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Prophylaxis and treatment of *Theileria orientalis*

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Abstract

Project P.PSH.0832 aimed to confirm; 1. the vector competence and transmission of *Theileria orientalis* ikeda by the 3-host tick *Haemaphysalis longicornis*; 2. whether chemotherapy could cure the carrier state in recovered cattle and, 3. whether mechanical infection with piroplasms of *T.orientalis* buffeli could protect against sporozoite challenge with *T.orientalis* ikeda.

Stabilate produced from infected *H.longicornis* salivary glands or ground up ticks both produced detectable infections with *T.orientalis* ikeda in naïve calves within 28 days after inoculation. This confirmed its vector competence and final host status.

Elimination of the carrier state in recovered cattle could not be achieved with buparvaquone (BPQ), Imidocarb, tulathromycin or oxytetracycline. While BPQ significantly reduced parasitosis over the first 2 weeks after administration, which offers clinical therapeutic benefits, *T.orientalis* ikeda DNA was still detectable by PCR, some 2 months later.

Inoculation of *T.orientalis* buffeli blood either intravenously or subcutaneously significantly reduced the first peak of parasitosis when calves were challenged with nymphal *H.longicornis* infested with *T.orientalis* ikeda. This result confirms speculation that carrier cattle possess some “premunity” that protects against the severity of repeated, seasonal tick challenges.

Executive summary

The intracellular protozoal parasite *Theileria orientalis* has rapidly spread across South-eastern Australia, substantially impacting local cattle industries since 2006. NSW-DPI have estimated an average cost of \$59K for dairy producers and \$11.6K for beef producers, which equates to AUD \$131/head for dairy cattle and AUD \$67/head for beef cattle for farms impacted by the parasite (Bailey 2012); all costing around \$20m pa nationally (Lane et al., 2015). Interestingly, several studies from Australia and New Zealand have indicated that the carrier state arising in recovered dairy cattle did not compromise subsequent productivity (Perrera et al., 2014; Lawrence et al., 2019).

Consequently, the Industry would benefit substantially from measures to reduce the impact of the initial infection either by means to control the vector or the early stages of the pathogenesis of the infection. For *Theileria parva*, the cause of East Coast Fever in Africa, this is achieved by the infect & treat (I&T) method, using long-acting oxytetracycline co-administered with *T. parva* stabilate, which attenuates the early stages of the infection (Radley et al., 1975a,b; Di Giulio et al., 2009). For the biological vector *Rhipicephalus appendiculatus*, constant dipping is required.

Current control measures for *T. orientalis* advocate exclusion of movement of naïve stock (from non-endemic regions) into endemic regions while current treatment of clinical theileriosis in Australia is limited to supportive therapy. The naphthoquinones, parvaquone and buparvaquone (BPQ), and the febrifuginone, halofuginone lactate, will cure clinical disease resulting from infection with *T. annulata* or *T. parva* (Schein & Voigt, 1979; McHardy, 1991). However, these are not registered for clinical use in Australia. The possible registration of BPQ is opposed by the grass-fed beef industry due to concerns about tissue residues, demonstrated in MLA project B.AHE.0194. Although mortality remains relatively low in endemic regions, naïve animals, including calves and introduced stock develop disease around 5-6 weeks after birth or entry. In endemic zones, recovered cattle remain asymptomatic carriers for at least 30 months, running the risk of tick infestation (Skilton et al., 2002). Recrudescence of clinical disease may be induced by transport stress as has occurred in the past with movement of cattle from coastal NSW to Narrabri and Terry Hie Hie, but carrier cattle calve successfully on their home farms.

This research project aimed to clarify:

- confirmation, vector competence and transmission by a/the biological vector;
- whether chemotherapy would “cure” the carrier state and diminish chances of relapse and transmission to ticks;
- whether mechanical infection with piroplasms could protect against sporozoite challenge (like the Babesial vaccines, MLA project B.AHE.0076); and,
- whether natural infection or mechanical immunisation could protect against sporozoite challenge with homologous or heterologous genotypes (or can immunisation with “ikeda” or “buffeli” protect against other pathogenic genotypes, eg “ikeda”).

The life cycle of the 3-host tick, *Haemaphysalis longicornis* was substantiated, with the entire generational life cycle of *H. longicornis* completed over ca. 4 months on dogs and cattle (Marendy et al., 2019). Feeding times for each stage ranged from 3-7 days. This “bush tick” was confirmed as the main biological vector for *T. orientalis* ikeda in the endemic regions (Hammer et al., 2015; Marendy et al., 2019), by means of trans-stadial transmission (infected larva to unfed nymph; infected nymph

to unfed adult). This was done by 2 methods. Firstly, stabilate was produced from salivary glands or whole ikeda-infected *H. longicornis* (“ground-up tick supernate” or “GUTS”) and injected subcutaneously into naïve animals, and secondly, infected, unfed nymphs and adult *H. longicornis* were secured under patches on naïve animals. Both methods induced PCR positivity for *T. orientalis* ikeda as well as clinical signs (decreased haematocrit and parasitaemias on blood smear). Like *T. parva*, sporozoites of *T. orientalis* also required around 3 days of tick feeding to mature before infection commenced. Depending on dose, recipients became PCR-positive within 10-28d, while haematocrit (PCV) declined and parasitaemias in blood smears were detected after 4 weeks of infestation. While it was initially anticipated that stabilate could be produced in sufficient quantities to conduct vaccine trials (akin to those used for *T. parva* in Africa), it was discovered that infection rates in *H. longicornis* were much lower than those for *T. parva* in *Rhipicephalus appendiculatus*. This precluded production of large batches of stabilate and experimental trials resorted to tick applications for infection and challenge. In additional studies, it was confirmed that while *H. longicornis* effectively transmitted *T. orientalis* ikeda, it was a less successful vector for *T. buffeli*, which is transmitted by *H. bancrofti* and *H. humerosa* (Stewart et al 1987a,b).

In a study at Gloucester (Swilks et al., 2017), naïve calves readily succumbed to infection within 1-2 months after birth or introduction to this region of endemic Theileriosis. In a parallel study on local calves and introduced cattle in this project at Dorrigo, similar kinetics of infection with several Theilerial genotypes were observed. Results indicated that infections with the virulent genotypes (ikeda and chitose) occurred earlier with maximal parasitosis around 5-6 weeks before declining, whereas parasitosis with *T. orientalis* (buffeli) was detectable in an increasing proportion of animals up to 3-4 months before stabilising. All animals remained carriers of all genotypes in the Ausdiagnostcs PCR kit for at least 6 months and >80% of ticks sampled from Dorrigo and Stroud contained DNA from 4 Theilerial genotypes (ikeda, chitose, Type 5 and buffeli).

In approaches to “cure” the carrier state, calves inoculated intravenously (i.v.) with blood stabilate (J36- ikeda) became PCR-positive over the next month before being allocated to treatments with BPQ, Imidocarb, long-acting oxytetracycline or tulathromycin (Draxxin®). A limited use permit was obtained for BPQ in April 2019 (PER85797). Only BPQ significantly reduced the parasitism as measured by reductions in parasite DNA copies over the following 3 weeks but this chemical did not eliminate the parasite over the following 3 months as determined by PCR. Two calves died from anaphylaxis following iv administration of infected blood, so that subcutaneous administration was tried and was also successful in producing detectable parasitosis (by PCR) in 3-4 weeks. With the delay with registration of BPQ, the availability of an alternative effective drug would have significant benefit for producers with clinical outbreaks or infections in valuable stock.

