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A novel *Sarcocystis neurona* genotype XIII is associated with severe encephalitis in an unexpectedly broad range of marine mammals from the northeastern Pacific Ocean



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ABSTRACT

Sarcocystis neurona is an important cause of protozoal encephalitis among marine mammals in the northeastern Pacific Ocean. To characterise the genetic type of *S. neurona* in this region, samples from 227 stranded marine mammals, most with clinical or pathological evidence of protozoal disease, were tested for the presence of coccidian parasites using a nested PCR assay. The frequency of *S. neurona* infection was 60% (136/227) among pinnipeds and cetaceans, including seven marine mammal species not previously known to be susceptible to infection by this parasite. Eight *S. neurona* fetal infections identified this coccidian parasite as capable of being transmitted transplacentally. Thirty-seven *S. neurona*-positive samples were multilocus sequence genotyped using three genetic markers: *SnSAG1-5-6*, *SnSAG3* and *SnSAG4*. A novel genotype, referred to as Type XIII within the *S. neurona* population genetic structure, has emerged recently in the northeastern Pacific Ocean and is significantly associated with an increased severity of protozoal encephalitis and mortality among multiple stranded marine mammal species.

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1. Introduction

The protozoan parasite *Sarcocystis neurona* is well recognised for causing equine protozoal myeloencephalitis (EPM), a severe neurological disease in horses (Dubey et al., 1991). In North America, the life cycle of *S. neurona* includes the Virginia opossum (*Didelphis virginiana*) as the definitive host (Fenger et al., 1995; Dubey and Lindsay, 1998) and a variety of mammals as intermediate hosts, including raccoons (*Procyon lotor*), nine-banded armadillos (*Dasypus novemcinctus*), dogs (*Canis familiaris*), and southern sea otters (Cheadle et al., 2001; Dubey et al., 2001a,b, 2003, 2006; Cooley et al., 2007). Opossums excrete infective sporocysts in their feces (Dubey et al., 2000), and infection in intermediate

hosts occurs when sporocysts are ingested. *Sarcocystis neurona* typically encysts in muscle cells of intermediate hosts without causing clinical disease, but may cause encephalomyelitis if the parasite migrates into the CNS (Dubey et al., 2000, 2001a).

The recent development of molecular genotyping markers has allowed for multilocus sequence typing (MLST) of *S. neurona* isolates in order to determine whether particular genotypes associate with disease (Rejmanek et al., 2010; Wendte et al., 2010b). *Sarcocystis neurona* is known to possess six major surface antigen genes, SnSAGs 1–6 (Ellison et al., 2002; Hyun et al., 2003; Howe et al., 2005; Crowds et al., 2008; Wendte et al., 2010b). Analysis for the presence or absence and allelic variation within these genes allows for classification of *S. neurona* isolates into genotypes or antigen genetic (Ag) types. Microsatellite (MS) markers are also used for the classification of epidemiological clones of *S. neurona*. In the United States, the population genetic structure of *S. neurona* is defined by 12 Ag types that further sub-divide into 22 MS types

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(Rejmanek et al., 2010; Wendte et al., 2010a,b). Not only has this classification system served to identify geographic clustering of genotypes, but it has revealed that genotypes are shared between terrestrial and marine species, supporting the idea of land-to-sea movement of this parasite (Sundar et al., 2008; Rejmanek et al., 2010; Wendte et al., 2010b), analogous to the related protozoal agents *Toxoplasma*, *Giardia* and *Cryptosporidium* (Miller et al., 2002, 2008; Fayer et al., 2004; Conrad et al., 2005).

In the Americas, certain *S. neurona* genotypes are thought to have emerged on the west coast via repeated introductions of opossums to California over many years (Grinnell et al., 1937; Rejmanek et al., 2010). Since the association between protozoa and fatal neurological disease in southern sea otters was first established (Thomas and Cole, 1996), *S. neurona* has been reported as an important cause of fatal encephalitis among marine mammals, including free-ranging Pacific harbour seals and sea otters off the coasts of California, Washington and Alaska, USA (Lapointe et al., 1998; Lindsay et al., 2000; Miller et al., 2001a,b; Kreuder et al., 2003; Thomas et al., 2007; Gibson et al., 2011). In 2004, an unusually pathogenic genotype of *S. neurona* (Type I) was identified as the primary cause of death in a mass mortality event that resulted in nearly 60 southern sea otter deaths in the space of 1 month (Miller et al., 2010; Wendte et al., 2010a). This was the first recorded apicomplexan-associated epizootic event in a marine mammal. Molecular analysis of isolates collected from the outbreak identified a genetically distinct clone that had expanded by self-mating in the opossum definitive host (Wendte et al., 2010a). This sexual expansion of distinct genotypes by uni-parental mating is now thought to play a key role in the maintenance of dominant lineages of *S. neurona* in nature (Wendte et al., 2010a).

