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Fatal hepatic sarcocystosis in three captive and one free-ranging pinniped

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ABSTRACT

Fatal hepatic sarcocystosis was diagnosed as the cause of death in four pinnipeds: two captive Hawaiian monk seals (*Monachus schauinslandi*), a captive, and a free-ranging California sea lion (*Zalophus californianus*). Based on necropsy, histopathology, electron microscopy and DNA sequencing, intralesional protozoal schizonts were determined to have caused the necrotizing hepatitis observed. Transmission Electron Microscopy (TEM) revealed schizonts similar to *Sarcocystis canis* in hepatocytes. PCR-DNA sequencing and phylogenetic analysis at the conserved *18S rRNA* and variable *ITS1* gene markers within the nuclear rRNA gene array from schizont-laden tissue established that the parasites were indistinguishable from *Sarcocystis canis* at the *18S rRNA* locus. However, six distinct single nucleotide polymorphisms (SNPs) were resolved at *ITS1* suggesting that the parasites infecting pinnipeds were distinct from *S. canis,* which commonly infects bears and dogs. We hypothesize that the parasite represents a novel *Sarcocystis* variant that we refer to as *S. canis*-like that infects pinnipeds. The definitive host of *S. canis* is enigmatic and its life cycle incomplete. These findings document a critical need to identify the life cycle(s), definitive host(s), and all susceptible marine and terrestrial intermediate hosts of *S. canis* and the *S. canis*-like variant infecting pinnipeds.

1. Introduction

Apicomplexan parasites within the genus *Sarcocystis* are diverse and more than 200 species have been identified. These parasites are heteroxenous and require at least two hosts to complete their life cycles. Sporocysts develop in the small intestine and are shed from the definitive host whereas transmissible sarcocysts develop largely in the muscle and brain tissues of intermediate hosts. The life cycle is typically completed when intermediate hosts act as prey for definitive hosts that ingest tissues containing mature sarcocysts (Dubey et al., 2016). While most *Sarcocystis* species utilize a particular definitive host and either an intermediate or group of related intermediate hosts, it has been demonstrated that rats and lizards can serve as monoxenous hosts and support both sarcocysts and sporocysts of the same *Sarcocystis* spp. (Matuschka and Bannert, 1989; Hu et al., 2011). It has also been suggested that some birds also support monoxenous transmission cycles (Juozaityte-Ngugu et al., 2021). Whereas *Sarcocystis canis* (like *Sarcocystis neurona*) exhibits an unusually wide range of intermediate hosts (Dubey et al., 2003). *Sarcocystis canis* was first described as the causative agent associated with encephalitis, hepatitis, and generalized coccidiosis in dogs (Dubey and Speer, 1991). Hepatitis associated with *S. canis* has also been diagnosed in a chinchilla (*Chinchilla laniger*), a horse (*Equus caballus*), black bears (*Ursus* americanus) (reviewed in Dubey et al., 2016; Lee et al., 2021) and other *Sarcocystis* parasites found to be infecting brown bears (*Sarcocsytis arctosi*) and polar bears (*Sarcocystis* spp.) have also been described (Dubey et al., 2007; Garner et al., 1997). Hepatic sarcocystosis in marine mammals is known to occur in both cetaceans, including striped dolphins (*Stenella coeruleoalba*) (Resendes et al., 2002; Giorda et al., 2021) and pinnipeds, including a California sea lion (*Zalophus californianus*) (Mense et al., 1992; Dubey et al., 2003),

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a Hawaiian monk seal (*Monachus schauinslandi*) (Yantis et al., 2003), a steller sea lion (*Eumetopias jubatus*) (Welsh et al., 2014), and a harbor seal (*Phoca vitulina*) (O'Byrne et al., 2021). Recently, the parasite infecting an Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) was genotyped using the bar-coding locus *Cytochrome Oxidase 1* (*CO1*) and phylogenetic analysis suggested that the parasite in cetaceans was distinct, but closely related to *S. canis* (Calero-Bernal et al., 2017). Currently, the life cycle of *S. canis* is incomplete. Further, the true extent of the host range of *S. canis,* as well as other species of *Sarcocystis* capable of causing fatal hepatic sarcocystosis in marine mammals, is unknown. The present paper describes an acute hepatic sarcocystosis in four pinnipeds, three of which were part of an outbreak that occurred at SeaWorld, San Antonio in 2010. All pinnipeds were infected with a novel variant of *S. canis,* which we refer to as *Sarcocystis canis*-like that infects pinnipeds.

