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"Molecular and Bioassay-Based Investigation of Bivalves as Transmission Vectors of Protozoal Encephalitis in Southern Sea Otters"

Objective 1:

An epidemiological study was conducted to determine whether *Toxoplasma gondii* protozoal brain infection and seropositivity in southern sea otters are associated with specific risk factors, including age, sex, geographic distribution and month of sampling. Miller et al. (2002) published the results of this study in the International Journal for Parasitology. ["Coastal freshwater runoff is a risk factor for *Toxoplasma gondii* infection of southern sea otters (*Enhydra lutris nereis*)". International Journal for Parasitology 32(8):997-1006; PDF attachment submitted with this report.]

In summary, specific evidence of coastal contamination of the marine ecosystem with the zoonotic protozoan parasite, *Toxoplasma gondii*, and extensive infection of southern sea otters (*Enhydra lutris nereis*) along the California coast was documented by this study. To investigate the extent of exposure and factors contributing to the apparent emergence of *T. gondii* in southern sea otters, we compiled environmental, demographic and serological data from 223 live and dead sea otters examined between 1997 and 2001. The *T. gondii* seroprevalence was 42% (49/116) for live otters, and 60% (66/107) for dead otters. Demographic and environmental data were examined for associations with *T. gondii* seropositivity, with the ultimate goal of identifying spatial clusters and demographic and environmental risk factors for *T. gondii* infection. Spatial analysis revealed clusters of *T. gondii*-seropositive sea otters at two locations along the coast, and one site with lower than expected *T. gondii* seroprevalence. Risk factors that were positively associated with *T. gondii* seropositivity in logistic regression analysis included male gender, older age and dead versus live status. Most importantly, otters sampled near areas of maximal freshwater runoff were 3 times more likely to be seropositive to *T. gondii* than otters sampled in areas of low flow. No association was found between seropositivity to *T. gondii* and human population density or exposure to sewage. This study provides evidence implicating land-based surface runoff as a source of *T. gondii* infection for marine mammals, specifically sea otters, and provides a convincing illustration of pathogen pollution in the marine ecosystem.

Objective 2:

Tank experiments were conducted at the Bodega Marine Laboratory to determine whether pathogen-free marine bivalves that are experimentally exposed to laboratory cultures of *T. gondii* oocysts will take up the parasite and maintain it in their tissues in an infective form. A manuscript by Arkush et al. reporting the results of these experiments was accepted for publication and is currently "in press" in the International Journal for Parasitology. ["Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*)"; PDF attachment of galley proofs submitted with this report.]

Our previous studies showed that *T. gondii* is associated with morbidity and mortality in a variety of marine mammals, including fatal meningoencephalitis in the southern sea otter (*Enhydra lutris nereis*). However, the source(s) of *T. gondii* infection and routes of transmission in the marine environment are unknown. We hypothesise that filter-feeding marine bivalve shellfish serve as paratenic hosts by assimilation and concentration of infective *T. gondii* oocysts and their subsequent predation by southern sea otters is a source of infection for these animals. To test this hypothesis, we developed a TaqMan PCR assay for detection of *T. gondii* ssrRNA and evaluated its usefulness for the detection of *T. gondii* in experimentally exposed mussels (*Mytilus galloprovincialis*) under laboratory conditions. *Toxoplasma gondii*-specific ssrRNA was detected in mussels as long as 21 days post-exposure to *T. gondii* oocysts. Parasite ssrRNA was most often detected in digestive gland homogenate (31 of 35, i.e. 89%) compared with haemolymph or gill homogenates. Parasite infectivity was confirmed using a mouse bioassay. Infections were detected in mice inoculated with any one of the mussel sample preparations (haemolymph, gill, or digestive gland), but only digestive gland samples remained bioassay-positive for at least 3 days post-exposure. For each time point, the total proportion of mice inoculated with each of the different tissues from *T. gondii*-exposed mussels was similar to the proportion of exposed mussels from the same treatment groups that were positive via TaqMan PCR. These experiments allowed us to establish the sampling protocols and TaqMan PCR assay conditions that were most applicable for use in testing field samples of free-living invertebrate prey species from high-risk coastal locations where *T. gondii* infections are prevalent in southern sea otters.

Objective 3.

The final objective of our study was to detect the presence of *T. gondii* parasites in samples of free-living marine bivalves collected from areas of clustering of sea otter protozoal brain infections, or from sites of significant sewage outfall or terrestrial runoff within the sea otter range. Invertebrate samples were collected from our two highest risk areas at Morro Bay/Cayucos and Elkhorn Slough/Moss Landing. Samples were taken from these sites after the first rains in November 2002 so that the filter-feeding invertebrates would have been exposed to freshwater. Bivalve collection sites were chosen based on the risk factor analysis in Objective 1, as well as potential contributing sources of cat feces, such as sewage treatment plants and freshwater outflow. Bivalve collection focused on mussels, specifically

Mytilus spp., which could be found across all the sites. A total of 342 mussels were collected from five or six points within the Moss Landing and Morro Bay area sites. As available, other bivalve and invertebrate species were also sampled, though they are not found at all six sites and thus would not give the valuable comparative data that the mussels provide. In the end, Macoma and Pismo clams as well as Emerita crabs and Innkeeper worms were collected from the two high- risk sites. TaqMan PCR testing was performed to amplify and detect *T. gondii* DNA in samples from 342 *Mytilus* spp. mussels and 203 other invertebrates that serve as sea otter prey species at these sites. Confirmation by sequence analysis of amplicon-positive samples is currently underway. Thus far our results have been encouraging and efforts are presently underway to identify and obtain funding to continue the collection and testing of bivalves for *T. gondii*.

This study provides evidence implicating freshwater surface runoff as a source of *T. gondii* infection for marine mammals, specifically sea otters, and illustrates the importance of pathogen pollution in the marine ecosystem. Our study results were reported nationally and internationally in peer-reviewed scientific publications and in newspapers and online science journals. (Please see National Sea Grant Project Questionnaire for specific references). Information regarding the *T. gondii* epidemiology in sea otters, development of quantitative molecular detection methods and the evaluation of bivalves, both as a source of *T. gondii* for otters and potential bio-indicators of fecal pollution, has been discussed with California Mussel Watch Program and California Regional Water Quality Control Board staff, as well as stakeholders in the "high-risk" Morro Bay area. These groups are actively working to facilitate our bivalve evaluation studies in coastal areas. Once validated, molecular pathogen detection in bivalves has future application in monitoring coastal water quality. In addition, serologic tests developed in this project are now available and being used as diagnostic tests by marine wildlife veterinarians and rehabilitation centers.

