

Invited review

Transmission of *Toxoplasma*: Clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment[☆]

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Abstract

Toxoplasma gondii affects a wide variety of hosts including threatened southern sea otters (*Enhydra lutris nereis*) which serve as sentinels for the detection of the parasite's transmission into marine ecosystems. Toxoplasmosis is a major cause of mortality and contributor to the slow rate of population recovery for southern sea otters in California. An updated seroprevalence analysis showed that 52% of 305 freshly dead, beachcast sea otters and 38% of 257 live sea otters sampled along the California coast from 1998 to 2004 were infected with *T. gondii*. Areas with high *T. gondii* exposure were predominantly sandy bays near urban centres with freshwater runoff. Genotypic characterisation of 15 new *T. gondii* isolates obtained from otters in 2004 identified only X alleles at *BI* and *SAG1*. A total of 38/50 or 72% of all otter isolates so far examined have been infected with a Type X strain. Type X isolates were also obtained from a Pacific harbor seal (*Phoca vitulina*) and California sea lion (*Zalophus californianus*). Molecular analysis using the C8 RAPD marker showed that the X isolates were more genetically heterogeneous than archetypal Type I, II and III genotypes of *T. gondii*. The origin and transmission of the Type X *T. gondii* genotype are not yet clear. Sea otters do not prey on known intermediate hosts for *T. gondii* and vertical transmission appears to play a minor role in maintaining infection in the populations. Therefore, the most likely source of infection is by infectious, environmentally resistant oocysts that are shed in the feces of felids and transported via freshwater runoff into the marine ecosystem. As nearshore predators, otters serve as sentinels of protozoal pathogen flow into the marine environment since they share the same environment and consume some of the same foods as humans. Investigation into the processes promoting *T. gondii* infections in sea otters will provide a better understanding of terrestrial parasite flow and the emergence of disease at the interface between wildlife, domestic animals and humans.

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1. Transmission of pathogenic protozoa at the human–domestic animal–wildlife interface

With a growing human population and changing demographics globally, the demarcations between urban

and rural communities and wildlife habitats are becoming less distinct. This has enhanced the potential for a flow of pathogens among ecosystems and species. Habitat fragmentation and degradation has increased contact among wildlife, people, and their pets; leading in some cases to declines in wildlife populations and increased pathogen exposure in humans, domestic animals, and wildlife (Daszak et al., 2000; 2001; Patz et al., 2000; 2004). Examples of these changes at the human–domestic animal–wildlife interface are clearly evident in coastal California where 60% of the state's 35.5 million residents live in 15

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coastal counties (<http://quickfacts.census.gov/qfd/states/06000.html>). Anthropogenic activities in this coastal area are contributing to environmental degradation, which combined with fecal pollution from humans and their animals, negatively impact water quality (Haile et al., 1999; Dwight et al., 2002; 2004). Among the waterborne biological contaminants of particular concern are zoonotic protozoal parasites, including *Toxoplasma gondii*, *Cryptosporidium* spp. and *Giardia* spp., whose oocyst or cyst stages are shed in the feces of terrestrial animals. For *T. gondii*, wild and domestic felids are the only known definitive hosts capable of shedding environmentally resistant oocysts that potentially can be transported into fresh and marine waters via sewage systems or stormwater drainage and freshwater runoff (Miller et al., 2002b; Fayer et al., 2004). There has been a focused effort over the past 5 years to study the impact of *T. gondii* infection on the southern sea otter (*Enhydra lutris nereis*) population in coastal California and to apply this knowledge to better understand the transmission of pathogenic protozoa at the human–domestic animal–wildlife interface.

Toxoplasma gondii is a ubiquitous protozoal parasite that infects a wide variety of animals, including humans. The biology and epidemiology of *T. gondii* have been well reviewed (Dubey and Beattie, 1988; Tenter et al., 2000). Infections in most immunologically normal humans are asymptomatic or result in an influenza-like illness, which often goes undiagnosed as toxoplasmosis (Frenkel, 1990; Tenter et al., 2000). Women who become infected for the first time during pregnancy and immunosuppressed patients are at serious risk from clinical toxoplasmosis. Initial infection with *T. gondii* during pregnancy can result in foetal death or symptoms, such as chorioretinitis and mental retardation, that are apparent at birth or develop later in childhood (Wilson et al., 1980; Guerina et al., 1994; McAuley et al., 1994). In the USA, it is estimated that between 400 and 4000 babies are born with congenital toxoplasmosis annually (Guerina et al., 1994; Lopez et al., 2000; Jones et al., 2003a). The incidence of congenital toxoplasmosis varies in different countries depending upon the prevailing infection risk in the area and the fraction of susceptible women (Stray-Pedersen, 1993). *Toxoplasma gondii* is recognised worldwide as a major cause of morbidity and mortality in AIDS patients, primarily as a result of encephalitis and cardiac disease (Luft and Remington, 1992; Renold et al., 1992; Hofman et al., 1993; Maturri et al., 1990). The recrudescence of latent infections can cause life-threatening disease in chemically immunosuppressed transplant patients (Frenkel, 1990; Ho-Yen, 1992). Latent *T. gondii* infections in adults have also been associated with schizophrenia, personality changes, and increased risk for traffic accidents due to delayed reaction times (Holliman, 1997; Flegr et al., 2000; 2002; Torrey and Yolken, 2003).

Humans become infected with *T. gondii* primarily by ingestion of either bradyzoite cysts in undercooked tissue

from infected intermediate hosts, such as pigs and sheep, or sporulated oocysts. In 1995, direct costs for medical treatment and productivity losses due to toxoplasmosis in the USA were estimated to be \$7.7 billion per annum (Buzby and Roberts, 1997). Although toxoplasmosis represents only 4.1% of hospitalisations due to foodborne illness per year, Mead et al. (1999) estimated that *T. gondii* accounts for 20.7% of deaths from foodborne illness; making *T. gondii* third on the list of the top foodborne pathogens causing human mortality in the USA. Despite a successful effort to reduce *T. gondii* infection in market swine in the USA (Lubroth et al., 1982; Dubey and Weigel, 1996), the prevalence, based on seropositivity of human *T. gondii* exposure, has not changed since 1988 (Jones et al., 2003a). One explanation for this persistence in exposure could be that environmental exposure to *T. gondii* oocysts is underestimated or increasing as a significant source of infection in this country.

