Detecting *Toxoplasma gondii* in the giant African snail (*Lissachatina fulica*) in O‘ahu, Hawai‘i

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Chapter 1

Evaluating if *Lissachatina fulica* can pass deactivated *Toxoplasma gondii* oocysts through their digestive system

Abstract

Toxoplasmosis is one of the most common parasitic foodborne diseases in the world and has severe negative impacts on humans and causes mortality in several wildlife species. Felids are the only definitive host of the disease-causing agent, *Toxoplasma gondii*, and once infected, shed millions of oocysts through their feces. Several mechanical vectors can disperse these oocysts, but it is unknown if invasive land snails can act as mechanical vectors. My goal was to demonstrate if *T. gondii* DNA and intact *T. gondii* oocysts can be detected in *Lissachatina fulica* feces to determine if *L. fulica* can act as a mechanical vector for *T. gondii*. To complete this goal, I fed 500 deactivated *T. gondii* oocysts to four *Lissachatina fulica* and used conventional PCR to detect the presence/absence of *T. gondii* DNA in the resulting feces. I used genetic sequencing to confirm positive samples and light microscopy to detect full *T. gondii* oocysts. The first fecal sample of each snail tested positive through conventional PCR and and successfully sequenced for *T. gondii* DNA. Oocysts were not found for any of the samples. However, successfully detecting *T. gondii* DNA in the fecal samples makes *L. fulica* a potential candidate for a biosentinel of *T. gondii* contamination. This would involve removing the destructive *L. fulica* from the environment, which would be helpful to several countries where *L. fulica* is invasive. *L. fulica* as a biosentinel could also be a solution to the difficulty of detecting *T. gondii* in environmental samples.

Introduction

Toxoplasmosis is one of the most common foodborne parasitic illnesses around the world (Montazeri et al. 2018) and one of the six neglected parasitic diseases in the United States (Centers for Disease Control and Prevention 2018). Approximately one third of the human population is estimated to have antibodies for toxoplasmosis (Woodhall et al. 2014) and many

wildlife species, such as New World monkeys (Epiphanio et al. 2003; Cedillo-Pelaez et al. 2011), marine mammals (Krusor et al. 2015; Dubey et al. 2006; Carlson-Bremer et al. 2015), Australian marsupials (Dubey and Crutchley 2008), and several bird species (Casagrande et al. 2015; Jokelainen and Vikoren 2014) are fatally impacted by the parasite. Humans are usually asymptomatic for the disease (Montazeri et al. 2018), though it can occasionally cause blindness (Park and Nam 2013; Jones and Holland 2010) and other severe symptoms in those who contract the disease congenitally (Chaudhry et al. 2014; Freppel et al. 2019) or while immunocompromised (Zhou et al. 2011; Wang et al. 2017).

Felids are the only definite host of *T. gondii*, and once infected, shed millions of oocysts into the environment (Hill and Dubey 2002). Each oocyst encases four sporozoites, which are the only free-living life stage of the parasite (Shapiro et al. 2019). If an animal ingests these oocysts, they can contract the disease (Center for Disease Control and Prevention 2018). Once ingested, sporozoites burst from the oocysts and become tachyzoites (Black and Boothroyd 2000; Dubey 2010), which asexually reproduce and form accumulations inside the neural and muscular tissue of the animal (Black and Boothroyd 2000). After forming these accumulations, the tachyzoites will become bradyzoites where they remain dormant throughout the animal's life (Center for Disease Control and Prevention 2018; Black and Boothroyd 2000). Predators, including humans, can become infected with toxoplasmosis after ingesting infected meat/prey (Black and Boothroyd 2000). If a felid ingests an infected animal, or ingests oocysts from the environment, *T. gondii* will sexually reproduce and create oocysts, completing the life cycle (Centers for Disease Control and Prevention 2018).

Several pathways can lead to toxoplasmosis infection, (Shapiro et al. 2019), such as consuming undercooked meat (Belluco et al. 2016), contaminated drinking water (Coupe et al.

2019; Bowie et al. 1997; de Moura et al. 2006), and unwashed foods such as fruits and vegetables (Pinto-Ferreira et al. 2019; Marques et al. 2020). Different organisms can even facilitate the distribution and transmission of *T. gondii* oocysts by acting as mechanical vectors. Some examples include different species of cockroaches (*Blatella germanica* and *Periplaneta americana*) and flies (*Musca domestica* and *Chrysomya megacephala*), which can carry infectious *T. gondii* oocysts on their bodies after exposure to infected feces (Graczyk et al. 2005). Dogs (*Canis lupus familiaris*) (Lindsay et al. 1997) and marine brown turban snails (*Chlorostoma brunnea, Chlorostoma montereyi and Promartynia pulligo*) (Krusor et al. 2015) can ingest and pass full *T. gondii* oocysts through their feces.

Other species could potentially act as mechanical vectors for *T. gondii*, but these areas are relatively unexplored or limited to one or two studies, which leaves a significant gap in our understanding of how *T. gondii* could be transmitted from environment to environment. One example is invasive land snails, which could ingest *T. gondii* oocysts from the soil and defecate them into new uncontaminated environments, causing concern to several different species of wildlife. Many invasive land snail species are found in locations where toxoplasmosis is known to negatively affect wildlife, such as Brazil (Gregoric et al. 2011; Albuquerque et al. 2008) and Australia (Blacket et al. 2016). Another example is the Hawaiian Islands, which have at least 63 non-native land snail species (Cowie et al. 1998), a large distribution of feral cats (*Felis catus*) (Hawai‘i Invasive Species Council 2020), and several important wildlife species fatally impacted by toxoplasmosis, such as the Hawaiian monk seal (Īlio-holo-i-ka-uaua; *Neomonachus schauinslandi*) (Honnold et al. 2005), the Hawaiian goose (Nēnē; *Branta sandvicensis*) (Work et al. 2002) and the Hawaiian crow (‘Alalā; *Corvus hawaiiensis*) (Work et al. 2000).

Since invasive land snails may share the same habitat as feral cats, it is possible that these snails are serving as mechanical vectors for *T. gondii* oocysts, which could increase the chance of transmission among Hawaiian wildlife. However, very few studies have explored this possibility and no studies have screened invasive snails for *T. gondii.* The University of Kansas Medical Center demonstrated that Puerto Rican land snails (*Caracolus caracolla*) can pass infectious *T. gondii* oocysts in their feces after ingesting infected cat feces, but the methodology for the experiment was vague and it was not stated how many snails were used for the experiment (Miller et al. 1972).

Because of the lack of knowledge of the role invasive land snails play in *T. gondii* dispersal and transmission, my goal was to determine if *T. gondii* DNA and full *T. gondii* oocysts could be detected in land snail feces. To accomplish my goal, my objectives were to 1) feed full *T. gondii* oocysts to a sample of invasive land snails and test the resulting feces for *T. gondii* DNA and 2) observe any positive fecal samples for full *T. gondii* oocysts. Based on previous findings (Krusor et al. 2015, Cong et al. 2021, Miller et al. 1972), I hypothesize *T. gondii* DNA and oocysts will be detected in land snail feces.

Methods

Sample Collection and Husbandry

To test my hypothesis, I collected three *Lissachatina fulica* from an area next to the Archie Baker Mini Park and one from the University of Hawai‘i at Manoa (UH Manoa) on the side of Maile Way to the right of Gilmore Hall in Honolulu, Hawai‘i. *L. fulica* was chosen because of their high abundance in Hawai‘i and invasive nature in several other countries (Lu et al. 2018). All snails were housed in the Malacology Department at the Bishop Museum. Snails were individually housed in plastic pipette containers (sanitized with 10% bleach water, rinsed

with boiling water, and wiped thoroughly with paper towels), sprayed with purified drinking water (bottled water), and fed a sweet potato aliquot weighing between 0.550-0.650 grams. All sweet potato pieces were microwaved with a small amount of tap water for about 1.5 minutes or until the pieces were slightly soft. Samples were weighed after being microwaved.

Feces was collected from the containers of each snail and observed daily for the first 72 hours. Fecal samples were collected with a small metal spatula (sanitized with 10% bleach water, rinsed with tap water, and dried with a paper towel between uses), placed in a 1.5 mL microcentrifuge tube, weighed, and stored in a freezer. Snails received a new sweet potato aliquot after fecal collection and were sprayed with bottled water if they appeared dry. Snails did not receive a new sweet potato aliquot after the final fecal collection during the first 72-hours. If any of the fecal samples were at least partially orange, I considered it a reasonable assumption that the snail's digestive tract was cleared. All four of the snails had at least one fecal sample that was partially orange by the end of the first 72 hours (See Figure 1.1).

Feeding Oocysts to the Snails

Inactivated *T. gondii* oocysts were provided by Dr. Karen Shapiro at the University of California at Davis (UC Davis) (See Appendix A). Oocysts (Type II strain M4) were isolated from cat feces of experimentally infected cats and sporulated before they were heat inactivated at 80°C for 20 minutes. Oocysts were suspended in 1 mL of PBS and shipped to the Pacific Center for Molecular Biodiversity (PCMB) at the Bishop Museum in Honolulu, Hawai‘i.

The plastic containers housing the four snails were sanitized with 10% bleach water, rinsed with tap water, and thoroughly dried with a paper towel. Snails were placed back in their containers and given a mashed sweet potato aliquot (between 0.550-0.650 grams) that was spiked with approximately 500 oocysts, so that snails had at least approximately 500 *T. gondii*

oocysts in their digestive system. The snails were kept for an additional 72-hours and the first and second fecal sample produced by each snail was collected in a 1.5 mL microcentrifuge tube, weighed, and stored in a freezer. After each fecal sample collection, each snails' container was rinsed with tap water and wiped with a paper towel. Each snail was returned to its enclosure and given a new sweet potato aliquot that was not spiked. Snails were not given a new sweet potato aliquot after the final fecal collection. Snails were euthanized after the final fecal sample was collected. Snails were placed in a plastic bag and put in the freezer.

