UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Prevalence of *Toxoplasma gondii* in commensal pest rodents at the National Zoological Garden in South Africa

by

Ndidzulafhi Terrence Thovhakale

Submitted in partial fulfilment of the requirements for the degree

MSc Veterinary Tropical Diseases in Global One Health

Department of Veterinary Tropical Diseases

Faculty of Veterinary Science

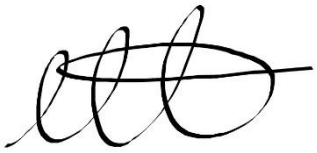
University of Pretoria

Supervisor: Prof Darshana Morar-Leather

October 2023

Declaration

I hereby declare that this mini dissertation, which I hereby submit for the Master of Science (Global One Health) degree in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, to be my own work and that this work has not been previously submitted by me for degree purposes at another tertiary institution.



Ndidzulafhi Terrence Thovhakale

Date 30 October 2023

Acknowledgements

I wish to express my sincere appreciation and gratitude to the following institutions and individuals:

- South African Agricultural Sector Education and Training Authority (AgriSETA) funding awarded to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria,
- South African National Biodiversity Institute (SANBI) National Zoological Garden for allowing me to utilise their facility for sample collection and processing.
- Prof Darshana Morar-Leather for supervising this study,
- Refilwe Bokaba, Madeli de Bruin and Christian Lewis, my co-workers, for providing technical support for the study,
- Prof Luis Neves for providing the rodent traps for this study ,
- Friends and family for their unwavering support.

Table of contents

List of Tables

List of figures

List of Abbreviations

AEC	Animal ethics committee
AIDS	Acquired immune deficiency syndrome
ARESC	Animal Research Ethics and Scientific Committee
DALRRD	Department of Agriculture, land reform and rural development
DALY	Disability-adjusted life years
DAT	Direct agglutination test
ELISA	Enzyme-linked immunosorbent assay
HIV	Human immunodeficiency virus
IFAT	Indirect immunofluorescence antibody test
IHAT	Indirect haemagglutination test
LAMP	Loop-mediated isothermal amplification
LAT	Latex agglutination test
MAT	Modified agglutination test
NZG	National Zoological Gardens
PCR	Polymerase chain reaction
PPE	Personal protective equipment
qPCR	Quantitative polymerase chain reaction
REC	Research ethics committee
SANBI	South African National Biodiversity Institute
SFDT	Sabin–Feldman dye test
UP	University of Pretoria

Dissertation Summary

Prevalence of *Toxoplasma gondii* in commensal pest rodents at the National Zoological Garden in South Africa

by

Ndidzulafhi Terrence Thovhakale

Supervisor: Prof. Darshana Morar-Leather

Co-workers: Ms Refilwe Bokaba, Ms. Madeli de Bruin and Mr. Christian Lewis

Degree: MSc (Global One Health)

Department: Veterinary Tropical Diseases

Toxoplasmosis is a zoonotic disease caused by the ubiquitous Apicomplexan protozoa *Toxoplasma gondii* (*T. gondii*). The overall epidemiology of *T. gondii* in Southern Africa is understudied. Although a few studies have documented its circulation in humans, domestic animals, and wild animals, these studies were limited in species diversity and geographical location. Rodents are intermediate hosts and are recognised as key reservoir hosts for *T. gondii*. Rodents play an important role in the maintenance and transmission of the parasite as they are preyed on by cats, the definitive hosts. *Toxoplasma gondii* infection rates in the local rodent population may reflect infection rates in cats. The aim of this study was to determine the prevalence of *T. gondii* in pest rodents within the South African National Biodiversity Institute National Zoological Garden (SANBI NZG). Furthermore, an attempt was made to confirm the presence of *T. gondii* DNA in the various rodents' tissues (brain, tongue, muscle, diaphragm, and heart) using quantitative polymerase chain reaction (qPCR). A total of 138 sera were tested for *T. gondii* antibodies using a commercial latex agglutination test. A cut-off titre ≥ 64 was used to distinguish between positive and negative cases. Ten samples were positive for *T. gondii* antibodies, bringing the overall prevalence to 7.25% (95%, CI= 3.53 – 12.92). Using the generalised linear model, there was a statistically significant ($p<0.00432$) positive correlation between presence of *T. gondii* antibodies and rodent body weight. No *T. gondii* DNA amplification was observed on the tissue samples from the ten *T. gondii* antibody positive rodents. The results of this study provide baseline knowledge about the role of rodents in the epidemiology of *T. gondii* natural infections, particularly in the human-wildlife interface.

Chapter 1: Introduction and Literature review

1.1. Etiological agent and a brief history

In 1908, Charles Nicolle and Louis Manceaux found an unknown protozoan parasite in the tissue of a common gundi (*Ctenodactylus gundi*) at the Pasteur Institute in Tunis, North Africa (Dubey, 2021; Nicolle & Manceaux, 1909). Nicolle originally misidentified the protozoan as a piroplasm, then Leishmania, but realised shortly after that the protozoan was a new organism, and he named it *Toxoplasma gondii* (Dubey, 2021; Nicolle & Manceaux, 1908). The genus name *Toxoplasma* was derived from the Greek word "*Toxo*", meaning bow or arc, owing to the crescent shape of the organism, and "*plasma*", meaning life or cell. The second part of the name, "*gondii*", is a misnomer of the word gundi, the species where the protozoan was first found (Dubey, 2021; Nicolle & Manceaux, 1909). In the same year, in Sao Paulo, Brazil, Alfonso Splendore reported finding the same protozoan organism in the tissues of a rabbit (Innes, 2010; Splendore, 1908). Following a growing interest in the study of *Toxoplasma gondii* (*T. gondii*) by several researchers, in 1937, Sabin and Olitsky obtained the first *T. gondii* isolate from an animal, while the first viable isolate from humans was obtained by Wolf *et al.,* (1939). Sabin then demonstrated that *T. gondii* isolates in humans and non-human animals were identical (Dubey, 2008). Over the following years, research on *T. gondii* revealed more information about the protozoan, such as; pathogenesis, transmission patterns, life cycle, and genetic diversity. Toxoplasmosis, a disease caused by the intracellular parasite, *T. gondii*, affects mainly terrestrial and aquatic mammalian and avian hosts (Dubey, 2021). It is currently the only organism in the genus *Toxoplasma* and belongs to the large phylum Apicomplexa, which includes other intracellular parasites such as *Plasmodium, Eimeria, Cryptosporidium, Neospora* and *Theileria* (Dubey, 2020).

1.2. The life cycle of *Toxoplasma gondii*

Members of the Felidae family, both domesticated and non-domesticated cats, are the only known definitive hosts for *T. gondii* (Figure 1) (Attias *et al*., 2020). The sexual cycle of *T. gondii* occurs exclusively in the intestinal epithelium of the definitive hosts with subsequent excretion of unsporulated oocysts in faeces (Attias *et al*., 2020). The life cycle of *T. gondii* involves three infective forms; tachyzoites, bradyzoites, and sporozoites (Dubey, 2021). Tachyzoites, also known as the proliferative or feeding form, can infect all cell types. This form of the parasite multiplies quickly and is usually the hallmark of an acute infection (Dubey, 2021; Attias *et al*., 2020). Tachyzoites subsequently differentiate into bradyzoites, which are encysted in tissues, forming tissue cysts that have a high affinity for muscle and neural tissues. The bradyzoite form multiplies slowly, usually characteristic of more chronic infection (Dubey, 2021; Attias *et al*., 2020). Sporozoites are the products of the sexual cycle of *T. gondii* in cats and are released within the oocysts, in the cat's faeces. (Attias *et al*., 2020; Dubey, 2020). In a recent study by Di Genova *et al*., (2019), the sexual development of *T. gondii* was artificially induced in laboratory mice.This was achieved by supplementing linoleic acid in the diet and inhibiting the activity of murine-delta-

6-desaturase in mice intestines (Hares *et al*., 2021; Di Genova *et al*., 2019). Cats are the only known mammal that lack delta-6-desaturase activity in their small intestines. In other mammals, delta-6-desaturase enzyme plays a role in converting linoleic acid to arachidonic acid. The build-up of linoleic acid, from the diet, in cats' small intestine is believed to act as a positive signal for *T. gondii* sexual development (Di Genova *et al*. 2019). All other mammals and avian species are intermediate hosts as the asexual phase of *T. gondii* occurs in them (Attias *et al*., 2020).

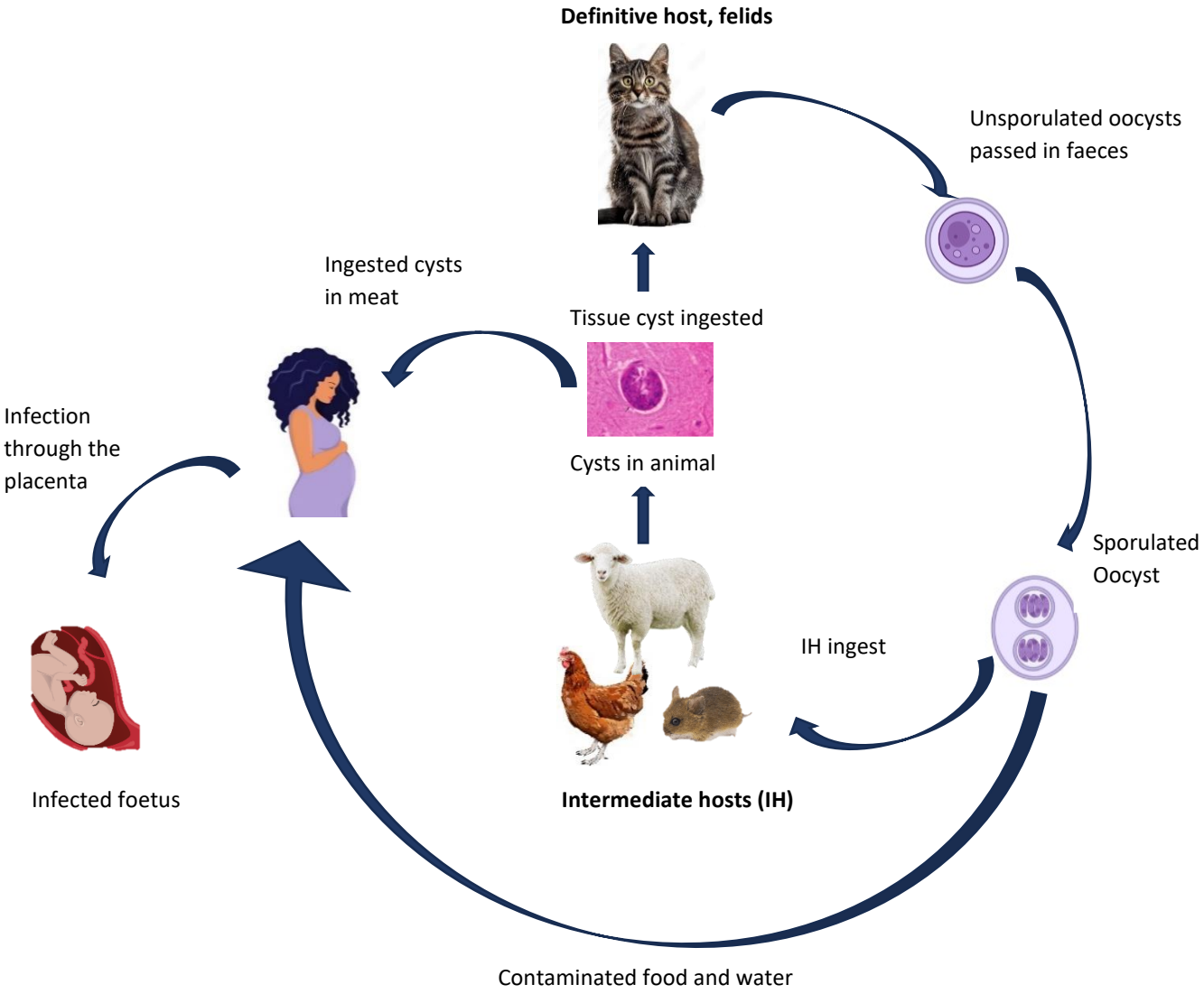


Figure 1: Life cycle of *Toxoplasma gondii* (Figure adapted from Almeria & Dubey, 2021)

1.3. *Toxoplasma gondii* in the definitive host

Felids can be infected by carnivorism of infected mammals, especially rodents and birds, or by ingesting sporulated oocysts from another feline. Although not common, transplacental and transmammary infection from a parasitaemic queen is possible (Cohen *et al*., 2016). Another potential source of infection is the consumption of unpasteurised milk or milk products by felines (Stelzer *et al*., 2019). After ingesting sporulated oocysts or tissue cysts from infected intermediate hosts, the infective forms i.e., bradyzoites and sporozoites, are released in the intestinal epithelium and enter enterocytes. The encysted bradyzoites are released from tissue cysts, while the sporozoites are released from the oocysts. Following the asexual and sexual reproductive cycle inside the definitive host, many unsporulated oocysts are passed in the feline faeces (Dubey, 2020). The unsporulated oocysts are transformed into a highly infectious sporulated form after being passed by the feline host. These sporulated oocysts contaminate the environment and remain infective for months or years (Dian *et al*., 2023; Barros *et al*., 2018)

1.4. *Toxoplasma gondii* in the intermediate host

All avian and mammal species can act as intermediate hosts for *T. gondii*. Infections with *T. gondii* can occur by via many pathways, the main routes of transmission are; eating undercooked or raw meat infested with tissue cysts, ingestion of food or water contaminated with *T. gondii* cysts, congenital or transplacental transmission from infected mother to child and, in humans, blood transfusions and organ transplantation of infected organs poses another potential source of transmission (Almeria & Dubey, 2021; Attias *et al*., 2020). All intermediate hosts are essentially dead-end hosts as they are unable to shed oocysts (Denk et al., 2022).

