

Concentration and retention of *Toxoplasma gondii* oocysts by marine snails demonstrate a novel mechanism for transmission of terrestrial zoonotic pathogens in coastal ecosystems

Colin Krusor,¹ Woutrina A. Smith,² M. Tim Tinker,^{3,4} Mary Silver,⁵ Patricia A. Conrad^{1,2} and Karen Shapiro^{1,2*}

¹Pathology, Microbiology, and Immunology, School of Veterinary Medicine and

²One Health Institute, University of California, Davis, CA, USA.

³Western Ecological Research Center, United States Geological Survey, Santa Cruz, CA, USA.

⁴Ecology and Evolutionary Biology and

⁵Ocean Sciences, University of California, Santa Cruz, CA, USA.

Summary

The parasite *Toxoplasma gondii* is an environmentally persistent pathogen that can cause fatal disease in humans, terrestrial warm-blooded animals and aquatic mammals. Although an association between *T. gondii* exposure and prey specialization on marine snails was identified in threatened California sea otters, the ability of kelp-dwelling snails to transmit terrestrially derived pathogens has not been previously investigated. The objective of this study was to measure concentration and retention of *T. gondii* by marine snails in laboratory aquaria, and to test for natural *T. gondii* contamination in field-collected snails. Following exposure to *T. gondii*-containing seawater, oocysts were detected by microscopy in snail faeces and tissues for 10 and 3 days respectively. Nested polymerase chain reaction was also applied as a method for confirming putative *T. gondii* oocysts detected in snail faeces and tissues by microscopy. *Toxoplasma gondii* was not detected in field-collected snails. Results suggest that turban snails are competent transport hosts for *T. gondii*. By concentrating oocysts in faecal pellets, snails may facilitate entry of *T. gondii* into the nearshore marine

food web. This novel mechanism also represents a general pathway by which marine transmission of terrestrially derived microorganisms can be mediated via pathogen concentration and retention by benthic invertebrates.

Introduction

Rivers and coastal seas provide valuable resources to humans and domestic animals, but may also serve as sinks for the myriad classes of pollutants that humans and domestic animals produce (Halpern *et al.*, 2008). Land-derived contaminants in coastal marine waters can threaten the very resources that sustain coastal communities, and these contaminants can expose humans, domestic animals and wildlife to disease risk. Of special concern are waterborne pathogen pollutants, which, unlike chemical pollutants, can reproduce and be maintained in infected hosts. Among the most troubling waterborne pathogen pollutants are those that possess two traits: host generalism and resistance to environmental insults. These adaptations enable a waterborne pathogen to endure biotic or abiotic transport over great distances, remain infectious in the environment for long periods of time and maintain reservoirs of infection in human, domestic animal and wildlife populations.

The protozoan parasite *Toxoplasma gondii* is a well-known example of an environmentally persistent, generalist waterborne parasite. No warm-blooded animal species is known to be entirely resistant to *T. gondii* infection. Wild and domestic felids are the only known definitive hosts for *T. gondii*. A single recently infected cat can shed hundreds of millions of oocysts into the environment (Fritz *et al.*, 2012a). Oocysts of *T. gondii* can survive and remain infectious in water or soil for months to years (Lindsay and Dubey, 2009; L  lu *et al.*, 2012). Although felids are the only known sources of *T. gondii* oocysts, infections have been identified in marine and terrestrial mammals in remote environments far from felid populations. Pinnipeds, sirenians, cetacians and polar bears acquire *T. gondii* infections (Dubey *et al.*, 2003; 2008; Gerber *et al.*, 2004; Cabezon *et al.*, 2011;

Received 4 March, 2015; revised 5 May, 2015; accepted 26 May, 2015. *For correspondence. E-mail kshapiro@ucdavis.edu; Tel. (+01) 530 219 5476; Fax +1 530 752 3349.

Alvarado-Esquivel *et al.*, 2012; Rengifo-Herrera *et al.*, 2012), and the parasite is a significant cause of mortality in the Southern sea otter population in California (Kreuder *et al.*, 2003; Miller *et al.*, 2004; Johnson *et al.*, 2009). Recent reports even describe detection of antibodies to *T. gondii* in flightless Galapagos cormorants (*Phalacrocorax harrisi*) that do not venture beyond a few hundred metres of the coast of the cat-free island of Fernandina (Deem *et al.*, 2010), and in pinnipeds and polar bears from the cat-free island of Svalbard, in the Arctic Ocean (Jensen *et al.*, 2010). Most of these intermediate hosts do not hunt or scavenge warm-blooded prey, so infection by ingestion of bradyzoite cysts in the tissues of other intermediate hosts (e.g., migrating birds) is unlikely. A growing body of evidence suggests that *T. gondii* infections in marine intermediate hosts are likely attributable to ingestion of oocysts that were transported to the ocean by overland watersheds (Conrad *et al.*, 2005; Miller *et al.*, 2008; VanWormer *et al.*, 2013b).

Mechanisms that might facilitate transmission of *T. gondii* oocysts within the marine environment are not yet well understood. Despite the large predicted environmental burden of this parasite in some terrestrial areas (Dabritz *et al.*, 2007; VanWormer *et al.*, 2013a), any oocysts transported in overland run-off would likely be reduced to low concentrations in the lower reaches of watersheds and in nearshore seawater by dilution and removal, especially where estuarine wetlands are present (Shapiro *et al.*, 2010; Hogan *et al.*, 2013; Simon *et al.*, 2013). However, if *T. gondii* oocysts are present in nearshore seawater, even at low concentrations, they might become accessible to sea otters and other marine hosts if they are first concentrated by biotic or abiotic processes and delivered into the marine food web via benthic or planktonic filter feeders or grazers. Recent research has demonstrated that *T. gondii* oocysts are efficiently captured from seawater by planktonic marine aggregates, a common constituent of nearshore marine waters (Shapiro *et al.*, 2012). Suspended aggregates are a major source of food for filter-feeding fish, benthic filter feeders and planktonic invertebrates, and settlement of marine aggregates delivers their contents to benthic grazers and detritivores. Among the harvesters of aggregates in nearshore marine waters are bivalves (Newell *et al.*, 2005; Kach and Ward, 2008; Ward and Kach, 2009). Bivalves are known to be able to obtain *T. gondii* oocysts from seawater, and they can be prey for sea otters (Miller *et al.*, 2008; Arkush *et al.*, 2003; Lindsay *et al.*, 2001; Lindsay *et al.*, 2004; Esmerini *et al.*, 2010; Estes *et al.*, 2003; Tinker *et al.*, 2008; Tinker *et al.*, 2012; Shapiro *et al.*, 2015). Bivalves therefore appear to present a potential means by which oocysts present in nearshore marine waters might become accessible to sea otters. However, predation upon bivalves has been identified as

lower risk foraging behaviour for Southern sea otters in California as compared with predation upon kelp-grazing marine snails, which is associated with an approximately 12-fold increased odds of *T. gondii* infection (Johnson *et al.*, 2009).

