TOXOPLASMA GONDII DETECTION FROM NATURALLY INFECTED CATS (*FELIS CATUS*) IN HAWAI‘I

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ABSTRACT

Toxoplasmosis is a disease affecting humans, mammals, and birds. The disease is caused by *Toxoplasma gondii*, a coccidian protozoan. Cats are the definitive host of *T. gondii* and can shed oocysts in their feces into the environment. To address these objectives, 120 soil samples and 60 fecal samples were collected from four cat colony sites at the University of Hawaiʻi at Mānoa, and nine fecal samples were obtained directly from cats from Kaʻena Point Natural Area Reserve on Oʻahu. *Toxoplasma gondii* DNA was extracted and amplified using standard PCR procedures. DNA was detected in five fecal samples from UHM and two fecal samples from Kaʻena Point NAR, though no soil samples were positive. The presence of *T. gondii* at the university suggests that cat colonies may cause potential health hazards urban areas and the presence of actively infected cats at the NAR poses a disease risk to native wildlife.

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bp – base pair

IgG – Immunoglobulin G test

IgM – Immunoglobulin M test

NAR – Natural Area Reserve

PCR – Polymerase Chain Reaction

SG – Specific Gravity

CHAPTER 1

INTRODUCTION

Overview of *Toxoplasma gondii*

Zoonotic diseases are a major threat to wildlife and human health, especially when the diseases have a high capacity to spill over into domestic animal and human populations, and vectors have high migration between urban and rural areas (Daszak et al. 2000; Weiss and McMichael 2004). Such diseases can become increasingly problematic in urban areas, especially as urban areas encroach into wildlife habitat, which can lead to increased human-wildlife interactions (Bradley and Altizer 2006). Wildlife that easily adapt to foraging in urban areas tend to form large aggregations where food is available (Bradley and Altizer 2006; Brittingham and Temple 1988; Jones and Reynolds 2008). These aggregations readily become reservoirs and vectors for parasites and diseases such as rabies, Lyme disease, mange, salmonella, and toxoplasmosis.

Toxoplasmosis is caused by *Toxoplasma gondii*, a tissue-cyst forming coccidian protozoan, which infects humans, mammals, and birds (Beazley and Egerman 1998; Tenter et al. 2000). The lifecycle of *T. gondii* consists of both sexual and asexual stages, with the former occurring only in the cat family Felidae, which are the definitive hosts, and the latter being the stage within intermediate hosts (Figure 1.1) (Dubey and Jones 2008). Felids also are the only host able to shed environmentally-resistant oocysts, one of three infectious stages, in their excrement. The infectious stages in the parasite's lifecycle are the tachyzoites (rapidly multiplying stage), bradyzoites (tissue cysts), and oocysts (the stage that is shed into the environment) (Dubey 2010b). Tachyzoites

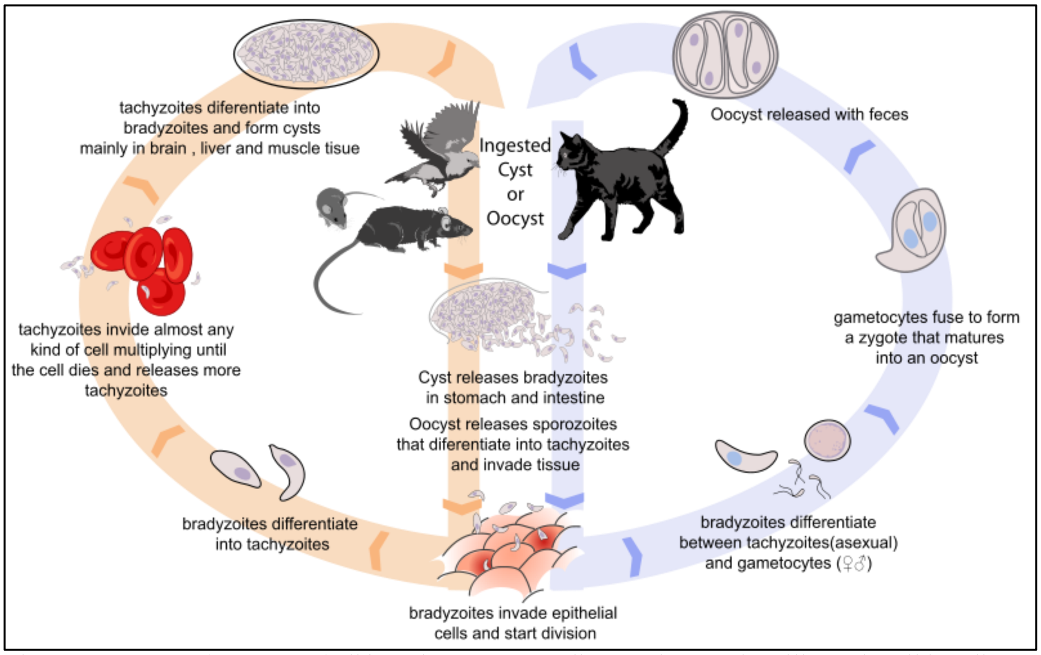

Figure 1.1: *Toxoplasma gondii* lifecycle. Image credit: Mariana Ruiz Villarreal, Wikimedia Commons.

penetrate a wide range of host cells and rapidly multiply asexually, while bradyzoites are found primarily in nervous and muscle tissues. Oocysts have an initial unsporulated stage that is excreted by felids, followed by an infective sporulated stage within one to five days of shedding (Dubey 2010b). Disease transmission usually occurs through ingestion of tissue cysts and/or tachyzoites in raw or undercooked meat, ingestion of feed or grass that have oocysts from felid feces, ingestion of fruits and vegetables that have grown in soil with oocysts, inhaling oocysts that have been aerosolized when soil or cat litter is highly disturbed, or drinking oocysts in contaminated water (Dubey and Jones 2008; Lass et al. 2011; Tenter et al. 2000; Teutsch et al. 1979).

Toxoplasma gondii oocysts are extremely resistant to most environmental conditions for long periods of time, and require extremely high temperatures to lyse (Dubey 2010b). Oocysts generally survive longest in warmer, humid climates in shaded areas within felid feces or soil, though there are examples of oocysts surviving varying conditions for long periods of time (Dubey 1998; Dubey 2010b; Frenkel et al. 1975; Lindsay et al. 2002; Lindsay and Dubey 2009). For example, oocysts have survived in feces outdoors in soil for 18 months in temperatures ranging from -20 to 35 ˚C in Kansas, and for up to 12 months in temperatures ranging from 15 to 30 ˚C in Costa Rica (Frenkel et al. 1975). Notably, oocysts have survived up to ~54 months in water at 4 ˚C and 18 months in sea water (Dubey 1998; Lindsay et al. 2003). Recently, Lélu et al. (2012) demonstrated that 7.4% of oocysts survived under dry conditions and 43.7% of oocysts survived under damp conditions after 100 days. Thus, *T. gondii* oocysts have been found to be resistant to a wide range of environmental conditions under which they can survive and have a minimum viability of 18 months.