1.5. *Toxoplasma gondii* in humans.

It is estimated that at least one-third of the human population have chronic *T. gondii* infections (Almeria & Dubey, 2021). Regional seroprevalence ranges from 0.5% to 87.7%, with African countries having the highest average seroprevalence at 61.4%, Oceania at 38.5%, South America at 31.2%, Europe at 29.6%, United States of America (USA)/Canada at 17.5% and Asia at 16.4% (Molan *et al*., 2019). *Toxoplasma gondii* has been reported to be one of the most important food- and water-borne parasites affecting both animals and humans (Almeria & Dubey, 2021). It is also recognised as one of the deadliest foodborne pathogens in the USA, with fatalities estimated at almost 400 per year (Almeria & Dubey, 2021; Jones *et al*., 2014). The disease burden of *T. gondii* infections, expressed in disability-adjusted life years (DALYs), is ranked high in both the USA and Netherlands (Almeria & Dubey, 2021).

Immunocompetent individuals infected with *T. gondii* are generally asymptomatic (Almeria & Dubey, 2021). Congenitally infected foetuses, neonates, and immunocompromised individuals (including HIV patients, chronic corticosteroid users, haematologic malignancies and transplant recipients) can develop severe toxoplasmosis with possible fatal outcomes (Almeria & Dubey

2021; Weiss & Dubey 2009).*Toxoplasma gondii* infections in humans causes various clinical symptoms, from a mild self-limiting illness to severe congenital infections and various neuropsychiatric disorders (Milne *et al*., 2020; El Bissati *et al*., 2018). Congenital toxoplasmosis occurs due to seronegative pregnant women acquiring a new infection during pregnancy. The severity of the clinical disease of the foetus depends on the time of infection in utero. Infections during weeks 10 to 24 of pregnancy result in highly severe clinical manifestations, while infections during weeks 26 to 40 result in subclinical disease, which often manifests or relapses later in life (Weiss & Dubey, 2009). Subclinical neonates that do not receive treatment tend to suffer from recurrent retinal disease and cognitive decline (McLeod *et al*., 2014). Congenital toxoplasmosis can present with subtle mild symptoms, or it can present with quite severe symptoms, including hydrocephalus, microcephalus, motor/tone abnormalities, central nervous system calcification, epilepsy, pneumonia, strabismus, premature births, sepsis, rash, petechia, blood and blood chemistry abnormalities, chorioretinitis, microphthalmia, retinal scar and many other manifestations (McLeod *et al*., 2014; Weiss & Dubey, 2009). Appropriate treatment of diagnosed pregnant women and neonates can change the course and severity of the clinical signs. Differential diagnoses for congenital toxoplasmosis include infections from rubella virus, syphilis bacteria, herpes simplex virus and cytomegalovirus (Weiss & Dubey, 2009).

Another clinical manifestation is ocular toxoplasmosis, and it is one of the most common causes of posterior uveitis (Kohler *et al*., 2023). Ocular toxoplasmosis can result from both congenitally acquired and postnatal acquired infections, with ocular disease being the most common manifestation of congenitally acquired infections. (Kohler *et al*., 2023; Hill & Dubey, 2002). Besides posterior uveitis, other lesions associated with ocular toxoplasmosis include; necrotising retinitis, retinochoroiditis, retinal vasculitis, and vitritis (Fabiani *et al*., 2022).

1.6. *Toxoplasma gondii* in rodents

Rodentia are the largest and most diverse order in the class Mammalia, accounting for over 40% of all mammals. Rodents are very well adapted and inhabit every corner of the continent except for Antarctica (Delaney *et al*., 2018). Rodents can harbour an excess of 85 zoonotic pathogens. Several zoonotic diseases, including yersiniosis, leptospirosis, salmonellosis, toxoplasmosis, and hantavirus disease, have been associated with rodents, mainly rats and mice (Hardgrove *et al*., 2021; Han *et al*., 2015). A study in South Africa found the following endoparasites of public health importance in free-roaming rodents; *Gangylonema sp*, *Trypanosoma lewisi*, *Hymenolepsis diminuta*, *Angiostrongylus cantonensis*, *T. gondii*, *Moniliformis moniliformis*, *Calodium hepaticum,* and *Hymenolepsis nana* (Archer *et al*., 2017).

Rodents are intermediate hosts for *T. gondii* and are recognised as key reservoir hosts for *T. gondii*. They play an important role in the maintenance and transmission of the parasite as they are preyed on by the definitive hosts of *T. gondii* (Dubey *et al*., 2021; Joachim *et al*., 2020). A study in a low cat density region detected *T. gondii* DNA in two out of six rodent foetuses,

confirming congenital transmission and suggesting that the parasite can be maintained in rodent populations without the definitive host (Thomasson et al., 2011). *Toxoplasma gondii* infection rates in the local rodent population may reflect infection rates in the local cat population. (Hill & Dubey, 2002). Interestingly, rodents infected with *T. gondii* have been reported to have altered behaviour; they lose their fear of cats and thus are more likely to be eaten, encouraging the cat-rodent cycle of the parasite (Webster, 2001).

The global seroprevalence for *T. gondii* in rodents has been calculated at 6% (Joachim et al., 2020). Two separate studies conducted in Durban, a coastal city in KwaZulu Natal, South Africa, found seroprevalence rates of 4% and 11.2% (Archer *et al*., 2017; Taylor *et al*., 2008). A recent study in Nigeria found a prevalence of up to 76.2% in wild rodents using nested PCR (Ode *et al.*, 2022).

1.7. *Toxoplasma gondii* in zoo animals

Zoos present quite a unique environment in that there is a dense co-habitation of species, coupled with overlapping close human interactions between the staff that work at the zoo and the public who visit the zoo (Hardgrove *et al*., 2021). Disease dynamics in these institutions can be extrapolated to other areas with an increased likelihood of close animal-human interaction. Toxoplasmosis should be considered a significant cause of sporadic animal deaths in zoos. The disease has been recognised as an essential threat to the conservation efforts of some endangered species (Denk *et al*., 2022; Liu *et al*., 2022). *Toxoplasma gondii* seroprevalence of close to 40% has been reported in animals from Taipei Zoo, one of the largest Zoos in Taiwan (Liu *et al*., 2022). A review study by Hardgrove et al., (2021) seroprevalence of up to 40% in wild rodents found in Zoos. Clinical toxoplasmosis in zoos is precipitated by the inherited stress of captivity, proximity to captive felids, and the common occurrence of feral cats and rodents in zoos, allowing the disease to circulate and progress within zoos (Dubey, 2022; Loyd & Hernandez, 2019).

Among zoo animals, new-world non-human primates, Prosimians, Australian marsupials, and Pallas' cats have been reported to be highly susceptible to the disease, whilst old-world monkeys, rats, cattle, and horses are resistant to infections (Denk *et al*., 2022; Dubey, 2022; Parameswaran *et al*., 2009). Other zoo collection species where toxoplasmosis was detected include members of the families; *Camelidae*, *Atelidae*, *Cebidae*, *Callitrichidae*, *Indriidae*, *Mustelidae*, *Musophaginae*, *Potoroidae*, *Dasyuridae*, *Pitheciidae*, *Lorisidae*, *Macropodidae*, *Tarsiidae*, *Ailuridae* and *Lemuridae*. (Hensel *et al*., 2023; Yang *et al*., 2023; Denk *et al*., 2022;). Given the nature of the parasite's life cycle, pest rodents and feral cat control significantly influence the spread of infections (Denk *et al*., 2022). Unfortunately, *T. gondii* infections in animals in zoos do not only affect the captive animals but pose a potential risk of exposure of zoo keepers and the public to *T. gondii* oocysts (Dubey, 2022; De Camps *et al*., 2008).

1.8. *Toxoplasma gondii* genetic diversity

The majority of *T. gondii* strains can be grouped into three clonal lineages, namely type I, II, and III (Howe & David Sibley, 1995; Su *et al*., 2002). Other rare strains are occasionally encountered and are classified as sexual recombinant strains, atypical or exotic strains (Ajzenberg *et al.*, 2004). A fourth clonal lineage, referred to as type XII, is the latest to be described and is commonly found in wildlife in North America (Khan *et al*., 2011). The predominant clonal lineages at the DNA sequence level are genetically similar, demonstrating only 1 to 2% differences, but these strains show strong phenotypic variations. There is no evidence that the various strains have different host preferences or species boundaries. There is also no substantial evidence that the various strains have geographical boundaries even though they can be found at a high prevalence in certain regions, (Ajzenberg *et al*., 2004; Su *et al*., 2002). Comparisons of strains from domestic populations versus strains from wild populations suggest more sexual recombinants in the wild populations, while domestic population strains remain largely clonal (Ajzenberg *et al*., 2004). Clonal type II strain is reported to be the most prevalent in humans and animals (Howe & David Sibley, 1995; Su et al., 2012). A study by Hammond-Aryee et al., (2016) revealed that type II was the predominant genotype in both animals and humans from Cape Town, South Africa (Du Plooy et al., 2023)

In experimental conditions, infections of clonal lineage type I strains were fatal to mice, while those of clonal lineage type II and III were less severe (Dubremetz & Lebrun, 2012), suggesting that clonal lineage type I strains were more virulent. This is further supported by reports that type I strains are the most frequently isolated strains in severe congenital toxoplasmosis and recurrent ocular toxoplasmosis in humans (Khan *et al*., 2005). Currently, the association between disease severity and type of the strain shows variable patterns and is believed to be a result of multiple factors, including genotype and the innate genetic resistance specific to the host species (Galal, *et al*., 2019 Brennan *et al*., 2016). The strains that cause non-lethal chronic infections are expected to persist in a population and are most likely to be found in high prevalence

1.9. *Toxoplasma gondii* infection diagnosis

The clinical signs for toxoplasmosis are non-specific, and there are no pathognomonic lesions for a definitive diagnosis (Hill & Dubey, 2002). Diagnosis can be achieved through microscopy, serological, or molecular methods (Dubey, 2021).

Histological methods involve direct visualisation of the *T. gondii* parasitic stages through microscopy. While highly specific, this method tends to have low sensitivity, as they rely on the sampled material having at least one of the three infective stages of the parasite present at the time of testing (Liyanage *et al*., 2021).

Serological assays

Multiple serological methods have been developed for the diagnosis of Toxoplasmosis. These tests detect the immunological footprint i.e., antibodies and antigens of *T. gondii*. *Toxoplasma gondii* antibodies can be found in; whole blood, serum, plasma, milk, body fluids, and meat juice. Various serologic assays are used to detect the different classes of *T. gondii* antibodies.

Sabin-Feldman Dye Test

This test was developed in 1948 and it revolutionised the diagnosis of *T. gondii* as it was the first test that could differentiate specific antibodies to *T. gondii*. The Sabin-Feldman dye test (SFDT) differentiates acute and latent infections (Liu *et al*., 2015; Reiter-Owona *et al*., 1999; Beverley & Beattie, 1952). The SFDT is one of the most reliable laboratory tests, with high specificity and sensitivity, except in ruminant and chicken sera. The test is often used in reference laboratories for testing *T. gondii* antibodies in humans (Reiter-Owona *et al*., 1999). The SFDT is performed by incubating live tachyzoites with the test serum and an accessory factor (human serum component), and adding Methylene blue, to the mixture. The tachyzoites that are not affected by the antibody are stained with Methylene blue uniformly, the tachyzoites affected by the antibody appear as “ghost” cells as they do not take up the Methylene blue dye. The SFDT is a relatively expensive test to perform, requires high level of expertise to operate and makes use of live virulent organisms that pose a potential risk to laboratory staff (de Vries & Volpe, 2018; Dubey, 2021).