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Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*)

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Abstract

Toxoplasma gondii is associated with morbidity and mortality in a variety of marine mammals, including fatal meningoencephalitis in the southern sea otter (*Enhydra lutris nereis*). The source(s) of *T. gondii* infection and routes of transmission in the marine environment are unknown. We hypothesise that filter-feeding marine bivalve shellfish serve as paratenic hosts by assimilation and concentration of infective *T. gondii* oocysts and their subsequent predation by southern sea otters is a source of infection for these animals. We developed a TaqMan PCR assay for detection of *T. gondii* ssrRNA and evaluated its usefulness for the detection of *T. gondii* in experimentally exposed mussels (*Mytilus galloprovincialis*) under laboratory conditions. *Toxoplasma gondii*-specific ssrRNA was detected in mussels as long as 21 days post-exposure to *T. gondii* oocysts. Parasite ssrRNA was most often detected in digestive gland homogenate (31 of 35, i.e. 89%) compared with haemolymph or gill homogenates. Parasite infectivity was confirmed using a mouse bioassay. Infections were detected in mice inoculated with any one of the mussel sample preparations (haemolymph, gill, or digestive gland), but only digestive gland samples remained bioassay-positive for at least 3 days post-exposure. For each time point, the total proportion of mice inoculated with each of the different tissues from *T. gondii*-exposed mussels was similar to the proportion of exposed mussels from the same treatment groups that were positive via TaqMan PCR. The TaqMan PCR assay described here is now being tested in field sampling of free-living invertebrate prey species from high-risk coastal locations where *T. gondii* infections are prevalent in southern sea otters.

Keywords: *Toxoplasma gondii*; Bivalve shellfish; Marine environment; Oocyst; Bioassay; TaqMan PCR

1. Introduction

Toxoplasma gondii is a protozoan parasite with a facultatively heteroxenous life cycle that potentially includes all warm-blooded animals (mammals and birds) as intermediate hosts and felids as definitive hosts. Felids shed oocysts which become infective through sporulation in the environment and subsequently are a potential source of infection for a wide variety of intermediate hosts, including humans and other felids (Tenter et al., 2000). Sporulation of oocysts is facilitated by aerogation, humidity, and warm temperature and is usually completed within 1–5 days in a temperate climate. Sporulated oocysts of *T. gondii* are environmentally resistant, retaining infectivity for at least 18 months in soil (Frenkel et al., 1975). Infections in terrestrial animals and humans have occurred as a consequence of exposure to sporulated oocysts in contaminated soil or fresh water (Frenkel and Dubey, 1972; Bowie et al., 1997; Aramini et al., 1999; Tenter et al., 2000).

There is also evidence of *T. gondii* infection in marine mammals, such as cetaceans (Cruickshank et al., 1990;

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Inskeep et al., 1990; Migaki et al., 1990; Mikaelian et al., 2000; Resendes et al., 2002), pinnipeds (Van Pelt and Dietrich, 1973; Migaki et al., 1977; Holshuh et al., 1985; Miller et al., 2001) and sirenians (Buergelt and Bonde, 1983), including the southern sea otter (*Enhydra lutris nereis*), which is a federally listed threatened species in the USA (Cole et al., 2000; Kreuder et al., in press). In a recent study on sea otters in California, *T. gondii* infection was detected in 36% of all dead sea otters by parasite isolation in cell culture and immunohistochemical examination of the brain (Miller et al., 2002a). The high incidence of exposure to *T. gondii* in Californian sea otters was verified serologically in a survey of 223 animals, which found that 42% of live sea otters and 62% of dead sea otters had *T. gondii* IFAT titres of $\geq$1:320 (Miller et al., 2002b). *Toxoplasma gondii* infections in adult sea otters can have serious consequences, as evidenced by recent findings that encephalitis due to *T. gondii* was the primary cause of mortality in 16.2% of California sea otters examined between 1998 and 2001, making it one of the top two causes of otter death during this period (Kreuder et al., in press). Thus far, the source(s) of *T. gondii* infection and routes of transmission to southern sea otters have not been established.

The most plausible explanation for the high number of southern sea otters infected by *T. gondii* off the coast of California is exposure to oocysts that are shed by felids and reach the ocean through streams, urban runoff and/or sewage effluent. Coastal freshwater runoff has been shown to be a risk factor for *T. gondii* infection in southern sea otters (Miller et al., 2002b). Oocysts are likely to be completely sporulated, and hence be infective, at the time they reach the ocean so that waterborne transmission to sea otters may occur through direct consumption of infective oocysts. However, the inevitable dilution of oocysts in fresh water questions that direct consumption of infective oocysts is a major route of transmission to sea otters, because infection doses received in this way are likely to be too low to cause disease in marine mammals.

Another scenario for the transmission of *T. gondii* in a marine environment may be that aquatic species, such as bivalve shellfish, serve as paratenic hosts through concentration of *T. gondii* oocysts, and that the predation of such hosts by southern sea otters results in infection doses high enough to cause disease in them. We hypothesise that infective oocysts of *T. gondii* in the marine environment are picked up by filter-feeding marine bivalves that are a major prey species of southern sea otters (Kvitek et al., 1998). To test this hypothesis we investigated the ability of shellfish to remove and concentrate *T. gondii* oocysts from seawater under controlled laboratory conditions. We examined the infectivity of oocyst-exposed mussels using a mouse bioassay, which is generally considered as the "gold standard" for detection of infective stages of *T. gondii*. However, mouse bioassays, while very sensitive, are also time consuming, expensive, and have the disadvantage of involving animal experiments. Therefore, we also describe the development and application of a TaqMan PCR assay for the detection of *T. gondii* ssrRNA in experimentally exposed mussels (*Mytilus galloprovincialis*). This TaqMan PCR detection method at the RNA level is rapid and sensitive, and potentially provides a new strategy for the detection of *T. gondii* in wild-caught bivalves in southern sea otter habitat.

2. Materials and methods

2.1. TaqMan PCR

2.1.1. Systems

TaqMan PCR assays (Applied Biosystems) for the present study were designed from published nucleotide sequences of *T. gondii* and a range of mussel species (GenBank accession numbers are given in brackets below). A TaqMan PCR system targeting the *T. gondii* ssrRNA was designed to detect *T. gondii* at the RNA level in tissue samples (Toxo18 TaqMan PCR system). The nucleotide sequences of the primers and probe were designed using the published sequence of the *T. gondii* ssrRNA gene (*T. gondii* 18S rRNA, U03070). In addition, a TaqMan PCR system was designed to target the ssrRNA of *M. galloprovincialis* (Myt18 TaqMan system; L33452), *Mytilus californianus* (L33449), *Mytilus edulis* (L78854), *Mytilus trossulus* (L33453) and *Geukensia denissa* (L22448) as an endogenous control to assess tissue integrity and RNA extraction efficiency. The Myt18 TaqMan system was designed not to cross-react with *T. gondii* ssrRNA sequences. A TaqMan PCR system targeting a portion of the B1 gene of *T. gondii* was also designed (ToxoB TaqMan PCR system; AF179871; Burg et al., 1989). For each target, two primers and an internal, fluorescently labelled TaqMan probe [5′ end, reporter dye 6-carboxyfluorescein; 3′ end, quencher dye 6-carboxytetramethylrhodamine] were designed using the Primer Express (Applied Biosystems) software (Table 1). The length of each PCR product was held very short (99 and 129 bp) to enable high amplification efficiencies. All TaqMan PCR systems were optimised according to a three point-protocol: (1) signal test to assess signal-to-noise ratio of the TaqMan probe fluorescent signal; (2) determination of amplification efficiency using a standard curve generated with plasmid DNA and/or genomic DNA diluted in 10-fold steps from a positive control in triplicate; and (3) analytical specificity by sequencing TaqMan PCR products. All samples collected during the course of the experiments were analysed for *T. gondii* RNA load.