2. Materials and methods

2.1. Animal history

During 1995, 12 underweight Hawaiian monk seal pups were taken from the French Frigate Shoals, Northwestern Hawaiian Islands, to facilities on the Hawaiian island of Oahu for rehabilitation and subsequent release. During rehabilitation, the animals developed ocular disease rendering them non-viable candidates for release back to the wild. In 1997, a 21-month-old female in this group exhibited signs of gastrointestinal disease and subsequently died from her illness. Post-mortem examination demonstrated good body condition, but with generalized icterus, severe acute necrotizing hepatitis with bile stasis and intralesional immature and mature schizonts. Schizont-laden merozoites in the hepatocytes were subsequently identified by transmission electron microscopy and immunohistochemistry to be similar to the schizonts of *S. canis* (Yantis et al., 2003), the etiologic agent of generalized coccidiosis in dogs (Dubey and Speer, 1991). The cause of death was attributed to this infection, along with a Gram-negative bacterial colitis.

In 1999, eight of these animals were transferred to SeaWorld San Antonio, in San Antonio, Texas, USA. All eight animals were housed in a single-species pool containing artificial salt water. During December of 2010, two of these monk seals (referred to as HMS 1 and HMS 2) died within three days of each other, each with a one-day history of sudden illness. Clinical evaluations on both animals included complete blood counts and serum chemistry analysis. Both animals demonstrated a moderate leukocytosis and elevations in alkaline phosphatase (ALP), aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). Both animals died spontaneously. On gross and histologic review, both HMS 1 and HMS 2 demonstrated similar hepatic pathology and the cause of death was attributed to necrotizing hepatitis and related concerns.

One week after the death of the second Hawaiian monk seal, a four-year-old, pregnant female California sea lion (CSL 1 in this study) also held captive at SeaWorld San Antonio demonstrated clinical illness with similar serum chemistry elevations in white blood cell count and hepatic enzymes (ALP, ALT, and AST). The animal had a mild clinical response to medication during treatment but subsequently died. Like HMS 1 and HMS 2, cause of death was attributed to necrotizing hepatitis.

In May of 2018, a free-ranging California sea lion (CSL 2 in this study) was found stranded on Newport Pier at Newport Beach, in California and brought to the Pacific Marine Mammal Center in Laguna Beach, California. During rehabilitation in captivity, the animal was clinically stable for three months. But in August of 2018, the animal deteriorated and analysis of its blood work showed elevations in white blood cell count, blood urea nitrogen, and hepatic enzymes (ALT, AST and Gamma-glutamyl transferase (GGT)). CSL-2 died shortly after beginning the treatment process. The primary cause of death was likewise identified to be necrotizing hepatitis.

2.2. DNA extraction and PCR amplification

Sections of formalin-fixed paraffin-embedded (FFPE) and/or frozen liver from all four animals were collected and sent to the Molecular Parasitology Section at the National Institutes of Health (NIH) in Bethesda, Maryland for molecular characterization. We also obtained a frozen liver section from a black bear that had hepatic sarcocystosis with visible schizonts present (Lee et al., 2021). DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA, USA) according to manufacturer instructions.

