Vol. 130: 165–175, 2018
https://doi.org/10.3354/dao03262

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published September 27

Toxoplasma gondii infection in stranded St. Lawrence Estuary beluga *Delphinapterus leucas* in Quebec, Canada

Asma Iqbal[1], Lena Measures[2], Stéphane Lair[3], Brent Dixon[1,*]

[1]Bureau of Microbial Hazards, Food Directorate, Health Canada, Ottawa, ON K1A 0K9, Canada
[2]Fisheries and Oceans Canada, Maurice Lamontagne Institute, Mont-Joli, QC G5H 3Z4, Canada
[3]Centre québécois sur la santé des animaux sauvages - Canadian Wildlife Health Cooperative, Faculté de médecine vétérinaire, Université de Montréal, St. Hyacinthe, QC J2S 2M2, Canada

ABSTRACT: The St. Lawrence Estuary (SLE) beluga *Delphinapterus leucas* in Quebec, Canada, is endangered due to intensive hunting in the 19th and 20th centuries and subsequent anthropogenic contamination and human activities in the region. Infectious disease is a primary cause of death in this population. The protozoan parasite *Toxoplasma gondii* is reported in numerous marine mammal species, including beluga. In the present study, 55 tissue samples (heart and brain) collected from 34 stranded SLE beluga were analysed by PCR followed by DNA sequencing and restriction fragment length polymorphism analysis (RFLP) to determine the PCR prevalence and genotypes of *T. gondii* in these beluga. Of 34 beluga tested, 44% were positive for *T. gondii* by PCR, with males having a higher prevalence of infection than females and with more infected neonates and juveniles than adults. Molecular analyses indicated that all *T. gondii* infecting stranded SLE beluga grouped into genotype II, which predominates in humans. While our results indicate that a high prevalence of stranded beluga are PCR-positive for *T. gondii* infection, very few deaths are attributed to toxoplasmosis based on published necropsy results. *Toxoplasma gondii* can cause a range of diseases, including neurological deficits, and more data are needed to investigate this parasite's effect on population recovery.

KEY WORDS: *Toxoplasma gondii* · Genotype · Beluga · *Delphinapterus leucas* · Pathology

INTRODUCTION

Toxoplasma gondii is an intracellular protozoan parasite that can infect humans and other warm-blooded vertebrates worldwide. Wild and domestic felids are the only known definitive hosts of *T. gondii* and are responsible for contaminating the environment with oocysts excreted in their faeces (Dubey 2010). Antibodies or tissue isolates of *T. gondii* are detected in numerous marine mammals, including pinnipeds, cetaceans and sea otters (Dubey 2010, Gibson et al. 2011). *T. gondii* infection is reported in beluga *Delphinapterus leucas* from the St. Lawrence Estuary (SLE) (De Guise et al. 1995, Mikaelian et al. 2000, Lair et al. 2016) and the Sea of Okhotsk (Alekseev et al. 2009), but not from Svalbard, Norway (Jensen et al. 2010) or Cook Inlet, Alaska (Burek-Huntington et al. 2015).

An estimated abundance of 900 to 1500 beluga inhabit the SLE in Quebec, Canada (Gosselin et al. 2014, 2017), and this isolated population is listed as 'endangered' under the Canadian Species at Risk Act (www.sararegistry.gc.ca). The failure of this population to recover following intensive hunting in the 19th and 20th centuries suggests that other limiting factors are present (Hammill et al. 2007, Lair et al. 2016) such as anthropogenic contaminants and other human activities (Martineau et al. 2002, Lair et al.

*Corresponding author: brent.dixon@canada.ca

2016) and ecological changes (Plourde et al. 2014, Starr et al. 2017). According to Lair et al. (2016), the 4 primary causes of death (CODs) among 222 stranded SLE beluga are infectious disease (pooled viral, bacterial and parasitic pathogens: 32%, dystocia and postpartum complications: 19%, malignant neoplasia: 14% and neonatal mortality: 8%), with 25% of CODs undetermined. While bacterial disease and verminous pneumonia are responsible for the majority of deaths due to infectious disease, a number of other pathogens also cause or contribute to death, including *T. gondii*. Toxoplasmosis was the primary cause of death in 2% of SLE beluga when all CODs were considered, and in 7% of the deaths caused by infectious disease only (Lair et al. 2016). Immunosuppression, caused by exposure to chemical contaminants such as polychlorinated biphenyls, or concurrent infections such as morbillivirus, is a potential risk factor for stranding with toxoplasmosis in cetaceans (Mikaelian et al. 2000, Di Guardo et al. 2013, Lair et al. 2016).