The second approach to diminish the impact of *T. orientalis* infection in naïve cattle and calves examined “immunisation” with buffeli-infected blood or ticks. Buffeli blood i.v. produced parasitosis by 4 weeks. Since *H. bancrofti* had a proven record for transmission of *T. orientalis* buffeli, (Stewart et al., 1987), infection was attempted. When these larval ticks failed to feed and died, infection was attempted with buffeli-infected, *H. longicornis* nymphs. When infection was not detected after 5 weeks, buffeli blood was given subcutaneously, producing parasitosis in 3-4 weeks. Following challenge with 200 ikeda-infected *H. longicornis* nymphs, infection was detected by PCR by day 13 after tick application. From 3 weeks after challenge, the pre-infestations significantly reduced the parasitosis over the ensuing 4 weeks by more than 80%, confirming some evidence from Japan and

Korea. This study is the first confirmation of speculations that the carrier state persisting in cattle which have recovered from the initial parasitosis with *Theileria* genotypes, establishes some type of “resistance” to subsequent, seasonal tick challenge. As suggested by de Vos (2011), this result would require confirmation in the field to determine dose rates and confirm efficacy and reliability. The procedure could be used for introduced stock (“pre-immunised” before transport into endemic zones). It would not be suitable for calves born in endemic regions as the infestation with virulent *Theileria* genotypes occurs too quickly. Introducing carrier animals to a region free from *T. orientalis*, but potentially harbouring a competent vector, would also amount to inadvertent introduction of this pathogen to a naïve population.

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1 Background

1.1 Theileriosis in Australasia

1.1.1 Impact and spread of the parasite- significance for the Industry

The intracellular protozoal parasite *Theileria orientalis* has rapidly spread across South-eastern Australia, substantially impacting local cattle industries since 2006. Given that *Theileria buffeli*, causing “benign theileriosis” had been present in Queensland since 1912 (Standfast et al., 1992; Stewart et al., 1996), the new syndrome was termed “Bovine Anaemia *Theileria orientalis* genotype” (BATOG; Izzo et al., 2010), until molecular techniques enabled discrimination of the *T. orientalis* genotypes and identified genotypes “ikeda” and “chitose” as those causing clinical disease (Kamau et al., 2011; Eamens et al., 2013a,b; Bogema et al., 2015). Historically, there was widespread taxonomic confusion regarding various Asian/Australasian Theilerial parasites. However, based on morphological and serological data and results from transmission experiments, it has been determined that all members of the *T. sergenti*, *T. buffeli*, and *T. orientalis* group be classified as a single species, *T. orientalis* (Stewart et al. 1996). The basis for the current classification uses the major piroplasm surface protein (MPSP), which is expressed in the intraerythrocytic stage of *T. orientalis* and conserved to some extent among different geographic isolates. It has been widely used for molecular epidemiological studies of *T. orientalis* and genetic diversity of *T. orientalis* in Japan (Ota et al., Sivakumar et al., 2012) Korea (Park et al., 2017), Kenya, and Australia (Kamau et al., 2011; Bogema et al., 2015). Currently, 11 genotypes of *T. orientalis* (type 1 or Chitose, type 2 or Ikeda, type 3 or buffeli, types 4–8, and N1-N3) had been identified based on MPSP gene sequences (Sivakumar et al. 2014; Bogema et al., 2015). Of these genotypes, 1 and 2 cause the majority of clinical disease in cattle.

Early studies indicated that *T. orientalis* prevalence was high in New South Wales (NSW, 23.7%), Queensland (Qld., 56.8%) and Victoria (Vic.), 34.0%, with variability among regions of each state. Genotype chitose was the most common and widespread type (19.1–43.7% per state), with buffeli present in all states at a lower prevalence (10.8–24.8% per state) when prevalence studies were performed by Eamens (2012) and Eamens et al. (2013a), similar to results reported by Kamau et al. (2011). Since its early detection, this parasite has spread through Victoria and across to Western Australia (WA) and east into New Zealand (NZ; Hammer et al., 2015; McFadden et al., 2011). NSW-DPI have estimated an average cost of \$59K for dairy producers and \$11.6K for beef producers, which equates to AUD \$131/head for dairy cattle and AUD \$67/head for beef cattle for farms impacted by the parasite (Bailey 2012), with similar losses reported from Victoria (Perrera et al., 2014). The total cost of Theileriosis was estimated at around \$20m pa nationally (Lane et al., 2015). Interestingly, several studies have indicated that the carrier state arising in recovered dairy cattle did not compromise subsequent productivity (Perrera et al., 2014; Lawrence et al., 2019).

Consequently, and since the effects of infection with virulent genotypes of *T. orientalis* on production is reported as minimal once cattle have survived the first acute phase of the infection by 6-8 weeks and entered carrier status (Lawrence et al., 2019), this research was directed towards blocking transmission of infection to naïve animals by either curing the carrier state or immunising naïve cattle. This was also influenced by the unavailability of buparvaquone (BPQ) for treatment in Australia. For tick control, the use of acaricides and integrated parasite management (IPM) has been

developed for effective control of *Rhipicephalus microplus* in Queensland, but is untried for management of 3 host ticks like *H. longicornis*. There is a concerted effort for new acaricides in this area, especially the isoxazolines so effective against ticks in companion animals, but external to the scope of this project.

1.1.2 Pathogenesis and transmission of Theileriosis in cattle

A thorough knowledge of transmission and pathogenesis underscores our ability to formulate rational control measures for disease, microbial or parasitic. For theilerial transmission and pathogenesis, sporozoites of *T. orientalis* inoculated from tick salivary glands during feeding 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 infestation, but are not responsible for the pathology associated with the infection, unlike other species of *Theileria* such as *T. parva* (Kawazu et al., 1991). Maturation of schizonts results in uninucleate merozoites which then escape from parasitised leucocytes and invade erythrocytes where the parasite develops into piroplasm. The invasion of red blood cells by merozoites takes place about 8-10 days post inoculation. The ensuing rounds of asexual reproduction are responsible for the febrile episodes and the clinical manifestations of *T. orientalis*, including the signs associated with anaemia (pale mucous membranes, icterus, tachycardia, tachypnoea, weakness; Izzo et al., 2011; Hammer et al., 2016; McFadden et al., 2015). Parasitosis in red cells was detected in blood smears 12-16 days after tick application for *T. orientalis buffeli* (Stewart et al, 1987), and approximately 20 days after ticks were seen for *T. orientalis ikeda* (Izzo et al., 2011), with variations in timing attributed to the quantum of infection. Consequently, clinical disease may not occur until around 4-6 weeks after infection, well after ticks had fed and gone. However, the timing is consistent with reports describing the introduction of susceptible stock into endemic areas (Eamens et al., 2013) and observations with calves (Hammer et al., 2015; Swilks et al., 2017).

While mechanical transmission with even small amounts of infected blood (0.1ml) results in detectable parasitosis (Hammer et al., 2016), trans-uterine and colostral transmission have also been suspected but not proven unequivocally (Hammer et al., 2016; Swilks et al., 2017). In Korea, intra-uterine infection with *T.orientalis [sergenti]* occurs readily, but does not protect against field challenge after birth (Minami et al., 1981; Onoe et al., 1994).

