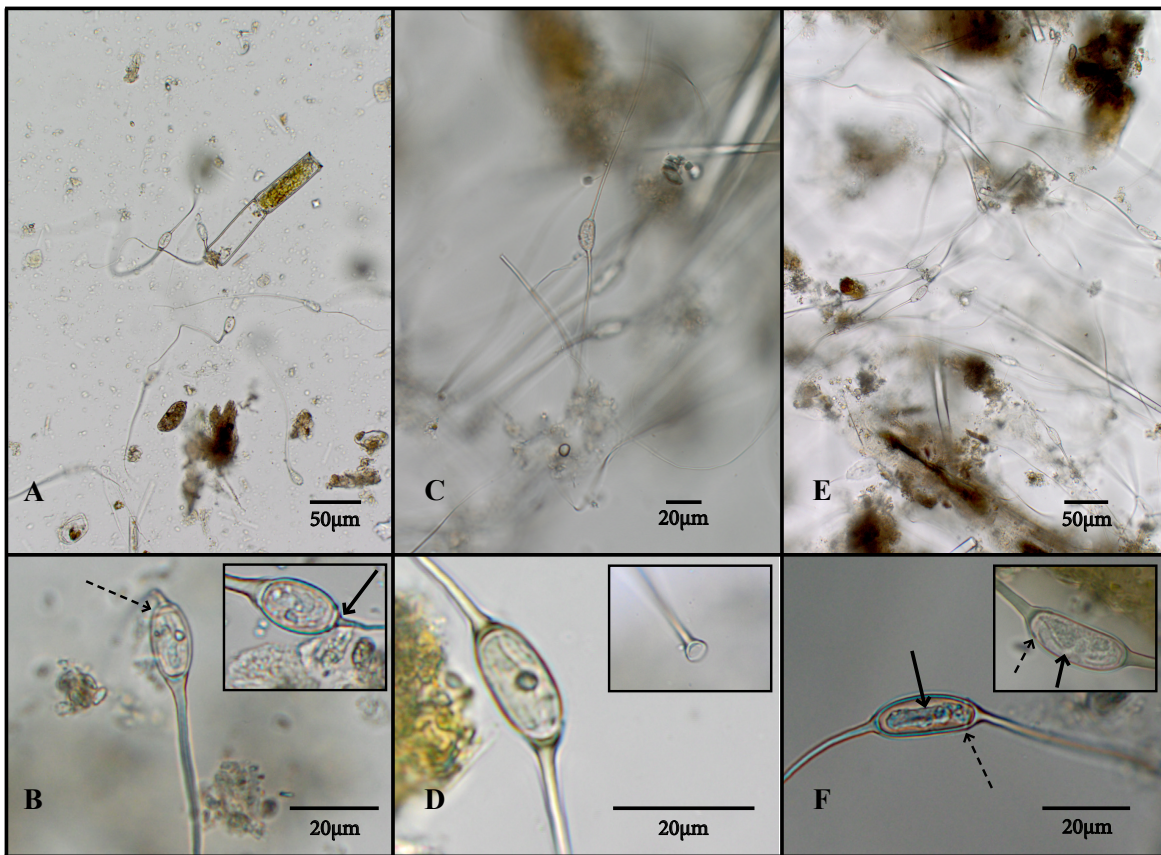


Figure 5. Trematode eggs with bipolar filaments of the Florida manatee (*Trichechus manatus latirostris*).

A, B= *Pulmonicola cochleotrema* eggs, operculum (dotted arrow) on end with smaller filament base (A) from a dead male juvenile (B) from a live male juvenile. (**Inset:** uneven base of bipolar filaments visible on higher magnification from a dead male adult; Smaller base width~ 1µm (solid arrow), Larger base width~ 4µm, Capsule length~ 18µm). **C, D= *Nudacotyle undicola*** eggs (C) from a dead female calf (D) from a live male adult, Base widths of bipolar filaments averaging 3.5µm, capsule length average= 18.5µm, miracidium beginning to form, (**Inset:** single filament broken off of capsule often noted from a live male calf). **E, F= *Moniligerum blairi*** eggs (E, F, and Inset) from dead female calf, Base widths of bipolar filaments are equal averaging 3.5µm, capsule length average= 22µm, (**F & Inset:** miracidia [solid arrows] and opercula [dotted arrow] visible on higher magnification).