Among the snail species exploited by Southern sea otters are *Chlorostoma brunnea*, *Chlorostoma montereyi* and *Promartynia pulligo* (Tinker *et al.*, 2012). These brown turban snails are kelp grazers, but they do not consume kelp blades in bulk. Rather, these snails superficially abrade kelp surfaces with a rasp-like radula, ingesting bryozoans, fungi and other epiphytes as well as some kelp tissue (McMillan, 2010; Mazzillo *et al.*, 2013). The snails also remove and ingest the mucus-like gel layer that covers kelp surfaces, as well as the particulate matter entrapped in this layer. It has recently been reported that when suspended in seawater, surrogate particles that have dimensions and surface properties similar to those of *T. gondii* oocysts adhere readily to the gel-like film naturally present on the blades of the giant kelp *Macrocystis pyrifera*, a dominant kelp species in coastal waters in California (Mazzillo *et al.*, 2013).

The objective of this study was to evaluate the capacity of marine snails to acquire, concentrate and retain *T. gondii* oocysts when they are present in seawater, as well as to develop methods that can be employed for surveillance of *T. gondii* in nearshore marine waters by sampling of marine snails. This research had two components: (i) laboratory exposure of captive marine snails to *T. gondii* oocysts and fluorescent microspheres (oocyst surrogates), followed by enumeration of oocysts and surrogate microspheres in snail tissue and faecal pellets at multiple time points for 2 weeks post-exposure and (ii) screening for naturally acquired *T. gondii* oocysts in snails collected from kelp forests near freshwater outflow sites along the California coast during wet and dry seasons.

Results

Exposure experiment: detection of T. gondii by microscopy

During the tank exposure experiment, 378 faecal samples were collected and 72 digestive/respiratory organ samples were obtained from snails by dissection. Oocysts were detected by microscopy up to 10 days post-exposure in faecal samples and 3 days in organ samples, and surrogate microspheres were detected up to 13 days post-exposure in faeces and 4 days in organ samples (Figs 1 and 2). The concentration of oocysts and surrogate microspheres detected in faecal samples collected immediately following snail exposure to spiked seawater were 152 and 235 times greater, respectively, than the concentrations of those particles in the seawater in the exposure containers (Fig. 3). Faecal concentrations of

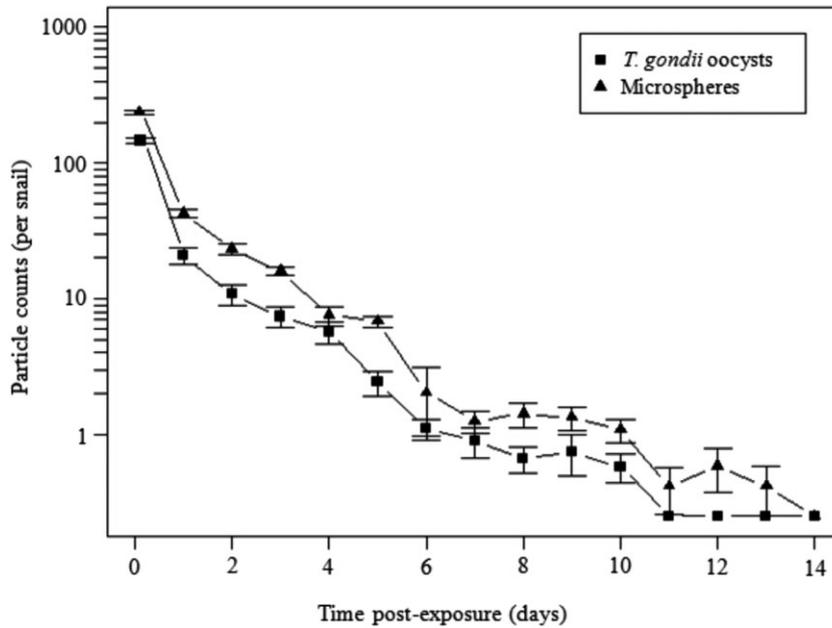


Fig. 1. Mean per-snail *Toxoplasma gondii* oocyst and surrogate microsphere counts in faecal homogenate at time points following cessation of exposure of brown turban snails to spiked seawater.

oocysts remained at least 10 times greater than the seawater in the exposure containers for 7 days post-exposure, and surrogate microspheres remained at least 10-fold more concentrated in faeces than in the exposure seawater for 10 days following exposure. No oocysts or microspheres were detected in faecal samples or organs from snails in the negative control group. Fisher's index of dispersion and likelihood ratio-based dispersion tests confirmed that count data for *T. gondii* oocysts and surrogate microspheres in snail faeces and organ homogenates were not consistent with a Poisson process, and Durbin–Watson testing confirmed the presence of significant serial autocorrelation. Count data for

T. gondii oocysts in snail faeces collected at 24 h intervals throughout the experiment are described by a negative binomial distribution having r and p parameters of 0.162 [standard deviation (SD) = 0.068] and 12.60 (SD = 8.13) respectively. Count data for surrogate microspheres in snail faeces from each daily collection during the experiment are described by a negative binomial distribution having r and p parameters of 0.235 (SD = 0.083) and 21.26 (SD = 11.40) respectively. Analysis of the count data for *T. gondii* oocysts and surrogate microspheres in snail faeces using Mann–Kendal tests corrected for serial autocorrelation confirmed that trends in these data are significant ($P \leq 0.001$ and $P \leq 0.01$ respectively).

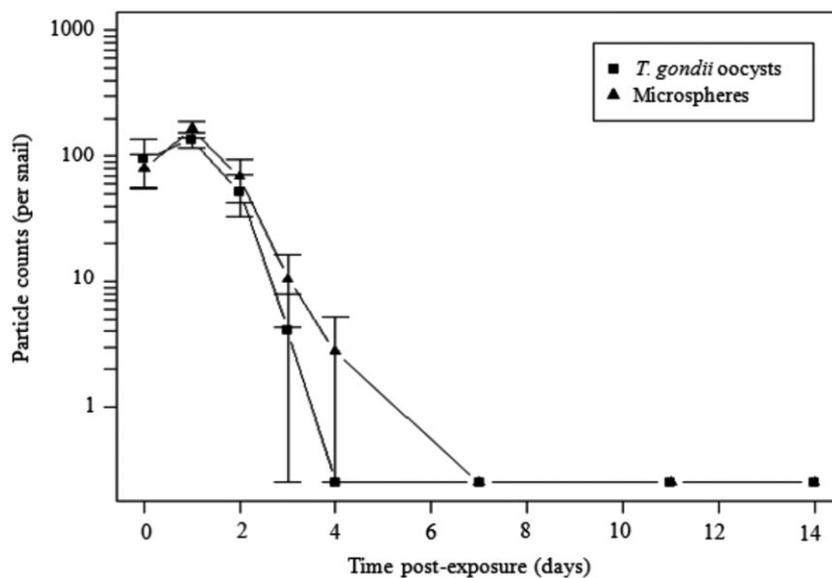


Fig. 2. Mean per-snail *Toxoplasma gondii* oocyst and surrogate microsphere counts in digestive and respiratory organ homogenate at time points following cessation of exposure of brown turban snails to spiked seawater.