Indirect Fluorescent Antibody Test

The Indirect fluorescent antibody test (IFAT) is a relatively complicated test, with low sensitivity and high specificity (Shaapan *et al*., 2008). Killed *T. gondii* tachyzoites are mixed with serum, fluorescent-labelled anti-species IgG is added in the mixture to enhance detection of the antibody. The test’s major drawbacks include; the need of a fluorescent microscope to view the results, use of species-specific conjugates and possibility of cross reaction with antinuclear antibodies and rheumatoid factor (Dubey, 2021).

Indirect Haemagglutination Test (IHAT)

Although the IHAT is very simple and inexpensive, it has limited use in clinical diagnosis of toxoplasmosis (Dubey, 2021; Ybañez *et al*., 2020). The test has a relative specificity of up to 100%, and a sensitivity of about 70% and thus, typically used in epidemiological surveys (Fernandes *et al*., 2019). Multiple variations of the test have been developed but the basis of how the test works is the same; red blood cells are coated with *T. gondii* tachyzoites antigen, and agglutination occurs if anti-*T. gondii* antibodies are introduced (Liu et al., 2015; Ybañez *et al*., 2020). The disadvantages of this test are that it often misses acute infections as it detects antibodies much later than the DT. Furthermore, it often fails to diagnose congenital infections (Dubey, 2021).

Modified agglutination test (MAT)

This test that is relatively simple to perform and accurate, often utilised for both laboratory diagnosis and epidemiological studies (Liu *et al*., 2015). It is also a favourable test as no special

instruments, conjugates or reagents are necessary (Dubey, 2021). The MAT detects only IgG antibodies and as such, may provide false negative results during early stages of acute infections (Dubey, 2021). To eliminate non-specific IgM or IgM-like substances the test sera is treated with 2-mercaptoethanol. The basis of the test is that formalin or acetone fixed *T. gondii* tachyzoites and diluted test sera are mixed in microtitre plates, a thin layer of agglutination will be seen in positive serum samples, whereas a compact pellet of precipitated tachyzoites at the bottom of the plate will be seen in negative serum samples (Liu *et al*., 2015).

Enzyme linked immunosorbent assay (ELISA)

The ELISA remains one of the most used immunoassays for the detection of *T. gondii* antibodies (Liyanage *et al*., 2021). Different types of ELISAs have been developed, but they all operate on the basis that antigen-antibody reactions are detected and amplified using a secondary covalently bonded enzyme-antibody molecules. A positive result (presence of antigen) is characterised by a colour change which can be read and quantified by a plate reader (Pettipher *et al*., 2005).

Latex agglutination Test (LAT)

The LAT is one of the indirect detection methods used for toxoplasmosis diagnosis in both humans and animals. It is a relatively simple test to perform that does not require species conjugates. The LAT utilises latex particles coated with soluble *T. gondii* antigens to demonstrate the presence of IgM and IgG antibodies. A positive test is characterised by an obvious agglutination observation when the antigen-coated latex particles and a positive serum are combined (de Barros *et al*., 2022; Liu *et al*., 2015).

Molecular assays

Molecular methods detect the parasites' nucleic acid. These techniques are routinely used in combination with either microscopic or serological methods. The most widely used techniques include conventional PCR, real-time PCR and Loop-mediated isothermal amplification (LAMP).

Conventional PCR

The technique works by specifically amplifying the parasite's DNA segments in a relatively short time. PCR targets that are routinely used for *T. gondii* detection include; the B1 gene, the 529 bp repeat element and the internal transcribed spacer (ITS-1) or 18S rDNA sequences (Liu *et al*., 2015).

Real-time PCR

Real time PCR also known as quantitative PCR (qPCR) can amplify relatively low concentrations (picograms-nanograms) of target DNA. In addition to primers, this technique utilises probes or intercalating dyes to measure the quantity of the amplification product at each cycle. The quantification of the starting DNA and amplification product is made possible by comparing it with a standard of known concentration (Liu *et al*., 2015). As it is a closed tube system, qPCR minimises the risk of contamination (Liu *et al*., 2015).

Loop-mediated isothermal amplification (LAMP)

A DNA amplification technique performed under isothermal conditions i.e., in a water bath or heat block. The technique utilises four primers that recognise six distinct regions of the target DNA. Several LAMP assays have been developed for the diagnosis of *T. gondii* in veterinary samples, medical samples, and water samples. Majority of these assays *T. gondii* SAG1, SAG 2, GRA1, B1, 529-bp repetitive element, oocyst wall protein (OWP) genes, and 18S rRNA (Liu *et al*., 2015).

1.10. Justification

According to management at the NZG, the facility has an established population of feral cats roaming between and within enclosures on the premises. The only population control measure currently in place, is sterilising all feral adult cats, if caught in routinely laid out traps. Once sterilised, a cat is taken back to the vicinity where it was caught. The rationale behind this control measure is to keep the feral cat numbers constant. As cats are highly territorial animals, only a certain number can co-exist within a certain proximity of each other. It is believed that constantly removing feral cats from the zoological garden would attract more feral cats from surrounding areas, whereby the animals keep returning.

According to the NZG, pest rodent populations are abundant, especially rats that roam freely across the grounds of the zoo. This abundance is likely promoted by accessibility to food/feed within the enclosures as well as ineffective pest control methods. The current control measure for these pests includes but is not limited to, the use of toxin-baited stations, which seems ineffective at eradicating the infestation. Given that a few species, such as kangaroo, ring-tailed lemur, and brown lemurs (unpublished data, Centre for Wildlife heath medical records), have been diagnosed with disseminated toxoplasmosis, the investigator wishes to establish the prevalence of *T. gondii* in pest rodents within this facility. *Toxoplasma gondii* strains and genotypes circulating within a rodent population may, in part, represent strains that will be transmitted to predators (mainly cats), contaminate the environment, and eventually result in transmission to humans. Understanding the role of rodents as reservoirs and studying the prevalence of *T. gondii* strain patterns in rodents may assist us in further understanding the epidemiology, risk, and control of *T. gondii* in humans and Zoo animal populations (Galal *et al*., 2019). This study intends to provide baseline knowledge about the role of rodents in the epidemiology of *T. gondii* natural infections, particularly in the wildlife-human interface.

Chapter 2: Materials and Methods

2.1. Study area

The South African National Biodiversity Institute National Zoological Garden (SANBI NZG) is located centrally within the City of Tshwane Metropolitan Municipality in the Gauteng Province of South Africa (Figure 2). The 85-hectare zoological garden hosts approximately 209 mammal species, 202 bird species, 190 fish species, four invertebrate species, and seven amphibian species. The Apies River flows through the NZG property, separating it into two distinct areas; low-lying flat areas near the city's central business district (CBD) and rocky, hilly terrain areas. Nearby establishments include schools, shopping centres, taxi ranks, houses, and residential apartments.

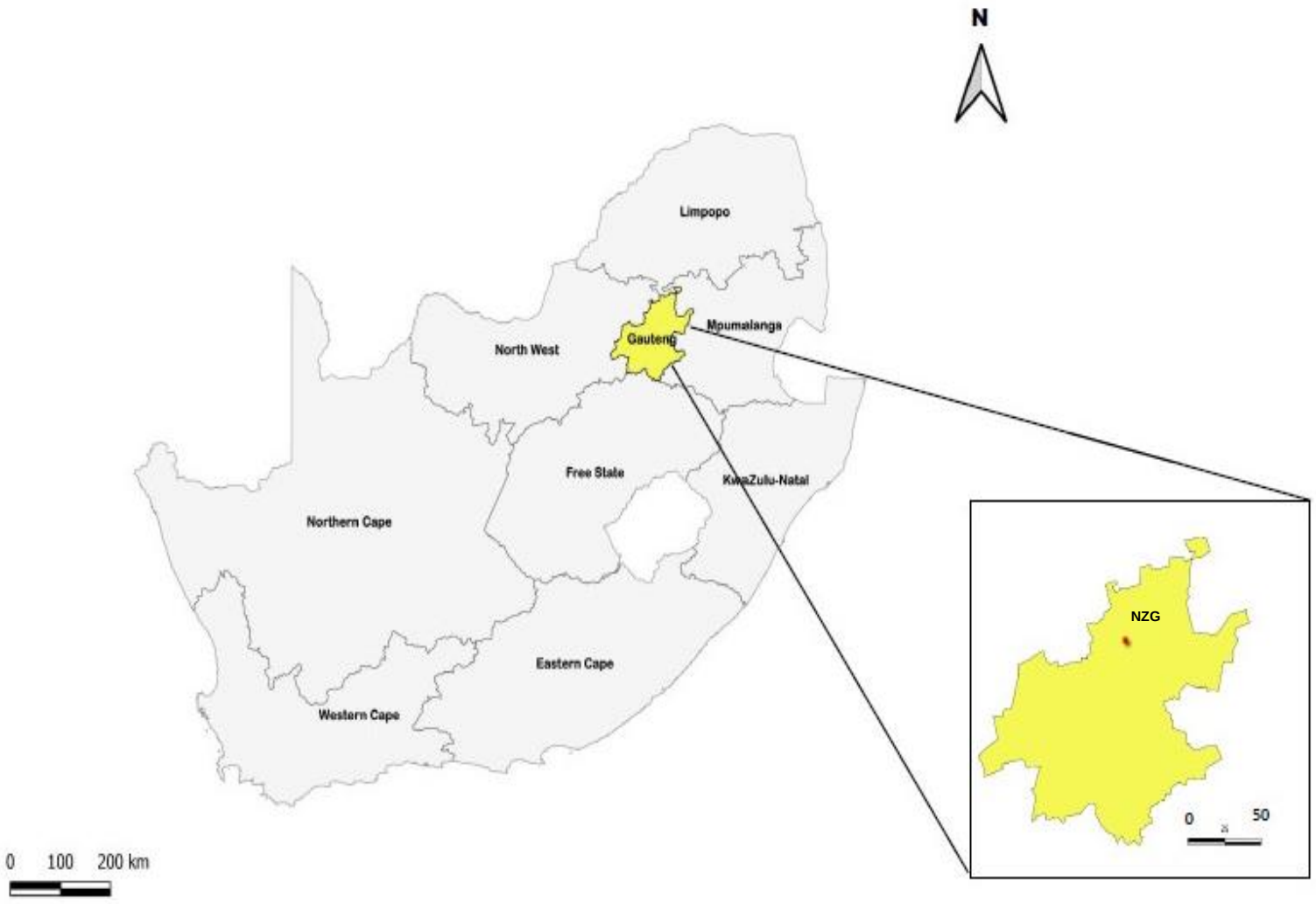


Figure 2: The above map shows the area where the NZG (Red dot) is located within South Africa

2.2. Study animals

The minimum number (n) of rodents sampled was guided by a study conducted by Joachim et al (2020), wherein the global seroprevalence for *T. gondii* in rodents was averaged at 6%. Two additional studies conducted in Durban, a coastal city in KwaZulu-Natal (KZN) province, South Africa, found a seroprevalence of 4% and 11.2% in Cato Crest and eThekwini Metropolitan area, respectively (Archer et al., 2017; Taylor et al., 2008). Based on these findings, a disease prevalence of approximately 10% (with a 95% level of confidence and 5% desired absolute precision) was expected from the current study. The formula below was used to calculate the minimum number of rodents required for this study (Thrusfield et al., 2018). A total of 138 rodents were sampled.

P = expected prevalence

d = desired absolute precision

n = required sample size

$$n = \frac{1.96^2 P(1-P)}{d^2}$$

$$n = \frac{1.96^2 * 0.1(1-0.1)}{0.05^2}$$

$n = 138{,}2976$

A minimum 138 rodents to be sampled to find at least the expected prevalence of 10%, given that we would like to achieve a 95% level of confidence and 5% desired absolute precision.

2.3. Animal sampling

The trapping of rodents was carried out from January 2023 until March 2023. These months fall within the summer-autumn seasons in the Southern Hemisphere. Rodents were trapped using Tomahawk[1] live traps (model 102XL), designed for humanely capturing smaller mammals. The traps were placed in close proximity to where clinical toxoplasmosis cases have been previously diagnosed (Centre for Wildlife Health medical records) and where rodents have been frequently seen. Table 1 below summarises all the trap sites and animal enclosures in the vicinity of the trap site.

[1] Tomahawk live traps. 6151 U.S. Hwy 51. Hazelhurst, WI 54531.

