Fluctuations in population size of *Theileria orientalis* Ikeda within the tick vector *Haemaphysalis longicornis* Neumann: an investigation into the life cycle of *T. orientalis* Ikeda.

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Abstract

Theileria orientalis Ikeda is a protozoan parasite of cattle that causes disease through the destruction of the host's erythrocytes. In 2012, the parasite caused an epidemic of bovine theileriosis in New Zealand at great economic cost to the farming community. As a result, a large number of scientific studies have been undertaken to understand the epidemiology of the *T. orientalis* Ikeda parasite in the hopes of mitigating the damage done by the parasite. An essential part of the epidemiology of any pathogen is the understanding of its life cycle and this holds true with *T. orientalis* Ikeda which, like other *Theileria* parasites, exhibits a complex life cycle involving multiple hosts.

In this thesis, fluctuations of *T. orientalis* Ikeda populations within nymph tick hosts were investigated over the course of six months. By doing so, this investigation highlights a part of the *T. orientalis* Ikeda lifecycle that is poorly covered in the scientific literature. The population of *T. orientalis* Ikeda within the tick was determined through qPCR analysis. Analysis of the qPCR results found that populations of *T. orientalis* Ikeda fluctuated greatly within the ticks over the course of six months.

Ticks infected with *Theileria* were procured through the development of a novel mass tick-rearing protocol: the artificial infestation of a cattle-beast with naïve ticks through the fixation of tick-containing ear-bags. A pilot study of the protocol showed that the fixation of ear-bags onto cattle using Kamar® adhesive did not negatively impact the welfare of the cattle involved. The subsequent field trial of the protocol resulted in the successful engorgement and infection of five thousand naïve tick larva. These results demonstrated a viable method to obtain *T. orientalis* Ikeda infected ticks that would be suitable for further research.

Also described in detail in this thesis are attempts at adapting the protocol of Krober and Guerin (2007) to create a silicone membrane for the artificial feeding of tick larvae on *Theileria*-infected blood without the involvement of live hosts. Despite multiple attempts, the experiments yielded no successful attachments of ticks onto the synthetic membranes. Failure here was attributed largely to the adapted protocol creating silicone membranes that were too thick for the larval ticks to successfully penetrate with their mouthparts. However, the *in vitro* feeding of ticks presents itself as a great potential contributor to future tick research. It is hoped that the knowledge gained from the repeated trials of the *in vitro* feeding apparatus in this experiment may help in the development of successful protocols in the future.

Acknowledgements

The completion of this thesis was a journey, one I could never have completed alone.

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Chapter 1: Introduction and Literature Review

1.1 Introduction

In early 2012, an epidemic of bovine theileriosis in New Zealand dealt a major financial blow to its pastoral farming sector (McFadden et al., 2015a). The bovine theileriosis was discovered to have been caused by the apicomplexan parasite *Theileria orientalis* Ikeda (Lawrence et al., 2015), which belongs to a branch of the *Theileria* genus known as the non-transforming *Theileria* (Watts et al., 2016). Other members of this group include *T. orientalis* Chitose and Buffeli variants, and as is true of the Chitose and Buffeli variants of *T. orientalis*, non-transforming *Theileria* are generally benign and cause no symptoms in infected cattle. In contrast, the group of *Theileria* species known as the 'transforming' *Theileria* are highly pathogenic and are recognised as major economic threats around the world. These transforming *Theileria* include the likes of *Theileria annulata* which causes tropical theileriosis, and *Theileria parva* which causes East Coast Fever (Schubert et al., 2010). The distinction between the two groups of *Theileria* parasites comes from 'transforming' *Theileria* species having the ability to 'hijack' the lymphocytes of their host animal. They do this by manipulating the genetic expression of the infected lymphocytes such that infected cells aid in the propagation of the *Theileria* parasite (Heussler et al., 2001). However, unlike most non- transforming *Theileria* species, *T. orientalis* Ikeda is a pathogenic *Theileria* species (Watts et al., 2016; Lawrence et al., 2019). Bovine theileriosis caused by the Ikeda strain of *T. orientalis* caused significant loss in body condition as well as abortion in pregnant cattle, with severe cases resulting in death (Watts et al., 2016).

Theileria orientalis Ikeda, like all *Theileria* species, spends a part of its lifecycle in an arthropod vector. In New Zealand, *T. orientalis* Ikeda is exclusively carried by the livestock tick *Haemaphysalis longicornis* (Neumann) - an ixodid tick with a three-host life cycle (Lawrence et al., 2019). In response to the 2012 epidemic, many scientific studies were launched into understanding both the *Theileria* parasite and its tick host so that their effect on livestock might be better mitigated in the future (Lawrence et al., 2018a; Lawrence et al., 2018b; Lawrence et al., 2019; Watts et al, 2016). As a part of that effort, this thesis seeks to detect changes, if any, in the concentration of *Theileria orientalis* Ikeda while it is inside its tick host *Haemaphysalis longicornis*. This first chapter will review the current literature regarding the parasite *T. orientalis* Ikeda and its vector *H. longicornis*.

1.2 Origins and life cycle

Theileria orientalis Ikeda is thought to have originated in Asia, occurring endemically in places such as China, Korea, and Japan (Watts et al., 2016). There it is spread proficiently by the tick *H. longicornis*, another endemic species (Heath, 2016). When first discovered in New Zealand, *T. orientalis* Ikeda was wrongly classified as a pathogenic strain of the cattle parasite *T. orientalis* Sergenti. It was only later through genetic studies that *T. orientalis* Ikeda was recognised as a separate parasite (Lawrence et al., 2018a); this distinction from *T. orientalis* Sergenti (now known to be a mixed infection of *T. orientalis* Ikeda and *T. orientalis*

Chitose (Lawrence at al., 2016)), allowed scientists to track its spread across the globe via the spread of *H. longicornis* (Watts et al., 2016).

The life cycle of *T. orientalis* Ikeda is spread between its two hosts: the cattle, and the tick *H. longicornis* (Lawrence et al., 2018). While research has shown that other hematophagous arthropods are able to mechanically transfer an infection of *T. orientalis* Ikeda from one animal to another (Hammer et al., 2016), the parasite is only able to complete the second half of its life cycle inside the salivary glands of the tick *H. longicornis* (Shaw and Young, 1994). *Theileria orientalis* Ikeda type begins its life cycle within the tick when it is ingested as part of the tick's blood meal. The parasite is ingested in the form of the piroplasm. Once inside the tick's midgut, the *Theileria* piroplasms migrate out of the midgut lumen and into the cells forming the midgut lining, where the piroplasms develop into gamonts. The gamonts inside the tick's gut cells release gametocytes which recombine sexually to form zygotes. Within the tick gut cells, the zygotes of *Theileria* develop into mobile kinetes; these kinetes migrate through the haemolymph of the tick and invade the salivary glands. Once inside the cells of the tick's salivary glands, the mobile kinetes develop into both sporokinetes (which in turn invade more salivary gland cells) and sporoblasts (Fuente and Kocan, 2003). The sporoblasts reside in the tick's salivary glands until sporogony is triggered by the release of salivary-gland growth hormones. This results in the release of thousands of sporozoites from the sporoblasts into the tick's saliva (Young et al., 1983).

The *Theileria* sporozoites are injected into the cattle's blood stream along with the tick's saliva when the tick feeds on the cattle host (Bishop et al., 2004). Once inside the cattle, the life cycle of *T. orientalis* Ikeda becomes less clear. In transforming *Theileria*, the mature schizonts in the altered lymphocytes undergo clonal expansion in the parasitized cells (Watts et al., 2016). Certain schizonts will undergo merogony and trigger apoptosis (programmed cell death) in the cells that they inhabit, releasing merozoites into the lymphatic system of the mammalian host. The merozoites migrate into the circulatory system where they invade erythrocytes and develop into piroplasms. Meanwhile, there is very little in the literature about where the sporozoites of the non-transforming *T. orientalis* Ikeda migrate to for their maturation into schizonts. According to Watts et al. (2016), *T. orientalis* Ikeda sporozoites have been detected in a variety of organs in infected animals, but mostly in the liver and kidneys. This has led to speculation that the sporozoites undergo transformation into schizonts in the liver and kidneys. Despite the difference in life cycle when first entering the cattle host, *T. orientalis* Ikeda piroplasms are nevertheless found in the host's circulatory system a few days after infection (Gias et al., 2016). When a hematophagous arthropod, such as the tick *H. longicornis*, feeds upon the cattle, it will inevitably take up a population of *T. orientalis* piroplasms in its blood meal (Figure 1.1).

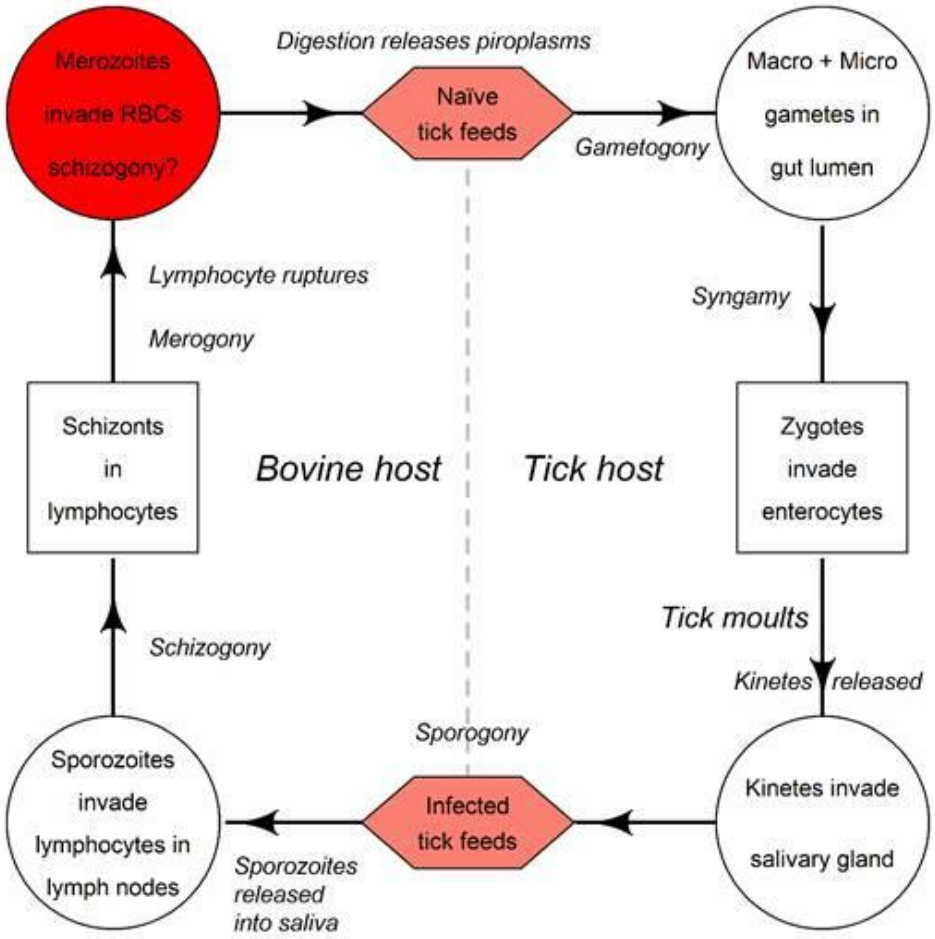


Figure 1.1: A simplified diagrammatic representation of the life cycle of *T. orientalis* Ikeda. Diagram provided by K. Lawrence in personal communication (20 Mar 2019).

1.3 Clinical signs and pathology

Research indicates that high stress environments such as those presented by the onset of spring (calving season) or those present during the long distance transport of cattle, increase the pathogenicity of *T. orientalis* Ikeda infections in cattle (Lawrence et al., 2016). Clinical signs in cattle infected by the parasite include lethargy, abortion of calves, fever, anaemia, and in the worst cases, death. However, should an animal make a full recovery from its infection the animal will have a life-long resistance to the parasite with low chances of relapse (Lawrence et al., 2016).

1.4 Diagnosis and the development of *Theileria* detection through qPCR assays

Traditionally, bovine theileriosis has been diagnosed through the use of Giemsa-stained blood smears and Polymerase Chain Reaction (PCR) amplification of *Theileria* MPSP (Major Piroplasm Surface Protein) genes from blood samples. *Theileria* piroplasms become visible once stained with Giemsa stain and viewed using a light microscope (Watts et al., 2016). However, the infection rates of the host erythrocytes are on average quite low - around

0.02-0.03% per smear; cattle with severe anaemia might yield an erythrocyte infection rate of higher than 1% (Watts et al., 2016). ELISA (enzyme linked immunosorbent assay) has also been used as a serological diagnostic test for theileriosis in cattle (Watts et al., 2016).