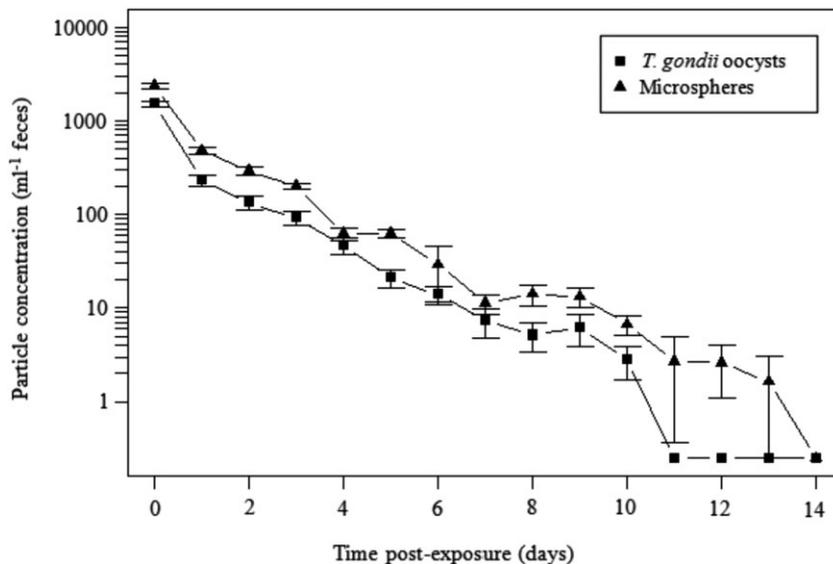


Fig. 3. *Toxoplasma gondii* oocyst and surrogate microsphere concentrations in snail faecal homogenate at time points following cessation of exposure of brown turban snails to spiked seawater. During the exposure period, *T. gondii* oocysts and surrogate microspheres were added at concentrations of 10 per millilitre of seawater. Thus, faecal pellets collected at t0 contained oocysts and microspheres at concentrations 2–3 orders of magnitude greater than those in surrounding seawater.

Corrected Mann–Kendal analysis of count data for *T. gondii* oocysts and surrogate microspheres in snail organ homogenate also detected significant trends ($P \leq 0.02$ and $P \leq 0.01$ respectively).

Exposure experiment: detection of T. gondii by polymerase chain reaction (PCR)

A total of 798 PCR reactions were performed on extracts from membrane filters containing snail faecal material. Amplification of *T. gondii* DNA was successful using the three nested primer sets targeting the B1, ITS1 and 529 bp repeat loci. However, nested PCR at these loci appeared to be less sensitive than epifluorescence microscopy for detection of *T. gondii* oocysts in snail faecal homogenate on membrane filters (Table S1; Fig. 4). The positive percent agreement (PPA) and nega-

tive percent agreement (NPA) values for each locus are shown in Table 1.

Observational study: detection of T. gondii by microscopy

A total of 227 snails were collected from the field for the purpose of detecting naturally acquired *T. gondii* oocysts in faecal samples and organ homogenate samples (Table S2). No *T. gondii* oocysts were detected by microscopy in faeces or organ samples from these snails. Positive and negative control samples supported the performance of the assay.

Discussion

The detection of *T. gondii* oocysts in the faeces, digestive and respiratory organs of *Chlorostoma* and *Promartynia*

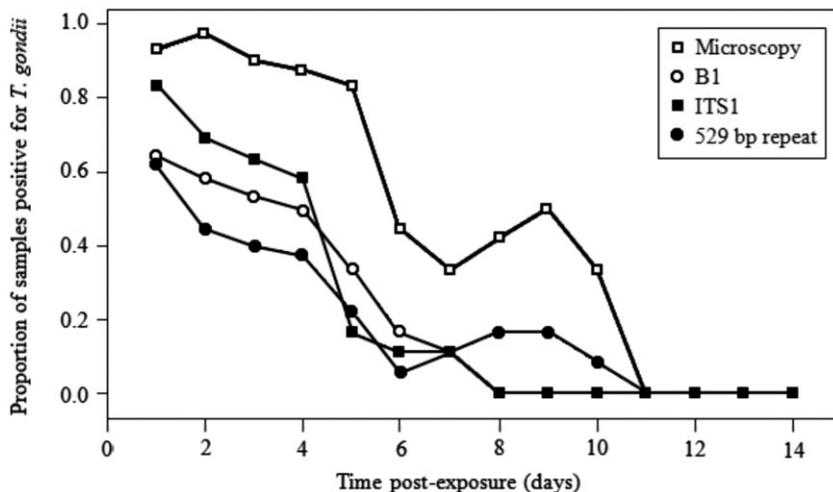


Fig. 4. Proportion of membrane-filtered faecal homogenate samples at each time point at which *Toxoplasma gondii* oocysts were detected by each of four detection methods: epifluorescence microscopy (\square), conventional PCR at the B1 locus (\circ), conventional PCR at the ITS1 locus (\blacksquare) and conventional PCR at the 529 bp repeat locus (\bullet).

Table 1. Positive percent agreement (PPA) and negative percent agreement (NPA) values, with epifluorescence microscopy as the reference, for *Toxoplasma gondii* detection via PCR on faecal material filtered onto membranes from the tank oocyst exposure experiment.

Primer set	PPA (95% CI)	NPA (95% CI)
B1	54.2 (46.3–61.9)	97.7 (91.0–99.6)
ITS1	57.8 (49.9–65.4)	95.3 (87.9–98.5)
529 bp repeat	42.2 (34.6–50.1)	100 (94.7–100)

CI, confidence interval.

snails exposed to spiked seawater presents compelling evidence that brown turban snails can acquire and retain *T. gondii* oocysts when present in the water column in kelp forests. When *T. gondii* oocyst inputs into the nearshore marine environment in California occur as transient pulses associated with rainfall and watershed discharge events, retention of oocysts by brown turban snails might substantially increase the duration of episodes of *T. gondii* infection risk to sea otters. Also, although it has been reported that the minimum infectious oral dose of *T. gondii* in mice and pigs might be as few as one oocyst, this number is unknown for sea otters (Dubey *et al.*, 1996; Dubey, 2006). It is likely that concentration of oocysts by brown turban snails increases the infection risk to sea otters by increasing the probability that any individual sea otter will acquire a dose of oocysts sufficient to cause infection. Further, by packaging oocysts at high concentrations in faecal pellets, brown turban snails might facilitate ingestion of oocysts by other benthic invertebrates such as filter feeders or detritivores that feed on organic material that includes invertebrate faeces. Brown turban snails may, therefore, serve as an entry point for *T. gondii* into the nearshore marine food web, and thereby ultimately promote infection of pinnipeds and other (even pelagic) intermediate hosts at higher trophic levels. In addition, by disseminating *T. gondii* oocysts (in faecal pellets) into seawater for up to 10 days following exposure, brown turban snails could increase the duration of *T. gondii* exposure risk for humans who use the nearshore marine environment for recreation or seafood harvest.

The timing of oocyst and microsphere gut passage by snails during the tank exposure experiment was longer than expected, based on previous studies (Miller *et al.*, 1972; Yee and Murray, 2004). For example, the gut passage time for *C. brunnea* was shown to be approximately 4 h after first access to food following a 2 day fast (Yee and Murray, 2004). A study of *T. gondii* host diversity found that oocysts continue to be detectable by latex agglutination in the faeces of Puerto Rican land snails for 2 days after ingestion of oocysts in cat faeces (Miller *et al.*, 1972). In the present study, oocysts were detected in faeces for up to 10 days post-exposure and

microspheres were detected in faeces up to 13 days following the end of the exposure period.

