IDENTIFICATION OF TWO NOVEL COCCIDIAN SPECIES SHED BY CALIFORNIA SEA LIONS (*ZALOPHUS CALIFORNIANUS*)

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ABSTRACT: Routine fecal examination revealed novel coccidian oocysts in asymptomatic California sea lions (*Zalophus californianus*) in a rehabilitation facility. Coccidian oocysts were observed in fecal samples collected from 15 of 410 California sea lions admitted to The Marine Mammal Center between April 2007 and October 2009. Phylogenetic analysis using the full ITS-1 region, partial small subunit 18S rDNA sequence, and the Apicomplexa rpoB region identified 2 distinct sequence clades, referred to as Coccidia A and Coccidia B, and placed them in the Sarcocystidae, grouped with the tissue-cyst–forming coccidia. Both sequence clades resolved as individual taxa at ITS-1 and rpoB and were most closely related to *Neospora caninum*. Coccidia A was identified in 11 and Coccidia B in 4 of 12 sea lion oocyst samples successfully sequenced (3 of those sea lions were co-infected with both parasites). Shedding of Coccidia A oocysts was not associated with age class, sex, or stranding location, but yearlings represented the majority of shedders (8/15). This is the first study to use molecular phylogenetics to identify and describe coccidian parasites shed by a marine mammal.

Coccidia represent the largest group of organisms within the Apicomplexa, and they have a wide range of animal hosts (Levine, 1988; Cox, 1994; Tenter et al., 2002), but there are relatively few reports documenting the coccidian parasites shed by marine mammals. Coccidia were first described in pinnipeds when 2 cases of clinical coccidiosis caused by *Eimeria phocae* in harbor seals (*Phoca vitulina*) were reported by Hsu, Melby, and Altman (1974) and Hsu, Melby, Altman, and Burek (1974). Subsequently, *E. phocae* was identified as the causative agent of hemorrhagic enteritis in harbor seals based upon necropsy and histopathologic findings (Munro and Synge, 1991; McClelland, 1993), and harbor seals were identified as the definitive host for the parasite (Van Bolhuis et al., 2007). Oocysts from 6 coccidian species were observed in fecal samples from free-ranging Antarctic seal species (Drozdz, 1987). Three of these coccidian species were named based on morphologic descriptions of sporulated oocysts, i.e., *Eimeria weddellii* and *Eimeria arctowski* from Weddell seals (*Leptonychotes weddellii*) and *Isospora miroungae* in elephant seals (*Mirounga leonina*). In addition, 3 other coccidian parasites were observed in feces from Weddell seals and crabeater seals (*Lobodon carcinophagus*), but these were not named due to a lack of data documenting sporulation.

The California sea lion (*Zalophus californianus*) inhabits the Pacific coast of North America, ranging from Mexico to Vancouver Island, Canada. While females and young animals remain near breeding grounds at the southern end of the range year-round, males migrate north during the non-breeding season. Commercial harvesting dramatically decreased the sea lion population at the turn of the 20th century. The population began recovering when commercial harvesting was banned in the early 1940s and has steadily increased at a rate of >5% per yr since the passage of The Marine Mammal Protection Act in 1972 (Barlow, 1995). Currently, the California sea lion population is considered to be at an optimal sustainable population level, with an estimated 238,000 animals (NOAA, 2010). The rescue and rehabilitation of marine mammals stranded along California's coast have provided a unique opportunity to discover novel diseases because rescued marine mammals are examined closely by medical staff, high-quality biological samples can be collected for diagnostic testing, and these animals usually have a higher prevalence of disease compared to the population at large (Gulland, 1999; Greig et al., 2005), thus providing a unique opportunity for pathogen discovery. The present paper describes the molecular characterization and epidemiology of 2 newly identified coccidian parasites in California sea lions admitted to The Marine Mammal Center (TMMC) between 2007 and 2009.

MATERIALS AND METHODS

Sample collection and processing

Fecal samples were collected from California sea lions that became stranded along the central and northern California coast (37°42′N, 123°05′W to 35°59′N, 121°30′W) between April 2007 and October 2009; samples were obtained during rescue, transport to, or rehabilitation at TMMC. Yearly, California sea lion admissions at TMMC range from 500 to 800 individuals. Fecal samples were collected from individual sea lions in 50-ml conical vials via rectal examination or from the pen floor, stored at 4 C at TMMC, and shipped with cold packs to the University of California, Davis, California. Samples were screened the next day for coccidial oocysts by flotation and microscopic examination, as previously described (Dabritz et al., 2007). When possible, additional fecal samples were collected from sea lions positive for coccidian parasites on initial sampling. For all positive fecal samples, oocysts were harvested according to methods described by Dabritz et al. (2007) for molecular characterization.

Oocyst characterization

DNA from harvested oocysts was extracted by incubation in boiling water for 1 min, followed by incubation in a dry ice and 95% ethanol bath for 1 min. This was repeated 3 times in order to crack the oocyst wall and obtain genetic material. Oocyst samples were subsequently processed using the Qiagen DNeasy Blood and Tissue DNA extraction kit (Qiagen, Valencia, California), following the manufacturer's tissue extraction protocol. DNA from coccidian cysts in sea lion tissues was processed as above and stored at −20 C until time of use.

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PCR was performed using nested primers anchored in conserved regions of the tissue-cyst–forming coccidia 18S and 5.8S rDNA regions that amplify across the variable ITS-1 region (Wendte et al., 2010). Sequence length polymorphism at the ITS-1 region facilitates rapid detection of multiple coccidian species by PCR. Nested PCR amplification was performed using 2 μl of extracted DNA for the external primer set, after which 1 μl of the external PCR reaction was used as DNA template for the internal reaction. Additional primers were utilized to further characterize the coccidia species. A hemi-nested set of primers located in an 18S rDNA region conserved among multiple coccidian species was utilized (18S-F: GCAAGGAAGTTTGAGGCAAT; 18S-R-Int: TCCTTCCTCTAAGT-GTTAAGGTTCA; 18S-R-Ext: TGCAGGTTCACCTACGGAAA). Primers targeting the RNA polymerase subunit beta of *Toxoplasma gondii* (ApiRPOBf: TAGTACATTAGAAATCCCTAAAC; ApiRPOBr: TCW-GTA TAAGGTCCTGTAGTTC) were used successfully to amplify coccidian DNA, as previously described (Wendte et al., 2010).

