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Epidemiology and transmission of *Toxoplasma gondii* oocysts at the human-animal-environment interface

By

SOPHIE ZHU

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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DAVIS

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Abstract

The ubiquitous zoonotic pathogen *Toxoplasma gondii* has a unique life cycle involving domestic and wild animal hosts in diverse ecosystems. While historically *T. gondii* was primarily considered a terrestrial pathogen, this parasite has gained prominence in recent years for causing morbidity and mortality in marine wildlife and via waterborne transmission to people. Domestic and wild felids are the only definitive hosts of *T. gondii* that shed the environmentally resistant stage of the parasite, oocysts, in their feces. Many questions remain on the broader host and environmental factors that influence oocyst shedding in naturally infected domestic and wild felids, which are increasingly important in light of current and future environmental changes that may alter *T. gondii* transmission. The overall goal of this dissertation was to advance knowledge regarding the epidemiology, transmission, and disease ecology of *T. gondii* oocysts in wildlife and human hosts.

In Chapter 1, I reviewed the current dynamics of oocyst shedding in domestic and wild felids and discussed key risk factors such as route of exposure, diet, and genotype. The difficulties of interpreting tests for detection of oocysts and *T. gondii* DNA, confirming parasite identity, repeat shedding, and the potential role of environmental contamination were also discussed.

In Chapter 2, I utilized a longitudinal field study of *T. gondii* in feces from free-ranging feral cat colonies near sea otter habitats in Monterey Bay to evaluate temporal trends in oocyst shedding and the presence of virulent *T. gondii* strains previously associated with sea otter mortality. *T. gondii* DNA prevalence in feces was high (25.9%) and was more likely to be detected in the wet season and in colonies with kittens. Strains previously implicated in sea otter mortalities from toxoplasmosis (Type X and X-variants) represented 14.8% of strains present in feral cat feces; other genotypes detected included Type I (59%), Type II/III (18.5%), and two atypical genotypes (7.4%) based on characterization at the B1 locus.

In Chapter 3, I evaluated climatic and anthropogenic risk factors for oocyst shedding in free-ranging domestic and wild felids using generalized linear mixed models. Human population density was associated with higher confirmed *T. gondii* shedding in free-ranging domestic cats and wild felids. Climate variables that reflected annual averages were not significant, however variables that reflected

smaller temporal changes such as mean temperature in the driest quarter and mean diurnal range were associated with oocyst shedding in wild and domestic felids respectively.

Finally, I developed a Bayesian quantitative risk analysis (QRA) model of oocyst vs. non-oocyst foodborne toxoplasmosis in Brazil in Chapter 4. Predicted likelihood of infections from oocysts on contaminated produce were significantly higher than infections from consumption of bradyzoites in contaminated meat. The importance of parameters such as oocyst removal efficiency and oocyst viability were identified in sensitivity analyses.

The results of this dissertation provide novel insight on the shedding behavior of *T. gondii* in definitive hosts, identification of anthropogenic predictors of oocyst shedding, and quantitative methods for evaluating foodborne risk of exposure to *T. gondii*. These insights will inform host and ecosystem level management strategies and areas of focus for future approaches to prevent *T. gondii* oocyst transmission and infection in animal populations and humans.

Introduction

Zoonotic disease transmission has become more common in recent decades due to anthropogenic development and human-caused climate change. These activities and fluctuations in climate have altered animal-pathogen interactions, destroyed habitats, challenged host resilience, and introduced new animal-human interactions that have contributed to more frequent infectious disease spillover events. Geographic distributions and prevalence of diseases will continue to change significantly without thoughtful interventions to mitigate spread and exposure.

Toxoplasma gondii is a zoonotic pathogen that can infect all warm-blooded vertebrates such as humans, birds, and mammals. While historically recognized as a terrestrial pathogen, *T. gondii* has gained prominence in recent years for its importance in aquatic environments as it is associated with morbidity and mortality in certain marine mammals, such as the threatened southern sea otter (*Enhydra lutris nereis*), and acute waterborne outbreaks in people. The success of *T. gondii* infection across a large number and diversity of hosts is in part attributed to the environmentally resistant stage, oocysts, which are only shed in the feces of domestic and wild felids and have the capacity to infect large numbers of hosts [1–3]. Oocyst shedding in domestic cats and select wild felid species has been studied in controlled laboratory studies, however, many questions remain regarding host and environmental risk factors for oocyst shedding in naturally infected felids. These factors are increasingly important in light of current and future environmental changes that may alter *T. gondii* transmission.

Life cycle and oocyst-borne transmission

The three infective stages of *T. gondii* are 1) tissue cyst forming bradyzoites (chronic infection), 2) fast-replicating tachyzoites (acute and recrudescent infections), and 3) sporozoites contained within oocysts. Sporozoites result from *T. gondii* sexual reproduction, which only occurs in wild and domestic felids, the definitive host. Felids shed oocysts in their feces for 5-21 days after initial infection, and an individual domestic cat can shed up to one billion oocysts in this period [4]. Once released in feces, oocysts can contaminate various matrices such as soil and water when transported by surface runoff driven by precipitation [5]. Oocyst sporulation, or maturation into an infective stage, occurs over 1-5 days

under standard ambient temperature (25° C) [6]. Sporulated oocysts are incredibly environmentally resistant and can withstand a wide range of temperatures (-20° C to 35° C) and artificial treatments including chlorination, ozonation, and ultraviolet radiation [7,8]. Oocysts may travel more easily across human-altered landscapes due to the lack of natural habitats that facilitate filtration and retainment of oocysts [9]. As the ultimate source of *T. gondii* to many hosts in lower trophic levels, oocysts are important in driving overall parasite transmission [10].

Definitive and intermediate hosts become infected with *T. gondii* after ingesting oocysts from soil, water, or food, eating tissue cysts in infected intermediate hosts, or via vertical transmission. After intermediate hosts ingest oocysts, the sporozoites excyst and start dividing as tachyzoites, which migrate and infect virtually every organ with predilection for neural and muscle tissues. As the immune response mounts against tachyzoites, these fast-replicated stages form tissue cysts packed with slowly dividing bradyzoites. Felids can become infected after ingesting tissue cysts in intermediate hosts or by directly ingesting sporulated oocysts in water or soil. Experimental evidence shows that infection and subsequent shedding are stage and not dose-dependent, meaning cats are more successfully infected via consumption of tissue cysts and shed more oocysts in feces following infection with this stage [11]. The experimental data aligns with the life cycle of *T. gondii* in natural conditions, where free-ranging felids consume tissue cysts in infected prey, oocysts are shed into the environment via feces, and naive prey become infected after consuming with oocyst-contaminated matrices. Congenital transmission typically occurs after primary infection during pregnancy, and these infections can lead to miscarriage, stillbirth, or developmental defects in intermediate hosts such as humans. *T. gondii* transmission through oocysts is less well-understood compared to bradyzoite ingestion and congenital transmission, and thus, addressing knowledge gaps for oocyst-borne transmission has immense benefits because oocysts in the environment drive both bradyzoite and vertical transmission [10].

Impacts on wildlife and human health

Threatened and endangered wildlife species including Nēnē geese (*Branta sandvicensis*), southern sea otters (*Enhydra lutris nereis*), and Hawaiian monk seals (*Neomonachus schauinslandi*) have experienced severe disease or mortality due to *T. gondii* infection [12–14]. Sea otters are a threatened subspecies whose current population number around 3,000. Common causes of death in this population include shark attacks, boat collisions, algal blooms, domoic acid exposure, and parasitic infection [15]. In California, *T. gondii* infections were responsible for 16.2% of deaths and were a secondary cause of death in an additional 11.4% of stranded sea otters that were examined between 1998 and 2001 [16]. Toxoplasmosis is not currently considered one of the primary threats to otter population recovery; however, increased human development and high intensity precipitation events due to climate change could alter the load of *T. gondii* oocysts that otters are exposed to in the environment [17]. Infections in marine mammals highlight the far-ranging impacts of *T. gondii* contamination in the environment by felids in terrestrial landscapes [5].

Roughly 30% of all humans are infected with *T. gondii,* and most people who are infected are asymptomatic or have mild, flu-like symptoms [18]. However, toxoplasmosis can prove fatal for immunocompromised individuals and developing fetuses [19]. Approximately 400-4,000 children are born with congenital toxoplasmosis in the US each year, which can lead to various severe sequelae [19]. Common symptoms can include chorioretinitis, intracranial calcification, hydrocephalus, ocular lesions, and intellectual disability [20]. Medications such as spiramycin and pyrimethamine can be administered early in pregnancy to reduce the likelihood of *T. gondii* transplacental infection or mitigate the severity of symptoms in the newborn [21]. However, detection and treatment early in pregnancy is crucial because the severity of congenital symptoms decreases with the timing of trimester of infection [22]. No human vaccines exist for toxoplasmosis, so prevention of transmission must occur through managing definitive hosts, filtration of water, proper food safety policies for meat and produce, and surveillance and screening of high-risk populations.

Climate and T. gondii

Climatic and environmental variables are increasingly important to understanding *T. gondii* transmission and infection. Temperature and precipitation are associated with the environmental contamination of oocysts because oocyst survival, sporulation, and persistence are temperature and moisture-dependent [23]. Sufficient rainfall and humidity ensure parasite survival in the environment and transport of oocysts to aquatic environments, where contamination of drinking water can lead to acute outbreaks in people [24,25]. Recent studies also report on the interconnected nature of climatic and ecological factors, such as temperature and trophic level, in predicting *T. gondii* prevalence in wild mammal and bird species [26,27]. There are also seasonal patterns of oocyst shedding among owned domestic cats; a recent study from Germany found that the proportion of fecal samples testing positive for *T. gondii* between January and June was lower than those collected between July and December [28]. Another study from Europe also found that fecal oocysts were more likely to be detected in summer (June to August) and autumn (September to November) compared to winter and spring [29]. Seasonal infection patterns also exist in humans; specifically, there is a higher incidence of infections among pregnant individuals in Austria and the Baltics in fall and winter [30,31]. Patterns of oocyst shedding in felids that live partially (pets) or exclusively outdoors (feral cats and wild felids) thus have important implications for exposure risk in intermediate hosts. One potential driver for seasonal oocyst shedding is feline reproduction. Cats require environmental conditions that allow for successful reproduction (temperatures warm enough for cats to initiate estrous) and sufficient levels of prey to raise one or multiple litters. In the northern hemisphere, the breeding season of domestic cats falls between April and October and corresponds with the patterns of oocyst shedding reported by Schares et al. [29], which could be due to the timing of exposure and shedding in naïve feline hosts. Shedding and host exposure are thus highly associated with the felid life cycle and presence of *T. gondii* oocysts in the environment.

Oocyst-borne transmission in food

The relative importance of oocyst vs. non-oocyst-borne *T. gondii* infections in humans has been a hotly debated topic, but recent findings have emphasized the growing importance of oocyst-borne infections. A large case-control study of pregnant women conducted in six major European cities at the

beginning of this century found that the majority (30-63%) of seropositive cases were attributed to eating uncooked or cured meats, while only 6-17% of cases were attributed to soil contact [32]. In contrast, more recent research from the 2010's demonstrated that oocysts were the primary source of *T. gondii* infection in North American outbreaks and in mothers of children with congenital toxoplasmosis [33]. Oocyst transmission is increasingly recognized as a significant contributor to total infections, and the majority of reported human outbreaks since 2000 were caused by oocysts, not tissue cysts [34]. The decrease in meat borne infections is a positive trend, but increased attention needs to be brought to the growing importance of infections from *T. gondii* oocysts through water, food, and soil. Even though drinking water treatment systems utilize a variety of chemical and physical treatments (chlorination, ozone, UV, filtration), adequately treated water is not available to many populations, especially in low- and middle-income countries [35]. Water is a common source of acute toxoplasmosis in Brazil [36] and was identified as the source of a large toxoplasmosis outbreak in Canada in the late 1990s [25]. Gaps in research targeting oocyst-borne toxoplasmosis include the availability of sensitive and specific detection methods, consistency of methods across studies, development and adherence to oocyst-specific inactivation methods in food, and greater societal awareness in high-risk areas.

Conclusion

Domestic and wild felids are both competent definitive hosts that help to drive *T. gondii* transmission to humans and other animals. It is important to consider the interconnected nature of *T. gondii*, felids, environmental conditions, and overlap with intermediate host populations to understand the risk of infection from environmental sources.

In order to mitigate health risks to people, domestic animals, and wildlife, future and ongoing research needs to address the frequency, relationship to climate factors, and consequences of oocyst shedding by free-ranging domestic cats and wild felids [10]. The overall goals of this dissertation were to advance knowledge regarding the epidemiology and disease ecology of *T. gondii* oocyst transmission in wildlife and human hosts. Specifically, I addressed four objectives: 1) assess current knowledge on risk factors for oocyst shedding by domestic and wild felids; 2) evaluate the seasonality and genotype of *T.*

gondii oocyst shedding in a longitudinal study of feral domestic cats near southern sea otter habitat; 3) assess the global effects of climate and human activity on *T. gondii* oocyst shedding in free-ranging domestic and wild felids; and 4) model oocyst and non-oocyst borne *T. gondii* foodborne infections in Brazil to identify regions and parameters that can be targeted through management and risk mitigation strategies. These distinct but interconnected studies provide novel insight into the potential roles of climate and management on *T. gondii* oocyst shedding and oocyst-borne transmission.

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Chapter 1 Dynamics and epidemiology of *Toxoplasma gondii* oocyst shedding in domestic and wild felids

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REVIEW

Dynamics and epidemiology of *Toxoplasma gondii* oocyst shedding in domestic and wild felids

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Abstract

Oocyst shedding in domestic and wild felids is a critical yet understudied topic in *Toxoplasma gondii* ecology and epidemiology that shapes human and animal disease burden. We synthesized published literature dating from the discovery of felids as the definitive hosts of *T. gondii* in the 1960s through March 2021 to examine shedding prevalence, oocyst genotypes, and risk factors for shedding. Oocyst shedding prevalence in many geographic regions exceeded the commonly accepted 1% reported for domestic cats; crude prevalence from cross-sectional field studies of domestic cat shedding ranged from 0% in Australia to 18.8% in Africa, with greater variation in reports of oocyst shedding in free-ranging, wild felids. Shedding in wild felid species has primarily been described in captive animals, with attempted detection of oocyst shedding reported in at least 31 species. Differences in lifestyle and diet play an important role in explaining shedding variation between free-ranging unowned domestic cats, owned domestic cats and wild felids. Additional risk factors for shedding include the route of infection, diet, age and immune status of the host. It is widely reported that cats only shed oocysts after initial infection with *T. gondii*, but experimental studies have shown that repeat oocyst shedding can occur. Factors associated with repeat shedding are common amongst free-ranging felids (domestic and wild), which are more likely to eat infected prey, be exposed to diverse *T. gondii* genotypes, and have coinfections with other parasites. Repeat shedding events could play a significant yet currently ignored role in shaping environmental oocyst loading with implications for human and animal exposure. Oocyst presence in the environment is closely linked to climate variables such as temperature and precipitation, so in quantifying risk of exposure, it is important to consider the burden of *T. gondii* oocysts that can accumulate over time in diverse environmental matrices and sites, as well as the spatial heterogeneity of free-ranging cat populations. Key directions for future research include investigating oocyst shedding in under-sampled regions, genotyping of oocysts detected in faeces and longitudinal studies of oocyst shedding in free-ranging felids.

KEYWORDS
domestic cats, felids, free-ranging, oocyst shedding, *Toxoplasma gondii*, transmission

1 | INTRODUCTION

Toxoplasma gondii is a ubiquitous, zoonotic, protozoan pathogen with a unique life cycle involving domestic and wild hosts in diverse ecosystems. Since its discovery in 1908, warm-blooded vertebrates including terrestrial and marine mammals, birds, and humans have been identified as intermediate hosts for *T. gondii* (Dubey, 2008). Cats (wild and domestic felids) are the only known definitive hosts of *T. gondii* that shed environmentally resistant oocysts into the environment (Dubey, 1998; Frenkel et al., 1969; Hutchison et al., 1969). Though laboratory studies have investigated infection routes and oocyst shedding in domestic cats and select wild felid species, many questions remain on the host, parasite and environmental factors that influence oocyst shedding in naturally infected domestic and wild felids. It is critical to understand the frequency and quantity of oocyst shedding in free-ranging domestic cats and wild felids as oocyst-borne infections also drive bradyzoite (tissue cyst) transmission, and oocysts in the environment can pose a significant health risk to people, domestic animals and wildlife (Shapiro et al., 2019).

Humans and animals can become infected with *T. gondii* via congenital transmission, ingestion of tissue cysts in infected meat or ingestion of oocysts in contaminated water, soil or food. In humans, it is estimated that 30% of the global population is infected with *T. gondii* (Flegr et al., 2014). Although most healthy people are asymptomatic, toxoplasmosis can prove fatal for immunocompromised individuals as well as developing fetuses (Jones et al., 2003). Atypical strains of *T. gondii* have been linked to severe symptoms and mortality even in immunocompetent people (Carme et al., 2009; Delhaes et al., 2010; Demar et al., 2012; Vaudaux et al., 2010), though few studies have looked at the association between disease severity and strain type. Asymptomatic infections are common in domestic animals and wildlife, but *T. gondii* remains an important cause of abortion in domestic sheep and goats (Stelzer et al., 2019). Mortality due to *T. gondii* is also a concern in threatened and endangered wildlife populations, including Nēnē geese (*Branta sandvicensis*), Southern sea otters (*Enhydra lutris nereis*), Hector's dolphins (*Cephalorhynchus hectori*) and Hawaiian monk seals (*Neomonachus schauinslandi*) (Barbieri et al., 2016; Duncanson et al., 2001; Roe et al., 2013; Shapiro, VanWormer, et al., 2019; Work et al., 2016). Marine mammal infections highlight the importance of oocyst-borne transmission as oocysts shed by felids in terrestrial landscapes can be carried via freshwater runoff to aquatic environments where *T. gondii* can contaminate food sources for people and wildlife (Shapiro et al., 2019). Compared with *T. gondii* transmission via tissue cyst ingestion or congenital transmission, the oocyst transmission route is the least studied, with critical knowledge gaps still remaining (Shapiro et al., 2019).

One aspect of *T. gondii* epidemiology that remains largely unexplored is the potential for repeat oocyst shedding in felids. Since most experimental studies were designed to observe a single oocyst shedding event after infection and field studies on oocyst shedding are typically cross sectional in design, the relative contribution of repeat shedding to the environmental load of oocysts remains unknown. In the limited number of laboratory repeat shedding studies that have been reported, domestic cats were shown to re-shed oocysts under specific conditions such as immune suppression, infection with a novel *T. gondii* genotype, or co-infection with other coccidian parasites (Chessum, 1972; Dubey, 1995; Dubey et al., 1977; Freyre et al., 2007; Malmasi et al., 2009; Zulpo et al., 2018). While experimental studies demonstrate that oocyst re-shedding is possible, there are no reports of re-shedding in free-ranging domestic or wild felids, and longitudinal studies of oocyst shedding in these hosts are rare.

Timing of initial infection, immune status, co-infection with other parasites and the genotypes of *T. gondii* to which a cat is exposed are all important risk factors to consider for oocyst shedding and re-shedding. Because an infected cat can shed hundreds of millions of oocysts per shedding event into the environment (Fritz et al., 2012), and oocysts remain a common source of infection for humans and many domestic and wild animals, it is important to understand the epidemiology of oocyst shedding in both domestic and wild felids. In this review, we identify current knowledge gaps by synthesizing the literature to date on oocyst shedding and re-shedding in domestic and wild felids, highlighting the geographical distribution of oocyst genotypes and identifying key directions for future research, especially in free-ranging domestic and wild felids.

2 | METHODS

Between February and March of 2020, we searched two databases – PubMed and Web of Science – for relevant publications, utilizing 12 sets of search terms related to *T. gondii*, felids and oocyst shedding. An additional sweep of the literature using this methodology was conducted in February 2021 prior to manuscript submission to account for new publications. These searches included the key word '*Toxoplasma*' in combination with these terms: 'felid', 'cat', 'shedding', 'genotype', 'repeat', 'transmission', 'captive', 'oocyst', 'feces' and 'zoo' (Supplemental Table 1). This initial search resulted in 2176 publications; a further 53 primary publications were found from the references of six relevant reviews (Amouei et al., 2020; Dabritz & Conrad, 2010; Dubey, 2008, 2018; Elmore et al., 2010; Torrey & Yolken, 2013), which resulted in a total of 2229 (Figure 1). After removing duplicates, the remaining 1118 unique publications were filtered for relevance through reading of abstracts, resulting in 296 publications.

Publications included in the final review reported evidence of oocyst shedding in at least one felid species detected by at least one commonly accepted diagnostic method (microscopy, polymerase chain reaction [PCR], and/or mouse bioassay). We included studies reporting *T. gondii*-like oocysts identified by microscopy in addition to studies with oocysts confirmed as *T. gondii* by bioassay or PCR to capture all possible information in the literature related to oocyst shedding. Crude pooled prevalence across studies was calculated for (1) confirmed *T. gondii* oocyst shedding and (2) combined *T. gondii* and *T. gondii*-like oocyst shedding (Table 1). Studies that did not confirm oocysts using PCR and/or bioassay could be identifying other closely related parasites such as *Hammondia* or *Besnoitia* instead of *T. gondii*. We excluded publications that did not describe oocyst shedding (e.g. serologic studies), soil studies, vaccination studies, studies of infection in other hosts and

TABLE 1 Definition of study types identified based on cat population sampled, number of studies within each category and crude confirmed and combined oocyst shedding prevalence. Confirmed oocyst shedding refers to studies that used PCR or bioassay to identify oocyst species. Combined prevalence also includes '*T. gondii* -like' oocysts which were identified using microscopy, which cannot discriminate between *T. gondii* and closely related apicomplexan parasites, especially *Hammondia* spp

Study type (number of studies)	Definition	Confirmed *T. gondii* oocyst shedding prevalence (samples positive/samples tested)	Combined *T. gondii* and *T. gondii*-like oocyst shedding prevalence (samples positive/samples tested)
Domestic cats – experimental ($n = 122$)	Infection of domestic cats with oocysts and or/infected tissue to induce oocyst shedding in an exclusively controlled, laboratory setting	52.1% (535/1026) 67 studies	52.6% (1042/1981) 120 studies[a]
Domestic cats – owned ($n = 58$)	A household owned or shelter cat that spends at least part of its life indoors, has the option to live indoors and is at least partially dependent on human caretakers	0.3% (370/109,859) 29 studies	0.4% (511/125,176) 57 studies
Domestic cats – unowned, free-ranging ($n = 69$)	An unowned domestic cat (including feral and stray cats) that spends its life exclusively outdoors. This includes animals that are fed by caretakers and/or independent cats eating hunted prey	3.2% (214/6635) 34 studies	4.1% (787/19,125) 68 studies
Domestic cats – experimental re-shedding ($n = 12$)	A domestic cat living in a laboratory setting with no outdoor access or access to wild prey	35.9% (37/103) 10 studies[b]	35.9% (37/103) 10 studies
Wild felids - captive ($n = 19$)	A wild felid that lives in captivity, either in a private collection or zoo and consumes prey exclusively provided by human caregivers. This also includes captive wild felids used temporarily in an experimental setting	1.8% (44/2506) 7 studies	2.0% (52/2606) 12 studies
Wild felids – free-ranging ($n = 14$)	A wild felid living in its natural habitat with minimal human contact, no supplemental feeding and no confinement	1.6% (5/322) 6 studies	2.4% (28/1177) 14 studies

[a]Not all studies had raw data available for calculation of oocyst shedding prevalence, so totals in each column may not reflect the total *n* listed in the study type column.
[b]Not all repeat shedding studies used molecular or bioassay confirmation of shed oocysts, but all cats were infected directly with *T. gondii* in a controlled environment.

studies of other protozoan parasites in cats. Papers that were inaccessible after extensive searching were also excluded ($n = 2$), resulting in 294 total publications.

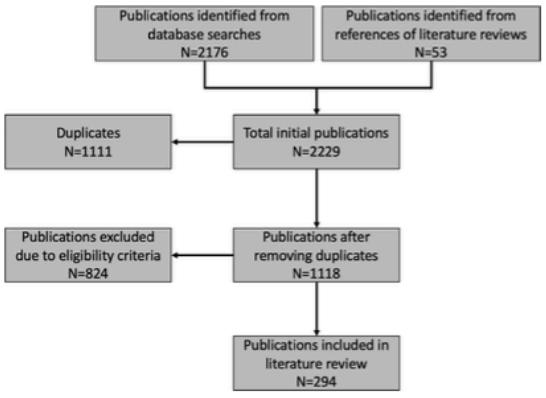

FIGURE 1 Summary of the literature review search process based on PubMed and Web of Science searches conducted in February–March 2020 and February 2021

Each publication was classified as containing data from domestic and/or wild felids. Studies that targeted shedding in domestic cats were further classified as experimental, owned, or unowned/free-ranging (Table 1). Studies that targeted shedding in wild felids were classified as captive or free-ranging. No language or time restrictions were set, and papers in languages other than English were translated using online translation tools and assistance from native speakers. For each eligible publication, we recorded year of publication, country of study, method of oocyst detection (microscopy, PCR, bioassay), number of felids or faeces sampled, oocyst shedding prevalence, genotype if available, and timeframe of sampling if available (Supplemental Tables 2 and 3).

3 | RESULTS AND DISCUSSION

3.1 | Global variation in *T. gondii* oocyst shedding prevalence and oocyst genotypes

Out of 294 relevant studies published between 1965 and 2020, there were 83 studies on oocyst shedding in free-ranging domestic cats ($n = 69$) or wild felids ($n = 14$) from 20 countries (Figures 2 and 3).

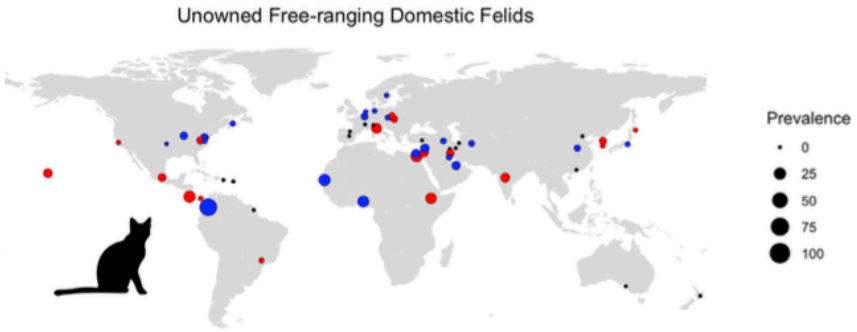

FIGURE 2 Map of confirmed *T. gondii* oocyst shedding prevalence (red) and *T. gondii*-like oocyst shedding prevalence (blue) in unowned, free-ranging domestic cats. Studies that did not detect confirmed *T. gondii* or *T. gondii*-like oocysts are indicated in black circles

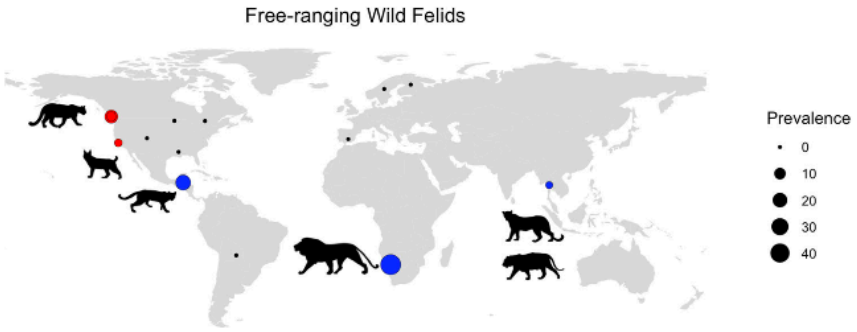

FIGURE 3 Map of confirmed *T. gondii* oocyst shedding prevalence (red) and microscopy-based *T. gondii*-like oocyst shedding prevalence (blue) in free-ranging wild felid species. Confirmed *T. gondii* or *T. gondii*-like oocyst shedding was documented in mountain lions (*Felis concolor*), bobcats (*Lynx rufus*), ocelots (*Felis pardalis*), lions (*Panthera leo*), leopards (*Panthera pardus*) and tigers (*Panthera tigris*). Two studies in British Colombia, Canada reported shedding in mountain lions which appear as two overlapping red circles in the figure. There was no observed oocyst shedding (0% shedding prevalence, black circles) in studies conducted in South America and Europe

Confirmed *T. gondii* oocyst shedding prevalence in free-ranging domestic cats was the highest in Africa (18.8%), with prevalence in Asia, Europe, North America and South America ranging between 0.7% and 3.4% (Table 2). The only studies of confirmed *T. gondii* oocyst shedding in free-ranging wild felids were conducted in North America, with prevalence ranging between 0% and 15.4% (Figure 3). The relative scarcity of oocyst shedding prevalence studies in free-ranging domestic cats ($n = 4$) and wild felids ($n = 2$) in South America is particularly concerning as congenital and ocular toxoplasmosis cases in this region are prevalent and appear to be more severe than in North America and Europe (Amouei et al., 2020; de-la-Torre et al., 2013; Vaudaux et al., 2010). Four domestic cat shedding studies were identified in island countries or continents where native wild felid species are absent, specifically New Zealand, Australia, and St. Kitts. In addition, two recent studies from the island of O'ahu (Hawaii, USA), where wild felids are also absent, detected *T. gondii* DNA in faeces from domestic cats (7% and 10.7% of cats tested), but these studies did not examine faeces microscopically for the presence of oocysts (Davis et al., 2018; Lepczyk et al., 2020).

Genotype of *T. gondii* infection can influence the severity of host disease and may also play a role in oocyst shedding dynamics. However, studies on the genotype of oocysts shed by captive or free-ranging domestic or wild felids are limited. Historically, *T. gondii* strains from humans and domestic animals sampled predominantly in North America and Europe were classified within three clonal types – I, II and III; recent global genetic characterizations of strains revealed a large number of atypical genotypes, with greater diversity in wildlife species and in certain geographic regions, including South America (Amouei et al., 2020; Howe & Sibley, 1995). Experimental studies that used domestic cats for bioassay of *T. gondii* cysts from other animals show that domestic cats can shed oocysts when infected with clonal (Types I, II and III) or atypical *T. gondii* genotypes; at least 30 unique genotypes were isolated in these bioassay studies (Supplemental Table 2). Certain genotypes of *T. gondii* may be more efficient at inducing oocyst shedding in wild or domestic hosts; in one study, wild felids that were experimentally infected with an atypical strain (LRH) shed oocysts, while those infected with an archetypal strain (Type III, M7741) did not (Miller et al., 1972).

TABLE 2 Confirmed *T. gondii* oocyst shedding prevalence and combined *T. gondii* and *T. gondii*-like oocyst shedding prevalence in free-ranging wild and domestic felids by geographic region

Region	Prevalence in wild felids		Prevalence in free-ranging domestic cats	
	Confirmed[a] *T. gondii* oocyst shedding	Combined *T. gondii* and *T. gondii*-like oocyst shedding	Confirmed *T. gondii* oocyst shedding	Combined *T. gondii* and *T. gondii*-like oocyst shedding
Africa	NA[b]	47.83%	18.8%	16.86%
Asia	NA	2.17%	3.09%	6.20%
Europe	NA	0.00%	2.24%	0.97%
North America	1.55%	1.23%	3.42%	2.35%
South America	NA	16.95%	0.74%	3.43%
Oceania	NA	NA	0.00%	0.00%

[a]Confirmed oocyst shedding refers to studies that used PCR or bioassay to identify oocyst species. '*T. gondii* -like' oocysts were identified using microscopy, which cannot discriminate between *T. gondii* and closely related apicomplexan parasites.
[b]NA = Not applicable: no studies were found that reported confirmed *T. gondii* or *T. gondii*-like oocyst shedding.

