



Type X *Toxoplasma gondii* in a wild mussel and terrestrial carnivores from coastal California: New linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters

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Abstract

Sea otters in California are commonly infected with *Toxoplasma gondii*. A unique Type X strain is responsible for 72% of otter infections, but its prevalence in terrestrial animals and marine invertebrates inhabiting the same area was unknown. Between 2000 and 2005, 45 terrestrial carnivores (lions, bobcats, domestic cats and foxes) and 1396 invertebrates (mussels, clams and worms) were screened for *T. gondii* using PCR and DNA sequencing to determine the phylogeographic distribution of *T. gondii* archetypal I, II, III and Type X genotypes. Marine bivalves have been shown to concentrate *T. gondii* oocysts in the laboratory, but a comprehensive survey of wild invertebrates has not been reported. A California mussel from an estuary draining into Monterey Bay was confirmed positive for Type X *T. gondii* by multilocus PCR and DNA sequencing at the *BI* and *SAG1* loci. This mussel was collected from nearshore marine waters just after the first significant rainfall event in the fall of 2002. Of 45 carnivores tested at the *BI*, *SAG1*, and *GRA6* typing loci, 15 had PCR-confirmed *T. gondii* infection; 11 possessed alleles consistent with infection by archetypal Type I, II or III strains and 4 possessed alleles consistent with Type X *T. gondii* infection. No non-canonical alleles were identified. The four *T. gondii* strains with Type X alleles were identified from two mountain lions, a bobcat and a fox residing in coastal watersheds adjacent to sea otter habitat near Monterey Bay and Estero Bay. Confirmation of Type X *T. gondii* in coastal-dwelling felids, canids, a marine bivalve and nearshore-dwelling sea otters supports the hypotheses that feline faecal contamination is flowing from land to sea through surface runoff, and that otters can be infected with *T. gondii* via consumption of filter-feeding marine invertebrates.

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Keywords: *Toxoplasma gondii*; Type X; *BI*; 18S rDNA; *SAG1*; *GRA6*; Otter; Felid; Mussel

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1. Introduction

The cosmopolitan parasite *Toxoplasma gondii* infects a wide range of intermediate hosts, including rodents, birds and mammals. The only animals known to serve as definitive hosts are wild and domestic felids (Tenter et al., 2000). *T. gondii* commonly infects marine mammals and is a pathogen of southern sea otters (*Enhydra lutris nereis*) and cetaceans (dolphins, porpoises and whales), causing central nervous system impairment, behavioural changes, abortion and foetal or neonatal death (Dubey et al., 2003b; Miller, 2008). A recent increase in pathogen-focused environmental research has contributed to enhanced public awareness of biological pollution impacting freshwater and coastal marine ecosystems (Fayer et al., 2004; Conrad et al., 2005; Miller et al., 2005a; Miller et al., 2006b). These include a number of well-documented, water-associated outbreaks of toxoplasmosis impacting humans Benenson et al., 1982; Bowie et al., 1997; Aramini et al., 1999; Tenter et al., 2000; Bahia-Oliveira et al., 2003; Dawson, 2005; de Moura et al., 2006; Palanisamy et al., 2006).

The high prevalence of *T. gondii* infection reported in marine species was unexpected, given that these animals rarely consume recognised intermediate hosts. It suggests that significant environmental exposure may be occurring via water contaminated with environmentally-resistant *T. gondii* oocysts shed in the faeces of infected cats. Soil contamination by *T. gondii* oocysts has been reported from areas where domestic cats are endemic (Ruiz and Frenkel 1980; Dabritz et al. 2007). Oocysts appear to be entering the nearshore marine environment through freshwater runoff (Miller et al., 2002b; Fayer et al., 2004). Upon reaching the ocean, these oocysts may be concentrated by filter-feeding invertebrates, thus serving as a source of infection for marine wildlife (Lindsay et al., 2001; Arkush et al., 2003). In the laboratory, California mussels (*Mytilus californianus*) and eastern oysters (*Crassostrea virginica*) have been shown to remove *T. gondii* oocysts from contaminated water through filter-feeding activity (Lindsay et al., 2001; Arkush et al., 2003; Lindsay et al., 2004). Oocysts taken up by these bivalves remained infectious for laboratory mice and were detectable by PCR 21 days postexposure (Lindsay et al. 2001; Arkush et al., 2003).

Prior studies characterising the range of *T. gondii* genotypes infecting animals and humans have revealed the predominance of three archetypal genotypes, designated Type I, II and III (Howe and Sibley, 1995; Grigg et al., 2001a; Su et al., 2003). The vast majority of human infections are caused by Type II *T. gondii* strains, which are abundant in nature and have a worldwide distribution (Howe and Sibley, 1995; Dardé, 1996). However, reports of non-archetypal, “atypical” genotypes have increased in recent years as a wide range of unique and comparatively pristine environmental niches have been explored (Grigg and Suzuki, 2003; Ajzenberg et al., 2004; Ferreira et al., 2004; Miller et al., 2004; Su et al., 2006). In these areas, atypical forms of *T. gondii* often dominate, in contrast to archetypal

lineages that are common in humans, domestic livestock and pets from more developed areas (Dubey et al., 2003a, 2004, 2005).

Recently, over 72% of *T. gondii*-positive southern sea otters from coastal California were found to be infected with an non-archetypal strain, designated Type X *T. gondii* (Conrad et al., 2005). To evaluate whether Type X *T. gondii* also infects terrestrial animals living along the adjacent shoreline, we tested tissues collected from animals recovered opportunistically along the central California coast between 2000 and 2005. We also tested marine and estuarine invertebrates obtained from coastal locations that were previously reported as high-risk areas for sea otter infection with *T. gondii*. Detection and genotyping of *T. gondii* was accomplished via multilocus PCR–RFLP analysis at the 18 S rDNA, *BI*, *SAG1* and *GRA6* loci, followed by DNA sequencing of *BI*, *SAG1* and/or *GRA6* amplicons for comparison with published *T. gondii* sequences. Fifteen terrestrial carnivores and a wild California mussel were confirmed positive for *T. gondii*. A range of *T. gondii* genotypes were detected in these animals, including the Type X clade that dominates in southern sea otters.

2. Materials and methods

2.1. Sample collection, serology and parasite isolation from terrestrial carnivores

Between 2000 and 2005, random-source terrestrial carnivores residing within 100 km of the California coast adjacent to the southern sea otter range were submitted for necropsy by collaborating agencies. Causes of death for animals enrolled in the study included vehicular collision, oil spill-associated mortality and euthanasia due to public safety concerns or depredation on livestock, pets or threatened and endangered wildlife. All animals were sampled opportunistically; none were euthanized for this study. Gross necropsy was performed, and blood and tissue samples were frozen and collected in formalin.