PCR conditions were as follows: 5 μl of 10× PCR buffer containing 15 mM MgCl$_2$ (Sigma-Aldrich, St. Louis, Missouri), 5 μl of 2 mM dNTPs mix, 20 pmol of each primer (forward and reverse), 2 U of DNA Taq polymerase (Sigma-Aldrich), and deionized water, for a total reaction volume of 50 μl. The PCR reaction conditions were as follows: initial denaturation step at 94 C for 5 min, followed by 35 cycles consisting of 94 C for 40 sec, 58 C for 40 sec, and 72 C for 90 sec, with a final extension for 10 min at 72 C. PCR products were separated via electrophoresis on 1% agarose gels stained with GelRed (Biotium, Inc., Hayward, California) and visualized under UV light. PCR positive samples were purified using ExoSap-It (USB Corporation, Cleveland, Ohio). Purified PCR samples were subjected to Sanger sequencing performed by the Genomics Unit at the Rocky Mountain Laboratories Research Technologies Section (Hamilton, Montana). Successful sequencing reactions were visualized, aligned, and compared with published GenBank sequences using the Lasergene SeqMan software (DNASTAR, Inc., Madison, Wisconsin).

Nucleotide sequence data newly reported in this paper are available in the GenBank database under the accession numbers GU936629–30 (18S-ITS-1) and HM173318–19 (rpoB).

Phylogenetic analysis

For the phylogenetic analysis, the alignment and its outgroup for the Apicomplexa DNA sequences encoding the small subunit 18S rDNA locus within the nuclear ribosomal gene complex were downloaded from the SILVA rDNA database (Pruesse, 2007). *Perkinsus chesapeake* and *Perkinsus marinus* (Alveolata, Perkinsida) were selected as an appropriate outgroup. The 18S rDNA sequences for the coccidia infecting California sea lions were aligned using the SINA Aligner tool (Pruesse, 2007). The subsequent alignment was manually edited. To provide representative coverage of the Apicomplexa, a maximum of 3 species per genus was chosen, giving a total of 40 taxa. For the ITS-1 and rpoB loci, Apicomplexan sequences were aligned using the MAFFT alignment tool (L-INS-I mode; version 6.707; Katoh, 2005).

Phylogenetic tree reconstruction and the evolutionary history for all 3 loci were inferred using 2 methods, neighbor-joining (Saitou and Nei, 1987) and minimum evolution (Rzhetsky and Nei, 1992). These methods were implemented by MEGA4 after deletion of gapped positions (Tamura et al., 2007). Each phylogeny was tested using 5,000 bootstrap replicates.

Necropsy, histology, immunohistochemistry, and serology

A gross necropsy was performed on 9 of the animals with positive fecal results that died or were killed at TMMC. Representative samples from all major tissues from 4 of these 9 cases were fixed in 10% neutral buffered formalin and sent to the Zoological Pathology Program, University of Illinois at Urbana–Champaign College of Veterinary Medicine, Champaign, Illinois, for processing and histopathologic evaluation. Tissues were processed for paraffin embedding, cut at 5 μm, and stained with hematoxylin and eosin (H&E). For tissues in which coccidian parasites were identified in H&E-stained sections, immunohistochemistry was performed for *Toxoplasma gondii* (rabbit polyclonal, AR125-5R; Biogenex Laboratories, Inc., San Ramon, California). Monoclonal clone 2G5-2T75 for *Sarcocystis neurona* and a rabbit polyclonal antibody for *Neospora caninum* were also employed.

Sera from sea lion cases 3–7, 10, 12, and 15 had titers to *T. gondii*, *S. neurona*, and *N. caninum* of <1:40 by indirect immunofluorescent antibody test (IFAT; Dierauf and Gulland, 2001; Miller et al., 2002). Sea lion case 1 had a titer to *T. gondii* of 1:320 and was not tested by IFAT for *S. neurona* and *N. caninum* due to lack of serum.

Epidemiology and statistical analysis

Upon admission to TMMC, individual sea lions received a unique identification number and were assigned to an age class based on length, stage of development of secondary sexual characteristics, and tooth wear (Evans and Robertson, 2001; Greig et al., 2005). Age classes for females were estimated as: pup = 0 to 1 yr; yearling = 1 to 2 yr; subadult = 2 to 5 yr; adult = ≥5 yr. Age classes for males were estimated as: pup = 0 to 1 yr; yearling = 1 to 2 yr; juvenile = 2 to 4 yr; subadult = 4 to 8 yr; adult = ≥8 yr. Sea lions were assigned a reason for stranding, categorized as malnutrition, leptospirosis, trauma, domoic acid toxicosis, cancer, and other, or unknown, causes (Greig et al., 2005). Final disposition for each animal in the study was recorded as placed at another captive facility, died, killed, or released. Risk factors for Coccidia A oocyst shedding in sea lions were evaluated using the chi-square test of independence or an exact test when 1 expected cell frequency was less than 5 (Daniel, 2005). Data analyses were performed using the statistical software package STATA 10 (StataCorp, College Station, Texas).

RESULTS

Oocyst characterization and phylogenetic analysis

In total, 410 individual sea lions were sampled for this study, and coccidian oocysts were identified in fecal samples from 15 individuals (Table I). Oocysts were round to ovoid in shape and approximately 11 to 14 μm in diameter (Fig. 1). Using standard sporulation conditions, including dilute sulfuric acid or potassium dichromate for up to 1 wk, neither condensation nor sporulation of the coccidian oocysts was observed. DNA was extracted from oocysts in samples from 12 of the positive California sea lions. PCR and subsequent DNA sequencing of part of the small subunit 18S rDNA and the entire ITS-1 region within the nuclear ribosomal gene complex identified 2 distinct DNA sequences that were homogeneous throughout, i.e., no dinucleotide sites; the 2 sequence classes were referred to as Coccidia A and Coccidia B.