The few field studies that characterized oocyst genotypes in our review also highlight the potential for domestic cats to contribute atypical as well as archetypal genotypes of *T. gondii* to environmental oocyst load. For owned domestic cats, nine studies provided molecular data on genotypes of *T. gondii* oocysts found in faeces (Supplemental Table 2), and the majority of these reports described Types I, II, III, and mixed II/III genotypes (Berger-Schoch et al., 2011; Dubey & Prowell, 2013; Frey et al., 2012; Herrmann et al., 2010; Jokelainen et al., 2012; Liang et al., 2016; Mancianti et al., 2015; Schares et al., 2008). One additional study reported a naturally infected owned cat with severe toxoplasmosis that shed oocysts characterized as ToxoDB Type #5 (also known as haplotype 12 or Type X) (Dubey & Prowell, 2013). While only eight studies of unowned, free-ranging domestic cats performed genotyping on faecal oocysts, half of them reported atypical or recombinant genotypes (Br1, ToxoDB #4, #7, #9, #11, #31, #54) (Supplemental Table 2). No studies have reported genotyping data on oocysts from captive or free-ranging wild felids, but atypical genotypes of *T. gondii* have been detected in tissue samples from wild felids (Dubey et al., 2013; VanWormer et al., 2014; Shapiro et al., 2019). Domestic cats living in and around wild felid habitats may acquire atypical genotypes from preying on animals in the overlapping food web or from contact with water and soil that are contaminated with oocysts from wild felids (Mercier et al., 2011; VanWormer et al., 2014). Existing shedding prevalence and oocyst genotype studies provide an important foundation for understanding the epidemiology of oocyst shedding in naturally infected felid hosts, but there is still a critical need for additional data on oocyst shedding prevalence and oocyst genotypes shed by domestic and wild felids globally.

3.2 | Challenges of oocyst detection and confirmation

Bioassays in cats or mice have been considered a gold standard for detection of viable *T. gondii* oocysts in diverse matrices. Given the high cost, time, labour and ethical considerations of bioassay experiments, many studies have implemented alternative strategies such as microscopy and/or molecular detection, which are considerably more affordable and feasible, though not without challenges. Microscopy offers the least costly approach for visualizing *T. gondii*-like oocysts in faeces; however, the identity of the parasite cannot be confirmed with microscopy alone given the virtually identical morphological appearance of closely related protozoans. Almost half of the 294 studies in this review used microscopy alone (48.3%, $n = 142$) for parasite identification, whereas 51.7% ($n = 152$) used either mouse bioassay or PCR to identify and confirm *T. gondii* in faeces. While most of these 152 studies also included microscopy, nine studies (5.9%) used only PCR, one study (0.7%) used only bioassay and one study used both PCR and bioassay (0.7%). A comparative analysis between copro-PCR and mouse bioassay using three seronegative female cats that were fed the VEG1 (Type III) strain found that copro-PCR was as sensitive and specific as mouse bioassay and provided much faster results than the 56-day duration required for the bioassay (Salant et al., 2010). However, PCR on faecal samples has limitations, especially when used without microscopy, as *T. gondii* DNA amplification may result from parasites in infected prey tissues that are passing through the gastrointestinal system, rather than true presence of oocysts (Poulle et al., 2016). Field studies of domestic and wild cats that exclusively use PCR for detection of *T. gondii* may overestimate the true oocyst shedding prevalence. A combination of testing methods (faecal floatation with microscopy + PCR or bioassay) provides more accurate identification of *T. gondii* oocysts in faeces.

3.3 | Risk factors for oocyst shedding in domestic and wild felids

Based on experimental studies, the commonly reported duration of oocyst shedding is approximately 1–2 weeks after initial infection, during which cats can excrete hundreds of millions and up to one billion oocysts (Dubey, 1995; Dubey & Frenkel, 1972; Fritz et al., 2012; Torrey & Yolken, 2013). In field studies, the majority of shedding data have focused on owned domestic cats; in our review, there were 511 confirmed *T. gondii* or *T. gondii*-like positives out of 125,176 faecal samples

(0.4%) from owned cats. The crude pooled prevalence of oocyst shedding in unowned, free-ranging domestic cat studies in this review was 4.1% (787 positive/19,125 samples over 68 studies), much higher than the commonly cited 1% for domestic cats in general (Dubey, 2008), and is likely a more realistic average shedding prevalence for unowned, free-ranging domestic cats.

Although public health messages often emphasize exposure to *T. gondii* from domestic cats, wild felids also contribute oocysts to the environment and have been associated with human outbreaks of toxoplasmosis in North and South America (Aramini et al., 1999; Carme et al., 2009). A total of 31 wild felid species and subspecies were represented across 33 publications in our review, and the most commonly studied species included bobcats (*Lynx rufus* $n = 8$), mountain lions or pumas (*Puma concolor*, $n = 8$), lions (*Panthera leo*, $n = 6$) and Pallas's cats (*Otocolobus manul*, n = 4) (Supplemental Table 3). As many wild felid species have not been sampled for oocyst shedding, the current list of *T. gondii* definitive host species is not exhaustive. Approximately two-thirds of the wild felid oocyst shedding studies focused on captive animals, which included both experimental infections and natural exposure in captivity (zoo or other managed setting) ($n = 19$). The majority of free-ranging wild felid studies (10/14) were conducted in North America or Europe, indicating a need for more research on *T. gondii* shedding in free-ranging wild felids globally (Figure 3). Oocyst shedding prevalence was only available for 26 of the 33 wild felid studies, 14 of which focused on free-ranging wild felids. Unfortunately, *T. gondii* oocyst confirmation by PCR or mouse bioassay was only available in six of these studies. The crude pooled oocyst shedding prevalence (confirmed *T. gondii* oocysts and *T. gondii*-like oocysts) across free-ranging wild felid studies was 2.4% (28 positive/1177 samples), though the individual study prevalences ranged from 0 to 47.8% (Figure 3; Supplemental Table 3). Shedding prevalence in free-ranging wild felids appears higher than in their domestic cousins, but the paucity of studies with confirmed *T. gondii* oocysts and studies in free-ranging felids currently limits broad comparisons between domestic and wild felid species. Collectively, however, the experimental and field studies on domestic and wild felids provide evidence for key host, parasite and environmental risk factors that can influence oocyst shedding including route of infection, felid diet, age and immune status.

3.3.1 | Route of infection

Experimental studies on the role of infection route in oocyst shedding show that infection and subsequent shedding are parasite stage rather than dose-dependent, meaning cats are more successfully infected and a higher percentage of animals will shed oocysts in faeces when they are exposed to *T. gondii* through consumption of bradyzoite tissue cysts than when they are infected with oocysts or tachyzoites (Dubey, 2002; 2006). The experimental data align with the life cycle of *T. gondii* in natural conditions, whereby free-ranging felids commonly consume tissue cysts in infected prey, shed oocysts into the environment, and infect prey species and other herbivorous or omnivorous intermediate hosts that ingest oocyst contaminated matrices.

3.3.2 | Felid diet (exposure to *T. gondii* through prey)

Wild felids and many unowned domestic cats subsist on wild prey, ranging from small mammals and birds to large herbivores that can serve as intermediate hosts for *T. gondii*. Because many cat owners let their cats roam outdoors, owned cats can also become infected through hunting wild prey. In addition to eating infected prey, domestic cats and captive wild felids can be fed raw meat contaminated with *T. gondii* and thereby shed oocysts. Pet cats consuming infected meat or prey may expose their owners, and improper disposal and management of their faeces outside can lead to contamination of soil, drinking or irrigation water.

Time spent outdoors is an important risk factor for oocyst shedding in domestic cats, given the potential exposure to oocyst-contaminated soil and water, as well as increased risk of consuming infected prey in unowned versus owned domestic cats. In California, confirmed oocyst shedding prevalences in feral cats associated with humans and unmanaged feral cats subsisting on wild prey were 0.5% and 5.9%, respectively (VanWormer et al., 2013). This difference in shedding prevalences is likely similar to, or even underestimates, the difference in oocyst shedding between owned and unowned domestic cats. In countries with high *T. gondii* infection levels reported in rodents and birds like Ethiopia and Egypt, researchers detected bioassay-confirmed oocyst shedding prevalences between 19% and 24% in free-ranging domestic cats (Dubey et al., 2013; Rifaat et al., 1976).

We know very little about relative differences in oocyst shedding among wild felids; however, a recent study found higher levels of confirmed *T. gondii* shedding in bobcats (6.3%) compared to mountain lions (2.0%) living in central California (VanWormer et al., 2013), with a similar trend detected in a 1976 survey of bobcats and mountain lions in the Western United States (Marchiondo et al., 1976). Bobcats eat higher numbers of smaller prey than mountain lions, which could lead to higher chances of consuming infected prey and exposure to novel, heterologous genotypes. However, given the small sample sizes in existing reports, larger and more geographically diverse studies of oocyst shedding in wild felids are needed to identify differences in oocyst shedding prevalence and risk factors among wild felid species and populations.

Differences in lifestyle and diet play a significant role in explaining oocyst shedding prevalences among owned and unowned domestic cats and wild felids, and the commonly cited 1% shedding prevalence for domestic cats may thus underestimate and overgeneralize oocyst shedding patterns. Management of unowned cat populations is an accepted practice in many countries, so understanding the contribution of each type of cat to the overall risk of environmental contamination with *T. gondii* is vital for decision-making regarding human health and wildlife conservation.

3.3.3 | Age

Some experimental studies suggest that young cats represent the highest risk group for oocyst shedding, with reported prevalences between 17.1% and 40% in domestic cats < 1 year old (Beelitz et al., 1992;

Knaus & Fehler, 1989). In experimental studies, adult cats can also shed oocysts after primary infection with *T. gondii*, with exposure to additional novel strains, and after losing immunity from a previous *T. gondii* infection (Zulpo et al., 2018). While age is an important risk factor for *T. gondii* infection, this association may be complicated by the fact that young cats are becoming exposed to *T. gondii* for the first time, which is highly correlated with age. Thus, it is difficult to tease out whether specific attributes of animal age, exposure in a naive host or a combination of these factors is responsible for higher shedding prevalence. Age is not a well described factor in most field shedding studies, but shedding has been reported in naturally infected adult domestic and wild felids (Mancianti et al., 2010; Pena et al., 2006; VanWormer et al., 2013). Particularly for free-ranging domestic or wild felids that subsist on prey and are likely to be exposed to *T. gondii* as young animals, it is unlikely that all reports of shedding in adult animals capture shedding after primary infection with *T. gondii*. Some documented oocyst shedding in adult animals may thus actually be instances of oocyst re-shedding.

3.3.4 | Immunosuppression

Experimental data on the role of immunosuppression in oocyst shedding is inconclusive. Inoculation of *T. gondii* in naïve cats 42 days after starting immunosuppressive therapeutics (e.g. cyclosporine) resulted in high oocyst shedding prevalence (100%, 10/10), but shedding prevalence was identical in animals given the placebo (100%, 10/10) and in animals given cyclosporine after *T. gondii* infection (100%, 10/10) (Lappin et al., 2015). However, immunosuppressive drugs may alter the quantity of oocysts shed and duration of shedding. Cats administered high doses of cyclosporine for the entirety of the experiment (126 days) had more severe symptoms of systemic toxoplasmosis and shed fewer oocysts over a shorter time period relative to the other two groups.

Cats that are infected with feline immunodeficiency virus (FIV) or feline leukemia virus (FeLV) can also be naturally immunosuppressed. Across three experimental studies of naïve cats challenged with *T. gondii* infected mice, 86.6% of FIV/FeLV-infected cats shed oocysts, but similar shedding levels and duration of shedding were observed in FIV/FeLV negative cats (Lappin et al., 1992, 1996; Patton et al., 1991). However, the experimental design may not fully capture the impacts of co-infection with immunosuppressive viruses on *T. gondii* shedding as these studies did not evaluate variable immunosuppression caused by different FIV and FeLV viruses, interaction with more virulent *T. gondii* genotypes than the isolates used (ME-49, goat isolate), or long-term effects of immunosuppression in relation to oocyst shedding dynamics. FIV, FeLV and *T. gondii* are more prevalent in free-ranging cats and owned cats with outdoor access, and the lifestyle factors of these cats could make it more likely for these animals to be exposed to more immunosuppressive viral strains and more diverse genotypes of *T. gondii* (Little et al., 2009; Norris et al., 2007).

3.4 | Frequency of oocyst shedding: Experimental and field evidence for repeat shedding

The commonly accepted assumption that cats only shed for a short period of time after initial infection has been reiterated in many publications, especially in studies of free-ranging domestic cats. Yet, we have known since at least 1972 that co-infection with another apicomplexan parasite, *Isospora felis, now called Cystoisospora felis*, induces re-shedding in domestic cats (Chessum, 1972; Sheffield & Melton, 1969). The order of infection plays a role in determining shedding, as cats experimentally infected with *C. felis* prior to *T. gondii* infection did not re-shed oocysts when challenged with *T. gondii*, while chronically *T. gondii*-infected cats did re-shed oocysts following subsequent infection with *C. felis* (Dubey, 1978). Subsequent experimental discoveries have also demonstrated oocyst re-shedding in cats after challenge with a heterologous (different) *T. gondii* genotype (Table 3). Heterologous challenge is an important mechanism for re-shedding due to relevant implications in natural settings, where diverse genotypes of *T. gondii* can be present in prey and the environment. Notably, data on repeat oocyst shedding in domestic cats are all from highly controlled laboratory studies that use a limited number of *T. gondii* strains representing mostly Types II and III genotypes (Table 3). If we compare these scenarios to free-ranging domestic and wild felids, which are likely to encounter multiple genotypes of *T. gondii* as well as other apicomplexan protozoa throughout their lifespan via consumption of infected prey, then the assumption of a single oocyst shedding period warrants legitimate questioning.

Studies of closely related protozoan parasites, such as *Neospora caninum*, also lend support to the possibility of repeat shedding; an experimental study in domestic dogs (a definitive host of *N. caninum*) showed re-shedding after an 8–18 month 'refractory period' (Gondim et al., 2005). Adult dogs in this study shed fewer oocysts than puppies, and this finding is echoed in the case of *T. gondii*, where there is debate about whether repeat shedding could actually contribute a significant number of oocysts to the environment. A recent study from Brazil showed that cats do indeed re-shed fewer oocysts per gram of faeces after re-infection (Zulpo et al., 2018). However, because adult cats produce more faeces than kittens, the total amount of oocysts shed is still high, especially when cats are exposed to heterologous strains.

Studies in wild felids also suggest that *T. gondii* oocyst repeat shedding can occur. A longitudinal, 54 week study of captive wild felids in a Czech zoo found *Toxoplasma*-like oocysts in faeces from a pair of wildcats (*Felis silvestris*), a 1-year old female wildcat, and a pair of Amur leopard cats (*Felis euptilurus*) multiple times over the course of a year (Lukešová & Literák, 1998). *T. gondii* oocysts were confirmed by mouse bioassay in weeks 2–3, 17 and 25 for the pair of wild felids, but faecal samples were pooled for both animals. The clearest evidence for repeat shedding in this study comes from the single wildcat, where bioassay-confirmed *T. gondii* oocysts were detected in weeks 2–3, 12 and 21. In a survey of a free-ranging lion pride (*Panthera leo*) in Namibia, *T. gondii*-like oocysts were found in faeces collected from the same two adult lions 6 months apart (Smith & Kok, 2006). While this could be indicative of repeat shedding in free-ranging wild felids, the identity of the para-

TABLE 3 Experimental studies reporting repeat *T. gondii* oocyst shedding (oocyst re-shedding) in domestic felids

Study	Microscopy	PCR	Mouse bioassay	Genotype (strain)	Re-shedding stimulus	Re-shedding prevalence (number of cats with re-shedding observed/ number of cats infected)
Kuhn and Weiland (1969)	Y	N	Y	Unknown	Homologous challenge	20% (1/5)
J. P. Dubey (1976)	Y	N	Y	III (M7741)	Spontaneous, *Cystoisospora felis*, *C. rivolta* superinfection	90% (9/10)
J. P. Dubey et al. (1977)	Y	N	Y	III (M7741), Unknown (CR6)	Homologous & heterologous challenge	0% (0/11)
J. P. Dubey (1978)	Y	N	N	III (M7741)	*C. felis* superinfection	43.8% (7/16)
Frenkel and Smith (1982)	Y	N	N	II (Gail), III (M7741)	Heterologous challenge	22.2% (2/11)
S. W. Davis and Dubey (1995)	Y	N	N	II (ME49)	Homologous challenge	0% (0/12)
J. P. Dubey (1995)	Y	N	N	II (ME49), BrIII (P89), Unknown (TS-2)	Heterologous challenge	44.4% (4/9)
J. P. Dubey et al. (1995)	Y	N	Y	II (ME49), BrIII (P89), Unknown (TS-2)	Heterologous challenge	44.4% (4/9)
Freyre et al. (2007)	Y	N	N	II (ME49), III (M7741)	Homologous challenge	11.1% (1/9)
Malmasi et al. (2009)	Y	N	N	II (Tehran)	Immune suppression (Dexamethasone)	66.6% (8/12)
Zulpo et al. (2018)	Y	Y	N	II (ME49, TgDoveBr1, TgDoveBr8), III (VEG)	Heterologous challenge	71.4% (5/7)

site was not confirmed by molecular tests or bioassay. As free-ranging domestic cats and wild species have lifestyle factors and exposures that would make them more likely to experience repeat shedding, longitudinal studies are necessary to clarify the frequency of repeat oocyst shedding in naturally infected wild and domestic felids and its contribution to environmental oocyst load.

3.5 | Climate, environment and oocysts

Climatic and environmental variables such as temperature and precipitation have become increasingly important to understanding the seasonal fluctuations of oocysts in the environment, as well as incidence of human toxoplasmosis cases in different seasons. Climate can directly influence oocyst-borne transmission because oocyst sporulation and persistence are temperature and moisture dependent (Dubey, 2016). However, there may also be seasonal differences in oocyst shedding. A study in Germany consisting of faecal samples from 18,259 domestic cats found that the proportion of *T. gondii* positive samples between January and June was significantly lower compared to the proportion of positive samples between July and December (Herrmann et al., 2010). Subsequent studies in Europe found that significantly more oocysts were detected in domestic cat faeces in summer (June to August) and autumn (September to November) than winter and spring, and that mean air temperature and North Atlantic Oscillation (a phenomenon of atmospheric pressure fluctuation) were sufficient explanatory variables for predicting the proportion of positive faecal samples (Schares et al., 2016). Climate factors will also affect the timing of feline reproduction and the number of litters a cat produces throughout the year: cats are constrained by environmental conditions that allow for successful reproduction (temperatures warm enough for cats to be in oestrous) and sufficient levels of prey. In the northern hemisphere, feline breeding season falls in the warmer months between April and October, which corresponds with the findings from Schares et al. (2016) in terms of the timing of exposure and shedding in naive hosts (kittens that start eating prey after weaning). Although precipitation was not as significant as temperature or atmospheric pressure in this study (Schares et al., 2016), rainfall may facilitate parasite persistence and transport of oocysts from faeces and soil to aquatic systems, including drinking water sources, as has occurred in North and South American waterborne outbreaks (Bahia-Oliveira et al., 2003; Bowie et al., 1997). Shedding and therefore human exposure are thus tightly linked to the felid life cycle and environmental persistence of *T. gondii* oocysts.

Studies of *T. gondii* in soil samples also enhance our understanding of seasonal patterns of oocyst shedding, though we do not yet know to what degree soil prevalence offers a meaningful comparison to faecal shedding prevalence and risk to human health. One California study only detected soil contamination with oocysts during the fall (November); soil samples from the same study sites tested negative for *T. gondii* in spring and summer sampling efforts (de Wit et al., 2020). Higher burden in soil likely means more cats are shedding, but interpretation of results is also dependent on the detection method used. Many soil studies rely on PCR to detect *T. gondii*, but the sole use of this method may be insufficient to make inferences about human risk because it can detect both viable and nonviable oocyst DNA. Finding DNA in

soil does not imply the presence of sporulated/infective oocysts; multiple techniques in combination or applications of parasite viability-discriminating methods in soil are required to improve the utility and interpretability of soil contamination studies.

4 | CONCLUSIONS

Domestic and wild felids can both serve as sources of oocysts that drive *T. gondii* transmission to humans and other animals. To understand their contributions to environmental oocyst contamination and toxoplasmosis in diverse locations, it is important to consider the species and numbers of felids present in a given area, their diet, habitat overlap among domestic and wild felids and overlap with humans or human-destined water and food sources. The potential for repeat oocyst shedding in free-ranging domestic and wild felids underscores the need for longitudinal field studies of domestic and wild felids. The human population is projected to grow for at least the next few decades, and as pet ownership becomes more common due to changing education and economic trends, the domestic cat population will also increase. This means more definitive hosts with the capacity to release large quantities of oocysts into the environment, leading to soil, water and food contamination that can result in toxoplasmosis in humans and other intermediate hosts (including livestock that can serve as sources of infection to people via consumption of undercooked meat). This is especially important for wildlife from areas without native felid species such as Australia, New Zealand and small Indo-Pacific islands. Introduction of domestic cats to felid-free regions has been profoundly detrimental to native wildlife populations, through direct impacts of predation and indirect but significant health impacts due to the pathogens they carry. The Hawaiian monk seal, Nēnē goose, kereru and brown kiwi are examples of wildlife from historically felid-free regions that have experienced fatal toxoplasmosis as a result of domestic cat introduction (Barbieri et al., 2016; L. Howe et al., 2014; Work et al., 2016).

As *T. gondii* oocyst shedding, especially in free-ranging felids, is still not well understood, there is a critical need for standardized detection methods, longitudinal studies and expanded geographic coverage. We recommend the use of faecal floatation and microscopy paired with molecular confirmation to identify *T. gondii* oocysts in faeces. This standardized diagnostic testing approach will facilitate confirmation of oocysts and comparability across studies. Expanded field studies on host (type of cat, age, co-infection with other parasites or immunosuppressive viruses) and environmental risk factors (season, habitat/land use, overlap with sympatric felid populations) for oocyst shedding will help to identify felids at highest risk of shedding. Field studies of oocyst shedding in free-ranging domestic and wild felids in under-represented geographic areas would also provide vital evidence on the presence and frequency of shedding. Longitudinal studies of free-ranging domestic and wild felids are needed to assess the potential for repeat shedding to contribute to environmental oocyst load. Whenever possible, field studies should include genotyping of oocysts shed to understand the spatial distribution of genotypes in the environment as well as potential relationships between genotype and quantity or frequency of shedding. Areas where more diverse felid species and potentially virulent, atypical *T. gondii* genotypes overlap are often the same regions that suffer high human seroprevalence and disease burden, highlighting the potential for future research on oocyst shedding to benefit human and animal health.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

ETHICAL STATEMENT

No ethical approval was required as this is a review article with no original research data.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplemental Table 1.1 Terminology and results of literature search process for manuscripts on *T. gondii* oocyst shedding by wild and domestic felids

Literature search terms	PubMed hits	Web of Science (WoS) hits
Toxoplasma+cat+shedding	88	198
Toxoplasma+felid+shedding	95	27
Toxoplasma+oocyst+shedding	114	132
Toxoplasma+felid+genotype	169	31
Toxoplasma+felid+repeat+shedding	1	0
Toxoplasma+domestic+repeat+shedding	0	0
Toxoplasma+wild+repeat+shedding	0	2
Toxoplasma+felid+zoo	53	26
Toxoplasma+felid+captive	48	36
Toxoplasma+felid+species	246	71
Toxoplasma+transmission+felid	469	71
Toxoplasma+felid+oocyst+feces	290	9
	1573	603
Manuscripts found from searches of relevant reviews		53
Total:		2229

Supplemental Table 1.2 Comprehensive list of experimental and observational domestic felid studies investigating *T. gondii* oocyst shedding

Reference	Study Location	†Study Type	Fecal flotation and microscopy	PCR	Bioassay	Genotype (strain)	§Oocyst shedding prevalence (%)
(Dubey, 1982)	USA	Experimental	Y	N	Y	‡NA	100 (1/1)
(Dubey, 1983)	USA	Experimental	Y	N	Y	ToxoDB #10	14.3 (1/7)
(Dubey, 1985)	USA	Experimental	Y	N	N	NA	69.23 (9/13)
(Dubey & Thulliez, 1993)	USA	Experimental	Y	N	N	ToxoDB #10	75 (3/4)
(Hilali et al., 1995)	Saudi Arabia	Experimental	Y	N	N	NA	100 (4/4)
(Dubey, Humphreys, et al., 1995)	USA	Experimental	Y	N	Y	NA	72.73 (8/11)
(Cole et al., 2000)	USA	Experimental	Y	N	Y	ToxoDB #1	50 (2/4)
(Dubey, Gamble, et al., 2002)	USA	Experimental	Y	N	Y	NA	92.73 (51/55)
(Dubey, Graham, et al., 2002)	Brazil	Experimental	Y	N	Y	ToxoDB #2, #10	48.15 (13/27)
(Dubey, Graham, da Silva, et al., 2003)	Brazil	Experimental	Y	N	Y	ToxoDB #2, #10	100 (13/13)
(Dubey, Graham, Dahl, Sreekumar, et al., 2003)	USA	Experimental	Y	N	Y	ToxoDB #1, #2	72.73 (8/11)
(Sreekumar et al., 2003)	India	Experimental	Y	N	Y	ToxoDB #1, #2	21.74 (5/23)
(Dubey, Graham, Dahl,	Egypt	Experimental	Y	N	N	ToxoDB #1, #2	20 (1/5)

Hilali, et al., 2003)							
(Dubey, Navarro, Graham, et al., 2003)	Brazil	Experimental	Y	N	Y	ToxoDB #10	75 (3/4)
(D. E. Hill et al., 2004)	USA	Experimental	Y	N	N	ToxoDB #2	40.63 (26/64)
(Dubey, Salant, et al., 2004)	Israel	Experimental	Y	N	Y	ToxoDB #1, #2	100 (2/2)
(Dubey, Graham, et al., 2004)	USA	Experimental	Y	N	Y	ToxoDB #1, #2, II+III recombinant	70 (7/10)
(Gajadhar et al., 2004)	USA	Experimental	Y	N	N	ToxoDB #2	100 (4/4)
(Dubey, Hill, et al., 2005)	USA	Experimental	Y	N	Y	ToxoDB #1, #2, #10	100.00 (7/7)
(Dubey, Rajapakse, et al., 2005)	Sri Lanka	Experimental	Y	N	Y	ToxoDB #1	33.33 (1/3)
(Dubey, Edelhofer, et al., 2005)	Austria	Experimental	Y	N	Y	ToxoDB #1	73.33 (11/15)
(Dubey, Lenhart, et al., 2005)	Venezuela	Experimental	Y	N	Y	ToxoDB #1, #2	38.89 (7/18)
(D. E. Hill et al., 2006)	USA	Experimental	Y	N	N	ToxoDB #2	100 (10/10)
(Dubey, Sundar, et al., 2006)	Nicaragua	Experimental	Y	N	Y	ToxoDB #2	33.33 (1/3)
(Dubey, Cortés-Vecino, Vargas-Duarte, et al.,	Colombia	Experimental	Y	N	Y	ToxoDB #9, #18, #29	100 (4/4)

2007)							
(Dubey, Sundar, Gennari, et al., 2007)	Brazil	Experimental	Y	N	Y	ToxoDB #2, #6, #7, #25, #28, #29, #30, #70, #77, #96, #105	50 (1/2)
(Dubey, Morales, Sundar, et al., 2007)	Costa Rica	Experimental	Y	N	Y	ToxoDB #1	100 (1/1)
(Dubey, Sundar, Nolden, et al., 2007)	USA	Experimental	Y	N	Y	ToxoDB #1, #32	28.57 (2/7)
(Dubey, Huong, Sundar, et al., 2007)	Vietnam	Experimental	Y	N	Y	ToxoDB #9, #18	100 (8/8)
(Shaapan & Ghazy, 2007)	Egypt	Experimental	Y	N	N	NA	100 (3/3)
(Dubey, Sundar, et al., 2008)	USA	Experimental	Y	N	Y	ToxoDB #1, #2, #3, #4, #9, #54, #72, #73, #131	68.63 (35/51)
(Prestrud et al., 2008)	Norway	Experimental	Y	N	Y	ToxoDB #1	100 (2/2)
(Dubey, Hill, et al., 2008)	USA	Experimental	Y	N	Y	ToxoDB #1, #113	68.75 (11/16)
(Dubey, Huong, et al., 2008)	Ghana, Indonesia, Poland, Vietnam, Italy	Experimental	Y	N	Y	ToxoDB #1, unknown	12.50 (2/16)
(Dubey & Crutchley, 2008)	USA	Experimental	Y	N	Y	ToxoDB #2	100 (1/1)

(Dubey, Velmurugan, et al., 2009)	Costa Rica	Experimental	Y	N	Y	ToxoDB #52	100 (1/1)
(Dubey, Mergl, et al., 2009)	Canada	Experimental	Y	N	Y	ToxoDB #3	100 (2/2)
(Forbes et al., 2009)	Canada	Experimental	Y	N	N	NA	13.33 (2/15)
(Dubey, Rajendran, Ferreira, et al., 2010)	USA	Experimental	Y	N	Y	ToxoDB #147	60 (3/5)
(Dubey, Rajendran, Costa, et al., 2010)	Brazil	Experimental	Y	N	Y	ToxoDB #59	100 (1/1)
(Frazão-Teixeira et al., 2011)	Brazil	Experimental	Y	N	Y	ToxoDB #1, #2, #4	100 (5/5)
(Dubey et al., 2011)	Brazil	Experimental	Y	N	Y	ToxoDB #2, #11	83.33 (5/6)
(Dubey et al., 2014)	USA	Experimental	Y	N	N	ToxoDB #167	25 (1/4)
(Dubey et al., 2015)	USA	Experimental	Y	N	Y	NA	79.31 (23/29)
(Verma et al., 2015)	USA	Experimental	Y	N	Y	ToxoDB #2, #249	100 (2/2)
(Verma et al., 2016)	USA	Experimental	Y	N	Y	ToxoDB #1, #2	100 (2/2)
(Dubey et al., 2016)	USA	Experimental	Y	N	N	NA	20.69 (6/29)
(Y.-R. Yang et al., 2017)	China	Experimental	Y	N	Y	ToxoDB #9	NA
(de Aluja & Aguila, 1977)	USA	Experimental	Y	N	N	ToxoDB #2	100 (25/25)
(Herrmann et al., 2012)	Germany	Experimental	Y	N	Y	Mixed II/III	100 (1/1)

						(TG-GER63)	
(Hutchison, 1965)	USA	Experimental	Y	N	Y	ToxoDB #1	100 (1/1)
(Dubey, 1968)	UK	Experimental	Y	N	N	ToxoDB #1	100 (2/2)
(Hutchison et al., 1968)	Denmark	Experimental	Y	N	Y	NA	32.73 (18/55)
(Sheffield & Melton, 1969)	USA	Experimental	Y	N	Y	ToxoDB #2	100 (4/4)
(Hutchison & Work, 1969)	UK	Experimental	Y	N	Y	NA	100 (2/2)
(Frenkel et al., 1969)	USA	Experimental	Y	N	Y	ToxoDB #2	100 (10/10)
(Hutchison et al., 1969)	UK	Experimental	Y	N	N	NA	100 (2/2)
(Dubey et al., 1970b)	USA	Experimental	Y	N	Y	ToxoDB #2	70 (35/50)
(Frenkel et al., 1970)	USA	Experimental	Y	N	Y	ToxoDB #2	77.78 (7/9)
(Witte & Piekarski, 1970)	Germany	Experimental	Y	N	N	ToxoDB #1	73.91 (17/23)
(Hutchison et al., 1970)	UK	Experimental	Y	N	N	NA	100 (2/2)
(Dubey et al., 1970a)	USA	Experimental	Y	N	Y	ToxoDB #2	88.46 (46/52)
(Overdulve, 1970)	Netherlands	Experimental	Y	N	Y	NA	100 (4/4)
(Piekarski & Witte, 1971)	Germany	Experimental	Y	N	Y	ToxoDB #10	69.56 (16/23)
(Hutchison et al., 1971)	UK	Experimental	Y	N	N	NA	52.94 (9/17)
(Kuhn et al., 1973)	Germany	Experimental	Y	N	N	NA	80 (4/5)
(Dubey &	USA	Experimental	N	N	Y	ToxoDB	100 (4/4)