Domestic cats (*Felis catus*) are one felid of particular concern due to their association with people. In urban settings, cats often form aggregates, or colonies, in areas where people regularly feed cats and shelter is available (Finkler et al. 2011; Natoli 1994). Feral (no contact with humans and food is from wild sources) and stray (some contact with humans and food may partially be from human sources) cats living freely outdoors tend to have higher instances of toxoplasmosis infection than pet cats since they are more likely to prey upon intermediate hosts (Dubey 2010b; Dubey and Jones 2008). Cats contract toxoplasmosis by ingesting prey items that have been infected by *T. gondii* and generally develop immunity to the protozoan after initial infection, though subsequent infections and reshedding of oocysts can occur (Dubey 2010b). Transmission from cats to other pets and wildlife does occur either when other animals ingest oocysts from the environment or when a predator consumes infected prey. Toxoplasmosis infections have been found in a wide range of animals and in most ecosystems, including pets (other cats, rabbits, etc.), livestock (pigs, sheep, chickens, etc.), wildlife (primates, aves, canids, etc.), marine species (pinnipeds, sea otters, cetateans, including polar bears), and endangered species (Dubey 2010b; Dubey and Jones 2008; Jensen et al. 2010; Tenter et al. 2000). While symptoms vary by species, lethargy and delayed reaction time are common and abortion and death sometimes occur (Dubey 2010b). Certain species can also be asymptomatic (latent) for toxoplasmosis infections, such as sheep, chickens, goats, and dogs (Dubey 1980; Dubey et al. 2007a; Dubey et al. 2007b; Dubey et al. 2007c; Dubey 2010a; Jacobs et al. 1963).

In humans toxoplasmosis can be contracted either congenitally (between mother and fetus) or clinically (infection from environmental exposure or ingesting infected meat), with most clinical cases being asymptomatic. However, symptoms are often severe in the fetus of pregnant or in individuals with compromised immune systems, either due to an immunodeficiency disease (e.g., AIDS) or undergoing immunotherapy (e.g., transplant patients) (Dubey and Jones 2008; Tenter et al. 2000). The typical manifestations of toxoplasmosis infection in people include ocular lesions and blindness, lymphadenopathy, encephalitis, sepsis syndrome, myocarditis, hepatitis, or death (Dubey 2010b; Dubey and Jones 2008; Tenter et al. 2000).

Although infections in healthy individuals have been considered asymptomatic, recent behavioral studies found behavioral changes in people with previous and current toxoplasmosis infections. For instance, women testing positive for latent toxoplasmosis (unknown previous infection without symptoms) had higher intelligence, were more apprehensive, self-reproaching, insecure, and may have had higher ergic tension (i.e. tense, impatient, frustrated) and radicalism (Flegr and Havlicek 1999). Similarly, Novotna et al. (2005) found men that were seropositive for toxoplasmosis (positive antibodies) were more reflective, reserved, slow, and controlled compared to men that tested negative for toxoplasmosis. Several other studies highlighted by Flegr (2007) consistently found that infected men were "more likely to disregard rules and were more expedient, suspicious, jealous, and dogmatic," while women were "more warm hearted, outgoing, conscientious, persistent, and moralistic." Furthermore, studies have found that people with latent toxoplasmosis were at a higher risk of traffic accidents, especially in people with recent or substantial infections, and had a higher rate of suicidal attempts (Flegr et al. 2002; Webster and McConkey 2010; Yagmur et al. 2010). Of considerable interest are the correlations between toxoplasmosis, schizophrenia, and brain cancer, which has been investigated by multiple studies in recent years (Thomas et al. 2011; Webster and McConkey 2010).

Scientists hypothesize that *T. gondii* alters intermediate host behavior to make predation by the definitive host more likely, ensuring the completion of the protozoan's lifecycle, termed the manipulation hypothesis (Berdoy et al. 2000; Lamberton et al. 2008). Specifically, both Berdoy et al. and Lamberton et al. demonstrated that rats infected with *T. gondii* lacked anti-predatory behaviors and were more likely to approach areas with the scent of cats, thereby possibly increasing predation. Furthermore, Pandrovszky et al. (2011) found that dopamine metabolism increased in mice experimentally infected with *T. gondii*, supporting the manipulation hypothesis. Increased dopamine metabolism may lead to behavioral changes in its host, such as alteration of the control of movements, reward behaviors, fear, and motivation.

Prevalence of toxoplasmosis infections in humans varies by location, and is often associated with cultural habits and hygiene (Dubey 2010b). Infections tend to be most prevalent in areas with warm climates, such as some South American countries. Countries with very high seropositivity (>70%) are generally between 30˚ North and South latitudes and include Brazil, Costa Rica, Cuba, Gabon, Ethiopia, and Indonesia. Notably, however, Turkey and Yugoslavia have also been found to have high seropositivity. Seroprevalence in the United States between 1999 and 2004 was estimated to be 9.0% in people between the ages of 12 and 49 years (Jones et al. 2007). Between 1988 and 1994, 14.1% of men and women over the age of 12 were seropositive in the US (Jones et al. 2007). The decreasing trend in toxoplasmosis infections in the US has been attributed to improved food handling and sanitation procedures, and improved education for at-risk groups. It is important to note that toxoplasmosis is a non-notifiable disease in the US, making the decrease in seroprevalence questionable as to whether or not reporting has decreased rather than incidence. Most infections now occur in adults and children that frequently come in contact with contaminated soil, handle cat feces (e.g. cleaning cat litter), or handle waste.

Detecting *Toxoplasma gondii* in the Environment

To date, there is no single method to detect *T. gondii*, or rather, there are a variety that differ based on sample mediums, including microscopy, antibody evaluation, bioassays, and molecular detection methods (specifically, DNA extraction and amplification using polymerase chain reaction [PCR]). Molecular detection methods have been found to be the most sensitive, least time consuming, and most economically feasible approach for detecting *T. gondii* oocysts (Garcia et al. 2008; Salant et al. 2010).

Different studies have used different methods for DNA extraction, depending upon such things as the medium (water, soil, tissue, or food) where *T. gondii* is found to occur (Dumètre and Dardè 2003). Specific procedures to extract DNA have included homogenizing the samples using agitation and washing with dispersion solution, filtration in gauze, sucrose floatation, and destroying oocysts with freeze-thaw events (Afonso et al. 2008), grinding with glass beads, and proteinase K digestion (Salant et al. 2007). In addition, several studies have also used commercial DNA extraction kits (Homan et al. 2000; Lass et al. 2009; Mancianti et al. 2010; Salant et al. 2007; and Salant et al. 2010). A complicating factor in analysis is that there are up to 245 known strains of *T. gondii*, many of which may amplify differently in PCRs (Aizenberg et al. 2004). Viable PCR markers were identified by Su et al. (2006) for 46 different strains of *T. gondii*, though at least 245 strains are known (Aizenberg et al. 2004).

In environmental samples, oocyst isolation would be expected to be highest when sampled directly from felid feces using PCR methodologies. However, relatively low percentages of feces analyzed across studies have tested positive for oocysts. Specifically, Pena et al. (2006) reported 1.2% of 237 samples were positive for *T. gondii* oocysts in cats from Brazil, Schares et al. (2008) reported 0.11% of 24,106 samples were positive in cats from Germany, Austria, France, and Switzerland, and Salant et al. (2007) reported 9% of 122 samples were positive in cats from Israel. Higher rates have been reported in samples tested with microscopy, which could be due to the similarity of *T. gondii* oocysts with other coccidians, such as *Hammondia hammondi* (Dubey 2010b).

Isolating oocysts from soil is extremely difficult as oocysts become integrated and disperse in the medium and may be protected by soil macroaggregates. Soil macroaggregates are between 0.25 and 2 mm in diameter and consist of clay particles (<0.002 mm) silt particles (0.002-0.05 mm), sand particles (0.05-2 mm), and various organic matter particles bonded together by plant roots, fungal hyphae, and microbial and plant polysaccharides. Because *T. gondii* oocysts are between 0.01 and 0.012 mm and have the potential to be protected within soil macroaggregates formed over time. Though water or mechanical dispersion methods may disperse most macroaggregates, dispersion solution may be required to break water-stable aggregates and several studies have used dispersion solutions to release oocysts from soil macroaggregates (Afonso et al. 2008, Lélu et al. 2011) though no study has been conducted to test whether different dispersion methods significantly increased oocyst recovery. Lélu et al. also demonstrated that simple sucrose floatation methods were highly successful in isolating oocysts from soil samples. Studies that have successfully isolated oocysts from soil samples using PCR methods have had positive samples ranging from 5.4% to 17.8% (Afonso et al. 2008; Du et al. 2012; Lass et al 2009).