Coccidia A and B possessed the same 18S rDNA sequence as that of *N. caninum* and *T. gondii*, but they were readily distinguished from *Sarcocystis* spp. across the ~320 nucleotides amplified. To visualize the relative position of Coccidia A and B within the Apicomplexa, SSU 18S rDNA sequences for the 2 coccidians were aligned against representative sequences across 40 taxa within the Apicomplexa and appropriate outgroups. The neighbor-joining and minimum evolution tree of the SSU 18S rDNA sequences established that Coccidia A and B possessed identical sequences to those of *T. gondii*, *N. caninum*, and other tissue-cyst–forming coccidia within the Sarcocystidae (Fig. 2).

The ITS-1 region in both Coccidia A and B was smaller (~350 bp) than other coccidians in GenBank, and BLASTN analysis established that Coccidia A and B possessed novel ITS-1 sequences that were significantly different from other coccidian species. To resolve the genera to which Coccidia A and Coccidia B were most closely associated, neighbor-joining and minimum evolution trees were constructed using the polymorphic ITS-1 and rpoB loci encoded within the nuclear ribosomal gene complex and apicoplast organelle, respectively. The ITS-1 sequence for Coccidia A was only 82% identical to *N. caninum* NCBovBA4 (FJ966046) (E score: 4e-57), whereas Coccidia B was significantly more polymorphic. Samples designated as Coccidia A or Coccidia B at the ITS-1 region likewise possessed distinct alleles at the rpoB

TABLE I. Demographic and stranding data for California sea lions (*Zalophus californianus*) with Coccidia A and/or Coccidia B oocysts observed in feces.

Case no.	TMMC ID	Admission date	Age class	Sex	Stranding location	Stranding diagnosis	Disposition	Histopathology available	Intestinal stages observed	Sequence clade
1	CSL 7361	8/25/2007	Yearling	Male	Marin	Malnutrition	Died	No		A
2	CSL 7508	10/15/2007	Yearling	Female	San Luis Obispo	Domoic acid toxicosis	Euthanized	No		A & B
3	CSL 7612	4/3/2008	Subadult	Female	San Luis Obispo	Abscess	Released	No		n/a*
4	CSL 7613	4/5/2008	Subadult	Female	Santa Cruz	Domoic acid toxicosis	Euthanized	No		n/a*
5	CSL 7614	4/4/2008	Subadult	Female	San Luis Obispo	Polymyositis	Euthanized	Yes	No	A
6	CSL 7678	6/5/2008	Yearling	Male	San Luis Obispo	Malnutrition	Released	No		A
7	CSL 7684	6/7/2008	Yearling	Male	Santa Cruz	Malnutrition	Released	No		A & B
8	CSL 7708	6/14/2008	Yearling	Female	San Luis Obispo	Malnutrition	Died	No		A
9	CSL 7775	7/23/2008	Yearling	Female	San Luis Obispo	Trauma	Euthanized	Yes	Yes	A
10	CSL 7788	7/28/2008	Juvenile	Male	San Francisco	Leptospirosis	Released	No		A
11	CSL 7815	8/6/2008	Juvenile	Male	Monterey	Leptospirosis	Released	No		A
12	CSL 7879	9/3/2008	Juvenile	Male	San Francisco	Leptospirosis	Euthanized	Yes	Yes	B
13	CSL 7953	10/5/2008	Yearling	Male	Monterey	Leptospirosis	Euthanized	No		A
14	CSL 7965	10/12/2008	Yearling	Male	Monterey	Trauma	Released	No		n/a*
15	CSL 7994	10/29/2008	Juvenile	Male	Monterey	Domoic acid toxicosis	Euthanized	Yes	Yes	A & B

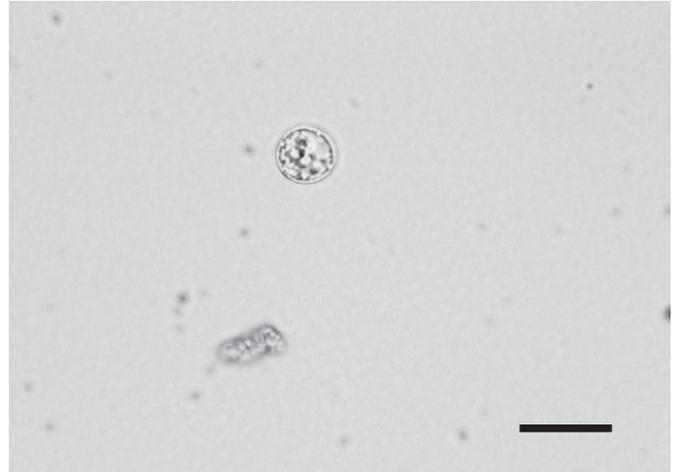* n/a, data not available.

FIGURE 1. Photomicrograph of an unsporulated oocyst, ×600, isolated from California sea lion (*Zalophus californianus*) feces and identified as Coccidia A through molecular phylogenetics. Coccidia A and B oocysts were morphologically similar, round to ovoid, and approximately 11 to 14 μm in diameter. Bar = 20 μm.

locus (Fig. 3). Individual trees constructed for each locus yielded similar tree topologies and resolved Coccidia A and B into individual taxa that grouped most closely with *Neospora* sp. (Fig. 3).

Of the 12 sea lion oocyst samples that were analyzed, 8 were found to harbor only Coccidia A (Table I). One sea lion oocyst sample contained only Coccidia B. Based on dinucleotide regions within a single sequence read, it was determined that 3 sea lion samples possessed alleles of both Coccidia A and Coccidia B. An additional sea lion oocyst sample could not be resolved to definitively harbor Coccidia A or Coccidia B due to poor sequence reads.