Sexual reproduction occurs in the final host, the tick. Since inoculation of parasitised bovine blood produces parasitosis but not clinical disease (Hammer et al., 2016), sexual reproduction in the tick may be required to restore or retain virulence. Since the 3-host tick, *H. longicornis* is one final host for *T. orientalis [ikeda]* (Heath, 2015, Marendy et al, 2019) and is widely distributed in Australia and NZ, breaking theilerial transmission requires attention to the vector. As protozoan parasites in 3-host ticks are transmitted trans-stadially, new infestations must be acquired for each new generation of ticks. The earliest infection occurs during feeding of larval ticks, so that only unfed nymphs and adults can transmit the infection. In this respect, it is not known if infected nymphs feeding on uninfected (second) hosts can retain infectivity through to the moult to the adult stage. Research in this project has also indicated that unfed, nymphal and adult ticks infected as larvae or nymphs, respectively, are variably PCR positive after the moult, but strongly positive for *Theileria* after 3 days of feeding on uninfected cattle. This need for 3-4 days to mature sporozoites is reported for *Ixodes scapularis* (Eisen, 2018) and for *T. parva*, where infected ticks are fed for 4 days on rabbits prior to

stabilate production (Kimbata & Silayo, 1997; Konnai et al., 2007). This also means that an ability to kill ticks within 3 days of attachment may block sporozoite transfer. However, to undertake infection and immunisation studies to alleviate clinical Theileriosis, vector competent ticks had to be definitively identified, grown and infected, so that either stabilate or infected ticks could be applied to cattle.

H. longicornis can complete its life-cycle on dogs and cattle in around 4 months under optimal conditions (Heath, 2015; Marendy et al., 2019), but seasonal factors extend the generational cycle to an approximately annual cycle, with larvae, nymphs and adults occurring around Autumn, winter and Spring, respectively. This coincides with peaks of infection in Victoria occurring around late Autumn and Spring, but also related to calving periods of stress (Jade Hammer, pers. comm). In other endemic regions like Dorrigo and Barrington, Theilerial infestations may occur throughout the year.

1.1.3 Immunisation against *Theileria orientalis* virulent genotypes

Some type and level of immunity exists in cattle which have recovered from the “first wave” of parasitosis which can produce clinical disease, but which usually slowly resolves around 2-3 months after infection (Jenkins et al., 2015, Perera et al., 2014). Recovered animals enter a persistent carrier state (Hammer et al. 2016). These cattle in endemic zones appear not to suffer recrudescence upon further seasonal tick challenge, so some form of “premunition” (Neal et al., 1969) interferes with the severity of ongoing challenge infestations.

For approaches to induce immunity against virulent genotypes of *T. orientalis* (ikedada and chitose), *T. parva* was used as the exemplar. For this parasite, cell-mediated immunity against the intracellular schizont stage was appreciated as the major protective immune response (Emery et al., 1982) and neutralising antibody also blocked sporozoite attachment (Nene & Morrison, 2016). Because of the requirements to induce cytotoxic T-lymphocytes for protection, active infection is needed, so attenuated vaccines have been the only approach inducing substantive protective immunity against *T. parva* (Radley et al., 1989; Emery et al., 1982). Similar approaches for *T. orientalis* would offer the greatest chances of success. A role for antibody appears less likely given that calves birthed from carrier dams develop clinical disease with 6 weeks of age despite receiving antibodies against piroplasmic antigens in colostrum (Swilks et al., 2017; Jenkins & Bogema, 2016; Hammer & Emery, unpublished).

Immunisation by intentional infection of cattle with blood containing *T. orientalis* was first reported in Japan in 1962 (Minami et al., 1981). A cryopreserved vaccine containing 2×10^8 infected red blood cells per dose “had an inhibitory effect on the clinical manifestation of theileriosis” with a need for proliferation of the inoculum (Ishihara, 1962) but this was not developed further. Production of an attenuated whole blood vaccine against *T. orientalis* [sergenti] occurred in Korea but outcomes were not reported and challenge appeared to use blood stabilate (Baek et al., 1992). Later, sonicated *T. orientalis* [sergenti] merozoites produced significant reductions in parasitosis after 3 months, among recipients receiving 2 doses of 100mg in complete Freund's adjuvant subcutaneously, 1 month apart, and subjected to field infestation from 2-5 months after initial vaccination (Baek et al., 1992). Unfortunately the trial was terminated after 5 months as all controls and 20% (4/20) vaccinates required treatment with diminazene (Berenil®) for anaemia (Baek et al., 1992). A recombinant MPSP

vaccine produced antibody but was not protective against field challenge with *T. orientalis* [sergenti] (Park et al., 1999). Further trials with recombinant antigens were summarised by Onuma et al. (1997) and research has been discontinued.

Given the trials in Korea and Japan, the long-standing presence of *T. orientalis* buffeli in Queensland and the lack of cases of *T. orientalis* ikeda in that State, a causal association was proposed (de Vos, 2011) and was investigated in this project. Considering that for *T. orientalis*, the merozoite stage in red cells was the major source of asexual reproduction (in contrast to the schizont stage for *T. parva*), it was speculated that immunisation with infected blood could protect against homologous or heterologous tick challenge, or perhaps complete infection with sporozoites was also needed. Inoculation of bovine blood containing *T. orientalis* ikeda induced a dose dependent parasitosis over time (Hammer et al., 2016). If infected blood proved protective in pen and field trials when this mode of immunisation did not cause clinical disease (Hammer et al., 2016), then combination with Babesial vaccines could be feasible.

1.1.4 Overarching aims of the Project

As presented below, the principal aims of the current project were to break transmission of Theilerial infection by a combined approach to prevent tick infection by resolving the carrier state in recovered animals and to reduce the severity of tick infection with virulent genotypes by prior immunisation with blood stages of the parasite or *T. orientalis* buffeli.

2 Project objectives

2.1 Aims of P.PSH.0832

The research in project P.PSH.0832 aimed to clarify:

1. vector competence and transmission by a/the biological vector (presumably *H. longicornis*);
2. whether chemotherapy would “cure” the carrier state and diminish chances of transmission and relapse; and,
3. whether mechanical infection with piroplasms could protect against sporozoite challenge (like the Babesial vaccines, B.AHE.0076); and,
 - whether natural infection or mechanical immunisation could protect against sporozoite challenge with homologous or heterologous genotypes (or can immunisation with various Theilerial genotypes such as “ikeda” or “buffeli” protect against other pathogenic genotypes, e.g. “ikeda”).

Successful outcomes were to be adopted into control measures on-farm.

All studies in the research project were conducted in accordance with approvals from the University of Sydney Animal Ethics Committee permits 2018/1328 and 2019/1517.

3 Methodology

3.1 Studies on *H. longicornis*

3.1.1 Life cycle studies and production of infected ticks

Engorged adult female *H. longicornis* ticks were sourced from Stroud NSW and eggs produced were sent to Elanco, Kemps Creek NSW. After hatching, half of the larval ticks were sent to the Queensland Department of Agriculture and Fisheries (QDAF), Biosecurity Sciences Labs (BSL), Coopers Plains, Qld. Subsequently, larval, nymphal and adult ticks were permitted to engorge on cattle (BSL) and dogs (Elanco) in pens or controlled environments and the time to “drop-off” was noted. The times and conditions for optimal survival from both egg hatching and stage-specific moults were determined under differing temperatures and humidities *in vitro* (Marendy et al., 2019).