Domestic and wild felids are the only definitive hosts of *T. gondii* in which sexual multiplication of the parasite results in the formation of oocysts that sporulate in the environment and are infective for susceptible hosts (Dubey et al., 1970). Cats become infected with *T. gondii* primarily by ingestion of either bradyzoite cysts in the tissues of infected intermediate hosts, such as rodents and birds, or sporulated oocysts from other cats (Dubey, 1998; 2002). Most cats infected by ingestion of tissue cysts shed oocysts within 3–10 days and may continue to shed for up to 20 days. In comparison, fewer (<30%) cats that ingest tachyzoites or oocysts become infected, generally with a longer prepatent period and shorter duration of shedding (Dubey and Frenkel, 1972; 1976). The first time cats are infected they can shed more than 100 million oocysts in their feces (Dubey et al., 1970; Dubey and Frenkel, 1972; Tenter et al., 2000). Cats may re-shed oocysts if they become infected with other benign coccidian parasites, are immunosuppressed or are re-exposed to *T. gondii* years after initial infection (Dubey and Frenkel, 1974; Dubey, 1976; 1995). The propensity of domestic cats to bury their feces and defecate in shady areas enhances the survival of oocysts, which can remain infective in soil up to 2 years under favourable climatic conditions (Yilmaz and Hopkins, 1972; Frenkel et al., 1975). Oocysts may sporulate within 24–48 h of defecation and are infectious to a wide variety of intermediate hosts (Dubey and Beattie, 1988; Tenter et al., 2000).

Recognition that *T. gondii* can be a significant waterborne pathogen via oocyst transmission has increased in recent years with human outbreaks of acute toxoplasmosis associated with exposure to contaminated water sources in Panama, Brazil and British Columbia (Benenson et al., 1982; Bowie et al., 1997; Tenter et al., 2000; Bahia-Oliveira et al., 2003; Dubey, 2004). An epidemiologic investigation showed that in 1995 an estimated 2894–7718 individuals were infected with *T. gondii* in a waterborne transmission outbreak in Vancouver, with 100 individuals who met the definition for an acute, outbreak-related case of toxoplasmosis. Epidemiologic findings suggested that the source of

infection was water from a reservoir that was contaminated with oocysts shed from a felid infected with *T. gondii* (Bowie et al., 1997). In the USA the size of the owned cat population has grown over 80% in the past decade. An estimated 32% of households in the USA own cats, and estimates of the owned cat population are greater than 78 million (http://www.petfoodinstitute.org/reference_pet_data.cfm). The size of the feral cat population is unknown, but estimated to be close to 73 million (Levy and Crawford, 2004). In contrast, the wild felid populations are comparatively small, with only 30,000 cougars (*Felis concolor*) in the USA (McCarthy, 2003) and an estimated 5100 in California (<http://www.dfg.ca.gov/lion/outdoor.lion.html>). Seroprevalence of *T. gondii* in wild felids has been investigated, but there are limited data on the dynamics of oocyst shedding in wild felids (Riemann et al., 1975; 1978; Franti et al., 1976; Marchiondo et al., 1976; Smith and

Frenkel, 1995; Aramini et al., 1998; 1999; Labelle et al., 2001; Zarnke et al., 2001; Kikuchi et al., 2004; Philippa et al., 2004; Riley et al., 2004). Surveys conducted on domestic cats indicate that from 8 to 74% of cats in the USA are infected with *T. gondii* based on seroprevalence data (Table 1) and up to 2% of cats may be shedding oocysts at any time (Dubey, 1973; Christie et al., 1976; Guterbock and Levine, 1977).

2. Sea otters as sentinels of protozoal pathogen pollution

The southern sea otter is a federally-listed threatened species located exclusively in the near coastal environment of central California. In addition to being a notable tourist attraction and icon of coastal California, these charismatic marine mammals serve several important ecological roles.

Table 1
Toxoplasma gondii serosurveys in cats from the USA

Location/state	No. cats tested	Seroprevalence ^a	Test	Cut-off ^b	Source of cats	Reference
IA	21	28.6% ^c	DT	1:16	Rural, collected for College of Medicine Animal Facility	McCulloch et al. (1964)
CA	32	25% (22–35%)	DT	1:32	Colony—18 from Central Valley, 14 from SFO Bay	Soave (1968)
NJ area	200	43%	IFAT	1:16	Not reported	McKinney (1973)
Kansas City, MO and IA	667	16% (8.6–57.9%)	DT	1:2	510 owned (MO), 157 stray (MO/IA)	Dubey (1973)
NM, AZ, CO	91	8%	DT	1:32 ^d	Not reported	Marchiondo et al. (1976)
Columbus, OH	1000	39%	IFAT	1:16	Shelter	Claus et al. (1977)
Atlanta, GA	39	23.1%	DT	1:2	32 owned, 7 feral ^e	Teutsch et al. (1979)
WA	87	31%	DT	1:4	87 shelter	Ladiges et al. (1982)
Baltimore, MD	650	14.5% (12.2–71.4%)	IFAT	1:32	600 shelter, 36 vet clinic, 14 trapped strays	Childs and Seegar (1986) Witt et al. (1989)
Athens, GA	188	60.7%	ELISA	1:64	81 healthy, 107 ill; veterinary teaching hospital	Lappin et al. (1989c)
OK	618	22% (10–24%)	LAT	1:16	Owned cats	Rodgers and Baldwin (1990)
IA	74	41.9%	MAT	1:32	Swine farms	Smith et al. (1992)
FL, GA, OH	124	74.2%	ELISA ^f	1:64	Veterinary teaching hospital cats with uveitis ^g	Lappin et al. (1992)
IL	391	68.3%	MAT	1:25	Swine farms	Dubey et al. (1995)
IA	20	80%	MAT	1:32	Trapped, free-ranging	Hill et al. (1998)
CO	206	23.6% (19.7–29.8%)	ELISA	1:64	129 owned, 77 shelter ^h	Hill et al. (2000)
OH	275	48%	MAT	1:25	197 owned, 78 feral	Dubey et al. (2002)
RI	200	42%	MAT	1:25	116 vet clinic, 84 shelter	DeFeo et al. (2002)
USA ⁱ	12,628	31.6% (16–44%)	ELISA	1:64	Clinically ill, veterinary teaching hospital	Vollaire et al. (2005)
Gainesville, FL	553	13.4%	ELISA	1:64	Feral	Luria et al. (2004)
NC	176	50.6% (34–63%)	MAT	1:25	76 owned, 100 feral	Nutter et al. (2004)

^a Percentages in parentheses represent seroprevalence ranges for different groups of cats in the study.

