ARTICLE

Molecular detection of *Toxoplasma gondii* in select cetaceans stranded in the Philippines in 2019

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Toxoplasma gondii infections affect marine mammal species worldwide. Investigating the presence of the protozoan parasite in marine mammals is crucial to understanding land-sea connection in relation to the movement of pathogenic and potentially pathogenic microorganisms in the marine environment. The main objective of this study was to detect *T. gondii*, through nested PCR targeting the *RE* gene of the parasite, in select cetaceans (n=19) that stranded in different parts of the Philippines from January to December 2019. *T. gondii* was detected in four cetaceans, specifically, in the brain tissue of a pantropical spotted dolphin (*Stenella attenuata*), brain and stomach tissues of a Cuvier's beaked whale (*Ziphius cavirostris*), brain and skeletal tissues of a pygmy sperm whale (*Kogia breviceps*), and lung tissue of another pantropical spotted dolphin. No statistically significant association was established between the stranding parameters and presence of *T. gondii* DNA in tissues of cetaceans. To the best knowledge of the authors, this study is the first to report the presence of *T. gondii* in a Cuvier's beaked whale (*Ziphius cavirostris*). The detection of *T. gondii* in deep dwelling cetacean species supports the claim that toxoplasmosis may have extended beyond coastlines where pathogen run-off is likely. *T. gondii* prevalence among cetaceans in the Philippines has received attention for the past five years, and there is a need to continue the surveillance of *T. gondii* among local cetacean populations given its implications in the conservation and management of these marine mammals.

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INRODUCTION

The occurrence of *Toxoplasma gondii* in marine mammals, particularly cetaceans, has been reported worldwide (e.g. Shapiro et al., 2018). The protozoan parasite has been detected in Hector's dolphins (*Cephalorhynchus hectori*) in New Zealand (Roe et al., 2013), Guiana dolphins (*Sotalia guianensis*) and

Amazon River dolphins (*Inia geoffrensis*) in Brazil (Santos et al., 2011; Marigo et al., 2013), Mediterranean fin whale (*Balaenoptera physalus*) and striped dolphins (*Stenella coeruleoalba*) in Italian coasts (Guardo et al., 2010; Profeta et al., 2015), and common bottlenose dolphins (*Tursiops truncatus*) in the Eastern Mediterranean Sea (Bigal et al., 2018), among others. It has also been detected in several cetaceans (*Stenella attenuata, Tursiops truncatus, T. aduncus, Kogia breviceps, Grampus griseus, Lagenodelphis hosei, S. longirostris, Globicephala macrorhynchus, S. coeruleoalba,* and *Mesoplodon* sp.*)* in the Philippines (Obusan et al., 2015; Obusan et al., 2019). Toxoplasmosis in cetaceans has often been considered a secondary disease, usually associated with immunosuppression, encephalitis, and abortion in stranded individuals (Grattarola et al., 2016; Mazzariol et al., 2012; Resendes et al., 2002).

T. gondii is among the most widespread parasites due to its wide range of host species and ability to be transmitted through diverse routes (Jeffers et al., 2018). The definitive host of *T. gondii* are cats and related wild felids that shed the unsporulated oocysts, contaminating soils, water bodies, and food items (Hanafi et al., 2014). The parasite has three infectious stages: the tachyzoite which is the proliferative stage, the bradyzoite which is found in tissues, and the sporozoite which is usually transmitted to hosts in external environments. The presence of *T. gondii* in marine mammal tissues presents a public health concern, as widespread consumption of the meat of these animals, especially in indigenous communities, provides an additional route for zoonotic transmission (VanWormer et al., 2013).

Toxoplasmosis can lead to serious illness and death especially among immunocompromised patients, making *T. gondii* a medically significant parasite. It causes birth defects as well as neurological and ocular diseases (Maubon et al., 2008; Montoya and Liesenfeld, 2004). Considering the extent of *T. gondii* infections worldwide, toxoplasmosis qualifies as a *One Health* disease, linking the health of domestic, terrestrial, and wildlife animals and their ecosystems (Aguirre et al., 2019).