No *T. gondii* oocysts were detected in faecal samples or organs from field-collected samples of snails; however, this finding is not unexpected, given the relatively small number of snails that were collected for this purpose ($n = 227$). Because individual sea otters are known to be capable of eating in excess of 10^3 turban snails per day, or $\sim 10^6$ snails every 2–3 years, a very low *T. gondii* prevalence in the turban snail population could be epidemiologically significant (Tinker *et al.*, 2008; Tinker *et al.*, 2012). Therefore, our failure to detect *T. gondii* in field-collected snails is not sufficient to discount the potential role of wild turban snails in transmission of this zoonotic parasite to sea otters. It is also worth noting that the actual prevalence of *T. gondii* oocysts in brown turban snails along the California coast during 2011 and 2012 could have been unusually low due to low rainfall and thus low watershed throughput. Rainfall in all California counties during the 2011–2012 water year (1 October 2011 to 30 September 2012) was less than half of the annual average by county, and one third of counties in the state experienced less than 25% of average rainfall (National Weather Service, 2005). The throughput of the Carmel River watershed (which drains into one of the coastal sites where snails were sampled for this study) at the Sleepy Hollow Weir gagging station during the 2011–2012 water year was 29% of the annual average for that site (Carmel River Fishery Report). Therefore, it is likely that the number of oocysts delivered to nearshore marine waters during our sampling period was anomalously small.

Including *T. gondii* surrogate microspheres in the snail laboratory exposure experiment enabled a comparison in gut transport behaviour between oocysts and inert (i.e. non-organic) polystyrene microspheres that have similar surface charge and hydrophobicity as viable oocysts (Shapiro *et al.*, 2009). The mean ratio of oocyst counts to microsphere counts in the same faecal samples from the tank exposure experiment was approximately 0.49 (SD = 0.131). That is, roughly two surrogate microspheres were detected in faecal samples for each detected oocyst. This difference is likely attributable almost entirely to a difference in detection sensitivity. The conspicuously unnatural brilliant green fluorescence of the Dragon Green microspheres is easily detectable by epifluorescence microscopy, even when the microspheres are partly obscured by snail faecal material, whereas the weaker indigo autofluorescence of *T. gondii* oocysts is more difficult to detect if obscured by the material of the faecal matrix and more difficult to distinguish from background fluorescence. In earlier experiments, the ratio of *T. gondii* oocyst counts to surrogate microsphere counts in snail faecal samples that had been directly spiked with oocysts and microspheres (the oocysts and microspheres

in those samples did not pass through a snail gut) was also approximately 0.49 (Supporting information). This congruence has two important implications. First, this finding suggests that any potential differences in physical and chemical features between *T. gondii* oocysts and surrogate microspheres do not affect rates of adhesion to kelp, rates of removal from kelp surfaces by snails or retention times in the snail gut. The surrogate microspheres used in this study are smooth, perfectly spherical and have protein-free surfaces, whereas *T. gondii* oocysts are ovoid and have irregular, proteinaceous surfaces (Possenti *et al.*, 2010; Fritz *et al.*, 2012b). If any mechanism within the transmission path that comprises adhesion to kelp and ingestion by snails was selective for properties specific to one of these two particle types (for example, if a component mechanism was dependent on protein-protein interactions at the particle surface), we would expect to see a larger ratio for detection of one particle type relative to the other in the exposure experiment data as compared with the spiked faeces data. Because no such difference is evident, these data suggest that acquisition, concentration and retention of particles by turban snails in nearshore marine waters is non-specific, at least within a range of particle variation that would encompass related protozoan parasites. These data also suggest that Dragon Green microspheres are similar enough to *T. gondii* oocysts to serve as surrogates not only for waterborne transport studies, as previously reported, but also for investigation of transmission through the marine food web (Shapiro *et al.*, 2009; 2010; 2012; Hogan *et al.*, 2013).

The second implication of this congruence is relevant to parasite viability following ingestion and excretion by a snail. The passage of intact oocysts at the expected recovery rate suggests that *T. gondii* oocysts remain undigested, and likely viable, in the snail digestive tract and in snail faecal pellets. Because only heat-inactivated oocysts were used, it was not possible to directly confirm *T. gondii* oocyst viability by bioassay in this study. However, the congruence between the detection ratios for the two particle types in samples from the tank exposure experiment and samples from the faecal spiking experiment suggest that *T. gondii* oocysts are not destroyed, and their autofluorescence is unaffected by conditions in the gut of *Chlorostoma* or *Promartynia* snails. Also, if conditions in the snail gut or in spiked faecal samples (e.g., enzymes) had a significant effect on the integrity or detectability of *T. gondii* oocysts, we would expect the duration of exposure to those conditions to be a determinant of the magnitude of the ratio of detected *T. gondii* oocysts to detected surrogate microspheres at each time point. However, the values of these ratios were not significantly associated with day of detection during this experiment. It has been demonstrated in prior studies that

T. gondii oocysts remain infectious after ingestion by earthworms, filter feeding fish, mussels and eastern oysters (Bettioli *et al.*, 2000; Lindsay *et al.*, 2001; 2004; Arkush *et al.*, 2003; Massie *et al.*, 2010).

Differences between *T. gondii* oocyst and surrogate microsphere counts in snail faecal samples and counts of those particles in snail organ homogenates are likely attributable to a difference in the sensitivities of the detection methods for these two matrices. Each snail organ homogenate sample contained a large amount of viscous and pigmented material that remained opaque and adhesive, even after papain digestion. Consequently, filtration of more than one third of the volume of each organ homogenate sample was impractical. This obstacle reduced the sensitivity of oocyst and microsphere detection in snail organ homogenate samples, as compared with their detection in faecal pellet homogenates.

The laboratory snail exposure experiment also provided an opportunity for comparing the sensitivity of microscopy and molecular assays for detection of *T. gondii* in complex environmental matrices. Results demonstrated consistently higher likelihood of detecting oocysts via microscopy in snail faecal and tissue homogenates. DNA amplification of *T. gondii* was included as a detection assay in this study because molecular confirmation is required for definitive identification of oocyst-like structures in environmental samples, as well as for downstream characterization of the parasite if identified in field-collected snails. The comparatively lower assay sensitivity of the nested PCR method that was applied in this study is likely a result of environmental inhibitors that were present in complex matrices such as faeces and gastric contents. The technique applied for parasite DNA extraction and PCR amplification is less likely to be implicated as a reason for lack of assay sensitivity, as similar methods were recently applied for testing mussel haemolymph for *T. gondii* where the limit of parasite detection was five oocysts per 1 ml of haemolymph (Shapiro *et al.*, 2015).