Frenkel, 1973)						#2	
(Kuhn et al., 1974)	Germany	Experimental	Y	N	Y	NA	60 (3/5)
(Dubey & Frenkel, 1976)	USA	Experimental	Y	N	N	ToxoDB #2	18.6 (8/43)
(Dubey, 1977)	USA	Experimental	Y	N	N	NA	16.36 (9/55)
(Janitschke, 1979)	Germany	Experimental	Y	N	N	ToxoDB #1	90 (9/10)
(A. W. C. A. Cornelissen & Overdulve, 1981)	Netherlands	Experimental	Y	N	Y	NA	87.50 (7/8)
(Hagiwara et al., 1981)	Japan	Experimental	Y	N	Y	NA	55.56 (5/9)
(HAGIWARA et al., 1981)	Japan	Experimental	Y	N	N	NA	33.33 (1/3)
(Frenkel & Smith, 1982)	USA	Reshedding	Y	N	N	ToxoDB #1, #2	16.67 (2/12)
(Schnieder, 1982)	Germany	Experimental	Y	N	N	NA	16.67 (1/6)
(Llorente Lacha et al., 1983)		Experimental	Y	N	N	NA	100 (2/2)
(Linek, 1985)	Germany	Experimental	Y	N	N	NA	9.09 (3/33)
(Krause, 1986)	Germany	Experimental	Y	N	N	NA	40 (2/5)
(Freyre et al., 1989)	USA	Experimental	Y	N	Y	ToxoDB #2	4 (1/25)
(Omata et al., 1990)	Japan	Experimental	Y	N	N	NA	100 (3/3)
(Patton et al., 1991)	USA	Experimental	Y	N	N	NA	100 (4/4)
(Frenkel et al., 1991)	USA	Experimental	Y	N	N	ToxoDB #2	16.22 (6/37)
(D. S. Lin &	USA	Experimental	Y	N	N	ToxoDB	100 (4/4)

Bowman, 1991)						#2	
(Lindsay et al., 1991)	USA	Experimental	Y	N	Y	ToxoDB #10	77.78 (7/9)
(Lappin et al., 1992)	USA	Experimental	Y	N	N	ToxoDB #1	60 (6/10)
(Dardé et al., 1992)	France	Experimental	Y	N	N	NA	80 (16/20)
(Freyre et al., 1993)	USA	Experimental	Y	N	N	ToxoDB #2	71.43 (5/7)
(Sato et al., 1993)	Japan	Experimental	Y	N	Y	NA	100 (9/9)
(Omata et al., 1994)	Japan	Experimental	Y	N	N	NA	13.33 (4/30)
(Dubey et al., 1995)	USA	Experimental	Y	N	N	NA	21.57 (11/51)
(Simon, 1995)	Germany	Experimental	Y	N	N	NA	100 (6/6)
(Omata et al., 1996)	Japan	Experimental	Y	N	Y	ToxoDB #10	85.71 (18/21)
(Dubey, 1996)	USA	Experimental	Y	N	N	ToxoDB #2	40.00 (8/20)
(Lappin et al., 1996)	USA	Experimental	Y	N	N	ToxoDB #1	100 (59/59)
(Dubey et al., 1997)	USA	Experimental	Y	N	N	ToxoDB #2	
(Burney et al., 1999)	USA	Experimental	Y	N	Y	ToxoDB #1	100 (5/5)
(Omata et al., 1999)	Japan	Experimental	Y	N	N	ToxoDB #1, #10	100 (4/4)
(Dubey, 2001)	USA	Experimental	Y	N	Y	ToxoDB #2	54.17 (13/24)
(Powell et al., 2001)	USA	Experimental	Y	N	N	ToxoDB #1	100 (6/6)
(Dubey, 2002)	USA	Experimental	Y	N	N	ToxoDB #1, #2	50 (13/26)

(Dubey, 2005)	USA	Experimental	Y	N	N	ToxoDB #2, #10	62.25 (94/151)
(Dubey, 2006)	USA	Experimental	Y	N	N	ToxoDB #10	36.5 (15/41)
(Garcia et al., 2007)	Brazil	Experimental	Y	N	N	ToxoDB #2, #10	63.64 (7/11)
(MOSALANEZHAD et al., 2007)	Iran	Experimental	Y	N	N	ToxoDB #1	100 (7/7)
(Meireles et al., 2008)	Brazil	Experimental	Y	N	N	ToxoDB #1	100 (4/4)
(Lélu et al., 2011)	France	Experimental	Y	N	N	ToxoDB #1	100 (1/1)
(Zulpo et al., 2012)	Brazil	Experimental	Y	N	N	ToxoDB #1, #10	100 (25/25)
(Fritz et al., 2012)	USA	Experimental	Y	N	N	ToxoDB #1	100 (5/5)
(Awobode et al., 2013)	USA	Experimental	Y	N	N	ToxoDB #1	20 (1/5)
(J. B. W. J. Cornelissen et al., 2014)	Netherlands	Experimental	Y	Y	N	ToxoDB #1	100 (15/15)
(Khan et al., 2014)	French Guiana	Experimental	Y	N	Y	ToxoDB #1, #2, #3	50 (7/14)
(Lappin et al., 2015)	USA	Experimental	Y	N	N	ToxoDB #1	100 (30/30)
(Chemoh et al., 2016)	Thailand	Experimental	Y	Y	N	NA	4.72 (12/254)
(Zulpo et al., 2017)	USA	Experimental	Y	Y	N	ToxoDB #2, #10	100 (12/12)
(Le Roux et al., 2020)	France	Experimental	Y	N	N	ToxoDB #10	100 (9/9)
(Bártová et al., 2003)	Czech Republic	Experimental	Y	N	Y	NA	100 (7/7)
(Kühn et al., 1973)	Germany	Experimental	Y	N	Y	NA	83.33 (10/12)

(Dabritz & Conrad, 2010)	USA	Experimental, Review	Y	N	N	NA	100 (6/6)
(Schreiber et al., 2020)	Switzerland	Experimental	Y	N	N	NA	66.7 (4/6)
(Salant et al., 2010)	Israel	Experimental	Y	Y	Y	ToxoDB #2	100 (3/3)
(Poulle et al., 2016)	USA	Experimental	N	Y	N	ToxoDB #1	0 (0/1)
(Janitschke & Jörren, 1972)	Germany	Owned	Y	N	Y	NA	83.33 (10/12)
(Christie et al., 1976)	USA	Owned	Y	N	Y	NA	1 (10/1000)
(Dubey et al., 1977)	USA	Owned	Y	N	Y	NA	0.70 (7/1000)
(Ladiges et al., 1982)	USA	Owned	Y	N	N	NA	16.09 (14/87)
(Fujinami et al., 1983)	Japan	Owned	Y	N	N	NA	0 (0/171)
(Svoboda & Svobodová, 1985)	Czech Republic	Owned	Y	N	Y	NA	75 (3/4)
(Gethings et al., 1987)	UK	Owned	Y	N	N	NA	0 (0/51)
(Svoboda et al., 1988)	Germany	Owned	Y	N	N	NA	1.29 (8/620)
(Knaus & Fehler, 1989)	Germany	Owned	Y	N	N	NA	1.12 (3/267)
(Dubey et al., 1990)	USA	Owned	Y	N	N	NA	100 (1/1)
(D.-S. Lin et al., 1990)	Taiwan	Owned	Y	N	N	NA	0 (0/96)
(Umeche, 1990)	Nigeria	Owned	Y	N	N	NA	0 (0/52)
(MacKnight & Robinson, 1992)	USA	Owned	Y	N	N	NA	6.54 (7/107)

(Beelitz et al., 1992)	Germany	Owned	Y	N	N	NA	17.1 (NA)
(Venturini et al., 1992)	Argentina	Owned	Y	N	Y	NA	2 (1/50)
(Dubey & Carpenter, 1993)	USA	Owned	Y	N	N	NA	26.67 (4/15)
(Epe et al., 1993)	Germany	Owned	Y	N	N	NA	0.61 (7/1147)
(Chong et al., 1993)	Singapore	Owned	Y	N	N	NA	0 (0/722)
(Edelhofer & Aspöck, 1996)	Austria	Owned	Y	N	N	NA	1.97 (27/1368)
(Svobodová et al., 1998)	Czech Republic	Owned	Y	N	Y	NA	0 (0/390)
(S. L. Hill et al., 2000)	USA	Owned	Y	N	N	NA	0 (0/206)
(Spain et al., 2001)	USA	Owned	Y	N	N	NA	1.14 (3/263)
(Rembiesa & Richardson, 2003)	USA	Owned	Y	N	N	NA	0.67 (3/450)
(Barutzki & Schaper, 2003)	Germany	Owned	Y	N	N	NA	0.89 (22/2473)
(Epe et al., 2004)	Germany	Owned	Y	N	N	NA	0.68 (3/441)
(López D et al., 2006)	Chile	Owned	Y	N	N	NA	4.35 (10/230)
(Schares et al., 2008)	Germany, France, etc.	Owned	Y	Y	N	ToxoDB #1	0.11 (26/24106)
(Herrmann et al., 2010)	Germany	Owned	Y	Y	Y	ToxoDB #1, #2, mixed, atypical	0.25 (46/18259)
(Berger-Schoch et al., 2011)	Switzerland	Owned	Y	Y	N	ToxoDB #1	0.40 (1/252)

(Lucio-Forster & Bowman, 2011)	USA	Owned	Y	N	N	NA	0.83 (11/1322)
(Ferreira et al., 2011)	Portugal	Owned	Y	Y	N	NA	4.55 (1/22)
(Jokelainen et al., 2012)	Finland	Owned	Y	Y	N	ToxoDB #1	0.76 (1/131)
(Frey et al., 2012)	Switzerland	Owned	Y	Y	N	ToxoDB #1	0.40 (1/252)
(Dubey & Prowell, 2013)	USA	Owned	Y	N	Y	ToxoDB #4	100 (1/1)
(Deksne et al., 2013)	Latvia	Owned	Y	N	N	NA	2.50 (2/80)
(Bastos et al., 2014)	Brazil	Owned	Y	N	N	NA	0 (0/54)
(Paris et al., 2014)	UK	Owned	Y	Y	N	NA	1.01 (11/1088)
(Esteves et al., 2014)	Portugal	Owned	N	Y	N	NA	35.56 (16/45)
(Mancianti et al., 2015)	Italy	Owned	Y	Y	N	ToxoDB #1, #2, #10	10.27 (15/146)
(Schares et al., 2016)	Germany	Owned	Y	Y	N	NA	0.14 (84/61224)
(Liang et al., 2016)	China	Owned	Y	N	Y	ToxoDB #1	4.35 (5/115)
(Veronesi et al., 2017)	Italy	Owned	Y	Y	N	NA	20.51 (16/78)
(YASA et al., 2017)	Turkey	Owned	Y	Y	N	NA	0 (0/102)
(Kostopoulou et al., 2017)	Greece	Owned	Y	N	N	NA	0.38 (1/264)
(Santoro et al., 2017)	Italy	Owned	N	Y	N	NA	16.88 (13/77)
(Nyambura Njuguna et al., 2017)	Kenya	Owned	Y	N	Y	NA	7.77 (8/103)

(Raue et al., 2017)	Germany	Owned	Y	N	N	NA	0.66 (6/903)
(Salman et al., 2018)	Japan	Owned	Y	N	N	NA	0.28 (1/351)
(Nabi et al., 2018)	Pakistan	Owned	Y	Y	N	NA	2.34 (11/470)
(Zottler et al., 2019)	Switzerland	Owned	Y	N	N	NA	0.60 (4/664)
(Nagamori et al., 2020)	USA	Owned	Y	N	N	NA	0.04 (1/2586)
(Dubey, Navarro, et al., 2004)	Brazil	Owned	Y	Y	N	ToxoDB #6, #8, #11, #14, #19, #21, #42, #47, #80, #104, #119, #120, #126	61.11 (33/54)
(Dubey et al., 1977)	Germany	Owned	Y	N	Y	NA	1.00 (5/502)
(Dabritz et al., 2007)	USA	Owned	Y	N	N	NA	0.92 (3/326)
(Bawm et al., 2020)	Myanmar	Owned	Y	Y	N	NA	20 (6/30)
(Izadi et al., 2020)	Iran	Owned	Y	Y	N	NA	22.50 (18/80)
(Wójcik-Fatla et al., 2020)	Poland	Owned	N	Y	N	NA	0 (0/65)
(Tan et al., 2020)	Malaysia	Owned	N	Y	N	NA	10.45 (21/201)
(Pampiglione et al., 1973)	Italy	Free-Ranging/Unowned	Y	N	Y	NA	0.40 (1/250)
(Ito et al., 1974)	Japan	Free-Ranging/Unowned	Y	N	N	NA	0.90 (4/446)

(Rifaat et al., 1976)	Egypt	Free-Ranging/Unowned	Y	N	Y	NA	24.24 (8/33)
(Zástěra et al., 1977)	Czech Republic	Free-Ranging/Unowned	Y	N	Y	NA	4.40 (4/91)
(Roudná, 1979)	Czech Republic	Free-Ranging/Unowned	Y	N	Y	NA	0.50 (1/202)
(Deeb et al., 1985)	Lebanon	Free-Ranging/Unowned	Y	N	N	NA	9.90 (31/313)
(Rothe et al., 1985)	Australia	Free-Ranging/Unowned	Y	N	Y	NA	0 (0/115)
(Childs & Seegar, 1986)	USA	Free-Ranging/Unowned	Y	N	N	NA	0.46 (3/650)
(Dubey et al., 1986)	USA	Free-Ranging/Unowned	Y	N	N	NA	6.25 (1/16)
(Svobodová & Svoboda, 1986)	Czech Republic	Free-Ranging/Unowned	Y	N	N	NA	1.29 (8/620)
(Oikawa et al., 1990)	Japan	Free-Ranging/Unowned	Y	N	Y	NA	0.30 (1/335)
(Langham & Charleston, 1990)	New Zealand	Free-Ranging/Unowned	Y	N	N	NA	0 (0/63)
(Guevara Collazo et al., 1990)	Mexico	Free-Ranging/Unowned	Y	N	N	NA	0 (0/200)
(Vanparijs et al., 1991)	Belgium	Free-Ranging/Unowned	Y	N	N	NA	0 (0/30)
(Shastri & Ratnaparkhi, 1992)	India	Free-Ranging/Unowned	Y	N	Y	NA	11.11 (1/9)

(Frenkel et al., 1995)	Panama	Free-Ranging/Unowned	Y	N	Y	NA	0.52 (2/383)
(Dubey, Weigel, et al., 1995)	USA	Free-Ranging/Unowned	Y	N	Y	NA	1.82 (5/274)
(de Aluja & Aguilar, 1977)	Mexico	Free-Ranging/Unowned	Y	N	Y	NA	7 (14/200)
(Montoya-Londoño et al., 1998)	Colombia	Free-Ranging/Unowned	Y	N	N	NA	66.67 (12/18)
(Mateus-Pinilla et al., 1999)	USA	Free-Ranging/Unowned	Y	N	N	NA	6.15 (11/179)
(Miró et al., 2004)	Spain	Free-Ranging/Unowned	Y	N	N	NA	0 (0/382)
(Robben et al., 2004)	Netherlands	Free-Ranging/Unowned	Y	N	N	NA	0.33 (1/305)
(Dubey, Gomez-Marin, et al., 2005)	Colombia	Free Ranging	Y	N	Y	NA	0 (1/143)
(Pena et al., 2006)	Brazil	Free-Ranging/Unowned	Y	N	Y	ToxoDB #2, #10	1.27 (3/237)
(Dubey, Su, et al., 2006)	Colombia	Free-Ranging/Unowned	Y	N	Y	NA	0 (0/170)
(Afonso et al., 2006)	France	Free-Ranging/Unowned	Y	N	N	NA	0 (0/322)
(Dubey, López-Torres, Sundar, et al., 2007)	USA	Free-Ranging/Unowned	Y	N	Y	NA	0 (0/6)
(Dubey, Zhu, Sundar, et al., 2007)	China	Free-Ranging/Unowned	Y	N	Y	ToxoDB #9, #11, #13, #18	0 (0/26)

(Salant et al., 2007)	Israel	Free-Ranging/Unowned	Y	Y	N	NA	9.02 (11/122)
(Hooshyar et al., 2007)	Iran	Free-Ranging/Unowned	Y	N	N	NA	0 (0/100)
(Karatepe et al., 2008)	Turkey	Free-Ranging/Unowned	Y	N	N	NA	0 (0/72)
(Dubey, Moura, et al., 2009)	Saint Kitts	Free-Ranging/Unowned	Y	N	Y	NA	0 (0/51)
(Sharif et al., 2009)	Iran	Free-Ranging/Unowned	Y	N	N	NA	0.00 (0/100)
(Mancianti et al., 2010)	Italy	Free-Ranging/Unowned	N	Y	N	NA	16 (8/50)
(Khalafalla, 2011)	Egypt	Free-Ranging/Unowned	Y	N	N	NA	8.85 (10/113)
(Stojanovic & Foley, 2011)	Canada	Free-Ranging/Unowned	Y	N	N	NA	1.28 (1/78)
(Becker et al., 2012)	Germany	Free-Ranging/Unowned	Y	N	N	NA	0.72 (6/837)
(Lilly & Wortham, 2013)	USA	Free-Ranging/Unowned	Y	Y	N	NA	6.12 (3/49)
(Dubey, Choudhary, et al., 2013)	Ethiopia	Free-Ranging/Unowned	Y	Y	N	ToxoDB #1, #2, #3, #9	14.58 (7/48)
(Spada et al., 2013)	Italy	Free-Ranging/Unowned	Y	N	N	NA	0 (0/139)
(Abdou et al., 2013)	Kuwait	Free-Ranging/Unowned	Y	N	N	NA	2.08 (5/240)

(Dubey, Darrington, et al., 2013)	Ethiopia	Free-Ranging/Unowned	Y	N	Y	ToxoDB #4, #7, #16, #80, #31, #54,	19.44 (7/36)
(Abu-Madi & Behnke, 2014)	Qatar	Free-Ranging/Unowned	Y	N	N	NA	9.14 (425/4652)
(Bajalan et al., 2016)	Iran	Free-Ranging/Unowned	Y	N	N	NA	0 (0/125)
(Jung et al., 2015)	Korea	Free-Ranging/Unowned	N	Y	Y	ToxoDB #1	4.67 (14/300)
(Y. Yang et al., 2015)	China	Free-Ranging/Unowned	Y	N	Y	ToxoDB #1	0.28 (1/360)
(Y. Yang & Liang, 2015)	China	Free-Ranging/Unowned	Y	N	N	NA	3.06 (11/360)
(Qian et al., 2012)	China	Free-Ranging/Unowned	Y	N	N	NA	0 (0/35)
(Ould Ahmed Salem et al., 2017)	Mauritania	Free-Ranging/Unowned	Y	N	N	NA	23 (23/100)
(Tavalla et al., 2017)	Iran	Free-Ranging/Unowned	Y	Y	N	ToxoDB #2, #10, I+III	7.20 (35/486)
(Poulle et al., 2017)	France	Free-Ranging/Unowned	N	Y	N	NA	1.6 (2/125)
(Grandi et al., 2017)	Sweden	Free-Ranging/Unowned	Y	N	N	NA	0.49 (1/205)
(Chemoh et al., 2018)	Thailand	Free-Ranging/Unowned	Y	Y	N	I/III, recom atypical	NA
(Taetzsch et al., 2018)	USA	Free-Ranging/Unowned	Y	N	N	NA	1.09 (3/275)

(Bastien et al., 2018)	France	Free-Ranging/Unowned	Y	N	N	NA	4.26 (6/141)
(Montoya et al., 2018)	Spain	Free-Ranging/Unowned	Y	N	N	NA	0 (0/263)
(Sroka et al., 2018)	Poland	Free-Ranging/Unowned	Y	Y	N	NA	2.44 (1/41)
(Nagamori et al., 2018)	USA	Free-Ranging/Unowned	Y	N	N	NA	0.12 (1/846)
(Khodaverdi & Razmi, 2019)	Iran	Free-Ranging/Unowned	Y	N	N	NA	2.52 (4/159)
(Ahn et al., 2019)	Korea	Free-Ranging/Unowned	Y	Y	N	NA	0.89 (5/563)
(Raeghi et al., 2011)	Iran	Free-Ranging/Unowned	Y	N	N	NA	2.31 (3/130)
(Wallace, 1973b)	USA	Free-Ranging/Unowned	Y	N	Y	NA	100 (1/1)
(Wallace, 1973a)	USA	Free-Ranging/Unowned	Y	N	Y	NA	0.75 (12/1604)
(Wallace, 1975)	USA	Free-Ranging/Unowned	Y	N	Y	NA	100 (1/1)
(Ruiz & Frenkel, 1980)	Costa Rica	Free-Ranging/Unowned	Y	N	Y	NA	23.21 (55/237)
(Awobode et al., 2020)	Nigeria	Free-Ranging/Unowned	Y	N	N	NA	21.43 (3/14)
(Blaizot et al., 2020)	French Guiana	Free-Ranging/Unowned	Y	N	N	NA	0 (0/12)

(Davis et al., 2018)	USA	Free-Ranging/Unowned	N	Y	N	NA	7.25 (5/69)
(Lepczyk et al., 2020)	USA	Free-Ranging/Unowned	N	Y	N	NA	10.71 (6/56)
(Chi et al., 2021)	Saint Kitts	Free-Ranging/Unowned	Y	Y	N	NA	0 (0/105)

†Study types: refer to definitions in Table 1. Experimental studies involve infection to induce oocyst shedding in a controlled, laboratory environment. Owned refers to studies of shedding in owned domestic cats. Free-ranging unowned refers to studies of unowned domestic cats that live outdoors exclusively.

‡The data were not reported or calculated, i.e. not genotyped, or not available in the case of raw oocyst shedding prevalence.

§Oocyst shedding prevalence includes both *T. gondii* and *T. gondii*-like oocysts as reported by each paper. Refer to the columns that detail methods of verification (floatation and microscopy, PCR, bioassay) to determine whether the oocyst shedding prevalence refers to *T. gondii* confirmed oocysts.

Supplemental Table 1.3 Oocyst shedding prevalence in captive and free-ranging wild felid species

Species	Study	Study type	Country	Microscopy†	PCR	Bioassay	Oocyst shedding prevalence
Lynx rufus, Felis yagouaroundi, Felis pardalis	(Frenkel, 1970)	Captive	USA	Y	N	N	NA‡
Felis bengalensis	(Janitschke & Werner, 1972)	Captive	Germany	Y	N	N	NA
Felis yagouaroundi, Felis pardalis	(Jewell et al., 1972)	Captive	Panama, Costa Rica	Y	N	Y	26.7% (4/15)
Lynx rufus, Acinonyx jubatus, Puma concolor	(Marchiondo et al., 1976)	Captive	USA	Y	N	Y	87.5% (7/8)
Oncifelis geoffroyi, Felis colocolo, Felis yagouaroundi	(Pizzi et al., 1978)	Captive	Argentina	Y	N	N	NA
Felis lybica, Felis manul, Panthera leo, Acinonyx jubatus	(Polomoshnov, 1979)	Captive	Russia	Y	N	Y	NA
Felis iriomotensis	(Akuzawa et al., 1987)	Captive	Japan	Y	N	Y	NA
Felis manul	(Dubey et al., 1988)	Captive	USA	Y	N	N	100% (1/1)
Panthera tigris	(Dorny & Fransen, 1989)	Captive	Belgium	Y	N	Y	NA
Panthera leo	(Ocholi et al., 1989)	Captive	Nigeria	Y	N	N	100% (2/2)

Felis silvestris, Felis euptilurus, Oncifelis geoffroyi	(Lukesˇová & Literák, 1998)	Captive	Czech Republic	Y	N	Y	1.0% (23/2287)
Felis manul	(Basso et al., 2005)	Captive	Austria	Y	N	Y	28.6% (2/7)
Acinonyx jubatus	(Lloyd & Stidworthy, 2007)	Captive	UAE	Y	N	N	0% (0/1)
Panthera leo	(Mukarati et al., 2013)	Captive	Zimbabwe	Y	N	N	16.7% (5/30)
Puma concolor	(J. P. Dubey, Alvarado-Esquivel, et al., 2013)	Captive	Mexico	Y	N	N	100% (1/1)
Felis yagouarundi , Puma concolor, Leopardus tigrinus, Leopardus pardalis, Caracal caracal, Lynx rufus, Panthera onca, Panthera pardus, Panthera tigris, Panthera leo	(Gomez-Rios et al., 2019)	Captive	Mexico	Y	Y	N	14.3% (5/35)
Panthera tigris	(Y. Yang et al., 2019)	Captive	China	Y	N	Y	0% (0/151)
Felis catus, Lynx rufus, Felis bengalensis, Puma concolor	(Miller et al., 1972)	Captive, Domestic felid	USA	Y	N	Y	NA

Felis catus, Acinonyx jubatus, Caracal caracal, Neofelis nebulosa, Otocolobus manul, Panthera leo, Panthera onca, Panthera pardus orientalis, Panthera pardus saxicolor, Panthera tigris altaica, Prionailurus viverrinus, Puma concolor, Puma concolor stanleyana, Uncia uncia	(Camps et al., 2008)	Captive, Domestic felid	USA	Y	N	N	0% (0/66)
Felis pardalis	(Patton et al., 1986)	Free-ranging	Belize	Y	N	N	22.2% (10/45)
Panthera pardus, Panthera tigris, Felis bengalensi, Neofelis nebulosa, Felis temminicki	(Patton & Rabinowitz, 1994)	Free-ranging	Thailand	Y	N	N	2.2% (2/92)
Puma concolor	(Aramini et al., 1998)	Free-ranging	Canada	Y	N	Y	15.4% (2/13)
Puma concolor	(Aramini et al., 1999)	Free-ranging	Canada	Y	N	Y	6.3% (1/16)

Felis pardalis, Oncifelis geoffroyi, Felis yagouaroundi	(Fiorello et al., 2006)	Free-ranging	Bolivia	Y	N	N	0% (0/14)
Lynx lynx	(Ryser-Degiorgis et al., 2006)	Free-ranging	Sweden	Y	N	N	0% (0/207)
Panthera leo	(Smith & Kok, 2006)	Free-ranging	Namibia	Y	N	N	47.8% (11/23)
Lynx pardinus	(García-Bocanegra et al., 2010)	Free-ranging	Spain	Y	N	N	0% (0/58)
Lynx rufus	(Carver et al., 2012)	Free-ranging	USA	Y	Y	N	0% (0/121)
Lynx lynx	(Jokelainen et al., 2013)	Free-ranging	Finland	Y	N	N	0% (0/332)
Lynx canadensis	(Simon et al., 2013)	Free-ranging	Canada	Y	N	N	0% (0/84)
Felis catus, Lynx rufus, Puma concolor	(VanWormer et al., 2013)	Free-ranging	USA	Y	N	N	0.5% (2/435)
Felis catus, Lynx rufus	(Verma et al., 2016)	Free-ranging	USA	Y	N	N	0% (0/50)
Lynx rufus	(Verma et al., 2017)	Free-ranging	USA	Y	N	Y	0% (0/35)

†We included ‘Toxoplasma-like' reports as microscopy-based studies cannot discriminate between *T. gondii* and closely related apicomplexan parasites, especially *Hammondia spp*.

‡NA refers to studies where oocyst shedding was searched for but actual prevalence was not obtained due to inability to access raw data from the original manuscript.

Chapter 2 High prevalence and diversity of *Toxoplasma gondii* DNA in feral cat feces from coastal California

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Abstract

Toxoplasma gondii is a zoonotic parasite that can cause severe morbidity and mortality in warm-blooded animals, including marine mammals such as sea otters. Free-ranging cats can shed environmentally resistant *T. gondii* oocysts in their feces, which are transported through rain-driven runoff from land to sea. Despite their large population sizes and ability to contribute to environmental oocyst contamination, there are limited studies on *T. gondii* oocyst shedding by free-ranging cats. We aimed to determine the frequency and genotypes of *T. gondii* oocysts shed by free-ranging domestic cats in central coastal California and evaluate whether genotypes shed in feces are similar to those identified in sea otters that died from fatal toxoplasmosis. We utilized a longitudinal field study of four free-ranging cat colonies to assess oocyst shedding prevalence using microscopy and molecular confirmation with polymerase chain reaction (PCR). *T. gondii* DNA was confirmed with primers targeting the ITS1 locus and positive samples were genotyped at the B1 locus. While oocysts were not visualized using microscopy (0/404), we detected *T. gondii* DNA in 25.9% (94/362) of fecal samples. We genotyped 27 samples at the B1 locus and characterized 13 samples at one or more loci using multi locus sequence typing (MLST). Parasite DNA detection was significantly higher during the wet season (16.3%, 59/362) compared to the dry

season (9.7%; 35/362), suggesting seasonal variation in *T. gondii* presence in feces. High diversity of *T. gondii* strains was characterized at the B1 locus, including atypical strains previously associated with sea otter mortalities. Free-ranging cats may thus play an important role in the transmission of virulent *T. gondii* genotypes that cause morbidity and mortality in marine wildlife. Management of free-ranging cat colonies could reduce environmental contamination with oocysts and subsequent *T. gondii* infection in endangered marine mammals and people.

Introduction

Toxoplasma gondii is a zoonotic apicomplexan parasite that infects warm-blooded vertebrates and causes morbidity and mortality in many species, including people and marine mammals. *T. gondii* has three primary life stages: tachyzoites, which propagate infection within the host; bradyzoites, which reside in tissue cysts during chronic infections; and sporozoites contained within oocysts, the environmental stage of the parasite [1]. *T. gondii* can only sexually reproduce in the intestine of its definitive hosts, domestic and wild felids, which are the only animals that shed oocysts in their feces. Oocysts are environmentally resistant and, when sporulated, can withstand extended exposure to high and low temperatures and UV from sunlight [2,3]. Surface runoff can mobilize *T. gondii* oocysts from soil into oceans or other bodies of water [4]. Waterborne oocysts can remain in the water column or may bioaccumulate in invertebrates such as mussels, snails, and oysters [5–7]. Marine mammals can directly or indirectly ingest oocysts from one of these pathways and become infected with *T. gondii*, which may lead to severe disease or even death [8].

In the 1990s, researchers discovered high numbers of fatal toxoplasmosis cases in the endangered southern sea otter (*Enhydra lutris nereis*) in California [8], and subsequent studies found that diet choice [9] and living near freshwater runoff [4,10] are risk factors for *T. gondii* infection. In parallel, other studies identified specific strains associated with severe disease and mortality in sea otters [11]. Despite this rich body of research on *T. gondii* infection in otters, critical information about the terrestrial origins of virulent *T. gondii* genotypes is lacking. Even though both domestic and wild felids can carry and shed *T. gondii*, domestic cats may contribute more to environmental oocyst load due to their large population

sizes [12]. Unfortunately, there are limited studies of oocyst shedding in free-ranging cats, none over a long period, and few that report the genotypes of *T. gondii* detected in feces. Since oocysts drive *T. gondii* infections [13], documenting shedding patterns in a natural setting is crucial to understanding oocyst exposure risk over time.