In response to the epidemic of theileriosis in New Zealand, Pulford et al. (2016) developed a qPCR diagnostic technique for *T. orientalis* Ikeda. Their team was able to design a probe complimentary to the amplicon of *T. orientalis* Ikeda that is specific to the MPSP (Pulford et al., 2016). The probe allows for the use of TaqMan® qPCR assays to detect the presence of *T. orientalis* Ikeda and differentiate it from the other strains of *T. orientalis* present in New Zealand livestock (Pulford et al., 2016). In the same year Gias et al. (2016) and McFadden et al. (2017) trialled successfully the new diagnostic TaqMan® qPCR assay developed by Pulford et al. (2016) in their study on anaemic New Zealand cattle. The new qPCR protocol is both faster to perform and more sensitive than the diagnostic techniques that have come before, being able to detect the presence of *Theileria* in the blood stream more than two weeks before the piroplasms reach visible density in Giemsa-stained blood smears (Gias et al., 2016).

1.5 Treatments and vaccination

The drug Buparvaquone is widely considered the most effective medical treatment for theileriosis (Watts et al., 2016). Buparvaquone inhibits the parasite's ability to proliferate in the host's body through the binding of several key *Theileria* protein receptors (Q_{01} and Q_{02}) (Muller et al., 2018). As such, Buparvaquone is sometimes used in *Theileria* inoculation treatments for cattle (also known as live-vaccination), where live *Theileria* sporozoites are artificially injected into the cattle and the subsequent infection is immediately treated with Buparvaquone (Mutugi et al., 1991; Darghouth, 2008). This method of *Theileria* inoculation treatment grants cattle life-long resistance to the *Theileria* parasite (Darghouth, 2008). While the majority of the literature focuses on the use of Buparvaquone in the treatment of tropical bovine theileriosis and *Theileria annulata* infections, the drug is also used in the treatment of oriental theileriosis caused by *T. orientalis* Ikeda infections. However, the usage of Buparvaquone is extremely limited in New Zealand - having only been allowed since 2016 - and it is banned in Australia; this is due to the drug having an extremely long hold over period in the tissue of the cattle that undergo treatment from it (McDougall et al., 2016). Studies have shown that the drug lasts an average of 147 days within the body and might still reside in detectable amounts within the milk up to a whole year after initial treatment (Watts et al., 2016). Furthermore, recent studies on *Theileria parva* suggest that there is now a growing resistance worldwide to the drug in *Theileria* populations (Mhadhbi et al., 2010). Other treatments for bovine theileriosis include mixed drug treatments involving halofuginones and primaquines; as well as the use of blood transfusions to offset the effects of the anaemia caused by the theileriosis (Watts et al., 2016).

There are currently no commercially available vaccines for *Theileria*. The nearest approximation to vaccination against bovine theileriosis is through live-vaccination. However, studies have shown that though the cattle that survive the inoculation become resistant to further infections, naïve ticks that feed on inoculated cattle are still able transmit the parasite to naïve cattle with dire consequences (Darghouth, 2008). Since direct

treatment for theileriosis is extremely limited, the current approach towards minimising the damage caused by *Theileria* is through prevention, not treatment. Globally, prevention of theileriosis often comes in the form of population control for the ticks that are vectors of the disease.

Population control for ticks has traditionally relied upon commercially available acaricides (Heath and Levot, 2015). These take the form of livestock dips or pour-on liquids with pyrethrins and organophosphates acting as the active ingredients (Heath et al., 1980). In New Zealand, tick burden on livestock is commonly treated through the use of flumethrin-based pour-on acaricides (Heath, 2016). These pour-on acaricides typically grant the recipient around six days of immunity from tick attachments. However, recent studies have indicated that the current use of acaricides in tick control might not be sustainable as ticks have become increasingly acaricide-resistant (Heath and Levot, 2015).

1.6 *Haemaphysalis longicornis*

Haemaphysalis longicornis (Neumann) is the only known exotic livestock tick in New Zealand (Lawrence et al., 2018). Thought to have originated in Asia, it is endemic to China, Japan and Korea; however, it can now be found in Australia, New Zealand, and North America (Heath, 2016). *H. longicornis* is both economically and medically important in the countries that it is found in, being able to vector a number of important pathogens of both human and livestock alike (Beard et al., 2018); recently, *H. longicornis* has been documented being the primary vector for a viral pathogen that causes a condition called "Severe fever and thrombocytopenia syndrome", currently found in China and the United States of America (Rainey et al., 2018). In New Zealand, *H. longicornis* acts as the main vector for the strains of *T. orientalis* found within the cattle population (Raghavan et al., 2018).

1.6.1 Life cycle of *H. longicornis*

Haemaphysalis longicornis has a three stage life cycle (Heath, 2016). The tick's life cycle begins with the egg stage - these are produced by the female either through sexual reproduction or parthenogenesis depending on the population. There are several differing populations of *H. longicornis* across the globe, with those in Asia and America belonging to the diploid population while triploid populations of *H. longicornis* are found in Korea and New Zealand (Heath, 2016).

The eggs of *H. longicornis* hatch into six-legged larvae. These larvae require only one blood meal before they moult into eight legged nymphs. Like the larvae, the nymphs require only one blood meal before moulting into adults. Female adult *H. longicornis* will feed to repletion before detaching from the host and seeking shelter in the pasture where they lay their eggs (Heath et al., 2016). Eggs of *H. longicornis* hatch in early spring with most ticks reaching the adult phase in early summer (Heath et al., 2016).

1.7 Aims and structure

While Lawrence et al (2018a; 2018b), McFadden et al (2011; 2015; 2017) and Watts et al (2016) deal extensively in the clinical signs and pathology of *T. orientalis* Ikeda in New Zealand cattle, very little research has been dedicated to its broader epidemiology (Watts et al. 2016). As mentioned earlier in this chapter, the life cycle of the parasite *T. orientalis* Ikeda is poorly understood. It is the purpose of this thesis to investigate the size of the population of *T. orientalis* Ikeda living inside the tick *H. longicornis* over the course of 6 months. By destructively sampling groups from a population of *T. orientalis* Ikeda-infected ticks for qPCR analysis, it is hoped that potential changes that happen to the population size of the *Theileria* parasite while inside the *H. longicornis* tick would be seen. Changes in Theileria population size may shed light on the activities of the Theileria while it is inside the tick. While past research (Shaw and Young, 1994) has shown that Theileria parasites undergo sporogony in reaction to the activation of the tick's salivary glands, this thesis focusses specifically on the changes in *Theileria* population while the tick is fasting.

1.8 Chapter outline:

Chapter 2: Introduction to methodology

In this chapter a brief introduction is provided for the two main tick-rearing methods explored in this thesis. The first method being that of in vitro feeding ticks using a silicone membrane; while the second method is the artificial infestation of cattle using tick-infested ear-bags. A brief history is provided for the two feeding methods and it is detailed how both live-animal feeding as well as in vitro feeding have progressed to this day.

Chapter 3: Experimental investigation of silicone artificial membranes for the mass-rearing of tick larvae

In this chapter, a detailed account is given for the investigation of in vitro feeding the tick *H. longicornis* using a silicone membrane. The methodology for the making of the membrane is explained in the light of past research, specifically that of Krober and Guerin (2007(a)). The discussion at the end of the chapter explores the possible reasons the in vitro feeding apparatus failed as well as briefly touching on possible avenues for future research.

Chapter 4: Development and implementation of ear-bags on cattle as a novel tick-rearing method

As conventional rearing of ticks on laboratory animals would not yield the *Theileria*-infected ticks needed for this study, a different approach was necessary. In this chapter, the development of the protocol for a novel tick-rearing method is detailed: the use of tick-infested ear-bags as a means to feed ticks on cattle infected by *T. orientalis* Ikeda.

A pilot study was conducted with the novel tick-feeding method to ensure that the ear-bags did not violate the welfare of the cattle they would be used on. This is the first half of the chapter. The second half of the chapter details the field trial of the ear-bags, the methodology and results, and concludes with a brief discussion of both potential upgrades for the ear bags as well as potential uses for the ear bags in field of tick research.

Chapter 5: Quantitative PCR analysis of *T. orientalis* Ikeda population within the hibernating tick *H. longicornis*

The TaqMan® PCR protocol for the detection of *T. orientalis* Ikeda DNA was developed by Pulford et al. (2016). This chapter details how the qPCR assay protocol developed by Pulford et al. (2016) was used in this study to ascertain the population size of the *T. orientalis* Ikeda parasites living inside the tick nymphs, as well as how that population changed in size over the course of 24 weeks.

The discussion at the end of the chapter postulates concerning the factors that may be involved in the changes of population size seen from the results. Three main factors are explored in detail: sampling bias, tick-pathogen interactions, and temperature-induced sporogony.

Chapter 6: General Discussion

In this chapter, the main points of the discussion of chapters four and five are summarised and avenues for future research is suggested. This chapter ends with the conclusion of the thesis.

Chapter 2: Introduction to Methodology

In order to accurately measure the population of *T. orientalis* Ikeda within the tick *H. longicornis*, a genetic testing method must be used to infer the amount of *Theileria* DNA present inside the tick, from which the number of *Theileria* organisms can then be quantified. PCR has long been used in the detection of *Theileria*, mostly as a diagnostic test for the presence of piroplasms in cattle blood samples (Bogema et al., 2015). However, due to the New Zealand epidemic of bovine theileriosis and the subsequent demand for diagnostic testing, a TaqMan® qPCR method for the rapid detection of *T. orientalis* Ikeda DNA was developed by Pulford et al. (2016) using primers and probes derived from the parasite's Major Piroplasm Surface Proteins (MPSP). The same protocol was adapted in this thesis and used in conjunction with the absolute quantification method described by Lawrence et al. (2018a) to infer the population of *T. orientalis* Ikeda present within the ticks used.

The main challenge in this investigation was the method in which *Theileria*-infected ticks were procured. It was decided that the collection of individual ticks off cattle beasts was not a viable method for acquiring *Theileria*-infected ticks. Too many confounding factors were present such as the diverse age demographic of the ticks collected; the duration ticks had been feeding on the cattle, and the potential for cattle to have had a previous *Theileria*-infection. These factors all potentially affect the population size of *T. orientalis* Ikeda existing within the tick (Cabezas-Cruz et al., 2019). In order to obtain ticks that were of uniform age and naïve to the parasite, larval ticks were obtained, with the knowledge that *Theileria* is transstadial- meaning it does not transmit between parents and offspring of the tick vectors (Ghosh and Azhahianambi, 2007). For the study to progress, a method of mass rearing ticks must be found that would also serve to infect the larval ticks with *T. orientalis* Ikeda.

The mass rearing of ticks has been an essential part of tick research since the early 20th century (Waladde et al., 1990). In this thesis, two approaches have been taken in regards to the mass-rearing of ticks: feeding ticks on live animals, and *in vitro* feeding using synthetic membranes. The infestation of live animals with ticks for the purpose of mass rearing is currently the standard practice, with many laboratories using mice, rats, and rabbits as tick hosts (Levin and Schumacher, 2016). Nuss et al. in their 2017 paper outlined the standard procedure for both the rearing of ticks on live hosts as well as the animal husbandry required for the feeding trials. An excerpt from their paper (Figures 2.1 and 2.2 below) has been included to give a better understanding of the apparatus required for tick-rearing on live hosts.

Figure 2.1: An excerpt from the paper by Nuss et al. (2017). This image shows the enclosures used by Nuss et al. in their tick-feeding trials. Reproduced with permission by Journal of Visualised Experiments.

