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INVESTIGATION OF *SARCOCYSTIS* SPP. INFECTION IN FREE-RANGING AMERICAN BLACK BEARS (*URSUS AMERICANUS*) AND GRIZZLY BEARS (*URSUS ARCTOS HORRIBILIS*) IN BRITISH COLUMBIA, CANADA

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ABSTRACT: *Sarcocystis* spp. are protozoan parasites that cause a spectrum of lesions in various hosts. Hepatic sarcocystosis and encephalitis have been described in captive American black bears (*Ursus americanus*) and polar bears (*Ursus maritimus*), and in a free-ranging grizzly bear (*Ursus arctos horribilis*), but have not previously been reported in free-ranging American black bears. This study aimed to characterize the presence and lesions associated with *Sarcocystis* spp. in free-ranging bears in British Columbia, Canada from samples submitted to the provincial diagnostic laboratory. From 2007 to 2019, 102 free-ranging American black bear and grizzly bear tissues were examined postmortem for sarcocystosis using histopathology and follow-up molecular diagnostics. Sarcocystosis was confirmed in 41 (40%) free-ranging bears including 39 American black bears and two grizzly bears. Microscopic lesions included multifocal necrotizing hepatitis, nonsuppurative encephalitis, and/or intramuscular sarcocysts with or without associated inflammation. Sarcocystosis was considered the cause of death in eight (20%) of these bears, exclusively in cubs of the year (<1 yr old). *Sarcocystis canis* was identified in 22/32 (69%) cases where molecular characterization was performed and was the etiologic agent associated with bears that died of sarcocystosis. Confirmed cases were distributed widely across British Columbia. While there was an alternate proximate cause of death in the other confirmed bears, sarcocystosis may have contributed. Age was a significant risk factor, with yearlings presenting more often with fulminant lesions; however, there was a sampling bias toward juvenile bear submissions due to size and ease of transport. Further research is needed to understand the disease epidemiology and significance to population health.

Key words: American black bear, encephalitis, free-ranging, grizzly bear, hepatitis, myositis, *Sarcocystis canis*, *Ursus americanus*.

INTRODUCTION

Apicomplexan parasites in the genus *Sarcocystis* commonly infect animals and have a complex epidemiology, life cycle, and taxonomy. Novel species and life cycles often remain enigmatic, and pathogenicity can vary widely. *Sarcocystis* spp. require an intermediate and definitive host to complete their life cycle (Dubey et al. 2016). Typically, asexual repro-

duction occurs in the tissues of an herbivore or omnivore intermediate host. When that animal is consumed by an appropriate definitive host, the parasite infects the intestinal tract and sexually multiplies, resulting in shedding of *Sarcocystis* sporocysts or oocysts in the feces. These are then infectious when consumed by an intermediate host. The parasite may cause morbidity and mortality in the intermediate host; disease severity can range from asymptomatic to fatal. Lesions depend on the animal species, intercurrent disease, immune status, parasite species and genotype, and stage of infection; they include myositis, encephalitis, and hepatitis (Dubey et al. 2014, 2015; Seguel et al. 2019).

Of the wild animals that can host *Sarcocystis* spp., bears (*Ursidae*) are known to be intermediate hosts of at least two species, with sarcocysts identified in the muscles of free-ranging bears (Crum et al. 1978). By observing the structural differences in the sarcocyst wall, and based on the species of bear in which sarcocysts were identified, prior studies classified these parasites into the two separate species, *Sarcocystis arctosi* and *Sarcocystis ursusi*, in the brown bear (*Ursus arctos*) and American black bear (*Ursus americanus*), respectively (Dubey et al. 2007, 2008). Bears also are aberrant hosts to *Sarcocystis canis* (Dubey and Speer 1991). *Sarcocystis canis* has caused fatal hepatic sarcocystosis in captive bears (Zeman et al. 1993) and a free-ranging grizzly bear (*Ursus arctos horribilis*; Britton et al. 2019) and hepatic sarcocystosis with encephalitis in two captive polar bears (*Ursus maritimus*; Garner et al. 1997) and an American black bear (Davies et al. 2011). The life cycle, distribution, risk factors, and overall health consequences associated with *S. canis* infection in bear populations is enigmatic.