Though several studies have attempted to isolate oocysts by inoculating water, two of three studies were able to isolate oocysts from drinking water taken from environmental sources (de Moura et al. 2006; Isaac-Renton 1998; Villena et al. 2004). Isolating oocysts from water is the most difficult since oocysts may be dispersed more readily as water moves and most studies that have attempted have not been successful (Aubert and Villena 2009; Isaac-Renton 1998; Yang et al. 2009). Villena et al. was able to detect *T. gondii* DNA in 8% of water samples, though no samples could be confirmed in mouse bioassays.

Toxoplasmosis in Hawai‘i

The state of Hawai‘i is well known for many things, including its year-round warm, humid climate and an overabundance of invasive and endangered species. Feral and stray cats occur in forests of Hawai‘i (Amarasekare 1994) and in multiple colonies found throughout urban areas in the islands (Lohr et al. 2013a). Because of the high number of feral cats, there are concerns over toxoplasmosis transmission to Hawai‘i's people, and native and endangered species. Feral cats on O‘ahu were first demonstrated to have toxoplasmosis in the early 1970s when Wallace (1971) isolated oocysts using microscopy and bioassays in mice. Wallace found that 0.5% of cats tested (1,023) were shedding oocysts, though antibodies were found in 20% of 522 cats tested using the Sabin-Feldman dye test. Likewise, on Hawai‘i Island, Danner et al. (2007) found 37.3% of 67 cats were seropositive for toxoplasmosis, with 3% of the cats tested were positive for new infections (IgM), 30% were positive for previous infections (IgG), and 5% tested were positive for both IgM and IgG.

Toxoplasmosis infections have been identified in a number of Hawaiian animals, including two endangered species (the Hawaiian crow [*Corvus hawaiiensis*] and the Hawaiian monk seal [*Monachus schauinslandi*]), one spinner dolphin (*Stenella longirostris*), and two other native bird species (the Hawaiian goose [*Branta sandvicensis*] and red-footed booby [*Sula sula*]) (Hawai‘i Department of Health 2000; Dubey and Jones 2008; Honnold et al. 2005; Migaki et al. 1990; Work et al. 2000, 2002). Within O‘ahu's livestock herds, 48.5% of 509 pigs tested positive for toxoplasmosis (Dubey and Jones 2008). Because Hawaiian monk seals are marine mammals, the infection infers *T. gondii* transmission through soil runoff, which may have further implications for ocean recreation users in Hawaiʻi and other wildlife. However, no studies have been conducted on environmental contamination of *T. gondii* oocysts in Hawaiʻi. Given the high cat densities in urban areas of Hawaiʻi, coupled with the large number of endangered animals, there is a critical need for understanding the prevalence of *T. gondii*.

According to the Hawaiʻi State Department of Health, there were two reported human cases of toxoplasmosis in the state of Hawaiʻi in 2010 (Hawaiʻi Department of Health 2010). The number of toxoplasmosis cases in the state has fluctuated over the past 10 years, exhibiting a decreasing trend. Specifically, in 2001 the number of reported cases was 22, compared to four in 2005. However, it is important to note that the U.S. Center for Disease Control and Prevention (CDC) states that toxoplasmosis is a non-notifiable disease, but one in which the CDC maintains surveillance. Because toxoplasmosis is generally asymptomatic, people may not be aware that they are infected. Therefore the decreasing trend in reported cases, though significant, does not reflect the prevalence of the disease in the environment. There are no data on the feral cat population in urban Hawaiʻi and feral cat colonies are generally allowed to proliferate, so it can be assumed that people using areas with feral cat colonies have the potential to be exposed to the disease.

Justification and Hypothesis

Although the likelihood of humans contracting toxoplasmosis has been decreasing in the US, there is concern among certain at-risk groups. In general, these groups include people with immunity disorders, pregnant women, landscaping personnel, and waste handling personnel that are in close proximity to cat colonies. Toxoplasmosis is also a threat to certain wildlife species, especially on islands where endemic species have evolved without exposure to toxoplasmosis. Hawaiʻi's main islands have both urban areas where cats often form colonies at feeding sites and remote locations where cats occur at lower densities, all within relatively close proximity of each other. The goal of this study was to determine if *T. gondii* occurs at both urban and remote sites in Hawaiʻi. Fecal samples from cats culled from Kaʻena Point NAR, and soil and fecal samples from colonies at the University of Hawaiʻi were tested to determine if the disease was present at both sites and the rates at which it occurs. The University of Hawaiʻi at Mānoa is located in Honolulu, Hawaiʻi (21˚ 18' 01" N, 157˚ 49' 23" W), a city with a human population of over 300,000 (United States Census Bureau 2012). The university's Office of Facilities and Grounds has reported approximately 13 colonies of feral cats that have established around the campus (unpublished data). There is a concern that the landscaping personnel may be coming in contact with *T. gondii* oocysts in soil. Another concern is that oocysts in the soil may be spreading to endemic and endangered species in the state. Kaʻena Point Natural Area Reserve (Kaʻena Point NAR) is located less than 65 kilometers (21˚ 34' 31" N, 158˚ 16' 27" W) from Honolulu. Though people often utilize the trail in the reserve, cats do not come in contact with people. Several species of concern occur at Kaʻena Point NAR and there are concerns that any of the animals utilizing the site may be at risk of infection, though no tests have been conducted.

The objectives of this study were to test 120 soil and 60 cat fecal samples from four randomly selected cat colonies from the University of Hawaiʻi at Mānoa, and to test cat feces from nine cats culled from within the Kaʻena Point NAR predator-proof fence for *T. gondii* oocysts using PCR. It has been estimated that 1% of cats are shedding oocysts at any point in time and a single cat is able to shed up to 80 million oocysts in a day for up to 13 days after initial infection (Dubey 2001; Dubey 2010). Given this estimation and the estimated number of cats occurring on the university campus (~250 cats [unpublished data]), it could be expected that approximately two cats could be shedding oocysts at any time throughout campus. Considering the sampling locations (~82 cats [unpublished data]), it would be likely that no cats (or 0.82 of a cat) would be shedding oocysts. Similarly, if this concept is applied to the small number of cats sampled from Ka‘ena Point NAR, no cats (or 0.09 of a cat) would be expected to shed oocysts at the time of sampling. The number of oocysts that could be expected to be occurring in soil at the university could also be extrapolated. Using a conservative estimate for the number of oocysts shed by a cat (~160 million), oocysts might be expected to be present in 0.00001% of the soil on campus (~6028536.3 m^3 of open soil and grass up to 10 cm depth). Given these extrapolations, it was hypothesized that the detection rate of *T. gondii* oocysts would be greater at the University of Hawai‘i at Mānoa than at Ka‘ena Point NAR, simply based on the number of samples available. However, it is important to note that cats at the university obtain a large portion of their food from human sources, rather than cats at Ka‘ena Point NAR that must hunt and scavenge for wild-based food. This will increase the likelihood that a cat at Ka‘ena Point NAR would be infected. Soil samples received a sucrose floatation treatment to concentrate oocysts and all samples were tested using a commercial DNA extraction kit and PCR. Management recommendations were made based on the results.