Table 1: Trap sites numbers and animal species associated with the trap site

Trap site no.	Animals/enclosures closest to trap site
1	Various hospital patients, including birds and carnivores
2	Chimpanzees (*Pan troglodytes*) enclosure
3	Parrots and macaw enclosures
4	Drills (*Mandrillus leucophaeus*), Patas monkeys (*Erythrocebus patas*), Lion-tailed macaques (*Macaca silenus*) and leopard (*Panthera pardus*) enclosure
5	Ring-tailed lemurs (*Lemur catta*) enclosure
6	Buff-cheeked gibbons (*Nomascus gabriellae*) enclosure
7	Ring-tailed lemurs *(Lemur catta*) enclosure
8	Western-lowland gorillas (*Gorilla gorilla gorilla*) enclosure
9	Lions (*Panthera leo*) and Tigers (*Panthera tigris*) enclosure
10	Black-tufted marmoset (*Callithrix penicillate*), Linne's two-toed sloth (*Choloepus didactylus*), Ring-tailed lemurs (*Lemur catta*) enclosures
11	Buff-cheeked gibbons (*Nomascus gabriellae*), Red-capped mangabey (*Cercocebus torquatus*) enclosures
12	Lar gibbons (*Hylobates lar*) enclosure
13	Common brown lemur (*Eulemur fulvis*) enclosure
14	Okapi (*Okapia johnstoni*), Sable antelope (*Hippotragus niger*), and Nyala (*Tragelaphus angasii*) enclosures
15	Capybara (*Hydrochoerus hydrochaeris*), Black-necked swan (*Cygnus melancoryphus*) enclosures

Figure 3 below shows the NZG's enclosure layout with the trap site numbers highlighted (in red).

Traps were left in the predetermined locations for at least four nights in a row before having been relocated to a new site if no signs of rodent activity, such as faecal droppings, displacement of the trap were seen. Number of traps placed per night ranged from 2 to 8, and was dependent on the area/enclosure of focus for that period. Different baits (e.g., peanut butter, peanuts, oats, shredded chicken, eggs, fruit, and vegetables) were trialled with varying degrees of success. Fourteen neonates found in nests during the demolition of certain enclosures for renovation purposes were also included in this study. Each rodent's sex, age group, weight, and species identification were recorded. The sex of the rodents, identified by the investigator, was based on the appearance of the external genitalia. The 14 neonates, approximately 10% of the sample population, were unsexed. The age group was based on the length, weight, and appearance of the rodent. The weight was gauged using a Richter scale[2] W113, x 0.005 kg scale. The principal investigator carried out rodent species identification based on the rodent's physical characteristics, such as specimen size, coat colour, length of the tail, and size and shape of ears, eyes, and head. All the rodents trapped overnight were collected in the morning and were

[2] Richter Scale Company (PTY) Ltd, Pretoria, South Africa.

translocated to the hospital for euthanasia and sampling. The rodents were placed in an air-tight gas induction chamber connected to an anaesthetic machine with a high-flow isoflurane vaporiser. The Isoflurane was turned on at 5% with an Oxygen flow rate of 1 litre/min. Once the animal was anaesthetised, it was removed from the chamber and transferred to a 250 mm x 360 mm zip lock sealing bag with an isoflurane-infused cotton wool ball. The highly concentrated isoflurane euthanased the rodent swiftly. Blood collection via cardiocentesis was performed immediately after the rodent was euthanased. The euthanased rodent was removed from the bag, and a necropsy (tissue sampling) was performed. The euthanasia process, rodent dissection, and tissue sample collection were carried out in a BSL2+ biosafety cabinet. Tissue samples collected during necropsies included tongue, heart, diaphragm, muscle, and brain tissues and were stored in 1.5 ml screw-top cryotubes, and kept frozen at -20℃ for further analysis. Whole blood was collected from the rodent into a 1.5 ml micro centrifuge (eppendorf) tube. The eppendorf tube was left upright, on a sample tray, in the fridge overnight. The samples were then centrifuged at 3200 x rpm for 10 min, following which the sera were decanted into a 2 ml screw-top cryotube for long-term storage at -20℃ for further analysis.

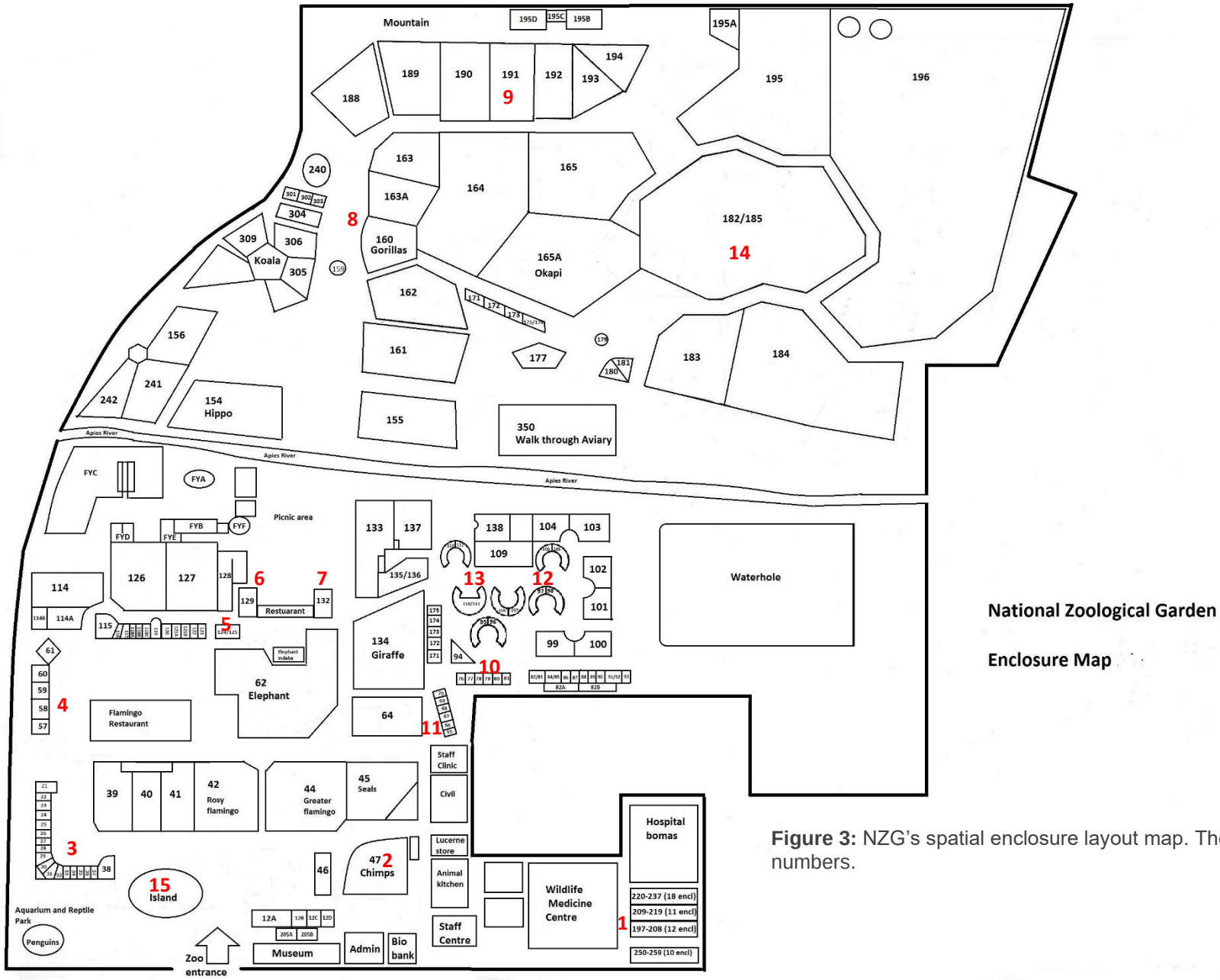


Figure 3: NZG's spatial enclosure layout map. The trap sites are indicated in red ink numbers.

2.4. Serological assay

For this study, the serological assay used to detect *T. gondii* antibodies was a commercially available latex agglutination test (LAT), Toxoreagent RST701, Mast Group LTD[3]. The assay was performed according to the manufacturer's instructions. Briefly, the assay was performed as follows; 25 µL diluent was first introduced to each well of a U-shaped bottom 96-microwell plate, then 1:8 diluted sera was introduced and double-diluted serially from 1:16 to 1:2048 dilutions. After that, 25 µL of *T. gondii* antigen-coated latex reagent was added to each well. The microwell plate was incubated overnight before the agglutination patterns were evaluated. Positive and negative controls were included in each plate. Results were interpreted as negative if a small and distinct circular sediment was formed at the bottom centre of the well. Positive results were recorded if varying degrees of agglutination at the bottom of the well were observed. *Toxoplasma gondii* positive cases were selected at cut-off titre ≥ 64, as was done in previous rodent studies by Buddhirongawatr et al. (2016) and Jittapalapong et al. (2011).

2.5. Tissue nucleic acid extraction

Approximately 50 mg of each tissue was diced using a scalpel blade. Nucleic acid extraction was performed using the Roche[4] High pure PCR template preparation kit. The extractions were performed according to the manufacturer's instructions. Briefly, tissue lysis buffer and proteinase K were added to 50 mg of diced tissue in a microcentrifuge tube. The mixture was incubated until the tissue was completely digested. A binding buffer was added with subsequent incubation, after which isopropanol was mixed, followed by the removal of insoluble tissue particles using a pipette. After centrifugation and a series of washes, the DNA was eluted and stored at -20℃ for later analysis. An estimation of nucleic acid concentration and purity of 16 randomly selected samples was deduced by direct spectrophotometric measurement using a Trinean Xpose™ spectrophotometer. The selected sample's purity average ratio was 1.77, and the average concentration was 88.59 ng/µl.

2.6. *Toxoplasma gondii DNA* detection

Molecular detection of *T. gondii* DNA in different tissues of *T. gondii* antibody positive animals was performed using a quantitative PCR (qPCR). The qPCR was performed as described by Kasper et al. (2009). The qPCR amplified an 81-base pair (bp) fragment of the 529-bp repeat element of unknown function in the *T. gondii* genome. The repeat element was amplified utilising a forward primer: ToxoRE f, reverse primer: ToxoRE r, and a TaqMan probe: ToxoRE p. The nucleotide sequences of the primers and probe is displayed in Table 2 below.

[3] Mast diagnostica GmbH, Germany.

[4] Roche Diagnostics GmbH. Sandhofer Strasse 116. 68305. Mannheim. Germany

Table 2: Primers and probe nucleotide sequences

Reagent name	Nucleotide sequence
ToxoRE f	5'-CACAGAAGGGACAGAAGT-3'
ToxoRE r	5'-CAGTCCTGATATCTCTCCTCCAAGA-3'
Taqman probe: ToxoRE p	FAM-5'-CTACAGACGCGATGCC-3'-BMN-MGB*

*FAM is a 6-carboxyfluorescein, and BMN-MGB is a non-fluorescent quencher (BMN-Q535) attached to a minor groove binder

The optimised reaction mixture composed of 2.5 µl of DNA template (sample DNA), 0.2 µM ToxoRE f, 0.2 µM ToxoRE r, 0.1 µM ToxoRE p, 12.5 µl TaqMan Universal Master-mix and 8.5 µl PCR grade water to bring the final reaction volume to 25 µl. All reactions were mixed in a MicroAmp™ fast optical 96-well reaction plate and the PCR was run in the StepOnePlus™ real-time PCR system[5]. The run set-up for the PCR machine is described in Table 3 below. Negative and positive controls (pure *Toxoplasma gondii* DNA) were included in the reaction plate.

Table 3: Run set-up for the qPCR machine

	Holding stage		**Cycling stage (No of cycles = 45)**		
	Stage 1	**Stage 2**	**Stage 1**	**Stage 2**	**Stage 3**
Temperature	50 ℃	95 ℃	95 ℃	62 ℃	68 ℃
Time	2 min	10 min	15 sec	15 sec	20 sec

Samples were identified as positive if C_t value was determined between cycle 1-39. Negative samples had undetermined C_t value.

2.7. Statistical analysis

All data, including date of trapping, trap site location, rodent species, weight, sex, amount of blood collected, amount of serum collected, and tissue types collected were recorded in a Microsoft excel sheet. Data analysis was conducted using R (R version 4.1.2, 2021), R studio (Rstudio team, 2021) and the epiR package. The statistical analysis was done using the generalised linear model (GLM) to analyse the relationship between seroprevalence and the three other variables; weight, age, and sex. Generalised linear model incorporates ANOVA, ANCOVA, MANOVA, MANCOVA, ordinary linear regression, t-test, and F-test. Values of $p \leq 0.05$ were considered significant.

