Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
International Journal for Parasitology 34 (2004) 275–284
www.parasitology-online.com

An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality☆

M.A. Miller[a,b,*], M.E. Grigg[c,d], C. Kreuder[b], E.R. James[d], A.C. Melli[b], P.R. Crosbie[e], D.A. Jessup[a], J.C. Boothroyd[c], D. Brownstein[a], P.A. Conrad[b]

[a]*California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, 1451 Shaffer Road, Santa Cruz, CA 95060, USA*
[b]*Wildlife Health Center, School of Veterinary Medicine, Old Davis Road, Davis, CA 95616, USA*
[c]*Department of Microbiology and Immunology, Stanford University School of Medicine, 299 Campus Drive, Stanford, CA 94305, USA*
[d]*Infectious Diseases, Departments of Medicine and Microbiology and Immunology, University of British Columbia, D452 HP East, VGH, 2733 Heather Street, Vancouver, BC, Canada V52 3J5*
[e]*Department of Biology, California State University-Fresno, 2555 East San Ramon Avenue, M/S SB73, Fresno, CA 93740-8034, USA*

Received 21 October 2003; received in revised form 15 December 2003; accepted 16 December 2003

Abstract

Toxoplasma gondii-associated meningoencephalitis is a significant disease of California sea otters (*Enhydra lutris nereis*), responsible for 16% of total mortality in fresh, beachcast carcasses. *Toxoplasma gondii* isolates were obtained from 35 California otters necropsied between 1998 and 2002. Based on multi-locus PCR-restriction fragment length polymorphism and DNA sequencing at conserved genes (*18S rDNA*, *ITS-1*) and polymorphic genes (*B1*, *SAG1*, *SAG3* and *GRA6*), two distinct genotypes were identified: type II and a novel genotype, here called type x, that possessed distinct alleles at three of the four polymorphic loci sequenced. The majority (60%) of sea otter *T. gondii* infections were of genotype x, with the remaining 40% being of genotype II. No type I or III genotypes were identified. Epidemiological methods were used to examine the relationship between isolated *T. gondii* genotype(s) and spatial and demographic risk factors, such as otter stranding location and sex, as well as specific outcomes related to pathogenicity, such as severity of brain inflammation on histopathology and *T. gondii*-associated mortality. Differences were identified with respect to *T. gondii* genotype and sea otter sex and stranding location along the California coast. Localised spatial clustering was detected for both type II (centred within Monterey Bay) and x (centred near Morro Bay)-infected otters. The Morro Bay cluster of type x-infected otters overlaps previously reported high-risk areas for sea otter infection and mortality due to *T. gondii*. Nine of the 12 otters that had *T. gondii*-associated meningoencephalitis as a primary cause of death were infected with type x parasites.

Keywords: *Toxoplasma gondii*; *SAG*; *B1*; *GRA6*; Type x; Sea otter; *Enhydra lutris*

1. Introduction

Protozoal meningoencephalitis was first recognised as a disease entity of California sea otters in 1992 (Thomas and Cole, 1996). Two species of protozoa have been linked with these fatal infections, namely *Toxoplasma gondii* and the closely related apicomplexan *Sarcocystis neurona* (Lindsay et al., 2000; Miller et al., 2001a,b; Kreuder et al., 2003). Based on the findings from immunohistochemistry and parasite isolation, *T. gondii* was present in brain tissue from 36% of California otters necropsied between 1998 and 2001, while 4% were infected with *S. neurona* (Miller et al., 2002b). *Toxoplasma gondii* infection with associated meningoencephalitis is now recognised as a major cause of death in subadult and prime-aged adult sea otters, accounting for 16% of total otter mortality (Kreuder et al., 2003). In contrast, healthy, immunocompetent adult humans and terrestrial animals generally have subclinical or mild *T. gondii* infections while infections of foetuses, neonates

☆ Nucleotide sequence data reported in this paper are available on GenBank, EMBL and DDDJ databases under the Accession Nos. AY488160–AY488170.

* Corresponding author. Address: California Department of Fish and Game, Marine Wildlife Veterinary Care and Research Center, 1451 Shaffer Road, Santa Cruz, CA 95060, USA. Tel.: +1-831-212-7007; fax: +1-831-469-1723.
E-mail address: mmiller@ospr.dfg.ca.gov (M.A. Miller).

0020-7519/$30.00
doi:10.1016/j.ijpara.2003.12.008

and aged or immunosuppressed individuals may be severe, especially if infected with atypical genotypes (Dubey and Beattie, 1988; Grigg et al., 2001b). Several explanations have been proposed for apparent high sea otter susceptibility to infectious agents such as *T. gondii*, including environmental pollutant exposure (Kanaan et al., 1998; Nakata et al., 1998), inbreeding depression (Larson et al., 2002) or immunosuppression (Kanaan et al., 1998). Possible cofactors for exacerbating *T. gondii* infection in otters have been discussed elsewhere (Cole et al., 2000; Miller et al., 2002b; Kreuder et al., 2003).

Strain variation or novel host–parasite interactions could also play a role in these infections. Although the genus *Toxoplasma* consists of only one species, significant intraspecific differences with respect to disease presentation exist (Sibley and Boothroyd, 1992; Howe and Sibley, 1995; Darde, 1996; Lehmann et al., 2000; Grigg et al., 2001a,b; Sibley et al., 2002). Strain classification is based on pathogenicity in mice as well as variability in specific genetic markers, including the *B1* and *SAG* genes. Studies employing these markers have confirmed a clonal population structure for the genus *Toxoplasma*, and revealed that the three dominant *T. gondii* genotypes (types I, II and III) evolved from one or more historically recent episodes of sexual recombination between two distinct *Toxoplasma* ancestral lineages within felid definitive hosts (Grigg et al., 2001a; Su et al., 2003; Volkman and Hartel, 2003). Additional genotypes are recognised, but appear to be less common in nature and are often restricted to particular ecological niches (Howe and Sibley, 1995; Darde, 1996; Grigg et al., 2001b). Based on PCR and restriction fragment length polymorphism (RFLP) analyses at two surface antigen genes (*SAG1* and *SAG2*) for 13 sea otter *T. gondii* isolates, Cole et al. (2000) reported that *T. gondii* strains obtained from sea otters were type II genotypes, but with some intrinsic genetic variation. RFLP analysis at the *SAG1* locus revealed heterogeneity, with 7 of 13 sea otter isolates expressing a new allele (designated 3) instead of the expected allele two which is typical of type II strains.