Fecal sample homogenization

Each fecal sample was homogenized in a mortar (sprayed with decontamination solution [1.5% sodium dichloro-s-triazinetrione, 1% NaOH {10 g/L}, 1% detergent], wiped with a paper towel, rinsed with sterile Nanopure water type I, and wiped with a paper towel between uses) with a metal spatula (sanitized in 10% bleach water, sterile Nanopure water type I, then dried with a paper towel between uses). A portion of the homogenized feces was placed inside a M-N bead beater tube type A, the first sample tube used in the Macherey-Nagel® Nucleospin™ DNA fecal kit, for molecular analysis. Portions were 50% of each fecal sample's total weight and placed in the freezer until the next step could be performed (See Appendix B). The rest of the homogenized sample was placed in the 1.5 mL microcentrifuge tube that held the fecal sample prior to homogenization. 1000 µL of sterile Nanopure water type I was pipetted into the mortar after the homogenized feces was removed, and the metal spatula was used to mix the water in the mortar to get as much feces off the sides of the mortar and spatula as possible. The same micropipette was used to place as much sample as possible into the 1.5 mL microcentrifuge tube with the rest of the microscopy sample. If there was no space left in the tube for the entire

sample, the rest of the sample was put in a new 1.5 mL microcentrifuge tube. The microscopy samples were placed in the freezer until fecal flotation and light microscopy could be performed.

Freeze/thaw methodology

All samples and a positive and negative control (positive controls contained approximately 100 *T. gondii* oocysts and negative controls contained 200 µL of autoclaved water) had 200 µL of autoclaved water added and were vortexed for approximately 10 seconds. After this, samples and controls were placed in a freezer (-80°C) for 10 minutes then into a hotblock (95°C) for 10 minutes interchangeably three times.

Molecular methodology

DNA extraction was performed following the instructions of the Macherey-Nagel® Nucleospin™ DNA fecal kit with modifications (see Appendix B). Genomic DNA (gDNA) was eluted using 50 µL of Buffer SE (elution buffer) at 95°C.

For all samples, gDNA was diluted 1:50 and amplified using conventional polymerase chain reaction (PCR) targeting the 529 bp repeat fragment (Krusor et al. 2015). Reactions were 25 µL in volume, containing 5 µL of template DNA, and a final concentration of 1X MangoMix™ (Bioline), and 0.2 µM forward primer Tox4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3'), and 0.2 µM reverse primer Tox5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') (Homan et al. 2000). Samples ran on a cycling program with samples held at 95°C for 5 minutes, 50°C for 1 minute, and 72°C for 1 minute for one cycle, followed by 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute for 45 cycles, and a final extension at 72°C for 10 minutes and 4°C for 3 minutes for one cycle. PCR product ran on a 2% agarose gel by electrophoresis. All gels contained approximately 1.5-3.0

μg/mL of ethidium bromide. 3 μL of EasyLadder I was used as the DNA molecular weight marker for each gel. All gels ran for 30 minutes on 130 Voltage.

Positive samples were sent to Eurofins in Louisville, Kentucky for DNA sequencing via Sanger sequencing with the reverse internal primer Tox5INT (5'-CTCCACTCTTCAATTCTCTCC-3') of a set of internal primers (Tox4INT and Tox5INT) designed for the 529 bp repeat fragment (Shapiro et al. 2010). DNA sequences were identified through NCBI BLAST and compared to databases in GenBank.

Fecal flotation and Light Microscopy

All samples that tested positive using molecular methodology were evaluated for *T. gondii* oocysts using fecal flotation and light microcopy, which was done at the Parasitology Laboratory at the College of Veterinary Medicine at Auburn University in Auburn, Alabama. Samples were taken from the freezer and thawed for 10 minutes. Each sample were pipetted into a plastic 15 mL falcon tube. The 1.5 mL microcentrifuge tubes were filled with 1000 μL of distilled water and vortexed for several seconds. The water was pipetted out of the microcentrifuge tube and placed in the corresponding 15 mL falcon tube. This was repeated once and the 1.5 mL tube was discarded. The falcon tube was filled with distilled water to the 14 mL mark, capped, and centrifuged at 2500 · g for 10 minutes. The supernatant was removed and the pellet was resuspended with Sheather's sucrose solution (specific gravity: 1.27). The falcon tube was capped and centrifuged at 2500 · g for 10 minutes. The falcon tubes were placed in a rack, sat for 5 minutes, and the top 20 μL was pipetted onto a microscope slide. Another 20 μL was taken off the top until 8 microscope slides were made per sample (See Appendix A and B).

The falcon tubes were capped and placed in the fridge for 5 days. The tubes were taken from the fridge and centrifuged at 250 · g for 10 minutes. The tubes were filled with Sheather's

sucrose solution to a reverse meniscus and a cover slip was placed on the top. The falcon tubes sat for 20 minutes and then the coverslips were placed on a microscope slide. All microscope slides were observed on 10x magnification and observed closer with 40x magnification when potential oocysts were found (Zajac et al. 2021) (See Appendix C). All samples were re-examined by the Parasitology Lab Research Assistant IV.

Results

Samples were considered positive when a band appeared within 500 bp on the agarose gel. Of the eight fecal samples, four amplified positive for *T. gondii* DNA (See Figure 1.2). All four samples were the first fecal sample collected for each snail. All four of these samples matched for the *T. gondii* repeat region on NCBI BLAST compared to databases in GenBank. No oocysts were observed during light microscopy for three of the samples. The results of the fourth sample are pending.

Discussion

Overall, I have demonstrated support for the first part of my hypothesis that *T. gondii* DNA can be detected in *L. fulica* feces, but not for intact *T. gondii* oocysts. A mechanical vector is characterized by physically carrying a disease-causing agent from one location to another, causing an increased chance of infection to wildlife species and human populations (Graczyk et al. 2005; Chalkowski et al. 2018). Finding *T. gondii* oocysts through light microscopy is considered a refutable standard for detecting the parasite (Liu et al. 2015) and differentiates the results from DNA, that could have come from degraded or digested oocysts, to intact oocysts that could theoretically cause illness if they were not heat inactivated. Therefore, the results of this project cannot conclude that *L. fulica* are mechanical vectors of *T. gondii*. However, since *T. gondii* DNA was detected using molecular methods, it is possible that *L. fulica* could be a

biosentinel for *T. gondii* to indicate environmental contamination. Several different species are biosentinels for *T. gondii*, such as arctic (*Vulpus lagopus*) and red foxes (*Vulpes vulpes*) (Bouchard et al. 2022), domestic dogs (Cabezon et al. 2010), and domestic chickens (*Gallus domesticus*) (Dubey et al. 2015; More et al. 2012). Molecular and serological methods are used to detect DNA or antibodies against *T. gondii* in the sentinel species, which can be used to determine areas of concern (Cabezon et al. 2010; Dubey et al. 2015) or rate of exposure over certain periods of time (More et al. 2012). This has recently been done with feral chickens (*Gallus gallus*) on Kauaʻi to understand environmental factors that could contribute to *T. gondii* prevalence in different environments (Chalkowski et al. 2020). Collecting *L. fulica* in addition to any future feral chicken surveys could provide a clearer picture of *T. gondii* prevalence throughout Hawaiʻi. For example, the results of the project revealed that the spiked snails passed *T. gondii* DNA exclusively in the first fecal sample after consuming oocysts. If this is a common characteristic of the species, it could be used to indicate recent shedding of *T. gondii* oocysts from feral cat definitive hosts, especially if snails are collected in feral cat defecation areas (Shapiro et a. 2019; Alfonso et a. 2008). However, this finding could also be because the spiked aliquots were sweet potato, which is a natural laxative, and might not accurately represent *T. gondii* DNA passing in the wild.

Regardless, using *L. fulica* as a biosentinel for *T. gondii* would be a useful utilization of the species, since environmental surveillance of *T. gondii* oocysts is difficult (Dumetre and Darde et al. 2003) and removing *L. fulica* from the Hawaiian environment is automatically beneficial, since the species is well established throughout the islands (Cowie et al. 1998) and is known to eat 500 different plant species (Meyer et al. 2008). Although complete eradication of *L. fulica* in Hawaiʻi would be incredibly costly and work intensive based on previous efforts in

areas such as Florida (Roda et al. 2016), having routine collections to survey an area for *T. gondii* contamination could offer some temporary relief throughout Hawai‘i. The methods used in this experiment to screen *L. fulica* are also similar to the methods used to screen several filter feeding biosentinels for *T. gondii*, such as oysters, clams, and several species of mussels (Coupe et al. 2018; Coupe et al. 2019; Staggs et al. 2015). These species are primarily used to detect contamination in marine and freshwater environments (Shapiro et al. 2019). Furthermore, throughout the duration of the project, *L. fulica* proved to be relatively easy to care for, especially within the constraints of a 72-hour period. They took up little space and provided feces almost immediately upon capture.

Although it is possible that *T. gondii* oocysts will be observed in the fourth fecal sample, oocysts were not observed in the three fecal samples to date. This differs from the findings of Miller et al. 1972 and Krusor et al. 2015, but there are several reasons for why this could be. One of these is that 500 oocysts might be sufficient for detecting *T. gondii* DNA in the invasive snail feces using molecular methods such as PCR and DNA sequencing, but not enough to detect oocysts through fecal flotation and light microscopy. Notably, Miller et al. 1972 fed their experimental snails infected cat feces. Since cats can shed millions of oocysts within 1-2 weeks after infection (Hill and Dubey 2002, Shapiro et al. 2019), it is very likely that tens of thousands of oocysts were present in the cat feces fed to the snails in Miller et al. 1972. Similarly, Krusor et al. 2015 exposed their experimental marine snails to a final concentration of 10,000 oocysts and microspheres per liter of water, which is much higher than the number of oocysts presented in the feeding trial. A second explanation is that since the *T. gondii* oocysts were sporulated prior to heat inactivation, it is possible that the oocysts wall disintegrated in the digestive system of the snails after the sweet potato aliquot was ingested, leaving only *T. gondii* DNA in fecal samples

(Dubey et al. 1998). Since Miller et al. 1972 fed infected cat feces to the snails, depending on how soon the feces was presented and how quickly the snails consumed the feces, it is possible that the oocysts inside the infected cat feces did not sporulate before consumption or that there were enough unsporulated oocysts eaten by the snails to detect with microscopy (Dubey et al. 1998; Attias et al. 2020; Dubey et al. 2010). A third explanation is that some microscopy samples were taken in and out of the freezer to place permanent labels on the 1.5 mL microcentrifuge tubes prior to fecal flotation. This could have simulated freeze/thaw techniques, which were designed to rupture the oocysts wall in the methodology of the project. Finally, it is possible that fecal flotation and light microscopy are producing false negatives and are less sensitive than molecular methods. Repeating the experiment using a larger number of oocysts and snails would be useful to explore these possibilities, as well as using both sporulated and unsporulated oocysts.