Genotype is an important factor that can impact the outcome of infection in sea otters. Atypical genotypes, namely Type X (ToxoDB #5) or X variants, were previously detected in otters that died from toxoplasmosis as a primary cause of death and from tissues of terrestrial domestic and wild felids [14]. Though the land-to-sea transmission of *T. gondii* is firmly established, the degree to which feral outdoor cats shed this genotype is unknown. While atypical *T. gondii* genotypes are found in domestic cats and wild felids [15–17], most molecular studies have focused on characterizing parasite DNA from the tissues of felids (bradyzoite cysts) – rather than oocysts from feces. Mixed *T. gondii* infection in cats (infection with multiple *T. gondii* genotypes) could result in different genotypes present in tissue relative to genotypes of oocysts shed in feces. Mixed infections with different genotypes have been identified in wild and domestic felids [18,19], and co-infection may be a common occurrence if these hosts encounter multiple *T. gondii* genotypes from the environment and infected prey throughout their lifetime. Detection of atypical genotypes of *T. gondii* from free-ranging domestic cat feces would further enhance understanding of the role these hosts play in land-to-sea transmission of virulent strains of *T. gondii* to marine environments.

Our objectives were to 1) determine the presence and prevalence of *T. gondii* oocyst shedding in free-ranging cat feces over time; and 2) characterize genotypes of *T. gondii* in free-ranging cat feces. We predicted that free-ranging cats shed *T. gondii* oocysts and that shedding prevalence would be higher in the wet versus dry season. We also predicted that atypical genotypes of *T. gondii* previously isolated from dead sea otters would be detected in free-ranging cat feces. Identifying the most relevant populations of cats that contribute virulent genotypes and when these oocysts enter the environment will allow for targeted management efforts to protect susceptible wildlife and human health.

Methods

Site identification and fecal collection

We identified four free-ranging cat colonies within watersheds bordering Monterey Bay coastal waters based on data from a previous study [20] and personal correspondence with animal control personnel from organizations near Santa Cruz and Monterey, CA. The four colonies were in Gilroy (Santa Clara County), Moss Landing (Monterey County), Aptos (Santa Cruz County), and Santa Cruz (Santa Cruz County). Three sites were located directly on the coastline, and one (Gilroy) was located inland (25.4 km from coast) in a watershed which drains directly into Monterey Bay. Monthly sampling was conducted every four to five weeks between July 2020 and August 2022 (24 months). Sampling did not occur in February or March 2021 due to COVID-19 restrictions, and sampling occurred twice in April 2022 due to the low sample size (n = 5) during the first collection trip earlier that month. We collected samples identified as domestic cat feces in plastic collection cups, transported them in a cooler with cold packs to UC Davis, and refrigerated them until processing the following day. Feces was identified as being from domestic cats based on visual presence of cats in the area, proximity to any feeding sites, and size and shape of the sample.

Sample size calculation

We determined the sample size for detection of disease (*T. gondii* oocyst shedding) in a large population, assuming test sensitivity of 90% and test specificity of 100%. The ability for *T. gondii* -like oocysts to be confirmed as *T. gondii* using PCR-based analysis and sequencing supports the assumed test specificity of 100%. Although the test sensitivity for detecting *T. gondii* -like oocysts using flotation and microscopy has not been reported, we detected oocyst shedding in experimentally and naturally infected domestic cats in previous studies with this approach [12,21]. We chose a conservative estimate of test sensitivity of 90%. In previous cross-sectional studies, ~2% of free-ranging domestic cats were shedding *T. gondii* -like oocysts at a given time [12]. Only half of those oocysts were confirmed as *T. gondii* using PCR, giving a conservative estimated shedding prevalence of 1%. Using power size calculations, a minimum of 332 fecal samples would be necessary to detect a shedding prevalence of 1% with the desired probability of 0.95 [22].

Fecal flotation

We used double centrifugation flotation to process fecal samples and examine specimens for *T. gondii* oocysts using both brightfield and UV epifluorescence at 100 X magnification. Any *T. gondii* -like oocysts were re-examined and measured at 200 X magnification. Two individuals screened each slide to improve the sensitivity of our microscopic examination, including diagnostic parasitologists with extensive experience identifying parasite ova (L. Camp or K. Shapiro). After microscopic examination, the cover slip was removed and rinsed into a clean 50 mL falcon tube and centrifuged to obtain a 100-500 µl pellet for nucleic acid extraction and PCR. Cover slips were not retained for all samples during the first three months of sampling (July - September 2020) due to lack of *T. gondii* oocyst visualization, however we began screening all samples with PCR from October 2020 onward as an amendment to our initial methodology. Flotations were performed with zinc sulfate solution (specific gravity (SG) 1.18) for the first six months of sampling (August 2020-January 2021) and subsequently (February 2021-August 2022) using a sucrose solution (SG 1.18) because crystal formation of zinc sulfate hindered screening when multiple personnel examined slides over time. All parasite ova were recorded when seen and categorized according to size and morphology (i.e. *T. gondii*-like, *Cystoisospora* spp.-like, *Toxocara* spp.-like).

DNA extraction and PCR

We centrifuged pellets obtained from washing fecal flotation coverslips at 2000 x g for 10 minutes and processed 100 µl for nucleic acid extraction according to the manufacturer's instructions for the DNeasy Blood and Tissue Kit (QIAGEN). Additionally, we performed a 4-minute freeze (liquid nitrogen -196°C), and a 4-minute thaw (boiling water, 100°C) cycle to rupture oocyst walls before adding proteinase K [23]. For initial screening of samples for apicomplexan parasite DNA, we used a conventional nested PCR targeting the internal transcribed spacer 1 (ITS-1) of the small subunit ribosomal RNA (SSU rRNA) [24]. The PCR reaction consisted of 18.6 µl (internal) or 21.6 µl (external) of DNase- and RNase-free distilled water, 0.5 µl each of 50 M forward and reverse primers, 0.8 µl of 10% Bovine Serum Albumin, 25 µl of 2X Amplitaq Gold 360MM, and 5 µl (external) or 2 µl (internal) of DNA template. Each PCR run contained one positive control (*Sarcocystis neurona*) and three negative controls (extraction reagents with

distilled water, PCR reagent control, and another PCR reagent control with distilled water added in the nested reaction step). PCR amplification products were separated and visualized with gel electrophoresis on a 2% agarose gel stained with Red Safe and viewed with a UV transilluminator. Samples that yielded no DNA amplification were run twice more for a total of triplicate attempts to increase the sensitivity of *T. gondii* DNA detection.

Since detecting *T. gondii* DNA is not indicative of parasite stage, and because previous studies have shown that bradyzoite DNA from infected prey can be detected from cat feces [25], we attempted to differentiate oocyst mRNA vs bradyzoite DNA using RT-qPCR targeting SporoSAG (a surface antigen glycoprotein found on the surface of sporozoites, the parasite stage contained within *T. gondii* oocysts) [26]. Starting in March 2022, we tested fecal samples that were *T. gondii* confirmed by extracting RNA and performed RT-qPCR in an attempt to detect sporozoite specific RNA.

Sequence analysis and* T. gondii *genotyping

We purified PCR products from amplified samples using the QIAquick Gel Extraction Kit (QIAGEN) and submitted them for sequencing at the UC Davis [UC]DNA Sequencing Facility. Using Geneious software (® R11 Biomatters Ltd., Auckland, New Zealand), we aligned the forward and reverse sequences and compared the consensus sequence with the GenBank database using the Basic Local Alignment Search Tool, BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and well characterized reference genotypes (Types I (RH strain), II (ME49 strain), III (CTG strain), and X (Bobcat strain)). To characterize *T. gondii* genotypes, we processed samples confirmed as *T. gondii* at the ITS-1 locus with PCR targeting the polymorphic B1 gene following previously published protocols [19]. We further performed multi-locus sequence typing (MLST) at 12 polymorphic loci on samples that were amplified at the B1 locus using previously published protocols and re-ran samples that did not amplify in duplicate [27]. Samples that exhibited novel single nucleotide polymorphisms (snps) were re-amplified if sufficient DNA remained and re-sequenced to confirm polymorphic nucleotides. For quality control, PCR reagent controls with and without added PCR-grade water were used with each run, as well as tachyzoite-derived *T. gondii* (RH strain Type I) as a positive control. Parasite genotype was first determined using virtual

restriction fragment length polymorphism (RFLP) at the B1 locus in Geneious. After comparing with reference strains, an RFLP genotype and variant specific B1 type were assigned [27,28].

Statistical analysis

Association between presence of any parasite ova during microscopy and *T. gondii* DNA detection was evaluated using the Chi-square test. We performed statistical analysis in R version 3.6.1 [29] and carried out spatial visualization using the *ggplot2*, *rnaturalearth*, and *ggspatial* packages [30–32]. Due to the small number of spatial clusters (n=4), we analyzed our data with a Bayesian approach to reduce bias in estimating parameter values using the package *brms* [33]. We fit models for *T. gondii* DNA detection in feces with the season of collection, kitten presence, and the number of adult cats as fixed effects, and year and site as random effects using a Bayesian mixed effects generalized linear regression model with a binomial distribution. Effective sample sizes from the posterior distribution ranged from 215 to 1474, and all predictors had R-hat values <1.01, indicating model convergence [33]. We used minimally informative priors for fixed effects (season, kitten presence, number of adults) to avoid overfitting; a normal distribution centered at 0 and a standard deviation of 10. Total monthly rainfall was used to characterize wet and dry seasons based on quantities measured by the University of California Santa Cruz Cooperative Extension between 2020 and 2022. The wet season began in any fall month (October-December) with more than 0.10 inches of rain and extended until the first month when spring rainfall fell below 0.10 inches. The dry season included all months outside of these criteria.

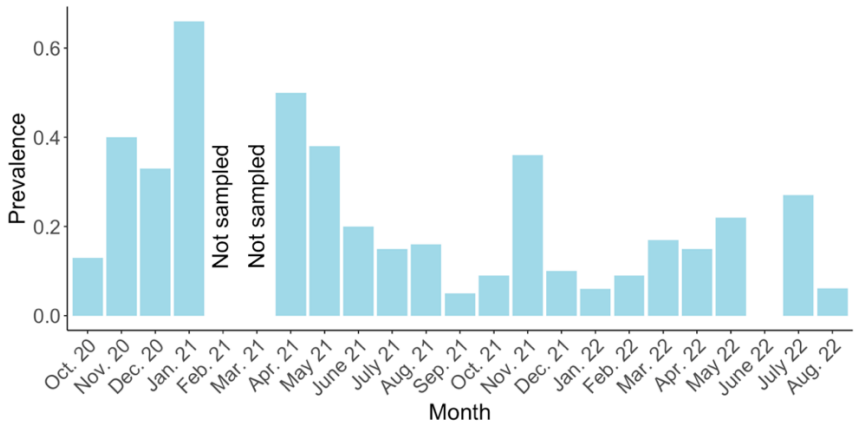

Figure 2.1 Seasonal variation of *T. gondii* DNA detection from feral cat feces (n=362). Parasites were not detected in any samples during June 2020 (0% prevalence).

Results

Over 24 months, we collected 404 fecal samples across four sites; 126 from Gilroy, 152 from Moss Landing, 68 from Aptos, and 58 from Santa Cruz (Table 2.1) [34]. No *T. gondii*-like oocysts were seen using microscopy, while ova of helminths such as *Toxocara cati*, and oocysts of other apicomplexans parasites (*Cystoisospora* spp., and *Sarcocystidae* spp.) were visualized (Supplemental Table 2.2). *T. gondii* DNA was detected in 94 fecal samples and confirmed with 100% identity when compared with publicly available *T. gondii* sequences. There was no association between suspected *Cystoisospora* spp. (p = 1), *Sarcocystidae* spp. (p = 1), or *Toxocara cati* (p = 0.91) visualization during microscopic examination and *T. gondii* DNA detection.

Table 2.1 Microscopy and DNA detection prevalence of *T. gondii* in feces from free-ranging feral cats in central coastal California

Site	*T. gondii*-like oocyst prevalence	Fecal DNA prevalence	Dry season prevalence	Wet season prevalence
Gilroy	0/126 (0%)	36/111 (32.4%)	15/66 (22.7%)	21/45 (46.7%)
Moss Landing	0/152 (0%)	27/139 (19.4%)	11/66 (16.7%)	16/73 (21.9%)
Aptos	0/68 (0%)	17/61 (27.9%)	6/34 (17.6%)	11/27 (40.7%)
Santa Cruz	0/58 (0%)	14/51 (27.5%)	3/27 (11.1%)	11/24 (45.8%)
Total	0/404 (0%)	94/362 (25.9%)	35/193 (18.1%)	59/169 (34.9%)

The overall fecal *T. gondii* DNA prevalence was 25.9% but ranged from a low of 19.4% in Moss Landing to a high of 32.4% in Gilroy (Table 2.1). Differences between DNA detection at sites and year of sampling were unlikely to be due to chance (Table 2.2). The output of the Bayesian generalized linear mixed-effects model showed that prevalence varied seasonally, with 3.42 times higher odds of detection in the wet versus dry season (Figure 2.1, Table 2.2). The presence of kittens was associated with 4.71 times higher odds of *T. gondii* DNA detection as compared to sites without kittens. The number of adult cats at the sampling site had a small association with lower DNA detection (OR=0.77). Spatially, more Type I and Type I variants were detected at the inland site versus coastal sites, which had higher proportions of Type X and X variant, as well as atypical genotypes (Figure 2.2).

Table 2.2 Results of Bayesian generalized linear mixed-effects regression model of *Toxoplasma gondii* DNA detected in feral cat feces (n = 362).

Variable	Odds Ratio (95% Credible Interval)
Random effect	
Site	1.39 (1.01, 3.78)*
Year	1.47 (1.01, 5.05)*
Fixed effects	
Rainy season (wet vs dry)	3.42 (1.97, 5.92)*
Adults (continuous)	0.77 (0.62, 0.96)*
Kitten presence (vs absence)	4.71 (2.03, 10.91)*

*Variables with lower and upper bounds of the 95% credible interval that do not cross 1 indicate significance.

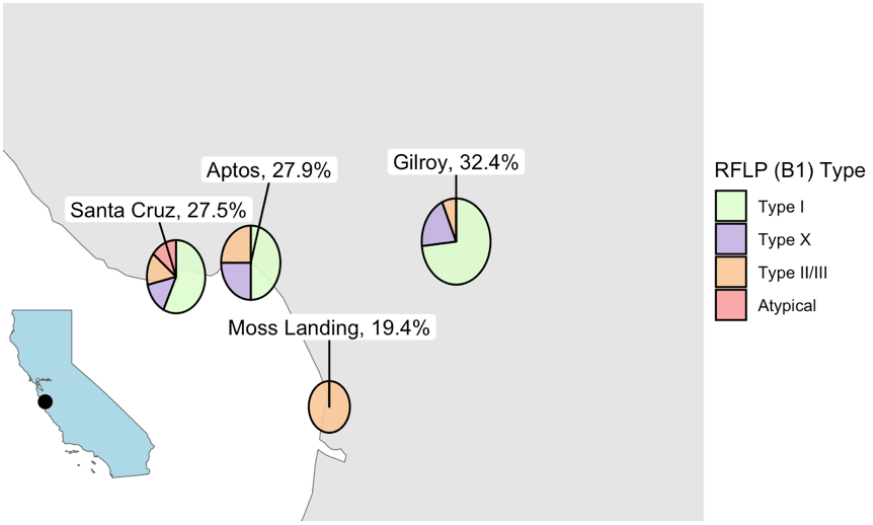

Figure 2.2 Prevalence of fecal samples that tested positive for *T. gondii* DNA in feral cats based in four colonies, and the relative distribution of parasite genotype as characterized via RFLP at the B1 locus. Relative pie chart size is proportional to the overall *T. gondii* DNA prevalence.

Of the 94 samples that tested positive for *T. gondii* at the ITS-1 locus, 27 were successfully amplified and genotyped at the B1 locus (28.7%), and 13 at one or more MLST loci (13.8%, Supplemental Table 2.1). No samples were amplified using RT-qPCR targeting SporoSAG mRNA. Of the 27 samples that amplified at the B1 locus, the majority of samples had alleles consistent with the Type I genotype (59%), followed by Type II/III (18.5%), Type X (14.8%), and two separate atypical cleaving patterns via RFLP (Atypical 1, 3.7%; Atypical 2, 3.7%) (Figure 2.3a). Using a variant specific typing

approach to characterize the B1 locus, these samples were further categorized into eight variant strains (Fig 2.3b) based on additional snps present in nucleotide sites not targeted by restriction enzymes [14]. Nine samples had novel snps and were categorized as variant strains according to B1, and a subset of these also had mixed base pairs at the B1 loci (as indicated by UPAC ambiguity codes S, Y, R, or M in Table 2.3). Five MLST samples had unique snps at either the SAG1, SAG3, or L358 locus (Supplemental Table 2.1). Only one sample (151) had confirmed novel snps at the L358 locus after re-sequencing.

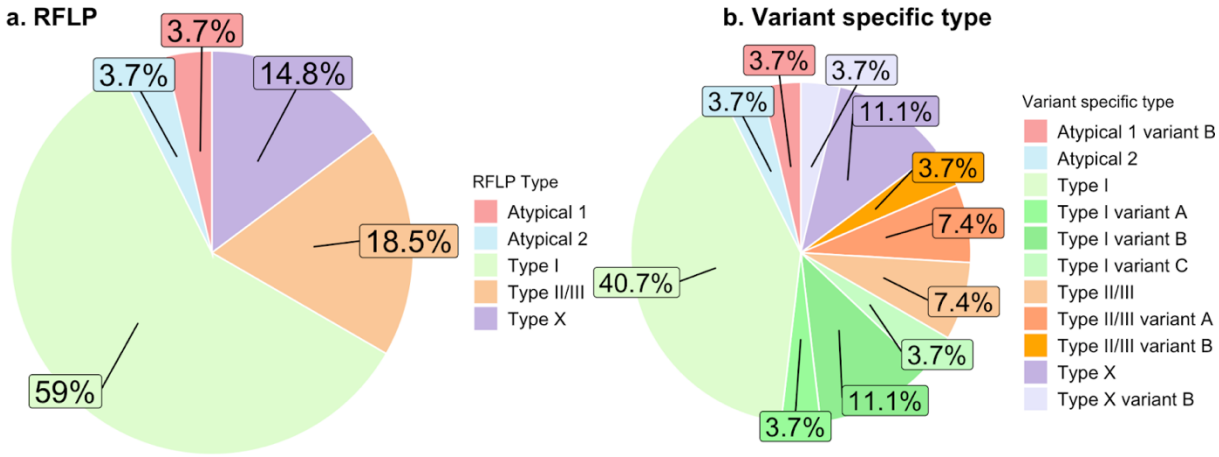

Figure 2.3a. Distribution of *T. gondii* genotypes detected in feral cat feces (n=27) as determined via RFLP at the B1 locus. 2.3b. Variant-specific MLST distribution at the B1 locus of *T. gondii* strains isolated from feral cat feces (n=27).

Discussion

We present evidence of a high prevalence (25.9%) of *T. gondii* DNA in feces from feral cats along the central California coastline near southern sea otter habitat. Lack of oocyst visualization in fecal samples that contain DNA from diverse genotypes of *T. gondii* is puzzling and may be due to several factors as discussed below. Notably, we detected atypical *T. gondii* genotypes from cat feces, showing that these genotypes are circulating within feral cat populations that can contribute oocysts to watersheds draining runoff to sea otter habitat. Our results support previous studies highlighting the importance of free-ranging cats in coastal California as a source of *T. gondii* transmission to susceptible hosts (including people) in terrestrial, coastal, and marine environments [10, 12, 20].

Table 2.3 Single nucleotide polymorphisms at the B1 locus from *T. gondii* detected in feral domestic cat feces in central coastal California. IUPAC codes designate mixed nucleotide bases (S=C or G, Y= C or T, R=A or G, and M=A or C). A slash (/) indicates that different nucleotides were identified on different sequencing attempts and therefore no definitive base(s) could be reported at that position for some samples.

Reference strains (Genbank accession no.)	**256**	**330**	**360**	**366* Xho**	**368* Xho**	**428**	**486**	**495**	**504*PmII**	**513**	**533**	**545**	**609**	**RFLP Type**	**MLST Type**
RH (AF179871)	A	T	C	T	C	T	T	G	G	T	A	C	A	I	I
ME49 (L49390.1)	A	T	C	T	C	T	T	G	S	T	A	C	A	II/III	II/III
CTG (AY143139.1)	A	T	C	T	C	T	T	G	S	T	A	C	A	II/III	II/III
Type X (bobcat, KM243024)	A	T	C	T	C	T	T	G	C	T	A	C	A	X	X
Type X variant (sea otter, MK988572)	A	T	S	T	C	T	T	G	C	T	A	C	A	X	X variant A
SR215 (KM243023)	A	T	C	Y	C	T	T	G	S	T	A	C	A	II/III	II/III
CR34 (KM243022)	A	T	C	C	C	T	T	G	G	T	C	C	A	Atypical 1	Atypical 1 Variant A
Fecal sample ID															
76, 111, 118, 119, 150, 165, 253, 287, 349, 351, 389	A	T	C	T	C	T	T	G	G	T	A	C	A	I	I
77** (OQ850747)	A	T	C	T	C	T	T	G	G	T	A	C	G	I	I variant A
87, 129	A	T	C	T	C	T	T	G	S	T	A	C	A	II/III	II/III
92, 113, 354	A	T	C	T	C	T	T	G	C	T	A	C	A	X	X
128** (OQ850748)	A	T	C	T	T	T	T	G	C	T	A	C	A	X	X var B
151	A	T	C	T	C	T	T	G	S/G	T/Y	A	C	R/G	II/III	II/III variant A
155^	A	T	C	T	C	T	T	G	S	T	A	C	R	II/III	II/III variant A
159** (OQ850749)	A	T	C	T	C	T	T	G	G	T	A	C	R	I	I variant B
161	A	T	C	T	C	T	T	G	G	T	A	C	R	I	I variant B
163	A	T	C	T/Y	C	T	T	G	G	T	A/M	C	R/G	I	I variant B
188** (OQ850750)	R	Y	C	T	C	Y	Y	R	G	T	A	C	G	I	I variant C
204** (OQ850751)	A	T	C	Y	C	T	T	G	S	T	M	C	A	II/III	II/III variant B
279	A	T	C	C	C	T	T	G	G	T	C	C/Y	A	Atypical 1	Atypical 1 variant B
296	A	T	C	C	C	T	T	G	C	T	A	C	A	Atypical 2	Atypical 2

*Restriction enzyme cleaving site used for RFLP genotype designation.

^Re-amplification not attempted due to the lack of extracted DNA.

**Unique/new variants that were successfully sequenced at least twice with confirmation of new snps and submitted to GenBank .

Prior studies of *T. gondii* oocyst shedding in felids have used a combination of microscopy and PCR to confirm the identity of visualized parasites (Figure 2.4). Using microscopy alone, *T. gondii* cannot be discriminated from closely related apicomplexan oocysts that may occur in felid feces, namely *Hammondia hammondi*, so either PCR or bioassay is necessary to confirm the identity of *T. gondii*-like oocysts [35]. The use of PCR alone is challenging to interpret because the detection of *T. gondii* DNA in cat feces does not necessarily indicate oocyst shedding; rather, it is possible to detect DNA from tissue cysts in recently consumed infected prey passing through the gut and into the feces [25].

Microscopy

PCR	Oocysts seen	No oocysts seen
+	True *T. gondii* oocyst shedding	- Oocysts degraded, DNA present - Low oocyst concentration - Bradyzoite DNA from prey passing through gut
-	*Hammondia hammondi* or *Besnoitia besnoiti*	*T. gondii* not detected

Figure 2.4 Interpretation of *T. gondii* detection results from cat feces using microscopy and PCR.

Other studies of *T. gondii* oocyst shedding have also contextualized their results in light of this critical methodological limitation [36, 37]. While we applied molecular detection and microscopy in parallel, we did not observe any *T. gondii*-like oocysts, which may be due to procedural error, low concentrations of oocysts in feces, oocyst degradation in the environment before fecal collection, or true absence of oocyst shedding. While procedural errors in the fecal flotation method are possible, we consider these less likely given decades of experience in our laboratory with recovering and identifying apicomplexan oocysts. In addition, our findings of other protozoan parasite oocysts in the same fecal samples (Supplemental Figure 2.2) suggests that the fecal flotation procedure for oocyst recovery was appropriate. We also attempted to isolate oocyst mRNA using RT-qPCR [26], which was used in order to

target a sporozoite-specific SAG protein mRNA. This technique could have allowed us to verify if our samples were from oocyst or bradyzoite DNA, however no samples were amplified using this method.

Our findings provide evidence for frequent dietary or environmental exposure to *T. gondii*. The high prevalence of *T. gondii* DNA detected in feces indicates that cats frequently come into contact with *T. gondii* in prey, water, or soil. We found seasonal variation in *T. gondii* detection in free-ranging cat feces, with higher likelihood of detection in the wet season. Seasonality of oocyst shedding and DNA detection may depend on location specific precipitation and temperature patterns. Among owned domestic cats in Germany, higher levels of *T. gondii* oocyst shedding were observed in summer and autumn (July-December) compared to winter and spring (January-June) [38]. Seasonal variation in oocyst shedding among owned domestic cats [39], seasonality of *T. gondii* detection in soil [20], and fluctuations in *T. gondii* infection in certain high-risk human populations (pregnant women [40, 41]) have been reported but it is unclear exactly how seasonality affects *T. gondii* infection and oocyst shedding in free-ranging domestic cats. Free-ranging feral cats' lifestyles and dietary habits make them one of the most likely groups to shed oocysts at any time of the year, partly because they are more likely to be immunocompromised or co-infected with other parasites.

Though we did not see *T. gondii*-like oocysts, we did observe other parasites, including *Cystoisospora* spp. and *Toxocara cati* (Supplemental Table 2.2, Supplemental Figure 2.2), the latter of which is an underappreciated zoonotic pathogen [42]. After infection, *T. cati* larvae can migrate to various organs and cause visceral larva migrans, a potentially severe and life-threatening disease in humans [43]. Knowing that feral cats are shedding zoonotic pathogens such as *T. cati* near human settlements is essential to convey to members of the general public, who may not be aware of this hazard. The presence of other parasites in feral cat feces can also inform another poorly understood aspect of *T. gondii* biology—the phenomenon of repeat shedding. Co-infection of *T. gondii* and other parasites like *C. felis* can induce repeat *T. gondii* oocyst shedding [44,45]. Though there was no association between visualizing other apicomplexans under microscopy and *T. gondii* DNA prevalence, detecting *Cystoisospora* spp. in feral cats suggests that this feral cat population may be more likely to experience

repeat shedding of *T. gondii*. In this study, we were not able to evaluate repeated presence of *T. gondii* DNA in feces from the same individual cat over time because data on individual cat identity for each fecal sample were not attainable.

Few studies have characterized the genotypes of *T. gondii* in free-ranging cat feces, and our study adds new data with relevant conservation implications. Importantly, we detected atypical genotypes of *T. gondii* present in cat feces, including types that were previously associated with fatal toxoplasmosis in sea otters, Type X and X variants [14]. These *T. gondii* genotypes were also previously detected from the tissues of nearby terrestrial domestic and wild felids, which points to the importance of assessing the terrestrial burden and source of *T. gondii*. Specifically, 4 out of 27 genotyped *T. gondii* samples from our study were Type X or X variants. Finding Type X and X variant *T. gondii* DNA in feces suggests that free-ranging feral cats are exposed to atypical *T. gondii* genotypes, most likely in prey, that can result in shedding of more virulent *T. gondii* oocysts, which was previously documented among owned domestic cats [16,46]. A newly described virulent genotype for sea otters in California, COUG, has a Type I allele at the B1 locus, which was the dominant B1 allele in our feral cat fecal samples [47]. We were unable to further characterize *T. gondii* in feral cat feces due to low quality/quantity of DNA, so the presence of COUG genotype has not been confirmed in feral cats at this time. The presence of Type I, I variants, and Type X in feral cat feces is also relevant to human health. Strains belonging to a Type I genotype are typically more virulent for humans and have been linked to more psychiatric and ocular disorders [48, 49]. Additionally, a recent outbreak of meat-borne toxoplasmosis in Wisconsin hunters (presumed to be immunocompetent) resulted from consuming venison that was infected with *T. gondii* tissue cysts characterized as Type X [50].

Due to the limited quantity and quality of DNA, we were unable to assign a ToxoDB ID to our sequenced samples as none amplified across more than 1-3 loci; our genotyping results were mostly limited to sequence analysis at the B1 locus. Only 13/27 samples that were sequenced at the B1 locus were successfully amplified and sequenced at one or more single copy loci commonly used in MLST, and only two samples amplified at three loci. The B1 locus is a multi-copy gene, so observing snps at several

sequence sites (Supplemental Figure 2.3) is difficult to interpret. These results indicate either (i) presence of more than one strain in gut content (oocysts or bradyzoites) or (ii) polymorphisms within the different copies of the B1 gene (which is estimated to have 35 copies [51]) within a single strain (Supplemental Figure 2.3). For example, Genotypes II and III have mixed bases at restriction cleaving sites that produce a predictable three band appearance following digestion with *Pml*I or *Xho*I, which allows for rapid discrimination from the more virulent Type I genotype [28].

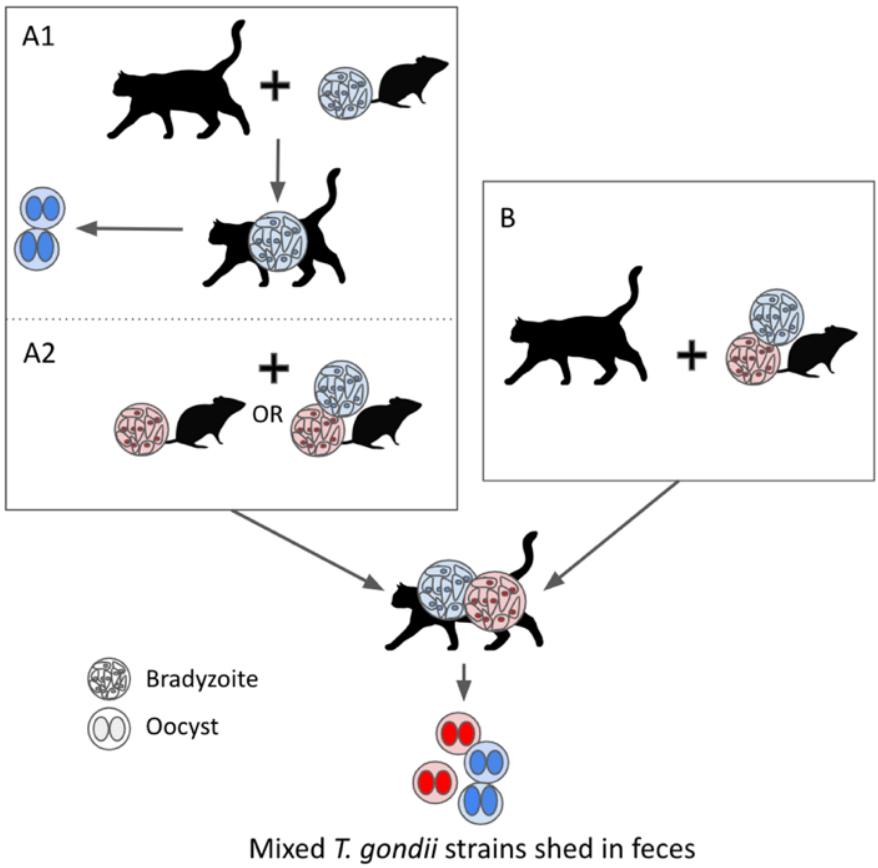

Figure 2.5 Mechanistic diagram of multiple *T. gondii* genotype exposure in felids and subsequent shedding of mixed strains. Red and blue indicate different *T. gondii* genotypes, and plus signs indicate consumption of *T. gondii* infected prey by felids. Mixed infection and shedding of more than one *T. gondii* strain can occur after animals are exposed to genotypes sequentially (Scenario A1 + A2), or simultaneously (Scenario B).