In 2008, *S. canis* was first recognized as a cause of mortality in a captive American black bear submitted to the British Columbia Provincial Animal Health Centre (AHC), Canada (Davies et al. 2011). Since then, an increasing number of free-ranging bears submitted to the laboratory have been diagnosed with fatal hepatic sarcocystosis. The objective of this case series is to describe the pathology, etiology, and basic epidemiology among cases of *Sarcocystis* spp. infection in wild bears from British Columbia submitted to the AHC from 2007–19.

MATERIALS AND METHODS

A total of 118 bears were submitted to the AHC from 2007–19. The inclusion range of this study was selected to start 1 yr before the first fatal bear sarcocystosis case diagnosed at the AHC, which occurred in 2008 (Davies et al. 2011). Of these, 16 cases were excluded because they were either found outside of British Columbia (n=5), were lacking tissues in the submission (n=4), were lacking a report (n=1), or the bear was captive in a zoo or similar facility (n=6). Cases included in this study had either carcasses or tissue samples submitted, and all had undergone postmortem examination (gross necropsy with or without histopathology, with ancillary testing as required) by a veterinary pathologist. Ages of the bears were estimated based on dentition (Marks and Erickson 1966; Willey 1974) and were categorized as either cub of the year (COY, <1 yr old), yearling (1 yr old), or adult/subadult (≥2 yr old).

A bear was considered confirmed for sarcocystosis if it had: 1) either visible tissue sarcocysts or a tissue that was positive for *Sarcocystis* spp. by PCR and subsequent DNA sequencing; and 2) one or more of the following histopathologic lesions: multifocal necrotizing hepatitis or hepatic necrosis, nonsuppurative myositis, and gliosis or encephalitis. A bear was considered presumptive for sarcocystosis if *Sarcocystis* spp. were not identified but there were compatible lesions without another attributable cause. Data on cases included location, date and cause of death, gross and microscopic lesions, species, age, sex, body condition, weight, and number of days spent in a wildlife rehabilitation facility, if applicable.

Molecular characterization of *Sarcocystis* spp.

Extraction of DNA, PCR, and sequencing were performed at the AHC as well as the National Institutes of Health (NIH) and University of California Davis (UCD). Tissue samples were stored at –20 C until processed.

At the AHC, samples were extracted by diluting tissue (1:10) in minimum essential media and homogenizing in a stomacher for 2 min at medium speed. Homogenized tissue was centrifuged briefly before DNA was extracted using the QIAamp® DNA Mini Kit (Qiagen Inc., Toronto, Ontario, Canada) tissue protocol. Extracted DNA was amplified by conventional heminested PCR assay targeting the small subunit 18S RNA gene of *Sarcocystis* spp. (Marsh 1996) or the cytochrome

c oxidase subunit 1 mitochondrial gene (CO1; Gjerde 2013). We performed PCR using the manufacturer-recommended protocol for illustra PuReTaq Ready-to-Go™ PCR bead (GE Healthcare, Mississauga, Ontario, Canada). We separated PCR amplicons on a 2% agarose gel stained with ethidium bromide and visualized them using an ultraviolet photodocumentation system. We cleaned amplicons prior to sequencing using Amicon Ultra centrifugal filters, 30K (Fisher Scientific, Ottawa, Ontario, Canada) following the manufacturer's user guide.

At the NIH and UCD, the internal transcribed spacer 1 (ITS1) primer sets for PCR were used to screen samples. Approximately 25 mg of each tissue type was digested overnight at 57 C with Proteinase K, and DNA was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Hilden, Germany) and then eluted in 40 µL of Qiagen EB buffer. Extracted DNA samples were stored at −20 C between PCR reactions. Previously published pan-coccidian ApiITS1 primers anchored in the 18S and 5.8S small subunit rDNA gene array were used to screen samples at NIH (Gibson et al. 2011) and UCD (Rejmanek et al. 2010). The ApiITS1 primers amplify across the ITS1 region to distinguish between closely related and novel species of protozoal parasites. All PCR reactions included a positive control (tachyzoite-derived DNA from a type I [RH] strain of *Toxoplasma gondii*) and a negative control of sterile water added to the master-mix reagents. We used nested PCR reactions, and reaction conditions (i.e., thermocycler settings) were done following Gibson et al. (2011) and Rejmanek et al. (2010). We visualized PCR amplicon products in gel-red (Biotium Inc., Hayward, California, USA) or RedSafe-stained 1% agarose gels (iNtRON Biotechnology, Daejeon, South Korea) and purified them using ExoSAP-IT (USB, Cleveland, Ohio, USA) for sequencing according to the manufacturer's instructions.