^b Dilution at and above which sera is determined positive for the specific dye test (DT), indirect fluorescent antibody test (IFAT), enzyme-linked immunoassay (ELISA), latex agglutination test (LAT) or modified agglutination test (MAT) used in serosurvey.

^c 40.6% using Dye test cutoff of 1:4.

^d Absorbed onto filter paper discs, lowest possible titer obtainable is 1:32.

^e Tested during an outbreak affecting 37 humans.

^f IgM, IgG or antigen positive as described in Lappin et al. (1989a,b).

^g Most cats were from Georgia.

^h Cats were also classified as follows: 88 with diarrhea, 106 without diarrhea, 12 unknown.

ⁱ Sera tested for IgM and IgG antibodies at Colorado State University from cats for whom clinical toxoplasmosis was a potential diagnosis, based on clinical presentation.

They act as a keystone species that helps to maintain coastal kelp forests by feeding on herbivorous sea urchins (Leighton, 1966; Parker and Kalvass, 1992). By controlling the destruction of kelp forests by grazing urchins, otters help maintain a diversity of forest inhabitants and ecosystem services, including protection of the coastline from erosion (Estes and Palmisano, 1974; Estes and Duggins, 1995; Jackson et al., 2001; Estes et al., 2004). As nearshore predators close to the top of the food chain, otters serve as sentinels and early indicators of environmental change. Living primarily within 0.5 km of the California coast, otters share the same environment and consume some of the same foods (e.g. mussels, clams and crabs) as humans (Riedman and Estes, 1990).

The southern sea otter population was diminished almost to the point of extinction by the fur trade in the 1800s. Despite federal protection since 1977, the population has been unable to maintain consistent recovery rates like those seen in northern sea otter populations. Current abundance counts indicate that there are only about 2800 sea otters in California (<http://www.werc.usgs.gov/otters/ca-surveydata.html>). Demographic models show that the depressed population growth rate cannot be accounted for by reduced birth rates or dispersal (Estes et al., 2003a; Gerber et al., 2004). As early as 1968, concern over the slow recovery of the southern sea otter population prompted the California Department of Fish and Game to start studying sea otter mortality. It was not until veterinary pathologists from the National Wildlife Health Center were added to the investigative team in 1992 that infectious diseases were identified as causing mortality in 38.5% of sea otters (Thomas and Cole, 1996). This unusual finding led to the development of a collaborative health investigation team led by California Department of Fish and Game and the Wildlife Health Center at the University of California (UC) at Davis School of Veterinary Medicine. The resulting necropsy program for southern sea otters, housed at the Marine Wildlife Veterinary Care and Research Center in Santa Cruz, California, is a model for investigating causes of mortality in threatened and endangered species. Currently, 40–50% of all estimated sea otter deaths in California are retrieved as beachcast carcasses (Estes et al., 2003a; Gerber et al., 2004) and examined to determine cause of death. All carcasses in fresh condition receive a complete pathological examination including whole body radiographs, histopathological examination of tissues, microbiological cultures, parasitological examinations, and toxicological evaluations. The findings are then shared with the collaborative team working to support sea otter recovery, including the United States Geological Survey—Biological Resource Division, Monterey Bay Aquarium, California Department of Fish and Game, UC Santa Cruz, The Marine Mammal Center and UC Davis.

A detailed analysis of causes of mortality, based on systematic necropsy of 105 otters recovered in California between 1998 and 2001, showed that disease played

a significant role in mortality, accounting for 63.8% of deaths of beachcast otters (Kreuder et al., 2003). In this more recent analysis, parasitic disease alone caused death in 38.1% of otters examined, with protozoal meningoencephalitis responsible for 28% of deaths (Kreuder et al., 2003), which is higher than the 1996 estimate of 8.5% (Thomas and Cole, 1996). Our studies showed that encephalitis due to brain infections with protozoan parasites was among the leading causes of southern sea otter mortality, paralleled only by peritonitis caused by acanthocephalan parasites (Kreuder et al., 2003). *Toxoplasma gondii* was the primary cause of death in 16.2% of otters and an additional 6.7% of deaths were attributed to brain infections with a closely related protozoan parasite, *Sarcocystis neurona*. In addition, infection with these parasites was significantly associated with the next most common cause of sea otter mortality—shark attack. Otters with moderate to severe *T. gondii* encephalitis were 3.7 times more likely to be attacked by sharks than otters without encephalitis (Kreuder et al., 2003). The high prevalence of pre-existing protozoal encephalitis in shark attacked otters suggests that they may exhibit aberrant behaviour, similar to findings in infected humans. Neurologic dysfunction might cause otters to be less able to evade attacks, to move offshore out of the protected areas, or to attract shark attention through abnormal movements and seizures. The majority of disease-caused deaths occurred in sub-adults and prime-age adults (Kreuder et al., 2003). Demographic analyses show that population growth rates are most susceptible to mortality within the sub-adult and adult female age class categories (T. Tinker, doctoral thesis, University of California, Santa Cruz, CA, 2004). Therefore, the high level of disease-related mortality in prime age adult southern sea otters is not compatible with recovery of this species.