For the single copy gene L358 targeted by MLST in sample 151, we had consistent mixed bases at specific sequence sites as confirmed by repeated sequencing. This observation likely represents the

presence of more than one strain in a fecal sample from one animal, as it is not feasible for a single copy gene to have two different bases at a given location. Mixed *T. gondii* genotype infections have previously been documented in felid tissues [18, 52] but to our knowledge, the presence of two different parasite strains from feral cat feces has not been documented in the literature. The presence of mixed strains in a cat fecal sample can indicate that a cat ate prey that was co-infected with two *T. gondii* strains and therefore has bradyzoites representing different strains passing through the gut, and/or a cat that is shedding *T. gondii* oocysts representing two different strains due to reactivation of previous infection following ingestion of a different genotype (Figure 2.5 Scenario A) or co-ingestion of two genotypes (that can result in sexual recombination) (Figure 2.5 Scenario B). Confirmation of mixed strains from feces is important as it demonstrates that diverse *T. gondii* genotypes are circulating in the environment and can increase the likelihood of oocyst re-shedding after cats come in contact with heterogenous strains.

Though not an aim of our study, our results demonstrate that both managed and unmanaged colonies had evidence of *T. gondii* DNA. One of our sites was near a private home with several supplemental feeding areas, one was in a city park with supplemental feeding stations, and the other two were near the beach without visible feeding stations despite extensive searching. Supplemental feeding could promote the survival and proliferation of larger colonies and increase predation of wildlife in an area [53, 54]. Cat management, especially of unowned free-ranging cats, may be vital to wildlife conservation efforts, as measures like trap-neuter-release (TNR) are ineffective in the long term [55–57] and do not prevent domestic cats from killing native birds and small mammals via predation in the short term [58, 59]. The aforementioned management techniques do not immediately address ongoing oocyst shedding and environmental contamination by feral cats. Keeping cats indoors can also be beneficial to cats, as many studies show lower seroprevalence of *T. gondii* [60] and longer life spans compared to outdoor animals [61]. It may be important to control current feral cat populations because natural mortality or artificial forces (TNR) may take too long to substantially reduce free-ranging cat population size in order to mitigate near-term impacts (hunting and pathogen transmission) to humans and native wildlife populations.

Our study demonstrates the importance of feral domestic cats as a population of felids that can contribute to the transmission of atypical and virulent genotypes of *T. gondii*. Although pet cats can shed *T. gondii* oocysts, between 63 and 80.6% of pet cats in the United States are reported to be indoor-only [61, 62]. Due to their large population sizes and association with human settlements, feral cats are more important sources of parasites than their wild felid and pet cat counterparts in certain areas, and likely contribute a large proportion of the environmental oocyst load. Large populations of feral cats and *T. gondii* contamination near sea otter habitats can pose a risk for endangered marine mammals. Feral cat management has many potential benefits to cat welfare, public health, and wildlife conservation. Dialogue between diverse stakeholders is ultimately needed in order to find viable, sustainable solutions that prioritize cat welfare and wildlife health.

Acknowledgments

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Supplemental Table 2.1 Results of attempted MLST sequencing of B1 positive feral cat fecal samples. Samples where amplification and/or sequencing were unsuccessful are indicated with "—".

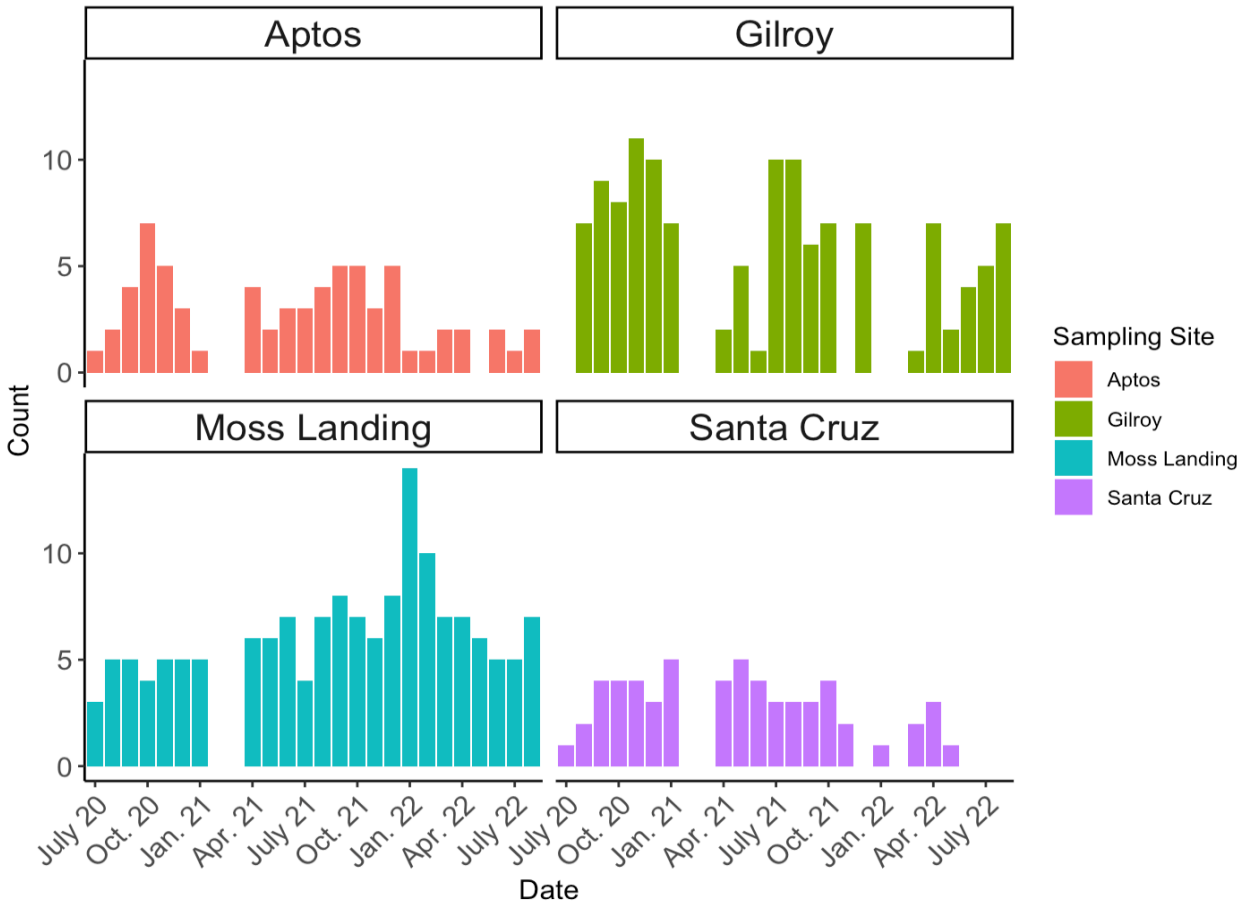
ID	B1	SAG 1	5'SAG2	3'SAG2	alt SAG2	SAG3	BTUB	GRA6	C22-8	C292	L358	PK1	Apico
76	Type I variant	—	—	—	—	—	—	—	—	—	—	—	—
77	Type I variant A	II/III	—	—	—	II/X	—	—	—	—	II	—	—
87	Type II/III	—	—	—	—	—	—	—	—	—	—	—	—
92	Type X	—	—	—	—	—	—	—	—	—	—	—	—
110*	—	—	—	—	—	—	—	—	—	—	III	—	—
111	Type I	II/III var	—	—	—	—	—	—	—	—	—	—	—
113	Type X	—	—	—	—	—	—	—	—	—	—	—	—
118	Type I	—	—	—	—	—	—	—	—	—	—	—	—
119	Type I	—	—	—	—	—	II	—	—	—	—	—	—
128	Type X var B	—	—	—	—	—	—	—	—	—	—	—	—
129	Type II/III	—	—	—	—	—	—	—	—	—	—	—	—
150	Type I	—	—	—	—	—	—	—	—	—	—	—	—
151^	Type II/III var B	—	—	—	—	—	—	—	—	—	II var	—	—
155	Type II/III var A	—	—	—	—	—	—	—	—	—	—	—	—
159	Type I variant B	—	—	—	—	—	—	—	—	—	—	—	—
161	Type I variant B	—	—	—	—	—	—	—	II/X	—	—	—	—
163	Type I variant B	II/III	—	—	—	—	—	—	—	—	—	—	—
165	Type I	—	—	—	—	I	—	—	—	—	—	—	—
188	Type I variant C	—	—	—	—	—	—	—	—	—	—	—	—
204	Type II/III var C	—	—	—	—	—	—	—	—	—	—	—	—
253	Type I	—	—	—	—	—	—	—	—	—	—	—	II
279	Atypical 1 var B	—	—	—	—	II/X var	—	—	—	—	—	—	—
287	Type I	—	II/III/X	—	II/X	II/X var	—	—	—	—	—	—	—
296	Atypical 2	—	—	—	—	II/X var	—	—	—	—	—	—	—
349	Type I	—	—	—	—	—	—	—	—	—	—	—	—
351	Type I	—	—	—	—	—	—	—	—	—	—	—	—
354	Type X	—	—	—	—	—	—	—	—	—	—	—	—
389	Type I	—	—	—	—	II/X	—	—	—	—	—	—	—

*Sample 110 amplified at the B1 locus, however sequencing for this sample failed. MLST was performed prior to receiving B1 results, and amplification and sequencing were successful at the L358 locus
^Variant snp confirmed via repeated sequencing at the L358 locus and submitted to GenBank (accession number OQ850752)

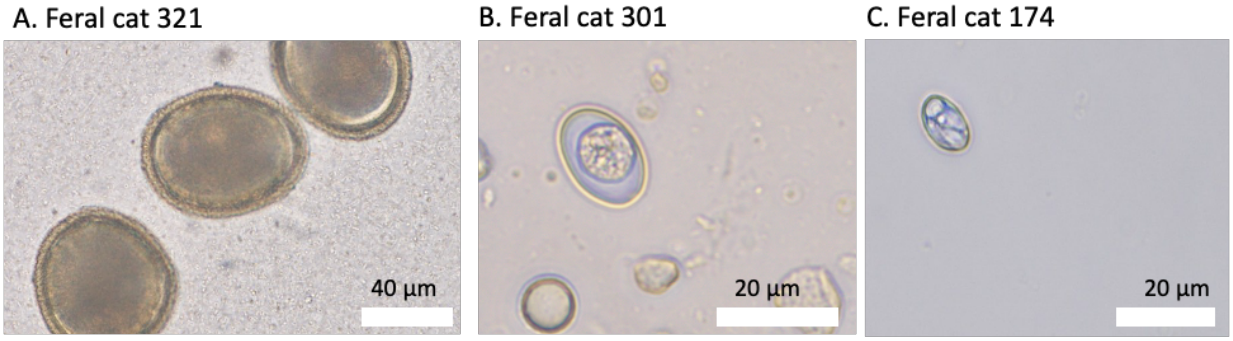
Supplemental Table 2.2. Fecal samples with microscopy and PCR DNA detection of protozoan oocysts and helminth ova collected from four feral cat colonies in the greater Monterey Bay area, July 2020 - August 2022. There was no association between microscopy detection of other apicomplexa and *T. gondii* DNA detection.

Parasite	Microscopy only (n = 404)	PCR only (n = 362)	Microscopy and PCR (n = 362)
Sarcocystidae spp.	3	17	0
Cystoisospora spp.	13	8	7
Toxocara cati	31	0	0

Supplemental Figure 2.1 Number of monthly fecal samples collected from four feral cat colonies (Aptos, Gilroy, Moss Landing, and Santa Cruz) from July 2020 - August 2022



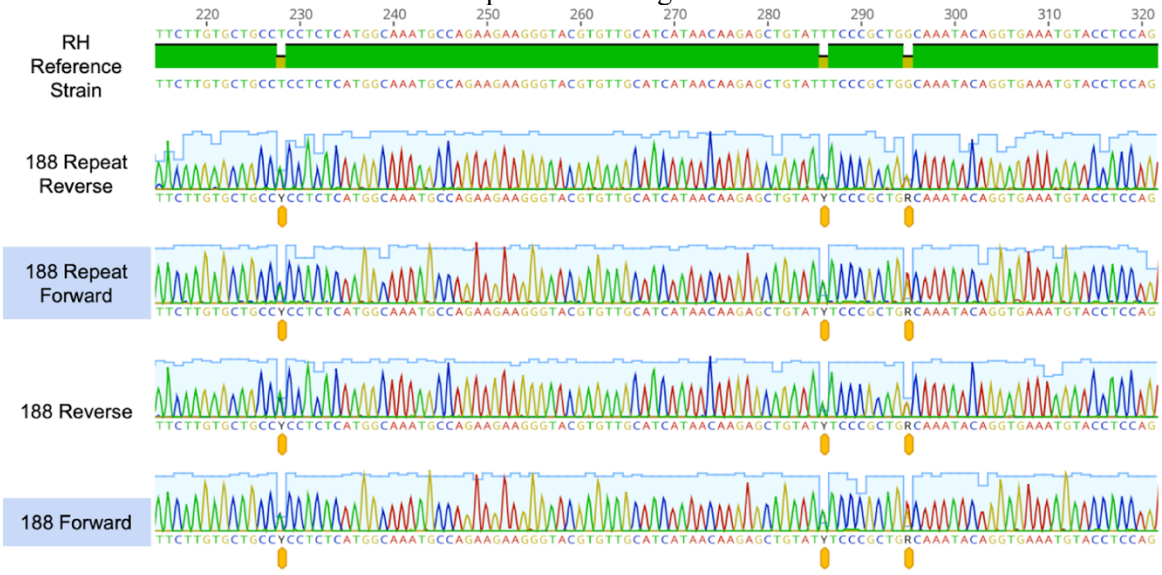
Supplemental Figure 2.2 Brightfield microscopy images of helminth ova and apicomplexan oocysts or sporocysts identified via double centrifugation floatation in feral cat feces collected from the greater Monterey Bay area between 2020 and 2022.



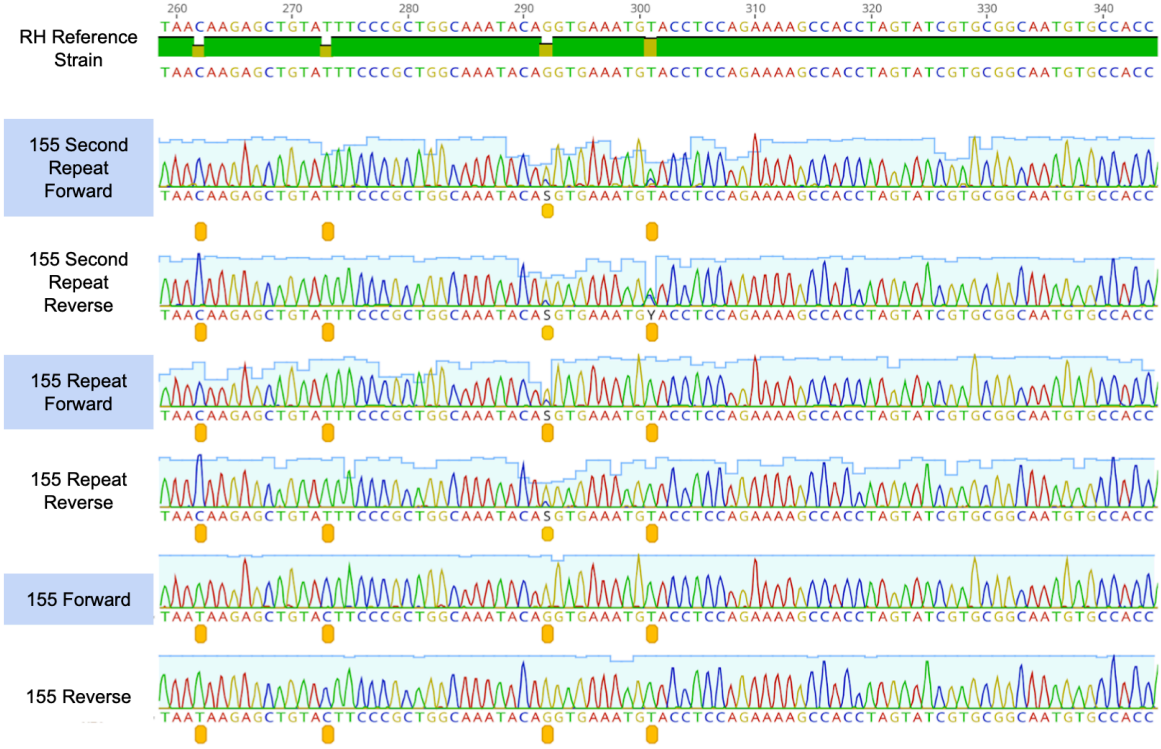
2A. *Toxocara cati* ova (40X). 2B. *Eimeria* spp.-like oocyst (40X). 2c. *Sarcocystis* spp.-like sporocyst (40X).

Supplemental Figure 2.3 Chromatograms of *T. gondii* DNA sequences targeting the B1 locus for a sample that had consistent single nucleotide polymorphisms (188) and a sample with inconsistent single nucleotide polymorphisms (151). Yellow bars under sequence nucleotide sites indicate locations of snps.

Sample 188. Consistent snps at positions 228, 286, and 294. Confirmation of mixed bases with repeat sequencing in this sample supports a strain that contains polymorphic nucleotide bases across multiple copies of the B1 gene.



Sample 151. Inconsistent snps at positions 262, 273, 292, and 301. Lack of consistency in sequencing results suggests presence more than one strain which could be the result of ingesting prey contaminated with different parasite strains or oocysts representing mixed genotypes (Figure 2.4).



Chapter 3 More people, more cats, more parasites: Human population density and temperature variation predict prevalence of Toxoplasma gondii oocyst shedding in free-ranging domestic and wild felids

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(Formatted for publication in PLOS One)

Abstract

Toxoplasma gondii is a ubiquitous zoonotic parasite that can infect warm-blooded vertebrates, including humans. Felids, the definitive hosts, drive *T. gondii* infections by shedding the environmentally resistant stage of the parasite (oocysts) in their feces. Few studies characterize the role of climate and anthropogenic factors in oocyst shedding among free-ranging felids, which are responsible for the majority of environmental contamination. We determined how climate and anthropogenic factors influence oocyst shedding in free-ranging domestic cats and wild felids using generalized linear mixed models. *T. gondii* oocyst shedding data from 47 studies were systematically reviewed and compiled for domestic cats and six wild felid species, encompassing 256 positives out of 9,635 total fecal samples. Shedding prevalence in domestic cats and wild felids was positively associated with human population density at the sampling location. Larger mean diurnal temperature range was associated with more shedding among domestic cats and warmer temperature in the driest quarter was associated with lower oocyst shedding in wild felids. Increasing human population density and temperature fluctuation can exacerbate environmental contamination with the protozoan parasite *T. gondii*. Management of free-

ranging domestic cats could lower the burden of environmental oocysts due to their large population sizes and affinity with human settlements.

Background

Climate change has not only led to devastating changes in ecosystem function and provided services but has also facilitated the emergence and/or expansion of many human and animal pathogens worldwide [1]. The reasons for disease emergence and expansion are complex and multifaceted but can include expanded pathogen or vector range, altered species interactions, and reduced host fitness [2,3]. Understanding the climactic and ecological drivers of pathogen spread is vital for the prevention and mitigation of diseases in both animal and human hosts. *Toxoplasma gondii* is a generalist zoonotic pathogen that can infect any warm-blooded vertebrate, including humans [4]; however, it can only sexually reproduce in its definitive hosts–domestic cats and wild felids [5]. The global distribution and large population of domestic cats (~600 million) [6], coupled with numerous parasite transmission pathways, contributes to *T. gondii's* ubiquitous nature and infection in hosts from remote oceanic islands to the Arctic Circle [7,8].

Toxoplasmosis can cause mild-to-severe disease in humans, domestic animals, and wildlife. Human infections are generally less common in most high-income nations, but toxoplasmosis is still a large contributor to disease burden in many regions. Disease burden is particularly high in South America, which has a high diversity of *T. gondii* genotypes, high levels of environmental contamination, and large populations of free-ranging domestic cats and wild felids [9]. Toxoplasmosis can also cause abortion in sheep and goats, which can be a financial burden for farmers [10]. In addition, marine wildlife can be vulnerable to *T. gondii* infections; oocysts accumulate in soil and flush into marine environments after rainfall, whereby they can contaminate prey sources consumed by susceptible hosts [11]. Domestic and wild felid species play a critical role in the ecology and epidemiology of *T. gondii* because they are the only source of oocysts, the parasite life stage that drives overall *T. gondii* transmission [12].

Cats typically shed oocysts for 1-3 weeks after primary infection, and research has been conducted in controlled, experimental settings to investigate risk factors for shedding in domestic cats,

which include route of infection, age, and immunosuppression [13–15]. Previous reports have suggested that 1% of all domestic cats are estimated to be shedding at any given point [16], though a recent meta-analysis reported a global pooled oocyst shedding prevalence of 2.6% (95% CI 1.9–3.3) for domestic cats [17]. The commonly cited 1% oocyst shedding prevalence likely underestimates shedding as it does not take into account the potential for re-shedding oocysts, and differences among cats, including diet and time spent outdoors. Outdoor pet cats, managed free-ranging cat colonies, truly feral/stray cats, and wild felid species that rely on partial or exclusively hunted prey have a higher risk of infection from consuming infected intermediate hosts and contact with oocyst-contaminated matrices. These felid populations are more frequently exposed to diverse genotypes of *T. gondii*, which may enhance the potential for initial and repeat oocyst shedding [18]. Less attention has been directed at investigating risk factors for shedding prevalence in free-ranging wild and domestic felids compared to owned domestic cats, despite the importance of the former groups in contributing to environmental contamination and toxoplasmosis transmission.

Temperature, precipitation, and humidity can impact oocyst sporulation and survival, oocyst transport/mobilization (via rainfall patterns), and felid host distribution, reproduction, and population size [19] (Supplemental Figure 3.1). Climate can also affect the life cycle and population dynamics of common prey species, which can provide favorable conditions for cat reproduction and increased numbers of young, naive definitive hosts susceptible to *T. gondii* infection [20].

Using published data on *T. gondii* oocyst shedding in free-ranging domestic and wild felids, we investigated whether climate and anthropogenic risk factors are predictive of shedding prevalence. Other studies have evaluated risk factors in a specific location, but to our knowledge, this study is the first to use a global dataset and consider both climate and anthropogenic factors. To investigate the impacts of temperature, precipitation, and anthropogenic activity on *T. gondii* oocyst shedding in free-ranging felids, we hypothesized that shedding will increase with warmer temperatures, higher precipitation, and in areas with higher human population density. Our study builds upon previous research by investigating risk factors for shedding in both free-ranging wild felids and domestic cats, the latter of which play a key role

in environmental contamination with oocysts in many areas and can be more readily targeted in management and policy efforts.

Methods

Literature search

Our analyses focused on confirmed *T. gondii* oocyst shedding in domestic and wild felids. A systematic review of the literature was performed in March 2020 using PubMed and Web of Science, with a subsequent sweep in July 2021 (n=2176). Twelve sets of search terms were used, always including '*Toxoplasma*' (See Supplemental Table 1 of Zhu et al. 2021 [21]). All studies through July 2021 were included, and no studies were excluded based on language of publication or location. Google Translate was used to translate and extract metadata as necessary. We obtained additional studies from the reference lists of reviews identified in this sweep along with a recent meta-analysis (n=51) [17], for a total of 2227 studies. Duplicates (n=1111), reports not able to be retrieved (n=2), and reports that did not focus on *T. gondii* or *T. gondii* -like oocyst shedding in felids were excluded (n=1003). Studies were restricted to oocyst shedding in free-ranging wild felids and unowned, free-ranging domestic cats, which include stray, semi-managed feral cats (i.e. unowned cats that are fed by humans but do not live indoors), and unmanaged feral cats that subsist on wild prey without human support. One study included cats euthanized at a humane shelter in Ohio [22]. According to a national database of 1,233 animal shelters in the US, the majority of live intakes for cats are strays, or unowned or free roaming animals [23]. For analysis, we assumed that the majority of unowned cats from the Ohio shelter would be stray or free-ranging, similar to values reported by the national database, though we acknowledge that we could not verify the exact origin of all animals. When fecal samples are screened using only fecal flotation and microscopy, *T. gondii* cannot be reliably distinguished from related apicomplexans, namely *Hammondia hammondi*. Confirmation of oocysts as *T. gondii* via PCR with sequencing or mouse bioassay is necessary to confirm parasite identity. We only included studies that verified oocyst presence with microscopy coupled with genetic parasite identification using PCR and/or mouse bioassay. After removing publications based on exclusion criteria for oocyst confirmation (n=64), 47 studies were included in the

final analysis (Supplemental Figure 3.2). For studies that met inclusion criteria, we extracted metadata including the year of publication, type of felid sampled (domestic/wild), country of study, continent, diagnostic method, number of positive fecal samples, total fecal samples tested, and approximate latitude and longitude of the sample site as reported by the authors for each study. We repeated this process in studies with multiple species of sampled felids. All data sources are provided in Supplemental Appendix 3.1 and a table with summarized metadata and all variables considered in analysis for each study was uploaded to DataDryad [24].

Our primary variables of interest were annual mean temperature, annual precipitation, and human population density. Additionally, we considered other climate variables, such as maximum temperature, mean diurnal temperature range and precipitation seasonality, as well as human activity variables such as habitat type and species richness for inclusion in subsequent models (Table 1). For each study, we used the R package 'raster' [25] with a World Geodetic System 1984 projection and a 2.5 km buffer to obtain location-specific data. Climate data (temperature and precipitation variables) was extracted at a 5 km resolution from the WorldClim 2.0 (1970-2000) dataset [26], while human population density and human footprint data at a 5 km resolution was extracted from the NASA Center for International Earth Science Information Network (CIESIN) [27]. Human population density was paired to each study by the closest time period (2000, 2005, 2010, 2015 and 2020). Habitat type was extracted at a resolution of 1 km, and species richness at 110 m from the International Union for Conservation of Nature (IUCN) [28] (Table 3.1).

Table 3.1 Description, rationale, and data source for variables assessed in univariable analysis and multivariable model building as predictors of *Toxoplasma gondii* oocyst shedding in free-ranging felids.

Variable Type	Rationale	Available data	Data source(s)
Temperature	Differences in temperature may alter oocyst survival [28]	Annual mean temperature **Mean diurnal range (D)** Isothermality Temperature seasonality Max temperature of warmest month	WorldClim [26]

		Min temperature of coldest month Temperature annual range Mean temperature of wettest quarter Mean temperature of coldest quarter **Mean temperature of driest quarter (W)** Mean temperature of wettest quarter	
Precipitation	Precipitation can affect oocyst survival and transport [29, 30]	Annual precipitation Precipitation of wettest month Precipitation of driest month Precipitation seasonality Precipitation of wettest quarter Precipitation of driest quarter Precipitation of warmest quarter Precipitation of coldest quarter Vapor pressure	WorldClim [26]
Human activity	Anthropogenic activities and human presence can alter landscapes in ways that lead to changes in domestic and wild felid population size and density, and diversity of intermediate hosts.	IUCN habitat type Species richness **Human population density (D/W)** Human footprint	IUCN [28[, NASA CIESIN Gridded Population of the World (GPW) v4 [27]

Variables included in final multivariable models for domestic (D) and wild (W) felids are bolded for emphasis.

Statistical analysis

We tested our hypotheses using generalized linear mixed models implemented in the R package 'glmmTMB' [32]. We chose to restrict our analysis to studies of confirmed *T. gondii* oocyst shedding (microscopy coupled with parasite confirmation via PCR and/or mouse bioassay) because climate and seasonality can have dissimilar relationships with shedding patterns of *T. gondii* and *T. gondii*-like oocysts (such as *H. hammondii*) [33]. *T. gondii* oocyst shedding prevalence was modeled as a proportion between 0 and 1 with a beta distribution. Exploratory analyses showed that the data were zero-inflated, resulting in the use of a zero-inflated beta distribution for further analyses. To account for other sources of variance such as spatial autocorrelation, we incorporated the study as a random effect in all multivariable models.

Model building and comparison were performed for domestic and wild felid data separately as the distribution of associated risk factors was different between the two groups based on descriptive statistics,

possibly resulting from a combination of sampling bias and biological differences in size, habitat, and dietary habits. The distribution of habitat type, species richness, and human population density for domestic and wild felids are summarized in Supplemental Figure 3.3. All variables were standardized and scaled prior to analyses. Variables were evaluated one at a time and considered for inclusion in multivariable models using a significance threshold of $p<0.10$. As there were multiple highly correlated variables used to measure temperature and precipitation, final variables were selected by category (temperature/ precipitation/anthropogenic activity) based on strength of significance using a threshold of $p<0.01$ (Supplemental Table 3.1). We calculated Pearson correlation coefficients between final variables; no variables tested had a coefficient above 0.4 (Supplemental Table 3.2). There were several temperature variables (e.g., temperature seasonality, temperature annual range, mean temperature in dry quarter) with $p<0.01$ for wild felids, and two temperature variables with $p<0.01$ for domestic cats, so we tested these factors in separate multivariable models and compared model AIC to determine final model composition. We did not evaluate interactions between candidate variables and our outcome because we expected linear relationships. The best model for each felid group (wild or domestic) was chosen based on AIC.

Results

The final dataset of only confirmed *T. gondii* oocysts included 51 groups of felids sampled from 47 studies (256 positives out of 9,635 fecal samples) (Supplemental Appendix 3.1) [24]. Sample size and oocyst shedding prevalence varied by felid type, with 37 out of 463 (8%) fecal samples testing positive from 12 groups of wild felid species sampled from 10 studies, and 219 out of 9,172 (2.4%) fecal samples testing positive from 39 groups of domestic cats sampled across 37 studies. The six wild felid species represented in our study include bobcat (*Lynx rufus*), cougar (*Felis concolor*), Geoffrey's cat (*Oncifelis geoffroyi*), Pampas cat (*Felis colocolo*), Iriomote cat (*Prionailurus iriomotensis*), and Pallas's cat (*Otocolobus manul*).

Domestic cat studies were primarily conducted in North America (n=11), Asia (n=11), and Europe (n=7), with fewer studies conducted in Africa (n=4), South America (n=3), and Oceania (n=2). The majority of shedding studies with confirmed *T. gondii* in feces from wild felids were conducted in

North America (n=7), with two studies conducted in Asia and one study in South America (Figure 3.1). The majority of domestic cat studies (n=29/38, 76%) were conducted in human-modified/urban environments. Non-urban habitats included forest-temperate (n=5), forest-subtropical/tropical moist lowland (n=1), forest-subtropical/tropical moist montane (n=1), savanna-dry (n=1), shrubland (n=1), shrubland-Mediterranean (n=2), and grassland - subtropical/tropical dry (n=1). Most wild felid studies were conducted in non-urban habitats, namely forest-temperate (n=5), shrubland-temperate (n=1), shrubland-subtropical/tropical dry (n=1), shrubland-Mediterranean (n=2), and grassland-temperate (n=1). Two groups of wild felids were sampled in agricultural environments.