Sera from necropsied animals were tested for the presence of IgG to *T. gondii* and *Neospora caninum* via indirect immunofluorescent antibody testing (IFAT) as previously described (Miller et al., 2002a). Sera from wild canids were tested using FITC-conjugated goat anti-canine IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania); sera from felids were tested using FITC-conjugated goat anti-cat IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania); and sea and river otter sera were tested using FITC-conjugated goat anti-ferret IgG (Bethyl Laboratories Incorporated, Montgomery, Texas). For all samples testing positive, endpoint titres were determined as the highest serum dilution exhibiting distinct outline fluorescence of formalin-fixed tachyzoites.

Brain, pectoralis muscle or tongue and myocardium were processed for PCR amplification, DNA sequencing and *T. gondii* genotype analysis as described below. Asep-

tically-collected brain from nonfrozen animals was homogenised and layered over uninfected cell monolayers as described (Miller et al., 2001). Where available these *T. gondii* isolates were used for PCR detection and genotyping in addition to cryopreserved tissues. Tissues from some animals were formalin-fixed, trimmed and processed into H&E sections that were examined microscopically.

2.2. Collection and sampling of nearshore marine invertebrates

All invertebrates sampled in this study were native or introduced species obtained from nearshore marine waters along the central California coast. Two regions were selected for invertebrate sampling: the Elkhorn Slough estuary near Moss Landing in Monterey Bay (36.8158N,

121.7918W) and the Morro Bay estuary in Estero Bay (35.3428N, 120.8138W) (Fig. 1). Both regions were reported as high-risk sites for sea otter infection by *T. gondii* (Miller et al., 2002b; Kreuder et al., 2003). Invertebrates in each area were sampled near heavy local freshwater outflows and large populations of outdoor cats, as determined through telephone surveys, counts and visual confirmation of outdoor feeding stations (Dabritz et al., 2006).

Sentinel mussels (*M. californianus*) were deployed and collected for *T. gondii* testing as described (Miller et al., 2005b). Other invertebrates sampled in addition to mussels included sand crabs (*Emerita analoga*), gaper clams (*Tresus nuttallii*), Pismo clams (*Tivela stultorum*) and fat innkeeper worms (*Urechis caupo*). These invertebrates are all sea otter prey items that are filter-feeders (Arkush et al., 2003) or seine-type detritivores (Osovitz and Julian, 2002). Mussels

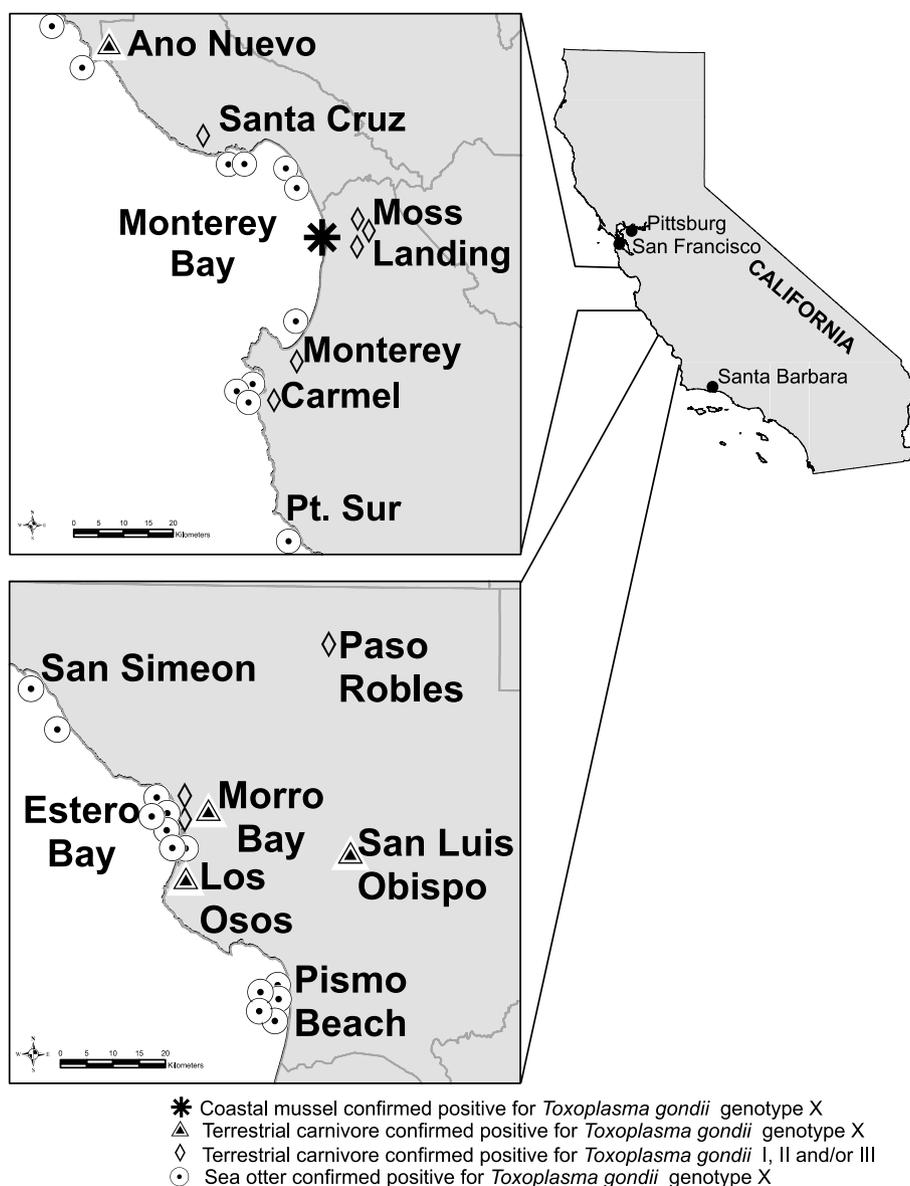


Fig. 1. Map of coastal California, showing sample locations and genotypes of *T. gondii* strains obtained from a wild mussel, terrestrial carnivores and sea otters.

were collected during the wet (November to May) and dry (June to October) seasons between 2002 and 2004; all other invertebrates were sampled only during the wet season. Pelletized hemocytes and digestive gland were collected from bivalves and the digestive tract was obtained from crabs and fat innkeeper worms. All samples were cryopreserved as described (Miller et al., 2005b).

