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Permalink
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Journal
International Journal for Parasitology Parasites and Wildlife, 5(1)

ISSN
2213-2244

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Publication Date
2016-04-01

DOI
10.1016/j.ijppaw.2015.11.003

Peer reviewed

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International Journal for Parasitology: Parasites and Wildlife 5 (2016) 5–16

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Detection and characterization of diverse coccidian protozoa shed by California sea lions

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A R T I C L E I N F O

Article history:
Received 21 September 2015
Received in revised form
24 November 2015
Accepted 26 November 2015

Keywords:
Tissue cyst-forming coccidia
Sarcocystidae
Neospora sp.
Sarcocystis neurona
California sea lions
Marine mammals

A B S T R A C T

Tissue-cyst forming coccidia in the family Sarcocystidae are etiologic agents of protozoal encephalitis in marine mammals including the federally listed Southern sea otter (*Enhydra lutris*). California sea lions (*Zalophus californianus*), whose coastal habitat overlaps with sea otters, are definitive hosts for coccidian protozoa provisionally named Coccidia A, B and C. While Coccidia A and B have unknown clinical effects on aquatic wildlife hosts, Coccidia C is associated with severe protozoal disease in harbor seals (*Phoca vitulina*). In this study, we conducted surveillance for protozoal infection and fecal shedding in hospitalized and free-ranging California sea lions on the Pacific Coast and examined oocyst morphology and phenotypic characteristics of isolates via mouse bioassay and cell culture. Coccidia A and B were shed in similar frequency, particularly by yearlings. Oocysts shed by one free-ranging sea lion sampled at Año Nuevo State Park in California were previously unidentified in sea lions and were most similar to coccidia infecting Guadalupe fur seals (*Arctocephalus townsendi*) diagnosed with protozoal disease in Oregon (USA). Sporulated Coccidia A and B oocysts did not replicate in three strains of mice or in African green monkey kidney cells. However, cultivation experiments revealed that the inoculum of fecally-derived Coccidia A and B oocysts additionally contained organisms with genetic and antigenic similarity to *Sarcocystis neurona*; despite the absence of detectable free sporocysts in fecal samples by microscopic examination. In addition to the further characterization of Coccidia A and B in free-ranging and hospitalized sea lions, these results provide evidence of a new role for sea lions as putative mechanical vectors of *S. neurona*, or *S. neurona*-like species. Future work is needed to clarify the distribution, taxonomical status, and pathogenesis of these parasites in sea lions and other marine mammals that share their the near-shore marine environment.

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1. Introduction

Coccidia are a diverse group of protozoan parasites within the phylum Apicomplexa and include pathogenic species of significance to animal and human health. Monoxenous coccidia, such as those in the genus *Eimeria* and *Isospora (syn. Atoxoplasma*), primarily parasitize a single host throughout their life cycle and include important avian pathogens (McDougald, 1998; Barta et al., 2005; Berto et al., 2011). Heteroxenous coccidian protozoa including *Toxoplasma gondii*, *Neospora caninum* and *Sarcocystis neurona* infect multiple hosts throughout their life cycle and their

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http://dx.doi.org/10.1016/j.ijppaw.2015.11.003
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transmission is facilitated by predator–prey relationships (Sibley, 2003). In the intermediate host (often herbivorous or omnivorous), infectious stages are formed within tissue cysts that can be ingested by the definitive host (often a carnivorous predator). The definitive host is so called because the parasite life cycle is perpetuated through sexual multiplication in these animals. Definitive hosts shed environmentally-resistant oocysts, (or sporocysts from sporulated oocysts in the case of *S. neurona*), which are infective after sporulation for intermediate hosts that ingest fecally-contaminated food or water (Sibley, 2003).

Although the only known definitive hosts of *T. gondii*, *N. caninum* and *S. neurona* are terrestrial animals, specifically felids, canids and opossums (*Didelphis* spp.), respectively, there is evidence that marine mammals can also become infected with these and other related coccidia (Dubey et al., 2000, 2001a, 2001b; Tenter et al., 2000; Dubey et al., 2003; Colegrove et al., 2011; Gibson et al., 2011; Carlson-Bremer et al., 2012a; Goodswen et al., 2013). The most likely modes of transmission of these pathogens to aquatic animals are via ingestion of water-borne oocysts or sporocysts originating in land-based surface runoff, or infected prey (Miller et al., 2002; Conrad et al., 2005; Massie et al., 2010; Gibson et al., 2011; Shapiro et al., 2012). One of the most serious consequences of *S. neurona* and *T. gondii* infection in marine mammals is fatal protozoal encephalitis which has been described most commonly in cetaceans, including Southern sea otters (*Enhydra lutris nereis*), California sea lions (*Zalophus californianus*) and Pacific harbor seals (*Phoca vitulana richardsi*) (Thomas and Cole, 1996; Lapointe et al., 1998; Cole et al., 2000; Miller et al., 2001a; Dubey et al., 2003; Kreuder et al., 2003; Miller et al., 2004, 2009, 2010; Gibson et al., 2011). More recently, severe myositis in a hospitalized California sea lion was recognized as a new clinical syndrome associated with *S. neurona* infection (Carlson-Bremer et al., 2012b). Pathogenic infections with other, less well characterized, tissue-encysting coccidia have also been documented in marine mammals in association with protozoal lymphadenitis, hepatitis, myocarditis, encephalitis and non-suppurative necrotizing meningoencephalitis (Dubey et al., 2003; Lapointe et al., 2003; Colegrove et al., 2011; Gibson et al., 2011).

California sea lions inhabit waters of the Pacific coast of North America between southwestern Canada and Baja California (Lowry et al., 1992). As long-lived coastal residents with large fat stores and piscivorous prey preferences that are shared with humans, sea lions have the potential to act as sentinel species, or indicators of aquatic ecosystem and human health (Bossart, 2011). During postmortem examination of rescued California sea lions that died at The Marine Mammal Center (TMMC, Sausalito, California), sexual and asexual stages of three novel coccidia with genetic similarity to *Neospora* spp. were identified in sea lion enterocytes, and these organisms were putatively named Coccidia A, B and C (Colegrove et al., 2011; Carlson-Bremer et al., 2012a). Subsequent fecal analysis of stranded and rehabilitated sea lions sampled between 2007 and 2009 confirmed that sea lions shed Coccidia A and B, particularly as yearlings, and that shedding of Coccidia A could be detected in feces for up to 22 days (Carlson-Bremer et al., 2012a). Evidence to date suggests that California sea lions act as both definitive and intermediate hosts of novel protozoa in the absence of clinical signs or pathologic evidence of disseminated infection. Yet, the identification of severe protozoal hepatitis, myocarditis and encephalitis in a neonatal harbor seal infected with Coccidia C is of particular concern because it indicates that sea lions may be capable of shedding coccidian protozoa that are pathogenic to harbor seals and perhaps other aquatic wildlife (Colegrove et al., 2011).

A better understanding of the biology, epidemiology, and pathogenesis of tissue-encysting coccidian organisms that parasitize marine mammals is needed to properly assess the risks and burden of protozoal disease in aquatic ecosystems such as the Pacific coast of North America. As part of ongoing health surveillance in aquatic species of the Pacific Ocean, and accompanying studies of pathogen pollution in near-coastal California waters, we examined the diversity of coccidian parasites shed by hospitalized and free-ranging sea lions at coastal haul-out locations in central California. In addition, we characterized the phenotypes of sea lion-derived coccidian protozoa in both a mouse model of pathogenesis and in mammalian cell culture.

2. Materials and methods

2.1. Sample collection

Staff at TMMC collected fecal samples from individual California sea lions (CSL) stranded along the central and northern California coast between September 2010 and May 2012 as previously described (Carlson-Bremer et al., 2012a). Samples were typically collected within the first week of hospital admission, although some animals were sampled up to 3 weeks later. Animals and corresponding fecal samples were assigned unique identity numbers (CSL-). Multiple samples from each animal were collected when available for up to 9 weeks. When coccidial oocysts were identified at TMMC, one or more fecal samples from an individual animal were shipped on cold packs to the University of California Davis for oocyst harvesting and genetic characterization. Age classifications for sea lions (adult, subadult, juvenile, yearling, and pup) were estimated as previously described (Carlson-Bremer et al., 2012a). Between May 2011 and October 2012, sea lion fecal samples from free-ranging animals were also collected from coastal haul out sites at Año Nuevo State Park (37.108, −122.337), White Rock (35.532, −121.088), and Seal Rock at Point Lobos (36.516, −122.336), placed into individual 50 mL conical vials and kept at 4 °C during transport to UC Davis for oocyst detection, harvesting and genetic characterization. Samples were identified according to location (Año-, WR- and PL-).