2.1.2. Sample preparation and processing

Tissue samples (20–50 mg) from exposed and control mussels were collected and stored at −20 °C until used. Before RNA extraction, the frozen tissues were transferred into 96-deep well plates containing two grinding beads

Table 1
Nucleotide sequences of PCR primers and TaqMan probes used to detect *Toxoplasma gondii* B1 gene, *T. gondii* ssrRNA or mussel ssrRNA

Target	Primer	Primer sequence	Length of PCR product (bp)	Probe	Probe sequence
Toxoplasma gondii B1 gene	ToxB-41f	5′-TCGAAGCTGAGATGCTCAAAGTC-3′	129	ToxB-69p	5′-FAM[a]-ACCGCGAGATGCACCCGCA-TAMRA[b]-3′
	ToxB-169r	5′-AATCCACGTCTGGGAAGAACTC-3′			
Toxoplasma gondii ssrRNA	Tox18-213f	5′-CCGGTGGTCCTCAGGTGAT-3′	120	Tox18-249p	5′-FAM-ATCGCGTTGACTTCGGTCTGCGAC-TAMRA-3′
	Tox18-332r	5′-TGCCACGGTAGTCCAATACAGTA-3′			
Mytilus/Geukensia ssrRNA	Myt18-412f	5′-CGGCTACCACATCCAAGGA-3′	99	Myt18-438p	5′-FAM-AGGCGCGCAAATTACCCACTCCTG-TAMRA-3′
	Myt18-510r	5′-GCCTCGAAAGAGTCCCGTATT-3′			

[a] FAM, 6-carboxyfluorescein.
[b] TAMRA, 6-carboxytetramethylrhodamine.

(4 mm diameter; SpexCertiprep, Metuchen, NJ, USA) and 800 μL of 1 × ABI lysis buffer (Applied Biosystems) in each sample well. Tissue samples were ground in a GenoGrinder2000 (SpexCertiprep) for 2 min at 1,500 strokes per min. After 30 min at 4 °C, total RNA was extracted from the tissue lysates using a 6700 Automated Nucleic Acid workstation (Applied Biosystems) according to the manufacturer's instructions. The RNA was eluted in 100 μL of RNA elution solution (Applied Biosystems).

Complementary DNA (cDNA) was synthesised using 100 U of SuperScript II (Invitrogen), 300 ng random hexadeoxyribonucleotide [pd$(N)_6$] primers, 10 U RNase inhibitor (RNaseOut) and 1 mM dNTPs (all from Invitrogen) in a final volume of 40 μL. The reverse transcription reaction proceeded for 50 min at 42 °C and was terminated by heating for 5 min to 95 °C and cooling on ice after addition of 10 μL of water.

Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR reagents (TaqMan Universal PCR Mastermix; Applied Biosystems) containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM dNTPs, 0.625 U DNA polymerase (AmpliTaq Gold; Applied Biosystems), 0.25 U AmpErase UNG and 5 μL of the cDNA sample in a final volume of 25 μL. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System; ABI). The manufacturer's default amplification conditions were used: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 60 s at 60 °C. A sample was considered TaqMan PCR-positive if the cycle threshold (CT) value (i.e., the PCR cycle at which the fluorescent intensity exceeded a threshold that was calculated based on the background fluorescent intensity between cycles three and 15) was <40.

2.2. Mussel collection and screening

Wild bay mussels (*M. galloprovincialis*) were collected from Tomales Bay, California, under a collection permit that was approved by the California Department of Fish and Game. The animals were held in a pathogen-containment system at the University of California-Davis, Bodega Marine Laboratory, Bodega Bay, California. Tanks were supplied with flow-through natural seawater (11–13 °C) filtered to 5 μm. The mussels were fed laboratory-derived phytoplankton (*Isochrysis galbana*) one to two times daily. Prior to each exposure experiment, 250 mussels were randomly selected for pre-screening for the detection of *T. gondii*. Animals were removed individually from the tanks and a small area of the left valve of each mussel was cleared of all encrusting detritus, bryozoans, and barnacles. For individual identification, a numbered tag was affixed to the shell using semi-permanent glue. A notch was formed in the shell of each mussel near the posterior axis using a triangular file. Using a 25-G, 1.5 inch needle and a 3-mL syringe, up to 500 μL of haemolymph was extracted from

the posterior adductor muscle. Samples were examined microscopically for the presence of haemocytes. The haemolymph was centrifuged at 16,000 × *g* to obtain a pellet of haemocytes and the fluid was discarded. The pellet fractions were tested for the detection of *T. gondii* by using the TaqMan ssrRNA PCR system.

2.3. Production of T. gondii oocysts

To obtain oocysts of *T. gondii*, specific pathogen-free (SPF) NMRI mice were orally inoculated with 1,000–1,500 oocysts of the AHC1 isolate of *T. gondii*. This isolate was obtained in Germany in 2000 from the brain of a naturally infected cat with toxoplasmic encephalitis. Mice were killed by cervical dislocation at 63 days p.i. [Batch 1] and 138 days p.i. [Batch 3]. The brains and hind limb muscles of five mice [Batch 1] and seven mice were [Batch 3] fed to SPF cats [Cat 22/013—Batch 1; Cat 28/110—Batch 3]. Cat faeces were examined daily by salt flotation to detect shedding of oocysts. Unsporulated oocysts were collected from faecal samples and enriched by flotation in saturated saline on days 4–11 p.i. [Batch 1] and days 5–10 p.i. [Batch 3]. Oocysts were washed three times by suspension in tap water and centrifugation to remove NaCl, and then suspended in tap water containing 2% sulphuric acid to prevent growth of bacteria and fungi. Sporulation was achieved by frequent aeration at 22 °C over 3–5 days. Individual batches of sporulated oocysts obtained from the same cat were combined and shipped to the University of California-Davis for exposure of mussels. A portion of these oocysts was used for spiking experiments.

2.4. Experimental design for oocyst exposure of mussels

2.4.1. Experiment 1

One hundred and eight mussels were randomly sampled from the population of mussels in the holding tanks found to be negative for *T. gondii*, and were transferred to a 15 °C cold room. Thirty-six of the mussels were placed in a 40-L tank containing 13 L of natural seawater (filtered to 10 μm). The remaining 72 mussels were placed in another 40-L tank containing 25 L of filtered seawater. A suspension of *T. gondii* oocysts (Batch 1; 1.5×10^7) in PBS was added to the tank containing 72 mussels, while the mussels in the other tank served as negative (non-exposed) controls. Both groups were held in their respective tanks for 6 h, and aeration was maintained in these tanks throughout the experiment. During the exposure period, strong aeration was maintained to provide water movement and continuous distribution of *T. gondii* oocysts throughout the tanks. After 6 h, the water from the control tank was collected and discarded. The water from the tank containing the exposed mussels was collected into 10-L carboys for tangential flow filtration and oocyst enumeration. Tangential flow filtration was conducted using the Millipore Pellicon cassette system (Millipore Corp.) as described by Isaac-Renton et al. (1986) but using a different filter (Durapore, PVDF VVPP, 0.1 μm; Millipore). Oocysts in the retentate were concentrated by filtration and enumerated by microscopic examination using a haemacytometer. All mussels were moved to new, clean 20-L tanks containing 15 L of filtered seawater (10 μm), two for the control and four for the exposed groups ($n = 18$). Partial water exchanges were conducted at 3, 8, 14, and 21 days post-exposure to maintain water quality, and the mussels were fed five times per week. Three mussels were randomly sampled from each of the tanks at 1, 3, 7, 14, 21, and 35 days post-exposure for TaqMan PCR analysis. These mussels were dissected using alcohol-flamed instruments, and haemolymph, gill, and digestive gland were collected in separate microcentrifuge tubes and held at −20 °C. Haemocyte pellets were obtained by centrifugation as described above prior to freezing.