2.8 Ethics approval and biosecurity

Approval to perform this project was granted by the Research Ethics Committee and the Animal Ethics Committee of the University of Pretoria, reference: REC 106-22 (Appendix IV and III

[5] Applied Biosystems, Randburg, South Africa.

respectively). Approval to perform this project at SANBI NZG was granted by the SANBI animal research ethics and scientific committee of SANBI, reference: SANBI/RES/P2022/17.

Section 20 approval under the Animal Diseases Act was also obtained, reference 12/11/1/1/18 (2570 AC)

Chapter 3: Results

3.1. Captured Rodents

A summary of the total number of rodents caught at each trapping site within the zoological garden is provided in Table 4 below. The best trapping outcomes were achieved when food that was normally fed to the exhibition animals close to the traps was offered as bait. The number of rodents caught on a particular day at each site depended on the following factors, but are not limited to them; weather conditions such as rain, or even the displacement of traps, whether intentional or accidental. The maximum number of rodents trapped, a total of 29, was at site 1, followed by 26 rodents at site 10. No rodents were trapped at sites 2, 9, and 14, although there was evidence of rodent activity near the traps around sites 2 and 14. There was no evidence of rodent activity around the traps at site 9.

Table 4: Total number of rodents sampled from each trap site over the sampling period and total number of positives rodents per site.

Trap site no.	Total No. of rodents sampled	Positive rodents per site
1	29	4
2	0	0
3	17	2
4	14	0
5	6	0
6	5	0
7	0	0
8	2	0
9	0	0
10	26	2
11	13	2
12	13	0
13	7	0
14	0	0
15	6	0
Total	**138**	**10**

3.2. Toxoplasma gondii antibodies in sampled rodents

\Ten of the 138 sera tested positive for *T. gondii* antibodies, bringing the overall seroprevalence to 7.25% (95%, CI= 3.53 – 12.92).

The specimens that tested positive were distributed throughout the various trap sites as follows; Four from site 1, two from site 3, two from site 10, and two from site 11.

3.2.1. Rodent age group distribution

The most prevalent age group of the sampled population was adults, 96 individuals, accounting for approximately 70% of the sample population, followed by 28 juveniles (~20%), and 14 newborns (~10%) (Figure 4A). Nine adults and one juvenile tested positive for *T. gondii* antibodies (Figure 4B). None of the newborn samples tested positive.

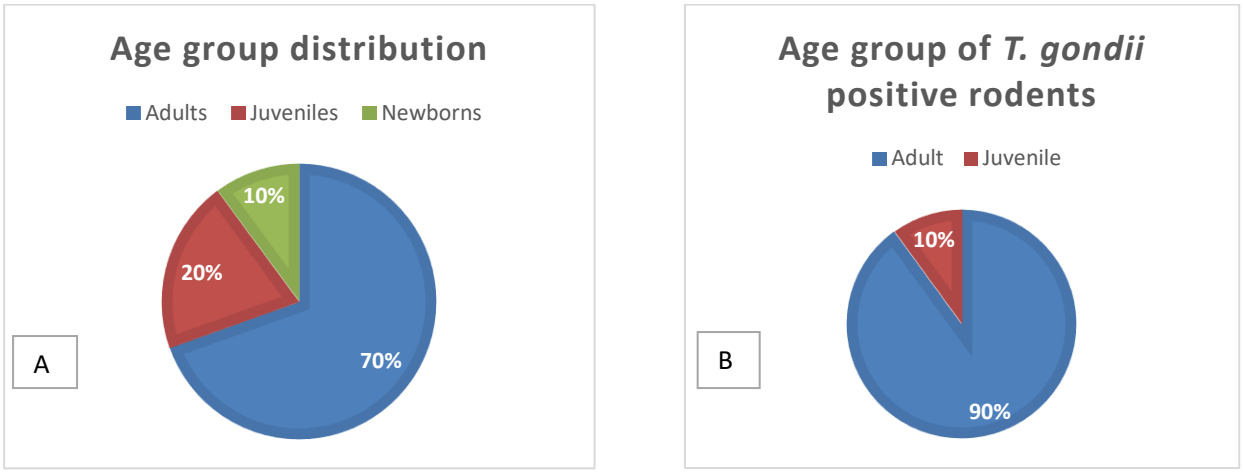


Figure 4: (A) Pie chart depicting the proportion of the rodents' age groups recorded throughout the study period. (B) Age group proportion of *T. gondii* positive rodents

Using GLM on R studio, the intercept had p-value of $1.17e^{-10}$, adult group with p-value 0.998, juvenile age group with p-value 0.335, and newborn age group with p-value 0.993

3.2.2. Rodent gender proportion

From the sampled rodent population, 73 individuals (~53%) were female, and 51 (~37%) were males. (Figure 5A). Upon further analysis of the sampled rodent population, it was found that 15 of the juveniles were female, and only 12 were male. A total of 58 of the adult rodents were female, while the remaining 39 were male. Of the ten rodents that had *T. gondii* antibodies, seven were male and the remaining three were female (Figure 5B)

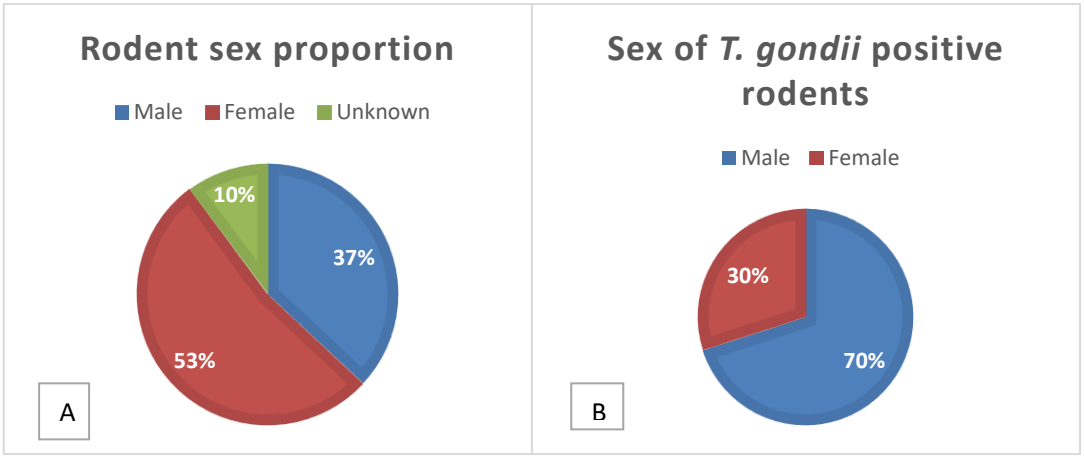


Figure 5: (A) Pie chart depicting the proportion of the rodents' sex recorded throughout the study period. (B) *T. gondii* antibody positive rodents sex proportion.

The female sex was chosen as the intercept with p-value of $9.17e^{-08}$, and the male sex with p-value of 0.0671.

3.2.3. Weights of rodents

Rodents of varying sizes were trapped and sampled. The recorded weights ranged from a minimum of 5 g and a maximum of 475 g. The average weight of the rodents was 150,4 g, with the first, second, and third quartiles at 70 g, 160 g, and 200 g, respectively. Six of the *T. gondii* antibody positive rodents were within the weight range 205-300 g. Two were within weight range 105-200 g, and one rodent in each of the 5-100 g and 405-500 g weight ranges (Figure 6).

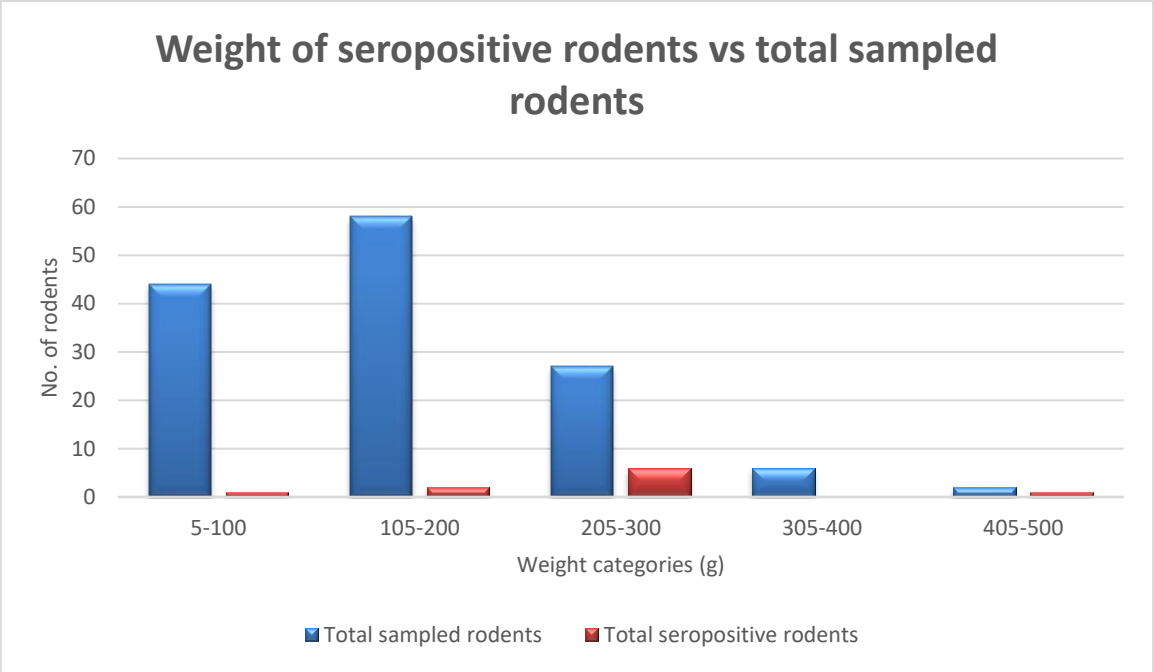


Figure 6: The weights of *T. gondii* antibody positive rodents in relation to the weight of all sampled rodents

Using GLM on R studio, the intercept had a p-value of $1.9e^{-07}$, and the weight of the rodent had a p-value of 0.00432.

3.3. *Toxoplasma gondii* DNA detection results

The qPCR was performed on all 52 samples (five tissues from each of the ten *T. gondii* antibody positive rodents and positive & negative controls). Significant amplification of *Toxoplasma* DNA was observed on the positive control, with a determined C_t value of 28.98. No parasite DNA amplification was observed on all the test samples and the negative control (figure 7).

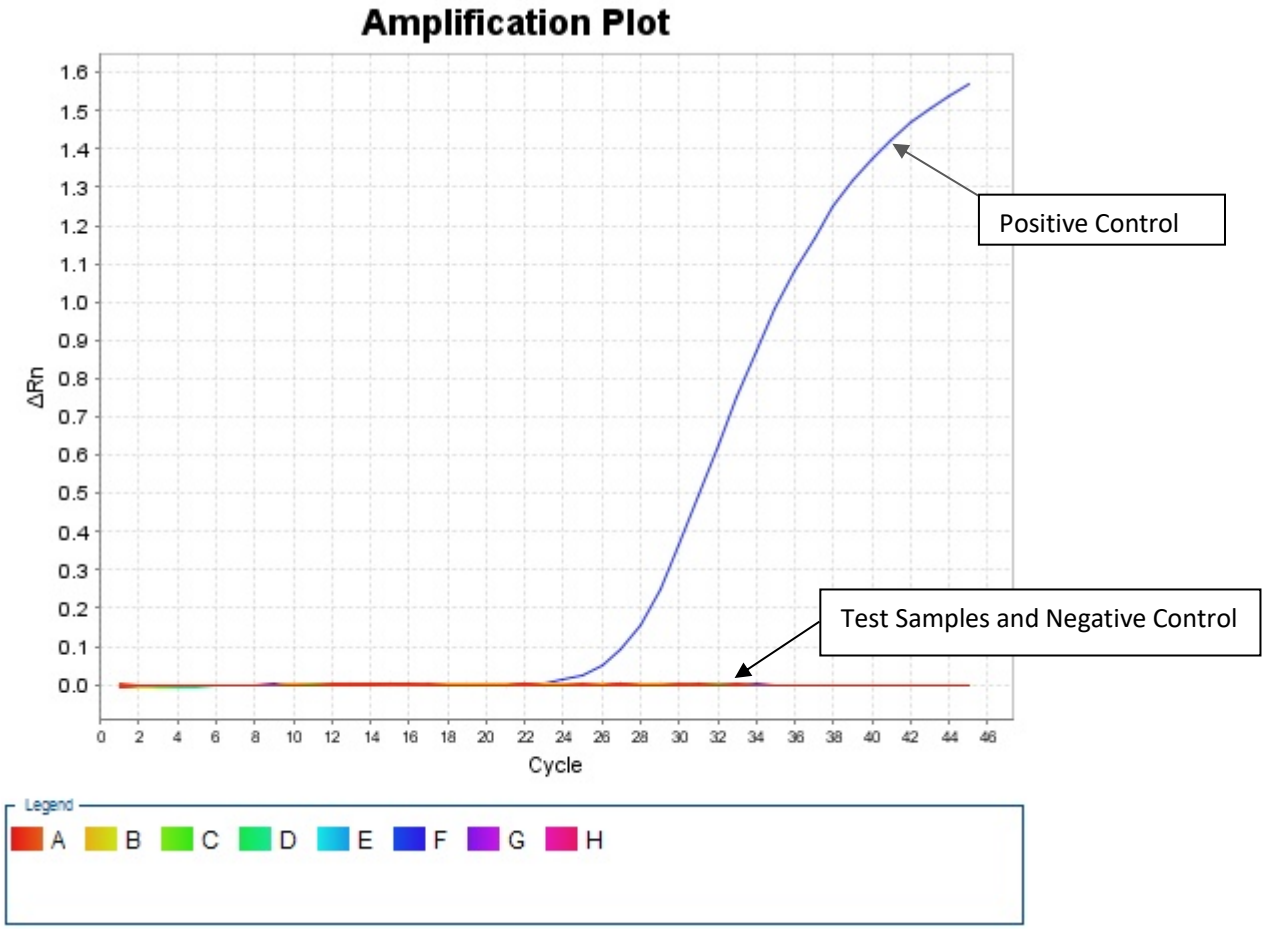


Figure 7: qPCR amplification plot of all *T. gondii* antibody positive tissues.