An additional component to the monitoring program focuses on capture, tagging and health screening of live, free-ranging sea otters. Serum samples are tested for *T. gondii*-specific antibodies using an indirect fluorescent antibody test (IFAT) that was validated and shown to have excellent sensitivity and moderate specificity (Miller et al., 2002a). Our experience has shown that the modified agglutination test (MAT) is less reliable than the IFAT when samples are haemolysed, which can occur when blood samples are collected from live or beachcast sea otters (Packham et al., 1998; Miller et al., 2002a). The cut-off titer and validation of the IFAT was determined by evaluating reactivity of serum collected from 77 fresh dead southern sea otters whose postmortem *T. gondii* infection status was confirmed by brain histopathology, immunohistochemistry and/or parasite isolation in cell culture. Animals that were considered negative for *Toxoplasma* for purposes of inclusion in the study had to be negative on all three tests to meet the inclusion criteria. These strict criteria for validation and testing were deemed the most appropriate for our epidemiologic studies in order to reduce the possibility

of overestimating the seroprevalence of infection. However, it remains possible that some otters are falsely classified as *T. gondii*-negative using the criteria above, especially if *T. gondii* cysts were present in brain tissue in low numbers or were present in tissues other than brain, and this is discussed later. For the purpose of diagnosis as a basis for making treatment decisions with clinically infected otters that are stranded or in captivity, the level and change in antibody levels as well as the presence of neurologic signs are also taken into consideration. A serosurvey conducted from 1997 to 2000 that used the strict IFAT validation previously described (Miller et al., 2002a) showed that 36% (29/80) of southern sea otters in California were seropositive for *T. gondii*, compared with 38% (8/21) of northern sea otters (*Enhydra lutris kenyoni*) from Washington and 0% (0/65) of otters (*Enhydra lutris lutris*) from Alaska (Hanni et al., 2003; Kreuder et al., 2003; Miller et al., 2002a,b). More recent serological survey results are discussed below.

3. Parasite biology indicates a land–sea connection

There are three possible routes by which sea otters could become infected with *T. gondii*; ingestion of sporulated oocysts, ingestion of bradyzoite cysts in the tissues of intermediate hosts or by vertical transmission. Although not proven, the former is more likely to be the primary mode of transmission because sea otters do not prey on warm blooded animals which are recognised intermediate hosts of *T. gondii*, but instead consume various species of

marine macroinvertebrates, such as bivalves, snails and crustaceans (Riedman and Estes, 1990). Similarly, the role of vertical transmission as the primary means of propagating *T. gondii* infection in sea otters is not supported by data from long-term serological screening (Fig. 1a) and in vitro parasite isolation from the brains of freshly dead otters (Fig. 1b). Between 1998 and April 2005, serum samples collected from 339 freshly dead otters submitted for necropsy in California were evaluated for the presence of antibodies to *T. gondii*, using the validated IFAT, as described (Miller et al., 2002a). Fig. 1a shows the proportion of seropositive animals stratified by sea otter age class. Nursing pups show an initial low peak in seropositivity to *T. gondii*, followed by a decline in the proportion of seropositive otters in the immature age class as nursing and parental care wanes. This initial low peak is presumed to be due to transplacental or transmammary transmission of maternal antibodies, although this means of antibody transmission has not yet been confirmed for sea otters. As immature otters begin to forage independently and mature into subadults and then adults, a progressive rise in the proportion of seropositive otters is observed, as would be expected if infection is acquired postnatally after foraging activity begins.

The trend is similar for long-term parasite isolation in cell culture from sea otters submitted for necropsy. Two hundred and seventy-two southern sea otters were necropsied during this same time period and had brain samples collected and cultured for the isolation of *T. gondii* in vitro using methods previously described (Miller et al.,

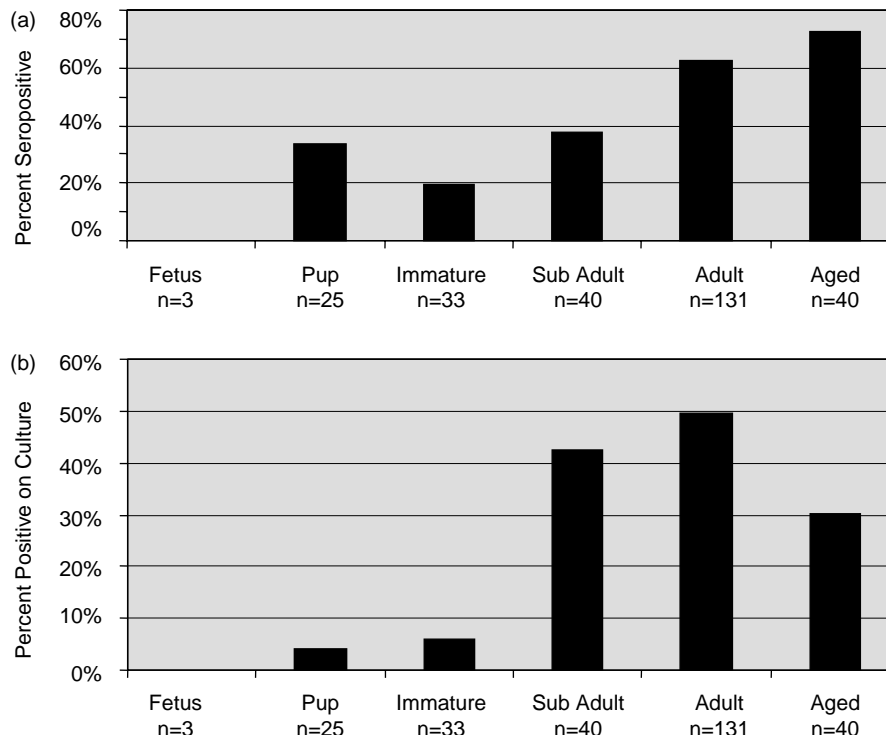


Fig. 1. Proportion by age class of southern sea otters necropsied 1998–2005 that were seropositive (a) or positive by culture isolation (b) for *Toxoplasma gondii*.