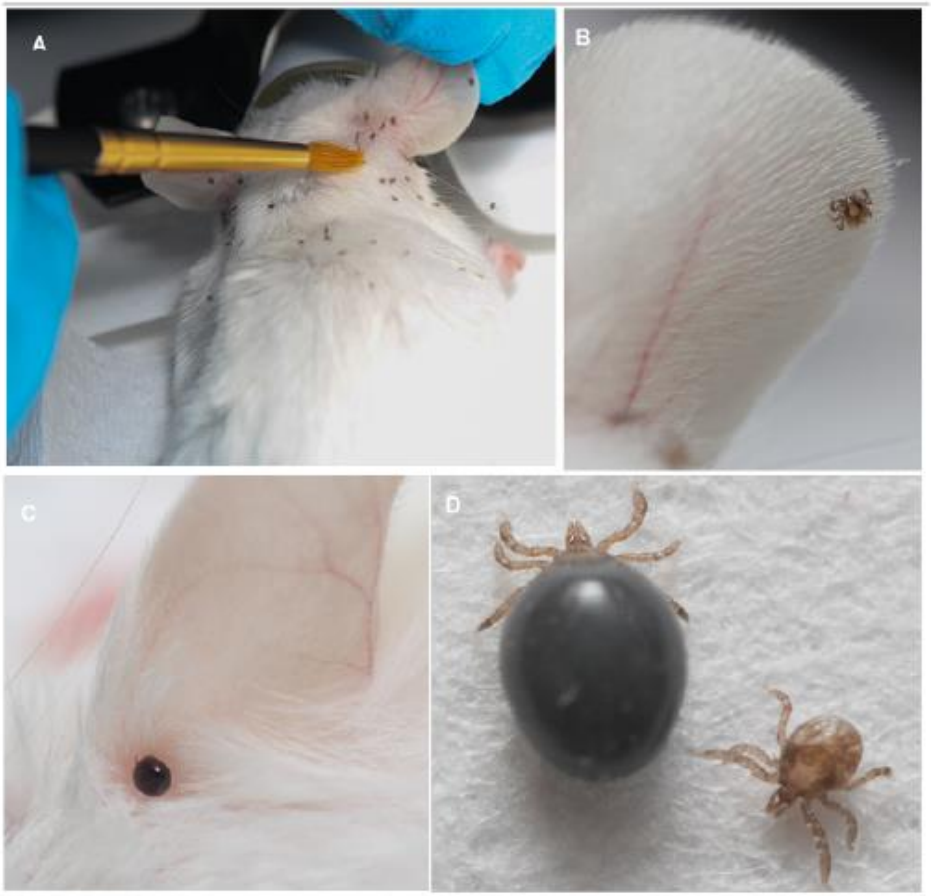


Figure 2.2: Images taken from the paper by Nuss et al. (2017) showing the application of larval ticks (Ixodes scapularis (Say) to sedated mouse for mass tick rearing. Reproduced with permission by Journal of Visualised Experiments.

The use of artificial membranes in *in vitro* tick-feeding apparatus appeared early on in the scientific literature of tick research (DeMeillon and Goldberg, 1947; Purnell and Joyer, 1970). The demand for an easy method of feeding and rearing ticks without the use of live animal hosts is high, especially in relation to the development and preparation of tick-vaccines (Trentelman et al., 2017). As acaricide resistance becomes increasingly common within tick populations around the world (Heath and Levot, 2015), the need for tick vaccines also increases. At the same time, with scientific research embracing the welfare of laboratory animals through the implementation of the 'reduce, replace, and refine' principles, *in vitro* feeding of ticks on artificial membranes becomes increasingly essential to ongoing tick research (Meng and Sluder, 2018, p. 188).

Initial research focused on the use of capillary tubes to feed ticks (Purnell and Joyer, 1970), although it was found that the method was unable to feed ticks to repletion. Later research on artificial tick-feeding membranes included investigation of paraffin membranes (Hokama et al., 1987), mouse and rabbit skin (Tajeri et al., 2016), and silicone membranes (Krober and Guerin, 2007(a)). Despite the multitude of protocols developed for the *in vitro* feeding and rearing of ticks on artificial membrane, no one method has risen to the popularity of the usage of live animals (Romano et al., 2018). A contributing factor to this may be the variety of challenges surrounding the use of artificial membranes such as cost of development, ease of production, and ease of implementation (Romano et al., 2018).

In this thesis, two methods of tick-rearing were investigated: in vitro feeding using a silicone membrane, and artificial infestation of a live host. Their methodology and the results will be discussed in detail in the following chapters

Chapter 3: Experimental investigation of silicone artificial membranes for the mass-rearing of tick larvae

3.1 Introduction

Rearing ticks on *T. orientalis* Ikeda infected blood was an essential component of this study into the effects of *Theileria orientalis* Ikeda type infection on *Haemaphysalis longicornis Neumann*. To this end, an attempt was made to develop a method for rearing ticks *in vitro*. The experimental method was based on that of Krober and Guerin (2007(a)) who developed an artificial membrane by bonding a silicone mixture with Kodak lens cleansing paper (Eastman Kodak, Rochester, NY, USA). Krober and Guerin then incorporated their silicone membrane into a feeding apparatus through which they successfully fed adults of the *tick Ixodes ricinus* (Linnaeus) (Figure 3.1). This method has since been replicated in other studies, albeit with differing success (Tajeri et al., 2016; Trentelman et al., 2017).

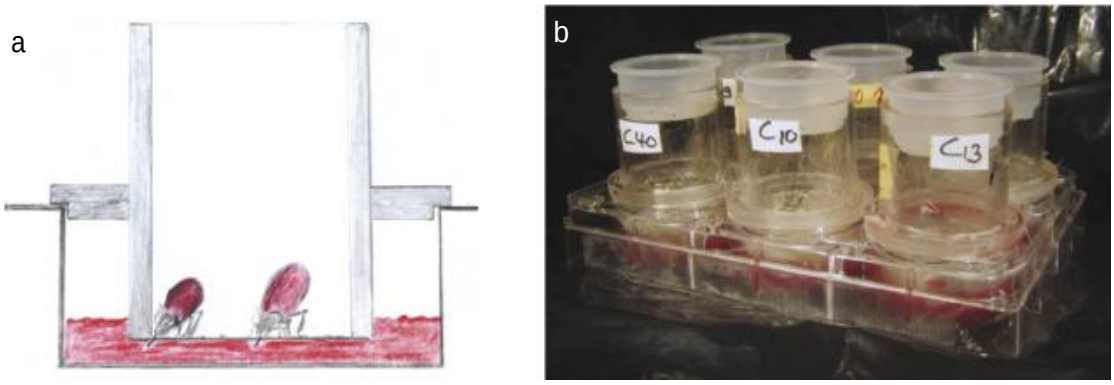


Figure 3.1: An excerpt taken from Krober and Guerin (2007). a) Their sketch of their feeding apparatus. b) The feeding apparatus inside the incubator. Reprinted with permission from Elsevier B.V.

This chapter covers the attempts at *in vitro* feeding larval *H. longicornis* ticks using a silicone membrane.

3.2 Method and materials

Development of silicone membranes:

The silicone-paper membrane was constructed as closely as possible following the products and method outlined by Krober and Guerin (2007(a)). However, due to the unavailability of some products, near equivalent products were substituted as follows: silicone oil (30% DC 200, viscosity ~10mPa's; Fluka, Switzerland) was replaced by 4.5 Silicone Grease Bayer (Sigma Aldritch, USA); and Kodak lens cleaning paper was replaced with Kimwipe (KIMTECH, New Zealand). In my modified method, a silicone paste was produced by mixing 15 g Silicone glue RTV-1 Elastosil® (Sigma Aldritch, USA), 4.5 Silicone Grease Bayer (Sigma Aldritch, USA), and 2.9 g Hexane. The paste was spread across a Kimwipe (KIMTECH, New Zealand) and

allowed to set for 24 hours. Of the feeding stimuli used by Krober and Guerin (2007(b)), only the cattle hair extract (a 0.5 mg lipid extracted from freshly shaven cow hair dissolved in 75 µL Dichloromethane) could be obtained. The mosquito netting and the plastic cross used in Krober and Guerin's protocol were not incorporated into the membranes used in this trial as the materials to make them were unavailable when the trials first began.

The *in vitro* apparatus was constructed using a micro-petri dish and a plastic tube of 5 cm length and 2 cm diameter. By drilling a hole in one end of the micro-petri dish, the plastic tube could be fitted through. Silicone membrane was then used to cover the end of the tube inside the micro-petri dish and the silicone membrane was held in place by a rubber band (Figure 3.2).

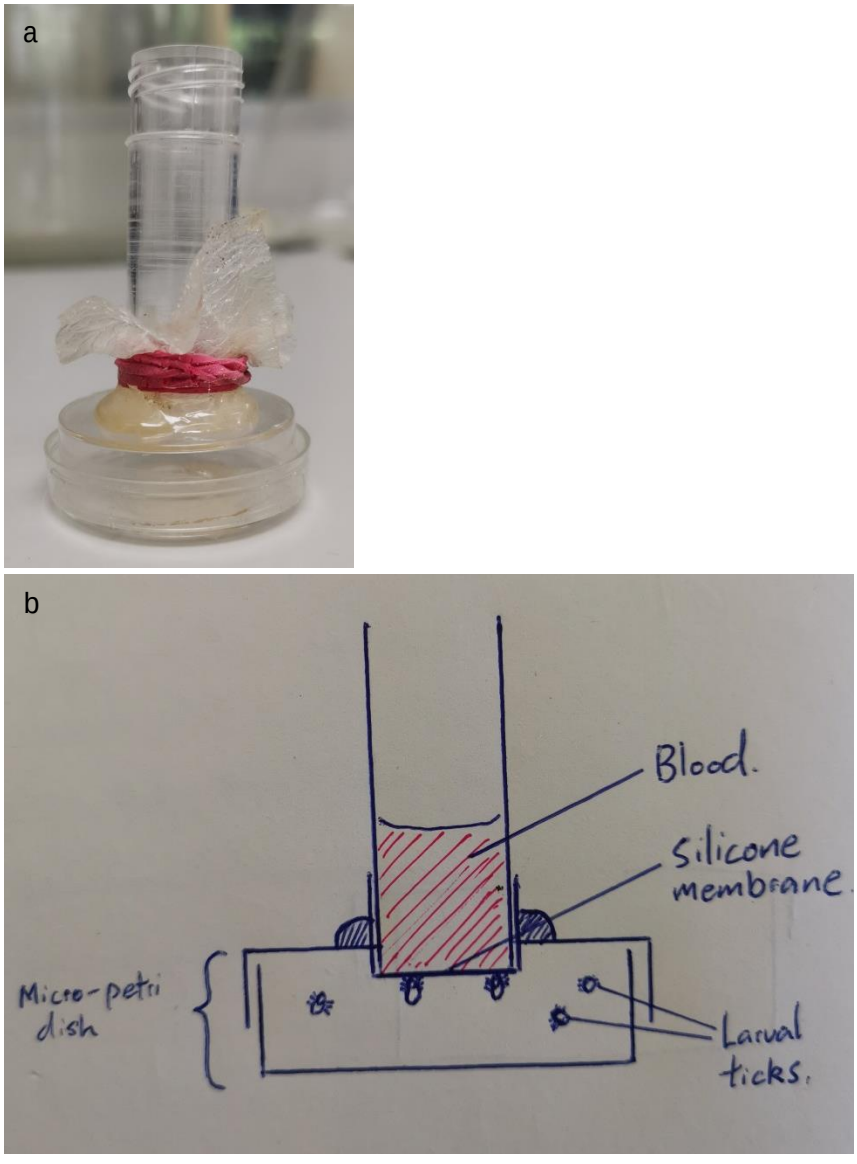


Figure 3.2: a) The feeding apparatus used in this study: the larval ticks are housed within the two halves of the micro-petri dish. This space is separated from the long plastic cylinder by the silicone membrane which has been fixed in place with a rubber band. b) An annotated sketch of the apparatus detailing the design.

Blood was contained inside the plastic tube while larval ticks were housed inside the micro-petri dish. The side of the silicone membrane adjacent to the ticks was brushed with cow hair extract.

The apparatus was kept at 35°C in an airtight container inside an incubator (Contherm Polar 1000C). The blood used in the trial was collected with permission from ANZCO Rangitikei (Bulls, New Zealand) and was stored in 10 ml EDTA vials. The blood within each tube was changed once every 24 hours and the apparatus was also checked for condensation as well as fungal growth.

Throughout the investigation, several different variations from the protocol provided by Krober and Guerin (2007) were trialled in an effort to produce a working apparatus. The summary of these trials can be found in Table 3.1. Of particular note is the incorporation of a hot plate and a magnetic stirrer to heat and move the blood. This was a necessary improvement since blood maintained at high temperature without rapid stirring would stagnate and separate into separate layers of what appeared to be red-tinged plasma and cells.

An annotated diagram of the apparatus after incorporation of a hot plate and magnetic stirrer can be found below in Figure 3.3).