During the past two decades, the geographic range of the opossum has edged northward. Accordingly, *S. neurona* infections have been detected in marine mammals stranding along the coast of the northeastern Pacific Ocean (Gibson et al., 2011). This region supports a variety of marine mammals including threatened and endangered species, so knowledge of the variables (e.g., pathogen prevalence, genotype and anthropogenic changes) associated with mortality is required for conservation efforts. Here, using advanced molecular techniques, we characterised the genotypes of *S. neurona* infecting marine mammals stranded along the coast of the northeastern Pacific Ocean. We identify the emergence of a new genotype of *S. neurona* (Type XIII), demonstrate that *S. neurona* is capable of transplacental transmission in both pinnipeds and cetaceans, and show that parasite genotype is associated with protozoal disease severity and related mortality in multiple marine mammal species.

2. Materials and methods

2.1. Sample collection

A total of 227 study subjects of beach-cast marine mammal carcasses were collected between 2004 and 2012 in two main areas: (i) the outer coastal regions ($n=131$) of Oregon ($n=9$), Washington ($n=120$), and southern British Columbia ($n=2$) and (ii) the inland coastal regions ($n=96$) of Washington ($n=93$) and southern British Columbia ($n=3$). Carcasses were opportunistically collected from these regions by the Washington Department of Fish and Wildlife, USA, Cascadia Research, USA and the Department of Fish and Oceans, Canada as part of a coastal health monitoring program by the Northwestern Marine Mammal Stranding Network, USA. This work was authorised under the Marine Mammal Protection Act (MMPA) Stranding Agreements and Section 109(h) (16 U.S.C. 1379(h)), USA. Additional samples

were acquired under a National Marine Fisheries Service (NMFS), USA MMPA Section 120 Letter of Authorisation and NMFS MMPA Scientific Research Permits 782-1702 and 13430. According to the Convention of International Trade of Endangered Species (CITES), both United States CITES export and Canadian CITES import permits were obtained in accordance with international protocols, and United States Fish and Wildlife declarations were completed prior to the transport of specimens across the international border.

Upon carcass collection, species identification and date were documented, and GPS coordinates (latitude/longitude) were recorded as an estimate for stranding location. Species were also categorised by stranding region. Individuals from Gertrude Island, USA and those stranding east of the entrance to the Strait of Juan de Fuca, USA were classified as inner coast, while individuals stranding along the Pacific Ocean coastline were classified as outer coast. Size of animal, sex and age class (fetus, pup/calf, sub-adult and adult, estimated using dentition) were determined at necropsy.

The majority of samples were from stranded animals with clinical or pathological evidence of protozoal disease, manifesting as: (i) observation of ante-mortem neurological behavior, or (ii) post-mortem gross lesions indicative of protozoal encephalitis. Additionally, Guadalupe fur seals and harbour porpoises that stranded during the study period were submitted as suspected protozoal cases due to region-wide unusual mortality events, and sea otters were submitted as suspected protozoal cases due to previous outbreaks of protozoal disease in sea otter populations. A subset of Pacific harbour seals without evidence of protozoal disease and a number of large cetaceans were also submitted.

Complete necropsies were performed on all marine mammal carcasses. Tissue samples for molecular detection of protozoal agents were sent to the Molecular Parasitology Section at the National Institutes of Health (NIH), USA where they were stored at -80°C until analysis. Following necropsy, a veterinary pathologist examined tissue samples by histology for the presence of parasites, inflammation and associated pathology in a blinded fashion. An encephalitis score (absent, mild to moderate or marked to severe) was assigned based on histological severity of the encephalitis due specifically to the presence and number of protozoa and the extent and severity of the associated inflammatory infiltrate detected in the brain of infected animals. A cause of death category (incidental, contributing or direct) was also assigned to each individual based on the anatomic location and density of infection, extent of inflammation and whether other agents (i.e., domoic acid, starvation, injury) may have influenced the overall contribution of *S. neurona* infection to the death of the animal, according to Gibson et al. (2011).

2.2. DNA extraction

DNA was extracted from tissue samples (including brain, heart, muscle and lymph node) according to the DNeasy Purification of Total DNA from Animal Tissues protocol (QIAGEN, Valencia, California, USA). Briefly, approximately 25 mg of tissue was digested overnight (approximately 20 h) at 55°C with Proteinase K and processed as described previously (Gibson et al., 2011). DNA extraction was performed on multiple (between 1 and 10) separate tissue sections from each individual. All extracted DNA was stored at -20°C until required for PCR analysis.

2.3. Molecular characterisation

Extracted DNA from tissue samples was used for multiple PCRs, with primer sets for the internal transcribed spacer 1 (*ITS1*) and *ITS1500* loci for initial coccidian screening. The *ITS1* locus is specific

for apicomplexan DNA and was used to differentiate between *S. neurona*, *Toxoplasma gondii* and *Neospora caninum* by their electrophoretic size polymorphism in a DNA agarose gel and by DNA sequencing (Gibson et al., 2011). The *ITS1500* locus is highly specific for the detection of *S. neurona* and *Sarcocystis falcatula* (Wendte et al., 2010b). For most tissue samples from carcasses collected between 2006 and 2009 ($n = 147$), PCRs at the *ITS1* and *ITS1500* loci were performed as part of the Gibson et al. (2011) study. Those samples were revisited here in order to determine the *S. neurona* genotype infecting those marine mammals. They were augmented with additional carcasses collected between 2005 and 2012 ($n = 80$), providing a total of 227 samples.