Pathology, immunohistochemistry, and serology

Four of the 9 sea lions (cases 5, 9, 12, and 15) shedding coccidian oocysts that died or were killed during this study had representative tissues submitted for histological examination (Table I). Enteric protozoal infection was identified in sea lions 9, 12, and 15 (Table I). In sea lions 9, 12, and 15, small numbers of protozoal schizonts and merozoites and rare sexual stages were found within the cytoplasm of enterocytes. Organisms were strongly immunoreactive to the *T. gondii* polyclonal antibody but negative using *S. neurona*– and *N. caninum*–specific polyclonal antibodies. Protozoal infection was only detected in the small intestine of these 3 cases; coccidian parasite stages were not observed in other tissues examined.

Immunohistochemistry staining for *T. gondii* and *S. neurona* was negative on intestinal sections from case 5. For cases 9, 12, and 15, intestinal protozoal infection was not clinically significant, and death was attributed to other causes. DNA sequences extracted from the jejunal tissue of 2 shedding sea lions, 12 and 15, were PCR-DNA sequence positive at the ITS-1 locus for Coccidia B, consistent with amplification results from the corresponding oocyst DNA samples (Colegrove et al., 2011).

Serum was available for cases 1, 3–7, 10, 12, and 15, and titers to *T. gondii*, *S. neurona*, and *N. caninum* were ≤1:40 for all individuals except case 1, in which the titer to *T. gondii* was 1:320 and IFATs for *S. neurona* and *N. caninum* were not performed.
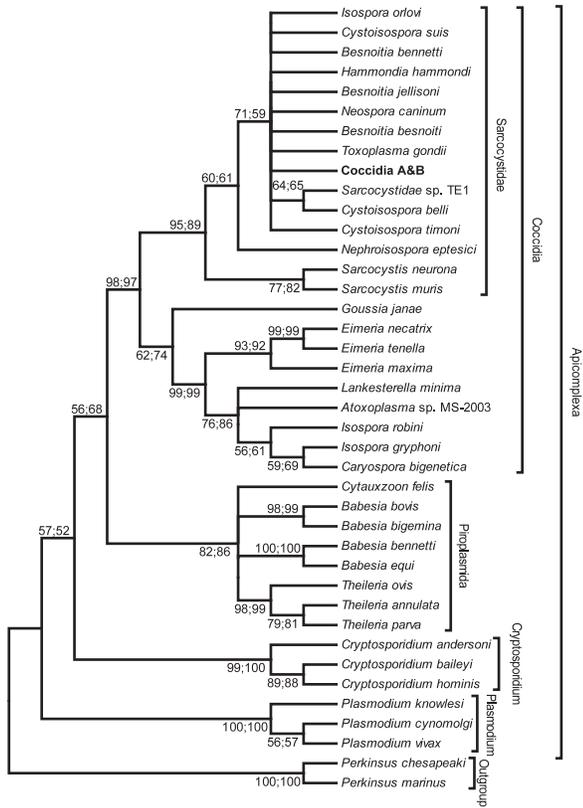
FIGURE 2. Neighbor-joining and minimum evolution SSU 18S rDNA tree. An alignment of a partial SSU 18S rDNA sequence was used to construct a phylogenetic tree to root the Coccidia A and B sequences within the Apicomplexa. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5,000 replicates) is shown next to the branches. Only those nodes with greater than 50% support are resolved.

Epizootiology

Of the 410 individual sea lions sampled during the study, 24 were sampled in 2007, 159 in 2008, and 224 in 2009. Most of the fecal samples were collected from yearling California sea lions during the spring and summer months, when admissions peak at TMMC (Figs. 4,5). The increase in admissions for pups, yearlings, and juveniles during this time reflects the relative abundance of these age classes in the population and the increased probability of young sea lions receiving veterinary care compared to subadult and adult sea lions. Therefore, opportunistic sampling at TMMC was biased by this correlation between season and age class. The median time between admission and fecal collection was 1 day (range 0 to 100 days). Multiple fecal samples (range 2–10 samples) were collected from 45 individual sea lions, with a median time of 4 days between fecal samples (range 4–56 days).

Prevalence of Coccidia A oocyst shedding in California sea lions was 2/24 (8.3%) in 2007, 9/155 (5.8%) in 2008, and 0/227 (0%) in 2009. For Coccidia B, oocyst shedding prevalence was 1/24 (4.2%) in 2007, 3/155 (1.9%) in 2008, and 0/227 (0%) in 2009. Table I shows stranding and demographic information for the 15 sea lions with coccidian oocysts positively identified in at least 1 fecal sample. Cases 3, 4, and 14 were not included in prevalence determinations for Coccidia A or B because their DNA was not available for analysis or coccidian species could not be resolved by

PCR and sequence analysis. Sea lion cases 1, 2, 7, and 11 had ≥2 fecal samples positive for coccidian oocysts, with the elapsed time between samples of 9, 22, 17, and 18 days, respectively. Based on our observed time between detection of positive fecal samples in the same individual, sea lions shed Coccidia A for at least 22 days. Duration of shedding of Coccidia B could not be estimated because repeated fecal samples were not collected from sea lions shedding only Coccidia B. Sea lions with a negative fecal sample at admission that were sampled again at a later date (n = 37) did not have a positive fecal sample on subsequent sampling.

The distribution of Coccidia A shedding by sea lions was evaluated among the sexes, age classes, and stranding month sampled. The month of stranding was not significantly associated with shedding of Coccidia A oocysts (χ^2 = 16.02, P = 0.11); however, more sea lions were observed shedding Coccidia A than expected for the months of April (1/5), June (3/81), and October (3/28) (Fig. 4). While shedding of Coccidia A oocysts was not statistically associated with age class (χ^2 = 4.49, P = 0.41), age class and month of stranding were strongly correlated (Spearman's rho = 0.54, $P \leq 0.005$), with the majority of pups stranding in May (62/67). Stranding of yearling sea lions peaked in June (73/169), while juvenile strandings peaked in August (28/82). Period prevalences for 2007 to 2009 by age class were 0% (0/67) for pups, 4.6% (8/172) for yearlings, 4.8% (4/84) for juveniles, 7.0% (3/43) for subadults, and 0% (0/41) for adults. Neither sex nor stranding location by county was associated with fecal shedding of Coccidia A oocysts (χ^2 = 0.23, P = 0.44 and χ^2 = 4.63, P = 0.61, respectively). The most common reasons for stranding in the study population were malnutrition (36%), trauma (19%), and leptospirosis (13%). The reason for stranding was not associated with shedding of Coccidia A oocysts (χ^2 = 6.21, P = 0.45). Of the 410 animals sampled for this study, 2 animals were placed at other facilities, 156 (36%) died, 135 (33%) were released, and 117 (29%) were killed. The final disposition was not associated with shedding (χ^2 = 2.44, P = 0.33). Similar analyses were not performed for Coccidia B because the parasite was only identified in oocyst samples from 3 sea lions during the entire study period.