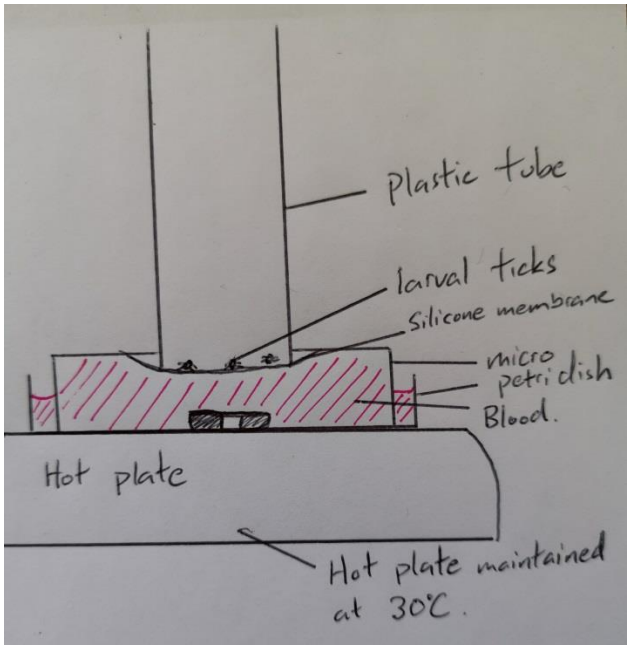


Figure 3.3: A variation of the original set up found in Figure 3. This set up allowed the blood to be heated independently from the rest of the apparatus to a temperature of 30°C while also stirring the blood to prevent stagnation.

All iterations of the apparatus were tested in triplicate (Table 3.1). Three sets of tick-feeding apparatus were run simultaneously for each iteration of the trial. All apparatus were checked for tick-attachment after 24 hours. If there was no attachment of ticks, the apparatus was then removed from the incubator and any live ticks were retrieved.

Table 3.1: Table summarising the multiple variations of Krober and Guerin's protocol (2007) that were attempted in this investigation of artificial feeding. Of particular note in these variations are those that involve animal skin and the trial where the apparatus was modified to accommodate the use of a magnetic stirrer.

	Standard silicone trial: silicone membrane with cattle hair attached scented with hair extract, placed at 30°C	
Trial:	Variation from the standard silicone trial described above:	Ticks involved in trial:
1	Temperature raised to 37°C	50
2	Temperature raised to 35°C	50
3	Temperature dropped to 25°C	50
4	Membrane scented with lard	50
5	Membrane scented with sweat (human)	50
6	More hexane used in setting mixture (thinner membrane)	50
7	Mouse skin used instead of silicone, no cattle hair or hair extract	50
8	Rabbit skin used instead of silicone, no cattle hair or hair extract	50
9	Apparatus suspended over hot water bath with blood stirred by magnetic paddle	50

Ticks:

Larval ticks were obtained by sourcing gravid female ticks from Limestone Downs (Port Waikato, New Zealand). Gravid female ticks were collected by the farmer and transported by car to Massey University (Manawatu, New Zealand). Upon arrival, gravid female ticks were sorted into small mesh top containers, with deceased and non-responsive ticks being discarded. The gravid female ticks were housed in small mesh top containers, with four females per container to avoid overcrowding. Gravid female ticks were kept at 24°C, 95% humidity, and constant darkness. Temperature and humidity regulation were achieved using an airtight container lined with wet paper towels and an incubator (Contherm Polar 1000C, Contherm Scientific Ltd, New Zealand) set at 24°C. The small mesh top containers which held the gravid females were placed inside the air-tight container, which itself was held in the incubator, helping to achieve the conditions of 24°C and 95% humidity.

Once egg-laying had finished (the process on average taking around a week), any dead or dying female ticks were removed from the mesh top containers and disposed of. This was to

prevent fungal growth on dead or dying female ticks from contaminating the eggs. Eggs were held at the same temperature until they had hatched into larvae, whereupon the temperature setting of the incubator was lowered to 20°C. Ticks continued to be regularly checked for fungal infection and water condensation.

3.3 Results

Attachment of *H. longicornis* on a silicone membrane

Nine triple replicated trials involving a total of around 1,500 *H. longicornis* larval ticks were conducted. There were no successful attachments of larvae to the silicone membrane at all, although tick probing behaviour was observed on several occasions (Table 3.2).

Various changes to the apparatus such as the changing of the temperature from 30-35°C, the impregnation of the silicone membrane with animal-hair extract made in accordance to the specifications of Krober and Guerin (2007) and the thinning of the silicone membrane did not induce any larval attachment to the silicone membrane.

Table 3.2: Table summarising the results obtained from the multiple variations of Krober and Guerin's (2007) protocol that were attempted in this investigation of artificial feeding.

	Standard silicone trial: silicone membrane with cattle hair attached, scented with hair extract, placed at 30°C		
Trial:	Variation from the standard silicone trial described above:	Ticks used per trial:	Results:
1	Temperature raised to 37°C	50	All ticks dead.
2	Temperature raised to 35°C	50	All ticks dead.
3	Temperature dropped to 25°C	50	Probing behaviour seen. No attachments.
4	Membrane scented with lard	50	Probing behaviour seen. No attachments.
5	Membrane scented with sweat (human).	50	No interest in membrane. No attachments.
6	More hexane used in setting mixture (thinner membrane)	50	No interest in membrane. No attachment.
7	Mouse skin used instead of silicone, no cattle hair or hair extract	50	Fungal growth. Mortality of ticks involved. Probing behaviours seen. No attachments.
8	Rabbit skin used instead of silicone, no cattle hair or hair extract	50	Fungal growth. Mortality of ticks involved. Probing behaviour seen. No attachments.
9	Apparatus suspended over hot water bath with blood stirred by magnetic paddle	50	Probing behaviour observed. No attachments. Tick mortality via condensation inside the plastic tube.

3.4 Discussion

Efforts to implement Krober and Guerin's (2007) protocol for the use of silicone membranes in an artificial feeding system were unsuccessful, with no ticks attaching to the membrane at any stage during the investigation. The most likely explanation for this is that the mouthparts of an adult *Ixodes ricinus* tick are significantly longer compared to that of the larval *H. longicornis* (Figure 3.4). The protocol for the feeding of ticks on a silicone

membrane developed by Krober and Guerin (2007) was optimised for the adults of the *I. ricinus* species. It is very likely then that the silicone membrane produced through the implementation of the protocol presented in their 2007 study was too thick for the larvae of *H. longicornis* to penetrate with their much shorter mouthparts. Probing behaviour from the larval ticks was seen in several trials suggesting that should the membrane have been of an appropriate thickness, feeding behaviour would have resulted from the tick's exposure to the apparatus.

The protocol for the development of a silicone membrane used in this investigation was not able to produce a membrane that would result in feeding; however, newer technologies may hold the key to unlocking better tick-feeding membranes.

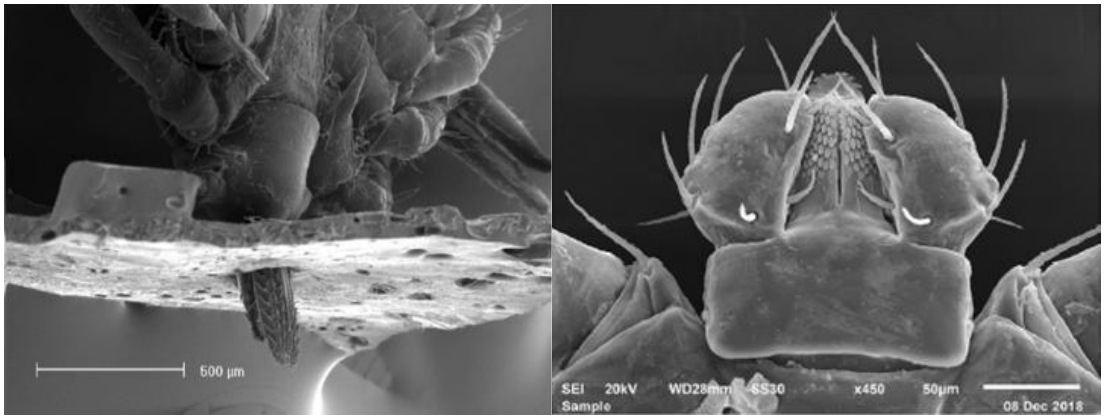


Figure 3.4: a) This is an electron micrograph of the mouthparts of the tick *I. ricinus* interacting with the silicone membrane that was developed Krober and Guerin (2007a). The scale bar shown at the bottom left of the image represents 500 µm. Reprinted with permission from Elsevier B.V. b) An excerpt from Egizi et al. (2018) (CC-BY). This is the electron micrograph of the mouth parts belonging to the tick *H. longicornis* while in the larval stage. The scale bar at the bottom right of the image represents 50 µm.

In recent years, with a new emphasis on moving away from animal testing, the field of biofilms and the creation of skin substitutes have advanced greatly (Kim et al., 2019). While current technologies, especially those involving hydrogel design, have yet to reach the capacity of reproducing anatomically accurate skin (Shao et al., 2020), progress within the field is rapid (Shao et al., 2020). With rudimentary skin-substitutes already available (Kim et al., 2019), it is not impossible to imagine the technology repurposed for the development of tick-rearing artificial membranes. The use of a skin substitute that consists of real cells and vascularisation (Kim et al., 2019) would bypass many of the limitations faced by current silicone membrane protocols such as the need for tick-stimulants, membrane thickness, and ease of production of the membrane. With the use of these new 3D bio-printing technologies, tick-feeding membranes could be made more efficiently and tailored to the tick species being fed.

The presence and growth of fungus on selected variations of the Krober and Guerin protocol, specifically those involving animal skin, has been a major contributor to the failure of this investigation. While other studies (Tajeri et al., 2016; Romano et al., 2018) have reported success in their use of animal-skin membranes in the *in vitro* feeding of ticks, their success was not replicated here. In particular, the protocol of Tajeri et al. (2016) was

followed in the preparation of the mouse skin used here, but fungal growth still occurred and subsequently resulted in the mortality of all larval ticks involved in that trial. From these results, it can be determined that the use of a sterile membrane (e.g. silicone) that has been treated with tick-stimuli would be better for the raising of ticks in the laboratory compared to animal-skin membranes that need additional treatment; after all, certain variants of the silicone membrane were able to induce probing behaviour from the larval ticks. Should the advent of 3D bio-printing make artificial cell membranes readily available for use in the feeding of ticks, there is no doubt that it would also provide a more efficient alternative to animal-skin membranes.

The development of artificial feeding systems opens up many avenues of tick research that otherwise might be considered unfeasible. This is especially evident in the research of tick vaccines (Krober and Guerin, 2007), tick-pathogen interactions and tick physiology. Therefore, future research is warranted in order to develop an artificial feeding system that is able to accommodate the physiological differences between tick species.

Chapter 4: Development and implementation of ear-bags on cattle as a novel tick-rearing method

4.1 Introduction to methodology

The ticks used in this study were not raised on laboratory animals. Rearing the larval ticks on mice and rabbits was not possible as neither the rats nor the rabbits carry *T. orientalis* Ikeda, and therefore could not infect the larvae with the parasite. Instead the ticks were raised through the artificial infestation of *Theileria*-infected calves. Of particular inspiration was the paper published by Ghosh and Azhahianambi in 2007 wherein they detailed their development for an attachable ear-bag for use in the tick infestation of rabbits and cattle (Figure 4.1). They detailed two makes of the ear-bags and its trials; the first trial was for the use of the ear-bag to feed larval ticks of the species *Hyalomma anatolicum anatolicum* on rabbits in the laboratory. The second trial focused on their usage of larger ear-bags to artificially infest cattle with the adult tick of the species *H. anatolicum anatolicum*. Both trials gave positive results with the majority of their ticks engorging and developing to the next stage of their life cycle.