For the production of ticks infected with genotypes of *T. orientalis*, batches of 200 (nymphs) or 1000 larval ticks were placed under calico patches which had been glued onto the backline of specifically infected, splenectomised (SplX) steers at the Tick Fever Research Centre (TFRC), Wacol, Qld. Infestation techniques were based on the methods of the International Livestock Research Institute, Nairobi, Kenya (ILRI; Naftaly Githaka, *pers comm*) and USDA Animal Disease Research Unit, Pullman, WA (Glen Scoles, *pers comm*). All tick life stages were incubated at 27°C and 85% relative humidity, conditions optimised for maintaining cultures of *Rhipicephalus (microplus) australis*.

3.1.2 Production and assay of infective stabilate

Initially, nymphal ticks were allowed to feed on an infected steer before being tested by PCR after moulting to unfed adults. These ticks tested negative for *T. orientalis* by PCR. To test infectivity for the production of stabilate, around 100 unfed, adult ticks, which had been infected with *T. orientalis* ikeda as nymphs, were placed under a flank-line patch onto an uninfected, splenectomised calf at TFRC and removed 3 days later. This followed similar feeding times on rabbits, enabling maturation of *T. parva* sporozoites in *Rhipicephalus appendiculatus* (Kimbata & Silayo, 1997). Subsequently, 5 days after tick application, the regional prefemoral lymph node was biopsied using a 19g needle into PBS-EDTA to search for schizonts. Blood smears were examined twice-weekly for the presence of piroplasms. This calf became positive for *T. orientalis* ikeda by blood smears after 13 days and samples of the 3 day-fed ticks were also positive for *T. orientalis* ikeda by PCR. This confirmed tick infectivity and the requirement for 3 days of feeding to mature sporozoites in infected ticks. Therefore, for stabilate production, around 3000 infected ticks were allowed to feed for 3 days on another uninfected calf before being removed and made into stabilate.

3.1.2.1 Stabilate from ticks

Ground-up tick supernatant fluid (GUTS) and salivary gland homogenates containing *T. orientalis* ikeda were produced according to the method described for *T. parva* in the OIE Terrestrial Handbook (2008, p799-80). Briefly, batches of 1000-1500 infected *H. longicornis* adult ticks were collected after 3 days of feeding. Ticks were disinfected in 70% ethanol and rinsed in distilled water before being partially “cut” through the caudal half of the abdomen and placed into a sterile glass homogeniser flask containing around 25ml of cold Hanks BSS (HBSS) with 10% bovine serum albumin (BSA; Australian sourced to avoid BSE biosecurity issues). The incisions allowed for easier grinding. Ticks

were then ground on ice for 2min using a tight fitting glass homogeniser before centrifugation at 50g for 5 min at 4°C and the supernatant fluid was harvested. An equal volume of cold 15% glycerol in HBBS-BSA was added dropwise while stirring on a magnetic stirrer, giving a final equivalent of 20 ticks /ml. The stabilate was aliquoted in 1.5ml volumes into cryovials (Nunc) before being gradually frozen, initially over 30-45min in the vapour phase of liquid nitrogen, then stored in liquid nitrogen until use.

3.1.2.2 Salivary gland stabilate

Tick salivary glands were dissected from around 1500 disinfected, washed ticks by the method of Patton et al., 2012 and progressively accumulated into 25ml cold HBBS-BSA in a glass homogenising flask. They were subsequently ground and processed as for GUTS above, frozen and stored in HBSS-BSA with 7.5% glycerol (final concentration) as 1.5ml aliquots in liquid nitrogen.

3.1.2.3. Stabilate infection and monitoring

Calves were purchased for Leppington Pastoral Co (LPC), Camden and housed on pasture at Pye Farm, Greendale, a University of Sydney Property. Calves were bled on arrival and tested by PCR to ensure they were negative for *T. orientalis* prior to any experiment. For infection, tick stabilate was thawed in water at room temperature immediately prior to inoculation. A volume of 1.5ml GUTS was inoculated subcutaneously (SC) into the right neck of each of 2 calves and a similar volume of the salivary gland stabilate was administered to an additional 2 calves. Both volumes were the equivalent of around 30 ticks. Calves were monitored daily, with 5ml blood collected twice weekly into EDTA vacutainers (Vacurette, Griener Bio-one) from 14 days post-inoculation.

This initial study was terminated at d28 when all calves tested positive by PCR, as BPQ was not available for any treatment.

3.1.2.4. Diagnostic PCR and estimation of Theilerial Gene copies

For all diagnostic PCR, DNA extraction was performed using the KingFisher® MO BIO PowerMag® Microbiome robotic program. For PCR, ten µL *T. orientalis* DNA template (MasterMix™) was used with the Easy-Plex™ Processor (AusDiagnostics Pty., Ltd., Australia) to conduct MT-PCR on 90µL DNA extracts for *T. orientalis* Buffeli, Ikeda, Chitose and type 5 according to the manufacturer's instructions. Briefly, this involves amplifying specific DNA sequences based on primers in the DNA template, where the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) detects the level of fluorescence emitted from the dye during amplification as a real-time PCR cycle threshold (Ct) value.

The levels of parasitosis were converted from the Ct values to gene copies per µl blood using spiked samples and sample volumes in the assays

3.1.2.5 Field studies of ticks from endemic regions

The possible production of GUTS from wild caught ticks was also investigated. In a preliminary investigation, 10 ticks were collected from cattle on a beef property at Stroud (Dr G.Brown) and 30 ticks from each of 4 farms at Dorrigo (courtesy of Dr. Chris Shirley and DVM students). The ticks were

confirmed as *H. longicornis*. Each tick was subsequently tested for Theilerial genotypes by PCR using the Ausdiagnostics kit.

Since >80% of ticks from Stroud and Dorrigo farms tested positive for multiple Theilerial genotypes (ikededa, chitose, buffeli and type5), a further 800 *H. longicornis* ticks were collected from Dorrigo and made into GUTS stabilate as above (60 vials x 1.5ml) as back-up for TFRC ticks.

3.2 Studies in cattle

3.2.1 Diagnosis and monitoring of Theilerial infections

From the initial study above (3.1.2.3), it was realised that the production of stabilate could not supply sufficient doses to conduct further experimental trials. So for the immunisation trial with *T. orientalis* buffeli, infected ticks were used. Following tick application (50 unfed adults or 100 unfed nymphs) under backline patches, calves were monitored daily, with 5ml blood collected weekly into EDTA vacutainers (Vacurette, Griener Bio-one) from d14 post-inoculation. Ticks were harvested from the patches on each calf, 6 days after application when the majority had completed engorgement. Ticks were collected into separate vials from each calf, counted and several placed into 100% ethanol for PCR, while the remaining engorged nymphs were sent to BSL for moulting into adults. For all diagnostic PCR, DNA extraction was performed as in 3.1.2.3.