Fatal infections in marine mammals involve hepatitis, nonsuppurative meningoencephalitis with severe neurological signs, adenalitis, lymphadenitis, interstitial pneumonia, thymitis, cerebral and placental necrosis, myocarditis and fatal disseminated toxoplasmosis (Migaki et al. 1990, Inskeep et al. 1990, Resendes et al. 2002, Dubey et al. 2003, 2008). Bradyzoites and tachyzoites were found in the brain, spleen, lymph nodes, adrenals, liver, mammary gland, lungs and thymus of 5 beluga which died of toxoplasmosis (De Guise et al. 1995, Mikaelian et al. 2000, Lair et al. 2016, S. Lair unpubl. data). Toxoplasmosis may cause neurological deficits with cognitive impairment and behavioural changes in marine mammals (Dubey & Odening 2001, Gajadhar et al. 2004) and increased death due to predation (Kreuder et al. 2003). If infection occurs early in gestation this can lead to severe consequences such as early embryonic death and resorption, fetal death and mummification, abortion, stillbirth and neonatal death in animals (Dubey 2010), including in marine mammals. Transplacental infections of *T. gondii* have been described previously in cetaceans (Inskeep et al. 1990, Jardine & Dubey 2002, Resendes et al. 2002). Vertical transmission of *T. gondii*, potentially useful in the marine environment, may be more important than previously believed (Worth et al. 2013).

T. gondii oocysts can remain infectious for at least 24 mo in seawater (Lindsay & Dubey 2009), and coastal freshwater runoff containing *T. gondii* oocysts is considered a potential source of contamination of the marine environment (Miller et al. 2002, Lair et al. 2016). While *T. gondii* has been detected in numerous marine invertebrates, most notably molluscan shellfish (Miller et al. 2008a, Shapiro et al. 2015, Staggs et al. 2015), SLE beluga are primarily piscivores, and invertebrates do not comprise an important part of the diet (Lesage 2014). Furthermore, no conclusive evidence exists for natural *T. gondii* infections in either freshwater or marine fish, and further studies on the role diet plays on *T. gondii* transmission in beluga are necessary.

Genotyping of *T. gondii* may provide some evidence for possible sources or severity of infection in beluga, but few studies have reported on the *T. gondii* genotypes found in marine mammals. In North America, genotypes of *T. gondii* identified in marine mammals include types I, II, X, and A (Conrad et al. 2005, Sundar et al. 2008, Dubey 2010, Gibson et al. 2011). Mixed or atypical infections of genotypes with unique alleles, combinations of alleles or multiple genotypes also occur in marine mammals (Gibson et al. 2011). Types X, A and II-like comprise a new 4th clonal lineage, haplogroup 12 (Dubey et al. 2011, Khan et al. 2011).

The objective of the present study is to determine the PCR prevalence and genotypes of *T. gondii* in stranded beluga from an isolated and at risk population in the SLE, Quebec, Canada, in order to determine age- and sex-based risk, and to identify possible sources of infection in these animals. This is the first study to genotype *T. gondii* in beluga.

MATERIALS AND METHODS

Sample collection

Drifting or beach-cast (stranded) SLE beluga were documented and validated by location and date using a network of observers. Carcasses were examined on the beach and, if accessible and not in advanced dcomposition, were transported to the Faculté de médecine vétérinaire, Université de Montréal, for complete necropsy to determine CODs using standard histologic techniques (see Lair et al. 2015, 2016). Animals were identified by sex, and age was determined by cutting longitudinal sections of teeth (dentine) and counting growth layer groups (GLGs) using GLG/1 year (Stewart et al. 2006). Animals were assigned to age class as follows: neonate (determined by the standard length, month of stranding and the presence of fetal structures such as unhealed umbilical cord stump and an open cardiac foramen ovale); juvenile (1–7 GLGs), and adult (8+ GLGs) (Lesage et al. 2014,

Lair et al. 2015, 2016). Carcass decomposition was classified as Code 1 to 5, where Code 1 is a live animal, Code 2 is a carcass in good condition (fresh), Code 3 is fair (decomposed, but organs basically intact), Code 4 is poor (advanced decomposition), and Code 5 is mummified or skeletal (Geraci & Lounsbury 2005). Code 1>2 refers to a stranded live animal which dies within hours on the beach.

During necropsies of stranded SLE beluga a total of 55 tissue samples were collected from 34 carcasses from 2009 to 2012, including heart (n = 33) and brain (cerebral cortex) (n = 22). These were all matched samples, i.e. heart and brain tissues were from the same animals. These samples were analyzed in the present study (i.e. separate from histopathologic analyses published by Lair et al. 2016).

Tissues were initially frozen at −20°C at the Faculté de médecine vétérinaire, Université de Montréal and then stored at −85°C at the Maurice Lamontagne Institute prior to shipping to Health Canada for laboratory analyses.