The PCR used is not species specific, therefore all PCR positive samples were subsequently Sanger sequenced for species-level identification. Sequences were visualized, manually quality checked, and trimmed using FinchTV (Geospize, PerkinElmer, Akron, Ohio, USA). Sequences were identified by alignment with known reference sequences and verified via a nucleotide BLAST search in the National Center for Biotechnology Information GenBank (NCBI 2020); MEGA X version 10.1.8 was used for aligning sequences (Kumar et al. 2018).

Epidemiologic and statistical analysis

To characterize risk factors for *Sarcocystis* spp. infection in free-ranging bears in British Columbia, we examined the distribution of age, sex, body condition, and species across *Sarcocystis* spp. status (positive versus negative, where positive included confirmed and presumptive cases) using bivariable logistic regression. A sensitivity analysis was done to assess the impact of including presumptive cases as positives by rerunning all bivariable tests with the four presumptive cases excluded. Finally, the distribution of sarcocystosis as the proximate cause of death across age groups was examined. All statistical analyses were completed using RStudio (R Development Core Team, Vienna, Austria) and R packages *stats* and *rstatix* with a significance level of $P<0.05$. The distribution of *Sarcocystis* spp.-confirmed, presumptive, and negative cases across British Columbia were mapped using ArcGIS version 10.7.1 (ESRI, Redlands, California, USA).

RESULTS

Of the 102 free-ranging bear cases, 41 (40%) had a complete necropsy at the AHC, 54 (53%) underwent field postmortem examination and only tissue samples were submitted, while seven (7%) were hunter-submitted skeletal muscle. The majority of bears (*n*=55; 54%) were euthanized by gunshot due to human safety concerns (*n*=24; 24%), disease or injury (*n*=22; 22%), or for other reasons (*n*=9; 9%). Other causes of mortality included legal harvesting (*n*=12; 12%), sarcocystosis confirmed or suspected by the case pathologist (*n*=9; 9%), trauma (*n*=7; 7%), emaciation (*n*=4; 4%), electrocution (*n*=2; 2%), intestinal intussusception (*n*=2; 2%), other diseases (*n*=6; 6%), or unknown (*n*=5; 5%). Most (*n*=97; 95%) were American black bears, with five (5%) being grizzly bears. The majority were COY (*n*=39; 38%), the others being yearlings (*n*=22; 22%) and adults or subadults (*n*=23; 23%). Forty-one bears (40%) were males and 35 (34%) were females. Age and sex were not recorded in 18 (18%) and 26 (25%) cases, respectively. While all bears were from free-ranging populations, 28 (27%) bears were housed at a wildlife rehabilitation facility prior to death for a median of 7 d (range: 1–250 d; *n*=15; 15%), or for an unknown period of time (*n*=13; 13%). Body condition at necropsy varied, most frequently poor (*n*=28; 27%), as defined by minimal internal fat stores, fol-

TABLE 1. Summary of histopathologic lesions and *Sarcocystis* spp. organisms observed in available tissues from confirmed sarcocystosis cases in American black bears (*Ursus americanus*) and grizzly bears (*Ursus arctos horribilis*) from British Columbia, Canada, 2007–19.

Tissues examined[a]			Total no. bears examined	Inflammation in all tissues examined[b]		Visible *Sarcocystis* spp. organisms[c] in all tissues examined	
Liver	Brain	Muscle		No. bears observed	% Bears examined	No. bears observed	% Bears examined
×			37	30	81	10	27
	×		23	12	52	0	0
		×	34	10	29	20	59
×	×		22	11	50	0	0
×		×	30	4	13	0	0
	×	×	21	2	10	0	0
×	×	×	20	1	5	0	0

[a] × = specific tissues examined in individual bear.

[b] Necrotizing hepatitis, nonsuppurative encephalitis, or nonsuppurative myositis.

[c] Intrahepatic schizonts or intramuscular sarcocysts.

lowed by good (n=27; 26%), moderate (n=18; 18%), or emaciated body condition (n=8; 8%); body condition was not recorded in 21 (21%) cases.