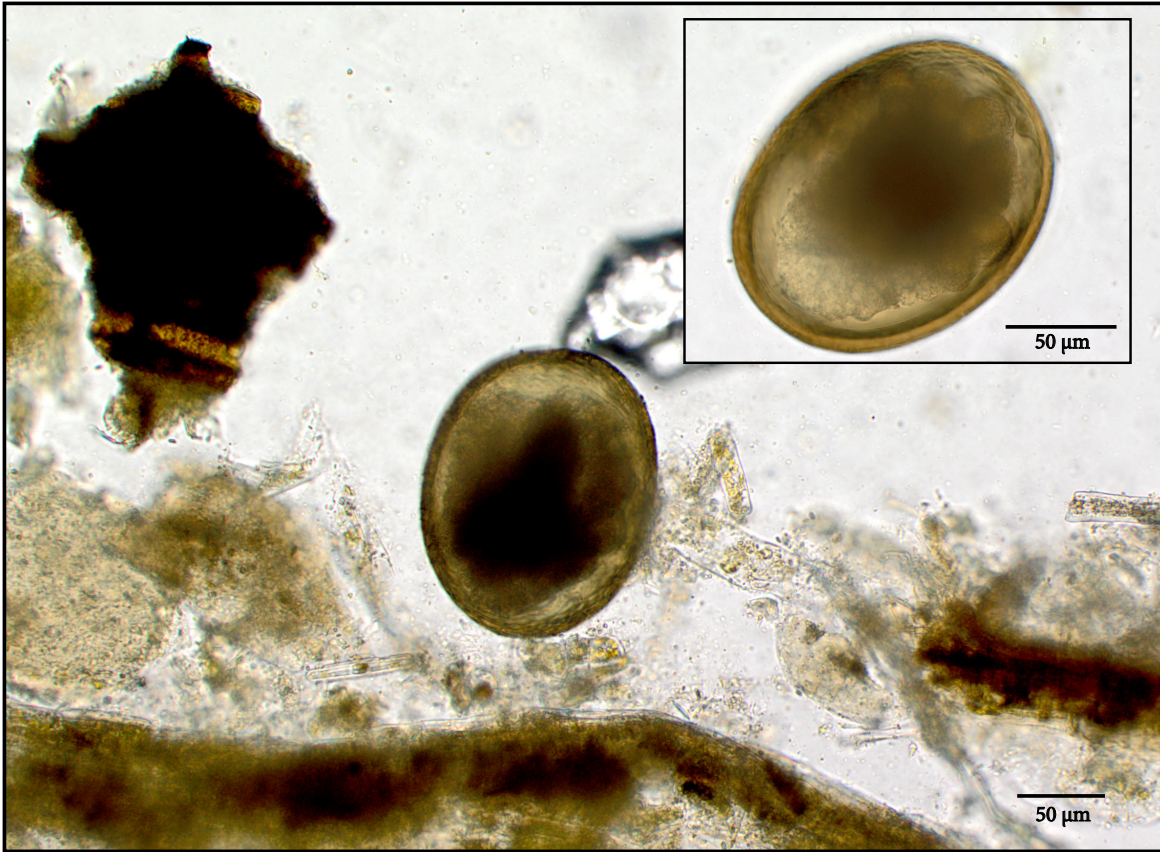


Figure 6. Ascarid eggs from the Florida manatee (*Trichechus manatus latirostris*). *Heterocheilus tunicatus* eggs (163 x 140μm) (Inset: internal morula visible on higher magnification). **Figure & Inset** from a live male adult.