PCR amplification was conducted according to Wendte et al. (2010b). DNA product was stored at 4 °C until gel electrophoresis on 0.8% agarose gels stained with Gel Red dye (Biotium, Inc., Hayward, California, USA) for visualisation under UV light. Positive amplicons were digested using Exo-SAP (USB Corporation, Cleveland, Ohio, USA) according to the manufacturer's instructions prior to DNA sequencing. DNA sequencing was carried out by Rocky Mountain Laboratory Genomics Unit DNA Sequencing Center, Division of Intramural Research (Hamilton, Montana, USA). The resulting sequences and polymorphisms were identified by alignment against a reference *S. neurona* isolate designated SN1 (Marsh et al., 1999) using the Seqman component of the Lasergene software. (DNASTAR, Inc., Madison, Wisconsin, USA). In order to decrease the chances of having false positive or false negative results, only specimens that tested *S. neurona* DNA-positive in a minimum of two tissue samples or negative in a minimum of two tissue samples were included in the study as cases and controls, respectively.

For samples with enough extracted DNA product remaining after initial PCR screening, molecular genotyping was performed. Three sets of nested surface antigen primers (for use in external and internal PCRs), SnSAG1-5-6, SnSAG3 and SnSAG4 were used, which allowed for *S. neurona* genotyping based on allele presence at SnSAGs 1, 5 and 6, and polymorphisms at specific nucleotide positions in SnSAG3 and SnSAG4 (Crowdus et al., 2008; Howe et al., 2008; Rejmanek et al., 2010; Wendte et al., 2010b). The SnSAG1-5-6 genes are differentially encoded among *S. neurona* strains, whereas SnSAG3 and SnSAG4 are polymorphic. The SnSAG2 gene is non-polymorphic (Rejmanek et al., 2010; Wendte et al., 2010b), and was not utilised in this study. In order to increase sensitivity, a set of forward and reverse internal primers were developed for SnSAG4 for use in a nested reaction, and a forward internal primer was developed for SnSAG1-5-6, for use in a hemi-nested reaction. The primers designed were as follows: SnSAG4 INTFWD – CAAGCAACAGCGTAACAGA, INTREV – CTC TCGCAACGGGAAGTTAG; SnSAG1-5-6 INTFWD – TGACACTCTGCT CYCCAGA.

External PCRs consisted of 3 μ L of sample DNA, 5 μ L of 10 \times PCR buffer containing 15 mM MgCl₂ (Sigma–Aldrich, St. Louis, Missouri, USA), 5 μ L of 2 mM dNTPs mix, 25 pmol of each primer (forward and reverse), 0.5 μ L of DNA Taq polymerase (Sigma–Aldrich) and deionised water in a total reaction volume of 50 μ L. PCR conditions included initial denaturation at 95 °C for 5 min; followed by 35 cycles consisting of 95 °C for 40 s, 58 °C for 40 s and 72 °C for 40 s; and a final extension for 10 min at 72 °C. All nested PCRs utilised 1 μ L of PCR product from the primary reaction as template, and reaction protocols and conditions were the same as for the initial PCR. Negative controls for both external and internal nested reactions consisted of molecular grade de-ionised water as well as DNA extracted from muscle tissue of an uninfected mouse. Product visualisation and DNA sequencing were performed as described above. Because immunosuppression due to morbillivirus infection can contribute to the development of protozoal disease, all animals were tested for morbillivirus infection by PCR (Haas et al., 1991).

2.4. Parasite isolation and microsatellite analysis

High resolution microsatellite typing is an excellent tool for identifying subtle genetic variations within Ag types, however it is an inherently less sensitive and more labor-intensive technique than nested MLST analysis. It requires sufficient parasite DNA from cloned isolates, which can only be achieved by culturing the parasite from infected tissues harvested from recently deceased animals. When fresh carcasses were available, parasites for MS typing were isolated according to Miller et al. (2004). Briefly, fresh brain tissue from stranded marine mammals with suspected protozoal infections were washed in antibiotic saline solution, trypsinised, disrupted by pipetting and seeded onto MA104 monkey kidney cell monolayers to isolate parasites. In total, six *S. neurona* isolates were recovered. Parasite DNA was extracted from parasite pellets and analysed at 12 MS markers according to Wendte et al. (2010b). MS allelic types were assigned based on the number of di-, tri- or complex-repeats obtained at each marker. The inheritance pattern of the repeats identified across the 12 MS markers was used to determine a multilocus MS type.

2.5. Data analysis

Statistical associations were evaluated between *S. neurona* genotype and putative risk factors including species, age class, sex, stranding year, stranding region (inner versus outer coast), and co-infection status (singular *S. neurona* infection versus co-infection with *T. gondii* in brain tissue), using the Fisher exact test and the chi square test for trend. Associations between *S. neurona* genotype and the encephalitis score and the cause of death category were evaluated on a subset of samples for which sufficient DNA was present to permit MLST genotyping using the two-sided Fisher exact test. *P* values < or =0.05 were considered statistically significant. Statistical analyses were conducted using Epi Info (version 7.1.2.0, Center for Disease Control, Atlanta, GA, USA), and STATA statistical software (version 12.1, StataCorp, College Station, TX, USA).