2.2. Oocyst harvesting, sporulation and excystation

Coccidial oocysts were harvested from fecal samples by a double centrifugation flotation method as described by Dabritz and coauthors (Dabritz et al., 2007) with some modifications. A detailed protocol for oocyst harvesting from sea lions is available in the supplemental information associated with this article (See Supplementary data). When quantities were sufficient, a portion of freshly-harvested oocysts were not frozen, but were reserved and sporulated for bioassay in mice and inoculation into cell cultures. Oocysts for cell culture were additionally excysted.

For sporulation, newly-harvested oocysts were washed two times in sterile PBS, and the final pellet resuspended in 5 mL PBS, to which was added Clorox bleach at a final concentration of 10%. Following 20 min incubation with occasional gentle mixing, the sample was washed 3 times in 50 mL molecular biology grade water, with each centrifugation at 1000 g for 10 min. After final centrifugation, supernatant was carefully removed and the pellet mixed with 3–5 mL PBS (higher volume for samples with higher oocyst concentration). Approximately 1 mL of oocyst suspension in PBS was added to 5 mL filtered seawater (0.22 μm, Acrodisc® Syringe Filters, Pall Corporation) with 250 μl amphotericin B (250 μg/mL) in a T-25 tissue culture flask with ventilated lid. Each flask was set on a gentle rocker at RT and visually inspected every 2 days by light microscopy for evidence of sporulation. Incubation continued until 45%–72% sporulation was observed and stabilized (typically 3–8 days). Sporulated oocysts were washed two times in ~40 mL sterile PBS, counted, and prepared for oral inoculation into mice.

For excystation and preparation for cell culture, sporulated oocysts were suspended in 0.5 mL warm (37 °C) Dulbecco's Minimum Essential Media (DMEM, Gibco), supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μG/ml streptomycin, 10 mM HEPES and 55 μM 2-mercaptethanol and transferred to 2 mL screw cap tubes containing 350 mg of 200–400 mm sterile acid-washed glass beads (ThermoFisher Scientific). Oocyst disruption and sporozoite release was accomplished through vortexing in the presence of glass beads for 20–30 s. To confirm the presence of free sporozoites, a 5 μL aliquot of the suspension was examined following each 10 s of vortexing. When sporozoites were visible, the sample was immediately transferred to monolayer cultures of embryonic monkey kidney (MA104) cells (BioWhittaker, Walkersville, MD) and incubated for 2 h at 37 °C in 5% CO_2, after which the inoculum was removed and replaced with fresh 37 °C prepared DMEM. Cells were checked 3 times weekly for parasite growth alongside uninoculated MA104 cells. When zoites became visible in cell culture, supernatant was collected and pelleted for DNA extraction, cryopreservation, and antigen slide preparation for serological testing.

2.3. Morphological analysis

During microscopic analysis of oocysts isolated from sea lion feces, multiple photomicrographs were taken at 400–1000× magnification using a camera (SPOT RT3 scientific digital CCD camera, SPOT Imaging Solutions ™, Sterling Heights, MI) mounted on a Zeiss Axioskop epifluorescent microscope equipped with a UV emission filter set (emitter 460/50 nm band pass filter; Chroma #11000 v3). Micrographs were used to measure the long axis and short axis lengths of oocysts (in unsporulated and sporulated forms) and sporocysts (in sporulated forms) using SPOT™ Advanced software (SPOT™ Imaging Solutions, Sterling Heights, MI, USA). The mean and standard deviation of 10–20 sporulated and unsporulated oocysts were calculated separately for each coccidian species. In oocyst-inoculated cell cultures, approximately 10 zoites per culture were measured at 200× magnification from photomicrographs using SPOT™ software.

2.4. Mouse bioassay

All animal experiments were conducted with the approval and oversight of the Institutional Animal Care and Use Committee at the University of California, Davis, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International (IACUC # 17501). Mouse strains were chosen based on known susceptibility to *T. gondii* (Rytel and Jones, 1966; Villegas et al., 2002; Gavrilescu and Denkers, 2003). Prior to inoculation, 6–8 week old female mice were screened by the indirect fluorescent antibody test (IFAT, see methods below) using a 1:40 serum dilution to ensure seronegativity to *T. gondii*, *N. caninum* and *S. neurona*. Approximately 1000 sporulated oocysts were inoculated into two mice of each genetic background: B6.129S7-*Ifng*^{tm1Ts}/J (C57BL/6 background), CBA/CaJ (The Jackson Laboratory, Bar Harbor, ME) and Swiss Webster (CFW®) (Charles River Laboratories, Wilmington, MA). Of the two mice in each genetic background group, one was orally inoculated by gastric lavage and the other was subcutaneously inoculated in the interscapular region as previously described (Fritz et al., 2012). Control mice of each genetic background were given inocula free of oocysts consisting of Clinicare Canine/Feline Liquid Diet (Abbott Health Care) for gastric lavage controls or PBS for subcutaneous controls. Mice were checked once per week for clinical signs of disease and were bled at 3 and 6 weeks post-infection for IFAT serology. Mice were humanely euthanized by CO_2 asphyxiation for necropsy at 50–70 days post-inoculation, at which time additional blood was collected from the heart cavity for IFAT serology. During necropsy, portions of the tongue, heart, and brain were collected, divided into three pieces, and frozen at −80 °C until DNA extraction. A portion of the mouse brains was homogenized, trypsinized and placed over monolayer cultures of MA104 cells for coccidial parasite detection as previously described (Miller et al., 2001b). Remaining mouse tissues were placed in 10% formalin and submitted to the California Animal Health and Food Safety laboratory (CAHFS, Davis, CA) for histological and immunohistochemical analysis.

2.5. Histopathology

The following mouse tissues were fixed in 10% neutral buffered formalin and examined histologically: liver, kidney, heart, lung, spleen, subcutaneous fat, skeletal muscle, pancreas, salivary gland, uterus, lymph node, thymus, multiple sections of gastrointestinal tract and brain, gonad, trachea, adrenal gland. Formalin-fixed tissues were trimmed, routinely processed for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin. Immunohistochemical staining for the presence of *T. gondii, S. neurona and N. caninum* antigen was performed as previously described (Miller et al., 2001b) on selected sections of formalin-fixed, paraffin-embedded tissues.

2.6. Indirect fluorescence antibody test (IFAT)

Antigen slides for IFAT analysis were prepared with parasites harvested from the supernatants of MA104 cultures containing visible zoites using previously described methods (Miller et al., 2001b). Mouse blood was collected by venipuncture, cardiac puncture or retro-orbital bleed, allowed to clot, and centrifuged at 1000 *g* for 10 min. Serum was removed and stored at −20 °C until testing. Sea lion serum was similarly separated after collection via venipuncture of the caudal gluteal vein during hospitalization at TMMC and was shipped to UC Davis for IFA testing. Serum was serially diluted in PBS from 1:40 to 1:81,920 and screened for immunoreactivity to *T. gondii*, *S. neurona* and *N. caninum* antigen using a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-canine IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Mouse sera were screened at a 1:40 dilution for immunoreactivity to antigens of the same three parasites using FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories). Test controls consisted of previously analyzed animal sera with titers ranging from 1:1280–1:10,240 (positive controls) or titers <1:40 (negative controls). Sea lion and mouse serum specimens that showed no reactivity at ≥1:40 dilution were considered test-negative. For the purpose of this study, sea lion sera that were reactive to *T. gondii* antigen at ≥1:640 dilution were considered test-positive to identify sea lions likely infected with *T. gondii*. Sea lion sera that were reactive to *S. neurona* antigen at ≥1:320 were considered test-positive as has been used previously to identify *S. neurona* infection in sympatric species (Kreuder et al., 2005; Mylniczenko et al., 2008).