DNA extraction

A minimum of 100–200 mg of each tissue was isolated and frozen in liquid nitrogen before being pulverized using a mortar and pestle. Samples were then transferred to microcentrifuge tubes and stored at −20°C for further processing. DNA was extracted using an Easy DNA™ Kit (Invitrogen), according to the manufacturer's protocol. DNA templates were stored at −20°C for analysis.

Amplification of the 529 bp fragment

PCR was used to amplify the 529 bp fragment (which is repeated 200- to 300-fold in the *Toxoplasma gondii* genome) according to Homan et al. (2000), using a 26-mer forward primer (Tox4) and a 26-mer reverse primer (Tox5) (Table 1). The reaction mixture was 50 µl, containing 200 µM of each of the 4 dNTPs (Promega), 0.2 µM of each of primers Tox4/Tox5 (Sigma), 2.5 mM $MgCl_2$ (Promega), 2.5 U of Go Taq Polymerase (Promega), 1× PCR buffer (PromegaI) and 2 µl of diluted (1:10) DNA template for each tissue sample.

Positive and negative DNA controls were included in all PCR amplifications. The positive control used was *T. gondii* type III DNA extracted from the brains of experimentally infected geese received from Dr. Emily Jenkins, Department of Veterinary

Table 1. Molecular markers used for genotyping the protozoan parasite *Toxoplasma gondii* in infected St. Lawrence Estuary beluga *Delphinapterus leucas*. na: not applicable

Marker (location)	External primers	Internal primers	Restriction enzyme(s)	Reference
529 bp	Tox4: CGCTGCAGGGAGGAAGACGAAAGTTG Tox5: CGCTGCAGACACAGTGCATCTGGATT	na	na	Homan et al. (2000)
B1 gene	B1outF: GGAACTGCATCCGTTCATGAG B1outR: TCTTTAAAGCGTTCGTGGTC	B1intF: TGCATAGGTTGCAGTCACTG B1intR: GGCGACCAATCTGCGAATACACC	na	Di Guardo et al. (2011)
5′-SAG2 (VIII)	F4: GCTACCTCGAACAGGAACAC R4: GCATCAACAGTCTTCGTTGC	F: GAAATGTTTCAGGTTGCTGC R2: GCAAGAGCGAACTTGAACAC	*Sau*3IA	Howe et al. (1997)
3′-SAG2 (VIII)	F3: TCTGTTCTCCGAAGTGACTCC R3: TCAAAGCGTGCATTATCGC	F2: ATTCTCATGCCTCCGCTTC R: AACGTTTCACGAAGGCACAC	*Hha*I	Howe et al. (1997)
GRA6 (XI)	FO: GGCAAACAAAACGAAGTG RO: CGACTACAAGACATAGAGTG	F: GTAGCGTGCTTGTTGGCGAC R: TACAAGACATAGAGTGCCCC	*Mse*I	Fazaeli et al. (2000)
BTUB (IX)	Btb (ext) F: TCCAAAATGAGAGAAATCGT Btb (ext) R: AAATTGAAATGACGGAAGAA	Btb-F: GAGGTCATCTCGGACGAACA Btb-R: TTGTAGGAACACCCGGACGC	*Bsi*EI, *Taq*I	Zhou et al. (2013), Khan et al. (2005)

Microbiology, University of Saskatchewan, and the negative control was DNase-free water in place of DNA template.

Amplification of the B1 gene

A region of approximately 95 bp within the B1 gene was amplified according to Di Guardo et al. (2011) by PCR using the primer pair B1outF and B1outR in a first round of PCR, followed by a second round using the primer set B1intF and B1intR (Table 1). The PCR mixture contained 1 µM of each primer, 1× PCR buffer, 1.5 mM of $MgCl_2$, 200 µM of dNTPs (Promega, Madison, WI) and 1.25 U of Taq DNA polymerase (Promega). The reaction volume was 50 µl, containing 2.0 µl of DNA extracts in the primary PCR and 4 µl of the diluted (1:10) first round PCR product was used as a template in the secondary PCR.

Genotyping using the SAG2 locus

Genotyping of *T. gondii* was based on DNA polymorphisms at the SAG2 locus, encoding the tachyzoite surface antigen p22. Samples were analyzed at the SAG2 locus by using a nested PCR approach that separately amplified the 5′ and 3′ ends of the locus. The 5′ end of the SAG2 locus was amplified according to Howe et al. (1997) in 2 stages, namely the primary reaction, using SAG2-F4 and R4 as forward and reverse primers, and the secondary reaction that amplified a fragment using the forward (SAG2-F) and reverse (SAG2-R2) primers (Table 1). The reaction was run in 50 µl containing 200 µM of each of the 4 dNTPs (Promega), 25 pmol of each primer SAG2-F4/R4 and SAG2-F/R2 (Sigma), 2.5 mM $MgCl_2$ (Promega), 2.5 U of Go Taq Polymerase (Promega) and 1× PCR buffer (Promega). Two µl of 1:10 diluted DNA template in DNase free water was used in the primary PCR, and 5 µl of the diluted first round PCR product was used as a template in the secondary PCR. The secondary PCR reagent concentrations were the same as those used in the primary PCR reaction.