Chapter 4: Discussion

Ten of the 138 rodents caught within zoo grounds had antibodies to *T. gondii,* bringing the prevalence to 7.25%. This prevalence coincides with the global rodent seroprevalence of 6%, as Joachim et al. (2020) reported in their systematic review and meta-analysis study (Joachim *et al*., 2020). Previous studies in rodents reported prevalence of 4% in Cato Crest, and 11.2% in eThekwini Metropolitan area (Archer *et al*., 2017; Taylor *et al*., 2008). A possible explanation for the relatively low prevalence in the current study is that the rodent traps were placed inside enclosures and night rooms to select rodents that interact closely with the zoo animals. This may have inadvertently been selected for rodents from areas where feral cats have no access; hence there may have been little to no environmental contamination by feline faecal-origin oocysts.

Eating unwashed vegetables and fruits has been identified as a risk factor for acquiring *T. gondii* infections in humans. A study by de Camps *et al*., (2008) showed a reduced risk of *T. gondii* infections in zoos that wash vegetables and fruits (De Camps *et al*., 2008). In this study, the participating zoo washed fruits and vegetables before offering them to animals, and because the pest rodents feed on food offered to exhibition animals, by extrapolation, rodent infections from oocysts contaminated fruit and vegetables will also be limited.

Another important source of infection for zoo collection animals, especially carnivorous species, is consumption of meat infected with *T. gondii* tissue cysts. As this meat also forms part of pest rodents' diet, it is a potential source of infections for pest rodents (Tidy *et al*., 2017). However, because the zoo in the present study almost exclusively feeds meat that has been frozen for prolonged periods, a procedure that is known to decrease infective tissue cysts (Cano-Terriza *et al*., 2020; Dubey, 1988), chances that rodents are infected this way are reduced. It must be noted that infection through ingestion of tissue cysts in the pest rodents remains one of the implicated methods of infection for the diagnosed primate's clinical cases in this zoo.

In the present study population, nine (90%) positive cases were adults, with the remaining 1 (10%) being a juvenile. The mean weight of the positive cases was calculated at 237.5 g. Using the generalised linear model, there was a statistically significant ($P \leq 0.00432$) positive correlation between *T. gondii* antibody presence and weight. These findings are similar to findings by Ayral *et al*., (2015), who found a positive correlation between testing positive for *T. gondii* and host body mass, further suggesting the detection of postnatal acquired infections.

The negative results (no amplification of *T. gondii* DNA) from the qPCR should be interpreted cautiously. The occurrence of infected tissues that were undetected by qPCR cannot be entirely ruled out. The tissues where DNA was extracted were minimal (with a maximum weight of approximately 50 mg). Given that the parasite distribution in tissue may be random and non-homogenous, coupled with the possibility of the low parasite density in tissues, it is possible that the parasite was present in the unexamined parts of the tissues (Peris *et al*., 2023). Galal *et al*.,

(2019) also demonstrated the poor correlation between serological results and DNA detection in small mammals. The investigator recommends screening the same samples with a highly specific test such as MAT to verify the results found.

This study assessed the proportion of the pest rodent population with *T. gondii* antibodies by serological methods and confirmation of the presence of *T. gondii* by molecular methods. Previous studies that have estimated the seroprevalence of *T. gondii* in rodents mainly focused on rodent populations located exclusively in the wild or residential areas. The current study, however, focused on rodents in a captive animal facility, the SANBI National Zoological Garden in Pretoria, South Africa, where there is constant human-animal interaction. Zoological Gardens present a closed, animal-dense environment where rodents can be predated upon by feral felids, captive felids, and other mammals such as primates. This study provides baseline data to assess the effect of rodents on *T. gondii* circulation in such an environment. Because rodents are preyed upon by the definitive hosts, these rodents can be a source of *T. gondii* infections for felids. Therefore, they can be an important indicator of environmental contamination by oocysts and an indicator of infection risk for felids (Gil et al., 2023).

4.1. Research limitations

This study confirmed the circulation of *T. gondii* within the rodent populations roaming within the National Zoological Garden. Despite the relatively low prevalence results, the author emphasises that this presents largely postnatal acquired infections. It would be worthwhile to perform *T. gondii* DNA detection methods, such as PCR, on all collected samples. This is because it is well known that viable *T. gondii* has been isolated from seronegative individuals in rats (Dubey *et al*., 2021). Congenitally infected rats have little to no immunological response and therefore test negative on serology (Dubey *et al*., 2021). The samples collected from this study will be ideal for studying the proportion of congenital infection in various age groups, from neonates to adults, were collected.

Chapter 5: Conclusion

This study found a low level of postnatal acquired *T. gondii* infections within the zoo pest rodent population. Such a low level of infection is unlikely to amplify the circulation of *T. gondii* within the zoo. Despite the low level of infection, this study confirmed the presence of *T. gondii* in the rodent population, and because of the natural habits of rodents, such as rapid proliferation and access to outdoor and indoor enclosures, it is imperative that strict outbreak prevention measures are maintained.

References

Ajzenberg, D., Bañuls, A. L., Su, C., Dumètre, A., Demar, M., Carme, B., & Dardé, M. L. (2004). Genetic diversity, clonality and sexuality in *Toxoplasma gondii*. *International Journal for Parasitology*, *34*(10), 1185–1196. https://doi.org/10.1016/j.ijpara.2004.06.007

Almeria, S., & Dubey, J. P. (2021). Foodborne transmission of *Toxoplasma gondii* infection in the last decade. An overview. *Research in Veterinary Science*, *135*, 371–385. https://doi.org/10.1016/J.RVSC.2020.10.019

Archer, C. E., Appleton, C. C., Mukaratirwa, S., Lamb, J., & Corrie Schoeman, M. (2017). Endo-parasites of public-health importance recovered from rodents in the Durban metropolitan area, South Africa. *Southern African Journal of Infectious Diseases*, *32*(2), 57–66. https://doi.org/10.1080/23120053.2016.1262579

Attias, M., Teixeira, D. E., Benchimol, M., Vommaro, R. C., Crepaldi, P. H., & De Souza, W. (2020). The life-cycle of Toxoplasma gondii reviewed using animations. In *Parasites and Vectors* (Vol. 13, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13071-020-04445-z

Ayral, F., Artois, J., Zilber, A. L., Widén, F., Pounder, K. C., Aubert, D., Bicout, D. J., & Artois, M. (2015). The relationship between socioeconomic indices and potentially zoonotic pathogens carried by wild Norway rats: A survey in Rhône, France (2010-2012). *Epidemiology and Infection*, *143*(3), 586–599. https://doi.org/10.1017/S0950268814001137

Barros, M., Cabezón, O., Dubey, J. P., Almería, S., Ribas, M. P., Escobar, L. E., Ramos, B., & Medina-Vogel, G. (2018). *Toxoplasma gondii* infection in wild mustelids and cats across an urban-rural gradient. *PLoS ONE*, *13*(6). https://doi.org/10.1371/journal.pone.0199085

Beverley, J. K., & Beattie, C. P. (1952). Standardization of the dye test for toxoplasmosis. *Journal of Clinical Pathology*, *5*(4), 350–353. https://doi.org/10.1136/jcp.5.4.350

Brennan, A., Donahoe, S. L., Beatty, J. A., Belov, K., Lindsay, S., Briscoe, K. A., Šlapeta, J., & Barrs, V. R. (2016). Comparison of genotypes of *Toxoplasma gondii* in domestic cats from Australia with latent infection or clinical toxoplasmosis. *Veterinary Parasitology*, *228*, 13–16. https://doi.org/10.1016/j.vetpar.2016.06.008

Cano-Terriza, D., Almería, S., Caballero-Gómez, J., Jiménez-Martín, D., Castro-Scholten, S., Dubey, J. P., & García-Bocanegra, I. (2020). Exposure to *Toxoplasma gondii* in zoo animals in Spain. *Preventive Veterinary Medicine*, *176*. https://doi.org/10.1016/j.prevetmed.2020.104930

Cohen, T. M., Blois, S., & Vince, A. R. (2016). Case Report Rapport de cas Fatal extraintestinal toxoplasmosis in a young male cat with enlarged mesenteric lymph nodes. In *Can Vet J* (Vol. 57).

de Barros, R. A. M., Torrecilhas, A. C., Marciano, M. A. M., Mazuz, M. L., Pereira-Chioccola, V. L., & Fux, B. (2022). Toxoplasmosis in Human and Animals Around the World. Diagnosis and Perspectives in the One Health Approach. In *Acta Tropica* (Vol. 231). Elsevier B.V. https://doi.org/10.1016/j.actatropica.2022.106432

De Camps, S., Dubey, J. P., & Saville, W. J. A. (2008). Seroepidemiology of *Toxoplasma gondii* in Zoo animals in selected Zoos in the midwestern United States. In *J. Parasitol* (Vol. 94, Issue 3). http://meridian.allenpress.com/journal-of-parasitology/article-pdf/94/3/648/2291712/ge-1453_1.pdf

de Vries, L. S., & Volpe, J. J. (2018). Viral, Protozoan, and Related Intracranial Infections. *Volpe's Neurology of the Newborn*, 973-1049.e18. https://doi.org/10.1016/B978-0-323-42876-7.00034-X

Delaney, M. A., Treuting, P. M., & Rothenburger, J. L. (2018). Rodentia. In *Pathology of Wildlife and Zoo Animals* (pp. 499–515). Elsevier. https://doi.org/10.1016/B978-0-12-805306-5.00020-1

Denk, D., De Neck, S., Khaliq, S., & Stidworthy, M. F. (2022). Toxoplasmosis in Zoo Animals: A Retrospective Pathology Review of 126 Cases. *Animals*, *12*(5). https://doi.org/10.3390/ani12050619

Di Genova, B. M., Wilson, S. K., Dubey, J. P., & Knoll, L. J. (2019). Intestinal delta-6-desaturase activity determines host range for Toxoplasma sexual reproduction. *PLoS Biology*, *17*(8). https://doi.org/10.1371/JOURNAL.PBIO.3000364

Dian, S., Ganiem, A. R., & Ekawardhani, S. (2023). Cerebral toxoplasmosis in HIV-infected patients: a review. In *Pathogens and Global Health* (Vol. 117, Issue 1, pp. 14–23). Taylor and Francis Ltd. https://doi.org/10.1080/20477724.2022.2083977

Du Plooy, I., Mlangeni, M., Christian, R., & Tsotetsi-Khambule, A. M. (2023). An African perspective on the genetic diversity of *Toxoplasma gondii*: A systematic review. In *Parasitology* (Vol. 150, Issue 7, pp. 551–578). Cambridge University Press. https://doi.org/10.1017/S0031182023000252

Dubey, J. (1988). Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with T. gondii oocysts and effect of freezing on viability of tissue cysts in pork. *AM. J. Vet. Res*, *49*, 910–913.