3.2.2 Chemotherapy of the carrier state

Twenty-five Holstein (neutered male) calves aged 4-5 months were purchased from LPC (Cobbitty) and housed at Pye Farm on pasture. Each calf was premedicated with anti-histamine coverage (100 mg of Histamil by intramuscular injection). After 30 min, each was given 1.5ml of *T. orientalis* ikeda infected blood stabilate intravenously (J-36, ex Camden via BSL/TFRC). Two calves died from a transfusion reaction (which had never occurred previously at Camden, although Jade Hammer had one at Bairnsdale). The study was commenced in July and this batch of calves exhibited a high prevalence of sub-clinical respiratory problems, so this may have been the cause, but antibodies to the donor may have been involved as well. The remaining calves were monitored for development of parasitism over the next month (Fig. 1), before being allocated to 5 groups and treated with BPO, Imadox, oxytetracycline or Draxxin (tulathromycin). Drug-induced reductions in parasitaemia were measured by PCR over the ensuing 3 weeks.

3.2.3 Immunisation with *T. buffeli*

Fifteen Holstein (neutered male) calves aged 4-5 months were purchased from LPC (Cobbitty) and housed at Pye Farm on pasture. Calves were weighed and bled to determine Theilerial status by PCR before being randomly assigned to 3 treatment groups, each of 5 animals. Group 1 calves were premedicated with 100 mg of the antihistamine chlorpheniramine maleate (Histamil) and 15mg dexamethasone (Dexapont) by intramuscular (i.m.) injection, to lower the risk of any transfusion reaction. After 30 min, each calf was given a 5ml intravenous (i.v.) infusion of fresh infected blood (ex TFRC steer 35884) containing 1.3×10^6 *T. orientalis* buffeli/ml. All calves became PCR positive within 28d. Group 2 calves were to be infested with 100 *H. bancrofti* (infected with *T. orientalis* buffeli as larvae) from TFRC under patches, but these ticks died on steer 35884. Consequently, *H. longicornis* larvae were fed on steer 35884, moulted, and 100 unfed nymphs were applied to each

calf in Group 2 (and removed after 6 days when they were engorged). When the 5 calves failed to become PCR positive after 5 weeks, 5ml of fresh blood infected with 9×10^8 *T. orientalis* buffeli per ml (ex steer 3584) was inoculated subcutaneously (S/C) into each calf. With this higher dose, all 5 calves were PCR positive for *T. orientalis* buffeli after 21d. Two calves in the control group tested as very low positive for *T. orientalis* ikeda before challenge (likely from *in utero* or colostral transfer) and were excluded from the control group analyses. These 2 were examined for any reduction post-challenge in comparisons to the remaining 3 uninfected control calves.

Twenty-eight days after the inoculation of Group 2 and 13 weeks after Group 1 calves received *T. orientalis* buffeli blood, 200 unfed, *H. longicornis* nymphs, previously infected with *T. orientalis* ikeda as larvae at TFRC, were placed under patches on each of the 15 calves (including the uninfected group 3 controls). All calves had become PCR positive for *T. orientalis* ikeda by day 12 after tick application. The ticks were collected from each calf after 6 days, counted and pooled, before posting to BSL to moult to adults. Unfed adult ticks (100 *H. longicornis*) were then applied to a further 3 naïve calves to determine if the original *T. orientalis* ikeda infection persisted through the nymphal stage on the uninfected calves and survived through the moult to adult ticks.

3.2.4 Field studies on the kinetics of Theileriosis in calves and imported cattle in endemic areas (Dorrigo).

In association with Dr Chris Shirley, (Dorrigo Vet), groups of 30 calves from the Spring calvings on 2 local properties were bled around 8 weeks after the start of calving in October 2017 and again in October 2019. Since calvings occurred over a 6-8 week period, ages of calves ranged from 4-10 weeks when blood samples were collected.

In a second study, 30 beef weaners from a non-endemic region were followed by bleeding and weight measures every 3 weeks for 6 months after introduction to a Dorrigo farm in late February 2020. These were used to compare the onset of Theileriosis and its impact on productivity and for comparison with the age-prevalence data from the calves in local properties above.

3.3 Statistical analyses

Statistical analysis was performed by Dr Evelyn Hall using GenStat where raw data were \log_e transformed and five linear mixed models for PCV, Ikeda and Buffeli gene copies per μL and Ct values were developed in each study as appropriate. The fixed effects were Day, Treatment and the interaction between Day and Treatment. The random effect was Animal ID. P-values were calculated for each fixed effect in each model to determine whether they were significant. For significant fixed effects, the differences in the predicted means for each factor level were compared to the Least Significant Differences (LSDs) at significance level 0.05 to determine whether pairwise comparisons were significant. If the interaction fixed effect was significant, no further pairwise comparisons were determined for the other fixed effects.

Predicted means for PCV, Ct values and gene copy per μL for Theilerial genotypes within treatment groups were presented with standard error (SE) bars.

4 Results

4.1 Production, infection and analysis of infected ticks and stabilate

The life cycle of *H. longicornis* on dogs and calves was found to be around 120 days (Table 1; Fig. 1).

Following application of 100 unfed adult ticks (infected with *T. orientalis* ikeda as nymphs) onto an uninfected, splenectomised calf, the host developed a detectable parasitaemia 13 days later, confirmed by PCR as *T. orientalis* ikeda genotype. Although biopsies of the prefemoral nodes were negative when taken 5d after application, piroplasms were detected in blood smears from day 13 post-application.

Table 1: Comparison of life-cycle timelines for *Haemaphysalis longicornis* in Australia, Japan and China.

Life cycle stage	Australia: Current study			Japan**		China***
	Cattle	Dogs		Range (days)	Average	Average
	Range (Days)	Range (days)	Average			
Oviposition to hatching	28	17-30	25	24-38	27.5	38.5
Resting (Larvae)	21	3-5	4	3-5	4	5.3
Feeding (larvae)	4-6	3-5	4	4-5	4.5	3.8
Larval moult	2-11	8-16	13.5	14-17	15.5	13.9
Hardening (nymph)	4-9	4-6	5	2-3	2.5	7.2
Feeding (nymph)	3-7	5-7	6	5-7	6	5.4
Nymphal moult	9-15	9-16	13	12-16	14	16.9
Resting (adults)		6-8	7	4-6	5	7.6
Feeding (adults)	3-6	6-11+	10	11-19	15	9.4
Preoviposition Period	4	4-7	6	3-7	5	7.8
Oviposition period	13	18-20	19	11-27	19	20
Numbers of eggs*	200		11.8		2024	
% hatch rate			97		94	
Generational cycle	97-128	83-131+	111		118	135.8

*Number of eggs deposited per mg of body weight.