3. Results

3.1. *Sarcocystis neurona* infections among stranded marine mammals from the northeastern Pacific Ocean

The overall frequency of *S. neurona* infection in our population of marine mammals exhibiting mainly pre- or post-mortem signs of neurological disease or stranding during peak seasons when neurological disease is observed was 60% (136/227). *Sarcocystis neurona* infection was documented in 63% (76/121) of Pacific harbour seals (*Phoca vitulina*), 70% (32/46) of Pacific harbour porpoises (*Phocoena phocoena*), 44% (11/25) of Guadalupe fur seals (*Arctocephalus townsendi*), 38% (3/8) of Steller sea lions (*Eumetopias jubatus*), 67% (4/6) of Northern sea otters (*Enhydra lutris*), 20% (1/5) of Northern fur seals (*Callorhinus ursinus*), 75% (3/4) of California sea lions (*Zalophus californianus*), and all (3/3) Northern elephant seals (*Mirounga angustirostris*), and all (1/1) of Pacific white-sided dolphins (*Lagenorhynchus obliquidens*). *Sarcocystis neurona* was also detected in a pregnant adult pygmy sperm whale (*Kogia breviceps*) as well as her second trimester fetus. Two killer whales (*Orcinus orca*), two gray whales, a Bryde's whale (*Balaenoptera brydei*), and a sperm whale (*Physeter macrocephalus*) were not found to be infected with *S. neurona*. Sixty-two percent (8/13) of fetuses, 50% (28/56) of pups/calves, 57% (39/69) of yearling/subadults, and 69% (61/89) of adults were positive for *S. neurona* by PCR. Sixty-three percent (63/100) of

females and 57% (71/124) of males were *S. neurona*-positive. In three individuals, the sex was not known.

Sarcocystis neurona infections were identified in 50% (48/96) of inner coast individuals and 67% (88/131) of outer coast individuals. Stranding region was analysed on a species-specific basis among residential species that had sufficient sample sizes (Pacific harbour seals and harbour porpoises). Pacific harbour seals were significantly more likely to be infected with *S. neurona* if stranded on the outer coast than if stranded on the inner coast ($P = 0.0002$) (Fig. 1). Samples analysed in 2006–2009 by Gibson et al. (2011) identified a high prevalence of co-infections with multiple coccidian species, as was likewise observed among the additional samples collected during 2005–2012 for the current study. In total, tissues from 66% (90/136) of *S. neurona*-positive individuals were infected with at least one additional coccidian parasite, including *T. gondii* (85/90), *N. caninum* (8/90), and other species within the

family Sarcocystidae (13/90) (*Sarcocystis canis*-like, *Sarcocystis cruzi*-like, *Sarcocystis columbae*-like, *Sarcocystis* sp. CRC-836, Coccidia A, B or C, among others). Nineteen individuals were infected with three or more coccidian species. All animals in this study were PCR negative for morbillivirus (Barrett et al., 1985), and there was no evidence of viral inclusions detected.

3.2. Molecular genetic analysis of *S. neurona* genotypes

Thirty-seven of the 136 *S. neurona*-infected individuals had sufficient DNA available to permit MLST analyses at the SnSAG1–5–6, SnSAG3 and SnSAG4 genes in order to determine the *Sarcocystis* genotype responsible for infection. In 26 individuals, all markers were successfully PCR amplified and DNA sequenced, permitting an MLST genotype designation (Table 1). Two genotypes were identified: genotype VI ($n = 15$), a widely distributed genotype