Dubey, J. P. (2008). The history of *Toxoplasma gondii* - The first 100 years. In *Journal of Eukaryotic Microbiology* (Vol. 55, Issue 6, pp. 467–475). https://doi.org/10.1111/j.1550-7408.2008.00345.x

Dubey, J. P. (2020). The history and life cycle of *Toxoplasma gondii*. *Toxoplasma Gondii: The Model Apicomplexan - Perspectives and Methods*, 1–19. https://doi.org/10.1016/B978-0-12-815041-2.00001-3

Dubey, J. P. (2021). Toxoplasmosis of Animals and Humans. In *Toxoplasmosis of Animals and Humans*. CRC Press. https://doi.org/10.1201/9781003199373

Dubey, J. P. (2022). Clinical toxoplasmosis in zoo animals and its management. *Emerging Animal Species*, *2*, 100002. https://doi.org/10.1016/j.eas.2022.100002

Dubey, J. P., Murata, F. H. A., Cerqueira-Cézar, C. K., Kwok, O. C. H., & Su, C. (2021). Epidemiological Significance of *Toxoplasma gondii* Infections in Wild Rodents: 2009-2020. In *Journal of Parasitology* (Vol. 107, Issue 2, pp. 182–204). American Society of Parasitologists. https://doi.org/10.1645/20-121

Dubremetz, J. F., & Lebrun, M. (2012). Virulence factors of *Toxoplasma gondii*. *Microbes and Infection*, *14*(15), 1403–1410. https://doi.org/10.1016/J.MICINF.2012.09.005

El Bissati, K., Levigne, P., Lykins, J., Adlaoui, E. B., Barkat, A., Berraho, A., Laboudi, M., El Mansouri, B., Ibrahimi, A., Rhajaoui, M., Quinn, F., Murugesan, M., Seghrouchni, F., Gómez-Marín, J. E., Peyron, F., & McLeod, R. (2018). Global initiative for congenital toxoplasmosis: an observational and international comparative clinical analysis. *Emerging Microbes and Infections*, *7*(1). https://doi.org/10.1038/s41426-018-0164-4

Fabiani, S., Caroselli, C., Menchini, M., Gabbriellini, G., Falcone, M., & Bruschi, F. (2022). Ocular toxoplasmosis, an overview focusing on clinical aspects. In *Acta Tropica* (Vol. 225). Elsevier B.V. https://doi.org/10.1016/j.actatropica.2021.106180

Fernandes, S., Brilhante-Simões, P., Coutinho, T., Cardoso, L., Dubey, J. P., & Lopes, A. P. (2019). Comparison of indirect and modified agglutination tests for detection of antibodies to Toxoplasma gondii in domestic cats. *Journal of Veterinary Diagnostic Investigation*, *31*(5), 774–777. https://doi.org/10.1177/1040638719868753

Galal, L., Hamidović, A., Dardé, M. L., & Mercier, M. (2019). Diversity of *Toxoplasma gondii* strains at the global level and its determinants. In *Food and Waterborne Parasitology* (Vol. 15). Elsevier Inc. https://doi.org/10.1016/j.fawpar.2019.e00052

Galal, L., Schares, G., Stragier, C., Vignoles, P., Brouat, C., Cuny, T., Dubois, C., Rohart, T., Glodas, C., Dardé, M. L., Kane, M., Niang, Y., Diallo, M., Sow, A., Aubert, D., Hamidović, A., Ajzenberg, D., & Mercier, A. (2019). Diversity of *Toxoplasma gondii* strains shaped by commensal communities of small mammals. *International Journal for Parasitology*, *49*(3–4), 267–275. https://doi.org/10.1016/j.ijpara.2018.11.004

Gil, M. P., Hegglin, D., Briner, T., Ruetten, M., Müller, N., Moré, G., Frey, C. F., Deplazes, P., & Basso, W. (2023). High prevalence rates of *Toxoplasma gondii* in cat-hunted small mammals - Evidence for

parasite induced behavioural manipulation in the natural environment? *International Journal for Parasitology: Parasites and Wildlife*, *20*, 108–116. https://doi.org/10.1016/j.ijppaw.2023.01.007

Hammond -Aryee, K., Van Helden, P., & Esser, M. (2016). *Seroprevalence and molecular epidemiology of Toxoplasma gondii in the western cape of South Africa*. https://scholar.sun.ac.za

Han, B. A., Schmidt, J. P., Bowden, S. E., Drake, J. M., Levin, S. A., & Designed, J. M. D. (2015). *Rodent reservoirs of future zoonotic diseases*. *112*(22), 7039–7044. https://doi.org/10.5061/dryad.7fh4q

Hardgrove, E., Zimmerman, D. M., von Fricken, M. E., & Deem, S. (2021). A scoping review of rodent-borne pathogen presence, exposure, and transmission at zoological institutions. In *Preventive Veterinary Medicine* (Vol. 193). Elsevier B.V. https://doi.org/10.1016/j.prevetmed.2021.105345

Hares, M. F., Tiffney, E. A., Johnston, L. J., Luu, L., Stewart, C. J., Flynn, R. J., & Coombes, J. L. (2021). Stem cell-derived enteroid cultures as a tool for dissecting host-parasite interactions in the small intestinal epithelium. In *Parasite Immunology* (Vol. 43, Issue 2). Blackwell Publishing Ltd. https://doi.org/10.1111/pim.12765

Hensel, M. E., Landsgaard, K., Wang, W., Neupane, S., Su, C., Blue-McClendon, A., Porter, B. F., Uzal, F. A., & Rech, R. (2023). Toxoplasmosis in ring-tailed lemurs (Lemur catta) and a peahen (Pavo cristatus) in a zoological collection caused by the common toxoplasma genotype in wild animals in the US. *Veterinary Parasitology: Regional Studies and Reports*, *41*, 100876. https://doi.org/10.1016/J.VPRSR.2023.100876

Hill, D., & Dubey, J. P. (2002). *Toxoplasma gondii*: Transmission, diagnosis, and prevention. *Clinical Microbiology and Infection*, *8*(10), 634–640. https://doi.org/10.1046/j.1469-0691.2002.00485.x

Howe, D. K., & David Sibley, L. (1995). *Toxoplasma gondii* Comprises Three Clonal Lineages: Correlation of Parasite Genotype with Human Disease. *The Journal of Infectious Diseases*, *172*(6). https://about.jstor.org/terms

Innes, E. A. (2010). A brief history and overview of *Toxoplasma gondii*. In *Zoonoses and Public Health* (Vol. 57, Issue 1, pp. 1–7). https://doi.org/10.1111/j.1863-2378.2009.01276.x

Joachim, A., Deksne, G., Liu, G.-H., Daryani, A., Galeh, T. M., Sarvi, S., Montazeri, M., Moosazadeh, M., Nakhaei, M., & Shariatzadeh, S. A. (2020). Global Status of *Toxoplasma gondii* Seroprevalence in Rodents: A Systematic Review and Meta-Analysis. *Frontiers in Veterinary Science | Www.Frontiersin.Org*, *1*, 461. https://doi.org/10.3389/fvets.2020.00461

Jones, J. L., Parise, M. E., & Fiore, A. E. (2014). Neglected parasitic infections in the United States: Toxoplasmosis. In *American Journal of Tropical Medicine and Hygiene* (Vol. 90, Issue 5, pp. 794–799). American Society of Tropical Medicine and Hygiene. https://doi.org/10.4269/ajtmh.13-0722

Khan, A., Dubey, J. P., Su, C., Ajioka, J. W., Rosenthal, B. M., & Sibley, L. D. (2011). Genetic analyses of atypical *Toxoplasma gondii* strains reveal a fourth clonal lineage in North America. *International Journal for Parasitology*, *41*(6), 645–655. https://doi.org/10.1016/j.ijpara.2011.01.005

Khan, A., Su, C., German, M., Storch, G. A., Clifford, D. B., & Sibley, L. D. (2005). Genotyping of *Toxoplasma gondii* strains from immunocompromised patients reveals high prevalence of type I strains. *Journal of Clinical Microbiology*, *43*(12), 5881–5887. https://doi.org/10.1128/JCM.43.12.5881-5887.2005

Kohler, J. M., Mammo, D. A., Bennett, S. R., & Davies, J. B. (2023). Primary ocular toxoplasmosis secondary to venison consumption. *American Journal of Ophthalmology Case Reports*, *29*. https://doi.org/10.1016/j.ajoc.2022.101776

Liu, Huang, W., Wang, S. L., Wang, S. L., Huang, P. Y., Lien, C. Y., Lai, Y. H., Wang, P. J., Wu, L. H., & Liao, A. T. (2022). Investigation of Toxoplasma infection in zoo animals using multispecies ELISA and GRA7 nested PCR. *BMC Veterinary Research*, *18*(1). https://doi.org/10.1186/s12917-022-03425-y

Liu, Q., Wang, Z. D., Huang, S. Y., & Zhu, X. Q. (2015). Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. In *Parasites and Vectors* (Vol. 8, Issue 1). BioMed Central Ltd. https://doi.org/10.1186/s13071-015-0902-6

Liyanage, K. L. D. T. D., Wiethoelter, A., Hufschmid, J., & Jabbar, A. (2021). Descriptive comparison of elisas for the detection of *Toxoplasma gondii* antibodies in animals: A systematic review. In *Pathogens* (Vol. 10, Issue 5). MDPI AG. https://doi.org/10.3390/pathogens10050605

Loyd, K. A. T., & Hernandez, S. M. (2019). Feral Cat Dilemma. *Fowler's Zoo and Wild Animal Medicine Current Therapy, Volume 9*, 104–109. https://doi.org/10.1016/B978-0-323-55228-8.00018-7

McLeod, R., Lykins, J., Gwendolyn Noble, A., Rabiah, P., Swisher, C. N., Heydemann, P. T., McLone, D., Frim, D., Withers, S., Clouser, F., & Boyer, K. (2014). Management of Congenital Toxoplasmosis. *Current Pediatrics Reports*, *2*(3), 166–194. https://doi.org/10.1007/s40124-014-0055-7

Milne, G., Webster, J. P., & Walker, M. (2020). *Toxoplasma gondii*: An Underestimated Threat? In *Trends in Parasitology* (Vol. 36, Issue 12, pp. 959–969). Elsevier Ltd. https://doi.org/10.1016/j.pt.2020.08.005

Molan, A., Nosaka, K., Hunter, M., & Wang. (2019). Global status of *Toxoplasma gondii* infection: systematic review and prevalence snapshots. In *Tropical Biomedicine* (Vol. 36, Issue 4). https://www.

Nicolle, C., & Manceaux, L. (1908). Sur une infection á corps de Leishman (ou organismes voisons) du gondi. *CR Acad Sci* , *147*, 763.

Nicolle, C., & Manceaux, L. (1909). Sur un protozoaire nouveau du gondi. *CR Acad Sci*, *148*, 369.

Ode, S., Jarikre, T., Jubril, A. J., Ularamu, H., Luka, P., Adamu, M., & Emikpe, B. (2022). High prevalence of *Toxoplasma gondii* in Nigerian wild rats by molecular detection. *Veterinary Parasitology: Regional Studies and Reports*, *35*. https://doi.org/10.1016/j.vprsr.2022.100776

Parameswaran, N., O'Handley, R. M., Grigg, M. E., Fenwick, S. G., & Thompson, R. C. A. (2009). Seroprevalence of *Toxoplasma gondii* in wild kangaroos using an ELISA. *Parasitology International*, *58*(2), 161–165. https://doi.org/10.1016/j.parint.2009.01.008

Peris, M. P., García, A. X., Castillo, J. A., Badiola, J. J., Halaihel, N., Serrano, M., & Gracia, M. J. (2023). *Toxoplasma gondii* in meat of adult sheep in Spain. *Food and Waterborne Parasitology*, e00203. https://doi.org/10.1016/j.fawpar.2023.e00203

Pettipher, G. L., Jay, J. M., & Wang, H. H. (2005). Microbiological Techniques. *Encyclopedia of Analytical Science: Second Edition*, 16–25. https://doi.org/10.1016/B0-12-369397-7/00374-5

Reiter-Owona, I., Petersen, E., Joynson, D., Aspo, H., Darde, M. L., Disko, R., Dreazen, O., Dumon, H., Grillo, R., Gross, U., Hayde, M., Holliman, R., Ho-Yen, D. O., Janitschke, K., Jenum, P. A., Naser, K., Olszewski, M., Thulliez, P., & Seitz, & H. M. (1999). The past and present role of the Sabin-Feldman dye test in the serodiagnosis of toxoplasmosis. *Bulletin of the World Health Organisation*, *77*(11), 929–935.