2.7. DNA extraction and PCR

Prior to DNA extraction, frozen, pelleted oocysts that had been harvested from sea lion feces were subjected to a single freeze–thaw cycle in liquid nitrogen and boiling water, 3 min each. DNA was extracted from oocysts and mouse tissues using the Qiagen DNeasy Blood and Tissue Kit using manufacturer's

recommendation for animal tissues. Two out of three mouse tissue sections were analyzed per tissue type per mouse, with preference for mice that demonstrated histological lesions, leading to a total of ~164 individual samples, including controls, which were extracted and analyzed by PCR. Negative controls (water) were included in the extraction process to monitor for environmental contamination. For molecular characterization of coccidian DNA in oocysts, mouse tissues and zoite cultures, nested PCR was performed using pan-coccidian primers that span the internal transcribed spacer 1 (ITS-1) region, and *S. neurona*/*S. falcatula*-specific primers ($ITS1_{500}$) that amplify ~500 bp of the ITS1 region in those organisms (Miller et al., 2009; Gibson et al., 2011). For characterization of *S. neurona*-like DNA, PCR protocols were employed to amplify microsatellites Sn7 (nested PCR) and Sn9 (hemi-nested PCR), (Asmundsson and Rosenthal, 2006; Rejmanek et al., 2010) and surface antigens SnSAG3, SnSAG4 (nested PCR) (Rejmanek et al., 2010) and either SnSAG1, SnSAG5, or SnSAG6 which amplify using snSAG1-5-6 primers (Wendte et al., 2010). Additional primers were used to amplify a secondary region of *S. neurona* ITS-1, approximately 150 bp upstream of the area amplified by $ITS1_{500}$ primers: SnITS1-F, CCGGGATGATGTCGTCAAG and SnITS1-R, ACAGATGATGTGCCCCGC (Carlson-Bremer, 2011).

All PCR reactions were performed using Qiagen HotStar Taq Plus (Qiagen, Valencia, CA) according to manufacturer's recommendations, utilizing 5 μL of template DNA. The following reaction conditions were used for all primer sets: initial denaturation at 95 °C for 5 min, followed by 35 cycles at 94 °C for 40 s, 58 °C for 40 s (external primers or non-nested PCR) or 59 °C for 40 s (internal primers for nested/hemi-nested PCR), and 72 °C for 90 s, followed by a 10 min extension at 72 °C. In the case of nested or hemi-nested PCR protocols, 1 μL of first round PCR product was added to the second round master mix. Positive controls for PCR included either *Hammondia heydorni* DNA isolated from a domestic dog, *Neospora hughesi* isolated from a horse, and/or *S. neurona* strain snUCD-1 (Rejmanek et al., 2010) isolated from a horse. Water was used as a negative control. PCR products were purified using the QiaQuick PCR purification kit (Qiagen) and were sequenced by the UCDNA Sequencing Facility (Davis, CA).

2.8. Sequence and statistical analysis

Forward and reverse sequences were assembled into contigs using Geneious Pro v. 5.3.4 (Drummond et al., 2009). Consensus sequences were aligned to reference sequences obtained from NCBI (http://www.ncbi.nlm.nih.gov/nuccore) using MUSCLE or MAFFT (Katoh et al., 2002; Edgar, 2004) with default parameters, followed by manual editing. Posterior sets of phylogenetic trees were generated using MrBayes (Huelsenbeck and Ronquist, 2001) implemented in Geneious Pro v. 5.3.4 and run for 1,000,000 generations using the GTR + G nucleotide substitution model. Trees were sampled every 1000 generations and 25% of trees were discarded from the initial burn-in period. Pairwise genetic distance values were determined through uncorrected-*p* distance matrices generated in PAUP* (Sinaeur Associates Inc.) using prepared alignments of 420 bp for ITS-1. Individual sequences were analyzed using BLAST (Basic Local Alignment Search Tool) available on the NCBI website http://blast.ncbi.nlm.nih.gov/Blast.cgi. Sequences for ITS-1 (accession nos. KP999999 and KP990539 for *T. gondii* M4 and Año11, respectively), and SnSAG3 (KR011915 for *Sarcocystis* sp. CSL10089/CSL10092) were deposited to GenBank.

Prevalence of oocyst shedding was calculated by sex and age class. Groups were compared for significant differences using the two-tailed two-sample test of proportions (Stata/IC v. 11.0, StataCorp LP, College Station, TX).

3. Results

3.1. Prevalence of Coccidia A and B shed by stranded California sea lions (CSL)

Fecal samples collected from 139 sea lions stranded in nine counties located on the California coast between 2010 and 2012 were screened upon hospitalization for the presence of coccidian oocysts. The majority of animals stranded in Monterey (n = 48), San Luis Obispo (n = 36) and Santa Cruz (n = 32) counties, followed by San Mateo (n = 7), San Francisco (n = 5), Sonoma (n = 4), Marin (n = 4), Mendocino (n = 2) and Humboldt (n = 1). Coccidian oocysts were identified in the feces of 16 of the 139 (11.5%) sea lions sampled the majority of which were from Monterey (8.3%, 4/48) and San Luis Obispo (13.9%, 5/36) counties. Only one of the 32 stranded animals (3.1%) from Santa Cruz County was shedding oocysts. Table 1 describes the clinical status and results of serological testing and coccidian oocyst DNA analysis for the 16 sea lions that had oocysts detected in their feces upon presentation at TMMC.

Oocysts observed in the feces of 16 stranded sea lions (Table 1) were 8–10 μm in diameter. *Sarcocystis* sp. sporocysts were not observed by light microscopy following fecal flotation in any of the sea lions sampled. A similar proportion of males (9/89) and females (7/51) shed oocysts ($P = 0.518$). Yearlings most commonly shed oocysts (8/33, 24.2%) followed by subadults (2/16, 12.5%), pups (2/19, 10.5%), juveniles (3/50, 6.0%) and adults (1/21, 4.8%). The proportion of yearlings shedding oocysts (8/33) was significantly greater than the proportion of all other age classes shedding oocysts (8/106; $P = 0.009$).

The phylogenetic tree in Fig. 1 shows the close relationship of coccidian DNA sequences amplified from fecal samples of the stranded hospitalized sea lions in this study (e.g. CSL NCMMC1047 Humboldt and CSL9878 Monterey) to *N. caninum* and DNA sequences amplified from marine mammal fecal samples and tissues in previous studies (Colegrove et al., 2011; Gibson et al., 2011; Carlson-Bremer et al., 2012a). In the 422 bp ITS-1 alignment, CSL NCMMC1047 was identical to Coccidia A (GenBank accession no. GU936629), and CSL9878 was identical to Coccidia B (GenBank accession no. GU936630). Consistent with earlier descriptions (Colegrove et al., 2011), Coccidia A (CSL NCMMC1047) and B (CSL9878) ITS-1 sequences shared 79.6% and 78.8% pairwise nucleotide similarity, respectively, to *N. caninum* and 74.1% and 71.8% similarity, respectively, to *T. gondii*.

Based on sequence analysis of the ITS-1 locus from the 16 hospitalized sea lions that were shedding coccidia during this study, 6 (37.5%) animals were confirmed to be shedding Coccidia A, 6 (37.5%) were shedding Coccidia B, and 2 (12.5%) were shedding both A and B simultaneously, indicating dual infections (Table 1). Sera from 5 (31.3%) of these sea lions had antibodies to *T. gondii* antigens detectable by IFAT, but only one of these animals (CSL9830) had an antibody titer above 1:640 (Table 1). One animal from Monterey (CSL9878) was seroreactive to *S. neurona* antigen at a 1:2560 dilution. No animals tested during this time period were seroreactive to *N. caninum* despite the apparent genetic relatedness of Coccidia A and B to *Neospora* spp. at the ITS-1 locus (Fig. 1) (Colegrove et al., 2011; Carlson-Bremer et al., 2012a).

Unsporulated oocysts had a round to ovoid shape and autofluoresced under UV illumination (Fig. 2) as observed for *T. gondii* and other coccidian oocysts (Lindquist et al., 2003). Unsporulated Coccidia A oocysts averaged 9.9 ± 0.59 μm in length and 9.5 ± 0.69 μm in width (n = 20 oocysts from CSL NCMMC 1047, measured at 200× magnification). Unsporulated Coccidia B oocysts averaged 8.9 ± 0.31 μm in length and 8.5 ± 0.51 μm in width (n = 20 oocysts from CSL9878, measured at 1000× magnification) (Fig. 2).

Table 1
Clinical status, serological results and coccidian sequence types shed by hospitalized California sea lions sampled between September 2010 and May 2012.