Genomic DNA was used in a nested Polymerase Chain Reaction (PCR) amplification at two nuclear ribosomal DNA fragments within the conserved *18S rRNA* and variable internal transcribed spacer-1 (*ITS1*) regions. The presence of *Sarcocystis* spp. DNA was detected using previously published *18S rRNA* and pan-Coccidian *ITS1* primers (Dubey et al., 2015). Specifically, hemi-nested *18S rRNA* gene primers that amplify an ~350 nucleotide fragment within the conserved 3' terminal portion of the locus were used on genomic DNA extracted from paraffin blocks as follows: 18S–F: GCAAGGAAGTTTGAGGCAAT; 18S-R-Int: TCCTTCCTCTAAGTGTTAAGGTTCA; 18S-R-Ext: TGCAGGTTCACCTACGGAAA (Carlson-Bremer et al., 2012). Nested primers that amplify nearly a full length fragment of the *18S rRNA* gene were used on genomic DNA extracted from frozen liver sections as follows: Fext-GGTTGATCCTGCCAGTAGTCA; Fint-TAAAGATTAAGCCATGCATGTC; Rext-CCTCTAAGTGTTAAGGTTCAC; and Rint-TACAAAGGGCAGGGACGTAA. The following pan-coccidian primers that amplify an ~892 nucleotide fragment across the *ITS1* region that are anchored in conserved portions of the *18S rRNA* gene and *5.8S rRNA* gene were as follows: ApiITS1Fext-TTACGTCCCTGCCCTTTGTA; ApiITS1Rext-TGCGTTCTTCATCGTTGCGC; ApiITS1Fint-GTGAACCTTAACACTTAGAGG; ApiITS1Rint-GAGCCAAGACATCCATTGCT (Gibson et al., 2011). Nested PCR amplifications were performed in 50 µl total reaction volumes containing template DNA (3 µl for external PCR and 1 µl for internal PCR), 50 pmol of forward and reverse primer, 1X *Taq* DNA Polymerase, and 10X PCR Reaction Buffer containing M_gCl_2 (Sigma Aldrich, St. Louis, MO, USA). The thermal cycler conditions were set for initial denaturation at 95 °C for 5 min; 35 cycles of amplification (95 °C for 40 s, 58 °C for 40 s, and 72 °C for 40 s) and final elongation at 72 °C for 10 min. PCR products were resolved on 0.8% agarose gel stained with GelRed (VWR, Radnor, PA, USA) and visualized under UV light.

2.3. DNA sequencing and phylogenetic analysis

High quality PCR products of *18S rRNA* and *ITS1* amplicons were sent for Sanger sequencing to the Genomics Unit within the Rocky Mountain Research Technologies Section (RTS) at Rocky Mountain Laboratories, NIH in Hamilton, Montana. Sequence chromatograms of forward and reverse reads from the PCR population were read and edited using *Geneious* version 2020.0.5 (Biomatters Ltd., Auckland, NZ). Consensus sequences were compared against the NCBI GenBank sequence database by BLASTn.

Sequences obtained at the *18S rRNA* and *ITS1* region from the pinnipeds in this study were aligned against an *S. canis* sample amplified from a black bear (*Ursus americanus*) at the *18S rRNA* (OR654898) and *ITS1* (OR336049) and other related *Sarcocystis* species downloaded from GenBank using Clustal Omega within *Geneious* version 2020.0.5. Neighbor-joining trees were constructed in *Geneious* version 2020.0.5 from resulting alignments using the Tamura-Nei genetic distance model to show phylogenetic relationships among various *Sarcocystis* species. A Neighbor-Joining bootstrap consensus tree was inferred from 1000 replicates.

3. Results

3.1. Histopathology

In this study, the liver of three captive (HMS 1, HMS 2, and CSL 1) and one free-ranging pinniped (CSL 2) had moderate to severe multifocal acute necrotizing hepatitis with associated scattered protozoal schizonts. Concurrently, there were moderate to severe diffuse hemosiderosis in all cases, with spleens demonstrating a multifocal hemosiderosis with a moderate focally extensive necrotizing splenitis. A moderate, diffuse acute pulmonary congestion was also identified in lung tissue. The liver of CSL 2 depicted numerous coalescing foci of acute necrosis (Fig. 1). Many of the larger caliber portal tracts were expanded by moderate amounts of fibrosis, tortuous biliary hyperplasia, and possessed elevated numbers of lymphocytes, plasma cells, and hemosiderin-laden macrophages. Widely distributed throughout the foci of necrosis were a myriad of protozoal schizonts of different sizes and developmental states with individual oval to round protozoal merozoites resolved. Within most schizonts, the oval to banana-shaped protozoa were arranged in flower-like radiating arrays. In some regions of necrosis, there was moderate fibrin deposition and acute hemorrhage. Kupffer cells had increased in frequency in affected areas and were often swollen and contained phagocytized debris. The morphology of the protozoa and character of the lesions were consistent with acute sarcocystosis with a *Sarcocystis canis*-like organism.