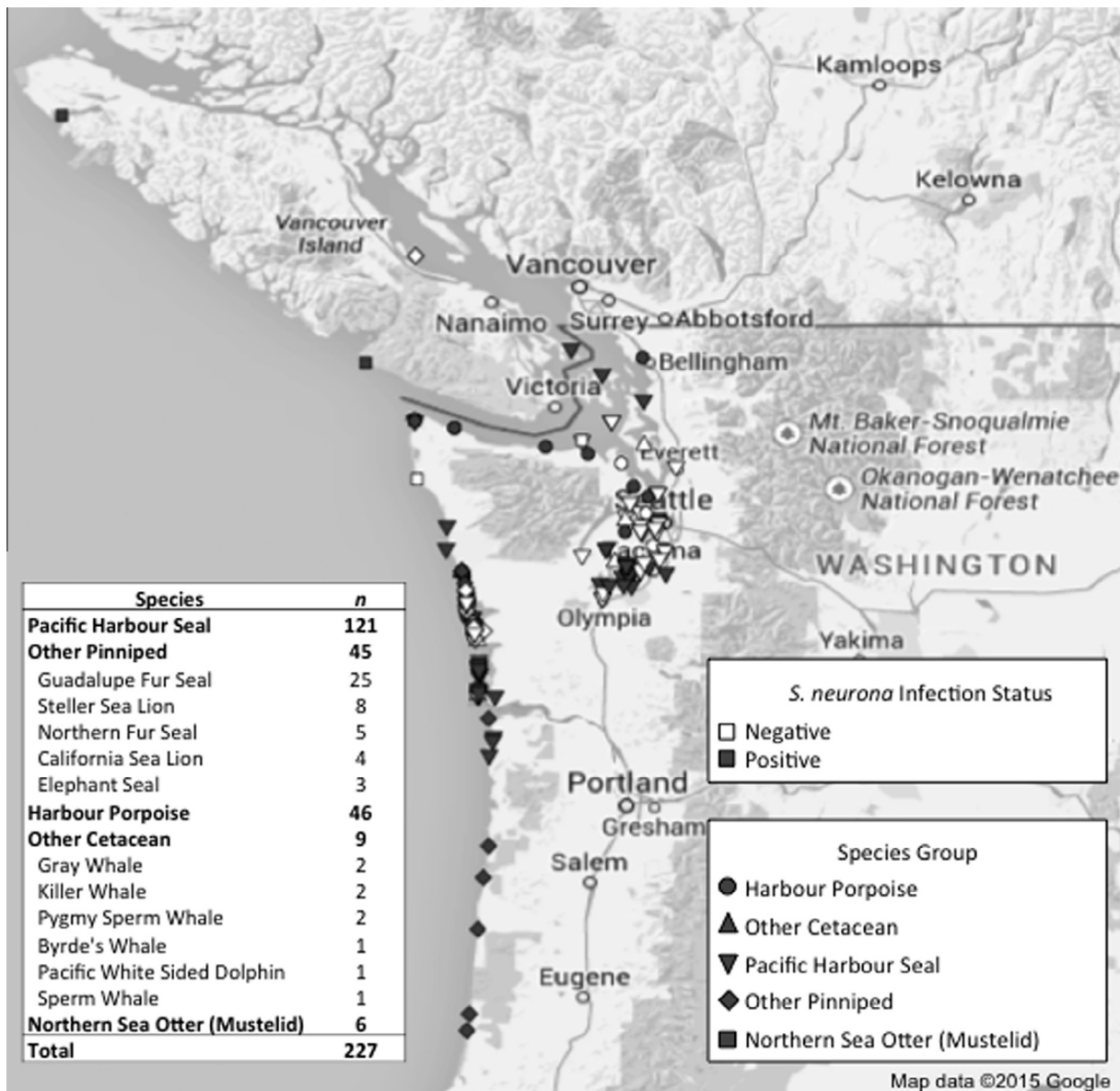


Fig. 1. Distribution of *Sarcocystis neurona* infection among 227 stranded marine mammals across Washington and Oregon, USA, and southern British Columbia, Canada. Each symbol represents a single sampled individual; individuals are designated by species group according to symbol and by infection status by shade (negative, white; infected, black). Pacific Harbour Seals ($n = 121$) and Harbour Porpoises ($n = 46$) represented the largest sample groups. Additional species were grouped by species type (pinniped, cetacean, mustelid) and group composition is shown above. Distribution maps were constructed using geographical coordinates collected at the time of sampling in R (Version 3.0.1). Base map data: ©2015 Google.

Table 1
Species, stranding year, *Sarcocystis neurona* surface antigen (SnSAG) sequencing and histopathological data for *S. neurona*-infected marine mammals stranded along the northeastern coast of the Pacific.