TMMC ID	Admit date	Sex	Age class	Stranding county	Preliminary diagnosis	Serology			Coccidian sequence type
						T. gondii	*S. neurona*	*N. caninum*	
CSL9830	9-Sep-2010	M	Juvenile	Santa Cruz	pneumonia, trauma (flipper), malnutrition, leptospirosis	1:1280	<1:40	<1:40	A
CSL9831	10-Sep-2010	M	Juvenile	Sonoma	trauma (eye and face), malnutrition, seizures, pneumonia	<1:40	<1:40	<1:40	A
CSL9878	16-Oct-2010	M	Yearling	Monterey	malnutrition, trauma	<1:40	1:2560	<1:40	B
CSL NCMMC1047	27-Jan-2011	M	Yearling	Humboldt	blind	1:40	<1:40	<1:40	A
CSL9930	25-Mar-2011	M	Pup	Mendocino	oil/tar, malnutrition	<1:40	<1:40	<1:40	B
CSL9939	14-May-2011	M	Pup	San Francisco	malnutrition, pneumonia	<1:40	<1:40	<1:40	Inadequate DNA
CSL9959	3-Jun-2011	F	Yearling	San Luis Obispo	malnutrition, abscess	<1:40	<1:40	<1:40	B
CSL9970	15-Jun-2011	F	Yearling	San Luis Obispo	malnutrition, abscess	<1:40	<1:40	<1:40	Inadequate DNA
CSL10089	30-Aug-2011	F	Yearling	San Luis Obispo	malnutrition, trauma (face)	1:320	<1:40	<1:40	A
CSL10092	31-Aug-2011	F	Yearling	San Mateo	abscess, malnutrition	1:80	<1:40	<1:40	A + B
CSL10100	3-Sep-2011	M	Yearling	Monterey	malnutrition	1:160	<1:40	<1:40	A + B
CSL10184	9-Oct-2011	F	Subadult	Marin	pneumonia, leptospirosis	<1:40	<1:40	<1:40	B
CSL10254	1-Nov-3011	F	Adult	San Luis Obispo	seizures, domoic acid toxicity (acute)	<1:40	<1:40	N/A	B
CSL10243	14-Nov-2011	M	Yearling	Monterey	entanglement	<1:40	<1:40	<1:40	A
CSL10266	7-Jan-2012	M	Juvenile	San Luis Obispo	trauma, malnutrition	<1:40	<1:40	<1:40	A
CSL10268	10-Jan-2012	F	Subadult	Monterey	trauma, osteomyelitis	<1:40	<1:40	<1:40	B

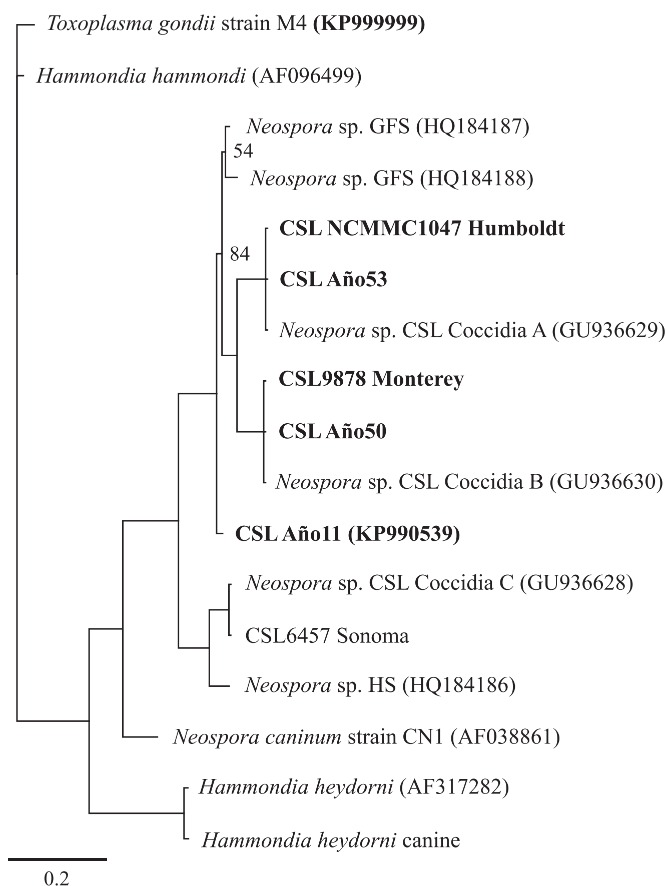

Fig. 1. Consensus Bayesian phylogenetic tree made from a 422 bp alignment of the partial internal transcribed spacer 1 region (ITS-1) sequence of coccidian parasite DNA amplified in California sea lion (CSL) fecal samples collected from stranded and free-ranging animals (bold). County of stranding in California is given for animals hospitalized at TMMC, and location of the Año Neuvo haul-out site is shown in Fig. 3. Included is an additional Coccidia C sequence amplified from intestinal tissue of a hospitalized sea lion (CSL6457; Colegrove et al., 2011). Newly-submitted GenBank accession numbers are also shown in bold. All posterior probabilities are equal to 100 unless otherwise indicated at tree nodes. HS, harbor seal; GFS, Guadalupe fur seal.

Coccidian oocysts A and B were previously identified in the feces of stranded California sea lions surveyed at TMMC between 2007 and 2009 (Carlson-Bremer et al., 2012a). Here, we summarized shedding prevalence for all sea lions sampled between 2007 and 2012 (Table 2). Over the six year period, 558 sea lions were sampled, 31 (5.6%) of which were shedding Coccidia A or B, in similar frequency. Yearlings comprised approximately 50% of the animals found shedding Coccidia A and B oocysts. Hospitalized stranded sea lions were typically diagnosed with malnutrition and trauma with no apparent clinical effects attributed to protozoal infection (Table 1).

3.2. Prevalence of Coccidia A, B and a unique coccidian species shed by free-ranging California sea lions

Between August 2011 and September 2012, California sea lion fecal samples were collected from coastal haul-out sites located at Año Nuevo State Reserve (n = 48), White Rock (n = 94) and Point Lobos (n = 70) and the samples were screened for oocysts. Sampling locations are shown in Fig. 3. The prevalence of oocysts identified by microscopy during isolation was 8.3% (4/48) at the Año Nuevo haul-out site, 3.2% (3/94) at White Rock, and 8.6% (6/70) at Point Lobos (Table 3). Oocysts observed were 8–10 μm in diameter. *Sarcocystis* spp. sporocysts were not observed by light microscopy following fecal flotation in any of the free-ranging sea lion samples. DNA amplification and sequence alignment revealed that both Coccidia A and B were present in fecal samples collected at Año Nuevo and Point Lobos, while only Coccidia A was identified in samples collected at White Rock (Table 3). Fecal sample 11 collected at Año Nuevo State Park (Año11) represented a novel sequence type with 97–98% pairwise similarity at the ITS-1 locus to previously published coccidian DNA isolates in Guadalupe fur seal tissues (see *Neospora* sp. GFS in Fig. 1) (Gibson et al., 2011). The CSL Año11 isolate had ~92% pairwise similarity to Coccidia A and B and shared even less similarity (87.7%) to Coccidia C at the ITS-1 locus. This sequence as well as representative sequences of Coccidia A and B DNA isolated from field-collected specimens (CSL Año53 and CSL Año50, respectively) are shown in Fig. 1, and the geographic distribution of coccidian oocyst species shed by free-ranging animals is shown in Fig. 3.

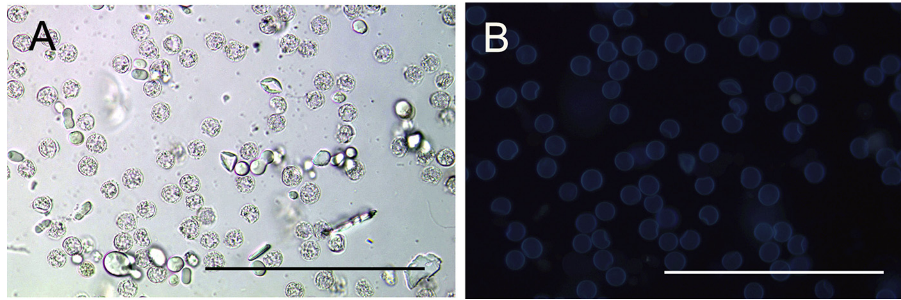

Fig. 2. Photomicrographs of Coccidia B isolated from California sea lion feces visualized under bright field illumination (A) or UV epifluorescence (B). Images A and B show unsporulated Coccidia B oocysts shed by CSL9878. Coccidia A oocysts (not shown) have similar morphology and epifluorescence. Scale bars are 100 μm.

Table 2
Summary of Coccidia A and B shedding by stranded California sea lions (CSL), 2007 to 2012.

Year	No. CSL sampled	No. CSL shedding oocysts	Prevalence	No. CSL shedding Coccidia A	No. CSL shedding Coccidia B
2007[a]	24	2	8.3%	2	1
2008[a]	156	13	8.3%	9	3
2009[a]	227	0	0.0%	0	0
2010	16	3	18.8%	2	1
2011	98	11	11.2%	5	6
2012	37	2	5.4%	1	1
Total	558	31	5.6%	19	12

[a] Samples obtained from 2007 to 2009 were reported previously (Carlson-Bremer et al., 2012b).