CHAPTER 2

TOXOPLASMA GONDII DETECTION FROM CAT COLONIES IN URBAN HAWAIʻI

Abstract

The presence of large numbers of feral cats (*Felis catus*) has raised concern both in terms of predation of native species and as vectors of disease in Hawaiʻi. One disease that has aroused a great deal of attention is toxoplasmosis, caused by the *Toxoplasma gondii* parasite. Because toxoplasmosis infects humans and native wildlife in Hawaiʻi, there is concern about its presence in the environment. Cats are the definitive host of *T. gondii* and are the only host that shed oocysts in their feces into the environment. The objectives of this study were to test soil for *T. gondii* oocysts at cat colony sites and fecal samples from feral cats in a natural area reserve using molecular identification methods. Soil and fecal samples were collected from cat colony sites at the University of Hawai`i at Mānoa and fecal samples from cats from Ka`ena Point Natural Area Reserve. Samples were tested for *T. gondii* oocysts using the QIAGEN QIAamp DNA Stool Mini Kit and amplified using standard PCR procedures. *Toxoplasma gondii* DNA was detected in five of 60 fecal samples from the University of Hawaiʻi at Mānoa, though no oocysts were detected from soil samples. Similarly, two of nine samples were positive for *T. gondii* DNA in cats from Ka`ena Point Natural Area Reserve. The presence of *T. gondii* at the university suggests that cat colonies may be a potential health hazards for landscaping personnel, students, staff, and visitors. Likewise, actively infected cats at Kaʻena Point Natural Area Reserve and other ground-nesting seabird colonies and marine mammal

haul-out sites pose a disease risk and should be considered when managing these areas. Because toxoplasmosis is a growing concern to both people and wildlife, further work is needed in determining it's pathway through the ecosystem and its longevity in the environment.

Key Words

Cat, *Felis catus*, Hawai‘i, natural area, oocyst, PCR, *Toxoplasma gondii*, toxoplasmosis

Introduction

Domestic cats (*Felis catus*) have proliferated in almost every environment, including arid deserts in Australia and sub-arctic/Antarctic islands (Nogales et al. 2004; Say et al. 2002). In urban settings, feral and stray cats often form aggregates, or colonies, in areas where people regularly feed cats and shelter is available (Finkler et al. 2011; Natoli 1994). Feral and stray cats living freely outdoors tend to have higher instances of toxoplasmosis infection than pet cats since they are more likely to prey upon intermediate hosts (Dubey 2010; Dubey and Jones 2008). *Toxoplasma gondii* is a tissue-cyst forming coccidian protozoan that causes the toxoplasmosis infection in humans, mammals, and birds. The lifecycle of *T. gondii* consists of both sexual and asexual stages, with members of the cat family (Felidae) being definitive hosts for the sexual stage. Felids are the only hosts to accommodate both the asexual and sexual stages of the protozoan and shed the environmentally-resistant oocysts in their excrement (Dubey and Jones 2008). Once oocysts are shed, transmission can occur through ingestion of oocysts in soil, grass, fruits and vegetables, inhalation, or in contaminated water (Dubey 2010; Dubey and Jones 2008; Tenter et al. 2000; Teutsch et al. 1979).

Oocysts are extremely resistant to most environmental conditions. For instance, oocysts have been shown to survive in fecal deposits in soil for 18 months outdoors in temperatures ranging from -20 to 35 ˚C and for two to 12 months outdoors in temperatures ranging from 15 to 30 ˚C (Frenkel et al. 1975). Notably, one study found oocyst survival to be up to approximately 54 months in water at 4 ˚C (Dubey 1998). Recently, Lélu et al. (2012) demonstrated that 7.4% of oocysts survived under dry conditions and 43.7% of oocysts survived under damp conditions after 100 days. However, oocysts are generally less likely to survive for long periods of time at colder temperatures, in dry climates, in water, and/or in direct sunlight, as compared to warmer temperatures, humid climates, in feces and soil, and/or in shade (Dubey 2010).

Toxoplasmosis in domestic cats (*Felis catus*) is of special concern as people coexist with cats both as pets and as a nuisance species. Feral (no contact with humans and food is from wild sources) and stray (some contact with humans and food may partially be from human sources) cats living freely outdoors tend to have higher instances of toxoplasmosis infection than pet cats since their diet consists of wild prey (Dubey 2010; Dubey and Jones 2008). Cats contract toxoplasmosis by ingesting prey items that have been infected by *T. gondii* and generally develop immunity to the protozoan after initial infection, though transmission occurs congenitally as well. Similarly, transmission from cats to other pets and wildlife occurs via ingestion of oocysts from the environment and when predators consume infected prey. Toxoplasmosis infections have been found in both domestic and wild animals, including marine mammals and several endangered species (Dubey 2010; Dubey and Jones 2008; Tenter et al. 2000). While symptoms vary by species, lethargy and delayed reaction time are common, and abortion and death sometimes occur. *Toxoplasma gondii* has also been linked to reduced fear and greater roaming behaviors in prey items (rats, mice, etc.) (Lamberton et al. 2008). A study by Prandovszky et al. (2011) has found that *T. gondii* infected mice had a significant increase in dopamine metabolism, supporting evidence of behavioral changes related to toxoplasmosis infection.

Oocysts entering the environment through felid feces are likely to become part of soil runoff into streams and near-shore areas (Miller et al. 2008). As a result, there have been significant cases of toxoplasmosis in marine mammals due to environmental exposure to the disease and consumption of mechanical hosts, such as bivalves and fish (Arkush et al. 2003; Massie et al. 2010; Miller et al. 2008). Transmission pathways for *T. gondii* infections in marine mammals and birds are poorly understood. Most studies reporting infections in marine mammals and birds are the result of finding sick or dead animals in the environment or from captive animals becoming sick, though one study experimentally infected grey seals (*Halichoerus grypus*) (Gajadhar et al. 2004). *Toxoplasma gondii* infections have been found in a number of marine mammals, including pinnipeds, cetaceans (primarily dolphins), sea otters, and manatees (Dubey 2010). However, few studies have investigated toxoplasmosis in seabirds, with the only examples from black-headed gulls (*Larus ridibundus*), common terns (*Sterna hirundo*), ring-billed gulls (*Larus delawarensis*), laughing gulls (*Larus atricilla*), red-footed boobies (*Sula sula*), and several penguin species (Burridge et al. 1979; Literák et al. 1992; Mason et al. 1991; Pak 1970, 1976 in Dubey 2010; Ratcliffe and Worth 1951; Work et al. 2002). Most marine mammals and birds do not prey on intermediate hosts of *T. gondii*, suggesting that transmission occurs in a different way.

The general consensus as to how toxoplasmosis is transmitted to marine mammals and certain marine birds is through soil runoff and oocyst uptake by marine bivalves and filter-feeding fish (Conrad et al. 2005). *Toxoplasma gondii* oocysts have been shown to survive up to 24 months in sea water at 4 ˚C (Lindsay and Dubey 2009) and there are several examples of *T. gondii* oocysts in bivalves and filter-feeding fish. Mediterranean mussels (*Mytilus galloprovincialis*) were experimentally exposed to oocysts and *T. gondii* DNA was detected in tissue up to 21 days post exposure, a wild California mussel (*Mytilus californianus*) was found with *T. gondii* Type X DNA, similar to infected southern sea otters (*Enhydra lutris nereis*) in the area, and DNA was detected in northern anchovies (*Engraulis mordax*) and Pacific sardines (*Sardinops sagax*) experimentally exposed to oocysts (Arkush et al. 2003; Massie et al. 2010; Miller et al. 2008). No bivalves or filter-feeding fish in the studies listed showed *T. gondii* infection, suggesting that they are mechanical hosts for toxoplasmosis in marine mammals and birds.

In humans, toxoplasmosis can be contracted either congenitally or clinically and most cases remain asymptomatic (latent). However, symptoms are severe in the fetus of pregnant women, often causing deformities and stillbirths, and in individuals with compromised immune systems, causing fever, seizures, other neurological problems, and possibly death (Dubey and Jones 2008; Tenter et al. 2000). Infections in healthy individuals have been considered asymptomatic, though recent studies have found associations between toxoplasmosis infection rates and behavioral differences, schizophrenia, higher risk for traffic accidents, and higher suicidal attempt rates (Flegr 2007; Flegr et al. 2002; Flegr and Havlicek 1999; Novotna et al. 2005; Webster and McConkey 2010; Yagmur et al. 2010). Although the likelihood of contracting toxoplasmosis has been decreasing in the US, there is concern among certain at-risk groups (Jones et al. 2007).