2.4.2. Experiment 2

Compared with the first experiment, samples were taken at earlier time points and additional mussels were included to assess *T. gondii* infectivity in mussel tissues using a mouse bioassay. One hundred and eighty pre-screened, *T. gondii*-negative mussels were transferred to a 15 °C cold room for exposure to *T. gondii* oocysts. Sixty mussels were placed into each of two 40-L tanks containing 10 L of natural seawater (filtered to 5 μm), and 30 mussels were placed into each of two 20-L tanks containing 5 L of filtered seawater. Prior to oocyst exposure, *I. galbana* algae were added to one large and one small tank to stimulate feeding activity, with the intent to possibly enhance *T. gondii* oocyst uptake. Hereafter, these mussels will be referred to as "fed" to indicate that they received *Isochrysis* during the exposure period. Immediately after adding the *Isochrysis*, a 1.6×10^5 suspension of *T. gondii* oocysts (Batch 3) in PBS was added to each of the two 40-L tanks, while the mussels contained in the 20-L tanks served as non-exposed controls. Mussel feeding activity was confirmed by visualising shell opening, mantle extension, and gradual disappearance of the green tint in the tanks containing *Isochrysis* algae. After 8 h, the water from the control tanks was collected and discarded. The water from the tanks containing the exposed mussels was collected into 10-L carboys for tangential flow filtration and oocyst enumeration as described above. All mussels were moved to new, clean 20-L tanks containing 15 L of filtered seawater (5 μm), two tanks each for the exposed ($n = 60$) and control ($n = 30$) groups. During the experiment, partial water exchanges were conducted at 3, 8, 16, and 21 days post-exposure to maintain water quality, and the mussels were fed five times per week. Mussels were sampled from each of the tanks at 3 and 6 h post-exposure, and then 1, 3, 7, 14, and 21 days post-exposure. At each time point, mussels were collected from each of the control ($n = 3$) and exposed ($n = 6$) groups for real-time PCR analysis. Haemocyte pellets, gill, and digestive gland were collected and frozen in microcentrifuge tubes at −20 °C.

2.5. Bioassay of bivalve tissues

In Experiment 2, three control and six *T. gondii*-exposed mussels were collected from the tanks at 6 h, 1 day, and 3 days post-exposure for assessment of *T. gondii* uptake and viability. The same samples (haemolymph, gill, and digestive gland) were collected as described above for TaqMan PCR analysis, except that each sample type was combined by treatment group. Immediately after collection, the haemolymph was concentrated as described above, and the resulting haemocyte pellet was suspended in 1 mL of saline containing antibiotics (Miller et al., 2001) and stored at 4 °C. Gill tissue was collected separately from each mussel and placed into a conical tube containing 5 mL of PBS (pH 7.4). The tubes were shaken vigorously for 15 s and then the tissues were removed, combined by treatment group and frozen at −20 °C. The supernatant from gill washings was combined by treatment group and centrifuged at 1,500 × *g* for 10 min. All but 1 mL of the supernatant was removed and discarded, an equal volume of antibiotic saline solution was added to the concentrated gill washing suspension and the samples were stored at 4 °C. Digestive gland tissues were collected and combined by treatment group in a mortar containing 2 mL of sterile PBS. The tissues were gently macerated with a pestle and the resulting homogenate was placed in a conical tube, vortexed, and centrifuged at 1,500 × *g* for 10 min. The supernatant was removed and discarded, and the tissue pellet was suspended in twice the equivalent volume of antibiotic saline and stored at 4 °C. These samples were used to examine the infectivity of mussel-retained *T. gondii* for mice.

One hundred and fifty-nine, 25–30 g, Swiss Webster female mice were housed three per cage and fed commercial feed pellets and water ad libitum. All mice were bled from the retroorbital complex prior to inoculation and again at the end of the experiment (at 28–29 days post-exposure), and serum was tested for *T. gondii* antibodies by an indirect fluorescent antibody test (IFAT) as previously described (Miller et al., 2001) except that the serial dilutions began at 1:5 and fluorescein isothiocyanate-conjugated goat anti-mouse IgG was used as secondary antibody. A conservative cut-off of ≥1:80 was used to define a seropositive result.

Mice were randomly allocated into three groups of 45 for each of three bivalve sample time points, i.e., 6 h, 1 day, and 3 days post-exposure. For each time point there were 15 different treatment groups, with three mice allocated per treatment group. The treatment groups consisted of mice that were either orally or s.c. inoculated with 0.2 mL of a haemocyte pellet, concentrated gill washing, or digestive gland homogenate (oral route of inoculation only) from *T. gondii*-exposed (fed or unfed) or unexposed (fed) bivalves. The same mussel tissues were used in the bioassays and the TaqMan PCR assays to allow comparisons between these two methods and to determine the optimal mussel tissue for *T. gondii* detection. Two routes of inoculation, oral and s.c., were included to evaluate potential variation in parasite development and hence detection. However, mice were not inoculated s.c. with mussel digestive gland samples to avoid potential introduction of gastrointestinal flora to the subcutis. To serve as positive controls for *T. gondii* exposure, 24 additional mice were inoculated via oral or s.c. routes with one of three doses (30, 300, or 3,000 oocysts per mouse) of *T. gondii* oocysts in sterile distilled water. Three mice were allocated for each oocyst control group, and six additional mice received distilled water only, either orally ($n = 3$) or s.c. ($n = 3$). Mice were monitored daily and were euthanised 28 or 29 days post-inoculation.

2.6. Examination of mice by immunohistochemistry and IFAT

Mice were euthanised (following University of California-approved protocols) and the thorax, abdomen, and calvarium were opened. Blood was collected from the mice either via retroorbital venipuncture (pre-exposure) or from the heart during necropsy, and the serum was evaluated for the presence of *T. gondii*-specific antibodies by IFAT as described above. For each mouse, brain, lung, liver, heart, spleen, tongue, and right quadriceps muscle were immersion-fixed in 10% neutral buffered formalin for 5–7 days, cut into 2–3 mm-thick slices and placed into two tissue cassettes. The trimmed mouse tissues were dehydrated using ethanol, paraffin-embedded using an automatic tissue processor, and 5 μm-thick tissue sections were cut using a rotary microtome, placed on glass slides, and deparaffinised. An immunoperoxidase procedure (Miller et al., 2001) was used to stain *T. gondii* parasites, if present, in the mouse tissues and iron haematoxylin was used as a counter-stain. Tissues from known infected and non-infected mice were used as positive and negative controls, respectively.