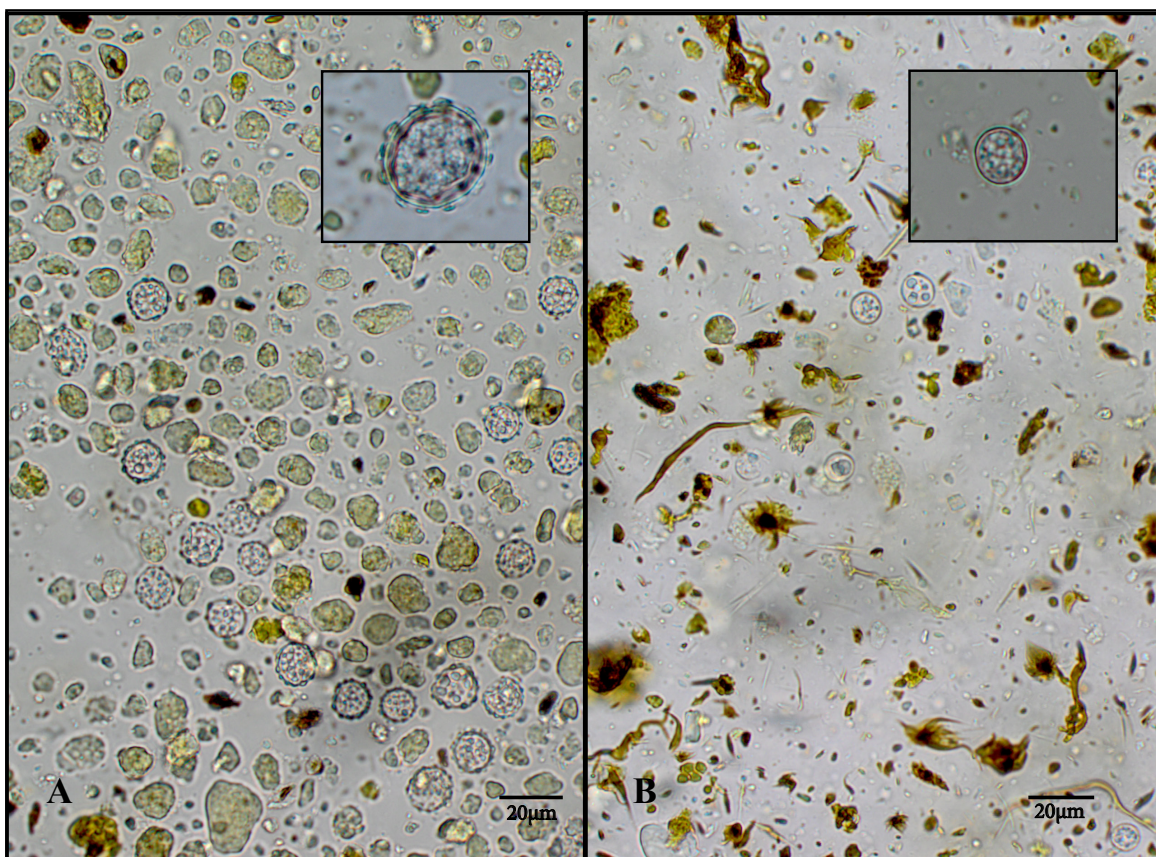


Figure 7. Protozoa from the Florida manatee (*Trichechus manatus latirostris*).

A= *Eimeria nodulosa* oocysts from a dead female juvenile (diameter ~15µm) (Inset: mushroom-like nodules covering oocyst shell visible on higher magnification from a live male adult). B= *Eimeria manatus* oocysts from a dead male juvenile (diameter range 8-10µm) (Inset: smooth oocyst shell visible on higher magnification from a live male adult).

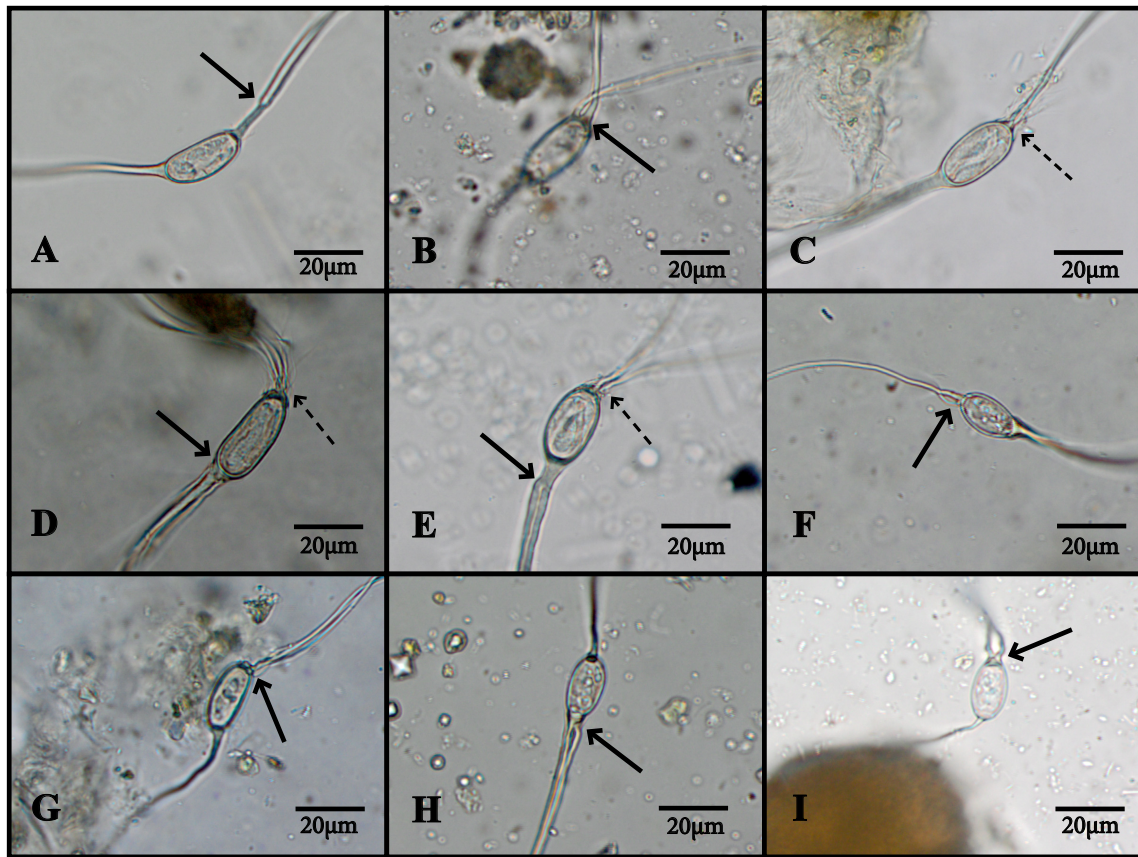


Figure 8. Atypical characteristics of trematode eggs from the Florida manatee (*Trichechus manatus latirostris*).