3.2. Transmission electron microscopy

Ultrastructurally, schizonts were present in the cytoplasm of the hepatocytes with no parasitophorous membrane. Merozoites contained small numbers of micronemes clustered near the parasite membrane, a conoid, and a prominent nucleus but no rhoptries could be resolved (Fig. 2). Parasites were not observed in other tissues, including brain, heart, lung, kidney, spleen, lymph nodes, tonsil, thymus, adrenal gland, pituitary gland, bladder, stomach, pancreas and intestines that were examined microscopically, nor were any sarcocysts identified.

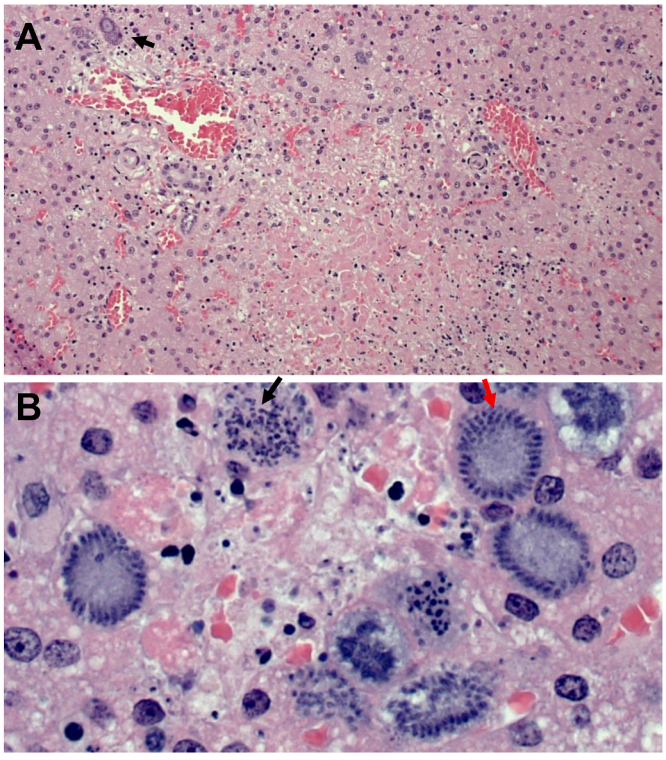

Fig. 1. Histologic liver sections of a California sea lion (*Zalophus californianus*) "CSL 2" with numerous coalescing foci of acute necrosis. **A.** Lower magnification of acute necrosis with *Sarcocystis* schizonts (black arrow). **B.** Higher magnification of mature protozoal schizonts with a rosette of merozoites (red arrow) and a schizont with greater than 30 free merozoites (black arrow) within a foci of hepatic necrosis.

3.3. DNA sequencing

PCR amplification and sequencing was successful at *ITS1* in the liver of all four pinnipeds from this study. A full length *18S rRNA* sequence (1600 nucleotides) was obtained only from the DNA extract from the frozen liver section obtained for CSL-2. For the other three samples, we only had DNA extracted from FFPE sections, and primers that amplify an ~350 nucleotide fragment of the 3' region of the *18S rRNA* gene resolved partial sequences that were identical to the sequence obtained from CSL-2. These latter sequences were excluded from the phylogenetic analysis. The full-length *18S rRNA* fragment from CSL 2 was deposited in NCBI GenBank under accession number OR339987. Consensus sequences obtained for *ITS1* (892 nucleotides) from all four animals in this study were deposited in NCBI GenBank under accession numbers OR339983-OR339986.

3.4. Phylogenetic analysis

Phylogenetic analysis based on a 994 nucleotide fragment within the *18S* rRNA sequences (Fig. 3) showed that the sequence type identified in the free-ranging California sea lion CSL 2 was identical to *S. arctosi* (EF564590) from a brown bear, *S. canis* (DQ146148) from a polar bear, and the sequence obtained for *S. canis* (OR654898) from a black bear. In contrast, at the *ITS1* locus, the phylogenetic analysis established that the sequences from the pinnipeds (HMS 1, HMS 2, CSL 1, and CSL 2), which all resolved as a single homozygous sequence type that was identical to each other, was genetically distinct from the *S. canis* sequences published for a polar bear (DQ176645) a black bear (MW136927) and the sequence obtained from the black bear sample sequenced in this study (OR336049) (Fig. 4). Specifically, the pinniped sequences possessed six