Under the *One Health* paradigm, marine mammals are recognized as sentinels for indicating the health of the marine environment (Bossart, 2011). Assessing the health status of stranded cetaceans can provide valuable information for evaluating the impacts of human activities including biological pollution to their populations in the wild. A recent study confirmed the land-sea connection in *T. gondii* infections affecting southern sea otter population (Vanwormer et al., 2016). Investigating the occurrence of *T. gondii* and other zoonotic parasites in marine mammal species is crucial to understanding the movement of pathogenic and potentially pathogenic microorganisms in the marine environment.

Diagnostic techniques for *T. gondii* include molecular, serological, and histological methods. Polymerase chain reaction (PCR) is used to detect the genes of *T. gondii*, such as *B1* gene and *RE* gene. Serological tests to detect specific antibody (IgG or IgM) against *T. gondii* is usually the initial and primary method of diagnosis (Dubey, 2016). Histopathological and immunohistochemistry techniques are also used to confirm toxoplasmosis by detecting the presence of the parasite in host tissues.

As part of cetacean health surveillance, this study generally aimed to detect the presence of *T. gondii* in select cetaceans that stranded in the Philippines from January to December 2019. Specifically, the study aimed to detect *T. gondii* in cetacean tissues through molecular method and find significant association between the occurrence of *T. gondii* in cetacean tissues and stranding event parameters such as stranding season and cetacean sex and age. The 14-year marine mammal stranding reports revealed increasing trend in the stranding events of cetaceans in the Philippines (Aragones and Laggui, 2019; Aragones et al., 2017). These events are opportunities to collect biological samples from local cetacean species, many of which are pelagic and deep diving species.

MATERIALS AND METHODS

Collection of tissue samples from cetaceans

The collection of tissue samples from cetaceans that stranded in the Philippines from January to December 2019 was done by the research teams of Microbial Ecology of Terrestrial and Aquatic Systems (METAS) Laboratory, Institute of Biology, and Marine Mammal Research and Stranding (MMRS) Laboratory, Institute of Environmental Science and Meteorology, University of the Philippines Diliman in collaboration with the Philippine Marine Mammal Stranding Network (PMMSN) and Bureau of Fisheries and Aquatic Resources, Department of Agriculture (BFAR-DA). When it is not possible for the researchers to go to the stranding site, collaborating veterinarians who were trained in microbiological sampling obtained and sent the specimens (frozen or in ethanol or formalin) to the laboratory. Biological specimens were collected based on animal disposition and physical preservation code system for marine mammals (Geraci and Lounsbury, 2005).

Molecular method for detecting *T. gondii*

DNA was extracted from cetacean tissues using a commercial kit (Wizard® Genomic DNA Purification Kit) following manufacturer's instructions. Briefly, 300 µL of tissue sample was transferred to 1.5 mL microcentrifuge tube and 900 µL of cell lysis solution was added. The mixture was incubated for 10 minutes and centrifuged at 13,250 x g for 20 seconds. The supernatant was discarded and 300 µL of nuclei lysis solution was added together with 1.5 µL of RNAse and 100 µL of protein precipitation solution. The solution was centrifuged in the same manner and the supernatant was collected. Finally, 70% ethanol was used to precipitate the DNA and stored with 100 µL DNA rehydrating solution.

To detect the *T. gondii* RE gene, nested Polymerase Chain Reaction (PCR) was performed using the primers (5′-TGACTCGGGCCCAGCTGCGTCTCCTCCCTTCGTCCAA GCCTCC-3') targeting 529 bp for the first round of nested PCR and primers (5′-AGGGACAGAAGTCGAAGGGGGCAGCCAAGCCGGAAA CATC -3') targeting 164 bp for the second round of nested PCR (Fallahi et al., 2014). The reaction mixture for first amplification was prepared containing 5 µL Taq DNA Pol 2.0 Master Mix (Lot No.5200300), 0.5 µL forward and 0.5 µL reverse primers, and 2.8 µL nucleotide free water at a final volume of 10 µL. For second round PCR amplification, the reaction mixture contained 3.4 µL nuclease free water, 5 µL master mix, and 0.5 µL forward and reverse primers. For amplification of *RE* gene (529 bp), the PCR conditions were: initial denaturation for 5 minutes at 94 °C followed by 30 cycles of denaturation for 20 seconds at 94 °C, annealing for 20 seconds at 55 °C, extension for 20 seconds at 72 °C, and final extension for 5 minutes at 72 °C. For the second round of amplification (164 bp), the conditions were: initial denaturation for 5 minutes at 94 °C followed by 35 cycles of denaturation for 20 seconds at 94 °C, annealing for 20 seconds at 55 °C, extension for 20 seconds at 72 °C, and final extension for 5 minutes at 72 °C.