Although the project succeeds in answering the first part of my hypothesis, there were limitations to the project that should be mentioned. The first limitation is that the sample size was very small which could cause statistical significance issues. The second is that there were large inconsistencies with the amount of feces taken from the homogenized sample for molecular analysis. If the experiment would be repeated, a sample size of 10 snails or higher would eliminate any problems of statistical significance and perhaps a set amount within 10% parameters for molecular analysis portions would eliminate inconsistencies with sample processing.

To the best of my knowledge, this is the first project that has fed *T. gondii* oocysts to *L. fulica* and used conventional PCR to test the resulting feces for *T. gondii* DNA. Further research should be performed to explore the potential reasons for why *T. gondii* oocysts were not

observed in snail fecal samples as well as to further observe the rate of *T. gondii* DNA passing in *L. fulica* to better understand their use as a biosentinel.

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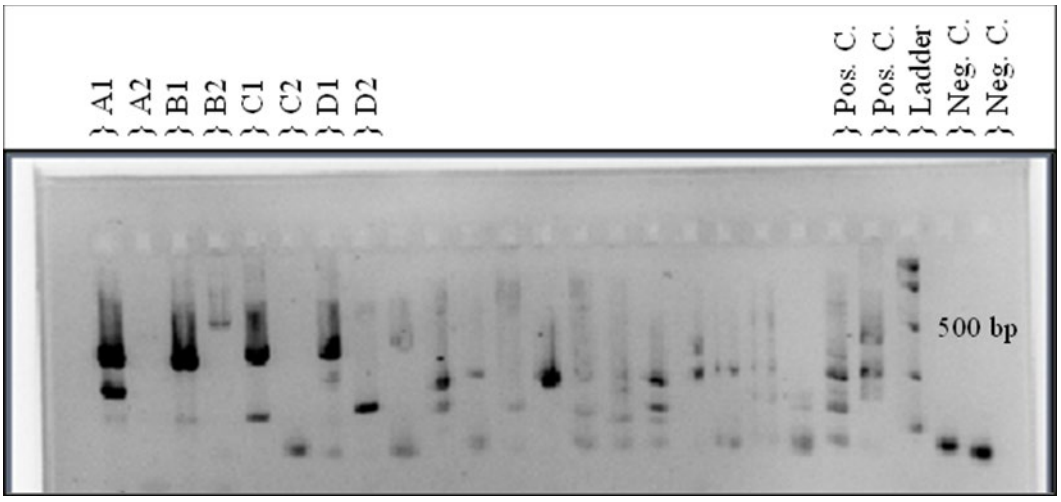
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Chapter 2

Testing for *Toxoplasma gondii* DNA in wild caught *Lissachatina fulica*

Abstract

Toxoplasmosis is one of the most common parasitic foodborne diseases in the world and has several transmission pathways due to the hardy nature of the oocysts in the environment. Felids are the only definitive host of the disease-causing agent, *Toxoplasma gondii*, and once infected, shed millions of oocysts through their feces. *T. gondii* can further disperse through several species of mechanical vectors, but no research has explored the detection of *T. gondii* in land snails. My goal was to demonstrate if *T. gondii* DNA and intact *T. gondii* oocysts can be detected in the feces of wild caught *Lissachatina fulica* feces, to determine if *L. fulica* can act as a mechanical vector for *T. gondii* in the wild. To accomplish my goal, I collected 127 *Lissachatina fulica* from three feral cat congregation sites in Honolulu, Hawai‘i and used conventional PCR targeting the 529 bp repeat fragment and nested PCR targeting the ITS1 region to detect the presence/absence of *T. gondii* DNA in the resulting feces. I used DNA sequencing to confirm positive samples and light microscopy to detect full *T. gondii* oocysts. Overall, 41 fecal samples tested positive through conventional PCR and 1 of these samples successfully sequenced for *T. gondii*. Four of the 41 positive samples were screened using nested PCR, of which all four were positive. One of these samples successfully sequenced for *T. gondii*. One of the samples was observed for full oocysts while the results of the second sample are pending. No oocysts were observed. Although the project does not demonstrate that *L. fulica* can be mechanical vectors, it does provide evidence that *L. fulica* could act as biosentinels for *T. gondii* contamination. This could be helpful in areas that are heavily invaded by *L. fulica* and provide a solution for the difficulty in detecting *T. gondii* from environmental samples.

Introduction

Toxoplasmosis is a common foodborne parasitic illness in many parts of the world (Flegr et al. 2014; Hill and Dubey 2002; Alizadeh et al. 2018) and in the United States alone, it infects 800,000 people each year (Division of Parasitic Diseases and Malaria, n.d.). No bird or mammal is shown to be immune to the disease (Attias et al. 2020), which can be fatal for several wildlife species, including New World monkeys (Epiphanio et al. 2003; Cedillo-Pelaez et al. 2011), Australian marsupials (Dubey and Crutchley 2008), and several species of marine pinnipeds (Krusor et al. 2015; Dubey et al. 2006) and birds (Casagrande et al. 2015; Jokelainen and Vikoren 2014). In humans, healthy individuals are usually asymptomatic to the disease (Montazeri et al. 2018; Yan et al. 2016), although occasionally it can cause blindness (Park and Nam 2013; Jones and Holland 2010), birth defects in congenitally infected individuals (Chaudhry et al. 2014; Alday and Doggett 2017) and encephalitis and mortality in immunocompromised individuals (Zhou et al. 2011; Wang et al. 2017; Mousavi et al. 2018).

Felids are the only definitive host of the disease-causing parasite, *Toxoplasma gondii*. Once infected, a felid can shed up to a billion *T. gondii* oocysts into the environment, each of which contain four sporozoites, the only free-living life stage of the parasite (Shapiro et al. 2019). When a mammal or bird ingests these oocysts, the sporozoites burst from the oocyst and become tachyzoites, which rapidly asexually reproduce and accumulate in neural and muscular tissue throughout the host's body (Black and Boothroyd 2000). After forming these accumulations, the tachyzoites become the final life stage of the parasite, bradyzoites (Attias et al. 2020). If a felid ingests an infected animal, these bradyzoites will sexually reproduce in the small intestine of the felid, creating oocysts and completing the life cycle (Black and Boothroyd 2000; Alizadeh et al. 2018; Centers for Disease Control and Prevention 2018).

T. gondii is perhaps one of the most successful protozoan parasites in the world (Djurkovic-Djakovic et al. 2019). Therefore, understanding the transmission pathways for the parasite are important for making good prevention management plans for human health and wildlife (Shapiro et al. 2019; Djurkovic-Djakovic et al. 2019). Several pathways have been described for *T. gondii* contraction, which tends to reference back to the hardy oocysts that are found in the environment (Hill and Dubey 2002; Yan et al. 2016; Dumetre et al. 2013). *T. gondii* oocysts can survive several severe conditions, such as high salinity (15 ppt) (Lindsay et al. 2003) and extreme variations in temperature (-20°C to 35°C in moist soils for up to 18 months) (Shapiro et al. 2019), making them available to a wide variety of intermediate hosts (Dumetre and Darde 2003; Freppel et al. 2019; Shapiro et al. 2019).

T. gondii oocysts can even disperse by biological means, such as through mechanical vectors (Chalkowski et al. 2018; Shapiro et al. 2019). Examples of these mechanical vectors include arthropods, such as earthworms, dung beetles (Hill and Dubey 2002), flies *(Musca domestica* and *Chrysomya megacephala*), and cockroaches (*Blatella germanica* and *Periplaneta americana*) (Graczyk et al. 2005), which can carry *T. gondii* oocysts on their bodies and distribute the oocysts to new environments (Shapiro et al. 2019; Hill and Dubey 2002; Graczyk et al. 2005). Other mechanical vectors, such as domestic dogs (*Canis lupus familiaris*) (Lindsay et al. 1997), and marine brown turban snails (*Chlorostoma brunnea, Chlorostoma montereyi and Promartynia pulligo*) (Krusor et al. 2015) can ingest full *T. gondii* oocysts and pass them through their feces intact.

Another example of a potential mechanical vector is invasive land snails. Several land snail species are invasive to many countries that house wildlife species negatively affected by *T. gondii*, such as Argentina (Gregoric et al. 2011), Brazil (Thiengo et al. 2007) and Australia

(Blacket et al. 2016). Another example is the Hawaiian Islands, which have at least 63 non-native land snail species distributed across the islands, including *Lissachatina fulica* (Cowie 1998), one of the most destructive invasive land snail species across the globe (Albuquerque et al. 2008; Sneha and Chakravarthi 2021). It is possible that if invasive land snails, such as *L. fulica*, spatially occur with felids in the Hawaiian Islands, they could ingest the oocysts from the contaminated environment and defecate them to different environments as they move. Similarly, it is also possible that animals that consume the snails could contract toxoplasmosis from oocysts potentially retained in the digestive system, similar to concerns with brown turban snails and the southern sea otter (*Enhydra lutris nereis*) (Krusor et al. 2015). This potential movement of *T. gondii* oocysts could be of concern to native Hawaiian wildlife, such as the Hawaiian monk seal (Īlio-holo-i-ka-uaua; *Neomonachus schauinslandi*) (Honnold et al. 2005), the Hawaiian goose (Nēnē; *Branta sandvicensis*) (Work et al. 2002) and the Hawaiian crow (ʻAlalā; *Corvus hawaiiensis*) (Work et al. 2000) which are fatally impacted by toxoplasmosis. However, little work has been done to evaluate land snails as mechanical vectors. The University of Kansas Medical Center demonstrating that Puerto Rican land snails (*Caracolus caracolla*) can pass infectious *T. gondii* oocysts in their feces after ingesting infected cat feces, but they did not explore this in wild caught snails (Miller et al. 1972). Understanding how invasive land snails could disperse *T. gondii* and aid in the transmission of oocysts could be an important step in protecting wildlife populations from preventable death.

Given the lack of knowledge in how invasive land snails could be mechanical vectors for *T. gondii*, my goal was to determine if *T. gondii* DNA and full intact oocysts can be detected in *L. fulica* feces from the wild. To complete my goal, I underwent two objectives: 1) to collect a sample of *L. fulica* from feral cat congregation areas in Hawaiʻi and test a portion of the resulting

feces for *T. gondii* DNA and 2) to observe the remaining feces of samples positive for *T. gondii* DNA for intact oocysts. Based on previous findings (Krusor et al. 2015, Cong et al. 2021, and Miller et al. 1972), I hypothesize that I will detect *T. gondii* DNA and oocysts in wild caught *L. fulica*.