2002a). Fig. 1b shows the proportion of these otters with confirmed *T. gondii* infections based on culture isolation. Due to their smaller size and tendency to decompose more quickly, acquisition of sea otter fetuses and pups for postmortem examination is uncommon as compared with the recovery of southern sea otters in older age categories. Even so, three fetuses and 25 pups, including stillborn neonates, were necropsied and screened for the presence of *T. gondii* by cell culture and histopathology. As a result, none of three aborted fetuses and only one of 25 freshly dead pups were found to be positive for *T. gondii* via culture and histopathology over 8 years of study, compared with two of 33 immature otters, 17 of 40 subadults, 65 of 131 adults and 12 of 40 aged adults. This positive correlation between otter age and the proportion of animals infected with *T. gondii* is more supportive of postnatally-acquired infection as a result of ingestion of infective *T. gondii* than it is of vertical transmission. From these data, which are the most extensive published to date, there appears to be no evidence to support the hypothesis that vertical transmission, although it likely occurs in sea otters, is a major contributor to the high prevalence of *T. gondii* infection observed in the southern sea otter population.

Investigation into the processes promoting *T. gondii* infections in sea otters may be our best opportunity to understand terrestrial parasite flow into the coastal marine system. In the absence of evidence that the high number of sea otters infected off the coast of California can be attributed either to the ingestion of prey containing tissue cysts or vertical transmission, the most likely source of infection is exposure to the environmentally resistant, infective oocysts of *T. gondii*, which can survive for months or years in contaminated soil, freshwater or seawater (Yilmaz and Hopkins, 1972; Frenkel et al., 1975; Dubey, 1998; Lindsay et al., 2003). *Toxoplasma gondii* oocysts may remain infective despite water chlorination or sewage processing (Aramini et al., 1999). Furthermore, the infectious dose for some species may be as low as one oocyst (Dubey et al., 1996). An epidemiologic study based on live and dead otter samples from 1998 to 2001 showed that otters sampled near areas of high freshwater runoff, such as Elkhorn Slough in Monterey Bay were 2.9 times more likely to be exposed to *T. gondii* than otters sampled in areas with low or medium freshwater runoff (Miller et al., 2002b). In addition, Morro Bay, south of Monterey Bay, was identified as an area of substantially increased risk for sea otter exposure to *T. gondii* (Miller et al., 2002b). A subsequent study revealed that there was also an increased risk for sea otter mortality due to *T. gondii* in the Morro Bay area (Kreuder et al., 2003).

After reaching the sea, oocysts may be concentrated by filter-feeding marine invertebrates that are prey for sea otters. Several studies have demonstrated that oocysts and cysts of pathogenic protozoa are concentrated by clams, mussels and oysters during filter-feeding activity (Fayer et al., 1998, 1999, 2003; Graczyk et al., 1998a–c, 1999a,b).

Recent studies in California showed that oocysts of *Cryptosporidium* spp. were detected in sentinel mussels (*Mytilus* spp.) at nearshore marine sites and clams (*Corbicula fluminea*) placed in rivers that flow into estuarine habitat for sea otters (Miller et al., 2005a,b). We have demonstrated that Pacific coast mussels (*Mytilus* sp.) remove and concentrate oocysts of *T. gondii* from contaminated water and that the parasites remain viable and infective within mussel tissues (Arkush et al., 2003). Similar studies showed that *T. gondii* could also be concentrated and remain viable in eastern oysters (Lindsay et al., 2004). Thus, raw shellfish could serve as a source of pathogenic protozoal infection for both marine mammals and humans. To maintain normal body weight and meet metabolic demands, sea otters consume approximately 25% of their body weight each day in invertebrate prey, such as mussels (Estes et al., 2003b). However, the identification of *T. gondii* in resident or sentinel bivalves in the wild has yet to be reported, so the role of these invertebrates in the transmission of oocysts to sea otters is unknown. Bivalves are likely not the only environmental source of infection for sea otters, as wide variation in individual prey preferences has been noted previously, and some otters consume few, if any, bivalves (Estes et al., 2003b). In addition, reports of infection based on histology and/or parasite isolation as well as seropositivity have been reported in a variety of marine mammal species that do not consume bivalves (Migaki et al., 1977; Holshuh et al., 1985; Inskoop et al., 1990; Miller et al., 2001; Dubey et al., 2003; Measures et al., 2004). Although not documented, other possible modes of *T. gondii* infection could be by the ingestion of oocysts in seawater, by grooming and ingesting oocysts collected on their fur or by ingestion of oocysts that are on or in prey other than bivalves. The mechanisms of transmission and extent of the intermediate host range for *T. gondii* requires further investigation.

4. Updated analysis of *T. gondii* exposure of live and dead otters

Between 1998 and 2004, 305 freshly beachcast sea otter carcasses were examined through the collaborative California sea otter recovery program described above. The resulting database also includes health and pathogen exposure information for live sea otters sampled along the California coast ($n=257$). These data are being used to identify spatial and temporal trends in age-specific survival rates and patterns of mortality in California sea otters on a continual basis. An important part of this health monitoring program has been to screen serum from both dead beachcast and live captured sea otters for *T. gondii* exposure using the IFAT (Miller et al., 2002a). For the period from 1998 to 2004, 38% of live otters and 52% of dead otters had a seropositive response to the *T. gondii* IFAT test using the established cut-off of greater than or equal to 1:320. As has

been shown previously (Miller et al., 2002b), adult and aged adult otters were significantly more likely to be exposed to *T. gondii* than immature and subadult otters. In fact, 46% of adult live otters were seropositive to *T. gondii*, compared with 19% of subadults and 4% of immatures ($P < 0.001$). Similarly, 65% of adult dead otters were seropositive to *T. gondii*, compared with 36% of subadults and 27% of immatures ($P < 0.001$). Increased seroprevalence in older age classes is typical of pathogens that cause persistent infection, as older otters are more likely to have come in contact with *T. gondii* during their lifetime.