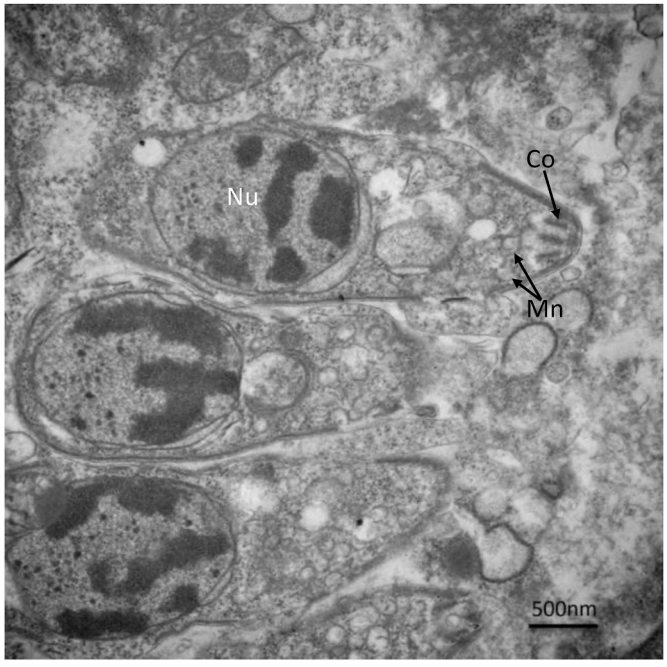

Fig. 2. TEM of a schizont with protozoa similar to *S. canis* in the hepatocyte of a California sea lion (*Zalophus californianus*) "CSL 1". Merozoites contained micronemes (Mn), a conoid (Co), and a prominent nucleus (Nu), but no rhoptries. Bar = 500 nm.

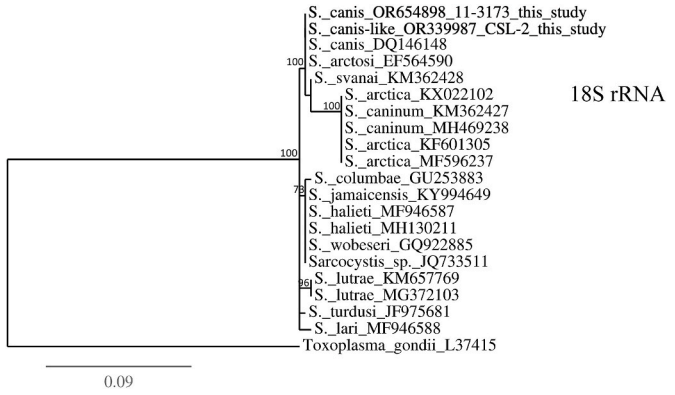

Fig. 3. Phylogenetic relationship of the *S. canis*-like variant isolate CSL-2 that infects pinnipeds from a California sea lion (*Zalophus californianus*) compared against *S. canis* from a black bear isolate 11–3173 (OR654898) and various other *Sarcocystis* spp. Within a 994 nucleotide fragment of the *18S* rRNA locus. Evolutionary distances were computed using the Tamura-Nei genetic distance model. A Neighbor-Joining bootstrap consensus tree was inferred from 1000 MUSCLE alignment iterations. Bootstrap percentage values are indicated at the branch points. *Toxoplasma gondii* was used as an outgroup.

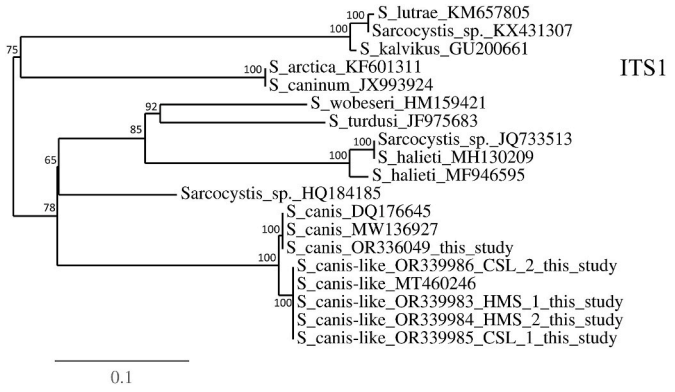

Fig. 4. Phylogenetic relationship among the *S. canis*-like variants from two Hawaiian monk seals (*Monachus schauinslandi*) and two California sea lions (*Zalophus californianus*), a Pacific harbor seal (MT460246) compared against *S. canis* sequences from two black bears (OR336049, MW136927), a polar bear (DQ176645) and various other *Sarcocystis* spp. at the complete *ITS1* locus. Evolutionary distances were computed using the Tamura-Nei genetic distance model. A Neighbor-Joining midpoint rooted bootstrap consensus tree was inferred from 1000 MUSCLE alignment iterations. Bootstrap percentage values are indicated at the branch points.