3.3. Mouse bioassay for Coccidia A and B

When sufficient quantities were available, oocysts harvested from fecal samples were sporulated and inoculated into mice to investigate coccidian parasite infectivity and pathogenesis. Sporulated Coccidia A oocysts were, on average, 9.8 ± 0.83 μm in length and 8.6 ± 0.68 μm in width, with sporocysts measuring 5.3 ± 0.69 μm long and 4.0 ± 0.53 μm wide (n = 20 oocysts from CSL NCMMC1047, measured at 400–1000× magnification) (Fig. 4A). Sporulated Coccidia B oocysts were 9.9 ± 0.88 μm in length and 9.0 ± 1.2 μm in width, with sporocysts measuring 6.0 ± 0.4 μm long and 4.6 ± 0.5 μm wide (n = 10 oocysts from CSL9878, measured at 400–1000× magnification) (Fig. 4B).

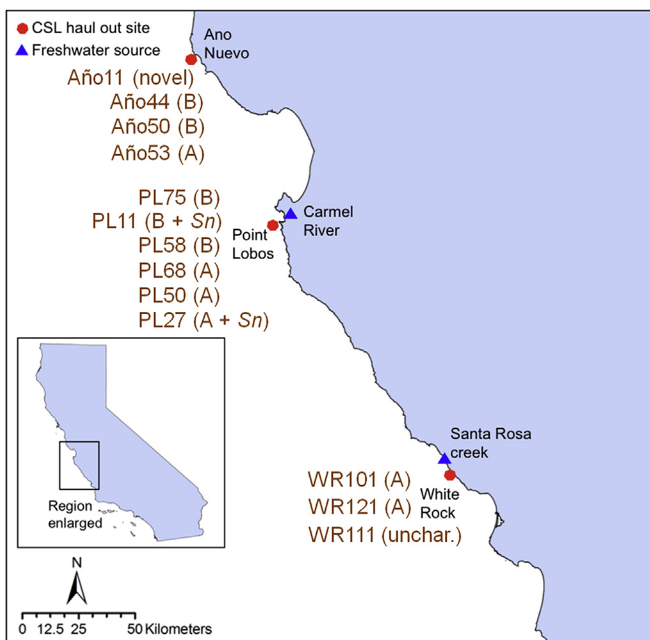

Fig. 3. Three California sea lion haul-out sites (red circles) on the central California coast where fecal samples were collected from free-ranging animals. Field sample identifiers for test-positive samples are given (Año-, PL- and WR-) at each location. Coccidian parasite genotypes and species amplified in sample DNA are indicated in parentheses: A, Coccidia A; B, Coccidia B; Sn, *Sarcocystis neurona*-like; unchar, uncharacterized species. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Sufficient quantities of oocysts from animals that were shedding either Coccidia A or B (or both) including CSL9830, CSL9878, CSL NCMMC1047, CSL10089, CSL10092, and CSL10100, were available for mouse bioassay. All mice were seronegative for *T. gondii*, *S. neurona* and *N. caninum* prior to inoculation and at 3 weeks and 6 weeks post-inoculation, as well as at the time of necropsy (~50–70 days post-inoculation). No clinical signs were observed in Coccidia A- and B- inoculated or mock-inoculated control mice throughout the observation period. Brain cultures were negative for parasite growth. Mouse tissues were generally unremarkable histologically, although one or more of the following histological changes were observed in approximately 40% of mice: slight nephritis, slight pneumonia, inflammation in the lung, spleen or lymph node, and steatitis. All mock-inoculated control mice had unremarkable tissues changes. Mouse brain, heart and tongue tissues tested negative for the presence of Coccidia A or B DNA using pan-coccidian primers that span the ITS-1 region.

3.4. Evidence of S. neurona in fecal samples from sea lions

3.4.1. Cell culture and serological examination

In an effort to propagate and isolate coccidian protozoa identified by light microscopy in sea lion feces, sporulated oocysts from CSL10089 (Coccidia A), CSL10092 (Coccidia A & B), CSL10100 (Coccidia A & B), and CSL10266 (Coccidia A) were prepared and inoculated into flasks containing MA104 cells. All cultures were fed and examined for the presence of live coccidian zoites three times per week. After 14 days, 21 days and 35 days of observation, cell cultures inoculated with sporulated oocysts from CSL10089, CSL10100, and CSL10092, respectively, showed propagating

Table 3
Prevalence and genetic diversity of coccidian species identified by microscopy in fecal samples of free-ranging California sea lions at coastal haul-out sites.

Location (Sample identifier)	No. samples examined for oocysts	Time period	No. oocyst-positive samples (Prevalence)	Sample ID (Coccidian species)
White Rock (WR-)	94	July 2011–August 2012	3 (3.2%)	WR101 (Coccidia A) WR111 (uncharacterized)[a] WR121 (Coccidia A)
Año Nuevo (Año-)	48	May 2011–October 2011	4 (8.3%)	Año11 (novel)[b] Año44 (Coccidia B) Año50 (Coccidia B) Año53 (Coccidia A)
Point Lobos (PL-)	70	August 2011–Sept 2012	6 (8.6%)	PL11 (Coccidia B + *S. neurona*-like[c]) PL 27 (Coccidia A + *S. neurona*-like[c]) PL50 (Coccidia A) PL58 (Coccidia B) PL68 (Coccidia A) PL75 (Coccidia B)
Total	244		13 (5.3%)	6 Coccidia A 5 Coccidia B 2 *S. neurona*-like 1 uncharacterized[a] 1 novel[b]

[a] Oocysts were identified by microscopy during isolation, but DNA was insufficient for molecular characterization.
[b] The amplified ITS-1 region of Año11 is genetically distinct from other coccidian isolates (See Fig. 1).
[c] The presence of *Sarcocystis neurona*-like DNA was confirmed by PCR and sequence analysis. Sporocysts were not identified in fecal samples by microscopy.

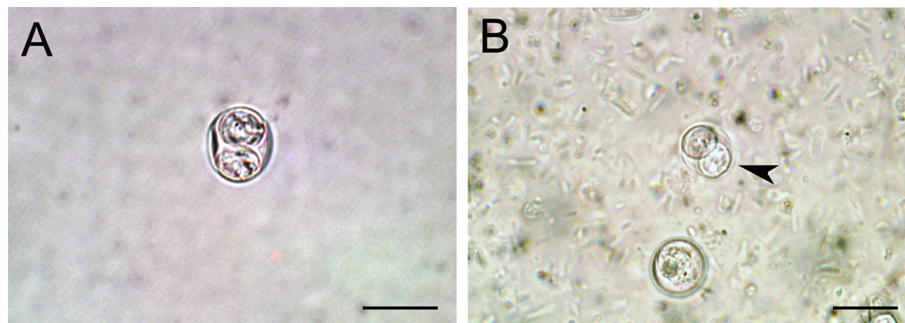

Fig. 4. Photomicrographs of sporulated oocysts of Coccidia A (A) and Coccidia B (B, arrowhead) isolated from fecal samples of California sea lions CSL NCMMC1047 and CSL9878. Scale bars are 10 μm.

organisms with morphologic similarity to *Sarcocystis* spp. (Table 4). Parasites were observed both extracellularly and within MA104 cells. Extracellular zoites were 8–11 μm in length and 1–2 μm in width when measured with SPOT™ Advanced software at 200× magnification. Schizonts were seen periodically as the culture matured and the number of free zoites increased; suggesting that these were merozoites produced by schizogony. Free zoites had active, circumaxial motility characteristic of *S. neurona* merozoites observed *in vitro* (Miller et al., 2001a). Similar organisms did not grow in cultures inoculated with sporulated oocysts from a fourth sea lion, CSL10266 or in un-inoculated cultures of MA104 cells (Table 4).

By IFAT, antigen slides prepared from zoite cultures of all three sea lions, CSL10089, CSL10092 and CSL10100, tested positive using serum from a horse with equine protozoal myeloencephalitis (Fig. 5A–C, Table 4). The horse's *S. neurona* infection was confirmed by IFAT and western blot on serum (1:320 titer to laboratory strain snUCD-1) and cerebral spinal fluid, and by immunohistochemistry used to detect *S. neurona* merozoites in the lumbar spine. In addition, all three zoite isolates from sea lion oocyst cultures reacted to sera from hospitalized sea lion CSL9878 (Table 4) which was itself seroreactive to merozoites of snUCD-1 (1:2560) (Table 1 and Fig. 5D). Sea lion culture antigen was IFAT-negative (<1:40) to goat serum containing *T. gondii*-reactive antibodies, bovine fetal serum containing *N. caninum*-reactive antibodies, and, as shown in Fig. 5E, to horse serum that was seronegative to *S. neurona* and *N. hughesi*.

