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Transmission of *Theileria orientalis* in

Australian Cattle.

Jade Hammer

A thesis submitted in fulfilment

of the requirements for the degree of

Master of Veterinary Science

Faculty of Veterinary Science

University of Sydney

2016
ABSTRACT

Background

Theileria are blood-borne intracellular protozoal parasites belonging to the phylum Apicomplexa. Previously considered a benign parasite in Australia, outbreaks of clinical disease resulting from Theileria orientalis genotypes have been reported in Australia since 2006. Since this time, outbreaks have become widespread in south-eastern Australia, resulting in significant adverse impacts on local dairy and beef industries. This project provides the first investigation into the possible biological and mechanical vectors involved in the rapid spread of the parasite. This includes identifying the likely biological vector tick, as well as assessing the role of biting arthropods (biting flies, lice, mosquitoes and ticks) and blood transmission during management procedures in the transmission of the disease. Transplacental and colostral transfer were also investigated.

Methods

To identify possible vectors for disease, ticks, biting flies, lice and mosquitoes were collected within active outbreak regions of Gippsland, Victoria. Ticks were collected from cattle and wildlife. Ticks were identified via DNA barcoding of the mitochondrial cytochrome oxidase I gene. Barcoded ticks were pooled according to species or phylogenetic group and tested for the presence of T. orientalis DNA and the genotypes Ikeda, Chitose and Buffeli using real-time PCR.

To further assess mechanical transmission and iatrogenic transmission through husbandry procedures, blood was collected from a T. orientalis Ikeda positive Angus steer, and cryopreserved in liquid nitrogen. Blood was also collected from a T. orientalis Ikeda positive Holstein-Friesian calf, but was not cryopreserved. Blood was then inoculated into the jugular vein of 9 calves in 3 treatment groups, each with 3 animals. Calves in group 1 received 10 ml
of cryopreserved blood, while those in groups 2 and 3 received 1 ml (fresh blood) and 0.1 ml (cryopreserved), respectively. An additional three animals remained as non-inoculated (negative) controls and the donor calf was also followed as a positive control. Blood was collected at intervals for 3 months, and analysed via qPCR for the presence of the parasite. Thirty samples of colostrum together with blood samples from the respective neonatal calves at 3-6 weeks along with their dams, from a commercial dairy herd within an endemic region in Gippsland, were collected and also tested for *T. orientalis* antigen and antibodies to the major piroplasm surface protein (MPSP).

To investigate transplacental transmission, 30 cows and their calves had blood collected within 24 hours of the cow giving birth. These EDTA samples were then tested via qPCR for *T. orientalis*.

**Results**

DNA barcoding and phylogenetic analysis identified ticks from the following species: *Haemaphysalis longicornis*, *Ixodes holocyclus*, *Ixodes cornuatus*, *Ixodes hirsti*, and *Bothriocroton concolor*. Additional *Haemaphysalis*, *Ixodes* and *Bothriocroton* spp. were also identified. Of the ticks investigated, only *H. longicornis* ticks from cattle carried theilerial DNA, with the genotypes Ikeda, Chitose and Buffeli represented. Mosquitoes collected in close proximity to outbreak herds included; *Aedes camptorhynchus*, *Aedes notoscriptus*, *Coquillettidia linealis*, *Culex australicus*, and *Culex molestus*. Low levels of *T. orientalis* Buffeli genotype were detected in some mosquito pools. The haematophagous flies tested negative. The batches of sucking louse (*Linognathus vituli*) from infected calves tested positive for *T. orientalis* Ikeda.

In the passive transfer trial, eight of the nine calves became positive for *T. orientalis*. Those calves given a high dose (10 ml) became positive 28 days following transfusion. Calves transfused with 1 ml became positive 41 days following transfusion, while 2 calves in group 3
became positive for the Ikeda genotype at 66 days, and the third animal became positive 98 days following transfusion. All negative control calves remained negative and the positive control calf remained positive.

Samples of colostrum were also shown to carry *T. orientalis*. Blood samples from calves receiving this colostrum were tested between the ages of 3-6 weeks and showed no transmission. However, the transfer of antibodies from colostrum was seen. Transplacental transmission was not observed in this study.

**Conclusions**

This is the first demonstration of a potential vector (*Haemaphysalis longicornis*) for *T. orientalis* in the current Australasian disease outbreak.

*T. orientalis* is capable of being transferred mechanically by intravenous inoculation with small volumes of blood. However, infection by this route does not appear to result in a high level of infection (as determined by qPCR) and did not result in any overt clinical signs. Animals infected by this means may play a significant role in the transmission of the disease by acting as asymptomatic carriers. Other modes of blood transfer, including biting arthropods and colostral transfer are also likely to play a role in transmission, and might help explain the rapid spread of the disease in Australasia. Transplacental transmission seems unlikely to occur.

**Publications arising from the research**

The results of this research project have been published in peer-reviewed journals and presented at several conferences, workshops and field days as detailed below:

**Peer Reviewed Journals:**


**Radio Interviews:**

- ABC Rural South Australia- Interviewed by Danielle Gindlay- 24th March 2015
- ABC Gippsland- Country Hour
- ABC Bega- Rural Report
- ABC Gippsland- South East NSW and Gippsland Rural Report
- ABC Gippsland Rural Report 16th February 2016
- ABC Victoria Country Hour 16th February 2016

**Major News Papers:**

- Stock and Land- “Warnings after theileriosis outbreak.”
- Stock and Land- “Bairnsdale vet ticked-off with blood disease.”
- Stock and Land- “Time to keep an eye out for ticks.”
- Stock Journal- “Vet warns of spike in Theileria cattle deaths.” Interview by Catherine Miller. 12 April 2015
- The Weekly Times- “Theileria cases are on the rise in East Gippsland.” Interview by Shannon Twomey August 28th 2014
- The Weekly Times Interview by Cassandra Zervos September 15th 2015
- Stock and Land- Interview by Laura Griffin 24th February 2016

**Television:**

- ABC 7:30 report- Interviewed by Xanthe Kleinig 9th March 2016
Local News Papers, Newsletters, and Online media:

- Macalister Demonstration Farm Newsletter 84. Interviewed by Maria Rose- Industry Development Officer
- The Land Multimedia- “Warning: beware of cattle ticks.” 30 March 2015
- The Australian Dairyfarmer- 30 March 2015
- Flock and Herd- “Theileria transplacental transmission.”
- Wn.com- News- “Victorian veterinarian Dr Jade Hammer says Theileria cattle disease is ‘of global significance.’”
- Turf Craft Multimedia
- Angus Australia- Website article
- Herefords Australia- Website article
- News Central by excess- Theileria, bovine, Australia
- How Now Gippy Cow Issue 192 Jan 2015- “Has your farm been impacted by the Theileria outbreaks?”
- Look For Diagnosis.com
- Aquatic Animal Health- “Subtropical disease in Victorian cattle.”
- The Cattle Site- “Theileriosis Disease Discovered in Australian cattle.”
- News Locker- Bairnsdale News- “Warning after theileriosis outbreaks.”

Public Talks:

- East Gippsland Beef Conference- 2014
- Macalister and East Gippsland Annual Meeting 2014
- Dairy Discussion group meeting- East Gippsland Jan 2014
- Dairy Discussion Group meeting- East Gippsland Jun 2014
- Dairy Discussion Group meeting- Macalister region 2014
- Post Grad Conference- University of Sydney 2014.
- United Dairyfarmers of Victoria general meeting 2014.
- MLA More Beef from Pastures South East Beef Innovation and Profit Drivers Days.
  Natural Resources South East- 2015
- Macalister and East Gippsland Annual Meeting 2015
- Dairy Discussion Group meeting- East Gippsland 2015
- Public Meeting Orbost- Snowy River Vet Clinic 2015
- Theileriosis workshop- Pan Pacific Veterinary Conference 2015
- Wairewa and District Landcare Group 2015
- Post Grad Conference- University of Sydney 2015
- Bairnsdale Beef Check Group, and Public Talk November 18 2015
- Joint meeting of United Dairy Farmers of Victoria, and Macalister and East Gippsland Dairy Extension Group December 2015
STATEMENT OF ORIGINALITY

The work presented in this thesis, is to the best of my knowledge, original and entirely my own and has not been submitted in any previous application for a degree at this or any other university. All sources used have been acknowledged.

Jade Hammer

Student Number 440096535
ACKNOWLEDGEMENTS

This research would not have been possible without the financial support of Meat and Livestock and The United Dairyfarmers of Victoria. Funding made available through collaboration with the Elizabeth Macarthur Agricultural Institute was also instrumental in the progression of the project.

I’d also like to acknowledge the support from the farmers in Gippsland. In particular I would like to acknowledge Craig and Teresa Hanrahan for allowing multiple blood and milk samples to be collected from their farm. I’m also very grateful to all our collectors and to those farmers who allowed trapping equipment on their properties.

I would also like to thank the Main Street Veterinary Clinic in Bairnsdale for allowing use of cryopreserving facilities, microscopy equipment, providing blood collection equipment, as well access to farmers affected with theileriosis. I’m also grateful to Luke Waddell and Dr. Derek Harms for assisting with sample collection.

I appreciate the help from Dr. Grant Rawlin who helped with initial conceptual discussions and for providing contacts within AgriBio. The type specimen of *H. longicornis* was kindly provided by Grant Rawlin and Mallik Malipatil. I would also like to acknowledge Phillip Carter for supply of cryopreservant and helpful discussions.

Finally, I’d like to thank the person who has been the most important contributor to the research; Prof. David Emery. David has been instrumental in conceptual discussions, study design, ethics approval application, manuscript drafting, funding applications and practical support. Aside from these things, David has been very encouraging and supportive throughout this time and has made himself readily available when needed in any aspect of the project. I can’t thank him enough.

**Declaration:** Dr. Cheryl Jenkins and Dr. Daniel Bogema undertook the qPCR and ELISA testing of samples provided for this research. I’d to thank them for their ongoing support and commitment to theilerial research.
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<td>PVP</td>
<td>Polyvinylpyrrolidone</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>fg/µL</td>
<td>Femtogram per microliter</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative Polymerase Chain Reaction</td>
</tr>
<tr>
<td>UIC</td>
<td>Universal Inhibition Control</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>2D-PAGE</td>
<td>Two-dimensional polyacrylamide gel electrophoresis</td>
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<td>p32</td>
<td>A universal <em>Theileria orientalis</em> gene</td>
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<tr>
<td>MPSP</td>
<td>Major piroplasm surface protein</td>
</tr>
<tr>
<td>DSn</td>
<td>Diagnostic sensitivity</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
</tr>
<tr>
<td>EMAI</td>
<td>Elizabeth Macarthur Agricultural Institute</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
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<td>H.</td>
<td><em>Haemaphysalis</em></td>
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<td>I.</td>
<td><em>Ixodes</em></td>
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<td>B.</td>
<td><em>Bothriocroton</em></td>
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Chapter 1: Literature Review

1.1 Introduction

*Theileria* parasites have a global distribution and vary greatly in pathogenicity, causing clinical disease ranging from benign to severe presentations and outcomes. The first outbreaks of disease associated with *Theileria* was in 1902 in Africa (Shaw 2002). Two diseases of global significance caused by *Theileria* are East Coast fever (*T. parva*) and tropical theileriosis (*T. annulata*). These rapidly spreading species are responsible for enormous economic losses with mortality as high as 80-100% from East Coast fever in newly introduced cattle (Shaw 2002). The total number of cattle deaths from East Coast Fever can approach a million per year, resulting in diminished food production and a negative impact on fragile economies (Shaw 2002). Although these species are exotic to Australia, this country now has its own pathogenic species of *Theileria* which has resulted in outbreaks of disease.