DISCUSSION

Analysis of partial small subunit 18S rDNA sequences from Coccidia A and B place them definitively in the Sarcocystidae, i.e., tissue-cyst–forming coccidia. This poses an interesting contrast to other coccidian parasites shed in the feces of marine mammals described in the literature, which have all been classified as species of *Eimeria* or *Isospora* (Hsu, Melby, and Altman, 1974; Lainson et al., 1983; Drodz, 1987; Upton et al., 1989; Munro and Synge, 1991; McClelland, 1993). These previous classifications were based upon morphologic descriptions of parasite stages and life-cycle characteristics, while in the present study, phylogenetic classification was based on molecular analysis of DNA sequences from 3 separate loci, all supporting the same position for Coccidia A and B within the Apicomplexa. These newly identified coccidian parasites of California sea lions may be distantly related to the previously described marine mammal *Eimeria* spp.; however, conclusions about relatedness among these parasites cannot be drawn until comparable molecular data on these *Eimeria* spp. from other marine mammals are available. Though morphologic and descriptive data place the coccidian parasites from other pinnipeds and sirineans within the Eimeriidae, future
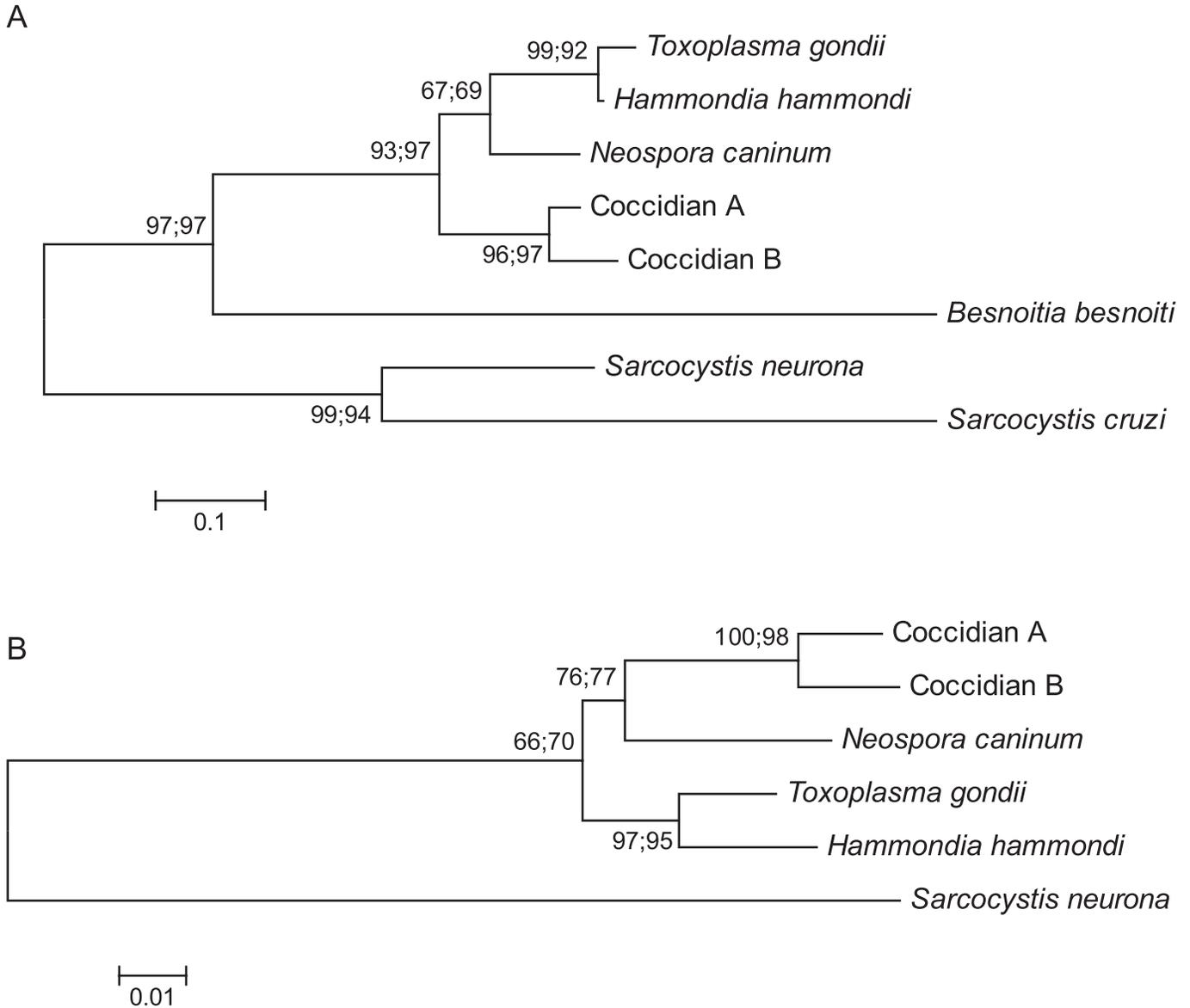
FIGURE 3.   Neighbor-joining and minimum evolution rpoB and ITS-1 trees. Alignment of the (A) ITS-1 and (B) rpoB DNA sequences was used to construct phylogenetic trees to root the Coccidia A and B sequences within the Sarcocystidae. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (5,000 replicates) is shown next to the branches. Only those nodes with greater than 50% support are resolved.

molecular data may support reclassification, similar to the reclassification of *Cystoisospora* spp. into the Sarcocystidae from the Eimeriidae (Samarasinghe et al., 2007).