In this study, multi-locus PCR-RFLP DNA sequence genotyping at the *B1*, *SAG1*, *SAG3* and *GRA6* genes detected allelic polymorphisms that identified two distinct genotypes among 35 *T. gondii* strains isolated from necropsied California sea otters. To investigate whether parasite genotype is a contributing factor in the high proportion of *T. gondii*-related mortality observed in California otters, we used epidemiological methods to examine the relationship between the isolated *T. gondii* genotype and specific infection-related outcomes, including parasite numbers in brain tissue, severity of brain lesions on histopathology and diagnosis of *T. gondii*-associated meningoencephalitis as a primary or contributing cause of death. We also analysed associations between the isolated *T. gondii* genotype and each otters stranding location along the central California coast.

2. Materials and methods

2.1. Sea otter necropsy and serological testing

All California sea otters (*Enhydra lutris nereis*) that were culture-positive for *T. gondii* (as described in Section 2.2) and were necropsied between 1998 and 2002 were included in this study. Otters were necropsied as previously described (Miller et al., 2002a; Kreuder et al., 2003), either at the Marine Wildlife Veterinary Care and Research Centre (MWVCRC) in Santa Cruz, CA, or at the University of California, Davis School of Veterinary Medicine. Where appropriate, swabs or tissues were collected at necropsy and submitted to the UC Davis Veterinary Medical Teaching Hospital for bacterial and/or fungal culture. Whole blood was collected postmortem, spun at 25,000 rev./min for 10 min and the resulting serum tested for the presence and titre of antibodies to *T. gondii* using an indirect immunofluorescent antibody test (IFAT) as previously described (Miller et al., 2002a). The antibody titre selected to establish *T. gondii* seropositivity ($\geq$1:320 serum dilution) was determined previously through test validation. The IFAT was used to compare the results between the postmortem *T. gondii* titre for each otter and its isolated *T. gondii* genotype. Infection with *T. gondii* was confirmed where possible by brain histopathology, immunohistochemistry and/or IFAT. *Toxoplasma gondii*-related meningoencephalitis was considered a primary or contributing cause of death if gross findings, radiographs, bacterial and fungal culture results, serological tests and histopathological examination of all major tissues revealed it to be one of the three most significant diagnostic abnormalities detected for that animal. Affected otters generally had moderate to severe lymphocytic to lymphoplasmacytic, variably necrotising meningoencephalitis affecting the meninges and neuropil, often with significant perivascular cuffing. Interpretation and scoring of sea otter brain tissues was completed prior to genotyping so that the pathologist was blinded to the *T. gondii* genotype(s) derived from each sea otter.

2.2. Parasite isolation and characterisation

At necropsy, fresh cerebrum and cerebellum were collected aseptically into sterile bags containing antibiotic saline solution (Miller et al., 2002a) and were shipped to Dr Conrad's laboratory at UC Davis for parasite isolation. For each necropsied sea otter, primary parasite isolation from trypsinised and non-trypsinised brain homogenate was attempted using embryonic monkey kidney (MA104) cell monolayers, as previously described (Miller et al., 2001a). All brain tissue-inoculated cell cultures were maintained for at least 30 days. *Toxoplasma gondii* isolates from sea otters were characterised via brain histopathology, immunohistochemistry and/or IFAT, as previously described (Miller et al., 2002a). Cryopreserved, *T. gondii*-infected MA104 cell pellets submitted for genotyping were used to inoculate

uninfected human foreskin fibroblasts in culture for parasite amplification prior to purification of genomic DNA.

2.3. Genotyping of isolates by PCR-RFLP and sequencing

The *T. gondii* genotype(s) for each isolate were determined by multi-locus PCR-RFLP analysis at the *B1* and *SAG1*, *SAG2* and *SAG3* genes (Burg et al., 1989; Howe and Sibley, 1995; Howe et al., 1997; Grigg et al., 2001b). Positive controls consisted of dilutions of known quantities of genomic DNA from well characterised *T. gondii* type I (RH), II (PRU) and III (CEP) strains. Negative controls consisted of deionised water and purified genomic DNA from non-infected human foreskin fibroblasts. Reaction mixtures and amplification conditions were according to published methods (Fazaeli et al., 2000; Grigg and Boothroyd, 2001; Grigg et al., 2001b). Briefly, 1 μl of template DNA was added to a total reaction volume of 50 μl, consisting of 5 μl of 10 × PCR buffer (Sigma PCR buffer containing 15 mM $MgCl_2$), 5 μl of 0.1 mM dNTP mix, 1 μl of 20 pmol forward primer and 1 μl of 20 pmol reverse primer and 1 U Taq polymerase. Template DNA was heated at 95 °C for 3 min prior to adding it to the reaction mixture. Amplification consisted of 35 PCR cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, with a post-PCR extension of 10 min at 72 °C. Amplification products were visualised using ethidium bromide staining on 1–2% agarose gels. PCR product from each reaction was digested with restriction enzymes (Table 1) to identify diagnostic restriction fragment patterns on subsequent agarose gels.

Genomic DNA from parasite isolates was PCR amplified for sequencing at the *18S* ribosomal DNA (*18S rDNA*) gene and the adjacent first internally transcribed spacer (*ITS-1*), as well as the *B1*, *SAG1*, *SAG3* and *GRA6* genes. Phylogenetically informative portions of the *18S* rDNA and *ITS-1* genes were PCR-amplified and sequenced as described previously (Miller et al., 2001b). Consensus sequences of *T. gondii 18S* rDNA and *ITS-1* DNA from each sea otter isolate were aligned with each other and with other *T. gondii* sequences for the same loci deposited in GenBank using Clustal X (Thompson et al., 1997). Subsequent hand editing was performed in MacClade 4.0 (Maddison, D., Maddison, W., 2000. MacClade 4: Analysis of Phylogeny and Character Evolution. Sinaeur, Sunderland, MA.).