1.2 *Theileria* classification

*Theileria* are blood-borne intracellular protozoal parasites belonging to the phylum *Apicomplexa*. The classification of the various genotypes of the parasite remains the topic of much conjecture (Uilenberg, Perie et al. 1985, Fujisaki, Kawazu et al. 1994, Gubbels, Hong et al. 2000). In particular, the more benign forms of bovine *Theileria* have been referred to differently throughout the world. The parasite group is known as *T. buffeli* in Australia, *T. sergenti* in Japan and *T. orientalis* in many other locations (Fujisaki, Kawazu et al. 1994). Attempts to classify *Theileria* have been based on a range of biological criteria. Uilenberg *et al* examined theilerial isolates from Australia, Britain, Iran, Japan, and the USA and compared them with isolates from Korea corresponding to *T. sergenti* reported in Russian literature (Yakimoff and Dekhtereff 1930); cited in (Uilenberg, Perie et al. 1985). Serological and
morphological comparisons showed all of these isolates to belong to one species, and named it
*T. orientalis* (Uilenberg, Perie et al. 1985). Another comparison of *T. sergenti/buffeli/orientalis*
by Gubbels, *et al.* (2000) using criteria of morphology, vector specificity, pathogenicity and
sequences of the 18S small subunit ribosomal RNA or major piroplasm protein also confirmed
the various isolates as the same species and suggested the name *Theileria buffeli*. This
supported the name proposed by (Callow 1984) and others (Stewart, Uilenberg et al. 1996).
Although the nomenclature of the *T. sergenti/buffeli/orientalis* group is still debated, it is now
generally agreed that *T. sergenti* is not acceptable as it has previously been used for a theilerial
species in sheep (Fujisaki, Kawazu et al. 1994, Stewart, Uilenberg et al. 1996, Gubbels, Hong
et al. 2000). Contradicting the combined grouping of *T. sergenti/buffeli/orientalis* is mounting
evidence for the separation of *T. sergenti* into a different taxonomical group to *T. orientalis/buffeli* (Fujisaki, Kawazu et al. 1994). Analysis of piroplasm proteins by 2D-PAGE
showed minor differences between *T. sergenti* and *T. orientalis/buffeli* (Sugimoto, Kawazu et
al. 1992). Differences in the subgeneric level of the vector ticks and tick transmission
experiments also support separation of *T. sergenti* (Fujisaki, Kawazu et al. 1994). For example,
the Australian tick *H. longicornis* could transmit only *T. sergenti* but could not transmit *T.
buffeli*, whereas Japanese *H. longicornis* could transmit both (Fujisaki, Kawazu et al. 1994). In
addition, serological comparisons of piroplasm antigens of *T. sergenti, T. buffeli* and *T.
orientalis* using ELISA and Western blotting in combination with 2D-PAGE also found
dissimilarities in proteins of 33-kDa of *T. sergenti* and the 32 and 34-kDa of *T. orientalis/buffeli*, suggesting that *T. sergenti* should be separated from *T. orientalis* and *T.
buffeli* (Kawazu, Sugimoto et al. 1992).

Despite the taxonomic discussions, the name *Theileria orientalis* is now widely applied to the
species present in Australia, New Zealand and throughout Asia (Kawazu, Kamio et al. 1999,
et al. 2011, Kamau et al 2011, Khukhuu, Lan et al. 2011, McFadden, Rawdon et al. 2011, Cufos, Jabbar et al. 2012, Yokoyama, Sivakumar et al. 2012, Eamens, Bailey et al. 2013, Eamens, Gonsalves et al. 2013). For this reason this thesis will refer to the *T. orientalis/buffeli/sergenti* group as *T. orientalis*. It is now appreciated that *T. orientalis* can be separated into several genotypes, namely, type 1 (Chitose), type 2 (Ikeda), type 3 (Buffeli), types 4-8 (Islam, Jabbar et al. 2011, Kamau et al 2011) and types N1-N3 (Khukhuu, Lan et al. 2011). The various genotypes have been associated with varying severity of clinical signs, and may account for the recent epidemic of severe disease and deaths in Australia (Kamau et al 2011) (see section 1.4 and 1.5).

### 1.3 *Theileria orientalis* life cycle

*T. orientalis* has a complex lifecycle and is highly adapted to invade and survive in both its vector and host. Although the vector has not been confirmed in Australia, the *T. orientalis* life cycle in Japan begins with the inoculation of sporozoites from the salivary gland of the tick vector as it feeds. Both nymphal and adult ticks are capable of injecting sporozoites of *Theileria parva* (Ochanda, Young et al. 1996), and this may apply for *T. orientalis*. Sporozoites then attach and invade host leucocytes and following division become schizonts. These can be seen transiently in lymph nodes, spleen, and liver with immunohistological staining around 10 days after infection, but are not responsible for the pathology associated with the infection, unlike other forms of *Theileria* such as *T. parva* (Kawazu, Sato et al. 1991, Shaw 2002). Asexual development occurs in a proportion of infected cells which results in uninucleate merozoites which then escape from parasitised leucocytes and invade erythrocytes where the parasite develops into piroplasms. The invasion of red blood cells by merozoites takes place about 10 days post inoculation, and is responsible for the febrile episodes and the clinical manifestations of *T. orientalis*, including the signs associated with anaemia (pale mucous membranes,
tachycardia, tachypnoea, weakness) (Hagiwara et al 1996, Sugimoto et al 2002). The analogous period with T. parva infection is also associated with the most severe inflammatory reactions as leucocytes rupture in submucosal tissues to release microschizonts (Morrison, Buscher et al. 1981). Although the mechanism of attachment and invasion of erythrocytes is poorly understood, high parasitaemia can result in severe anaemia and death of the host (Hagiwara et al 1996). The pathogenesis of anaemia is mostly the result of extravascular removal of parasitised cells but non-parasitised red cells in infected calves also exhibit an increased turnover (Yagi, Ito et al. 1991). The time between inoculation of sporozoites and the detection of piroplasms (prepatent period) and clinical signs varies considerably. The prepatent period has been shown to vary from 7-10 days in Korea (Baek et al 2003), while in Australia, 14-47 days (Riek 1982), 12-16 days (Stewart, de Vos et al. 1987) were reported after tick application, and approximately 20 days after ticks were seen (Izzo, Poe et al. 2010). Therefore, clinical disease may not develop for at least 1 month after removal of putatively infected ticks (Izzo, Poe et al. 2010) and this may depend on the quantum of infection.

Tick hosts become infected by ingesting piroplasms from cattle blood when feeding. Infected red cells are lysed in the gut lumen where the released piroplasms differentiate into gametocytes. Fertilisation follows and the resulting zygotes invade the gut epithelium during the ticks’ moulting process. At this stage the parasite is diploid, whereas for most of the cycle the parasite is haploid. Within the gut epithelium, the parasite then differentiates into motile kinetes and invades the tick’s salivary glands. The parasite then undergoes asexual multiplication resulting in thousands of sporozoites, ready to be discharged from the salivary glands into a new bovine host (Takahashi, Kawai et al. 1993). Transmission within ticks is trans-stadial and no transovarial transmission occurs (Callow and Hoyte 1961, Riek 1982, Stewart, de Vos et al. 1987, Shaw 2002). It has been demonstrated that transplacental and
mechanical transmission also occurs and this will be addressed in section 1.8 (Fujisaki, Kamio et al. 1993, Baek et al 2003).

1.4 The History and Manifestations of theileriosis


Clinical signs of *T. orientalis* are mostly associated with the sequellae from anaemia. These signs can include depression, weakness, anorexia, pale mucous membranes, lymph node swelling, tachypnoea, tachycardia, dyspnoea, pneumonia, jaundice, late-term abortions, dystocia, still births, pyrexia, metritis, and mortality (Izzo, Poe et al. 2010, Islam, Jabbar et al. 2011, McFadden, Rawdon et al. 2011). Clinical presentations vary depending on the stage of the disease as well as host factors. For example, rectal temperatures increase to 40.2 - 41.3°C from days 9-14 after tick infestation and regional lymph nodes enlarge 8-12 days after tick attachment when schizogony is occurring, (Stewart, De Vos et al. 1987) presumably associated with the destruction of parasitised cells. Despite the array of clinical presentations, diagnosis of clinical theileriosis is readily achieved from a combination of clinical signs, a low packed cell volume (PCV) indicating anaemia, and confirmation by a blood smear showing theilerial piroplasms.
PCR testing for p32 gene DNA has been shown to be much more sensitive than clinical pathology in detecting *T. orientalis* infections (Eamens, Gonsalves et al. 2013). In this study, 50% of blood smears that were negative for theilerial piroplasms were found to be positive with molecular testing for theilerial DNA (Eamens, Bailey et al. 2013). It was shown that approximately 90% of smear positive samples were positive in the p32 and Ikeda assays (Eamens et al. 2013). The majority (235/243, 97%) of the samples considered smear positive were confirmed as PCR positive in the p32 assay, and all of these were positive in the Ikeda PCR. In addition, the p32 PCR detected 33/69 (48%) of smear negative samples as positive for *T. orientalis*, and 39/69 (57%) of smear negative were confirmed to contain Ikeda (Eamens, Gonsalves et al. 2013). This finding is supported by (Noaman 2014) who found that smear testing for *T. annulata* exhibited 57% diagnostic sensitivity (DSn) and 99% diagnostic specificity (DSp) compared with 100% DSp and DSn for PCR testing (Noaman 2014). Smear examinations can be subjective due to the great variation in piroplasm morphology and parasitaemia. This is especially the case where the parasitaemia is low, reducing DSn (Eamens, Bailey et al. 2013). Baek et al (2003) confirmed the PCR method to be specific with no amplification detected from other common bovine haemoparasites, including *Anaplasma centrale* and *Babesia ovata* (Baek et al 2003). The DSn of PCR was found to be approximately 4.5 parasites per ml$^{-1}$ of blood, or equivalent to a parasitaemia of 0.00009% (Tanaka, Matsuba et al. 1992, Baek et al 2003). A Victorian study also found PCR detection to be far more sensitive as the DSn of blood smears was calculated to be only 38.7% (95% CI 32-46%) (Perera, Gasser et al. 2013).

### 1.5 Australian outbreaks of clinical theileriosis and its Impacts

*Theileria* has been recognised in Australia since 1910 (Seddon 1952) and is found in all states except Tasmania (Izzo, Poe et al. 2010). Until recently *T. orientalis* genotypes have been
considered benign (Kamau et al 2011, Cufos, Jabbar et al. 2012). The first clinical cases in Australia were reported in Queensland in the 1960s, and it was recognised that under “certain undefined conditions,” the organism could become highly virulent (Seddon 1966, Kamau et al 2011). Despite three cases of severe natural infection being recorded in the early 1960s, it was assumed that the disease was of minor economic importance in Australia (Rogers and Callow 1966). This changed after 2006, when a large number of outbreaks of clinical disease were reported in New South Wales, Victoria, Western Australia, (Islam, Jabbar et al. 2011, Thomson 2013) and more recently in South Australia (Unknown 2014). The emergence of clinical theileriosis and associated mortalities in Australia is of increasing concern for the local beef and dairy industries (Izzo, Poe et al. 2010, Islam, Jabbar et al. 2011).

The economic impacts of the recent Australian outbreaks are difficult to quantify. A recent study in Australia has shown significant negative impacts on milk production. The study has shown that at 100 days of lactation, and at 305 days of lactation, *Theileria* positive cows produced significantly less milk, milk fat, and milk protein compared with cattle that did not carry the parasite (Perera, Gasser et al. 2014). Based on this reduced production, an annual economic loss of AUD $202 per head was estimated. This figure does not take into account the death of animals which was estimated to be an average of AUD $1,800 per head. Interestingly, this study did not show any statistically difference in reproductive performance between *Theileria* infected and non-infected cattle (Perera, Gasser et al. 2014). Another study investigating production losses from *Theileria* in Victoria found marked milk reduction in cattle aged 4-7 years with an average drop in production of 300 litres in the first 50 days of lactation (Read 2013). New South Wales Primary Industries have estimated an average cost of $58,916 for dairy producers and $11,662 for beef producers, which equates to AUD $131/head for dairy cattle and AUD $67/head for beef cattle for farm impacted by the parasite (Bailey 2012). A recent survey revealed theileriosis mainly affected cows of more than two years of
age prior to calving. It was shown that of the 18 affected farms investigated, abortions were recorded in 55.5% and deaths in 72.2% of farms (Perera, Gasser et al. 2013). Therefore the full impact of the parasites in herds need to consider multiple factors including; cattle deaths, abortions, decreased milk production, reduced weight gains, veterinary services, and medications (Baek et al 2003). The disease has been estimated to cost the livestock industries in Japan and Korea around USD $100 million annually (Yokoyama, Sivakumar et al. 2012).