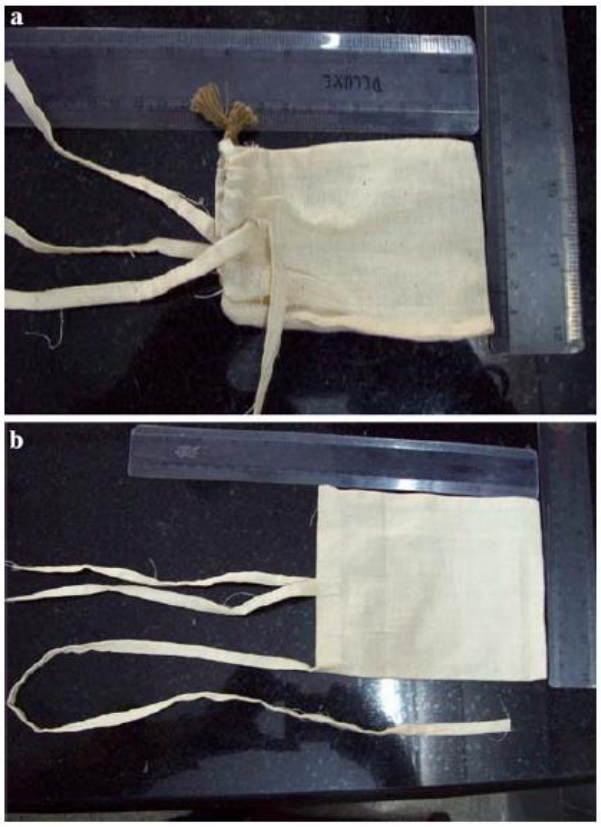


Figure 4.1: An excerpt from the paper by Ghosh and Azhahianambi (2007). This image shows the design of the ear-bags that were tied to the heads of the animals in their experiment. The bags housed the ticks, which attached once the ear-bag was secured to the face of the animals used in the trial. Reproduced with permission from Springer Science.

This experiment would similarly use ear-bags to achieve the *en masse* rearing of the cattle tick *H. longicornis*. The ear-bags would allow for the simultaneous rearing and infection of the naïve larval ticks. Additionally, this experiment seeks to improve on the design of the ear-bags used by Ghosh and Azhahianambi (2007). Their ear-bags were not sealed, rather they were held in place by string that was tied across the animal's face. The attachment of the ear-bags, though firm, still allows for the potential entry of foreign ticks into the selected population of laboratory raised ticks.

This chapter covers the development of a novel tick-rearing method through the use of glue-on ear-bags. There are two main parts to the chapter: the first half of the chapter covers the pilot study, which investigates the welfare of the cattle while they are wearing the ear bags. The second half of this chapter covers the field trial of the ear-bags, where the ear-bags are used to successfully feed a large population of larval ticks to repletion.

4.2 Pilot study

4.2.1 Introduction and materials

To facilitate the natural infection of larval ticks (*H. longicornis*) with the protozoan parasite *T. orientalis* Ikeda this study developed a protocol for the attachment of tick-infested cotton bags to the ears of *Theileria*-positive calves. However, concerns about the welfare of calves involved in the proposed study were raised by Massey University Animal Ethics Committee (MUAEC). In particular the committee expressed concern about the potential damage and discomfort that could be caused by the Kamar® adhesive used to attach the bags to the calf ears, and the potential discomfort stemming from the ear-bags themselves. To satisfy these legitimate concerns, a pilot study of the ear-bags was conducted on a small number of calves belonging to Mrs. B. Adams, an equine research technician at Massey University. With her permission, three calves were attached with the ear-bags using Kamar® adhesive. No ticks were used in the pilot study since the study was conducted solely to assess the impact of the adhesive and the ear-bags on animal welfare.

Study site:

The pilot study was conducted on a lifestyle property in Rongotea 40°16'28.2"S 175°25'49.7"E

Ear-bags and glue:

Ear-bags were constructed using cotton sheets sourced from Spotlight (New Zealand). The cotton sheets on average were 15 cm x 8 cm. The sheets were constructed into the form of a bag using hot glue (Figure 4.2).

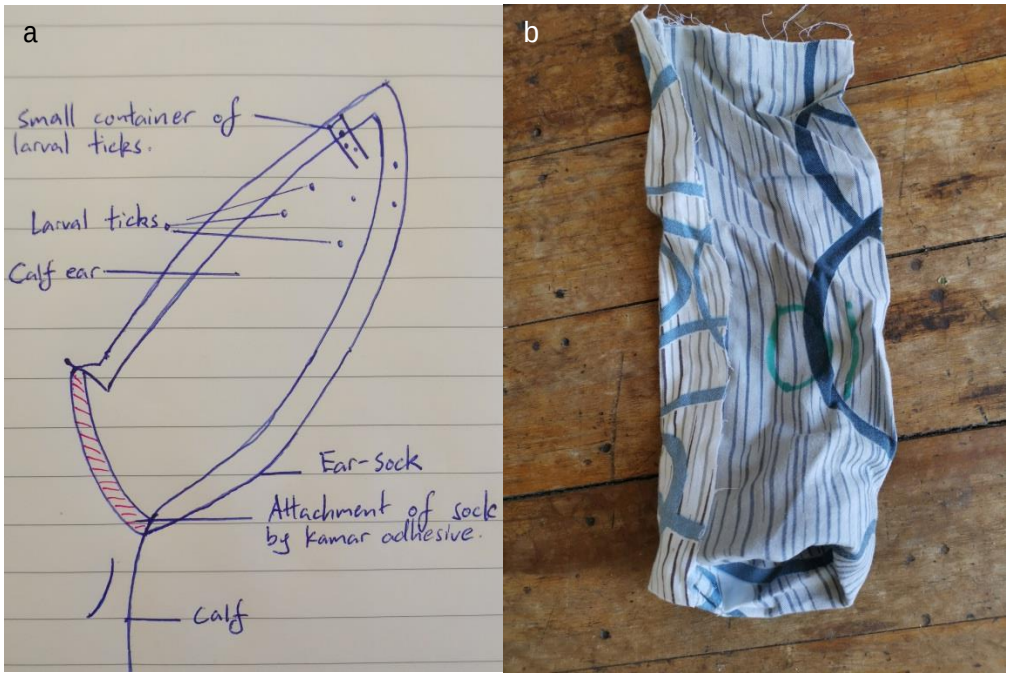


Figure 4.2. a) Conceptual drawing of the apparatus b) Photograph taken of a finished product. Please refer to Figure 4.3 for how it looks on the calves themselves.

The adhesive used to attach the ear-bags to the calf ears was Kamar® adhesive (Kamar Products, Inc. IN, USA), sourced through LIC, New Zealand.

Calves:

On 11 December 2018, three calves were selected from a group of five calves on a small farm in Rongotea. The calves were gently restrained for ear-bag attachment by securing their heads using a halter at the end of a vet race; the ear-bags were then attached using Kamar® adhesive (Figure 4.3).

Figure 4.3: One of the calves involved in the pilot study for the ear-bag protocol, with ear-bag in place. In the picture, the calf has been gently restrained using a head halter so that the ear-bag could be applied.

Once the ear-bags were attached, the calves were allowed to mingle back with their group. The group of calves were then allowed to roam freely in their paddock and provided with water and food *ad libitum*. After a 12 hour acclimation period, the three calves were monitored for four hours a day (10 am to 2 pm) over the course of two days for behaviours that might indicate discomfort resulting from the attached ear-bags.

Four behaviours indicating possible discomfort and four behaviours indicating comfort were decided upon. The four behaviours indicating discomfort were: ear twitching, pacing, head shaking, and scratching. The four behaviours indicating comfort/acceptance of the ear-bags were: eating and drinking, lying down, grooming, and playing. Observation began an hour after arrival on the farm to facilitate the acclimatisation of the calves to the observer.

Observations of the calves' behaviours were then recorded every 20 minutes, with the most commonly displayed behaviour that the calves had been exhibiting over that 20 minute period being noted in a data collection table (Table 4.1). The calves were yarded again on 14 December, however only one ear-bag could be easily removed at that time; the other two ear-bags were left on until 17 December when they were removed easily.

Table 4.1: Table used for data collection, showing the behaviours indicating discomfort and comfort against the time for which they were observed. Each hour of observation is divided into three 20 minute slots. Each slot represents an observation time; at the end of each 20 minutes the most commonly displayed behaviour over the last 20 minutes for each calf is noted down on the data collection table. The time of the observation is displayed on the data collection sheet e.g.: 10am (00) is 10:00 am; 10am (20) is 10:20 am; 10am (40) is 10:40 am and so on. Each behaviour count will be marked down as an 'I'. For example, should the calves have been seen eating, resting and grooming at 11:20 am then an 'I' would be marked in the respective rows under the column 11am (20).

	10am			11am			12pm			1pm			2pm/ final observation
Time:	00	20	40	00	20	40	00	20	40	00	20	40	00
Scratching													
Ear-twitching													
Pacing													
Head shaking													
Eating													
Resting													
Drinking													
Grooming													

4.2.2 Results

The first set of data were collected prior to the ear-bags being attached to provide baseline data on the calves' normal behaviour, for comparison with the data collected after the ear-bags were attached (Table 4.2).

Behaviour counts:

Table 4.2: The behaviour counts taken on the first day of sampling: before the ear-bags were attached to the calves.

Behaviour count on Day 1. No ear-bags have been attached.													
	10am			11am			12pm			1pm			2pm/ final observation
Time:	00	20	40	00	20	40	00	20	40	00	20	40	00
Scratching													
Ear-twitching													
Pacing											II		
Head shaking													
Eating	I	I		I	I		I				I		
Resting	II	II	III	II	II	III		III	III	III		III	III
Drinking							I						
Grooming							I						

The behaviour count shown above in Table 4.2 acts as the control group. There were a total of 39 behaviours recorded over the course of 4 hours. Each behaviour count is marked down as an 'I'. Of the 39 behaviour counts, there were only two counts of non-benign behaviour. The ear-bags were attached to the calves after the last observations were made at 2 pm. The next observation period occurred one day after the ear-bags were attached.

Table 4.3 includes a total of 39 behaviour counts taken over the course of 4 hours, at which point the ear-bags had attached for 20-24 hours. Over the course of the observed time, the calves did not exhibit any behaviour that indicated possible discomfort.

Table 4.3: The behaviour counts taken 20 hours after the application of the ear-bags onto the calves.

Behaviour count on day 2. Ear-bags have been attached for 20 hours.													
	10 am			11am			12pm			1pm			2pm/ final observation
Time:	00	20	40	00	20	40	00	20	40	00	20	40	00
Scratching													
Ear-twitching													
Pacing													
Head shaking													
Eating	II	I	I				I			I	III	I	
Resting		II	II	I	III	III	II	II	I	II		II	III
Drinking	I							I	II				
Grooming				II									

The behavioural data collected in Table 4.2 and 4.3 have been summarised in the Table 4.4 below.

During the control period, the calves spent 95% of their time exhibiting behaviours considered benign, with only two counts of pacing happening during the day (Tables 4.2 and 4.4). When the ear-bags were in place, the calves exhibited behaviours considered benign 100% of the observed time (Tables 4.3 and 4.4).

When the ear-bags were removed from the calves four days later, no injuries such as inflammation or the breaking of skin was seen on the ears. The Kamar® adhesive did not damage the skin where it was applied. Their ears were sprayed with Tetravet anti-septic spray (Tetravet™, New Zealand) in accordance with MUAEC regulations.

Table 4.4: Summary of the behavioural counts collected when ear-bags had not been attached, and when the ear-bags had been attached for 20-24 hours

Primary behaviour displayed per 20 minute interval, over 4 hours:	Total counts of behaviour:	
	Day1 (No ear-bags)	Day2 (Ear-bags attached)
Scratching	0	0
Ear-twitching	0	0
Pacing	2	0
Head-shaking	0	0
Eating	6	10
Resting	29	23
Drinking	1	4
Grooming	1	2

4.2.3 Discussion

This pilot trial provided compelling evidence for MUAEC that the welfare of the calves which had ear-bags attached was not compromised. Although the pilot study operated with an extremely limited sample size, MUAEC deemed that the data collected from the behaviour counts gave good confidence that the Kamar® adhesive was safe to use on the calves and that the ear-bags would not compromise the welfare of the calves. With their permission, the proposed protocol (Protocol 18/93) was allowed to proceed to field trials.

4.3: Field study for the use of ear-bags in the feeding of ticks

4.3.1 Materials

Study site:

The field experiment was conducted on a farm on Cemetery Road, Sanson (40°12'07.8"S 175°26'59.7"E), with approval from Massey University Animal Ethics Committee, Protocol 18/93.

