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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.

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

1. Introduction

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

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

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

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

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

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

2. Materials and methods

2.1. Study population

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

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

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

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

2.2. Serum collection and testing, live and dead otters

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

2.3. Definition of risk factors

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

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

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

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

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

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

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

2.4. Univariate analysis of risk factors

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

2.5. Spatial analysis

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

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

2.6. Logistic regression analysis

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

3. Results

3.1. Seroprevalence

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

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

3.2. Risk factors

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

3.3. Spatial analysis

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

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

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

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

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

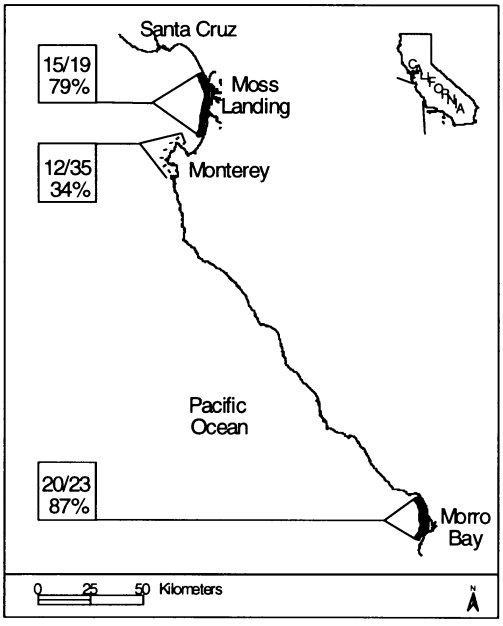

Fig. 1. Spatial clusters with higher (dark lines) or lower (dotted line) than expected proportions of sea otters that were seropositive for *Toxoplasma gondii*.

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

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

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

3.4. Logistic regression analysis

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

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

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

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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