** Kitaoka (1961), cited by Hoogstraal *etal.*, (1968); *** Lui & Jiang (1998)

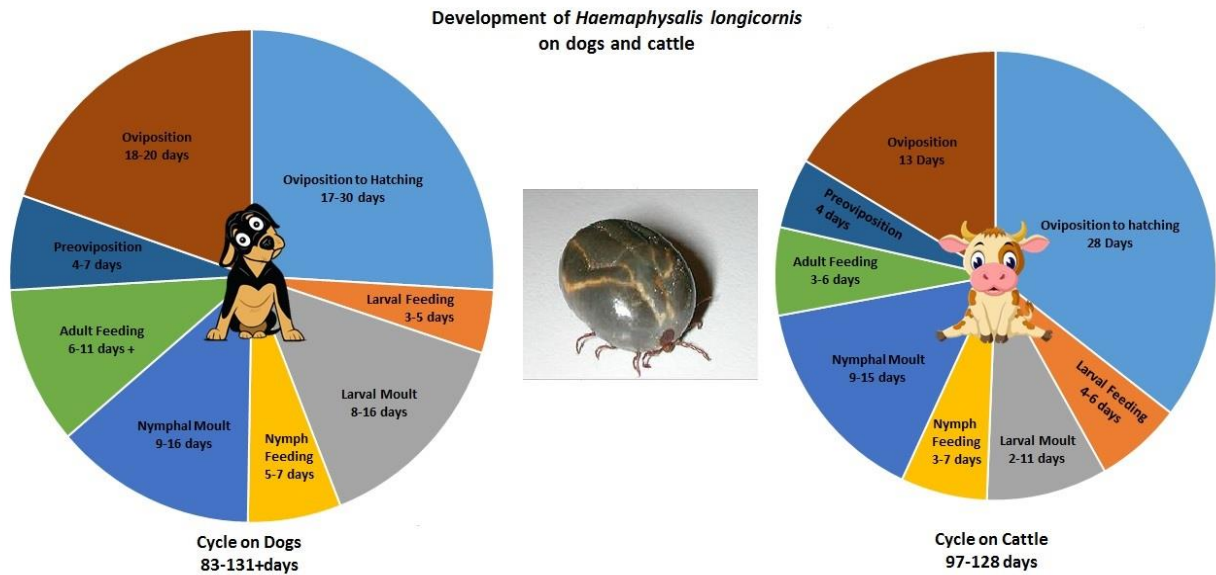


Figure 1. The development of *H. longicornis* on dogs and cattle (from Mareudy et al., 2019)

The subcutaneous inoculation of GUTS or salivary glands equivalent to around 30 ticks produced parasitosis detectable by PCR, in all 4 calves within 26 days. Three calves became positive for *T. orientalis* ikeida on days 22 (both salivary glands and 1 GUTS) and day 26 (GUTS). Clinical disease was avoided as the study was terminated at this point as a permit to use buparvaquone for treatment had not been issued. Throughout the same 26-day period, blood smears were all negative for piroplasms and the haematocrit remained unchanged.

These 2 trials confirmed that *H. longicornis* could act as the final (biological) host for *T. orientalis* ikeida and was a competent biological vector (Mareudy et al., 2019).

4.2 Chemotherapy of the carrier state

Calves infected i.v. with blood stabilate (J36- ikeda) became PCR-positive over the next month (Fig. 2) before being allocated to treatments with BPQ, Imadox, long-acting oxytetracycline or Draxxin. A limited use permit was obtained for BPQ in April 2019 (PER85797). The results determined by Restricted Maximum Likelihood (REML), indicated that only BPQ significantly reduced parasite DNA copies over the following 3 weeks (Fig. 3). When tested by PCR at 16 weeks after treatment, all 3 calves receiving BPQ were still PCR-positive for *T. orientalis* ikeda, indicating that while BPQ initially and significantly reduced parasitosis, it did not eliminate the carrier state.

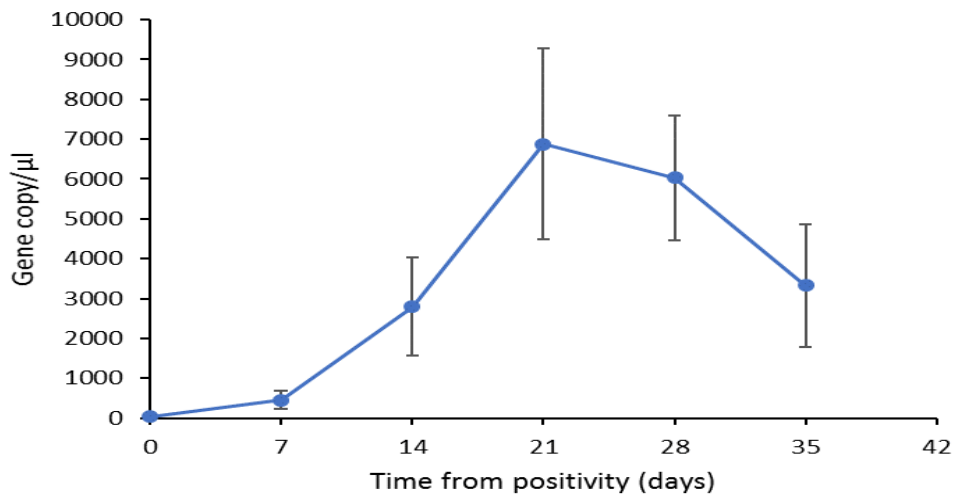


Figure 2: The level of parasitosis with *T. orientalis* ikeda (gene copies DNA / microlitre) in blood of calves after initial detection of the Theilerial parasitism following inoculation of infected (ikeda) blood.

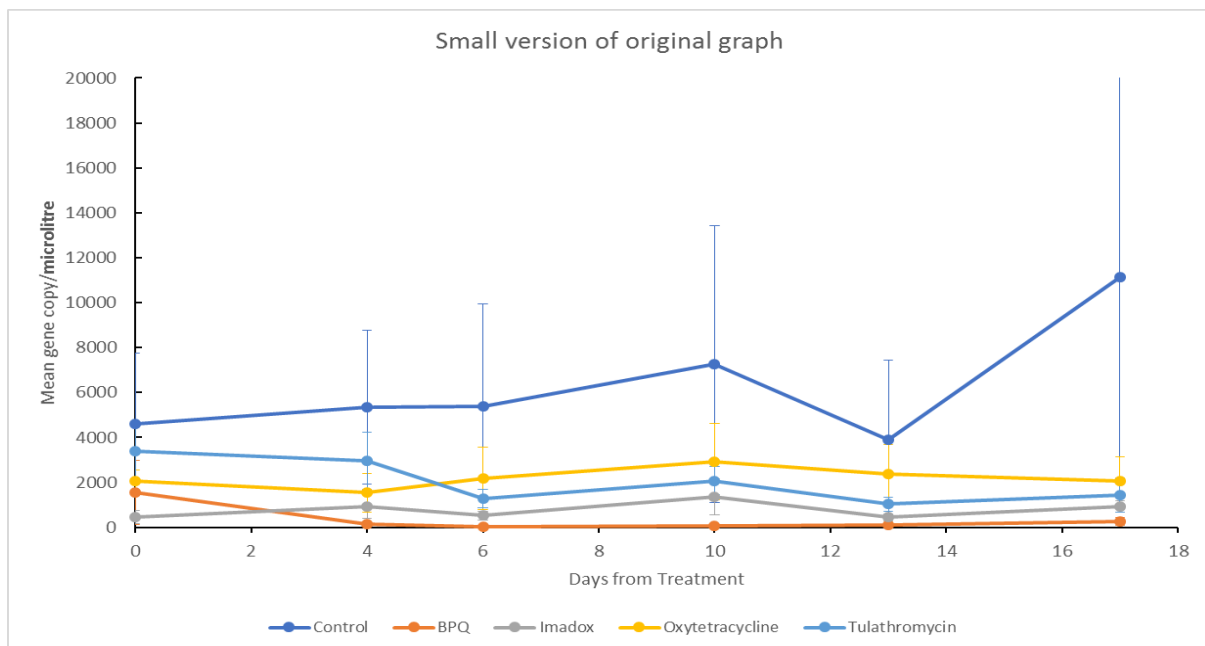


Figure 3. Levels of parasitosis (gene copies DNA/microlitre) in parasitised calves following treatments as indicated (from Laura Kerrison and Savannah Spillet, DVM3 research projects 2018).