2.3. RNA extraction and *T. gondii* detection from invertebrates using the TaqMan assay

We previously developed and validated TaqMan PCR assays for detection of *T. gondii* 18S rDNA/RNA and *BI* genes using mussels experimentally exposed to oocysts (Arkush et al., 2003). Based on results from this prior study, the TaqMan 18S rRNA assay was selected as the initial screening test for all wild invertebrates. RNA was extracted from digestive tissue (50 mg) or pelleted haemolymph (50–100 µl) and cDNA synthesised using published protocols (Arkush et al., 2003). Real-time PCR was carried out in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Samples were considered positive if the cycle threshold (C_T) value (i.e. the PCR cycle at which the fluorescence intensity exceeded the background intensity) was <40. Purified cDNA from invertebrates testing positive for *T. gondii* via the TaqMan assay was submitted to an independent laboratory for confirmation of PCR results and genotyping, as outlined below. Negative controls included PBS and haemolymph or digestive gland from known-negative mussels. Positive controls consisted of *T. gondii* oocysts and *T. gondii* tachyzoites (both genotype II) in haemolymph or PBS. An additional internal control consisted of purified *T. gondii* cDNA from a Type II isolate. A TaqMan PCR system (Clam18 TaqMan) was used to target the 18S rRNA gene of *M. californianus*, *M. galloprovincialis*, *M. californianus*, *M. edulis*, *M. trossulus*, *Geukensia denissa* and *Corbicula fluminea* as an endogenous control to assess tissue integrity and RNA extraction efficiency, as described (Miller et al., 2006a).

2.4. *Toxoplasma gondii* genotype determination

DNA was extracted from multiple 25 mg aliquots of brain and/or muscle or infected cell pellets from terrestrial carnivores and screened for *T. gondii* via PCR amplification and RFLP analysis of the *T. gondii* *BI*, *SAG1* and *GRA6* gene products, as described (Burg et al., 1989; Grigg and Boothroyd, 2001; Miller et al., 2004). A phenol/chloroform extraction of DNA was performed on tissue solubilised in 10 mM TRIS–EDTA–lithium chloride–Triton X-100 extraction buffer (Grigg et al., 2001b). Amplification and gel electrophoresis of the *T. gondii* *BI* gene locus was used for initial detection of *T. gondii*-infected animals (Grigg and Boothroyd, 2001). Each assay was run at least twice on separate aliquots of brain or muscle before an animal was considered negative, because the *T. gondii* tissue burdens were expected to be low in naturally-infected terrestrial

animals (Dubey et al., 1999). Samples testing positive at the *BI* locus were analysed by RFLP and DNA sequencing to establish the *T. gondii* genotype present. All *BI*-positive samples were then subjected to PCR amplification using nested primers at the single copy *SAG1* and *GRA6* loci to attempt multi-locus genotyping of infected tissues (Grigg and Boothroyd, 2001; Miller et al., 2004; Conrad et al., 2005). Two nested primer pairs were used for all PCR reactions and gDNA (carnivores) or cDNA (invertebrates) served as templates for the reaction. Samples spiked with purified gDNA or cDNA from well characterised *T. gondii* Type II strains served as positive controls. Negative controls consisted of deionized water and genomic DNA from PCR-negative terrestrial carnivores. Reaction mixtures and amplification conditions were as described (Miller et al., 2004). Amplification products were visualised using ethidium bromide-staining in a 1–2% agarose gel. Purified PCR product from each reaction was digested with restriction enzymes to identify diagnostic restriction fragment patterns on agarose gels. DNA sequence analysis was completed for selected cases to confirm the genotype present in each sample, as described (Miller et al., 2004).

3. Results

3.1. Terrestrial carnivores: sampling, molecular characterisation and genotyping

Forty-five coastal-dwelling terrestrial carnivores were sampled opportunistically between 2000 and 2005 (three bobcats, 26 mountain lions, five domestic cats, nine red foxes and two river otters), including a mixture of juveniles and adults of both sexes (Table 1). Most were in good nutritional condition and significant gross lesions were related to vehicular impact, petroleum product exposure or euthanasia. IFAT titres *T. gondii* ranged from <40 to 10,240, with titres ≥ 320 considered seropositive (Miller et al., 2002a). Over half of the animals (56%) were seropositive, and 69% had titres ≥ 160 (Table 1). Fifteen carnivores (33%) were PCR-positive for *T. gondii* using highly sensitive *BI* primers. Based on the serological data, additional animals were probably infected, but *T. gondii* was not detected, possibly due to low levels of parasitism and the limited mass of tissue that could be analysed using PCR. No carnivores had IFAT titres for *N. caninum* >40, nor were *N. caninum* infections identified by PCR using panspecific 18S rDNA primers (data not shown).

For the 15 carnivores with *BI* PCR-confirmed *T. gondii* infection, nine were submitted for parasite isolation and four yielded isolates in cell culture (Table 1). Animals positive for *T. gondii* by PCR–RFLP included 5/27 (18%) mountain lions, 2/3 (67%) bobcats, 3/7 (43%) domestic cats, 3/11 (27%) red foxes and 2/2 (100%) river otters. All 15 animals testing positive for *T. gondii* by *BI* gene amplification were also evaluated using the single copy *SAG1* and *GRA6* typing loci; only eight of these animals (53%) were positive at *GRA6* and four were positive at *SAG1* (Table 1), confirm-

Table 1
Results of serology, parasite isolation, DNA PCR and genotypic analysis for 45 terrestrial carnivores from coastal California (2000–2005)

