Characterization and Epidemiologic Investigation of Apicomplexan Parasites Associated With Meningoencephalitis in Southern Sea Otters (*Enhydra lutris nereis*) and Pacific Harbor Seals (*Phoca vitulina richardsi*)

BY

MELISSA ANN MILLER

DVM (University of California, Davis) 1994

DISSERTATION

Submitted in partial satisfaction for the requirements for the degree of

DOCTOR OF PHILOSOPHY
in
COMPARATIVE PATHOLOGY

in the

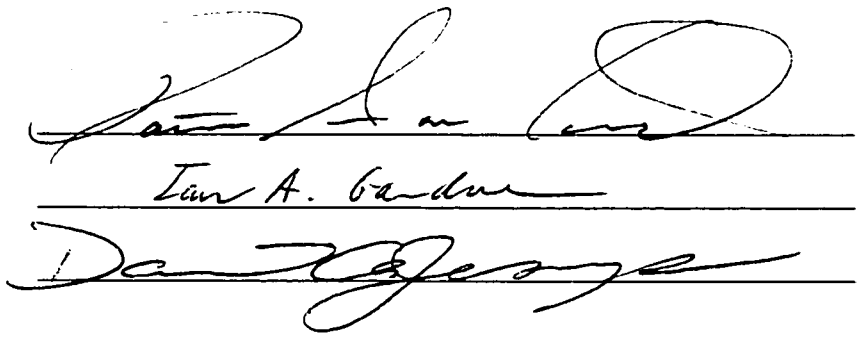
OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:



Committee in Charge

2002

Melissa Ann Miller
December, 2002
Comparative Pathology

Characterization and Epidemiologic Investigation of Apicomplexan Parasites Associated With Meningoencephalitis in Southern Sea Otters (*Enhydra lutris nereis*) and Pacific Harbor Seals (*Phoca vitulina richardsi*)

ABSTRACT

Protozoal meningoencephalitis was first recognized as a cause of mortality for southern (California) sea otters and harbor seals in 1992. My dissertation research to characterize these infections was initiated in 1998, with the specific goals to: 1.) isolate and confirm the identity of these protozoal parasites 2.) investigate the relationship between protozoal parasites of otters and seals and those associated with illness of terrestrial animals and humans 3.) develop and validate serodiagnostic tests, and 4.) evaluate potential risk factors for protozoal exposure. Between 1998 and 2002, 180 otters and 20 seals were necropsied, 400 otters and 100 seals were evaluated serologically and 55 protozoal isolates were obtained. Our isolates were characterized antigenically, molecularly and ultrastructurally, and were found to be indistinguishable from *Toxoplasma gondii* and *Sarcocystis neurona*. Over 36% of otters examined between 1997 and 2001 were infected with *T. gondii* at necropsy, while 4% were infected with *S. neurona*. Otter and seal sera were assessed for *T. gondii* and *S. neurona* antibodies using indirect fluorescent antibody tests (IFATs), and the otter *T. gondii* IFAT was validated using serum from confirmed-infected otters. Sera from California, Washington and Alaska otters were evaluated. The proportion of *T. gondii*-positive otters was comparable for California and (36%) and Washington (38%), whereas 0% of Alaskan otters were seropositive. An epidemiological study was performed to assess live and dead sea otter data for associations between *T. gondii* IFAT seropositivity and defined risk factors for *T. gondii* infection. Seropositivity was positively correlated with male gender, increasing age and dead versus live status. Spatial analysis revealed two clusters of *T. gondii*-seropositive otters and one cluster of seronegative otters. Otters at one high-risk site were 9 times more likely to be seropositive to *T. gondii* than otters sampled at other sites. No association was found between *T. gondii* seropositivity and human population density or sewage exposure. Otters sampled near maximal freshwater runoff were 3 times more likely to be seropositive to *T. gondii* than otters sampled in areas of low flow. This study provides evidence that surface runoff is a source for *T. gondii* infection for southern sea otters.

Patricia A. Conrad, DVM, PhD
Professor of Parasitology
Major Advisor

ACKNOWLEDGEMENTS

First I must acknowledge and most humbly thank my major professor and friend, Patricia Conrad. A better graduate student then myself likely exists, and in fact is probably not even rare! A better graduate mentor does not exist. Patricia Conrad is and will always remain a "glass-half-full" person, the consummate cheerleader and team-builder, and she provided the inspiration, energy and good humor to sustain me through the early stages of this graduate program, when I feared we'd never get this important research funded, through the middle, when I thought I'd never get this important work done, right on through to the end, when I still thought I'd never get it done! I have learned a lot from this journey, most of it good, all of it important. Pat knows the difference between doing things for her mentees, and enabling and inspiring them to achieve for themselves. To that end, she has helped me to become a good writer and much better scientist by patiently providing excellent, constructive feedback on each and every one of the many writes and re-writes of study proposals, grant applications, scholarship applications and manuscripts. When I had no funding early in the course of my studies, Pat provided the seed money to get things rolling. When I stumbled, many times, personally and professionally, she helped me to get back on the right path. Pat gives far more of herself to her graduate students than any other professor I have ever seen, and does so with endless enthusiasm and good humor. Patricia Conrad, I thank you for making room for me in your lab, and also for making space for me in your heart.

David Jessup. I idolized you and others of your ilk when I was a speck, working my way up the food chain in veterinary school and you were one of the world's original wildlife veterinarians! You were what I wanted to be, way back then. It was a dream that

seemed so far out of reach. You helped to make my dream happen, in its own way and time, and for that I thank you. You should know that you are one of the most courageous men I have ever seen, and I respect you for continually "daring to be different"! Now you are my boss and friend. I hope to see that relationship grow as we continue to nurture and support this collaborative research team.

Dr Jonna Mazet: Thanks for always making space for me on "the team", for providing encouragement and support, and for helping me to find and regain my way. Thanks also for your honesty and courage, which are inspiring.

I would like to especially thank Drs Jessup, Mazet and Conrad, whose broad-mindedness and stalwart support allowed me to complete my dissertation research while employed full-time working my "dream job" as a veterinary pathologist and researcher for CDFG and the UCD Wildlife Health Center. This has been a difficult road, in part due to unforeseen "bumps", but they have remained patient, kind supportive and enthusiastic throughout, and unfailingly served as my cheering section when I, for a short while, lost my way.

Dr Ian Gardner: I have learned a great deal about epidemiology and the business of good science from you, and a lot about life as well. I thank you very much for those lessons, and for your encouragement, support and critical review of this work.

Dr Bradd Barr: You are an excellent pathologist, and I respect you very much. Thank you for your encouragement, support and critical reviews.

Heartfelt thanks go to Dr Frances Gulland, for her constant support, incredible positive energy and encouragement. This project would never have happened without Frances's mentorship, leadership and facilitation.

Dr Linda Lowenstine: Thank you very much for your early encouragement and support, and for showing me the difference between good and outstanding work as a wildlife pathologist.

To Dr Paul Crosbie, Professor of Parasitology, Fresno Sate University, who oh-so patiently and humorously helped me to learn to be a good scientist and author.

To Carla Crane for helping me to learn the beaurocratic side of grant writing, with patience, good humor and easy grace.

Dr Chris Kreuder: You made epidemiology fun, alive and very real for me. Thank you for your friendship, kindness and good cheer.

To Erin Dodd. You are one classy lady. We could all learn a lot from you. I am honored to have been a part of your life, and hope that as you go out in the world you will one day look back on this whole crazy experience, laugh and be glad that you were here!

To Mike Harris and Jack Ames: They don't call you guys the "A Team" for nothing! Thanks for the "real world" education in otter biology and for the patient and detailed perusals of manuscripts and grant proposals, the pep talks, the interesting insights into sea otter digestive function and the pitchers of margaritas.

Dr Anne Kjemtrup: You and Pat were cut from the same cloth. Thanks for the PCR lessons, the coaching for my oral exam and your unfailing good cheer.

To Grace Lee. Your name characterizes you well. Thanks for patiently answering a million questions, and for always making time for folks like me.

To Karen Worcester and David Paradies, the most effective "effector arm" folks I have ever met. You have restored my faith in state government! I look forward to many other chapters together in this long path of exploration.

Thanks also to the clinical veterinarians in California who helped to facilitate our work, and who worked late nights, weekends and holidays in hopes of saving otters and seals with protozoal meningoencephalitis. They succeeded. They include Drs Mike Murray, Frances Gulland, Marty Haulena, Dave Casper, Debbie Fauquier, Shawn Johnson, Tom Riedarsen, Judy St. Leger and Jim McBain.

This work could not have been completed without the sustained effort of staff and volunteers from California Department of Fish and Game (CDFG), the Monterey Bay Aquarium (MBA), The Marine Mammal Center (TMMC), United States Geological Survey, US Fish and Wildlife Service, UC Santa Cruz, Moss Landing Marine Laboratories and other members of the California Marine Mammal Stranding Network. Their dedication and caring is evident in the excellent quality of the samples received for necropsy and serological testing. Very special thanks go to Dave Jessup, Jack Ames, Mike Harris, Erin Dodd, Heather Harris, Jack Hawkes, Debbie Brownstein, Bryant Austin, Frank Wilhelm, Nancy Christian, Laura Yeates, Christine Arnold and Sharon Toy-Choutka of CDFG, Mike Murray, Michelle Staedler, Andy Johnson, Sue Campbell and Karl Mayer of MBA, Frances Gulland, Marty Haulena, Sue Pemberton and Bill and Jennifer Lazelere of TMMC, Brian Hatfield, Mike Kenner, Tim Tinker and Jim Estes of USGS, Greg Sanders of USFWS, Dave Casper of UCSC and Stori Oates and Jim Harvey of MLML. Likewise, portions of this research completed at UCD were significantly assisted by Karen Sverlow, Andrea Packham, Ann Melli, Paul Crosbie, Tom Robinson, Anne Kjemtrup, Grace Lee and Antoinette Marsh. Veterinary students Woutrina Smith, Amy Wells, Astrid Pulley and many others assisted on late-night otter necropsies. Jim

Estes, Stori Oates, Jim Harvey, Jim Bodkin, Tracey Goldstein, Dave Jessup, Krista Hanni and Shawn Larson and many others facilitated sample acquisition.

To my family: Mom, Dad, Sara, Dane, Kevin, Dane E., Seth and Taylor, Daniel, Berta, Martha and Leah for being there. To Phil: A journey well begun, and not regretted. Thank you for believing in me early on. To Dr David Ebert who refused to let me stay discouraged for any reason. And finally, to the late Dr Richard Stroud, Professor Emeritus, Animal Science, University of New Hampshire, who first taught me the pure joys of good science and good teaching: Thank you for your mentorship. I will never forget you.

This work was completed at the UCD Department of Pathology, Microbiology and Immunology, the Veterinary Medical Teaching Hospital and the California Animal Health and Food Safety Laboratory, University of California, Davis, as well as the Marine Wildlife Veterinary Care and Research Facility, California Department of Fish and Game in Santa Cruz, California. This research was supported through the National Sea Grant Program, Department of Commerce, PKD Trust, the San Francisco Foundation, the Marine Mammal Center, Morris Animal Foundation, UCD Wildlife Health Center, the California Department of Fish and Game, the Lindbergh Foundation, UCD Genetic Resource Foundation, and Friends of the Sea Otter.

Last, but never, never least, I must acknowledge the kindness and love for sea otters of two individuals, Phil and Karen Drayer, who made this work possible and allowed me to follow my dream of becoming a wildlife pathologist.

This dissertation is dedicated to Phil and Karen Drayer

Thank you for making this research possible,
for your unwavering dedication to southern sea otter research and conservation,
for your friendship and support
and for making my dreams come true.

TABLE OF CONTENTS

Chapter 1

Introduction

INTRODUCTION

(i) Chapter Overview

This chapter provides an overview of the biology and molecular characterization of two species of Apicomplexan protozoa, *Toxoplasma gondii* and *Sarcocystis neurona* (Class: *Sporozoa*, Order: *Eimeriorina*, Family: *Sarcocystidae*). Both parasites have been implicated in cases of fatal meningoencephalitis in free-living southern sea otters (*Enhydra lutris nereis*) and Pacific harbor seals (*Phoca vitulina richardsi*) along the central California coast. Information about the biology and life cycles of these two coastal marine mammal species relevant to this research project is also discussed.

(ii) History of Protozoal Encephalitis in Pacific Coast Sea Otters and Harbor Seals

Numerous anecdotal reports of *T. gondii* infection and *T. gondii*-associated pathology in marine mammals exist in the scientific literature (reviewed in Chapters 2 and 3), but the environmental significance of this parasite was overlooked until it was reported as a large-scale pathogen of southern sea otters (Thomas and Cole, 1996). Protozoal meningoencephalitis was first recognized as a cause of mortality in southern sea otters (*E. l. nereis*), a federally listed threatened species, and sympatric Pacific harbor seals from California in 1992 (Thomas and Cole, 1996; Lapointe et al., 1998). Both free-ranging and captive sea otters and seals were affected (Thomas and Cole, 1996; Lapointe et al., 1998; Rosonke et al., 1999), and two distinct types of intracellular protozoal parasites were implicated in these infections. In all reported cases, affected animals were either found dead or died shortly after hospitalization. Affected otters and seals exhibited

severe neurological disease, including muscle tremors, seizures, deficits in ambulation or proprioception, paresis, pupillary mydriasis and obtundation (Lapointe et al., 1998; Rosonke et al., 1999; Murray, Gulland, Haulena et al., unpublished data). Until recently, neurological signs, once apparent, were invariably progressive and the disease fatal despite aggressive medical intervention. Microscopic examination of tissue from these animals revealed moderate to severe meningoencephalitis in association with proliferating intracellular protozoal parasites. In 1998 a long-term study was initiated as my dissertation project, to better understand protozoal brain infections of California sea otters and harbor seals.

One of the two parasites implicated in these fatal infections was a parasite similar or identical to *Toxoplasma gondii*, a well-recognized terrestrial pathogen of domestic animals and humans (Thomas and Cole, 1996). Based on the examination of freshly dead otters collected from coastal California between 1992 and 1995, 8.5% of total sea otter mortality was attributed to protozoal meningoencephalitis, with the majority of cases attributed to a *Toxoplasma gondii*-like parasite (Thomas and Cole, 1996). This finding was surprising, given that the only animal hosts known to shed the tough, environmentally-resistant oocyst stage of *T. gondii* were felids, including domestic cats. If this parasite was indeed *T. gondii*, how could these oocysts be entering the nearshore marine environment in sufficient quantities to affect significant numbers of sea otters?

At about the same time, a second parasite, similar or identical to *Sarcocystis neurona* was found to be the major cause of mortality for adult Pacific harbor seals that were hospitalized at the world's largest marine mammal stranding and recovery center, located in northern California (Lapointe et al., 1998). Based on histopathology, electron

microscopy and preliminary antigenic characterization, this parasite appeared to be similar or identical to *Sarcocystis neurona*, the causative agent of equine protozoal myeloencephalitis, or EPM (Simpson and Mayhew, 1980; Davis et al., 1991; Dubey et al., 1991; MacKay et al., 1992). Interestingly, during this same time period, fatal meningoencephalitis was also reported in a captive northern sea otter (*E. l. kenyoni*) housed at a private aquarium in central Oregon (Rosonke et al., 1999), and in a captive harbor seal housed at a private zoo in the central valley of California (Lapointe et al., 1998). A *S. neurona*-like agent was also implicated in those infections. As with the *Toxoplasma*-like parasite, infections of coastal marine mammals at multiple locations along the Pacific coast with a *S. neurona*-like parasite was unexpected, given that the only animal hosts known to shed environmentally-resistant sporocysts of *S. neurona* in their feces were new world opossums (eg *Didelphis virginiana* and *D. albiventris*). The ability of opossums to serve as definitive hosts for *S. neurona* was confirmed by Fenger et al., 1995, Dubey and Lindsey, 1998 and Dubey et al., 2001a, 2001c, 2001d.

In 1998, when this dissertation research was initiated, both *T. gondii* and *S. neurona* were implicated as the cause of fatal meningoencephalitis in California sea otters and harbor seals. However, all earlier reports were based on histopathology, electron microscopy and limited serological testing. Isolation, molecular characterization and comparison of these parasites to similar parasites isolated from terrestrial animals and humans had not been reported. Thus *in vitro* isolation and confirmation of the identity of these parasites via molecular techniques was an important first step in investigating this apparent land-sea connection, and these efforts served as an initial focus of my PhD research. Parasite isolation from aseptically-collected otter and harbor seal brain tissue

was coupled with detailed postmortem examinations, and isolated parasites were characterized using antigenic, ultrastructural and molecular techniques. Antigenic characterization consisted of immunohistochemistry and immunofluorescent antibody tests (IFATs) for *T. gondii* and *S. neurona*. Ultrastructural characterization was completed for selected isolates using transmission electron microscopy of parasite-infected cell cultures. Molecular characterization was performed via amplification and sequencing of two phylogenetically informative segments of parasite nuclear DNA. A second research goal was to compare the taxonomic relationship between *T. gondii* and *S. neurona*-like isolates from otters and seals with those derived from terrestrial animals and humans. Investigation of this molecular relationship was critical to provide clues as to the possible source(s) of these pathogenic parasites in the nearshore marine environment.

The final phase of this research was aimed at developing the tools and knowledge to better define risk factors for *T. gondii* and *S. neurona* infection of otters and seals in the marine environment. Through this work, we hoped to learn more about the frequency of these infections in wild otter and seal populations, and to begin to investigate the association between parasite infection and disease, discernable either clinically or on histopathology.

(iii) Hypotheses and Objectives

The overall goal of this doctoral research project to was test five hypotheses concerning protozoal infections in Southern (California) sea otters and harbor seals:

HYPOTHESIS I: *Toxoplasma gondii and Sarcocystis neurona are present in brain tissue of California sea otters and harbor seals.*

HYPOTHESIS II: *T. gondii and S. neurona isolates from sea otters and harbor seals are indistinguishable from isolates of the same species that infect humans and/or terrestrial animals.*

HYPOTHESIS III: *T. gondii brain infections in sea otters are associated with the presence of detectable parasite-specific serum antibodies*

HYPOTHESIS IV: *Seroprevalence to T. gondii varies between geographically distinct sea otter populations in California, Washington and Alaska.*

HYPOTHESIS V: *Spatial, demographic and environmental "risk factors" exist for sea otter exposure to T. gondii in California.*

The specific objectives of my dissertation research were designed to address these hypotheses:

OBJECTIVES

1. *To confirm the identity of protozoa infecting sea otter and harbor seal brain tissue, using ultrastructural, immunologic and molecular techniques.*

2. *To investigate the phylogenetic relationship between protozoal parasites isolated from sea otter and seal brain tissue and morphologically similar protozoans associated with illness and mortality of terrestrial animals and humans.*

3. *To develop and, where possible, validate serodiagnostic tests for the protozoal parasites associated with encephalitis in sea otters.*

4. *To use these validated tests to screen geographically distinct sea otter populations in California, Washington and Alaska.*

5. *To evaluate potential demographic, spatial and environmental risk factors for T. gondii exposure and disease.*

Each hypothesis and the corresponding specific objectives are addressed in detail in subsequent chapters. Hypotheses I and II (Objectives 1 and 2) are addressed in Chapter 2: *Isolation and characterization of Sarcocystis neurona from a southern sea otter,* and Chapter 3: *Isolation and characterization of parasitic protozoa from a Pacific harbor seal*. Hypotheses III and IV (Objectives 3 and 4) are addressed in Chapter 4: *Evaluation of an indirect fluorescent antibody test for detection of Toxoplasma gondii in sea otters.* Hypothesis V (Objective 5) is addressed in Chapter 5: *Freshwater runoff is a risk factor for Toxoplasma gondii infection in sea otters*. Chapter 6: *Summary and Conclusions,* summarizes the important findings of this dissertation, provides additional details

concerning patterns of *T. gondii* and *S. neurona* infection in sea otters and harbor seals and suggests directions for future research.

The original intent of this dissertation research was to explore the origin and identity of protozoal brain infections of both sea otters and sympatric harbor seals. However, obtaining sufficient numbers of harbor seals for necropsy proved difficult. Although harbor seals are numerous in California, few adult animals are submitted to local wildlife rehabilitation facilities. Because sea otters are a federally-listed threatened species, a rangewide, multi-agency carcass recovery system exists that facilitates rapid collection and postmortem examination. Thus, sea otters became the main focus of this research. To best understand the significance of protozoal brain infections in southern sea otters and seals, it is important first to review the basic biology and food habits of these nearshore-dwelling marine mammals.

(iv) Biology of Southern Sea Otters

The sea otter (*Enhydra lutris*) is the smaller of two species of marine otters recognized worldwide, and the world's smallest marine mammal (Reynolds et al., 1999). Adult male sea otters may exceed 30 kg body weight and 129 cm total body length (Reidman and Estes, 1990). Three subspecies of *E. lutris* are recognized: *E. l. lutris* from southeast Russia, *E. l. kenyoni* from coastal Alaska, Canada and Washington and *E. l. nereis* from California (Riedman and Estes, 1990; Wilson et al., 1991; Anderson et al., 1996). Only *E. l. nereis*, the southern sea otter is federally listed as a threatened species, although the Aleutian subpopulation of the Alaskan sea otter is currently in severe decline (Estes et al., 1998), and may become federally listed in the near future. Southern

sea otters, which are found only along the central coast of California, are distinguished from their northern counterparts by their smaller size and by genetic and skeletal markers (Riedman and Estes, 1990; Wilson et al., 1991; Anderson et al., 1996; Bodkin et al., 1999).

Historically, southern sea otters were present throughout coastal California, and extended well into Mexico along the Baja Peninsula. However, sea otters have the densest fur of any marine mammal, over 160,000 hairs/ cm^2 (Reidman and Estes, 1990), and this attribute led to widespread exploitation of sea otters for their luxurious pelts from the 1700s through 1911 (Estes, 1980). Drastic population reductions coincided with the advent and growth of the Pacific maritime fur trade, and resulted in the near-extirpation of this species throughout its range. Protection was first afforded with the International Fur Seal Treaty in 1911 and California state protection (1913), and was later strengthened by the Marine Mammal Protection Act (1972) and listing under the Endangered Species Act (listed in 1977). However, these protective measures almost came too late: by the beginning of the 20^{th} century, the number of southern sea otters had declined precipitously, and for years this species was thought to be extinct (Estes, 1980; Reidman and Estes, 1990). However, in 1938 a few otters were observed along the remote Big Sur Coast of central California (Fisher, 1942). This remnant population, numbering perhaps as few as 50 animals, has provided the nucleus for southern sea otter repopulation. Since 1990, annual counts of the southern sea otter population have ranged between 1,680 and 2,377 animals, occupying a 600 km range that extends from Half Moon Bay south to near Santa Barbara, and spans the central third of the California coast.

Female southern sea otters mature at approximately 3 years of age and thereafter produce one pup annually. Pupping occurs throughout the year, with seasonal peaks in spring and fall. The smaller pupping peak in fall has been attributed to early pup loss during the spring, resulting in repeat breeding of spring-peak females (Reidman and Estes, 1990). A single pup is born in the water with a thick natal pelage that facilitates floating (Reidman and Estes, 1990). Lactating females have two inguinal teats. Lactation, rather than pregnancy appears to be a major physiological stressor for reproductive-aged females (Estes, 1980; Miller, unpublished necropsy data). Pups are fully weaned and maternal care ceases at approximately six months of age (Estes, 1980). Within days of weaning her pup, the female enters estrus and breeds. Male southern sea otters bite and grasp the female's nose during copulation (Reidman and Estes, 1990). Gestational length averages 6 months, and consists of approximately two months of delayed implantation and four months of fetal development post-implantation (Reidman and Estes, 1990).

Polygynous adult male otters establish and defend territories within or adjacent to female home ranges, and defend the territory and females against subdominant males (Estes, 1980; Jameson, 1989). Annual seasonal (fall and winter) movements of males from the central (female-dominated) portions of sea otter range to the northern and southern range peripheries are well documented (Ralls et al., 1996). Females are more sedentary, but rare, wide-ranging movements of either gender have been documented (Pederson and Stout, 1963; Leatherwood et al., 1978).

Despite attaining fully protected state (1913) and federal (1977) status, southern sea otter populations have not recovered at the same rate as their northern counterparts, with consistently lower annual population growth rates of 5-7% for California otters,

compared to 17-20% for Alaskan otters (Reidman and Estes, 1990). There is additional reason for concern: since 1995, annual rangewide sea otter counts have suggested a stagnant or declining southern sea otter population (USGS, unpublished data). The reduced population growth rates and recent population declines observed for southern sea otters have been attributed to increased mortality, rather than decreased fecundity. Also of concern is the observation, based on postmortem examination of freshly dead otters, that approximately 40 to 45% of southern sea otter mortality may be attributable to infectious agents (Thomas and Cole, 1996; Kreuder et al., 2003). This pattern of mortality is not typical of a healthy, free-ranging wildlife population (Daszak et al., 2000; Daszak et al., 2001).

Sea otters are a nearshore marine mammal and typically spend all of their time within 1-2 km of the shoreline. These animals have broad, muscular jaws and bunodont-style molars designed to crush the shells of hard prey items (Reidman and Estes, 1990). Use of tools such as rocks, bottles and bricks for opening hard-shelled prey is well documented. Tools are placed on the ventral chest, and the prey item is pounded against the rock or brick repeatedly using both front paws. The tool as well as prey items may be tucked within a large flap of skin inside of each front leg during foraging dives (Reidman and Estes, 1990).

A wide variety of prey species are consumed by southern sea otters. Their diet consists primarily of benthic and kelp canopy invertebrates such as shellfish, urchins, abalone, snails and worms (Estes et al., 1986). Squid and octopus are also avidly consumed. Southern sea otters forage in many types of habitat, including rocky shorelines, sandy areas, kelp beds and mud-bottom estuarine environments (Estes, 1980;

Kvitek and Oliver, 1987; Reidman and Estes, 1990). Individual sea otters may develop strong preferences for one or more prey species, and as a result will preferentially occupy coastal habitats where these food items occur (Reidman and Estes, 1990). Adult females appear to teach their prey species selection, foraging-style and habitat preferences to their pups (Reidman and Estes, 1990). The sea otter is considered a keystone species: by preying on kelp-grazing macroinvertebrates, such as urchins and snails, sea otters may help promote the growth and expansion of kelp forests (Dayton, 1975; Estes and Steinberg, 1988).

Sea otters lack a dermal blubber layer and rely instead on a dense, air-insulated fur coat for warmth (Reidman and Estes, 1990). Because of their small size and lack of a blubber layer, sea otters have a high metabolic rate: each otter requires a mass of prey equivalent to 25 to 30% of their body weight each day to maintain their weight and basal metabolic rate (Reidman and Estes, 1990). This is equivalent to 7 kg of shellfish per day for an average 28 kg adult male otter. Factors such as the high energy requirements of sea otters, their reliance in filter-feeding invertebrates as food and their tendency to feed near the shoreline may collectively place otters at high risk for exposure to terrestrial-origin chemical and biological pollutants.

(v) Biology of Pacific Harbor Seals

The Pacific harbor seal (*Phoca vitulina richardsi*) is one of 5 subspecies of harbor seals present worldwide (King, 1983; Reeves et al., 1992; Burns, 2002). Unlike southern sea otters, Pacific harbor seals are numerous throughout their range, which extends from the Aleutian Islands to the Baja Peninsula in Mexico, and overlaps that of the southern

sea otter in central California (Burns, 2002). Although sympatric, Pacific harbor seals are vastly different from southern sea otters in their food habits and habitat use. Unlike sea otters, Pacific harbor seals often feed ≥5 km offshore, including over the continental shelf, deep coastal basins and submarine canyons, and preferred prey consists of rockfish, herring, anchovies, flatfishes and squid (Pitcher, 1980; Brown and Mate, 1983; Harvey et al., 1995; Oxman, 1995; Euguchi, 1998). Harbor seals tend to feed in deeper water (to 484 meters), often at night, and rest on shore during daylight hours (Eguchi, 1998).

Harbor seals are more gregarious than sea otters, and high densities of animals may assemble in communal resting (haul-out) and pupping areas. Unlike otters, harbor seal breeding and pupping are synchronized between females. Pupping occurs in March in Central California, with 10 to 15 kilogram pups born on shore on protected beaches and inlets (Temte, 1986; Reeves et al., 1992; Burns, 2002; Greig, 2002). Also unlike otters, female harbor seals often leave pups in these communal areas and travel offshore to forage. Harbor seal pups are weaned at 4 to 6 weeks of age (Thompson et al., 1994; Burns, 2002; Greig, 2002). Like otters, female harbor seals enter estrus at the time of pup weaning, and experience delayed implantation. The total gestation period is approximately 10.5 months, which consists of about 2.5 months of pre-implantation pregnancy and 8 months of post-implantation gestation (Reeves et al., 1992; Burns, 2002). Harbor seals mature at 3 to 5 years of age. Adult males weigh 55 kg and adult females weigh 45 kg (Bigg, 1969). Dominant male harbor seals establish and defend underwater territories, often just offshore from major haul-out and pupping areas (Hayes, 2002). Breeding occurs underwater within these territories (Hayes, 2002).

Harbor seal numbers in California appear to be increasing. The total population of Pacific coast harbor seals is estimated at around 150,000 individuals (Caretta et al., 2001; Burns, 2002). Sea otters and harbor seals can often be found in close proximity to each other, and both species may share the same haul-out sites. Interaction between the species is common, and usually brief, although sea otter copulation with, and sexual aggression toward harbor seals, particularly harbor seal pups, has occasionally been reported (Hatfield et al., 1994; Miller, Oates, Harvey et al., unpublished necropsy data).

(vi) Biology of Toxoplasma gondii

The genus *Toxoplasma* consists of a single species, *T. gondii*, which has three genotypically-distinct strains (Type I, II and III), as determined by PCR and restriction fragment length polymorphisms (RFLP) (Howe and Sibley, 1994; Howe and Sibley, 1995; Howe et al., 1997; Grigg et al., 2001). *Toxoplasma gondii* is a single-celled apicomplexan protozoan parasite (Nicolle and Manceaux, 1909) with a heteroxenous life cycle (Tenter et al., 2000). Virtually any vertebrate, including humans, birds, rodents, and sheep may serve as intermediate hosts (Dubey, 1987; Tenter et al., 2000). However, only felids (cats) serve as definitive hosts and support the sexual phase of the parasite's life cycle, resulting in the production of infective oocysts, which are shed in their feces (Frenkel et al., 1970; Dubey, 1987; Dubey and Beattie, 1988; Dubey, 1993; Tenter et al., 2000). Cats are infected primarily through consumption of bradyzoite cysts in muscle, brain or other tissues of infected intermediate hosts (eg mice and birds), or via consumption of sporulated oocysts derived from the feces of another infected cat (Dubey, 1987; Dubey and Beattie, 1988). Cases of transplacental or transmammary transmission

are occasionally reported from various hosts (Dubey and Beattie, 1988; Dubey, 1993; Tenter et al., 2000).

Once ingested, sporozoites of *T. gondii* invade the cat's intestinal epithelium and multiply rapidly. Parasite multiplication first proceeds asexually and then sexually (via gamogony), culminating in the production of micro- and macrogametocytes. These gametocytes fuse to form zygotes, which form a hard outer shell and pass out in the cat's feces as tough, environmentally-resistant oocysts. The interval between parasite ingestion and fecal patency depends on a number of factors, including the parasite stage ingested, host age and level of immunocompetence, and history of previous exposure. The shortest prepatent period occurs in young, naïve cats that ingest infective bradyzoites present within tissue cysts in the flesh of intermediate hosts (Dubey, 1987). For these cats, the prepatent period may be as short as 2 to 3 days (Frenkel and Dubey, 1972). After defecation, these oocysts can sporulate (form sporozoites) under optimal conditions and become infective within 1 to 5 days (Frenkel and Dubey, 1972; Dubey, 1987). One infected cat may shed 100 million infective oocysts during an average patency period of 10 to 14 days (Tentor et al, 2000). Infected cats that shed oocysts typically exhibit no clinical signs. Once present in the environment, protozoal oocysts, cysts and sporocysts may remain infective for months or years, and are resistant to refrigeration, freezing, drying and many common disinfectant processes (Frenkel and Dubey, 1972; Yilmaz et al., 1972; Dubey, 1987; Tenter et al., 2000; Lindsay et al, 2002), including most municipal surfacewater treatment procedures (Weniger et al., 1983; Bowie et al., 1997; Isaac-Renton et al., 1998; Chauret et al., 1999; Wiandt et al., 2000; Payment et al., 2001; Quinonez-Diaz et al., 2001; Thurston et al., 2001; Lindsay et al, 2002). An important role

for surfacewater runoff in dissemination of *T. gondii* oocysts is increasingly recognized (Benenson et al., 1982; Mullens, 1996; Bowie et al., 1997; Aramini et al., 1998; Isaac-Renton et al., 1998; Aramini et al., 1999; Slifiko et al., 2000; Tenter et al., 2000). Intermediate hosts are infected via the consumption of bradyzoite cysts or sporulated oocysts. The infective dose can be as low as a single sporulated oocyst (Dubey et al., 1996b, 1996d). Potential routes of oocyst exposure for intermediate hosts include fecally-contaminated soil, food or water (Tenter et al., 2000). Once ingested, these parasites leave the intestinal tract and spread systemically, multiplying asexually as invasive, rapidly multiplying tachyzoites (Dubey, 1987). Depending on host immunocompetence, *T. gondii* strain and other factors, clinical signs may be absent during acute infection, or may consist of fever, myalgia and lymphadenopathy. Occasionally acute infections are fatal, especially for infected fetuses, immunosuppressed individuals or highly susceptible species such as Australian marsupials (Tenter et al., 2000). As specific host cell-mediated immunity is generated, there is a conversion of the rapidly multiplying tachyzoites to slower, less active bradyzoites (Frenkel and Dubey, 1972; Dubey, 1987; Dubey and Beattie, 1988). Bradyzoite-filled tissue cysts develop, primarily in the brain, spinal cord, skeletal muscle, and myocardium (Dubey, 1987; Dubey and Beattie, 1988). Tissue cysts may remain infective for months or years and may persist for the life of the host (Dubey, 1988; Tenter et al., 2000). Once tissue cysts are formed, infection becomes chronic, perhaps lifelong (Dubey, 1987; Dubey and Beattie, 1988). Recrudescence of active infection is thought to occur in chronically infected humans and other animals when host cell-mediated immunity wanes (Dubey, 1988; Gross et al., 1996).

(vii) Biology of Sarcocystis neurona

The genus *Sarcocystis* is large and diverse, with many as yet undescribed and uncharacterized species (Dubey, 1991; Dubey et al., 1994). The life cycle for *Sarcocystis* spp. is also heteroxenous. For many well described *Sarcocystis* spp., the most direct life cycle consists of a carnivore/omnivore-herbivore cycle in nature (eg coyote-deer), with the carnivore or omnivore serving as a definitive host, and an herbivore serving as an intermediate host (Dubey et al., 1994). In contrast to the genus *Toxoplasma*, the genus *Sarcocystis* consists of numerous species, each of which utilize different definitive and intermediate hosts to complete their life cycles (Dubey et al., 1994). Unlike *Toxoplasma*, the intermediate host range for *Sarcocystis* spp. is typically narrow, perhaps as few as one to two species, while the definitive host range is broad (Dubey 1993; Dubey et al., 2001b).

Sarcocystis neurona is an apicomplexan, heteroxenous protozoan similar to *T. gondii* in many respects, with a few important differences (Dubey, 1991). As a newly recognized species (Davis et al., 1991; Dubey et al., 1991), the life cycle for *S. neurona* is still being elucidated. However, a number of species, including mink, skunks, raccoons, armadillos and opossums appear to be capable of serving as intermediate hosts (Dubey and Hedstrom, 1993; Dubey et al., 1996a; Dubey and Lindsay, 1998; Cheadle et al., 2001a, 2001b; Dubey et al., 2001a, 2001c; Hamir and Dubey, 2001;Tanhauser et al., 2001). New world opossums (*Didelphis virginiana* and *D. albiventris*) are the only confirmed definitive hosts (Fenger et al., 1997; Dubey et al., 2001a, 2001d). Additional, unrecognized definitive hosts may also exist. This parasite first gained wide recognition as a pathogen of horses, and is the most frequent cause of the progressive neurological

disease equine protozoal myeloencephalitis (EPM) (Simpson and Mayhew, 1980; Davis et al., 1991; Dubey et al., 1991; MacKay et al., 1992).

(viii) Differences between T. gondii and S. neurona

The life cycle of *S. neurona* is quite different from *T. gondii*. The infective stage for *S. neurona* is a sporocyst, because the oocysts form, sporulate, and commonly break open to release sporocysts containing sporozoites prior to being shed in the feces of infected opossums (Dubey and Lindsay, 1998; Dubey et al., 2001a, 2001d). Thus, while *T. gondii* oocysts are not infective when passed in the feces, *S. neurona* sporocysts and sporulated oocysts are immediately infective when defecated. Like the cat, the opossum appears to be an asymptomatic host for *S. neurona* infection and shedding, and may shed large numbers of sporocysts without clinical signs (Dubey et al., 2001a).

Another important difference between *T. gondii* and *S. neurona* is in the type of asexual reproduction that occurs within host cells outside of the gastrointestinal tract. For *T. gondii* division occurs by endodyogeny, in which two parasites are formed by lateral division within a single "mother" parasite (Dubey, 1993). Asexual division of *S. neurona* occurs by endopolyogeny, in which daughter parasites bud externally from a single, central mother cell, forming a flower-like radial arrangement called a rosette-form schizont (Dubey, 1993). The invasive stages resulting from asexual division of *S. neurona* are called merozoites, because they are the product of schizogony, and to distinguish them from the tachyzoites and bradyzoites formed by asexual reproduction of *T. gondii* (Dubey, 1993). In contrast to *T. gondii* tachyzoites and bradyzoites, *S. neurona* merozoites do not possess rhoptries (Dubey et al., 2001b). In addition, while *T. gondii*

tachyzoites and bradyzoites are formed directly during asexual division, *Sarcocystis* spp. merozoites may transition through an intermediate stage, called a metrocyte (Dubey, 1993).

(ix) Molecular characterization of protozoa

The main goal of molecular characterization was to investigate whether the parasites isolated from sea otter and Pacific harbor seal brain tissue were similar or identical to the well-characterized protozoa *T. gondii* and *S. neurona*. With this goal in mind, two different DNA segments were selected for amplification and sequencing. The first was the18s ribosomal subunit DNA (18s rDNA), and the second was the adjacent, first internal transcribed spacer (ITS-1).