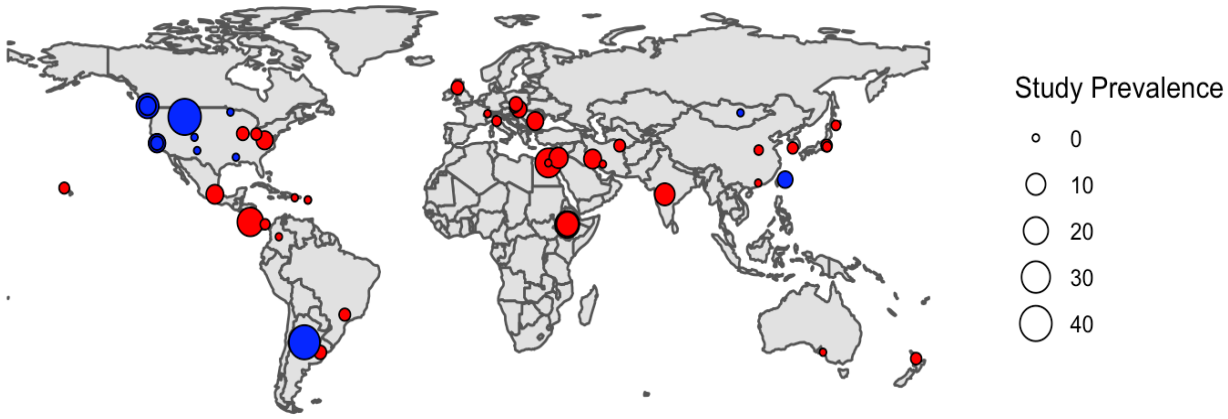

Figure 3.1 Map of confirmed *T. gondii* oocyst shedding prevalence in free-ranging domestic cats (red) and wild felid species (blue) in studies conducted from 1973 - 2021. Modified from Zhu et al. 2021 [21] and included for illustrative purposes. Map created using 'rnaturalearth' package [34].

Based on univariable analysis, four variables were retained in multivariable model building for free-ranging domestic cats (mean diurnal temperature range, isothermality, precipitation seasonality, human population density) and four variables for wild felids (temperature seasonality, temperature annual range, mean temperature in dry quarter, human population density). A total of twelve linear models were considered to explain confirmed *T. gondii* oocyst shedding in domestic cats (Supplemental Table 3.3). The top model, based on AIC, showed a significant, positive effect of human population density (OR 1.38, p=0.0014) and mean diurnal temperature range (OR 1.63, p=0.0007) on *T. gondii* oocyst shedding prevalence in domestic felids. Eight models were considered to explain oocyst shedding in wild felids

(Supplemental Table 3.4). Between two wild felid models with the same AIC value, we chose the model with population density and mean temperature in the driest quarter because it had no significant deviations for actual vs. predicted residuals. The top model showed a positive association between human population density (OR 340, p=1.54 e-11) and a negative association between mean temperature in the driest quarter (OR 0.26, p<2e-16) and oocyst shedding in wild felids.

Table 3.2 Final multivariable model structure and effect size (β and OR) for evaluation of hypotheses between climate (temperature, precipitation) and anthropogenic risk factors and domestic cat and wild felid oocyst shedding. Study was also included as a random effect in both models for domestic and wild felids to account for other unaccounted sources of variance.

Model type	Model variable(s)	Beta	OR (95% CI)	p-value
Domestic cat[a]	Human population density	0.32	1.38 (1.13, 1.68)	0.0014
	Mean diurnal range	0.49	1.63 (1.22, 2.17)	0.0007
Wild felid[a]	Human population density	5.34	340 (62, 1856)	1.54 e-11
	Mean temperature in dry quarter	-1.34	0.26 (0.19, 0.36)	2 e-16

[a]Study was also included as a random effect in both models for domestic and wild felids to account for other unaccounted sources of variance.

Discussion

We found a significant positive association between human population density and *T. gondii* oocyst shedding prevalence in a global dataset of free-ranging domestic cats and six wild felid species (Supplemental Appendix 3.1) [24]. Other variables of interest, namely total annual precipitation and mean annual temperature, were not associated with *T. gondii* oocyst shedding prevalence. Although lower prevalence of oocyst shedding has been reported in domestic cats versus wild felids [17, 21], their large population sizes and association with human populations mean that they are an important source of *T. gondii* oocysts for wildlife and people. As global estimates of domestic cat population density do not exist, human population density offers a proxy within our models as higher human density and activity can result in the release of unwanted pets, more outdoor pet cats, pet cats escaping, and proffered feeding to feral cat colonies. Additionally, increasing human activity can lead to landscape changes that may

facilitate increased environmental oocyst contamination [35]. Taken together, these factors can alter the epidemiology and transmission of *T. gondii* by facilitating the increased abundance of free-ranging domestic cats that contribute to oocyst contamination in the environment.

Our main finding that human density is significantly associated with oocyst shedding prevalence in domestic and wild felids is consistent with a recent study that found associations between human population density and *T. gondii* seroprevalence in wild mammals [36]. Association with human settlements introduces a multitude of behaviors that benefit feral cats through the provision of additional food, deterrents to native predators, and protection against the elements, which in turn could help to maintain and even increase colony and overall cat population size. Human populations are concentrated in urban areas, which have increased availability of food (trash, intentional food sources like bird feeders) that can attract urban wildlife like rats and birds [37]. Higher prey populations can support larger populations of free-ranging cats, so even low seroprevalence rates in prey (<1%) can result in widespread cat infection with *T. gondii* due to the sheer number of prey consumed and high likelihood of consuming infected prey over time [38]. Though access to additional anthropogenic food could reduce prey consumption by urban cats, direct and indirect contact (such as sharing fecal latrines) among individuals can facilitate pathogen transmission in dense urban cat populations [39]. Wild felids that live near urban-rural interfaces have increased opportunities to share a food web with domestic felids and can be exposed to infected commensal intermediate wildlife hosts like rodents as well as *T. gondii* strains commonly associated with domestic cats in addition to wild strains. Oocyst spread is also facilitated in human-dominated landscapes. Modified surfaces like roads allow surface water runoff to transport oocysts easily from land into sources of water shared by humans and animals [30], whereas natural cover and vegetation can reduce oocyst delivery by filtering pathogens in runoff [40].

Oocyst load in the environment is not sufficient by itself to estimate infection risk because host and environmental factors can interact to influence location-specific risk. Our measured outcome was the prevalence of *T. gondii* oocyst shedding (generally only occurring for 1-2 weeks), not the prevalence of *T. gondii* infection, which is more easily captured due to presence of antibodies due to lifelong infection.

Prior studies of *T. gondii* in wild mammals found a positive association between mean annual temperature and seroprevalence, however, infection in intermediate hosts and shedding by definitive hosts are related but separate phenomena that may be impacted differentially by climate, especially at different time scales [36]. Climate data sets used in this study spanned a 30 year period and were not necessarily designed to assess seasonality [26]. While our analysis did not reveal clear relationships between oocyst shedding and mean annual temperature or precipitation, warmer temperatures and higher humidity are linked to higher *T. gondii* infection prevalence in humans and livestock [41, 42], and higher cat seroprevalence has been linked to greater local rainfall [43]. Mechanistically we still do not understand how variables such as mean diurnal temperature range directly influence oocyst shedding, however we recommend that the impacts of climate on *T. gondii* oocyst shedding should be assessed in longitudinal studies at the local scale in order to gain a clearer picture of this relationship.

One limitation of our analysis is that many primary literature sources did not perform serological testing to discriminate between animals with previous evidence of exposure (e.g. antibodies produced against *T. gondii*). In two of the original studies [44, 45], most of the domestic and wild felids were seropositive, indicating that they had most likely already shed oocysts at least once. Though seropositive cats can shed oocysts [21, 46], studies with seropositive animals may be less likely to capture oocyst shedding than studies sampling naïve animals. Two other limitations of this study and similar reports on *T. gondii* oocyst shedding are geographic and species-based sampling bias, and inconsistency of detection methods used to determine oocyst shedding. Due to the small sample size and large confidence interval for effect size in wild felids (62-1856), we caution that our wild felid results may be incredibly sensitive to sampling bias and may need further investigation to determine the true association with human population density. Despite the diversity of *T. gondii* genotypes present in South America, oocyst shedding in this region remains critically understudied. Studies are also sparse in Africa and Asia, even though both regions have multiple wild felid species and high projected human population growth [47]. Africa is consistently reported to have the highest confirmed and non-confirmed oocyst shedding prevalence compared to other continents [17, 21] (Figure 3.1). As human population density continues to

increase in these regions, it is necessary to monitor environmental *T. gondii* oocyst contamination as it is relevant to human and wildlife health [48]. Future studies should also consider socioeconomic status (SES) to understand how people can be disproportionately affected by urbanization and disease transmission. Wealthy neighborhoods often have sufficient resources to manage populations of unwanted rodents through culling and proper sanitation measures, which indirectly helps to deter stray and feral cats. Water infrastructure in resource-limited countries can also be heterogeneous. In Brazil, richer individuals may have access to treated and/or bottled water while those living in favelas (urban slums) do not [49]. Similar observations have been reported for *Toxocara* infections in the boroughs of New York City [50], increased *T. gondii* seropositivity among lower SES groups in the US [51], and higher rates of maternal exposure to *T. gondii* and congenital toxoplasmosis in Brazil among lower SES groups [52]. The challenge of variable fecal testing methods and sampling bias exacerbates inequality, as there are more microscopy-based studies in low and middle-income countries (LMICs) compared with North America and Europe. From an equity lens, microscopy-based studies are biased toward LMIC laboratories that have less funding and resources compared to laboratories in high-income nations that are well-resourced, well-financed, and have higher training ability that can perform more molecular and bioassay studies. We thus have a clearer picture of *T. gondii* transmission in regions that are relatively low risk for *T. gondii* exposure and an ambiguous picture where high-quality data may actually be more valuable to protect human public health and vulnerable animal populations.

Identification of predictive and/or protective factors for oocyst shedding in free-ranging wild and domestic felids is highly relevant for relevant stakeholders and policymakers to make informed decisions for human and animal health. Our results reinforce the role that free-ranging domestic cats have in contributing to biological contamination of *T. gondii* oocysts, and therefore the need to target management (cat removal) and/or more holistic landscape-based interventions (wetland restoration, vegetation buffers) that can reduce transport of oocysts [40,53]. Overall, we recommend that more studies be conducted in locations where *T. gondii* is highly endemic but understudied such as South America and Africa, the utilization of molecular methods paired with microscopy to verify parasite identity and

characterize oocyst genotypes, and more sampling of wild felid species. Most regions are under sampled for wild felids, despite the fact that a higher proportion of these animals are thought to be shedding at any given point in time. We do not attempt to prove a causal relationship between human population density and oocyst shedding prevalence, however, the association between human density and oocyst shedding suggests that focusing on free-ranging feral cats as the source of environmental oocyst contamination is an important disease management strategy. Feral cat management can serve to reduce the risk of toxoplasmosis in humans, livestock, and wildlife, and will also have added benefits for reducing predation of native wildlife species [54]. We emphasize the need to address major data gaps and potential strategies for monitoring *T. gondii* oocyst shedding prevalence in free-ranging domestic cat and wild felid populations. Human-caused climate change and urbanization may create more ideal environmental conditions for a generalist pathogen and its disease ecology, which can be relevant for other infectious diseases that are influenced by human population growth and increased urbanization.

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Data accessibility

The data that support the findings of this study are archived on Dryad Digital Repository: doi.org/10.25338/B8H63B

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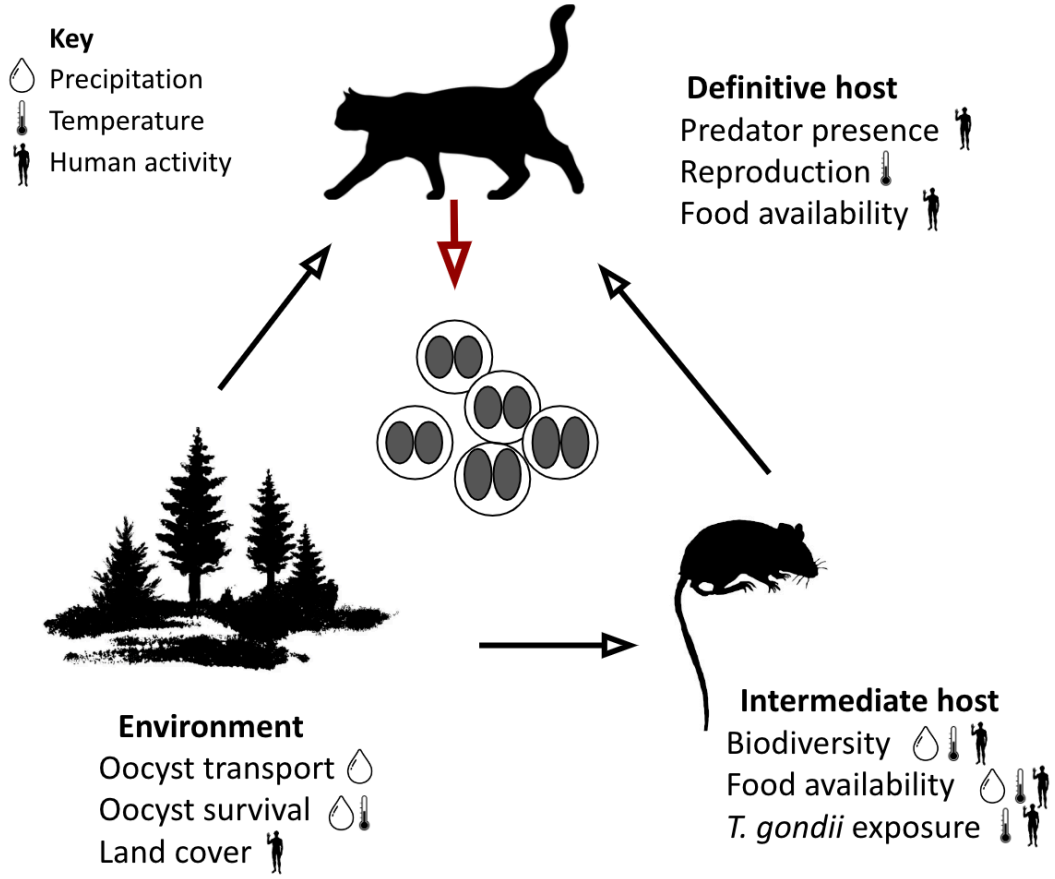
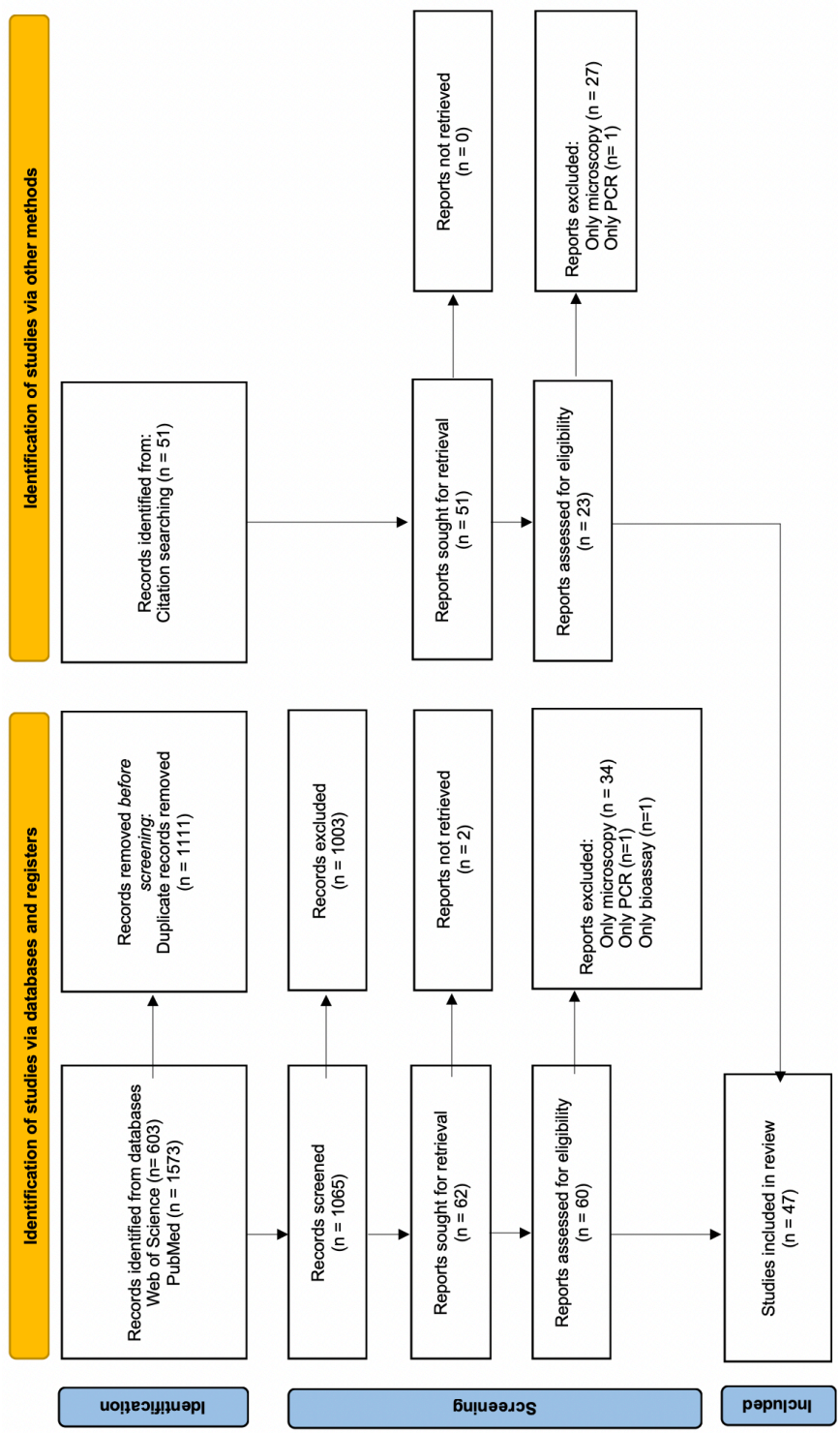
Supplemental Figure 3.1 Mechanistic framework of pathways in which climate and anthropogenic factors can influence Toxoplasma gondii oocyst shedding in free-ranging domestic cats and wild felids.



Each parameter that can affect T. gondii transmission (and thus felid oocyst shedding) can be influenced by precipitation, temperature and/or human activity, as noted by the symbols listed in the key.

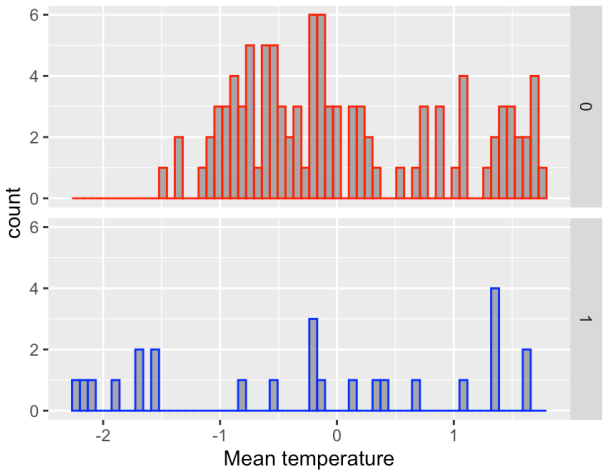
Supplemental Figure 3.2 Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) 2020 flow diagram.



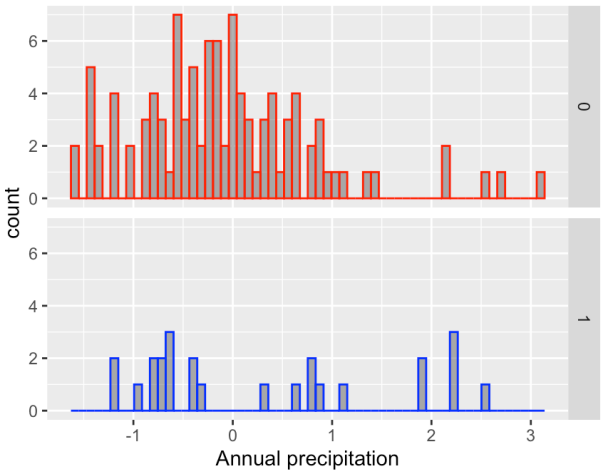
The following PRISMA flow diagram describes the identification, screening, and inclusion process for the 47 studies analyzed in this manuscript. *From*: Page MJ, McKenzie JE, Bossuyt PM, Boutron I, Hoffmann TC, Mulrow CD, et al. The PRISMA 2020 statement: an updated guideline for reporting systematic reviews. BMJ 2021;372:n71. doi: 10.1136/bmj.n71. For more information, visit: http://www.prisma-statement.org/

Supplemental Figure 3.3 Distribution of scaled and standardized a) annual mean temperature (°C), b) annual precipitation (mm), c) IUCN habitat type, d) species richness, and e) human population density (people/km^2) in studies reporting the prevalence of oocyst shedding by free-ranging domestic cats (red) and wild felids (blue).

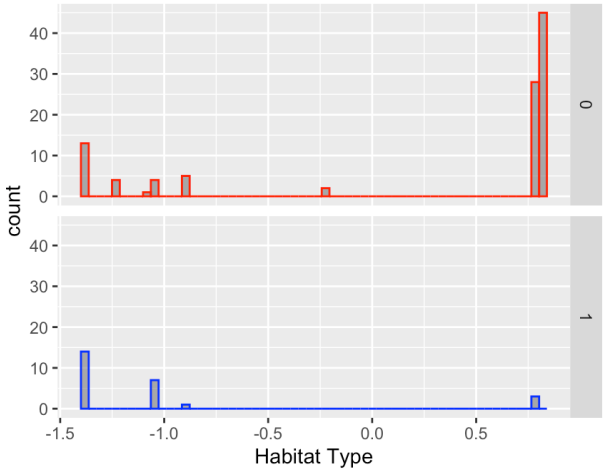
a) Annual mean temperature (°C)



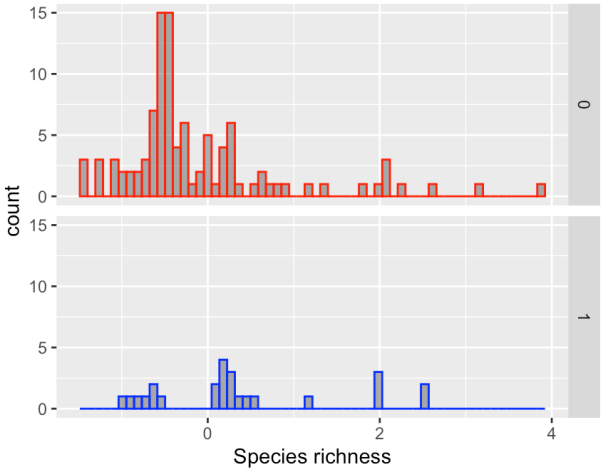
b) Annual precipitation (mm)



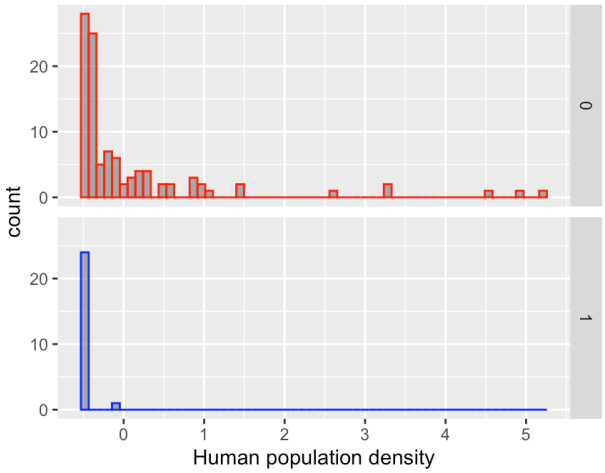
c) IUCN habitat type



d) Species richness



e) Human population density (people/km^2)


count
20
10
0
0
20
10
0
0
1
2
3
4
5
Human population density
0
1

Supplemental Table 3.1 Univariable analysis of putative risk factors for *T. gondii* oocyst shedding in free-ranging domestic and wild felids. Coefficients are shown as betas with a 95% confidence interval and p-value. Variables in bold were the most significant as determined by p-value and were evaluated in subsequent multivariable models.

Confirmed *T. gondii* studies (n=47, n felid groups = 50)

Variable Name	Effect Size Domestic (n=38)	p-value	Effect Size Wild (n=12)	p-value
Annual mean temperature	0.30 (-0.07, 0.66)	0.11	-0.33 (-1.00, 0.35)	0.34
Mean diurnal range	**0.52 (0.21, 0.82)**	**0.00073**	-0.33 (-0.10, 1.26)	0.09
Isothermality	**0.30 (0.03, 0.57)**	**0.0015**	-0.28 (-1.17, 0.62)	0.544
Temperature seasonality	-0.25 (-0.55, 0.05)	0.10	**1.52 (0.71, 2.34)**	**0.00025**
Max warm month	0.24 (-0.12, 0.60)	0.19	0.09 (-0.67, 0.84)	0.82
Min cold month	0.22 (-0.10, 0.55)	0.17	-0.74 (-1.50, 0.30)	0.06
Temperature annual range	-0.12 (-0.45, 0.20)	0.47	**1.25 (0.74, 1.76)**	**1.38e-06**
Mean wet quarter	0.03 (-0.003, 0.06)	0.078	-0.06 (-0.14, 0.02)	0.14
Mean dry quarter	0.27 (-0.07, 0.60)	0.115	**-1.06 (-1.69, -0.43)**	**0.00099**
Annual precipitation	-0.20 (-0.69, 0.29)	0.42	-0.08 (-0.53, 0.37)	0.73
Precipitation seasonality	**0.50 (0.14, 0.87)**	**0.007**	-0.33 (-1.09, 0.43)	0.39
Precipitation warm quarter	-0.29 (-0.62, 0.05)	0.09	0.06 (-0.41, 0.53)	0.80
Precipitation cold quarter	-0.18 (-0.65, 0.30)	0.46	-0.25 (-0.81, 0.30)	0.37
Precipitation wet quarter	0.0007 (-0.0008, 0.002)	0.42	-0.0005 (-0.003, 0.002)	0.70
Precipitation dry quarter	-0.47 (-0.90, -0.05)	0.027	-0.05 (-0.42, 0.33)	0.81
Precipitation wet month	0.0015 (-0.003, 0.006)	0.57	-0.002 (-0.009, 0.005)	0.64
Precipitation dry month	-0.44 (-0.87, -0.004)	0.048	-0.05 (-0.42, 0.32)	0.79
Vapor pressure	0.09 (-0.22, 0.40)	0.57	-0.05 (-1.15, 1.06)	0.94
Human footprint	0.33 (-0.14, 0.80)	0.17	0.26 (-0.78, 1.30)	0.63
Habitat type	0.02 (-0.14, 0.18)	0.77	0.06 (-0.29, 0.41)	0.74
Human population density	**0.40 (0.17, 0.63)**	**0.00058**	**4.14 (-0.10, 8.39)**	**0.056**

Species richness	0.20 (-0.09, 0.49)	0.18	0.48 (-0.78, 1.75)	0.45
Latitude	-0.003 (-0.015, 0.009)	0.60	-0.02 (-0.04, 0.008)	0.20

Supplemental Table 3.2 Pearson correlation coefficients of significant univariable variables prior to multivariable model building. 2a. reflects coefficients between variables assessed for free-ranging domestic cats, and 2b. reflects coefficients between variables assessed for wild felids. All temperature variables for wild felids (temperature seasonality, temperature annual range, mean temperature in driest quarter) were tested in separate models due to the high level of correlation between candidate variables.
a. Domestic

	Isothermality	Mean diurnal range	Precipitation seasonality	Human population density
Isothermality				
Mean diurnal range	0.12			
Precipitation seasonality	0.31	0.67		
Human population density	-0.0096	0.12	0.21	

b. Wild

	Temperature seasonality	Temperature annual range	Mean temp. dry quarter	Human population density
Temperature seasonality				
Temperature annual range	0.94			
Mean temp. dry quarter	-0.56	-0.47		
Human population density	-0.015	0.004	0.20	

Supplemental Table 3.3 Model selection results based on Akaike information criterion (AIC) for testing hypotheses for climate (temperature and precipitation) and anthropogenic (human population density) factors associated with *Toxoplasma gondii* oocyst shedding prevalence in free-ranging domestic cats. ΔAIC = change in AIC relative to the final model.

		AIC	ΔAIC
(1|study) + human population density + meandiurnalrange	Final model	-80.4	0
(1|study) + human population density + meandiurnalrange + precipitation seasonality		-79.4	1
(1|study) + human population density + isothermality + precipitation seasonality		-77.9	2.6
(1|study) + human population density + precipitation seasonality		-77.9	2.6
(1|study) + meandiurnalrange		-76.6	3.9
(1|study) + meandiurnalrange + precipitation seasonality		-75.7	4.8
(1|study) + precipitation seasonality + isothermality		-75.0	5.5
(1|study) + human population density + isothermality		-75.0	5.5
(1|study) + precipitation seasonality		-74.3	6.2
(1|study) + human population density		-73.5	6.9
(1|study) + isothermality		-72.0	8.5
(1|study)		-69.7	10.8

Supplemental Table 3.4 Wild felid model selection results based on Akaike information criterion (AIC) for testing hypotheses for climate (temperature and precipitation) and anthropogenic (human population density) factors associated with *Toxoplasma gondii* oocyst shedding prevalence in free-ranging wild felids. ΔAIC = change in AIC relative to the final model.

		AIC	ΔAIC
(1|study) + human population density + meandryquarter	Final model	-1.1	0
(1|study) + human population density + temp seasonality		-1.1	0
(1|study) + human population density + temp annual range		1.7	2.9
(1|study) + temp annual range		5	6.2
(1|study) + temp seasonality		7.8	9
(1|study) + meandryquarter		8.9	10.1
(1|study) + human population density		12.4	13.5
(1|study)		12.8	14

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Chapter 4 Quantitative risk analysis of oocyst versus bradyzoite foodborne transmission of *Toxoplasma gondii* in Brazil

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(Formatted for publication in "*Pathogens*")

Abstract

Toxoplasma gondii is a globally distributed zoonotic protozoan parasite. Infection with *T. gondii* can cause congenital toxoplasmosis in developing fetuses and acute outbreaks in adults, and disease burden is especially high in South America. Prior studies found that the environmental stage of *T. gondii*, oocysts, are an important source of infections within Brazil, however, no studies have quantified this risk relative to other parasite stages. We developed a Bayesian quantitative risk assessment (QRA) to estimate the relative attribution of the two primary parasite stages (bradyzoite and oocyst) that can be transmitted in foods to people in Brazil. Oocyst contamination in fruits and greens contributed significantly more to overall estimated *T. gondii* infections than bradyzoite-contaminated foods (beef, pork, poultry). In sensitivity analysis, treatment, i.e. cooking temperature for meat and washing efficiency for produce, most

strongly affected the estimated *T. gondii* infection incidence rate. Due to lack of regional food contamination prevalence data and high level of uncertainty in many model parameters, this analysis provides an initial estimate of the relative importance of food products. Important knowledge gaps for oocyst-borne infections were identified and can inform future studies to improve risk assessments and effective policy actions to reduce human toxoplasmosis in Brazil.

Introduction

Toxoplasma gondii is a ubiquitous protozoan pathogen that has three infectious life stages: tachyzoites, bradyzoites in tissue cysts, and sporozoites in oocysts. The definitive hosts of *T. gondii,* domestic and wild felids, are the only animals that can shed environmentally resistant oocysts in their feces. Although most infections are asymptomatic, *T. gondii* can cause mild to severe disease in intermediate hosts, including humans [1]. Infection usually occurs via three pathways: 1) by eating meat from infected animals that harbor *T. gondii* tissue cysts, 2) ingesting oocysts in contaminated soil, water, shellfish or fresh produce [2], or 3) via vertical transmission of tachyzoites across the placenta after primary infection or maternal reinfection during pregnancy [3,4]. Because infection with *T. gondii* is lifelong, if a previously infected individual becomes immunosuppressed, *T. gondii* can reactivate and cause encephalitis, pneumonia, and even death [5]. Vertical transmission during pregnancy can cause congenital toxoplasmosis (CT), which can lead to spontaneous abortion, stillbirth, or ocular and developmental disease in the developing fetus. The current estimated global annual disease burden for CT is 1.15 million disability-adjusted life years (DALYs) [6]. Preventing exposure for high-risk groups (i.e. immunocompromised individuals and pregnant women) is vital because there are no human vaccines, nor effective treatments that can eliminate the chronic bradyzoite cysts from tissues of infected individuals.