Methods

Sample Collection and Husbandry

To test my hypotheses, I collected 127 *L. fulica* from three collection sites in Honolulu, Hawai‘i. *L. fulica* was chosen because of their high abundance in Hawai‘i and invasive nature in several other countries (Lu et al. 2018). I collected 58 snails (33 snails on December 13, 2020 and 25 snails on December 16, 2020) from the University of Hawai‘i at Manoa (UH Manoa) on the side of Maile Way to the right of Gilmore Hall, 58 snails (30 snails on December 21, 2020 and 28 on January 3, 2021) from an area directly beside the Archie Baker Mini Park, and 61 snails on January 10, 2021 from various locations in the Honolulu Zoo (the cheetah enclosure, the garden beside the petting zoo, the garden by the ectotherm exhibits, the garden by the bird enclosure, and inside ectotherm exhibit 6, ectotherm 10-23) (see Figure 2.1).

Sample sites were selected based on sightings of feral cats and feral cat feedings by locals spotted by Bishop Museum staff and experts. The Honolulu Zoo was selected to determine if *T. gondii* could be detected in snails found on zoo grounds, which could be a concern to the species housed there. No feral cats were seen during field work at these sites. Evidence of feral cat presence was observed at UH Manoa (See Figure 2.2) and the Honolulu Zoo (See Figure 2.1).

Snails were individually housed in plastic pipette containers (sanitized with 10% bleach water, rinsed with hot tap water, and dried thoroughly with a paper towel) and kept for 72-hours without food. All the feces produced in the container within the 72-hour period was collected

from each snail. Feces was collected every day and placed in a 1.5 mL microcentrifuge tube. Snails were sprayed with sterile Nanopure water type I if they appeared dry. There were 12 snails that did not provide enough feces for molecular processing after 72 hours and were kept for an additional 24-hours to produce another fecal sample. A small metal spatula was used to scoop the fecal samples out of the plastic containers (sanitized with decontamination solution (1.5% sodium dichloro-s-triazinetrione, 1% NaOH [10 g/L], 1% detergent), rinsed with hot tap water, and wiped with a paper towel between uses). Fecal samples were stored in a freezer.

Snails were all housed in the Bishop Museum Guest Cottage on the Bishop Museum campus. Approximately 30 snails were kept in the cottage at a time. After the final fecal sample was collected, the snails were placed in a plastic bag and put in a freezer for euthanasia. The pipette containers and lids were soaked in hot water and decontamination solution. Containers and lids were rinsed with hot tap water and placed on a paper towel to air dry. They were used to house the next collection of snails. If the pipette containers were not completely dry before use, they were wiped or blotted on a paper towel.

Of the 177 snails, 50 were stricken from the data. 45 of these snails were kept for two additional 24-hour periods and were fed a small piece of lettuce to keep the snails digestive tracks moving. Since these lettuce aliquots were not measured, these snails were stricken from the data. An additional 5 snails were stricken from the data due to one of the following conditions: they died before I could collect feces over a 72-hour period, they failed to produce any feces, or a human error occurred that either made the sample unusable or unidentifiable. In total, 127 snails were used in the wild survey experiment (39 from UH Manoa, 28 from the Archie Baker Mini Park, and 60 from the Honolulu Zoo).

Fecal sample homogenization

All the samples for each snail were placed in a mortar (sprayed with decontamination solution [1.5% sodium dichloro-s-triazinetrione, 1% NaOH {10 g/L}, 1% detergent], wiped with a paper towel, rinsed with sterile Nanopure water type I, and wiped with a paper towel between uses) and homogenized together using a metal spatula (sanitized in 10% bleach water, sterile Nanopure water type I, then dried with a paper towel between uses). A portion of homogenized sample was placed in the first sample tube used in the Macherey-Nagel® NucleoSpin™ DNA fecal mini kit (M-N bead beater tubes type A) for molecular analysis. Samples were weighed and placed in the freezer until freeze/thaw and molecular analysis could be performed.

The remaining homogenized feces was placed in one of the 1.5 mL microcentrifuge tubes used to hold the samples prior to homogenization. 1000 µL of sterile Nanopure water type I was pipetted into the mortar. The water was mixed in the mortar to get as much of the feces off the sides of the mortar and spatula as possible. The same pipette tip was used to take as much of the water and feces mixture from the mortar as possible and place it in the 1.5 mL tube with the rest of the microscopy sample. In some circumstances, a new 1.5 mL tube was needed to hold all the microscopy sample. All microscopy samples were placed in the freezer until light microscopy was performed. Homogenization methodology was modified twice before this method was decided on (See Appendix B).

Samples that were used for molecular analysis were between 0.160-0.260 grams in weight. Of the 127 samples, 34 (26.771%) weighed outside of this range. Of these 34 samples, 20 weighed above this range (between 0.262-0.311 grams) and 14 samples weighed below this range (between 0.008-0.148 grams) (See Appendix B).

Freeze/thaw methodology

Samples for molecular analysis were placed in a -20°C freezer for 10 minutes and then immediately in a 95°C hotblock for 10 minutes interchangeably 3 times. If DNA extraction could not be done on the samples immediately after freeze/thaw, the samples were kept in the freezer on their third round until DNA extraction could be performed. Prior to DNA extraction, samples were placed in the 95°C hotblock for 10 minutes to complete freeze/thaw cycle (See Appendix B). Positive controls, consisting of *T. gondii* oocysts, underwent freeze/thaw separately (See Appendix B). A positive control was placed in the hot block with samples to complete the cycle prior to DNA extraction. *T. gondii* oocysts were provided by Dr. Karen Shapiro at the University of California at Davis (UC Davis) (See Appendix A). Oocysts (Type II strain M4) were isolated from cat feces of experimentally infected cats and sporulated before they were heat inactivated at 80°C for 20 minutes. Oocysts were suspended in 1 mL of PBS and shipped to the Pacific Center for Molecular Biodiversity (PCMB).

Molecular Methodology

All samples and controls underwent DNA extraction using the Macherey-Nagel® Nucleospin™ DNA fecal kit using the standard instructions with some modifications (See Appendix B). Negative controls consisted of 200 µL of autoclaved water. The first 16 samples were eluted with 30 µL of elution buffer (Buffer SE) at 95°C. The rest of the samples were eluted with 50 µL of elution buffer (Buffer SE) at 95°C (See Appendix A).

The gDNA for each sample was diluted 1:50 and amplified using conventional polymerase chain reaction (PCR) targeting the 529 bp repeat fragment (Krusor et al. 2015). Each sample reaction was in a 25 µL volume, containing 5 µL of template DNA, and a final concentration of 1X MangoMix™ (Bioline), and 0.2 µM forward external primer Tox4 (5'-

CGCTGCAGGGAGGAAGACGAAAGTTG-3'), and 0.2 µM reverse external primer Tox5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') (Homan et al. 2000). Samples ran on a cycling program with samples held at 95°C for 5 minutes, 50°C for 1 minute, and 72°C for 1 minute for one cycle, followed by 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute for 45 cycles, and a final extension at 72°C for 10 minutes and 4°C for 3 minutes for one cycle. Conditions for reactions using the internal primers, Tox4INT and Tox5INT (Shapiro et al. 2010) are still being optimized. PCR product ran on a 2% agarose gel by electrophoresis. All gels contained approximately 1.5-3.0 µg/mL of ethidium bromide and EasyLadder I was used as the DNA molecular weight marker. All gels ran for 30 minutes on 130 Voltage.

Four positive samples for PCR using the external primers targeting the 529 bp repeat fragment as stated above underwent a second nested PCR targeting the ITS1 region (Krusor et al. 2015). Sample gDNA was diluted 1:50 and reactions were in 20 µL volumes, containing 2 µL of template DNA and a final concentration of 1X MangoMix™ (Bioline), and 0.15 µM forward external primer ITS1DF (5'-TACCGATTGAGTGTTCCGGTG-3') and 0.15 µM reverse external primer ITS1DR (5'-GCAATTCACATTGCGTTTCGC-3') (Rejmanek et al. 2009). Samples ran on a cycling program with samples held at 95°C for 5 minutes, 56°C for 1 minute, and 72°C for 1 minute for one cycle, followed by 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute for 45 cycles, and a final extension at 72°C for 10 minutes and 4°C for 3 minutes for one cycle. Positive samples underwent a nested PCR under the same conditions using 2 µL of PCR amplicon and the forward internal primer ITSdiF (5'-CGTAACAAGGTTTCCGTAGG-3') and ITS1diR (5'-TTCATCGTTGCGCGAGCCAAG-3') (Rejmanek et al. 2009). PCR product ran on a 1.5% agarose gel by electrophoresis. All gels

contained approximately 1.5-3.0 µg/mL of ethidium bromide and EasyLadder I was used as the DNA molecular weight marker.

Positive samples were sent to Eurofins in Louisville, Kentucky for DNA sequencing via Sanger sequencing. The sample that amplified for the 529 bp repeat fragment was sent with the reverse primer Tox5INT (5'-CTCCACTCTTCAATTCTCTCC-3') of a set of internal primers (Tox4INT and Tox5INT) (Shapiro et al. 2010). The sample that amplified for the ITS1 region was sent with both internal primers for the ITS1 region (Rejmanek et al. 2009). DNA sequences were identified through NCBI BLAST and compared to databases in GenBank.

Fecal Flotation and Light microscopy

All samples that tested positive using molecular methodology were evaluated for *T. gondii* oocysts using fecal flotation and light microcopy, which was done at the Parasitology Laboratory at the College of Veterinary Medicine at Auburn University in Auburn, Alabama. These samples were taken from the freezer and thawed for 10 minutes. Samples were pipetted into a glass 15 mL tube. The 1.5 mL microcentrifuge tubes that held the sample was filled with 1000 µL of distilled water and vortexed for several seconds. The distilled water was pipetted out of the tube and placed in the corresponding 15 mL glass tube. This was repeated once, and the 1.5 mL tube was discarded. The glass tubes were filled with 10-15 mL of distilled water and centrifuged at 390 · g for 5 minutes. The supernatant was removed, and the pellet was resuspended with 5-7 mL of Sheather's sucrose solution (specific gravity: 1.27). If needed, the pellet was broken up with a wooden applicator. The glass tubes were placed in a centrifuge and filled with Sheather's solution to a reverse meniscus. A coverslip was placed on the top and the glass tubes were centrifuged at 250 · g for 10 minutes. The glass tubes were taken from the centrifuge and the cover slips were placed on a microscope slide. Microscope slides were

observed on 10x magnification and observed closer with 40x magnification when potential oocysts were found (Zajac et al. 2021) (See Appendix C). Samples were re-evaluated by the Parasitology Lab Research Assistant IV.