Because otter captures, strandings and age classes were not distributed evenly along the coast, geographic differences were evaluated by multivariate logistic regression so that findings could be adjusted by age and live/dead status. Significant differences in risk of exposure to this pathogen in live and dead otters among different areas within the sea otter range are evident. Otters from the Elkhorn Slough/Moss Landing area (in the centre of Monterey Bay) and otters from Morro Bay had the highest levels of exposure to *T. gondii* (Fig. 2). Specifically, otters from the Elkhorn Slough area were six times as likely (95% Confidence Interval (CI): 1.9–20.0) and otters from San Simeon to Morro Bay were five times as likely (95% CI: 1.9–16.2) to have been exposed to *T. gondii* than otters from the more remote and rocky Big Sur coast. Otters from the northern half of Monterey Bay (Santa Cruz to the Pajaro River outlet) and otters from south of Pismo Beach had moderate levels of exposure and were four times as likely (95% CI: 1.3–10.8) and three times as likely

(95% CI: 1.1–9.6), respectively, to have been exposed to *T. gondii* compared to otters from the Big Sur coast (Fig. 2). Areas with high *T. gondii* exposure are predominantly sandy bays near urban centres with freshwater runoff. As has been demonstrated previously (Miller et al., 2002b), increased risk of exposure in these areas may be associated with proximity to high levels of freshwater runoff although additional processes that promote high *T. gondii* exposure in otters are currently being investigated. Interestingly, only one of the 16 otters captured at San Nicolas Island was exposed to *T. gondii*. A small population of translocated otters resides at San Nicolas Island, which is an island located approximately 100 km west of Los Angeles with a very small human community and very few introduced cats (<12 individuals).

5. Discovery of a unique clade of *T. gondii* genotypes infecting marine mammals

The morbidity and mortality seen with *T. gondii* infections in sea otters are in marked contrast to the subclinical or mild infections seen in most immunocompetent humans and terrestrial animals. Proposed explanations for the apparent high sea otter susceptibility to infectious diseases include environmental pollutant exposure (Kanaan et al., 1998; Nakata et al., 1998), inbreeding depression (Larson et al., 2002), and immunosuppression (Kanaan et al., 1998). *Toxoplasma gondii* strain variation or novel host-parasite interactions could also play a role in these infections. Significant intraspecific differences in respect to clinical presentation of toxoplasmosis exist and may be explained in part by infection with atypical genotypes (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Darde, 1996; Lehmann et al., 2000; Grigg et al., 2001a,b; Sibley et al., 2002).

Toxoplasma gondii is the sole species in the genus and is composed of three major genotypes (>94% of all isolates), designated as Types I–III, that have emerged as the dominant strains worldwide (Howe and Sibley, 1995; Grigg et al., 2001b; Su et al., 2003; Volkman and Hartl, 2003). In nature, these genotypes represent the successful recombinants from a sexual cross (or crosses) between two distinct lineages of *Toxoplasma*. Hence, these three lines share the same set of just two alleles that have reassorted in different combinations across all genetic loci thus far investigated (Grigg et al., 2001b). Type II strains are most common in nature and have been isolated from a wide variety of intermediate hosts, but sampling has been largely biased towards parasites recovered from humans and their domestic animals (Howe and Sibley, 1995; Darde, 1996).

Recently, we genotyped *T. gondii* isolates from California sea otters with toxoplasmic encephalitis and began to establish a database of genotypes found in the marine environment so as to investigate the relationship between these and isolates from terrestrial animals. Multi-locus PCR restriction fragment length polymorphism



Fig. 2. California range of the southern sea otter illustrating coastal areas of low, moderate and high seroprevalence of *Toxoplasma gondii* in live and dead sea otters surveyed 1998–2004.

(PCR-RFLP) analyses with limited DNA sequence analysis was applied against 35 *T. gondii* isolates from otter brain tissue, and these analyses identified two distinct lines of *T. gondii* associated with California otters (Miller et al., 2004). Forty percent were infected with the common zoonotic Type II strain whereas 60% were infected with a genotype, now designated as Type X, which possessed novel alleles at three genetic loci quite different from the alleles found in the Type I, II and III lines. No Type I or III strains were identified among the isolates examined. There was an unexpected difference in the geographic distribution of Type II and X isolates from otters in this study. Type II genotypes predominated in the northern half of the sea otter range, whereas otters from the southern half of the range were eight times more likely to be infected with Type X *T. gondii* (Miller et al., 2004). A statistically significant spatial cluster of Type X-infected otters was detected near Morro Bay; the same location that had been identified as a high risk site for sea otter infection with and mortality due to *T. gondii* in previous studies (Miller et al., 2002; Kreuder et al., 2003). However, an association between isolate genotype and pathogenicity was not statistically significant in this study and will require further investigation.

Since, reporting the initial characterisation of sea otter isolates (Miller et al., 2004), we have expanded our analysis to include 15 additional isolates obtained from otters in 2004 and two other marine mammal species. Multi-locus PCR-RFLP analysis on the additional 15 sea otter isolates identified only the Type X allele at both *BI* and *SAG1* (unpublished results). Combining these with the isolates from our previous study brings the total of Type X isolates to 38/50 or 72% of all otter isolates thus far examined by molecular genotyping techniques.

The incidence and seroprevalence of *Toxoplasma* infection in other marine mammals is well documented and recently reviewed (Dubey et al., 2003) but as yet, no molecular genotyping analyses have been reported. To ask whether Type X strains are infecting marine mammals other than southern sea otters, multi-locus PCR-RFLP analyses using the *BI*, *SAG1*, and *GRA6* markers was carried out on *Toxoplasma* isolates collected from a Pacific harbor seal (*Phoca vitulina richardsi*) (Miller et al., 2001) and a California sea lion (*Zalophus californianus*). Both pinnipeds stranded along the California coast within the southern sea otter range, but these species do not typically share prey species with sea otters (Riedman and Estes, 1990). At *BI* and *SAG1*, RFLP analysis identified the Type X allele for both the harbor seal and sea lion isolates, indicating that these strains were not archetypal and thus could not be related to the Type II genotype that predominates world-wide (data not shown). PCR-RFLP analysis at *GRA6* identified the Type II strain allele, which is consistent with these strains having a Type X genotype since *Mse* I digestion cannot distinguish between the Type II and X alleles, but does distinguish the Type I allele from Type III allele, each of which give a different banding pattern distinct from Type II and X. To establish that

these isolates possessed a Type X allele, direct DNA sequencing was performed on the *GRA6* PCR amplification products (GenBank AY964058 Ca Sea Lion; AY964059 Harbor Seal; AY964060 SO 3160). Over the ~600 nucleotide PCR product, both isolates possessed the same allele, and it was identical to the Type X allele previously identified in the sea otter isolate 3160 (Miller et al., 2004). This data-set strongly suggests that the harbor seal and sea lion were infected with the same Type X genotype causing mortality in southern sea otters.