A total of 41 bears (40%) met the case definition for confirmed sarcocystosis, while four (4%) were presumptive. Of the confirmed cases, 39 (95%) were black bears, two (5%) were grizzly bears. Fourteen (34%) were yearlings, 13 (32%) were COY, five (12%) were ≥2 yr old, and nine (22%) were of undetermined age. Three (7%) bears were especially small based on weight and size for their corresponding age (Marks and Erickson 1966). In five (12%) bears there was marked generalized icterus.

A summary of lesions found in sarcocystosis-confirmed bears is detailed in Table 1 and Supplementary Material Table S1. Necrotizing hepatitis (Fig. 1A), multifocal nonsuppurative encephalitis without visible parasites (Fig. 1B), intramuscular sarcocysts with no associated inflammation (Fig. 1C), and nonsuppurative myositis (Fig. 1D) were observed histologically. Myositis was only observed with intralesional sarcocysts. Affected striated muscle included tongue, diaphragm, and appendicular musculature. In cases with skeletal muscle sections from multiple anatomic sites, the myositis tended to be most severe in the tongue. No bears had both histologically visible intrahepatic schizonts and intramuscular sarcocysts (Table 1).

Tissue samples from 32/45 (71%) bears with sarcocystosis-consistent histopathologic lesions were screened using molecular markers (ITS1, 18S, or CO1). Of these, 30 (94%) bears had *Sarcocystis* spp. DNA present based on PCR amplification and DNA sequencing (Supplementary Material Tables S1, S2). Sixteen bears (53%) had only *S. canis* (GenBank accession no. MW136927) as identified by DNA sequencing. Four bears were co-infected with *S. canis* and one other coccidian parasite, either *T. gondii* (n=2; 7%) or a *Cystoisospora*-like sp. (n=2; 7%; GenBank no. MW136925). Two (7%) bears were infected with a *Sarcocystis* sp. that belonged to a clade most closely associated with *Sarcocystis felis*, but was unique (no. MW136928). In six (20%) bears the parasite could only be identified to the genus (*Sarcocystis*). Three cases were negative by PCR for one tissue screened, although there were histopathologic lesions consistent with sarcocystosis and visible intramuscular sarcocysts in those cases.

Confirmed sarcocystosis cases were distributed throughout British Columbia, although most submissions were near the coast (Fig. 2).