The molecular analyses utilized here allowed us to rapidly identify 2 closely related, and newly recognized, coccidian parasites in California sea lions. Such techniques permit efficient exploration of parasite diversity, which would not have been easily done using morphologic descriptions alone. Given that Coccidia A and B are closely related, and that co-infection with both species was observed in 3 of the 15 sea lions with oocysts observed in feces, it may be that the 2 parasites share similar life cycles and that routes of exposure and infection in sea lions are similar.

Unlike the *E. phocae* infections previously reported in harbor seals in multiple research and rehabilitation facilities, these coccidial infections in California sea lions were not associated with an outbreak of coccidiosis in this rehabilitation hospital. The 15 sea lions with coccidian oocysts observed in fecal samples were sampled shortly after admission over a 14-mo period, indicating that sea lions are likely naturally infected with these parasites in the wild. Furthermore, none of these individuals exhibited signs of clinical disease associated with the coccidial infection. Sexually replicating stages seen in the histology of intestinal sections from cases 9, 12, and 15 were not associated with severe pathologic changes. The lack of clinical signs in stranded sea lions shedding coccidian oocysts on admission suggests that the parasites were not a significant cause of morbidity in this population.

Coccidian parasites have complex life cycles and can be directly transmitted between individuals or indirectly transmitted through a paratenic or intermediate host (Dubey and Greene, 1998;
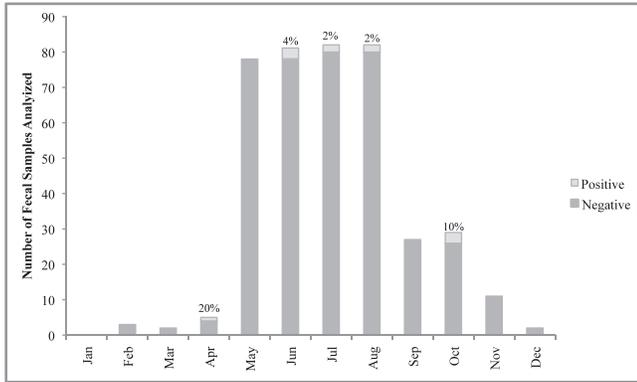
FIGURE 4. Number of fecal samples collected from California sea lions (*Zalophus californianus*) admitted to The Marine Mammal Center (Sausalito, California) and analyzed for coccidian oocysts between 2007 and 2009, stratified by month. For months in which Coccidia A oocysts were detected in fecal samples, the corresponding prevalence is reported at the top of columns.

Duszynski et al., 1998; Odening, 1998; Innes, 2009). Although our fecal sampling effort was focused on newly admitted animals, repeated sampling was performed when possible on animals that were not shedding upon admission. The presence of active infection in sections of the jejunum and oocyst shedding rule out the sea lion as a paratenic host, and recent work from Colegrove et al. (2011) identified systemic infections indicating that California sea lions likely serve as both definitive and intermediate hosts for these coccidia.

Shedding of coccidian oocysts may be infrequent given that only 4 out of 15 shedding sea lions had a second positive fecal sample. No animal was observed to begin shedding coccidian oocysts after being housed with other sea lions during the course of their stay in rehabilitation, which could occur if Coccidia A, or B, or both, were directly transmitted between sea lions. Immunity due to previous infection, low pathogenicity, inadequate infectious dose, or inadequate prepatent period before exposed animals were released could account for a failure to observe direct transmission of these coccidians between sea lions. In
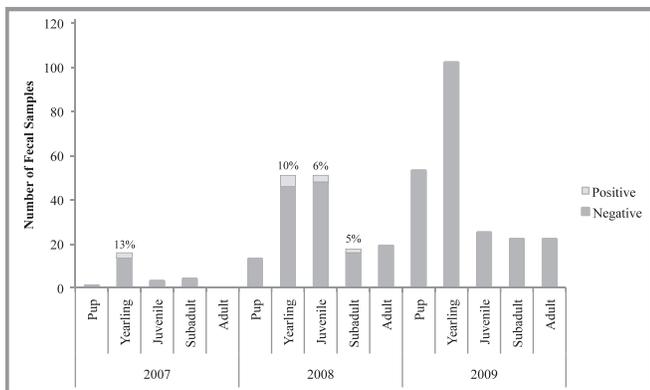

FIGURE 5. Number of fecal samples collected from California sea lions (*Zalophus californianus*) admitted to The Marine Mammal Center and analyzed for coccidian parasites between 2007 and 2009, stratified by year and age class. For strata in which Coccidia A oocysts were identified in fecal samples, the corresponding prevalence of Coccidia A fecal shedding is reported at the top of the column.

addition, all observed oocysts from sea lion feces were unsporulated; the rehabilitation setting may not have provided adequate conditions for sporulation, which may be required for infectivity. Coccidia A and B are closely related to other tissue-cyst–forming coccidia, many of which have a multihost life cycle, and for some, the tissue-cyst stages are more infective than the oocysts (Lindsay et al., 1997; Dubey, 2009; Kiehl et al., 2010); it is possible that the parasites in this study also have a more complex parasite-host life cycle, with a requisite intermediate or paratenic host stage, or the stages within the life cycle have variable infectivities.

McClelland (1993) postulated that *E. phocae* was directly transmitted between harbor seals on land at haul-out sites and would be more likely to cause disease in weakened or malnourished young animals. A similar scenario in the sea lion population would put the youngest sea lion age class, pups less than 1 yr of age, at greatest risk for shedding, since pups are most likely to strand due to malnutrition and spend the most time hauled out. In the present study, pups were not observed to shed oocysts, and the majority of California sea lions shedding coccidian oocysts were yearlings (8/15). These results are consistent with infection by environmental exposure to oocysts or by ingestion of prey containing oocysts or tissue-cyst stages as young sea lions leave the rookery and begin foraging on their own after being weaned by mothers who, by then, would be expecting a new pup. This type of exposure could also explain the observed prevalence of oocyst shedding in the summer months, since births and, therefore, weaning occur between late spring and summer. Sea lion prey base does not change with age; therefore, the lack of observed shedding in adults supports exposure in the younger age classes, after which individuals would then acquire immunity and be less likely to shed oocysts as adults.