4.3 Immunisation with *T. orientalis buffeli*

4.3.1 *T. orientalis buffeli*

Both intravenous (iv) and subcutaneous (sc) inoculation of bovine blood infected with *T.orientalis buffeli* produced parasitosis detectable by PCR within 4 weeks Inoculation. Over the 13 weeks before challenge, the parasitosis in Group 1 (IV) reached a mean peak of 5,063 GC/μl of *T.orientalis buffeli* by 5 weeks, decreasing and stabilising between means of 1200 and 878 GC/μl, 13 weeks after initial inoculation (Table 2). By comparison, at the time of challenge, 4 weeks after inoculation SC, Group 2 calves had a mean parasitosis of 1547 GC/μl (Table 2). Following the tick challenge, the parasitosis with *T.orientalis buffeli* declined steadily in both Groups 1 and 2 over the next 60 days to <2500 GC/μl in both groups.

Following the initial inoculations of blood into Group 1, it was revealed that 2 calves in the control Group 3 exhibited pre-existing low parasitoses with *T.orientalis ikeda* of <500 GC/μl, presumably from infections acquired *in utero* or postnatally. This meant that these 2 calves were excluded from the control group (3 calves) for statistical analysis, but were included for analysis as a separate group (of 2 calves) after tick infestation.

Table 2. Parasitosis of *T.orientalis buffeli* from calves in Groups 1 & 2 after inoculation of infested blood.

Group treatment	Days after tick infestation								
	-62*	-56	-28	0	12	18	35	47	62
IV	9144 +/- 85	5063 +/- 72	1292 +/- 21	876 +/- 0.67	133 +/- 0.65	3 +/- 0.2	3 +/- 0.2	3 +/- 0.3	2 +/- 0.2
SC	na	na	na	1547 +/-0.7	157 +/- 0.65	20 +/- 0.65	24 +/- 0.6	20 +/- 0.4	12 +/- 0.4

Results are expressed as mean GC/μl blood +/- SE.

* 4 weeks after inoculation of blood IV; na; not applicable

4.3.2 *T. orientalis ikeda*

4.3.2.1 Changes in parasitosis

All 15 calves became positive for *T.orientalis ikeda* within 12 days after infestation (dai) from the application of the 200 infected *H.longicornis* nymphs. The parasitosis in the control groups followed a typical pattern in peaking around 5 weeks (39 dai) after infestation at a mean GC/μl of 69,734 before declining to <2000 GC/μl blood by 62 dai (Fig.4, Table 3). The parasitosis in the 3 treatment groups were significantly reduced between 30 and 85% during the first wave of parasitaemia from

25-39 dai (Fig. 4, Table 3). In parallel, the PCV in the control group decreased by 16-20% to a mean of 25% by 39 dai, significantly reduced compared to the SC immunised group 2 (Table 3) and remaining significantly lower than group 2 up to 62 dai (Table 3).

Table 3. Parasitosis of *T.orientalis* ikeda in treatment groups after challenge with infested *H.longicornis*.

Groups	Day 12	Day 18	Day 25	Day 32	Day 39	Day 47	Day 62
Control (3)	189 (97) ^{ab}	9180 (4690) ^a	33134 (18585) ^a	30303 (15500) ^a	68734 (8969) ^a	18251 (9150) ^a	84 (588) ^a
IV (5)	94 (37) ^a	2800 (1110) ^a	1124 (445) ^c	3446 (1360) ^b	9740 (6414) ^b	10027 (6253) ^a	2855 (1588) ^b
*Controls +ve (2)	1181 (835) ^b	9063 (6409) ^a	6741 (4767) ^b	11464 (8107) ^b	6815 (4374) ^b	810 (632) ^b	422 (228) ^a
SC (5)	488 (193) ^b	9200 (3640) ^a	4020 (1590) ^b	3648 (1440) ^b	9154 (9180) ^b	3637 (4224) ^a	432 (331) ^a

Results are expressed as mean GC/μl blood +/- SE.

Within columns, data with different superscripts are significantly different (p<0.05).

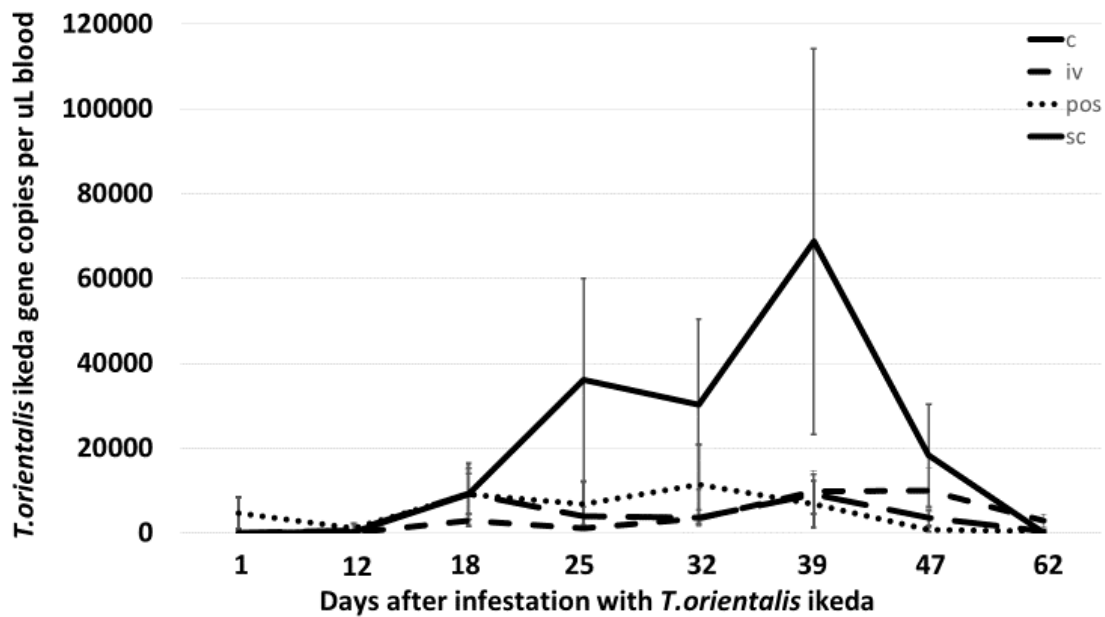


Figure 4. Group mean parasitoses for *T.orientalis* ikeda (GC/ per μ l blood +/- SD) following infestation with 200 *H.longicornis* nymphs (see M&M, section 2.3.3). Group comparisons include: uninfected controls, (3 calves, solid line); previously infected controls (2 calves, dotted line); groups of 5 calves immunised with *T.orientalis* buffeli either IV (short dashes) or SC (long dash).

4.3.2.2 Changes in Haematocrit (Packed Cell Volume, PCV)

Overall, the PCV across all treatment groups decreased but stayed within the reference range of 24-46% for cattle. Within this range, a late decline in the Control group occurred after 32 DAI (Fig. 5). Consequently, the interaction between treatment and time was significant ($p < 0.001$) where the effect of treatment diminishes the rate of decline in PCV. On 32 DAI, the control group PCV ($25 \pm 1.5\%$) was significantly lower ($p < 0.05$) than the IV group ($29.5 \pm 1.7\%$; Table 4).