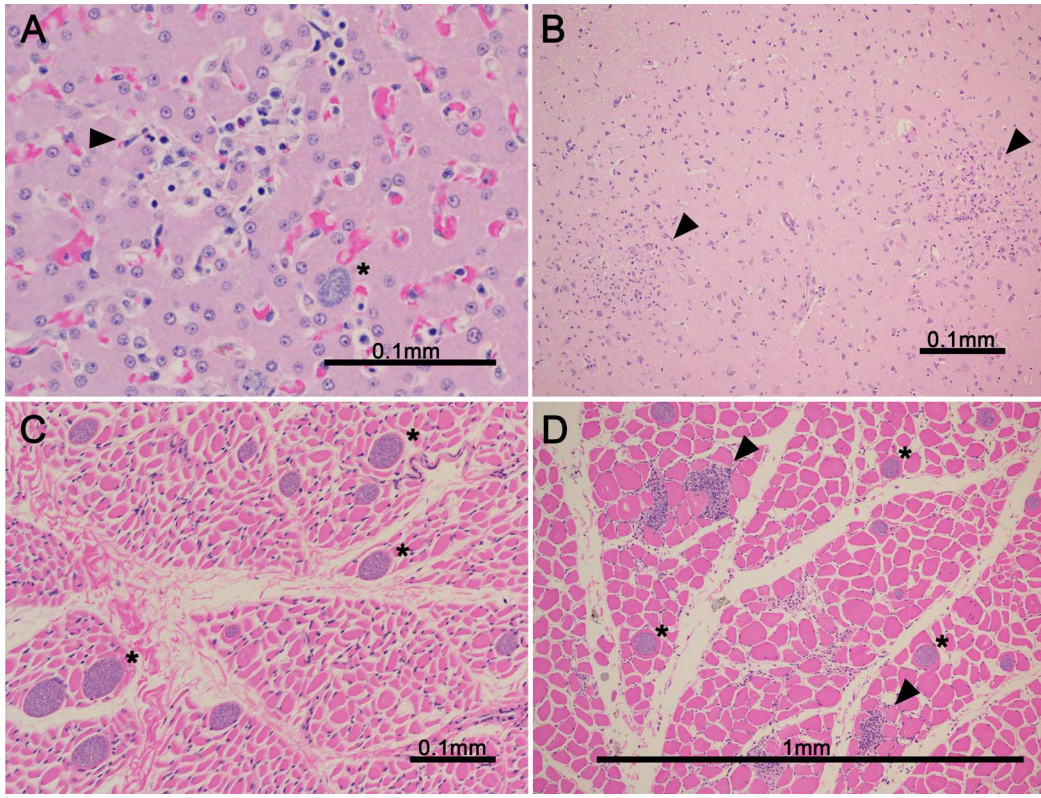

FIGURE 1. Histologic lesions associated with sarcocystosis in free-ranging American black ears (*Ursus americanus*) and grizzly bears (*Ursus arctos horribilis*) in British Columbia, Canada from 2007–19. American black bear tissues. H&E stain. (A) Liver. Necrosis and inflammatory infiltrate (arrow) with a meront (asterisk). (B) Brain. Microgliosis (arrows). (C) Skeletal muscle. Intramuscular sarcocysts (asterisks) without inflammation. (D) Semimembranosus muscle. Multifocal nonsuppurative myositis (arrows) with several adjacent intramuscular sarcocysts (asterisks).

Annual prevalence among all bears submitted to the lab ranged from 14% to 80%, with a median of 44% (Fig. 3). Yearlings had greater odds of sarcocystosis (odds ratio=4.41, 95% confidence interval=1.3–16.4) compared to bears ≥2 yr old; however, there were no significant differences between COY and yearlings or between COY and bears ≥2 yr old. Sarcocystosis was identified as a cause of death exclusively among COY. Sex, body condition, and species were not associated with confirmed *Sarcocystis* spp. infection.

DISCUSSION

This case series demonstrates that sarcocystosis is common among wild bears submitted to the AHC. The highest risk of infection occurred in yearling bears while mortality occurred exclusively in COY; cases were distributed throughout British Columbia, and *S. canis* was the most common pathogen. Bears are probably an intermediate host for *S. canis*, and the constellation of lesions observed represents stages in the progression of infection. It appears that following ingestion of oocysts, sporozoites excyst in the intestine and migrate into tissues. This occurs in bears primarily in the liver, where they develop into meronts and undergo one to several rounds of merogony, releasing merozoites into circulation. Necrotizing hepatitis with visible meronts was documented in 10 of the examined bears. Merozoites then circulate, often in monocytes, and enter striated myocytes and mature to sarcocysts, resulting in visible