Results

Samples were considered positive for conventional PCR when a band appeared within 500 bp on the agarose gel (Homan et al. 2000). Of the 127 samples, 41 were positive for the 529 bp repeat fragment using the external primers (11 from UH Manoa, 10 from the Archie Baker Mini Park, and 20 from the Honolulu Zoo). Of the 20 positive samples collected from the Honolulu Zoo, four were collected from the garden next to the cheetah enclosure, two were from the garden near the petting zoo, five were from the garden outside the bird enclosure, four were outside the ectotherm exhibit, and five were inside ectotherm enclosure 6 (one in ectotherm 10, 11, 20, and two in ectotherm 17). Almost all these positive samples had additional non-positive strands of DNA that was amplified, which would inhibit DNA sequencing. One sample did not have additional strands and could be sent for DNA sequencing (Honolulu Zoo, bird enclosure garden).

Four of the 41 positive samples were amplified for the ITS1 region using nested PCR (all from Makiki Park) and were considered positive when a band appeared within 480-500 bp on the agarose gel (Tirosh-Levy et al. 2020). Gel pictures for the nested PCR was much cleaner with very few additional DNA strands. Three of the four samples could be sent for DNA sequencing. The results for the remaining 37 samples are pending.

Of the four samples sent for DNA sequencing, two matched for *T. gondii* on NCBI BLAST. The sample that amplified for the 529 bp repeat fragment locus matched at 95.06% similarity and the sample that amplified for the ITS1 region matched at 93% similarity. No

oocysts were detected for the sample that amplified the 529 bp repeat fragment locus. The results of the second sample that amplified for the ITS1 region is pending.

Discussion

Overall, I have shown support for the first part of my hypothesis, that *T. gondii* DNA can be detected in *L. fulica* feces, but not for full intact *T. gondii* oocysts. Although one sample that was amplified for the 529 bp repeat fragment was successfully sequenced for *T. gondii* DNA, nested PCR targeting the ITS1 region was more successful with eliminating excess non-positive DNA bands present in the sample, preventing inhibition during DNA sequencing. However, of the four samples that were amplified using the ITS1 region, only one successfully sequenced for *T. gondii* DNA. This indicates specificity issues with the ITS1 primers. The excess DNA bands found in the samples could be because of the large number of plant species that *L. fulica* are known to consume, as well as the feces of other animals, dead animals, and other dead *L. fulica* (Meyer et al. 2008). This results in a large amount of DNA in the samples. This could also be the reason for a high rate of DNA sequencing failure for the samples amplified for the ITS1 region. However, since *T. gondii* oocysts were not observed in the fecal sample I screened with microscopy to this point, there is not sufficient evidence that *L. fulica* can act as a mechanical vector for *T. gondii*. Finding *T. gondii* oocysts through light microscopy is considered a refutable standard for detecting the parasite (Liu et al. 2015), which differentiates the results between environmental DNA or DNA from degraded oocysts from intact oocysts that could theoretically cause illness. However, since the research shows that *T. gondii* DNA can be detected in wild *L. fulica* fecal samples, it is possible that *L. fulica* could serve as a biosentinel for *T. gondii*.

Several different species are used as biosentinels for *T. gondii*, such as arctic (*Vulpus lagopus*) and red foxes (*Vulpes vulpes*) (Bouchard et al. 2022), domestic dogs (Cabezon et al.

2010), and domestic chickens (*Gallus domesticus*) (Dubey et al. 2015; More et al. 2012). Molecular and serological methods are used to detect *T. gondii* DNA or antibodies against *T. gondii* in the sentinel species, which can be used to determine areas of concern (Cabezon et al. 2010; Dubey et al. 2015) or rate of exposure over certain periods of time (More et al. 2012). This has recently been done with feral chickens (*Gallus gallus*) on Kaua‘i to understand environmental factors that could contribute to *T. gondii* prevalence in different environments (Chalkowski et al. 2020). Using the methods described in this research, collecting and screening *L. fulica* for *T. gondii* could provide important additional information of prevalence, environmental factors, and recent shedding of oocysts, particularly if snails are collected near feral cat defecation areas. Since cats tend to defecate in the same areas (Shapiro et a. 2019; Alfonso et al. 2008), collecting *L. fulica* from identified latrines might be an efficient way to monitor patterns and timing of shedding, especially since environmental surveillance of *T. gondii* is difficult (Dumetre and Darde et al. 2003).

Removing *L. fulica* from the Hawaiian environment is automatically beneficial since the species is well established (Cowie et al. 1998) and considered a pest (Albuquerque et al. 2007). Although complete eradication of *L. fulica* in Hawai‘i would be incredibly costly and work intensive based on previous efforts in areas such as Florida (Roda et al. 2016), routine removals would provide relief for plant and native land snail species that might be negatively affected throughout Hawai‘i (Yeung et al. 2019). Molecular analysis has also been used to screen several species of filter feeding biosentinels for *T. gondii* in marine and freshwater environments (Shapiro et al. 2019) such as oysters, clams, and several species of mussels (Coupe et al. 2018; Coupe et al. 2019; Staggs et al. 2015). Furthermore, throughout the duration of the project, *L.*

fulica proved to be relatively easy to care for, especially within the constraints of a 72-hour period. They took up little space and provided feces almost immediately upon capture.

The results of the project reveal an apparent need to continue surveillance efforts in Hawai‘i, since one of the two fecal samples that was confirmed to contain *T. gondii* DNA came from a snail that was found at the Honolulu Zoo. Although no oocysts were found in the fecal sample, the presence of DNA indicates that *T. gondii* could be prevalent in the area. Given that native Hawaiian wildlife are housed at the Honolulu Zoo and is also an attraction for human populations, using invasive species as biosentinels in vulnerable locations could be a biological "win/win" in areas such as the Hawaiian Islands.

Although *T. gondii* oocysts could be observed in the fecal sample that is pending for microscopy results, I was not able to find oocysts in the fecal sample I screened. There are several reasons for why this could be. One is that there may have been enough oocysts shed in the fecal samples to detect using molecular methods, but not through light microscopy. During PCR optimizations, I discovered that as few as 100 oocysts could be detected in *L. fulica* feces. However, it is estimated that there needs to be at least 1000 oocysts per gram of feces in order to visualize oocysts during light microscopy (Dubey 2010). The microscopy sample that was observed was approximately 1.087 grams in weight, but it's possible that less than 1000 oocysts were present. A second explanation is that some microscopy samples were taken in and out of the freezer to place permanent labels on the 1.5 mL microcentrifuge tubes that contained the smaples. This could have simulated freeze/thaw techniques, which were designed to rupture the oocysts wall in the methodology of the project. Thirdly, it is also possible that fecal flotation and light microscopy are producing false negatives and are less sensitive than molecular methods.

Perhaps feeding a sample of snails a large amount of sporulated and unsporulated *T. gondii* oocysts would be useful for discovering what limitations are responsible for these findings.

Although the results were ultimately successful in answering the first part of my hypothesis, there were limitations to the project which compromised its ability to explain other relevant questions. The first was that the range for determining the size of homogenized feces for molecular analysis had a very large range and many samples fell outside of this range, resulting in an inconsistency between samples and making the project unable to determine the prevalence of *T. gondii* in the sample sites. Ideally, if the experiment was to be repeated, feces taken for molecular analysis would fall within a specific target weight within a 10% margin, eliminating any issues of inconsistency.

To the best of my knowledge, this is the first research project screening wild caught *L. fulica* for *T. gondii* DNA and a promising start to the possibility of using *L. fulica* as a biosentinel for *T. gondii*. Efforts to continue fecal flotation and light microscopy are underway. More specific primers could be explored to eliminate extra DNA that inhibits PCR and DNA sequencing, or even a metagenomics approach could be informative. Other research projects could identify locations of priority in Hawai‘i or feral cat latrines for *L. fulica* screening or to explore the potential reasons for why *T. gondii* oocysts were not observed in snail fecal samples.

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List of Tables

Table 2.1. Fecal samples that tested positive for *T. gondii* DNA.

	Total # of Snails/Fecal samples	Conventional PCR (529 bp repeat fragment)	Nested PCR (ITS1 region)*	DNA Sequencing	Light Microscopy
UH Manoa	39	11	0	0	0
Archi Baker Mini Park	28	10	4	1	**
Honolulu Zoo	61	20	0	1	0
Total	127	41	4	2	0

* Results are pending for 37 samples. 4 samples have been screened for this locus and are represented on this table.
**Results are pending

List of Figures


Petting Zoo garden
Kapahulu Ave
Honolulu Zoo
Bird enclosure
Cheetah exhibit garden
Ectotherm exhibit garden
Ectotherm exhibits
Google Earth
1000 ft
N

Appendix A

Research Project Optimization

Fecal homogenization using a mortar – Two fecal samples were collected from a Bishop Museum Malacology Department *L. fulica* "mascot." The "mascot" was caught in the wild and hand raised in the malacology department for at least 12 months. The snail was fed human grade leftover fruits and vegetables. The two fecal samples were homogenized together in a mortar (sprayed with decontamination solution [1.5% sodium dichloro-s-triazinetrione, 1% NaOH {10 g/L}, 1% detergent], wiped with a paper towel, rinsed with sterile Nanopure water type I, and wiped with a paper towel between uses) and metal spatula (sanitized in 10% bleach water, sterile Nanopure water type I, then dried with a paper towel between uses) and split into three equal parts (two samples were 0.152 grams and the third sample was 0.151 grams). Each part was placed in a new 1.5 mL microcentrifuge tube. One was spiked with approximately 100 oocysts (0.152 g), the other with approximately 20 oocysts (0.152 g), and the third was not spiked with any oocysts (0.151 g). The samples underwent freeze/thaw, the second protocol for DNA extraction (See Appendix C) and were amplified using conventional PCR targeting the 529 bp repeat fragment (See Chapter 2). The two spiked samples amplified positive for *T. gondii* DNA and the control sample was negative. The sample that was spiked with approximately 100 oocysts successfully sequenced for *T. gondii*. I considered this sufficient confirmation that *T. gondii* DNA in *L. fulica* feces can be detected when using a mortar to homogenize fecal samples.