<i>T. gondii</i> IFAT titre	Sample size	Species distribution	Animals PCR-positive for <i>T. gondii</i>	Nearest major bay	<i>T. gondii</i> observed on histopathology	Parasite isolated	DNA sequencing			<i>T. gondii</i> genotype
							B1	GRA6	SAG1	
10,240	1	Bobcat	Bobcat 1	Monterey	No	<i>T. gondii</i>	X	X	X	X
5120	3	Domestic cats (2), red fox	Domestic cat 1	Monterey	No	–	II or III	II	–	II
			Domestic cat 2	Monterey	No	–	II or III	II	–	II
2560	6	Mountain lions (3), red fox (2), domestic cat	Red fox 9	Estero	No	<i>T. gondii</i>	II or III	II	II	II
1280	2	Bobcat (2)	Bobcat 2	Monterey	No	nd	II or III	–	–	II or III
640	11	Mountain lions (9), red fox (2)	Mtn Lion 2	Estero	No	nd	X	nd	–	X
			Mtn Lion 7	Estero	No	–	II or III	II	–	II
			Red fox 6	Estero	No	nd	II or III	–	–	II or III
			Red fox 7	Estero	No	–	X	X	–	X
320	2	Mountain lion, red fox	Mtn lion 5	Monterey	No	nd	I	nd	–	I
160	6	Mountain lions (4), river otter, bobcat	River otter 1	San Francisco	Yes	<i>T. gondii</i>	II or III	II	II	II
80	4	Mountain lions (3), red fox	None	–	–	–	–	–	–	–
40	5	Mountain lions (4), river otter	Mtn lion 25	Monterey	No	nd	I	nd	–	I
			Mtn lion 15	Estero	No	nd	X	nd	–	X
			River otter 2	San Francisco	No	<i>T. gondii</i>	II or III	II	II	II
<40	5	Mountain lions (2), domestic cat (2), red fox	Domestic cat 3	Monterey	No	–	I	–	–	I
Total:	45		15							

nd = not done (carcass frozen, moderately decomposed or insufficient sample size).

“–”: negative.

ing that low concentrations of *T. gondii* DNA were present in the animal tissues. H&E-stained tissue sections were examined for all 15 PCR-positive animals, but *T. gondii* tissue cysts were only observed in tissues of one animal, a river otter. Most animals exhibited mild lymphadenopathy and lymphoplasmacytic meningitis or meningoencephalitis supportive of chronic *T. gondii* infection.

Based on multi-locus PCR-RFLP and DNA sequencing analyses using the *BI*, *SAG1* and *GRA6* loci (Table 1), the most common *T. gondii* strains identified infecting terrestrial carnivores were archetypal Type II strains ($n = 6$). Three carnivores were infected with *T. gondii* I strains that possessed Type alleles and two additional animals were likely infected with either Type II or Type III strains (these animals could not be assigned definitive genotypes because PCR amplification at the *GRA6* and *SAG1* loci was unsuccessful) (Table 1). Infections with Type X *T. gondii* strains were confirmed in four terrestrial carnivores: two mountain lions, a bobcat and a red fox. None of the seven domestic cats sampled in this study were positive for *T. gondii* strains that possessed Type X alleles and no carnivores were infected with more than one *T. gondii* genotype.

Recovery locations for the *T. gondii*-positive terrestrial carnivores are depicted in Fig. 1, along with stranding locations for selected *T. gondii* Type X- and Type II-infected sea otters recovered over the same area and time period. All terrestrial carnivores infected with Type II strains were recovered near Monterey Bay, but carnivores infected with strains bearing Type X alleles were recovered from both the Estero Bay and Monterey Bay regions. Three of the four Type X-infected terrestrial carnivores were found within 1 km of the ocean (Table 1).

3.2. Nearshore marine invertebrates: sampling, molecular characterisation and genotyping

Between 2002 and 2004, 1396 marine and estuarine invertebrates were sampled, including 530 from Monterey

Bay and 866 from Estero Bay. Sample sizes and spatial distributions of invertebrates screened for *T. gondii* are summarised in Table 2. In prior studies (Miller et al., 2005b; Miller et al., 2006b), the highest protozoal pathogen detection was noted for invertebrates collected during the rainy season, so wet season samples ($n = 1126$) were prioritized for *T. gondii* testing in the present study (81%). The majority of tested samples were bivalve, crab or worm digestive tract. Haemolymph was tested for an additional 222 mussels and 56 clams (Table 2), but all were negative for *T. gondii*. In contrast, C_T values for digestive tract from one outplanted mussel and one resident sand crab were positive for *T. gondii* ($<40 C_T$) by the TaqMan assay. The TaqMan-positive mussel was collected during the early rainy season (December) in Elkhorn Slough, near the town of Moss Landing in Monterey Bay (Fig. 1), while the positive sand crab was collected during the same period from Morro Bay Harbor in Estero Bay. No additional source material (digestive tract or haemolymph) was available from these invertebrates, so TaqMan testing was repeated using purified cDNA. The sand crab was above threshold levels for *T. gondii* on a second TaqMan assay and no sample remained for further evaluation. The mussel digestive gland remained PCR-positive on a second TaqMan assay. The presence of *T. gondii* DNA in the mussel tissue was confirmed by a second, independent laboratory and multi-locus PCR-RFLP analyses performed at the *BI* (digested with PmlI and XhoI) and *SAG1* (digested with HaeII and Sau96A) loci identified a Type X *T. gondii* RFLP pattern (Fig. 2). No amplification product was detected when *GRA6* PCR primers were applied. DNA sequencing of the *BI* and *SAG1* amplicons confirmed that the parasite present in the bivalve possessed identical sequences to Type X strains of *T. gondii* previously isolated from southern sea otters (Miller et al., 2004). Type X *T. gondii* had not been handled in the laboratory where RNA extraction, cDNA preparation and TaqMan amplification was performed prior to this study.

Table 2

Geographic and temporal distributions of California coastal marine invertebrates tested for *T. gondii* using PCR and genotypic analysis

Invertebrate species	Sampling period			Total
	Wet season, 2002–2003	Dry season 2003	Wet season, 2003–2004	
<i>Mussels:</i>				
Estero Bay	216(12) ^a	150(90) ^a	368	734
Monterey Bay	135 ^b	120(120) ^a	120	375
Total mussels:	351	270	488	1,109
<i>Other invertebrates:</i>				
Estero Bay	117 ^b (13) ^a	0	15	132
Monterey Bay	140(43) ^a	0	15	155
Total, other invertebrates:	257	0	30	287
Total, all invertebrates:(all locations)	608	270	518	1396

^a Number of samples (in parentheses) from each group evaluated using haemolymph instead of digestive tract.

^b Groups with a single sample testing positive for *T. gondii* using the TaqMan PCR assay. The positive samples were digestive tract from a sand crab (Morro Bay) and a bay mussel (moss landing). The sand crab was negative for *T. gondii* on subsequent testing. However, the mussel was confirmed positive for *T. gondii*.