Ribosomes are present in all organisms and form an essential cellular organelle required for the synthesis of cell proteins (Hillis and Dixon, 1991). The DNA encoding eukaryote ribosomal RNA is present in the nucleus as approximately 100 to 300 identical or nearly identical copies, usually arranged as a tandem array (Guay et al., 1992; Palumbi et al., 1996). Each tandem repeat is separated by a nontranscribed spacer (NTS). Each ribosomal DNA gene is made up of 7 distinct parts (Appendix 1). Each part serves a different function, either encoding the ribosomal RNA, acting as a noncoding spacer between segments of coding DNA, or directing post-transcriptional ribosomal RNA processing (Hillis and Dixon, 1991; Palumbi et al., 1996). Each eukaryote ribosomal DNA gene contains three segments of coding DNA, the 18s, 5.8s and 28s DNA. The "s" denotes the sedimentation velocity of each DNA segment in Svedberg units, a measurement based on comparative sedimentation rates under centrifugation. Thus the

number of Svedberg units provides an index of the relative size of each DNA molecule, with higher Svedberg numbers indicating larger molecules (Palumbi et al., 1996). Each coding DNA segment is flanked on either side by noncoding DNA that acts as spacer DNA and also contains information for correct processing of the RNA transcript.

Processed ribosomal RNA forms the functional ribosome required for protein synthesis by the host cell. Each eukaryote ribosome is composed of two parts. The large ribosomal subunit is derived from host cell-derived proteins complexed with rRNA assembled from the 5.8s and 28s ribosomal DNA segments. The smaller ribosomal subunit on which translation of mRNA to amino acids and proteins occurs is composed of host cell-derived proteins complexed with ribosomal RNA from the18s rDNA segment.

The 18s rDNA is a 1,800 nucleotide (nt) segment of nuclear DNA that has been widely used by molecular scientists for phylogenetic studies, because of its high copy number and large size, which facilitate amplification and detection (Hillis and Dixon, 1991; Guay et al., 1992). Also, because it codes for ribosomal RNA that serves a critical function in guiding cellular protein synthesis, the 18s rDNA is one of the slowest evolving nuclear genes, and has been highly conserved throughout evolutionary time and across diverse taxonomic lineages (Hillis and Dixon, 1991). This has resulted in the retention of highly conserved regions that are optimal for universal primer design (Hillis and Dixon, 1991). As a result, universal primers are available that amplify 18s rDNA from organisms as diverse as fungi and mammals (Gagnon et al., 1993; Palumbi et al., 1996; Kjemtrup et al., 2000). Numerous complete or partial 18s rDNA sequences from apicomplexan parasites have been published and are searchable in computerized DNA sequence databanks such as GenBank. This greatly facilitates phylogenetic comparison

between 18s rDNA sequences derived from well-characterized organisms and those derived from unknown or novel organisms (Gagnon et al., 1993; Fenger et al., 1994; Holmdahl et al., 1994; Ellis et al., 1995; Marsh et al., 1995, 1996; Johnson et al., 1997). Because of their highly conserved nature, 18s rDNA sequences are best used to examine ancient (eg Precambrian) evolutionary divergences, and to investigate relationships between organisms at higher taxonomic levels such as family, genus and species (Hillis and Dixon, 1991; Hillis et al., 1996). Less restricted, or variable regions also exist within the 18s rDNA genome, especially toward the 5' end of the molecule, and these variable regions are often helpful in distinguishing fine differences between closely related taxa (Torczynski et al., 1983; Dams et al., 1988). In the present study, the first 530 bp of the 5' 18s rDNA encompassing many of the most variable regions were amplified, sequenced and compared to similar 18s rDNA sequences available on GenBank. This 5' region of the 18s rDNA was selected because it was found to be especially phylogenetically informative in studies of related protozoans (Ellis et al., 1994; Luton et al., 1995; Marsh et al., 1995; Payne and Ellis, 1996; Barta, 1997; Carreno and Barta, 1999; Marsh et al., 1999; Carreno et al., 2001; Kjemtrup et al., 2000). DNA amplification was accomplished using a two-step nested PCR reaction utilizing universal primers (see Chapters 2 and 3 for details). However, the 18s rDNA, as a highly conserved and slowly evolving gene, provides scant data to discern fine differences (eg subspecies and strains) between closely related organisms (Torczynski et al., 1983; Dams et al., 1988). For this reason, a second, more rapidly evolving DNA sequence was also amplified and sequenced.

The adjacent ITS-1 DNA is a small to medium-sized segment of non-coding DNA that acts as a spacer between the 18s and 5.8s rDNA (Appendix 1). At its 3' end, the

ITS-1 contains information to guide the correct processing of the adjacent 5.8s ribosomal RNA, and appears to have a role in the early assembly events of the ribosome (Hillis and Dixon, 1991; Hillis et al., 1996; Homan et al., 1997). Because the ITS-1 represents spacer, or non-coding DNA, it is less highly conserved and is under less evolutionary pressure to retain the same size and DNA sequence as the18s rDNA throughout evolutionary time (Hillis and Dixon, 1991; Homan et al., 1997).

The ITS-1 is characterized by significant variation between species, but remarkable homogeneity within most species examined to date (Hyde, 1990; Barta, 1997; Marsh et al., 1998). For example, the *T. gondii* ITS-1 DNA is approximately 392 bp long, while the *S. neurona* ITS-1 DNA is approximately 1,053 bp (Marsh et al., 1999; Miller et al., 2001a, 2001b). This difference is attributable to variation in the number of repeated domains between the ITS-1 DNA of different species (Hillis and Dixon, 1991; Homan et al., 1997). Thus the ITS-1, even though it is adjacent to the highly conserved 18s rDNA, is a more rapidly evolving DNA segment. As a result, it can be used to help explore more recent evolutionary divergences (eg Cenozoic) between taxonomic groups, such as between species or in a few cases, strains (Hillis and Dixon, 1991; Holmdahl and Mattson, 1996; Homan et al., 1997; Marsh et al., 1999).

For selected protozoal isolates derived from otter and seal brain tissue, we amplified and sequenced the entire ITS-1 DNA molecule, and then compared our sequences to available GenBank ITS-1 sequences for *T. gondii*, *S. neurona* and related apicomplexan parasites. At the time that this study was initiated, parasites similar or identical to *T. gondii* and *S. neurona* had been implicated as a cause of mortality in California sea otters and harbor seals (Thomas and Cole, 1996; Lapointe et al., 1998), but

parasite isolation and antigenic and molecular characterization had not been reported. Thus the isolation and characterization of these parasites and the development of these techniques became the initial focus of my doctoral research.

(x) Serological Test Development

One of the research objectives was to develop serological tests that could be used to screen live and dead otters and seals for evidence of protozoal infection. Once developed and validated, these tests could be utilized by veterinarians to expedite diagnosis and treatment of live animals with protozoal meningoencephalitis. In addition, these serological tests could be used to address specific hypotheses regarding risk factors for environmental exposure, and to investigate the relationships between *T. gondii* and *S. neurona* infection and disease.

A variety of serological tests are available for detecting protozoal infections in animals and humans (Desmonts et al., 1986; Wilson et al., 1990; Yamane et al., 1993; Dubey et al., 1995; Dubey et al., 1996b, 1996c; Packham et al., 1998; Rossano et al., 2000). The choice of which assay to select depends on the purpose for testing, the parasite species and its pathophysiology and limitations on test and sample conditions. Other factors to consider include assay cost, rapidity and the desired form of the test results (eg quantitative versus binary [positive or negative]). Serologic assays for apicomplexan parasites such as *T. gondii*, *S. neurona* and *N. caninum* are reviewed and compared in Desmonts et al. (1980); Dubey et al. (1995) and Packham et al. (1998). Commonly-used agglutination tests for diagnosis of *T. gondii* infection include the direct agglutination (DAT), modified agglutination (MAT) and latex agglutination (LAT) tests.

These tests all share three attributes: availability on a collaborative or commercial basis, ease of use and ability to screen animals for which no species-specific linking antibodies are available. However, none of these tests have been validated for testing marine mammal serum. In addition, serum antibody titers may be artificially elevated or decreased by the presence of hemolysis or excessive cellular debris in test samples (Dubey et al., 1996b; Packham et al., 1998). MATs have been used to detect antibodies to *T. gondii* using harbor seal, other phocid and cetacean sera (Oksanen et al., 1998; Lambourne et al., 2001). The LAT has also been used to detect *T. gondii* antibodies in river otter serum (Tocidlowski et. al., 1997).

Other types of serologic tests that have been used to detect apicomplexan infections include enzyme-linked immunosorbent assays (ELISA) and Western blots (Conrad et al., 1993; Packham et al., 1998). Because 96-well plates are used, the ELISA lends itself well to automation, and can be less labor intensive than other types of serological assays. However, ELISA-based assays, like agglutination tests, may suffer from problems with sensitivity, specificity and increased background, especially when used to evaluate hemolyzed, or cellular debris-rich serum samples (Wilson et al., 1990; Dubey et al., 1996b; Packham et al., 1998). The Western blot test can be used to help discern differences between related organisms, and has been utilized to confirm *S. neurona* infection in California harbor seals (Lapointe et al., 1998). However, this test is labor intensive and expensive, and users may encounter problems with specificity when attempting to discern between closely related taxa (Rossano et al., 2000; Marsh et al., 2001).

Each type of serological test has inherent strengths and weaknesses. A serologic test should be selected that best complements the anticipated testing conditions and project goals. For this dissertation research, we were utilizing otter and seal serum collected at necropsy, or during field sampling of live animals, where prolonged intervals between sample collection and processing could occur. Concerns about serum hemolysis were a deciding factor in our decision to develop immunofluorescent antibody tests (IFATs) to screen sea otters and harbor seals for exposure to *T. gondii* and *S. neurona*. Because of the fluorescence-based reporting system used in IFATs, they are less susceptible to error due to hemolysis or cellular debris than agglutination and ELISA-based assays (Dubey et al., 1996b; Packham et al., 1998). In addition, test sensitivity and specificity were shown to be good, and cross-reactivity minimal when IFATs were utilized to detect the presence of serum antibodies to *T. gondii* and related apicomplexan parasites (Dubey et al., 1996b; Packham et al, 1998). Unfortunately, IFATs are more labor intensive and subjective in their interpretation than ELISAs and agglutination tests (Dubey et al., 1996b; Packham et al., 1998). Thus, standardizing conditions for test preparation and interpretation is essential (Packham et al., 1998). Because species-specific secondary antibodies were not available, these tests were developed using commercially available antisera raised against ferrets (for sea otters) and canines (for harbor seals). Polyclonal fluorescence (FITC)-labeled anti-ferret (otters) or anti-canine (seals) antisera were used as the assay reporter system (see Chapters 3 and 4 for details).

IFATs for detecting circulating IgG to *T. gondii* and *S. neurona* in otters and seals were developed, and the sea otter *T. gondii* IFAT was validated using positive and

negative control sera. *Toxoplasma gondii* infection was confirmed via immunohistochemical identification of parasites and/or *in vitro* isolation of parasites from brain tissue.

The validated *T. gondii* IFAT was used to screen serum collected from free-living otters in California, Washington and Alaska. The proportion of seropositive otters from each geographically distinct sea otter population was compared, as well as the proportion of seropositive dead versus live California sea otters (Chapter 4). The IFATs for *S. neurona* and *N. caninum* were not validated because of the low number of positive control animals identified during this study.

(xi) Risk Factors for Sea Otter Exposure to Toxoplasma gondii

Techniques for quantitatively estimating risk of exposure to infectious agents are frequently utilized in veterinary and human medicine (Kulldorf and Nagarwalla, 1995; Morris et al., 1995; Weigel et al., 1995; Yanga et al., 1995; Carpenter and Gardner, 1996; Atwill et al., 1997; Bobic et al., 1998; Kulldorf et al., 1998; Greiner and Gardner 2000; Saville et al., 2000a, 2000b; Carpenter, 2001). A variety of potential risk factors may be evaluated using epidemiological techniques, including demographic factors such as gender, and environmental factors such as association of the presence of disease with certain habitats. Because many diseases cluster in both time and space, investigation of spatio-temporal patterns of infection may provide important clues regarding disease transmission and may facilitate control (Carpenter, 2001). Quantitative techniques for assessing risk of infection or disease are not commonly applied to wildlife populations (Atwill et al., 1997; Ley et al., 1997; Hartup et al., 1998), in part because of the difficulty of obtaining sufficient sample size to achieve adequate statistical power. However, when

specific risk factors are identified in wildlife populations, these data may indicate new and important pathways for investigation, or suggest routes for intervention to lessen the impact of pathogens on wildlife populations (Hartup et al., 1998).

A final goal of my dissertation research was to investigate potential risk factors for *T. gondii* exposure for sea otters, so that patterns of exposure in the nearshore marine environment of California could be better understood. This study is described in Chapter 5. Three major categories of risk factors were assessed: demographic, environmental and spatial risk factors. Demographic data were collected at the time of capture or necropsy, and included gender, age class, total length, and sampling date. Environmental data included the proximity of each otter's sample location to large freshwater outflows (eg streams and rivers), municipal sewage outfalls or areas of dense human settlement. Finally, the location of each otter at the time of live-sampling or carcass recovery was analyzed to detect spatial clustering of *T. gondii*-seropositive or seronegative otters along the California coast. The overall goal of the risk study was to identify specific factors that were associated with increased or decreased odds of *T. gondii* exposure, and to provide preliminary data for subsequent, site-focused research.

(xii) Literature Cited

Anderson, C. G., J. L. Gittleman, K. P. Koepfli and R. K. Wayne. 1996. Sea otter systematics and conservation: which are critical subspecies? Endangered Species Update: Conservation and management of the Southern Sea otter. 13: 6-10.

Aramini, J. J., C. Stephen and J. P. Dubey. 1998. *Toxoplasma gondii* in Vancouver Island cougars (*Felis concolor vancouverensis*): serology and oocyst shedding. J. Parasitology. 84: 438-440.

____, _____, _____, C. Engelstoft, H. Schwantje and C. S. Ribble. 1999. Potential contamination of drinking water with *Toxoplasma gondii* oocysts. Epid. and Infect. 122: 305-315.

Atwill, E. R., R. A. Sweitzer, M. D. Pereira, I. Gardner, D. Van Vuren and W. M. Boyce. 1997. Prevalence and associated risk factors for shedding *Cryptosporidium parvum* oocysts and *Giardia* cysts within feral pig populations in California. Appl. and Envir. Microbiol. 63: 3946-3949.

Barta, J. R. 1997. Investigating phylogenetic relationships within the Apicomplexa using sequence data: The search for homology. Methods in Enzymol. 13: 81-88.

Beneson, M. W., E. T. Takafuji, S. M. Lemon, R. L. Greenup and A. J. Sulzer. 1982. Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water. New. Engl. J. Med. 307: 666-669.

Bigg, M.A. 1969. The harbour seal in British Columbia. Bull. Fish. Res. Bd. Can. 172: 33 Pp.

Bobic, B., I. Jevremovic, J. Marinkovic, D. Sibalic and O. Djurkovic-Djakovic. 1998. Risk factors for *Toxoplasma* infection in a reproductive age female population in the area of Belgrade, Yugoslvia. Eur. J. Epidemiol. 14: 605-610.

Bodkin, J. L., B. E. Ballachey, M. A. Cronin and K. T. Scribner. 1999. Population demographics and genetic diversity in remnant and translocated populations of sea otters. Conservat. Biol. 13: 1378-1385.

Bowie, W. R., A. S. King, D. H. Walker, J. L. Isaac-Renton, A. Bell, S. B. Eng and S. A. Marion. 1997. Outbreak of toxoplasmosis associated with municipal drinking water. Lancet. 350:173-177.

Brown, R.F. and B.R. Mate. 1983. Abundance, movements, and feeding habits of harbor seals, *Phoca vitulina*, at Netarts and Tillamook Bays, Oregon. Fishery Bull. 81: 291-301.

Burns, J. J. 2002. Harbor seal and spotted seal. *IN*. Encyclopedia of marine mammals. W. A. Perrin, B. Wursig and J. G. M. Thewissen, Eds. Academic Press. San Diego, CA. pp 552-560.

Carpenter, T. and I. A. Gardner. 1996. Simulation modeling to determine herd-level predictive values and sensitivity based on individual-animal test sensitivity and specificity and sample size. Prevent. Vet. Med. 27: 57-66.

_____. 2001. Methods to investigate spatial and temporal clustering in veterinary epidemiology. Prevent. Vet. Med. 48: 303-320.

Carreno, R. A., and J. R. Barta. 1999. An eimeriid origin of isosporoid coccidian with steida bodies as shown by phylogenetic analysis of small subunit ribosomal RNA gene sequences. J Parasitol. 85: 77-83

_____, N. J. Pokorny, H. Lee, J. T. Trevors and S. A. Grandis. 2001. Phenotypic and genotypic characterization of *Cryptosporidium* species and isolates. J. Indust. Microbiol. and Biotech. 26: 95-106.

Carretta, J.V., J. Barlow, K.A. Forney, M.M. Muto and J. Baker. 2001. U.S. Pacific Marine Mammal Stock Assessments: 2001. U.S. Department of Commerce, NOAA Technical Memorandum NMFS-SWFSC-317. 280 Pp.

Chauret, C., S. Springthorpe and S. Sattar. 1999. Fate of *Cryptosporidium* oocysts, *Giardia* cysts and microbial indicators during wastewater treatment and anerobic sludge digestion. Canad. J. Microbiol. 45: 257-262.

Cheadle, M. A., S. M. Tanhauser, J. B. Dame, D. C. Sellon, M. Hines, P. E. Ginn, R. J. MacKay and E. C. Greiner. 2001a. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31: 330-335.

_____, C. A. Yowell, D. C. Sellon, M. Hines, P. E. Ginn, A. E. Marsh, J. B. Dame and E. C. Greiner. 2001b. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31: 330-335.

Conrad, P. A., K. Sverlow, M. Anderson, J. Rowe, R. Bondurant, G. Tuter, R. Breitmeyer, C. Palmer, M. Thurmond, A. Ardans, J. P. Dubey, G. Duhamel, and B. Barr. 1993. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. J. Vet. Diag. Invest. 5: 572-578.

Dams, E. L., Y. Van De Peer, J. Neefs, G. Smits, I. Vandenbempt and R. De Wachter. 1988. Comparison of small ribosomal subunit RNA sequence. Nucleic Acids Res. 16: r87-r173.

Daszak, P., A. A. Cunningham and A. D. Hyatt. 2000. Emerging infectious diseases of wildlife: Threats to biodiversity and human health. Science. 287: 443-449.

_____, _____ and _____. 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Tropica. 78:103-116.

Davis, S. W., B. N. Daft and J. P. Dubey. 1991. *Sarcocystis neurona* cultured from a horse with equine protozoal myeloencephalitis. Equine Vet. J. 23: 315-317.

Dayton, P. K. 1975. Experimental studies of algal canopy interactions in a sea otter-dominated kelp community at Amchitka Island, Alaska. Proc. Calif. Acad. Sci. 40: 429-437.

Desmonts, G., and J. S. Remington. 1980. Direct agglutination test for diagnosis of Toxoplasma infection: Method for increasing sensitivity and specificity. J. Clin. Micro. 11: 562-568.

Dubey, J. P. 1987. Toxoplasmosis. *IN.* Vet. Clin. North Am.: Small An. Prac. 17: 1389-1405.

_____. 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of cysts in pork. Am. J. Vet. Res. 49: 910-913.

_____ and C. P. Beattie. 1988. Toxoplasmosis of animals and man. CRC Press. Boca Raton, Florida. 220pp.

_____, S. W. Davis, C. A. Speer, D. W. Bowman, A. de LaHunta, D. E. Granstrom, M. J. Topper, A. N. Hamir, J. F. Cummings and M. M. Suter. 1991. *Sarcocystis neurona* N. sp. (*Protozoa:Apicomplexa*), the etiologic agent of equine protozoal myeloencephalitis. J. Parasitol. 77: 212-218.

_____. 1993. *Toxoplasma, Neospora, Sarcocystis* and other tissue cyst-forming coccidia of humans and animals. *IN.* Krier, J. P., Editor. Parasitic Protozoa, Second Edition. Acdemic Press, Inc. San Diego, CA. 158pp.

_____ and O. R. Hedstrom. 1993. Meningoencephalitis in mink associated with a *Sarcocystis neurona*-like organism. J. Vet. Diag. Invest. 5: 467-471.

_____, C. A. Speer and R. Fayer. 1994. Sarcocystosis of animals and man. CRC Press, Inc. Boca Raton, Florida. 215 pp.

_____, P. Thuillez and E. C. Powell. 1995. *Toxplasma gondii* in Iowa sows: Comparison of antibody titers to isolation of *T. gondii* by bioassay in mice and cats. J. Parasitol. 81: 48-53.

_____, A. N. Hamir, M. Neizgoda and C. E. Rupprecht. 1996a. A *Sarcocystis neurona*-like organism associated with encephalitis in a striped skunk (*Mephitis mephitis*). J. Parasitol. 82: 172-174.

_____, C. D. Andrews, P. Lind, O. C. Kwok, P. Thuillez and J. K. Lunney. 1996b. Antibody responses measured by various serologic tests in pigs orally inoculated with low numbers of *Toxoplasma gondii* cysts. Am. J. Vet. Res. 57: 1733-1737.

_____, D. S. Lindsay, D. S. Adams, J. M. Gay, T. M. Baszler, B. L. Blagburn, and P. Thuillez. 1996c. Serologic responses of cattle and other animals infected with *Neosproa caninum*. Am. J. Vet. Res. 57: 329-336.

_____, J. K. Lunney, S. K. Shen, O. C. Kwok, D. A. Ashford and P. Thuillez. 1996d. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. J. Parasitol. 82: 438-443.

_____ and D. S. Lindsay. 1998. Isolation in immunodeficient mice of *Sarcocystis neurona* from opossum (*Didelphis virginiana*) faeces, and its differentiation from *Sarcocystis falcatula*. Int. J. Parasitol. 28: 1823-1828.

_____, _____, C. E. Kerber, N. Kasai, H. F. Pena, S. M. Gennari, O. C. Kwok, S. K. Shen and B. M. Rosenthal. 2001a. First isolation of *Sarcocystis neurona* from the South American opossum (*Didelphis albiventris*) from Brazil. Vet. Parasitol. 95: 295-304.

_____, _____, W. J. Saville, S. M. Reed, D. E. Granstrom and C. A. Speer. 2001b. A review of *Sarcocystis neurona* and equine protozoal myeloencephalitis (EPM). Vet. Parasitol. 95: 89-131.

_____, A. C. Rosypal, B. M. Rosenthal, N. J. Thomas, D. S. Lindsay, J. F. Stanek, S. M. Reed and W. J. Saville. 2001c. *Sarcocystis neurona* infections in sea otter (*Enhydra lutris*): Evidence for natural infection with sarcocysts and transmission of infection to opossums. J. Parasitol. 87: 1387-1393.

_____, W. J. Saville, J. F. Stanek, D. S. Lindsay, B.M. Rosenthal, M. J. Oglesbee, A. C. Rosypal, C. J. Njoku, R. W. Stich, O. C. Kwok, A. N. Hamir and S. M. Reed. 2001d. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): Evidence for natural infection with sarcocysts, transmission to opossums (*Didelphis virginina*), and experimental induction of neurologic disease in raccoons. Vet. Parasitol. 100: 117-129.

Ellis, J. T., K. Luton, P. R. Baverstock, P. Brindley, K. A. Nimmo, and A. M. Johnson. 1994. The phylogeny of *Neospora caninum*. Molec. and Biochem. Parasitol. 64: 303-311.

_____, _____, _____, G. Whitworth, A. M. Tenter and A. M. Johnson. 1995. Phylogentic relationships between *Toxoplasma* and *Sarcocystis* deduced from a comparison of 18s rDNA sequences. Parasitol. 110: 521-528.

Estes, J. 1980. *Enhydra lutris*. Mammal. Species. 133: 1-8.

_____, K. E. Underwood and M. J. Karmann. 1986. Activity-time budgets of sea otters in California. J. Wildl. Manage. 50: 626-636.

_____ and P. D. Steinberg. 1988. Predation, herbivory and kelp evolution. Paleobiol. 14: 19-36.

_____, M. T. Tinker, T. M. Williams and D. F. Doak. 1998. Killer whale predation on sea otters linking oceanic and nearshore ecosystems. Science. 282: 473-476.

Eguchi, T. 1998. Morphology of the Pacific harbor seal (*Phoca vitulina richardsi*) using Elkhorn Slough, California, and their movements and diving behavior in the Monterey Bay area. [MSc thesis], Moss Landing Marine Laboratories, California State University, Fresno. 105 Pp.

Fenger, C. K., D. E. Granstrom, J. L. Langemeier, A. Gajadhar, G. Cothran, R. R. Tramontin, S. Stamper, and J. P. Dubey. 1994. Phylogenetic relationship of *Sarcocystis neurona* to other members of the family *Sarcocystidae* based on small subunit ribosomal RNA gene sequence. J. Parasitol. 80: 966-975.

_____, _____, _____, S. Stamper, J. M. Donahue, J. S. Patterson, A. A. Gajadhar, J. V. Marteniuk, Z. Xiaomin and J. P. Dubey. 1995. Identification of opossums (*Didelphis virginiana*) as the putative host of *Sarcocystis neurona*. J. Parasitol. 81: 916-919.

_____, _____, A. A. Gajadar, N. M. Williams, S. A. McCrillis, S. Stamper, J. L. Langemier, and J. P. Dubey. 1997 . Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis sp.* sporocysts from the opossum (*Didelphis virginiana*). Vet. Parasitol. 68: 199-213.

Fisher, E. M. 1942. Prices of sea otter pelts. Calif. Fish and Game. 27: 261-300.

Frenkel, J. K., J. P. Dubey and N. Miller. 1970. *Toxoplasma gondii* in cats: Fecal stages identified as coccidian oocysts. Science. 167: 893-896.

_____ and _____. 1972. Toxoplasmosis and its prevention in cats and man. J. Infect. Dis. 126: 664-673.

Gagnon, S., R. C. Levesque, M. L. Sogin and A. A. Gadjadhar. 1993. Molecular cloning, complete sequence of the small subunit ribosomal RNA coding region and phylogeny of *Toxoplasma gondii*. Molec. and Biochem. Parasitol. 60: 145-148.

Greig, D. 2002. Pregnancy and parturition rates of harbor seals in Monterey Bay, California. [MSc thesis], Moss Landing Marine Laboratories, San Jose State University. 68 Pp.

Griener M. and I. A. Gardner. 2000. Application of diagnostic tests in veterinary epidemiologic studies. Prevent. Vet. Med. 45: 43-59.

Grigg, M., S. Bonnefoy, A. B. Hehl, Y. Suzuki and J. C. Boothroyd. 2001. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. Science. 294: 161-165.

Gross, U., W. Bohne, M. Soete and J. F. Dubremetz. 1996. Developmental differentiation between tachyzoites and bradyzoites of *Toxoplasma gondii*. Parasitol. Today. 12: 30-33.

Guay, J. M., A. Huot, S. Gagnon and R. C. Levesque. 1992. Physical and genetic mapping of cloned ribosomal DNA from *Toxoplasma gondii*: Primary and secondary structure of the 5S gene. Gene. 114: 165-171.

Hamir, A. N. and J. P. Dubey. 2001. Myocarditis and encephalitis associated with *Sarcocystis neurona* infection in raccoons (*Procyon lotor*). Vet. Parasitol. 95: 335-340.

Hartup, B., H. O. Mohammed, G. V. Kollias and A. A. Dhondt. 1998. Risk factors associated with mycoplasmal conjunctivitis in house finches. J. Wildl. Dis. 34: 281-288.

Harvey, J.T., R.C. Helm and G.V. Morejohn. 1995. Food habits of harbor seals inhabiting Elkhorn Slough, California. Calif. Fish and Game. 81: 1-9.

Hatfield, B., R. J. Jameson, T. G. Murphey and D. D. Woodard. 1994. Atypical interactions between male southern sea otters and pinnipeds. Marine Mamm. Sci. 10: 111-114.

Hayes, S.A. 2002. Mating strategies and reproductive success of the male Pacific harbor seal (*Phoca vitulina richardsi*). [PhD dissertation], University of California, Santa Cruz. 158 Pp.

Hillis, M. D. and M. T. Dixon. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quar. Rev. Biol. 66: 411-453.

_____, B. K. Mable, A. Larson, S. K. Davis and E. A. Zimmer, 1996. Chapter 9: Nucleic acids IV: Sequencing and cloning. *IN*: Molecular Systematics. Second edition. D. M. Hillis, C. Moritz, and B. K. Mable, Eds. Sinauer Associates, Inc., Sunderland, Massachusetts, U.S. pp.321-381.

Holmdahl, O. J. M., J. G. Mattson , A. Uggla, and K. E. Johansson. 1994. The phylogeny of *Neospora caninum* and *Toxoplasma gondii* based on ribosomal RNA sequences. Fed. Eur. Microbiol. Soc. (FEMS) Microbiol. Letters 119: 187-192.

_____, and _____. 1996. Rapid and sensitive identification of *Neospora caninum* by *in vitro* amplification of the internal transcribed spacer 1. Parasitol. 112: 177-182.

Homan, W. L., L. Limper, M. Verlaan, A. Borst, M. Vercammen, F. van Knapen. 1997. Comparison of the internal transcribed spacer, ITS 1, from *Toxoplasma gondii* isolates and *Neospora caninum*. Parasitol. Res. 83: 285-289.

Howe D. K. and L. D. Sibley. 1994. *Toxoplasma gondii*: Analysis of different laboratory stocks of the RH strain reveals genetic heterogeneity. Exper. Parasitol. 78: 242-245.

_____, and _____. 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. J. Infect. Dis. 172: 1561-1566.

_____, S. Honore, F. Derouin, and L. D. Sibley. 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35: 1411-1414.

Hyde, J. E. 1990. Molecular Parasitology. Open University Press. Milton Keynes, UK. 93pp.

Isaac-Renton, J., W. R. Bowie, A. King, G. S. Irwin, C. S. Ong, C. P. Fung, M. O. Shokeir and J. P. Dubey. 1998. Detection of *Toxoplasma gondii* oocysts in drinking water. Appl. Envir. Microbiol. 64: 2278-2280.

Jameson, R. L. 1989. Movements, home range and territories of male sea otters off central California. Marine Mamm. Sci. 5: 159-172.

Johnson, A. M., S. Illana, J. P. Dubey and J. P. Dame. 1987. *Toxoplasma gondii* and *Hammondia hammondi*: DNA comparison using cloned rRNA gene probes. Exper. Parasitol. 63: 272-278.

King, J.E. 1991. Seals of the world. Second Edition. Cornell University Press. New York. NY. 240 Pp.

Kjemtrup, A. M., J. Thomford, T. Robinson and P. A. Conrad. 2000. Phylogentic relationships of human and wildlife piroplasm isolates in the western United States inferred from the 18s nuclear small subunit ribosomal RNA gene. Parasitol. 120: 487-493.

Kreuder, C., M. A. Miller, D. A. Jessup, L. J. Lowenstine, M. D. Harris, J. A. Ames, T. E. Carpenter, P. A. Conrad and J. K. Mazet. Recent patterns of mortality in southern sea otters (*Enhydra lutris nereis*). Submitted to J. Wildl. Dis.

Kulldorf, M. and N. Nagarwalla. 1995. Spatial disease clusters: detection and inference. Statist. Med. 14: 799-810.

_____, W. F. Atlas, E. J. Feuer, B. A. Miller and C. R. Key. 1998. Evaluating cluster alarms: a space-time scan statistic and brain cancer in Los Alamos. Am. J. Publ. Health. 88: 1377-1380.

Kvitek, R. G. and J. S. Oliver. 1987. Chapter 3: Sea otter foraging habits ans effects on prey populations and communities in soft-bottom environments. *IN*. The Community Ecology of Sea Otters. G. R. Van Blaricom and J. A. Estes, Eds. Springer-Verlag Press. Berlin, Germany. Pp 22-47.

Lamborn, D. M., S. J. Jeffries and J. P. Dubey. 2001. Seroprevalence of *Toxoplasma gondii* in harbor seals (*Phoca vitulina*) in southern Puget Sound, Washington. J. Parasitol. 87: 1196-1198.

Lapointe, J. M, P. J. Duignan, A. E. Marsh, F. M. Gulland, B. C. Barr, D. K. Naydan, D. P. King, C. A. Farman, K. A. Burek Huntingdon, and L. J. Lowenstine. 1998. Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific Harbor Seals (*Phoca vitulina richardsi*). J. Parasitol. 84: 1184-1189.

Leatherwood, S., L. J. Harrington-Coulombe and C. L. Hubbs. 1978. Relict survival of the sea otter in central California and evidence of its recent redispersal south of Point Conception. Bull. of the South. Calif. Acad. Sci. 77: 109-115.

Ley, D. H., J. E. Berkhoff, K. Joyner, L. Powers and S. Levenson. 1997. Molecular epidemiologic investigations of *Mycoplasma gallisepticum* conjunctivitis in songbirds by random amplified polymorphic DNA analyses. Emerg. Infect. Dis. 3: 375-380.

Lindsay, D. S., B. L. Blagburn and J. P. Dubey. 2002. Survival of nonsporulated *Toxoplasma gondii* oocysts under refrigerator conditions. Vet. Parasitol. 103: 309-313.

Luton, K, M. Gleeson, and A. M. Johnson. 1995. rRNA gene sequence heterogeneity among *Toxoplasma gondii* strains. Parasitol. Res. 81: 310-315.

MacKay, R. J., Davis, S. W. and J. P. Dubey. 1992. Equine protozoal myeloencephalitis. Compend. Contin. Educ. Pract. Vet. 14: 1359-1367.

Marsh, A. E., B. C. Barr, K. Sverlow, M. Ho, J. P. Dubey, and P. A. Conrad. 1995. Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. J. Parasitol. 81: 530-535.

_____, _____, J. Madigan, J. Lakritz, and P. A. Conrad. 1996. Sequence analysis and polymerase chain reaction amplification of small subunit ribosomal DNA from *Sarcocystis neurona*. Am. J. Vet. Res. 57: 975-981.

_____, _____, A. E. Packham, and P. A. Conrad. 1998. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). J. Parasitol. 84: 983-991.

_____, _____, L. Tell, D. D. Bowman, P. A. Conrad, C. Ketcherside, and T. Green. 1999. Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocystis neurona*. J. Parasitol. 85: 50-57.

_____, P. J. Johnson, J. Ramos-Vera and G. C. Johnson. 2001. Characterization of a *Sarcocystis neurona* isolate from a Missouri horse with equine protozoal myeloencephalitis. Vet Parasitol. 95: 143-154.

Miller, M. A., P. R. Crosbie, K. Sverlow, K. Hanni, B. C. Barr, N. Kock, M. J. Murray, L. J. Lowenstine, and P. A. Conrad. 2001a. Isolation and characterization of *Sarcocystis* from brain tissue of a free-living southern sea otter (*Enhydra lutris nereis*) with fatal meningoencephalitis. Parasitol Res. 87: 252-257.

_____, K. W. Sverlow, P. R. Crosbie, B. C. Barr, L. J. Lowenstine, F. M. Gulland, A. Packham and P. A. Conrad. 2001b. Isolation and characterization of parasitic protozoa from a Pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalitis. J. Parasitol. 87: 816-822.

Morris, C. R., I. A. Gardner, S. K. Hietala, T. E. Carpenter, R. J. Anderson and K. M. Parker. 1995. Seroepidemiologic study of natural transmission of *Mycoplasma hyopneumoniae* in a swine herd. Prevent. Vet. Med. 21: 323-337.

Mullens, A. 1996. "I think we have a problem in Victoria": MDs respond quickly to toxoplasmosis outbreak in B. C. Can. Med. Assoc. J. 154: 1721-1724.

Nicolle, C. and L. Manceaux. 1908. Sur une infection a corps de Leishman (ou organisms viosins) du gondii. C. R. Hebd. Seances Acad. Sci. 147: 763-766.

Oksanen, A., M. Tryland, K. Johnsen and J. P. Dubey. 1998. Serosurvey of *Toxoplasma gondii* in North Atlantic marine mammals by use of agglutination test employing whole tachyzoites and dithiothreitol. Compar. Immunol., Microbiol. and Infect. Dis. 21: 107-114.

Oxman, D.S. 1995. Seasonal abundance, movements, and food habits of harbor seals (*Phoca vitulina richardsi*) in Elkhorn Slough, California. [MSc thesis], Moss Landing Marine Laboratories, California State University, Stanislaus. 126 Pp.

Packham, A. E., K. W. Sverlow, P. A. Conrad, E. F. Loomis, J. D. Rowe, M. L. Anderson, A. E. Marsh, C. Cray, and B. C. Barr. 1998. A modified agglutination test for *Neospora*: Development, optimization and comparison to the IFAT and ELISA. Clin. and Diag. Lab. Immunol. 5: 467-473.

Palumbi, S. R. 1996. Chapter 7: Nucleic acids II: The polymerase chain reaction. *IN*: Molecular Systematics. Second edition. D. M. Hillis, C. Moritz, and B. K. Mable, Eds. Sinauer Associates, Inc., Sunderland, Massachusetts, U.S. Pp. 205-247.

Payment, P., R. Plante, and P. Cejka. 2001. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Canad. J. Microbiol. 47: 188-193.

Payne, S., and J. Ellis. 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. Int. J. Parasitol. 26: 347-351.

Pederson, R. J. and J. Stout. 1963. Oregon sea otter sightings. J. Mammal. 44: 415.

Pitcher, K.W. 1980. Food of the harbor seal, *Phoca vitulina richardsi*, in the Gulf of Alaska. NOAA Fish Bull. 78: 544-549.

Quinonez-Diaz, M. J., M. M. Karpiscak, E. D. Ellman and C. P. Gerba. 2001. Removal of pathogenic and indicator microorganisms by a constructed wetland receiving untreated domestic wastewater. J. of Envir. Sci. Health, Part A: Toxic Hazardous Substances in the Environment. 36: 1311-1320.

Ralls, K. T. C. Eagle and D. B. Siniff. 1996. Movement and spatial use patterns of California sea otters. Canad. J. Zool. 74: 1841-1849.

Reeves, R.R., B.S. Stewart and S. Leatherwood. 1992. The Sierra Club handbook of seals and sirenians. Sierra Club Books. San Francisco, CA. 359 Pp.