Ticks:

Larval ticks were obtained by sourcing gravid female ticks from Limestone Downs located 13 km south of Port Waikato (latitude 37°49′S, longitude 174°77′E). The gravid female ticks were collected, with the farmer's permission, off the cattle as they came in for milking. The gravid ticks were then transported by car in large air-tight boxes inside a chiller bin filled with ice to the Parasitology Laboratory in the School of Veterinary Science at Massey University (Palmerston North, New Zealand). The transportation process took approximately 6 hours and any ticks thought to be deceased upon arrival were removed from the trial. The gravid female ticks were housed in small mesh top containers, with four females per container to avoid overcrowding. Temperature and humidity regulation were achieved through the use of an airtight container lined with wet paper towels and an incubator (Contherm Polar 1000C, Contherm Scientific Ltd, New Zealand) set at 24°C. The small mesh top containers which held the gravid females were placed inside the air-tight container, which itself was held in the incubator, helping to achieve the conditions of 24°C and 95% humidity (Figure 4.4).

Once egg-laying had finished (this process taking around a week), the dead or dying female ticks were removed from the mesh top containers and disposed of to prevent fungal growth on the eggs. Eggs were held at the same temperature until they had hatched into larvae, whereupon the temperature setting of the incubator was lowered to 20°C.

Larval ticks were transported to and from the farm in Cemetery Road in an airtight container lined with wet paper towels. Estimated travel time was around 40 minutes. Any ticks thought to be deceased were removed from the trial. Fed larval ticks were held in larger mesh top containers inside a new air-tight container. The aim of the air-tight container, and keeping this air-tight container in the incubator was to maintain the conditions at 20°C at 85% humidity. The humidity was decreased (through the use of less wet paper towels) as fungal growth had become a problem for the fed larval ticks in the previous container despite regular cleaning.

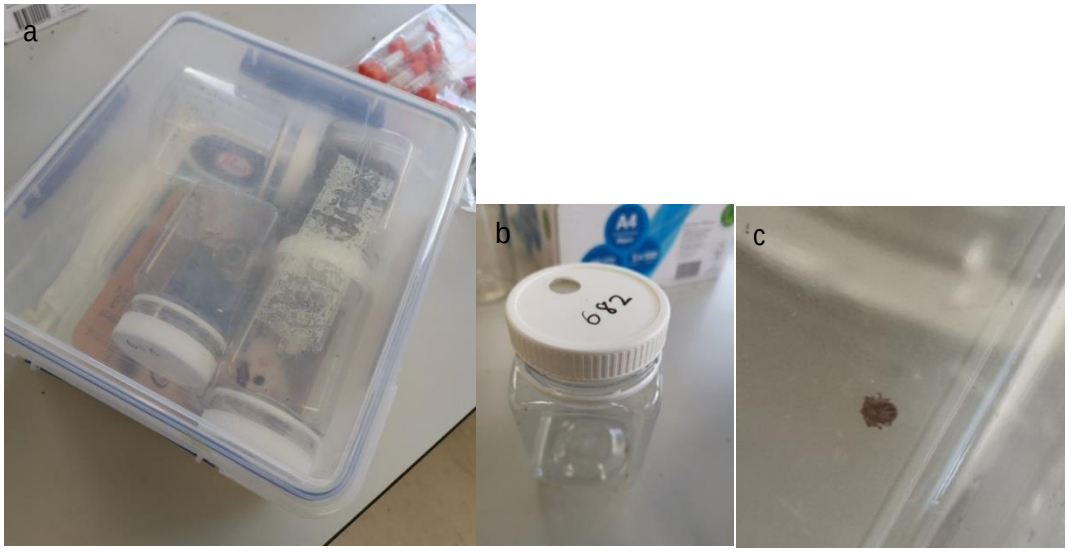


Figure 4.4: a) The airtight container containing the smaller mesh top containers; b) the mesh top container; c) an *H. longicornis* nymph.

Calves:

Twelve calves were selected randomly from a group of 6-month old calves and 10 ml blood samples were collected into EDTA vacutainers by a Massey University veterinarian (Kevin Lawrence). A pooled PCR test for *Theileria orientalis* Ikeda was carried out on the blood samples through IDEXX Laboratories (NZ) and confirmed that infection was present in the mob. While it could not be certain that all calves were infected prior to conducting the experiment, the history and time of year strongly suggested that they would be. This was confirmed retrospectively when individual PCR testing was carried out on stored samples from these calves and all tested positive. The blood samples were stored at -20 °C after the pooled test until molecular testing.

After the ear-bags were attached to the 12 selected calves, they were allowed to mingle back with their original group. The 12 selected calves were allowed to roam freely in their paddock with food and water provided *ad libitum* for the duration of the study.

Ear-bags and glue:

The ear-bags used in this trial were made from size 10 cotton 'Business Socks' sourced from Kmart (New Zealand) (Figure 4.5). Cotton socks were used instead of the handmade cotton bags with permission from MUAEC; the cotton socks were considered to be less susceptible to breaking at the seams and less likely to possess hidden holes from which larval ticks might escape.

Figure 4.5: The cotton socks purchased from Kmart that were used in lieu of the handmade cotton ear-bags.

The adhesive used to attach the ear-bags to the calf ears was the same that was used in the trial: Kamar® adhesive (Kamar Products, Inc. IN, USA), sourced through LIC, New Zealand.

4.3.2 Methodology:

The twelve calves were restrained with the use of a head bail on the cattle crush, positioned on the end of a vet race. Further control was obtained by using a halter to tie the calf's head around to the right, thus presenting the left ear for sock attachment. While immobilized, the surface of the ear was lightly clipped to reduce the hair length. Kamar® adhesive was then applied in a ring around the base of the calf's ear and the sock, with ticks inside, was attached using Kamar® adhesive. The larval ticks (around 1,000 per sock) were kept inside the sock by trapping them in a small lidded vial and attaching it to the inside of the sock using cyanoacrylate (Gorilla Glue Company™, OH, United States). Once the sock was attached to the ear of the calf, the lid of the vial was manually removed by manipulating it through the sock, allowing the larval ticks to exit onto the calf's ear (Figure 14a).

After 5 days, the bags were removed from the calves by gently peeling off the sock while the calves were immobilized in the cattle crush. Removal of the socks did not damage or cause bleeding of the ears for any of the calves involved. Each sock was then placed in an air-tight container, which in turn was kept in an ice-filled cooler until the samples reached the laboratory (Figure 4.6b).

Inside the laboratory, the engorged larval ticks were collected from their respective bags and placed in an incubator set to 20°C and 90% humidity.

Figure 4.6: a) An artificially tick-infested calf wearing its tick-infested ear-bag. The ear-bag is fixed to the calf at the base of its ear using Kamar® adhesive. b) The small containers that housed the fed larvae as they were transported back to the Parasitology Laboratory, Massey University, Palmerston North.

Feeding of *H. longicornis* larvae on calves:

Larvae were fed on calves as discussed in section 4.3 on 4 April 2019. Twenty-four hours were given for the larvae to attach to the calf once the infested bags were attached to the calf's ears. *H. longicornis* larvae feed to repletion after 6-7 days (Heath, 2010). Of the 12 calves infested with *H. longicornis*, four calves had their bags removed after 5 days while the rest had their bags removed after seven days.

Rearing of larvae until moulting:

Engorged larvae were sorted from their bags according to the calf they had fed on. They were placed in separate containers and kept at a constant 24°C and 85% humidity until moulting occurred. Moulting was confirmed to have occurred six weeks after the engorged larvae had been collected from the calves, with the newly moulted nymphs gathering around the lids of the containers.

4.3.3 Results:

The first removal yielded around 400 engorged larvae in total from the four bags removed; with each sock originally containing an estimated 500 to 700 larvae, the yield of the first harvest of ticks was around 20%.

During the removal of bags on day 5, many ticks were seen to still be attached to the calves. The rest of the infested ear-bags were removed seven days after the trial had begun.

However, due to a communication error with the farm staff, the calves had been treated with pesticide and the final collection yielded no live ticks.

4.3.4 Discussion:

Despite the unfortunate loss of most of the ticks submitted for rearing, the four ear-bags that were removed before the loss of the other ear-bags to pesticide yielded a large amount of engorged larval ticks. Because of this, it is within reason to deem that the novel method of tick-rearing via ear-bag is a success.

It is possible for the method to be further refined through the use of different materials in the construction of the ear-bag. While the majority of tick mortality in this study can be attributed to the application of pesticide on the calves, a small percentage of ticks appeared to have been drowned by the water-logged cotton socks after rainfall occurred between the first and second collection. It is possible that by making the ear-bag out of water resistant/ non-absorbent material such as polyester the mortality of many of the ticks could have been prevented. A further moderation to the ear-bags that could potential prevent tick mortality is a second lining of plastic outside the cloth, such a lining can in theory protect the ticks inside the ear-bags not only from the rain but also desiccation in the event of dry, hot weather. Weather conditions allowing for desiccation of the ticks did not occur during the trial period of the ear-bags and thus it is unknown how much protection the cotton bags would have granted the ticks inside.

Future applications:

The issue of mass rearing ticks has long been the topic of scientific studies (Romano et al. 2018). While the use of laboratory animals has been and still is the main avenue of rearing ticks for research, limitations such as animal welfare and the specificity of tick-vectored parasites (as was encountered in this study) meant that in vitro feeding of ticks using an artificial membrane had the potential to be a more viable option for future research. However, despite successful implementations of artificial membranes in the feeding of ticks in the 20th century (Waladde et al., 1990) and later on with Krober and Guerin (2007), and Trentelmann et al. (2017), the ability to raise large populations of ticks using in vitro feeding methods is still absent from most laboratories. Romano et al. (2018) in his review commented that the use of in vitro feeding methods for tick-raising is hindered in its application due to either a high cost in materials or the difficulty in successfully operating an apparatus once constructed.

It is possible that the glue-on ear-bags detailed in this study can find relevance as a more widely used way of mass-rearing ticks due to both its ease of set up and low cost, as well as its compliance with animal welfare.

Chapter 5: Quantitative PCR analysis of *T. orientalis* Ikeda population within the hibernating tick *H. longicornis*.

5.1 Introduction:

In 2016, Pulford et al. developed a protocol for the detection of *T. orientalis* Ikeda in samples of cattle blood using TaqMan® qPCR as a means of fast and sensitive diagnosis. The protocol was then used by Gias et al. (2016) as well as Lawrence et al. (2018a) in the detection of *T. orientalis* Ikeda to great success. Because of the common usage of Pulford et al.'s protocol as well as the lack of any alternatives, this study also utilises the qPCR protocol set out by Pulford et al (2016). Specifically this includes the base pair sequences used for the probes and the forwards and reverse primers.

Through the use of qPCR analysis, the amount of *T. orientalis* Ikeda DNA in each individual sample, and thus the amount of individual *T. orientalis* Ikeda organisms per tick, can be accurately calculated. By plotting the results of each qPCR performance, the average population of *T. orientalis* Ikeda inside an individual *H. longicornis* tick.

This chapter covers the qPCR analysis of the ticks gathered from the ear-bags described in chapter 4.

5.2 Methodology and Materials:

Ticks:

Engorged larval ticks (*H. longicornis*) obtained from the ear-bags were kept in calf-specific mesh-top bottles which were placed inside a larger air-tight container lined with wet paper towels. The entire set up was incubated at 20°C in constant darkness. The constant darkness and the relatively low temperature of the incubator were employed to ensure that the ticks were induced into torpor (Heath, 2016). This was to make sure that the ticks stayed alive for as long as possible so that any changes in the population of *T. orientalis* Ikeda within them would be properly expressed.

A sample of 10 engorged larval ticks were frozen before they could molt into nymphs and kept as sample A. This would represent the baseline concentration of *T. orientalis* Ikeda present inside the tick after feeding to repletion. Apart from the first sample of ticks, the population of engorged larvae *H. longicornis* was kept at high humidity, constant darkness and 20°C for six weeks before sampling began so that all ticks would have time to molt and go into induced torpor.

DNA Extraction:

Theileria DNA was extracted from ten randomly selected infected nymphs using the methodology outlined by Guerrero et al. (2001). The ticks were frozen in liquid nitrogen before being crushed inside a micro-tube using a micro-pestle. A volume of 100 µl of

extraction solution (100 nm Tris HCL, 500nm HCL) was then added to the crushed ticks and the whole mixture heated to 100°C for 3 minutes in a heating block. The solution was then diluted 1:10 with nuclease free water. This was regarded as a single sample.

The process of DNA extraction was repeated on the population every three weeks until seven samples had been collected. The first sample, labelled A1, was collected on the day the ticks were removed from the calves. The next sample D1 was collected two months after sample A1 was collected to ensure that all fed larvae had moulted into nymphs. Samples E1 to I1 were collected in chronological order, with three weeks between each sample's collections and samples D1 and E1.