The recent outbreaks of theileriosis stimulated efforts to determine the causative agent, and the genotype *T. orientalis* Ikeda was soon implicated (Islam, Jabbar et al. 2011, Kamau et al 2011). In addition, it was found that *T. orientalis* Chitose genotype had entered the southern, more temperate area of Victoria and with Ikeda, was linked to a severe outbreak of theileriosis on a beef farm (Islam, Jabbar et al. 2011). A prevalence study of genotypes detected in New South Wales showed that Ikeda accounted for 46%, Chitose 46% and Buffeli 17.9% of infections (Kamau et al 2011). It was also found that the majority of cattle had mixed infections of Ikeda and Chitose (Kamau et al 2011). This was not the case in Queensland where the percentage of Chitose was 52.9%, Ikeda 5.9%, and Buffeli was 58.8%. The majority of cows in Queensland had mixed Buffeli and Chitose genotypes (Kamau et al 2011). Overall, the research effort confirmed the presence of four theilerial genotypes: *T. orientalis* (Buffeli), *T. orientalis* (Ikeda), *T. orientalis* (Chitose) and *T. orientalis* type 4 (MPSP) or type C (SSU rRNA) in Australia (Kamau et al 2011). In Victoria, Ikeda accounted for 91.1%, Chitose 32.9%, Buffeli 2.4%, and Type 5, 1.4% of blood tested (Perera, Gasser et al. 2013). Mixed infections with genotypes Ikeda and Chitose were common and seen in 21.6% of blood tested (Perera, Gasser et al. 2013).

The method of introduction of *Theileria* to Australia is uncertain and may have occurred with the introduction of *Haemaphysalis longicornis* ticks into Australia (Seddon 1952, Hoogstraal, Roberts et al. 1968, Stewart, de Vos et al. 1987). This may have coincided with the introduction
of cattle from Japan carrying *Haemaphysalis longicornis* ticks (Stewart, Standfast et al. 1992). However, based on local transmission studies, the role of *H. longicornis* as a vector was problematic (Stewart, de Vos et al. 1987). It was also recognised that the introduction of *Theileria* to Australia on imported British cattle must have occurred on numerous occasions (Stewart, de Vos et al. 1987). It has been speculated that the Ikeda genotype was introduced to Australia from cattle imported from Eastern Asia, (Perera, Gasser et al. 2015) and the introduction of these pathogenic genotypes to Victoria was a result of the introduction of infected cattle from endemic areas of NSW into naïve Victorian herds. A change in weather events including high rainfall in summer and high humidity in 2011-12 led to increased tick reproduction and activity in Victoria increasing the transmission of these pathogenic genotypes (Perera, Gasser et al. 2013).

While the introduction of *T. orientalis* remains uncertain, so does an explanation for the emergence of more pathogenic genotypes. It has been speculated that antigenic variation might have enhanced the virulence of the parasite (Kamau et al 2011). The occurrence of the previously unreported Ikeda and type 4 (type C) isolates may be a result of a silent mutation but this has not been identified (Kamau et al 2011). It is known that the Ikeda genotype is associated with more clinical cases (severe disease) than Chitose and Buffeli in Australia, and this also occurs internationally where clinical cases of *T. orientalis* have been reported (Yokoyama, Sivakumar et al. 2012, Eamens, Gonsalves et al. 2013). It has also been demonstrated the Ikeda type is more virulent, and is reflected in an increasing prevalence compared to the Buffeli genotype (Eamens, Bailey et al. 2013). It was speculated that an increased growth rate or rate of transmission of Ikeda type, or failure of the host immune system to clear this genotype accounted for the increased virulence (Eamens, Bailey et al. 2013). In addition to being more pathogenic, it has been proposed that in particular herds, the Ikeda genotype appears more virulent and might outcompete other less pathogenic and benign
genotypes (Eamens, Bailey et al. 2013). The mechanism of this natural selection might be a result of co-infection with Ikeda and other genotypes where interaction, synergy and or interference between parasite types could result in the loss of a genotype (Kamau, Salim et al. 2011). It has been demonstrated that experimentally infected cattle show loss of *T. buffeli* infection after a few weeks in the presence of Ikeda and Chitose infection (Kamau, Salim et al. 2011), suggesting strongly that the latter two genotypes are more “virulent”.

### 1.6 Vectors of *T. orientalis* in Australia

Transmission experiments involving *Theileria* in Australia occurred prior to clinical outbreaks of the disease. The vectors for *T. orientalis* Ikeda genotype have not been determined in Australia. However, the findings from older Australian transmission experiments may also apply to the newly identified strains. The ticks *Rhipicephalus australis* (syn. *Boophilus microplus*) and *Haemaphysalis bispinosa* were once implicated as vectors in Australia (Seddon 1952), but this is no longer the case (Callow and Hoyte 1961, Riek 1982). Many attempts to obtain transovarial and stage to stage (transtadial) transmission with *B. microplus* were not successful despite feeding larval, nymph and adult stages of ticks on animals harbouring parasitaemia (Callow and Hoyte 1961, Riek 1982).

While *R. australis* is unlikely to be a vector in Australia, other tick species have been demonstrated to transmit the infection. An earlier study showed *H. longicornis* is a highly successful vector for *T. buffeli*, as is *H. bancrofti*, while *I. holocyclus* and *A. triguttatum* have failed to transfer infection (Riek 1982). In Queensland, *H. bancrofti*, and *H. humerosa*, but not *H. longicornis* was shown to be vectors (Stewart, de Vos et al. 1987). However in southern New South Wales and Victoria, *H. bancrofti*, and *H. humerosa* are non-existent or rare and other vectors are assumed to be involved (Watts, Playford et al. 2015). Despite multiple attempts, a later study failed to transmit the parasite with *H. longicornis* although infective
parasites could be retained in *H. humerosa* between moults from larva to nymph and from nymph to adult (Stewart, de Vos et al. 1987). Experimental transmission studies in Japan employing the Japanese Ikeda genotype of *T. orientalis* with *H. longicornis* sourced from both Australian and Japanese populations were successful. Indeed, these early studies were used as one criterion to differentiate the Australian “*T. buffeli*” (now referred to as *T. orientalis* Buffeli) from “*T. sergenti*” which encompasses the Ikeda genotype (Fujisaki, Kawazu et al. 1994). In separate experiments, the native Australian wallaby tick, *H. bancrofti*, was shown to transmit both *T. orientalis* Ikeda (Fujisaki, Kawazu et al. 1994), and a strain of *T. orientalis* sourced from Queensland and presumed to be the Buffeli type “Warwick strain” (Stewart, Devos et al. 1989). Vector studies overseas have also identified other possible transmission vectors in *H. megaspinosa, H. douglasi, I. persulcatus, and I. ovatus* (Yokoyama, Sivakumar et al. 2012). In New Zealand, only *H. longicornis* appears available as the tick vector (Watts et al. 2015).

### 1.7 Ticks in Gippsland Victoria

*Haemaphysalis longicornis*, a known vector tick for *T. orientalis* internationally, has a complex life cycle. It has a wide host range, and a distribution along the coastal strip from south east Queensland to the Victoria border with rare occurrences in other locations in Victoria (Roberts 1970). It has also been reported in locations in south west Western Australia (Besier and Wroth 1985). It is a three host tick with larva, nymph, and adult engorging for approximately 7 days before dropping from the host (Cane 2010). Female ticks lay up to 2000 eggs in late spring and early summer and these hatch in 60-90 days depending on environmental conditions. Larvae have been shown to survive up to 217 days, nymphs 263 days and adults 249 days without feeding (Cane 2010). Most adults are seen in January and February. Reproduction is via parthenogenesis, making male adults rarely seen.
Other ticks recorded from cattle in Victoria include; *Ixodes cornuatus, Ixodes holocyclus, Amblyomma moreliae, Aponomma concolor, Aponomma fimbriatum, Aponomma hydrosauri,* and *Rhipicephalus sanguineus* (Roberts 1970). Of these ticks, *I. cornuatus* and *I. holocyclus* are known to be present in Eastern Victoria where this research was conducted (Jackson, Beveridge et al. 2007). In addition, *Amblyomma moreliae* has also been recorded in Gippsland (Roberts 1970). *Aponomma concolor* is mostly seen on echidnas, *Aponomma hydrosauri* is mostly found on reptiles, and *Rhipicephalus sanguineus* is mostly seen on dogs, but the occurrences of all of these ticks on cattle are rare (Roberts 1970). Based on this data, likely vectors on *T. orientalis* might include *H. longicornis, I. cornuatus, I. holocyclus,* and *A. moreliae.* Despite the fact that both *H. bancrofti* and *H. humerosa* are apparently competent vectors for *T. orientalis* under experimental conditions, the range of both of these species is limited to the more temperate and tropical climates of northern New South Wales, Queensland and the “Top End” and are therefore unlikely to act as vectors in Victoria.

### 1.8 Mechanical and vertical transmission

Unequivocal evidence for mechanical transmission of *T. orientalis* is lacking. A single attempt to transmit *T. mutans* mechanically by *Stomoxys calcitrans* and by needle puncture failed in Australia (Seddon 1952). However, although the precise details are unknown, it was found that *T. mutans* is readily transmissible by blood inoculation (volumes and route unknown), inducing a “thermic” reaction with large numbers of parasites in the peripheral blood (Seddon 1952). It has been hypothesised that mechanical transfer of theilerial piroplasms from one animal to another could result in disease. This may occur from vaccination needles and the proboscis of biting flies (Heath 2013). There is limited evidence to support this, however a Japanese study found that a species of Tabanidae (*Tabanus trigeminus*), under “certain” (unspecified) conditions can mechanically transfer *Theileria*; (cited in (Fujisaki, Kamio et al. (Fujisaki, Kamio et al. 2013)).
Fujisaki (1993) reported that splenectomised calves acquired *T. orientalis* Ikeda from lice previously fed on infested host (Fujisaki, Kami o et al. 1993). A calf naturally infested with lice (*Linognathus vituli*) was artificially infected with *T. orientalis* Ikeda by subcutaneous inoculation of a sporozoite suspension derived from *Haemaphysalis longicornis* (Kamio, Fujisaki et al. 1989). Around 100-200 female *L. vituli* were collected from this calf when the parasitaemia ranged from 1.8-6.5%, 37-41 days after the inoculation. These lice were transferred to a negative calf for 18 days, and theilerial piroplasms were detected in blood smears at 49 days after louse application. A second trial fed lice on a calf from 83-91 days after inoculation of sporozoites when it had a parasitaemia of 3.1-4.4% over that period. These lice were transferred to another *Theileria* negative calf for 20 days and piroplasms were detected in this recipient at 33 days with a peak parasitaemia of 14.5% at 65 days. Around 30 ticks of a known vector (*H. cornigera*) were allowed to feed (from days 52 to 60) on one recipient that was infected by the lice. Thirty days after moulting, these ticks had developed sporozoites in their salivary glands. By contrast, the lice were likely to be mechanical rather than a biological vector (Fujisaki, Kami et al. 1993), as dissection of the salivary glands and guts only showed piroplasms in the gut sections. This was elegantly demonstrated when lice on a known *Theileria* negative calf were collected and transferred to a rabbit. Blood collected from this rabbit showed anti-bovine-immunoglobulin antibodies 12-13 days after attachment, indicating that regurgitation is the likely mechanism of transfer of *T. orientalis* Ikeda by *L. vituli* (Fujisaki, Kami et al. 1993). This was also supported indirectly by studies where mechanical transmission with male ticks was attempted but was not successful (Riek 1982).

Vertical transmission of *T. orientalis* has also been confirmed. Baek *et al* (2003) used 6 heifers known to be negative for *Theileria* and artificially inseminated prior to infestation with known positive ticks (*H. longicornis*) (Baek et al 2003). All heifers were positive for *Theileria* by
29 periphery blood smears, 7 to 10 days after exposure. Of the 6 heifers, 2 aborted at 6 and 7 months, one had her calf delivered by caesarean section at 8 months and the other 3 calved normally. All 6 dams, and calves including the 2 aborted foetuses had samples of blood, liver, spleen and lymph nodes collected. These samples were all examined by parasitology, serology and PCR. One calf had negative results on blood examination but was positive on PCR. All other samples were positive for Theileria confirming vertical transmission (Baek et al 2003). Transfer from dam to calf through colostrum is another possible mode of transmission but no previous studies have investigated this possibility. Another study also observed possible prenatal infections of T. orientalis (Onoe, Sugimoto et al. 1994). This study found 5 out of 100 blood samples of 1 or 2 day old calves were positive for Theileria with parasitaemia levels between 0.01 and 0.06% (Onoe, Sugimoto et al. 1994). Supporting this finding is the previous demonstration of prenatal infections of piroplasmosis in Babesia bovis and Babesia equi (Trueman and McLennan 1987, Dewaal 1992).