single nucleotide polymorphisms (SNPs) that distinguished their sequence haplotype from that of *S. canis* from the polar bear and two black bears (Table 1). Of note, a published sequence from another pinniped, specifically a Pacific harbor seal (MT460246), was identical to the four pinniped sequences obtained in this study.

4. Discussion

Acute hepatic sarcocystosis was diagnosed in four pinnipeds described in this study based on hepatic lesions and morphology of the parasite by histopathology and ultrastructural analyses. The identity of the parasite was confirmed to belong to the genus *Sarcocystis*, and our results suggest that it is distinct from *S. canis* and represents a novel variant according to sequences at *ITS1* from schizont-laden tissues of *S. canis* (DQ17765) sequenced from a polar bear and *S. canis* from two

Table 1
Diversity and frequency of single-nucleotide polymorphisms displayed by *Sarcocystis canis* isolates from two Black Bears (*Ursus americanus*) and the *Sarcocystis* canis-like variants that infected pinnipeds including two California sea lions (*Zalophus californianus*), two Hawaiian monk seals (*Monachus schauinslandi*) and a Pacific harbor seal (*Phoca vitulina*) at the complete *ITS1* locus. Sequences are compared to the *S. canis* isolate from a polar bear (DQ176645).

	ITS1 nucleotide position in reference *S. canis* DQ176645					
	252	282	537	543	572	635
	C	T	C	G	T	A
Isolate						
S. canis Black Bear_OR336049	.	.	.	.	.	.
S. canis Black Bear_MW136927	.	.	.	.	.	.
S. canis-like CSL-1_OR339985	G	C	T	T	C	T
S. canis-like CSL-2_OR339986	G	C	T	T	C	T
S. canis-like HMS-1_OR339983	G	C	T	T	C	T
S. canis-like HMS-2_OR339984	G	C	T	T	C	T
S. canis-like Harbor Seal MT460246	G	C	T	T	C	T

black bears (OR336049; MW136927). We refer to this variant sequence type as *S. canis*-like because it possessed six distinct SNPs among all pinnipeds investigated. Like *S. canis,* which causes acute hepatic sarcocystosis in different species of ursids and canids, this variant sequence type caused similar pathology in two different pinniped species examined herein. Specifically, among captive Hawaiian monk seals and a California sea lion during an outbreak at an aquarium facility, as well as a free-ranging California sea lion.

A *Sarcocystis canis*-like parasite has been described previously that was associated with mortality in a Hawaiian monk seal (Yantis et al., 2003). Of particular interest is that two of these animals herein were held with the affected animal 13 years earlier, when the index case occurred. This association raises the question of pathogenesis. Were these animals infected 13 years earlier and they represent a recrudescent infection? Recrudescence is unlikely because only the asexual life forms were identified in the monk seals. Therefore, recent exposure and infection are the most parsimonious explanation, and suggest that Hawaiian monk seals are at an increased risk of mortality once infected by this organism. The separate finding that the same *S. canis*-like variant infected and caused hepatic sarcocystosis in a free-ranging California sea lion (CSL 2) further substantiates this perspective, that a novel *S. canis*-like variant is capable of causing significant disease in pinnipeds. Future studies should characterize the *S. canis*-like variant using an increased number of molecular markers to differentiate this protozoan parasite from *S. canis,* the agent causing hepatic sarcocystosis in canids and ursids, and identify the life cycle and definitive host of this parasite species. Interestingly, a recent report identified the same *ITS1* sequence type in muscle tissue recovered from a stranded Pacific harbor seal with no evidence of hepatic sarcocystosis (O'Byrne et al., 2021). Whether sarcocysts were present in the skeletal muscle was not reported, so it is not clear whether harbor seals represent relevant intermediate hosts for this S. canis-like variant which appears to infect a broad range of pinniped species.