Animal ID	Species	Stranding year	Genotype	SnSAG1–5–6	SnSAG3							SnSAG4	Protozoal encephalitis	Cause of death	Brain coinfection	Used in statistical analysis	
					239	503	504	505	506	735	1057						
SN1	Reference		II	1	C	A	T	A	T	T	C	C					
ZC1	California Sea Lion	2008	ind	1								C	Mild/Moderate	Contributing	-	n	
PP1	Harbour Porpoise	2007	VI	5	C	A	T	A	T	T	C	G	Mild/Moderate	Direct	-	y	
PP2	Harbour Porpoise	2008	VI	5	C	A	T	A	T	T	C	G	Mild/Moderate	Incidental	-	y	
PP3	Harbour Porpoise	2008	VI	5	C	A	T	A	T	T	C	G	Mild/Moderate	Direct	-	y	
PP4	Harbour Porpoise	2008	VI	5	C	A	T	A	T	T	C	G	Marked/Severe	Direct	Coccidia C	n	
PP5	Harbour Porpoise	2008	VI	5	C	A	T	A	T	T	C	G	Mild/Moderate	Direct	<i>Toxoplasma gondii</i>	n	
PP6	Harbour Porpoise	2008	VI	5	C	A	T	A	T	T	C	G	Mild/Moderate	Contributing	-	y	
PP7	Harbour Porpoise	2009	XIII	1	G	-	-	-	-	C	T	C	Mild/Moderate	Direct	-	y	
PP8	Harbour Porpoise	2009	ind	1								C	Contributing	<i>T. gondii</i>	n		
PP9	Harbour Porpoise	2009	VI	5	C	A	T	A	T	T	C	G	Absent	Contributing	-	y	
PP10	Harbour Porpoise	2009	VI	5	C	A	T	A	T	T	C	G	Absent	Incidental	-	y	
PP11	Harbour Porpoise	2011	VI	5	C	A	T	A	T	T	C	G	Marked/Severe	Contributing	<i>T. gondii</i>	n	
PP12	Harbour Porpoise	2012	VI	5	C	A	T	A	T	T	C	G	Absent	Incidental	-	y	
PV1	Pacific Harbour Seal	2005	ind	1								C	Mild/Moderate	Incidental	-	n	
PV2	Pacific Harbour Seal	2007	ind									G	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV3	Pacific Harbour Seal	2007	VI	5	C	A	T	A	T	T	C	G	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV4	Pacific Harbour Seal	2008	ind	1								C	Absent	Incidental	-	n	
PV5	Pacific Harbour Seal	2008	XIII	1	G	-	-	-	-	C	T	C	Mild/Moderate	Contributing	-	n	
PV6	Pacific Harbour Seal	2008	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV7	Pacific Harbour Seal	2008	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV8	Pacific Harbour Seal	2009	ind		C	A	T	A	T	T	C	G	Mild/Moderate	Incidental	-	n	
PV9	Pacific Harbour Seal	2009	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV10	Pacific Harbour Seal	2009	ind									G	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV11	Pacific Harbour Seal	2009	VI	5	C	A	T	A	T	T	C	G			-	n	
PV12	Pacific Harbour Seal	2009	VI	5	C	A	T	A	T	T	C	G	Absent	Incidental	-	n	
PV13	Pacific Harbour Seal	2009	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV14	Pacific Harbour Seal	2009	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV15	Pacific Harbour Seal	2010	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV16	Pacific Harbour Seal	2011	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV17	Pacific Harbour Seal	2011	ind		C	A	T	A	T	T	C	G	Marked/Severe	Contributing	<i>T. gondii</i>	n	
PV18	Pacific Harbour Seal	2011	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	
PV19	Pacific Harbour Seal	2011	ind		C	A	T	A	T	T	C	G	Marked/Severe	Direct	-	n	
PV20	Pacific Harbour Seal	2012	ind		G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	n	
PV21	Pacific Harbour Seal	2012	ind		C	A	T	A	T	T	C	G	Marked/Severe	Direct	-	n	
PV22	Pacific Harbour Seal	2012	VI	5	C	A	T	A	T	T	C	G	Marked/Severe	Direct	<i>T. gondii</i>	n	
PV23	Pacific Harbour Seal	2012	VI	5	C	A	T	A	T	T	C	G	Marked/Severe	Direct	-	y	
EJ1	Steller Sea Lion	2008	XIII	1	G	-	-	-	-	C	T	C	Marked/Severe	Direct	-	y	

“-” represents an insertion or deletion at SnSAG3. Only specimens possessing full multilocus sequence types (MLST) were used for statistical analysis. ind, indeterminate; unable to assign a strain genotype.

The protozoal encephalitis category was assigned based on the histological severity of the encephalitis due specifically to the protozoal infection.

The cause of death category was assigned based on the overall contribution of *S. neurona* infection to the death of the animal.

Empty cells represent a lack of available data.

Table 2
Microsatellite (MS) sequencing of *Sarcocystis neurona* (Sn) strains from six isolates from marine mammals stranded along the coast of the northeastern Pacific Ocean.

Sample	Year	Source	Ag Type	MS Type	MS locus											Total nt count
					Sn2 (GT) _n	Sn3 (AT) _n	Sn4 (CA) _n	Sn5 (CA) _n	Sn7 (CA) _n	Sn8 (CA) _n	Sn9 (GT) _n	Sn10 (AT) _n	Sn11 (CA) _n	Sn1520 (CTA) _n	Sn1863 (AC) _n	
SO3106	1999	Wendte et al., 2010a	II	r	9	12	12	9	17	10	17	11	13	9	17	120
PP11	2011	This study	VI	r	10	11	*	9	*	10	14	10	*	14	16	81
PV16	2011	this study	XIII	hh	9	10	*	10	*	10	14	9	14	10	22	81
PV17	2011	this study	ind	u	10	*	*	9	18	10	14	9	*	*	15	
PV18	2011	this study	XIII	hh	9	10	*	10	18	*	14	9	14	10	22	
PV19	2011	this study	ind	u	10	*	13	9	18	10	14	9	14	14	15	
PV22	2012	this study	VI	p'	10	11	13	9	18	10	14	10	14	15	16	

SO, Sea Otter; PP, Harbour Porpoise; PV, Harbour Seal; nt, nucleotide.

*Unable to assign numerical value for the number of repeats at the MS locus.

ind, indeterminate; unable to assign a strain genotype.

Specimens are grayscale shaded to reflect different antigen (Ag) and MS types.

Empty cells represent a lack of available data.

SO3106 serves as a reference sequence (Wendte et al., 2010a).

common throughout the United States, and genotype XIII ($n = 11$), a novel genotype not previously observed. For the remaining 11 *S. neurona*-infected individuals, it was not possible to assign a genotype due to a lack of sequence information at one or more of the SnSAG genes.