1.9 Objectives of this study

The primary objective of this study is to gain a better understanding of the transmission of T. orientalis to assist in underscoring formulation of control and prevention measures in integrated parasite management. To this end, collection and identification of possible mechanical and intermediate hosts including ticks, biting flies, lice and mosquitoes across Gippsland to gain a current knowledge of the species now present in these areas was undertaken. These haematophagous arthropods were tested for Theileria DNA to determine which species are capable of picking up Theileria and estimate their parasite "load" and whether they could act as mechanical or biological vectors of the disease.

The development of reliable recommendations for livestock producers to counter disease outbreaks has been hampered by the lack of understanding of the parasite epidemiology and
pathogenesis in Australia. Standard husbandry practices that include blood transfer can include re-using castration knives, vaccination/medication needles, ear notches, rectal examination gloves and injury during transport and yarding. Current measures recommend washing and disinfecting castration knives, avoiding multiple use of needles or where impractical for herd vaccination, to use sharp needles and change these regularly to minimise blood transfer (Bailey 2015).

This study is the first to examine whether blood inoculation can mechanically transfer an Australian strain of *T. orientalis* and the quantities that may be involved. The results are interpreted for the epidemiological risks posed for animal husbandry practices, and biting arthropods. The possibilities of transplacental and colostral transfer of infection is also investigated.
Chapter 2: Methods and materials

2.1 Collection and identification of biting arthropods

2.1.1 Collection and identification of ticks

For this study, 220 ticks were collected from a variety of livestock and wildlife hosts over a large geographical region of Victoria (Figure 1) by local veterinary practices, wildlife carers, farmers and members of the public. Ticks were predominantly adult parasites, although some larval and nymph stage ticks were also collected, and ranged from unengorged through to fully engorged.

Figure 1 - Distribution of *Haemapsalis longicornis* in Australia

Map of Australia showing the known range of *H. longicornis* as described in Riek (1986). An enlarged map of the state of Victoria is also shown with the geographic locations, from which ticks and other arthropods were collected, highlighted (closed circles). The location of the likely entry point of *T. orientalis* Ikeda into the state of Victoria is also shown (open circle).
All ticks were frozen and capture data recorded. Preliminary identification of ticks was undertaken morphologically using identification charts (Roberts 1970). All ticks collected were subsequently transported on dry ice to the laboratory for molecular identification. Ticks were photographed, in order to maintain a record of sample morphology, and samples for which morphological identification was uncertain (n = 135), were subjected to DNA barcoding. A specimen of *H. longicornis* tick from an Australian reference collection was also barcoded for comparison with the field-collected ticks.

An individual leg was dissected from each tick for PCR testing and the legs were washed with PBS to remove any adherent host material. DNA was extracted from the tick legs using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions and with a 100µL elution volume. The mitochondrial cytochrome oxidase I (COI) genes were amplified using the metazoan “barcoding” primers of Folmer (Folmer, Black et al. 1994). PCR amplification was performed in a total volume of 25 µL comprising 1 × Immolase reaction buffer, 2.0 mM MgCl₂, 200 µM dNTPs, 400 nM each of the LCO1490 and HCO2198 primers and 1 U of Immolase DNA polymerase (Bioline). PCR products were visualised on a 0.5 × TBE-1.5% agarose gel and of the 135 ticks tested, 105 yielded sufficient amplicon for downstream sequencing. PCR products were purified using the Qiaquick PCR purification kit (Qiagen) and subjected to Big Dye terminator sequencing at the Australian Genome Research Facility (AGRF). Contiguous sequences were assembled using Geneious Version 7.1.5 (Biomatters, Auckland, New Zealand) and compared with existing tick COI sequences in GenBank using the Basic Local Alignment Search Tool (BLAST).

### 2.1.2 Collection of haematophagous flies

Haematophagous flies were collected during suitable weather during summer 2013/14, autumn 2014 and the summer of 2014/15 in locations within one kilometre of outbreak farms. Two Nzi
traps ([www.nzitrap.com](http://www.nzitrap.com)) were set up using dry ice and 1-Octen-3-ol as an attractant (Figure 2A). Dry ice was made using a dry ice maker from a carbon dioxide cylinder as shown in Figure 2B.

**Figure 2A - Nzi trap used for collection of biting flies**

One of the two Nzi traps set up in close proximity to outbreak herds. A canister containing dry ice is shown, holes in the bottom of this canister allows escape of carbon dioxide. This along with the chemical attractant 1-octen-3-ol placed on the grass below the trap attracts flies. Flies enter at the bottom of the trap and naturally fly upwards into the collection point shown at the top of the figure.

**Figure 2B - Dry ice maker**

Carbon dioxide cylinder and a dry ice maker used to produce the dry ice used in the fly and mosquito traps.
2.1.3 Collection and identification of mosquitoes

A total of 4 (UV) light traps (Australian Entomology Supplies, Model PR101) were used for the collection of mosquitoes (Figure 3).

**Figure 3 - Light and carbon dioxide trap used for collection of mosquitoes**

One of the 4 mosquito traps that was set up on properties that have experienced outbreaks of clinical disease associated with *T. orientalis*. The canister at the top contained dry ice and vents at the bottom of the canister allowed escape of carbon dioxide. A small light near the battery also helped attract mosquitoes close to a fan which blows the mosquitoes into the net at the bottom. The air current prevents mosquitoes flying out the trap.

Traps were set on properties where clinical cases of theileriosis had been identified, or located in close proximity (not more than 1 km) from such a herd. Each trap was baited with carbon dioxide from dry ice. Mosquitoes were identified morphologically using a dichotomous key (Russell 1996).
2.1.4 Collection of sucking lice

Hundreds of the sucking louse (*Linognathus vituli*) were collected from two calves naturally infested on a property in Fernbank, Victoria (37°48'21.9"S 147°19'41.7"E). Blood was collected from two of these calves and lice were collected with a flea comb and identified morphologically. Capture data was recorded for all arthropods, which were then frozen and stored at -80°C until required.

2.2 Testing collected ticks, flies, mosquitos and lice for *T. orientalis*

Ticks grouped by species or phylogenetic cluster, and where possible by host and location, were dissected for quantitative PCR (qPCR) analysis by removal and collection of the capitulum and the anterior portion of the scutum. For mosquitoes and tabanid flies, tissue from the abdomen and thorax was extracted.

Mosquitoes were also tested in 10-15 mg pools. Thirteen pools of mosquitoes were tested (approximately 10 mosquitoes per pool). All remaining mosquitoes were batch tested. DNA extraction was performed as described above for the tick legs, except that each tissue sample was homogenised prior to extraction with a sterile microfuge pestle. Mosquitoes and march flies (*Dasybasis* sp.) were batch tested.

Tick tissue was pooled in groups of 1-10 samples depending on phylogenetic group but also on tissue volume so as not to exceed a total of 25 mg of tissue per pool.

Seven pools of lice, each containing 25 lice from each calf were tested. Hundreds of other lice from the same donor were tested in a bulk pool. Each pool of lice was homogenized prior to extraction with a sterile microfuge pestle. DNA extraction was performed as described above for the tick legs.
Quantitative PCR (qPCR) was performed at EMAI, Menangle, NSW using a validated qPCR assay for *T. orientalis* detection and genotype differentiation as described previously (Bogema, Deutscher et al. 2014). This assay was used for quantification of the total load of *T. orientalis* (Universal) and to semi-quantitatively determine the relative proportions of the Ikeda and Chitose genotypes (UIC assay). DNA extracts were also tested for the Buffeli genotype of *T. orientalis* using a semi-quantitative singleplex qPCR (B assay). For this assay, the same reagents and instrumentation described in Bogema et al. 2015 (Bogema, Deutscher et al. 2015) were used, however the probe mix was substituted for a single probe designed based on an *in silico* analysis to specifically target the Buffeli genotype (5′ - FAM-CTCCTTTGCAGTATTCTTCTATCTC-BHQ1 - 3’). The analytical specificity of the Buffeli probe for this genotype was assessed using plasmids containing Ikeda, Chitose or Buffeli MPSP gene inserts and also DNA extracts from *T. orientalis*-negative cattle and cattle infected with the closely related parasites, *Babesia bovis* and *B. bigemina*, all as described in Bogema et al. (2015). The limit of detection (LOD) was defined as the limit where 95% of assays were successful and was experimentally determined by testing 8 replicates of Buffeli plasmid DNA at dilutions 15000, 1500, 150, 50, 15, 5, 1.5 and 0.5 gene copies/µL (GC/µL), followed by Probit analysis. The efficacy of the Buffeli singleplex assay was confirmed on bovine blood extracts previously demonstrated to be positive for this genotype (Eamens, Bailey et al. 2013). A standard curve generated from a 10-fold dilution series of a plasmid containing the Buffeli MPSP gene (Bogema, Deutscher et al. 2015), ranged from $1.5 \times 10^1$ to $1.5 \times 10^7$ to MPSP GC/µL and was included in each run. Quality acceptance parameters for standard curves were an $R^2$ value $> 0.98$ and an amplification efficiency between 90 and 110%.

**2.3 Mechanical transmission**

This part of the study was performed with approval by the Animal Ethics Committee, University of Sydney, project number 673.
2.3.1 Calf husbandry

2.3.1.1 Calf selection and testing

Thirteen bucket reared Holstein-Friesian steers aged 6 months were sourced from a commercial dairy herd near Bairnsdale in Victoria (37°49’27.1”S 147°31’47.3”E). They were separated from the dam within 24 hours of birth after receiving adequate colostrum. They were immediately blood tested to confirm a negative status for *T. orientalis*, and moved to a calf shed. During the following 12 weeks, the calves were fed milk and grain, ear tagged, vaccinated, castrated, drenched and de-horned. The first calf was born on the 3/8/2014 and the final calf was born 10 days later on the 13/8/2014. These calves were re-tested for *T. orientalis* prior to the start of the trial and one calf had become positive in the clinically relevant range (and remained as a positive control). Calves all tested negative for Bovine Viral Diarrhoea Virus by an ear notch test for antigen (IDEXX; BVDV Ag Point-of-Care Test). All calves were then re-located to the study location.

2.3.1.2 Study location

A 2.5 acre block in Clifton Creek, Victoria was selected as the study location (37°44’03.6”S 147°39’31.6”E). This block mimics a typical grazing block for the area (Figure 4A). A slightly undulating block with a large central dam is surrounded by house blocks on two sides, a road on one side and extensive unstocked farm-land other the other side. The block had had no cattle grazing on it in the previous 2 years. The entire block is overlooked by home of the examiner, which enabled close monitoring of the calves. Calf handling facilities were constructed, including a 6m x 6m shelter shed providing protection from the prevailing winds (Figure 4B). Approximately half of the grazing area was irrigated throughout the trial periods in order to maintain green grass feed. The trial was undertaken during summer and autumn.
Figure 4A – Study location

Irrigated pasture block used as the study location.

Figure 4B – Calf Shelter

A six meter by six meter calf shelter and wood shavings as a bedding substrate. Providing a wind and rain shelter from the prevailing weather.

2.3.1.3 Feed and Handling

Calves were acclimatised to the property and handling facilities for 2 weeks prior to the start of the trials. Calves were free to move throughout the block, shed and handling facilities at all times. Free access to irrigated pasture formed the majority of the diet, however, three times a week, calves were fed 20kg of Heifer Developer Pellets (Barastoc) after being blood tested. Calves were examined for ticks on a daily basis.
2.3.2 Collection and storage of theilerial blood

A recumbent Angus steer exhibiting clinical signs of theileriosis (tachypnoea, tachycardia, pale mucous membranes, weakness), had a packed cell volume of 8% and piroplasms in a blood smear. Diagnosis was confirmed by PCR and the genotype was confirmed as *T. orientalis* Ikeda. The quantitative PCR (qPCR) of this stablate was 1678.99 femtograms/microlitre (fg/µL) (251,849 gene copy number) for universal *T. orientalis*, and 1570.78 fg/µL Ikeda genotype. This parasitaemia is considered in the clinically relevant range (Bogema, Deutscher et al. 2015).