Table 1: Stranded cetaceans that were sampled for *T. gondii*. The PMMSN Code includes first letters of scientific names, latest count of stranding for such species in the region, region, and date; these data are included in the PMMSN database of marine mammal strandings in the Philippines.

Sample Code	PMMSN Code	Common Name	Species	Region Collected	Date Collected (d/m/year)	Season	Sex	Age
S1	Pe01R11090219	melon-headed whale	*Peponocephala electra*	XI	9/2/2019	NE	F	A
S2	Kb10R11120219	pygmy sperm whale	*Kogia breviceps*	XI	12/2/2019	NE	F	A
S3	Sb05R2010319	rough-toothed dolphin	*Steno bredanensis*	II	1/3/2019	NE	M	A
S4	Be01R2100319	Bryde's whale	*Balaenoptera edeni*	II	10/3/2019	NE	M	SA
S5	Zc01R11150319	Cuvier's beaked whale	*Ziphius cavirostris*	XI	15/3/2019	NE	F	A
S6	Fa01R8160319	pygmy killer whale	*Feresa attenuata*	XIII	16/3/2019	NE	M	A
S7	Tt02R6270319	common bottlenose dolphin	*Tursiops truncatus*	VI	27/3/2019	NE	M	A
S8	Sa05R4A090419	pantropical spotted dolphin	*Stenella attenuata*	IV-A	9/4/2019	IMSW	F	SA
S9	Kb03R4A100419	pygmy sperm whale	*Kogia breviceps*	IV-A	10/4/2019	IMSW	M	A
S10	Gg03R3100119	Risso's dolphin	*Grampus griseus*	III	10/1/2019	NE	M	A
S11	Gg04R4B090519	Risso's dolphin	*Grampus griseus*	IV-B	9/5/2019	IMSW	M	A
S12	Kb08R1110519	pygmy sperm whale	*Kogia breviceps*	I	11/5/2019	IMSW	F	A
S13	Sb20R1200519	rough-toothed dolphin	*Steno bredanensis*	I	20/5/2019	IMSW	M	A
S14	Zc02R11300719	Cuvier's beaked whale	*Ziphius cavirostris*	XI	30/7/2019	SW	F	A
S15	Sa26R1131119	pantropical spotted dolphin	*Stenella attenuata*	II	13/11/2019	SW	M	C
S16	Pe06R8070819	melon-headed whale	*Peponocephala electra*	VIII	7/8/2019	SW	M	SA
S17	Gg04R4B090519	Risso's dolphin	*Grampus griseus*	IV-B	9/5/2019	SW	F	A
S18	Gm07R4B051119	short finned pilot whale	*Globicephala macrorhynchus*	IV-B	5/11/19	IMNE	M	A
S19	Lh02R13011119	Fraser's dolphin	*Lagenodelphis hosei*	XIII	1/11/19	IMNE	*	A

Legend:

Season – NE (Northeast monsoon), SW (Southwest monsoon), IMNE (Inter-monsoon before Northeast), IMSW (Inter-monsoon before Southwest). Sex – F (Female), M (Male), * (Undetermined). Age Group – A (Adult), SA (Sub adult), C (Calf), * (Undetermined)

PCR products were subjected to electrophoresis on 1.5% agarose gel containing Gel-Red in TAE (Tris-acetate-EDTA) buffer at 8 V/cm. Gels were viewed under Gel Doc Bio-Rad to observe the target DNA bands.

Statistical analysis

Chi-square test was used to find significant association between the presence of *T. gondii RE* gene in tissues of cetaceans and their stranding parameters (i.e., cetacean sex, age group, stranding season) using NTM SPSS Statistics 20.