Since high resolution MS typing is a less sensitive technique that cannot readily be set up in a nested or multiplexed PCR configuration, this technique was only applied against the six *S. neurona* isolates that were recovered by cell culture (Table 2). The two isolates identified as Type VI had different MS types, p' and r. In contrast, the two Type XIII isolates shared the same novel MS type, hh. The other two isolates (PV17, PV19) did not amplify at the SnSAG1, SnSAG5 or SnSAG6 gene. However, they both possessed the same MS type, u, and were presumed to be either Ag type VI or VII based on alleles present at the SnSAG3 and SnSAG4 genes. This Ag/MS type combination has been identified previously in an infected southern sea otter from Monterey CA, USA (Wendte et al., 2010a).

3.3. Risk factors for parasite infection

Genotype XIII was significantly more common in harbour seals and genotype VI was significantly more common in harbour porpoises ($P = 0.012$), although each genotype was capable of infecting both pinnipeds and cetaceans. Age class, sex, stranding region, stranding year and co-infection status did not differ among animals infected with genotype XIII compared with genotype VI, although insufficient power may have precluded determining significance. Genotype XIII was first identified in stranded animals in 2008, and all 11 genotype XIII individuals were collected from the outer coast of Washington State.

Of the 26 individuals to whom an MLST genotype was assigned, one individual (PV11; genotype VI) was excluded from the encephalitis and cause of death analyses due to a lack of histopathological data, and two more individuals (PV12, genotype VI; and PV5, genotype XIII) were excluded because they were infected transplacentally and did not represent environmentally-acquired infections. Another seven individuals were excluded from the pathological analyses due to brain co-infections with other protozoan species based on evidence that co-infections with both *S. neurona* and *T. gondii* are associated with increased encephalitis and mortality (Gibson et al., 2011). Among the remaining 16 singly-infected animals, those infected with genotype XIII ($n = 8$) were significantly more likely to develop a marked or severe encephalitis than those animals infected with genotype VI ($n = 8$) ($P = 0.01$). Accordingly, cause of death by *S. neurona* was significant among genotype XIII infected individuals – all animals died directly as a result of their *S. neurona* infection, whereas the majority of genotype VI infected individuals did not ($P = 0.03$). When the seven individuals with brain co-infections

were also considered, those infected with genotype XIII ($n = 10$) all died directly as a result of their *S. neurona* infection and were still significantly more likely to develop severe encephalitis than those infected with genotype VI ($n = 13$) ($P = 0.05$).

4. Discussion

Sarcocystis neurona has previously been documented infecting sea otters, harbour porpoises and harbour seals (Wendte et al., 2010a). This study establishes that *S. neurona* is capable of infecting a wider range of marine pinnipeds and cetaceans, including a Steller sea lion, Guadalupe fur seal, Northern elephant seal, Northern fur seal, pygmy sperm whale and Pacific white-sided dolphin. The molecular genetic analyses identified three MS genetic signatures among two genotype VI isolates and two additional isolates that did not PCR amplify at the SnSAG1-5-6 locus (Table 2). All of these Ag/MS genotypes have been identified previously among northeastern Pacific Ocean marine mammals and intermediate terrestrial hosts throughout the United States (Wendte et al., 2010a). In contrast, a new, unique molecular signature (Ag type XIII, MS type hh) was identified in this study and is responsible for fatal infections among both pinnipeds and cetaceans in the northeastern Pacific Ocean. All but one individual with single genotype XIII infections, and all individuals with co-infections that included genotype XIII, had marked/severe protozoal encephalitis and all died of protozoal disease, demonstrating that this genotype is highly pathogenic and is an important cause of mortality in marine mammals. Although protozoal disease has been seen in association with immunosuppression due to morbillivirus infection (Haas et al., 1991), all animals in this study were PCR-negative for this virus. The mortalities appear to be attributed to genotype XIII, however, other disease processes potentially contributing to immunosuppression were not screened for in this study. *Sarcocystis neurona* Type XIII was first detected in the northeastern Pacific Ocean in 2008 and is likely an emerging disease in this region. Infections with this parasite genotype have now occurred over several years, indicating a persistent source of infection that has the potential to cause long-term impacts on marine mammal populations, and possibly be even more detrimental than the Type I epizootic, which emerged in 2004 and caused mass mortality among sea otters off the coast of California before subsequently disappearing (Miller et al., 2010; Wendte et al., 2010a).

The population genetic structure of *S. neurona* is not complex. In the United States it is largely comprised of 12 MLST Ag types that appear largely clonal in origin (Wendte et al., 2010a). Despite this relative lack of overall diversity, local populations of *S. neurona* are known to exhibit substantial smaller-scale variability, as evidenced by the application of MS markers (Asmundsson et al., 2006; Sundar et al., 2008; Wendte et al., 2010a). The discovery of a highly pathogenic genotype of *S. neurona* infecting pinnipeds and cetaceans in