Reynolds, J. E., D. K. Odell and S. A. Rommel. 1999. Chapter 1: Marine mammals of the world. *IN*. Biology of Marine Mammals. J. E. Reynolds and S. A. Rommel, Eds. Smithsonian Institution Press. Washington D. C. Pp.1-14.

Riedman, M. L. and J. A. Estes. 1990. The sea otter (*Enhydra lutris*): Behavior, ecology and natural history. United States Fish and Wildlife Service Biological Report. 90: 126pp.

Rosonke, B. J, S. R. Brown, S. J. Tornquist, S. P. Snyder, M. M. Garner, and L. L. Blythe. 1999. Encephalomyelitis associated with a *Sarcocystis neurona*-like organism in a sea otter. J. Am. Vet. Med. Assoc. 215: 1839-1842.

Rossano, M. G., L. S. Mansfiled, J. B. Kaneene, A. J. Murphy, C. M. Brown, H. C. Schott and J. C. Fox. 2000. Improvement of Western blot test specificity for detecting equine serum antibodies to *Sarcocystis neurona*. Vet. Diag. Invest. 12: 28-32.

Saville, W. J., P. S. Morley, S. M. Reed, D. E. Granstrom, C. W. Kohn, K. W. Hinchcliff and T. E. Whittum. 2000a. Evaluation of risk factors associated with clinical improvement and survival of horses with equine protozoal myeloencephalitis. J. Am. Vet. Med. Assoc. 217: 1181-1185.

_____, S. M. Reed, P. S. Morley, D. E. Granstrom, C. W. Kohn, K. W. Hinchcliff and T. E. Whittum. 2000b. Analysis of risk factors for the development of equine protozoal myeloencephalitis in horses. J. Am. Vet. Med. Assoc. 217: 1174-1180.

Simpson, C. F. and I. G. Mayhew. 1980. Evidence for *Sarcocystis* as the etiological agent of equine protozoal encephalitis. J. Protozool. 27: 288-292.

Slifiko, T. R., H. V. Smith and J. B. Rose. 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30: 1379-1393.

Tanhauser, S. M., M. A. Cheadle, E. T. Massey, B. A. Mayer, D. E. Schroedter, J. B. Dame, E. C. Greiner and R. J. MacKay. 2001. The nine-banded armadillo (*Dasypus novemcinctus*) is naturally infected with *Sarcocystis neurona*. Int. J. Parasitol. 31: 325-329.

Temte, J. L. 1986. Photoperiod and the timing of pupping in the Pacific harbor seal (*Phoca vitulina richardsi*) with notes on reproduction in northern fur seals and Dall's porpoises. [MSc thesis], Oregon State Univ. Corvallis, OR.

Tenter, A. M., A. R. Heckenroth and L. W. Weiss. 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30: 1217-1258.

Thomas, N. J, and R. A. Cole. 1996. The risk of disease and threats to the wild population. Endangered Species Update Special Issue: Conservation and management of the southern sea otter. 13: 23-27.

Thompson, P.M., K.M. Kovacs and B.J. McConnell.1994. Natal dispersal of harbor seals (*Phoca vitulina*) from breeding sites in Orkney, Scotland. J. Zool. Lond. 234: 668-673.

Thurston, J. A., C. P. Gerba, K. E. Foster and M. M. Karpiscak. 2001. Fate of indicator microorganisms, *Giardia* and *Cryptosporidium* in subsurface flow constructed wetlands. Water Res. 35: 1547-1551.

Tocidlowski, M. E., M. R. Lappin, P. W. Summer and M. K. Stoskopf. 1997. Serologic survey for toxoplasmosis in river otters. J. Wildl. Dis. 33: 649-652.

Torczynski, R., A. P. Bollon and M. Fluke. 1982. The complete nucleotide sequence of the rat 18s ribosomal RNA gene and comparison with the respective yeast and frog genes. Nuc. Acids Res. 11: 4879-4890.

Weigel, R. M., J. P. Dubey, A. M. Siegel, U. D. Kitron, A. Manelli, M. A. Mitchell, N. E. Mateus-Pinilla, P. Thulliez, S. K. Shen, O. C. Kwok and K. S. Todd. 1995. Risk factors for transmission of *Toxoplasma gondii* on swine farms in Illinois. J. Parasitol. 81: 736-741.

Weniger, B. G., M. J. Blaser, J. Gedrose, E. C. Lippy and D. D. Juranek. 1983. An outbreak of waterborne giardiasis associated with heavy water runoff due to warm weather and volcanic ashfall. Am. J. Pub. Health. 73: 868-872.

Wiandt, S., A. M. Grimason, B. Baleux and J. Bontoux. 2000. Efficiency of wastewater treatment plants at removing *Giardia* sp. cysts in southern France. Schriftenr. Ver. Wasser Boden Lufthyg. 105: 35-42.

Wilson, M., D. A. Ware and D. D. Juranek. 1990. Serologic aspects of toxoplasmosis. J. Am. Vet. Med. Assoc. 196: 277-281.

Wilson, D. E., M. A. Bogan, A. M. Burdin, amd M. K. Marinov. 1991. Geographic variation in sea otters, *Enhydra lutris*. J. Mammal. 72: 22-36.

Yamane, I., J. W. Thomford, I. A. Gardner, J. P. Dubey, M. Levy and P. A. Conrad. 1993. Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. Am. J. Vet. Res. 54:1579-1584.

Yanga, S. T., I. A. Gardner, H. S. Hurd. K. A. Eernisse and P. Willeburg. 1995. Mangement and demographic factors associated with seropositivity to transmissible gastroenteritis virus in US swine herds, 1980-1990. Prevent. Vet. Med. 24: 213-228.

Yilmaz, S. M. and S. H. Hopkins. 1972. Effects of different conditions on duration of infectivity of *Toxoplasma gondii* oocysts. J. Parasitol. 58: 938-939.

Chapter 2

Isolation and characterization of *Sarcocystis neurona* from a southern sea otter

Parasitol Res (2001) 87: 252–257

RAPID COMMUNICATION

Melissa A. Miller · Paul R. Crosbie · Karen Sverlow
Krista Hanni · Bradd C. Barr · Nancy Kock
Michael J. Murray · Linda J. Lowenstine
Patricia A. Conrad

Isolation and characterization of *Sarcocystis* from brain tissue of a free-living southern sea otter (*Enhydra lutris nereis*) with fatal meningoencephalitis

Received: 15 September 2000 / Accepted: 10 October 2000

Abstract A protozoan was isolated in cell culture from the brain of a free-ranging sea otter with fatal meningoencephalitis. The biological history of this otter, a study animal being monitored via an intraperitoneal radio transmitter, is summarized. Histologically, protozoal parasites were associated with areas of brain inflammation and necrosis in the cerebrum and cerebellum. Morphology and measurements of fixed, Giemsa-stained protozoal zoites growing on coverslips were consistent with *Sarcocystis*. These parasites reacted only with polyclonal antisera raised against *S. neurona* on immunohistochemistry. Cell culture-derived zoites reacted strongly with polyclonal antiserum to *S. neurona* on indirect fluorescent antibody tests. Amplification of portions of the 18S ribosomal DNA and the adjacent first internal transcribed spacer were performed. The resulting sequences were compared with published sequences from similar apicomplexan protozoa. This isolate (SO SN_1), was indistinguishable from *S. neurona*, based on parasite morphology, antigenic reactivity and molecular characterization.

M. A. Miller · L. J. Lowenstine · P. A. Conrad (✉) B. C. Barr
Department of Pathology, Microbiology and Immunology,
School of Veterinary Medicine, University of California,
Davis, 1 Shields Avenue, Davis, CA 95616-8739, USA
e-mail: paconrad@ucdavis.edu
Tel.: +1-530-7527210; Fax: +1-530-7523349

P. R. Crosbie
Department of Biology, California State University,
Fresno, 2555 E. San Ramon, Fresno, CA 93740, USA

K. Sverlow
CAHFS, University of California,
P.O. Box 1770, Davis, CA 95617, USA

K. Hanni
Wildlife Health Center, School of Veterinary Medicine,
University of California, Davis, 1 Shields Avenue,
Davis, CA 95616, USA

N. Kock
Marine Wildlife Veterinary Care and Research Center,
California Department of Fish and Game,
1451 Shaffer Road, Santa Cruz, CA 95060, USA

M. J. Murray
Monterey Bay Aquarium, 886 Cannery Row, Monterey,
CA 93940-1085, USA

Introduction

Protozoal brain infections in marine mammals have been reported previously in cetaceans, mustelids, pinnipeds and sirenians (Van Pelt et al. 1973; Migaki et al. 1977; Buergelt et al. 1983; Holshuh et al. 1985; Cruickshank et al. 1990; Inskeep et al. 1990; Migaki et al. 1990; Thomas and Cole 1996; Lapointe et al. 1998, Oksanen et al. 1998; Rosonke et al. 1999; Cole et al. 2000; Lindsay et al. 2000; Mikaelian et al. 2000). Cases have been reported from both captive (Lapointe et al. 1998; Rosonke et al. 1999) and free-ranging (Buergelt et al. 1983; Holshuh et al. 1985) marine mammals. The parasite implicated in most cases was *Toxoplasma gondii* (Van Pelt et al. 1973; Migaki et al. 1977; Buergelt et al. 1983; Holshuh et al. 1985; Cruickshank et al. 1990; Inskeep et al. 1990; Migaki et al. 1990; Di Guardo et al. 1995). However, reports were generally anecdotal and most did not include parasite isolation and characterization techniques.

Since that time, protozoal encephalitis has been recognized as a significant cause of mortality in free-living marine mammals from coastal California. In 1998, *Sarcocystis neurona* was reported from seven Pacific harbor seals (*Phoca vitulina richardsi*) with fatal meningoencephalitis (Lapointe et al. 1998). These harbor seals had died between 1992 and 1998. And all were recovered from the California coastline between Sea Ranch, Mendocino County and metropolitan San Francisco. Western blotting revealed that four of five seals tested had serum antibodies reactive to antigens from *S. neurona*. Protozoa in brain tissue from all cases stained positively by immunohistochemistry, using antisera raised against *S. neurona* antigens, but parasite isolation was not attempted. In September, 1998, we examined a

wild, adult female harbor seal from Zmudowski State Beach, in the mid-coastal region of Monterey Bay, California. This seal was found alive, but died within hours of hospitalization. Clinical signs prior to death consisted of seizures, tremors and obtundation. Histological examination of the cerebrum and cerebellum revealed severe non-suppurative meningoencephalitis; and two distinct protozoan parasites were isolated from the brain tissue and cerebrospinal fluid (Miller et al. 2001). These parasites were separated by mouse inoculation and limiting dilution. They were found to be indistinguishable from *S. neurona* and *T. gondii*, using immunohistochemistry, serology (indirect fluorescent antibody tests; IFAT) and polymerase chain reaction (PCR) amplification and sequencing of two different DNA fragments.

Protozoal meningoencephalitis was first reported in southern sea otters (*Enhydra lutris nereis*) from California in 1996 (Thomas and Cole 1996). This disease syndrome was soon recognized as a significant source of southern sea otter mortality, responsible for the deaths of 8.5% of fresh dead California otters examined by the National Wildlife Health Center (Thomas and Cole 1996). Initial parasite characterization was suggestive of *T. gondii* infection in most cases. However infection by another, as yet uncharacterized, protozoan was suspected (Thomas and Cole 1996). In 1998, a captive northern sea otter (*E. l. kenyoni*), housed at a public aquarium in coastal Oregon, died after developing progressive neurological deficits (Rosonke et al. 1999). Postmortem examination revealed non-suppurative meningoencephalitis, associated with large numbers of protozoal parasites that stained strongly positive for *S. neurona* on immunohistochemistry. Seropositivity was confirmed by Western blot analysis, but parasite isolation and characterization were not performed.

In November 1999, Lindsay et al. (2000) isolated *S. neurona* from a wild, juvenile male southern sea otter from northern Monterey Bay. A 1,100-bp fragment of the first internal transcribed spacer (ITS 1) region was amplified, followed by restriction enzyme digestion to determine the phylogenetic relationship between their protozoal isolate and *S. neurona* obtained from horses. Sequence analysis of the partial 18S and entire ITS portions of the parasite DNA were not reported. No data were available on the animal prior to hospitalization.

In May, 1999, we isolated a protozoal parasite from a free-living juvenile male sea otter that was recovered from the same geographic area as the infected harbor seal discussed above. Brain histology revealed multifocal encephalitis and meningitis with moderate numbers of intralesional protozoal parasites. This sea otter had been implanted with an abdominal radio transmitter to facilitate frequent observation for studies on food habits and post-weaning survival. It had been monitored at intervals of 1 to 3 days for 34 days prior to being found dead. Extensive information was collected on the animal's location, behavior, daily movements, prey species consumption and interactions with other otters. The present paper summarizes the biological data from this sea otter, together with the results of our efforts to isolate and characterize the protozoan parasite seen in association with lesions in its brain tissue.

Materials and methods

Case history

A male southern sea otter pup was found stranded on San Carlos Beach, Monterey, California on December 1, 1998. There were no signs of illness or injury, but the pup was exhausted and partially emaciated (6.2 kg). It was estimated to be 16 weeks old. Due to the pup's young age and poor nutritional condition, it was presumed that the otter had become separated from its mother and was unable to forage for itself. On admission to the Monterey Bay Aquarium veterinary facilities, preliminary laboratory data (complete blood count and blood chemistry panel) were unremarkable.

Initial prophylactic therapy consisted of subcutaneous fluids (lactated Ringer's solution), antibiotics (procaine/benzathine penicillin) and gastric mucosal protectants (cimetidine). Standard rehabilitation procedures were followed for this animal during 84 days of hospitalization (Williams et al. 1995), during which it was held in seawater tanks maintained as a flow-through, sand-filtered seawater system. Seawater supplying the pools was obtained from a standpipe located 300 m offshore in water approximately 20 m deep. While hospitalized, the otter was fed a variety of prey items, consisting of previously frozen surf clam foot and adductor muscle (*Spisula solidissima*), shrimp (*Penaeus vannamei*), Icelandic cod (*Gadus morhua*), squid (*Loligo opalescens*), both fresh and frozen whole manila clams (*Tapes* sp.), and fresh, live cancer crab (*Cancer anntenarius*), all purchased from commercial vendors.

No behavioral or physical abnormalities were detected during the period of captive rehabilitation, and normal rates of weight gain and behavioral development were observed. A radiotransmitter was placed in the abdomen by ventral midline celiotomy on January 21, 1999, using standard anesthetic procedures (oxymorphone/diazepam/isoflurane). Intra-operative serum chemistry and complete blood counts were unremarkable and post-surgical recovery was uneventful.

The animal was released at Seal Bend, Elkhorn Slough, Monterey County, California, on February 23, 1999. At the time of release, the otter was in excellent nutritional condition (11.4 kg) and met all appropriate weight and behavioral criteria for its sex and age (Williams et al. 1995). Elkhorn Slough is composed of shallow tidal channels with a sand or mud bottom and is commonly used by sea otters for resting and foraging (Silberstein and Campbell 1989). Invertebrate prey species found in the slough include mussels (*Mytilus edulis*), Washington clams (*Saxidomus nuttallii*), gaper clams (*Tresus nuttallii*), sand crabs (*Pachygrapsus* spp. and *Hemigrapsus* spp.) and fat inkeeper worms (*Urechis caupo*; Morris et al. 1990). The slough is a seasonal estuary, with minimal freshwater inflows during periods of low precipitation (i.e. summer and fall). Adjacent terrestrial habitat consists of areas of salt marsh (designated as a wildlife refuge), agricultural fields, a commercial dairy and scattered private residences. Behavioral observations were obtained at intervals of 24–48 h, until the animal was found dead 34 days after initial release.

Postmortem examination and immunohistochemistry

The otter was found dead in a tidal channel at Seal Bend on March 28, 1999, and was transported to the Marine Wildlife Veterinary Care and Research Center, operated by the California Department of Fish and Game in Santa Cruz, California, where it was necropsied upon arrival. The estimated postmortem interval was 3 days. Selected tissues were immersion-fixed in 10% neutral buffered formalin. Trimmed, formalin-fixed tissues were processed and embedded

in paraffin. Five µm sections were cut, stained and examined using a light microscope. In addition, 4 µm sections of cerebrum, cerebellum, medulla, lung, liver, spleen, pancreas, hilar lymph node, thymus, testicle and epididymis were immunohistochemically stained, using rabbit-derived polyclonal antisera to *Sarcocystis neurona* (UCD-SN1), *Toxoplasma gondii* (ME49), and *Neospora caninum* (BPA 1), as previously described (Lapointe et al. 1998).

Protozoal parasite culture

Brain tissue was collected aseptically at the time of necropsy and was processed for parasite isolation in stationary cell cultures of monkey kidney cells (MA104; Biowhittaker, Walkersville, Maryland; Conrad et al. 1993a; Marsh et al. 1998). All cultures were maintained at 37 °C in 5% CO_2 and were checked three times weekly under an inverted microscope for signs of parasite growth. The medium was renewed three times each week, on alternating days. Isolated parasites were maintained in cell culture and passaged to uninfected monolayers (Conrad et al. 1993a).

Indirect fluorescent antibody testing

An IFAT was performed as previously described (Conrad et al. 1993b) to antigenically characterize the protozoal parasites obtained from the sea otter. Briefly, protozoal zoites derived from the sea otter isolate were harvested by cell scraping and centrifugation, and were then diluted to 5,000 zoites/µl. Aliquots (10 µl) of the parasite suspension were dotted onto multi-well IFAT slides (Erie Scientific, Portsmouth, N.H.), formalin-fixed and air-dried. The same panel of rabbit-derived polyclonal antisera to *S. neurona*, *T. gondii* and *N. caninum* zoite antigens utilized for immunohistochemistry was also used for IFAT; and a 1:300 dilution of fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG was used as a secondary antibody. Zoites derived from fully characterized protozoal isolates from domestic animals and humans served as positive and negative controls. The last well exhibiting distinct whole parasite fluorescence was the reported titer.

Molecular characterization

Molecular characterization of isolated parasites was accomplished by PCR amplification and subsequent cycle-sequencing of two nuclear genetic markers: a portion of the 18S ribosomal DNA (rDNA) and the entire adjacent ITS-1, as previously described (Marsh et al. 1995, 1996, 1999). Genomic DNA was extracted and purified from zoites grown in cell culture using an IsoQuick nucleic acid extraction kit (ORCA Research, Bothell, Wash.). The nucleotide sequences of complementary strands were aligned and compared; and a contiguous nucleotide sequence was determined for each gene fragment, using the Sequencher 3.0 program (GeneCodes, Ann Arbor, Mich.).

Published sequences of 18S rDNA and ITS1 + 5.8S rDNA from protozoal parasites of domestic animals and humans were imported from GenBank, and then aligned and compared with the sequences obtained from the sea otter isolate (designated SO SN_1). ClustalW (Thompson et al. 1994) was used to perform initial alignments; and subsequent editing and positional analysis were conducted using MacClade 3.7 (Maddison and Maddison 1992).

Results

Case history

During post-release radio monitoring, the otter was never observed to move more than 2 km from the point of release at Seal Bend, Elkhorn Slough, nor to venture into the open ocean 2 km away. Several instances of contact with other otters were observed and this otter often rafted with other otters when sleeping. The animal's foraging activity was focused near the south bank of Elkhorn Slough, near a commercial dairy located at the shoreline. Foraging behavior was observed on several occasions and the animal appeared to be developing a normal foraging pattern and maintaining its body weight. Favored prey items included small mussels, clams and crabs. No abnormal behavior was observed at any time during the next 34 days of observation. The last period of monitoring was 4 days prior to the otter being found dead on March 28, 1999. On that day, the telemetry signal obtained was typical of an animal alive and resting in the water; however visual observation was not recorded at the time.

Postmortem examination and immunohistochemistry

Gross postmortem findings consisted of moderate emaciation, characterized by symmetrical muscle thinning and serous atrophy of body fat. Body weight on recovery was 9.7 kg, reflecting approximately 15% weight loss since release. No evidence of trauma was noted, and postmortem radiographs were unremarkable. The gastrointestinal tract was empty and low numbers of acanthocephalan parasites (*Corynosoma* sp.) were attached to the intestinal mucosa. A mixture of sand and mud was present in the trachea and bronchi of the right lung field. Moderate expansion of the pulmonary interlobular septae by serosanguinous fluid was noted. Several hundred milliliters of similar fluid were present in the thoracic cavity. The brain appeared soft grossly, but was otherwise unremarkable. Moderate postmortem decomposition was noted.

Microscopic examination of sections of cerebrum, cerebellum and brainstem revealed numerous small nodules of non-suppurative inflammation and gliosis within both gray and white matter tracts, with moderate numbers of intralesional free zoites and intracellular protozoal schizonts. Some schizonts were arranged in a prominent rosette pattern. Prominent perivascular cuffs of mononuclear cells were present in the meninges and superficial neuropil. The inflammation was most severe in sections of cerebellum. The spinal cord was not available for examination.

Parasite profiles in sections of cerebrum and cerebellum stained strongly positive using antiserum to *S. neurona* on immunohistochemistry. Positively stained protozoal schizonts and free zoites were associated with areas of inflamed neuropil. These same parasites did not stain when *T. gondii* or *N. caninum* antiserum was utilized. All other tissues were negative for staining for all three parasites.

Protozoal parasite culture

A protozoal parasite was first detected in MA104 cell cultures 42 days after initial culture inoculation with

trypsinized cerebrum and cerebellum from the otter. Both intracellular schizonts and free zoites were observed in this culture, with some schizonts arranged in a prominent rosette pattern. The free zoites were long and slender (averaging 4 × 1 μm on fixed, Giemsa-stained slides, with a range of 3–5 μm × 0.8–1.5 μm, $n = 30$ zoites), with active, circum axial motility, similar to previous isolates of *Sarcocystis neurona* that we obtained from horses and harbor seals (K. Sverlow and M. Miller, unpublished data).

Indirect fluorescent antibody testing

Antigenic characterization of purified zoites from this isolate revealed strong positive staining with *S. neurona* antisera (1:2,560 titer by IFAT). Reactivity to *T. gondii* and *N. caninum* antisera were below initial screening values (<1:40 for each, titer by IFAT). Based on parasite morphology in cell culture and the results of antigenic characterization, this sea otter isolate was designated sea otter *S. neurona* 1 (SO SN_1).

Molecular characterization

We successfully obtained sequences of both 18S rDNA and the adjacent ITS 1 from protozoal isolate SO SN_1. The 18S rDNA amplification product was 530 nucleotides long and extended from the 5′ end of the 18S rDNA. The ITS 1 amplification product was 1,053 nucleotides in length, encompassing the entire ITS 1 DNA segment. Both sequences were deposited in GenBank: 18S rDNA as GenBank accession number AY009112 and ITS 1 as GenBank accession number AY009113. The 18S sequence was indistinguishable from the published 18S sequences of *S. neurona* obtained from horses (GenBank accession number U33149; Dame et al. 1995) and harbor seals (GenBank accession number AF252406; Miller et al. 2001); and the ITS 1 sequence was indistinguishable from published ITS 1 sequences from horses (GenBank accession number AF081944; Marsh et al. 1999) and harbor seals (GenBank accession number AF252407; Miller et al. 2001). Based on these direct comparisons of the 18S rDNA and ITS 1 sequences, the SO SN_1 isolate from the sea otter was genetically indistinguishable from *S. neurona*.

Conclusions

How sea otters become infected with *Sarcocystis neurona* is unknown. The opossum (*Didelphis virginiana*) is recognized as a definitive host of *S. neurona*, shedding sporocysts infective for horses (Fenger et al. 1995). However other, unrecognized definitive hosts may also exist. Opossums were introduced to California in 1910 (Jameson and Peeters 1988) and are present in the vicinity of Elkhorn Slough, where this sea otter lived and foraged. Sea otters began using Elkhorn Slough as a resting and foraging area around 1984 (Kvitek and Oliver 1988; Kvitek et al. 1988). The recent recognition of severe encephalitis associated with *S. neurona*-like parasites in sea otters and harbor seals in coastal California may be due to increased exposure to *S. neurona* sporocysts shed by introduced opossums.

These sporocysts may be present in surface runoff (Benenson et al. 1982; Aramini et al. 1999) and could potentially be concentrated through the filter-feeding activity of bivalve prey species fed upon by sea otters. Protozoal cysts/oocysts of *Giardia*, *Cryptosporidium* and *Cyclospora* can be concentrated within filter-feeding bivalves, both experimentally and under natural conditions (Fayer et al. 1998; Graczyk et al. 1998a, b, 1999a, b). Many types of bivalves, including mussels, clams and oysters, have been shown to efficiently remove and concentrate protozoal cysts/oocysts when exposed to contaminated water. Mussels and clams represent key prey items for sea otters (Kvitek and Oliver 1988; Kvitek et al. 1988). California sea otters spend most of their time in the water and consume up to 25% of their body weight each day in bivalves and crustaceans (Reidman and Estes 1990). Thus contamination of marine bivalves by oocysts derived from sewage or surface runoff may be an effective mechanism for protozoal infection of sea otters.

The length of time between exposure to sporocysts and development of clinical signs in sea otters is unknown, so the location of initial exposure to *S. neurona* for this otter is uncertain. A tissue cyst stage has never been detected for *S. neurona*, but we cannot discount the possibility of reactivation of a latent infection. This otter was clinically normal throughout its period of captivity, remained clinically normal for at least one month post-release and was never observed to venture outside of Elkhorn Slough. Therefore, it is likely that the animal was exposed to *S. neurona* sporocysts while living and feeding within the slough, and not during the preceding period of captive hospitalization. Since 1999, two additional sea otters have stranded in the vicinity of Elkhorn Slough with clinical signs suggestive of protozoal encephalitis. These otters exhibited marked elevation in IFAT titers to both *Toxoplasma gondii* and *S. neurona*; and they exhibited partial to complete resolution of neurological deficits after prolonged antiprotozoal therapy.

In 1998, we isolated a parasite indistinguishable from *S. neurona* from an adult female harbor seal which stranded approximately 3 km from the stranding location of this otter (Miller et al. 2001). Large numbers of harbor seals use the mud banks in Elkhorn Slough as a resting site during the day and leave the area at night to feed on squid and fish in nearby Monterey Bay. Harbor seals do not typically consume bivalves, so alternative routes of protozoal exposure, such as direct contact with contaminated mud or water, are also possible. Future epidemiological research may implicate Elkhorn Slough

as a relatively "high risk" area for protozoal infection for sea otters and seals.

This paper reports the successful isolation and both antigenic and molecular characterization of an *S. neurona*-like parasite from a free-ranging southern sea otter with fatal meningoencephalitis. These results build on previous data presented by Lindsay et al. (2000), in which molecular characterization was based upon restriction fragment length polymorphism analysis only. In the current paper, phylogenetically informative regions of the 18 S rDNA and ITS1 nuclear DNA from our isolate were amplified, sequenced and directly compared to published sequences for *S. neurona* from horses. Based on parasite morphology in cell culture, immunohistochemistry, antigenic characterization and DNA amplification and sequencing, this protozoal isolate, designated SO SN_1, appears antigenically and molecularly distinct from *T. gondii* and *Neospora caninum*, but is indistinguishable from *S. neurona* from horses. Additional data are presented on the affected sea otter's biological history; and we describe a possible focus of sea otter and harbor seal protozoal infection at Elkhorn Slough, California.

Acknowledgements This research was supported in part by funding from the Lindbergh Foundation, the Morris Animal Foundation, the PKD Trust, the UCD Genetic Resources Conservation Program and the California Department of Fish and Game. The authors wish to acknowledge the excellent technical assistance of staff and volunteers from the Monterey Bay Aquarium, Monterey, California, especially Michelle Staedler, Julie Stewart and Andy Johnson. The advice and support of Anne Kjemtrup, Tom Robinson and Grace Lee for assistance in molecular characterization are deeply appreciated. Phil Chiu of the California Animal Health Food and Safety Laboratory provided invaluable assistance for immunohistochemistry. We thank David Sibley of the Washington University School of Medicine and Antoinette Marsh of the University of Columbia, Missouri, for their advice and assistance in obtaining isolates. These experiments comply with current laws in the United States.

References

Aramini JJ, Stephen C, Dubey JP, Engelstoft C, Schwantje H, Ribble CS (1999) Potential contamination of drinking water with *Toxoplasma gondii* oocysts. Epidemiol Infect 122: 305–315

Benenson MW, Takafuji ET, Lemon SM, Greenup RL, Sulzer AJ (1982) Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water. N Engl J Med 307: 666–669

Buergelt CD, Bonde RK (1983) Toxoplasmic meningoencephalitis in a West Indian manatee. J Am Vet Med Assoc 183: 1294–1296

Cole RA, Lindsay DS, Roderick CL, Dubey JP, Thomas NJ, Baeten LA (2000) Biological and molecular characterizations of *Toxoplasma gondii* obtained from southern sea otters (*Enhydra lutris nereis*). J Parasitol 86: 526–530

Conrad PA, Barr BC, Sverlow KW, Anderson MA, Daft B, Kinde H, Dubey JP, Munson L, Ardans A (1993a) In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology 106: 239–249

Conrad PA, Sverlow K, Anderson M, Rowe J, Bondurant R, Tuter G, Breitmeyer R, Palmer C, Thurmond M, Ardans A, Dubey JP, Duhamel G, Barr B (1993b) Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. J Vet Diagn Invest 5: 572–578

Cruickshank JJ, Haines DM, Palmer NC, St. Aubin DJ (1990) Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Can Vet J 31: 213–215

Dame JB, Mackay RJ, Yowell CA, Cutler TJ, Marsh A, Greiner EC (1995) *Sarcocystis falcatula* from passerine and psittacine birds: synonymy with *Sarcocystis neurona*, agent of equine protozoal myeloencephalitis. J Parasitol 81: 930–935

Di Guardo G, Corradi A, Agrimi U, Zizzo N, Morelli L, Perillo A, Kramer L, Cabassi E, Kennedy S (1995) Neuropathological lesions in cetaceans found stranded from 1991 to 1993 on the coast of Italy. Eur J Vet Pathol 1: 51–57

Fayer R, Graczyk TK, Lewis EJ, Trout JM, Farley CA (1998) Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl Environ Microbiol 64: 1070–1074

Fenger CK, Granstrom DE, Langemeier JL, Stamper S, Donahue JM, Patterson JS, Gajadhar AA, Marteniuk JV, Xiaomin Z, Dubey JP (1995) Identification of opossums (*Didelphis virginiana*) as the putative definitive host of *S. neurona*. J Parasitol 81: 916–919

Graczyk TK, Farley CA, Fayer R, Lewis EJ, Trout JM (1998a) Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissues of eastern oysters (*Crassostrea virginica*) carrying principal oyster infectious diseases. J Parasitol 84: 1039–1042

Graczyk TK, Ortega YR, Conn DB (1998b) Recovery of waterborne oocysts of *Cyclospora cayetanensis* by asian freshwater clams (*Corbicula fluminea*). Am J Trop Med Hyg 59: 928–932

Graczyk TK, Fayer R, Conn DB, Lewis EJ (1999a) Evaluation of the recovery of waterborne *Giardia* cysts by freshwater clams and cyst detection in clam tissue. Parasitol Res 85: 30–34

Graczyk TK, Fayer R, Lewis EJ, Trout JM, Fairley CA (1999b) *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol Res 85: 518–521

Holshuh HJ, Sherrod AE, Taylor CR, Andrews BF, Howard EB (1985) Toxoplasmosis in a feral northern fur seal. J Am Vet Med Assoc 187: 1229–1230

Inskeep W, Gardner CH, Harris RK, Dubey JP, Goldstone RT (1990) Toxoplamosis in Atlantic bottle-nose dolphins (*Tursiops truncatus*). J Wildl Dis 26: 377–382

Jameson EW, Peeters HE (1988) California mammals. University of California Press, Berkeley

Kvitek RG, Oliver JS (1988) Sea otter foraging habits and effects on prey populations and communities in soft-bottom environments. In: Van Blaricoum GR, Estes JS (eds) The community ecology of sea otters. (Ecological studies, vol 65) Springer, Berlin Heidelberg New York, p 22–47

Kvitek RG, Fukayama AK, Anderson BS, Grimm BM (1988) Sea otter foraging on deep-burrowing bivalves in a California central lagoon. Mar Biol 98: 157–167

Lapointe JM, Duignan PJ, Marsh AE, Gulland FM, Barr BC, Naydan DK, King DP, Farman CA, Burek Huntingdon KA, Lowenstine LJ (1998) Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific harbor seals (*Phoca vitulina richardsi*). J Parasitol 84: 1184–1189

Lindsay DS, Thomas NJ, Dubey DP (2000) Biological characterization *of Sarcocystis neurona* isolated from a southern sea otter (*Enhydra lutris nereis*). Int J Parasitol 30: 617–624

Maddison WP, Maddison DR (1992) MacClade: analysis of phylogeny and character evolution, ver 3.07. Sinauer Associates, Sunderland

Marsh AE, Barr BC, Sverlow K, Ho M, Dubey JP, Conrad PA (1995) Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. J Parasitol 81: 530–535

Marsh AE, Barr BC, Madigan J, Lakritz J, Conrad PA (1996) Sequence analysis and polymerase chain reaction amplification of small subunit ribosomal DNA from *Sarcocystis neurona*. Am J Vet Res 57: 975–981

Marsh AE, Barr BC, Packham AE, Conrad PA (1998) Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). J Parasitol 83: 1189–1192

Chapter 3

Isolation and characterization of parasitic protozoa from a Pacific harbor seal

ISOLATION AND CHARACTERIZATION OF TWO PARASITIC PROTOZOA FROM A PACIFIC HARBOR SEAL (*PHOCA VITULINA RICHARDSI*) WITH MENINGOENCEPHALOMYELITIS

Melissa A. Miller, Karen Sverlow*, Paul R. Crosbie†, Bradd C. Barr*, Linda J. Lowenstine, Frances M. Gulland‡, Andrea Packham*, and Patricia A. Conrad§

Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, California 95616-8739

ABSTRACT: Two species of protozoans were isolated from a harbor seal with fatal meningoencephalitis. Serologic reactivity was detected to both *Sarcocystis neurona* and *Toxoplasma gondii*. Parasites associated with brain inflammation and necrosis reacted only with immunohistochemical stains utilizing polyclonal antisera raised against *Sarcocystis neurona*. However, 2 distinct parasites were observed in cell cultures derived from the seal's brain tissue. These parasites were separated by mouse passage and limiting dilution. Purified zoites from 1 isolate (HS1) reacted strongly with polyclonal antiserum to *S. neurona* and with the harbor seal's own serum (1:2,560 for each) on indirect immunofluorescent antibody tests (IFAT), but weakly to antisera to *T. gondii* and *Neospora caninum* (1:40). Zoites from the second isolate (HS2) reacted positively with *T. gondii* polyclonal antiserum (1:81,920) and with the harbor seal's own serum (1:640), but weakly to *S. neurona* and *N. caninum* antisera (1:80 or less). Amplification and sequence analysis of protozoal DNA encoding portions of the 18s ribosomal RNA (18s rDNA) and the adjacent first internal transcribed spacer (ITS1) were performed for both isolates, and resulting sequences were compared to those from similar protozoans. Based on molecular characterization, parasite morphology, serologic reactivity, histology, and immunohistochemistry, HS1 was indistinguishable from *S. neurona*, and HS2 was indistinguishable from *T. gondii*.

Protozoan infections of the brain have been diagnosed in wild and captive marine mammals worldwide. Cetaceans, pinnipeds, sirenians, and sea otters have all been found to be infected (Van Pelt et al., 1973; Buergelt and Bonde, 1983; Holshuh et al., 1985; Cruickshank et al., 1990; Inskeep et al., 1990; Migaki et al., 1990; Di Guardo et al., 1995; Thomas and Cole, 1996; Lapointe et al., 1998; Oksanen et al., 1998; Cole et al., 2000; Lindsay et al., 2000; Mikaelian et al., 2000). A number of protozoans, including *Toxoplasma gondii* and *Sarcocystis neurona* have been implicated in these infections, but precise molecular and antigenic characterization of these parasites has been limited. In some cases, protozoan infections have been associated with concurrent infection by other agents, such as morbillivirus or staphylococcal bacteria (Van Pelt and Dietrich, 1973; Di Guardo et al., 1995). However in other cases, no other contributing factor was found, and the protozoan infection was felt to be the primary cause of morbidity or mortality (Lapointe et al., 1998; Rosonke et al., 1999; Cole et al., 2000). Pacific harbor seals (*Phoca vitulina richardsi*) occur along the entire coast of California. Although California populations of harbor seals appear stable, diseases that affect these animals may have great importance for management of less numerous sympatric species, such as threatened southern sea otters (*Enhydra lutris nereis*).

Protozoan meningoencephalitis was first recognized as a cause of mortality in Pacific harbor seals from Northern California in 1992 (Lapointe et al., 1998). Affected animals were found dead or stranded, with weakness, depression, head tremors, mydriasis, and seizures, and usually died within hours of hospitalization. Common to all 7 reported cases was severe nonsuppurative meningoencephalitis, associated with proliferating protozoal schizonts and zoites. Preliminary evaluations implicated *S. neurona* as the causative agent in all cases (Lapointe et al., 1998). However as yet, the parasite has not been isolated and compared with *S. neurona* isolates from horses.

In the present paper, in vitro cultivation and characterization of 2 distinct protozoal parasites isolated from brain tissue and cerebrospinal fluid (CSF) of a harbor seal with fatal meningoencephalitis are reported. Antigenic analysis and molecular techniques were used to directly compare these protozoan parasites with those obtained from domestic animals and humans.