Using a CPDA-1 single blood-pack Unit (Fenwal International Inc.), 250 ml of blood was collected from the jugular vein of this steer. Once collected, 2.5 ml blood was decanted into each of 5 ml cryotubes. These cryotubes were then filled with 2.5 ml of the cryopreservant polyvinylpyrrolidone (PVP 40,000; pH 7.2). High molecular weight PVP was used as it is nontoxic to tick borne parasites and less hazardous to humans than Dimethylsulphoxide (DMSO). This cryopreservant was supplied by the Tick Fever Centre in Wacol, Queensland.

The blood was then placed in the vapour phase of liquid nitrogen for 15 minutes before being lowered into the liquid nitrogen for storage.

A second inoculum of blood was obtained from a 6 month old calf, diagnosed by PCR as infected with *T. orientalis* Ikeda. This calf showed no obvious clinical signs of theileriosis apart from being much smaller than calves of the same age. One ml of blood was collected into each of three syringes containing CPDA-1 anticoagulant. This blood was not stored but was used immediately for inoculation into the 3 calves in group 2. The qPCR of this stablate was 1029.33 fg/µL (154,400 gene copy number) for universal *T. orientalis*, and 954.23 fg/µL Ikeda genotype. This parasitaemia is considered in the clinically relevant range (Bogema, Deutscher et al. 2015).
2.3.3 Transmission and monitoring protocol

Calves were randomly assigned to one of four treatment groups. Cryopreserved blood was thawed rapidly in a water bath at 37ºC for 5 min before being transfused via the jugular vein into the recipients. This fast thawing rate is based on studies looking at the optimal thawing rate for *B. bigemina*, which was less damaging and resulted in the shortest prepatent period (Dalgliesh and Mellors 1974).

Group 1 (Calves H1, H2, H3) were inoculated intravenously with 20 ml of inoculum containing 10 ml cryopreserved, infected blood. Based on the gene copy number of this inoculum, the total number of parasites transferred was approximately 2.5 billion. The 3 calves in Group 2 (Calves M1, M2, M3) each received 1 ml of fresh blood (approx. 154.4 million parasites). This fresh blood was collected in syringes containing 0.14 ml of anticoagulant (CPD-1) drawn up and mixed with one ml of blood collected from the jugular vein of the positive control calf. Group 3 calves (Calves L1, L2, L3) each received 0.2 ml of inoculum, containing 0.1 ml cryopreserved blood (approx. 25.2 million parasites). Group 4 contained 3 negative control calves and the positive donor calf. The 3 negative control calves acted as sentinel animals for active transmission by ticks and other possible vectors.

Calves were yarded, clinically examined and bled for PCR testing to ensure they were negative for *T. orientalis* prior to transfusion. Thirty minutes before inoculation of blood, each calf in treatment groups received 100 mg of the antihistamine chlorpheniramine maleate (Histamil) by intramuscular injection, to lower the risk of any transfusion reaction. Animals were monitored for an hour following infusion when a post-inoculation blood sample was taken, but adverse reactions were not observed.

To monitor the progress of the trial, calves were yarded daily. During these times, observations of behaviour, gait, appetite and respiration were made. To detect theilerial infection, blood was
collected from the tail veins of all calves into 5 ml EDTA blood tubes three times weekly (Sundays, Tuesdays, Thursdays) for 13 weeks. During these times, calves were hand fed (1.5 kgs Barastoc Heifer Developer Pellets) and rectal temperatures were also taken. Collected blood was frozen, and sent in bulk to be tested via qPCR for the presence of *T. orientalis* and genotype differentiation.

### 2.3.4 Blood storage and testing

Blood collected was immediately frozen and sent in batches to Elizabeth Macarthur Agricultural Institute.

qPCR was performed using a validated multiplex qPCR assay for *T. orientalis* detection and genotype differentiation as described previously (Bogema, Deutscher et al. 2015). This assay was used for quantification of the infection intensity of *T. orientalis* (Universal) and to semi-quantitatively determine the relative proportions of the Ikeda and Chitose genotypes (UIC assay). DNA extracts were also tested for the Buffeli genotype of *T. orientalis* using a semi-quantitative singleplex qPCR (B assay) as described by Bogema et al. 2015 (Bogema, Deutscher et al. 2015).

### 2.4 Colostral transmission

Thirty cows from a commercial dairy herd within the endemic region of Bairnsdale, Victoria were randomly selected. Colostrum was collected from each quarter into a single sterile container within 24 hours of giving birth. The samples were immediately frozen and transported for testing by qPCR in bulk for *T. orientalis* using the same assay as described previously. Samples were collected over a 17 day period (24/07/15 to 06/08/15).

Blood samples were collected from all dams who had colostrum tested 3-6 weeks after giving birth. Blood was collected into an EDTA and a plain tube from the tail vein. The calves of these cows, who received colostrum were tested at the same time as the cow when their ages ranged
from 3 to 6 weeks. Of the 30 calves born, only female calves were available at the time of blood testing as the male calves had been sold. A total of 19 female calves were available for testing.

2.5 Transplacental transmission

2.5.1 Sample collection

The same commercial dairy herd as used for the colostral sampling was used to investigate transplacental transmission. Over a 2 week period, thirty cows and their calves had 5 ml of blood collected into EDTA blood tubes and 5 ml of blood collected into a plain tube. Tail veins were used for sampling the blood from the cows, and jugular vein was used to collect blood from the new born calves. PCV was measured and samples were then frozen and sent for qPCR analysis from the EDTA blood samples as described previously. In addition, ELISA antibody testing was carried out on all dams and their calves using the clotted blood samples.

2.5.2 ELISA antibody testing

ELISA antigen preparation

ELISA assays were conducted at EMAI, Menangle, NSW. MPSP genes amplified from each of the *T. orientalis* Ikeda, Chitose and Buffeli MPSP types were cloned into the Champion pET100 D-TOPO expression vector (Invitrogen, Carlsbad, Calif., USA) according to the manufacturer’s instructions. Primers used for PCR amplification of the MPSP gene fragments were as follows (the CACC overhang required for directional cloning are underlined and the artificial stop codon for terminating translation is shown in bold): Ts-Bc: 5' CACC-TGC TCT GCA ACC GCA GAG 3', Ts-Cc: 5' CACC-TTC CTC ATC GTC TCT GCA ACT 3', Ts-Ic: 5' CACC-ATC GTC TCT GCT ACC GCC GC 3' and Ts-Rc: 5' CTA TGT GAG ACT CAA TGC GCC TA 3'. PCR products were amplified in 50 µL volumes using 1 × *Pwo* polymerase reaction buffer and 1U *Pwo* polymerase (Roche, Basel, Switzerland), 100 µM dNTPs and 10
µM primers. The Ts-Bc, Ts-Cc and Ts-Ic (forward) primers were each paired with the Ts-Rc (reverse) primer in amplification of the Ikeda, Chitose and Buffeli MPSP genes respectively. Antigen purification and dialysis was performed as previously described (Jenkins, Wilton et al. 2006). Antigen concentration was estimated using the bichinchoninic acid (BCA) assay (Thermo Scientific, Rockford, IL, USA). Stock antigens were stored at -20°C as an equimolar cocktail of the 3 MPSP antigens.

**ELISA assay**

ELISA plates (Linbro/ Titerette E.I.A Cat no. 76-381-04, MP Biomedicals LLC., Santa Ana, CA, USA) were coated with 1 µg of antigen in 100 µL carbonate coating buffer (pH 9.6) per well and incubated overnight at room temperature (22°C) in a humid chamber. All washing steps were performed as five washes with Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBST) using an automatic 96-well ELISA washer. After coating, plates were washed in TBST and then blocked with 1% bovine serum albumin (BSA) in TBS for 1 h. After washing with TBST, 100 µL of each test serum sample and control sera diluted 1:100 in 1% BSA-TBST were added to each well and incubated for 1 h. After washing in TBST, 100 µL of monoclonal anti-bovine IgG (clone BG-18 alkaline phosphatase conjugate, Sigma) diluted 1:20 000 in 1% BSA-TBST was added to each well and incubated for 1 h. The plates were washed and developed with BluePhos alkaline phosphatase substrate (KPL). Plates were read 610 nm (A610) on an xMark plate reader (Bio-Rad Laboratories, Hercules, CA, USA). Optical density (OD) at 610 nm was determined for each plate when the positive control to negative control serum had a mean OD610 ratio of >5 and the OD610 of the negative serum was < 0.15. Results were expressed as an ELISA ratio (ER: mean OD test serum/mean OD of the negative control serum). Sera with an ER < 2 were considered negative and an ER ≥ 2 as positive. These results were shown previously to give a specificity of 98% and sensitivity of 67% in comparison to PCR testing (Eamens and Jenkins 2013).
Chapter 3: Results

3.1 DNA Barcoding of ticks

A total of 81 tick COI barcodes were identified; 56 from ticks of the *Haemaphysalis* genus, 18 from *Ixodes* spp. and 7 from *Bothriocroton* spp. A phylogenetic tree constructed from a MUSCLE alignment of these sequences and GenBank reference sequences is shown in Figure 5. Tick barcode sequences clustered into 9 major clades, 8 with high (>97%) bootstrap support. Species identified included *H. longicornis*, *B. concolor*, *I. holocyclus*, *I. hirsti* and *I. cornuatus*. The remaining 4 clades identified also clustered within the genera *Haemaphysalis*, *Bothriocroton* and *Ixodes*, however these tick samples could not be confidently assigned to particular species. There was moderate bootstrap support for a relationship between *Haemaphysalis* ticks collected from koalas (*Phascolarctos cinereus victor*) and the species *H. doenitzi* and *H. humerosa*. Ticks collected from the common wombat (*Vombatus ursinus*) were of the *Bothriocroton* genus but formed a distinct cluster from *B. concolor* (the Echidna tick), and *B. undatum* and *B. hydrosauri*, which are typically found on reptiles. A further cluster of sequences forming a sister group to *I. hirsti* was identified from both koalas (*P. cinereus victor*) and brush-tailed possums (*Trichosurus vulpecula*). Tick sample 30-1, also collected from *T. vulpecula*, was most closely related to members of the *Ixodes* genus, but did not cluster with any other species for which sequence data was available.

Figure 5 - Phylogenetic tree of ticks investigated

Phylogenetic tree based on mitochondrial cytochrome oxidase I (COI) gene sequences of ticks collected in this study along with reference tick COI sequences sourced from Genbank. The phylogenetic tree is based on a MUSCLE alignment of the sequences in which gap positions were trimmed and was constructed using the Maximum Likelihood method based on the model of Tamura and Nei, within the program MEGA v6 (Tamura and Nei 1993). The tree with the highest log likelihood is shown. Bootstrap replications (1000) were performed, which are expressed on each node as a percentage. The scale bar indicates the number of nucleotide substitutions
per site. Accession numbers for references sequences are indicated. Sequences derived from this study were deposited in GenBank. Species (or clades) identified in this study are indicated on the right.
3.2 Identification of lice, flies and mosquitoes

Thousands of lice were collected and identified as the long-nosed sucking cattle louse *Lingognathus vituli*. Approximately seven thousand mosquitoes were collected on outbreak farms or within close proximity. The mosquitoes identified include; *Aedes camptorhynchus, Aedes notoscriptus, Coquillettidia linealis, Culex australicus,* and *Culex molestus*. The biting fly collected was the March fly (*Dasybasis sp.*) tested. Only 28 of these flies were collected and tested for *T. orientalis*.