RESULTS AND DISCUSSION

Profile of stranded cetaceans

A total of 19 select cetaceans (with codes S1-S19) that stranded in the Philippines (Fig. 1) were sampled for detection of *T. gondii*. Eleven of these cetaceans were males and seven were females, while the sex of one individual was undetermined. The sex of cetaceans is determined by observing the distance between the anal and uro-genital openings (and presence of mammary slits for females) found in the ventral section of the animal, which is sometimes difficult to perform in some live cases, thus the failure of responders to record the data in the field. As for the age group of the cetaceans, 15 were adults, three were sub-adults, and one was a calf (Table 1). All these individuals were involved in single stranding events.

PCR analysis

Molecular detection targeting the *T. gondii RE* gene in brain, cardiac, skeletal, kidney, liver, intestine, stomach, lung, and blood tissues revealed four cetaceans have tissue/s positive for the parasite: two pantropical spotted dolphins (S8, S15), one pygmy sperm whale (S9), and one Cuvier's beaked whale (S14). Specifically, *T. gondii* was detected in brain tissues of S8, S9, and S14, skeletal tissue of S9 (Fig. 2), lung tissue of S15, and stomach tissue of S14 (Table 2). The presence of the parasite's

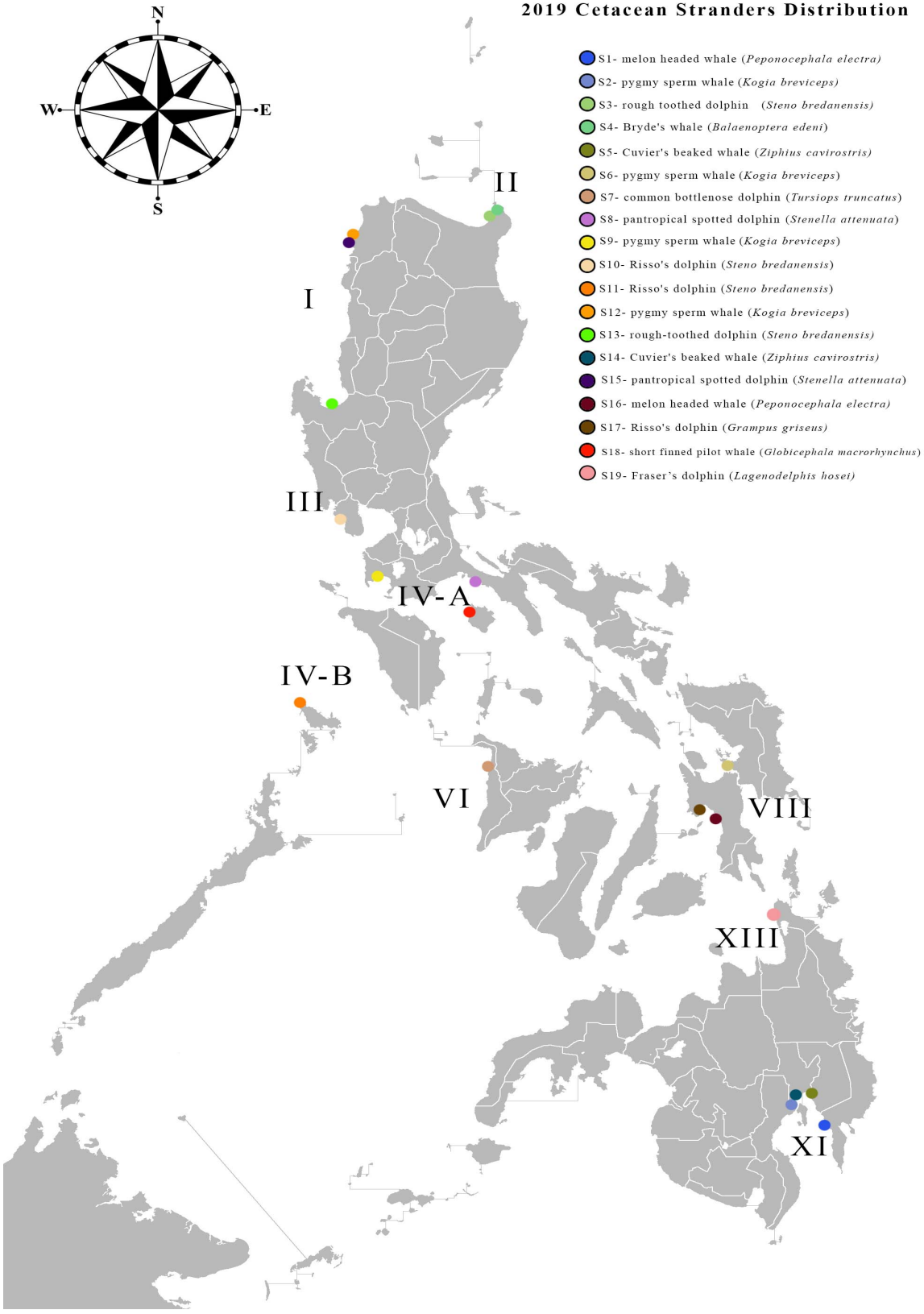

Figure 1: Distribution map of stranded cetaceans sampled for the study.

genetic material may be associated with acute infection while its presence in more than one tissue suggests disseminated toxoplasmosis. However, further investigation is needed to confirm these using other methods of detection such as histopathology and antibody detection.

Overall, *T. gondii* was detected in 21% of 19 select cetaceans that stranded during the year 2019. Previous studies reported the local detection of the parasite in 71% of 28 cetaceans that stranded from 2016-2018 (Obusan et al., 2019) and in 3% of 23 cetaceans that stranded from 2012-2013 (Obusan et al, 2015). The differences in the prevalence could be due to the detection

Table 2: Molecular detection of *T. gondii* targeting the *RE* gene.