Extensive sequence analysis at multiple genetic loci among Type I, II and III strains collected throughout the world from different animal hosts has identified only two alleles and limited genetic diversity among archetypal lines. In the absence of this type of exhaustive molecular analysis for the marine Type X isolates, we applied a number of previously published random amplified polymorphic DNA (RAPD) markers against a subset of 24 *T. gondii* marine isolates (18 X and four II sea otter isolates, one X harbor seal, and one X sea lion isolate) and compared their molecular fingerprints against four Type I (RH, GT-1, CT-1, OH3), three Type II (76K, PRU, BEV), three Type III (CEP, POE, C56), and a II/III recombinant (SOU) strains to explore the extent of genetic diversity in the X isolates compared with the I, II and III archetypes. This new analysis was undertaken chiefly to investigate whether intra-type variation could be detected among the marine X isolates. RAPD markers provide a rapid snapshot of genetic variation and can be used to discern taxonomic relatedness among strains to provide meaningful data on their genetic relationship. This technique utilizes a single primer, usually 10 nucleotides in length, and serves ostensibly as a DNA fingerprinting assay. RAPD analyses typically generate molecular signatures of a reproducible number of distinct PCR products depending on the source of genomic DNA provided. Thus, the banding patterns identified serve as diagnostic markers for strain genotyping and can rapidly assess the extent of genetic variability within a cohort of isolates.

Seven RAPD primers have previously been applied against *T. gondii* isolates with varying degrees of success (Guo et al., 1997; Ferreira et al., 2004). Of these, we applied the primers B5, B12, C8, and C20 (Guo et al., 1997) against our cohort of 20 marine X isolates versus a collection of archetypal strains. Primers B5, B12, and C20 each gave a characteristic signature profile of PCR amplification products depending on the source of genomic DNA provided (data not shown). However, absolute reproducibility of the banding patterns identified between successive PCR amplifications for each source of genomic DNA was problematic even though general differences between Types I–III from X were readily discernible. In contrast, the C8 primer was particularly useful, and all parasite DNA preparations gave highly reproducible banding patterns between successive amplifications. Previously unpublished data showed that Type I, II, III, and II/III recombinant isolates clustered together with strong bands at 1.4 and 0.8 kb, and these could

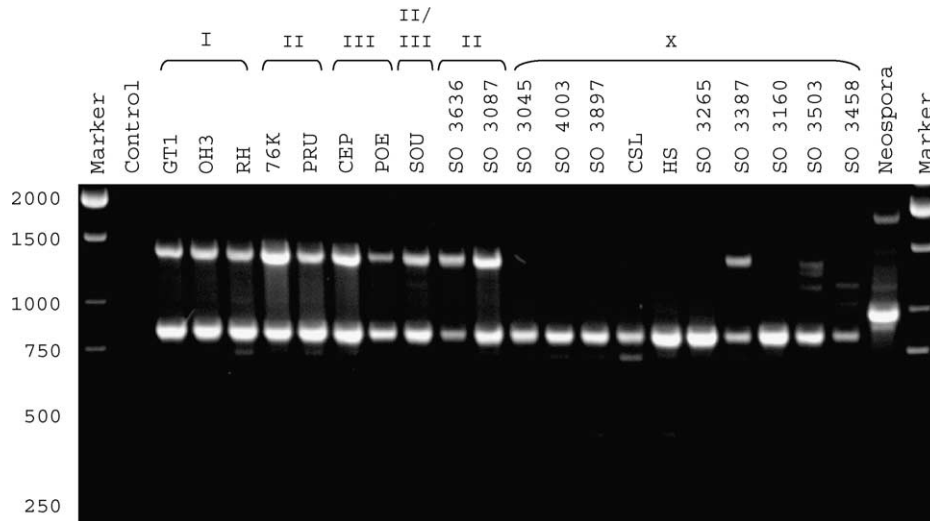


Fig. 3. Random amplified polymorphic DNA (RAPD) profile of *Toxoplasma gondii* genomic DNA amplified with the C8 primer. Genomic DNA was extracted from 35 *Toxoplasma* isolates and one *Neospora caninum* isolate was amplified using the single C8 oligonucleotide 5'-TGGACCGGTG-3'. Only a subset of 20 *Toxoplasma* isolates are shown. Parasites were solubilised in a 50 mM Tris-HCl pH 8.0, 62.5 mM EDTA, 2.5 M lithium chloride, 4% Triton X-100 solution and DNA was extracted by phenol/chloroform extraction followed by ethanol precipitation. The PCR amplification was performed as described (Guo et al., 1997). Briefly, a 2 min initial denaturation at 94 °C was followed by 45 cycles of 1 min at 94 °C, 1 min at 36 °C, 2 min at 72 °C, and then a final 10 min 72 °C extension. Products were separated on 1.4% agarose gels and visualised using ethidium bromide. A nil genomic DNA control was included to ensure that the bands visualised were not the result of primer artifacts. *Neospora* DNA served as an outgroup control and showed a different molecular signature of bands from the *Toxoplasma* isolates.

be readily distinguished from the X isolates (Fig. 3). The archetypal II and III isolates possessed a 0.4 kb band which was determined in previous studies (Guo et al., 1997) to be a diagnostic band capable of discriminating virulent from avirulent strains of *T. gondii*. Our analyses likewise showed that none of the four Type I virulent strains possessed the 0.4 kb band (Fig. 3; data not shown). The four Type II sea otter isolates (only SO 3636 and SO 3087 shown in Fig. 3) gave the same molecular banding pattern as archetypal Type II strains. All 20 X isolates possessed the 0.8 kb band, and none possessed the upper 1.4 kb band (data for 10 are shown in Fig. 3). Nine of the 20 X isolates possessed bands at 0.4 and 0.75 kb (data for 5 shown in Fig. 3). What was particularly interesting was the ability to detect genetic variability within the X isolates using the C8 marker. Otter isolate 3387 possessed a unique band at 1.3 kb, while otter isolates 3503 and 3458 had a series of reproducible bands between 1 and 1.4 kb and were distinct from all other isolates. These results strongly suggest that the X isolates are more genetically diverse than the archetypal lines, at least using the C8 RAPD marker. *Neospora* was included as an outgroup, and RAPD amplification of this genomic DNA preparation yielded a molecular signature distinct from that seen with *Toxoplasma* genomic DNA.