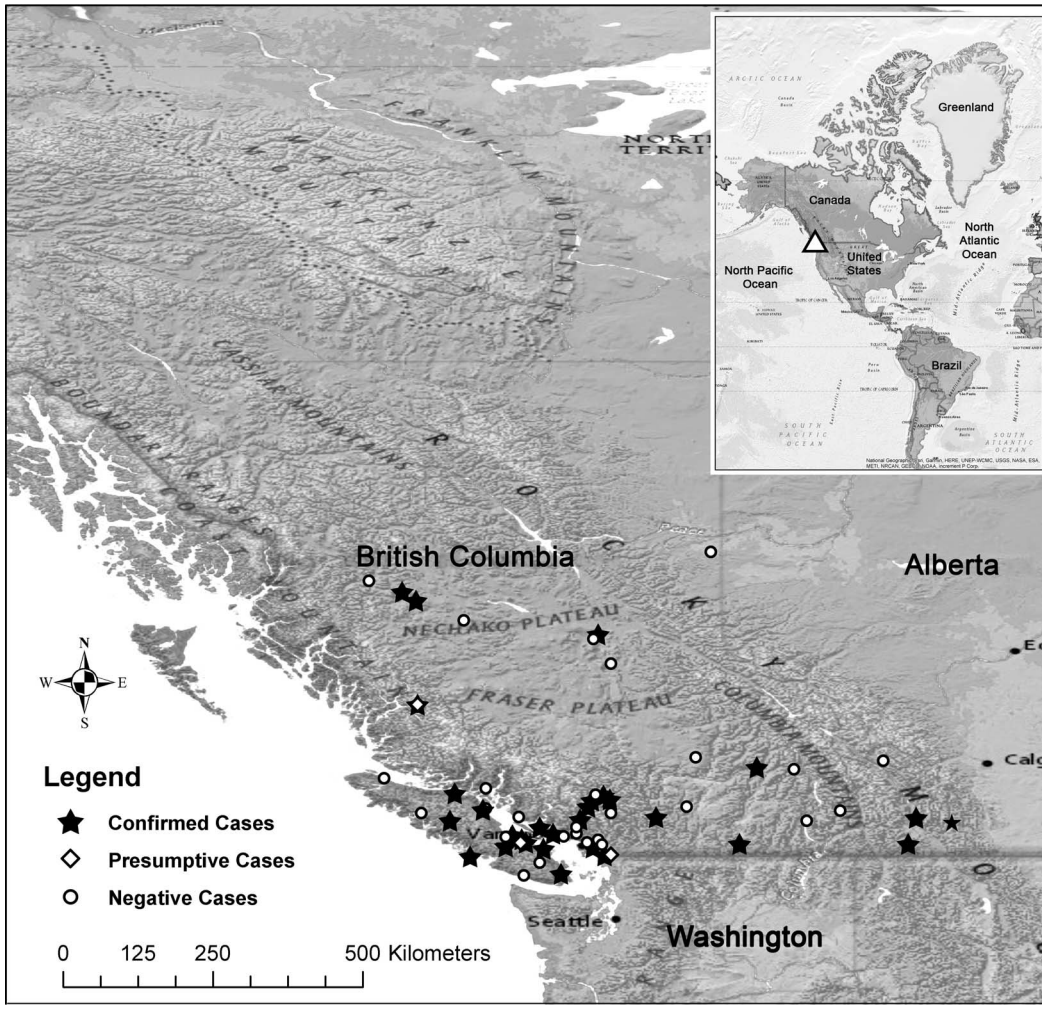

FIGURE 2. Distribution of *Sarcocystis* spp. cases in free-ranging American black bears (*Ursus americanus*) and grizzly bears (*Ursus arctos horribilis*) in British Columbia, Canada from 2007–19. Geographic region where death of the animal occurred for confirmed (stars), presumptive (diamonds), and negative cases (circles) across the province. No location information was available for 19 cases. Map inset indicates the location of Vancouver, British Columbia (triangle) within North America. Maps were created using ArcGIS software (ESRI, Redlands, California, USA).

sarcocysts in skeletal muscle cells. The lack of visible sarcocysts in hepatocytes in bears with visible intramuscular sarcocysts is consistent with this stage of disease progression.

Fatal hepatitis with encephalitis associated with *S. canis* has previously been reported in captive American black bears (Zeman et al. 1993; Davies et al. 2011) and polar bears (Garner et al. 1997), with one report in a free-ranging grizzly bear COY from British Columbia (Britton et al. 2019), which is included as part of this case series. Myositis is commonly observed in herbivore intermediate hosts for other *Sarcocystis* spp. (Gabor et al. 2010; Vangeel et al. 2013). Fatal sarcocystosis has not been previously described in free-ranging American black bears, nor has *Sarcocystis* spp.-associated myositis been described in any bear species to our knowledge. Lesions in bears of this study were predominantly associated with *S. canis*, although there did not appear to be any distinct histopathologic