2.4. Data compilation and analysis

The two-sided χ^2 test of independence was used to compare associations between the isolated *T. gondii* genotype and demographic factors such as sea otter sex, age and stranding location. The χ^2 test was also used to compare associations between the *T. gondii* genotype and potential outcome variables for *T. gondii*-infected otters. Outcome variables that were evaluated included IFAT titre, severity of brain inflammation and tissue necrosis, number of *T. gondii* cysts on histopathology, whether *T. gondii* meningoencephalitis was the primary cause of death and whether meningoencephalitis played any role in death (as a primary or contributing cause of death). Stranding location was defined as each sea otter's coastal location to the nearest 0.5 km, along a hand-smoothed line set offshore at approximately 55 m (30 fathoms) depth. The sea otter range was also subdivided as north (Half Moon Bay to the northern Big Sur Coast) or south (southern Big Sur coast to Santa Barbara) for some portions of the study. Parasite numbers as well as the extent and type of inflammation were scored after examining five to eight 5.0 μm thick H and E sections of cerebrum, cerebellum, mesencephalon and brainstem from each otter (Kreuder et al., 2003).

Table 1
Polymorphic surface antigens utilised for *Toxoplasma gondii* strain typing

Toxoplasma antigen locus	Chromosome	Restriction enzyme	*Toxoplasma* genotype[a]				
			I	II[b]	III	IV	x[b]
B1	IX	*Xho* I	−	±	±	−	−
		Pml I	−	±	±	−	+
SAG1	VIII	*Sau* 96I	+	−	−	+	+
		Hae II	−	+	+	−	+
SAG2A	VIII	*Sau* 3AI	−	−	+	+	−
		Hha I	−	+	−	−	+
SAG3	II	*Nci* I	++	−	+	+	−
		Alw NI	−	+	−	−	+
GRA6	nd	*Mse* I	+	++	++	nd	++

[a] Groupings are based on restriction fragment length polymorphisms resulting from cleavage by the listed restriction enzyme(s) according to the following references: *B1* (Grigg and Boothroyd, 2001); *SAG1* (Cole et. al., 2000); *SAG2A* (Howe et al., 1997); *SAG3* (Grigg et al., 2001b); *GRA6* (Fazaeli et al., 2000). nd, not determined; minus (−) indicates no digestion; plus (+) indicates digestion; (±) at the 35-fold repeat *B1* gene indicates that only a proportion of the PCR amplification product is digested while the rest remains undigested (Fig. 1).

[b] Established genotypes present in California sea otters.

Fisher exact test was used instead of the χ^2 test when the expected frequency for any variable distribution fell below five. Comparison among outcome variables (*T. gondii* titre category, tissue cyst number, severity of meningoencephalitis, whether meningoencephalitis played a role in death as a primary or contributing cause and whether *T. gondii* meningoencephalitis was determined to be the primary cause of death) was stratified by any variable significantly associated with strain type to evaluate confounding and effect modification. SPSS software version 11.0 (www.spss.com, SPSS Inc., Chicago, IL) was used for all univariate and stratified comparisons of groups. Multivariate logistic regression methods were precluded by small sample size.

The spatial distribution of stranding location for otters with each different *T. gondii* genotype was evaluated using the spatial scan statistic (Kulldorf and Nagarwalla, 1995). Data were tested for both high- and low-risk clusters and clusters were considered to be significant at $P < 0.10$. Spatial analysis was conducted using statistical software (Kulldorf M, Information Management Services I, Software for spatial and space-time scan statistics, 2002, SaTScan v. 3.1).

3. Results

3.1. Sea otter necropsy, parasite isolation and histopathological examination

Toxoplasma gondii isolates were obtained from 35 California sea otters necropsied between June 1998 and June 2002. *Toxoplasma gondii* brain infection was confirmed via histopathology and immunohistochemistry, postmortem serum testing for the presence and titre of antibodies to *T. gondii* and *S. neurona*, and by molecular genotyping at a minimum of three genetic loci. On microscopic examination of brain tissue, 10 otters had moderate to high numbers of tissue cysts within the neuropil, five otters had low numbers of cysts and 20 otters from which *T. gondii* was isolated had no visible tissue cysts on histopathology. For two otters, concurrent brain infection with *S. neurona* was detected, based on findings from histopathology, IFAT and/or parasite isolation in cell culture. To eliminate interpretation error due to concurrent protozoal brain infections, both animals were excluded from statistical analyses of brain inflammation and specific cause of death. Excluding those two cases, lymphocytic to lymphoplasmacytic, variably necrotising meningoencephalitis was minimal or absent for 14 *T. gondii*-infected otters, mild for eight otters and moderate to severe for 11 otters. Meningoencephalitis attributed to *T. gondii* was considered the primary cause of death for 12/33 sea otters enrolled in the study, and was considered as a primary or contributing cause of death for 18/33 otters.

3.2. Genotyping of isolates by PCR-RFLP and DNA sequencing

Isolates of *T. gondii* from six otters that stranded between May and July, 1998 were subjected to PCR amplification and sequencing analysis for genotyping. Partial sequences were obtained for *18S* rDNA, encompassing the variable portions of this highly conserved gene. After alignment and editing, these partial sequences were each 530 bp (GenBank Accession Nos. AY488160–AY488165). All six *18S* rDNA sequences were identical to each other and with all comparison sequences obtained from GenBank. Partial *ITS-1* sequences were obtained from five of these same six isolates. After alignment and editing, these sequences were each 454 bp (GenBank Accession Nos. AY488166–AY488170) and were identical to each other as well as to all comparison sequences for *T. gondii ITS-1* from GenBank. Thus, analysis at more polymorphic loci was required to distinguish between the various sea otter isolates. Multi-locus PCR-RFLP analyses at *B1* and surface antigen genes (*SAG1*, *SAG2A* and *SAG3*) were completed for all 35 sea otter isolates. RFLP analysis at *SAG2A* and *SAG3* identified the type II allele for all isolates. At *B1* and *SAG1*, 14 out of 35 isolates genotyped as type II strains, with no type I or III genotypes identified among the 35 isolates examined. For 21 sea otter isolates (60%), a novel RFLP pattern was observed at *B1* and *SAG1* that was distinct from the archetypal type I, II and III lineages and these isolates were hereafter designated as genotype (type) x (Table 1).