3.4.2. PCR and sequence analysis

As soon as zoites were observed, supernatant from the MA104 cell cultures inoculated with sporulated, excysted oocysts from the fecal samples of CSL10089, CSL10100 and CSL10092 were collected for molecular characterization approximately once per week for 11–14 weeks. Pan-coccidian primers that span the ITS-1 region produced a ~1000 bp band when DNA was amplified from zoites taken from cultures from the 3 sea lions throughout their collection period. This is the expected band size for amplification of *S. neurona* DNA using pan-coccidian ITS-1 primers (Gibson et al., 2011). In addition, a second band ~400 bp in size was amplified simultaneously in culture supernatant of CSL10100 and CSL10092 using the pan-coccidian primers, but only in the first week of sample collection following zoite observation (Table 4). Four hundred basepairs is the expected band size for amplification of Coccidia A, B or C using pan-coccidian ITS-1 primers (Colegrove et al., 2011; Gibson et al., 2011). In culture CSL10266, in which no zoites (only oocysts) were observed, only the ~400 bp band was amplified by PCR during the first two weeks of incubation. Subsequent supernatant samples of culture CSL10266 and all uninoculated MA104 control cultures were PCR-negative (Table 4).

Sequence analysis confirmed that the ~400 bp amplicon DNA generated in early sampling of cultures CSL10100, CSL10092 and CSL10266 was identical to DNA amplified from frozen, pelleted oocysts originally harvested from the same animal; namely, Coccidia A alone (in the case of CSL10092 and CSL10266) or a dual

Table 4
Characterization of zoites grown in MA104 cell cultures inoculated with sporulated, excysted oocysts of Coccidia A and B from California sea lion (CSL-) fecal samples 10089, 10092, 10100 and 10266. Antigenic reactivity of zoites to *Sarcocystis neurona* antisera from a horse and a sea lion (CSL 9878) by indirect fluorescent antibody test (IFAT). PCR amplification of zoite DNA confirmed that zoites were similar to *S. neurona* (Sn).

	CSL10089	CSL10092	CSL10100	CSL10266	MA104 (control)
Fecal oocyst coccidian sequence type[a]	A	A & B	A & B	A	NT
Zoites observed in MA104 cell culture	+	+	+	−	−
IFAT results with antigen slides of CSL zoites in MA104 cell culture					
S. neurona antisera					
Horse 681441	1:320	1:160	1:320	NT	NT
CSL9878	1:10,240	1:1280	1:640	NT	NT
PCR results with approximate amplicon size (coccidian species/genotype[b])					
Genetic locus/primers					
ITS-1 (pan-coccidian)[c]	1000 bp (Sn)	1000 bp (Sn)/ 400 bp (Coccidia A)	1000 bp (Sn)/ 400 bp (Coccidia A + B)	400 bp (Coccidia A)	Negative
$ITS1_{500}$ (*S. neurona/falcatula*)	500 bp (Sn)	500 bp (Sn)	500 bp (Sn)	Negative	Negative
Sn7	150 bp (Sn/CA_{17})	150 bp (Sn/CA_{17})	150 bp (Sn/CA_{17})	NT	NT
Sn9	150 bp (Sn/GT_{18})	150 bp (Sn/GT_{18})	150 bp (Sn/GT_{18})	NT	NT
Sn-ITS	600 bp (Sn)	600 bp (Sn)	600 bp (Sn)	NT	NT
SnSAG 1-5-6	1000 bp (Sn)	1000 bp (Sn)	1000 bp (Sn)	NT	NT
SnSAG3	1000 bp (Sn)	1000 bp (Sn)	1000 bp (Sn)	NT	NT
SnSAG4	1000 bp (Sn)	1000 bp (Sn)	1000 bp (Sn)	NT	NT

NT = not tested.
[a] Coccidian species determined based on analysis of DNA from frozen pelleted oocsyts from feces and not culture inoculum, using ITS-1 locus PCR and sequencing.
[b] Based on genetic similarity across unambiguous sequence.
[c] 400 bp amplicon was only observed the first 1–2 wks post-inoculation. The 1000 bp PCR products were amplified continuously in CSL10089, CSL10092, and CSL10100 cultures.

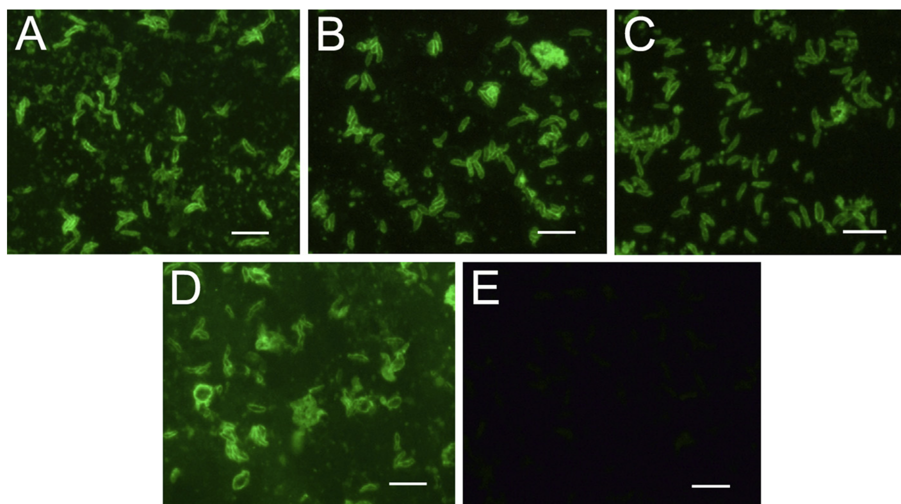

Fig. 5. Indirect fluorescence antibody test of California sea lion sporulated oocyst cultures. Zoites derived from cultivated oocysts from animal CSL10089 (A), CSL10092 (B), and CSL10100 (C) were tested using serum from a *Sarcocystis neurona*-infected horse. Image D shows reactivity of serum from the *S. neurona*-infected horse to *S. neurona* culture isolate snUCD-1. Image E shows a lack of seroreactivity of negative control serum from a *S. neurona*-negative horse to zoite culture from CSL10089. Scale bars are 15 μm.

infection with Coccidia A and B (in the case of CSL10100) (Table 4). By BLAST analysis, good quality partial sequences (~700 bp; abbreviated due to the presence of a homopolymer region) generated from the ~1000 bp amplicon in CSL10089, CSL10092 and CSL10100 zoite cultures were 99% similar to S. neurona DNA isolated from horses (AF204230 & AF081944) and from a skunk (AY082648) (See Supplementary data). Dinucleotide mixtures in the ITS-1 sequences indicated the presence of at least two *S. neurona*-like genotypes in the sea lion cultures. These polymorphic regions shared nucleotide similarity to horse strains snUCD-1 (AF081944) and SN-MU1 (AF204230), sea otter strains SO3639 (DQ084486) and SO5259, and a skunk isolate (AY082648) (See Supplementary data).

To further characterize the *S. neurona*-like organisms cultured from sea lion feces, we amplified seven additional genetic loci in DNA extracted from zoites in the sea lion oocyst culture supernatant. Table 4 displays the PCR and sequencing results for these additional loci. Using $ITS1_{500}$ primers, with known specificity for *S. neurona* or *Sarcocystis falcatula* (Gibson et al., 2011), a 500 bp product was consistently amplified in all culture supernatant samples from CSL10089, CSL10092, CSL10100 in which zoites had been observed. By BLAST analysis, good quality partial $ITS1_{500}$ amplicon sequences (~300 bp, abbreviated due to the presence of a homopolymer region) were 98% identical to *S. neurona* isolated in Southern sea otters including isolates SO3528, SO3539, SO3485, and SO3501 (GenBank DQ084485 – DQ084488). Microsatellite primers amplified ~150 bp products containing di-nucleotide repeats CA_{17} (Sn7) and GT_{18} (Sn9) found in *S. neurona* strain SN-MU1 and other *S. neurona* strains isolated from opossums, sea otters, and cats (Rejmanek et al., 2010). Merozoite surface antigen SAG1 gene sequences of CSL10089, CSL10092 and CSL10100, amplified by SnSAG 1-5-6 primers, were 100% identical across 959 bp to

S. neurona strain snUCD-1 (AF401682) (Ellison et al., 2002) and 99% similar across 968 bp to the representative sequence of the snSAG1 allele amplified in samples from terrestrial and marine mammals (GQ851951) (Wendte et al., 2010). Sea lion cultures CSL10089, CSL10092 and CSL10100 were identical to each other across a 948 bp sequence of the merozoite surface antigen gene SnSAG4 and 100% identical to sequences of *S. neurona* isolated from a sea otter (SO3106, GQ851957) and an opossum (OP134, GQ386979).