The 3′ end of the SAG2 locus was similarly analyzed with the primers SAG2-F3 and SAG2-R3 for the initial amplification, and the internal primers SAG2-F2 and SAG2-R (Table 1) for the second round of amplification. Two µl of diluted DNA template was used in the primary PCR, and 2 µl of the diluted first round PCR product was used as template in the secondary PCR. The primary and secondary PCR reagent concentrations and amplification conditions were the same as those used in the 5′-SAG2 PCR. Primers were selected to separately amplify the 3′ and 5′ ends of the *T. gondii* SAG2 locus, resulting in 241 bp and 221 bp products respectively.

Genotyping using the GRA6 gene

Nested PCR was performed to amplify the coding region of the GRA6 gene according to Zakimi et al. (2006). PCR amplification was performed with 2 µl of DNA template in 50 µl of a reaction mixture containing 5× GoTaq Flexi buffer (Promega). Two mM $MgCl_2$ (Promega), 200 µM of each of the 4 dNTPs (Promega), 50 pmol of each primer (Sigma) and 1.25 U of Go Taq Hot Start Polymerase (Promega). The PCR primer pair was designed from the GRA6 gene sequence, GRA6FO and GRA6RO used in primary PCR (Table 1). Two µl of 1:10 diluted primary PCR product was used as a template in the secondary PCR using the internal primers described by Fazaeli et al. (2000), GRA6F/GRA6R (Table 1).

Genotyping using the BTUB gene

Genotyping using the beta-tubulin (BTUB) gene of *T. gondii* was performed by PCR amplification according to Zhou et al. (2013) and Khan et al. (2005). The initial round of amplification with the external primers Btb (ext) F/ Btb (ext) R (Table 1) was carried out in 50 µl of mixture containing 5× GoTaq Flexi buffer (Promega), 2.5 mM $MgCl_2$ (Promega), 200 µM each of the dNTPs (Promega), 1 µg BSA (New England BioLabs), 0.4 µM each of the forward and reverse primers, 1.25 U of Go Taq Hot Start Polymerase (Promega) and 2 µl of DNA. PCR products were diluted 1:10 and used for a second round of amplification with the internal primers Btb-F and Btb-R in a 50 µl volume containing the same PCR reagent concentrations described previously, with the exception that 1.5 mM $MgCl_2$ was added and no BSA was used in the round two PCR mixture. The second round amplification protocol was 94°C for 4 min followed by 30 cycles of 94°C for 30 sec, 60°C for 1 min, and 72°C for 2 min, with an over-extension step at 72°C for 5 min.

Analysis of PCR amplification products

The quality and band intensity of all multilocus nested PCR amplicons were examined on 1.5%

agarose gels containing GelRed (5 µl 100 ml^{-1}) (Biotium) and were run for 40 min at 120V with 1× TE buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) and visualized under ultraviolet light.

Restriction fragment length polymorphism (RFLP) analyses were performed on PCR-positive samples in order to determine the *T. gondii* genotype (i.e. type I, II or III). Specifically, amplicons resulting from PCR amplification at the 5′-SAG2, 3′-SAG2, GRA6, and BTUB loci were incubated with the appropriate restriction enzymes (Table 1) according to the manufacturer's instructions (New England BioLabs). The digested PCR products were visualized by electrophoresis on 2.5 to 3.0% agarose gels containing GelRed.

DNA sequence analysis

PCR amplification products were purified using a Mini Elute PCR purification kit (Qiagen) according to the manufacturer's protocol. The PCR product of *T. gondii* SAG2 amplification was subjected to bi-directional, automated sequencing (ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems) using the same primers employed in the secondary PCR. Nucleotide sequences were aligned for comparison using 'Clustal W' from Bio-Edit Sequence Alignment Editor, and neighbour-joining trees were constructed from the aligned sequences using MEGA5 software.