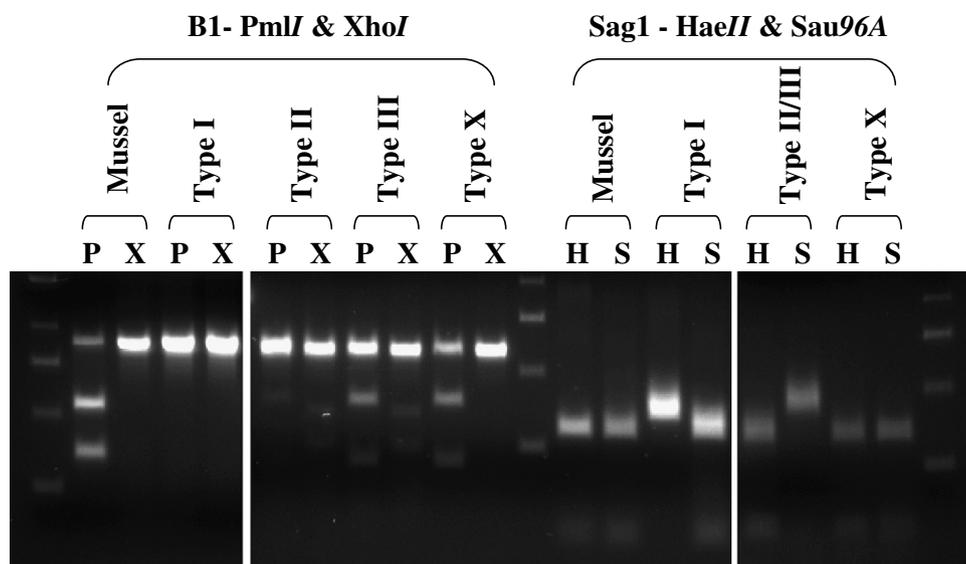


Fig. 2. PCR detection and genotyping of *T. gondii* from digestive gland of a wild California mussel (*M. californianus*). *B1* and *SAG1* PCR–RFLP analyses distinguish Type X *T. gondii* alleles from archetypal Type I, II and III strain alleles. mRNA isolated from a wild California mussel was converted to cDNA and subjected to two rounds of PCR amplification using nested sets of primers against the *T. gondii* *B1* and *SAG1* genes. Parasite DNA amplified using *B1* primers was digested with PmlI (P) and XhoI (X), whereas DNA amplified using *SAG1* primers was digested with Hae II (H) and Sau 96A (S) prior to separation in 1% ethidium bromide-stained agarose gels for visualization. Representative digests of Type I, II, III, and X strains are shown against the digests from the *T. gondii* PCR-amplified products derived from the California mussel. The mussel possessed a Type X RFLP pattern at both loci, and DNA sequencing confirmed the existence of canonical Type X alleles.

4. Discussion

Here we report the first confirmation of Type X *T. gondii* in terrestrial carnivores and a wild mussel occupying the same region of coastal California as Type X-infected southern sea otters. Forty-five terrestrial carnivores were screened for *T. gondii* using PCR–RFLP and DNA sequencing. *T. gondii* infection was found to be common, with 56% of animals having *T. gondii* IFAT titres ≥ 320 and 33% with PCR-confirmed *T. gondii* infections. Archetypal Type I, II and/or III strains were identified in 11/15 (73%) PCR-positive carnivores. Type X strains, known to cause most *T. gondii* infections in threatened southern sea otters (Miller et al., 2004; Conrad et al., 2005) were also identified in 4/15 (27%) terrestrial carnivores. Three of the Type X-infected carnivores were wild felids (two mountain lions and a bobcat), but no domestic cats were Type X-positive. Examination of larger samples of wild and domestic felids will help clarify these initial findings. If Type X strains are detected more commonly from wild felids in subsequent studies, this could suggest that these animals are more important land-based sources of *T. gondii* for marine wildlife than are domestic cats. In a large-scale outbreak of human toxoplasmosis in Pacific coastal Canada, exposure to *T. gondii* in drinking water was linked to oocyst-shedding by wild felids (Bowie et al., 1997; Aramini et al., 1999).

Based on serological data, several carnivores had high antibody titres to *T. gondii*, but brain tissue was negative by parasite isolation and PCR. This finding is not surprising, given the comparatively low pathogenicity of *T. gondii*

for North American felids and canids (Dubey and Beattie, 1988; Dubey 1993; Dubey et al., 1999) and the small tissue volumes that can be analysed using PCR. Our success rate for isolation of *T. gondii* from brain tissue was also low for these terrestrial animals (12.5%), when compared to equivalent sea otter tissues (35%) processed in the same laboratory (data not shown). The low parasite tissue burden in these terrestrial carnivores was also confirmed by histopathological examination: Of 14 PCR-positive animals examined microscopically, only one had visible *T. gondii* parasites.

The mild climate of the central California coast may help contribute to the high prevalence of *T. gondii* infection reported for marine mammals residing just offshore by facilitating oocyst survival. In addition, this region is bordered by mountains that receive ≥ 75 in. (≥ 190 cm) of precipitation/year (Miller et al., 2002b). Most local watersheds have been heavily impacted by human development, with reductions in natural wetlands that buffer surface water flow and reduce particulate discharges. In addition, extensive conversion of the nearshore landscape to impervious surfaces (streets, roads, sidewalks and parking lots) and increased density of pet and feral cats may accompany coastal urbanization. Past research has documented enhanced aqueous transport of pet waste from urbanised communities with increased hardscape (Young and Thackston, 1999; Mallin et al., 2000). In one recent study, domestic feline faecal deposition in communities adjacent to Estero Bay was conservatively estimated at 107 metric tonnes/year, or 26 kg/ha (Dabritz et al., 2006). Using data from homeowner surveys and faecal transects, Dabritz

et al. (2007) estimated that 29–244 billion oocysts are shed by domestic cats annually in the Estero Bay region, which translates to an annual deposition of 94–434 oocysts/m².

Marine invertebrates were examined for the presence of *T. gondii* oocysts to help trace potential sources and pathways for *T. gondii* oocyst infection of marine wildlife. Bivalves represent a major prey item for southern sea otters and are efficient concentrators of suspended particulate matter, when present in faecally contaminated coastal freshwater runoff (Roper and Hickey, 1995). Nearshore-dwelling marine and estuarine invertebrates serve as efficient biological filters for *T. gondii*, *Cryptosporidium* and *Giardia* oocysts or cysts (Lindsay et al., 2001; Arkush et al., 2003; Fayer et al., 2004; Miller et al., 2006a). In the laboratory, *T. gondii* oocysts accumulate and remain infective in tissues, digestive gland and haemolymph of bivalves, including oysters, clams and mussels that may be consumed raw or undercooked by humans (Lindsay et al., 2001; Arkush et al., 2003; Fayer et al., 2004; Lindsay et al., 2004).