All tissue sections were examined on a compound microscope at a magnification of 400-fold and 1,000-fold for the presence of stained parasites, inflammation, or other lesions. If any tissue was positive for immunoperoxidase-labelled parasites or if the serological examination resulted in a titre of ≥1:80 in post-exposure serum, that mouse was considered positive for *T. gondii* infection. If all tissues on both slides were negative for parasites and there was no evidence of seroconversion based on IFAT results, the mouse was considered negative for *T. gondii* infection. All microscopic slides were interpreted by a pathologist who was blinded to the mussel treatment groups, the PCR results for *T. gondii*-exposed and control mussel tissue and the results of mouse serological testing for *T. gondii.*

2.7. Statistical analysis

The percentage of positive samples among tanks and among sampling times was compared by Pearsons χ^2 test. McNemar's χ^2 test was used to compare the percentage of ssrRNA-positive results from different tissues

(haemolymph, gill, digestive gland) of exposed mussels. P values <0.05 were considered significant.

3. Results

3.1. Validation of real-time TaqMan PCR systems

The two TaqMan PCR systems specific for B1 and ssrRNA were validated for amplification efficiency, analytical sensitivity and analytical specificity. Both TaqMan PCR systems amplified *T. gondii* DNA extracted from types I, II, and III with high amplification efficiency (>95%). Using cloned B1 and ssrRNA TaqMan PCR products to generate standard curves with 10-fold diluted plasmids, both systems had a reproducible analytical sensitivity of ten molecules (Fig. 1). Amplification efficiencies obtained on plasmids containing *T. gondii* sequences and DNA extracted from *T. gondii* oocysts were within a 10% range difference (Fig. 1). Both the B1 and ssrRNA TaqMan PCR systems recognised DNA extracted from isolates of *T. gondii* types I, II and III. Analytical specificity was confirmed by sequencing the TaqMan PCR products. In addition, specificity was tested using DNA extracted from other apicomplexan organisms including *Cryptosporidium parvum*, *Sarcocystis neurona*, *Sarcocystis falcatula*, *Sarcocystis cruzi*, *Sarcocystis arienticanis*, *Sarcocystis miescheriana*, *Sarcocystis tenella*, *Sarcocystis gigantean*, *Sarcocystis muris*, *Neospora caninum*, and *Neospora hughesi*. DNA extracted from these apicomplexan organisms tested negative with both *T. gondii* TaqMan PCR systems, but tested positive with specific TaqMan PCR systems for *Cryptosporidium*, *Sarcocystis* and *Neospora*.

The ToxoB TaqMan PCR was compared to a conventional B1 specific *Toxoplasma* PCR system as described

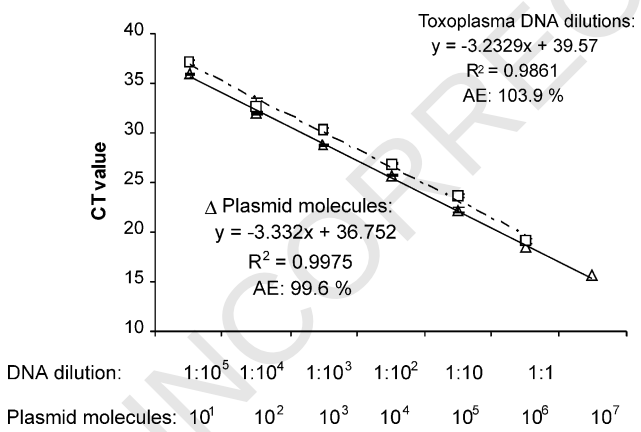

Fig. 1. Linearity of TaqMan PCR is shown using dilutions of plasmid (□, six log decades) obtained by cloning B1 TaqMan PCR products and of target genomic DNA extracted from *Toxoplasma gondii* isolates (△, five log decades). Amplification efficiency (AE) of *Toxoplasma gondii* DNA (□) and standard plasmid (△) is calculated based on the slope of the standard curves using the formula: $E = 10^{1/-s}-1$, where $E(100)$ is the % efficiency and s is the slope of the standard curve.

(Burg et al., 1989) by spiking dilutions of known numbers of sporulated oocysts into different tissues of *M. galloprovincialis* tissue (gill, haemolymph, and digestive gland). Parallel analysis performed on extracted gDNA showed a 10- to 100-fold increased sensitivity of the TaqMan PCR when compared to the conventional gel-electrophoresis PCR protocol (results not shown).

The Myt18 TaqMan PCR system specific for ssrRNA of *M. galloprovincialis* was used to assess the RNA quality extracted from the tissue samples. Validation of the Myt18 TaqMan PCR system confirmed high amplification efficiency (96%) and no cross-reactivity when tested against DNA extracted from *T. gondii* oocysts.

3.2. Experiment 1

Following tangential flow filtration, an estimated total of 20,266 oocysts were recovered from the exposure water 6 h after the onset of the experiment. This represented an approximately 740-fold reduction in oocyst number from the initial inoculation of the tank with 1.5×10^7 *T. gondii* oocysts. In both experiments, *T. gondii*-exposed and control mussels were scored as positive if *T. gondii*-specific ssrRNA was detected in any of the samples tested, i.e., haemocyte pellet, gill supernatant, or digestive gland homogenate. *Toxoplasma gondii* ssrRNA was detected in 50% of the mussels at 1 day post-exposure, in 25 and 17% of the mussels at 3 and 7 days post-exposure, respectively, and then in 33% of the mussels at 21 days post-exposure (Table 2). Overall, 21% (15 of 72) of mussels were *T. gondii*-positive when samples were tested at different time points over the 35 day experimental period. *Toxoplasma gondii*-specific ssrRNA was not detected in any control (non-exposed) mussels.

The proportions of positive samples (i.e., any tissue was positive) in the four tanks containing the *T. gondii*-exposed mussels did not differ significantly ($P = 0.852$): Tank 1 (five of 60), Tank 2 (three of 60), Tank 3 (four of 59), and Tank 4 (three of 60) and hence the data were combined for subsequent analyses. There was a difference in the percentage of positives detected per time point ($P = 0.06$) but it was not significant. More positives were detected in samples of haemolymph ($n = 8$) than digestive gland ($n = 5$) or gill ($n = 3$) but the difference was not statistically significant.