A= Trematode egg with split filament (solid arrow), capsule=22.47µm. **B=** Trematode egg with extra polar filament on one end (solid arrow) from dead female calf, capsule=18µm. **C=** Trematode egg with several small hair-like projections (dotted arrow), capsule=17µm. **D=** Trematode egg with three filaments on one end (solid arrow) and small hair-like projections (dotted arrow), capsule=19µm. **E=** Trematode egg with split filament (solid arrow) on one end and small hair-like projections (dotted arrow), capsule=22.99µm. **F=** Trematode egg with two filaments on one end (solid arrow) from a dead female juvenile, capsule=17.19µm. **G=** Trematode egg with multiple small hair-like projections on one end (solid arrow), capsule=20.9µm. **H=** Trematode egg with two filaments on one end (solid arrow), capsule=18µm. **I=** Trematode egg with two filaments on one end (solid arrow) from a dead juvenile, capsule=19µm. **A, C-E** from same dead female calf. **G & H** from same dead male juvenile.

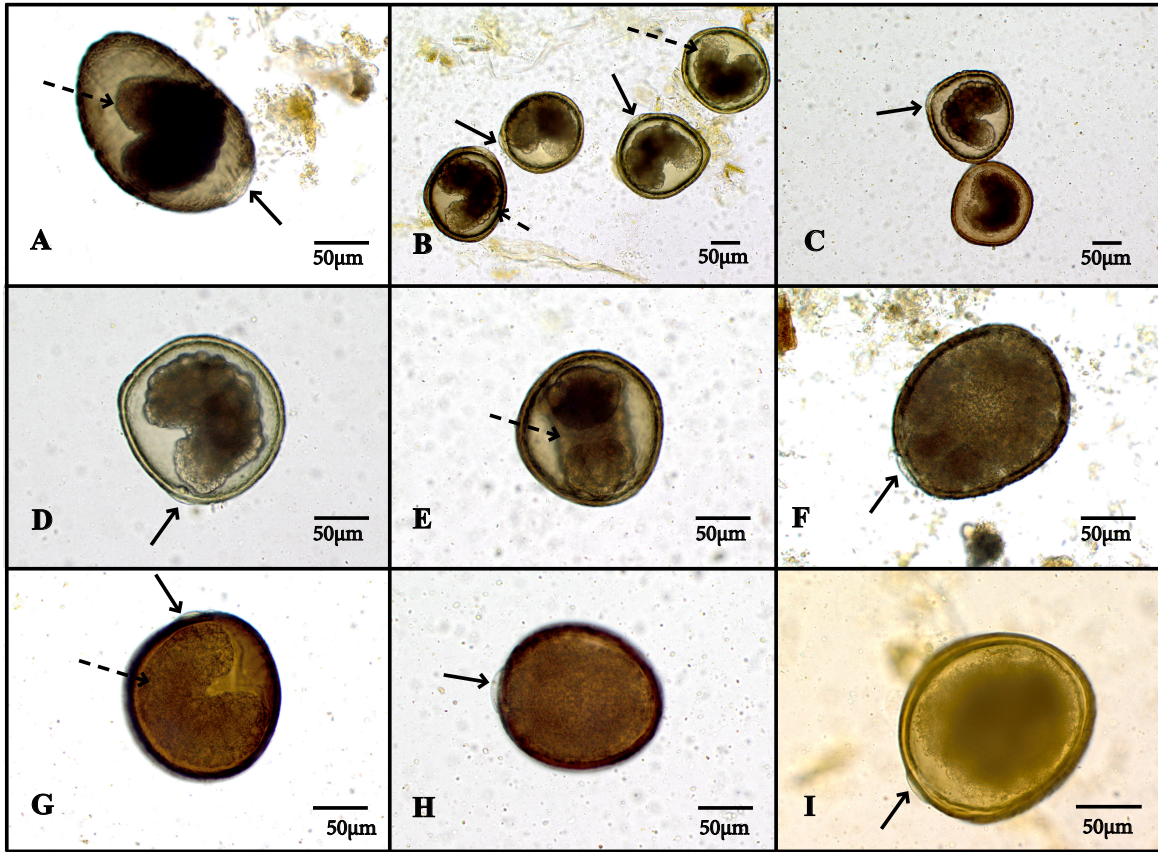


Figure 9. Atypical characteristics of ascarid eggs from the Florida manatee (*Trichechus manatus latirostris*)

All eggs assumed to be *Heterocheilus tunicatus* and average size= 163 x 140µm. Solid arrows= suspected micropyle and cap. Dashed arrows= larval development within egg. **A** from a dead juvenile male. **B & I** from a live male adult. **C & E** from a live male adult. **D** from a live female adult. **F** from a dead male juvenile. **G** from a live male adult. **H** from a live male juvenile.