Like most oceanic islands, Felids did not occur in Hawai‘i until European explorers and settlers introduced domesticated cats (*Felis catus*) to the islands (Berger 1981). Feral and stray cats occur in forests of Hawai‘i (Amarasekare 1994) and in multiple colonies found throughout urban areas in the islands (Lohr et al. 2013a). Hawai‘i's high abundance of cats and favorable climate for the survival of *T. gondii* oocysts has important implications for Hawai‘i's already decreasing native wildlife abundance (Duffy and Capece 2012; Lohr et al. 2013a). Because of the high number of feral cats there are concerns over toxoplasmosis transmission to the people of Hawai‘i, and native and endangered species. For example, on Mauna Kea, Hawai‘i, a volcano with forests consisting of many endangered bird species, 37.3% of cats were seropositive for toxoplasmosis (Danner et al. 2007). Similarly, 0.5% of stray cats from O‘ahu tested were shedding oocysts and 20% had antibodies were found (Wallace 1971). Toxoplasmosis infections have been identified in a number of Hawaiian animals, including endangered species (Hawaiian crow [*Corvus hawaiiensis*], Hawaiian goose [*Branta sandvicensis*], and Hawaiian monk seal [*Monachus schauinslandi*]), spinner dolphin (*Stenella longirostris*), red-footed booby (*Sula sula*) and in livestock (Dubey and Jones 2008; Hawai‘i Department of Health 2000; Honnold et al. 2005; Work et al. 2000, 2002). Instances of *T. gondii* infection in marine mammals suggests that *T. gondii* transmission may be occurring through soil runoff containing oocysts, which has further implications for ocean recreation users in Hawai‘i and other wildlife (Miller et al. 2008).

Although the likelihood of humans contracting toxoplasmosis has been decreasing in the US, there is concern among certain at-risk groups. In general, these groups include people with immunity disorders, pregnant women, landscaping personnel, and waste handling personnel that are in close proximity to cat colonies. Toxoplasmosis is also a threat to certain wildlife species, especially on islands where endemic species have evolved without exposure to toxoplasmosis. Hawai‘i’s main islands have both urban areas where cats often form colonies at feeding sites, and remote locations where cats occur at lower densities, all within relatively close proximity of each other. The goal of this study was to determine if *T. gondii* occurs at both urban and remote sites in Hawai‘i. Fecal samples from cats culled from Ka‘ena Point NAR, and soil and fecal samples from colonies at the University of Hawai‘i were tested to determine if the disease was present at both sites and the rates at which it occurs. The University of Hawai‘i at Mānoa is located in Honolulu, Hawai‘i (21˚ 18' 01" N, 157˚ 49' 23" W), a city with a human population of over 300,000 (United States Census Bureau 2012). The university’s Office

Figure 2.1 here

of Facilities and Grounds has reported approximately 13 colonies of feral cats that have established around the campus (unpublished data). There is a concern that the landscaping personnel may be coming in contact with *T. gondii* oocysts in soil. Another concern is that oocysts in the soil may be spreading to endemic and endangered species in the state. Kaʻena Point Natural Area Reserve (Kaʻena Point NAR) is located less than 65 kilometers (21˚ 34' 31" N, 158˚ 16' 27" W) from Honolulu. Though people often utilize the trail in the reserve, cats do not come in contact with people. Several species of concern occur at Kaʻena Point NAR and there are concerns that any of the animals utilizing the site may be at risk of infection, though no tests have been conducted.

The objectives of this study were to test 120 soil and 60 cat fecal samples from four randomly selected cat colonies from the University of Hawaiʻi at Mānoa, and to test cat feces from nine cats culled from within the Kaʻena Point NAR predator-proof fence for *T. gondii* oocysts using PCR. It has been estimated that 1% of cats are shedding oocysts at any point in time and a single cat is able to shed up to 80 million oocysts in a day for up to 13 days after initial infection (Dubey 2001; Dubey 2010). Given this estimation and the estimated number of cats occurring on the university campus (~250 cats [unpublished data]), it could be expected that approximately two cats could be shedding oocysts at any time throughout campus. Considering the sampling locations (~82 cats [unpublished data]), it would be likely that no cats (or 0.82 of a cat) would be shedding oocysts. Similarly, if this concept is applied to the small number of cats sampled from Kaʻena Point NAR, no cats (or 0.09 of a cat) would be expected to shed oocysts at the time of sampling. The number of oocysts that could be expected to be occurring in soil at the university could also be extrapolated. Using a conservative estimate for the number of oocysts shed by a cat (~160 million), oocysts might be expected to be present in 1.4% of the soil on campus (~6,028,536.3 m^3 of open soil and grass up to 10 cm depth), assuming that oocysts are evenly distributed and have not been removed or degraded. Given these extrapolations, it was hypothesized that the detection rate of *T. gondii* oocysts would be greater at the University of Hawaiʻi at Mānoa than at Kaʻena Point NAR, simply based on the number of samples available. However, it is important to note that cats at the university obtain a large portion of their food from human sources, rather than cats at Kaʻena Point NAR that must hunt and scavenge for wild-based food. This will increase the likelihood that a cat at Kaʻena Point NAR would be infected. Soil samples received a sucrose floatation treatment to concentrate oocysts and all samples were tested using a commercial DNA extraction kit and PCR. Management recommendations were made based on the results.

Materials and Methods

Site Description

To address the objectives of this study, stray cat colonies on the University of Hawaiʻi at Mānoa main campus in Honolulu, Hawaiʻi and feral cats from Kaʻena Point NAR were tested for *T. gondii* oocysts. The campus currently supports a population of feral cats that are managed by cat colony caregivers. Cat colonies occur in areas where people regularly feed cats. Four of the 13 university cat colonies were randomly selected for soil sampling (Figure 2.2). The four sites varied in both the type of vegetation and surface material as well as the number of cats generally found in them. Specifically, sites ranged from 13 to 36 cats, on average, with all sites having areas of vegetation that the cats utilized. Cat numbers are based on observations between 6:00 pm and 7:00 pm on

Figure 2.2 here

days with favorable weather as part of a regular monitoring program (unpublished data). Ka‘ena Point NAR is approximately 0.24 km^2 and is located at the north-western end of the Wai‘anae mountain range on the island of O‘ahu, Hawai‘i. It is a breeding and nesting location for Laysan albatross (*Phoebastria immutabilis*) and wedge-tailed shearwaters (*Puffinus pacificus*), and is frequented by the critically endangered Hawaiian monk seal (*Monachus schauinslandi*). All non-native animals were removed from within a predator-proof fence in 2012 as part of the establishment of a predator free Natural Area Reserve site, and a total of nine cats were removed and frozen for later analysis (Lohr et al. 2013b).