MATERIALS AND METHODS

Postmortem examination and immunohistochemistry

On 8 September 1998, an adult female Pacific harbor seal was found depressed, seizuring, and unresponsive on Zmudowski State Beach, 32 km northwest of Monterey, California, and was transported to a local rehabilitation facility. The 65-kg female harbor seal was in poor nutritional condition, with bilateral mydriasis and poor pupillary light responses. She died shortly after admission and was shipped on ice to the University of California Veterinary Medical Teaching Hospital for necropsy. Blood was collected by cardiocentesis prior to necropsy examination. A detailed gross postmortem examination was performed, including bacterial cultures from samples of lung, brain, spleen, bronchus, and colon, and tissues were collected for histopathological examination. The following tissues were fixed in 10% neutral buffered formalin: brain, spinal cord, multiple skeletal muscles, myocardium, diaphragm, tongue, spleen, kidney, liver, adrenal gland, lung, and multiple lymph nodes. Formalin-fixed tissues were trimmed, routinely processed for paraffin embedding, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E). Immunohistochemical stains were performed on selected sections of formalin-fixed, paraffin-embedded brain, spinal cord, myocardium, skeletal muscle, tongue, liver, adrenal gland, and lung. Polyclonal antisera were produced by inoculation of rabbits with isolates of *S. neurona* (UCD-SN1), *Sarcocystis falcatula* (UCD-SF1), and *Neospora caninum* (BPA1) (Conrad, Sverlow et al., 1993; Marsh et al., 1996, 1998; Packham et al., 1998). Polyclonal *T. gondii* antibodies were prepared via rabbit inoculation as follows: a 3.5-kg adult female New Zealand White rabbit was injected subcutaneously (s.c.) with 3.5×10^8 killed, culture-derived *T. gondii* (ME49) tachyzoites raised on monkey kidney (MA104) cells; 37 days later, the rabbit was inoculated s.c. with

Received 11 July 2000; revised 14 December 2000; accepted 14 December 2000.

*California Animal Health and Food Safety Laboratory, University of California, Davis, School of Veterinary Medicine, P.O. Box 1770, Davis, California 95617-1770.

†Department of Biology, California State University, Fresno, 2555 E. San Ramon, Fresno, California 93740.

‡The Marine Mammal Center, Marin Headlands, 1065 Fort Cronkhite, Sausalito, California 94965.

§Corresponding author.

100 live, culture-derived *T. gondii* (ME49) tachyzoites grown in guinea pig lung fibroblasts (ATCC CCL158); 29 days after live tachyzoite inoculation, the rabbit was euthanized and exsanguinated.

The Dako EnVision + System HRP (AEC) Rabbit Kit (Dako Corporation, Carpinteria, California) was used to visualize sites of antibody binding. Paraffin sections (3 μm) were incubated for 10 min with 3% hydrogen peroxide in absolute methanol to block endogenous peroxidase, placed in pepsin digestion solution for 15 min at 37 C, then washed in tap water for 5 min. The slides were soaked in phosphate-buffered saline (PBS) for 3 min and placed in blocking solution (PBS/0.5% casein) for 10 min at room temperature to prevent nonspecific antibody binding. Primary antibodies were applied for 30 min at room temperature, followed by a PBS wash. The following optimal primary antibody dilutions were used: 1:1,600 for *S. neurona*, 1:1,600 for *S. falcatula*, 1:1,500 for *T. gondii*, and 1:400 for *N. caninum*. Next, all slides were treated with Dako EnVision labeled polymer HAP solution from the Dako kit for 30 min at room temperature then washed with PBS. Dako EnVision AEC substrate chromogen solution was applied to each slide for 10 min at room temperature, followed by rinses in PBS and then running water. Mayer's hematoxylin was used as a counterstain. Tissue blocks containing protozoa from experimentally infected mice (*T. gondii*, *N. caninum*, and *S. neurona*) or budgerigars (*S. falcatula*) were used as controls. These tissue sets were used as positive controls for antisera raised against the same parasites and negative controls for antisera raised against the other parasites.

Serology

Serum from the harbor seal was examined for the presence of antibodies to *T. gondii*, *N. caninum*, and *S. neurona* using indirect immunofluorescent antibody tests (IFAT). In these tests, culture-derived zoites of *S. neurona* (UCD-SN1), *T. gondii* (ME49), or *N. caninum* (BPA1) originally isolated from domestic animals or humans were harvested by scraping when >50% of the monolayer cells were infected. *Sarcocystis neurona*-infected cells were centrifuged at 1,500 *g* for 10 min, and the resulting pellet was resuspended in 3 ml sterile PBS. The cell suspension was passed 3 times through a 22-gauge needle to disrupt the cells. The suspended *S. neurona* zoites were adjusted to a final concentration of 5,000 zoites/μl PBS and dotted onto each of 12, 4-mm wells of IFAT slides (Erie Scientific, Portsmouth, New Hampshire). The *T. gondii* and *N. caninum* parasite suspensions were passed through a PD10 Sephadex (Sigma, St. Louis, Missouri) column to remove feeder layer cells. The effluent was centrifuged again, and the purified zoite pellet was resuspended to a final concentration of approximately 5,000 zoites/μl in sterile PBS, dotted onto IFAT slides, and air-dried. Resulting slides were fixed in 10% neutral buffered formalin for 10 min, rinsed in PBS, air-dried, and stored at −70 C until used.

The harbor seal's serum was diluted in PBS by doubling dilutions from 1:40 to 1:81,920, and 10 μl of each dilution was placed in separate wells of the IFAT slides. After incubation for 1 hr at 37 C, the slides were washed 3 times in PBS. A 1:100 dilution (10 μl) of FITC-conjugated rabbit anti-canine IgG (Affini-Pure, Jackson Immunoresearch, West Grove, Pennsylvania) was added to each well. After an additional 1 hr of incubation, the slides were washed 3 times and mounted in buffered (pH 8.5) glycerol. The wells were examined using fluorescence microscopy at ×200, and the last well with distinct parasite outline fluorescence was the reported titer.

Parasite isolation

CSF and brain tissue were collected aseptically at the time of necropsy and used for parasite isolation in stationary cultures of monkey kidney cells (MA104, BioWhittaker, Walkersville, Maryland) using methods modified from Conrad, Barr et al. (1993) and Marsh et al. (1997). Briefly, pooled samples of cerebrum and cerebellum were placed in chilled, sterile antibiotic saline (0.85% saline with 100 IU/ml penicillin G and 100 μg/ml streptomycin) for transport to the culture laboratory. After decanting the antibiotic saline, the tissues were homogenized using a 12 ml syringe with a 14-gauge needle. Brain homogenate (1 ml) was added directly to 5 ml of protozoal culture medium (Dulbecco's modified essential medium [DMEM], supplemented with 10% heat-inactivated bovine serum, 2 mM L-glutamine, and 50 IU/ml penicillin G and 50 μg/ml streptomycin) in a T25 flask containing 90% confluent MA104 cells. Additionally, 1 ml of CSF was applied directly to the MA104 cells in a T25 flask.

All flasks were incubated for 2 hr at 37 C in 5% CO_2. A second 1-ml aliquot of brain homogenate was placed in a tube containing 10 ml of 0.05% trypsin and was incubated at 37 C for 1 hr. The digested material was centrifuged for 10 min at 1,500 *g*, and the resulting pellet was resuspended in a minimal volume of warm medium and added to a T25 flask containing MA104 cells. After incubation, the medium mixed with homogenate or CSF was removed and discarded, and the flasks were gently rinsed 2 times with warm media. After the second rinse, 5 ml of warm medium was placed in each flask, and the cultures were returned to the incubator. An additional isolation attempt was made following storage of the brain homogenate at 4 C for 7 days. The stored homogenate was trypsinized and inoculated onto MA104 cells as described above.

To prevent cross-contamination of parasites, uninfected MA104 cells were maintained in a separate incubator from infected cell cultures, and distinct parasite isolates in cell culture were maintained either in separate incubators or on different shelves within the same incubator. Sterile disposable pipettes were used to add fresh culture medium to infected or uninfected cells, or for passage of parasites. All cell cultures, infected and noninfected, were maintained at 37 C in 5% CO_2 and were checked 3 times weekly for signs of parasite growth or contamination when the media was changed.

Isolated parasites were maintained in cell culture and passaged to uninfected monolayers as previously described (Conrad, Barr et al., 1993). The nontrypsinized brain homogenate and the stored, trypsinized homogenate both produced 2 morphologically distinct types of zoites in cell culture. The 2 zoite types, designated HS1 and HS2, were separated by passage in mice and in vitro cultivation after limiting dilution.

Separation of parasites by mouse passage and in vitro cultivation

To purify a *T. gondii*-like isolate (HS2) that predominated in brain-derived cultures after multiple passages, live zoites obtained from passage (P)22 (420 days) cell cultures were inoculated into BALB/c mice. Infected MA104 cells were harvested by scraping and were passed 3 times through a 22-gauge needle to disrupt the cells. Resulting free tachyzoites were counted with a hemacytometer and adjusted to 2.5×10^3 parasites/ml of sterile PBS. Female BALB/c mice weighing ~25 g were inoculated s.c. with 500 (n = 2) or 5,000 (n = 2) live tachyzoites and were maintained ad libitum on pelletized feed and water. On post-inoculation day (PID) 10, 1 mouse receiving 5,000 tachyzoites was moribund and was euthanized. Lung and spleen were removed and homogenized as a single tissue pool. Half of the brain was processed as a separate tissue pool, and the other half was immersion-fixed in formalin for histopathology and immunohistochemistry. Each homogenized tissue pool was inoculated into 2 T25 flasks containing uninfected MA104 cells and incubated as described above.

The remaining mice were given 0.5 mg/ml sulfadiazene (Sigma) in water. On PID 14, the other mouse injected with 5,000 tachyzoites was moribund. It was bled and euthanized, and tissue cultures were inoculated with separate pools of homogenized lung and spleen and homogenized brain, as described above. The mice given 500 tachyzoites were continued on sulfadiazene and euthanized on PID 56. Serum was collected postmortem, and lung, spleen, and brain tissue were collected and fixed in 10% formalin.

To purify a *S. neurona*-like parasite (HS1), which predominated in early passages of brain-derived cultures, cryopreserved and infected MA104 cells obtained from P1 of nontrypsinized seal brain homogenate were resuscitated from liquid nitrogen and placed in a flask of uninfected MA104 cells. The resulting infected cell culture was trypsinized, and a 24-well microtiter plate was inoculated with sequential dilutions (1:10–1:640) of parasite-infected cells. After 7 days of growth, zoites from a single area in the highest dilution well with visible parasites were scraped and passed into a flask containing uninfected MA104 cells. This flask was observed 3 times weekly for signs of parasite growth.

Antigenic characterization

Purified zoites of HS1 and HS2 were harvested from infected MA104 cells by scraping when >50% of cells were parasitized. *Toxoplasma*

THE JOURNAL OF PARASITOLOGY, VOL. 87, NO. 4, AUGUST 2001

gondii like zoites were purified via a PD10 Sephadex column as described. *Sarcocystis neurona* like whole-cell suspensions were expressed 3 times through a 22-gauge needle to lyse the cells and purify the zoites. The final parasite concentration of both isolates was adjusted to 5,000 zoites/μl, and 10-μl aliquots of each were dotted separately onto IFAT slides, formalin-fixed, and air-dried. The same panel of rabbit-origin polyclonal antisera used for immunohistochemistry and serology as positive controls was used for antigenic characterization of the harbor seal–derived protozoal isolates. The purified zoites derived from the 2 harbor seal isolates were reacted with the previously described rabbit polyclonal antisera raised to *S. neurona*, *T. gondii*, and *N. caninum*, as well as the harbor seal's own serum. A 1:300 dilution of FITC-conjugated goat anti-rabbit IgG or a 1:100 dilution of FITC-conjugated rabbit anti-dog IgG (harbor seal serum only) were used as secondary antibodies. Following incubation with these antibodies, the slides were examined using a fluorescence microscope at ×200. The last well exhibiting distinct parasite outline fluorescence was the reported titer. Positive controls consisted of similar slides containing zoites prepared from fully characterized protozoal isolates from domestic animals and humans.

DNA extraction, gene amplification, sequencing, and protozoan molecular identification

Molecular characterization of the protozoan isolates was accomplished by polymerase chain reaction (PCR) amplification and subsequent cycle sequencing of 2 nuclear genetic markers, a segment of 18S ribosomal DNA (rDNA), and the adjacent first internal transcribed spacer (ITS1). Genomic DNA from zoites grown in cell culture was extracted using an IsoQuick nucleic acid extraction kit (ORCA Research Inc., Bothell, Washington). The universal primers A and B were used to PCR-amplify the 18S rDNA, as previously described (Marsh et al., 1995, 1996). These amplifications and subsequent cycle-sequencing reactions were performed in thin-walled microtubes in a Perkin Elmer GeneAmp 2400 thermocycler (Perkin Elmer Corporation, Foster City, California). Amplified DNA was purified using a QIAquick PCR purification kit (Qiagen Inc., Chatsworth, California) or Centri-sep columns (Princeton Separations, Adelphia, New Jersey). Dye-deoxy terminator sequencing reactions were performed on both chains of the molecule using the universal primers A and C under conditions reported previously (Marsh et al., 1995, 1996). Subsequent sequence visualization was performed in an ABI Prism 377 Sequencer (Perkin Elmer). The nucleotide sequences of complementary strands were aligned and compared, and a contiguous nucleotide sequence determined for each gene fragment, using the computer program Sequencher 3.0 (GeneCodes, Ann Arbor, Michigan).

Primers ITS2 and ITS5 were used to PCR-amplify the entire ITS1 region of HS1 and HS2 (Marsh et al., 1998, 1999). DNA was purified as described above for 18S rDNA. Sequencing reactions were performed in the GeneAmp 2400 using the 2 amplification primers and additional internal primers PN1, PN2 (Holmdahl and Mattson, 1996), ITS-snFi, ITS-snRi2, ITSbFor, and ITSbrev (Marsh et al., 1999). A single contiguous sequence representing the entire ITS1 and the 5′ end of 5.8S rDNA was assembled for each protozoan isolate by alignment and comparison of the sequenced fragments using Sequencher 3.0.

The final 18S rDNA and ITS1 + 5.8S rDNA sequences were separately aligned with previously reported published sequences of the same genes (imported from GenBank) from protozoan parasites of domestic animals and humans to facilitate generic, interspecific, and intraspecific comparison (Marsh et al., 1998). Initial alignments were performed in ClustalW (Thompson et al., 1994), with subsequent editing and positional analysis conducted using MacClade 3.7 (Maddison and Maddison, 1992).

RESULTS

Postmortem examination and immunohistochemistry

Gross necropsy of the harbor seal revealed only mild gastrointestinal endoparasitism (*Anasakis* sp. and unspeciated acanthocephalans). No bacterial pathogens were isolated from samples of lung, brain, spleen, and colon. No gross brain or spinal cord abnormalities were observed. CSF collected postmortem via cisternal tap was slightly cloudy. Histopathological examination of the central nervous system revealed necrotizing meningoencephalomyelitis with moderate numbers of intralesional zoites, intracytoplasmic protozoal schizonts, and rare rosette-form schizonts. These lesions extended from the rostral cerebrum to the distal spinal cord, but were most severe in the caudal cerebrum and cerebellum, and encompassed both gray and white matter. The inflammatory infiltrate consisted of small lymphocytes, with fewer histiocytes and neutrophils, arranged as perivascular cuffs and scattered nodular aggregates within the neuropil and meninges. In severely affected areas of the brain and spinal cord, the nodular aggregates were confluent with inflammation surrounding adjacent cuffed vessels. Mild multifocal lymphohistiocytic adrenalitis and hepatitis were also present.

Immunohistochemical stains using *S. neurona* antiserum revealed strong positive staining of schizonts, rosette forms, and extracytoplasmic free zoites in the cerebrum, cerebellum, and cervical spinal cord. Rare positively stained parasites were observed in the lumbosacral spinal cord and in capillaries within the lung. All other tissues were negative for immunohistochemical staining using antiserum to *S. neurona*. No parasites were found that stained positively using polyclonal antibodies to *T. gondii* or *N. caninum*.

Serology

The harbor seal's serum showed a high level of antibody reactivity to *S. neurona* (1:20,480) and moderate reactivity to *T. gondii* antigens (1:1,280) by IFAT. There was a 1:160 antibody titer by IFAT to *N. caninum*, which in cattle is considered to be below the cut-off value for this test (Conrad, Sverlow et al., 1993).

Parasite isolation

Intracellular protozoal parasites were successfully isolated from CSF, nontrypsinized brain homogenate, and trypsinized brain homogenate from the harbor seal. All isolates were obtained in October 1998, within 4 wk of cell inoculation with brain tissue or CSF. Parasites were observed in cell cultures inoculated with CSF 14 days after inoculation, in flasks inoculated with nontrypsinized brain tissue 15 days after inoculation, and in flasks inoculated with trypsinized brain tissue 11 days after inoculation. Initial observations of live, parasite-infected cells in DMEM revealed hundreds of circumaxially motile, slender, extracellular zoites ($\bar{x}$ = 4.8 μm long, SD = 0.7; $\bar{x}$ = 1.8 μm wide, SD = 0.3 on fixed, Giemsa-stained slides, n = 30 zoites). Intracellular parasite clusters were highly refractile within the cytoplasm. These size and motility characteristics were consistent with previous isolates of *S. neurona* obtained from domestic animals (K. Sverlow, pers. obs.). However, by P15, this nontrypsinized isolate contained a mixture of zoites of 2 different lengths, shapes, and motilities. Some zoites were slender, refractile, and highly motile, similar to those described above. Others were less refractile when present in the cell cytoplasm, and free zoites were shorter and stouter ($\bar{x}$ = 3.5 μm long, SD = 0.5; $\bar{x}$ = 2.2 μm wide, SD = 0.4, on fixed, Giemsa-stained slides, n = 30 zoites) than described above. When free in DMEM, these short zoites exhibited no circumaxial motion and aggregated in clusters on the cell culture surface. These

TABLE I. Comparative reactivity of polyclonal antisera to harbor seal isolates and characterized protozoans.*

Antigen (isolate)	Polyclonal antisera to specific isolates				
	Sarcocystis neurona (UCD-SN1)	*S. falcatula* (UCD-SF1)	*Neospora caninum* (BPA1)	*Toxoplasma gondii* (ME49)	Harbor seal (postmortem serum)
S. neurona (HS1)	2,560	2,560	40	40	2,560
T. gondii (HS2)	80	80	40	81,920	640
S. neurona (SN UCD-1)	2,560	2,560	40	40	2,560
N. caninum (BPA1)	80	40	20,480	80	80
T. gondii (ME49)	<40	<40	<40	20,480	160

* Polyclonal antisera were produced in rabbits inoculated with specific zoite isolates. Antigenic reactivity was tested via an indirect fluorescent antibody test (IFAT). Reciprocal antibody titers are shown.

characteristics are similar to *T. gondii* isolates obtained from domestic animals and sea otters (K. Sverlow, pers. obs.). Brain homogenate that was refrigerated for 7 days and then trypsinized produced a similar mixed culture of large slender and short stout zoites. In both cases, the proportion of *T. gondii*–like parasites increased over time. By P22 (420 days after inoculation), parasites corresponding to the *Sarcocystis*-like morphology were no longer observed in either culture. These observations, combined with the results of serologic testing suggested that the harbor seal had been infected with 2 different brain parasites: 1 similar to *S. neurona* and 1 similar to *T. gondii*.

Separation of parasites by mouse passage and in vitro cultivation

Live zoites from a late passage, nontrypsinized cell culture with predominantly *T. gondii*–like parasites were inoculated into BALB/c mice. Mice injected with 5,000 zoites s.c. became depressed and lethargic postinfection and were euthanized. Gross necropsy revealed splenomegaly and pulmonary pallor. Histologic examination of lung, brain, and liver revealed nonsuppurative inflammation with large numbers of intralesional protozoal parasites. These parasites stained strongly positive on immunohistochemistry using rabbit antiserum raised against *T. gondii* but were negative when antisera raised against *S. neurona* or *N. caninum* were used. Noninfected MA104 cells were inoculated with pooled tissues from these mice, and 3 days later, short, stout, relatively nonmotile tachyzoites consistent with *T. gondii* were observed. These purified parasites were maintained in cell culture and passaged to uninfected monolayers. This mouse-derived *T. gondii* like isolate, designated HS2, has been maintained in continuous cell culture for over 18 mo and contains only zoites consistent with *T. gondii* as determined by morphology, antigenic reactivity, and DNA sequence analysis as described below. Serum collected PID 56 from a mouse inoculated with 500 tachyzoites s.c. of HS2 showed a 1:10,240 titer to *T. gondii*, a 1:320 titer to *N. caninum*, and a <1:40 titer to *S. neurona* by IFAT.

Sarcocystis neurona–like parasites were isolated in pure culture through limiting dilution of a low passage (P1) *S. neurona*–like dominant mixed culture derived from nontrypsinized harbor seal brain tissue. This purified isolate, designated HS1, has been continuously maintained in culture for over 18 mo and contains only parasites consistent with *S. neurona* as determined by morphology, antigenic reactivity with specific antisera and DNA sequence analysis as described below.

Antigenic characterization

Zoites of isolate HS1 reacted strongly to the harbor seal's own serum (1:2,560) and with rabbit-origin polyclonal antibodies to *S. neurona* (1:2,560) and *S. falcatula* (1:2,560), but weakly with all other polyclonal antisera (Table I). Zoites of HS2 reacted positively (Table I) with the harbor seal's own serum (1:640) and even more strongly with *T. gondii* polyclonal antiserum (1:81,920) (Table I). Antigenic reactivity of the HS2 isolate with rabbit-origin polyclonal antisera to *S. neurona* and *S. falcatula* were below initial screening values (1:80 each). The responses of both isolates to rabbit polyclonal antiserum raised against *N. caninum* were below initial screening values (1:40 each).

DNA extraction, gene amplification, sequencing, and protozoan molecular identification

Genomic DNA was successfully extracted from both protozoal isolates. Subsequently, partial sequences of 18s rDNA and the complete ITS1 from both HS1 and HS2 were obtained. The 18S rDNA partial sequence of isolate HS1 was 530 nucleotides long and spanned the most variable portions of the 18s rDNA (HS1 18s rDNA: GenBank accession AF252406). Following alignment, this sequence was determined to be identical to the same portions of 2 sequences of *S. neurona* 18S rDNA derived from isolates recovered from a horse in Kentucky (isolate SN5, GenBank accession U07812 [Fenger et al., 1994]) and a horse in California (isolate UCD-SN1, GenBank accession U33149 [Dame et al., 1995]).

Amplification and sequencing of the entire ITS1 region of HS1 yielded a 1,053-bp sequence (HS1 ITS1: GenBank accession AF252407). This sequence was aligned with the ITS1 sequence of *S. neurona* isolate UCD-SN1, GenBank accession AF081944 (Marsh et al., 1999). A transition (T–C) at position 1,015 of the alignment was the sole difference between the sequences.

The 18S rDNA sequence of isolate HS2 was 530 base pairs (bp) long (HS2 18s rDNA: Genbank accession AF291184). This sequence was aligned with and compared to all available *T. gondii* 18S rDNA sequences from GenBank, including accession nos. L37415 (ME49 strain [Luton et al., 1995]), U12138 (S48 strain [Luton et al., 1995]), U03070 (RH European strain [Holmdahl et al., 1994]), and L24381 (RH Australian strain [Ellis et al., 1994]). No differences were found between the 18s rDNA from HS1 and 18s rDNA sequences from

THE JOURNAL OF PARASITOLOGY, VOL. 87, NO. 4, AUGUST 2001

other strains of *T. gondii* isolated from domestic animals and humans.

A 495-bp sequence for HS2 encompassing the 3′ end of 18S rDNA, the entire ITS1 region, and the 5′ end of 5.8S rDNA (HS2 ITS1; GenBank accession AF252408) was also obtained. This sequence was aligned with contiguous portions of already resolved *T. gondii* sequences, GenBank accession nos. L49390 (ME49 strain [Payne and Ellis, 1996]), X75430 (Sailie strain), X75429 (RH strain), and U16161 (RH strain [Holmdahl and Mattson, 1996]). The final edited alignment was 440 bp in length, corresponding to positions 1–392 of ITS1 (the entire region), and positions 1–48 of 5.8S rDNA. The sequence derived from HS2 had a single unique difference from the other *T. gondii* sequences, an A–T transversion at position 426 of the alignment. Otherwise, the sequence was identical to U16161 (RH strain) but shared a difference, an A–G transition at position 436 of the alignment, between this strain and the remaining 3 strains. There were 2 other positional differences between the sequence X75430 (the avirulent Sailie strain) and the other 4 sequences: 2 C–T transitions at positions 20 and 120.

DISCUSSION

This paper describes successful in vitro cultivation and characterization of 2 species of protozoans from the brain of a wild harbor seal with fatal meningoencephalomyelitis. These parasites were characterized serologically, immunohistochemically, antigenically, and molecularly and were found to be indistinguishable from *S. neurona* and *T. gondii* obtained from domestic animals and humans. Elevations of serum antibodies directed against *S. neurona* and *T. gondii* antigens were detected in serum collected from the seal at the time of death, and a mixed population of parasites was repeatedly isolated in cell culture from brain homogenate. The 2 parasites, HS1 and HS2, were separated by mouse inoculation and limiting dilution and have been maintained in continuous cell culture since October 1998.

Although 2 species of parasites were isolated from brain tissue of a seal, only parasites with morphology and staining characteristics consistent with *S. neurona* were observed on histopathology and immunohistochemistry. Sections of brain and spinal cord contained parasite profiles that stained strongly positive using polyclonal antibodies to *S. neurona* and were closely associated with foci of inflammation and necrosis. Parasite profiles consistent with *T. gondii* were not detected in any tissue, including brain and spinal cord. *Toxoplasma gondii*–like parasites have also been isolated from seropositive, but histologically and immunohistochemically negative, sea otters in California (M. Miller, pers. obs.).

This seal appears to have died due to encephalitis associated with a *S. neurona* like parasite infection but was also harboring an inapparent or latent *T. gondii* like parasite infection that was detected only through serological testing and parasite culture. This result was surprising, given observations of parasite growth in cell culture (e.g., in coinfected cell cultures). The *T. gondii* like parasite (HS2) dominated over the *S. neurona* like parasite (HS1) over time in all cases (M. Miller, pers. obs.). The apparent cause of death of this seal was massive proliferation of *S. neurona* parasites with associated inflammation and necrosis of brain tissue and spinal cord. Therefore, the relationship between protozoal isolate growth characteristics in the present cell culture system and relative pathogenicity to the host seal does not appear comparable.

How the harbor seal became infected with these parasites is unknown. Opossums are a definitive host for *S. neurona* (Fenger et al., 1997) and are present in coastal habitats in California as an introduced species. Harbor seal exposure to *S. neurona* sporocysts or sporulated oocysts may occur via exposure to feces in land-based runoff or sewage, or by ingestion of unknown paratenic or intermediate hosts. Alternatively, additional definitive hosts may exist, and the possibility of a marine life cycle of this parasite should not be discounted.

Felids are the only recognized definitive hosts for *T. gondii*. Unsporulated (noninfective) oocysts are shed in cat feces, and these oocysts become sporulated (infective) 1 to 5 days after being shed, depending upon environmental conditions (Dubey, 1994). *Toxoplasma gondii* affects a wide range of intermediate hosts, including sheep, goats, pigs, and humans (Dubey, 1994). Presumably, harbor seals are exposed, and serve as intermediate hosts after contact with sporulated oocysts from cat feces in terrestrial runoff or sewage. It is possible that prey species may concentrate protozoal oocysts or sporocysts, thus facilitating transmission. If so, then infection of harbor seals and sympatric sea otters by *T. gondii* may have important, as yet unrecognized, health implications for humans, who consume some of the same coastal marine prey species.

Common pathogenic effects of toxoplasmal infection in humans and animals that serve as intermediate hosts include placentitis, abortion, and chorioretinitis (Dubey, 1994), but mild or subclinical infections are also common. However, if the infected animal becomes immunosuppressed, as may occur with AIDS in humans, encysted *T. gondii* parasites may be reactivated, causing life-threatening encephalitis. Fatal *T. gondii* encephalitis has been identified in marine mammals, including sea otters (Thomas and Cole, 1996; Cole et al., 2000), cetaceans (Innskeep et al., 1990; Migaki et al., 1990), and pinnipeds (Migaki et al., 1977; Holshuh et al., 1985). In this harbor seal's case the *T. gondii* infection appeared to be latent, detectable only through serology and parasite cultivation from brain tissue.

The reason for the 4-fold higher reactivity of the harbor seal's own serum to purified HS1 antigens versus HS2 antigens is not known. The immunological response of harbor seals to these parasites has not been characterized, and little is known about their relative antigenicity in seals. Harbor seals may prove to be exquisitely sensitive to *S. neurona* infection and more resistant to infection by *T. gondii*. Alternatively, the HS2 infection may have occurred some time previously, and the remaining *T. gondii*–like parasites were encysted in the brain tissue in low numbers. Thus, parasite detection via histopathology and immunohistochemistry would have been more difficult and the antigenic stimulus low, resulting in a low titer. In contrast, the HS1 infection may represent an acute, severe infection, with large numbers of *S. neurona*–like parasites present in close proximity to areas of brain necrosis and inflammation, resulting in a high titer. It is also possible that these isolates represent types or strains of *S. neurona* and *T. gondii* with differing pathogenicity from other recognized isolates.

The *S. neurona* and *T. gondii* like parasites isolated from this harbor seal may prove to be genetically distinct from similar parasites infecting domestic animals and humans, but signifi-

cant differences were not detected using the 18S and ITS1 ribosomal DNA molecular markers employed in this study. These markers were selected for amplification and sequencing of our isolates based on the availability of previously published sequences and their utility in molecular characterization and phylogenetic studies of parasitic protozoa, particularly apicomplexans. The 18S rDNA is moderately conserved in both prokaryotes and eukaryotes, compared with the adjacent, noncoding ITS1, which expresses greater variability (Hillis and Dixon, 1991; Marsh et al., 1995; Holmdahl and Mattson, 1996; Homan et al., 1997; Marsh et al., 1998). Our data suggest that HS1 and UCD-SN1 are closely related isolates of the same parasite, *S. neurona*, and that HS2 is closely related to the RH strain of *T. gondii*.

Molecular analysis of 13 isolates of *T. gondii* obtained from sea otters in California revealed that all were type II strains (Cole et al., 2000). This typing system is based on identification of restriction fragment length polymorphisms (RFLP) at the SAG2 locus, which encodes tachyzoite surface antigen P22 (Howe et al., 1997). Type II strains of *T. gondii* are implicated in the majority of human cases of toxoplasmosis (Howe and Sibley, 1994; Howe et al., 1997). However, the RH strain of *T. gondii*, also isolated from a human, is a type I strain, most commonly associated with human congenital infections (Howe and Sibley, 1994; Howe et al., 1997). The strain type of the harbor seal *T. gondii*-like isolate HS2 is currently unknown. Future research will involve direct comparison of our isolates with other isolates of *T. gondii* and *S. neurona* by RFLP.

This paper describes the in vitro isolation and molecular and antigenic characterization of 2 species of protozoans from a wild harbor seal with fatal meningoencephalomyelitis. This is the first dual-species protozoan brain infection documented in a marine mammal, and the first isolation of *S. neurona* and *T. gondii*-like parasites from a harbor seal. Recently, *S. neurona* has been isolated from 2 different southern sea otters with fatal encephalitis (Lindsay et al., 2000; Miller et al., 2001), and additional cases have been detected via brain histopathology and immunohistochemistry (M. Miller, pers. obs.). The present study includes the longest continuous cultivation of *S. neurona*-like parasites from any marine mammal and the first report of the recovery of live *S. neurona*-like parasites from cerebrospinal fluid (CSF). No detectable antigenic differences, and minimal nucleotide sequence differences were detected between these isolates and *S. neurona* and *T. gondii* isolated from domestic animals or humans. Future epidemiological investigations of *S. neurona* and *T. gondii* infection in harbor seals and sympatric sea otters will help to identify risk factors for protozoal infection and disease, including possible land- or marine-based sources of oocysts.

ACKNOWLEDGMENTS

This research was supported in part by funding from the PKD Trust, the Lindbergh Foundation, the Morris Animal Foundation, the UCD Genetic Resources Conservation Program, and the California Department of Fish and Game. The authors acknowledge the excellent technical assistance of staff and volunteers from The Marine Mammal Center, Sausalito, California, for rapid notification and transport of suspect cases of protozoal encephalitis in harbor seals and for postmortem cardiocentesis. The advice and support of Anne Kjemtrup, Tom Robinson, and Grace Lee for assistance in molecular characterization are deeply appreciated. Phil Chiu of the California Animal Health Food Safety Laboratory provided assistance for immunohistochemical staining. David Sibley of Washington University School of Medicine kindly provided *T. gondii* tachyzoites, and Antoinette Marsh of the University of Columbia, Missouri, provided antiserum to *S. falcatula* for antigenic characterization, as well as the UCD-SN1 *S. neurona* isolate. This paper is dedicated to Jo Miller.

LITERATURE CITED

BUERGELT, C. D., AND R. K. BONDE. 1983. Toxoplasmic meningoencephalitis in a West Indian Manatee. Journal of the American Veterinary Medical Association **183:** 1294–1296.

COLE, R. A., D. S. LINDSAY, C. L. RODERICK, J. P. DUBEY, N. J. THOMAS, AND L. A. BAETEN. 2000. Biological and molecular characterizations of *Toxoplasma gondii* obtained from southern sea otters (*Enhydra lutris nereis*). Journal of Parasitology. **86:** 526–530.

CONRAD, P. A., B. C. BARR, K. W. SVERLOW, M. A. ANDERSON, B. DAFT, H. KINDE, J. P. DUBEY, L. MUNSON, AND A. ARDANS. 1993. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology **106:** 239–249.

———, K. SVERLOW, M. ANDERSON, J. ROWE, R. BONDURANT, G. TUTER, R. BREITMEYER, C. PALMER, M. THURMOND, A. ARDANS, J. P. DUBEY, G. DUHAMEL, AND B. BARR. 1993. Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. Journal of Veterinary Diagnostic Investigation **5:** 572–578.

CRUICKSHANK, J. J., D. M. HAINES, N. C. PALMER, AND D. J. ST. ALBIN. 1990. Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Canadian Veterinary Journal **31:** 213–215.

DAME, J. B., R. J. MACKAY, C. A. YOWELL, T. J. CUTLER, A. MARSH, AND E. C. GREINER. 1995. *Sarcocystis falcatula* from passerine and psittacine birds: Synonymy with *Sarcocystis neurona*, agent of equine protozoal myeloencephalitis. Journal of Parasitology **81:** 930–935.

DI GUARDO, G., A. CORRADI, U., AGRIMI, N. ZIZZO, L. MORELLI, A. PERILLO, L. KRAMER, E. CABASSI, AND S. KENNEDY. 1995. Neuropathological lesions in cetaceans found stranded from 1991 to 1993 on the coasts of Italy. European Journal of Veterinary Pathology **1:** 51–57.

DUBEY, J. P. 1994. Toxoplasmosis. Journal of the Veterinary Medical Association. **205:** 1593–1598.

ELLIS, J. T., K. LUTON, P. R. BAVERSTOCK, P. BRINDLEY, K. A. NIMMO, AND A. M. JOHNSON. 1994. The phylogeny of *Neospora caninum*. Molecular and Biochemical Parasitology **64:** 303–311.

FENGER, C. K., D. E. GRANSTROM, A. A. GAJADAR, N. M. WILLIAMS, S. A. MCCRILLIS, S. STAMPER, J. L. LANGEMIER, AND J. P. DUBEY. 1997. Experimental induction of equine protozoal myeloencephalitis in horses using *Sarcocystis* sp. sporocysts from the opossum (*Didelphis virginiana*). Veterinary Parasitology **68:** 199–213.

———, ———, J. L. LANGEMEIER, A. GAJADHAR, G. COTHRAN, R. R. TRAMONTIN, S. STAMPER, AND J. P. DUBEY. 1994. Phylogenetic relationship of *Sarcocystis neurona* to other members of the family Sarcocystidae based on small subunit ribosomal RNA gene sequence. Journal of Parasitology **80:** 966–975.

HILLIS, M. D., AND M. T. DIXON. 1991. Ribosomal DNA: Molecular evolution and phylogenetic inference. The Quarterly Review of Biology **66:** 411–453.

HOLMDAHL, O. J. M., AND J. G. MATTSON. 1996. Rapid and sensitive identification of *Neospora caninum* by in vitro amplification of the internal transcribed spacer 1. Parasitology **112:** 177–182.

———, ———, A. UGGLA, AND K. E. JOHANSSON. 1994. The phylogeny of *Neospora caninum* and *Toxoplasma gondii* based on ribosomal RNA sequences. Federation of European Microbiological Societies Microbiology Letters **119:** 187–192.

HOLSHUH, H. J., A. E. SHERROD, C. R. TAYLOR, B. F. ANDREWS, AND E. B. HOWARD. 1985. Toxoplasmosis in a feral northern fur seal. Journal of the Veterinary Medical Association **187:** 1229–1230.

HOMAN, W. L., L. LIMPER, M. VERLAAN, A. BORST, M. VERCAMMEN, AND F. VAN KNAPEN. 1997. Comparison of the internal transcribed spacer, ITS 1, from *Toxoplasma gondii* isolates and *Neospora caninum*. Parasitology Research **83:** 285–289.

HOWE D. K., AND L. D. SIBLEY. 1994. *Toxoplasma gondii:* Analysis of

different laboratory stocks of the RH strain reveals genetic heterogeneity. Experimental Parasitology **78:** 242–245.

———, S. HONORE, F. DEROUIN, AND L. D. SIBLEY. 1997. Determination of genotypes of *Toxoplasma gondii* strains isolated from patients with toxoplasmosis. Journal of Clinical Microbiology **35:** 1411–1414.

INSKEEP, W., C. H. GARDNER, R. K. HARRIS, J. P. DUBEY, AND R. T. GOLDSTONE. 1990. Toxoplamosis in Atlantic bottle-nose dolphins (*Tursiops truncatus*). Journal of Wildlife Diseases **26:** 377–382.

LAPOINTE, J. M. P. J. DUIGNAN, A. E. MARSH, F. M. GULLAND, B. C. BARR, D. K. NAYDAN, D. P. KING, C. A. FARMAN, K. A. BUREK HUNTINGDON, AND L. J. LOWENSTINE. 1998. Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific Harbor Seals (*Phoca vitulina richardsi*). Journal of Parasitology **84:** 1184–1189.

LINDSAY, D. S., N. J. THOMAS, AND D. P. DUBEY. 2000. Biological characterization of *Sarcocystis neurona* isolated from a southern sea otter (*Enhydra lutris nereis*). International Journal for Parasitology **30:** 617–624.

LUTON, K., M. GLEESON, AND A. M. JOHNSON. 1995. rRNA gene sequence heterogeneity among *Toxoplasma gondii* strains. Parasitology Research **81:** 310–315.

MADDISON, W. P., AND D. R. MADDISON. 1992. MacClade: Analysis of phylogeny and character evolution. Version 3.07. Sinauer Associates, Sunderland, Massachusetts.