Code	Common Name	Scientific Name	Cetacean Tissues for *T. gondii RE* gene Detection								
			Brain	Cardiac	Kidney	Skeletal	Liver	Intestine	Lungs	Stomach	Blood
S1	melon-headed whale	*Peponocephala electra*	-	-	-	-	*	*	*	*	*
S5	Cuvier's beaked whale	*Ziphius cavirostris*	-	-	-	-	NT	NT	NT	*	*
S8	pantropical spotted dolphin	*Stenella attenuata*	+	-	-	-	NT	*	*	*	NT
S9	pygmy sperm whale	*Kogia breviceps*	+	-	NT	+	NT	*	*	*	NT
S14	Cuvier's beaked whale	*Ziphius cavirostris*	+	NT	NT	-	-	-	NT	+	*
S15	pantropical spotted dolphin	*Stenella attenuata*	*	*	NT	*	NT	*	+	-	*
Total number of tissues tested for *T.gondii RE* gene			5	5	6	6	5	2	3	2	4

Legend: + positive for *T. gondii* DNA, - negative for *T. gondii* DNA, * no available biological sample, NT Not tested

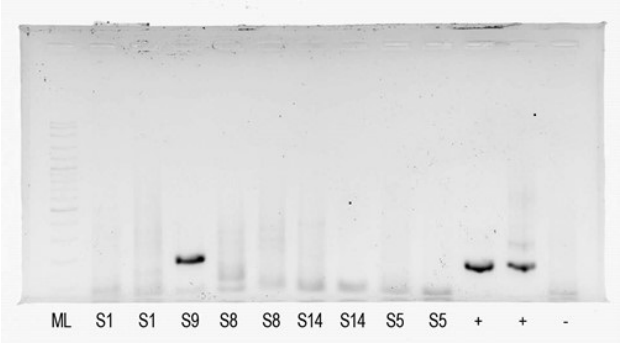

Figure 2: Molecular detection of *T. gondii* DNA in skeletal tissue of cetaceans (ML molecular ladder, + positive control, - negative control).

techniques employed; the present study only used PCR while the other studies used both PCR and serological assays.

Cetacean species with *T. gondii*

Species found to harbor *T. gondii* were pantropical spotted dolphin (*Stenella attenuata*), pygmy sperm whale (*Kogia breviceps)* and Cuvier's beaked whale (*Ziphius cavirostris)*. The pantropical spotted dolphin is one of the most abundant dolphins in the Eastern Tropical Pacific. They are mostly found offshore but can be found close to the shore where deep water approaches the coast (e.g., Hawaiian Islands, off Taiwan, and in the Philippines). Those found offshore feed mainly on epi- and meso-pelagic fishes, squid, and crustaceans, while those that stay near shore are thought to feed on larger and tougher fishes (Jefferson et al., 2008). Cuvier's beaked whale is more commonly found in deep-water and rarely nearshore. These species can be found in waters more than 200 meters deep, which they prefer for feeding (Heyning et al., 2009). The pygmy sperm whale is known for uncommon sightings and also thrive in deep waters with its diet consisting mainly of deep-water cephalopods. In general, knowledge regarding the ecology and behavior as well as other information about these species were obtained from stranded specimens.