To ensure my sanitization methods were efficiently preventing possible contamination between samples, approximately 100 *T. gondii* oocysts were pipetted into a cleaned mortar (sprayed with decontamination solution [1.5% sodium dichloro-s-triazinetrione, 1% NaOH {10 g/L}, 1% detergent], wiped with a paper towel, rinsed with sterile Nanopure water type I, and

wiped with a paper towel between uses) and "homogenized" several times with a cleaned metal spatula (sanitized in 10% bleach water, sterile Nanopure water type I, then dried with a paper towel between uses). A new pipette tip was used to transfer as much of the solution as possible into a M-N bead beater tube type A. The mortar was sanitized and the process was repeated using 1000 µL of sterile Nanopure water type I. This was the negative control. The two samples underwent freeze/thaw, DNA extraction using the third DNA extraction protocol (See Appendix C) and were amplified using conventional PCR targeting the 529 bp repeat fragment. The sample of 100 oocysts amplified positive for *T. gondii* DNA and the negative control did not amplify. I considered this sufficient confirmation that my sanitization methods were preventing contamination between samples.

DNA extraction – All the DNA extraction positives for the 127 wild caught fecal samples underwent conventional PCR targeting the 529 bp repeat fragment (Krusor et al. 2015) (See Chapter 2). There were 10 positive controls. Six had approximately 100 oocysts and four had approximately 50 oocysts. Dilutions were not made prior to PCR. All six of the controls that had approximately 100 oocysts amplified and the four controls that had approximately 50 oocysts did not amplify (See Appendix B). The six controls that amplified positive successfully sequenced for *T. gondii* DNA. I considered this confirmation that *T. gondii* DNA can be detected using my DNA extraction protocols as long as at least 100 oocysts were present.

To confirm that 100 oocysts in *L. fulica* feces could be detected, A control *L. fulica* fecal sample was spiked with approximately 100 oocysts and underwent freeze/thaw and molecular methodology (See Appendix A, Chapter 2, "Fecal homogenization using a mortar") The sample amplified and successfully sequenced for *T. gondii* DNA. I considered this confirmation that *T.*

gondii DNA can be detected in *L. fulica* feces using my DNA extraction protocols as long as at least 100 oocysts were present.

Conventional PCR (529 bp repeat region) – The first 100 samples underwent conventional PCR with the first 27 reactions occurring in a 25 µL volume, containing 5 µL of template DNA, and a final concentration of 1X MangoMix™ (Bioline), and 0.2 µM forward external primer Tox4 (5'-CGCTGCAGGGAGGAAGACGAAAGTTG-3'), and 0.2 µM reverse external primer Tox5 (5'-CGCTGCAGACACAGTGCATCTGGATT-3') (Homan et al. 2000). The second 73 reactions occurred in 15 µL volumes, containing 3 µL of template DNA, and a final concentration of 1X MangoMix™ (Bioline), and 0.2 µM of both primers. The gDNA was not diluted for these samples and the thermocycling program held samples at 95°C for 5 minutes, 50°C for 1 minute, and 72°C for 1 minute for one cycle, followed by 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute for 45 cycles, and for a final extension at 72°C for 10 minutes and 4°C for 3 minutes for one cycle. From these 100 samples, three positive samples, a negative sample, and positive controls underwent several conventional PCRs to eliminate excess additional nonpositive DNA bands. Although I was not able to completely eliminate junk bands, the clearest PCR positive bands occurred when the gDNA for samples were diluted 1:50, positive controls were not diluted, and reactions occurred in 25 µL volumes with 5 µL of template DNA, and the cycling program was kept the same as it was when the first 100 samples were initially screened.

Nested PCR (ITS1 region) – Five positive controls and three positive samples using conventional PCR targeting the 529 bp repeat region underwent nested PCR targeting the ITS1 region (Krusor et al. 2015). The gDNA of the five positive samples were diluted 1:50 and reactions were in 20 µL volumes, containing 2 µL of template DNA and a final concentration of 1X MangoMix™

(Bioline), and 0.15 µM forward external primer ITS1DF (5'-TACCGATTGAGTGTTCCGGTG-3') and 0.15 µM reverse external primer ITS1DR (5'-GCAATTCACATTGCGTTTCGC-3') (Rejmanek et al. 2009). Samples ran on a cycling program with samples held at 95°C for 5 minutes, 56°C for 1 minute, and 72°C for 1 minute for one cycle, followed by 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute for 45 cycles, and a final extension at 72°C for 10 minutes and 4°C for 3 minutes for one cycle. Positive samples underwent a nested PCR under the same conditions using 2 µL of PCR amplicon and the forward internal primer ITSdiF (5'-CGTAACAAGGTTTCCGTAGG-3') and ITS1diR (5'-TTCATCGTTGCGCGAGCCAAG-3') (Rejmanek et al. 2009). Positive bands were clear with very little excess nonpositive DNA bands, therefore this methodology will be used to screen the remaining positive samples.

Fecal flotation optimization – Two methods were designed for fecal flotation. The first method was tested with a fecal sample that amplified and sequenced positive for *T. gondii*. I was unable the use the sample in the study. This sample underwent the first protocol for fecal flotation. No *T. gondii* oocysts were observed, but there were other parasite egg-like life forms observed on the resulting slide. This method was replaced with the second fecal flotation method since there was concern that oocysts could be missed by taking off the top 20 µL of the sample suspended in the falcon tube (See Appendix C). The second method is the standard method used for *T. gondii* oocysts detection in the Parasitology Lab at the College of Veterinary Medicine at Auburn University in Auburn, Alabama

Appendix B

Exceptions and Modifications

Exceptions

Chapter 1:

Fecal sample homogenization – Portions taken for molecular analysis from homogenized fecal samples were planned to weigh 50% of the total weight of the sample. Percentages of the portions taken from each sample are inconsistent due to human error (See Table A.1).

Table A.1. The actual percentages taken for molecular analysis

	Snail 1 – 1st fecal sample	Snail 1 – 2nd fecal sample	Snail 2 – 1st fecal sample	Snail 2 – 2nd fecal sample	Snail 3 – 1st fecal sample	Snail 3 – 2nd fecal sample	Snail 4 – 1st fecal sample	Snail 4 – 2nd fecal sample
Total Weight	Un-known	0.154 g	0.224 g	0.227 g	0.358 g	0.081 g	0.226 g	0.251 g
Amount taken for Mol. Analysis	0.069 g	0.064 g	0.086 g	0.142 g	0.177 g	0.041 g	0.125 g	0.124 g
% of total weight	Un-known	41.558%	38.392%	62.555%	49.441%	50.617%	55.309%	49.402%

Fecal flotation/light microscopy – Microscopy samples underwent fecal flotation using the first fecal flotation protocol (See Appendix C). Afterwards, the falcon tubes were capped and placed in the fridge for 5 days. The samples were resuspended in Sheather's sugar solution to the 14 mL mark. Samples were spun down at 250 · g for 10 minutes. The samples were filled with Sheather's sugar solution to a reverse meniscus and a microscope slide was placed on top. Samples sat for 20 minutes before slides were made. All microscopy samples underwent fecal flotation using the second fecal flotation protocol from this point onward.

Chapter 2:

Fecal sample homogenization – Portions taken from homogenized feces for molecular analysis was planned to weigh within 0.180-0.220 grams, according to the Macherey-Nagel® Nucleospin™ DNA fecal kit standard instructions. Samples were inconsistent with this range, due to human error. Of the 127 samples, 73.22% percent of samples weighed within 0.160-0.260 grams, 15.748% was above this range (0.262-0.311 grams) and 11.023% percent was below this range (0.008-0.148).

Freeze/thaw methodology – During freeze/thaw, 21 of the 127 samples (16.535%) underwent an additional 2 periods in the freezer and hotblock each (in the freezer and hotblock 5 times overall), of which 1 of these samples was confirmed to have *T. gondii* DNA.

Positive and negative controls for DNA extraction – For the first 15 samples, the DNA extraction positive control was a small piece of *L. fulica* tissue, which was provided by PCMB. The piece of tissue was prepared according to steps 3-7 from the protocol used for isolating DNA from snail tissue (see Appendix C). After this, the tissue underwent DNA extraction according to the first protocol with the 15 samples (see Appendix C). This was the only time *L. fulica* tissue was used as the positive control for DNA extraction.

There were 10 DNA extraction positive controls for the 127 samples; six of these had approximately 100 oocysts and four had approximately 50 oocysts. The four controls that had approximately 50 oocysts never amplified a positive band. There were 61 samples that had these as their DNA extraction controls, one of which was confirmed to have *T. gondii* DNA.

Modifications

Chapter 1:

DNA extraction – All samples underwent DNA extraction using the third DNA extraction protocol (See Appendix C).

DNA extraction protocols – The third DNA extraction protocol followed the standard instructions provided by the Macherey-Nagel® NucleoSpin™ DNA fecal mini kit with some modifications. The first involved adding 10 µL of Proteinase K to each sample, shaking horizontally for 2-3 seconds, and incubating for 20 minutes at 70°C (vortexted every 10 minutes) (steps 7-8) (See Appendix C). This step derived from the supplementary protocol for isolating genomic DNA from chicken feces. The second is that 200 µL of autoclaved water was added to each sample and control prior to freeze/thaw, and 700 µL of ST1 was added after freeze/thaw (steps 1-3).

Chapter 2:

Fecal sample homogenization – fecal sample homogenization was modified twice before the final method was decided on:

- First 15 samples – This methodology was used for the first 15 of the 127 fecal samples (11.811%). All the fecal samples for each snail were submerged in 4 mL of sterile Nanopure water type I in a 15 mL falcon tube. The samples were vigorously homogenized with a bulb pipette and a vortex mixer. 1000 µL of the homogenized solution was taken from the falcon tube with the same bulb pipette and put in a clean 1.5 mL microcentrifuge tube. The 1.5 mL microcentrifuge tubes were centrifuged at 14,000 · g for 10 minutes and the supernatant was removed with a micropipette. After this, the 1.5 mL tube was weighed. If the sample was

too light, another 1000 µL of homogenized solution was put in the corresponding tube and the tube was centrifuged again. If the sample was too heavy, a cleaned metal spatula (sanitized with decontamination solution and wiped with a paper towel) was used to scoop a small part of the pellet out of the 1.5 mL microcentrifuge tube and put back in the falcon tube. Once the pellet was between 0.160–0.260 grams of weight, the sample was put in the freezer for molecular analysis. The rest of the homogenized solution in the falcon tube was placed in a new 1.5 mL microcentrifuge tube using the bulb pipette. These samples were not spun down and were also placed in the freezer until microscopy. Using this method, it took over 40 minutes to finish 15 samples, which was impractical and time consuming.