Since the true extent of polymorphism between alleles cannot be fully assessed by RFLP analysis, it was unclear how this atypical genotype related to archetypal *T. gondii* alleles. Thus, DNA sequencing at the highly polymorphic loci *B1*, *SAG1* and *SAG3* was completed for two type x and one type II sea otter isolates. At *SAG3* all sea otter isolates possessed the type II strain allele when compared with those from archetypal strains (data not shown). The *SAG1* locus is comprised of over 1.3 kb of coding and non-coding sequence containing 15 polymorphic sites. However, only two alleles have been identified among archetypal lines that distinguish type I strains from type II and III strains (which possess identical sequences). For both sea otter type x isolates, the sequence at the *SAG1* locus was identical to those obtained for type II and III genotypes except that at position 211, a C-for-T substitution identified a *Sau*96 I restriction site that was the basis for the unique RFLP pattern that has been previously described (Cole et al., 2000; Fig. 1). At the 35-fold tandemly arrayed *B1* gene, over 600 nucleotides of high fidelity sequence was obtained for the two type x isolates and three differences were identified with respect to archetypal type II alleles. At nucleotide position 504, a C was uniformly present in type x resulting in complete digestion with the restriction enzyme *Pml* I (Fig. 1); type II strains had a mix of C and G at this position in the 35 repeats. Similarly, at position 366 type x strains

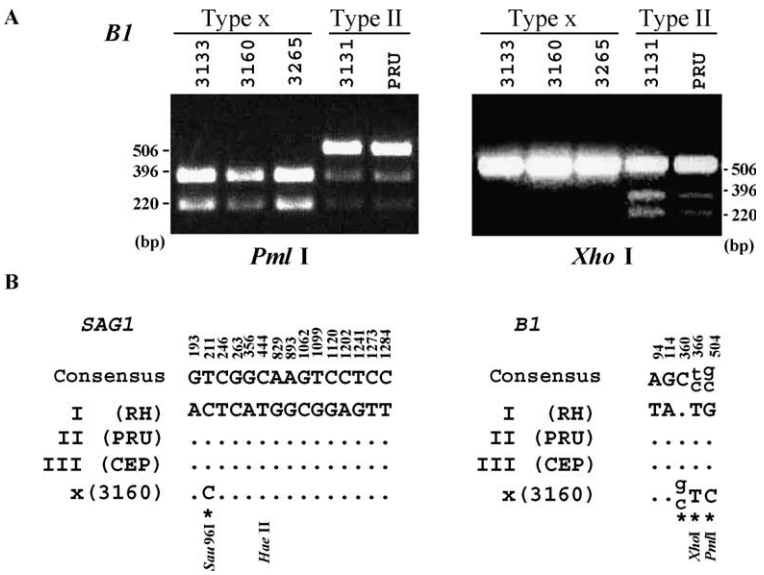

Fig. 1. (A) *B1* PCR-restriction fragment length polymorphism analysis distinguishes type x from type II strains of *Toxoplasma*. *B1* PCR-amplified DNA from all 35 sea otter *Toxoplasma* isolates were digested with either *Pml* I or *Xho* I and separated in 1% ethidium-bromide stained agarose gels for visualisation. A representative digest of three type x (sea otters 3133, 3160, 3265) and two type II (sea otter 3131, strain PRU) genotypes is shown. (B) DNA sequence analysis at *SAG1* and *B1*. Polymorphic sites that exist between the three archetypal type I (RH), II (PRU) and III (CEP) lineages were compared with sequences obtained from two type x and one type II sea otter genotypes. The consensus sequence was defined as the nucleotide common to at least two of the three archetypes. Periods (·) indicate identity with the consensus sequence. The numerical positions annotated refer to the numbered sites in the published sequences (GenBank Accession Nos. X14080 and AF179871, respectively). Sites demarcated by an asterisk (∗) indicate polymorphic sites that distinguish type x alleles from those possessed by archetypal I, II and III genotypes. Nucleotide positions 360, 366, and 504 at *B1* possess polymorphic sites at which each of two nucleotides were observed at significant levels (the upper letter represents the more abundant nucleotide) in the sequence electropherograms.

had a uniform T, whereas type II strains had a mix of T and C (a C is needed for *Xho* I digestion). At nucleotide position 360, the *B1* gene repeats in type x isolates possessed a G or a C vs. a uniform C in type II. Thus, DNA sequencing of type x vs. type II isolates identified three polymorphisms at the *B1* locus and one at the *SAG1* locus.

The relationship among alleles across many genetic loci provides a window into the evolutionary origins of different genotypes. To distinguish whether type x strains possessed alleles consistent with these isolates existing as a genetically drifted type II lineage versus a novel genotype that possessed a different mix of alleles across genetic loci, additional sequencing was performed at the highly polymorphic *GRA6* locus. PCR-RFLP analysis at *GRA6* tentatively identified a type II lineage allele among the 35 sea otter isolates (Fig. 2). RFLP analyses were not sufficiently resolved, however, so DNA sequencing was performed on type II and x sea otter isolates. Surprisingly, while the type II sea otter isolate possessed the expected type II lineage allele, the type x allele was entirely novel (Fig. 2). The type x allele possessed two insertions, a unique three base pair GAT insertion and a 15 base pair insertion that is present in the type I and III lineage alleles, as well as many unique polymorphic nucleotide sites that readily distinguished the type x allele from the type II lineage allele. Of importance, the nucleotide polymorphism that was responsible for the type II vs. I and III allele designation (identified by *Mse* I digestion) was shared by the type x allele (Fig. 2). These results thus provide compelling evidence that the type x isolates are new, apparently recombinant, non-archetypal genotypes that are genetically distinct from the type II lineage.