3.3 qPCR results

3.3.1 qPCR results for mosquitos, lice and flies

Of the 13 pools of mosquitoes tested, one was weakly positive for *T. orientalis* (41 GC/µL), with only the Buffeli genotype identified over the limit of detection of the assay. Batch testing of the remaining mosquitoes also resulted in a weak positive (20 GC/µL) for *T. orientalis* with no genotypes exceeding the limits of detection for the respective assays. Biting flies (*n*=28) were found to be negative for *T. orientalis* and all genotypes, while all 14 pools of *Linognathus vituli* lice tested positive for *T. orientalis* Ikeda. Blood collected from the two lousy calves were positive for *T. orientalis* with infection intensities of 89.07 fg/µL (Calf 1), and 143.85 fg/µL (Calf 2). The average infection intensity from the batches of lice from calf one was 0.33 fg/µL (49.50 gene copies) for universal *T. orientalis* and 1.14 fg/µL (171.72 gene copies) for the Ikeda genotype. The average intensity from the batches of lice from calf two was 0.24 fg/µL (36.44 gene copies) for universal *T. orientalis* and 0.47 fg/µL (46.22 gene copies) for the Ikeda genotype. The infection intensities for Ikeda correspond to low range. All lice tested negative for the Chitose and Buffeli genotypes.
3.3.2 qPCR results for ticks

Amongst the ticks examined, only *H. longicornis* was found to be positive for theilerial DNA. As shown in Table 1, ticks sourced over a large geographical area were found to harbour all 3 genotypes of *T. orientalis* examined (Ikeda, Chitose and Buffeli). The Ikeda genotype was found in all pools of ticks testing positive for *T. orientalis*. The Buffeli and Chitose genotypes were found in all, and all but two, of the *T. orientalis*-positive pools respectively. Quantitative data indicated that parasite load was highest in ticks sourced from Won Wron and Yarram and in most cases, the Ikeda genotype was present in higher concentrations than either the Chitose or Buffeli types (Table 1).
Table 1 - Tick theilerial PCR results

<table>
<thead>
<tr>
<th>Tick Species or Clade</th>
<th>No. Ticks / Pool</th>
<th>Host – Location</th>
<th>qPCR result (GC/µL DNA extract)</th>
<th>T. orientalis</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle - Won Wron</td>
<td>8.7 × 10^3 + (7.4 × 10^3) - + (2.7 × 10^3)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle - Won Wron</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle - Won Wron</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle - Won Wron</td>
<td>1.5 × 10^4 + (7.1 × 10^3) + (2.9 × 10^3) + (4.5 × 10^3)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>6</td>
<td>Cattle - Won Wron</td>
<td>4.2 × 10^3 + (1.5 × 10^3) + (1.5 × 10^3) + (1.1 × 10^3)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>6</td>
<td>Cattle - Won Wron</td>
<td>1.0 × 10^4 + (4.6 × 10^3) + (1.8 × 10^3) + (3.3 × 10^3)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle - Warragul, Bairnsdale</td>
<td>1.9 × 10^2 + (6.3 × 10^1) + (1.0 × 10^2) + (3.5 × 10^1)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>5</td>
<td>Cattle – Yarram</td>
<td>4.1 × 10^4 + (3.6 × 10^4) - + (1.4 × 10^4)</td>
<td></td>
</tr>
<tr>
<td>H. longicornis</td>
<td>4</td>
<td>Cattle – Yarram</td>
<td>9.2 × 10^2 + (4.2 × 10^2) + (2.5 × 10^2) + (2.5 × 10^2)</td>
<td></td>
</tr>
<tr>
<td>H. doenitzi clade</td>
<td>5</td>
<td>Koala - Raymond Island</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>H. doenitzi clade</td>
<td>3</td>
<td>Koala - Raymond Island</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>I. holocyclus</td>
<td>6</td>
<td>Cattle – Bairnsdale Dogs – Bairnsdale</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>I. cornuatus</td>
<td>10</td>
<td>Dog – Bairnsdale</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>I. cornuatus</td>
<td>2</td>
<td>Wombat - Raymond Island</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>Ixodes sp. (clade 2)</td>
<td>5</td>
<td>Possum, Koala Raymond Island, Bairnsdale</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>Ixodes sp. (clade 1)</td>
<td>3</td>
<td>Koalas – Bairnsdale</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>Ixodes sp. (clade 1)</td>
<td>1</td>
<td>Possum - Fernbank</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>B. concolor</td>
<td>5</td>
<td>Echidnas – Heyfield</td>
<td>0 - - -</td>
<td></td>
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<tr>
<td>B. concolor</td>
<td>5</td>
<td>Echidnas – Heyfield</td>
<td>0 - - -</td>
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<tr>
<td>B. concolor</td>
<td>2</td>
<td>Echidnas -Bairnsdale, Orbost</td>
<td>0 - - -</td>
<td></td>
</tr>
<tr>
<td>Bothriocroton sp. Clade</td>
<td>3</td>
<td>Wombat- Bairnsdale</td>
<td>0 - - -</td>
<td></td>
</tr>
</tbody>
</table>

Collected tick species, location and results for universal T. orientalis and genotype specific qPCR
### 3.3.3 qPCR results for transplacental transmission

Table 2: Table showing PCV, ELISA and qPCR results for Universal as well as Ikeda, Chitose, and Buffeli genotypes for cattle and their calves within 24 hours of giving birth. Highlighted in bold are the abnormal PCV and ELISA results.

<table>
<thead>
<tr>
<th>Dam ID</th>
<th>Calf PCV</th>
<th>Dam PCV</th>
<th>ELISA ratio Dam</th>
<th>ELISA ratio Calf</th>
<th>Copy Number-Universal</th>
<th>Copy Number-Ikeda</th>
<th>Copy Number-Chitose</th>
<th>Copy Number-Buffeli</th>
</tr>
</thead>
<tbody>
<tr>
<td>1786</td>
<td>39%</td>
<td>36%</td>
<td>0.82</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1003</td>
<td>30%</td>
<td>37%</td>
<td>0.72</td>
<td>0.79</td>
<td>2237</td>
<td>2042</td>
<td>1546</td>
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</tr>
<tr>
<td>1519</td>
<td>48%</td>
<td>35%</td>
<td>0.69</td>
<td>0.33</td>
<td>4844</td>
<td>4228</td>
<td>2963</td>
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</tr>
<tr>
<td>1832</td>
<td>41%</td>
<td>38%</td>
<td><strong>2.34</strong></td>
<td>0.66</td>
<td>57304</td>
<td>96469</td>
<td></td>
<td></td>
</tr>
<tr>
<td>545</td>
<td>38%</td>
<td>38%</td>
<td>NS</td>
<td>0.91</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>781</td>
<td><strong>24%</strong></td>
<td>39%</td>
<td><strong>2.56</strong></td>
<td><strong>4.91</strong></td>
<td>14844</td>
<td>22753</td>
<td></td>
<td></td>
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<tr>
<td>771</td>
<td>32%</td>
<td>34%</td>
<td>0.49</td>
<td>0.46</td>
<td>4067</td>
<td>6365</td>
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<tr>
<td>639</td>
<td>48%</td>
<td>35%</td>
<td>0.57</td>
<td>0.53</td>
<td>10093</td>
<td>13628</td>
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<td>709</td>
<td>36%</td>
<td>37%</td>
<td>0.68</td>
<td>0.59</td>
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<tr>
<td>564</td>
<td>42%</td>
<td>34%</td>
<td>1.45</td>
<td>0.73</td>
<td>1854</td>
<td>958</td>
<td>522</td>
<td>1567</td>
</tr>
<tr>
<td>345</td>
<td>46%</td>
<td>40%</td>
<td>0.38</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>804</td>
<td>39%</td>
<td>38%</td>
<td>1.40</td>
<td>0.43</td>
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<tr>
<td>741</td>
<td>32%</td>
<td>36%</td>
<td>0.62</td>
<td>1.16</td>
<td>17601</td>
<td>17039</td>
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<tr>
<td>646</td>
<td>49%</td>
<td>41%</td>
<td>1.03</td>
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<tr>
<td>568</td>
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<td>31%</td>
<td>0.40</td>
<td>0.33</td>
<td>4118</td>
<td>178</td>
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<tr>
<td>717</td>
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<td>31%</td>
<td>1.15</td>
<td>0.54</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>538</td>
<td>40%</td>
<td>39%</td>
<td>0.57</td>
<td>0.34</td>
<td>7249</td>
<td>22121</td>
<td></td>
<td></td>
</tr>
<tr>
<td>788</td>
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<td>36%</td>
<td>0.92</td>
<td>1.53</td>
<td>22399</td>
<td>33938</td>
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<tr>
<td>794</td>
<td>50%</td>
<td>32%</td>
<td>0.62</td>
<td>0.59</td>
<td>50380</td>
<td>45103</td>
<td>27673</td>
<td>8430</td>
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<tr>
<td>726</td>
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<td>40%</td>
<td>0.47</td>
<td>0.47</td>
<td></td>
<td></td>
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<tr>
<td>702</td>
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<td>38%</td>
<td>1.01</td>
<td>1.15</td>
<td>28149</td>
<td>42783</td>
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</tr>
<tr>
<td>604</td>
<td>37%</td>
<td>31%</td>
<td>0.62</td>
<td>0.70</td>
<td>72049</td>
<td>82804</td>
<td>3904</td>
<td>1294</td>
</tr>
<tr>
<td>720</td>
<td>36%</td>
<td>36%</td>
<td>0.51</td>
<td>0.34</td>
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<tr>
<td>812</td>
<td>32%</td>
<td>39%</td>
<td>0.64</td>
<td>0.86</td>
<td>119475</td>
<td>178026</td>
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<td>658</td>
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<tr>
<td>630</td>
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<td>38%</td>
<td>1.21</td>
<td>1.57</td>
<td>16873</td>
<td>24225</td>
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<tr>
<td>758</td>
<td>42%</td>
<td>36%</td>
<td>0.49</td>
<td>0.34</td>
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</tr>
<tr>
<td>819</td>
<td>28%</td>
<td>30%</td>
<td>0.47</td>
<td>0.36</td>
<td>1720</td>
<td>1839</td>
<td></td>
<td></td>
</tr>
<tr>
<td>644</td>
<td>32%</td>
<td>37%</td>
<td>0.59</td>
<td>1.24</td>
<td>67887</td>
<td>84894</td>
<td></td>
<td></td>
</tr>
<tr>
<td>537</td>
<td>36%</td>
<td>29%</td>
<td>0.56</td>
<td>0.75</td>
<td>4743</td>
<td>57733</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
As shown in table 2, 63% of cattle tested positive for *T. orientalis* with Ikeda genotype accounting for 84%, Chitose 37% and Buffel 32% of infections. Thirty-six percent of infections were mixed infections. Out of the PCR positive cattle, only 2 dams and one calf tested ELISA positive for antibodies. Only one calf was anaemic from all of the samples collected, and this calf was positive for antibodies and had a mother also testing positive for both theilerial DNA (qPCR) and antibodies for *T. orientalis*.

### 3.3.4 Colostral transmission

Of the 30 colostrum samples collected 4 tested positive, and a further 11 had amplification but was below the diagnostic threshold of the assay (Bogema, Deutscher et al. 2015). In addition 6 samples of colostrum tested positive for theilerial antibodies. Of these 6 calves who received antibodies in colostrum, only one tested positive for antibodies. Of the 4 samples of colostrum that tested positive for *T. orientalis* no evidence of transmission to calves was demonstrated. No calves tested positive for *T. orientalis*. Sixteen of the 30 dams tested positive for *T. orientalis*. Of these 16 Dams, 67% were positive for Ikeda genotype only, 25% had a mixed infection of Ikeda and Buffel, and one dam tested for Chitone only as shown in Table 3.
Table 3- Colostral transmission trial results. qPCR and ELISA results on colostrum, and bloods from Dams and Calves.

KEY: I=Ikeda, C=Chitose, B=Buffeli, T=Threshold, +=Positive result, -=Negative result, N/A=Male calf not available to retest as they had been sold by the time of blood testing, Units=Gene copies/uL

<table>
<thead>
<tr>
<th>Cow ID</th>
<th>Colostrum Universal</th>
<th>Colostrum qPCR</th>
<th>Colostrum ELISA</th>
<th>Dam Universal</th>
<th>Dam ELISA</th>
<th>Calf qPCR</th>
<th>Calf ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td>783</td>
<td>0</td>
<td>-</td>
<td>+ 3.26</td>
<td>14638 I, B</td>
<td>1.86</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>733</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>698</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>&lt;T</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
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3.4 Mechanical transmission results

Eight of the nine inoculated calves became positive for theilerial DNA during the trial period. Calf H2, who was transfused 10 ml of infected cryopreserved blood became clinically ill within 2 hours of transfusion. The calf became lethargic and recumbent for approximately 16 hours, exhibiting tachycardia, tachypnoea, and pyrexia (40.7 ºC). This calf recovered but tested negative for theilerial DNA for the length of the trial.