Animals shedding coccidian oocysts were found stranded in 5 different coastal counties, from Marin County in the north to Monterey County in the south. These results suggest that coccidian parasite distribution overlaps with a large part of the sea lion range in California. However, adult male California sea lions easily travel 100 km in a day (Wright et al., 2010), and, based on the results of the repeated fecal sampling of sea lions in this study, shedding duration may last between 9 and 22 days. It is possible that if prepatency and patency periods of sea lion coccidian parasites are prolonged, there could be significant geographical displacement of a sea lion after exposure and infection. Therefore, shedding of coccidian parasites at the time of stranding may not correlate with, or reflect, the geographical location of infection.

Although sampling wild animals in a rehabilitation setting can lead to overestimates in disease prevalence, our inferences regarding coccidian oocyst shedding were based on samples obtained on admission to TMMC and are, therefore, likely to be more representative of pathogen prevalence in the free-ranging sea lions among the age classes sampled in this study. However, our sampling scheme was opportunistic, and we may have missed demographic groups in the free-ranging population with higher or lower risk. We suspect that our prevalence estimates actually miscalculate the true shedding prevalence in the wild population because individuals may have been misclassified as negative due to the less than perfect sensitivity of our diagnostic method (Dabritz et al., 2007). Based upon histologic findings from routine necropsy, at least 3 additional sea lions that became stranded during the study period had sexual stages of coccidian parasites

present in intestinal tissue stages, but oocysts were not found on fecal examination in these animals.

Sampling efforts were increased in 2009, particularly in our yearling age class, which was determined to be at higher risk for shedding coccidian oocysts based on preliminary data. The goal of increased sampling was to obtain more oocysts to further describe and characterize the newly discovered sea lion parasites; however, the interannual variability in oocyst shedding prevalence was greater than expected, and no oocysts were detected in 2009. Interannual variability may be a characteristic of coccidian parasite infection in sea lions and may occur in response to large-scale ecological shifts in the ocean environment or changes in prey availability. The California sea lion population experienced the early effects of an El Niño year in 2009, with large geographic shifts in the population and an increase in pup and yearling strandings due to malnutrition as food sources became scarce (Ono et al., 1987; Greig et al., 2005). The geographic and potential prey shifts in the sea lion population in response to ecosystem changes may have disrupted environmental exposure patterns and disrupted the natural life cycle of these newly identified coccidian parasites, leading to a decrease in shedding prevalence.

This is the first publication of oocysts from pinniped coccidian parasites since Drodz (1987) observed and described coccidian oocysts in ice seals and the first to use molecular phylogenetics to identify and describe the parasites. Furthermore, it is an epizootiological study of a coccidian parasite shed by a marine mammal, made possible through the information and biological samples available at The Marine Mammal Center. This interdisciplinary approach to coccidian disease ecology paves the way to explore the relatively unknown world of marine mammal coccidian parasites and begin to understand coccidian species diversity, coccidian parasite life cycles within the marine ecosystem, and the evolutionary links to other terrestrial tissue-cyst–forming coccidian parasites.

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LITERATURE CITED

Barlow, J., R. L. Brownell Jr., D. P. DeMaster, K. A. Forney, M. S. Lowry, S. Osmek, T. J. Ragen, R. R. Reeves, and R. J. Small. 1995. U.S. Pacific marine mammal stock assessments. NOAA Technical Memorandum NMFSSWFSC219. U.S. Department of Commerce, La Jolla, California, 162 p.

Colegrove, K. M., M. E. Grigg, D. Carlson-Bremer, R. H. Miller, F. M. D. Gulland, D. J. P. Ferguson, D. Rejmanek, B. C. Barr, R. Nordhausen, A. C. Melli, and P. A. Conrad. 2011. Discovery of three novel coccidian parasites infecting California sea lions (*Zalophus californianus*) with evidence of sexual replication and interspecies pathogenicity. Journal of Parasitology 97: 868–877.

Cox, F. E. G. 1994. The evolutionary expansion of the Sporozoa. International Journal for Parasitology 24: 1301–1316.

Dabritz, H. A., M. A. Miller, E. R. Atwill, I. A. Gardner, C. M. Leutenegger, A. C. Melli, and P. A. Conrad. 2007. Detection of *Toxoplasma gondii*–like oocysts in cat feces and estimates of the environmental oocyst burden. Journal of the American Veterinary Medical Association 231: 1676–1684.

Daniel, W. W. 2005. Biostatistics: A foundation for analysis in the health sciences, 8th ed. John Wiley & Sons, Hoboken, New Jersey, 783 p.

Dierauf, L. A., and F. M. D. Gulland. 2001. CRC Handbook of marine mammal medicine, 2nd ed. CRC Press, Boca Raton, Florida, 1120 p.

Drodz, J. 1987. Oocysts of six new Coccidiomorpha species from pinnipeds of King George Island (South Shetlands, Antarctica). Acta Protozoologica 26: 263–266.

Dubey, J. P. 2009. The evolution of the knowledge of cat and dog coccidian. Parasitology 136: 1469–1475.

———, and C. E. Greene. 1998. Enteric coccidiosis. *In* Infectious diseases of the dog and cat, 2nd ed., C. E. Greene (ed.). W. B. Saunders, Philadelphia, Pennsylvania, p. 510–518.

Duszynski, D. W., S. Upton, and L. Couch. 1998. The coccidia of the world: A unified database. *In* Proceedings of the IX International Congress of Parasitology (Makuhari Messe, Chiba, Japan), I. Tada, S. Kojima, and M. Tsuji (eds.). Monduzzi Editore SpA, Bologna, Italy. p. 1067–1071.