MARSH, A. E., B. C. BARR, J. MADIGAN, J. LAKRITZ, AND P. A. CONRAD. 1996. Sequence analysis and polymerase chain reaction amplification of small subunit ribosomal DNA from *Sarcocystis neurona*. American Journal of Veterinary Research **57:** 975–981.

———, ———, A. E. PACKHAM, AND P. A. CONRAD. 1998. Description of a new *Neospora* species (Protozoa: Apicomplexa: Sarcocystidae). Journal of Parasitology **84:** 983–991.

———, ———, K. SVERLOW, M. HO, J. P. DUBEY, AND P. A. CONRAD. 1995. Sequence analysis and comparison of ribosomal DNA from bovine *Neospora* to similar coccidial parasites. Journal of Parasitology **81:** 530–535.

———, ———, L. TELL, D. D. BOWMAN, P. A. CONRAD, C. KETCHERSIDE, AND T. GREEN. 1999. Comparison of the internal transcribed spacer, ITS-1, from *Sarcocystis falcatula* isolates and *Sarcocystis neurona*. Journal of Parasitology **85:** 750–757.

———, ———, L. TELL, M. KOSKI, E. GREINER, J. DAME, AND P. A. CONRAD. 1997. In vitro cultivation and experimental inoculation of *Sarcocystis falcatula* and *Sarcocystis neurona* merozoites into budgerigars (*Melopssitacus undulatus*) Journal of Parasitology **83:** 1189–1192.

MIGAKI, G., J. F. ALLEN, AND H. W. CASEY. 1977. Toxoplasmosis in a California sea lion (*Zalophus californianus*). American Journal of Veterinary Research **38:** 135–136.

———, T. R. SAWA, AND J. P. DUBEY. 1990. Fatal disseminated toxoplasmosis in a spinner dolphin (*Stenella longirostris*). Veterinary Pathology **27:** 463–464.

MIKALELIAN, J., J. BOISCLAIR, J. P. DUBEY, S. KENNEDY, AND D. MARTINEAU. 2000. Toxoplasmosis in beluga whales (*Dephinapterus leucas*) from the St. Lawrence estuary: Two case reports and a serological survey. Journal of Comparative Pathology **122:** 73–76.

MILLER, M. A., P. C. CROSBIE, K. SVERLOW, K. HANNI, B. C. BARR, N. KOCK, M. J. MURRAY, L. J. LOWENSTINE, AND P. A. CONRAD. 2001. Isolation and characterization of *Sarcocystis* from brain tissue of a free-living southern sea otter (*Enhydra lutris nereis*) with fatal meningoencephalitis. Parasitology Research. **87:** 252–257.

OKSANEN, A., M. TRYLAND, K. JOHNSEN, AND J. P. DUBEY. 1998. Serosurvey of *Toxoplasma gondii* in North Atlantic marine mammals by use of agglutination test employing whole tachyzoites and dithiothreitol. Comparative Immunology, Microbiology and Infectious Diseases **21:** 107–114.

PACKHAM, A. E., K. W. SVERLOW, P. A. CONRAD, E. F. LOOMIS, J. D. ROWE, M. L. ANDERSON, A. E. MARSH, C. CRAY, AND B. C. BARR. 1998. A modified agglutination test for *Neospora*: Development, optimization and comparison to the IFAT and ELISA. Clinical and Diagnostic Laboratory Immunology **5:** 467–473.

PAYNE, S., AND J. ELLIS. 1996. Detection of *Neospora caninum* DNA by the polymerase chain reaction. International Journal for Parasitology **26:** 347–351.

RATCLIFF, H. L., AND C. B. WORTH. 1951. Toxoplasmosis in captive wild birds and mammals. American Journal of Pathology **27:** 665–667.

ROSONKE, B. J, S. R. BROWN, S. J. TORNQUIST, S. P. SNYDER, M. M. GARNER, AND L. L. BLYTHE. 1999. Encephalomyelitis associated with a *Sarcocystis neurona*-like organism in a sea otter. Journal of the American Veterinary Medical Association **215:** 1839–1842.

THOMAS, N. J, AND R. A. COLE. 1996. The risk of disease and threats to the wild population. Conservation and management of the southern sea otter. Endangered Species Update (Special Issue) **13:** 23–27.

THOMPSON, J. D., D. G. HIGGINS, AND T. J. GIBSON. 1994. Clustal W—Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research **22:** 4673–4680.

U.S. FISH AND WILDLIFE SERVICE. 1995. Draft southern sea otter recovery plan. USFWS. Ventura, California, 41 p.

VAN PELT, R. W., AND R. A. DIETRICH. 1973. Staphylococcal infection and toxoplasmosis in a young harbor seal. Journal of Wildlife Diseases **9:** 258–261.

Chapter 4

Evaluation of an indirect fluorescent antibody test (IFAT) for detection of *Toxoplasma gondii* in sea otters

EVALUATION OF AN INDIRECT FLUORESCENT ANTIBODY TEST (IFAT) FOR DEMONSTRATION OF ANTIBODIES TO *TOXOPLASMA GONDII* IN THE SEA OTTER (*ENHYDRA LUTRIS*)

M. A. Miller, I. A. Gardner*, A. Packham, J. K. Mazet*, K. D. Hanni*, D. Jessup†, J. Estes‡, R. Jameson§, E. Dodd†, B. C. Barr, L. J. Lowenstine, F. M. Gulland‖, and P. A. Conrad

Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, California 95616. *e-mail: paconrad@ucdavis.edu*

ABSTRACT: An indirect fluorescent antibody test (IFAT) for detection of *Toxoplasma gondii* infection was validated using serum from 77 necropsied southern sea otters (*Enhydra lutris nereis*) whose *T. gondii* infection status was determined through immunohistochemistry and parasite isolation in cell culture. Twenty-eight otters (36%) were positive for *T. gondii* by immunohistochemistry or parasite isolation or both, whereas 49 (64%) were negative by both tests. At a cutoff of 1:320, combined values for IFAT sensitivity and specificity were maximized at 96.4 and 59.2 %, respectively. The area under the receiver-operating characteristic curve for the IFAT was 0.84. A titer of 1:320 was used as cutoff when screening serum collected from live-sampled sea otters from California (n = 80), Washington (n = 21), and Alaska (n = 65) for *T. gondii* infection. Thirty-six percent (29 out of 80) of California sea otters (*E. lutris nereis*) and 38% (8 out of 21) of Washington sea otters (*E. lutris kenyoni*) were seropositive for *T. gondii*, compared with 0% (0 out of 65) of Alaskan sea otters (*E. lutris kenyoni*).

After near-extinction from overhunting in the maritime fur trade, the southern sea otter (*Enhydra lutris nereis*; Wilson et al., 1991) population has recovered slowly, punctuated by several periods of decline (Estes, 1990). The sluggish growth and recent decline of this population appear to be the consequence of elevated mortality (Estes et al., 1996; Monson et al., 2000). A high proportion of southern sea otter mortality (40%) has been attributed to infectious disease (Thomas and Cole, 1996). Protozoal meningoencephalitis is a frequent postmortem finding in southern sea otters stranded along the California coast (Thomas and Cole, 1996). Two different protozoa, *Toxoplasma gondii* and *Sarcocystis neurona*, have been isolated from affected animals (Thomas and Cole, 1996; Cole et al., 2000; Lindsay et al., 2000; Miller, Crosbie et al., 2001) and are considered to be an important cause of mortality. Isolates of *T. gondii* obtained from otters were similar or identical to those isolated from terrestrial animals and humans, and produced oocysts in cats fed mice inoculated with brain tissue from infected otters (Cole et al., 2000). Most reports on *T. gondii* infection in North American marine mammals provide detailed information on histopathological abnormalities (Van Pelt and Dieterich, 1973; Migaki et al., 1977; Buergelt and Bonde, 1983; Holshuh et al., 1985; Cruickshank et al., 1990; Inskeep et al., 1990; Migaki et al., 1990) but do not include evaluation of antibody response to *T. gondii*. Existing *T. gondii* serological surveys of marine mammals in North America have been limited to screening individuals diagnosed with fatal protozoal meningoencephalitis or opportunistically collected serum samples with limited internal controls (Lapointe et al., 1998; Rosonke et al., 1999; Lindsay et al., 2000; Mikalelian et al., 2000; Miller, Sverlow et al., 2001). To date, no studies on the true prevalence or seroprevalence (or both) of *T. gondii* in sea otters in different geographical areas has been reported.

In the present study, an indirect fluorescent antibody test (IFAT) for diagnosis of *T. gondii* infection in sea otters was validated. The IFAT was developed in response to the need for a screening test that could be used on hemolyzed serum samples, such as those collected at necropsy or samples from live animals with hemolysis attributable to conditions of collection, transport, or storage. Sera collected from necropsied southern sea otters with confirmed *T. gondii* infection status were used to validate this test and to examine the relationship between the *T. gondii* IFAT titer and extent of sample hemolysis. In addition, *T. gondii* IFAT seroprevalence was compared across 3 geographically distinct populations of sea otters, i.e., live-sampled southern sea otters from California and northern sea otters (*E. lutris kenyoni*; Wilson et al., 1991) from Alaska and Washington.

MATERIALS AND METHODS

Postmortem examination

Between February 1997 and February 2001, 96 freshly dead (<72 hr postmortem) southern sea otters from central California were examined at the Marine Wildlife Veterinary Care and Research Center, California Department of Fish and Game (CDFG), in Santa Cruz, California. A detailed necropsy was performed on each animal, and selected tissues were screened for pathogenic bacteria or fungi. Otter age was recorded on the basis of previously established length, body weight, and dentition criteria (Morejohn et al., 1975). Serum was separated from whole blood via centrifugation and stored at −70 C until tested. The extent of hemolysis (hemolysis score) was noted for each serum sample tested: 0 = no hemolysis (clear serum with no red discoloration), 1 = minimal hemolysis (red-tinged but clear samples), 2 = moderate hemolysis (deep red and opaque serum samples), or 3 = marked hemolysis (dark red to black and opaque, sometimes with a viscous consistency).

Tissues were immersion-fixed in 10% neutral buffered formalin, trimmed, dehydrated through a graded series of ethanol and xylol, and embedded in paraffin. Five-micrometer sections were cut using a rotary microtome, deparaffinized, stained with hematoxylin and eosin (H&E), and examined using a compound microscope. The primary and contributing causes of death were determined through gross necropsy, histopathology, bacterial culture, and other techniques, as required. From

Received 16 July 2001; revised 28 November 2001; accepted 1 December 2001.

* Department of Medicine and Epidemiology, 1 Shields Avenue, School of Veterinary Medicine, University of California, Davis, California, 95616.

† Marine Wildlife Veterinary Care and Research Center, California Department of Fish and Game, 1451 Shaffer Road, Santa Cruz, California 95060.

‡ Long Marine Laboratory, University of California, Santa Cruz, Santa Cruz, California 95060.

§ Western Ecological Research Center, USGS, 200 SW 35th Street c/o EPA, Corvallis, Oregon 97333.

‖ The Marine Mammal Center, Marin Headlands, 1065 Fort Cronkite, Sausalito, California 94965.

each otter, at least 7 5-μm-thick HE-stained sections of brain were examined, including frontal cortex, rostral hippocampus, temporal cortex, cerebellum, medulla, and brainstem. In most cases, multiple sections of spinal cord were also examined.

Immunohistochemistry

From each otter, 2 blocks of brain tissue containing at least 1 section each of cerebrum and cerebellum were selected for immunohistochemistry. The blocks that were selected were those with visible protozoan parasites or the greatest degree of nonsuppurative inflammation on histopathological examination or both. All immunohistochemical slides were prepared at the California Animal Health and Food Safety Laboratory, Davis, California, using standardized techniques, equipment, and reagents. Immunohistochemical stainings for *T. gondii*, *Neospora caninum*, and *S. neurona* were performed as described (Miller, Sverlow et al., 2001). Slides were evaluated by a pathologist with no prior knowledge of each otter's IFAT titer or parasite isolation status. An otter was considered positive on immunohistochemistry only if stained parasites were observed, and both the external and internal portions of the organisms were stained using the immunoperoxidase technique.

Parasite isolation

At necropsy, samples of cerebrum and cerebellum were collected aseptically into chilled, sterile antibiotic saline solution (0.85% saline with 100 IU/ml penicillin G and 100 μg/ml streptomycin), homogenized, and placed over stationary cultures of monkey kidney (MA104) cells (BioWhittaker, Walkersville, Maryland) as previously described (Miller, Crosbie et al., 2001; Miller, Sverlow et al., 2001). Both trypsinized and nontrypsinized brain homogenates were processed for cell culture inoculation, and cell cultures were maintained as described by Conrad et al. (1993). Cell cultures were considered positive when characteristic refractile intracytoplasmic protozoal cysts or motile extracellular zoite stages, or both, were first observed. The identity of each protozoan isolate was confirmed through parasite morphology in cell culture, antigenic characterization, molecular characterization, or electron microscopy or both as previously described (Miller, Crosbie et al. 2001; Miller, Sverlow et al., 2001). Unless visibly contaminated, cell cultures were maintained for at least 1 mo after brain tissue inoculation before being deemed negative and discarded. Cell cultures that were parasite-negative when discarded (because of fungal or bacterial contamination) within 21 days of inoculation were considered indeterminate with respect to protozoal infection status. These cases were excluded from the IFAT evaluation unless the *T. gondii* brain immunohistochemistry result was positive.

Determination of *T. gondii* infection status for necropsied sea otters

The positive gold standards for *T. gondii* infection in sea otters were direct isolation and characterization of *T. gondii* in cell culture from brain homogenate, or observation of positively stained parasites with morphology consistent with *T. gondii* on immunohistochemistry, or both. The negative gold standards for *T. gondii*-free status in brain tissue were absence of detectable protozoan parasite stages via protozoal isolation and via immunohistochemistry.

IFAT

The IFAT was evaluated as a screening test for *T. gondii* by comparing serum IFAT titers to each otter's *T. gondii* infection status, as determined above. The IFAT was performed as previously described (Miller, Sverlow et al., 2001), except that 10 μl of a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-ferret IgG (Bethyl Laboratories, Montgomery, Texas) was substituted for the FITC-conjugated rabbit anti-canine IgG used for the seal IFAT. Endpoint titers were determined by serial dilution from 1:80 to 1:81,920. Wells were examined using fluorescence microscopy at ×200, and the last well with distinct parasite outline fluorescence was the reported titer. All completed IFAT slides were assessed by the same observer to ensure consistency of interpretation.

The overall accuracy of the IFAT and the optimal cutoff titer was assessed by receiver-operating characteristic (ROC) analysis (Greiner et al., 2000) using MedCalc Version 6.0 (Mariakerke, Belgium). Ninety-five percent confidence intervals [CI] for IFAT sensitivity and specificity at the selected cutoff value were calculated by exact binomial methods (Greiner and Gardner, 2000) using Epi Info (Version 6.04; Centers for Disease Control and Prevention, Atlanta, Georgia). For serum collected from both live and dead otters, a Kruskal–Wallis test was used to determine whether degree of sample hemolysis and resulting *T. gondii* IFAT titers were associated. To assess the repeatability of the IFAT, slides were screened in a blinded fashion by 2 readers, and results were compared. The between-reader repeatability of the IFAT was assessed using a weighted kappa value (Fleiss, 1981).

Serologic screening of otters from California, Washington, and Alaska

Between 1997 and 2000, serum was obtained from live-sampled, presumably healthy sea otters from central California (*E. lutris nereis*), coastal Washington (*E. lutris kenyoni*), and coastal Alaska (*E. lutris kenyoni*) (Anderson et al., 1996). Animals were captured using dip nets, tangle nets, or Wilson traps as part of population biology studies by personnel of the Biological Resources Division, United States Geological Survey (USGS-BRD), and the CDFG. Samples were obtained for both sexes and all age classes, during all seasons (California and Washington) or only summer (Alaska), and from diverse geographic areas and marine habitats.

Live-sampled California sea otters (n = 80) were sampled between March 1998 and March 2000 in the vicinity of Monterey Bay, California (36.5500°–36.9667°N, 121.0334°–121.7500°W). Most otters were sampled within 5 km of coastal cities such as Santa Cruz, Capitola, Monterey, and Pacific Grove. Twenty-one serum samples were obtained from live-sampled sea otters from coastal Washington between February 1997 and August 1998. Most serum samples (n = 20) were obtained from otters located within the coastal boundaries of Olympic National Park, extending from Cape Alava (48.1696°N, 124.7314°W) to the Chilean Memorial (47.9650°N, 124.6653°W). This is an isolated stretch of wilderness along the northwestern coast of Washington, reachable only by foot or boat. One additional otter was sampled at Waadah Island (48.3846°N, 124.6056°W). Sixty-five otter serum samples were collected from coastal Alaska during the summer of 1997. Otters were captured near Adak (52.0000°N, 176.5000°W; n = 33), Kiska (52.0000°N, 177.5000°W; n = 14), and Kanaga Islands (52.0000°N, 177.0000°W; n = 7) in the western Aleutian Island chain and at Elfin Cove (58.1667°N, 136.3334°W; n = 11) in southeastern Alaska. Small towns with less than 500 people are found on Adak Island and at Elfin Cove. All sea otters were captured ≥10 km from these towns.

A physical examination was performed on all sampled otters, during which animals were weighed to the nearest 0.1 kg and their age class and sex determined. The age of each captured otter was estimated on the basis of pelage, tooth wear, weight, length, and reproductive status characteristics (Morejohn et al., 1975). Age classes were defined as pup (0–6 mo), immature (>6 to 12 mo), subadult (>1 to 3 yr), and adult (>3 yr). Serum samples obtained from live otters were screened using the same IFAT technique that was used for samples obtained from dead sea otters. The extent of hemolysis of each sample was recorded, and all samples were held at −70 C before testing.

Chi-square tests were used to compare seroprevalence of *T. gondii* among live-sampled sea otters in California, Washington, and Alaska, as well as between the live-sampled otters and the freshly dead otters with known *T. gondii* infection status. Otters were considered seropositive if their IFAT titer was ≥1:320, the optimal cutoff titer as determined in the validation study.

RESULTS

Postmortem examination

From February 1997 to February 2001, 96 freshly dead southern sea otters were examined grossly and via histopathology. Serum was obtained for IFAT screening from 84 of 96 otters. For 7 of the 84 otters, IFAT testing was completed, and immunohistochemistry was negative for *T. gondii*, but parasite isolation from the otter brain tissue was not attempted. Parasite isolation was not done when zoonotic pathogens such as *Coc-*

cidiodes immitis were suspected, because of chain-of-custody restrictions or as a result of logistical problems with sample shipment and processing, e.g., lost samples. These 7 cases were classified as indeterminate for *T. gondii* infection and were excluded from IFAT evaluation. For the 7 indeterminate cases, the *T. gondii* IFAT titers were <1:80 (n = 4), 1:160 (n = 1), 1:640 (n = 1), and 1:1,280 (n = 1). Thus, 77 of the freshly dead otters were evaluated for *T. gondii* infection status by immunohistochemistry and parasite culture and also had sufficient serum for IFAT screening. Adults, subadults, immatures, and pups made up 61, 12, 18, and 9% of the sample, respectively. Forty-seven percent (36 out of 77) of the otters were females.

Immunohistochemistry

Thirteen of 77 freshly dead otters were positive for *T. gondii* on brain immunohistochemistry. Immunohistochemical staining in brain tissue encompassed entire parasites, and no unstained protozoa were observed in slides prepared using rabbit antisera to *T. gondii*. *Toxoplasma gondii*-stained protozoa did not show evidence of cross-reactivity with *S. neurona* or *N. caninum* polyclonal antiserum.

Parasite isolation

For 77 culture attempts, the median interval between gross necropsy with brain tissue collection and inoculation of monolayer cultures with brain homogenate was 2 days (range <1 to 21 days). Twenty-four separate isolates of *T. gondii* were obtained from 75 culture attempts with sea otter brain homogenate, an isolation ratio of 32%. Four additional otters for which parasite isolation was negative (n = 1) or the monolayer cultures were contaminated with bacteria (n = 1) or parasite isolation was not attempted (n = 2) were parasite-positive on immunchistochemistry. Thus, 36% of the otters (28 out of 77) were shown to be infected with *T. gondii* at the time of necropsy, and 49 otters were noninfected on the basis of negative results on both immunohistochemistry and parasite isolation.

Of 24 isolates of *T. gondii*, 14 were obtained only from trypsinized brain tissue, 3 were obtained from both trypsinized and nontrypsinized brain tissue, and 4 were obtained from nontrypsinized brain tissue only. For the remaining 3 animals, the tissue treatment protocol was not recorded. The median time from cell inoculation to initial detection of parasites in cell culture was 14 days (range 3–35 days). Brain homogenate-inoculated cells were maintained in culture for a median of 31 days before being considered negative for parasites and discarded. However, 7 cultures were discarded within 9–21 days of inoculation because of fungal or bacterial contamination.

Determination of *T. gondii* infection status for necropsied otters

Of 24 otters positive for *T. gondii* on parasite isolation, only 11 (42%) were also detected by brain immunohistochemistry. In contrast, only 2 otters for which parasite isolation was negative were *T. gondii*-positive on immunohistochemistry. For 1 of these 2 cases, the inoculated cells were discarded because of bacterial contamination, presumably resulting in a false-negative isolation attempt.

TABLE I. Distribution of *Toxoplasma gondii* indirect fluorescent antibody test (IFAT) titers by protozoal infection status, as determined by parasite isolation and immunohistochemistry.

T. gondii IFAT titer	*T. gondii* infected	Noninfected
≥20,480	5	0
10,240	1	0
5,120	3	1
2,560	4	1
1,280	4	10
640	6	4
320	4	4
160	1	5
80	0	5
<80	0	19
Total	28	49

IFAT

End point *T. gondii* titers of otters ranged from <1:80 to 1:81,920 (Table I), and the area under the ROC curve was 0.84 (95% CI = 0.74–0.92). The maximal combined values for test sensitivity and specificity occurred at a cutoff of 1:320. At this cutoff, the *T. gondii* IFAT was highly sensitive (96.4%; 95% CI = 81.7–99.9%), but only moderately specific (59.2%; 95% CI = 44.2-73.0 %), resulting in 1 false-negative and 20 false-positive diagnoses of *T. gondii* infection. At a cutoff of 1:320, 47 of the 77 otters (61%) were seropositive for *T. gondii*. Increasing the cutoff to 1:640 resulted in a sensitivity of 82.1% (95% CI = 63.1–93.9%) and specificity of 75.0% (95% CI = 60.4–86.4%).

Between-reader comparisons of IFAT reproducibility were restricted to samples with at least 1 end-point titer falling within 2 dilutions of the selected test cutoff of 1:320, e.g., samples with end point titers ≥1:80, but not greater than 1:1,280. In addition, if the primary reader determined the final titer value to be 1:1,280, the other reader had to agree to within ±2 serum dilutions for ≥90% of the tested samples. The IFAT results for 61 samples met the above restrictions (at least 1 end point titer ≥1:80 but not greater than 1:1,280) and were read out by both primary and second readers. For 93% of these samples (57 out of 61), the end point titers of both readers were within 2 serum dilutions of each other. The weighted kappa value for between-reader comparisons was 0.41, which indicated moderate agreement beyond chance.

For serum from freshly dead and live otters, the median hemolysis scores were 1 (range 0–3) and 0 (range 0–2), respectively. The degree of serum hemolysis was not statistically associated with end point *T. gondii* IFAT titers. This finding was the same for serum collected from dead (P = 0.846, n = 66) and live (P = 0.204, n = 166) sea otters.

Serologic screening of otters from California, Washington, and Alaska

Summary demographic and serological data for otters from California, Washington, and Alaska are shown in Table II. The sample collection periods, sex, and age distribution were comparable for the 3 populations. Using the established cutoff of

TABLE II. Summary of demographic data and *Toxoplasma gondii* indirect fluorescent antibody test results for sea otters (*Enhydra lutris*) from California, Washington, and Alaska.

	Necropsied, California	Live sampled, California	Live sampled, Washington	Live sampled, Alaska
Number sampled	77	80	21	65
Interval	1997–2001	1998–2000	1997–1998	1997
Females (%)	47	65	76	72
Adults (%)	61	65	62	85
Subadults (%)	12	9	19	10
Immatures and pups (%)	27	26	19	5
Seropositive (≥1:320)	61	36	38	0

1:320, 36% of the serum samples obtained from live-sampled California sea otters (29 out of 80) were seropositive for *T. gondii* on IFAT. For the live-sampled California otters, 61% (49 out of 80) had *T. gondii* IFAT titers ≤1:80. Twelve of the 80 live-sampled California otters had *T. gondii* IFAT titers of 1:160 or 1:320, and 19 otters had IFAT titers ≥1:1,280. Eight of the 21 Washington otter serum samples (38%) were positive (≥1:320) for *T. gondii* on IFAT. Observed *T. gondii* IFAT titers were <1:80 (n = 11), 1:80 (n = 1), 1:160 (n = 1), 1:320 (n = 2), 1:640 (n = 4), and 1:1,280 (n = 2). None of the serum samples from Alaska sea otters (0 out of 65) was positive. Of the 65 samples screened, 58 (89%) had *T. gondii* IFAT titers <1:80, 3 (5%) had 1:80, and 4 (6%) had 1:160.

Comparisons of seroprevalence indicated no significant difference ($P = 0.880$) in *T. gondii* IFAT seroprevalence between live-sampled *E. lutris nereis* from coastal California (36%) and live-sampled *E. lutris kenyoni* from Washington (38%). However, the *T. gondii* seroprevalence for both California and Washington otters was significantly greater than that of *E. lutris kenyoni* from Alaska ($P = 0.0001$ for both comparisons). In addition, the *T. gondii* IFAT seroprevalence was lower ($P = 0.002$) in the live-sampled California otters (36%) than in the dead otters (61%) used in the validation study.

DISCUSSION

The IFAT was validated using a cross-sectional sample of 77 freshly dead sea otters whose true *T. gondii* infection status was determined by immunohistochemistry and parasite isolation in cell culture. All otters were screened for *T. gondii* regardless of the apparent cause of death, and serum samples collected at the time of necropsy were used to compare *T. gondii* antibody titers with the results of the postmortem screening tests. Because the southern sea otter is a federally listed threatened species (Anderson et al., 1996), invasive sampling of live otters to confirm *T. gondii* infection for test validation was neither permissible nor desirable.

In the present study, the combined gold standard for *T. gondii*-free status was absence of detectable protozoan parasites via both cell culture and brain immunohistochemistry. It is recognized that some otters might have been incorrectly classified as negative for *T. gondii* infection on parasite isolation or immunohistochemistry, in part because of the small volume of brain tissue used in these tests. Also, false-negative results might have occurred if an isolate was missed in cell culture or was not detected because of fungal or bacterial contamination of cultures. In addition, some otters might have had *T. gondii* present in tissues other than brain.

Pretreatment of brain homogenate with trypsin before cell inoculation resulted in more successful isolation of *T. gondii* isolates than nontrypsinized homogenates obtained from the same otters. The additional trypsinization step likely facilitated the release of infective parasite stages from tissue cysts present in brain homogenate and minimized the likelihood of missing infected animals that had few parasites present.

When the results of *T. gondii* immunohistochemistry and parasite isolation were compared for the same otters, immunohistochemistry was less sensitive for parasite detection than for parasite isolation. This result is not surprising, given the sparse distribution of *T. gondii* on brain histopathology and the large difference in the volume of brain tissue screened via the 2 tests; <0.25 g of brain tissue (7–10 5-μm-thick histological sections) was examined from each otter via immunohistochemistry, compared with 2–4 g of brain homogenate layered over cells in culture. Examination of additional brain sections by immunohistochemistry might increase the sensitivity of this method, but numerous tissue sections would need to be examined to equal the volume of brain tissue screened via parasite isolation. Although mouse bioassay has been shown to be more effective in some situations (Dubey et al., 1995) when used in combination with tissue histopathology and immunohistochemistry, as in the present study, isolation in cell culture is an effective means of parasite detection. In addition, it obviates the need and expense of laboratory animal care and animal sacrifice.

At a cutoff titer of 1:320, the IFAT had high sensitivity (96.4%) but only moderate specificity (59.2%). However, IFAT specificity was likely underestimated because of the inclusion of unrecognized *T. gondii*-infected otters as gold standard negatives for the reasons outlined above. Some otters in the sample that were negative for *T. gondii* on parasite isolation and immunohistochemistry had high *T. gondii* titers, e.g., ≥1:2,560, and were likely to have been falsely classified as noninfected. This phenomenon has been noted for serologic tests for *T. gondii* in pigs when an imperfectly sensitive but perfectly specific gold standard (mouse bioassay) was used (I. Gardner, unpubl.).

One potential disadvantage of IFAT tests is the subjective element of test interpretation. To minimize variation caused by differences in interpretation between different test readers, a single reader was employed to interpret all IFAT results for this study. The *T. gondii* IFAT performed adequately on hemolyzed serum samples, and IFAT titers were not associated with the

extent of serum hemolysis. Thus, the *T. gondii* IFAT should be appropriate for screening serum samples collected at necropsy as well as samples from live otters where hemolysis is a concern. Other serological tests for protozoa such as the modified agglutination test are more sensitive to serum hemolysis, potentially resulting in false-positive test results (Packham et al., 1998).

In addition to validation of the IFAT, the present study provided important data regarding *T. gondii* infections in California sea otters. On the basis of the results of immunohistochemistry and parasite isolation, 36% of the freshly dead otters in the California sample were infected with *T. gondii*. This is likely an underestimate of the true prevalence of infection because some infections were not detected. In spite of this possibility, our findings indicate that *T. gondii* is prevalent and highlights the need for studies on modes of transmission of the parasite in the marine environment.

The validated *T. gondii* IFAT has several potential applications. First, it will be valuable as a diagnostic test for hospitalized sea otters with neurological disease consistent with protozoal meningoencephalitis. Second, it will facilitate investigation of risk factors for *T. gondii* infection in live-sampled otters. The cutoff titers selected for *T. gondii* seropositivity in each case will depend on assessment of the "cost" of a false-positive and false-negative diagnosis and the true prevalence of *T. gondii* infection in the population. For example, the cost of a false-negative diagnosis of toxoplasmosis is high in an otter with severe neurological disease compared with that of a false-positive diagnosis. Although some *T. gondii*-infected otters do not exhibit neurological disease, no southern sea otter is reported to have survived clinical toxoplasmosis without antiprotozoal therapy. In contrast, the risk of adverse effects caused by short-term antiprotozoal therapy in sea otters, either infected or uninfected, appears to be low. Thus, an IFAT with optimal sensitivity is best for diagnostic screening for *T. gondii* infection in otters with neurological disease. However, a clinical diagnosis of toxoplasmosis should be based on demonstration of a rising *T. gondii* titer, or clinical response to antiprotozoal therapy, or both.

A key goal in screening serum from live-sampled sea otters is to identify differences in *T. gondii* infection or seroprevalence among geographically distinct otter groups and investigate risk factors for *T. gondii* infection. The cost of a false-positive or false-negative diagnosis in an epidemiological study is inclusion of animals in the wrong infection group for analysis; imperfect sensitivity and specificity usually result in nondifferential misclassification of odds ratio estimates for risk factors, e.g., bias toward finding no effect. Thus, consideration of both test sensitivity and specificity is important for interpreting IFAT data that are used in epidemiological studies. On the basis of the gold standards used in this study, the maximal combined values for IFAT sensitivity and specificity occurred at a cutoff value of 1:320 as *T. gondii*-positive. This value was selected for screening sera collected from live-sampled sea otters, recognizing that the *T. gondii* infection status of some otters could be misclassified.

Although the populations had similar age and sex distributions (Table II), IFAT seroprevalence of *T. gondii* in California sea otters was greater for freshly dead (61%) than for live-sampled otters (36%). The reasons for this difference are speculative. The higher proportion of seropositive dead otters might reflect spectrum-of-disease bias because otters with more severe *T. gondii* infections might be more likely to die than otters with less extensive infections. Alternatively, concurrent immunosuppressive disease (Kannan et al., 1998) might result in reactivation of chronic, inapparent *T. gondii* infection and a change in corresponding IFAT titers. Otters may also have an increased risk of mortality resulting from seemingly unrelated factors when infected by *T. gondii*. For example, some otters with gross evidence of mortality resulting from boat strike or shark predation had *T. gondii*-related meningoencephalitis on histopathology and immunohistochemistry (M. Miller, pers. obs.). Neurological abnormalities induced by protozoal brain infections might make otters more susceptible to predation or trauma and render them less able to forage effectively or avoid danger, thus indirectly resulting in mortality. Severe clinical deficits, including seizures, apparent blindness, and obtundation have been observed in stranded sea otters and harbor seals with *T. gondii*-associated meningoencephalitis (Lapointe et al., 1998; Lindsay et al., 2000; Miller, Crosbie et al., 2001; Miller, Sverlow et al., 2001). Finally, otters that die from other causes may have a lower probability of appearing as beach-cast carcasses.

The *T. gondii* seroprevalence in live-sampled sea otters from coastal California and Washington was similar, but was greater than the *T. gondii* seroprevalence observed in Alaskan otters. Geographical differences in *T. gondii* seroprevalence might be attributable to differential *T. gondii* exposure, unrecognized environmental factors affecting *T. gondii* survival in the nearshore environment, differences in otter susceptibility, differences in paratenic prey species abundance, or other, unrecognized, factors. Geographical comparisons of *T. gondii* seroprevalence between populations or individuals must be interpreted with caution because of the chronicity of *T. gondii* infection, the large distances traveled by some otters within their respective ranges, and the unknown mechanisms of *T. gondii* transmission in sea otters. All 3 otter groups (California, Washington, and Alaska) are widely separated geographically, and movement of animals between the groups is expected to be minimal. However, rare, long-distance movements of otters have been reported (Pederson and Stout, 1963; Leatherwood et al., 1978).

In conclusion, the sensitivity and specificity of an IFAT for diagnosis of *T. gondii* infection in sea otters were estimated, and the IFAT was used to compare seroprevalence of *T. gondii* infection in live-sampled sea otters from California, Alaska, and Washington. All sampled Alaskan otters were seronegative for *T. gondii*, but ≥36% of live-sampled otters from California and Washington were seropositive at ≥1:320 serum dilution. It is proposed the IFAT will be useful for screening sea otters with neurological disease for *T. gondii* infection, so that antiprotozoal therapy can be expedited. In addition, it will facilitate epidemiological studies of risk factors for *T. gondii* exposure in live-sampled otters. This is important for the conservation of the threatened southern sea otter: Elevated mortality is felt to be a key factor in the failure of this population to recover; infectious disease appears to be an important cause of the elevated mortality, and processes leading to *T. gondii* exposure and death in otters are largely unstudied.

ACKNOWLEDGMENTS

This research was supported in part through funding from the UC Davis Wildlife Health Center, PKD Trust, the Lindbergh Foundation,

Friends of the Sea Otter, the Morris Animal Foundation, the San Francisco Foundation, The Marine Mammal Center (TMMC), and the CDFG. The authors wish to acknowledge the excellent assistance of staff and volunteers from CDFG, TMMC, the Monterey Bay Aquarium (MBA), and USGS-BRD for sea otter carcass recovery. The authors would like to thank E. Correa, B. Brault, H. Harris, J. Hawkes, S. Toy-Choutka, P. Chiu, J. Ames, M. Harris, M. Kenner, B. Hatfield, J. Bodkin, M. Ziccardi, P. Yochem, M. Murray, M. Staedler, A. Johnson, and M. Haulena for sample collection, processing, or assistance with data compilation.

LITERATURE CITED

ANDERSON, C. G., J. L. GITTLEMAN, K. P. KOEPFLI, AND R. K. WAYNE. 1996. Sea otter systematics and conservation: Which are critical subspecies? Endangered Species Update Special Issue: Conservation and Management of the Southern Sea Otter **13:** 6–10.

BUERGELT, C. D., AND R. K. BONDE. 1983. Toxoplasmic meningoencephalitis in a West Indian Manatee. Journal of the American Veterinary Medical Association **183:** 1294–1296.

COLE, R. A., D. S. LINDSAY, C. L. RODERICK, J. P. DUBEY, N. J. THOMAS, AND L. A. BAETEN. 2000. Biological and molecular characterizations of *Toxoplasma gondii* obtained from southern sea otters (*Enhydra lutris nereis*). Journal of Parasitology **86:** 526–530.

CONRAD, P. A., B. C. BARR, K. W. SVERLOW, M. A. ANDERSON, B. DAFT, H. KINDE, J. P. DUBEY, L. MUNSON, AND A. ARDANS. 1993. In vitro isolation and characterization of a *Neospora* sp. from aborted bovine foetuses. Parasitology **106:** 239–249.

CRUICKSHANK, J. J., D. M. HAINES, N. C. PALMER, AND D. J. ST. AUBIN. 1990. Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Canadian Veterinary Journal **31:** 213–215.

DUBEY, J. P., P. THULLIEZ, AND E. C. POWELL. 1995. *Toxoplasma gondii* in Iowa sows: Comparison of antibody titers to isolation of *T. gondii* by bioassays in mice and cats. Journal of Parasitology **81:** 48–53.

ESTES, J. A. 1990. Growth and equilibrium in sea otter populations. Journal of Animal Ecology **59:** 385–401.

ESTES, J. A., D. F. DOAK, J. R. BODKIN, R. J. JAMESON, D. MONSON, J. WATT, AND M. T. TINKER. 1996. Comparative demography of sea otter populations. Endangered Species Update **13:** 11–13.

FLEISS, J. L. 1981. Statistical methods for rates and proportions, 2nd ed. John Wiley, New York, 321 p.

GREINER, M., AND I. A. GARDNER. 2000. Epidemiologic issues in the validation of veterinary diagnostic tests. Preventive Veterinary Medicine **45:** 3–22.

———, D. PEIFFER, AND R. D. SMITH. 2000. Principles and practical application of the receiver-operating characteristic analysis for diagnostic tests. Preventive Veterinary Medicine **45:** 23–41.

HOLSHUH, H. J., A. E. SHERROD, C. R. TAYLOR, B. F. ANDREWS, AND E. B. HOWARD. 1985. Toxoplasmosis in a feral northern fur seal. Journal of the American Veterinary Medical Association **187:** 1229–1230.

INSKEEP, W., C. H. GARDNER, R. K. HARRIS, J. P. DUBEY, AND R. T. GOLDSTONE. 1990. Toxoplamosis in Atlantic bottlenose dolphins (*Tursiops truncatus*). Journal of Wildlife Diseases **26:** 377–382.