- Second 38 samples – The next 38 of the 127 fecal samples (29.921%) underwent this methodology. All the fecal samples of each snail were placed in a mortar and homogenized together using a metal spatula. Using the same metal spatula, a portion of the homogenized sample was placed in one of the original 1.5 mL microcentrifuge tubes that held the fecal samples prior to homogenization. The tube was weighed and either more homogenized feces was added to the tube or taken away using the same metal spatula until it reached the correct weight of 0.160-0.260 grams. Once this range was reached, the tube was relabeled and placed in a freezer for molecular analysis. The rest of the homogenized sample in the mortar was placed in another one of the 1.5 mL microcentrifuge tubes used to hold the fecal samples prior to homogenization. This would be the microscopy sample. After the microscopy sample was taken out of the mortar, 1000 µL of

sterile Nanopure water type I was pipetted into the mortar. The same metal spatula was used to mix the water in the mortar to get as much of the feces off the sides of the mortar and metal spatula as possible. The same pipette tip was used to take as much of the water and feces mixture from the mortar as possible and place it in the 1.5 mL microcentrifuge tube with the rest of the microscopy sample. In some circumstances, a new 1.5 mL microcentrifuge tube was needed to hold the entire microscopy sample. Microscopy samples were placed in the freezer until microscopy could be done. For 54 samples (42.519%) the sterile Nanopure water type I was not pipetted into the mortar, but a small unmeasured portion was poured into the mortar. This was changed to pipetting 1000 µL of water into the mortar to keep samples consistently processed. This is the process used to process all microscopy samples for the rest of this research project.

Although this method was much more efficient time wise, there was a concern that a good portion of the sample was still inside the 1.5 mL microcentrifuge tube after transferring the sample to the M-N bead beater tube type A, which is the first tube that samples are placed in when undergoing DNA extraction using the Macherey-Nagel® NucleoSpin™ DNA fecal mini kit. This was accomplished through steps 2-5 of the second DNA extraction protocol (See Appendix C). To maximize the amount of molecular sample that underwent DNA extraction, another modification was made for the last 74 samples.

- Final 74 samples – For the final 74 fecal samples, feces were homogenized according to the previous method. However, instead of placing a portion of homogenized feces inside one of the original 1.5 mL microcentrifuge tubes, it was

put directly into a M-N bead beater tube type A. These M-N bead beater tubes type A were weighed, labeled, and placed in a freezer until freeze/thaw and DNA extraction was performed. Freeze/thaw was performed on the samples in the M-N bead beater tubes type A. This is the final method used to homogenize fecal samples and the standard method for processing *L. fulica* fecal samples for the rest of this research project

DNA extraction – The first 15 samples underwent DNA extraction according to the first DNA extraction protocol (See Appendix C). The next 38 samples underwent DNA extraction according to the second DNA extraction protocol (See Appendix C). The final 74 samples underwent DNA extraction according to the second DNA extraction protocol, excluding steps 2-5. Instead, after step 1, each sample and control had 500 µL of ST1 and 200 µL of autoclaved water added and samples immediately continued the protocol at step 6.

DNA extraction protocols – The DNA extraction protocols followed the standard instructions provided by the Macherey-Nagel® NucleoSpin™ DNA fecal mini kit with some modifications. The first modification involved adding 20 µL of Proteinase K to each sample, shaking horizontally for 2-3 seconds, and incubating for 30 minutes at 70°C (vortexted every 10 minutes) (step 9-10 for the first DNA extraction protocol). This was modified further to 10 µL of Proteinase K and incubated for 20 minutes (vortexed every 10 minutes) (step 9-10 for the second DNA extraction protocol and step 7-8 for the third DNA extraction protocol) (See Appendix C). This step derived from the supplementary protocol for isolating genomic DNA from chicken feces. The second modification is that instead of 1 mL of ST1 added to the samples as stated in the first DNA extraction protocol (step 6), 700 µL of ST1 and 200 µL of autoclaved water were added to the samples in the second protocol (step 2-5).

Appendix C

Protocols

First protocol for DNA extraction:

M-N NucleoSpin DNA from Stool samples

Isolating T. gondii DNA from giant African snail feces

1. Pool three fecal samples from each snail in a labeled 15 polypropylene tube. Let thaw for 1 minute
2. Put **4 mL** of holy water in the 15 mL tube and homogenize the three stool samples vigorously.
3. Take **1 mL** of homogenized feces from the 15 mL tube and place it in a **MN Bead Tube Type A**. Centrifuge at 14,000 x g for 10 minutes.
4. Remove and discard the supernatant without disturbing the pellet
5. Save the 3mL of homogenized feces in the 15 mL tube for microscopy
6. Add **1 mL** of **Buffer ST1** to the bead tube containing the pellet
7. Place the bead tube in the RT bead beater rack and vortex for **10 minutes** at **max speed**
8. Centrifuge the bead tube for 5 seconds at 13,000 x g to spin down foam
9. Add **20 µl** Proteinase K and vortex (or shake horizontally) for 2-3 seconds.
10. Incubate at 70C for 30 minutes, invert the tube every 10 minutes to mix the solution.
11. Label a 1.5 mL tube during this time which will be used for step 29.
12. Centrifuge for 3 minutes at 13,000 x g
13. Transfer **600 µl** of the supernatant to a 2 mL tube. If there is less supernatant than 600 µl**,** transfer as much of the supernatant as you can without disturbing the pellet.
14. Add **100 µl** of **buffer ST2** to the 2 mL tube and vortex for 5 seconds
15. Centrifuge the 2 mL tube for 3 minutes at 13,000 x g.
16. Place a NucleoSpin Inhibitor Removal Column (red ring) in a collection tube (2mL, lid)
17. Place **550 µl** of supernatant from the 2 mL tube onto the NucleoSpin Inhibitor Removal Column (if there is less than 550 µl of supernatant, transfer as much as possible without disturbing the pellet)
18. Close the lid of the Removal Column and centrifuge for 1 min at 13,000 x g
19. Discard the Removal Column (if there is still a pellet in the flowthrough, take the supernatant and place in a new 2 mL tube)
20. Add **200 µl of buffer ST3** to the collection tube and vortex for 5 seconds
21. Place a NucleoSpin DNA Stool Column (green ring) in a collection tube (2mL, no lid)
22. Load **700 µl of sample** onto the column and centrifuge for 1 minute at 13,000 x g.
23. Discard flowthrough and place the column back into the collection tube
24. Add **600 µl** of **buffer ST3** to the column and centrifuge for 1 minute at 13,000 x g
25. Discard flowthrough and put **550 µl** of **buffer ST4** onto the column and centrifuge for 1 minute at 13,000 x g

26. Discard flowthrough and put **700 µl** of **Buffer ST5** onto the column and vortex for 2 seconds. Then centrifuge for 1 minute at 13,000 x g.
27. Discard flowthrough and put **700 µl** of **buffer ST5** onto the column and centrifuge for 1 minute and 13,000 x g.
28. Discard flowthrough and centrifuge for 2 minutes at 13,000 x g.
29. Place the column into a labeled 1.5 mL tube from step 11 and put **30 µl** of **95C holy water** onto the column and centrifuge for 1 minute at 13,000 x g.
30. Discard the column and vortex the 1.5 mL tube for 2 seconds

Second protocol for DNA extraction:

M-N NucleoSpin DNA from Stool samples

Modified for L. fulica feces to screen for *T. gondii*

Make sure Buffer ST5 contains ethanol before first use

Set Heatblocks to 95°C and 70°C

Label all tubes needed at the start if possible, including final elution tubes

1. Freeze/thaw fecal samples for DNA extraction at least **3 times** (-20°C 10 min:95°C 10 min)
2. Add **500µl** of **Buffer ST1** to each 200-250mg sample
3. Transfer all the sample/ST1 mixture to a **M-N bead beater tube type A** using a 1000µl tip
4. Add another **200µl** of **Buffer ST1** and **200µl** of **sterile water** to the original 1.5 ml microcentrifuge tube
5. Vortex to resuspend any remaining feces/cysts – transfer to the type A tube with pipette.
6. Shake to mix the samples and beads. Incubate samples at **70°C** for **5 min** (vortex after 2.5 min)
7. Place samples in bead beater rack (**balance and level**) and beat for **10 min (2X 5 min)** at **1400**
8. Centrifuge at **13,000 x g** for **5 s** to spin down foam
9. Add **10µl** of **Proteinase K** and mix briefly
10. Incubate at **70°C** for **20 min**. vortex every **10 min**.
11. After incubation, centrifuge at **13,000 x g** for **3 min**
12. Transfer **600µl** of the supernatant to a **1.5 mL** tube. (DO NOT DISTURB PELLET)
    Note: Transfer as much of the supernatant as possible if less than **600µl**
13. Add **100µl** of **buffer ST2** and vortex for **5 s**.
14. Incubate at **4°C** for **5 min**
15. Centrifuge at **13,000 x g** for **3 min**.
16. Transfer **550µl** of supernatant into the NucleoSpin Inhibitor Removal Column (**RED RING**)
    Note: Transfer as much of the supernatant as possible if less than 550 µl
17. Centrifuge at **13,000 x g** for **3 min**.
18. Discard the Removal Column and if a pellet is visible, transfer supernatant to a new 1.5 mL tube.
19. Add 200µl of Buffer ST3 and vortex for 5 s.
20. Transfer 700µl of sample to NucleoSpin DNA Stool Column (**GREEN RING**) and centrifuge at **13,000 x g** for **1 min**.
21. Discard flowthrough and place the column back into the collection tube
22. Add **600µl** of **Buffer ST3** to the column and centrifuge at **13,000 x g** for **1 min**
23. Discard flowthrough and add **550µl** of **Buffer ST4** onto the column and centrifuge at **13,000 x g** for **1 min**
24. Discard flowthrough and add **700µl** of **Buffer ST5** to the column and vortex for **2 s**
25. Centrifuge at **13,000 x g** for **1 min**

26. Discard flowthrough and add **700µl** of **Buffer ST5** to the column and centrifuge at **13,000 x g** for **1 min**
27. Discard flowthrough and centrifuge at **13,000 x g** for **2 min** to dry membrane.
28. Place the column into a labeled **1.5mL** tube (step 1) and add **50µl** of **95°C Buffer SE** to the center of the membrane
29. Incubate at **RT** for **1 min** and centrifuge at **13,000 x g** for **1 min**.
30. Discard the column and vortex the 1.5ml for 2 sec.

Note – for the last 87 samples in Chapter 1, steps 3 and 5 were skipped.