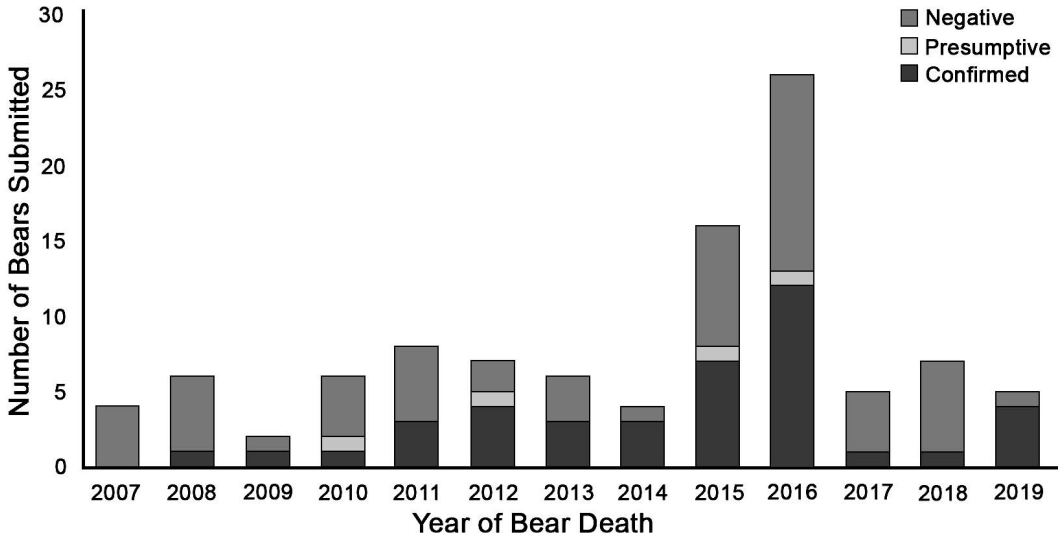

FIGURE 3. Sarcocystosis cases diagnosed in free-ranging American black bears (*Ursus americanus*) and grizzly bears (*Ursus arctos horribilis*) submitted to the provincial diagnostic laboratory annually from 2007–19 in British Columbia, Canada. Confirmed, presumptive, and negative cases for sarcocystosis are stacked in bars by corresponding year of bear mortality.

differences among the lesions associated with *S. canis, S. felis*-like sp., or a co-infection with other protozoal parasites such as *T. gondii* or *Cystoisospora*-like sp. Further, it is unclear to what extent the co-infecting coccidian species or other intercurrent disease contributed to the histopathologic lesions.

While *Sarcocystis* spp. infection was identified in 40% of all free-ranging bear cases submitted between 2007 and 2019, sarcocystosis as the proximate cause of mortality was not as common—occurring in only 7% of total free-ranging bear submissions and exclusively in COY. Although rarely the proximal cause of death in other published case series or individual reports, it is possible that myocellular sarcocystosis contributed directly or indirectly to death. For example, the myositis seen in 10 of the bears may have contributed to an inability to prehend or swallow food, leading to emaciation or increased garbage-seeking, with a consequence of euthanasia for animal welfare and human safety. Involvement of the diaphragm may have impeded normal respiratory efforts, while rhabdomyocytolysis may have contributed to pain or discomfort, with myoglobinemia and possible myoglobinuria. Many of these juvenile bears were considered orphans. It is possible that neural sarcocystosis may increase the likelihood of maternal abandonment and therefore death, due to unusual behavior and poor growth. Natural mortality may be underrepresented given that the majority of bears submitted were euthanized for various enforcement or conservation concerns. Similarly, although morbidity and mortality appears to be more common in younger animals, there may be a sampling bias toward COY and yearlings because of requests for submission and because their smaller size allows easier transport to the laboratory.

Little is known regarding the life cycle or distribution of *S. canis*. Dogs can be clinically affected, resulting in severe hepatitis and encephalitis, (Dubey and Speer 1991), similar to in bears. Hepatic *S. canis* schizonts in dogs have been speculated to occur prior to sarcocyst formation in muscle (Dubey and Speer 1991), and future studies are required to determine if *S. canis* follows a comparable pathogenesis in bears. To date, *S. canis*-like protozoa have also been found to be associated with hepatitis in marine mammals (Dubey et al. 2003; Yantis et al. 2003; Welsh et al. 2014). These reports indicate that *S.*

canis probably has a wide geographic distribution and numerous intermediate hosts, but the definitive host has not been identified.

Importantly, many of the submissions received in this study were aggregated near coastal areas. While it is possible that this reflects a greater abundance of bears near the coast, there is a lack of data on bear abundance throughout British Columbia. The measures of bear population density that do exist are fragmented and are unlikely to be representative (Mowat et al. 2005). Further, these areas may also have higher human population density in the province. As such, it is not possible to say whether these greater numbers of submissions reflect bear abundance or human density. It will be important for future studies aimed at understanding the ecology of *Sarcocystis* spp. in free-ranging bears to incorporate information on bear demographics in order to systematically sample bears and derive accurate prevalence and distribution data.

Our data suggest that sarcocystosis may be an important disease for juvenile free-ranging bears in British Columbia. Further work is needed to understand the health and conservation impacts of disease associated with *Sarcocystis* spp. as well as its epidemiology. It will also be important to identify the definitive host and to characterize the life cycle of the parasite in British Columbia in order to better understand, monitor, and mitigate risks associated with sarcocystosis in bears.

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SUPPLEMENTARY MATERIAL

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