Quantitative PCR:

Quantitative PCR (qPCR) on the extracted *Theileria* DNA was performed using the primers and probe previously described for *T. orientalis* by Pulford et al. (2016), with all primers and probes sourced from integrated DNA Technologies (Iowa, USA).

The qPCR was performed using the following conditions: 1X PerfeCTa qPCR ToughMix (Quantabio, Massachusetts, USA), 500 nM of each primer, 400 nM of probe (the probes used are described in Table 5.1, and 5 µl of extracted DNA to make a final volume of 20 µl with nuclease free water.

Table 5.1: Primer and probe sequences for the analysis of *T. orientalis* Ikeda via TaqMan® qPCR (Pulford et al. 2016).

Name	Primer/Probe	Sequence (5' – 3')
NZIke-1F	Forward primer	AGTTAACGCCACCGCAGCCG
NZIke-1R	Reverse primer	ACGCGGTATCCCTCTTCGGCA
NZIke-probe	Probe	/56-FAM/CGCCTCAAACGCCAACGACG/3BHQ_1/

Analyses of the DNA samples A1 to I1 (representing the seven samples taken in chronological order) were performed in triplicate to mitigate the effect of possible outliers.

Thermal cycling was performed in a Mic qPCR thermalcycler (BioMolecular Systems, Upper Coomera, Queensland, Australia). Thermal cycling conditions were as follows; 95°C for 2 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds, with fluorescence capture on the 60°C step. Standard curves were produced utilizing a serial dilution of a plasmid from a previously produced clone of a section of the *T. orientalis* Ikeda MPSP (Major Piroplasm Surface Protein) gene (provided by K. Gedye), and ranged from 100

pg/µL to 10 fg/µL of the plasmid. The results of the qPCR assays were analysed using the Mic Software (BioMolecular Systems, Upper Coomera, Queensland, Australia). The standards were also the positive controls, with the negative control being PCR grade water.

Organism count for *Theileria* DNA extract from tick nymphs:

To obtain data regarding the exact number of *Theileria* organisms per µL, each qPCR performance was run with a serial ten-fold dilution of five Ikeda plasmid DNA standards: 100 pg/µL to 100 fg/µL. The mic PCR machine used these standards to quantify the concentration of *Theileria* DNA in each run - in this case, the gene-copies of Ikeda MPSP. As the gene sequence of MPSP is a single copy gene, the concentrations calculated by the mic PCR machine give a direct indication of the number of *T. orientalis* organisms per µL.

The specific number of *Theileria* organisms can be calculated by multiplying the molarity of the *Theileria* DNA in the qPCR sample with Avogadro's number (Lawrence, 2018a). These calculations were done using the website https://cels.uri.edu/gsc/cndna.html, created by Andrew Staroscik. The website assumes that average weight of a DNA base pair to be 650 Daltons, which in turn means that one mole of a base pair is 650 g. Thus, by using the length of the base pair being amplified and the amount of DNA detected in the qPCR run, the exact amount of organism can be estimated.

Of the 100 µL of the original extraction solution (10 dead ticks crushed in 100 µl of tick buffer solution made up of Tris HCL and KCL), 10µL was taken and diluted in 100 µl of nuclease-free water to give a 1:10 dilution (110 µL in total). Of this diluted solution, 5 µL was then used in the qPCR process.

To obtain the number of *T. orientalis* Ikeda organisms per tick, the number of organisms calculated using the website was multiplied by the dilution factor of 440 to give the total number of organisms present (440x where x is the number of organisms) in the 110 µL solution *i.e.*: the solution representing the DNA extracted from one tick.

5.3 Results:

All samples were amplified, with Cq values (Cycle quantification values) between 24.35 and 31.02- relating to *Theileria* concentration 0.00041 ng/µL to 0.052 ng/µL. Absolute quantification of *T. orientalis Ikeda* load in ticks is shown in Table 5.2.

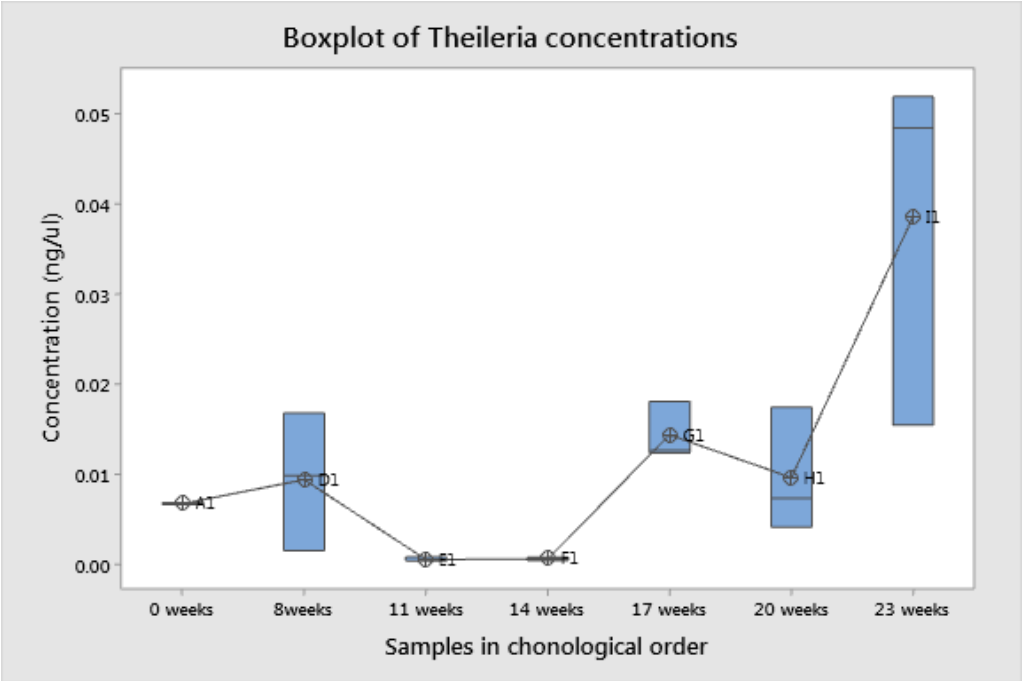
Absolute quantification:

Table 5.2: Summary of the results of the qPCR reactions. Organisms (*T. orientalis* Ikeda) / µL represent the concentration of *T. orientalis* Ikeda found in the 20µL qPCR reaction well. Organisms/ tick were obtained through multiplying Organisms/µL with the dilution factor of 440 to get 440x (where x is the number of organisms), where 'organisms' refers exclusively to *T. orientalis* Ikeda.

qPCR sample:	Concentration ng/ µL: run#1	Run#2	Run#3	Average concentration: ng/µL	Average organisms/ µL	Average organisms /Tick
A1	0.0069	0.0067	0.0068	0.0068	$7.03*10^{7}$	$3.21*10^{10}$
D1	0.0099	0.017	0.0016	0.0094	$9.82*10^{7}$	$4.32*10^{10}$
E1	0.00041	0.00087	0.00041	0.00056	$5.85*10^{6}$	$2.57*10^{9}$
F1	0.00084	0.00048	0.00065	0.00066	$6.85*10^{6}$	$3.01*10^{9}$
G1	0.013	0.012	0.018	0.014	$1.50*10^{8}$	$6.60*10^{10}$
H1	0.0074	0.0042	0.017	0.0097	$1.01*10^{8}$	$4.44*10^{10}$
I1	0.048	0.052	0.015	0.039	$4.02*10^{8}$	$1.77*10^{11}$

Sample I1 tested for the highest number of *T. orientalis* Ikeda organisms per tick out of all the samples tested. The lowest number of organisms per tick was found in sample E1 which yielded an average of $2.57*10^{9}$/tick organisms per tick tested. The number of organisms per tick rose from $3.21*10^{10}$/tick in the initial sample AI to $4.32*10^{10}$/tick in sample D1. The number of *T. orientalis* Ikeda organisms then drops to a low of $2.57*10^{9}$/tick in sample E1. The average number of organisms per tick then rises to $6.60*10^{10}$ in sample F1. A drop in the average number of organisms per tick occurs in sample H1- a decrease from $6.60*10^{10}$ to $4.44*10^{10}$. However, the average number of organisms rises again in the last sample where sample I1 records the highest average number of *T. orientalis* Ikeda organisms per tick at $1.77*10^{11}$ per tick.

Theileria concentrations over time:



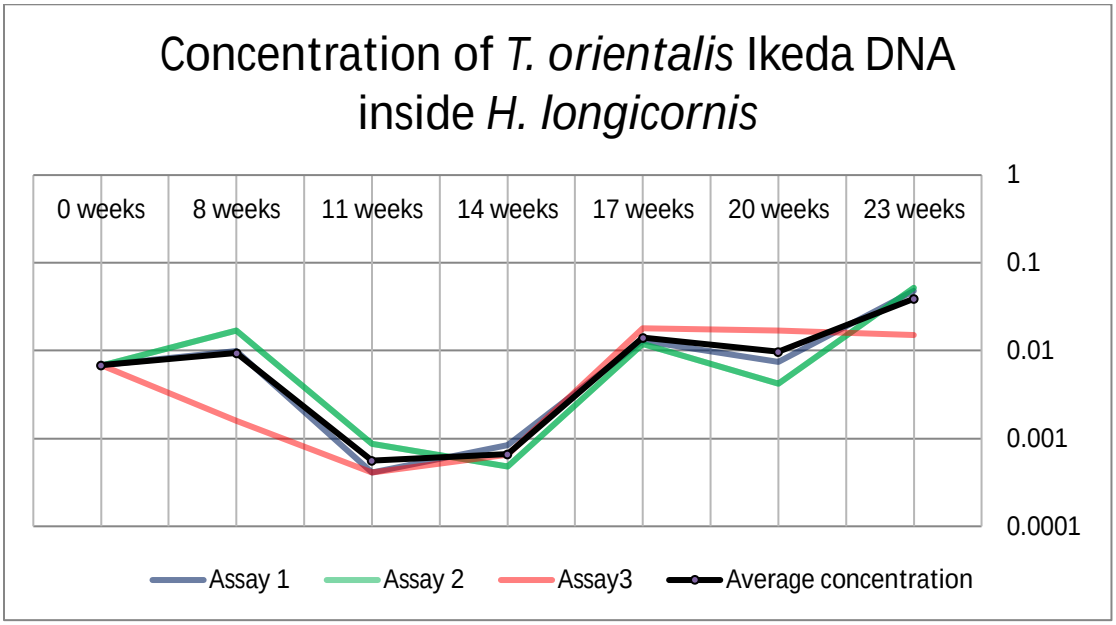


Figure 5.1: a) Time progression of the mean concentration (with error bars) of *T. orientalis* Ikeda DNA per sample over time, as evaluated by qPCR. The samples in the graph are labelled A1, D1, E1, F1, G1, H1, and I1; with each sample consisting of 5µL of DNA extracted from 10 H. longicornis ticks. The dots on the box plot represent the average concentration of each sample, with n=3. The horizontal line within each box represents the median concentration of that respective sample. b) Line graph of the concentrations of *T. orientalis* Ikeda. The concentrations have been plotted against a $\log^{10}$ y-axis while the x-axis is the passage of time. As each sample was run in triplicate, each sample yielded three assays. Each assay is represented by a line on the line graph with the mean of the assays being represented in black.

The highest average concentration of *T. orientalis* Ikeda DNA was I1, with a concentration of 0.039 ng/µL. The lowest average concentration of *Theileria* DNA was found in sample E1, with a value of 0.00056 ng/µL.

The average starting concentration of *T. orientalis* Ikeda DNA inside the ticks are represented both by A1 and D1 (Figure 5.1); where A1 represents the average concentration of *Theileria* DNA inside a *H. longicornis* tick upon engorgement as larvae, and D1 represents the average concentration of *T. orientalis* Ikeda DNA inside a tick once it has engorged and moulted into its nymph form. There is a decrease in the concentration of *T. orientalis* Ikeda DNA between A1 and E1 with the mean *T. orientalis* Ikeda concentration falling by 0.0067 ng/μL. The concentration of *T. orientalis* Ikeda DNA inside the ticks rises between the samples F1 and G1, with an increase in average *Theileria* DNA concentration by 0.014 ng/μL. There is a drop in mean *T. orientalis* Ikeda concentration between G1 and H1 of 0.0043 ng/μL. There is a sharp increase in *Theileria* concentration in the ticks between samples H1 and the last sample, I1, with the increase in concentration being 0.029 ng/μL of *T. orientalis* Ikeda DNA. For further information, please refer to the qPCR report attached under appendix A.