RESULTS

Of 34 stranded beluga in this study, 23 (68%) were female ranging in age from <1 to 68 yr and 11 (32%) were male from <1 to 55 yr of age (Table 2) and included neonates (4), juveniles (8) and adults (22). The greatest number of beluga, 11 (32%), were stranded in the months of July and August. Causes of death in these animals included trauma, reproductive problems (neonatal mortality, dystocia, postpartum complications) and infections. Cause of death could not be determined for 6 animals (18%) (Table 2). Preservation of carcasses (Code) varied from 1 to 4. One neonate stranded alive but died within hours (Code 1>2) (Table 2).

Fifteen (44%) of the 34 stranded beluga were *Toxoplasma gondii*-positive by PCR, including 64% of males and 35% of females, and 50% of neonates, 63% of juveniles and 36% of adults. Twenty-nine percent of the 55 beluga tissue samples were *T. gondii*-positive by multilocus PCR, including 33% of heart tissue samples and 23% of brain tissue samples (Table 3).

Four of 55 (7%) tissue samples were positive for both 529 bp and B1 genes, and 4 (7%), 15 (27%), 5 (9%) and 4 (7%) samples were positive by nested PCR using 5′-SAG2, 3′-SAG2, GRA6 and BTUB genes, respectively.

In order to determine the SAG2 genotypes/alleles of *T. gondii* isolates from SLE beluga, a total of 10 isolates were successfully sequenced in both directions. From BLAST results, there was high (99%) sequence similarity with published sequences of *T. gondii* SAG2. SAG2 *T. gondii* sequences from stranded beluga all grouped into type II and were highly similar to the BEVERLEY and COUGAR strains. A phylogenetic tree was constructed using the *T. gondii* SLE beluga isolates plus sequences from well-characterized type I (RH) and type III (NED) *T. gondii* strains (obtained from GenBank), using the neighbour-joining method (Fig. 1). There was 100% agreement between the genotyping results obtained by sequencing and by RFLP.

DISCUSSION

The present study identified a high PCR prevalence (44%) of *Toxoplasma gondii* in beluga stranded in the SLE. By comparison, Mikaelian et al. (2000), using a modified agglutination test, observed that 27% of stranded SLE beluga were seropositive to *T. gondii*, with 2 deaths attributed to toxoplasmosis involving a 2 yr old juvenile male (also seropositive) and a 51 yr old female (not tested serologically) (ages corrected in Lair et al. 2016). An additional 3 fatal cases of toxoplasmosis in stranded SLE beluga (2 adult females, 12 and 42 yr old; and 1 adult male, 35 yr old) were identified (Lair et al. 2016). In contrast, only 5% of beluga in the Sea of Okhotsk were seropositive for *T. gondii* using an immune enzyme assay (Alekseev et al. 2009).

More male beluga were PCR-positive in the present study (64%) than female (35%). The differences observed may be attributed to exposure and susceptibility due to differences in body mass (Lair et al. 2016), diet and foraging behaviour (Lesage 2014), endocrine and immune interactions (Klein 2004), nutritional and reproductive status (Zuk & McKean 1996, Lesage et al. 2014, Lair et al. 2016) and contaminant concentrations (Lebeuf et al. 2014a,b), which may affect immune competence. While the small sample size precluded statistical analyses, PCR prevalence was also higher in neonates (50%) and

Table 2. Estimated age (GLGs: growth layer groups), sex, carcass condition code (according to Geraci & Lounsbury 2005) and cause of death (according to Lair et al. 2016) in individual stranded St. Lawrence Estuary beluga *Delphinapterus leucas*, with results of PCR-testing for the protozoan parasite *Toxoplasma gondii* (H: heart; B: brain)