Third protocol for DNA extraction:

M-N NucleoSpin DNA from Stool samples

Modified for L. fulica feces to screen for *T. gondii*

Make sure **Buffer ST5** contains ethanol before first use

Set Heatblocks to **95°C** and **70°C**

Make sure an aliquot of **buffer SE** is in the **95°C** hotblock

Label all tubes needed at the start if possible, including final elution tubes

1. Add **200µl** of **sterile water** to each 200-250mg fecal sample. Vortex for 10 seconds.
2. Freeze/thaw fecal samples for DNA extraction at least **3 times** (-80°C 10 min:95°C 10 min)
3. Add **700µl** of **Buffer ST1** to each sample.
4. Shake to mix the samples and beads. Incubate samples at **70°C** for **5 min** (vortex after 2.5 min)
5. Place samples in bead beater rack (**balance and level**) and beat for **10 min (2X 5 min)** at **1400**
6. Centrifuge at **13,000 x g** for **5 s** to spin down foam
7. Add **10µl** of **Proteinase K** and mix briefly
8. Incubate at **70°C** for **20 min**. Vortex every **10 min**.
9. After incubation, centrifuge at **13,000 x g** for **3 min**
10. Transfer **600µl** of the supernatant to a **1.5 mL** tube. (DO NOT DISTURB PELLET)
    Note: Transfer as much of the supernatant as possible if less than **600µl**
11. Add **100µl** of **buffer ST2** and vortex for **5 s**.
12. Incubate at **4°C** for **5 min**
13. Centrifuge at **13,000 x g** for **3 min**.
14. Transfer **550µl** of supernatant into the NucleoSpin Inhibitor Removal Column (**RED RING**)
    Note: Transfer as much of the supernatant as possible if less than 550 µl
15. Centrifuge at **13,000 x g** for **1 min**.
16. Discard the Removal Column and if a pellet is visible, transfer supernatant to a new **1.5 mL** tube.
17. Add **200µl** of **Buffer ST3** and vortex for **5 s**.
18. Transfer **700µl** of sample to NucleoSpin DNA Stool Column (**GREEN RING**) and centrifuge at **13,000 x g** for **1 min**.
19. Discard flowthrough and place the column back into the collection tube
20. Add **600µl** of **Buffer ST3** to the column and centrifuge at **13,000 x g** for **1 min**
21. Discard flowthrough and add **550µl** of **Buffer ST4** onto the column and centrifuge at **13,000 x g** for **1 min**
22. Discard flowthrough and add **700µl** of **Buffer ST5** to the column and vortex for **2 s**
23. Centrifuge at **13,000 x g** for **1 min**

24. Discard flowthrough and add **700µl** of **Buffer ST5** to the column and centrifuge at **13,000 x g** for **1 min**
25. Discard flowthrough and centrifuge at **13,000 x g** for **2 min** to dry membrane.
26. Place the column into a labeled **1.5mL** tube (step 1) and add **50µl** of **95°C Buffer SE** to the center of the membrane
27. Incubate at **RT** for **1 min** and centrifuge at **13,000 x g** for **1 min**.
28. Discard the column and vortex the **1.5ml** for **2 sec**.

Protocol for isolating DNA from snail tissue:

M-N NucleoSpin DNA Isolation

Gastropod Protocol

Check Prot-k stocks.
If needed, add **2.5** ml of Proteinase buffer to **50** mg powder Prot-K (Store 500 µl aliquots -20°C)
Make sure buffer **B5** has ethanol added to it (**160** ml of EtOH to **40** ml of **B5** concentrate)
Preheat aliquot of buffer **BE** to 70 °C

1. Prepare **55 °C** hotblock – note you will need it set to **70 °C** for the second day.
2. a. If tissues already subsampled and in **T1** add **15 µl Proteinase K** (20mg/ml) to each tube, then go to **step 7**

   b. If subsampling tissue, label a 1.5 ml tube for each sample being extracted and add **215 µl** of **FRESHLY MADE T1 buffer/Prot-K** (200 ml T1 buffer with 15 µl Proteinase K (20mg/ml)) solution to each tube. *(do not mix T1 buffer/Prot-K more than 15 min prior to use).* **Go to step 3**
3. Using a sterile razor, cut a ~**5-10 mg** piece of foot tissue (ca. 3 x 3 x 3 mm) and place in a drop of sterile H_2O on glass plate.
4. Repeat for each snail being extracted and allow tissue to soak in the H_2O for ~**1 min**.
5. Place tissue on piece of weigh paper (cut into 1/8 strips) and fold the weigh paper over the tissue. Crush the tissue using a hammer or blunt object.
6. Using a sterile toothpick remove the crushed tissue from the weigh paper and place into the 1.5 ml tube containing the T1/Prot-K solution.
7. **Vortex** each sample and place in the 55°C hotblock and incubate for at **least 1hr** or until all tissue has dissolved (**Overnight preferred**). Vortex samples every 10-15 min.
8. Once all tissue has dissolved, vortex for 20 sec and add **200 µl** of **buffer B3** to each sample. It is important that the B3 buffer and sample be mixed thoroughly and immediately.
9. Vortex vigorously and then incubate samples for **10 min at 70°C.**
10. After incubation vortex briefly and add **210 µl of 100% EtOH** (from -20 °C). Vortex ~ 20 sec. If precipitate forms make sure it is all added to the column in the next step.
11. Transfer all of mixture from previous step into the labeled **NucleoSpin column** inside a labeled 2 ml collection tube provided with kit. Incubate at RT for **2 min**.
12. Centrifuge at ≥ 11 000 x g (~ **10 200 rpm**) for **1 min**. Discard flow through and place column back in the same collection tube.
13. Add **500 µl** of **Buffer BW** and centrifuge for **1 min** at ≥ 11 000 x g (~ **10 200 rpm**). Discard flow through and place back into collection tube.
14. Add **600 µl** of **Buffer B5**. Centrifuge for **1min** at ≥ 11 000 x g (~ **10 200 rpm**). Discard flow through and place column back in the same tube.
15. Centrifuge for 1 min at ≥ 11 000 x g (~ **10 200 rpm**) to remove residual EtOH. Discard flow through and collection tube. Remove column carefully after this step to make sure you don't carry over any flow through from the previous step. The EtOH from previous step can contaminate DNA and prevent amplification later.
16. Place NuceloSpin column in a clean (sterile) labeled 1.5 ml microcentrifuge tube.

17. Add 50 µl of pre-warmed **Buffer BE (70°C)** directly on the membrane of the column. Incubate at RT for ~3 min and spin at ≥ 11 000 x g (~ **10 200 rpm**) for 1 min to elute DNA.
18. Repeat step 17 with 50 µl of warm **Holy H_2O**.
19. Spec and store DNA at 4°C if doing PCR that day or the next. If not doing PCR until later store at -20°C until needed for PCR. **Long term (>6 months) storage at -80 °C.**

First Protocol for fecal flotation:

T. gondii oocysts from *L. fulica* feces

NOTE - This methodology is for samples that have arrived with no preservative and/or nanopure water

Fecal Flotation Methodology:

1. Gather all the 1.5ml tubes of feces for each snail and place them in a rack. Let them thaw for 10 minutes
2. For tubes with only feces and no water, scoop the feces into a 15mL falcon tube with a 1mL pipette tip. Fill the 1.5mL tubes with distilled or autoclaved water and vortex and shake the 1.5mL tubes vertically for several seconds
3. Pipette the water out of the 1.5mL tubes and place in the corresponding 15mL falcon tube. Discard the 1.5mL tubes
4. For tubes with feces and water, pipette the sample into the same 15mL falcon tube with a 1mL pipette tip. Fill the 1.5mL tubes with distilled or autoclaved water and vortex and shake the 1.5mL tubes vertically for several seconds
5. Pipette the water out of the 1.5mL tubes and place in the corresponding 15mL falcon tube.
6. Repeat steps 2-5 two times for each 1.5mL tube. Discard the 1.5mL tubes when done
7. Spin the falcon tubes down at 2500 x g for 10 minutes (Sroka et al. 2018)
8. Take off the supernatant and resuspend the pellet in sucrose solution to the 14mL mark. Vortex the pellet in the sucrose solution for several seconds.
9. Spin the falcon tube down at 2500 x g for 10 minutes
10. Put the falcon tubes in a rack and let them sit for 5 minutes (source)
11. While the falcon tubes are sitting, label two microscope slides for each sample with a sharpie "Sample number – Initials and date" and "Sample number-B – initials and date"
12. Take the top 20µL off the sucrose solution with a micropipette and put on the labeled microscope slide. Take the next 20µL off the top and place on the microscope slide labeled with a B
13. Cover both with a cover slip and observe under 100x with light microscopy
14. If no oocysts are observed, make the slides permanent for future checking and clean the falcon tubes with hot soapy water
15. If oocysts are observed, take a picture and make the slide permanent for future viewing or pictures
16. Wash all supplies with hot soapy water. Remember to properly dispose of biohazard waste and sharps

Second protocol for fecal flotation:

T. gondii oocysts from *L. fulica* feces

NOTE - This is the final methodology that was used for all samples (four exceptions)

Fecal Flotation Methodology:

1. Gather all the 1.5ml tubes of feces for each snail and place them in a rack. Let them thaw for 10 minutes
2. **For tubes with only feces inside**, scoop the feces into a 15mL glass tube with a 1mL pipette tip.
3. Fill the 1.5mL tubes with distilled water to the 1000µL mark. Vortex the tubes and shake for several seconds
4. Pipette the water out of the 1.5mL tubes and place in the corresponding 15mL glass tube. Repeat steps 3 and 4 once, then discard the 1.5mL tubes
5. **For tubes with feces and water inside**, pipette the sample into the same 15mL glass tube with a 1mL pipette tip.
6. Fill the 1.5mL tubes with distilled water to the 1000µL mark. Vortex the tubes and shake for several seconds
7. Pipette the water out of the 1.5mL tubes and place in the corresponding 15mL glass tube. Repeat steps 6 and 7 once, then discard the 1.5mL tubes
8. Centrifuge the glass tubes at 390 x g for 5 minutes (Source)
9. Take off the supernatant with a bulb pipette and resuspend the pellet in Sheather's sucrose solution to about two-thirds the pellet's volume and break up the pellet with a wooden applicator stick.
10. Place the glass tubes in the centrifuge and fill the glass tubes to a reverse meniscus with the sucrose solution
11. Carefully place a coverslip on the tube and tap gently to assure good contact
12. Centrifuge the glass tubes at 250 x g for 10 minutes
13. While waiting, label microscope slides for each sample
14. Put the glass tubes in a rack and put the coverslip on the appropriate microscope slide
15. Observe slides at 10x and observe further with 40x when a potential oocysts is discovered
16. If no oocysts are observed, make the slides permanent for future checking and discard the glass tubes
17. If oocysts are observed, take a picture and make the slide permanent for future viewing or pictures