KANNAN, K., K. S. GURUGE, N. J. THOMAS, S. TANABE, AND J. P. GIESY. 1998. Butyltin residues in southern sea otters (*Enhydra lutris nereis*) found dead along California coastal waters. Environmental Science and Technology **32:** 1169–1175.

LAPOINTE, J. M., P. J. DUIGNAN, A. E. MARSH, F. M. GULLAND, B. C. BARR, D. K. NAYDAN, D. P. KING, C. A. FARMAN, K. A. BUREK HUNTINGDON, AND L. J. LOWENSTINE. 1998. Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific Harbor Seals (*Phoca vitulina richardsi*). Journal of Parasitology **84:** 1184–1189.

LEATHERWOOD, S., L. J. HARRINGTON-COULOMBE, AND C. L. HUBBS. 1978. Relict survival of the sea otter in central California and evidence of its recent redispersal south of Point Conception. Bulletin of the Southern California Academy of Sciences **77:** 109–115.

LINDSAY, D. S., N. J. THOMAS, AND D. P. DUBEY. 2000. Biological characterization of *Sarcocystis neurona* isolated from a southern sea otter (*Enhydra lutris nereis*). International Journal of Parasitology **30:** 617–624.

MIGAKI, G., J. F. ALLEN, AND H. W. CASEY. 1977. Toxoplasmosis in a California sea lion (*Zalophus californianus*). American Journal of Veterinary Research **38:** 135–136.

———, T. R. SAWA, AND J. P. DUBEY. 1990. Fatal disseminated toxoplasmosis in a spinner dolphin (*Stenella longirostris*). Veterinary Pathology **27:** 463–464.

MIKALELIAN, J., J. BOISCLAIR, J. P. DUBEY, S. KENNEDY, AND D. MARTINEAU. 2000. Toxoplasmosis in beluga whales (*Dephinapterus leucas*) from the St. Lawrence estuary: Two case reports and a serological survey. Journal of Comparative Pathology **122:** 73–76.

MILLER, M. A., P. R. CROSBIE, K. W. SVERLOW, K. HANNI, B. C. BARR, N. KOCK, M. J. MURRAY, L. J. LOWENSTINE, A. PACKHAM, AND P. A. CONRAD. 2001. Isolation and characterization of *Sarcocystis* from brain tissue of a free living southern sea otter (*Enhydra lutris nereis*) with fatal meningoencephalitis. Parasitology Research **87:** 252–257.

———, K. W. SVERLOW, P. R. CROSBIE, B. C. BARR, L. J. LOWENSTINE, F. M. GULLAND, A. PACKHAM, AND P. A. CONRAD. 2001. Isolation and characterization of two parasitic protozoa from a pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalomyelitis. Journal of Parasitology **87:** 816–822.

MONSON, D., J. A. ESTES, D. B. SINIFF, AND J. L. BODKIN. 2000. Life history plasticity and population regulation in sea otters. Oikos **90:** 457–468.

MOREJOHN, G. V., J. A. AMES, AND D. B. LEWIS. 1975. Post mortem studies of sea otters, *Enhydra lutris l*, California. Marine Resources Technical Report #30. California Department of Fish and Game Long Beach, California, 82 p.

PACKHAM, A. E., K. W. SVERLOW, P. A. CONRAD, E. F. LOOMIS, J. D. ROWE, M. L. ANDERSON, A. E. MARSH, C. CRAY, AND B. C. BARR. 1998. A modified agglutination test for *Neospora*: Development, optimization and comparison to the IFAT and ELISA. Clinical Diagnostic Laboratory Immunology **5:** 467–473.

PEDERSON, R. J., AND J. STOUT. 1963. Oregon sea otter sighting. Journal of Mammalogy **44:** 415.

ROSONKE, B. J., S. R. BROWN, S. J. TORNQUIST, S. P. SNYDER, M. M. GARNER, AND L. L. BLYTHE. 1999. Encephalomyelitis associated with a *Sarcocystis neurona*-like organism in a sea otter. Journal of the American Veterinary Medical Association **215:** 1839–1842.

THOMAS, N. J., AND R. A. COLE. 1996. The risk of disease and threats to the wild population. Endangered Species Update Special Issue: Conservation and Management of the Southern Sea Otter **13:** 23–27.

VAN PELT, R. W., AND R. A. DIETRICH. 1973. Staphylococcal infection and toxoplasmosis in a young harbor seal. Journal of Wildlife Diseases **9:** 258–261.

WILSON, D. E., M. A. BOGAN, R. L. BROWNELL JR., A. M. BURDIN, AND M. K. MIMINOV. 1991. Geographic variation in sea otters, *Enhydra lutris*. Journal of Mammology **72:** 22–36.

Chapter 5

Freshwater runoff is a risk factor for *Toxoplasma gondii* infection in sea otters

ELSEVIER

Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*)

M.A. Miller[a], I.A. Gardner[b], C. Kreuder[c], D.M. Paradies[d], K.R. Worcester[e], D.A. Jessup[f], E. Dodd[f], M.D. Harris[f], J.A. Ames[f], A.E. Packham[a], P.A. Conrad[a,*]

[a]*Department of Pathology, Microbiology and Immunology, 1 Shields Avenue, School of Veterinary Medicine, University of California, Davis, CA 95616, USA*
[b]*Department of Medicine and Epidemiology, 1 Shields Avenue, School of Veterinary Medicine, University of California, Davis, CA 95616, USA*
[c]*Wildlife Health Center, TB 128, Old Davis Road, School of Veterinary Medicine, University of California, Davis, CA 95616, USA*
[d]*Bay Foundation of Morro Bay, 601 Embarcadero, Suite 11, Morro Bay, CA 93442, USA*
[e]*Central Coast Regional Water Quality Control Board, 81 Higuera St., Suite 200, San Luis Obispo, CA 93401, USA*
[f]*California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, 1451 Shaffer Road, Santa Cruz, CA 95060, USA*

Received 22 January 2002; received in revised form 8 April 2002; accepted 8 April 2002

Abstract

The association among anthropogenic environmental disturbance, pathogen pollution and the emergence of infectious diseases in wildlife has been postulated, but not always well supported by epidemiologic data. Specific evidence of coastal contamination of the marine ecosystem with the zoonotic protozoan parasite, *Toxoplasma gondii*, and extensive infection of southern sea otters (*Enhydra lutris nereis*) along the California coast was documented by this study. To investigate the extent of exposure and factors contributing to the apparent emergence of *T. gondii* in southern sea otters, we compiled environmental, demographic and serological data from 223 live and dead sea otters examined between 1997 and 2001. The *T. gondii* seroprevalence was 42% (49/116) for live otters, and 62% (66/107) for dead otters. Demographic and environmental data were examined for associations with *T. gondii* seropositivity, with the ultimate goal of identifying spatial clusters and demographic and environmental risk factors for *T. gondii* infection. Spatial analysis revealed clusters of *T. gondii*-seropositive sea otters at two locations along the coast, and one site with lower than expected *T. gondii* seroprevalence. Risk factors that were positively associated with *T. gondii* seropositivity in logistic regression analysis included male gender, older age and otters sampled from the Morro Bay region of California. Most importantly, otters sampled near areas of maximal freshwater runoff were approximately three times more likely to be seropositive to *T. gondii* than otters sampled in areas of low flow. No association was found between seropositivity to *T. gondii* and human population density or exposure to sewage. This study provides evidence implicating land-based surface runoff as a source of *T. gondii* infection for marine mammals, specifically sea otters, and provides a convincing illustration of pathogen pollution in the marine ecosystem.

Keywords: *Toxoplasma gondii*; *Enhydra lutris*; Sea otter; Risk factor; Spatial analysis; Runoff

1. Introduction

Growing evidence supports the link between human environmental disturbance and emerging infectious diseases of wildlife populations (Daszak et al., 2001). More than any other animal species, humans impact the environment locally, regionally and globally, inducing atmospheric, hydrological and biochemical changes that can be detected in the most remote regions of the planet. Anthropogenic environmental changes may promote the emergence of pathogens through the transportation and introduction of infectious agents or hosts to new environments, through manipulation of local ecosystems to favour the proliferation or prolonged survival of infectious agents, or by facilitating new host–pathogen interactions. These emerging infectious diseases in turn pose threats to ecosystem biodiversity and human health.

The protozoan parasite *Toxoplasma gondii* is a recognised pathogen of humans and terrestrial animals. This parasite has a two-host life cycle, with many animals, including mice, birds, domestic livestock and humans serving as potential intermediate hosts (Frenkel and Dubey, 1972). In the intermediate host, invasive stages of *T. gondii* may spread throughout the muscles, nervous system and other tissues, forming long-lived tissue cysts. However, the only animals known to shed oocysts in their faeces are felids, most importantly domestic cats. These oocyst-shedding

* Corresponding author. Tel.: +1-530-752-3349.
E-mail address: paconrad@ucdavis.edu (P.A. Conrad).

definitive hosts are infected through oocyst exposure, or by consumption of infected intermediate hosts.

The most common routes of *T. gondii* infection for humans are through exposure to oocysts in contaminated soil, transplacental transmission or by consumption of uncooked or undercooked meat containing encysted parasites (Frenkel and Dubey, 1972). However, recent evidence indicates that waterborne *T. gondii* exposure is more common than previously recognised, and may represent an important source of human infection (Bowie et al., 1997; Aramini et al., 1999; Tenter et al., 2000). These waterborne infections probably result from exposure to infective oocysts in polluted water, but it is also possible that aquatic species serve as intermediate or paratenic hosts.

Increasing recognition of *T. gondii* infection in diverse species of marine mammals, including cetaceans (Cruickshank et al., 1990; Inskeep et al., 1990; Migaki et al., 1990; Mikelian et al., 2000), pinnipeds (Van Pelt and Dietrich, 1973; Migaki et al., 1977; Holshuh et al., 1985; Miller et al., 2001) and sirenians (Buergelt and Bonde, 1983) provides compelling evidence for marine dispersal of this terrestrial pathogen. Until recently, most reports consisted of case studies on individual *T. gondii*-infected animals. However, the recent recognition of numerous fatal *T. gondii* brain infections in southern sea otters (*Enhydra lutris nereis*) from California (Thomas and Cole, 1996; Cole et al., 2000) prompted concerns about the emergence of *T. gondii* as a significant marine pathogen. Whether the emergence of *T. gondii* infection in sea otters is attributable to increasing prevalence, increased surveillance, or both, is unknown. For California otters examined between 1992 and 1995, Thomas and Cole (1996) attributed 8.5% of total sea otter mortality to protozoal meningoencephalitis. Using parasite isolation in cell culture and brain immunohistochemistry, we recently discovered that 36% (28/77) of freshly dead sea otters were infected with *T. gondii* at the time of postmortem examination (Miller et al., 2002), suggesting that *T. gondii* infection is common in southern sea otters.

Sea otters are a unique marine mammal species because they live, reproduce and feed almost exclusively in the nearshore marine environment, often within 0.5 km of the shoreline (Riedman and Estes, 1990). As a federally listed threatened species with evidence of recent population declines, the high prevalence of *T. gondii* infection in southern sea otters is of concern. To investigate the apparent emergence of *T. gondii* as a pathogen of southern sea otters, we determined seroprevalence in live and dead sea otters examined between 1997 and 2001 using an indirect fluorescent antibody test (IFAT) which was recently validated for sea otters (Miller et al., 2002). Additional coastal environmental data, including location and volumes of river and stream runoff, municipal sewage outfall and human coastal population density were assembled from federal and state sources. The compiled demographic and environmental data were examined for statistical associations with *T. gondii* seropositivity in sea otters. Our working hypotheses were that *T. gondii* exposure in sea otters would be positively correlated with age class, total length, body weight, nutritional condition, coastal human population density and areas of maximal sewage and freshwater outflow. Because we focussed on *T. gondii* seropositivity, not *T. gondii*-induced disease for the present study, we expected to find no relationship between seropositivity and dead versus live status at time of sampling. Through spatial analysis we hoped to detect high and low risk areas for *T. gondii* seroprevalence that could provide optimal sampling locations for future research on routes and mechanisms of *T. gondii* exposure in sea otters.

2. Materials and methods

2.1. Study population

Data from 223 live- and dead-sampled otters were included in the study (Table 1). Throughout the study period, yearly rangewide counts identified <2,300 sea otters along the central coast of California (United Sates Geological Survey unpublished technical report). Southern sea otters currently range from Half Moon Bay south to Santa Barbara, California, a distance of approximately 661 km. Data on each otter's gender, age class, stranding or sampling location and other factors, as defined below, were recorded at the time of capture or necropsy.

Table 1
Demographic characteristics of live and dead California sea otters enrolled in risk factor study (1997–2001)

Live/dead status	Gender	Age class			
		Pup/immature	Subadult	Adult/aged adult	Total
Live	Male	14 (29%)	2 (4%)	32 (67%)	48
	Female	7 (10%)	7 (10%)	54 (80%)	68
		21 (18%)	9 (8%)	86 (74%)	116
Dead	Male	15 (24%)	8 (13%)	39 (63%)	62
	Female	13 (29%)	7 (15%)	25 (56%)	45
		28 (26%)	15 (14%)	64 (60%)	107
Total		49	24	150	223

Dead sea otters ($n = 107$) were collected along the central California coast, transported to the California Department of Fish and Game Marine Wildlife Veterinary Care and Research Center in Santa Cruz, California and necropsied as described (Miller et al., 2002). All freshly dead (postmortem interval <72 h) otters examined between January 1997 and June 2001 with available serum were included in the study. Live-sampled southern sea otters ($n = 116$) were captured at various locations between January 1997 and June 2001. Live-sampled otters received flipper tags prior to release to prevent inadvertent repeat sampling. For live-sampled otters, the sample location, gender distribution and sample dates were influenced by ongoing research projects, permit-related sampling restrictions and weather conditions.

2.2. Serum collection and testing, live and dead otters

Blood was obtained from live-sampled otters by jugular venipuncture and from necropsied otters by collection from the heart and great vessels. Whole blood was allowed to clot, centrifuged at $1,500 \times g$ for 10 min. and stored at −70°C until tested. Serum samples were screened for *T. gondii* using an IFAT and endpoint titres were determined through serial dilution (Miller et al., 2001, 2002). An IFAT cutoff of ≥1:320 was previously determined to be optimal for detecting *T. gondii* infection in southern sea otters of known *T. gondii* infection status (Miller et al., 2002), thus this cutoff was used in the present study. Confirmation of *T. gondii* infection in live-sampled otters was not possible by non-invasive methods other than serology. However, previous studies showed good correlation of IFAT results with *T. gondii* infection status (Miller et al., 2002).

2.3. Definition of risk factors

The following potential risk factors were selected for evaluation of associations with *T. gondii* seropositivity: gender, live versus dead status at time of sampling, age class, body weight (kg), body length (cm), length–weight ratio, nutritional condition score, sample or stranding location, coastal human population density and sampling location proximity to river and stream outflow locations, or municipal sewage treatment plant outfall locations.

The sea otter age classifications used in this study were based on total body length, dentition and pelage characteristics, as described by Morejohn et al. (1975). Three age categories were used for live and dead otters: pups plus immatures, subadults and adults plus aged adults. The youngest and oldest age classes were collapsed into single categories because of differences in age class assessment criteria for live and dead otters. Nutritional condition was assessed only for dead otters, and categories were defined as follows: emaciated, no discernable body fat; thin, minimal body fat (e.g. hocks only); fair, scant subcutaneous body fat (e.g. hocks and hips); moderate, moderate subcutaneous body fat distributed throughout subcutis and abundant, abundant subcutaneous body fat. Total body length was measured as flat linear distance (cm) from the tip of the nose to the fleshy tip of the tail. Length–weight ratio was the ratio of total length to body weight in kilograms. Correlations among the age and gender-related biological factors were assessed using several techniques, as outlined below.

To assign a numerical value for each otter's stranding or sampling location, the central California coastline encompassing the southern sea otter range (661 km) was divided into 0.5 km increments and was assigned a numerical value, starting with 1 to the north, and ending at 1,322 to the south (California Department of Fish and Game, unpublished data). Each point was mapped in reference to prominent coastal geographical features along a hand-smoothed contour line, set offshore at 5 fathoms depth. All live or dead otters sampled along the coastline were assigned to the closest 0.5 km site, based on their location at the time of carcass recovery or capture. These locational data were converted to latitude and longitude values and were used for all subsequent spatial analyses.

Data for human population density along the central California coast were compiled from United States 2000 census data (http://www.geographynetwork.com). Population density was reported as the number of human beings per square mile, using the following five groups: 0–100; >100–1,000; >1,000–3,000; >3,000–6,000 and >6,000. Each 0.5 km coastal point within the southern sea otter range was assigned the human population density score of the adjacent coastal 2000 census tract. All dead- and live-sampled otters were assigned the appropriate score, based on their location at the time of recovery or sampling.

Quantification of freshwater outflow along the central California coast was done using a geographic information system (GIS) map marked with the marine outfall location of each stream or river along the central California coast. All watersheds drained by unique rivers or streams (delineated by CalWater 2.2 GIS data and US EPA Reach File 3 GIS data) were included in this study. Relative discharge from each watershed was estimated using the 60-year average rainfall data (Central Coast Regional Water Quality Control Board), expressed as areas of equal rainfall, or isohyets, in conjunction with the boundaries and total area of each watershed. Since the amount of precipitation lost to impoundment, ground absorption or other factors could not be accurately determined for each watershed, the theoretical maximum flow values (average precipitation per unit area, times total acreage) were used. The relative contributions of water impoundment, irrigation and other exogenous factors were assumed to be equal across all watersheds. The relative exposure to stream and river outflow was determined for each 0.5 km otter sample point described above. An exponential dilution model was used to predict the influence of runoff from each river and stream, with each successive 0.5 km coastal point assigned a calculated value for magnitude of freshwater influence. Sample point values were determined by weighting both the sample point's proximity to each river or stream mouth and total annual

outflow (e.g. 0–10,000; 10,001–100,000 or 100,001–1,000,000 acre-ft/year). Wherever the influences of two rivers or streams overlapped, their weighted flow values were combined at each applicable 0.5 km point. This freshwater outflow model assumed that outflow from rivers is mixed with salt water at a rate that varies exponentially with distance from the point of entry. Freshwater influence was presumed to be negligible when the magnitude of freshwater outfall was less than 10,000 acre-ft per year at a given 0.5 km coastal point.

The proximity of each otter's sampling site to the location of the nearest major municipal sewage outfall was determined using similar techniques as for freshwater outflows. Sewage plant discharge locations and volumes were obtained from National Pollutant Discharge System permit records (California Central Coast Regional Water Quality Control Board). For each treatment facility, total yearly marine discharge (acre-ft per year) was assessed. Areas of coastal influence of treatment plant discharges were estimated by mapping each sewage outfall pipe's discharge location using the 0.5 km coastal sampling units described above. The combined influences of proximity and effluent volume exposure were calculated using an exponential dilution model, with the exposure values recalculated for each sequential 0.5 km sampling location from the sewage outfall pipe. Sewage influence was categorised as <1; 1–4,000 or 4,001–8,000 acre-ft per year. When two sewage treatment plants were discharging in close proximity to each other, their numerical values for total flow were added at each affected 0.5 km site. For both sewage outfalls and freshwater flows, no attempt was made to correct for seasonal variation in volume discharged at each site or local effects attributable to wind, marine currents or coastal geography.

2.4. Univariate analysis of risk factors

Chi-square tests were used to determine univariate associations between *T. gondii* serological status and categorical risk factors (e.g. gender and age class) in otters. *t*-Tests were used to determine associations between *T. gondii* serological status and continuous risk factors (e.g. body weight and total length). P values <0.05 were considered statistically significant. Odds ratios and 95% confidence interval (CI) were calculated for categorical risk factors. All analyses were done using SPSS Graduate Pack, version 10.0 (SPSS Inc.).

2.5. Spatial analysis

The spatial relationship between *T. gondii* serological status in otters and sample location was evaluated using SaTScan (http://www.nic.nih.gov/prevention/bb/satscan.html), version 2.1. A Bernoulli-based (Kulldorf and Nagarwalla, 1996), purely spatial equation for probability was selected for the analyses because of differences in sample collection periods between the live and dead otter groups, and due to the binary character of the data (e.g. seropositive or seronegative). Data from live and dead otters were analysed separately and were combined for spatial analyses. The data were analysed for both higher and lower than expected clusters of *T. gondii* seropositivity, recognising that both regions would be of interest in subsequent studies on routes and mechanisms of sea otter infection by *T. gondii*. A second spatial analysis was performed to examine in more detail potential spatial clusters within the Monterey Bay region. Only data points located within the greater Monterey Bay region (0.5 km markers 256–390) were included in this second, smaller-scale spatial analysis. A P value of <0.1 was considered statistically significant for detecting spatial clusters with increased or decreased risk for *T. gondii* seropositivity.

As a second technique to examine the data for spatial associations between stranding or sampling location and *T. gondii* seropositivity, the central coast of California was divided into 22 segments, with the points of separation delineated by coastal geographical features (e.g. peninsulas) or points of transition between rural and urban areas. Proportions of seropositive otters among regions were compared to supplement our findings derived from SaTScan spatial analyses.

2.6. Logistic regression analysis

Relationships between potential demographic, environmental and spatial risk factors and seropositivity to *T. gondii* were further assessed by logistic regression. The logistic regression equation was developed using SPSS Graduate Pack, version 10.0, (SPSS Inc.). Logistic modelling followed recommended procedures (Hosmer and Lemeshow, 2000) and considered all biologically plausible risk factors using forwards and backwards selection of factors. For the logistic regression analysis, serological data for live and dead otters were pooled to maximise sample size. Overall fit of the final logistic equation was assessed using Hosmer–Lemeshow goodness-of-fit statistics. Adjusted odds ratios and 95% CIs were calculated to measure the strength of association between each risk factor in the equation and serological status for *T. gondii*.

3. Results

3.1. Seroprevalence

The *T. gondii* seroprevalence was 42% (49/116) for live otters and 62% (66/107) for dead otters using an IFAT cutoff titre of $\geq$1:320 as positive. Reciprocal IFAT titres ranged from 80 to 20,480 for both live and dead otters. Gender and age distributions differed between the live and dead otters (Table 1). Live-sampled otters had a higher proportion of females ($P = 0.013$) and young age classes ($P = 0.068$) compared with dead otters. These differences between the two groups were accounted for in the logistic regression analysis of risk factors. The proportion of seropositive otters for each study year ranged from 25% (1997; $n = 4$) to 75%

(1998; $n = 44$). However, variation in the proportion of seropositive otters was not significant among study years ($P = 0.8$).

3.2. Risk factors

Based on univariate analyses, seropositivity to *T. gondii* was positively associated with male gender, increasing age class and dead versus live status at time of sampling ($P \leq 0.05$) (Table 2). The odds of *T. gondii* seropositivity for females were approximately one half of those for males. Dead-sampled otters were 2.2 times more likely to be seropositive for *T. gondii*, when compared with live-sampled otters. Surprisingly, no association was detected between nutritional condition and seropositivity to *T. gondii* ($P = 0.100$). However, nutritional condition was assessed only for dead otters. Similarly, seropositivity to *T. gondii* was not significantly associated with human population density ($P = 0.293$), or proximity to sewage outfalls ($P = 0.955$), but was highly correlated with freshwater flow ($P < 0.001$). Highly significant associations were detected between increasing body weight and total length and *T. gondii* seropositivity ($P < 0.001$). Mean (±SEM) body weight and length of seropositive otters (20.6 ± 0.6 kg and 118.9 ± 1.2 cm, respectively) were significantly greater ($P < 0.001$) than the corresponding measurements for seronegative otters (15.8 ± 0.7 kg and 107.6 ± 2.0 cm, respectively). An inverse relationship was detected between seropositivity to *T. gondii* and the calculated length–weight ratio ($P < 0.001$). Seropositive otters had a significantly lower length–weight ratio (6.6 ± 0.3) than seronegative otters (8.7 ± 0.5). This result is not surprising, however, given that length–weight ratio was also found to correlate inversely with sea otter age (data not shown).

3.3. Spatial analysis

Spatial analysis of pooled live and dead otter serological data revealed a large cluster of *T. gondii*-seropositive otters (20/23, or 87% seropositive) within a 20 km coastal region centred on the towns of Morro Bay and Cayucas, California (35.361°N, 120.870°W) (Fig. 1). Otters sampled from this area were nearly twice as likely to be seropositive to *T. gondii* as expected, and this difference was statistically significant ($P = 0.082$).

For otters sampled within Monterey Bay, a second potential cluster of *T. gondii* seropositivity was detected within a 27 km region centred on Elkhorn Slough and the small town of Moss Landing (36.790°N, 121.799°W) (Fig. 1). Nearly 79% (15/19) of otters sampled within this spatial cluster were seropositive for *T. gondii*, and otters sampled within 10 km of Elkhorn Slough were 1.5 times more likely to be

Table 2
Categorical risk factors for seropositivity to *Toxoplasma gondii* in California sea otters (1997–2001), univariate analysis[a]

Risk factor	Group	Percentage seropositive for *T. gondii*	Odds ratio	95% CI	Chi-square *P*-value
Gender	Male	59 ($n = 110$)	1.00	–	0.027
	Female	44 ($n = 113$)	0.55	0.32–0.94	
Age class	Immature	20 ($n = 49$)	1.00	–	<0.001
	Subadult	54 ($n = 24$)	4.61	1.41–15.42	
	Adult	61 ($n = 150$)	6.19	2.72–14.40	
Live–dead status	Alive	42 ($n = 116$)	1.00	–	0.004
	Dead	60 ($n = 107$)	2.20	1.29–3.76	
Nutritional condition[b] (based on subcutaneous body fat)	Abundant	75 ($n = 20$)	1.00	–	0.100
	Moderate	78 ($n = 13$)	1.11	0.17–7.71	
	Fair	40 ($n = 10$)	0.22	0.03–1.44	
	Thin	68 ($n = 25$)	0.71	0.16–3.15	
	Emaciated	49 ($n = 37$)	0.32	0.08–1.20	
Human population (no. of humans per square mile)	<100	65 ($n = 49$)	1.00	–	0.293
	100–1,000	46 ($n = 63$)	0.45	0.19–1.05	
	1,000–3,000	47 ($n = 53$)	0.47	0.20–1.13	
	3,000–6,000	50 ($n = 16$)	0.53	0.15–1.92	
	>6,000	50 ($n = 42$)	0.53	0.21–1.34	
Sewage outfall exposure (acre-ft/year)	Low	51 ($n = 214$)	1.00	–	0.955
	Medium	57 ($n = 7$)	1.26	0.23–7.29	
	Heavy	50 ($n = 2$)	0.95	0.03–35.07	
Freshwater outflow exposure (acre-ft/year)	Low	41 ($n = 121$)	1.00	–	<0.001
	Medium	45 ($n = 60$)	1.16	0.59–2.27	
	Heavy	76 ($n = 42$)	4.54	1.93–10.93	

[a] Analysis includes IFAT results from both dead ($n = 107$) and live ($n = 116$) otters.
[b] Nutritional condition data were only available for dead otters, and were not assessed for two otters.

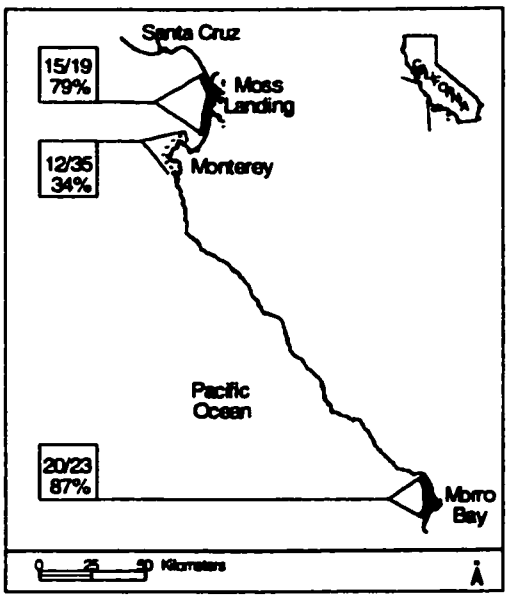


Fig. 1. Spatial clusters with higher (dark lines) or lower (dotted line) than expected proportions of sea otters that were seropositive for *Toxoplasma gondii*.

seropositive than for all otters combined. However, this difference was not statistically significant ($P = 0.997$). Spatial analysis was repeated on a smaller scale to further examine this potential cluster of seropositive otters. Analysis of pooled live and dead otter serology data for the greater Monterey Bay region again revealed a spatial cluster overlapping the Elkhorn Slough/Moss Landing site (36.634°N, 121.918°W). This spatial cluster from the more restricted spatial analysis more closely approached statistical significance ($P = 0.224$, data not shown).

A region of low *T. gondii* seropositivity was detected for otters sampled within a 28 km region encompassing the tip and southern portion of Monterey Peninsula (36.579°N, 121.980°W) (Fig. 1). Live and dead otters sampled from within this region were half as likely to be seropositive to *T. gondii* as expected, and this difference was statistically significant ($P = 0.007$). Separate univariate analyses of the 22 major coastal segments (as described in Section 2) supported our findings from spatial analyses, with higher than expected proportions of seropositive otters detected in the vicinity of Morro Bay (78%, $n = 26$) and Elkhorn Slough (74%, $n = 19$), with lower than expected proportion of seropositive otters detected in the vicinity of south Monterey Peninsula (34%, $n = 35$).

To further evaluate the clusters of seropositive and seronegative otters detected through spatial analysis, locations of all otters (live and dead) were coded as follows: 1 = all otters sampled within the Elkhorn Slough spatial cluster, 2 = all otters sampled within the Morro Bay spatial cluster, 3 = all otters sampled within the south Monterey Peninsula cluster and 4 = all otters sampled at sites falling outside of these spatial clusters. The resulting data were incorporated into a logistic model to determine if associations between the sample location and other risk factors could explain the observed spatial clustering.

3.4. Logistic regression analysis

The goal of logistic regression analysis was to simultaneously investigate the relative contributions of the various risk factors to *T. gondii* seropositivity, while adjusting for differences between sample populations. The final logistic equation identified significant associations between *T. gondii* seropositivity in relation to otter gender, age class, sampling location and maximal freshwater outflow (Table 3). The Hosmer–Lemeshow goodness-of-fit P value of the final logistic equation was $P = 0.96$, which indicated excellent fit between the observed data and the model. A slight protective effect was attributed to female gender, younger

Table 3
Logistic regression of risk factors for seropositivity to *Toxoplasma gondii* for California sea otters (1997–2001)[a]

Risk factor		Adjusted odds ratio	95% CI	Significance (P)
Gender	Male	1.00		
	Female	0.49	0.26–0.93	0.028
Age class	Pup/immature	1.00		
	Subadult	8.08	2.21–29.62	0.002
	Adult	14.61	5.10–41.84	<0.001
Status at time of sampling	Alive	1.00		
	Dead	1.85	0.88–3.89	0.103
Sampling location	All other sites	1.00		
	Morro Bay	9.31	2.26–38.31	0.002
Freshwater outflow exposure	Light	1.00		
	Medium	1.07	0.48–2.4	0.876
	Heavy	2.90	1.21–6.9	0.017

[a] Analysis includes IFAT results from both dead ($n = 107$) and live ($n = 116$) otters.

age class and otters that were sampled at points distant from Morro Bay. After accounting for the effects of age class, gender and sampling location, the adjusted odds ratio for *T. gondii* seropositivity for dead-sampled otters was still almost twice that for live otters (1.85:1). However, these findings were not significant ($P = 0.103$).

In contrast, significantly increased odds of *T. gondii* seropositivity were detected for otters sampled near maximal (heavy) freshwater outfalls (Table 3). Based on our analysis, the odds of *T. gondii* seropositivity were highest for adult male sea otters sampled from areas of central California with maximal freshwater outflow, especially those sampled near Morro Bay/Cayucas. No significant associations with *T. gondii* seropositivity were found in relation to sewage flow, either by univariate analysis (Table 2) or by logistic regression analysis ($P > 0.1$, data not shown). However, 96% of our otter samples (214/223) were obtained from coastal areas with minimal values for municipal sewage exposure.

4. Discussion

The overall goal of the present study was to investigate the apparent emergence of *T. gondii* infections in southern sea otters from California. Between 1997 and 2001, we collected serum from 223 live and dead sea otters. The current California sea otter population is approximately 2,300 animals. Using a *T. gondii* IFAT that was previously validated for sea otters, we determined that 42% (49/116) of live otters, and 62% (66/107) of fresh dead California otters were seropositive for *T. gondii* at the time of sampling. Our specific objective was to evaluate our sea otter serological and demographic data, along with coastal environmental data for potential demographic, spatial or environmental factors associated with an increased risk of *T. gondii* seropositivity in sea otters. The data were also examined for factors associated with a lower than expected risk of *T. gondii* seropositivity, as both types of risk factors would provide important clues regarding the route and mechanisms of sea otter infection by *T. gondii*.

A number of obstacles, including misidentification of exposure location, incorrect classification of demographic or serological data and laboratory error could have inhibited our ability to detect risk factor associations. Unavoidable misclassification of data might have occurred due to wide-ranging movements of some otters with chronic *T. gondii* infections, postmortem carcass drift, error in identification of seropositive or seronegative otters (false positives or false negatives), laboratory error in sample processing or interpretation, and incorrect categorisation of age class or other demographic data. Despite these obstacles, we were able to identify statistically significant demographic, spatial and environmental associations, as outlined below. These associations provide strong evidence to support the suspected land-based origin of *T. gondii* infections in sea otters, and reveal new avenues for scientific investigation. We believe that the true associations may be even stronger, but were partially masked by suspected non-differential misclassification of data due to the factors listed above.

At the onset of the study we did not hypothesise that otter gender would be associated with seropositivity to *T. gondii*. However, male otters were almost twice as likely as females to be seropositive (Table 2), possibly due to behavioural differences. Variation in home range size and seasonal movements are recognised, and males are more likely to travel long distances in their efforts to establish and defend territories (Jameson, 1989; Ralls et al., 1996). Thus spatial associations identified in female otters may more accurately reflect local exposure conditions than similar data derived from more wide-ranging males. Conversely, if *T. gondii* contamination of the nearshore marine environment occurs as multiple areas of point-source contamination, then wide-ranging males would be more likely to come into contact with one or more of these contaminated areas during their lifetime.

We hypothesised that increasing sea otter age would increase the risk of seropositivity to *T. gondii*. As with humans and terrestrial animals (Dubey, 1987; Guerina, 1994; Esteban-Redondo et al., 1999), *T. gondii* infection in otters is likely to be prolonged, perhaps lifelong, as a result of tissue cyst formation. Assuming the temporal risk of *T. gondii* exposure remains relatively constant, then the probability of otter infection and seropositivity increases the longer an animal lives. All indices of age employed in the present study (age class, body weight, total length and length–weight ratio) yielded similar associations with seropositivity. Otters that were older, heavier and longer were far more likely to be seropositive to *T. gondii*. The present study did not account for potential foetal loss or neonatal mortality attributable to transplacental infection by *T. gondii*. Such infections have been documented in humans and domestic animals, and may contribute significantly to foetal loss and neonatal mortality (Guerina, 1994; Buxton, 1998). Transplacental transmission of *T. gondii* in sea otters has not been documented, but could easily be missed due to uterine resorption or lower carcass recovery rates for affected foetuses and neonates, when compared with larger, more obvious carcasses of subadult and adult otters.

The focus of the present study was on seropositivity to *T. gondii*, not disease attributable to *T. gondii* infection. Thus we expected to find minimal association between live or dead otter status at the time of sampling and *T. gondii* serostatus, after adjusting for age and gender differences. However, we found that dead otters were more than twice as likely to be seropositive to *T. gondii*, when compared with live otters in our univariate analysis ($P = 0.004$). Increased odds of seropositivity for dead otters might be attributed to increased risk of mortality for *T. gondii*-exposed otters, due to the direct or indirect effects of *T. gondii* infection. Other studies have documented *T. gondii* encephalitis as an important cause of sea otter mortality

(Thomas and Cole, 1996). When live–dead status at time of sampling was incorporated into a logistic model, the adjusted odds ratio for seropositivity for dead otters was approximately twice that for live otters (Table 3). However, this difference was not found to be significant ($P = 0.103$) when other factors such as gender, age class, sampling location and freshwater flow exposure were accounted for in the model. This suggests that associations between some or all of these factors may have contributed to the variation in *T. gondii* seropositivity observed between the live- and dead-sampled sea otter groups.

Our working hypothesis was that *T. gondii*-positive sea otters would be in poorer nutritional condition than seronegative otters, because *T. gondii* infection could result in impairment of vision or compromised brain, heart or muscle function, leading to impaired foraging efficiency and emaciation. Univariate analysis revealed no statistical association between nutritional condition and *T. gondii* serostatus. However, nutritional condition was only assessed for dead otters at necropsy, not live otters, and many other causes of death may be associated with poor nutritional condition.

We speculated that exposure to surface runoff and sewage would be maximal in areas of high human density. Thus increased flow of *T. gondii*-contaminated water into the nearshore marine environment would be expected near densely settled areas, and would be reflected as a higher proportion of seropositive sea otters. However, our assumption that human population density could serve as an index of maximal surface runoff or sewage outfall was incorrect. Negative correlations were detected between freshwater outflow (e.g. runoff) and coastal human population density, and between sewage outfall and coastal human population density ($P < 0.05$, data not shown), suggesting that regions of maximal freshwater and sewage outflow were preferentially located in areas of low human population density. In addition, variation in inland human population density, which may have contributed directly to coastal freshwater outflow, and indirectly to coastal sewage outflow, were not assessed. Thus, the relationship between human population density and *T. gondii* exposure in sea otters should be investigated using techniques other than those utilised in the present study.

The relationship between areas of increased human density and domestic cat density in California is unknown, but it seems logical to assume that increased numbers of feral and domestic cats could be associated with areas of human development. However, feral cats were also detected in regions of moderate to low human density, such as the vicinity of Elkhorn Slough and Morro Bay (Miller, unpublished data).