Beluga no.	Date found stranded	Age (GLGs)	Age class	Sex	Code	PCR results (tissue)	Cause of death
DL-01-2009	Jun 16, 2009	12	Adult	F	2	Negative	Dilated cardiomyopathy
DL-02-2009	Jul 29, 2009	1	Juvenile	M	2.5	Positive (H)	Suppurative encephalitis
DL-03-2009	Aug 1, 2009	49	Adult	M	3	Negative	Protozoan pneumonia (*Kyaroikeus cetarius* probable)
DL-04-2009	Sep 3, 2009	0	Neonate	M	2	Positive (H)	Trapped in fishing gear
DL-05-2009	Sep 15, 2009	48	Adult	F	2	Negative	Not determined
DL-01-2010	Mar 9, 2010	2	Juvenile	F	2	Negative	Verminous bronchopneumonia
DL-02-2010	Mar 21, 2010	1	Juvenile	F	2	Positive (H)	Verminous bronchopneumonia
DL-03-2010	Apr 30, 2010	7	Juvenile	F	4	Negative	Wooden foreign body in blow hole
DL-04-2010	Jun 9, 2010	42	Adult	F	3.5	Positive (H)	Toxoplasmosis
DL-05-2010	Jun 10, 2010	51	Adult	F	2.5	Positive (H)	Rectal perforation
DL-06-2010	Jul 16, 2010	18	Adult	F	2.5	Negative	Post-partum complications
DL-07-2010	Jul 31, 2010	40	Adult	F	2	Negative	Dystocia
DL-08-2010	Aug 29, 2010	31	Adult	F	3	Negative	Dystocia
DL-09-2010	Nov 4, 2010	1	Juvenile	M	2	Positive (H)	Verminous bronchopneumonia
DL-10-2010	Dec 31, 2010	2	Juvenile	M	2	Positive (H)	Systemic herpesviral infection
DL-11-2010	Dec 31, 2010	35	Adult	F	2	Negative	Primary starvation
DL-01-2011	Apr 7, 2011	2	Juvenile	M	2.5	Negative	Boat trauma
DL-02-2011	May 19, 2011	44	Adult	F	2	Positive (H, B)	Dystocia
DL-03-2011	Jun 8, 2011	30	Adult	F	3	Positive (B)	Dystocia
DL-04-2011	Jul 12, 2011	56	Adult	F	2	Positive (H)	Adenocarcinoma of the adrenal gland/ lymphangiosarcoma
DL-05-2011	Aug 9, 2011	14	Adult	F	2.5	Negative	Dystocia
DL-06-2011	Sep 10, 2011	68	Adult	F	2	Positive (B)	Primary starvation
DL-07-2011	Oct 9, 2011	55	Adult	M	4	Positive (B)	Not determined
DL-08-2011	Oct 28, 2011	22	Adult	F	2.5	Negative	Verminous bronchopneumonia
DL-09-2011	Nov 9, 2011	43	Adult	M	2.5	Negative	Not determined
DL-01-2012	May 19, 2012	26	Adult	F	2.5	Negative	Post-partum complications
DL-02-2012	May 27, 2012	23	Adult	M	3	Negative	Verminous bronchopneumonia
DL-03-2012	Jul 16, 2012	21	Adult	F	3	Negative	Not determined
DL-04-2012	Aug 12, 2012	0	Neonate	F	1>2	Negative	Neonatal mortality
DL-05-2012	Aug 4, 2012	0	Neonate	M	4	Positive (B)	Neonatal mortality
DL-06-2012	Aug 23, 2012	0	Neonate	F	4	Negative	Neonatal mortality
DL-07-2012	Oct 3, 2012	3	Juvenile	F	3	Positive (H)	Verminous bronchopneumonia
DL-08-2012	Nov 5, 2012	53	Adult	M	3	Positive (H)	Not determined
DL-09-2012	Nov 7, 2012	30	Adult	F	3.5	Negative	Not determined

juveniles (63%) than in adults (36%), suggesting the possibility of transplacental or transmammary transmission of *T. gondii* in the animals tested.

In the present study, heart tissues were more often found to be PCR-positive than brain tissues. In experimentally inoculated geese, heart and brain tissues had substantially higher detection probabilities for DNA of *T. gondii* than other tissues, and were considered the best tissues to test in surveillance studies (Elmore et al. 2016). Of the different *T. gondii* genes amplified, the 3′-SAG2 gene resulted in the highest number of PCR-positives, and the amplification of this gene should be considered whenever only a single gene is used in detection. However, since the amplification of other genes occasionally resulted in PCR-positives when 3′-SAG2 was negative, it may be prudent to perform multilocus PCR whenever possible.

To our knowledge, the present study is the first report on the molecular characterization of *T. gondii* in beluga. Using DNA sequencing and RFLP, 10 PCR-positive samples were successfully genotyped, with type II being the only genotype identified. Most human infections in North America and Europe are thought to be caused by type II *T. gondii,* which is also common in livestock in these regions (Sibley et al. 2009). The virulence of the different *T. gondii* genotypes is only well described in mice, in which type I strains cause lethal infection even at low inocula, while types II and III are much less virulent

Table 3. Results of PCR-testing for the parasite *Toxoplasma gondii* in St. Lawrence Estuary beluga *Delphinapterus leucas* tissues. Beluga ID nos. as in Table 2. na: sample not available