In this study, one of 1396 (0.007%) wild invertebrates collected between 2002 and 2004 was confirmed positive for *T. gondii* by PCR–RFLP and DNA sequencing: Type X *T. gondii* was identified in the digestive gland of a California mussel by TaqMan and conventional PCR techniques. The PCR-positive mussel was collected from Elkhorn Slough, a known “high-risk” area for *T. gondii* infection in sea otters (Miller et al., 2002b). Type X *T. gondii* was also detected in terrestrial carnivores living near Estero Bay, a second area of increased risk for sea otter infection by *T. gondii*, especially infection by Type X strains (Kreuder et al., 2003; Miller et al., 2004). Conversely, the predominance of archetypal Type II *T. gondii* infections in terrestrial carnivores residing near Monterey Bay is the same spatial trend identified for sea otters in a prior study (Miller et al., 2004).

A sand crab was also positive on initial TaqMan testing, but the presence of *T. gondii* was not confirmed with available sample material. Interestingly, both the crab and the mussel were collected in early December, just after the first seasonal flush of faecally-laden freshwater runoff. In addition, both invertebrates were obtained from partially enclosed embayments characterised by prolonged stormwater retention due to restricted tidal exchange (Elkhorn Slough/Moss Landing and Morro Bay – Fig. 1). Collectively, these findings suggest that future efforts to detect *T. gondii* in wild invertebrates should emphasise samples collected (1) within or near embayments and (2) during the early wet season, soon after the “first flush” of faecally-laden stormwater runoff reaches the ocean. Similar findings were reported for detection of *Cryptosporidium*, *Giardia* and faecal bacteria in wild mussels and other invertebrates from the same region in prior studies (Miller et al., 2006a; Miller et al., 2006b).

The low prevalence of confirmed, *T. gondii* PCR-positive wild invertebrates detected in this study suggests that the probability of an otter becoming infected with *T. gondii*

through prey ingestion is low. However, several unique attributes of sea otter biology may contribute substantially to this risk: In addition to a tendency to feed directly along the shoreline, often within or adjacent to stormwater discharges, southern sea otters consume 25% of their body weight in prey each day (Reidman and Estes, 1990), or about 2190 kg/otter/year for each adult (24 kg) otter. Medium-sized, California mussels average 40 g (data not shown). If mussels or similar bivalves represented 50% of a sea otter’s diet, each otter would consume at least 76 mussels/day, or 27,582/year. Multiplying this yearly bivalve consumption by the proportion of wild mussels confirmed positive for *T. gondii* in the present study (0.007%), provides a conservative estimate of 60 *T. gondii*-contaminated mussels consumed yearly by each adult California otter, or perhaps 600 or more over an animal’s lifespan. Ingestion of as little as one *T. gondii* oocyst is capable of causing infection in terrestrial animals (Dubey et al., 1996, 1997). Thus, the potential for contaminated invertebrate prey to serve as an efficient vehicle for exposure of southern sea otters to *T. gondii* is biologically plausible. Recent studies highlighting the tendency for marine fish, amphipods, worms, sea urchins, bivalves and barnacles to bioconcentrate viruses (Enriquez et al., 1992), bacteria Miller et al., 2006b, diatoms and algae (Lefebvre et al., 1999), plastic debris (Thompson et al., 2004) and even toilet paper fibres (Delmas and Regis, 1986) underscore the diverse, but highly important pathways for trophic transfer of particulate pathogens like *T. gondii* to marine vertebrates.

As expected, application of the previously validated *T. gondii* BI TaqMan assay (Arkush et al., 2003) to testing naturally-exposed invertebrates posed significant technical challenges, including PCR inhibition by shellfish tissues, inter-area variation in mussel filtration and diet and regional differences in water quality, flow and sedimentation. Other factors that could have impacted assay performance include differences in oocyst age, sporulation, viability and genotype. Finally, because of environmental dilution, the concentration of *T. gondii* oocysts encountered by wild invertebrates in surrounding water is certainly much lower than for the laboratory-exposed bivalves that were utilised for test validation. The development and application of oocyst- and cyst-specific antibodies have greatly facilitated the detection of related protozoans *Cryptosporidium* and *Giardia* in filter-feeding invertebrates and environmental samples Pereira et al., 1999; Miller et al., 2005a; Miller et al., 2006a). Similar reagents are not yet available to facilitate detection of *T. gondii*.

As high-capacity consumers of nearshore marine invertebrates, California sea otters are ideal upper trophic level sentinels of particulate pathogen exposure. The detection of high levels of biological pathogens in sea otters is concerning, both for the conservation of a federally protected, threatened species and because sea otters may be serving as highly sensitive indicators of under-recognised health risks to humans from consumption of raw or undercooked

marine foods. Precise tracing of pathogen flow from land to sea is challenging because of the diverse processes affecting microbial dissemination. However, in this study, temporal and spatial connections were established for Type X *T. gondii* infections of terrestrial and marine wildlife. The non-archetypal strain of *T. gondii* that dominates in local sea otters has now been identified in both coastal-dwelling terrestrial carnivores and a wild marine invertebrate from the same environment. The putative land-to-sea flow of *T. gondii* and related pathogens highlights the importance of both monitoring and mitigating biological pathogen transfer originating from nonpoint source pollution.