5.4 Discussion

The aim of this study was to investigate whether the quantity of the apicomplexan parasite *Theileria orientalis* Ikeda type inside its tick vector *Haemaphysalis longicornis* changed over time, when the host was subjected to an extended period of fasting. The qPCR results indicated that the levels of *T. orientalis* Ikeda type inside the tick fluctuated over time. Although the aetiology is unknown, these results are in broad agreement with Young et al. (1984). In comparison to the non-transforming *Theileria,* the research available on the more pathogenic transforming *Theileria* is comparatively extensive. Assuming that approximations can be made between the physiology of transforming and non-transforming *Theileria*, extrapolations can be made from the available research to give an explanation for the observed results.

5.4.1 Random error due to disproportionately high parasite load

A possible contributor to the fluctuations (Figure 5.1) seen in the *Theileria* population inside the tick is sampling error. A study by Watts and Walker (2000) found that the distribution of parasite load in a population of ticks infected by *Theileria* was not evenly distributed, but aggregated in a few individuals that contained a disproportionately large number of *Theileria* organisms, while the rest had a very small *Theileria* count (Watts and Walker, 2000). Although Watts and Walker (2000) concerned itself only with *Theileria parva*, the phenomenon might also occur with *T. orientalis* Ikeda. The current study attempted to correct for uneven distribution of *Theileria* infection across tick hosts by measuring the average load of ten ticks at each test. Even so, 10 ticks is not a huge number and it is possible that individuals with very high parasite loads could still bias the results. The sporadic inclusion of such super-infected individuals in certain test samples could easily explain the fluctuating levels of *Theileria* DNA detected by the qPCR runs.

In order to remove this bias from further experiments, a method needs to be developed to standardise the infectious dose that each tick acquires. A possible method would be through the use of an artificial feeding system. Using the protocol developed by Krober and Guerin (2007), a silicone membrane based *in vitro* feeding apparatus would allow for the ticks to feed on blood with known parasite load. Furthermore, when the infected ticks feed on the silicone membrane, the saliva of the ticks could potentially be sampled and thus allowing their parasite load to be determined. However, this methodology requires further research as the protocol outlined by Krober and Guerin (2007) was trialled as part of this study without success. Further study is needed to produce a silicone membrane that can facilitate the feeding of larval ticks of the species *H. longicornis*.

5.4.2 Tick-pathogen interactions

In addition to sampling bias, the fluctuating numbers of *Theileria* organisms within the tick could have been caused by the tick-pathogen interactions that occur within the tick. Studies into the relationship between vectors and the pathogens they carry have shown that the interactions between the host organism and the pathogen are seldom mutualistic (Walker, 1990). A study done by Watt and Walker (2000) on the ticks *Rhipicephalus appendiculatus* (Neumann) that carry *Theileria parva* found that those with high parasite loads had lower

fitness compared to those that were free of the parasite. This included higher mortality rates, failure to moult from nymphs into adults, and the production of non-viable eggs (Watt and Walker, 2000). These findings are further reinforced by the study done by Fuente and Kocan (2003) where it was discovered that *Theileria* parasites, when entering their mobile kinete phase, selectively parasitize salivary gland cells that contain the proteins that the ticks use to create their cement cones when feeding. These cells, while containing the proteins needed for cement cone production, also act as food stores for the tick when undergoing extended periods of fasting. Thus, the parasitization of these cells by *Theileria* renders the tick unable to cope with prolonged food shortages (Fuente and Kocan, 2003).

In the tick, the main barrier against invasion from pathogenic microbes is the midgut (Hajdusek et al., 2013). The tick employs a wide array of defence mechanisms in the midgut, not only to supress the immune-bodies of the host animal, but also from invading pathogens such as *Theileria* (Hernandez et al., 2019). However, due to the eight weeks between collection of the first and second sample, it is unlikely that the effect of the midgut immune response can be seen from the data collected. This is because *Theileria* has been recorded to have a maximum survival time of 2-3 days inside the midgut before either being killed by the immune factors present or migrating into the midgut cells and completing sexual recombination (Shaw and Young, 1994). As the samples were comprised solely of moulted nymphs, it is safe to assume that all mobile kinetes in the tick have had sufficient time to metamorphosis into either sporokinetes or sporoblasts (Hajdusek et al., 2013).

Therefore, the fluctuating levels of *Theileria* deal only with the last two stages of the *Theileria's* life cycle inside the tick. According to the literature, a number of tick immune factors might still be at work on the *Theileria*, even as it resides inside the tick's salivary gland cells. Ticks possess a strong humoral immune response - these being a wide variety of antigens, antimicrobial enzymes and the like (Hernandez et al., 2019). While *Theileria* and other vectored parasites are known to take over the redox capabilities of tick cells, preventing them from undergoing apoptosis (Huessler et al., 2001), it is still possible for the immune responses to target the infected cells. The destruction of infected salivary gland cells and its contents by the tick immune system is a likely candidate for the cause of the dips in *Theileria* concentration within the tick that were seen in this study.

5.4.3 Adaptive immunity of the *Theileria* parasite

The interactions between the pathogen *T. orientalis* Ikeda and its tick vector *H. longicornis* provides an explanation not only for decreases in the *Theileria* population, but also for the increases. As an arthropod, its immune system is classified as 'innate' as opposed to 'adaptive' (Hernandez et al., 2019). Therefore, the tick's immune system is extremely limited in its ability to tailor its response to specific invading pathogens in the way mammalian immune responses work. It is suspected that *Theileria* is able to bypass the strong but static immune response of the tick due to its long history of coevolution as pathogen and vector (Cabezas-cruz et al., 2019). Furthermore, studies have shown that protozoan parasites are able to adapt to their host's immune system over time via the changing of their surface proteins. A study done by Matetovici et al. (2019) on Tsetse flies (*Glossina* spp.) found that the trypanosomes (*Trypanosoma* spp.) that use them as a vector adapt over time to the

immune system of the flies. Similar studies on *Theileria parva* suggest that the same adaptations may occur for *Theileria* parasites within the tick (Cabezas-Cruz et al., 2019). Such phenomenon would explain not only the dips in *Theileria* seen from the data, but also why the decrease in *Theileria* parasites within the tick does not continue over the course of the experiment.

5.4.4 Specific temperature-induced sporogony

Although *T. orientalis* Ikeda may adapt to the immune responses of the tick *H. longicornis*, that alone does not fully explain the large increases of *Theileria* population seen in the results. Therefore, it is likely that an additional factor is involved - temperature dependent sporogony. There is only one true multiplication step in the *Theileria's* life cycle within the tick: sporogony (Bishop et al., 2004). Sporogony occurs in response to the development of the tick's salivary glands as it prepares to attach to a mammalian host (Shaw and Young, 1994). However, studies suggest that sporogony can be artificially triggered through the keeping of ticks at a constant high temperature (Young and Leitch, 1981; Bishop et al., 2004). In a study done by Young et al. (1984), it was shown that sporogony could be induced in *Theileria parva* sporoblasts. The ticks were kept at varying temperatures, resulting in sporogony being triggered in ticks being kept at 23°C and 28°C. The ticks in this experiment were kept at a constant 20°C, which may have been enough to trigger sporogony in the *T. orientalis* Ikeda sporoblasts. Coupled with the possibility of *T. orientalis* Ikeda developing resistance to the tick's immune system, the artificial triggering of sporogony through temperature would offer a valid explanation for the fluctuating levels of *T. orientalis* Ikeda observed over time in the tick nymphs.

Chapter 6: General Discussion

6.1 Summary of chapters and synthesis:

The purpose of this thesis was the investigation of the population size of *T. orientalis* Ikeda inside the tick *H. longicornis* and how it changes over the course of twenty-four weeks while the ticks are fasting. The mass-rearing protocol used to facilitate the collection of *Theileria*-positive *H. longicornis* ticks is detailed in chapter four, while the results of the qPCR analysis and subsequent analysis are found in chapter five. Both chapters contain material that may have wide ranging applications or implications for future scientific research in both ticks and *Theileria*, and these are explored in full below.

The results of the qPCR analysis in chapter five, the population of *T. orientalis* Ikeda, have three major potential influences. These are: Sampling bias, tick-pathogen interactions, and temperature-induced sporogony. A study by Watts and Walker (2000) found that the distribution of *T. parva* within a population of *R. appendiculatus* was not uniform, but rather formed spikes of highly parasitized individual ticks while other ticks had on average a low *T. parva* count. Although neither the tick species nor the species of *Theileria* are the shared between the study done by Watts and Walker (2000) and this thesis, their results may be extrapolated to cover the subjects of this study as it concerns the relationship between a vectoring tick and the *Theileria* that lives inside.

Tick-pathogen interactions present a much more complex explanation to the results of the qPCR analysis as studies have shown that these interactions can both cause increase and decrease in the resident population of *Theileria* inside its tick host. The study done by Hernandez et al (2019) highlights the fact that ticks possess an immune reaction to invading pathogens- including *Theileria*. Such immune responses may be a factor in the decreases in population of *T. orientalis* Ikeda as shown in the results of chapter five. Conversely, studies done by both Huessler et al. (2001) and Cabezas-cruz et al. (2019) highlight the ability of tick-vectored protozoans (such as *Anaplasma phagocytophilum* and multiple species of *Theileria*) to manipulate the transcriptional activity of the tick cells they infect to prevent detection by the immune system.

Temperature induced sporogony is a phenomenon observed by Young et al. (1984) in *T. parva* populations within the tick species *R. appendiculatus*. Normally, sporogony of the Theileria within a tick vector is triggered only through the activity of the tick's salivary glands; however, Young et al. (1984) successfully induced sporogony of *T. parva* through the incubation of the tick vector at specific temperatures. Once again, extrapolation of this observation is possible due to the similar relationship between *T. orientalis* Ikeda and *H. longicornis,* and *R. appendiculatus* and *T. parva*.

These three potential factors all present possible avenues for future research. The plausibility of the extrapolation of data and observations from other studies can be verified through the use of qPCR assays. In the case of sampling bias, qPCR assays should be performed on individual ticks in a population to verify the existence of individuals with

disproportionately high *Theileria* populations- as was observed by Watts and Walker with *R. appendiculatus*.

In the case of tick-pathogen interactions, to verify the possibility of the tick's immune response as well as the pathogen's subsequent adaptation, qPCR runs must be performed on fasting *Theileria*-infected ticks to quantify the expression of the tick's immune-regulating genes over time. Further qPCR runs should be performed on the expression of various surface proteins of the *Theileria* parasite to determine if the parasite does indeed change its surface proteins in adaptation to the tick's immune response. These avenues of research should be pursued in the future so that a better understanding can be reached regarding the tick-pathogen relationship that exists between the tick *H. longicornis* and *Theileria orientalis* Ikeda.

Lastly, the phenomenon of temperature induced sporogony in *H. longicornis* with *T. orientalis* Ikeda should be investigated through the incubation of fasting ticks at the specific temperatures set forth by Young et al. (1984)- these being 18°C, 24°C and 33°C for *T. parva*. Subsequent analysis of the fasting *H. longicornis* ticks using TaqMan® PCR would be able to determine if the extrapolation between *T. parva* and *T. orientalis* Ikeda is plausible in this area of their epidemiology.

While the novel tick-rearing protocol set out in chapter four is tangential to the topic of *T. orientalis* Ikeda population dynamics, it is nevertheless important as the facilitator of these experiments. As mentioned in the Chapter 4 discussion, the development of a novel tick-rearing protocol that is low cost and simple in set up has wide ranging applications in tick research.

6.2 Conclusion

Fluctuations were observed in the population of *T. orientalis* Ikeda over time in parasitizing *H. longicornis* ticks (Figure 5.1). Any one or combination of several factors may explain this. While the existence of sampling bias cannot be ruled out entirely, research conducted on other *Theileria* variants suggest that while inside the tick vector, *T. orientalis* Ikeda is subject to a range of tick-pathogen interactions revolving around the tick's immune response. These tick-pathogen interactions may explain both the increases and decreases in the *T. orientalis* Ikeda population. Additionally, temperature induced sporogony may have amplified the increases in *T. orientalis* Ikeda population that may have occurred as a result of the tick-pathogen interactions.

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