The habit of staying near-shore is purported to be a contributing factor to the susceptibility of some cetacean species to *T. gondii* infection. A recent study by Diaz-Delgado et al., (2020) documented a case of acute systemic toxoplasmosis that caused the demise of a Bryde's whale stranded in Brazil. The source of *T. gondii* infection in this particular animal is unknown, but staying along the coastlines might have exposed it to pathogens through land-based effluents. VanWormer et al. (2016) found that watersheds characterized by higher level of coastal development are associated with regions of increased incidence of marine mammal infection by *T. gondii*. The occurrence of the parasites in marine mammals indicates the extent of land based biological pollution as well as impacts of anthropogenic changes to regional watersheds (Shapiro et al., 2018). Interesting to note here though that *T. gondii* was not detected in Risso's and common bottlenose dolphins. Both species are pelagic but are known to wander near islands with deep waters.

Pantropical spotted dolphins and pygmy sperm whales were reported in previous studies (Obusan et al., 2015; Obusan et al., 2019) as among cetacean species in the Philippines found to have *T. gondii* (Table 3). To the best knowledge of the authors, the present study is the first to report the detection of the parasite in a Cuvier's beaked whale (*Ziphius cavirostris*). The species is an addition to the growing list of deep offshore cetacean species documented to have *T. gondii*, and supports the hypothesis that the extent *T. gondii* infection has extended beyond coastlines where pathogen run-off is likely. Furthermore, this suggests that this parasite might be coupled in the complex food chains of the oceans.

Transmission of *T. gondii* to marine mammals and potential risks to humans

The widely accepted mode of transmission of *T. gondii* oocysts from land to sea is through freshwater run-off. Native wild felids, as well as introduced pet and un-owned or feral domestic cats, have the potential to shed massive quantities of oocysts in terrestrial and aquatic habitats (Alfonso et al., 2010). Freshwater runoff carrying oocyst contaminated waters from felids feces may explain *T. gondii* transmission into the marine environment (Santos et al., 2011; Marigo et al., 2013; Vanwormer et al., 2013; van de Velde et al., 2016). It has been demonstrated that *T. gondii* oocyst can sporulate and remain infectious in seawater for two years at 4°C and for half-year at room temperature (Lindsay and Dubey, 2009).

Another mechanism of *T. gondii* transmission is through consumption of prey. Prey species of marine mammals such as anchovies, sardines, and bivalves were found to harbor viable *T. gondii* oocysts and have the potential to incorporate *T. gondii* in the marine food web (Massie et al., 2010). The decline in the population of southern sea otters in California (Miller et al., 2008) was linked to widespread infection of *T. gondii* facilitated by the consumption of prey species that harbor the parasite.

Table 3: *T. gondii* detection in cetacean species of the Philippines.

Common name	Species	Obusan et al., (2015) study	Obusan et al., (2019) study	This study
pygmy sperm whale	*Kogia breviceps*	+	+	+
pantropical spotted dolphin	*Stenella attenuata*	+	+	+
beaked whale	*Mesoplodon* sp.	+	*	*
Indopacific bottlenose dolphin	*Tursiops aduncus*		*	*
Risso's dolphin	*Grampus griseus*	*	+	-
Fraser's dolphin	*Lagenodelphis hosei*	*	+	-
spinner dolphin	*Stenella longirostris*	-	+	*
melon headed whale	*Peponocephala electra*	*	+	-
striped dolphin	*Stenella coeruleoalba*	*	+	*
Bryde's whale	*Balaenoptera edeni*	*	+	-
rough toothed dolphin	*Steno bredanensis*	-	+	-
Cuvier's beaked whale	*Ziphius cavirostris*	*	*	+
short finned pilot whale	*Globicephala macrorhynchus*	-	+	-

Legend: + detected *T. gondii*, - did not detect *T. gondii*, * no available/qualified biological sample