Because the life cycle of *S. canis* is not known, diagnoses remain reliant on serologic and molecular tests, rendering unequivocal species and taxonomic classification challenging (Dubey et al., 2006). Some attempts at speciation have resorted to the name "*S. canis*–like organism" in diagnoses for cetaceans (striped dolphins and an Indo-Pacific bottlenose dolphin) (Resendes et al., 2002; Giorda et al., 2021; Calero-Bernal et al., 2017, respectively), a pinniped (Hawaiian monk seal) (Yantis et al., 2003), and ursine species (black bears, polar bears, and a free-ranging grizzly bear cub) (Davies et al., 2011, Garner et al., 1997; Britton et al., 2019, respectively). However, the ambiguous

classification using names such as "*S. canis*" and "*S. canis*–like" in existing literature have contributed significant confusion. It is unclear whether *S. canis* and *S. canis*-like species are synonymous but named as "variant" or "like" solely because they were recovered from different animal species. For example, molecular evaluation of schizont-laden liver tissue from a Steller sea lion at the conserved *18S rRNA* locus revealed 100% identify with an 866 nucleotide fragment recovered from ursids and canids as *S. canis* (Welsh et al., 2014), but no additional analyses were performed using other genetic markers. Whereas molecular characterization of protozoal infected tissue from a bottlenose dolphin at *CO1*, found only 98% identity with *S. canis* (DQ146148) from a polar bear, prompting these investigators to refer to the organism as *S. canis*–like (Calero-Bernal et al., 2017). It is important to note that such *S. canis*–like organisms remain unresolved and support the notion that samples should be typed at several markers such as *CO1, RpoB, 18S rRNA,* and *ITS1* to verify the taxonomic classification of the parasite present, in addition to ultrastructural characterization of the sarcocyst stage.

The confusion pertaining to taxonomic classification within *Sarcocystis* spp. largely stems from the fact that parasites within the genus are generally considered to be specialists, with narrow intermediate host ranges, such that the mere presence of a sarcocyst or schizont in a different animal species has previously been thought to be sufficient to resolve a new species type. However, it is increasingly evident that some species of *Sarcocystis* possess broad intermediate host ranges (*e.g.*, *S. canis, S. neurona, S. falcatula, S. lutrae, S. halieti,* and *S. calchasi*) and require us to rethink whether the discovery of a *Sarcocystis* parasite within a specific animal host is sufficient to support a new species designation. For example, the discovery of an *S. canis*–like organism infecting different bear species has resulted in the description of *S. arctosi* for *Sarcocysitis* parasites infecting brown bears, *S. canis* for those infecting polar bears and *S. ursusi* for those producing sarcocysts in black bears, despite the fact that *S. arctosi* and *S. canis* possess identical sequences at both the *18S rRNA* and *ITS1* genetic markers (Dubey et al., 2007). A similar situation exists for *S. caninum*, a parasite that forms sarcocysts in the muscles of dogs (Dubey et al., 2015) versus *S. arctica,* a parasite that forms sarcocysts in Arctic and Red foxes (Gjerde and Schulze, 2014; Pavlasek and Maca, 2017). Both parasites also possess identical sequences at the *18S rRNA* and *ITS1* genetic markers. Additional research is therefore needed to confirm or clarify whether *S. canis*–like organisms infecting black bears, polar bears, and grizzly bears are in fact the same etiologic agent, and synonymous with the *S. canis*-like parasites infecting pinnipeds and cetaceans.

Our data herein support the designation that the *Sarcocystis* parasite infecting pinnipeds is distinct from *S. canis* because it possesses a unique *ITS1* sequence type that is invariant among different species of pinnipeds but variant from *S. canis* sequences recovered from dogs and bears. Hence the designation *S. canis*-like variant infecting pinnipeds is being used to reflect a close ancestry with canids and ursids, but also to distinguish the sequence type recovered from infected pinnipeds as unique. Without a detailed analysis of the ultrastructure of the transmissible sarcocyst stage, or the discovery of the animal species serving as intermediate or definitive hosts, it is not possible to support a new species classification for the parasite sequence type identified herein that was responsible for causing fatal hepatic sarcocystosis in pinnipeds. These two marine species, like horses in the case of the parasite *Sarcocystis neurona,* are likely aberrant hosts for the *S. canis*-like variant that infected these pinnipeds.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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