Brazil is one of the most populous countries in South America, with approximately 213 million residents and is characterized by high geographic heterogeneity, ranging from equatorial rainforests in the Amazon Basin to tropical savannahs in Central Brazil and even more diverse microclimates [7]. Toxoplasmosis disease burden is high in Brazil due to a combination of high environmental contamination, the presence of diverse, virulent genotypes of *T. gondii*, and poor or unreliable water

quality [8]. The incidence of CT in Brazil is 2 per 1000 births [6] and the prevalence of ocular toxoplasmosis can be as high as 1 in 6 in certain regions [9]. In Europe, meat-borne toxoplasmosis is perceived as a larger hazard (vs oocyst ingestion) based on source attribution studies of pregnant women in the early 2000s, where 30-63% of seropositive cases were attributed to eating uncooked or cured meats, while only 6-17% of cases were attributed to soil contact [10]. In contrast, all reported outbreaks in Brazil with known or suspected routes of transmission since 2000 have been attributed to oocyst-based transmission [11]. Income inequality and disparity is a persistent social and economic issue, with additional consequences for health outcomes. Infection from oocysts may represent a higher proportion of total infections in Brazil due to the disproportionate exposure to waterborne pathogens for individuals from lower socioeconomic groups, who often have limited access to treated drinking water [12,13]. The combination of ecological, environmental, and socioeconomic factors makes Brazil a vital location to study food and waterborne *T. gondii* transmission.

To prioritize intervention strategies, it is essential to identify the relative risk of exposure from different infection pathways. Various toxoplasmosis outbreaks have been reported worldwide, including in Canada and the United States, however, the majority of recent acute outbreaks have been reported in Brazil [14–16]. Common source attribution techniques used for outbreaks are epidemiological studies, outbreak investigation, expert elicitation, and risk assessment [17]. A recent expert elicitation by the WHO found that between 27-77% of acquired toxoplasmosis cases in South America are due to foodborne transmission [18]. Within foodborne transmission, few studies compare the importance of different food products such as meat and produce. Quantitative risk assessment (QRA), a method to estimate the potential risk of microbial exposure, has been applied to meat-borne transmission of *T. gondii* in China, Italy, England, the Netherlands, and the United States, but to our knowledge, no studies have compared the relative risk of bradyzoite and oocyst-borne transmission from food [19–24]. QRA assessments utilize results from surveys, prevalence studies, and dose-response studies in order to integrate data into models that will be used to guide decision-making. Data on parasite prevalence are available for many meat products, but water, soil, and produce are matrices where *T. gondii* prevalence

data are incredibly sparse; a recent systematic review found only 23 articles on the prevalence of *T. gondii* in fresh produce and 40 articles on *T. gondii oocyst* detection in water [25]. Of these studies, only 6 studies on water and 2 studies on produce were conducted in Brazil.

Given the limited knowledge surrounding the relative risk of oocyst foodborne *T. gondii* transmission, our main objective was to develop a Bayesian QRA model to estimate the risk associated with *T. gondii* exposure via ingestion of bradyzoites and oocysts from common foods in Brazil. Our aim was to characterize and compare the relative risk of oocyst vs non-oocyst infections and make management recommendations for transmission pathways of highest concern to people in Brazil.

Methods

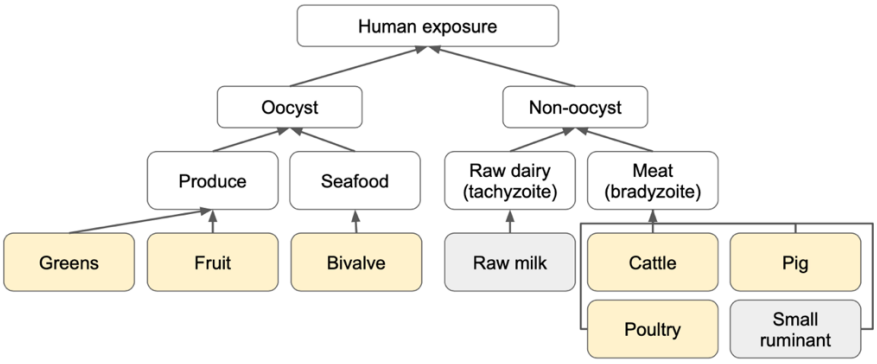

Figure 4.1 Summary of *T. gondii* transmission pathways by parasite stage and inclusion for consideration in the risk assessment model. Food groups evaluated in our models are highlighted in yellow.

The stochastic Bayesian QRA that we applied for characterizing the risk of *T. gondii* exposure consists of four distinct steps: 1) hazard identification (Figure 4.1), 2) exposure assessment, 3) dose-response, and 4) characterization of risk from each transmission pathway (oocyst or bradyzoite) repeated for each food type (Figure 4.2). The final outcome of the model was an adjusted incidence rate per 1000 people per day, which accounts for immunity among Brazilians previously exposed to *T. gondii*. Given limited data on *T. gondii* across the various food matrices tested, a Bayesian risk assessment approach was selected to evaluate the probability of oocyst and bradyzoite *T. gondii* transmission in Brazil. Sensitivity and scenario analysis were also performed to evaluate a range of possible outcomes given different initial assumptions. All analyses were performed in R using the 'rjags' package [26] to interface

between R and JAGS, a clone of BUGS (Bayesian analysis Using Gibbs Sampling). This framework can be adapted for future use when more data on regional and food-specific *T. gondii* prevalence are generated.

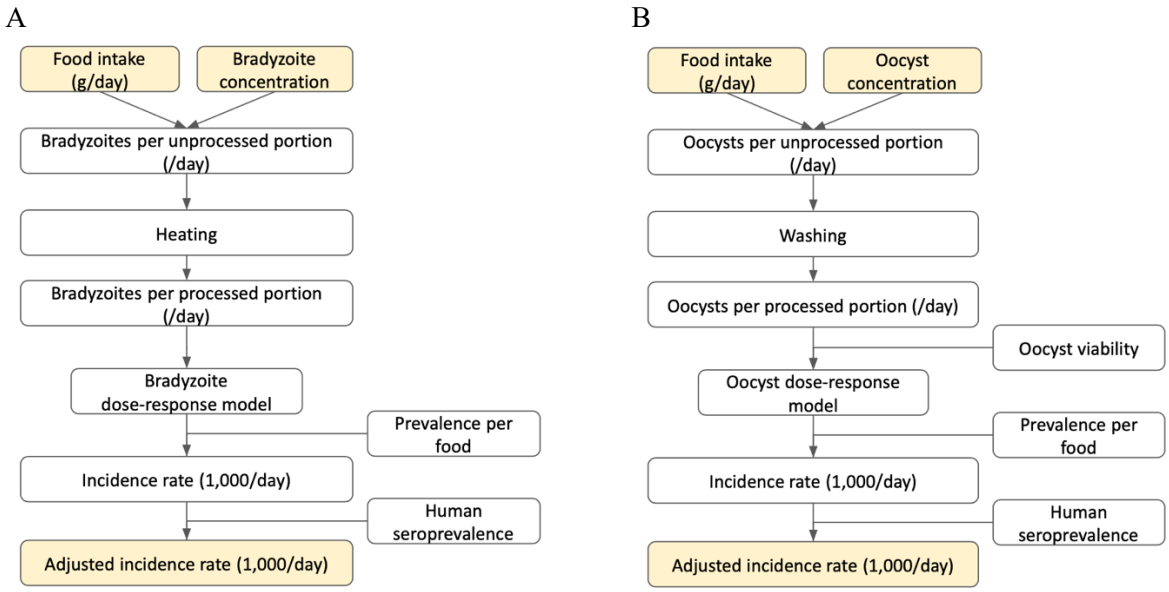

Figure 4.2 Conceptual framework for *T. gondii* infection via A) oocyst-borne and B) bradyzoite-borne food exposure in Brazil.

2.1. Hazard identification

T. gondii foodborne exposure is broadly divided into two categories, non-oocyst and oocyst-borne foods (Figure 4.1). For foods contaminated with parasite stages that are not oocysts, we distinguish between meat-borne bradyzoite cyst infection from beef, pork, poultry or ovine/caprine meat, and tachyzoite infections from unpasteurized dairy products. Oocyst-borne infections can result from consumption of contaminated produce (fruits and vegetables), seafood (fish and/or shellfish), as well as water. Our study focused on the foodborne route to tease out food preferences and preparation practices that can be targeted for mitigating foodborne disease, and thus did not include water as a potential source of exposure. Infections due to tachyzoites in raw dairy and bradyzoites from small ruminants were excluded from analysis due to the lack of consumption and dose-response data in Brazil.

Table 4.1 Model parameters and equations used in QRA of foodborne toxoplasmosis in Brazil

Variable	Equation/Distribution/Value	Reference
Exposure assessment bradyzoite		
N1: n log10-transformed bradyzoites/100g unprocessed meat	*N1* ~ Beta general (shape 1 = 6.5, shape 2 = 5.7, min = 0, max = 6.8) *transformed= Beta(0.955, 0.838)	[22]
N2: n bradyzoite consumed/meat type	*N2* ~ Poisson ($\lambda = C_{meat}$ x ($10^{N1*6.8}$/ 100))	[22]
C: food consumption amount (g)	Table S1	IGBE [32]
N3: n bradyzoites after home cooking per consumed portion	N3=N2 X RF(T)	[22]
RF(T): temperature reduction factor	RF(T)=D(T)/D(T0)	[22]
D(T): dose of bradyzoites	D(T)=-ln(1-P0(T))/r0	[22]
P0(T): probability of infection in mice	P0(T) = 1/ (1+e^-(44.181-0.834XT)	[23]
r0: *T. gondii* infection probability of a single bradyzoite in mice	r0 = 0.011	[23]
T: Temperature C	T~Laplace (m = location, s = dispersion) Pork m=71.11, s=9.88 Beef & sheep m= 71.11, s=9.82 Poultry m=75.56, s=9.31	[23]
T0: temperature before cooking (C)	T0= 25	[23]
Exposure assessment oocyst		
N4: n oocysts/g unprocessed produce	*N4* ~ Beta (shape 1 = 0.105, shape 2 = 0.702)	Fitted data from Marques et al. [33]
N5: n oocysts consumed/produce type	*N5* ~ Poisson (λ = Cproduce * *N4* * 180)	[22]
N6: n oocysts after washing W(T): washing	N6 X W(T) W(T) ~ Beta (1, 0.57)	Distribution fit from Temesgen et al. 2021 [34]
Dose-response		
P_1: probability of human infection (per meat type in one day)	P_1 = 1-e(-r1 * N3)	[35]
r1: probability of single bradyzoite initiating *T. gondii* infection in humans	r1 = 0.001535	[35]

P_2: probability of human infection (per produce type in one day)	$P_2 = 1\text{-}e(-r2 * N6)$	[36]
r2: probability of single oocyst initiating *T. gondii* infection in humans	r2=0.46	[36]
Risk characterization		
P_3: probability of infection through consumption of food product in human population (/meat or produce type per day)	$P_3 = P_1$ or P_2 x P_{food} x $(1\text{-}P_{human})$	[22]
P_{food} : prevalence per food type. Overall averaged prevalences for the entirety of Brazil were used due to the low number of available studies.	P_{food} : Supplemental Table 4.2	See references in Supplemental Table 4.2
P_{human} : seroprevalence of human population	P_{human} : Unif (0.215, 0.974)	[8]

2.2. Exposure assessment

Data on average daily consumption of specific foods that were included in our study were obtained from the 2017-2018 edition of Pesquisa de Orçamentos Familiares (POF, Family Budget Research) [27], which is administered by the Brazilian Institute of Geography and Statistics (IBGE). The data (mean grams consumed/day) focus on a subset of the Brazilian population aged 10 years and older and encompass differences in urban and rural areas across all Brazilian states. The 26 states and one federal district are commonly grouped into five regions based on geographic, social, and economic factors as used by the IBGE; north, northeast, center-west, southeast, and south (Figure 4.3).

2.2.1 Food intake quantity (g/day)

Bradyzoite exposure - Meat

Daily average per capita consumption of specific meat products from the 2017-2018 IBGE POF survey were grouped for analysis purposes under the source animal (Supplemental Table 4.1). Three broad categories were used; beef, pork, and poultry. Lamb and mutton were not incorporated into the model due to a lack of data on consumption from IBGE, and relatively low reported level of consumption (1.26kg/year) according to the Organization for Economic Co-operation and Development [28].

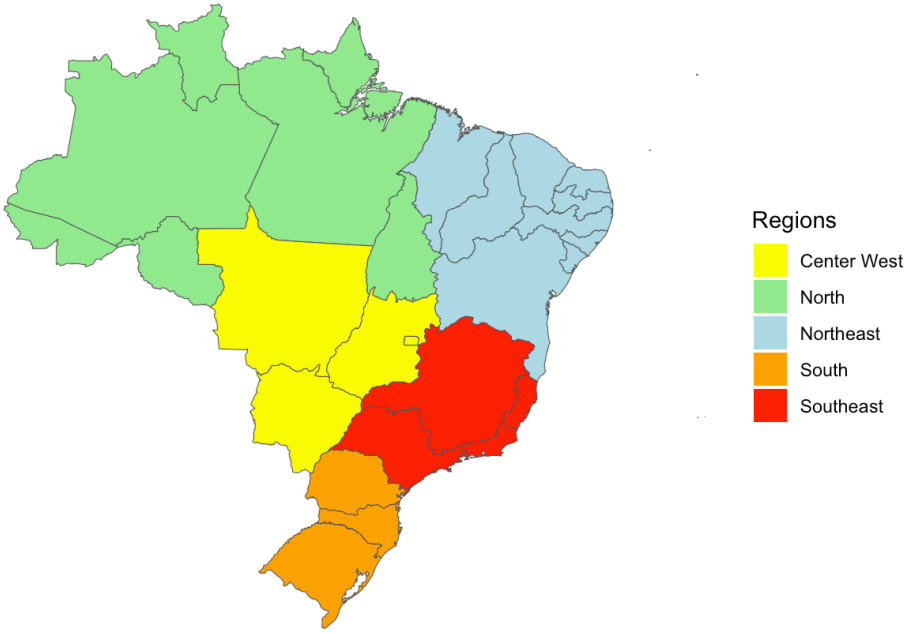

Figure 4.3 The five major regions of Brazil as classified by the Brazilian Institute of Statistics (IBGE). States are grouped based on similar geographical and socioeconomic characteristics, though there is still high heterogeneity both between and within regions.

Oocyst exposure - Fresh produce

For this study, we focused on fruits and vegetables that were previously associated with reports of *T. gondii* infections, i.e. salad mix, leafy greens, and açai berries [29,30]. Salad mix, leafy greens, and cabbage were combined under total "greens" consumption to reduce the number of categories in modeling. Total consumption of either greens or fruit was considered rather than consumption of individual products (mixed salad, lettuce).

Oocyst exposure - Seafood

Shellfish and fish can serve as mechanical vectors of *T. gondii* oocysts, passively concentrating oocysts in tissues or gastrointestinal tract without serving as a true intermediate host for the parasite [31]. Because fish digestive tracts or gills (where oocysts may be present) are generally removed before eating, the risk of oocyst exposure from fish is estimated to be lower than that of shellfish that are usually eaten whole and sometimes raw. Shellfish consumption is not evenly distributed across Brazil, nor was consumption data available for shellfish from the IBGE. To demonstrate how our model can be applied

towards a food product with limited data and to assess the potential role of raw shellfish consumption in foodborne toxoplasmosis risk, we obtained Pacific and native oyster production data from the state of Santa Catarina, which is the only state with industrial scale shellfish production, and converted it to estimated consumption for the Southern region of Brazil (Supplemental Table 4.2). Due to the limited data on shellfish consumption and oocyst concentration, we only assessed shellfish as a food source in specific scenario analysis, not in the baseline model.

2.2.2 Parasite concentration

The estimate of bradyzoite cyst concentration per gram of meat from infected animals was obtained from a study of naturally infected sheep in the Netherlands; although different hosts have different propensities to form cysts, this is one of the only published studies of cyst concentration in livestock and these estimates were used by other assessments of meat-borne *T. gondii* transmission [37]. The log10-transformed concentration of bradyzoites in an infected animal was described by a generalized beta distribution with shape α1 of 6.5, α2 of 5.7, a minimum of 0, and a maximum of 6.8 (mean 3.6; 3,981 bradyzoites per 100 g) as used by Opsteegh et al. and Deng et al. [22,23]. Because the JAGS framework does not support the generalized beta distribution by default, we used normalization to convert this to a beta distribution N1.1, where N1 is the original distribution, a is the minimum of N1, and b is the maximum of N1 [38].

$$\text{N1.1} \sim \frac{N1-a}{b-a}$$

$$\text{N1.1} \sim \frac{N1}{6.8} = \text{N1} \sim \frac{(6.5,5.7,0,6.8)}{6.8}$$

$$\text{N1.1} = \text{beta}(0.955, 0.838) * 6.8$$

Samples drawn from this distribution were then multiplied by 6.8 (the maximum concentration from the generalized beta distribution N1) to convert the values back into the proper log10-transformed concentration (Table 4.1).

As no studies of oocyst concentration on produce in Brazil exist, *T. gondii* oocyst concentration on fruits and vegetables was estimated using data from Spain and Portugal [33]. The theoretical

distribution according to the skewness-kurtosis graph from the R package 'fitdistrplus' [39] showed the data was beta distributed. Oocyst concentrations for fruits and vegetables were transformed into a proportion by dividing by the maximum concentration per gram of produce plus 0.1 (179.9 oocysts/g + 0.1 = 180) in order to be fit to a beta distribution. The transformed data followed a beta distribution with shape 1 = 0.26 and shape 2 = 0.70, which we used for both fruit and greens due to the small sample size for both types of produce. Samples drawn from this distribution were multiplied by 180 (maximum concentration from N4) to convert the values from proportions back into concentrations.

To date, there have been no reports in the literature that quantified oocyst concentrations in harvested shellfish, however experiments in our laboratory [40] found that the minimum limit of detection was 5 *T. gondii* oocysts per oyster. Another study in São Paulo demonstrated that the limit of detection by nested PCR was 100 oocysts [41], which we selected as a conservative upper limit of detection for studies reporting presence of the parasite. Assuming that the average edible weight of Pacific oysters (*Crassostrea gigas*), the primary shellfish species produced in Brazil, is ~20g [42], the maximum concentration of oocysts would be 100/20 or 5/gram; because we did not have data to fit a distribution, we used a uniform distribution with range 0.1-5 oocysts per consumed mussel for this parameter.

2.2.3 Parasites per unprocessed portion

The number of parasites per unprocessed portion of each food item tested were calculated with the following equations from Table 1. The number of bradyzoites (*N2*) follows a Poisson distribution with λ = the amount of each type of meat consumed multiplied by 10 to the power of bradyzoite concentration divided by 100 [22]:

$$N2 \sim \text{Poisson}\ (\lambda = C_{meat} \times (10^{N1}/\ 100))$$

The number of oocysts (*N5*) was also assumed to be Poisson distributed with λ calculated as the product of the amount of produce consumed multiplied by produce oocyst concentration:

$$N5 \sim \text{Poisson}\ (\lambda = C_{produce} * N4)$$

2.2.4 Parasites per processed portion

To account for loss of bradyzoite infectivity in cooked meat, the number of bradyzoites in infected meat that was cooked was calculated using the methods of Deng et al., who multiplied the number of bradyzoites in unprocessed meat by a temperature reduction factor [23], RF (Table 4.1).

Similarly, to account for the physical removal of oocysts from washed produce, the number of oocysts on washed produce was calculated in a similar manner using N6: N5 X W(T), where the number of oocysts remaining on washed produce (N6) was equal to the product of oocysts on unwashed produce (N5) times the proportion of oocysts remaining after washing, W(T). As there are no previous studies to our knowledge that have described the effect of washing on oocyst removal, we used washing data for *Cyclospora cayetanensis* from berries [34]. Sporulated *T. gondii* oocysts (10-12 μm) are similar to the size of *Cyclospora* oocysts (7.5-10 μm). The proportion of *Cyclospora* oocysts remaining after washing with water for 1 minute was fit to a distribution using the package 'fitdistrplus' [39] and followed a beta distribution with shape $\alpha 1=1$ and $\alpha 2$ of 0.57. No reduction factor (washing) or heat inactivation parameter was used for shellfish because oysters are often eaten whole and raw.

2.3. Dose-response assessment

The dose of potentially infective *T. gondii* oocysts consumed through food was calculated by multiplying the amount of food matrix consumed (grams) by *T. gondii* stage concentration (per grams) and contamination prevalence for each food product. Dose-response equations from previous *T. gondii* bradyzoite-borne and oocyst-borne risk assessments on mice and rats were used as no human dose-response experiments exist [22]. The probability that a single bradyzoite will lead to infection, also known as single hit probability of infection (r1), is 0.001535 (Table 4.1). The single hit probability of oocysts used in dose response equations was obtained from a recent study that used scaled and combined mouse, rat, and pig data [36]. A bradyzoite viability parameter in meat products was not considered because all meat was assumed to be consumed fresh; freezing meat for an extended period of time can inactivate bradyzoites [43]. In contrast, not all *T. gondii* oocysts that are present on food matrices are sporulated or viable, and there are no studies reported to date that have tested for oocyst viability on produce in field studies. As a conservative estimate for oocyst viability, we assumed that only 50% of

oocysts present in consumed foods were viable in our baseline model. The probability of illness from bradyzoites and oocysts were calculated with P_1 and P_2 respectively (Table 4.1).

2.4. Risk characterization

2.4.1 Prevalence of contamination by food product

T. gondii contamination across different types of food items varies geographically, so we collected estimates specific to Brazil whenever possible, and fit data to distributions to capture variability across studies (Supplemental Table 4.2). When prevalence estimates in Brazil were not available, we used data from other countries, as was the case for fruit.

2.4.2 Adjusted incidence rate

Many Brazilians have previously been infected with *T. gondii* and are likely to be immune to new infection in our analysis. To account for this, we calculated an adjusted incident exposure rate that accounts for prior immunity. Seroprevalence is highly variable within Brazil, but we obtained a pooled estimate of 28.6% (17535/61283) for the average seroprevalence of T. gondii in Brazil from a recent meta-analysis [52]. Most studies from the meta-analysis focused on women of reproductive age, but for the purposes of our study we assumed this prevalence was similar in the general population [52]. The number of infections in each region of Brazil was calculated by multiplying the exposure incidence rate/1000 people per day by a factor of 0.714 to estimate the number of new infections per year in the susceptible population.

2.5. Data analysis

Analysis was implemented in R version 4.2.2 using the packages ‘rjags’ and ‘coda’ [26,53]. The mean number of cases of oocyst or bradyzoite-borne toxoplasmosis from each source was calculated for the general population with 4 chains, 10,000 samples for adaptation, 50,000 iterations, and a burn-in interval of 5000. Autocorrelation and model convergence were assessed by looking at the trace and gelman plots (Supplemental Figure 4.3, 4.4).

2.6. Sensitivity and scenario analysis

Consumption amount, food prevalence, cooking temperature (meat), or washing (produce) were varied in sensitivity analysis of each parameter from -50 to +50% of the baseline estimate. Mean total cases were calculated using consumption quantities averaged over all five regions (Supplemental Table 4.1). A total of four scenarios were evaluated: 1) a lower concentration of bradyzoites in beef (1/100) compared to other meat products, 2) a produce washing efficiency that increases oocyst removal by 25%, 3) a decrease of oocyst viability by 50%, and 4) incorporation of shellfish as another food source of infectious oocysts (Table 4.2). Many *T. gondii* experts do not believe that beef is a significant source of exposure due to the difficulty in isolating viable infectious parasites from cattle muscle tissues [44,54]. The first scenario explores one potential mechanism which may explain the lower source attribution of beef by lowering bradyzoite concentration. In scenario 2, we explored how improved washing efficiency alone could reduce parasite exposure by highlighting a food preparation practice that can be easily implemented by consumers. In scenario 3, we assumed that oocyst viability was half of the baseline model (50% viable), or 25% overall oocyst viability. In scenario 4, shellfish consumption was added based on production patterns and dietary habits from correspondence with collaborators in the state of Rio de Janeiro (P. Souto Rodrigues, R. DaMatta, and L. Bahia-Oliveira).

Table 4.2 Description of scenarios assessed in QRA model of foodborne toxoplasmosis burden in Brazil

Scenarios	Definition
Baseline model	Baseline consumption of food products averaged over all five regions of Brazil to compare against other scenarios
1) Lower bradyzoite concentration in beef	Bradyzoite concentration for beef lowered to 1/100 that of other meat species to reflect lower estimated tissue cyst burden in cattle
2) Improved washing efficiency	Washing oocysts off of produce increased by 25% from baseline
3) Decreased oocyst viability	Oocyst viability assumed to be half as viable from baseline, 25% viability overall
4) Inclusion of shellfish	Oyster consumption added as another source of oocyst foodborne transmission due to its growing popularity. Production data was obtained for the South of Brazil and converted to consumption quantity (g) (Supplemental Table 4.3).

Results

Mean incidence of estimated foodborne *T. gondii* infections per 1000 people per day that were estimated in our risk analysis are summarized in Table 4.3 and Figure 4.4. Across the five regions of Brazil, the estimated range of mean incidence was higher for oocyst-borne infections (leafy greens 7.58-9.68 and fruit 11.76-48.13, respectively) as compared with bradyzoite-borne infections from meat (beef 3.58-6.32, pork 0.97-2.03, and poultry 0.11-0.17). Overall, estimated incidence of *T. gondii* infection was highest in the North, and oocyst-borne infections from fruit contributed the most to the risk of foodborne infection in this region. Risk estimates for oocyst-borne infections from greens and fruit had very large credible intervals (CI); for example, the incidence of infection per 1000 people from fruit in the north region of Brazil was 48.13, but the 95% CI ranged from 0 to 314.2 (Table 4.3).

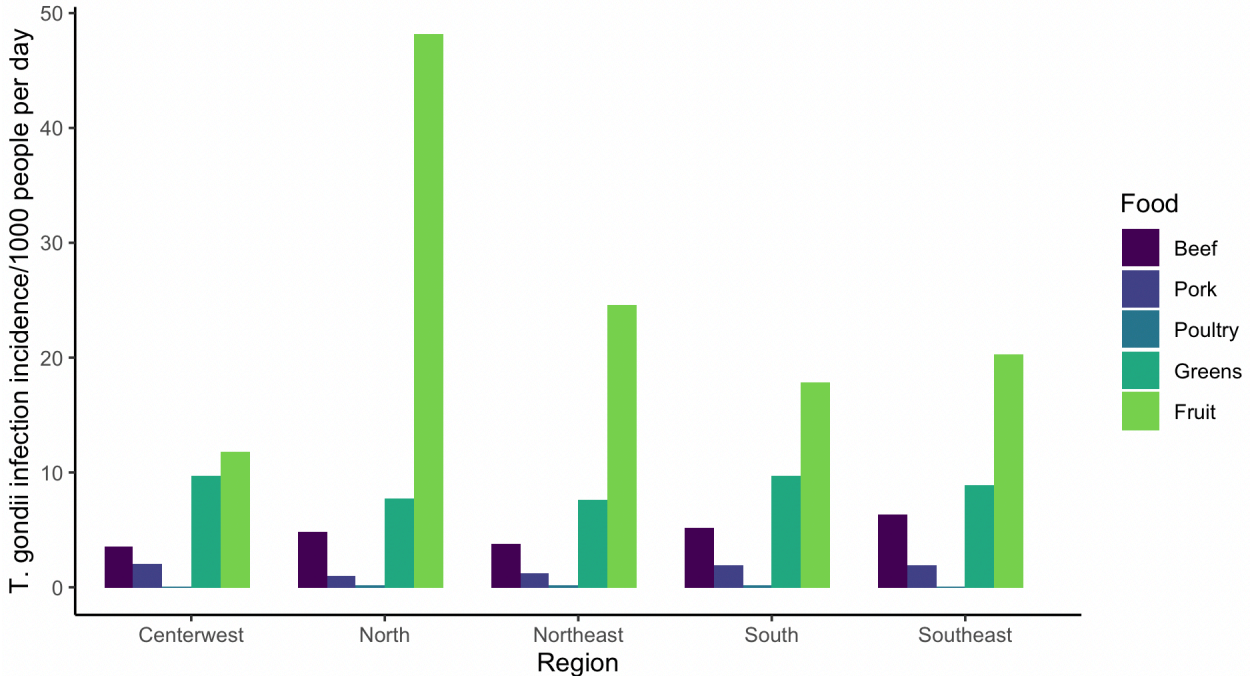

Figure 4.4 Baseline results for risk analysis estimating the mean oocyst and bradyzoite foodborne *T. gondii* infection incidence/1000 people per day in five regions of Brazil.

3.1 Sensitivity analysis

We varied parasite concentration, consumption quantity, *T. gondii* prevalence in food, and heating or washing efficiency to evaluate effects of these parameters on the estimated incidence of foodborne infection. Our sensitivity analysis demonstrated that heating and washing efficiency were the most

important variables affecting our incidence estimates for *T. gondii* infection from bradyzoites and oocysts, respectively (Supplemental Figure 4.1).

3.2 Scenario analysis

In addition to the baseline model that estimated *T. gondii* infection incidence by region, several scenarios were chosen to evaluate the role of specific foods and processing methods that may be of high interest to food producers and/or consumers (Figure S2). In the first scenario, reducing the concentration of bradyzoites in beef compared to other meat types reduced estimated incidence from beef consumption compared to the baseline model (-99%). When oocyst washing efficiency was improved by 25%, the estimated infections per 1000 persons per day were greatly reduced from both greens (-35%) and fruit (-40%). A reduction in the number of oocysts that were viable on produce also reduced infections from greens (-14%) and fruit (-10%), and thus total infections, however this reduction was less pronounced as compared with the oocyst washing efficiency scenario. Finally, we explored how other food sources such as shellfish can play an important role in local risk for foodborne *T. gondii* exposure. Although the average estimated consumption of shellfish was low, incorporating this seafood as a product did increase total incidence slightly (+0.6%).

Table 4.3 Baseline model with estimated incidence of foodborne toxoplasmosis per 1000 people, 95% credible interval [CI], and percentage of total infections by region.

	North		Northeast		Center west		Southeast		South	
Food	Mean [95% CI]	%	Mean [95% CI]	%	Mean [95% CI]	%	Mean [95% CI]	%	Mean [95% CI]	%
Beef	4.81 [1e-06, 46.1]	7.8	3.78 [0, 48]	10.1	6.32 [4e-08, 59.4]	16.9	5.19 [0, 53.4]	14.9	3.58 [8e-07, 39.6]	13.2
Pork	0.97 [0, 12.7]	1.6	1.28 [0, 16.3]	3.4	1.91 [0, 20.3]	5.1	1.91 [0, 20.7]	5.5	2.03 [0, 23.6]	7.5
Poultry	0.13 [1e-07, 1.3]	0.2	0.17 [0, 1.8]	0.5	0.11 [0, 1.1]	0.3	0.15 [0, 1.36]	0.4	0.11 [0, 1.1]	0.4
Greens	7.69 [0, 60.2]	12.5	7.58 [0, 57.4]	20.3	8.86 [0, 71.9]	23.6	9.68 [0, 70.4]	27.9	9.67 [0, 80.3]	35.6
Fruit	48.13 [0, 314.2]	77.9	24.62 [0, 259.5]	65.7	20.31 [0, 179.5]	54.1	17.85 [0, 198.1]	51.3	11.76 [0, 118.1]	43.3

Total	61.73	37.43	37.51	34.78	27.15

Discussion

In this study, we present the first risk analysis model designed to quantify and discriminate the risk of oocyst and bradyzoite foodborne transmission in Brazil. Exposure assessments for oocysts are especially needed as a recent review of the literature found that out of seven Brazilian outbreaks that occurred between 2000-2018, 42.8% were attributed to meat and 28.6% were attributed to produce, highlighting the importance of oocyst exposure [11]. The remainder of recent outbreaks were due to water (14.3%) and soil (14.3%), which further highlight the importance of oocyst exposure to human infection. Congenital and acute toxoplasmosis disease burden are both incredibly high in Brazil [8,9,55], and quantitative assessment tools are necessary in order to improve food safety practices that can help reduce risk of exposure.