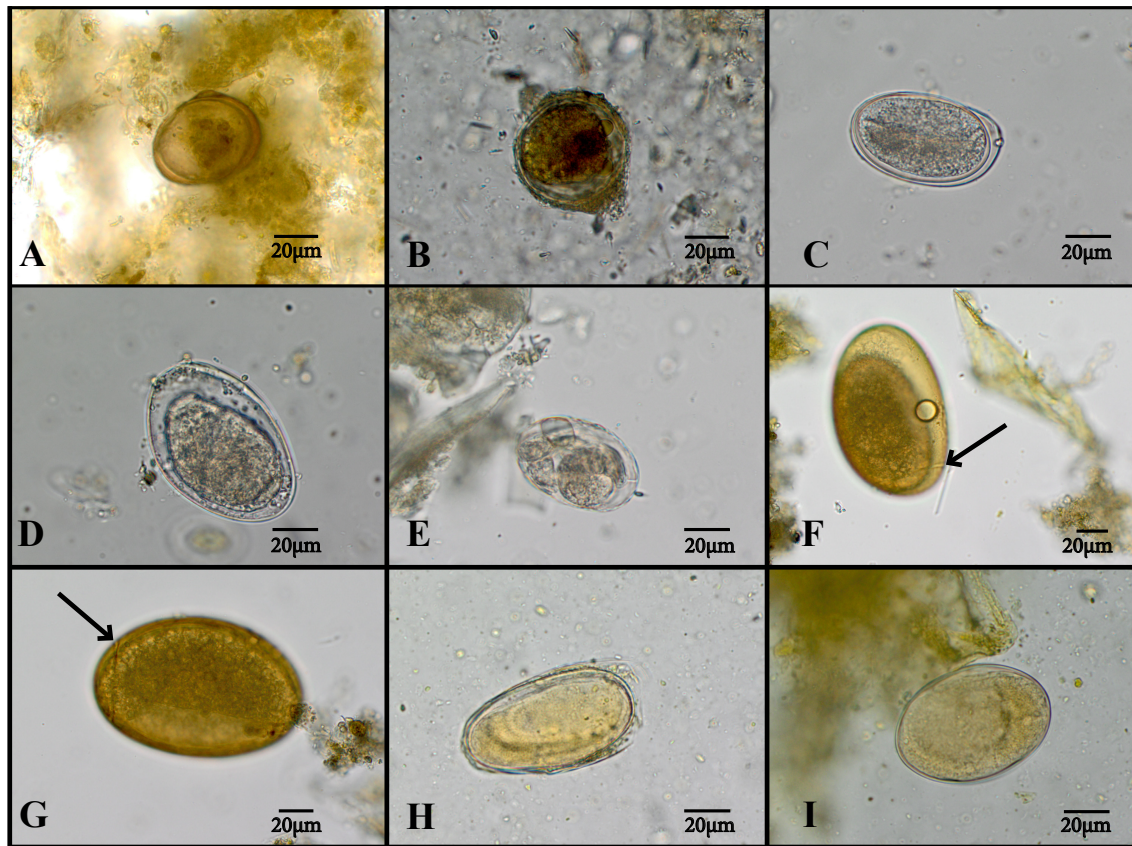


Figure 10. Unidentified helminth eggs from the Florida manatee (*Trichechus manatus latirostris*).

A= ascarid-type egg with visible (50 x 42µm). **B**= ascarid-type egg (diameter~50µm) from a dead female juvenile. **C**= hookworm-type egg (64 x 39µm) from a live male adult. **D**= unknown trematode-type egg (76 x 41µm). **E**= larvated egg (56 x 40µm) from a live male juvenile. **F**= unknown trematode-type egg with operculum (arrow; 120 x 80 µm). **G**= unknown trematode-type egg with operculum (arrow; 111 x 71µm). **H**= unknown egg (80 x 55µm) from a live male adult. **I**= unknown trematode-type egg (70 x 50µm). **A & D** from same live male juvenile. **F,G, I** from same live male adult.