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References

- Ajzenberg, D., Bañuls, A.L., Su, C., Dumetre, A., Demar, M., Carme, B., Dardé, M.L., 2004. Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *Int. J. Parasitol.* 34, 1185–1196.
- 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, 305–315.
- Arkush, K.D., Miller, M.A., Leutenegger, C.M., Gardner, I.A., Packham, A.E., Heckerth, A.R., Tenter, A.M., Barr, B.C., Conrad, P.A., 2003. Molecular and bioassay-detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*). *Int. J. Parasitol.* 33, 1087–1097.
- Bahia-Oliveira, L.M., Jone, G., Azevedo-Silva, L.M., Alves, C.C.F., Orefice, F., Addiss, D.G., 2003. Highly endemic, waterborne toxoplasmosis in north Rio de Janeiro state, Brazil. *Emerg. Infect. Dis.* 9, 55–62.
- Benenson, M.W., Takafuji, E.T., Lemon, S.M., Greenup, R.L., Sulzer, A.J., 1982. Oocyst-transmitted toxoplasmosis associated with ingestion of contaminated water. *N. Engl. J. Med.* 307, 666–669.
- 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, 173–177.
- Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. *J. Clin. Microbiol.* 27, 1787–1792.
- Conrad, P.A., Miller, M.A., Kreuder, C., James, E.R., Mazet, J., Dabritz, H., Jessup, D., Gulland, F., Grigg, M., 2005. Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. *Int. J. Parasitol.* 35, 1155–1168.
- Dabritz, H.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Conrad, P.A., 2006. Outdoor fecal deposition by free-roaming cats and attitudes of cat owners and nonowners toward stray pets, wildlife, and water pollution. *J. Am. Vet. Med. Assoc.* 229, 74–81.
- Dabritz, H.A., Miller, M.A., Atwill, E.R., Gardner, I.A., Leutenegger, C.M., Melli, A.C., Conrad, P.A., 2007. Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *J. Am. Vet. Med. Assoc.* 231, 1676–1684.
- Dardé, M.L., 1996. Biodiversity in *Toxoplasma gondii*. *Curr. Top. Microbiol. Immunol.* 219, 27–41.
- Dawson, D., 2005. Foodborne protozoal parasites. *Int. J. Food Microbiol.* 103, 207–227.
- de Moura, L., Bahia-Oliveira, L.M., Wada, M.Y., Jones, J.L., Tuboi, S.H., Carmo, E.H., Ramalho, W.M., Camargo, N.J., Trevisan, R., Graca, R.M., da Silva, A.J., Moura, I., Dubey, J.P., Garrett, D.O., 2006. Waterborne toxoplasmosis, Brazil, from field to gene. *Emerg. Infect. Dis.* 12, 326–329.
- Delmas, P., Regis, M.B., 1986. Preliminary findings on the contents of the edible urchin *Paracentrotus lividus* (Lamarck) when subjected to the influence of domestic effluents [Donnés préliminaires sur le contenu digestif de l'oursin comestible *Paracentrotus lividus* (Lamarck) soumis à l'influence d'effluents domestiques]. *Mar. Environ. Res.* 20, 197–220.
- Dubey, J.P., 1993. *Toxoplasma*, *Neospora*, *Sarcocystis* and other tissue cyst-forming coccidia of humans and animals, second ed.. In: Krier, J.P. (Ed.), *Parasitic Protozoa*, vol. 6 Academic Press, Inc., San Diego, CA, p. 158.
- Dubey, J.P., Beattie, C.P., 1988. *Toxoplasmosis of Animals and Man*. CRC Press, Boca Raton, Florida (pp. 220).
- Dubey, J.P., Lunney, J.K., Shen, S.K., Kwok, O.C., Ashford, D.A., Thulliez, P., 1996. Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. *J. Parasitol.* 82, 438–443.
- Dubey, J.P., Speer, C.A., Shen, S.K., Kwok, O.C., Blixt, J.A., 1997. Oocyst-induced murine toxoplasmosis: life cycle, pathogenicity, and stage conversion in mice fed *Toxoplasma gondii* oocysts. *J. Parasitol.* 83, 870–882.
- Dubey, J.P., Storandt, S.T., Kwok, O.C.H., Thulliez, P., Kazacos, K.R., 1999. *Toxoplasma gondii* antibodies in naturally exposed wild coyotes, red foxes, and gray foxes and serologic diagnosis of toxoplasmosis in red foxes fed *T. gondii* oocysts and tissue cysts. *J. Parasitol.* 85, 240–243.
- Dubey, J.P., Graham, D.H., Dahl, E., Streekmar, C., Lehman, T., Davis, M.F., Morishita, T.Y., 2003a. *Toxoplasma gondii* isolates from free-ranging chickens from the United States. *J. Parasitol.* 89, 1060–1062.
- Dubey, J.P., Zarnke, R.L., Thomas, N.J., Wong, S.K., Van Bonn, W., Briggs, M., Davis, J.W., Ewing, R., Mense, M., Kwok, O.C., Romand, S., Thulliez, P., 2003b. *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. *Vet. Parasitol.* 116, 275–296.
- Dubey, J.P., Salant, H., Streekmar, C., Dhal, E., Vianna, M.C., Shen, S.K., Kwok, O.C., Spira, D., Hamburger, J., Lehmann, T.V., 2004. High prevalence of *Toxoplasma gondii* in a commercial flock of chickens in Israel, and public health implications of free-range farming. *Vet. Parasitol.* 121, 317–322.
- Dubey, J.P., Graham, D.H., De Young, R.W., Dahl, E., Eberhard, M.L., Nace, E.K., Won, K., Bishop, H., Punksody, G., Streekmar, C., Vianna, M.C., Shen, S.K., Kwok, O.C., Summers, J.A., Demarais, S., Hill, D.E., Chirukandoth, S., Dubey, J.P., 2005. Biology and epide-