In conclusion, by acquiring, concentrating and retaining particles that have become attached to the surfaces of kelp blades, and by packaging these particles in faecal pellets, kelp-grazing snails provide *T. gondii* oocysts an entry into the kelp forest food web. Because kelp forests are nurseries for some species of pelagic fish and are seasonally visited by migratory fish and marine mammals, the kelp-mediated capture of terrestrial parasites by grazing snails appears to offer a mechanism that could account for *T. gondii* infections not only in sea otters, but also in pinnipeds and their predators, bottlenose dolphins and other marine intermediate hosts. This research appears to be the first study to demonstrate that marine gastropods can serve as transport hosts for pathogens of terrestrial origin. It seems unlikely that the transmission mechanism described here is unique to the specific

organisms that were the focus of this work. This novel mechanism appears to present a non-specific shuttle that can transmit not only *T. gondii* oocysts, but perhaps other terrestrial protozoan pathogens (i.e., *Cryptosporidium* spp., *Giardia* spp. and *Sarcocystis* spp.) to marine animals, and potentially to humans who use nearshore marine waters for recreation and seafood harvest.

Experimental procedures

Preparation of T. gondii oocysts and surrogate microspheres

Oocysts of *T. gondii* (Type II, strain M4) were produced as previously described by Fritz and colleagues (2012a). Oocysts used for the present work were inactivated by heating to 80°C for 20 min as a biosafety measure. Autofluorescent Dragon Green carboxylate-modified polystyrene microspheres were also used (Bangs Laboratory, Fishers, IN, USA; product number FCO7F/5493). These microspheres are similar in surface charge, hydrophobicity, shape, size and density to viable *T. gondii* oocysts (10.35 µm diameter, 1.06 g cm⁻³ specific gravity), and they have been validated and employed previously as surrogates for *T. gondii* oocysts in waterborne transport studies (Shapiro *et al.*, 2009; 2010; 2012; Hogan *et al.*, 2013). Surrogate microspheres were employed alongside heat-inactivated oocysts to evaluate whether *T. gondii* oocysts are digested or preferentially sequestered (as compared with inert particles) during passage through the snail gut. Heat inactivation of oocysts was performed to minimize the risk of exposure to laboratory personnel due to the large volumes of water that were spiked with oocysts for the snail exposure experiments.

Tank exposure experiment

A cross-sectional sample of 81 brown turban snails was collected by divers from kelp surfaces at a depth of approximately 10 m near Cambria, California, on 10 September 2012 (additional detail provided in Supporting information). The sample contained species distribution that was representative of that at the collection site and, included individuals of a spectrum of sizes (mean mass 12.8 g) and three species: *C. brunnea* (37 individuals), *C. montereyi* (10 individuals) and *P. pulligo* (34 individuals). The snails were shipped overnight to the laboratory, where they were rinsed and sorted by species. A total of 24 snails were then allocated to each of three polyethylene tubs containing 4 l of continuously aerated pre-filtered (0.2 µm) seawater, such that each tub contained the same species distribution as that collected from the field (11 *C. brunnea*, 3 *C. montereyi* and 10 *P. pulligo*). Kelp blades from the field collection site were rinsed and blotted with paper towels, then transferred to each tub. The tubs were maintained at 10–12°C for the entire duration of the experiment. All faecal materials from the snails in each tub were removed and half of the water volume was replaced at 24 h intervals for 72 h of acclimation. Two of the tubs were then spiked with heat-inactivated *T. gondii* oocysts and surrogate microspheres to a final concentration of 10⁴ per litre of each particle type. Fresh kelp was added to all three tubs. A

fourth 4 l tub served as a snail-free control tub and contained pre-filtered and aerated seawater, kelp and heat-inactivated oocysts and surrogate microspheres at 10⁴ per litre.

Twenty-four hours following spiking of the exposure tubs, three snails were selected from each tub and moved to a 1 l bottle for anaesthesia, euthanasia and dissection, as described below. Digestive tracts and respiratory organs were harvested from each snail. The remaining 21 snails in each tub were rinsed and transferred to individual 1 l acrylic boxes containing continuously aerated 800 ml filtered seawater and a fresh piece of washed kelp. No exchange of water occurred between boxes. All of the faecal material produced by the snails was collected from the bottom of the three snail tubs. A volume of seawater equal to the mean of the volumes of faecal suspension collected from the snail-containing tubs was removed from the bottom of the snail-free control tub.

Twenty-four hours after relocation of snails to individual boxes, a second sample of six exposed and three non-exposed control snails was selected and dissected from the 63 individual boxes. Similar subsets of nine snails were removed for collection of digestive and respiratory organs at six subsequent time points: 2, 3, 4, 7, 11 and 14 days following relocation to individual boxes. At 24 h intervals throughout the experiment, all kelp and faecal material were removed, each box was emptied and rinsed, and each snail was rinsed. Each box was then refilled with pre-filtered seawater and fresh kelp was added.

Collection, filtration and microscopy of faecal samples

Collected faecal suspensions were transferred to conical-bottom of 50 ml polypropylene tubes, and homogenized by repeated passage through 15 gauge needles. An aliquot of faecal homogenate was then removed from each tube and diluted for membrane filtration and epifluorescence microscopy. Oocysts and surrogate microspheres were enumerated in snail faeces by vacuum filtration of syringe-homogenized samples through mixed cellulose ester filter membranes with a 25 mm diameter and 5 µm pore size (Millipore cat. no. SMWP02500). Filters were then examined and oocysts and surrogates enumerated using a Zeiss Axioskop epifluorescence microscope (both particle types were visualized simultaneously using a mercury vapour emitter, 460/50 nm band-pass filter; Chroma 11 000 v3) at 200×. This method has been previously evaluated for detection and enumeration of *T. gondii* oocysts and surrogate microspheres in water (Shapiro *et al.*, 2010). Filtration continued for each sample until at least five *T. gondii* oocysts and surrogate microspheres had been observed by microscopy. The total numbers of *T. gondii* oocysts and surrogate microspheres in each sample were then estimated from the number observed in the fraction processed. Samples containing fewer than five *T. gondii* oocysts and five surrogate microspheres were filtered and examined in their entirety.

Dissection and collection of digestive and respiratory organs

Dissection of snails was performed following anaesthesia and euthanasia. Magnesium chloride was chosen as the anaesthetic agent for its safety, simplicity of use and efficacy

for gastropods and marine invertebrates (Runham *et al.*, 1965; Messenger *et al.*, 1985; Arafa *et al.*, 2007; Cooper, 2011; Maheux *et al.*, 2011). For anaesthesia, snails were placed in bottles containing 1 l of seawater in which 45 g of magnesium chloride had been dissolved. These bottles were then gradually chilled and maintained at 4°C for 12 h. Rapid onset of flaccid paralysis without aversive retraction into the shell preceded the drop in water temperature and confirmed the action of the agent. After 12 h, 800 ml of the anaesthetic solution was removed, ethanol was added to a final concentration of 10% (w/v), and the snails were maintained in this solution for 1 h at 4°C for euthanasia. Euthanized snails were dissected by cracking the shell with a bench-top vise and separating the gills and the entire digestive tract from other organs. Each sample of respiratory and digestive organs was stored initially at 4°C in a 1.5 ml tube with 200–500 µl of 70% ethanol. These organ samples were then transferred to 15 ml conical-bottom polypropylene tubes, deionized water was added to a total volume of 4 ml and the samples were enzymatically digested with papain. Preliminary experiments were performed in order to optimize and validate the papain digestion protocol (Supporting information). Papain solutions were prepared fresh each day by incubation of 0.05 g papain with 0.01 gram L-cysteine in 11 ml 0.05 M buffered EDTA (pH 8) for 20 min at 40°C. The activated papain solutions were then added to organ homogenate samples and incubated at 65°C for 5 h prior to membrane filtration and microscopy. Each digested organ homogenate sample was filtered through multiple 5 µm mixed cellulose membranes until a minimum of five *T. gondii* oocysts and five surrogate microspheres were observed by epifluorescence microscopy, or until one third of the sample (5 ml) had been filtered. Due to the large numbers of membrane filters required for processing of the viscous and pigmented snail organ homogenates, it was not possible to filter these samples in their entirety.