The prevalence of *T. gondii* among wild animals including cetaceans, is a public health concern. Consumption of meat with *T. gondii* cysts remains to be a risk factor for humans, as oral route is considered a major source of *T. gondii* infection (Montoya and Liesenfeld, 2004). Out of 25 toxoplasmosis outbreaks reported, 24% (6/25) were associated with ingestion of tissue cysts from undercooked or raw meat in Brazil (Ferreira et al., 2018). In the Philippines, existing laws such as Republic Acts 8550 and 9147, and Fisheries Administrative Order No. 185, prohibit the trade and consumption of marine mammal meat. However, the risk remains since there are reports that illegal hunting and selling of cetacean meat exists in remote areas (pers comm., BFAR Region V) and the meat of stranded cetaceans may be consumed (Reyes, 2019).

The occurrence of *T. gondii* in marine mammals indicates the extent of land-based biological pollution as well as anthropogenic impacts to watersheds (Shapiro et al., 2018). The prevalence of the parasite among cetaceans suggests that toxoplasmosis may be circulating among marine life, including species that are directly associated to humans. Only in recent years have scientists shed light on the potential role of seafood consumption in the transmission of *T. gondii* (Esmerini et al., 2010; Marino et al., 2019). Studies involving the detection of *T. gondii* oocysts in local seafood such as mussels oysters, and fishes (as mechanical vectors) may help provide the link between the presence of this parasite in cetaceans and possible sources of infection.

Association of cetacean stranding parameters and *T. gondii* detection

Chi square test of association between the presence of *T. gondii RE* gene and stranding parameters yielded a p-value of 0.465 for gender, 0.135 for stranding season, and 0.102 for age group of cetaceans (α=0.05).

Shapiro et al., (2018) identified the risk factors for marine mammals associated with *T. gondii* infection, and these include diet, age, sex, location. The present study found no significant association between the detection of *T. gondii RE* gene and stranding parameters (cetacean sex, age group, stranding season). However, the present finding is limited to the number of cetaceans that were responded in one year. For finding significant associations, a long-term study involving more cetacean species is recommended. Previous studies elsewhere reported a significant association between the detection or infection of *T. gondii* and biological characteristics of marine mammal species. The prime aged adult Californian sea otters, where *T. gondii* in marine mammals was first detected, are said to be more susceptible to infection compared to juvenile otters (Kreuder et al., 2003). The same study also reported that male marine mammals have higher likelihood of exposure to parasites such as *T. gondii*, due to larger body mass and caloric demand. In terms of seasons, it is hypothesized that increased rainfall puts marine mammals at a higher risk since runoff of oocysts is very likely (Shapiro et al., 2018).

For future studies, we recommend the concurrent use of other methods to corroborate the detection of *T. gondii* by PCR in cetaceans. For example, avidity test and/or Enzyme Linked Immunosorbent Assay (ELISA) may be used to detect IgA while immunohistochemistry staining may be used to detect the parasites' cysts in tissues. A combination of detection methods is needed to confirm acute or chronic infection as well as disseminated toxoplasmosis in stranded cetaceans and their counterparts in the wild.

In cases wherein necropsy is conducted as part of the cetacean stranding response, documentation of clinical manifestations in relation to toxoplasmosis such as meningoencephalitis, should be done to substantiate the results of detection assays. Other tissues should be collected and tested. Moreover, the determination of the specific genotype of *T. gondii* circulating among local cetaceans is recommended to further characterize the nature of infection in marine wildlife.

CONCLUSION

The study investigated the prevalence of *T. gondii* in select cetaceans that stranded in the Philippines in 2019. Three species of cetaceans (*Kogia breviceps, Stenella attenuata*, and *Ziphius cavirostris),* represented by four stranded individuals, tested positive for *T. gondii RE* gene through molecular detection by

nested PCR. As cetaceans are difficult to observe and sample, the information generated from the stranded individuals indicate the health status of their wild populations which are facing the impacts of anthropogenic activities such as land-sea movement of biological pollutants.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

CONTRIBUTION OF INDIVIDUAL AUTHORS

RJCDG, LVA, and MCMO designed the methodology. LVA led the cetacean stranding response. LVA and MCMO received funding for the study. MCMO and RJCDG performed the laboratory procedures. MCMO, LVA and RJCDG collated and analyzed all the data, interpreted the results, and prepared the manuscript. All authors have read and approved the final version of the manuscript.

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