3.3. Experiment 2

Following tangential flow filtration, an estimated total of 5,135 oocysts were recovered from the exposure water in tanks with algae and only 880 oocysts were recovered from the exposure water without algae 8 h after the onset of the experiment. However, the latter sample contained more debris that impeded the accurate enumeration of oocysts. Overall this represented an approximately 50-fold reduction in oocyst numbers from the initial inoculation of the tanks

Table 2
Detection of *Toxoplasma gondii* ssrRNA in tissues of the bay mussels (*Mytilus galloprovincialis*) sampled at different time points following experimental exposure to *T. gondii* oocysts

Time point post-exposure	Exposed groups				Control groups	
	Unfed mussels[a]		Fed mussels[a]		Unfed mussels[a]	Fed mussels[a]
	No. of positive mussels (*n*)	Positive tissue[b]	No. of positive mussels (*n*)[c]	Positive tissue[b,c]	No. of positive mussels (*n*)	No. of positive mussels (*n*)[c]
Experiment 1						
1 day	6 (12)	4 H, 1 G, 1 DG	ND	ND	0 (3)	ND
3 days	3 (12)	3 DG	ND	ND	0 (3)	ND
7 days	2 (12)	2 G	ND	ND	0 (3)	ND
14 days	0 (12)	–	ND	ND	0 (3)	ND
21 days	4 (12)	3 H, 1 H + DG	ND	ND	0 (3)	ND
35 days	0 (12)	–	ND	ND	0 (3)	ND
Experiment 2						
3 h	6 (6)	1 H + DG, 1 G, 4 DG	3 (6)	1 H + DG, 2 DG	0 (3)	0 (3)
6 h	6 (6)	2 H + DG, 1 H + G, 1 G + DG, 2 DG	6 (6)	1 G, 2 H + G + DG, 1 G + DG, 2 DG	0 (3)	0 (3)
1 day	3 (6)	3 DG	5 (6)	1 H + G + DG, 4 DG	0 (3)	0 (3)
3 days	3 (6)	1 G, 2 DG	1 (6)	1 DG	0 (3)	0 (3)
7 days	1 (6)	1 DG	1 (6)	1 DG	0 (3)	0 (3)
14 days	0 (6)	–	0 (6)	–	0 (3)	0 (3)
21 days	0 (6)	–	0 (6)	–	0 (3)	0 (3)

[a] Mussels were combined by treatment group. Mussels fed live cultures of *Isochrysis galbana* during the exposure period are labelled "fed", whereas unfed groups received only *T. gondii* oocysts.
[b] H, haemolymph; G, gill; DG, digestive gland. +, more than one tissue in the same mussel was positive.
[c] ND, not done.

with 3.2×10^5 *T. gondii* oocysts. *Toxoplasma gondii*-specific ssrRNA was detected in tissues from experimentally exposed mussels at all time points up to 7 days post-exposure but not in the 14 and 21 days post-exposure samples (Table 2). During the challenge period, *T. gondii*-specific ssrRNA was detected in 75 and 100% of mussels collected from the exposure tanks at 3 and 6 h, respectively. In the following week, detection diminished, with 67% at 1 day, 33% at 3 days, and 17% at 7 days post-exposure. Overall, 58% (35 of 60) of mussels were *T. gondii*-positive by TaqMan PCR. Parasite ssrRNA was most often detected in digestive gland homogenate (31 of 35; 89%). *Toxoplasma gondii*-specific ssrRNA was not detected in any non-exposed mussels at any time point of this experiment.

As in Experiment 1, there was no effect of tank (data not shown) and results from the tanks containing the exposed mussels were combined for subsequent analyses. Comparisons between different tissues (60 matched sets of tissues from 30 mussels in each of the two tanks) were based on the results of samples collected from 3 h to 7 days post-exposure because there were no positive samples from animals collected on days 14 and 21 post-exposure. Positive molecular detection of *T. gondii* was more frequent in samples of digestive gland (31 of 60) than samples of gill (nine of 60; $P < 0.001$) or haemolymph (eight of 60; $P < 0.001$), whereas there was no difference in the detection rate in haemolymph versus gill ($P = 1.0$).

Results of the mouse bioassays using samples collected in Experiment 2 are shown in Table 3. All of the mice remained clinically normal throughout the 28–29 days of post-exposure care, except for one mouse that was found dead 8 days post-exposure. Gross and histopathologic examination of this mouse indicated that disseminated lymphosarcoma was the likely cause of death and there was no evidence of *T. gondii* infection. All mice were seronegative for *T. gondii* (IFAT titres $\leq$1:5) prior to exposure to bivalve tissues or haemolymph. Mice were considered bioassay-positive for *T. gondii* if an IFAT titre $\geq$1:80 was detected, and/or if microscopic examination of tissues revealed the presence of positively stained protozoal parasites on immunohistochemistry. Bioassay-positive mice were detected in all three treatment groups (gill, haemolymph, and digestive gland) at 6 h post-exposure (Table 3), but only digestive gland remained bioassay-positive over the longer post-exposure periods (up to 3 days). Eighty-three percent of the mice given gill homogenate from mussels collected at 6 h post-exposure by either oral or s.c. inoculation became infected (10 of 12), compared to 50% (six of 12) of the mice inoculated orally or s.c. with mussel haemolymph, and 33% (two of six) of mice inoculated orally with digestive gland. Over all three time points (6 h, 1 day, and 3 days), 12 of 18 (67%) mice orally inoculated with digestive gland derived from *T. gondii*-exposed mussels were bioassay-positive for *T. gondii*. For control mice that were inoculated with

Table 3
Mouse bioassay of tissues from mussels (*Mytilus galloprovincialis*) after experimental exposure to *Toxoplasma gondii* oocysts

Samples inoculated into mice		Route of inoculation into mice	Mouse[b]		
Type of mussel tissue or number of oocysts	Time post-exposure to *T. gondii*		1	2	3
Gill	6 h[a]	Oral	+	+	+
	6 h	Oral	+	+	+
	6 h[a]	s.c.	+	+	+
	6 h	s.c.	+	−	−
	1 day[a]	Oral	−	−	−
	1 day	Oral	−	−	−
	1 day[a]	s.c.	−	−	−
	1 day	s.c.	−	−	−
	3 days[a]	Oral	−	−	−
	3 days	Oral	−	−	−
	3 days[a]	s.c.	−	−	−
	3 days	s.c.	−	−	−
Haemolymph	6 h[a]	Oral	−	−	−
	6 h	Oral	+	+	−
	6 h[a]	s.c.	−	+	+
	6 h	s.c.	+	+	−
	1 day[a]	Oral	−	−	−
	1 day	Oral	−	−	−
	1 day[a]	s.c.	−	−	−
	1 day	s.c.	−	−	−
	3 days[a]	Oral	−	−	−
	3 days	Oral	−	−	−
	3 days[a]	s.c.	−	−	−
	3 days	s.c.	−	−	−
Digestive gland	6 h[a]	Oral	−	+	+
	6 h	Oral	−	−	−
	1 day[a]	Oral	+	+	+
	1 day	Oral	−[c]	+	+
	3 days[a]	Oral	+	+	−
	3 days	Oral	+	+	+
30 oocysts	–	Oral	−	+	−
300 oocysts	–	Oral	−	−	−
3,000 oocysts	–	Oral	−	−	−
30 oocysts	–	s.c.	−	−	+
300 oocysts	–	s.c.	+	+	+
3,000 oocysts	–	s.c.	+	+	+

[a] Samples taken from mussels that were fed *Isochrysis galbana* during the 8 h exposure.
[b] +, *T. gondii* was detected in mouse tissues by immunohistochemistry and/or IFAT titre was ≥1:80. −, *T. gondii* was not detected in mouse tissues by immunohistochemistry and IFAT titre was ≤1:5.
[c] Mouse died at 8 days p.i. from unrelated causes (lymphosarcoma).

a predetermined number (30, 300, or 3,000) of oocysts either s.c. or orally, a larger proportion of bioassay-positive mice were detected for mice inoculated s.c. versus orally (Table 3). Seventy-eight percent (seven of nine) of mice inoculated s.c. with purified *T. gondii* oocysts in distilled water were positive, compared with 11% (one of nine) of mice inoculated orally. No non-exposed mice were bioassay-positive for *T. gondii*.