Sample Collection and Processing

Soil at the university is an inceptisol from the Makiki stony clay loam series with 0% to 3% slope (Natural Resource Conservation Service 2009). However, because most of the grounds have been modified through construction and landscaping the soil at the four sites is highly mixed. A total of 30 soil samples were randomly collected from a

1 m × 1 m grid sampling scheme at each colony from the main campus, yielding a grand total of 120 soil samples. Soil was collected up to approximately 10 cm depth using a surface soil probe. Collected soil also included rocks (>2 mm diameter), plant material, and surface organic matter since as *T. gondii* oocysts could potentially become part of any aggregate containing any of these materials. Oocysts were recovered from soil samples using a sucrose floatation method based on methodology described by Lélu et al. (2011). Specifically, ~20 g of soil was added to a 50 mL cone centrifuge tube along with 20 ml of deionized water was and then vortexed for 2 min to homogenize and disperse water-soluble aggregates. Samples were then centrifuged at 1500 × G for 10 min, followed by discarding of the supernatant, and the addition of 20 ml of sucrose solution with a specific gravity of 1.15 (*T. gondii* oocysts have a density of 1.10 SG; Dubey et al. 1970). The sample was then vortexed for 2 min to ensure thorough mixing and centrifuged for 10 min. The supernatant was poured equally into two 50 mL cone centrifuge tubes and 35 mL of deionized water was added, bringing the density of the solution to 1.05 SG. Samples were centrifuged for 10 min at 1500 × G to pellet oocysts and the supernatant from each centrifuge tube from each centrifuge tube was poured equally into another 50 mL cone centrifuge tube (four cone centrifuge tubes per sample) along with 20 ml of deionized water to bring the solution density to near 1.00 SG. Samples were shaken and centrifuged for 10 min at 1500 × G. Supernatant from each tube was carefully discarded, ensuring that the pellet remained. The pellet from each 50 mL cone centrifuge tube was pooled and 200 µL of the pellet was reserved in a 2.0 mL microcentrifuge tube and stored at -20 ˚C.

Defecation sites at each colony on the university campus were targeted and 15 fecal samples were collected from each colony. Fecal samples were both wet and dry though attempts were made to collect as many wet samples as possible before collecting dry samples. All fecal samples were collected the same day and double bagged in zip-top bags. Approximately 200 mg of feces was placed into 2 mL microcentrifuge tubes for DNA extraction and stored at -20 ˚C until use. Cats from Ka‘ena Point NAR were thawed in October 2012 for gut content analysis (Lohr et al. 2013b) and removal of feces. Fecal samples were collected from the lower large intestine of nine cats culled for predator. Fecal samples collected from the University of Hawai‘i at Mānoa and Ka‘ena Point NAR were stored at 4 ˚C until used.

Molecular Analysis

DNA was extracted using the commercial kit QIAGEN QIAamp® DNA Stool Mini Kit (QIAGEN, USA) according to manufacturer's instruction (QIAGEN 2010). The QIAamp® DNA Stool Mini Kit was designed for purification of DNA from stool samples and utilizes a reagent that removes compounds that can degrade DNA and inhibit enzymatic replication. DNA elute of each sample was stored at -20 ˚C. *Toxoplasma gondii* specific detection was conducted by polymerase chain reaction (PCR) targeting the GRA6 gene using the primers reported by Fazaeli et al. (2000): GRA6 forward (5' TTT CCG AGC AGG TGA CCT 3') and GRA6 reverse (5' TCG CCG AAG AGT TGA CAT AG 3'). The amplification mixture contained 3.0 µL 5x GoTAQ buffer (Promega), 1.5 µL 1% bovine serum albumin, 1.2 µL $MgCl_2$ (25mM), 0.3 µM of each primer, 2.4 µL dNTP, and 0.2 µL TAQ polymerase (Promega), and 1 µL DNA template for a reaction mixture volume of 15 µL. PCR amplification was performed for 30 cycles (MJ Research

DNA Engine PTC-200). Denaturation occurred at 95 ˚C for 2 min and 93 ˚C for one minute, annealing occurred at 60.8 ˚C for 30 sec, and elongation occurred at 72 ˚C for one minute. PCR products were examined by electrophoresis using 1.5% agarose gel stained with ethidium bromide and visualized under UV. Samples were compared using *T. gondii* RH strain (Advanced Biotechnologies, Inc., USA) as a positive control.

Results

One-hundred twenty soil samples and 60 fecal samples from cat colonies at the University of Hawai‘i at Mānoa, and nine fecal samples from feral cats culled from Ka‘ena Point NAR were tested for *T. gondii* oocysts using PCR targeting the GRA6 gene. Of the University of Hawai‘i at Mānoa samples, five fecal samples were positive for *T. gondii* DNA (Figure 2.3), while no soil samples were positive. Oocysts were isolated

Figure 2.3 here

from feces collected from two sites that had kittens present at the time of sampling. Three samples were positive for oocysts from site 3 (Figure 2.3a) and two samples were positive from site 4 (Figure 2.3b). PCRs from site 3 amplified at different bp sizes, possibly from the presence of multiple strains present, and site 4 shows multiple bands for positive samples, though this is not out of the ordinary for *T. gondii* based on previous literature (Su et al. 2006). From the cats culled from Ka‘ena Point NAR, two were positive for *T. gondii* DNA (Figure 2.4). The light band in lane 3 may indicate that the cat was in the later stages of shedding oocysts (less DNA available for amplification), while lane 5 shows a very strong band.

Figure 2.4 here

Discussion

Toxoplasma gondii oocysts were found in 8.3% of fecal samples tested from cat colonies at the University of Hawai‘i at Mānoa and in 22% of fecal samples from feral cats culled from Ka‘ena Point NAR. When sampling naturally infected cat feces using molecular detection, studies have found between 0.11-9% positive (Pena et al. 2006; Salant et al. 2007; Schares et al. 2008). Pena et al. reported 1.2% of 237 samples were positive for *T. gondii* oocysts in cats from Brazil, Schares et al. reported 0.11% of 24,106 samples were positive in cats from Germany, Austria, France, and Switzerland, and Salant et al. reported 9% of 122 samples were positive in cats from Israel. Other studies have attempted to detect *T. gondii* from naturally infected soil using molecular detection and have ranged between 5.4-17.8% in temperate environments (Afonso et al. 2008; Du et al. 2012; Lass et al. 2009). Afonso et al. reported a detection rate of 5.4% from 55 samples during the first collection period and 12.9% from 62 samples collected from the second sampling period in France, Du et al. reported 16.3% from 252 samples collected in China, and Lass et al. reported 17.8% from 101 samples collected in Poland. Notably, Afonso et al. demonstrated that positive samples only occurred at defecation sites. Although the percent positive at Ka‘ena Point NAR is higher than some locations, it is important to note the small sample size. Regardless, the findings of *T. gondii* at a site of high conservation value are very important because of the risk of transmission to native, endemic, and endangered wildlife. It is important to note that there has not been a study testing oocyst in feral cats with no human contact. Other studies using similar methods either took samples from owned cats (Schares et al. 2008) or did not indicate whether or not the cats were truly feral (no contact with humans).

One confounding factor of investigating toxoplasmosis is that only 1% of cats are estimated to be shedding oocysts at any point in time (Dubey 2010). Thus, a larger sample size and focusing on defecation sites may have increased the likelihood of detecting oocysts. There were several limitations for this study. Soil sampling effort could have been concentrated at defecation sites, little information is known about the age distribution and previous infection rates in the cats on the university campus, and there may also have been limitations with interpreting results, the commercial DNA kits, and PCR. There are up to 245 known strains of *T. gondii*, many of which may amplify differently in PCRs and no studies have been conducted to identify the common strains in Hawai‘i (Aizenberg et al. 2004). The commercial DNA kit may not have effectively lysed all oocysts due to the resistant nature of *T. gondii*. The commercial DNA kit also may not have effectively removed all enzymes and inhibitors that could reduce the amount of samples positive. It is important to note that samples could have also been from the same cat as no attempts were made to differentiate fecal samples from each individual cat in each colony and the purpose of the study was to detect *T. gondii* in the environment. It is also important to note that the two sites that were positive for *T. gondii* had kittens present. In general, kittens do not have high seropositivity (Dubey 2010) as the likelihood of infection and antibody development increases with age. Serological methods may have indicated whether the cats tested had current and/or previous infections; however, it was outside the scope of this study. The intent of this study was to assess the presence of oocysts entering the environment and becoming a risk to native species.