3.3. Associations between T. gondii genotype and selected risk factors

Univariate analysis revealed a significant association between the isolated *T. gondii* genotype and each otter's carcass recovery location (Table 2). While otters infected with genotype x were found throughout the sea otter range, otters stranding in the southern half of the range (within or near Estero Bay) were eight times (95% CI 1.4–45.1) more likely to be infected with genotype x *T. gondii* than otters stranding in the northern half of the range (within or near Monterey Bay; $P = 0.011$). Of 14 *T. gondii*-infected otters stranding in the southern half of the range, most (83%) were infected with the type x genotype (Table 2). Spatial analysis also revealed a significant cluster of genotype x isolates centred on the smaller estuary of Morro Bay, located within Estero Bay in the southern aspect of the sea otter range (35.391998 N, 120.872002 W, $P = 0.07$). This spatial cluster of genotype x-infected sea otters extended 75 km southward from the municipal pier at Cayucos, CA to the northern end of Pismo State Beach. All (9/9) freshly dead, *T. gondii*-positive otters recovered from this region were infected with *T. gondii* genotype x strains.

In contrast, nearly all (86%, or 12/14) otters that were positive for *T. gondii* genotype II were recovered in

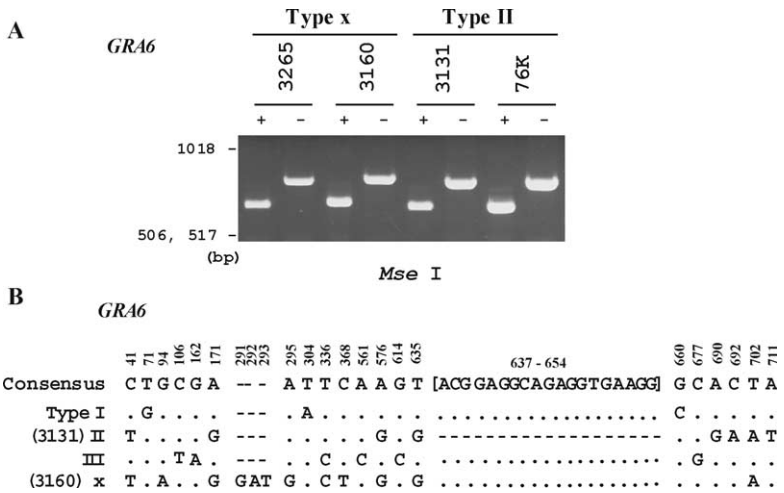

Fig. 2. (A) *GRA6* PCR-restriction fragment length polymorphism analysis of sea otter isolates. *Mse* I digestion of *GRA6* PCR-amplified DNA from all 35 sea otter isolates carried out identified a type II lineage allele expressed by the isolates, and a representative digest for two type x and one type II sea otter isolate is shown. *Mse* I digestion distinguishes between *GRA6* alleles possessed by archetypal strains I, II and III (Fazaeli et al., 2000); plus (+) indicates digestion with *Mse* I, minus (−) indicates undigested PCR amplified *GRA6* DNA. (B) DNA sequence analysis at *GRA6* identifies a unique allele. Polymorphic sites among archetypal lineages (Fig. 1) were compared with sequences obtained from one type x and one type II sea otter isolate. Consensus sequence was defined as the nucleotide common to at least two of the three archetypes. Dashes (−) indicate deletions and periods (·) indicate identical nucleotides with the consensus sequence. The numerical positions annotated refer to the numbered sites in the published sequences (GenBank Accession Nos. AF239283 [RH], AF239284 [BEV] and AF239286 [NED]).

the northern half of the range. Spatial analysis revealed a significant cluster (90%, or 10/11) of genotype II-infected otters from Monterey Bay (centred at 36.920002 N, 121.865997 W, $P = 0.005$). This spatial cluster of genotype II-infected otters spanned 70 km, extending from Natural Bridges State Park in Santa Cruz southward to Point Alones in Monterey.

Univariate analysis also revealed a significant association between the *T. gondii* genotype and sea otter sex (Table 2). Females were nearly five times (95% CI 1.05–22.0) more likely to be infected with genotype x strains than were males ($P = 0.036$). When sea otter sex was stratified by stranding location (north or south), differences between gender groups remained marginally significant for otters stranding in the northern half of the range ($P = 0.087$), but not significant for otters stranding in the south ($P = 0.473$), suggesting that females were more likely to be infected with genotype x in the north, but that genotype x infection was common in both males and females in the south. The age class of otters infected with *T. gondii* genotypes II (3/14 immature otters and 8/21 adults) and x (11/14 immature otters and 13/21 adults) did not differ significantly ($P = 0.461$). Serum was available for testing from 33 of the 35 otters from which *T. gondii* was isolated and genotyped. No significant associations were detected between the *T. gondii* genotype isolated from brain tissue and each otter's postmortem IFAT titre ($P = 0.434$).

In humans and animals, *T. gondii*-associated brain inflammation is typically characterised as lymphocytic to lymphoplasmacytic meningoencephalitis with variable tissue necrosis and dystrophic mineralisation, sometimes accompanied by round to elliptical, thin-walled tissue cysts and/or intracellular or free zoites (Dubey and Frenkel, 1988). Characteristic inflammatory brain lesions were present in 63% of genotype x-infected sea otters, but only in 50% of genotype II-infected sea otters, although this difference was not statistically significant (Table 2). Similarly, no significant association was detected between the genotype isolated from brain tissue and the number of *T. gondii* tissue cysts observed in brain tissue on histopathology ($P = 0.791$; Table 2).