PCR detection of *T. gondii* on filter membranes

Because *T. gondii* oocyst-like structures in environmental samples that are identified morphologically by microscopy would require molecular confirmation for conclusive identification, DNA amplification by PCR was validated as a secondary detection method. Preliminary experiments were first performed in order to validate and optimize a protocol for detection of *T. gondii* oocysts in snail faeces by PCR (Supporting information). Filter membranes containing snail faecal homogenate collected during the tank exposure experiment were examined by microscopy as described above. Material entrained on each membrane filter was then liberated from the filter by acetone dissolution of the membrane according to modifications to previously reported methods (Aldom and Chagla, 1995; Graczyk *et al.*, 1997). The coverslip was removed from each slide-mounted filter with forceps, and placed upside down in a clean plastic box. With a scalpel, each filter was lifted, folded in half and transferred to a 1.5 ml tube. Each coverslip was then held over the corresponding tube and washed with 600 µl acetone. After 10 min of centrifugation at 14 000 × *g*, the top 400 µl of supernatant was removed, 400 µl of ethanol was added and the tubes were centrifuged again. The top 400 µl of supernatant was then removed again from each tube, 400 µl of deionized water was

added and the tubes were centrifuged a third time. Following the third centrifugation, 400 µl of supernatant was removed from each tube, and the tubes were then sealed and subjected to two freeze–thaw cycles in liquid nitrogen and 95°C water. Column purification of the DNA in each sample was performed using the Qiagen DNEasy Blood and Tissue kit (Qiagen cat. no. 69504), and the columns were eluted with 30 µl of 95°C water. Each volume of eluate was divided into three 10 µl aliquots, and each of these was allocated to a PCR targeting one of three loci: B1, ITS1 or a 529 bp repeat element fragment (Table S4). Amplification reaction volumes were 50 µl each, containing MgCl₂ (15 mM), BSA (16 mg), dNTPs (2.5 mM), forward and reverse primers (500 nM each), Taq polymerase (1.5 U), and DNA template (10 µl of extracted DNA for the external reaction, 2 µl of amplicon for the internal reaction). The amplicon from the second-round amplification was identified by electrophoresis in 2% agarose gels containing ethidium bromide. Positive control samples of *T. gondii* tachyzoite DNA (Type II strain M4 propagated in cell culture) and negative control samples of deionized water were included in all reactions.

Field sample collection and processing

To test for the presence of *T. gondii* in wild-caught snails, 6 samples of brown turban snails were collected in the field from kelp surfaces at depths between 3 and 10 m by divers and shipped on ice overnight to the laboratory (Table S2). The species identities of the snails were recorded in the laboratory, and they were weighed and transferred to 1 l jars of pre-filtered (0.2 µm) seawater maintained at 10–13°C. Kelp blades obtained from the snail collection sites were provided in each jar, and the seawater was continuously aerated. After 24 h, all faecal pellets were removed from each jar using a serological pipet, and the snails were removed for anaesthesia, euthanasia and dissection. Processing of these samples and detection of *T. gondii* oocysts by microscopy was performed according to the protocols described above.

Statistical analyses

Data collected included the species identity and mass of each snail in the experimental and observational samples, daily faecal production for each snail, oocyst and surrogate microsphere counts for faecal samples and organ samples from the tank exposure experiment, and PCR results for filter extracts, faecal homogenates and organ homogenates. The data were compiled and imported into R version 2.14.0 for analyses (R Core Development Team, 2011). Fisher's index of dispersion and a likelihood ratio overdispersion test were used to assess overdispersion in the microscopy data sets, and the Durbin–Watson test was used to measure autocorrelation. Mean *T. gondii* oocyst and surrogate microsphere counts in snail faeces and organ homogenates were plotted as functions of time post-exposure and a modified Mann–Kendal test ('mktrend', Santander Meteorology Group), corrected for autocorrelation, was used to detect significant trends ($\alpha = 0.05$). A maximum likelihood method was used to derive the parameters (*r* and *p*) for negative binomial distributions fitted against data sets for *T. gondii* oocyst counts and surrogate microsphere counts in snail

faeces. PPA and NPA were calculated for comparisons of microscopy to PCR at each locus.

Acknowledgements

Funding for this work was provided by the National Science Foundation (NSF) Ecology of Infectious Disease program (OCE-1065990). We are grateful to the field teams that supported collections of snails, and specifically acknowledge Don Canestro, Michael Kenner, Mark Kocina, Jim Webb, Matt Smith, Brian Hatfield, Aiko Adell, Joe Tomoleoni, Ben Weitzman, Zach Randell and the Ken Norris Rancho Marino Reserve. We also greatly appreciate assistance from David Dann and Marcel Losekoot in obtaining kelp and filtered seawater for this study. Any use of trade, product or firm names in this publication is for descriptive purposes only and does not imply endorsement by the U.S. government.