Shaapan, R. M., El-Nawawi, F. A., & Tawfik, M. A. A. (2008). Sensitivity and specificity of various serological tests for the detection of *Toxoplasma gondii* infection in naturally infected sheep. *Veterinary Parasitology*, *153*(3–4), 359–362. https://doi.org/10.1016/J.VETPAR.2008.02.016

Splendore, A. (1908). Un nuovo protozoa parassita de' conigli. incontrato nelle lesioni anatomiche d'une malattiache ricorda in molti punti il Kala-azar dell' uomo. *Rev Soc Scient Sao Paulo*, *3*, 109–112.

Stelzer, S., Basso, W., Benavides Silván, J., Ortega-Mora, L. M., Maksimov, P., Gethmann, J., Conraths, F. J., & Schares, G. (2019). *Toxoplasma gondii* infection and toxoplasmosis in farm animals: Risk factors and economic impact. In *Food and Waterborne Parasitology* (Vol. 15). Elsevier Inc. https://doi.org/10.1016/j.fawpar.2019.e00037

Su, C., Howe, D., Dubey, J., Ajioka, J., & David Sibley, L. (2002). Identification of quantitative trait loci controlling acute virulence in *Toxoplasma gondii*. *Proc Natl Acad Sci USA*, *99*(16), 10753–10758.

Su, C., Khan, A., Zhou, P., Majumdar, D., Ajzenberg, D., Dardé, M.-L., Zhu, X.-Q., Ajioka, J. W., Rosenthal, B. M., Dubey, J. P., & David Sibley, L. (2012). Globally diverse *Toxoplasma gondii* isolates comprise six major clades originating from a small number of distinct ancestral lineages. *Proc Natl Acad Sci USA*, *109*(15), 5844–5849. https://doi.org/10.1073/pnas.1203190109

Taylor, P. J., ARNTZEN, L., HAYTER, M., ILES, M., FREAN, J., & BELMAIN, S. (2008). Understanding and managing sanitary risks due to rodent zoonoses in an African city: beyond the Boston Model. *Integrative Zoology*, *3*(1), 38–50. https://doi.org/10.1111/j.1749-4877.2008.00072.x

Thomasson, D., Wright, E. A., Hughes, J. M., Dodd, N. S., Cox, A. P., Boyce, K., Gerwash, O., Abushahma, M., Lun, Z. R., Murphy, R. G., Rogan, M. T., & Hide, G. (2011). Prevalence and co-infection of *Toxoplasma gondii* and Neospora caninum in Apodemus sylvaticus in an area relatively free of cats. *Parasitology*, *138*(9), 1117–1123. https://doi.org/10.1017/S0031182011000904

Thrusfield, M., Christley, R., Brown, H., Diggle, P. J., French, N., Howe, K., Kelly, L., O'Connor, A., Sargeant, J., & Wood, H. (2018). *Veterinary Epidemiology* (Fourth). John Wiley & Sons Ltd.

Tidy, A., Fangueiro, S., Dubey, J. P., Cardoso, L., & Lopes, A. P. (2017). Seroepidemiology and risk assessment of *Toxoplasma gondii* infection in captive wild birds and mammals in two zoos in the North of Portugal. *Veterinary Parasitology*, *235*, 47–52. https://doi.org/10.1016/j.vetpar.2017.01.004

Webster, J. P. (2001). Rats, cats, people, and parasites: the impact of latent toxoplasmosis on behaviour. *Microbes and Infection*, *3*(12), 1037–1045. https://doi.org/10.1016/S1286-4579(01)01459-9

Weiss, L. M., & Dubey, J. P. (2009). Toxoplasmosis: A history of clinical observations. In *International Journal for Parasitology* (Vol. 39, Issue 8, pp. 895–901). https://doi.org/10.1016/j.ijpara.2009.02.004

Wolf, A., Cowen, D., & Paige, B. (1939). Human Toxoplasmosis: Occurence in infants as an Encephalomyelitis verification by transmission to animals. *Science*, *89*(2306), 226–227.

Yang, L., Ren, H., Zhu, N., Mao, G., Li, J., Su, C., Jiang, Y., & Yang, Y. (2023). Epidemiology and isolation of viable *Toxoplasma gondii* strain from macropods. *Heliyon*, *9*(3), e13960. https://doi.org/10.1016/J.HELIYON.2023.E13960

Ybañez, R. H. D., Ybañez, A. P., & Nishikawa, Y. (2020). Review on the Current Trends of Toxoplasmosis Serodiagnosis in Humans. In *Frontiers in Cellular and Infection Microbiology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fcimb.2020.00204

Appendix I: SANBI ARESC Approval

SANBI/RES/P2022/17

SANBI Biodiversity for Life
South African National Biodiversity Institute

01 December 2022

Dr Terrence Thovhakale
NZG

OUTCOME OF RESUBMITTED RESEARCH PROPOSAL

This letter serves to inform you that your resubmitted research proposal titled "Seroprevalence and genetic characterisation of *Toxoplasma gondii* in feral rodents in a zoological garden in South Africa" is **approved** by the SANBI NZG Animal Research Ethics and Scientific Committee (ARESC).

The following provisos should be taken into consideration:

1. Inform the ARESC of completion or termination (with reason) of your research.
2. Submission of an annual progress report in November of each year. Failure to submit a progress report may result in approval to be withdrawn.
3. Submission of a written request for any extension or modification of the research project.
4. SANBI should be acknowledged in all reports, scientific publications, and conference contributions.

IMPORTANT: It is your responsibility to ensure compliance with Section 20 of the Animal Diseases Act 1984 (Act 35 of 84) that applies to "investigation, experiment or research" or any other relevant permits. For any adverse event during the research, you need to complete the adverse event form and submit it within 3 working days.

The research proposal has been registered on the database as P2022/17. Please use this project number in all future correspondence.

Thank you for making use of SANBI NZG as a research platform.

Yours sincerely

Prof Antoinette Kotze
Chairperson: SANBI NZG Animal Research Ethics & Scientific Committee

Appendix II: DALRRD Section 20 Approval

agriculture, land reform & rural development
Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

2030 NDP

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Ms Marna Laing • Tel: +27 12 319 7532 • E-mail: MarnaL@dalrrd.gov.za Website: www.dalrrd.gov.za
Reference: 12/11/1/1/18 (2570 AC)

Dr Ndidzulafhi Terrence Thovhakale
SANBI National Zoological Gardens
Pretoria
E-mail: sean.terrence@yahoo.com ; darshana.morar@up.ac.za

Dear Dr Thovhakale,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application dated 21 July 2022 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other Act of the Republic of South Africa;
2. The research project is approved as per the application form dated 21 July 2022 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
3. Ethics approval must be obtained prior to the start of the study;
4. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
5. An on-site audit by the Directorate: Animal Health Laboratory Inspection Team has been scheduled for the SANBI National Zoological Gardens, Pretoria, in 2023. Failure to have the facilities audited or should any major non-compliances be found during the audit, will immediately result in this Section 20 approval becoming nil and void. Note that the approvals contained within this permit may be subject to change, depending on the findings of the audit;
6. Rodents (as per the species list provided) may be captured in the SANBI National Zoological Gardens, Pretoria, for use in this study;
7. It is the responsibility of the researcher and relevant laboratory or facility managers to ensure

SUBJECT: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO. 35 OF 1984) 12/11/1/5/8 (2631KL)

that the human safety aspects of this research project are adequately addressed;

8. Rodents may be euthanised in the Centre for Wildlife Health animal clinic. Serum, muscle, tongue, heart and diaphragm samples may be collected from the rodents, as per the SOP provided, under a biosafety cabinet class II in the DNA extraction Laboratory (NZG 20002742) of the NZG;
9. Serum samples may be serologically tested for *Toxoplasma gondii* antibodies under a biosafety cabinet class II in the DNA extraction Laboratory (NZG 20002742) of the NZG;
10. DNA may be extracted (from the samples listed above) in the DNA extraction Laboratory (NZG 20002742) of the NZG and sent to the Research and Training Laboratories (2-44, 2-46, 2-51, 2-58) of the Department of Veterinary Tropical Diseases, University of Pretoria for molecular analyses of *Toxoplasma gondii* and for rodent identification;
11. Samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);
12. All potentially infectious waste from the research project must be removed and disposed of by a waste disposal company registered to remove biohazardous waste;
13. Muscle, tongue, heart and diaphragm samples that tested positive for Toxoplasmosis may be stored under access control in the -20°C freezers of the SANBI NZG biobank;
14. Samples or material may not be outsourced or used for further/ other research without prior written approval from the Director: Animal Health;
15. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to MarnaL@dalrrd.gov.za;
16. Records must be kept for five years for auditing purposes.

Title of research/study: *"Seroprevalence and genetic characteristic of Toxoplasma gondii in rodents captured at the NZG Pretoria South Africa"*
Researcher: Dr Ndidzulafhi Terrence Thovhakale
Institution: Centre for Wildlife Health, SANBI National Zoological Gardens, Pretoria in collaboration with Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria
Permit Expiry Date: June 2024
Our ref Number: 12/11/1/1/18 (2570 AC)
Your ref: -

Kind regards,

DR MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2022 -12- 05

Appendix III: UP AEC Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

6 December 2022

Approval Certificate with Conditions
New Application

AEC Reference No.: REC106-22
Title: Seroprevalence and genetic characterisation of Toxoplasma gondii in feral rodents in a zoological garden in South Africa
Researcher: Dr NT Thovhakale
Student's Supervisor: Dr D Morar-Leather

Dear Dr NT Thovhakale,

The New Application as supported by documents received between 2022-08-12 and 2022-11-22 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2022-11-22.

Please note the following about your conditional ethics approval: Applicant to report back on **ALL species/numbers** caught as an annual renewal report (not just the one's sampled and euthanized) excel document to be provided.

1. The use of species is approved:

Species	Number
Rodents - Various	138
Samples	Number
Rodents - Blood (Samples from live animals)	138
Rodents - brain (Samples from live animals)	138
Rodents - diaphragm (Samples from live animals)	138
Rodents - heart (Samples from live animals)	138
Rodents - muscle tissue (Samples from live animals)	138
Rodents - spleen (Samples from live animals)	138
Rodents - tongue (Samples from live animals)	138

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2023-12-06.
3. Please remember to use your protocol number (REC106-22) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5. **All incidents** must be reported by the PI by email to Ms Marleze Rheeder (AEC Coordinator) within 3 days, and must be subsequently submitted electronically on the application system within 14 days.
6. The committee also requests that you record major procedures undertaken during your study for own-archiving, using any available digital recording system that captures in adequate quality, as it may be required if the committee needs to evaluate a complaint. However, if the committee has monitored the procedure previously or if it is generally can be considered routine, such recording will not be required.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8434
Fax +27 12 529 8321
Email: marleze.rheeder@up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

approval by the Committee.

We wish you the best with your research.

Yours sincerely

Prof A Tordiffe
DEPUTY CHAIRMAN: UP-Animal Ethics Committee

Appendix IV: UP REC Approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Research Ethics Committee

12 September 2022

CONDITIONALLY APPROVAL

Ethics Reference No	**REC106-22**
Protocol Title	**Seroprevalence and genetic characterisation of Toxoplasma gondii in feral rodents in a zoological garden in South Africa**
Principal Investigator	**Dr NT Thovhakale**
Supervisors	**Dr D Morar-Leather**

Dear Dr NT Thovhakale**,**

We are pleased to inform you that your submission has been conditionally approved by the Faculty of Veterinary Sciences Research Ethics committee, subject to other relevant approvals.

Please note the following about your ethics approval:

1. Please use your reference number (REC106-22) on any documents or correspondence with the Research Ethics Committee regarding your research.
2. Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
3. Please note that ethical approval is granted for the duration of the research as stipulated in the original application for post graduate studies (e.g. Honours studies: 1 year, Masters studies: two years, and PhD studies: three years) and should be extended when the approval period lapses.
4. The digital archiving of data is a requirement of the University of Pretoria. The data should be accessible in the event of an enquiry or further analysis of the data.

Ethics approval is subject to the following:

1. The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.
2. **Applications using Animals:** FVS ethics recommendation does not imply that AEC approval is granted. The application has been pre-screened and recommended for review by the AEC. Research may not proceed until AEC approval is granted.

Conditionally approved (pending obtaining ALL other relevant approvals).

We wish you the best with your research.

Yours sincerely

PROF M. OOSTHUIZEN
Chairperson: Research Ethics Committee

100 YEARS OF VETERINARY EDUCATION

Room 6-6, Arnold Theiler Building
University of Pretoria, Faculty of Veterinary Science
Private Bag X04, Onderstepoort, 0110, South Africa
Tel +27 (0)12 529 8390
Email marie.watson-kriek@up.ac.za
www.up.ac.za