Estimated incidence rates for *T. gondii* exposure were simulated for five regions of Brazil to identify the relative importance of different foods as sources of infection in regions that may have different dietary habits. Beef contributed the most to meat-borne infection in all regions, which agrees with the findings of a Dutch QRA but not with a more recent QRA performed for China [22,23]. These differences may depend heavily on food consumption preferences between countries; even within our analysis, estimated incidence from a given food group could be 1.5-2 times higher between the lowest and highest regions (Table 4.3). Regional heterogeneity of infections was also present in a recent retrospective analysis of infant mortality associated with congenital toxoplasmosis in Brazil, suggesting that regional exposure risk differences can play a role in pathogen transmission [56]. Predicted infections from oocyst-borne foods such as fresh produce (i.e. fruits and greens) were up to 100 times greater than infections from eating meat (Table 4.3), which may be an overestimate of the actual relative importance of produce due to assumptions made in our baseline model.

Sensitivity analyses demonstrated that oocyst concentration on fresh produce had a substantial effect on estimated risk. Our estimates of this parameter were from a single study in Portugal and Spain

that quantified the number of oocysts on fresh leafy greens and berries from local suppliers/markets [33]. We pooled data for *T. gondii* oocyst count on greens and fruit due to the limited number of samples, which may not accurately capture differences in oocyst load among different types of produce. The presence and amount of parasite contamination can vary dramatically across geographic regions, and can be affected by produce source, washing prior to sale, storage conditions, vendor knowledge on food safety, as well as produce type [57–59]. Produce contamination by *T. gondii* during cultivation or transport cannot be controlled by consumers, so this is an aspect of food production and processing that could be more tightly regulated by improvements to food safety practices and guidelines. In tandem with production level changes, improved education and awareness can lead consumers to alter their individual food consumption and preparation practices in order to reduce *T. gondii* oocyst exposure.

Under experimental conditions, a single oocyst can cause *T. gondii* infection in an intermediate host such as a rodent or pig, though the exact infectious dose for humans is unknown [36,60]. Additionally, not all oocysts in the environment are viable and infective, or able to lead to excystation, cell invasion and replication in a host. The proportion of oocysts that are viable or infective on produce was another parameter where we lack robust quantitative estimates. In soil, oocyst viability can range from 7.4% in dry conditions to 43.7% in damp conditions after 100 days [61]. As many vegetables are grown in soil, these values could be used to inform estimates of oocyst concentration on leafy greens. New detection methods for distinguishing between viable and non-viable oocysts in water exist, but many of these techniques are not yet widely available or used across studies that estimate *T. gondii* oocyst contamination in foods [62]. Like Deng et al. we assumed a baseline viability of 50% but recognize that this assumption may overestimate the true oocyst viability/infectivity on fruits, vegetables, and within shellfish tissues, and thus oocyst-borne infections in our model [63].

Washing produce in order to remove oocysts was the most influential variable for oocyst-borne infections in our sensitivity analyses, but similar to oocyst concentration, there were few published sources that we could use in determining accurate distributions for model inputs. The study that we referenced looked at the percentage of parasites removed (*Cyclospora cayetanensis*) from blueberries and

raspberries using different methods such as washing in running water, washing with vinegar, and using a salad spinner after washing [34]. Because the size and surface properties of different parasite oocysts can vary drastically, *T. gondii* oocysts may actually be more or less resistant to removal by washing compared to *Cyclospora*. Variables such as berry type, washing duration, tap water quality, water infrastructure quality, and parasite type could all change the proportion of oocyst removal due to washing. We assumed that most households in Brazil would wash their produce with tap water but testing how washing and other treatments such as ultraviolet light disinfection (UV) can remove or inactivate *T. gondii* oocysts on produce can enhance our understanding of risk in realistic settings. The exact method of parasite treatment may be more or less relevant depending on the location of study, as methods such as UV may be less realistic for widespread application in LMICs.

We recognize that *T. gondii* prevalence in different foods will vary across regions within a country or even locally. Brazil is the largest country by land mass and population size in South America, and a handful of studies cannot cover the breadth of variation in *T. gondii* contamination patterns across different food matrices and subsequent *T. gondii* exposure. For example, *T. gondii* seroprevalence in the state of Minas Gerais can range from 2.7 to 31.4%, a 10-fold difference [64,65]. Regional or individual food preparation, consumption preferences, and socioeconomic status can also alter exposure risk [52,66]. In addition to heterogeneity in regional parasite prevalence in foods, the consumption data we used from IGBE is supposed to be representative of consumption patterns by region, but these values are a population average. The results presented in this study describe average relative risk, not absolute risk, and are better suited to guide general public health recommendations rather than individual change in food preferences.

We explored structural assumptions of our model in scenario analysis, namely reducing bradyzoite concentration in beef, increasing washing efficiency for produce, lower oocyst viability, and inclusion of shellfish as an additional food group. Although beef was the primary source of toxoplasmosis from meat, we acknowledge that this may overrepresent the actual risk of infection due to the low numbers of viable bradyzoites that are isolated from beef compared to meat from other livestock species

[44]. Similar to Deng et al., we found that when beef concentration was reduced to 1/100 of other meat, toxoplasmosis incidence decreased to be more on par with pork and poultry [23]. Both increased oocyst removal from washing and an assumption of lower oocyst viability reduced estimated toxoplasmosis incidence, though washing was more effective (Supplemental Figure 4.2). This suggests that more empirical information on viability and washing may need to be gathered, and that in the meantime washing with clean water sources (free of *T. gondii* oocyst contamination) could be prioritized in dissemination of proper food safety guidelines to consumers. Shellfish consumption is a risk factor for toxoplasmosis as they are generally consumed whole and may be eaten raw, putting consumers at a greater risk of exposure to *T. gondii* compared to foods which are treated to remove or inactivate parasites [67]. We explored the role of shellfish consumption preferences in the south of Brazil, the main region where shellfish is produced commercially. As shellfish consumption only represented a small fraction of the food consumed on a daily basis (0.185 g/person/day Supplemental Table 4.3), this food accounted for 0.6% of infections in scenario four, which was roughly the same percentage as poultry (0.2%). Though the predicted risk of infections from shellfish in our model was small, production of shellfish in Brazil has increased in the last decade as its popularity has grown among consumers. If shellfish becomes a more lucrative and sought-after product within and outside of southern Brazil, infections from this food type may become more common. There are no official reports of shellfish consumption because current production and consumption are still low, however this example illustrates how we can use modeling to explore relative patterns and potential scenarios as food preferences change or as more data are produced from on-going research.

Oocyst genotype and virulence were not accounted for in our model, but it is important to note that not all *T. gondii* genotypes may be equally efficient at causing severe symptoms in humans [68]. Because South America has high *T. gondii* genetic diversity, there are many opportunities for novel and repeated exposure to virulent genotypes [69,70]. Prior exposure to *T. gondii* in Brazil is common; up to 70% of pregnant women are seropositive in regional surveys [52]. Infection with one genotype may not be protective against future *T. gondii* infections with different parasite genotypes; mixed infections in

humans can lead to systemic illness [4,71,72]. Thus, a level of immunity that completely protects against reinfection in the Brazilian population is not likely to be reached due to the diversity of circulating strains. This may be one of the limiting intrinsic factors of our model.

Though previous epidemiological studies in Brazil have highlighted the importance of waterborne transmission, we were unable to evaluate water as a source of *T. gondii* oocysts due to the limited data on *T. gondii* prevalence and oocyst concentration in water. Additionally, we considered regional patterns of food consumption, whereas water is essential and has less geographic variability in consumption. The ability of contaminated water to cause large scale outbreaks in Brazil and elsewhere cannot be underestimated [12–14]. Numerous waterborne outbreaks have been reported in Brazil since 1966, including a 2018 outbreak with at least 2270 suspected cases, which was the largest oocyst-associated outbreak reported to date [16]. One of the reasons that waterborne transmission is so common is variability in the quality of drinking water available to many Brazilians; only up to 83.3% of the population has access to safe drinking water [73]. There are huge disparities in the availability and quality of clean water in Brazil across socioeconomic levels [74]. Aside from a lack of knowledge of proper food safety, unreliable or nonexistent access to treated water can put individuals from poorer socioeconomic backgrounds at a higher risk for *T. gondii* exposure. Mitigating transmission in poorer and/or rural areas of Brazil may require greater attention and resources in order to equitably address disease prevention. Improving existing infrastructure to increase access to clean, potable water can have cascading benefits to *T. gondii* transmission as a whole by reducing waterborne exposure and allowing for increased removal of oocysts from produce when tap water is used for washing food.

We developed a novel QRA model for relative comparison of oocyst and bradyzoite-borne *T. gondii* infections from food in Brazil. Predicted infections from produce were ~8-13 times higher than infections from meat sources across different regions. Modeling studies which identify potential knowledge gaps are critical for exploring appropriate risk mitigation at different food production and preparation steps. For example, in addition to sampling food products to obtain *T. gondii* contamination prevalence, studies that identify sources of oocyst contamination at farms, produce processing, or

transport can be conducted in tandem. Oocyst-borne transmission of *T. gondii* should be given greater attention and resources for intervention and management, and avenues for further research include investigations on oocyst concentration and viability, oocyst removal and inactivation methods, contamination prevalence, and characterization of different parasite of genotypes in common food products. As more data on these variables, especially contamination prevalence and oocyst removal methods become available, we can continue to use this modeling framework in Brazil and in other countries to obtain more accurate estimates of *T. gondii* infection risk from oocyst-contaminated foods as compared with meat-borne infections.

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Supplemental Table 4.1 Average daily consumption of food products (g) by region, data from 2017-2018 IGBE POF survey [32]

Stage	Food	North	Northeast	Southeast	South	Centerwest	Average (used in sensitivity and scenario analysis)
Bradyzoite	Bovine	58.6	41.5	48.4	50.8	80.7	56
	Porcine*	15.3	15.1	20.3	29.5	31.5	22.34
	Poultry	51.8	58.7	44.9	37.4	35.8	45.72
Oocyst	Greens	12.3	14	28.8	22.5	37.7	23.06
	Fruit	45.4	1.6	1.1	0.4	2.6	10.22

*Porcine meat included pork as well as specific pork derivatives such as linguiça (smoke-cured pork sausage), mortadella (heat-cured pork), and ham [75,76].

Supplemental Table 4.2 Prevalence of *T. gondii* in food by product type

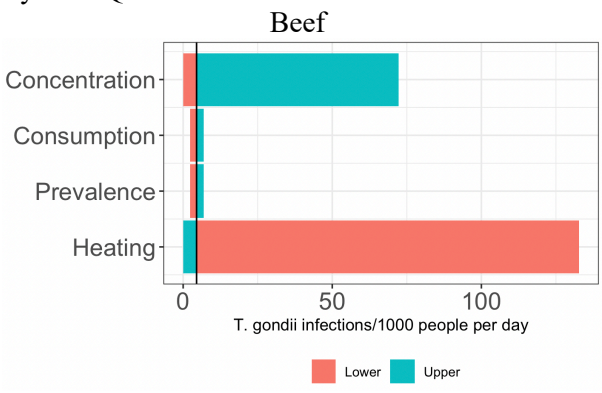
Food	Distribution	Sources
Meat - Bovine	Beta ~ (0.74, 1.90)	Brazil [44]
Meat - Porcine	Beta ~ (1.12, 3.87)	Brazil [45]
Meat - Poultry	Beta ~ (1.19, 2.14)	Brazil [46]
Produce - Greens	Beta ~ (0.7, 19.9)	Brazil [47-50]
Produce - Fruit	Beta ~ (0.66, 3.56)	Columbia [51], Spain/ Portugal [33]
Seafood - Bivalves*	Beta ~ (24.7, 1112.8)	Brazil [12]

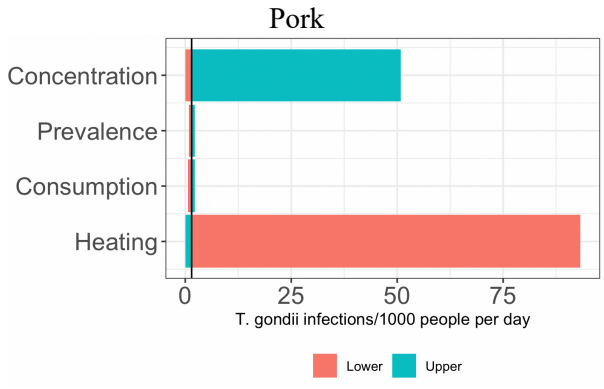
*Values used in Scenario 4 only

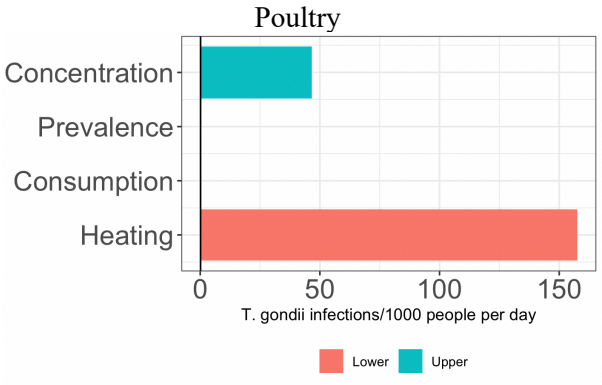
Supplemental Table 4.3 Declared federal production of oysters in Santa Catarina, Brazil in 2020. These numbers represent a conservative estimate of the true production as producers are taxed on harvest amounts in federal waters. Values are shown in tons produced and amount of edible product in kilograms (tons * 0.2) calculated using the USDA Handbook on Weights, Measures, and Conversion Factors for Agricultural Commodities and Their Products [77]. Grams per person per day was calculated by dividing the edible weight by the estimated number of adult residents in the Southern region of Brazil that consume shellfish. This figure was estimated by multiplying the proportion of people over 15 (0.789 [78]) by 57.38% (percentage of people who report eating seafood at least once a month [79]) and by the number of residents; 30,192,315 [7].

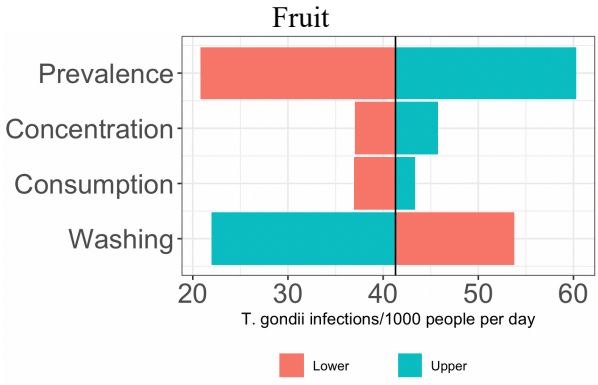
Seafood type	Tons produced	Edible weight (kg)	g/person/year	g/person/day
Pacific oyster (*Crassostrea gigas*)	2215.5	886200	64.8	0.18
Native oyster (*Crassostrea spp.*)	65.01	26004	1.9	0.005
Total	2280.51	912204	66.7	0.185

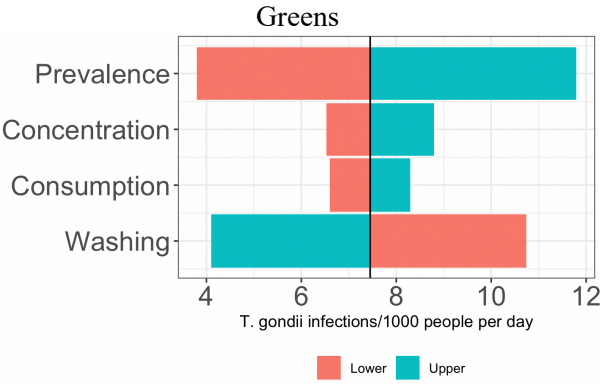
Supplemental Figure 4.1 Sensitivity analysis visualized through tornado plots for model parameters in Bayesian QRA model.



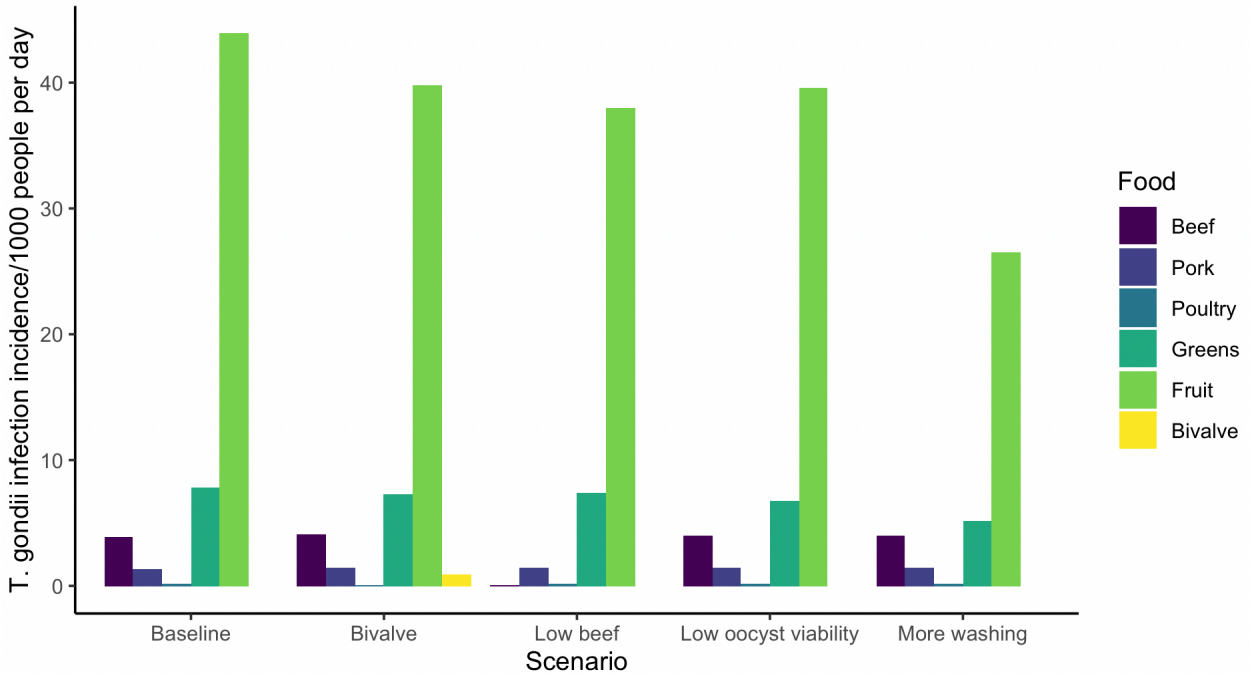




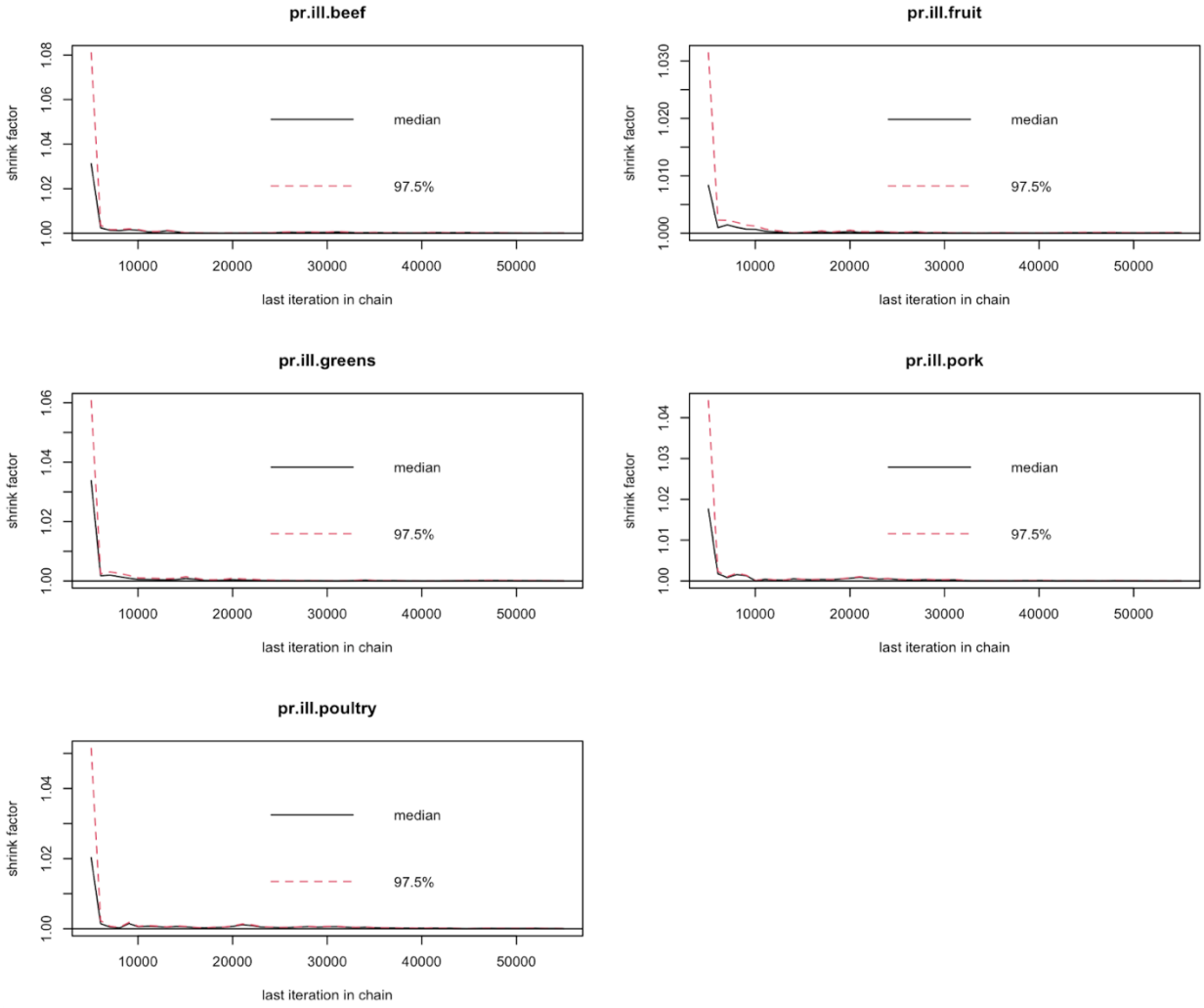




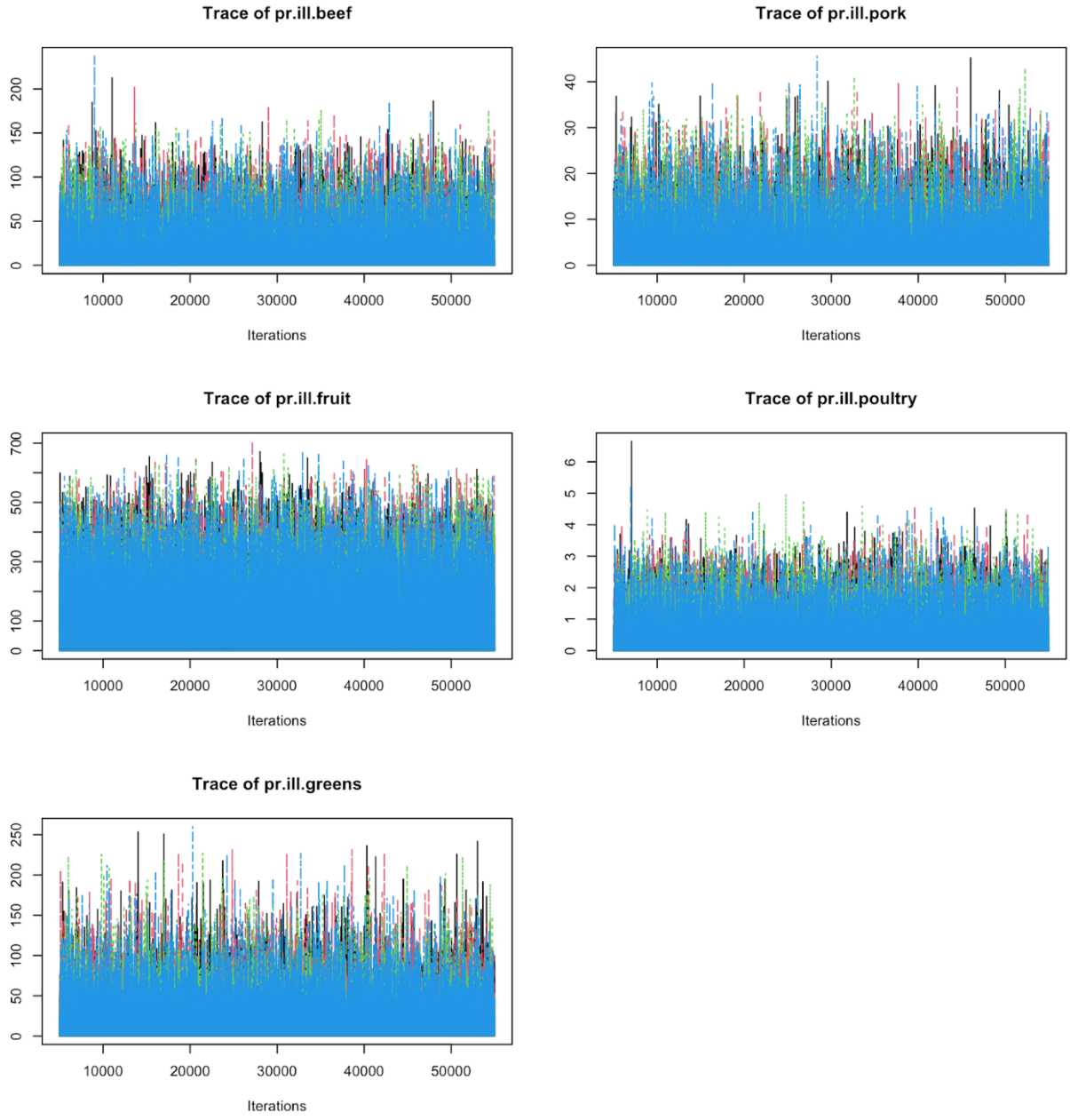
Supplemental Figure 4.2 Scenario analysis results for putative scenarios of interest. Food consumption was averaged over all five regions of Brazil to home in on simulated change due to parameter assumptions. Baseline: consumption of food products averaged across all regions of Brazil. Bivalve: Oocyst consumption added as an additional source of foodborne exposure to *T. gondii.* Low beef: Bradyzoite concentration lowered to 1/100 compared to that of other meat products. Low oocyst viability: Oocyst viability halved from baseline to 25%. More washing: Washing produce increased by 25% from baseline.



Supplemental Figure 4.3 Gelman plots for Bayesian QRA model for the Northern region of Brazil, indicating lack of autocorrelation and presence of convergence.



Supplemental Figure 4.4 Trace plots for Bayesian QRA model for the Northern region of Brazil, indicating adequate mixing and presence of convergence.



Conclusions

This dissertation addresses current gaps in *Toxoplasma gondii* transmission dynamics in diverse ecosystems and populations in California and Brazil using a combination of empirical, statistical, and modeling approaches. As *T. gondii* oocysts drive transmission of toxoplasmosis as a whole, knowledge gained from these studies will benefit both animal and human health.

In Chapter 1, we used a longitudinal field survey of feral cat colonies in the greater Monterey Bay region to assess temporal trends in *T. gondii* prevalence and genotypes shed in feces. Few studies have documented oocyst shedding in feral cats, a felid population that likely accounts for a large percentage of overall environmental oocyst contamination, especially near urbanized areas. Genotype can be instrumental in determining disease outcome and link sources of definitive host populations and infections in intermediate hosts, yet genotype detection from felid feces is uncommon due to the difficulties in obtaining high quality samples from 'dirty' matrices (feces, soil, water). We did not detect any *T. gondii* oocysts over 24 months of sampling, which could be due to low sensitivity of detection using microscopy, low probability of obtaining fecal samples right after primary infection, or oocyst degradation in the environment. However, we were able to detect *T. gondii* DNA in a surprisingly high proportion (25.9%) of samples tested with PCR. The majority of strains had alleles consistent with Type I or Type I variants at the B1 gene, but the second most common strains were atypical Type X and X variants, which were previously associated with mortality in southern sea otters. *T. gondii* DNA detection was high across sampling sites (19.4-32.4%) and was higher in the wet season versus dry season (18.1 vs. 34.9%). Taken together, these results show that feral cats are exposed to a diversity of *T. gondii* genotypes in the environment, and that they have the potential to shed virulent strains in feces. No guidelines currently exist to regulate feral cat populations on the basis of zoonotic disease transmission, but our study could justify future management efforts that aim to reduce the burden of toxoplasmosis in susceptible wildlife as well as humans.

Next, I sought to evaluate the impacts of climate and anthropogenic activities on *T. gondii* oocyst shedding globally using data from free-ranging domestic and wild felids. Prior studies found positive

associations between annual mean temperature and human population density and *T. gondii* seroprevalence in wild birds and mammals. Oocyst shedding typically only lasts for 1-2 weeks, compared with lifelong tissue infection and serological detection of circulating antibodies in definitive and intermediate hosts. Still, we found that higher human population density was associated with increased *T. gondii* oocyst shedding in free-ranging domestic cats and wild felids, which warrants further investigation as human populations and urbanization continue to increase. We also found relationships between *T. gondii* oocyst shedding and temperature variables such as mean diurnal range (domestic cats) and mean temperature in the driest quarter (wild felids). These variables reflect temperature fluctuations and seasonality rather than average temperature trends. Most *T. gondii* oocyst shedding studies have not been designed to assess relationships with changes in temperature and precipitation over time, and the mechanisms by which climate may affect oocyst shedding, sporulation, and survival are still not well understood. Further studies of the influence of global change (temperature, precipitation, habitat alterations, anthropogenic influence) on *T. gondii* oocyst shedding and transmission are needed to understand how exposure risk for humans and other vertebrate species may change over time.

Finally, we evaluated the risk of oocyst-borne and bradyzoite-borne *T. gondii* infections in Brazil, a high-risk region of South America. Brazil has many factors that contribute to high toxoplasmosis burden, including diverse, virulent *T. gondii* genotypes, large populations of free-ranging domestic cats and wild felids, and inequalities in clean water access. In recent years, oocyst transmission has been found to be the cause of the majority of outbreaks and infections, including water-borne outbreaks in Brazil. Oocyst transmission is largely understudied with regard to food safety, so we conducted a Bayesian quantitative risk assessment to compare the risk of *T. gondii* exposure from different foods. Incidence of toxoplasmosis was estimated to be highest from oocyst sources such as fruit and leafy greens. In sensitivity analysis, parasite treatment through heating of bradyzoites (cooking meat at proper temperatures) and washing oocyst-contaminated foods were identified as the most important parameter that influence predicted numbers of new *T. gondii* infections. In scenario analysis, we used this

framework to illustrate how foods that are growing in popularity, such as açai, can drastically affect total incidence.

Taken together, the results of this dissertation highlight the importance of *T. gondii* oocyst transmission across species and geographical regions. These findings can be used to guide management strategies, highlight key regions and populations for surveillance, and inform food safety decisions. Such interventions and public health practices will only become more important in light of ongoing anthropogenic changes affecting *T. gondii* ecology, transmission and epidemiology.