- miology of *Toxoplasma gondii* in man and animals. *Anim. Health Res. Rev.* 6, 41–61.
- Enriquez, R., Frösner, G.G., Hochstein-Mintzel, V., Riedemann, S., Reinhardt, G., 1992. Accumulation and persistence of hepatitis A virus in mussels. *J. Med. Virol.* 37, 174–179.
- Fayer, R., Dubey, J.P., Lindsay, D.S., 2004. Zoonotic protozoa: from land to sea. *Trends Parasitol.* 20, 531–536.
- Ferreira, A.M., Vitor, R.W., Carneiro, A.C., Brandao, G.P., Melo, M.N., 2004. Genetic variability of Brazilian *Toxoplasma gondii* strains detected by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) and simple sequence repeat anchored PCR (SSR-PCR). *Infect. Genet. Evol.* 4, 131–142.
- Grigg, M.E., Boothroyd, J.C., 2001. Rapid identification of virulent Type I strains of the protozoan pathogen *Toxoplasma gondii* by PCR-restriction fragment length polymorphism analysis at the *BI* gene. *J. Clin. Microbiol.* 39, 398–400.
- Grigg, M.E., Suzuki, Y., 2003. Sexual recombination and clonal evolution of virulence in *Toxoplasma*. *Microbes and Infect.* 5, 685–690.
- Grigg, M.E., Bonnefoy, S., Hehl, A.B., Suzuki, Y., Boothroyd, J.C., 2001a. Success and virulence in *Toxoplasma* as the result of sexual recombination between two distinct ancestries. *Science* 294, 161–165.
- Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001b. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. *J. Infect. Dis.* 184, 633.
- Howe, D.K., Sibley, L.D., 1995. *Toxoplasma gondii* comprises three clonal lineages: correlation of parasite genotype with human disease. *J. Infect. Dis.* 172, 1561–1566.
- Kreuder, C., Miller, M.A., Jessup, D.A., Lowenstine, L.J., Harris, M.D., Ames, J.A., Carpenter, T.E., Conrad, P.A., Mazet, J.A., 2003. Recent patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2001. *J. Wildl. Dis.* 39, 495–509.
- Lefebvre, K.A., Powell, C.L., Busman, M., Doucette, G.J., Moeller, P.D., Silver, J.B., Hughes, M.P., Singaram, S., Silver, M.W., Tjeerdema, R.S., 1999. Detection of domoic acid in northern anchovies and California sea lions associated with an unusual mortality event. *Nat. Toxins* 7, 85–92.
- Lindsay, D.S., Phelps, K.K., Smith, S.A., Flick, G., Dubey, J.P., 2001. Removal of *Toxoplasma gondii* oocysts from seawater by eastern oysters (*Crassostrea virginica*). *J. Eukaryot. Microbiol.*, 197S–198S.
- Lindsay, D.S., Collins, M.V., Mitchell, S.M., Welch, C.N., Rosypal, A.C., Flick, G.J., Zajac, A.M., Lindquist, A., Dubey, J.P., 2004. Survival of *Toxoplasma gondii* oocysts in eastern oysters (*Crassostrea virginica*). *J. Parasitol.* 90, 1054–1057.
- Mallin, M.A., Williams, K.E., Esham, E.C., Lowe, R.P., 2000. Effect of human development on bacteriological water quality in coastal watersheds. *Ecol. Appl.* 10, 1047–1056.
- Miller, M.A., 2008. Tissue cyst-forming coccidia of marine mammals. In: Fowler, M., Miller, E. (Eds.), *Zoo and Wild Animal Medicine, Current Therapy*, sixth ed., W.B. Saunders Press. Philadelphia, PA, pp. 319–340.
- 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 two parasitic protozoa from a pacific harbor seal (*Phoca vitulina richardsi*) with meningoencephalomyelitis. *J. Parasitol.* 87, 816–822.
- Miller, M.A., Gardner, I.A., Packham, A., Mazet, J.K., Hanni, K.D., Jessup, D.A., Estes, J., Jameson, R., Dodd, E., Barr, B.C., Lowenstine, L.J., Gulland, F.M., Conrad, P.A., 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.
- Miller, M.A., Gardner, I.A., Kreuder, C., Paradies, D.M., Worcester, K.R., Jessup, D.A., Dodd, E., Harris, M.D., Ames, J.A., Packham, A.E., Conrad, P.A., 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.
- Miller, M.A., Grigg, M.E., Kreuder, C., James, E.R., Melli, A.C., Crosbie, P.R., Jessup, D.A., Boothroyd, J.C., Brownstein, D., Conrad, P.A., 2004. An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. *Int. J. Parasitol.* 34, 275–284.
- Miller, W.A., Atwill, E.R., Gardner, I.A., Miller, M.A., Fritz, H.M., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2005a. Clams (*Corbicula fluminea*) as bioindicators of fecal contamination with *Cryptosporidium* and *Giardia* spp. in freshwater ecosystems of California. *Int. J. Parasitol.* 35, 673–684.
- Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, E.R., Harris, M.D., Ames, J.A., Jessup, D.A., Melli, A.C., Paradies, D., Worcester, K., Olin, P., Barnes, N., Conrad, P.A., 2005b. New genotypes and factors associated with *Cryptosporidium* detection in mussels, (*Mytilus* spp.) along the California coast. *Int. J. Parasitol.* 35, 1103–1113.
- Miller, W.A., Gardner, I.A., Leutenegger, C.M., Miller, M.A., Atwill, E.R., Hedrick, R.P., Melli, A.C., Barnes, N.M., Conrad, P.A., 2006a. Evaluation of methods for improved detection of *Cryptosporidium* spp. in mussels (*Mytilus californianus*). *J. Microbiol. Methods* 65, 367–379.
- Miller, W.A., Miller, M.A., Gardner, I.A., Atwill, R.E., Byrne, B., Jang, S.S., Harris, M., Ames, J., Jessup, D.A., Paradies, D., Worcester, K., Melli, A.C., Conrad, P.A., 2006b. *Salmonella* spp., *Vibrio* spp., *Clostridium perfringens*, and *Plesiomonas shigelloides* in marine and freshwater invertebrates from coastal California ecosystems. *Microb. Ecol.* 52, 198–206.
- Osovitz, C.J., Julian, D., 2002. Burrow irrigation behavior of *Urechis caupo*, a filter-feeding marine invertebrate, in its natural habitat. *Mar. Ecol.-Prog. Ser.* 245, 149–155.
- Palanisamy, M., Madhavan, B., Balasundaram, M.B., Andavar, R., Venkatapathy, N., 2006. Outbreak of ocular toxoplasmosis in Coimbatore, India. *Indian J. Ophthalmol.* 54, 129–131.
- Pereira, M.D.G., Atwill, E.R., Jones, T., 1999. Comparison of sensitivity of immunofluorescent microscopy to that of a combination of immunofluorescent microscopy and immunomagnetic separation for detection of *Cryptosporidium parvum* in adult bovine feces. *Appl. Environ. Microbiol.* 65, 3226–3239.
- Roper, D.S., Hickey, C.W., 1995. Effects of food and silt on filtration, respiration and condition of the freshwater mussel *Hyridella menziesi* (Unionacea: Hyriidae): Implications for bioaccumulation. *Hydrobiologia* 312, 17–25.
- Ruiz, A., Frenkel, J.K., 1980. *Toxoplasma gondii* in Costa Rican cats. *Am. J. Trop. Med. Hyg.* 2, 1150–1160.
- Su, C., Evans, D., Cole, R., Kissinger, J., Ajioka, J., Sibley, L., 2003. Recent Expansion of *Toxoplasma* through enhanced oral transmission. *Science* 299, 414–416.
- Su, C., Zhang, X., Dubey, J.P., 2006. Genotyping of *Toxoplasma gondii* by multilocus PCR–RFLP markers: a high resolution and simple method for identification of parasites. *Int. J. Parasitol.* 36, 841–848.
- Tenter, A.M., Heckeroth, A.R., Weiss, L.M., 2000. *Toxoplasma gondii*: from animals to humans. *Int. J. Parasitol.* 30, 1217–1258.
- Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., McGonigle, D., Russell, A.E., 2004. Lost at sea: where is all the plastic? *Science* 304, 838.
- Young, K.D., Thackston, E.L., 1999. Housing density and bacterial loading in urban streams. *J. Environ. Eng.* 125, 1177–1180.