The results of this study show that *T. gondii* is present at the University of Hawaiʻi at Mānoa. Though *T. gondii* was not isolated from soil in this study, it was present in cat feces at the sampling sites and seroprevalence tests would show true infection levels in colony cats. The presence of *T. gondii* has the potential to pose a health threat to the staff, faculty, students, and cat caregivers at the university if they come into direct contact with infected soil or feces and may potentially enter the environment due to the proximity of the university to a stream, possibly infecting ocean recreation users and marine mammals and birds. Studies have indicated that oocysts entering the ocean through soil runoff may be ingested by filter-feeding invertebrates and fish. Though no studies have indicated trophic accumulation of oocysts in marine life, there may be a potential for such a relationship in Hawaiʻi. The most prevalent filter-feeding and detritivore invertebrates in Hawaiʻi include coral, and various species of molluscs, crustaceans, and polychaete worms. With the exception of coral, no other filter-feeding or detritus-feeding invertebrate occur at high densities as they do in higher latitudes. These are also not usual prey items for marine birds and mammals that occur in Hawaiʻi; however, they are the prey of fish and cephalopod species that Hawaiʻi's marine birds and mammals prey on. The people of Hawaiʻi also consume many coastal fish and invertebrate species, including ʻopihi (Family Patellidae), a filter-feeding limpet often eaten raw. Research on how *T. gondii* oocysts move in tropical marine environments is greatly needed. The university administration will need to consider the implications of toxoplasmosis infection in its community.

In the past, oocysts entering the environment at Kaʻena Point NAR could have had significant negative effects on the wildlife that occurred there. Both Laysan albatross and wedge-tail shearwater adults and chicks may have ingested cat feces or soil containing oocysts and became infected. Hawaiian monk seals were likely to become infected from oocysts in soil runoff around the nearshore area. Oocysts may also have had the potential to accumulate in the prey of the marine birds and mammals. However, the true risk to these three species is unknown since little information is known on how long *T. gondii* oocysts could persist in the environment at Kaʻena Point NAR. Since the removal of all cats from within the predator-proof fence, it can be assumed that the risk of toxoplasmosis has significantly decreased, though the findings of this study suggest the need to keep the fence intact as even the removal of the cats may not have removed the disease.

Studies have indicated that oocysts entering the ocean through soil runoff may be ingested by filter-feeding invertebrates and fish (Arkush et al. 2003; Conrad et al. 2005; Massie et al. 2010; Miller et al. 2008). Though no studies have indicated trophic accumulation of oocysts in marine life, there may be a potential for such a relationship. The most prevalent filter-feeding and detritivore invertebrates in Hawai‘i include coral, and various species of molluscs, crustaceans, and polychaete worms. With the exception of coral, no other filter-feeding fish or invertebrates occur at high densities as they do in higher latitudes. These are also not usual prey items for marine birds and mammals that occur in Hawai‘i, though they are the prey of fish and cephalopod species that Hawai‘i’s marine birds and mammals prey on. The people of Hawai‘i also consume many coastal fish and invertebrate species, including ‘opihi (Family Patellidae), a filter-feeding limpet often eaten raw. This could also apply to other tropical location and research on how *T. gondii* oocysts move in tropical marine environments is greatly needed.

Managing for the presence of *T. gondii* in the environment will prove difficult. Removal of cats may stop *T. gondii* oocyst inputs into soil, but oocysts can persist in the environment for years and removing cats may not be socially acceptable. Consideration should also be given to other zoonotic diseases that may be present in cats. Other zoonotic diseases associated with cats that may be of concern include murine typhus, bartonellosis, roundworms and hookworms, and other protozoan infections, many of which occur in cat feces or are carried by cat fleas. Though there is no evidence that these diseases are present in cats in Hawai‘i, they can be prevalent in areas with high cat densities. Given the high cat densities in Hawai‘i, coupled with the large number of endangered animals, there is a critical need for understanding the prevalence of *T. gondii* and how the disease is moving through the environment.

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Figures

Figure 2.1: Trail camera image of feral cat at Ka‘ena Point NAR peering into a wedge-tailed shearwater (*Puffinus pacificus*) burrow. Photo courtesy of Pacific Rim Conservation.

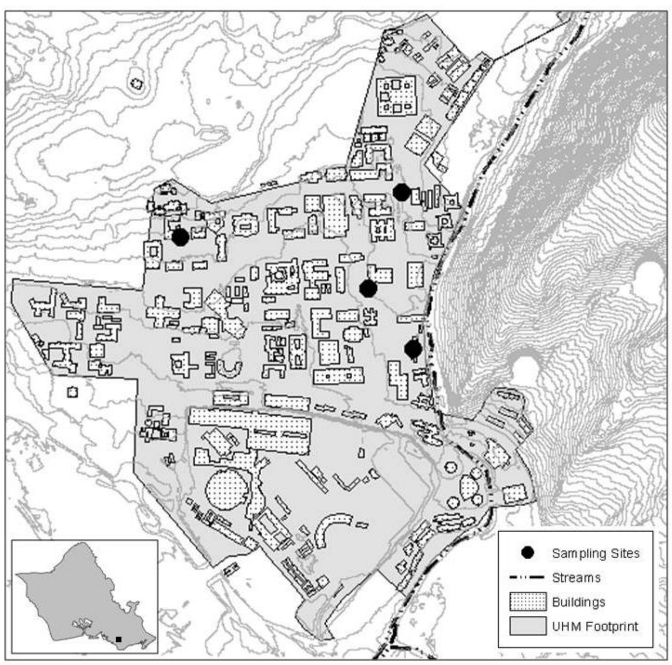

Figure 2.2: Map of the University of Hawai‘i at Mānoa with sampling locations.

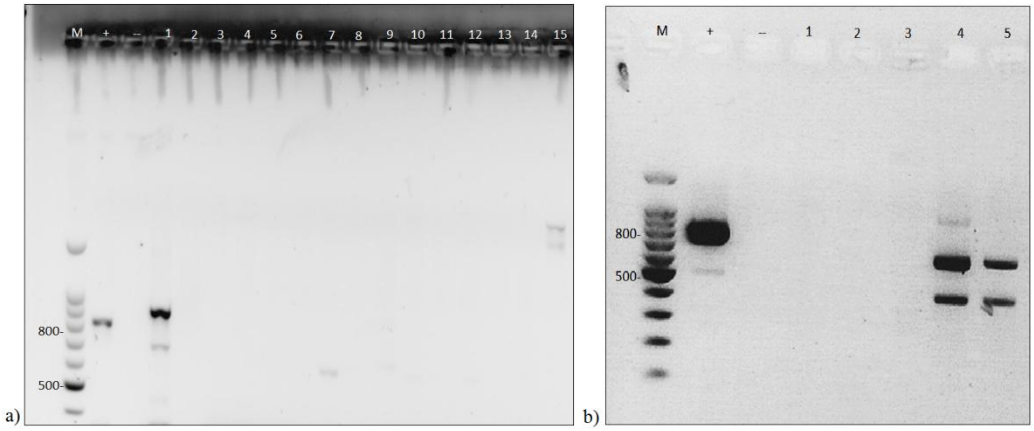

Figure 2.3: Gel electrophoresis of fecal samples positive for *T. gondii* targeting the GRA6 gene for the collected from sites 3 and 4 at the University of Hawaiʻi at Mānoa: a) Site 3 - Lane M is a 100 bp marker, lane + is the *T. gondii* RH strain positive control, lane -- is a negative control, and lanes 1, 7, and 15 are positive samples, though lanes 7 and 15 are faint; b) Site 4 - Lane M is a 100 bp marker, lane + is the *T. gondii* RH strain positive control, lane -- is a negative control, and lanes 4 and 5 are positive samples.