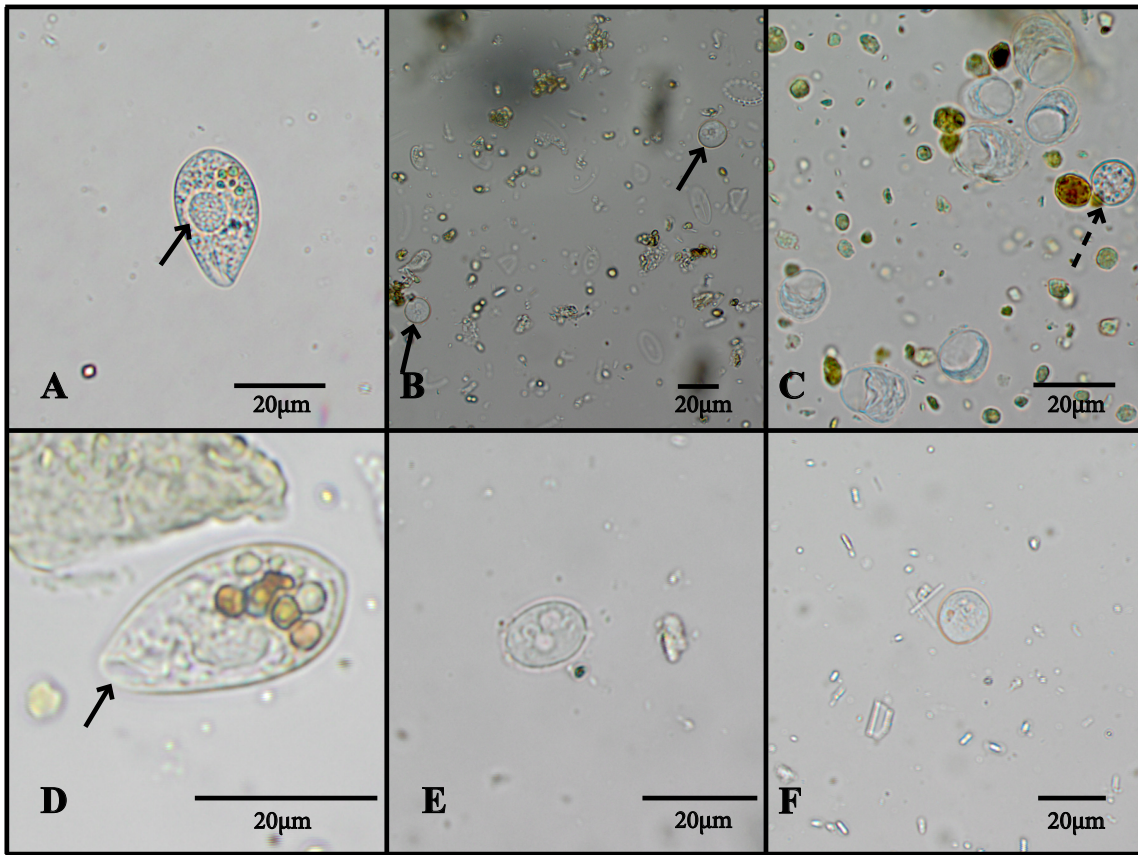


Figure 11. Unidentified cysts from the Florida manatee (*Trichechus manatus latirostris*). **A=** Ameoba or ciliate (length= 31.5µm) with large nucleus (arrow). **B=** Small cysts visible on 10x (length range 15µm- 18µm) with 2-5 nuclei. **C=** Small collapsed cysts, length range 15µm- 18µm (dotted arrow= *Eimeria manatus* oocyst). **D=** Ameoba or ciliate from A with fold (arrow) easily visible on higher magnification. **E, F=** Small cysts on higher magnification with 3 nuclei clearly visible in each. All pictures from live male adults; **A & D** from same live male adult, **C & E** from same live male adult.