In the present study we hypothesised that *T. gondii* seropositivity in otters would be associated with exposure to coastal plumes of municipal sewage. Potential sources of *T. gondii* in sewage include flushable cat litter or skimmed cat faeces that have been disposed into toilets. Common techniques for primary and secondary sewage processing may not kill protozoan oocysts or sporocysts (Payment et al., 2001), and may even enhance their infectivity (e.g. by aeration) prior to wastewater release. We found no evidence of a relationship between seropositivity to *T. gondii* and exposure to municipal sewage. This may be because the major municipal sewage outfalls are located far offshore (e.g. 0.5–5 km), and nearly all (96%) otters were sampled at locations >5 km from the nearest major municipal sewage outfall. Thus exposure of sea otters to sewage plumes derived from major municipal sources was considered to be low in the present study. It is important to note that the potential negative impacts of exposure to non-municipal sewage, such as boat bilge discharge and seepage from broken sewage pipes or septic tanks, were not addressed, because these smaller, intermittent sources of faecal waste are more difficult to detect and monitor. The same is true for small sources of freshwater outflow, such as municipal surface water runoff. However, the cumulative importance of these smaller sources of polluted water in transporting *T. gondii* oocysts from contaminated litter, lawns, gardens, sidewalks and streets into the nearshore marine environment could be significant, and should not be discounted. Collectively, these smaller point sources of marine contamination may have important, as yet unrecognised deleterious effects on sea otter health. Potential negative impacts of sea otter exposure to sewage should be investigated by targeted sampling of animals from sewage-impacted and sewage-free areas.

We hypothesised that *T. gondii* seropositivity would be associated with exposure to high volumes of freshwater outflow, because environmentally resistant *T. gondii* oocysts present in cat faeces could be efficiently transported to the nearshore marine environment by surface runoff. If this is true, then otters living in or near large plumes of contaminated freshwater would be at increased risk for *T. gondii* exposure. In California, surface water runoff is conducted to coastal streams, or directly to the ocean from lawns, streets and open land via storm drains, ditches and culvert pipes, with essentially no pre-treatment. Significant surface water contamination by *T. gondii* oocysts was demonstrated previously in British Columbia, Canada, where a large-scale outbreak of human toxoplasmosis led to the discovery of contamination of a public water supply, presumably by feline faeces (Aramini et al., 1999). Coastal freshwater outflow, as calculated in this study, is roughly analogous to maximal terrestrial surface water runoff. When adjusted for variation attributable to gender, age class, live–dead status at time of sampling and high or low risk sites detected through spatial analysis, a strong association was detected between *T. gondii* seropositivity in otters and locations of maximal freshwater outflow along the coast. Otters sampled at these maximal flow sites were nearly three times more likely to be seropositive to *T. gondii* than those sampled at low flow sites. This association between maximal surface runoff and *T. gondii* seropositivity in sea otters suggests a significant role for freshwater runoff in the transmission of *T. gondii* to sea otters. In addition to terrestrial-

origin input of infective protozoan oocysts, these freshwater plumes might also enhance sea otter *T. gondii* exposure through other means, perhaps by enhancing oocyst survival in the nearshore marine environment, or by creating optimal habitat for otter prey species that may serve as efficient intermediate or paratenic hosts.

Spatial analysis was conducted to detect clusters of seropositive and seronegative otters, and to develop hypotheses about site-specific risk factors for *T. gondii* exposure. For example, spatial clustering of seropositive sea otters might be associated with localised *T. gondii* oocyst contamination through rivers, streams or other point sources. However, the spatial analyses did not adjust for demographic and environmental exposure variables in the population-at-risk. To adjust for potential variation in these factors, our results from spatial analysis were examined in relation to freshwater flow by univariate analysis, and were incorporated into the final logistic regression model. Most (89%) of the otters ($n = 19$) sampled in the vicinity of Elkhorn Slough were exposed to maximal freshwater flow, thus explaining the increased proportion of seropositive otters sampled at this site. Similarly, the low risk spatial cluster centred on south Monterey Peninsula could be attributable to low freshwater flow exposure, as 98% of sampled otters ($n = 60$) from this region were exposed to low or moderate freshwater flow. In addition, over 78% of the south Monterey Peninsula otters were live-sampled, which could have biased the sampling towards a higher proportion of seronegative animals.

The relationship between freshwater flow exposure and *T. gondii* seropositivity was less clear for otters living in the vicinity of Morro Bay/Cayucas. Otters sampled from this region were evenly divided between low ($n = 8$), moderate ($n = 9$) and heavy ($n = 7$) freshwater exposure. Even after variation in freshwater flow, gender, age class, and live–dead status were accounted for in the logistic model, otters sampled at this location were nine times more likely to be seropositive for *T. gondii* ($P < 0.001$). Analysis of protozoan isolates obtained from necropsied otters revealed a similar trend, with 67% of otters (12/18) recovered from the Morro Bay/Cayucas region found to be infected with *T. gondii*, compared with 27% infection (16/59) on average for the other freshly dead otters necropsied at our facility (Miller, unpublished data).

Unrecognised factors appear to be contributing to the increased risk for *T. gondii* exposure in otters sampled from the Morro Bay/Cayucas region. Interestingly, this is the only region within southern sea otter range where primary treated municipal sewage is permitted to be discharged into the nearshore marine environment. Any causal relationship remains to be established. The present study design did not allow for an in-depth evaluation of the potential effects of sewage, since nearly all otters in the study were sampled at sites >5 km away from municipal sewage outfall locations. To exclude sewage as a risk factor for *T. gondii* exposure, targeted sampling of otters should be completed in known sewage-impacted areas, as well as sites distant from any recognised sewage input. Coastal geography, winds, tides and marine currents may also play a role in locally concentrating oocysts that have gained access to the nearshore environment. A large enclosed harbour (Morro Bay) is located near the centre of this region, and is widely used by otters for foraging and resting. This harbour has relatively low freshwater input and has a narrow opening to the ocean. Thus the normal flushing action of waves, storms and tidal changes may be minimised at this site. In addition, feral cats are present at sites immediately adjacent to the enclosed harbour and open ocean in this vicinity (Harris, personal observations). Studies in progress now may help to better define the sources and risk factors for *T. gondii* infection for sea otters for this high-risk area.

The marine source of *T. gondii* exposure for sea otters is not known. One possible route is through direct ingestion of infective oocysts present in contaminated water. However, infective oocysts might also be efficiently concentrated and transmitted to sea otters through filter-feeding activity of benthic invertebrates, as has been demonstrated previously for related pathogenic protozoa (e.g. *Cryptosporidium* and *Giardia*) (Graczyk et al., 1999a,b; Tamburrini and Pozio, 1999). Filter-feeding benthic invertebrates, such as clams and mussels are a common prey source for southern sea otters (Kvitek et al., 1988; Riedman and Estes, 1990). Because sea otters feed almost exclusively in the nearshore marine environment and consume approximately 25% of their body weight each day in filter-feeding benthic invertebrates and other prey (Riedman and Estes, 1990), these invertebrates could serve as an efficient route of *T. gondii* uptake and dissemination to sea otters. If confirmed, these findings would help explain the high proportions of *T. gondii*-infected (36%) and seropositive (42% for live, 62% for dead) otters sampled along the central coast of California. Since humans consume the same or similar invertebrate species, including clams and mussels, confirmation of *T. gondii* contamination of nearshore benthic invertebrates would have significant human health implications.

This study provides compelling evidence implicating land-based surface runoff as a source of *T. gondii* infection for sea otters, and is an excellent illustration of pathogen pollution in the nearshore marine environment. Nearshore marine contamination through surface runoff would most likely result from transport and nearshore marine deposition of feline faeces, which may contain millions of infective *T. gondii* oocysts (Frenkel and Dubey, 1972). Collectively, our findings suggest that the interplay between surface runoff, coastal geography and coastal development may play an important role in *T. gondii* exposure for southern sea otters.

Acknowledgements

This research was supported in part through funding from the National Sea Grant Program (NA06RG0142 R/CZ-169), PKD Trust, the Morris Animal Foundation, the San Fran-

cisco Foundation, UC Davis Wildlife Health Center, the Lindbergh Foundation, Friends of the Sea Otter, the Marine Mammal Center and the California Department of Fish and Game. The authors wish to acknowledge the excellent assistance of staff and volunteers from the California Department of Fish and Game, the Marine Mammal Center, the Monterey Bay Aquarium and USGS-BRD for sea otter carcass recovery. Ann Melli, Eva Correa, Sara Miller, Miles Reed, Jack Hawkes, Heather Harris and Debbie Scher assisted with sample collection, sample processing and data compilation. Data for sewage, stream and river outfalls were compiled by the California Central Coast Regional Water Quality Control Board from numerous federal and state sources.

References

Aramini, J.J., Stephen, C., Dubey, J.P., Engelstoft, C., Schwantje, H., Ribble, C.S., 1999. Potential contamination of drinking water with *Toxoplasma gondii* oocysts. Epidemiol. Infect. 122, 305–15.

Bowie, W.R., King, A.S., Walker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., Marion, S.A., 1997. Outbreak of toxoplasmosis associated with municipal drinking water. Lancet 350, 173–7.

Buergelt, C.D., Bonde, R.K., 1983. Toxoplasmic meningoencephalitis in a West Indian Manatee. J. Am. Vet. Med. Assoc. 183, 1294–6.

Buxton, D., 1998. Protozoan infections (*Toxplasma gondii*,*Ne ospora caninum* and *Sarcocystis spp.*) in sheep and goats: recent advances. Vet. Res. 29, 289–310.

Cole, R.A., Lindsay, D.S., Roderick, C.L., Dubey, J.P., Thomas, N.J., Baeten, L.A., 2000. Biological and molecular characterizations of *Toxoplasma gondii* obtained from southern sea otters (*Enhydra lutris nereis*). J. Parasitol. 86, 526–30.

Cruickshank, J.J., Haines, D.M., Palmer, N.C., St. Aubin, D.J., 1990. Cysts of a *Toxoplasma*-like organism in an Atlantic bottlenose dolphin. Can. Vet. J. 31, 213–5.

Daszak, P., Cunningham, A.A., Hyatt, A.D., 2001. Anthropogenic environmental change and the emergence of infectious diseases in wildlife. Acta Trop. 78, 103–16.

Dubey, J.P., 1987. Toxoplasmosis in goats. Agri-Practice: Caprine Parasitol. 8, 43–44, 46–47, 49–52.

Esteban-Redondo, I., Maley, S.W., Thomson, K., Nicoll, S., Wright, S., Buxton, D., Innes, E.A., 1999. Detection of *T. gondii* in tissues of sheep and cattle following oral infection. Vet. Parasitol. 86, 155–71.

Frenkel, J.K., Dubey, J.P., 1972. Toxoplasmosis and its prevention in cats and man. J. Infect. Dis. 126, 664–73.

Graczyk, T.K., Fayer, R., Conn, D.B., Lewis, E.J., 1999a. Evaluation of the recovery of waterborne *Giardia* cysts by freshwater clams and cyst detection in clam tissue. Parasitol. Res. 85, 30–34.

Graczyk, T.K., Fayer, R., Lewis, E.J., Trout, J.M., Farley, C.A., 1999b. *Cryptosporidium* oocyts in bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol. Res. 85, 518–21.

Guerina, N.G., 1994. Congenital infection with *Toxoplasma gondii*. Pediatr. Ann. 23, 138–51.

Holshuh, H.J., Sherrod, A.E., Taylor, C.R., Andrews, B.F., Howard, E.B., 1985. Toxoplasmosis in a feral northern fur seal. J. Am. Vet. Med. Assoc. 187, 1229–30.

Hosmer, D.W., Lemeshow, S., 2000. Applied Logistic Regression, 2nd ed. Wiley, New York, NY 373pp..

Inskeep, W., Gardner, C.H., Harris, R.K., Dubey, J.P., Goldstone, R.T., 1990. Toxoplamosis in Atlantic bottlenose dolphins (*Tursiops truncatus*). J. Wildl. Dis. 26, 377–82.

Jameson, R.L., 1989. Movements, home range and territories of male sea otters off central California. Mar. Mamm. Sci. 5, 159–72.

Kulldorf, M., Nagarwalla, N., 1996. Spatial disease clusters: detection and inference. Stat. Med. 14, 799–810.

Kvitek, R.G., Fukayama, A.K., Anderson, B.S., Grimm, B.K., 1988. Sea otter foraging on deep-burrowing bivalves in a California coastal lagoon. Mar. Biol. 98, 157–67.

Migaki, G., Allen, J.F., Casey, H.W., 1977. Toxoplasmosis in a California sea lion (*Zalophus californianus*). Am. J. Vet. Res. 38, 135–6.

Migaki, G., Sawa, T.R., Dubey, J.P., 1990. Fatal disseminated toxoplasmosis in a spinner dolphin (*Stenella longirostris*). Vet. Pathol. 27, 463–4.

Mikelian, J., Boisclair, J., Dubey, J.P., Kennedy, S., Martineau, D., 2000. Toxoplasmosis in beluga whales (*Dephinapterus leucas*) from the St. Lawrence estuary: two case reports and a serological survey. J. Comp. Pathol. 122, 73–76.

Miller, M.A., Sverlow, K.W., Crosbie, P.R., Barr, B.C., Lowenstine, L.J., Gulland, F.M., Packham, A., Conrad, P.A., 2001. Isolation and characterization of parasitic protozoa from a pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalitis. J. Parasitol. 87, 816–22.

Miller, M.A., Gardner, I.A., Packham, A., Mazet, J.K., Hanni, K.D., Jessup, D., Estes, J., Jameson, R., Dodd, E., Barr, B.C., Lowenstine, L.J., Gulland, F.M., Conrad, P.A., 2002. Evaluation of an indirect fluorescent antibody test (IFAT) for demonstration of antibodies to *Toxoplasma gondii* in the sea otter (*Enhydra lutris*). J. Parasitol. (in press).

Morejohn, G.V., Ames, J.A., Lewis, D.B., 1975. Post mortem studies of sea otters, *Enhydra lutris l.*. California Marine Resources Tech. Report #30. California Department of Fish and Game, Long Beach, CA, 82pp.

Payment, P., Plante, R., Cejka, P., 2001. Removal of indicator bacteria, human enteric viruses, *Giardia* cysts, and *Cryptosporidium* oocysts at a large wastewater primary treatment facility. Can. J. Microbiol. 47, 188–93.

Ralls, K., Eagle, T.C., Siniff, D.B., 1996. Movement and spatial use patterns of California sea otters. Can. J. Zool. 74, 1841–9.

Riedman, M.L., Estes, J.A., 1990. The sea otter (*Enhydra lutris*): behavior, ecology and natural history. United States Fish and Wildlife Service Biological Report, 90, 126pp.

Tamburrini, A., Pozio, E., 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int. J. Parasitol. 29, 711–5.

Tenter, A.M., Heckenroth, A.R., Weiss, L.W., 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30, 1121–1258.

Thomas, N.J., Cole, R.A., 1996. The risk of disease and threats to the wild population. Endangered Species Update Special Issue: conservation and management of the southern sea otter, 13, 23–27.

Van Pelt, R.W., Dietrich, R.A., 1973. Staphylococcal infection and toxoplasmosis in a young harbor seal. J. Wildl. Dis. 9, 258–61.

Chapter 6

Summary and Conclusions

SUMMARY AND CONCLUSIONS

(i) Parasite Isolation

This research confirmed the identity of two apicomplexan protozoal parasites associated with fatal meningoencephalitis of southern sea otters and Pacific harbor seals. Parasite isolation in cell culture from aseptically-collected cerebrum, cerebellum, and cerebrospinal fluid (CSF) from otters and seals has yielded over 55 isolates of two distinct species of protozoans (Miller, Conrad et al., unpublished data), including one isolate of *S. neurona* from seal CSF (Chapter 3). In most cases, parasite isolation in cell culture was combined with microscopic examination of major tissues and brain immunohistochemistry, utilizing antisera raised against well-characterized laboratory strains of *T. gondii* and *S. neurona*.

Between 1998 and 2002, over 180 sea otters and 20 harbor seals were examined, and brain tissue was submitted for parasite isolation from 135 sea otters and 15 harbor seals. Protozoal isolation in cell culture was attempted for nearly all fresh dead sea otters received for necropsy, regardless of clinical history. The frequency with which we identified *T. gondii* and *S. neurona* infections from sea otters was surprising, considering that we were screening marine mammals. As of September, 2002, we have obtained 43 isolates of putative *T. gondii* and 10 isolates of putative *S. neurona* from southern sea otters (Miller and Conrad, unpublished data). For otters, the isolation frequency for putative *T. gondii* from 1998 to 2002 was 31.9% (43 isolates obtained from 135 attempts).

In contrast, *T. gondii* was isolated from only one harbor seal. The isolation frequency of *T. gondii* for harbor seals (6.6%) was 5 times lower than that for sea otters

sampled from the same range and same time period (31.9%). However, our sample size for harbor seals was small (n = 15). An hypothesis for this discrepancy in isolation rates between otters and seals is discussed below. For three otters and one harbor seal, both *T. gondii* and *S. neurona* were isolated simultaneously from brain tissue. Thus 2.2% of sea otters and 6.6% of harbor seals had brain infections consisting of both *S. neurona* and *T. gondii* at the time of necropsy (Chapter 3; Miller, Conrad et al., unpublished data).

The proportion of otters from which *S. neurona* was isolated increased from the initial 1998-2001 study (4%) to 7.4% between 1998 and 2002 (10 isolates obtained from 135 attempts) (Miller and Conrad, unpublished data). This was due to a large number of new *S. neurona* meningoencephalitis cases detected in 2001 after the initial study was completed. This did not continue as a trend in 2002, with only one new case detected.

Between 1998 and 2002, *S. neurona* was isolated from 26.7% of harbor seals (4 isolates obtained from 15 attempts) (Miller and Conrad, unpublished data). This represents a biased sample, since protozoal encephalitis-suspect adult harbor seals were preferentially submitted for necropsy and parasite isolation. However, *S. neurona*-associated encephalitis is the primary cause of mortality for adult harbor seals at the wildlife rehabilitation facility submitting these cases (F. Gulland, pers. commun.).

Detailed study of protozoal brain infections of harbor seals was hampered by low sample size. However, an excellent review of 6 free-ranging harbor seal *S. neurona*-suspect cases was reported by Lapointe and colleagues, 1998. Although harbor seals are common throughout California, few adult seals were admitted to local rehabilitation facilities for veterinary care or postmortem examination during the course of this study. There is a strong bias in carcass recovery effort for sea otters, a federally-listed

threatened species, versus harbor seals, which are common. Even so, the number of live and dead-strandings for adult and subadult harbor seals seemed surprisingly low. Reasons for the discrepancy between the number of adult and subadult harbor seal strandings (low) and seal population size (large) are unclear. However, this discrepancy has also been reported for a related phocid, the elephant seal (*Mirounga angustirostris*) (F. Gulland, pers. commun.). Adult elephant seals feed far off the California shoreline, but are locally numerous during molting and breeding (Bowen and Siniff, 1999). However, few subadult and adult elephant seals were recovered sick or dead outside of communal breeding and molting areas (F. Gulland, pers. commun.). Possibly phocids such as harbor seals and elephant seals avoid land-strandings when sick or injured, and may sink when they die. In contrast, sea otters, as the species with the highest lung volume per unit body weight of any marine mammal (Tarasoff and Kooyman, 1973), will often float when they die at the water's surface. Large predators such as sharks will prey on sick or injured phocids, and may also scavenge carcasses of these blubber-rich animals after death. Although shark attack is a common cause of mortality for sea otters, consumption of any portion of the carcass by sharks is rare (Ames et al., 1996; Kreuder et al., 2003).

(ii) Isolate Characterization

For most of our *T. gondii* isolates, the isolate identity was confirmed by serology, histopathology, immunohistochemistry and/or amplification and restriction fragment length polymorphism (RFLP) analysis of the *T. gondii* B1 or SAG genes (Miller, Grigg, Boothroyd and Conrad, unpublished data). For 6 selected *T. gondii* isolates, 5 from sea otters and 1 from a harbor seal, parasite identity was also confirmed by amplification and

sequencing of phylogenetically informative portions of the 18s rDNA and adjacent first internal transcribed spacer (ITS-1) (Miller et al., 2001b; Miller, Conrad and Crosbie, unpublished data). For *T. gondii* and *S. neurona* 18s and ITS-1 rDNA, amplification was completed via a nested PCR reaction utilizing both forward and reverse primers. The characterization of an isolate of *T. gondii* from a harbor seal was reported in Chapter 3. Interestingly, the seal *T. gondii* isolate was obtained concurrently with an *S. neurona* isolate. No *T. gondii* parasites were observed on histopathology or immunohistochemistry of brain tissue from this animal. Based on necropsy and histopathology, the primary cause of death for this seal was *S. neurona*-associated encephalitis, and the *T. gondii* infection was considered incidental.

The genus *Toxoplasma* consists of a single species, *T. gondii*, consisting primarily of three strains (Type I, II and III), as determined by PCR, DNA sequencing and RFLP (Howe and Sibley, 1995; Howe et al., 1997; Grigg et al., 2001a, 2001b, 2001c). *Toxoplasma gondii* strains are classified according to variation in specific genetic markers, such as the B1, BRS4 and SAG genes (Lehman et al., 2000; Lekutis et al., 2001; Grigg et al., 2001a) and also based on their pathogenicity in mice (Grigg et al., 2001a). Type I strains are typically more pathogenic in mice, with small doses resulting in rapid mortality. In contrast, type II and III strains are less pathogenic in mice and result in chronic infections, often with abundant production of tissue cysts in the central nervous system (Grigg et al., 2001a). Type I and type II *T. gondii* strains are associated with most reports of human clinical toxoplasmosis; however, all three strains have been associated with human disease (Howe and Sibley, 1995; Howe et al., 1997). Cole and colleagues (2000) recently reported that sea otter *T. gondii* isolates that they tested were

type II strains, based on limited RFLP analysis of SAG genes. A manuscript describing the molecular characterization and strain typing of our *T. gondii* isolates is in preparation. Associations between the *T. gondii* strain and the sea otter's stranding location, cause of death and IFAT titer will be assessed. It will be important also to compare the strains of *T. gondii* isolated from sea otters and those obtained from domestic felids along the adjacent coastline, especially in high-risk areas for *T. gondii* exposure for sea otters.

Amplification and sequence analysis of 18s and ITS-1 rDNA was completed for two *S. neurona* isolates, one from a sea otter and one from a harbor seal. The characterization of these *S. neurona* isolates from otters and seals was described in detail in Chapters 2 and 3. We have since obtained 12 additional isolates of putative *S. neurona*. Although characterized via histopathology, serology and in some cases immunohisto-chemistry, these 12 new isolates from otters and seals are tentatively identified as *S. neurona* until molecular characterization is completed.

Using the antigenic, ultrastructural and molecular characterization techniques outlined in the preceding chapters, the apicomplexan parasites isolated from otters and seals were indistinguishable from *T. gondii* and *S. neurona* parasites derived from terrestrial animals and humans. It is important to note, however, that these data represent just one portion of the information denoting an organism's taxonomic status (Kunz, 2002; Tenter et al., 2002). Fine discrimination between marine mammal, human and terrestrial animal isolates would be facilitated by evaluating sequences of other, less highly conserved nuclear and extra-nuclear DNA molecules, including microsatellite DNA (Blackston et al., 2001; Ajzenberg et al., 2002), intergenic spacer regions (Fazaeli et al., 2000) and mitochondrial DNA. These rapidly evolving sites are especially useful for

examining recent evolutionary divergences. The recent detection of a third, unidentified species of protozoan associated with fatal encephalitis in a Pacific harbor seal from California suggests that additional apicomplexan species may cause meningoencephalitis in California marine mammals (Lapointe et al., 2003).

(iii) Serological Test Development and Validation

In the present study, serological tests (IFATs) were developed to facilitate the detection of *T. gondii* and *S. neurona* infection in live and dead otters and seals. The IFAT for *T. gondii* in sea otters was validated using serum from necropsied sea otters whose *T. gondii* infection status was confirmed through immunohistochemistry and parasite isolation. The *T. gondii* IFAT was highly sensitive (96.4%), but only moderately specific (59.2%). However, the specificity was probably artifactually lowered by inclusion of unrecognized false-negative otters in the non-infected category. The extent of serum hemolysis did not correlate with the *T. gondii* IFAT titer. Thus the *T. gondii* IFAT for sea otters appears to be relatively unaffected by postmortem hemolysis.

During four years of study, no *Neospora caninum* infections were detected in sea otters or harbor seals through parasite isolation, PCR, immunohistochemistry, or serological testing. Animals with positive titers to *T. gondii* on IFAT had low titers to *N. caninum* (usually <1:80), suggesting that cross reactivity with these closely related apicomplexans was not a significant obstacle for interpretation of the *T. gondii* IFAT. However, the lack of positive control *N. caninum* cases precludes validation of an *N. caninum* IFAT for otters and seals.

(iv) Using IFATs to Test Otter and Seal Serum

Serum samples from over 400 live and necropsied sea otters from differing geographic locations were evaluated during the course of this study. In addition, serum samples were tested from over 80 California harbor seals. Numerous otters and a few seals had detectable antibody titers for *T. gondii* and *S. neurona*, ranging as high as 1:80,000 serum dilution. In many cases, high titers correlated with evidence of brain infection, as determined by parasite isolation and histopathology. However, validation of *S. neurona* IFATs for otters and seals cannot be completed at present due to sample size limitations. Validation of the harbor seal IFAT for *T. gondii* and the harbor seal and sea otter IFATs for *S. neurona* will be completed when sera from sufficient numbers of positive controls are accumulated to permit statistical evaluation. One potential pitfall to keep in mind as test validation proceeds is the detection of a second, putative *Sarcocystis* spp. in sea otter skeletal muscle, as described below (Miller and Conrad, unpublished necropsy data). This finding raises the potential for false-positive *S. neurona* IFAT titers due to antigenic cross-reactivity. To address this question, specific muscles have been routinely collected at necropsy, and the presence or absence of these large intramuscular protozoal cysts is noted on the necropsy reports so that evaluation of these findings in relation to the *S. neurona* titer, parasite isolation and brain histopathology will be facilitated during *S. neurona* IFAT validation for sea otters.

Once validated, the *T. gondii* IFAT was used to screen serum obtained from live-captured and presumably healthy otters in California, Washington and Alaska (Miller et al., 2002a). The proportion of otters positive for *T. gondii* was comparable for otters sampled in California and Washington (36% and 38%, respectively), but both were

higher than for otters sampled in Alaska (0% of 60 serum samples were positive for *T. gondii*). In this preliminary study, all of the sea otters from Alaska were sampled in remote areas. Other studies suggest that *T. gondii* is present in terrestrial Alaskan wildlife (Zarnke et al., 2000). Therefore, additional serum samples should be screened from Alaskan otters living in closer proximity to humans, their pets, and terrestrial wildlife, such as in the vicinity of the Kenai peninsula and Prince William sound.

(v) Risk Factors for Toxoplasma Exposure

An important goal of this research was to explore *T. gondii* infection patterns in free-living otter populations, and to investigate potential demographic, environmental and spatial risk factors for protozoal infection. Thus, the epidemiological relationships reported in Chapters 4 and 5 are those between *T. gondii* seropositive otters and potential risk factors, such as location, gender, age class, sample date and level of exposure to specific environmental factors.

Serological data from live and dead otters were examined for associations between *T. gondii* seropositivity and potential demographic, environmental and spatial risk factors for *T. gondii* infection (Miller et al., 2002b; Chapter 5). *Toxoplasma* infection was more frequent in older otters, males, and otters sampled at necropsy. Male otters range more widely than females to establish and defend territories (Pederson and Stout, 1963), and older otters would have more opportunity to be exposed to *T. gondii* over their longer lifespan. Thus subadult to adult female otters may be better targets than adult males for future studies aimed at pinpointing specific environmental origins of *T. gondii* exposure. A high proportion of dead-sampled, seropositive southern sea otters was

detected between 1998 and 2001 (61%), compared to live-sampled otters (36%) (Chapter 4). This difference was also detected in the risk factor study (Chapter 5), and may indicate an increased risk of mortality for otters infected by *T. gondii*. However, this difference in seropositivity between live-sampled and dead otters was not statistically significant when the data was adjusted for variation attributable to gender, age class and other risk factors. These findings are discussed in Chapter 5.

Environmental data evaluated in the study included the proximity of each otter's sample location to municipal sewage outfalls, major sources of freshwater runoff and centers of dense human settlement. Otters sampled near areas of maximal freshwater flow were almost 3 times more likely to be seropositive to *T. gondii* than otters sampled in areas of low flow, suggesting that surface runoff is a significant risk factor for *T. gondii* infection of California's sea otters. No statistical association was detected between seropositivity to *T. gondii* and human population density or exposure to sewage. The relationship, if any, between exposure to municipal sewage and *T. gondii* exposure requires clarification, because most of the otters enrolled in this study were sampled at sites distant from major municipal sewage outfalls. Similarly, the study design and data resulting from this study did not permit an accurate evaluation of the relationship between human population density and sea otter *T. gondii* exposure. To address these questions, future studies should be designed to sample sea otters that are inhabiting sewage-impacted and non-sewage-impacted coastal areas, and from regions of high and low human density.

Spatial analysis revealed clustering of *T. gondii*-seropositive otters at two locations along the coast. A single cluster of seronegative otters was detected at the south

end of Monterey peninsula. The two high risk locations were centered near Moss Landing, in the northern half of sea otter range, and Morro Bay, located in the southern half of California sea otter range. On spatial analysis, only the Morro Bay site was significant at $p \leq 0.1$. However, our sample size ($n = 223$) was small for performing spatial analyses for samples spread over 600 km of California coastline.

The data were examined for interactions between risk factors as a possible explanation of the observed spatial clustering. Otters living near the Moss Landing high risk site were exposed to high freshwater runoff, which accounted for some of the apparent clustering of seropositive otters at this site. Similarly, otters living near the low risk site (south Monterey peninsula) were exposed to low freshwater flow, which could explain why fewer seropositive animals were detected in this area. In addition, both sites were also biased with respect to gender and age, due to selective sampling of otters during live-capture operations. Otters sampled near Moss Landing were more likely to be adult and male. Adult male otters were the age and gender group most likely to be infected with *T. gondii* (Chapter 5). South Monterey peninsula is a prime pupping area, and a high proportion of young otters and adult females were sampled at this site, resulting in some age and gender bias toward groups that were more likely to be seronegative to *T. gondii*. Thus, while relative freshwater flow was felt to be a major contributor to the clustering of *T. gondii*-exposed otters at these sites, some effect due to age and gender bias was also noted. This does not mean that the clustering effect was not real: it simply means that the spatial clustering that we detected at each site could be explained by the relative contributions of other important risk factors.

In contrast, contributions of other risk factors did not fully explain the large cluster of *T. gondii*-seropositive otters detected near Morro Bay. Even after adjusting for variation due to freshwater flow, age and gender bias, otters sampled near Morro Bay were 9 times more likely to be seropositive to *T. gondii* when compared to other locations. Thus, unrecognized risk factor(s) appear to be significantly increasing the risk of *T. gondii* infection for sea otters sampled in this region. This finding highlights the need for site-specific investigation of *T. gondii* exposure for sea otters living near Morro Bay.

One interesting common denominator between the two high-risk sites detected in this study is the presence of a large, electricity-generating power plant in the immediate vicinity of each site. At both sites the power plant is permitted to utilize seawater for cooling purposes, and the heated seawater is then discharged within 150 meters of the shoreline. Warm water is a possible factor enhancing *T. gondii* oocyst survival (Tenter et al., 2000; Slifko et al., 2000). It is possible that these nearshore warmwater plumes also enhance the survival of favored sea otter prey species that effectively concentrate *T. gondii*, or serve as intermediate or paratenic hosts. Interestingly, both high-risk sites are centered on large, partially enclosed sloughs (Moss Landing) or bays (Morro Bay), while the low-risk site (Monterey peninsula) faces the open ocean. Enclosed embayments or sloughs may facilitate local concentration of *T. gondii* parasites that reach the nearshore marine environment. Also, within these enclosed sloughs and embayments are freshwater inflows that may lower salinity and perhaps enhance oocyst survival. Other factors that may contribute to the enhanced risk of *T. gondii* exposure observed at these sites include coastal topography, wind and marine currents, which collectively could concentrate

particulate pollutants, including oocysts, along the shoreline, and within these enclosed areas. In addition, both sites are recognized areas of concentration for anthropogenic chemical pollutants, such as butyltins and DDT, which could have immunological effects at high levels of exposure (Kanaan et al., 1998; Nakata et al., 1998). It would be helpful to examine the relationship between water temperature, salinity, hydrological conditions, marine fauna and *T. gondii* oocyst survival, and to examine the relationship between specific tissue pollutant burdens and mortality due to *T. gondii*. Comparison with low-risk sites may provide additional clues as to the sources and mechanisms of sea otter exposure to *T. gondii*.

(vi) Associations Between Protozoal Infection and Disease

For this research, relationships between protozoal infection and disease were not emphasized, because we felt that the most important initial questions to address concerned tracing the environmental sources of these pathogens to the nearshore marine environment. However, brain histopathology was examined and scored for all necropsied otters and seals, and these data have been utilized for studies on the relationship between *T. gondii* infection and disease (Kreuder et al., 2003). Based upon histopathological examination of brain tissue, three interesting and potentially important differences were noted between *T. gondii* and *S. neurona* brain infections of otters and seals (Miller, unpublished necropsy data; Kreuder et al., 2003).

1. The inflammatory infiltrate and lesion distribution for *T. gondii*-infected otters was different than that for *S. neurona*-infected otters and seals:

In otters and seals, the inflammatory response to *T. gondii* was invariably nonsuppurative, dominated by small lymphocytes, with fewer admixed macrophages and plasma cells. The presence of numerous small lymphocytes in the meninges and within perivascular cuffs was suggestive of, but not definitive for brain infection by *T. gondii* in otters. The distribution of the inflammatory response within the neuropil (brain tissue) was best characterized as a "shotgun pattern", with one to many nodular foci of nonsuppurative inflammation, tissue necrosis and parasite proliferation occurring in a random, often asymmetric pattern. Perivascular cuffs were often located adjacent to nodules of inflammation. Although parasites and inflammation were identified within the spinal cord, significant lesions were most common within the brain and brainstem. Rare foci of microcavitation and/or mineralization of the neuropil were observed in *T. gondii*-infected otters, similar to those reported for congenital *T. gondii* brain infections in humans (Dubey and Beattie, 1988). Some otters with *T. gondii* brain infections also had nonsuppurative myositis or myocarditis (Miller et al., unpublished necropsy data; Kreuder et al., 2003). However, parasites were rarely observed in skeletal muscle or myocardium.

In contrast, the inflammatory infiltrate associated with *S. neurona* brain infection for both otters and seals was more neutrophilic; lymphocytes, plasma cells and macrophages dominated, but low to moderate numbers of neutrophils were also scattered throughout. The pattern of inflammation in *S. neurona*-associated encephalitis was more severe, diffuse and necrotizing than was typical for *T. gondii* infections. The lesions were often most severe in the cerebellum and brainstem, although parasites were present throughout the central nervous system on immunohistochemistry. This pattern of lesion

distribution has been reported previously for otters and seals with *S. neurona*-associated encephalitis (Lapointe et al., 1998; Rosonke et al., 1999). On histopathology, the detection of parasites and inflammation outside of the central nervous system was more common in *S. neurona*-infected otters and seals than those infected with *T. gondii*.

2. The histological response to *T. gondii* brain infection was variable, compared with *S. neurona* brain infection:

All otters or seals in this study with confirmed *S. neurona* infections exhibited moderate to severe meningoencephalitis on brain histopathology, leaving little doubt as to the primary cause of death. In contrast, for *T. gondii*-infected otters and a seal, the relationship between protozoal brain infection (as determined by histopathology, immunohistochemistry and/or parasite isolation) and disease (as determined by reported clinical signs and histopathology) was less clear-cut. Some otters had *T. gondii* cysts and minimal inflammation, whereas in other cases cyst stages were associated with moderate to severe inflammation and necrosis. Our aggregate clinical and histopathological data for *T. gondii* in southern sea otters suggests a range in severity of response to infection by *T. gondii* from asymptomatic, incidental infection to fatal, *T. gondii*-associated meningoencephalitis. Thus definitive diagnosis of *T. gondii*-associated meningoencephalitis as a cause of death was often more difficult than that for *S. neurona*. This finding is not unexpected: for many other intermediate hosts for *T. gondii*, including humans, pigs and sheep, asymptomatic to mild infections are common, and life threatening infections are rare in immunocompetent hosts (Sibley et al., 1999; Yap et al, 1999; Sibley et al., 2002; Su et al., 2002). What is unusual, however, is the high

proportion of sea otters with significant encephalitis in association with either *T. gondii* or *S. neurona* infection, as discussed below.

As a result of these observations and concerns, rigorous diagnostic criteria were developed to define a "case" of *T. gondii*-associated meningoencephalitis: *Toxoplasma gondii*-associated meningoencephalitis was defined as a primary or contributing cause of death only when multifocal nonsuppurative inflammation was present within the neuropil in combination with *T. gondii* tissue cysts and, rarely, zoites. Otters with nonsuppurative inflammation that was confined to the leptomeninges or Virchow-Robbins spaces (eg perivascular cuffs) were not classified as *T. gondii*-associated meningoencephalitis, even if *T. gondii* bradyzoite cysts were identified within the neuropil. This was done to avoid accidental misclassification of other potential causes of nonsuppurative inflammation (eg unrecognized viral or fungal infections) as *T. gondii*-associated meningoencephalitis. This rigorous classification system may have resulted in under-reporting of the true impact of *T. gondii* on southern sea otter mortality. Even so, the proportion of otters diagnosed with histologically significant apicomplexan protozoal brain infections was high: a collaborative survey of recent (1998-2001) causes of sea otter mortality was completed using the sea otter necropsy data generated during this dissertation research as part of ongoing necropsy examinations of southern sea otters by the California Department of Fish and Game (Kreuder et al., 2003). Despite imposing the rigorous diagnostic criteria described above, 16.2% of otters were found to have died directly due to *T. gondii* infection, another 11.4% of otters had *T. gondii*-related meningoencephalitis as a contributing cause of death and 6.7% of otters died due to *S. neurona*-related meningoencephalitis. Thus the total proportion of otters diagnosed with moderate to

severe apicomplexan protozoal infections at necropsy was over 34% (Kreuder et al., 2003). Interestingly, a significant number of otters (64.3%) that had died acutely due to traumatic events, such as white shark predation or boat strike exhibited moderate to severe encephalitis at the time of necropsy. In all cases, the brain lesions appeared to be subacute to chronic. This suggests that pre-existing protozoal brain infection may enhance an affected otter's risk of traumatic death, possibly due to detrimental effects on behavior, locomotion or visual acuity (Kreuder et al., 2003). Significant neurological deficits, including obtundation, tremors, ambulatory deficits, paresis and apparent blindness have been observed antemortem in sea otters with necropsy-confirmed protozoal encephalitis (Rosonke et al., 1999; Lindsay et al., 2000; Murray, Haulena, Gulland et al., unpublished data).