the northeastern Pacific Ocean is of particular interest. Current efforts to sequence the Type XIII genome are ongoing to determine the genetic history of the strain and whether it is a recent strain that has emerged by sexual recombination in its definitive opossum host from a cross between strains of *S. neurona* circulating in the United States. Previous studies have demonstrated that experimental crosses between avirulent strains of the closely related coccidian parasite, *T. gondii*, are capable of producing offspring more virulent than either parent, and that this appears to be occurring in nature (Grigg and Sundar, 2009; Grigg et al., 2001; Wendte et al., 2010a). Hence, sexual reproduction has the potential to rapidly produce new gene combinations that can lead to a spectrum of disease, ranging from largely incidental infections to mass mortality events (Miller et al., 2004; Wendte et al., 2010a). In this context, the *S. neurona* 2004 outbreak in southern sea otters was determined to be the result of infection by a single, distinct genotype (Ag type I, MS type c), that was amplified by its opossum definitive host (Miller et al., 2010; Wendte et al., 2010a). Since the discovery of Type XIII among marine mammals stranding along the coast of the northeastern Pacific Ocean, this genotype has yet to be detected in the definitive opossum host. Work is ongoing to determine the source or reservoir of infection for this highly pathogenic genotype (to marine pinnipeds and cetaceans) among the definitive host, as well as known *S. neurona* intermediate hosts.

The mechanism for *S. neurona* transmission in the marine environment is unclear. The widely accepted hypothesis is that the definitive host, the Virginia opossum, sheds the infective stage of the parasite on land, which then enters the marine environment through freshwater runoff, a mechanism that has also been proposed for a variety of similar protozoal pathogens (Miller et al., 2002, 2008; Fayer et al., 2004; Conrad et al., 2005; Shapiro et al., 2012). There is evidence that near-shore filter-feeding invertebrates can concentrate the closely related protozoan *T. gondii*, which may then be consumed by intermediate hosts (Lindsay et al., 2001; Miller et al., 2008). Furthermore, *S. neurona* infection in sea otters has been linked to consumption of certain prey, including filter-feeding bivalves and soft-sediment invertebrates, as well as to sandy foraging habitat (Johnson et al., 2008). Filter-feeding fish, including northern anchovies and Pacific sardines, have been shown experimentally to ingest *T. gondii* oocysts, which remained infective over time, perhaps suggesting that filter-feeding fish could function as transport hosts for protozoal infection of marine mammals (Massie et al., 2010).

In our sample population, harbour seals stranding along the outer coast were significantly more likely to be infected with *S. neurona* than those along the inner coast, while infection in another resident species, the harbour porpoise, was evenly distributed. The model of land-to-sea transmission is supported here by the fact that harbour seals spend a significant portion of their lives on land (whereas harbour porpoises are restricted to the water), thus increasing exposure to potential land-based pathogens. While harbour porpoises and harbour seals have been known to consume both pelagic and benthic fishes, with substantial overlap in prey species (Pacific herring, walleye Pollock, Pacific sand lance, eelpout) (Brown and Mate, 1983; Walker et al., 1998; Lance et al., 2007), harbour seals are also known to consume a high proportion of benthic and epibenthic prey including rockfish spp., sculpins and several flatfish spp.) (Brown and Mate, 1983; Lance et al., 2007). Thus, differences in diet between these two species could also contribute to discrepancies in *S. neurona* infection, if certain prey species are indeed acting as transport hosts. Examination of samples from a broader geographical range including land-based mammals could determine whether genotype XIII exists outside the outer coastal waters of Washington State or if there may be a point source of infection in this region. The fact that the infective oocyst stage of similar protozoan parasites can remain viable in

the environment for extended periods of time (Lindsay et al., 2003) suggests that factors such as location in the water column, bottom sediment, current and weather may also play a role in sporocyst distribution and subsequent infection of marine mammals.

Vertical transmission is an important route of infection for *N. caninum* (Anderson et al., 1997; Davison et al., 1999; Moré et al., 2009), and has been demonstrated to occur for *T. gondii*, and some *Sarcocystis* spp. (Moré et al., 2009), however, fetal infection with *S. neurona* has not previously been documented (Duarte et al., 2004). Our study has identified fetal infection with *S. neurona* in a Steller sea lion, a harbour porpoise, five harbour seals and a pygmy sperm whale. Fetuses from the harbour porpoise, the pygmy sperm whale and a harbour seal were recovered post-mortem from the mother's uterus, and both mother and fetus were infected with *S. neurona*. The remaining five fetuses were beach-cast carcasses, too young to have been viable, and thus were determined to have become infected trans-placentally. These findings indicate that vertical transmission of *S. neurona* infection may exist as a new threat to the population health of listed and/or recovering pinnipeds and cetaceans by decreasing their reproductive fecundity.

During recent decades, small mammals including the Virginia opossum have been observed to shift their geographic ranges northwards (Myers et al., 2009), both due to increases in global surface temperature (Alley et al., 2007), and likely increased availability of human-associated food resources (Kanda, 2005). As the range of the opossum expands, new *S. neurona* genotypes such as Type XIII may emerge to cause infection in susceptible host species not previously exposed to these pathogens such as marine mammals. Although some of the marine mammal species in our study, such as the Pacific harbour seal and the harbour porpoise, have stable population growth, pathogens that are prevalent in less abundant species may have negative consequences on population health and reproductive fitness, particularly in heavily impacted ecosystems. Better understanding of the genetic variation of pathogens such as *S. neurona*, their capacity to cause reproductive loss, and how this variation influences host specificity and pathogenicity, will allow for enhanced appreciation of the population-level risks associated with *S. neurona* infection in marine mammals.

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