3.4.1 High dose group

Calves in the high dose group (10 ml of cryopreserved blood) became positive for theilerial DNA at 28 days (4 weeks) following transfusion and remained positive until the end of the monitoring period (Figure 6). The maximum infection intensity from this group was 517.35 fg/µL (77602 gene copy number) for universal *T. orientalis*. This corresponded to 1446.36 fg/µL for the Ikeda genotype.

Figure 6 – Gene copy number per microliter for the high dose group at days post inoculation.
3.4.2 Medium dose group

Calves in the medium dose group (1 ml of fresh blood) all became positive at 41 days (almost 6 weeks) following transfusion and remained positive until the end of the monitoring period (Figure 7). The maximum infection intensity for this group was 113.06 fg/µL (16959 gene copy number) for universal *T. orientalis*. This corresponded to 152.58 fg/µL for the Ikeda genotype.

Figure 7 – Gene copy number per microliter for the medium dose group at days post inoculation.

3.4.3 Low dose group

Calves in the low dose group (0.1 ml of cryopreserved blood) also became positive (Figure 8). Calves L1 and L2 became positive at day 66 (9.5 weeks), while calf L3 was first positive at day 98 (14 weeks). The maximum infection intensity for this group was 12.58 fg/µL (1187
gene copy number) for universal *T. orientalis*. This corresponded to 17.24 fg/µL for the Ikeda genotype.

Figure 8 – Gene copy number per microliter for the low dose group at days post inoculation.

### 3.4.4 Control group

All negative control calves remained negative throughout the trial period and daily tick searches on all calves failed to detect ticks. The positive control calf remained positive throughout trial, although the infection intensity changed throughout the trial period (Figure 9).
Figure 9 – Gene copy number per microliter for the positive control calf over time.
Chapter 4: Discussion

A thorough knowledge of disease epidemiology is required to formulate rational and effective control measures to mitigate spread and limit production losses and deaths. To this end, this study reports the first intensive study addressing the active, passive and mechanical transmission of recently introduced *T. orientalis* in Australia. The epidemiology of the *T. orientalis* outbreaks in south-eastern Australia is largely unknown, although outbreaks in Victoria are believed to be linked to introduction of infected cattle from New South Wales to a farm near Seymour (Islam, Jabbar et al. 2011), at the periphery of the known range of *H. longicornis* (Figure 1).

Detection of infection: To date much of the diagnosis of theileriosis has relied in clinical signs and blood smears. The more sensitive PCR has been developed in response to increasing need to keep abreast of the expanding front of the infection. The analytical specificity and sensitivity of the *T. orientalis* multiplex UIC assay has been reported previously (Bogema, Deutscher et al. 2015). The analytical specificity of the semi-quantitative singleplex qPCR for Buffeli genotype testing (B assay) was confirmed using both plasmids containing MPSP genes and Buffeli positive and negative EDTA blood extracts. The limit of detection of the Universal, Ikeda and Chitose components of the UIC assay are 17 (8-45), 27 (8-90) and 20 (6-65) GC/µL respectively (Bogema, Deutscher et al. 2015). The limit of detection of the B assay used in this study was found to be similar at 20 (9-43) GC/µL. Samples yielding amplification above these levels are reported as positive (Table 1). The districts of Won Wron, Yarram, Bairnsdale and Warragul (Figure 1), are all areas in which clinical outbreaks of theileriosis have been recently reported (Perera, Gasser et al. 2013, Read 2013). In 2013, a serological and haematological survey found evidence of theilerial infection in 10 of 15 herds from these regions of Gippsland (Read 2013). The Ikeda genotype, which has caused clinical outbreaks in Australia (Kamau et al 2011, Eamens, Bailey et al. 2013, Perera, Gasser et al. 2013), was found in all pools of ticks
testing positive for *T. orientalis*. Quantitative data indicated that parasite load was highest in ticks sourced from Won Wron and Yarram and in most cases, the Ikeda genotype was present in higher concentrations than either the Chitose or Buffeli types (Table 1). Because pools of ticks were tested, this may equate to high levels of the Ikeda parasite in individual ticks or an increased prevalence of the Ikeda type within multiple ticks. These results are consistent with prior studies on blood samples collected from cattle in Eastern Victoria, in which all three of these genotypes were variably detected in disparate herds (Perera, Gasser et al. 2013, Read 2013).

**Vectors and intermediate hosts:** Prior evidence for the transmission of *T. orientalis* by *Haemaphysalis* ticks in Australia has been contradictory, but *H. longicornis* ticks sourced from both Japan and Australia have been shown experimentally to transmit the Japanese *T. orientalis* Ikeda genotype trans-stadially (Fujisaki, Kawazu et al. 1994). Indeed, the *H. longicornis* barcoding sequences obtained in this study suggest that the *H. longicornis* populations in Eastern Victoria are relatively homogenous and are closely related to those in Asia. Interestingly, the known range of *H. longicornis* in Australia closely mirrors areas in which outbreaks of clinical theileriosis have occurred, including an isolated population of this species in the south west of Western Australia (Figure 1). It could be presumed from the prior data that *H. longicornis* is a likely vector for *T. orientalis* Ikeda in Australia and the detection of *T. orientalis* Ikeda DNA in ticks from various regions in Victoria supports this view. Based on previous studies, the ability of *H. longicornis* to transmit *T. orientalis* Buffeli is less certain (Stewart, de Vos et al. 1987). It is noteworthy therefore, that the Buffeli genotype (as well as the Chitose genotype) was detected within *H. longicornis* ticks along with the Ikeda genotype. This study also revealed *H. longicornis* as the major tick species found on cattle in Victorian herds suffering recent clinical outbreaks, further implicating this species as a likely vector of bovine theileriosis. More extensive analysis of the proportions of the *T. orientalis* genotypes
within individual ticks would be of future interest to determine whether particular genotypes are selected during the tick phase of the parasite’s lifecycle.

Other *Haemaphysalis* and *Ixodes* spp. have recently been implicated in transmission of *T. orientalis* in the Eastern Hokkaido and Okinawa prefectures via molecular screening of tick species for the presence of the parasite (Yokoyama, Sivakumar et al. 2012). Interestingly, we did not detect theilerial DNA in any other tick species. New Zealand has experienced very similar outbreaks of *T. orientalis* linked to the Ikeda genotype since 2012 (Watts, Playford et al. 2015). While there are a number of potential vectors for transmission of *T. orientalis* in Australia, New Zealand only has one live-stock-infesting tick- *H. longicornis* (Heath 2015), making this the likely vector in New Zealand, and also increasing the likelihood of *H. longicornis* being the vector in Southern Australia.

**Passive transfer with infected blood:** The results of this study has shown that theilerial infections can be readily established after inoculation of fresh or cryopreserved, infected, bovine blood. Fresh blood was used as a medium dose (1 ml) in an attempt to determine if the cryopreservation process of the high and low dose transferred affected the infectivity of the inoculum. The relationship between inoculated blood volume and time to patency clearly indicated the inverse dose-response and that the cryopreservation process preserved the viability of the piroplasms. From the level of donor parasitism (around 2%), the transfer of around $10^7$ parasitised erythrocytes (in 0.1 ml) can establish a parasitosis with sufficient quantum to be detected and graded by qPCR as a low-level infection (Bogema, Deutscher et al. 2015). The diagnostic threshold or sensitivity of the test used in this trial was 30 gene copies per microliter of blood. This is equivalent to 30 parasites per microliter of blood. Amplifications below this threshold were considered negative and so the times post inoculation where calves became positive accurately show the dose-response relationship for infection.
Animals were kept under typical field conditions in an endemic region of Victoria during a time when the region experienced very low numbers of ticks. Animals were kept in typical conditions as opposed to housed pens to mimic the more strenuous physiological conditions experienced in the field versus pen studies. Daily tick searches throughout the trial failed to detect any ticks, and no active transmission occurred as demonstrated by the 3 control calves that failed to become positive for *T. orientalis*. The other calf which failed to become positive during the trial period was calf H2. This calf became lethargic soon after transfusion and developed pyrexia. The cause of these symptoms was not determined although it was considered to be a transfusion reaction. This calf was monitored closely but no treatment other than the antihistamine given prior to transfusion was given. This calf returned to normal within 24 hours and remained clinically normal throughout the trial period. This calf failed to become positive following transfusion possibly a result of parasite clearance resulting from the pyrexia following transfusion.

The peak infection intensity for each treatment group corresponded to a low to moderate infection. The genotype *T. orientalis* Ikeda was detected at each of these peak infection levels. This corresponds to the genotype present in the inoculum, and so in this trial no genotypic switching was observed. A number of studies have suggested that the dominant parasite population can change (Perera, Gasser et al. 2014, Jenkins, Micallef et al. 2015), even during persistent infection in cattle over several months (Kubota, Sugimoto et al. 1996). Vector and host immunological factors are thought to result in genotype switching and these are likely to play a role in parasite persistence within mammalian hosts and its transmission from tick vector (Kubota, Sugimoto et al. 1996, Jenkins, Micallef et al. 2015). Although genotypic switching was not observed in this study, it seems to be another mechanism for the persistence and spread of theileriosis.
**Risks of mechanical transfer:** Routine husbandry practices whereby blood is transferred between animals is a concern for the mechanical transmission of *Theileria* without the need of an intermediate host. Piroplasms could be transferred from one animal to another iatrogenically when vaccinating a mob of cattle, or giving medications to cattle by using the same needle between animals. Other examples of blood transfer between cattle could include contaminated castration knives, ear notching procedures, as well as injury sustained during yarding and transport of cattle. We have shown that *T. orientalis* can be transmitted though the lowest dose examined in this trial (0.1 ml), so that the risk of mechanical transmission through husbandry practices cannot be excluded. The risk of iatrogenic transmission would depend on the volume and parasitaemia of the blood transferred and on the ability of the parasite to survive outside a host before being inoculated into a susceptible animal.

As has been demonstrated for African trypanosomes (Desquesnes and Dia 2003), mechanical transmission through biting arthropods is also a possible risk factor for the transmission of *T. orientalis*. Hematophagous insects; including flies, lice and mosquitos as well as non-biological vector ticks could have the potential to transmit *Theileria*. Regurgitation of part of a previous blood meal or passive transfer of blood on mouth parts are possible modes of transmission depending on both the level of parasitaemia in the donor and the cumulative volumes inoculated or transferred by the biting arthropods. It has been shown that approximately 1.3% of gut content in one species of tick can be regurgitated during a blood meal (Connat 1991), leading to possible transfer for piroplasms. We detected DNA of *T. orientalis* in at least one pool of 100 mosquitos captured in close proximity to herds experiencing clinical cases of theileriosis. Blood meals examined from two species of mosquitos ranged from 1 nl to 6 µl (Konishi 1989), but it was not determined whether any was regurgitated while feeding on a second host. Two pools of mosquitoes were weakly positive for *T. orientalis*; however because these samples were close to the limit of detection of the
assay, only the Buffeli genotype was identified in one of the pools. Further research is required to confirm their role as possible mechanical transmitters, as has been demonstrated for lice in Japan (Fujisaki, Kamio et al. 1993). The number of haematophagous flies caught on the outbreak farms was too low to be conclusive, and lice should also not be ruled out as a possible vector, even though no herd in the outbreak regions investigated showed evidence of louse infestations at the time of theilerial diagnosis. An understanding of the epidemiology, including the identification of vector(s), mechanical and intermediate hosts, is essential to stem the outbreaks of this emerging disease.