Evans, K., and K. Robertson. 2001. A note on the preparation of sperm whale (*Physeter macrocephalus*) teeth for age determination. Journal of Cetacean Research & Management 3: 101–107.

Greig, D. J., F. M. D. Gulland, and C. Kreuder. 2005. A decade of live California sea lion (*Zalophus californianus*) strandings along the central California coast: Causes and trends, 1991–2000. Aquatic Mammals 31: 11–22.

Gulland, F. M. D. 1999. Stranded seals: Important sentinels. Journal of the American Veterinary Medical Association 214: 1191–1192.

Hsu, C. K., E. C. Melby, and N. H. Altman. 1974. *Eimeria phocae* sp. n. from the harbor seal (*Phoca vitulina concolor*). Journal for Parasitology 60: 399–401.

———, ———, ———, and J. D. Burek. 1974. Coccidiosis in harbor seals. Journal of the American Veterinary Medical Association 164: 700–701.

Innes, E. A. 2009. A brief history and overview of *Toxoplasma gondii*. Zoonoses and Public Health 57: 1–7.

Katoh, K., K. Kuma, H. Toh, and T. Miyata. 2005. MAFFT version 5: Improvement in accuracy of multiple sequence alignment. Nucleic Acids Research 33: 511–518.

Kiehl, E., A. O. Heydorn, E. Schein, K. A. S. Al-Rasheid, J. Selmair, F. Abdel-Ghaffar, and H. Mehlhorn. 2010. Molecular biological comparison of different *Besnoitia* species and stages from different countries. Parasitology Research 106: 889–894.

Lainson, R., R. D. Naiff, R. C. Best, and J. J. Shaw. 1983. *Eimeria trichechi* n. sp. from the Amazonian manatee, *Trichechus inunguis* (Mammalia: Sirenia). Systematic Parasitology 5: 287–289.

Levine, N. D. 1988. The protozoan phylum Apicomplexa, 2 volumes. CRC Press, Boca Raton, Florida, 240 p.

Lindsay, D. S., J. P. Dubey, and B. L. Blagburn. 1997. Biology of *Isospora* spp. from humans, nonhuman primates and domestic animals. Clinical Microbiology Reviews 10: 19–34.

McClelland, G. 1993. *Eimeria phocae* in harbor seals from Sable Island, Canada. Diseases of Aquatic Animals 17: 1–8.

Miller, M. M., I. A. Gardner, A. Packham, J. K. Mazet, K. D. Hanni, D. Jessup, J. Estes, R. Jameson, E. Dodd, B. C. Barr, et al. 2002. Evaluation of an indirect fluorescent antibody test (IFAT) for demonstration of antibodies to *Toxoplasma gondii* in the sea otter (*Enhydra lutris*). Journal of Parasitology 88: 594–599.

Munro, R., and B. Synge. 1991. Coccidiosis in seals. Veterinary Record 129: 179–180.

National Oceanic and Atmospheric Association (NOAA). 2010. California sea lion (*Zalophus californianus*). Available at: http://www.nmfs.noaa.gov/pr/species/mammals/pinnipeds/californiasealion.htm. Accessed 28 April 2010.

ODENING, K. 1998. The present state of species-systematics in *Sarcocystis* Lankester, 1882 (Protista, Sporozoa, Coccidia). Systematic Parasitology **41:** 209–233.

ONO, K. A., D. J. BONESS, AND O. T. OFTEDAL. 1987. The effect of a natural environmental disturbance on maternal investment and pup behavior in the California sea lion. Behavioral Ecology and Sociobiology **21:** 109–111.

PRUESSE, E., C. QUAST, K. KNITTEL, B. FUCHS, W. LUDWIG, J. PEPLIES, AND F. O. GLÖCKNER. 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research **35:** 7188–7196.

RZHETSKY, A., AND M. NEI. 1992. A simple method for estimating and testing minimum evolution trees. Molecular Biology and Evolution **9:** 945–967.

SAITOU, N., AND M. NEI. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Molecular Biology and Evolution **4:** 406–425.

SAMARASINGHE, B., J. JOHNSON, AND U. RYAN. 2007. Phylogenetic analysis of *Cystoisospora* species at the rRNA ITS1 locus and development of a PCR-RFLP assay. Experimental Parasitology **118:** 592–595.

TAMURA, K., J. DUDLEY, M. NEI, AND S. KUMAR. 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Molecular Biology and Evolution **24:** 1596–1599.

TENTER, A. M., J. R. BARTA, I. BEVERIDGE, D. W. DUSZYNSKI, H. MEHLHORN, D. A. MORRISON, R. C. A. THOMPSON, AND P. A. CONRAD. 2002. The conceptual basis for a new classification of the coccidian. International Journal for Parasitology **32:** 595–616.

UPTON, S. J., D. K ODELL, G. D. BOSSART, AND M. T. WALSH. 1989. Description of the oocysts of two new species of *Eimeria* (Apicomplexa: Eimeriidae) from the Florida manatee, *Trichechus manatus* (Sirenia: Trichechidae). Journal of Protozoology **36:** 87–90.

VAN BOLHUIS, G. H., J. D. W. PHILIPPA, A. A. GAJADHAR, A. E. M. E. OSTERHAUS, AND T. KUIKEN. 2007. Fatal enterocolitis in harbor seals (*Phoca vitulina*) caused by infection with *Eimeria phocae*. Veterinary Record: Journal of the British Veterinary Association **160:** 297–300.

WENDTE, J. M., M. A. MILLER, A. K. NANDRA, S. M. PEAT, P. R. CROSBIE, P. A. CONRAD, AND M. E. GRIGG. 2010. Limited genetic diversity among *Sarcocystis neurona* strains infecting southern sea otters precludes distinction between marine and terrestrial isolates. Veterinary Parasitology **169:** 37–44.

WRIGHT, B. E., M. J. TENNIS, AND R. F. BROWN. 2010. Movements of male California sea lions captured in the Columbia River. Northwest Sciences **84:** 60–72.