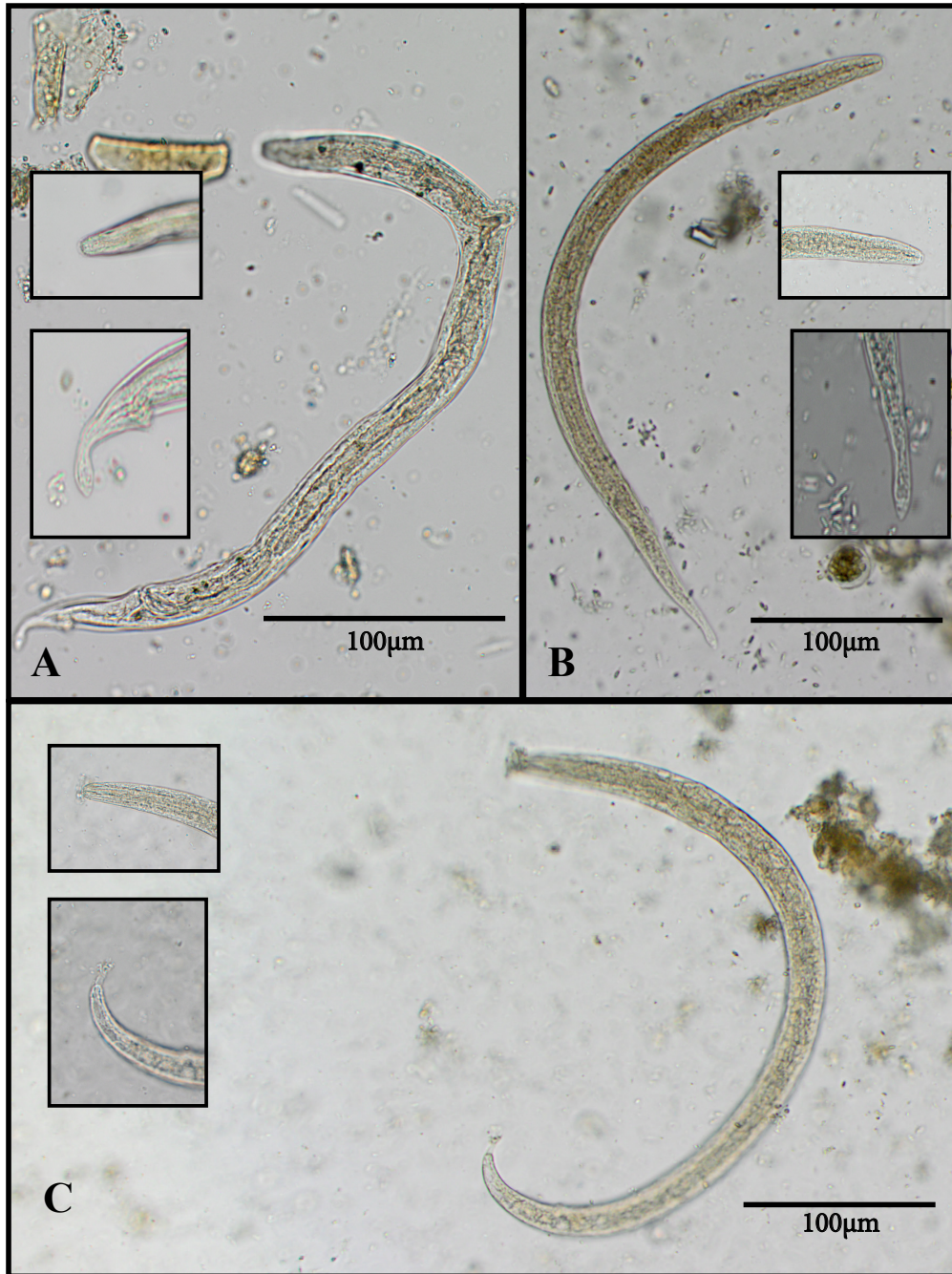


Figure 12. Unidentified larvae from the Florida manatee (*Trichechus manatus latirostris*). A= unknown larva from a live male adult. (**Inset:** anterior and posterior morphology on higher magnification), Length ~387µm. B= unknown larva (**Inset:** anterior and posterior morphology on higher magnification), Length ~ 424.5µm. C= unknown larva (**Inset:** anterior and posterior morphology on higher magnification), Length ~474µm. **B & C** from same live female adult.

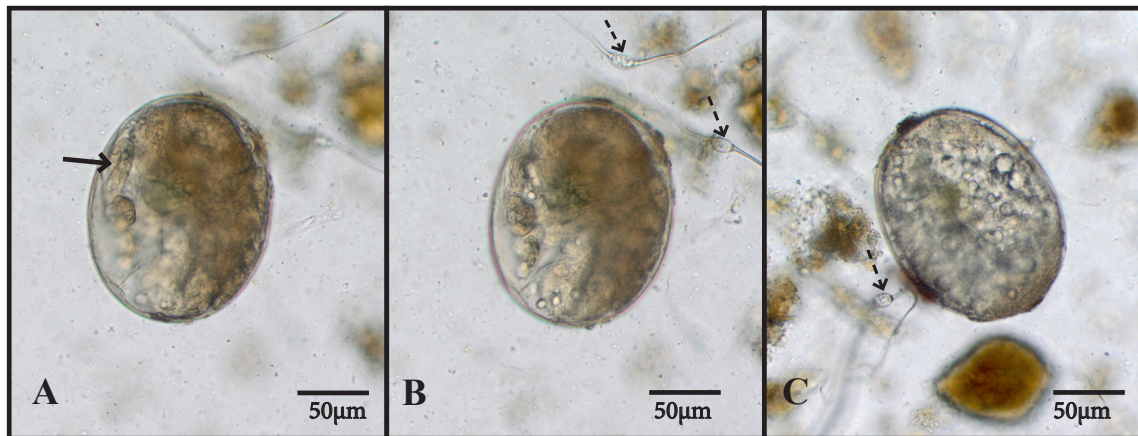


Figure 13. Unidentified arthropod eggs from the Florida manatee (*Trichechus manatus latirostris*).

Arthropod eggs measuring 160 x 128µm. **A**= shows appendages within egg shell (arrow). **B**= shows more appendages within egg shell (dotted arrows= *Pulmonicola cochleotrema* eggs). **C**= shows “knobs” on outer egg shell (dotted arrow= *Pulmonicola cochleotrema* egg). All pictures from same dead male juvenile.

CHAPTER IV
Conclusion

Toxoplasma gondii is a parasite of felids that causes disease in a wide range of intermediate hosts (IH) such as the West Indian manatee (*Trichechus manatus*). Toxoplasmosis has been detected in many species of marine mammals both through serologic and histologic methods (Aguirre *et al.* 2007, Alvarado-Esquivel *et al.* 2012, Cabezon *et al.* 2004, Conrad *et al.* 2005, Delgado *et al.* 2013, Forman *et al.* 2009, Lambourn *et al.* 2001, Mathews *et al.* 2012, Measures *et al.* 2004, Murata *et al.* 2004, Oksanen *et al.* 1998, Omata *et al.* 2005, Pretti *et al.* 2010, Santos *et al.* 2011, Sulzner *et al.* 2012). Given that serum from previously documented toxoplasmosis manatee cases was not collected we were unable to determine serological cut off values for manatees. Our findings highlight a need to routinely collect sera from necropsied marine mammals in order to combine serological testing with necropsy findings and provide validation of serologic tests that can be subsequently used for antemortem detection of *T. gondii*.