Compared to genotype II, more genotype x-infected otters had *T. gondii*-associated meningoencephalitis as a primary cause of death, although this difference was not statistically significant ($P = 0.126$). *T. gondii*-associated meningoencephalitis was the primary cause of death for 47% (9/19) of otters from which type x strains were isolated, compared to only 21% (3/14) of otters infected with type II genotypes (Table 2). When both primary and contributing causes of death were considered (Table 2), *T. gondii*-associated meningoencephalitis was a primary or contributing cause of death for 63% (12/19) of otters from which genotype x was isolated, compared with only 50% (7/14) of otters from which genotype II was isolated ($P = 0.450$).

4. Discussion

This study reveals new information on genotypes of *T. gondii* infecting California sea otters. In addition we explore associations between the *T. gondii* genotypes isolated from brain tissue at necropsy and otter stranding location, sex and age, as well as standardised assessments of *T. gondii*-associated pathogenicity, such as tissue cyst

Table 2
Number of sea otters (*Enhydra lutris neries*) infected with each *Toxoplasma gondii* genotype: distribution among demographic risk factors and indicators of pathogenicity

Risk factor	*Toxoplasma gondii*	
	Genotype II ($n = 14$)	Genotype x ($n = 21$)[a]
Otter carcass recovery location[b]		
a. North coast	12	9
b. South coast	2	12
Otter sex[c]		
a. Male	11	9
b. Female	3	12
Number of T. gondii-like tissue cysts in brain		
a. None	9	11
b. Low numbers	2	3
c. Moderate to high numbers	3	7
Severity of T. gondii-associated inflammation and tissue necrosis (brain)		
a. None	7	7[a]
b. Mild	4	4[a]
c. Moderate to severe	3	8[a]
Otters with T. gondii as a		
a. Primary or contributing cause of death	6	12[a]
a. Primary cause of death	3	9[a]

[a] For indicated proportions, $n = 19$ because two *Sarcocystis neurona*-infected sea otters were excluded from scoring of *T. gondii*-associated brain inflammation and cause of death due to high potential for interpretation error.
[b] Significant difference detected between locations ($P = 0.011$).
[c] Significant difference detected between sexes ($P = 0.036$).

density, severity of histopathological lesions in brain tissue and cause of death. Genotyping was completed for 35 *T. gondii* isolates and no differences were detected at the *18S* rDNA and *ITS-1* loci. However, based on molecular genotyping methods at polymorphic *B1*, *SAG1, SAG3* and *GRA6* genes, 40% ($n = 14$) of the sea otter isolates were type II strains, whereas 60% ($n = 21$) comprised a genetically distinct genotype, designated type x. DNA sequencing at *B1*, *SAG1* and *GRA6* showed that type x strains are genetically distinct from type II strains. Given the unique allele at the highly polymorphic *GRA6* gene, it is highly unlikely that type x strains are variant type II strains that have undergone slight genetic drift, nor is it likely that type x strains represent a new mix of the archetypal alleles as is seen with types I–IV. Further sequence analysis at additional loci will be needed to determine how type x genotypes are related to the archetypal lines that predominate in the majority of hosts so far studied worldwide. Interestingly, *T. gondii* strains bearing a type x *SAG1* allele by RFLP analysis have been reported elsewhere for *Toxoplasma* isolates derived from a turkey, two deer and two humans in the southeastern United States (Howe and Sibley, 1995) and in sea otters (Cole et al., 2000). However, DNA sequencing at the *SAG1* and *GRA6* loci were not performed for these isolates, so it is unclear at present whether these strains represent type x strains identical to those that are infecting the California otters described in this study.

Little is known about the distribution and biology of 'native' *T. gondii* genotypes in terrestrial animals living along the Pacific coast of North America. Interestingly, exposure to heavy freshwater runoff was previously identified as a significant risk factor for *T. gondii* infection in California sea otters (Miller et al., 2002b). Upon gaining access to the nearshore marine environment, type x parasites could possess traits that render them more capable of infecting marine mammals. Since the sea otter diet consists primarily of filter-feeding invertebrates (Reidman and Estes, 1990) and *T. gondii* oocyst uptake by estuarine and marine bivalves has been demonstrated under laboratory conditions (Lindsay et al., 2001; Arkush et al., 2003), the most plausible route of sea otter infection is through consumption of infective oocysts in ingested prey. In both laboratory studies, mice treated with *T. gondii*-exposed bivalve tissues or fluids became infected. The predominance of type x strains in California sea otters might thus be explained by a predominance of this unusual genotype in terrestrial animals living along the California coast, increased capacity for oocyst production or extended patentcy in the definitive felid host and/or increased ability of type x strains to survive and remain infective in marine water or invertebrate prey species. Studies are in progress now to investigate the biological potential of this unusual *T. gondii* strain and to genotype *T. gondii* isolates obtained from terrestrial wildlife and domestic animals from coastal California.

The predominance of the unusual genotype x parasites in the majority of *T. gondii*-infected sea otters was markedly different from the expected frequencies for strains identified from other studies of both human and animal infections. Several reports have shown that the substantial majority of infections (~60–80%) in warm-blooded vertebrates are of type II parasites, and of the remaining isolates identified, the majority are of either type I or III parasites (Howe and Sibley,1995; Darde, 1996; Howe et al., 1997; Boothroyd and Grigg, 2002). However, when particular ecological niches are sampled (i.e. outbreaks in Erechim, Brazil and Victoria, Canada which are known or suspected to be caused by type I strains) or the strains responsible for infection of patients experiencing atypical disease presentations are identified (i.e. ocular toxoplasmosis by type IV strains), a skewing of the expected frequencies has been documented (Bowie et al., 1997; Grigg et al., 2001b). A separate study of *T. gondii* infection of California sea otters by Cole et al. (2000) also identified the type x *SAG1* allele in the majority of isolates obtained from infected otters. In our study, type x-infected sea otters were more common (e.g. 21 genotype

x, compared with 14 genotype II isolates), more widespread, and were present throughout the northern and southern halves of the sea otter range, while genotype II-infected otters were most commonly detected within Monterey Bay.