References

- Aldom, J., and Chagla, A. (1995) Recovery of *Cryptosporidium* oocysts from water by a membrane filter dissolution method. *Lett Appl Microbiol* **20**: 186–187.
- Alvarado-Esquivel, C., Sánchez-Okrucky, R., and Dubey, J. (2012) Serological evidence of *Toxoplasma gondii* infection in captive marine mammals in Mexico. *Vet Parasitol* **184**: 321–324.
- Arafa, S., Sadok, S., and Abed, A.E. (2007) Assessment of magnesium chloride as an anaesthetic for adult sea urchins (*Paracentrotus lividus*): incidence on mortality and spawning. *Aquac Res* **38**: 1673–1678.
- Arkush, K.D., Miller, M.A., Leutenegger, C.M., Gardner, I.A., Packham, A.E., Heckerroth, A.R., *et al.* (2003) Molecular and bioassay-based detection of *Toxoplasma gondii* oocyst uptake by mussels (*Mytilus galloprovincialis*). *Int J Parasitol* **33**: 1087–1097.
- Bettiol, S.S., Obendorf, D.L., Nowarkowski, M., Milstein, T., and Goldsmid, J.M. (2000) Earthworms as paratenic hosts of toxoplasmosis in eastern barred bandicoots in Tasmania. *J Wildl Dis* **36**: 145–148.
- Cabezón, O., Hall, A., Vincent, C., Pabón, M., García-Bocanegra, I., Dubey, J., and Almería, S. (2011) Seroprevalence of *Toxoplasma gondii* in North-eastern Atlantic harbor seal (*Phoca vitulina vitulina*) and grey seal (*Halichoerus grypus*). *Vet Parasitol* **179**: 253–256.
- Carmel River Fishery Report (2012) Carmel River Watershed Conservancy [WWW document]. URL <http://carmelriverwatershed.org/uncategorized/2012/11/carmel-river-fishery-report-for-september-2012/>.
- Conrad, P., Miller, M., Kreuder, C., James, E., Mazet, J., Dabritz, H., *et al.* (2005) Transmission of *Toxoplasma*: clues from the study of sea otters as sentinels of *Toxoplasma gondii* flow into the marine environment. *Int J Parasitol* **35**: 1155–1168.
- Cooper, J.E. (2011) Anesthesia, analgesia, and euthanasia of invertebrates. *ILAR J* **52**: 196–204.
- Dabritz, H.A., Miller, M.A., Atwill, E.R., Gardner, I.A., Leutenegger, C.M., Melli, A.C., and Conrad, P.A. (2007) Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden. *J Am Vet Med Assoc* **231**: 1676–1684.
- Deem, S.L., Merkel, J., Ballweber, L., Vargas, F.H., Cruz, M.B., and Parker, P.G. (2010) Exposure to *Toxoplasma gondii* in Galapagos penguins (*Spheniscus mendiculus*) and flightless cormorants (*Phalacrocorax harrisi*) in the Galapagos Islands, Ecuador. *J Wildl Dis* **46**: 1005–1011.
- Dubey, J. (2006) Comparative infectivity of oocysts and bradyzoites of *Toxoplasma gondii* for intermediate (mice) and definitive (cats) hosts. *Vet Parasitol* **140**: 69–75.
- Dubey, J., Lunney, J., Shen, S., Kwok, O., Ashford, D., and Thulliez, P. (1996) Infectivity of low numbers of *Toxoplasma gondii* oocysts to pigs. *J Parasitol* **82**: 438–443.
- Dubey, J., Zarnke, R., Thomas, N., Wong, S., Van Bonn, W., Briggs, M., *et al.* (2003) *Toxoplasma gondii*, *Neospora caninum*, *Sarcocystis neurona*, and *Sarcocystis canis*-like infections in marine mammals. *Vet Parasitol* **116**: 275–296.
- Dubey, J., Fair, P., Sundar, N., Velmurugan, G., Kwok, O.H., McFee, W., *et al.* (2008) Isolation of *Toxoplasma gondii* from bottlenose dolphins (*Tursiops truncatus*). *J Parasitol* **94**: 821–823.
- Esmerini, P.O., Gennari, S.M., and Pena, H.F. (2010) Analysis of marine bivalve shellfish from the fish market in Santos city, Sao Paulo state, Brazil, for *Toxoplasma gondii*. *Vet Parasitol* **170**: 8–13.
- Estes, J., Riedman, M., Staedler, M., Tinker, M., and Lyon, B. (2003) Individual variation in prey selection by sea otters: patterns, causes and implications. *J Anim Ecol* **72**: 144–155.
- Fritz, H., Barr, B., Packham, A., Melli, A., and Conrad, P. (2012a) Methods to produce and safely work with large numbers of *Toxoplasma gondii* oocysts and bradyzoite cysts. *J Microbiol Methods* **88**: 47–52.
- Fritz, H.M., Bowyer, P.W., Bogyo, M., Conrad, P.A., and Boothroyd, J.C. (2012b) Proteomic analysis of fractionated *Toxoplasma* oocysts reveals clues to their environmental resistance. *PLoS ONE* **7**: e29955.
- Gerber, L.R., Tinker, M.T., Doak, D.F., Estes, J.A., and Jessup, D.A. (2004) Mortality sensitivity in life-stage simulation analysis: a case study of southern sea otters. *Ecol Appl* **14**: 1554–1565.
- Graczyk, T.K., Fayer, R., Cranfield, M.R., and Owens, R. (1997) *Cryptosporidium parvum* oocysts recovered from water by the membrane filter dissolution method retain their infectivity. *J Parasitol* **83**: 111–114.
- Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C.V., Micheli, F., D'Agrosa, C., *et al.* (2008) A global map of human impact on marine ecosystems. *Science* **319**: 948–952.
- Hogan, J.N., Daniels, M.E., Watson, F.G., Oates, S.C., Miller, M.A., Conrad, P.A., *et al.* (2013) Hydrologic and vegetative removal of *Cryptosporidium parvum*, *Giardia lamblia*, and *Toxoplasma gondii* surrogate microspheres in coastal wetlands. *Appl Environ Microbiol* **79**: 1859–1865.
- Jensen, S., Aars, J., Lydersen, C., Kovacs, K., and Åsbakk, K. (2010) The prevalence of *Toxoplasma gondii* in polar bears and their marine mammal prey: evidence for a marine transmission pathway? *Polar Biol* **33**: 599–606.
- Johnson, C.K., Tinker, M.T., Estes, J.A., Conrad, P.A., Staedler, M., Miller, M.A., *et al.* (2009) Prey choice and habitat use drive sea otter pathogen exposure in a resource-limited coastal system. *PNAS* **106**: 2242–2247.