A unique sequence in sea lion *S. neurona*-like isolates was identified in the merozoite surface antigen 3 (SnSAG3) locus in which CSL10089 was confirmed by bi-directional sequencing to contain an AT insertion at positions 519–520 (Table 5). The same insert was observed in single direction sequencing for CSL10092 but could not be confirmed in CSL10100 due to poor sequence quality. The same insert was not identified in SnSAG3 sequences available in GenBank originating in a variety of host species including sea otters, opossums and horses (Table 5). Lack of the position 519–520 insert in laboratory strain SnUCD-1, derived from a horse in California (Table 5), ruled out the possibility that *S. neurona* growth in sea lion oocyst cultures was due to laboratory contamination. At other polymorphic SnSAG3 nucleotide positions, including 239 and 1059, sea lion *S. neurona*-like isolates were similar to published opossum and sea otter isolates (Table 5).

3.5. Evidence of S. neurona shedding by free-ranging sea lions

In light of the unexpected findings revealed during cell culture analysis of fecal samples from stranded hospitalized sea lions, we screened all DNA extracted from frozen, pelleted oocysts of both hospitalized and field-collected fecal samples using *S. neurona*/*S. falcatula*-specific $ITS1_{500}$ primers. While none of the additional frozen, pelleted oocyst samples collected from hospitalized animals were PCR-positive for *S. neurona*-like DNA (including those remaining for CSL10089, CSL10092 and CSL10100), two fecal samples collected at Point Lobos (Fig. 3, samples PL11 and PL27) produced ~500 bp products in the $ITS1_{500}$ PCR reaction (Table 3). Additional primers (Sn-ITS) targeting the ITS-1 region of *S. neurona* produced amplicons with sequences identical to hospitalized sea lion zoites collected in the present study as well as *S. neurona* isolated in other animals (See Supplementary data). All other attempts to amplify *S. neurona* in samples PL11 and PL27 using alternative primers failed with the exception of microsatellite Sn7 which, in sample PL27, produced a sequence containing the di-nucleotide repeat CA_{17} found in *S. neurona* and zoites isolated in CSL10089, CSL10092 and CSL10100 (Table 4).

Table 5
Nucleotide polymorphisms identified in the *Sarcocystis neurona* merozoite surface antigen 3 (SnSAG3) locus amplified from zoites grown in MA104 cell cultures inoculated with sporulated, excysted oocysts from California sea lion (CSL) fecal samples. Sequences from the CSL-derived zoites are aligned with GenBank sequences of *S. neurona* isolates from sea otter, opossum and horse tissues.

GenBank accession no.	Isolate ID[b]	SnSAG3 nucleotide position number[a]					
		239	519	520	521	522	1059
KR011915 (this study)	CSL10089	G	A	T	A	T	T
KR011915	CSL10092	G	A	T	A	T	T
Unpublished	snUCD-1 (P83)	G	–	–	A	T	T
GQ386975	OP134	G	–	–	A	T	T
GQ851954	SO3106	G	–	–	A	T	T
GQ386978	SO5283	G	–	–	–	–	T

[a] Nucleotide position numbers based on SnSAG3 nucleotide sequence amplified from an opossum isolate of *S. neurona* OP134 (GenBank accession No. GQ386975). Insertion/deletion position numbers are based on numbering reported by Rejmanek et al., 2010.
[b] SO-, sea otter; OP-, opossum; CSL-, California sea lion. Laboratory strain snUCD-1 is an isolate of *S. neurona* derived from a horse with equine protozoal myeloencephalitis (EPM). The nucleotide sequence was obtained at culture passage number 83.

4. Discussion

The investigation of infectious diseases in coastal aquatic wildlife allows us to probe the relationships between terrestrial-origin pathogen distribution, patterns of overland water runoff, and the risk of pathogen pollution in the coastal and ocean environment. The present research was undertaken to investigate the role of California sea lions in the dispersal and life cycle of terrestrial and aquatic coccidian parasites that pose a risk to marine mammal health, and to genetically and phenotypically characterize protozoa shed by sea lions.

4.1. Sea lions shed Coccidia A, B and CSL Año11, possibly of aquatic origin

Building on data acquired from previous protozoal pathogen surveillance efforts in California sea lions from the coastal waters of California (Carlson-Bremer et al., 2012a), we confirmed that oocysts (8–10 μm in size) that are morphologically similar to, but genetically distinct from, *T. gondii* and *N. caninum* are frequently shed by stranded sea lions that are hospitalized and by free-ranging populations. Genetic variability in the partial ITS-1 region of these Coccidia A and B isolates was not observed within this study or in previous reports (Colegrove et al., 2011; Carlson-Bremer et al., 2012a). Consistent with other studies of stranded sea lions, shedding of Coccidia A and B occurred primarily in yearlings, and those shedding oocysts had stranded due to causes other than protozoal infection. While our data further establish that sea lions are naturally asymptomatic definitive hosts of Coccidia A and B, the pathogenic potential of these protozoa in other aquatic species requires further investigation, particularly considering the known virulence of Coccidia C and other novel tissue-cyst forming coccidia in harbor seals and Guadalupe fur seals (Colegrove et al., 2011; Gibson et al., 2011).

Phylogenetic analyses of the ITS-1 locus performed here and elsewhere (Gibson et al., 2011; Carlson-Bremer et al., 2012a) demonstrate that Coccidia A, B, C, CSL Año11, and isolates from a harbor seal and Guadalupe fur seals share a common ancestor with *N. caninum*. Morphologically, however, Coccidia A and B measured in this study were smaller than *N. caninum* oocysts and had <80% pairwise similarity to *N. caninum* at the ITS-1 locus. While unsporulated Coccidia A and B oocysts shed by sea lions are 8–10 μm in diameter, unsporulated *N. caninum* oocysts shed by dogs are typically 10–11 μm in diameter (McAllister et al., 1998). Sporulated *N. caninum* oocysts are reportedly an average of 11.7 × 11.3 μm (Lindsay et al., 1999) while sporulated forms of Coccidia A and B are an average of 9.8 × 8.6 μm, and 9.9 × 9.0 μm, respectively. By comparison, unsporulated *T. gondii* oocysts are 10 × 12 μm in diameter and sporulated forms are 11 × 13 μm in diameter (Dubey et al., 1998). More detailed genetic and morphologic studies (e.g. whole genome sequencing, electron microscopy, and histological examination of life stages *in situ*) are necessary to clarify the taxonomy of novel coccidia identified in aquatic animals, and will likely reveal that these organisms belong to either new *Neospora* species or a new genus within the family Sarcocystidae.

4.2. MA104 cells and laboratory mice are not susceptible to Coccidia A and B

At the phenotypic level, we observed that the sporulated oocysts of Coccidia A and B were not cultivable in the African green

monkey kidney cell line, MA104, commonly used to propagate other tissue-cyst-forming coccidia including *T. gondii*, *N. caninum* and *S. neurona* (Miller et al., 2001a, 2004; Holmberg et al., 2006). While Coccidia A DNA was detectable by PCR in a culture prepared from oocyst isolate CSL10266 for up to two weeks after inoculation, no protozoal organisms were ever observed. In MA104 cultures of oocyst isolates CSL10092 and CSL10100, Coccidia A and B DNA as well as *S. neurona*-like DNA were amplified for up to 5–6 weeks post-inoculation; after which time only *S. neurona*-like DNA and live *S. neurona*-like zoites were detected for the duration of observation. While it is possible in the latter cases that Coccidia A and B did not replicate *in vitro* due to competition from the *Sarcosystsis* sp. during co-culture, we hypothesize, rather, that the MA104 cell line may not be susceptible to infection with Coccidia A and B, or the culture conditions were not optimal for these parasites. Other cell culture systems should be investigated for their propagation, particularly in the interest of developing a serological assay for the purpose of screening marine mammals for infections with these and other potentially pathogenic protozoa.