6. Insights into *T. gondii* transmission from sea otter studies

Results from these recent studies of toxoplasmosis in southern sea otters provide some valuable clues to better

understand the risks of *T. gondii* transmission to humans, as well as wildlife. The case has been made that since sea otters do not prey on known intermediate hosts for *T. gondii*, the most likely source of their infection is by ingestion of environmentally resistant oocysts that are shed in the feces of felids and transported via freshwater runoff into the marine ecosystem. Concentration of oocysts within filter-feeding marine bivalves that serve as prey may play a role in transmission; however, this may not be the only mechanism by which otters acquire infections, as discussed above. If oocysts are the primary or exclusive source of infection, then the high seroprevalence and mortality rates due to *T. gondii* infection in otters indicates that oocyst contamination of the terrestrial, freshwater and marine environments may be greater than presently presumed by the general public and physicians. In the USA, routine surveillance for *T. gondii* infection in the human population does not occur. There appears to be very little awareness by the general public or physicians about the risks of infection resulting from environmental contamination with oocysts or by oocyst ingestion from contaminated water or bivalves, such as raw oysters or mussels (Jones et al., 2003b).

The identification of 'high risk' sites of *T. gondii* infection in sea otters provides an opportunity for more focused investigations into the ecology of *T. gondii* at these sites. Some of the important questions that should be addressed in these investigations include: (i) does exposure to oocysts that are shed in the feces of terrestrial felids entirely account for the high proportion of infected sea otters and if so, what factors affect transmission? (ii) can ecological models be developed to predict the risk of

T. gondii infection in cats, humans, and wildlife? and (iii) what is the relative impact of domestic cats, both owned and feral, as well as wild felids on environmental contamination with *T. gondii* oocysts and can that impact be reduced? These questions are of ever increasing relevance as domestic cat populations in many areas, including coastal California, continue to rise. Reliable scientific data and careful consideration is required to determine the potential impact of existing and proposed changes in how domestic cats are managed. For example, in all but a few communities in California (as in most areas globally), owned cats are not required to be licenced. Many domestic cats have extensive outdoor access which allows them to prey on wild rodents and birds that may be infected with *T. gondii*, as well as defecate in any convenient location. In addition, some brands of cat litter are now marketed as being 'ecologically friendly' and suitable for composting or flushing down the toilet. Oocysts may survive up to 18 months in soil under favourable conditions (Frenkel et al., 1975), and wastewater treatment practices are not designed to destroy the highly resistant oocysts of *T. gondii*. Until reliable methods to inactivate oocysts in wastewater are developed, feces disposed directly into the toilet or in flushable cat litter must also be investigated as a possible source of oocysts. Current recommendations are for cat owners to bag and dispose of cat feces in approved sanitary landfills where runoff is controlled (<http://www.seaotterresearch.org/>). Both research and education to inform the public of scientific findings will be required to reduce risk of *T. gondii* exposure.

The discovery that a high proportion of sea otters infected with the new Type X genotype of *T. gondii* raises many intriguing questions about the distribution and infectivity of Type X parasites in otters and other animals. Is the Type X genotype circulating as a particularly successful genotype in the marine ecosystem, or are humans and other terrestrial animals in these coastal areas equally susceptible and likewise infected with Type X strains? Interestingly, RFLP analysis at *SAG1* has identified a number of strains that bear an allele consistent with the Type X genotype in isolates collected from wild animals and two humans in the southeastern United States (Howe and Sibley, 1995). Whether these terrestrial isolates actually possess Type X genotypes identical to those infecting marine mammals is not yet clear. To establish whether Type X strains are widespread in nature, molecular characterisation of *T. gondii* isolates from a variety of hosts in different geographic locations will be required to provide the insight necessary to determine the ecology and transmission of this fascinating new genotype in this highly successful protozoan parasite. Hence, investigating the processes promoting *T. gondii* infections in sea otters will likely provide a better understanding of terrestrial parasite flow and the emergence of disease at the interface between wildlife, domestic animals and humans.

It is important to recognise that the source of infection for otters is unknown. Discovering the source and transmission

dynamics of *T. gondii* infection in the marine environment is critical if we are to better understand the parasite and its impact on otter survival. Because otters share the near-shore environment where humans recreate and harvest food, people are likewise potentially at risk of developing toxoplasmosis in the same way as otters. A reasonable and testable hypothesis is that *T. gondii* infection is via ingestion of environmentally resistant oocysts secreted by terrestrial felids, but this rationale presupposes that the intermediate host range for *T. gondii* is restricted to warm-blooded animals. The question of whether non-mammal marine species could also serve as intermediate hosts for *T. gondii* replication is unknown. No reports have been published whether it is possible for *T. gondii* to infect and/or replicate in fish, amphibians, invertebrates and/or other aquatic animals in either freshwater or marine ecosystems. If these atypical hosts were shown to be sources of infection for otters, then it would be possible to invoke a marine transmission cycle as a potential source for the high rates of *T. gondii* seropositive marine animals.

Sea otters are only one of many animals susceptible to infection and disease with *T. gondii*. However, as a flagship wildlife species and an icon of coastal California, the health of this population is of public concern. Therefore, these charismatic animals have helped draw widespread attention to the disease risks associated with waterborne pathogen pollution and toxoplasmosis. The toxoplasmosis problem in sea otters provides a unique opportunity to inform the public about the impact of domestic animals, such as owned and feral cats, on the health of both wildlife and humans. The investment of federal and state funds to support health monitoring for threatened southern sea otters has made it possible to validate diagnostic tests, molecularly characterise novel pathogenic isolates and establish a comprehensive data base to support detailed investigations on the ecology of *T. gondii* at the wildlife–domestic animal–human interface on a scale that has not been possible previously. The outcome of these studies needs to focus on the application of new knowledge to develop strategies for the reduction of environmental contamination with oocysts and the risks of *T. gondii* exposure for wild and domestic animals, as well as humans.

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