Year of collection	Heart tissue			Brain tissue		
	No. tested	No. positive (beluga no.)	No. negative (beluga no.)	No. tested	No. positive (beluga no.)	No. negative (beluga no.)
2009	5	2 (DL-02-2009) (DL-04-2009)	3 (DL-01-2009) (DL-03-2009) (DL-05-2009)	5	0	5 (DL-01-2009) (DL-02-2009) (DL-03-2009) (DL-04-2009) (DL-05-2009)
2010	11	5 (DL-02-2010) (DL-04-2010) (DL-05-2010) (DL-09-2010) (DL-10-2010)	6 (DL-01-2010) (DL-03-2010) (DL-06-2010) (DL-07-2010) (DL-08-2010) (DL-11-2010)	na	na	na
2011	9	2 (DL-02-2011) (DL-04-2011)	7 (DL-01-2011) (DL-03-2011) (DL-05-2011) (DL-06-2011) (DL-07-2011) (DL-08-2011) (DL-09-2011)	9	4 (DL-02-2011) (DL-03-2011) (DL-06-2011) (DL-07-2011)	5 (DL-01-2011) (DL-04-2011) (DL-05-2011) (DL-08-2011) (DL-09-2011)
2012	8	2 (DL-07-2012) (DL-08-2012)	6 (DL-01-2012) (DL-02-2012) (DL-03-2012) (DL-04-2012) (DL-06-2012) (DL-09-2012)	8	1 (DL-05-2012)	7 (DL-01-2012) (DL-02-2012) (DL-03-2012) (DL-04-2012) (DL-06-2012) (DL-07-2012) (DL-09-2012)
Total	33	11 (33%)	22 (67%)	22	5 (23%)	17 (77%)

(Sibley et al. 2009). Since type II is so common in humans and animals in North America, it is not possible to attribute any particular source of infection in the SLE beluga.

It is important that the source(s) of *T. gondii* infection in SLE beluga be identified and mitigated if possible. Domestic and feral cats are likely the most important source of oocysts contaminating marine environments, either through defecation within the watershed or through storm sewers. The disposal of cat faeces into toilets may also contribute to contamination of the environment in some areas populated by humans, with wildlife playing a greater role in areas unpopulated by humans (Lafferty 2015). While post-treatment analyses of sewage effluent discharged into the St. Lawrence River upstream of the SLE beluga habitat by Montreal's primary physicochemical sewage treatment plant indicated the removal of some waterborne parasites and faecal coliforms (Payment et al. 2001), no analyses for *T. gondii* in the effluent

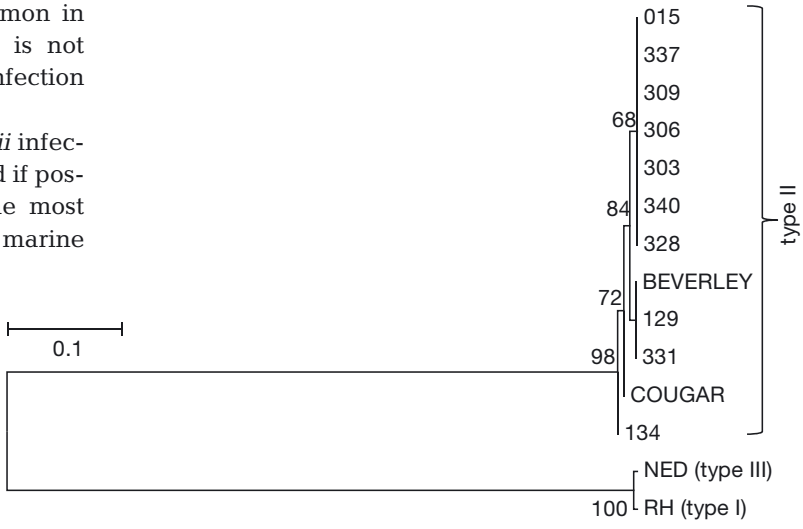

Fig. 1. Phylogenetic analysis of *Toxoplasma gondii* SAG2 sequence data using neighbour-joining analysis. Sequences from the present study (015, 129, 134, 303, 306, 309, 328, 331, 337 and 340) as well as reference strain RH (type I) and NED (type III) are indicated. Evolutionary distances were computed using the Kimura 2-parameter method, numbers at nodes represent percentage occurrence of clades in 1000 bootstrap replications of data

were performed. It is likely, however, that cat faeces are present in raw sewage. This plant now uses ozone treatment of effluent before discharge into the environment but ozone does not inactivate *T. gondii* oocysts (Dumètre et al. 2008). In Quebec, 14% (12/84) (Simon et al. 2013) and 44% (47/106) (Labelle et al. 2001) of lynx *Lynx canadensis* and 40% (4/10) of bobcat *Lynx rufus* (Simon et al. 2013) were seropositive for *T. gondii* using the modified agglutination test. Bobcats have a more restricted distribution and are less abundant than lynx, which are widespread throughout Quebec but cyclic in abundance. Domestic cats are considered more important sources for environmental loading of *T. gondii* than wild felids (Dubey & Odening 2001). While an estimated 1.45 million house cats are owned in Quebec based on a 2008 survey (Léger Marketing 2008), the number of feral cats is unknown but is estimated to be as high as 435 000 to 725 000 (Massé et al. 2012). Minimizing contamination of the environment from domestic cat faeces through education of cat owners to keep cats indoors at all times and to avoid disposing of cat litter in toilets, and eliminating feral cat populations, can serve to reduce this source of oocysts in areas populated by humans, thus helping to protect the SLE beluga as well as the health of humans, domestic and wild animals from this pathogenic protozoan.