3. The parasite numbers and stages present within the neuropil differed between *T. gondii* and *S. neurona*-infected otters and seals:

The predominant parasite stages visible on histopathology and immunohistochemistry within the neuropil of most *T. gondii*-infected sea otters were thin-walled, often angular-edged tissue cysts, suggesting that in many cases mortality was associated with chronic, perhaps recrudescent *T. gondii* infection. Electron microscopic examination of these structures in affected brain tissue should be performed to confirm their identity as true bradyzoite cysts. According to the scientific literature, the presence of bradyzoite tissue cysts is indicative of chronic *T. gondii* infection, and these quiescent cysts do not typically elicit a significant inflammatory response (Dubey, 1987; Dubey and Beattie, 1988). For necropsied sea otters, tissue cysts were occasionally observed in areas of the

neuropil with no adjacent inflammatory infiltrate. However, these cysts were more often located within, or at the periphery of foci of nonsuppurative inflammation and tissue necrosis. On rare occasions, brain immunohistochemistry demonstrated the presence of free or intracytoplasmic zoites, but typically only cysts were stained with *T. gondii* antiserum. These lesions may represent foci of parasite recrudescence, or ongoing, low-grade parasite turnover. Smoldering infections may be tolerated by the infected host, either until a critical portion of the neuropil is affected, or when the aggregate volume of tissue damage results in life-threatening clinical disease. This is especially concerning for sea otters, a species with high metabolic and nutritional requirements where even minor impacts on foraging efficiency could have rapid and devastating effects. *Toxoplasma gondii* tissue cysts or other parasite stages were only rarely identified outside of the central nervous system. However, immunohistochemistry of tissues other than brain was not performed routinely in this study, and it is possible that some stages were not detected on histopathologic examination.

Parasite stages observed in association with *S. neurona* brain infections were numerous and diverse, but cyst stages were never observed within the neuropil. Parasite forms commonly observed in the brain included extracytoplasmic merozoites and intracytoplasmic schizonts of widely varying morphology, including classic rosette-form schizonts. On immunohistochemistry, *S. neurona* parasites were often numerous within the brain and spinal cord. On histopathology, identification of *S. neurona* parasites in tissues outside of the central nervous system was reasonably common, especially within the lungs, lymph nodes and spleen (Miller, unpublished necropsy data). Some otters with *S. neurona*-associated encephalitis also had small, round to elliptical, multi-chambered,

intracellular cysts averaging 22 x 10 μm (n = 10) within skeletal muscles and/or myocardium (Miller, unpublished necropsy data). Both the cyst wall and internal zoite stages of these small cysts stained faintly on *S. neurona* immunohistochemistry, and thus could be *S. neurona* cysts, as reported previously (Rosonke et al., 1999; Dubey et al., 2001b). Intramuscular cyst stages similar to those described above were also observed in skeletal and cardiac muscle of harbor seals with *S. neurona*-associated meningoencephalitis (Lapointe et al., 1998; Miller, unpublished necropsy data). Thus, both sea otters and harbor seals may serve as intermediate hosts for *S. neurona*. Similar muscle cysts have been described from *S. neurona*-infected domestic cats (Dubey et al., 2001a), raccoons (Dubey et al., 2001c), skunks (Cheadle et al., 2001b) and armadillos (Cheadle et al., 2001a; Tanhauser et al., 2001). Cyst stages have not been reported to occur within the neuropil of otters, seals or other animals infected with *S. neurona*.

Large, elliptical, thick-walled cysts averaging 138 x 81 μm (n = 10), often with prominent external cyst wall projections and internal septations were observed within both northern (*E. l. kenyoni*) and southern sea otter skeletal muscle, and, rarely, myocardium (Miller, unpublished necropsy data). Moderate cross reactivity to *S. neurona* antiserum was demonstrated on immunohistochemistry: antibodies raised to *S. neurona* reacted with the outer cyst wall, but not the zoites within these large intramuscular cysts. Similar large intramuscular cyst stages have been described from the skeletal muscle of river otters (*Lutra lutra*) in Sweden (Wahlstrom et al., 1999). The taxonomic identity and biology of these parasites is unknown, but they probably represent additional *Sarcocystis* spp. infecting sea otters. Molecular and ultrastructural characterization of both types of

cysts from skeletal muscle and myocardium would be helpful in confirming the sea otter's role as an intermediate host for *S. neurona* and related *Sarcocystis* spp.

(vii) Summary and Future Directions

Collectively our findings on histopathology and immunohistochemistry suggest that *S. neurona* infections are often acutely to subacutely fatal to otters and seals, while fatal *T. gondii* meningoencephalitis may be attributed to smoldering or recrudescent infections. A study on the relationship between clinical signs, IFAT serology, histopathology, and results of parasite isolation in otters and seals is planned to examine these relationships in greater detail. Interestingly, sea otter mortality due to *S. neurona*-related meningoencephalitis was found to be strongly seasonal (peaking in late spring and early summer at the conclusion of the rainy season), while *T. gondii*-related otter mortality and seropositivity were not seasonal (Kreuder et al., 2003).

Many hypotheses have been suggested for the high proportion of southern sea otter mortality attributed to *T. gondii*, including genetic restriction or "bottleneck effect", immunosuppression due to exposure to anthropogenic chemical pollutants, novel host-parasite interactions and heavy parasite exposure (Leatherwood et al., 1978; Anderson et al., 1996; Thomas and Cole, 1996; Kanaan et al., 1998; Nakata et al., 1998; Bodkin et al., 1999). However, no studies to systematically examine these interactions have been reported.

The host-parasite relationship in *T. gondii* infection is exceedingly complex and is only now beginning to be understood. In terrestrial animals and humans, both the infected host and the infecting parasite appear to play a role in maintaining the "status quo",

which, if successful for *T. gondii*-infected animals leads to chronic, asymptomatic infection. Host cell mediated immunity, specifically the interplay between T cell helper subset 1 (TH1) and TH2-mediated cellular proliferation and cytokine production, plays an important role in achieving and maintaining this delicate host-parasite balance (Sibley et al., 1999; Suzuki, 1999; Yap and Scher, 1999; Barragan and Sibley, 2002; Brake, 2002; Innes et al., 2002; Johnson et al., 2002; Sibley et al., 2002; Su et al., 2002). Several types of host cells and cytokines play a critical role, both in containing the parasites and moderating the host immune response. Key cells involved in this response in humans and terrestrial animals include T lymphocytes, macrophages and natural killer (NK) cells (Sibley et al., 1999; Yap and Scher, 1999; Barragan and Sibley, 2002; Brake, 2002; Innes et al., 2002; Johnson et al., 2002; Sibley et al., 2002; Su et al., 2002). Important cytokines include gamma interferon, interleukin (IL) 12 and IL-10 (Suzuki, 1999; Yap and Scher, 1999; Sarciron et al., 2000). A change in parasite competence, or host immunocompetence or immunoreactivity could lead to fatal infection by one of two routes. A tip of the scales in one direction could lead to uncontrolled parasite proliferation, resulting in significant cell damage and death due to tissue necrosis. If the scales are tipped in the opposite direction, the result could be unregulated inflammation, leading to the same ultimate outcome: significant tissue destruction and death. Studies to compare the demographic, nutritional and genetic characteristics of sea otters that have died due to *T. gondii* infection with those that have died due to other causes would be invaluable in helping us to understand why *T. gondii* is such a common cause of morbidity and mortality for southern sea otters. It would also be helpful to characterize the host cells and cytokines involved in fatal and nonfatal protozoal brain infections. Studies

characterizing the variability of the host major histocompatibility (MHC) I and MHC II gene complexes are in progress. These studies could provide important insights as to whether threatened southern sea otters suffer from "genetic bottleneck" effect due to the small size of the founder population (approximately 50 animals) in the early 1900s (Leatherwood et al., 1978). Genetic studies using other loci have supported the concept that the southern sea otter gene pool may be restricted, especially when compared to northern sea otter populations (Anderson et al., 1996; Bodkin et al., 1999; Wilson et al., 1991).

Preliminary research also suggests an association between high tissue burdens of anthropogenic chemical pollutants, specifically butyltins and DDT, and sea otter mortality due to infectious disease (Kanaan et al., 1998; Nakata et al., 1998). However, research utilizing gender, age and site-matched controls to examine the specific relationship between mortality due to *T. gondii* or *S. neurona* infection and specific tissue pollutant burdens has not been reported.

The concept of novel host-parasite interactions as a cause of increased southern sea otter mortality at first seems far-fetched, since wild felids, the definitive hosts for *T. gondii*, were historically present within the southern sea otter range. *Toxoplasma gondii* infection has been confirmed in mountain lions (*Felis concolor*), lynx (*Lynx canadensis*) and bobcats (*Lynx rufus*) (Reimann et al., 1974, 1975; Oertley et al., 1980; Patton et al., 1986; Dubey et al., 1987; Dreeson, 1990; Smith and Frenkel, 1995; Aramini et al., 1998; Labelle et al., 2001; Zarnke et al., 2001). However, the number of felids living near the coast has undoubtedly increased by several orders of magnitude in association with human settlement, especially in the form of pet and feral cats. In addition, humans have

greatly modified the coastal landscape, drastically reducing natural wetlands and expediting the flow of surfacewater to the nearshore marine environment. Thus without the filtering and buffering effects of natural wetlands, chemical or biological pollutants present in terrestrial surface waters may remain more concentrated, and be more efficiently transported into the nearshore marine environment. Another important consideration is that the definitive host for *S. neurona*, the Virginia opossum (*Didelphis virginiana*) (Fenger et al., 1995), is a new face in the coastal California landscape. These new world marsupials were introduced to coastal California at the beginning of the 20th century (Allen, 1901).

To review, the oocyst or sporocyst stages of *T. gondii* and *S. neurona* appear to be spread exclusively by terrestrial animals, notably felids for *T. gondii* and opossums for *S. neurona*. Recognized intermediate hosts of both parasites such as rodents and birds are rarely, if ever consumed by sea otters (Riedman and Estes, 1988), and their consumption could not explain the high numbers of seropositive and infection-positive otters detected in this study. Taken collectively, the high seroprevalence of live, wild southern sea otters to *T. gondii* (42%), frequent isolation of *T. gondii* from otter brain tissue (≥32%) and reports of dual *T. gondii* and *S. neurona* infections in both sea otters and harbor seals (Lindsay et al., 2001b; Miller et al., 2001b; Miller and Conrad, unpublished necropsy data) suggest a significant exposure to these protozoa in the central California coastal marine environment. Compelling evidence to support the hypothesis of terrestrial-origin infection includes the very narrow definitive host ranges for both *T. gondii* (felids) and *S. neurona* (opossums). Cole and colleagues (2001) demonstrated that *T. gondii* isolated from sea otter brain tissue was infective for cats, and resulted in fecal shedding of

oocysts. Based on antigenic, ultrastructural and molecular characterization, we found our marine mammal isolates of *T. gondii* and *S. neurona* were indistinguishable from *T. gondii* and *S. neurona* isolated from terrestrial animals and humans. There was a strong seasonal correlation to *S. neurona* infections otters and seals, with most cases reported during or after seasons of maximal precipitation and runoff (Kreuder et al., 2003). In addition, our epidemiological research provides statistical evidence implicating terrestrial runoff as a likely source for *T. gondii* infections of sea otters (Chapter 5: Miller et al., 2002b).

In-depth investigations of the relationships between terrestrial and marine isolates of *T. gondii* and *S. neurona* should be continued by sampling cats, opossums and sea otter prey species within high-risk areas, and comparing the parasite genotypes and strains derived from these exogenous sources with those isolated locally from sea otters. These studies could help to confirm that *T. gondii* and *S. neurona* are reaching the nearshore marine environment as a result of water pollution by biological pathogens derived from the terrestrial environment. Further work is needed to accomplish this task, especially regarding the capability of marine organisms to serve as intermediate or paratenic hosts for *T. gondii* and *S. neurona*. This hypothesis could be addressed experimentally by examining other marine mammals, fish and invertebrates for *T. gondii* and *S. neurona* using, histopathology, immunohistochemistry and molecular techniques.

Although the sample sizes were small, the frequency of isolation of *T. gondii* from necropsied sea otters (32%) was approximately 5 times higher than for harbor seals (6.6%) sampled in the same geographic area. In addition, recent evaluation of 70 harbor seal serum samples collected from the vicinity of Moss Landing, a high-risk site for *T.*

gondii infection of sea otters (Chapter 5), revealed few seals with titers to *T. gondii* or *S. neurona* (Conrad, Miller, Oates, Harvey, Gulland et al., unpublished data). One explanation for this apparent discrepancy may be differences in prey species selection and feeding habits between these two species. Sea otters reside and feed entirely within the nearshore marine environment, often within 2 km of the shoreline. Although harbor seals rest and haul out on land, often in close proximity to sea otters, much of their feeding activity occurs offshore in deep water. Primary prey species consumed by southern sea otters include nearshore benthic and kelp canopy invertebrates, many of which are filter feeders, and otters consume a mass of prey equivalent to 25% of their body weight each day (Reidman and Estes, 1990). In contrast, harbor seals feed primarily on midlevel and benthic fish, shrimp and squid (Burns, 2002). Because they have a well-developed blubber layer to aid in thermoregulation, harbor seals do not require such a large amount of prey relative to body weight as sea otters (Burns, 2002). Thus differences in prey species selection and volume of consumption may account for the apparent difference in *T. gondii* exposure between sea otters and sympatric harbor seals.

Significant potential exists for marine invertebrates to remove and concentrate *T. gondii* and *S. neurona* oocysts or sporocysts, and thus act as a source of protozoal infection for sea otters and humans. Atlantic coast benthic invertebrates such as mussels, clams and oysters have been shown to concentrate related protozoa, such as *Giardia* spp., *Cryptosporidium* spp. and *Cyclospora* spp. (Fayer et al., 1998, 1999; Graczyk et al., 1998a, 1998b, 1998c, 1999a, 1999b; Tamburrini and Pozio, 1999). These findings have been confirmed in both experimentally-exposed and free-living marine, estuarine and freshwater bivalves (Fayer et al., 1998, 1999; Graczyk et al., 1998a, 1998b, 1998c,

1999a, 1999b; Tamburrini and Pozio, 1999). Recently, uptake of *T. gondii* oocysts from contaminated water by eastern oysters was confirmed in a laboratory setting, and oocyst viability was confirmed by mouse bioassay (Lindsay et al., 2001a). We have obtained funding to examine *T. gondii* uptake by experimentally-exposed and free-living Pacific coast marine bivalves. Site selection for monitoring free-living bivalves will be determined in part through identification of high-risk areas for *T. gondii* infection in sea otters, presented in chapter 5 of this dissertation.

(viii) Significant Contributions of This Dissertation Research

This research has contributed to the body of knowledge concerning protozoal brain infections of California marine mammals, and the larger environmental implications of these infections. It has provided preliminary data to suggest that this problem, initially detected in sea otters and seals, may have important and far-reaching implications for marine ecosystem health, and perhaps for human health. When this research was initiated in 1998, the scientific data concerning protozoal brain infections in otters and seals were sparse. Two parasites had been implicated in these infections in California sea otters and harbor seals, and scientists suspected the parasites might be *Toxoplasma gondii* and *Sarcocystis neurona*. However, this tentative identification had not been confirmed, and *in vitro* isolation of the parasites in cell culture was not reported. We had no data about the prevalence or incidence of infection in natural populations, or the relationship between protozoal infection and disease. During the course of this study, some significant milestones have been achieved. Specifically, we have:

1. Isolated *T. gondii* and *S. neurona* parasites from otter and seal brain tissue, and confirmed that these parasites are responsible for sea otter and harbor seal brain infections in California.

2. Using antigenic, ultrastructural and molecular techniques, compared *T. gondii* and *S. neurona* isolates obtained from otters and seals with those from humans and terrestrial animals, and demonstrated that there were no significant differences.

3. Developed serological tests (IFATs) to identify otters and seals infected with *T. gondii* and *S. neurona*, and validated the *T. gondii* IFAT for sea otters.

4. Compared sera collected from 3 geographically distinct sea otter populations in California, Washington and Alaska for seroprevalence to *T. gondii*, using the validated IFAT.

5. Evaluated potential demographic, geographic and environmental risk factors for southern sea otter exposure to *T. gondii*.

6. Provided diagnostic tests (IFATs) which were utilized by the wildlife veterinary community to more rapidly diagnose and treat otters and seals that were clinically affected by *T. gondii* and *S. neurona*.

7. Raised the awareness of the public and scientific community concerning potentially harmful impacts of biological pollutants.

8. Helped to stimulate the formation of an interdisciplinary collaborative research network which includes members from academia, state and federal government and private facilities entrusted with the care and conservation of marine wildlife and coastal water quality.

9. Revealed new avenues for scientific investigation.

Each new discovery has lead to new questions that beg to be addressed. Future lines of investigation include exploration of the potential of marine bivalves to concentrate and remove *T. gondii*, related protozoans and bacteria through filter-feeding activity. Areas where additional research would be illuminating include studies involving strain typing of *T. gondii* and *S. neurona* isolates obtained from otters and seals and the association of strain type with the cause of death, stranding location and antibody titer. Comparison of marine isolates with those isolated from nearshore definitive hosts, such as cats and opossums, would help to clarify potential pathways for exposure. Characterization of the genetic variation and immune response in fatal and nonfatal protozoal brain infections might provide important clues regarding host susceptibility. Studies of nearshore ecological, hydrological and hydrodynamic conditions could help to clarify why otters living in certain locations are at higher risk for *T. gondii* exposure. Our collaborative pursuit of these important questions will provide information to enhance sea

otter conservation and may underscore the impact that human beings have on the marine environment.

(ix) Literature Cited

Ajzenberg, D., A. L. Banuls, M. Tibayrenc and M. L. Darde. 2002. Microsatellite analysis of *Toxoplasma gondii* shows considerable polymorphism structured into two main clonal groups. Int J. Parasitol. 32: 27-38.

Allen, J. A. 1901. A preliminary study on the North American opossums of the genus *Didelphis*. Bull. Am. Mus. of Nat. Hist. 14: 149-188.

Ames, J. A., J. J. Geibel, F. E. Wendell and C. A. Patton. 1996. White shark-inflicted wounds of sea otters in California, 1968-1992. *IN*. Great white sharks: The biology of *Charcharodon carcharias*. P. E. Klimley and D. G. Ainsly, Eds. Pp. 309-316.

Anderson, C. G., J. L. Gittleman, K. P. Koepfli and R. K. Wayne. 1996. Sea otter systematics and conservation: which are critical subspecies? Endangered Species Update: Conservation and management of the Southern Sea otter. 13: 6-10.

Aramini, J. J., C. Stephen and J. P. Dubey. 1998. *Toxoplasma gondii* in Vancouver Island cougars (*Felis concolor vancouverensis*): serology and oocyst shedding. J. Parasitol. 84: 438-440.

Barragan, A. and L. D. Sibley. 2002. Transepithelial migration of *Toxoplasma gondii* is linked to parasite motility and virulence. J. Exp. Med. 195: 1625-1633.

Blackston, C. R., J. P. Dubey, E. Dotson, C. Su, P. Thuillez, D. Sibley and T. Lehmann. 2001. High-resolution typing of *Toxoplasma gondii* using microsatellite loci. J. Parasitol. 87: 1472-1475.

Bodkin, J. L., B. E. Ballachey, M. A. Cronin and K. T. Scribner. 1999. Population demographics and genetic diversity in remnant and translocated populations of sea otters. Conservat. Biol. 13: 1378-1385.

Bowen, W. D. and D. B. Siniff. 1999. Distribution, population ecology and feeding ecology of marine mammals. *IN*. Biology of marine mammals. J. E. Reynolds and S. A. Rommmel, Eds. Smithsonian Institution Press, Washington D.C. Pp. 430-433.

Brake, D. A. 2002. Vaccinology for control of apicomplexan parasites: a simplified language of immune programming and its use in vaccine design. Int. J. Parasitol. 32: 509-515.

Burns, J. J. 2002. Harbor seal and spotted seal. *IN*. Encyclopedia of marine mammals. W.

A. Perrin, B. Wursig and J. G. M. Thewissen, Eds. Academic Press. San Diego, CA. Pp 552-560.

Cole, R. A., D. S. Lindsay, C. L. Roderick, J. P. Dubey, N. J. Thomas, and L. A. Baeten. 2000. Biological and molecular characterizations of *Toxoplasma gondii* obtained from southern sea otters (*Enhydra lutris nereis*). J. Parasitol. 86: 526-530.

Cheadle, M. A., S. M. Tanhauser, J. B. Dame, D. C. Sellon, M. Hines, P. E. Ginn, R. J. MacKay and E. C. Greiner. 2001a. The nine-banded armadillo (*Dasypus novemcinctus*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31: 330-335.

_____, C. A. Yowell, D. C. Sellon, M. Hines, P. E. Ginn, A. E. Marsh, J. B. Dame and E. C. Greiner. 2001b. The striped skunk (*Mephitis mephitis*) is an intermediate host for *Sarcocystis neurona*. Int. J. Parasitol. 31: 843-849.

Dreeson, D. W. 1990. *Toxoplasma gondii* infections in wildlife. J. Am. Vet. Med. Assoc. 196: 274-276.

Dubey, J. P. 1987. Toxoplasmosis. *IN*. Vet. Clin. North Am.: Small An. Prac. 17: 1389-1405.

_____, W. J. Quinn and D. Weinandy. 1987. Fatal neonatal toxoplasmosis in a bobcat. J. Wildl. Dis. 23: 324-327.

_____ and C. P. Beattie. 1988. Toxoplasmosis of animals and man. CRC Press. Boca Raton, Florida. 220 pp.

_____, D. S. Lindsay, D. Fritz and C. A. Speer. 2001a. Structure of *Sarcocystis neurona* sarcocysts. J. Parasitol. 87: 1323-1327.

_____, A. C. Rosypal, B. M. Rosenthal, N. J. Thomas, D. S. Lindsay, J. F. Stanek, S. M. Reed and W. J. Saville. 2001b. *Sarcocystis neurona* infections in sea otter (*Enhydra lutris*): Evidence for natural infection with sarcocysts and transmission of infection to opossums. J. Parasitol. 87: 1387-1393.

_____, W. J. Saville, J. F. Stanek, D. S. Lindsay, B. M. Rosenthal, M. J. Oglesbee, A. C. Rosypal, C. J. Njoku, R. W. Stich, O. C. Kwok, A. N. Hamir and S. M. Reed. 2001c. *Sarcocystis neurona* infections in raccoons (*Procyon lotor*): Evidence for natural infection with sarcocysts, transmission to opossums (*Didelphis virginina*), and experimental induction of neurologic disease in raccoons. Vet. Parasitol. 100: 117-129.

Fayer, R., T. K. Graczyk, E. J. Lewis, J. M. Trout, and C. A. Farley. 1998. Survival of infectious *Cryptosporidium parvum* oocysts in seawater and eastern oysters (*Crassostrea virginica*) in the Chesapeake Bay. Appl. and Envir. Microbiol. 64: 1070-1074.

_____, E. J. Lewis, J. M. Trout, T. K. Graczyk, M. C. Jenkins, J. Higgins, L. Xiao, and A. A. Lal. 1999. *Cryptosporidium parvum* in oysters from commercial harvesting sites in the Chesapeake Bay. Emerg. Infect. Dis. 5: 706-710.

Fazaeli, A., P. E. Carter and T. H. Pennington. 2000. Intergenic spacer (IGS) polymorphism: A new genetic marker for differentiation of *Toxoplasma gondii* strains and *Neospora caninum*. J. Parasitol. 86: 716-723.

Fenger, C. K., D. E. Granstrom, J. L. Langemeier, S. Stamper, J. M. Donahue, J. S. Patterson, A. A. Gajadhar, J. V. Marteniuk, Z. Xiaomin and J. P. Dubey. 1995. Identification of opossums (*Didelphis virginiana*) as the putative host of *Sarcocystis neurona*. J. Parasitol. 81: 916-919.

Graczyk, T. K., C. A. Farley, R. Fayer, E. J. Lewis, and J. M. Trout. 1998a. Detection of *Cryptosporidium* oocysts and *Giardia* cysts in the tissues of eastern oysters (*Crassostrea virginica*) carrying principal oyster infectious diseases. J. Parasitol. 84: 1039-1042.

_____, R. Fayer, M. R. Cranfield, and D. B. Conn. 1998b. Recovery of waterborne *Cryptosporidium parvum* oocysts by freshwater benthic clams (*Corbicula fluminea*). Appl. and Envir. Microbiol. 64: 427-430.

_____, Y. R. Ortega, and D. B. Conn. 1998c. Recovery of waterborne oocysts of *Cyclospora cayetanensis* by Asian freshwater clams (*Corbicula fluminea*). Am. J. Trop.Med. Hyg. 59: 928-932.

_____, R. Fayer, D. B. Conn, and E. J. Lewis. 1999a. Evaluation of the recovery of waterborne *Giardia* cysts by freshwater clams and cyst detection in clam tissue. Parasitol. Res. 85: 30-34.

_____, _____, E. J. Lewis, J. M. Trout, and C. A. Farley. 1999b. *Cryptosporidium* oocysts in Bent mussels (*Ischadium recurvum*) in the Chesapeake Bay. Parasitol. Res. 85: 518-521.

Grigg, M. S. and J. C. Boothroyd. 2001a. Rapid identification of virulent type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism at the B1 gene. J. Clin. Microbiol. 39: 398-400.

_____, Jyotsom, G. and J. C. Boothroyd. 2001b. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184: 633-639.

_____, S. Bonnefoy, A. B. Hehl, Y. Suzuki and J. C. Boothroyd. 2001c. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distict ancestries. Science. 294: 161-165.

Howe, D. K., and L. D. Sibley. 1995. *Toxoplasma gondii* comprises three clonal lineages: clorrelation of parasite genotype with human disease. J. Infect. Dis. 172: 1561-1566.

_____, S. Honore, F. Deroin and L. D. Sibley. 1997. Determination of genotypes of *Toxoplasma* strains isolated from patients with toxoplasmosis. J. Clin. Microbiol. 35: 1411-1444.

Innes, E. A., A. G. Adrianarivo, C. Bjorkman, P. A. Conrad and D. J. Williams. 2002. Immune responses to *Neospora caninum* and prospects for vaccination. Trends in Parasitol. In Press.

Johnson, J., C. W. Roberts, C. Pope, F. Roberts, M. J. Kirisits, R. Estes, E. Mui, T. Kreiger, C. R. Brown, J. Forman and R. McLeod. 2002. *In vitro* correlates of Ld-restricted resistance to toxoplasmic encephalitis and their critical dependence on parasite strain. J. Immunol. 169: 966-973.

Kanaan, K., K. S. Guruge, N. J. Thomas, S. Tanabe and J. P. Giesy. 1998. Butyltin residues in southern sea otters (*Enhydra lutris nereis*) found dead along California coastal waters. Envir. Sci. and Technol. 32: 1169-1175.

Kreuder, C., M. A. Miller, D. A. Jessup, L. J. Lowenstine, M. D. Harris, J. A. Ames, T. E. Carpenter, P. A. Conrad and J. K. Mazet. Recent patterns of mortality in southern sea otters (*Enhydra lutris nereis*). Submitted to J. Wildl. Dis.

Kunz, W. 2002. When is a parasite species a species? Trends in Parasitol. 18: 121-125.

Labelle, P., J. P. Dubey, I. Mikalian, N. Blnachette, R. Lafond, S. St-Onge and D. Martineau. 2001. Seroprevalence of antibodies to *Toxoplasma gondii* in lynx (*Lynx canadensis*) and bobcats (*L. rufus*) from Quebec, Canada. J. Parasitol. 87: 1194-1196.

Lapointe, J. M, P. J. Duignan, A. E. Marsh, F. M. Gulland, B. C. Barr, D. K. Naydan, D. P. King, C. A. Farman, K. A. Burek Huntingdon, and L. J. Lowenstine. 1998. Meningoencephalitis due to a *Sarcocystis neurona*-like protozoan in Pacific Harbor Seals (*Phoca vitulina richardsi*). J. Parasitol. 84: 1184-1189.

_____, _____, B. C. Barr, D. W. MacPherson, F. M. Gulland and J. P. Dubey. Meningoencephalitis associated with an unidentified apicomplexan protozoan in a Pacific harbor seal. Submitted: J. Parasitol.

Leatherwood, S., L. J. Harrington-Coulombe and C. L. Hubbs. 1978. Relict survival of the sea otter in central California and evidence of its recent redispersal south of Point Conception. Bull. of the South. Calif. Acad. Sci. 77: 109-115.

Lehmann, T., C. R. Blackston, S. F. Parmley, J. S. Remington and J. P. Dubey. 2000. Strain typing of *Toxoplasma gondii*: Comparison of antigen-coding and housekeeping genes. J. Parasitol. 86: 960-971.

Lekutis, C., D. J. Ferguson, M. E. Grigg, M. Camps and J. C. Boothroyd. 2001. Surface antigens of *Toxoplasma gondii*: variations on a theme. Int. J. Parasitol. 31: 1285-1292.

Lindsay, D. S., N. J. Thomas, and D. P. Dubey. 2000. Biological characterization of *Sarcocystis neurona* isolated from a southern sea otter (*Enhydra lutris nereis*). Int. J. Parasitol. 30: 617-624.

_____, K. K. Phelps, S. A. Smith, G. Flick and J. P. Dubey. 2001a. Removal of *Toxoplasma gondii* oocysts from sea water by Eastern Oysters (*Crassostrea virginica*). Journal of Eukaryotic Microbiology. 197S-198S.

_____, N. J. Thomas, A. C. Rosypal and D. P. Dubey. 2001b. Dual *Sarcocystis neurona* and *Toxoplasma gondii* infection in a northern sea otter from Washington state, USA. Vet. Parasitol. 97: 319-327.

Miller, M. A., K. W. Sverlow, P. R. Crosbie, B. C. Barr, L. J. Lowenstine, F. M. Gulland, A. Packham and P. A. Conrad. 2001b. Isolation and characterization of parasitic protozoa from a Pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalitis. J. Parasitol. 87: 816-822.

_____, I. A. Gardner, A. Packham, J. K. Mazet, K. D. Hanni, D. Jessup, J. Estes, R. Jameson, E. Dodd, B. C. Barr, L. J. Lowenstine, F. M. Gulland and P. A. Conrad. 2002a. Evaluation of an indirect fluorescent antibody test (IFAT) for demonstration of antibodies to *Toxoplasma gondii* in the sea otter (*Enhydra lutris*). J. Parasitol. 88: 594-599.

_____, _____, C. Kreuder, D.ave M. Paradies, K.aren R.Worcester, D. A. Jessup, E. Dodd, M.ike D. Harris, J. A.ack Ames, A. E. Packham and P. A. Conrad. 2002b. Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*). Int. J. Parasitol 32: 997-1006.

Nakata, H., K. Kanaan, L. Jing, N. Thomas, S. Tanabe and J. P. Giesy. 1998. Accumulation pattern of organochlorine pesticides and polychlorinated biphenlyls in southern sea otters (*Enhydra lutris nereis*) found stranded along coastal California, USA. Envir. Pollut. 103: 45-53.

Oertley, K. D. and K. W. Walls. 1980. Prevalence of antibodies to *Toxoplasma gondii* among bobcats of West Virginia and Georgia. J. Am. Vet. Med. Assoc. 177: 852-853.

Patton, S., A. Rabinowitz, and S. Randolph. A coprological survey of parasites of wild neotropical felidae. J. Parasitol. 72: 517-521.

Pederson, R. J. and J. Stout. 1963. Oregon sea otter sighting. J. Mammal. 44: 415.

Riedman, M. L. and J. A. Estes. 1988. Predation on seabirds by sea otters. Canad. J. Zool. 66: 1396-1402.

Riedman, M. L. and J. A. Estes. 1990. The sea otter (*Enhydra lutris*): Behavior, ecology and natural history. United States Fish and Wildlife Service Biological Report. 90: 126 pp.

Reimann, H. P., D. E. Behymer, M. E. Fowler, T. Schultz, A. Lock, J. G. Orthoefer, S. Silverman and C. E. Franti. 1974. Prevalence of antibodies to *Toxoplasma gondii* in captive exotic animals. J. Am. Vet. Med. Assoc. 165: 798-800.

_____, J. A. Howarth, R. Ruppanner, C. E. Franti and D. E. Behmer. 1975. *Toxoplasma* antibodies among bobcats and other carnivores of Northern California, USA. J. Wildl. Dis. 11: 272-276.

Rosonke, B. J, S. R. Brown, S. J. Tornquist, S. P. Snyder, M. M. Garner, and L. L. Blythe. 1999. Encephalomyelitis associated with a *Sarcocystis neurona*-like organism in a sea otter. J. Am. Vet. Med. Assoc. 215: 1839-1842.

Sarciron, M. E., and A. Gherardi. 2000. Cytokines involved in toxoplasmic encephalitis. Scand. J. Immunol. 52: 534-543.

Sibley, L. D., D. Mordue and D. K. Howe. 1999. Experimental approaches to understanding virulence in toxoplasmosis. Immunobiol. 201: 210-224.

_____, _____, S. Chunlei, P. M. Robben and D. K. Howe. 2002. Genetic approaches to studying virulence and pathogenesis in *Toxoplasma gondii*. Philosoph. Transacts. Royal Soc. Lond. 357: 1417.

Slifiko, T. R., H. V. Smith and J. B. Rose. 2000. Emerging parasite zoonoses associated with water and food. Int. J. Parasitol. 30: 1379-1393.

Smith, D. D. and J. K. Frenkel. Prevalence of antibodies to *Toxoplasma gondii* in wild mammals of Missouri and East central Kansas: Biologic and ecologic considerations of transmission. J. Wildl. Dis. 31: 15-21.

Su, C., D. K. Howe, J. P. Dubey, J. W. Ajioka and L. D. Sibley. 2002. Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. Procs. Nat. Acad. Sci. 99: 10753-10758.

Suzuki, Y. 1999. Genes, cells and cytokines in resistance against development of toxoplasmic encephalitis. Immunobiol. 201: 255-271.

Tamburrini, A. and E. Pozio. 1999. Long-term survival of *Cryptosporidium parvum* oocysts in seawater and in experimentally infected mussels (*Mytilus galloprovincialis*). Int. J. Parasitol. 29: 711-715.

Tanhauser, S. M., M. A. Cheadle, E. T. Massey, B. A. Mayer, D. E. Schroedter, J. B.

Dame, E. C. Greiner and R. J. MacKay. 2001. The nine-banded armadillo (*Dasypus novemcinctus*) is naturally infected with *Sarcocystis neurona*. Int. J. Parasitol. 31: 325-329.

Tarasoff, R. J. and G. L. Kooyman. 1973. Observations on the anatomy of the respiratory system of the river otter, sea otter and harp seal. II: The trachea and bronchial tree. Canad. J. Zool. 51: 171-177.

Tenter, A. M., A. R. Heckenroth and L. W. Weiss. 2000. *Toxoplasma gondii*: from animals to humans. Int. J. Parasitol. 30: 1217-1258.

_____, J. R. Barta, I. Beveridge, D. W. Duszynski, H. Melhorn, D. A. Morrison, R. C. Thompson and P. A. Conrad. 2002. The conceptual basis for a new classification of the coccidia. Int. J. Parasitol. 32: 595-616.

Thomas, N. J. and R. A. Cole 1996. Risk of disease and threats to wild populations. Endangered Species Update. 13:23-27.

Wahlstrom, K. T., Nikkila and A. Uggla. *Sarcocystis* species in skeletal muscle of otter (*Lutra lutra*). Parasitol. 118: 59-62.

Wilson, D. E., M. A Bogan, A. M. Burdin and M. K. Marinov. 1991. Geographic variation in sea otters, *Enhydra lutris*. J. Mammal. 72: 22-36.

Yap, G. S. and A. Scher. 1999. Cell-mediated immunity to *Toxoplasma gondii*: Initiation, regulation and effector function. Immunobiol. 201: 240-247.

Zarnke, R., J. P. Dubey, O. C. Kwok and J. M. Ver Hoef. 2000. Serologic survey for *Toxoplasma gondii* in selected wildlife species from Alaska. J. Wildl. Dis. 36: 219-224.

_____, _____, J. M. Ver Hoef, M. E. McNay and O. C. Kwok. 2001. Serologic survey for *Toxoplasma gondii* in lynx from interior Alaska. J. Wildl. Dis. 37: 36-38.

Appendix

The ribosomal RNA gene and first internally transcribed spacer

Figure Legend:

5' = 5 prime (upstream) end of DNA molecule

3' = 3 prime (downstream) end of DNA molecule

NTS = nontranscribed spacer

ETS = external transcribed spacer

18s = DNA coding for small ribosomal subunit (1,800 base pairs [bp] long)

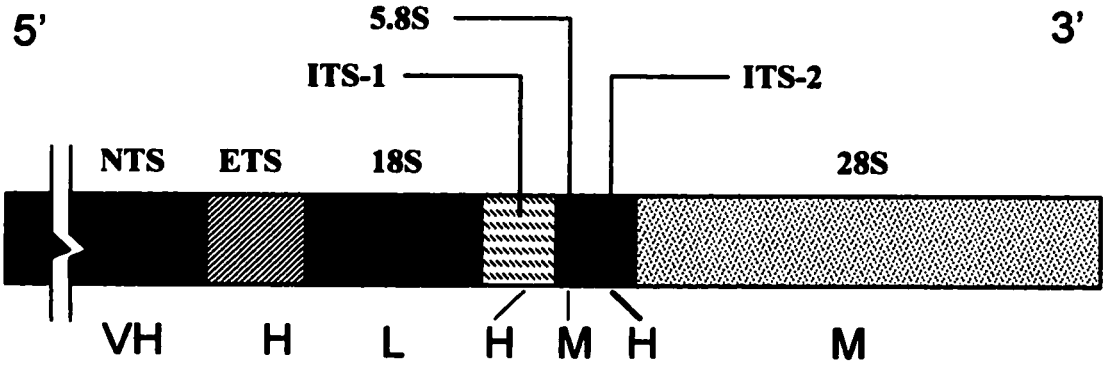
ITS-1 = first internal transcribed spacer

5.8s = DNA coding for 5.8s portion of large ribosomal subunit (160 bp long)

28s = DNA coding for 28s portion of large ribosomal subunit (4,000 bp long)

Comparative DNA sequence variability: L = low, M = medium, V = high and VH = very high

Eukaryote 18s DNA Structure



Ribosomal Structure