The proportion of infected mice derived from fed or unfed mussels was comparable at all time points [6 h (10 versus eight), 1 day (three versus two) and 3 days (two versus three)]. Thus, feeding mussels *Isochrysis* immediately prior to *T. gondii* oocyst exposure did not enhance infectivity of shellfish tissues for mice.

3.4. Correlation between bioassay and TaqMan PCR results

For each time point (6 h, 1 day, and 3 days post-exposure of mussels), the total proportion of haemolymph, gill, or digestive gland-exposed mice infected with *T. gondii*, as detected by bioassay, was similar to the proportion of exposed mussels that were positive by TaqMan PCR. In total, 60% (18 of 30) of mice exposed to mussel tissues collected at 6 h post-exposure to *T. gondii* were bioassay-positive for *T. gondii*, compared to 58% (21 of 36) of mussels tested during the same post-exposure period for the presence of *T. gondii* ssrRNA (combined data from 3 and 6 h time points). At 1 day post-exposure of mussels to *T. gondii*, 17% (five of 30) of mice were bioassay-positive, while 22% (eight of 37) of mussels were positive for *T. gondii* ssrRNA. Finally, at 3 days post-exposure of mussels to *T. gondii*, 17% (five of 30) of mice were positive for *T. gondii* by bioassay, compared to 11% (four of 36) of mussels tested from the same post-exposure period for the presence of *T. gondii* ssrRNA.

4. Discussion

In the present study, we showed that mussels (*M. galloprovincialis*) can remove and concentrate *T. gondii* from oocyst-contaminated water, and demonstrated that *T. gondii*, once present in bivalve tissues and haemolymph, remains infectious for mice. Additionally, we have developed a molecular-based method for detecting *T. gondii* at the RNA level in shellfish tissues.

Bivalve shellfish, including *Mytilus* spp., have been shown to bioaccumulate various substances including PCB and trace metals (Nelson et al., 1995) and are routinely used to identify contaminated marine habitats in monitoring programmmes such as the U.S. Environmental Protection Agency's Mussel Watch (Farrington et al., 1987). In previous studies, Atlantic coast shellfish have been shown to concentrate protozoans such as *C. parvum*, *Giardia duodenalis*, and *Cyclospora cayetanensis*, following the discharge of runoff or sewage effluent (Fayer et al., 1998; Graczyk et al., 1998b, 1999b,c). Bivalves can process large volumes of water through filter-feeding activity (2.5 L per bivalve per hour; Roper and Hickey, 1995), and are capable of recovery of infectious stages of protozoa from experimentally contaminated water (Fayer et al., 1997; Graczyk et al., 1998a, 1999a). Filtration rate in *M. edulis* has been estimated to be approximately 1.5 L per hour

(Foster-Smith, 1975). Concentration and slow depuration of pathogenic protozoa has been demonstrated in free-living shellfish collected from commercial harvesting sites. Eastern oysters (*Crassostrea virginica*) collected from six sites near wastewater outfalls or cattle farms were found to contain infective *C. parvum* oocysts (Fayer et al., 1998, 1999). Bent mussels (*Ischadium recurvum*) and Asian freshwater clams (*Corbicula fluminea*) were also found to concentrate pathogenic protozoa in their tissues (Graczyk et al., 1998a, 1999a,b). As demonstrated in the present study, the ability of mussels to concentrate *T. gondii* oocysts is comparable to what has been reported for retention of *C. cayetanensis* oocysts by Asian freshwater clams, where oocysts were detected in haemolymph and gill tissues up to 13 days post-exposure (Graczyk et al., 1998b). Similarly, in experimental exposures of the Eastern oyster to *C. parvum*, oocysts were detected as long as 1 month post-exposure in both gill washings and haemolymph (within haemocytes) by immunofluorescence (Fayer et al., 1997).

The duration of *T. gondii* oocyst infectivity in seawater is unknown, but *C. parvum* oocysts have been shown to survive in seawater for up to 1 year and can be filtered out by mussels (*M. galloprovincialis*), retaining infectivity for mice up to 14 days (Tamburrini and Pozio, 1999). In this report, we have demonstrated that experimentally exposed mussels can retain *T. gondii* infectivity up to 21 days post-exposure. At the earliest time points post-exposure evaluated here (i.e., 3 and 6 h after the onset of exposure), *T. gondii* ssrRNA was detected in gill tissue, haemolymph and digestive gland (Table 2). At later time points, *T. gondii* was detected more frequently in the mussel digestive gland. Infectivity for mice followed the same temporal pattern (Table 3). For both fed and unfed mussels, a number of mice inoculated with haemolymph and gill collected from *T. gondii*-exposed mussels at 6 h post-exposure were bioassay-positive. However, when mice were inoculated with gill homogenate or haemocyte pellets from mussels from the same group (i.e., fed or unfed), but later than 6 h post-exposure, no bioassay-positive animals were detected, suggesting that most of the depuration or clearance of *T. gondii* oocysts from gill and haemolymph occurred within the first 24 h post-exposure. This was true for mice inoculated both orally and s.c. with haemolymph and gill. In contrast, mice inoculated orally with mussel digestive gland homogenate were positive at all time points evaluated (6 h, 1 day, and 3 days post-exposure). In fact, the proportion of positive mice was higher when they were inoculated with digestive gland homogenate derived from mussels on day 1 post-exposure (five of six) than at 6 h (two of six), suggesting that ingested *T. gondii* oocysts may become concentrated in the digestive gland around 24 h post-exposure. Thus, digestive gland may prove to be a better sample to test than gill or haemolymph for field monitoring of shellfish exposed to contaminated water.

Historically, the mouse bioassay has been regarded as the most sensitive method for detecting infectious *T. gondii* parasites. Subcutaneous inoculation of mice with sporulated oocysts was shown by Dubey et al. (1997) to be a more sensitive method for detecting infectivity than oral administration. This proved to be the case in our control experimental inoculations in which mice orally administered high doses of oocysts without bivalve tissue did not become infected, whereas mice receiving subcutaneous inoculations of the three different doses of oocysts showed evidence of infection. Oocysts administered orally may have passed quickly through these fasted mice, or infections may have occurred but been undetectable by serology and histology in the 28–29 day period of this experiment. This did not appear to be a problem when mice were infected orally with tissues from bivalves exposed to oocysts in the tank experiment. Recently, Eastern oysters were shown to remove *T. gondii* oocysts from seawater under laboratory conditions (Lindsay et al., 2001). In that report, oocyst uptake was confirmed using only mouse bioassay. Seventeen per cent (five of 29) of the mice fed infected oyster tissues were noted to have *T. gondii* infections, though it is unclear if these infections were confirmed by histological examination, immunohistochemistry, serology, or a combination of tests. Our molecular assay has the advantage of being a rapid, less expensive and humane method of detecting the presence of *T. gondii* parasites; though it does not determine viability. Furthermore, sensitivity and specificity testing of this TaqMan PCR method is currently underway with the goal of screening shellfish harvested from areas of sea otter habitat (Conrad and Leutenegger, unpublished results). Once sufficiently validated, such a test could also be used to monitor *T. gondii* contamination of commercially harvested shellfish destined for human consumption. Several other real-time PCR assays have been described for the amplification of *T. gondii* DNA, including those that target the *T. gondii* B1 gene and mRNA expression of *T. gondii* stage-specific genes (Bell and Ranford-Cartwright, 2002). Comparisons between these assays to determine their relative advantages for the detection of *T. gondii* in bivalve tissues would be of future interest.