Dietary sources of *T. gondii* infection in SLE beluga are poorly understood. Mollusc shells have been found in some beluga stomachs but invertebrates are considered an insignificant part of their diet. These mollusc shells likely originate from the stomach and intestine of fish who consume molluscs rather than from molluscs directly consumed by beluga (Vladykov 1946; Kleinenberg et al. 1964). Thus, *T. gondii* oocysts in mollusc tissues in the gut of flatfish, sand lance or other molluscivorous fish may be transferred, secondarily, to beluga or other marine mammals such as pinnipeds (also exposed naturally to *T. gondii*; Measures et al. 2004), that feed on these fish. The existence of true *T. gondii* infections in fish (i.e. presence of tissue cysts) remains questionable and further study on the role that fish may play in the transmission of *T. gondii* is warranted.

A variety of threats are identified as possibly limiting the recovery of the SLE beluga population (Lebeuf et al. 2014a,b, Lesage 2014, Ménard et al. 2014, Plourde et al. 2014, Mosnier et al. 2015, Lair et al. 2016, Chion et al. 2017, Starr et al. 2017), but it is difficult to establish cause and effect or to mitigate some of them (Hammill et al. 2007, Lair et al. 2016, Williams et al. 2017). There is too little data to detect any temporal trend in mortalities due to toxoplasmosis, but the recent increase in mortalities of neonates as well as increasing cases of parturition-related mortalities of pregnant females, particularly from 2010 to 2012 (Lair et al. 2016), are significant threats to recovery of this endangered population. In the present study, the cause of death of 3 of the 4 stranded neonates was classified as neonatal mortality, which is defined as a dependent calf dead at or during the first week after birth without significant underlying disease processes, and believed to be due to abandonment, separation, lack of maternal care or mortality or morbidity of the dam. Two neonates were infected with *T. gondii*, likely through transplacental transmission. One neonate (PCR-positive but with no *T. gondii* visible on histologic section) was dead of asphyxia from entrapment in fishing gear (see Lair et al. 2016 for histopathologic methods). As latent infections of *T. gondii* are known to cause behavioural changes in humans and rodents (Flegr 2013) and possibly other animals (Gajadhar et al. 2004, Namroodi et al. 2016 and citations therein), SLE beluga with *T. gondii* infections may be at increased risk of accidents such as net entrapment or ship collision. From 1983 to 2007 the number of dead neonates varied annually from 0 to 3 (median = 1) but increased significantly thereafter, ranging from 4 to 16 (median = 6) for the period 2008 to 2014 (Gosselin et al. 2017). In addition, neurotoxins (saxitoxin) in the marine environment have also been implicated in the mortality of both adult beluga and neonates in the SLE (Lesage et al. 2014, Lair et al. 2016, Starr et al. 2017), and chronic exposure to disturbance, chemical contaminants and infectious pathogens likely place a significant immunologic and energetic burden on individual beluga (Owens & Wilson 1999).

CONCLUSIONS

The present study demonstrated a high PCR prevalence of *Toxoplasma gondii* in stranded SLE beluga, and molecular analyses indicated that type II, which predominates in humans and livestock in North America, was the only genotype present. While domestic and feral cats are the most likely source of oocysts contaminating marine environments, there may be other dietary sources of *T. gondii* infection in SLE beluga, and it is important that these sources be identified and mitigated. Neonate and juvenile SLE beluga were found to be more often infected than adults, and necropsy and PCR results suggested transplacental transmission of *T.*

gondii. Transplacental, and possibly transmammary, transmission and reproductive impairment due to *T. gondii* in marine mammals is poorly documented but increasing evidence suggests that this protozoan parasite could affect some cetacean populations (Miller et al. 2008b). While neonate mortality due to *T. gondii* may be contributing to the lack of recovery of the SLE beluga population, a variety of threats to this population have been identified, and further investigations are needed.

Acknowledgements. This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Visiting Fellowships in Canadian Government Laboratories Program (A.I.), and Fisheries and Oceans Canada. We thank Carl Guimond and Pierre Béland (Les Industries Filmar), and the Réseau québécois d'urgences pour les mammifères marins for locating, collecting and transporting beluga carcasses.

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Editorial responsibility: Stephen Raverty, Abbotsford, British Columbia, Canada

Submitted: August 22, 2017; Accepted: June 17, 2018
Proofs received from author(s): August 28, 2018