The sucking louse of cattle, *Linognathus vituli* is a widespread parasite that has been shown to transmit *Theileria* to splenectomised calves when previously fed on infected cattle (Fujisaki, Kamio et al. 1993). We present the first evidence of *Linognathus vituli* being able to carry detectable amounts of *T. orientalis* Ikeda in Australia. Fujisaki (1993), artificially infected a calf with *T. orientalis* Ikeda by subcutaneous inoculation of a sporozoite suspension derived from *Haemaphysalis longicornis* (Kamio, Fujisaki et al. 1989). Around 100-200 female lice, *Linognathus vituli*, fed and were collected from this calf 37-41 days after infection when the parasitaemia ranged from 6.5-1.8%. After these lice were placed onto a negative calf for 18 days, theilerial piroplasms were detected in blood smears at 49 days after application. In a second trial, lice were fed on a calf from 83-91 days after inoculation of sporozoites when the parasitaemia ranged from 4.4-3.1%. These lice were transferred to another negative calf for 20 days and piroplasms were detected in this recipient after 33 days (Fujisaki, Kamio et al. 1993), a similar prepatent period to the high dose inoculum in the current study. However, the diagnostic sensitivity of the qPCR is around 30 gene copies ml⁻¹ blood while a 1% parasitaemia approximates to around 7000 gene copies ml⁻¹ and a lag period of around 10 days to detection (Eamens, Gonsalves et al. 2013). This would suggest that the prepatent period of the high dose inoculum in the present study would be around 38 days if blood smears were used for diagnosis.
While splenectomy undoubtedly facilitated the establishment of the louse-borne infection as did intravenous inoculation in our trial, both confirmed the ability to transfer the parasite with piroplasmic stages of *T. orientalis*.

The ramifications of mechanically-transferred parasitoses for the epidemiology of theileriosis are obvious if these could produce viable infections in tick vectors. This was shown in a second louse experiment (Fujisaki, Kamio et al. 1993) when around 30 nymphal stages of the tick *H. cornigera* were allowed to feed on the infected calf between days 52 and 60 after louse application and when the calf was parasitaemic. Thirty days after moulting, these ticks had developed sporozoites in their salivary glands (Fujisaki, Kamio et al. 1993), indicating the infectivity of mechanically transferred infection for tick vectors. By contrast, the lice were considered to be a mechanical rather than a biological vector (Fujisaki, Kamio et al. 1993), as dissection of the salivary glands and guts only showed piroplasms in the gut sections. This was elegantly demonstrated when lice on a known negative calf were collected and transferred to a rabbit which produced anti-bovine-immunoglobulin antibodies within 12-13 days after attachment, indicating that regurgitation is the likely mechanism for transfer of *T. orientalis* Ikeda by *L. vituli* (Fujisaki, Kamio et al. 1993). This conclusion was also supported indirectly by studies where mechanical transmission with male ticks was attempted but was not successful (Riek 1982). The amount of blood regurgitated by *L. vituli* was not calculated, but was sufficient to transmit infection. By comparison, female human head lice (*Pediculus humanus capitis*) ingest an average blood meal of 0.158 μL (Speare, Canyon et al. 2006), and they feed multiple times a day. Calculations of the blood volumes and the numbers of piroplasms transferred in these and the current study were considered too speculative to provide any meaningful comparisons.

*Stomoxys* (Diptera: Muscidae) are obligate blood-sucking insects that are known mechanical vectors for viral, bacterial, rickettsial, protozoal, and helminth pathogens (Baldacchino,
Muenworn et al. 2013). Blood can be inoculated from contents remaining on their mouthparts when injecting saliva prior to blood-sucking, but they are also capable of regurgitating digestive tract contents (Baldacchino, Muenworn et al. 2013). We failed to detect *T. orientalis* in biting flies in an outbreak region however further research in the role of biting flies is warranted as our sample size was relatively small.

Although we have shown the minimum infectious dose of *T. orientalis* is very low, the risk of mechanical spread from biting arthropods would be highly dependent on several principal factors to provide a sufficient quantum of piroplasms to initiate a detectable (and transferrable) infection. These would include the prevalence of infection in the herd and the parasitaemia of infected donors, the volume of blood inoculated into the naïve host, the parasite load from the previous blood meal and the number of biting arthropods. Finally, the ability of *T. orientalis* to remain viable between feeds and the subcutaneous inoculation of this blood would also affect the chances of transmission, as would the ability of flying arthropods (flies and mosquitoes) to find a recipient in a short period of time or to be transferred by contact (lice). The sexual phase of the *T. orientalis* lifecycle occurs within vector ticks, allowing genetic recombination to occur. The genetic diversity generated within the parasite population by passage through arthropod vectors is believed to be an important immune evasion mechanism in a number of apicomplexans including the theileriae (McKeever 2006, Walker, Katzer et al. 2006, MacHugh, Weir et al. 2011). However, mechanical transmission results in the direct transfer of haploid phase piroplasms from host to host, thereby bypassing the sexual phase of the lifecycle. The inability of the parasite to genetically recombine therefore would be expected to reduce overall diversity within parasite population. Thus, extensive mechanical transfer of the parasite would be expected to decrease the ability of the parasite to evade the host immune system. Therefore, since the parasitosis persists in the recipient, mechanical transmission may
help to explain the rapid spread of the parasite over large geographical areas. However, it is likely that biological vector ticks are essential to maintain virulence and pathogenicity.

**Vertical and passive transmission**: Given the small volume of blood required for the transmission of *T. orientalis* other biological modes of transmission should be considered. One such method is the transmission between dams and their calves. Transplacental transmission has been demonstrated on a small scale with calves and dams tested by PCR, and were found to be positive following artificial transmission of *Theileria* to the dams (Baek et al, 2003). Another study supporting intra-uterine transmission found 1 or 2 day old calves were microscopically positive for *Theileria*-like organisms with a parasitaemia levels between 0.01 and 0.06% (Onoe, Sugimoto et al. 1994). This study failed to support this finding. Despite nineteen of the thirty cows (63%) tested positive for *T. orientalis* no calf was positive within 24 hours of birth. Interestingly, only 2 of the PCR positive cows produced antibodies for *Theileria* and of those, only one transferred these antibodies to the calf- presumably via colostrum. The calf that tested positive for antibodies via ELISA testing was the only calf who had a subnormal PCV of 24%. Anaemia at birth is categorized as a PCV of less than 25% (Radostits, Done et al. 2007, Ramin, Asri-Rezaei et al. 2014). These abnormal results are highlighted in Table 2. The lack of antibodies (ELISA ratio >2), in clinically normal cattle suggests no humoral immunity, and so it is possible these infected cattle mount a cell-mediated responses or did not suffer cellular breakdown that would expose Theileria to the host immune system. Further research is required into immune responses to *T. orientalis*, as a small scale trial by Glassop and Kerr showed a high level of antibody production in cows that were recently infected (Glassop and Kerr 2013), which is in contrast to this endemic herd. The ELISA used in this study is the same as that used by Glassop and Kerr, and detects antibodies against the major piroplasm surface protein, so unless there is an antibody response to that protein, the samples will test negative. Although this protein is immunodominant, and is very highly
expressed, it is possible that not all animals seroconvert to MPSP. It seems that in newly infected herds an IgG response dominates, but over several months, serum ELISA titres decline in the absence of re-infection (Eamens et al 2013).

Blood testing for this part of the study also highlighted the prevalence of the genotypes in this herd. Of the 63% of cattle who tested positive for universal *T. orientalis*, the Ikeda genotype was evident in 84%, 37% Chitose, and 32% Buffeli. Mixed infections were seen in 36% of positive cases. Of the mixed infections 29% were Ikeda-Chitose, 14% Chitose-Buffeli, 14% Ikeda-Buffeli, and 42% had mixture of Ikeda-Chitose-Buffeli. This dairy herd was previously involved as one of 5 herds in the Bairnsdale region prevalence study (Perera, Gasser et al. 2015). This study showed that this region in Gippsland has the highest prevalence (63.8%) of *T. orientalis* in south-eastern Victoria (Perera, Gasser et al. 2015). Two years after the blood for this study was taken, this study show the prevalence remains similar at 63%. This may indicate that the parasite is now endemic and has reached a steady state.

Aside from transplacental transmission which appears to occur from aborted foetuses that were diagnosed as parasite positive (Baek et al 2003), transmission could conceivably occur through blood transfer in colostrum. Private veterinary practitioners are commonly witnessing morbidity and high mortality in calves aged between 6-14 weeks of age on properties where theileriosis has previously been diagnosed (Eastwood 2013). This is consistent with the pre-patent period for *T. orientalis*. This would suggest that calves may be becoming infected at or soon after birth. Given the typical husbandry practices of farms (calves reared in sheds) it is unlikely that there is exposure to ticks at this stage of life. Calves born in winter also can show infection at around 6 weeks of age- a time of the year where ticks and other biting arthropods are rarely seen. Given these observation, colostral transfer is a possible mode of transmission within 24 hours of birth.
This study is the first to report qPCR analysis results of *T. orientalis* in colostrum. We have shown that colostrum can contain significant quantum of parasites as determined by qPCR analysis. Of the 30 samples of colostrum tested, only 4 contained significant amounts of *T. orientalis*, and calves receiving this colostrum within 6 hours of birth failed to become positive. From the samples collected, on this commercial dairy farm, 53% of cows tested positive for *T. orientalis*. Erythrocytes are commonly present in colostrum within the first 24 hours from birth, however the fact that only a small number positive dams contained *T. orientalis* in their colostrum is likely due to the fact that the negative colostrum samples simply did not contain blood.

The separation of the maternal and foetal blood supply by the syndesmochorial placentation in cattle prevents transfer of macromolecules into the foetal circulation. However, immunoglobulins, cytokines, leukocytes, and functionally viable maternal cells (possibly including memory T cells), have all been demonstrated to be transferred via colostrum into the neonatal bloodstream (Donovan, Reber et al. 2007, Reber, Donovan et al. 2008). Parasitised red cells could potentially transfer into a calf in the first 24 hours of life, releasing piroplasms. The capacity for ingested piroplasms to transfer into recipient erythrocytes is not known, but macroschizonts of *T. parva* within cultured lymphoblasts can transfer into recipient lymphocytes with an efficiency of around 10^-5 when inoculated into fully allogenic (unrelated) recipients (Emery, Morrison et al. 1982). Therefore it may be possible for other life stages of *T. orientalis* to be transmitted via colostrum. Although no calves tested positive for *T. orientalis* when tested at 3-6 weeks of age, the number of positive colostral samples transferred to calves in this trial was too low to rule out the possibility of colostral transmission.

The research is the first to examine the presence of antibodies in colostrum and the possible transfer to calves. Six of the 30 samples contained *T. orientalis* antibodies. As shown in table one, only two cows tested ELISA positive. This is likely a result of colostrum containing higher
concentrations of immunoglobulins than serum, as demonstrated in a number of studies (Beer, Billingh.Re et al. 1974, Baumrucker, Burkett et al. 2010, Jenvey, Weir et al. 2015). One of the 30 cows tested was positive for *T. orientalis* in both serum and colostrum. This cow also contained antibodies in both serum and colostrum, and was the only cow to have a calf test positive for *T. orientalis* antibodies. Although the numbers investigated in this trial was small, it nevertheless shows that antibodies can be transmitted via colostrum to calves, however this transfer of immunity seems to occur infrequently. Erythrocytes are commonly present in colostrum, and within the first 24 hours from birth, parasitised red cells could potentially transfer into a calf, releasing piroplasms.

**Chapter 5: Conclusion**

This thesis presents the first data identifying DNA from *T. orientalis* in *H. longicornis* from herds with recent outbreaks of clinical theileriosis. This adds to the body of international evidence for its role as an intermediate host in the life cycle and transmission of the parasite. Further studies are required to demonstrate the sporozoite stage of the parasite within *H. longicornis* and to confirm transmission of the various *T. orientalis* genotypes from *H. longicornis* to cattle.

*T. orientalis* can be transmitted by low volumes of blood transferring from infected to naïve animals. Although this mode of transmission does not appear to result in significant disease, parasitaemia can persist and allow for low-grade carriers of the parasites to occur within herds. This raises the possibility of iatrogenic transmission through husbandry practices and the likelihood that biting arthropods can act as mechanical vectors as distinct from obligatory intermediate hosts. Mechanical transmission might help to explain the epidemiology of the rapid spread of *T. orientalis* and associated clinical outbreaks in southern Australia and elsewhere.
Colostral transmission could play a role in disease transmission, while transplacental transmission does not seem likely. This study is the first to report *T. orientalis* in colostrum, and also the first to show antibodies in colostrum. However, the transmission of immune defences from dam to calf via colostrum seems to be poor.

**BIBLIOGRAPHY**