Epidemiologic studies on *T. gondii* in marine mammals are extremely important in the conservation of aquatic animals (Shapiro *et al.* 2010a, 2010b, 2014). Our study is the first attempt to obtain information on *T. gondii* epidemiology in the Antillean and Florida manatees. By incorporating direct oocysts detection from felid feces, environmental testing of the manatee's primary food source (sea grass), and serologic and histologic screening of the manatees themselves, we investigated the eco-epidemiology of toxoplasmosis in marine mammals. Excluding the one inconclusive serologic test from a dead Antillean manatee from Puerto Rico, the seroprevalence of *T. gondii* in the Antillean manatees from Puerto Rico (n=5) was 0% as was the seroprevalence in the Florida manatees (n=353). No *T. gondii* oocysts were found in the felid samples from Florida. However, Luria *et al.* (2004) found an 11% seroprevalence to *T. gondii* in Florida cats indicating that these seropositive cats were infected and previously shed oocysts in the environment. Although we failed to collect feces from the free-roaming cats in Puerto Rico, we report a 16% seroprevalence to *T. gondii* in the serum samples from these populations. Given that oocysts are only shed from felids for a short period, serological testing of cats provides a more accurate representation of infection prevalence in felids. Unfortunately without oocysts, we were not able to genotype the parasites which would have provided the first step in constructing an epidemiological molecular link between feral cat populations and toxoplasmosis in manatees. Despite this, the detection of *T. gondii* antibodies in the felid populations indicates that these animals shed oocysts prior to sampling, which supports an epidemiological connection that necessitates further disease monitoring in the manatee populations. No *T. gondii* was found in the 33 seagrass samples collected from seagrass beds around Puerto Rico's main island. Unfortunately, we were unable to secure permits for seagrass collection in Florida waters. Future manatee toxoplasma research projects should include investigations of seagrass from areas surrounding coastal development and feral cat populations as well as less developed areas to understand what anthropogenic factors are associated with parasite transmission and thus identify potential mitigation steps to minimize the transmission.

Despite the previous studies on manatee parasites detected in the feces, our results show that there are still many parasite eggs/oocysts that we are not able to identify (Bando *et al.* 2014; Upton *et al.* 1989). Future studies should focus on antemortem parasite detection in the manatees as well as combining classical parasitology and molecular techniques. Determining clinical signs and the extent of morbidity caused by parasites is difficult but it is critical to understanding how various parasite loads and coinfections affect the manatees. Our results

demonstrated that all animals had multiple parasite species. Deciphering the exact life cycles can provide biologists with insight into modes of infection which could lead to better management strategies to reduce contamination and exposure to the parasites and limit their transmission. Thus, studies involving parasite development from the egg stage to adult is warranted.

Given the concerns for manatee population health both in Florida and Puerto Rico, investigating the causes of morbidity and mortality in these animals is vital to conserving their numbers. Antemortem manatee parasite prevalence can provide valuable baseline information for these animals. Parasite pathogenicity is nearly impossible to determine without experimental infection studies; however, observational studies of live manatees could provide more inference to clinical signs associated with infections diagnosed antemortem. Additionally, more research on toxoplasmosis in both manatee subspecies is essential to determining future disease impacts on these subspecies populations.

Marine mammals with the ability for terrestrial locomotion (e.g. sea otters), those with coastal plain habitats, and captive marine mammals are likely most at risk for toxoplasmosis because of their proximity to the completely terrestrial feline definitive host (DH) and freshwater run-off. The manatee populations with the highest prevalence to *T. gondii* are captive animals in close proximity to uncontrolled feral cat populations (Alvarado-Esquivel *et al.* 2012, Dubey *et al.* 2009). Captivity, either in situ (constrained in natural environment) or ex situ (held in facility tanks in an unnatural environment), is likely a mitigating factor for toxoplasmosis (Attademo *et al.* 2016). Methods for mitigating *T. gondii* transmission to manatee populations should primarily focus on feral cat population controls specific to manatee habitats. Furthermore, an intensive survey of *T. gondii* in free-roaming cats in proximity to the watershed regions is warranted. Environmental testing in coastal areas immediately following optimal weather patterns for freshwater runoff would maximize *T. gondii* detection and pinpoint locations for marine contamination. Knowing which habitats are most frequently utilized by manatees would allow biologists to produce targeted recommendations for costal development and feral cat removal to attempt to minimize *T. gondii* transmission.

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VITA

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