In the first experiment, only 50% of the mussels sampled at day 1 post-exposure were infected, and the infection rate declined subsequently, with no infected mussels detected by day 14. Notably, *T. gondii*-positive mussels were detected again at 21 days post-exposure (see Table 2). It is possible that oocysts were released by some mussels, circulated in the tank water, and were taken up again by other mussels. Alternatively, the failure to detect *T. gondii*-positive mussels at 14 days could indicate that those particular mussels were never infected or that they cleared the parasite prior to being sampled. We conducted the second experiment to (a) include earlier sampling time points, (b) determine if we could enhance filtration rate, and thus oocyst uptake, by

feeding the mussels during the exposure period, and (c) conduct a mouse bioassay concurrently with sampling for TaqMan PCR analysis. Indeed, inclusion of earlier time points enhanced the detection rate, both in terms of absolute numbers of infected mussels and the number of mussels in which more than one tissue was positive (Table 2). Most of the mussels in which multiple tissues were positive were sampled during the earliest time points evaluated (i.e., 3 and 6 h post-exposure), suggesting that once the mussels filter the oocysts out of the water, presumably as food particles, these oocysts can be found in various systems associated with feeding. In the Eastern oyster, putative food particles are subjected to a digestive and transport process that includes phagocytosis by haemocytes (Galtsoff, 1964; Kennedy et al., 1996). Feng et al. (1977) reported that haemocytes travel in both directions across the epithelial lining of the alimentary tract in *Crassostrea gigas*, transporting pinocytosed material. The aggressive phagocytic activity of Eastern oyster haemocytes, specifically the internalisation of *C. parvum*, has been described (Graczyk et al., 1997). Presumably, the detection of *T. gondii* in the concentrated fraction of the haemolymph prepared in this study is a consequence of a similar process. Feeding the mussels during the exposure period did not affect oocyst detection, though clearance of the algae from the water was noted visually.

In the present study, we showed that common Pacific coast invertebrates can remove and concentrate viable *T. gondii* oocysts from contaminated water in a laboratory setting. The parasite remained viable within mussel tissues and haemolymph, and was infectious for mice. Both oral and s.c. routes of inoculation of infected mussel tissues were sufficient to establish *T. gondii* infections in mice. This finding supports our working hypothesis that sea otters may become infected with *T. gondii* by consuming oocyst-contaminated marine bivalves from polluted water. Previous studies have identified a link between heavy surface runoff and *T. gondii* infection in sea otters (Miller et al., 2002b). Many of these "high-outflow" areas also support large populations of filter-feeding bivalves, and within these regions a large proportion of a sea otter's diet may be composed of mid-level and benthic filter-feeding invertebrates. If concentration of *T. gondii* oocysts by marine bivalves is confirmed in a field setting, these findings may help to explain the unusually high proportions of sea otters infected with *T. gondii* (42–62%) along the central coast of California, especially within areas of high coastal runoff (Miller et al. 2002b). *Mytilus* spp. and other shellfish are also consumed by humans. Therefore, the potential human health risks of the findings reported herein should not be underestimated and merit further investigation. The TaqMan PCR assay developed and evaluated in this study may prove to be a valuable method for the identification of other marine hosts and routes of *T. gondii* transfer.

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

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Abstract

The association among anthropogenic environmental disturbance, pathogen pollution and the emergence of infectious diseases in wildlife has been postulated, but not always well supported by epidemiologic data. Specific evidence of coastal contamination of the marine ecosystem with the zoonotic protozoan parasite, *Toxoplasma gondii*, and extensive infection of southern sea otters (*Enhydra lutris nereis*) along the California coast was documented by this study. To investigate the extent of exposure and factors contributing to the apparent emergence of *T. gondii* in southern sea otters, we compiled environmental, demographic and serological data from 223 live and dead sea otters examined between 1997 and 2001. The *T. gondii* seroprevalence was 42% (49/116) for live otters, and 62% (66/107) for dead otters. Demographic and environmental data were examined for associations with *T. gondii* seropositivity, with the ultimate goal of identifying spatial clusters and demographic and environmental risk factors for *T. gondii* infection. Spatial analysis revealed clusters of *T. gondii*-seropositive sea otters at two locations along the coast, and one site with lower than expected *T. gondii* seroprevalence. Risk factors that were positively associated with *T. gondii* seropositivity in logistic regression analysis included male gender, older age and otters sampled from the Morro Bay region of California. Most importantly, otters sampled near areas of maximal freshwater runoff were approximately three times more likely to be seropositive to *T. gondii* than otters sampled in areas of low flow. No association was found between seropositivity to *T. gondii* and human population density or exposure to sewage. This study provides evidence implicating land-based surface runoff as a source of *T. gondii* infection for marine mammals, specifically sea otters, and provides a convincing illustration of pathogen pollution in the marine ecosystem. © 2002 Published by Elsevier Science Ltd. on behalf of Australian Society for Parasitology Inc.

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Study population

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

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

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

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

2.2. Serum collection and testing, live and dead otters

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

2.3. Definition of risk factors

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

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

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

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

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

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

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

2.4. Univariate analysis of risk factors

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

2.5. Spatial analysis

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

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

2.6. Logistic regression analysis

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

3. Results

3.1. Seroprevalence

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

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

3.2. Risk factors

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

3.3. Spatial analysis

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

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

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

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

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

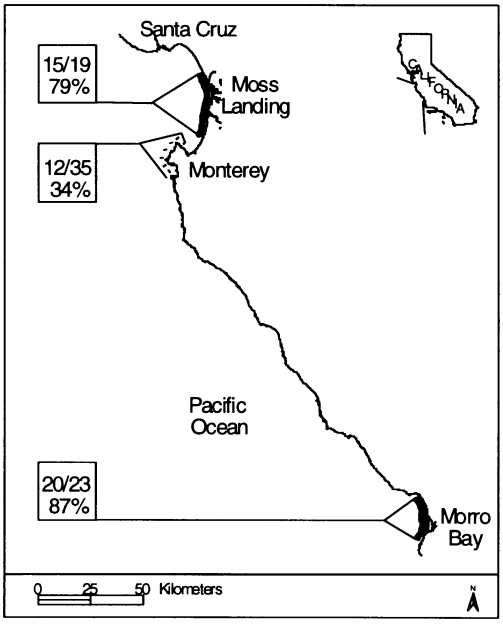

Figure 1: Spatial clusters with higher (▬) or lower(·) than expected proportions of sea otters that were seropositive for Toxoplasma gondii

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

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

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

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

3.4. Logistic regression analysis

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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