The clustering of type II isolates within a small area of Monterey Bay was surprising and might indicate a localised point source exposure to type II strains. Indeed, all but two type II isolates (12/14) were obtained from sea otters stranding within this area. Elkhorn Slough is roughly at the centre of this spatial cluster, and otters stranding near the slough were found to be at slightly higher risk for *T. gondii* infection in a previous study (Miller et al., 2002b). The other two type II isolates were obtained from a subadult and an aged adult male sea otter, each recovered dead in the southern portion of the sea otter range. Male sea otters express less site fidelity than females and are more likely to range widely in their efforts to establish and defend territories (Reidman and Estes, 1990). In addition, annual movements of males toward the northern and southern range peripheries are recognised (Reidman and Estes, 1990). Thus it is possible that these two otters became infected by genotype II *T. gondii* in Monterey Bay, prior to migration toward the southern part of the sea otter range where they were recovered. Detailed analysis of these genotypes using highly polymorphic micro and/or macrosatellite loci should help to establish the degree of genetic heterogeneity among *T. gondii* type II isolates derived from sea otters stranding at different locations along the coast, and thus could support or refute our hypothesis of a localised point source of genotype II exposure within Monterey Bay.

In contrast, spatial analysis revealed that the Morro Bay region is a high-risk area for genotype x *T. gondii* infections, with 100% (9/9) of locally stranded otters found to be infected with genotype x. Interestingly, for 12 otters in this study with fatal, *T. gondii*-related meningoencephalitis, half (six) were recovered from within 50 km of Morro Bay, and five of these six otters were infected with genotype x *T. gondii*. Two previous epidemiological studies have identified the southern sea otter range, particularly the area around Morro Bay as a high-risk area for *T. gondii*-related sea otter infection and mortality (Miller et al., 2002a,b; Kreuder et al., 2003). Both studies concluded that additional, as yet unrecognised risk factors (or cofactors) in addition to those that were evaluated were felt to be playing a role in this localised spatial clustering. Although our analyses were inconclusive, infection with type x strains may be one factor contributing to the high proportion of *T. gondii*-associated infection and mortality observed for otters living in the southern portion of the sea otter range. As with the observed Monterey Bay spatial cluster of genotype II-infected otters, as yet unrecognised cofactors (outlined below) and sampling error may be contributing to the genotype-specific clustering observed in this study.

Factors contributing to spatial 'hot spots' of *T. gondii* infection and disease worldwide are often attributed to a unique local combination of environmental, host and parasite-specific characteristics. All are equally plausible factors that should be considered in continuing investigations of *T. gondii*-associated infection and mortality of California sea otters. Potential environmental factors include local variation in winds, tides, coastal geography, precipitation, felid density, water temperature, environmental pollutant levels or marine currents. Potential otter-specific risk factors include age, nutritional or reproductive status, genetic factors, presence of unrecognised immunosuppressive agents and concurrent disease. Potential parasite-specific factors include variations in parasite prevalence, infectivity and oocyst production, survival or pathogenicity as previously discussed. To our knowledge, there are insufficient data at present to conclude with confidence which combination(s) of these factors are most critical in the distribution and pathogenesis of *T. gondii*-related sea otter infection and disease. Focused studies on individual risk factors as well as large-scale epidemiological studies are needed to elucidate the roles of these potential cofactors.

In the present study we addressed one small part of this complex puzzle; variation in *T. gondii* genotype and its relation to selected risk factors for *T. gondii*-associated infection and disease. Studies in mice have shown that infection with different *T. gondii* genotypes result in dramatically different disease outcomes. For instance, type I strains are highly virulent in mice, whereas type II strains are less virulent but are often associated with high cyst burdens and significant meningoencephalitis (Suzuki et al., 1989; Sibley and Boothroyd, 1992; Howe and Sibley, 1995). Thus, parasite genotype could be one risk factor for sea otter infection with and mortality due to *T. gondii*. Our data set for comparisons of pathogenicity (33 *T. gondii*-infected otters) may have been too small to establish statistical significance. However, this study provides evidence to suggest that the infecting *T. gondii* genotype could be a contributing factor, worthy of more in-depth study. Both genotype x and II strains were associated with substantial brain lesions and mortality in some infected sea otters. However, otters infected with type x strains tended to have moderate to severe meningoencephalitis on histopathology more frequently than otters infected with type II strains. In addition, more type x-infected otters (47%) had *T. gondii*-associated meningoencephalitis as a primary cause of death, when compared with type II-infected otters (21%). Thus, type x *T. gondii* could be more likely to cause meningoencephalitis, although statistically significant differences were not identified in this study.

In addition to small sample size, other factors could have inhibited our ability to detect significant associations between the *T. gondii* genotype isolated and potential risk factors such as otter location and primary cause of death. Otters may not strand in the same location where they initially became infected with *T. gondii*, and otter carcasses may continue to move after death due to the action of winds and tides prior to carcass recovery. In addition, *T. gondii*

infections are typically chronic and otters are a long-lived species, both of which negatively may impact efforts to identify localised point sources of *T. gondii* exposure. These factors would all tend to promote non-differential misclassification of the data, and could hinder our ability to detect significant associations, where present. Other considerations include potential cofactors for exacerbation of infections, as discussed above, laboratory error, or reporting error (e.g. errors in recording of stranding location). It is unlikely that simultaneous infection of a single sea otter with multiple genotypes of *T. gondii* was missed because low-passage cell pellets were selected for genotyping (thus maximising our potential for detecting mixed infections, if present) and *B1* and *SAG* PCR-RFLP primers were utilised that can distinguish between type x, type II and other genotypes, if present in the sample.