Coccidia A and B did not cause disease in mice with known susceptibility to *T. gondii* including B6.129S7-*Ifng*^{tm1Ts}/J, CBA/CaJ and Swiss Webster (CFW®) (Rytel and Jones, 1966; Villegas et al., 2002; Gavrilescu and Denkers, 2003). Surprisingly, Coccidia A and B were not pathogenic to CBA/CaJ mice despite previous studies showing that CBA/Ca mice can be used as a model of *N. caninum* pathogenesis (McGuire et al., 1997; Rettigner et al., 2004). These findings, taken together with the lack of growth in MA104 cells or reactivity of serum from Coccidia A- and B-infected sea lions with *N. caninum*, *T. gondii* or *S. neurona* antigen by IFAT, is further evidence of the taxonomic divergence of the sea lion parasites from known tissue-cyst forming coccidia with terrestrial life cycles.

4.3. California sea lions shed low levels of S. neurona or an S. neurona-like species

A significant outcome of this study was the discovery of *S. neurona* or *S. neurona*-like zoites growing in cell cultures inoculated with fecal material (including sporulated, excysted oocysts of Coccidia A and B) prepared from three stranded hospitalized sea lions sampled between 2010 and 2012. The cultivation of live protozoal zoites with strong genetic similarity to *S. neurona* across multiple genes during parasite isolation from sea lion feces, particularly in the absence of microscopic evidence of *Sarcocystis* sp. sporocysts in the fecal samples, is puzzling. A possible explanation for these findings is that samples taken for microscopic observation during fecal flotation only represent a small portion of the overall feces sampled and the overall pool of oocysts being shed. If the original pool of isolated protozoa from feces consisted of a mixture of variable numbers of coccidian oocysts (Coccidia A and/or B) and sporocysts (*S. neurona*-like), it is possible that the subdivision of the oocyst pool for flotation and microscopy, pelleting/freezing, sporulation for mouse bioassay, and sporulation/excystation for cell culture, could explain discrepancies in the ability to detect different parasites, depending on the sensitivity of each assay. We hypothesize that a small number of *S. neurona*-like sporocysts existed in the pool of protozoa prepared for cell culture, but were not present in the subsamples designated for microscopy, pelleting and freezing or mouse bioassay. In addition, *in vitro* cultivation may have facilitated the expansion of small numbers of *S. neurona* sporozoites that were able to develop into schizonts that produced merozoites.

The isolation of *S. neurona*-like DNA from feces of two free-ranging sea lions at Point Lobos indicates that the organism may occur naturally in sea lions, as opposed to occurring via nosocomial exposure in the hospitalized setting. Exposure of sea lions to *S. neurona*, or an *S. neurona*-like organism, most likely occurs in coastal waters contaminated with opossum feces transported from land-to-sea via freshwater run-off (Carlson-Bremer et al., 2012b; Shapiro et al., 2012) or may be concentrated in sea lion prey, as has been shown with *T. gondii* shed by terrestrial felids (Miller et al., 2002; Johnson et al., 2009). Although the extent of environmental contamination with *S. neurona* sporocysts has not been documented, infections in Southern sea otters and a number of other marine mammals of the Pacific Northwest have been reported (Miller et al., 2001a; Miller et al., 2001b, 2009, 2010; Kreuder et al., 2003; Barbosa et al., 2015).

All mouse tissues were PCR-negative for *S. neurona* using $ITS1_{500}$ primers, indicating that the sea lion *S. neurona*-like sporocysts were either not present in the inoculum, were present but below the threshold of infection, or the mice utilized were not susceptible. Because of the similarity of Coccidia A and B to *T. gondii*, mice in this study were selected for their susceptibility to *T. gondii*, not *S. neurona*. Future studies aimed at describing the phenotype of *Sarcocystis* spp. shed by sea lions would benefit from the utilization of interferon gamma gene knock-out mice (e.g. C57BL/6-black) with well-characterized susceptibility to *S. neurona* (Dubey et al., 2013).

Sarcocystis neurona is the etiologic agent of equine protozoal myeloencephalitis – a serious and progressively debilitating neurological disease in horses– and is one of several coccidian parasites known to cause protozoal encephalitis in Pacific harbor seals, southern sea otters, and other marine mammals (Lapointe et al., 1998; Rosonke et al., 1999; Lindsay et al., 2000, 2001; Miller et al., 2001a; Lapointe et al., 2003; Thomas et al., 2007; Gibson et al., 2011; Dubey et al., 2015). Although not frequently reported in the literature, when *S. neurona* infections do occur in California sea lions, they are either clinically irrelevant (Gibson et al., 2011) or associated with myositis (Carlson-Bremer et al., 2012b). Tissue cysts caused by other *Sarcocystis* spp., including *Sarcocystis canis*-like organisms, have been detected in sea lion muscle (Mense et al., 1992; Miller, 2008) and liver tissue (Mense et al., 1992; Dubey et al., 2003). The three animals that shed *S. neurona*-like protozoa were admitted to TMMC for malnutrition or trauma, and had no overt signs of protozoal infection. Lack of serum titers to *S. neurona* in these animals indicated that they had not yet mounted a detectable antibody response, or that they never had systemic infections. An elevated *S. neurona* titer (1:2560) was detected in another hospitalized sea lion, CSL9878, but this animal also had no signs of disease and was not shedding detectable levels of *S. neurona*-like sporocysts or DNA at the time of sampling for this study.

Overall, findings from this study suggest that California sea lions are likely acting as mechanical vectors of *S. neurona* or an *S. neurona*-like organism, similar to the way canines can mechanically transmit *T. gondii* (Lindsay et al., 1997). Using a mouse bioassay to detect the presence of infectious *T. gondii* oocysts in canine tissues and feces, Lindsay et al. (1997) found that “sporulated *T. gondii* oocysts can traverse the canine intestinal tract and be excreted in an infectious state”. Several experimental outcomes indicate that an S. *neurona*-like organism is excreted by sea lions in low concentrations, which is expected during mechanical transmission: (1) *S. neurona*-like sporocysts were not observed by light microscopy following fecal flotation (2) *S. neurona*-like DNA was not amplified from concentrated oocysts (pellets) isolated from hospitalized sea lion fecal samples, (3) *S. neurona*-like DNA was only amplified by PCR on high copy number genes in concentrated oocysts isolated from feces of free-ranging animals, and (4) *S. neurona*-like merozoites were only observed after long-term cultivation in MA104 cells.

The genetic similarity of sea lion *S. neurona*-like isolates to previously characterized protozoa in aquatic and terrestrial animals

across much of the genome is not surprising considering the extensive overlap in genetic identity in *S. neurona* isolates from the tissues of opossums, sea otters and horses that was previously demonstrated (Rejmanek et al., 2010; Wendte et al., 2010). The key SnSAG3 polymorphism that we identified in the zoites from sea lion fecal samples may represent a novel *S. neurona* genotype which, in the future, may facilitate identification of the origin of infection. This is particularly important as we seek to understand the flow of terrestrial pathogens into oceans and risk factors for *S. neurona* infections in other aquatic species. Continued investigations into the pathogenesis of *S. neurona* in sea lions through necropsy and systematic surveillance for *S. neurona* exposure, shedding, and disease will help clarify the importance of this host species in parasite transmission within the Pacific coastal ecosystem.

In conclusion, continued surveillance for tissue-cyst forming coccidia in marine mammals should remain a priority for programs that monitor the population health of aquatic wildlife. Efforts to explore the distribution, pathogenesis and genetic diversity of Sarcocystidae in land and sea isolates promise to improve our understanding of the interconnectedness between terrestrial and aquatic microbial diseases, and to direct interventions that will benefit our oceans and their inhabitants.

5. Acknowledgments

Funding for this work was provided by the National Science Foundation (NSF) Ecology of Infectious Disease program (OCE-1065990). We are very grateful for the help of Jennifer Soper (TMMC), as well as Tom Saicheur, Beatriz Aguilar, and Terra Berardi (UC Davis) for sample processing and diagnostics in the laboratory. Many thanks go to Aiko Nakashima, Beatriz Aguilar, Terra Berardi, Don Canestro, Brian Hatfield, Mike Kenner, Holly MacCormick, Pat Morris, Zachary Randell, Tim Tinker, Joe Tomoleoni, Ben Weitzman, Collin Kruzor who worked to collect sea lion fecal samples in the field; and to the California Animal Health and Food Safety laboratory for providing horse serum. Collection of CSL feces from the White Rock haul-out site was facilitated through the University of California Ken Norris Rancho Marino Reserve.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ijppaw.2015.11.003.

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