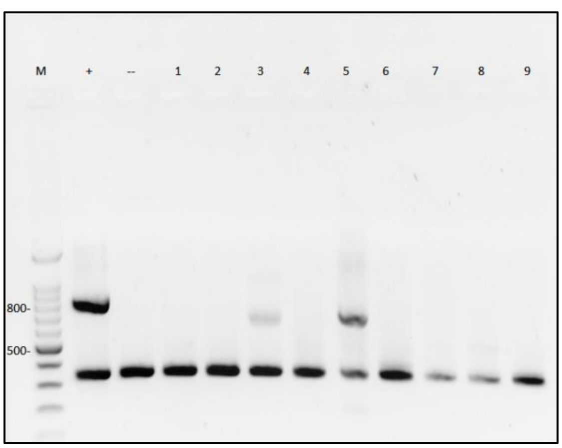

Figure 2.4: Gel electrophoresis of fecal samples positive for *T. gondii* targeting the GRA6 gene for the collected from Kaʻena Point Natural Area Reserve. Lane M is a 100 bp marker, lane + is the *T. gondii* RH strain positive control, lane – is a negative control, and lanes 3 and 5 are positive fecal samples.

CHAPTER 3

CONCLUSION, IMPLICATIONS AND FUTURE DIRECTIONS

The purpose of this project was to test naturally infected environmental samples for *Toxoplasma gondii* oocysts. This is the first study to successfully test environmental samples in Hawaiʻi for *T. gondii*, though no soil samples were positive for oocysts. The reason for this is unclear, however, fecal samples collected from the university were from defecation sites and fecal samples collected from cats from Kaʻena Point Natural Area Reserve were low enough in the cat's digestive tract to assume that they would be environmental samples. Soil and fecal samples were collected from the University of Hawaiʻi at Mānoa, where cats (*Felis catus*) are regularly fed and from colonies. Fecal samples were also collected from nine feral cats from Kaʻena Point NAR, where there is little to no contact with humans. It was hypothesized that the detection rate of *T. gondii* oocysts would be greater at the University of Hawaiʻi at Mānoa than at Kaʻena Point NAR, simply based on the number of samples available. However, cats at the university obtain a large portion of their food from human sources, rather than cats at Kaʻena Point NAR that must hunt and scavenge for food, possibly increasing the likelihood that a cat at Kaʻena Point NAR would be infected. All samples were tested using a commercial DNA extraction kit (QIAGEN QIAamp® Stool Mini Kit, QIAGEN, USA) and PCR amplification methodologies targeting the GRA6 gene.

The results of this project did not support the hypothesis and instead showed the opposite, even with the small sample size from Kaʻena Point NAR. From the university, five of 60 (8.3%) fecal samples were positive for *T. gondii*, while two (22%) of the nine cats tested from Ka‘ena Point NAR were positive for *T. gondii* oocysts. Cats residing in colonies in urban areas tend to concentrate defecation in specific locations, thereby concentrating oocysts. This can be hazardous to people working with soil, children playing in soil, anyone breathing oocysts aerosolized in dust, and for nearshore areas where people may recreate or wildlife occur. At protected areas, wildlife may ingest feces directly or soil and plant matter that have oocysts. This can be especially problematic for endangered and protected species since *T. gondii* infections can reduce birth rates and kill adults. The results of this study may also indicate higher toxoplasmosis rates in cats found in tropical climates.

Oocysts entering the nearshore areas of Hawai‘i presents an interesting case for transmission pathways in coastal areas. Studies have indicated that oocysts entering the ocean through soil runoff may be ingested by filter-feeding invertebrates and fish and the most prevalent filter-feeding and detritivore invertebrates in Hawai‘i include coral, and various species of molluscs, crustaceans, and polychaete worms (Arkush et al. 2003; Massie et al. 2010; Miller et al. 2008). With the exception of coral, no other filter-feeding or detritus-feeding invertebrate occur at high densities as they do in higher latitudes and Hawai‘i lacks large schools of filter-feeding fish near coastlines. These are also not usual prey items for marine birds and mammals that occur in Hawai‘i; however they are the prey of fish and cephalopod species that marine birds and mammals occurring in Hawai‘i prey on. The people of Hawai‘i also consume many coastal fish and invertebrate species, including ‘opihi (Family Patellidae), a filter-feeding limpet often eaten raw. Though no studies have indicated trophic accumulation of oocysts in marine life, there may be a potential for such a relationship in Hawai‘i.

The presence of *T. gondii* in soils has the potential to pose a health threat to the staff, faculty, students, and cat caregivers at the university if they come into direct contact with infected soil and ingest the oocysts. The university administration will need to consider the implications of toxoplasmosis infection in its community if *T. gondii* oocysts are detected on the campus. An important step would be to test cats and landscaping personnel for toxoplasmosis seropositivity. Though there is no guarantee that any antibodies present for landscaping personnel is related to the university, a high enough occurrence in personnel may suggest that the infections came from the university. Seroprevalence rates in cats will indicate true toxoplasmosis rates for cats at the university and would suggest risk to the university community and personnel.

Managing for any toxoplasmosis may prove difficult. Removal of cats from a location may stop *T. gondii* oocyst inputs into soil, but oocysts can persist in the environment for years. Social impacts should also be considered as individuals may be resistant to removal of cats. Given the high cat densities in urban areas of Hawaiʻi, coupled with the large number of endangered animals, there is a critical need for understanding the prevalence of *T. gondii* in the environment and the risk it poses to people and wildlife in Hawaiʻi and throughout the world. Little information is known about *T. gondii* risk and management in tropical locations around the world as most research that has been conducted has looked at toxoplasmosis infection risk for people and most instances of toxoplasmosis in wildlife have been case studies, rather than infection risk for wildlife, marine species, or the environment.

Several limitations exist for this study, including a lack of knowledge about the population of cats at the university and *T. gondii* strains found in Hawaiʻi, limitations with the commercial DNA extraction kits and PCR, and sampling design. Further study is required to determine if cat population dynamics at cat colonies in Hawai‘i have an effect on *T. gondii* infection rates. The common strains of *T. gondii* have not been identified for Hawai‘i, making primer design difficult. The DNA extraction kit may not have lysed highly resistant oocysts or fully removed inhibitors and enzymes that affect PCR results. Finally, Afonso et al. (2008) demonstrated that oocysts were only extracted from cat defecation sites when the entire study area was sampled during two sampling events (5.4% and 12.9%). The prevalence of *T. gondii* at the university may have been higher if sampling was concentrated at defecation sites. However, there was interest in whether positive samples would be found where landscaping personnel often work, so a grid sampling scheme was used. The university was both a good and poor proxy for other urban areas in Hawai‘i. The university is frequented by people and is home to several colonies of feral and stray cats that are cared for by individuals of the community. It is possible that toxoplasmosis rates may differ across colonies in Hawai‘i. There is some debate as to whether managed colonies have high rates of toxoplasmosis and a study that compares toxoplasmosis rates and management intensity would help inform management practices.

Further research needs to be conducted on this subject in Hawai‘i. Little is known about the strains of *T. gondii* found in the state. Work can be done to identify common *T. gondii* strains found in Hawai‘i, which would allow for better primer design and confirm positive results. Further work should be conducted to test survival of oocysts under arid conditions and develop sensitive methods for isolating oocysts from water should also be tested for sampling streams and estuaries for *T. gondii*. This would help to inform the conditions under which *T. gondii* may have high transmission probability to marine life and ocean users. Transmission pathways and movement through trophic systems in marine environments are also not well understood. Different trophic systems occur at different marine ecosystems and knowing how oocysts might move through the system would help to answer how certain marine mammals and birds are infected with toxoplasmosis. This project is the first to detect *T. gondii* oocysts in the environment from both colony cats and feral cats in Hawaiʻi and will hopefully inform future projects and management in Hawaiʻi and other tropical locations around the world.

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