In this study we report the genotyping of 35 California sea otter-derived *T. gondii* isolates obtained between 1998 and 2002. All isolates were clustered into one of two main clonal groups: type II and type x strains. The type x strains were distinct from type II strains because of sequence divergences at the *B1*, *SAG1* and *GRA6* loci. Both strains were associated with substantial brain lesions and mortality in some otters. Low sample size and the fact that type x and II strains were not equally represented in the northern and southern regions may have hindered our ability to detect significant differences among groups. Type x strains were found to predominate in California otters, and this unusual genotype might represent an endemic *T. gondii* strain within this species. In contrast, the type II strains were less numerous in sea otters and were spatially clustered within the Monterey Bay region, possibly due to a comparatively recent point source introduction of a novel *T. gondii* genotype into sea otters, a marine species that inhabits an environment where substantial ecological barriers to new genotypic introduction likely exist. The identification and spatial mapping of these two genotypes, as presented in this study, will help to stimulate research aimed at better understanding the pathogenicity of *T. gondii* in sea otters. Future studies aimed at more precisely characterising the molecular fingerprint, pathogenicity and spatial distribution of *T. gondii* genotypes from sea otters should help to clarify whether parasite genotype contributes to the severity of brain inflammation and/or sea otter mortality. In addition, the application of DNA fingerprinting techniques such as microsatellite analysis will allow us to more precisely compare sea otter isolates to *T. gondii* isolated from humans or animals living in the adjacent terrestrial environment of California and may help to clarify routes and mechanisms of land–sea transfer of this putative biological pollutant.

Acknowledgements

This research was supported in part through funding from the PKD Trust, National Sea Grant, Morris Animal Foundation, the Lindbergh Foundation, The UC Davis Wildlife Health Centre, the Marine Mammal Centre, the California Department of Fish and Game, NSERC, the National Institutes of Health (AI21423) and a grant from the Alameda County District Attorney's Office (Food Safety Initiative). For assistance with carcass recovery, sample collection, shipment and processing, and DNA sequencing, we thank employees and volunteers at the California Department of Fish and Game, especially Dr Nancy Kock, Erin Dodd, Eva Berberich, Sharon Toy-Choutka, Jack Ames and Mike Harris, employees of Monterey Bay Aquarium, especially Dr Mike Murray, Michelle Staedler and Andy Johnson, employees of the United States Geological Survey and United States Fish and Wildlife Service, UC Davis faculty and staff, especially Linda Lowenstine, Jonna Mazet, Andrea Packham, Karen Sverlow, Tom Robinson, Anne Kjemtrup and Grace Lee and employees and volunteers of the Marine Mammal Centre, especially Drs Frances Gulland and Marty Haulena. MEG is a Scholar of the Michael Smith Foundation for Health Research.

References

Arkush, K.D., Miller, M.A., Leutenegger, C.M., Gardner, I.A., Packham, A.E., Heckeroth, A.R., Tenter, A.M., Barr, B.C., Conrad, P.A., 2003. Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*). Int. J. Parasitol. 33, 1087–1097.

Boothroyd, J.C., Grigg, M.E., 2002. Population biology of *Toxoplasma gondii* and its relevance to human infection: do different strains cause different disease? Curr. Opin. Microbiol. 5, 438–442.

Bowie, W.R., King, A.S., Werker, D.H., Isaac-Renton, J.L., Bell, A., Eng, S.B., Marion, S.A., 1997. Outbreak of toxoplasmosis associated with municipal drinking water. The B.C. *Toxoplasma* investigation team. Lancet 350, 173–177.

Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii*, by polymerase chain reaction. J. Clin. Microbiol. 27, 1787–1792.

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

Darde, M.L., 1996. Biodiversity in *Toxoplasma gondii*. Curr. Top. Microbiol. Immunol. 219, 27–41.

Dubey, J.P., Beattie, C.P., 1988. Toxoplasmosis of animals and man. CRC Press, Boca Raton, FL.

Fazaeli, A., Carter, P.E., Darde, M.L., Pennington, T.H., 2000. Molecular typing of *Toxoplasma gondii* strains by GRA6 gene sequence analysis. Int. J. Parasitol. 30, 637–642.

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

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

Grigg, M.E., Ganatra, J., Boothroyd, J.C., Margolis, T.P., 2001b. Unusual abundance of atypical strains associated with human ocular toxoplasmosis. J. Infect. Dis. 184, 633.

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

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

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

Kreuder, C., Miller, M.A., Jessup, D.A., Lowenstine, L.J., Harris, M.D., Ames, J.A., Carpenter, T.E., Conrad, P.A., Mazet, J.K., 2003. Recent patterns of mortality in southern sea otters (*Enhydra lutris nereis*). J. Wildl. Dis. 39, 495–509.

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

Larson, S., Jameson, R., Etnier, M., Flemming, M., Bentzen, P., 2002. Loss of genetic diversity in sea otters (*Enhydra lutris*) associated with the fur trade of the 18th and 19th centuries. Mol. Ecol. 11, 1899–1899.

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

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

Lindsay, D.S., Phelps, K.K., Smith, S.A., Flick, G., Dubey, J.P., 2001. Removal of *Toxoplasma gondii* oocysts from sea water by Eastern Oysters (*Crassostrea virginica*). J. Eukaryot. Microbiol., 197S–198S.

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

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

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

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

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

Riedman, M.L., Estes, J.A., 1990. The sea otter (*Enhydra lutris*): behavior, ecology and natural history. USFWS Biol. Rep., 90.

Sibley, L.D., Boothroyd, J.C., 1992. Virulent strains of *T. gondii*, a single clonal lineage. Nature 359, 82–85.

Sibley, D.L., Mordue, D.G., Su, C., Robben, P.M., Howe, D.K., 2002. Genetic approaches to studying virulence and pathogenesis. Philos. Trans. R. Soc. Lond. Ser. B 357, 81–88.

Su, C., Evans, D., Cole, R.H., Kissingger, J.C., Ajioka, J.W., Sibley, L.D., 2003. Recent expansion of *Toxoplasma* through enhanced oral transmission. Science 299, 414–416.

Suzuki, Y., Conley, F.K., Remington, J.S., 1989. Differences in virulence and development of encephalitis during chronic infection vary with the strain of *Toxoplasma gondii*. J. Infect. Dis. 159, 790–794.

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

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F., Higgins, D.G., 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 24, 4876–4882.

Volkman, S., Hartl, D.L., 2003. A game of cat and mouth. Science 299, 353–354.