- Kach, D.J., and Ward, J.E. (2008) The role of marine aggregates in the ingestion of picoplankton-size particles by suspension-feeding molluscs. *Marine Biol* **153**: 797–805.
- Kreuder, C., Miller, M., Jessup, D., Lowenstine, L., Harris, M., Ames, J., et al. (2003) Patterns of mortality in southern sea otters (*Enhydra lutris nereis*) from 1998–2001. *J Wildl Dis* **39**: 495–509.
- Lélu, M., Villena, I., Dardé, M.-L., Aubert, D., Geers, R., Dupuis, E., et al. (2012) Quantitative estimation of the viability of *Toxoplasma gondii* oocysts in soil. *Appl Environ Microbiol* **78**: 5127–5132.
- Lindsay, D.S., and Dubey, J. (2009) Long-term survival of *Toxoplasma gondii* sporulated oocysts in seawater. *J Parasitol* **95**: 1019–1020.
- Lindsay, D.S., Phelps, K.K., Smith, S.A., Flick, G., Sumner, S.S., and Dubey, J. (2001) Removal of *Toxoplasma gondii* oocysts from sea water by eastern oysters (*Crassostrea virginica*). *J Eukaryot Microbiol* **48**: 197s–198s.
- Lindsay, D.S., Collins, M.V., Mitchell, S.M., Wetch, C.N., Rosypal, A.C., Flick, G.J., et al. (2004) Survival of *Toxoplasma gondii* oocysts in Eastern oysters (*Crassostrea virginica*). *J Parasitol* **90**: 1054–1057.
- McMillan, S.M. (2010) Trophic interactions among *Chlorostoma brunnea*, *Macrocystis pyrifera*, and fungi. In: San José State University.
- Maheux, A.F., Bissonnette, L., Boissinot, M., Bernier, J.-L.T., Huppé, V., Picard, F.J., et al. (2011) Rapid concentration and molecular enrichment approach for sensitive detection of *Escherichia coli*/*Shigella* in potable water samples. *Appl Environ Microbiol* **77**: 6199–6207. 02337-02310.
- Massie, G.N., Ware, M.W., Villegas, E.N., and Black, M.W. (2010) Uptake and transmission of *Toxoplasma gondii* oocysts by migratory, filter-feeding fish. *Vet Parasitol* **169**: 296–303.
- Mazzillo, F.F., Shapiro, K., and Silver, M.W. (2013) A new pathogen transmission mechanism in the ocean: the case of sea otter exposure to the land-parasite *Toxoplasma gondii*. *PLoS ONE* **8**: e82477.
- Messenger, J., Nixon, M., and Ryan, K. (1985) Magnesium chloride as an anaesthetic for cephalopods. *Comp Biochem Physiol C* **82**: 203–205.
- Miller, M., Miller, W., Conrad, P., James, E., Melli, A., Leutenegger, C., et al. (2008) Type X *Toxoplasma gondii* in a wild mussel and terrestrial carnivores from coastal California: new linkages between terrestrial mammals, runoff and toxoplasmosis of sea otters. *Int J Parasitol* **38**: 1319–1328.
- Miller, M.A., Grigg, M.E., Kreuder, C., James, E., Melli, A., Crosbie, P., et al. (2004) An unusual genotype of *Toxoplasma gondii* is common in California sea otters (*Enhydra lutris nereis*) and is a cause of mortality. *Int J Parasitol* **34**: 275–284.
- Miller, N.L., Frenkel, J., and Dubey, J. (1972) Oral infections with *Toxoplasma* cysts and oocysts in felines, other mammals, and in birds. *J Parasitol* **58**: 928–937.
- National Weather Service California Nevada River Forecast Center (2005) National Oceanic and Atmospheric Administration [WWW document]. URL http://www.cnrfc.noaa.gov/rainfall_data.php.
- Newell, C.R., Piiskaln, C., Robinson, S., and MacDonald, B. (2005) The contribution of marine snow to the particle food supply of the benthic suspension feeder, *Mytilus edulis*. *J Exp Mar Bio Ecol* **321**: 109–124.
- Possenti, A., Cherchi, S., Bertuccini, L., Pozio, E., Dubey, J., and Spano, F. (2010) Molecular characterisation of a novel family of cysteine-rich proteins of *Toxoplasma gondii* and ultrastructural evidence of oocyst wall localisation. *Int J Parasitol* **40**: 1639–1649.
- R Development Core Team (2011) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0.
- Rengifo-Herrera, C., Ortega-Mora, L.M., Álvarez-García, G., Gómez-Bautista, M., García-Párraga, D., García-Peña, F.J., and Pedraza-Díaz, S. (2012) Detection of *Toxoplasma gondii* antibodies in Antarctic pinnipeds. *Vet Parasitol* **190**: 259–262.
- Runham, N., Isarankura, K., and Smith, B. (1965) Methods for narcotizing and anaesthetizing gastropods. *Malacologia* **2**: 231–238.
- Shapiro, K., Largier, J., Mazet, J.A., Bernt, W., Ell, J.R., Melli, A.C., and Conrad, P.A. (2009) Surface properties of *Toxoplasma gondii* oocysts and surrogate microspheres. *Appl Environ Microbiol* **75**: 1185–1191.
- Shapiro, K., Conrad, P.A., Mazet, J.A., Wallender, W.W., Miller, W.A., and Largier, J.L. (2010) Effect of estuarine wetland degradation on transport of *Toxoplasma gondii* surrogates from land to sea. *Appl Environ Microbiol* **76**: 6821–6828.
- Shapiro, K., Mazet, J.A., Schriewer, A., Wuertz, S., Fritz, H., Miller, W.A., et al. (2010) Detection of *Toxoplasma gondii* oocysts and surrogate microspheres in water using ultrafiltration and capsule filtration. *Water Res* **44**: 893–903.
- Shapiro, K., Silver, M.W., Largier, J.L., Conrad, P.A., and Mazet, J.A. (2012) Association of *Toxoplasma gondii* oocysts with fresh, estuarine, and marine macroaggregates. *Limnol Oceanogr* **57**: 449–456.
- Shapiro, K., VanWormer, E., Aguilar, B., and Conrad, P.A. (2015) Surveillance for *Toxoplasma gondii* in California mussels (*Mytilus californianus*) reveals transmission of atypical genotypes from land to sea. *Environ Microbiol* **17**: 4177–4188.
- Simon, A., Rousseau, A.N., Savary, S., Bigras-Poulin, M., and Ogden, N.H. (2013) Hydrological modelling of *Toxoplasma gondii* oocysts transport to investigate contaminated snowmelt runoff as a potential source of infection for marine mammals in the Canadian Arctic. *J Environ Manage* **127**: 150–161.
- Tinker, M.T., Bentall, G., and Estes, J.A. (2008) Food limitation leads to behavioral diversification and dietary specialization in sea otters. *PNAS* **105**: 560–565.
- Tinker, M.T., Guimarães, P.R., Novak, M., Marquitti, F.M.D., Bodkin, J.L., Staedler, M., et al. (2012) Structure and mechanism of diet specialisation: testing models of individual variation in resource use with sea otters. *Ecol Lett* **15**: 475–483.
- VanWormer, E., Conrad, P.A., Miller, M.A., Melli, A.C., Carpenter, T.E., and Mazet, J.A. (2013a) *Toxoplasma gondii*, source to sea: higher contribution of domestic felids to terrestrial parasite loading despite lower infection prevalence. *Ecohealth* **10**: 277–289.
- VanWormer, E., Fritz, H., Shapiro, K., Mazet, J.A., and Conrad, P.A. (2013b) Molecules to modeling: *Toxoplasma*

gondii oocysts at the human–animal–environment interface. *Comp Immunol Microbiol Infect Dis* **36**: 217–231.

Ward, J.E., and Kach, D.J. (2009) Marine aggregates facilitate ingestion of nanoparticles by suspension-feeding bivalves. *Mar Environ Res* **68**: 137–142.

Yee, E., and Murray, S. (2004) Effects of temperature on activity, food consumption rates, and gut passage times of seaweed-eating *Tegula* species (Trochidae) from California. *Marine Biol* **145**: 895–903.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Mean *Toxoplasma gondii* oocyst counts for samples maintained under specific papain concentration and incubation times in preliminary experiments. Data trends for samples treated at the lower papain concentration or without papain were not significant ($\alpha = 0.05$).

Fig. S2. Mean surrogate microspheres counts for samples maintained under specific papain concentration and incubation

times in preliminary experiments. Data trends for samples treated at the lower papain concentration or without papain were not significant ($\alpha = 0.05$).

Table S1. Comparison of the numbers of membranes containing filtered snail fecal material from the tank exposure experiment that had detectable *Toxoplasma gondii* oocysts via epifluorescence microscopy or nested PCR at the B1, ITS1, and 529 bp repetitive loci.

Table S2. Six cross-sectional samples of snails were collected by divers from kelp surfaces for laboratory exposure to *Toxoplasma gondii* (Sample 6) and for observational studies (Samples 1–6).

Table S3. Composition of samples in the papain concentration and incubation time experiment.

Table S4. Sequence data for primers used for detection of *Toxoplasma gondii* by nested polymerase chain reaction (PCR).

Table S5. Detection of *Toxoplasma gondii* via nested PCR in spiked samples of snail feces and organ homogenate.

Table S6. Detection of *Toxoplasma gondii* via nested PCR in spiked samples of snail feces on membrane filters.