Table 4. Changes in haematocrit in treatment groups after challenge with infested *H.longicornis*.

Groups	Day 12	Day 18	Day 25	Day 32	Day 39	Day 47	Day 62
Control (3)	32 ^a	38.3 ^a	31.7 ^a	25 ^a	26.7 ^a	27 ^a	27.3 ^a
IV (5)	27.8	30.8	32 ^a	29.8 ^b	30.2 ^{ab}	30.4 ^{ab}	28.2 ^{ab}
*Controls +ve (2)	35 ^a	33.5 ^a	36 ^a	27.5 ^{ab}	29.5 ^{ab}	32 ^{ab}	31.5 ^b
SC (5)	28.8	33.6	31.6 ^a	28.6 ^{ab}	31.6 ^b	33.2 ^b	31.8 ^c

Results are expressed as % PCV.

Within columns, data with different superscripts are significantly different ($p < 0.05$).

4.4 Kinetics of Theilerial infestations in endemic regions (Dorrigo)

4.4.1 Theilerial parasitosis in Dorrigo calves

Parasitosis in 30 calves born in 2017 on each of the 2 properties at Dorrigo are shown in Fig. 5, and a similar trend occurred in calves born at the same farms in 2019. All calves were infected with *T.orientalis* ikeda with the youngest at 5 weeks showing the highest levels of parasitosis as determined by PCR and expressed as Theilerial DNA copies/ μ l blood. There were no significant correlations between the parasitosis with Theilerial genotypes (Table 5), but as calves aged, there was a trend for lower levels of parasitosis (Fig. 5A-B). In contrast, the levels of parasitosis with *T.orientalis* buffeli increased with age and the proportion of calves infected also increased to around 80% in animals by 4 months of age (Fig. 5C-D). However, the gene copies of *T.orientalis* buffeli were around 3-fold lower than for *T.orientalis* ikeda (Fig. 5C-D).

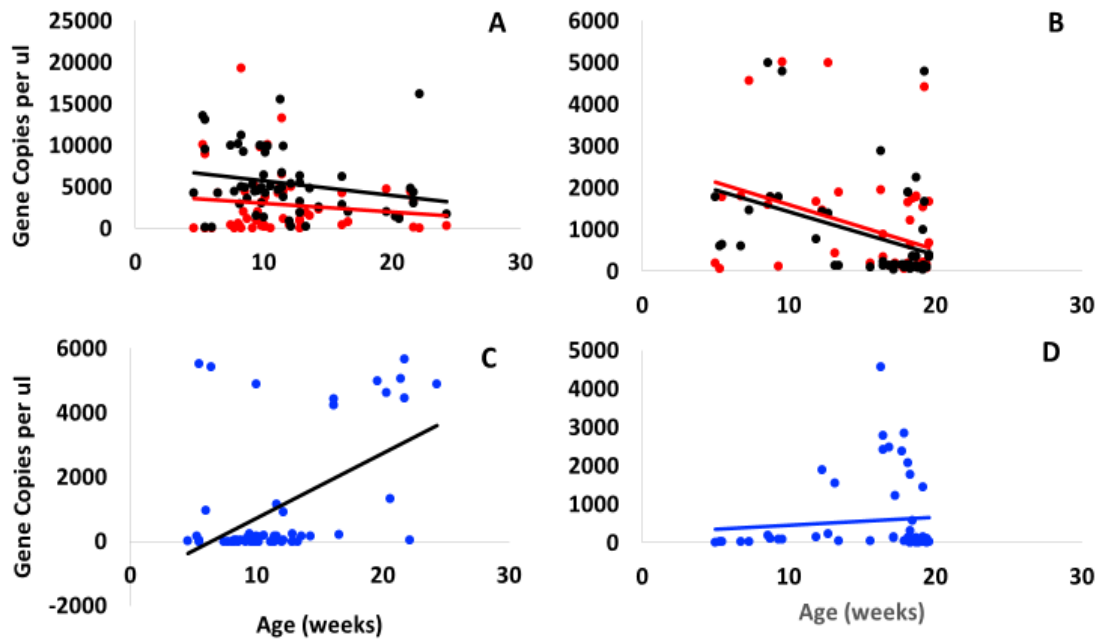


Figure 5. Parasitosis expressed as gene copies / μl (GC/ μl) in blood of calves born into 2 Dorrigo farms in in 2017 (left) and 2019 (right). Comparisons include: (A) *T.orientalis* genotypes ikeda (black) and chitose (red) in 2019 (top left); (B) *T.orientalis* genotypes ikeda (black) and chitose (red) in 2019 (top right); (C) *T.orientalis* buffeli (blue) in 2017 (bottom left); (D) *T.orientalis* buffeli (blue) in 2017 (bottom right). Lines of best fit (see Table 5) are displayed into each section. Chantelle Loo and Shaojing Yang, DVM3 research projects).

Table 5. Equations for lines of best fit describing correlations between age and parasitosis with Theilerial genotypes in Dorrigo calves

Year	Theilerial genotype	Line of best fit (Y=)	Correlation (R^2)
2017	Ikeda	$176.12x + 7477.4$	0.0174
	Chitose	$107.34x + 4104.3$	0.0186
	buffeli	$202.69x - 1309$	0.2541
2019	Ikeda	$-176.12x + 7477.4$	0.0471
	Chitose	$-107.64x + 2671.6$	0.135
	buffeli	$20.398x + 255.54$	0.0078

4.4.2 Theilerial parasitosis in introduced naïve cattle following arrival at Dorrigo

The levels of Theilerial parasitism and average daily liveweight gain (ADLG) in 30 introduced weaners is depicted in Fig. 6. Animals became infected with *T. orientalis* within 3 weeks after arrival as detected by PCR. The parasitosis for *T. orientalis* ikeda and chitose was maximal at the 6 and 9 week bleeds. A sharp decline in ADLG and a parallel but later decline in blood packed red cell volume (PCV)

was evident following and through this initial parasitaemia, particularly around the 2-3 months after arrival (Figs. 6&7). Subsequently, the parasitosis decreased and ADLG and PCV increased by 3 months after arrival (Fig. 6). In contrast, the prevalence of *T. orientalis buffeli* increased more slowly, with infections in around 70% of the cattle by 3 months after arrival (Figs. 6&7) and a lower level of parasitosis (Fig. 6).

In both introduced naïve cattle and calves born into endemic regions of Theileriosis, infection occurs rapidly, most rapidly for *T. orientalis ikeda* and chitose genotypes, and much more slowly with *T. orientalis buffeli* (Fig. 6). Once the initial wave of parasitosis passed after 2-3 months, both PCV and ALDG improved towards regional benchmarks of around 32 and 0.8 kg/day, respectively, in May. ALDG declined again over the winter months while the parasitosis remained relatively stable (Fig. 7). PCVs recovered in the majority of the cattle by 4 months and for the remainder of the observation period towards mid-August, 6 months after arrival (Fig. 6).

However, the mean weight at arrival was 155kg. Based on an expected ADLG of 0.8kg/day, the mean expected weight after 90 days would be 227kg. Since the mean observed weight at this time was 205kg, the Theilerial infestation cost around 22kg over this period. As the PCV rose, the second reduction in ADLG from June to August was likely due to declining nutrition. The expected liveweight gain over these 3 months was 45kg, some 25kg short of expected gains at 0.8kg/day.

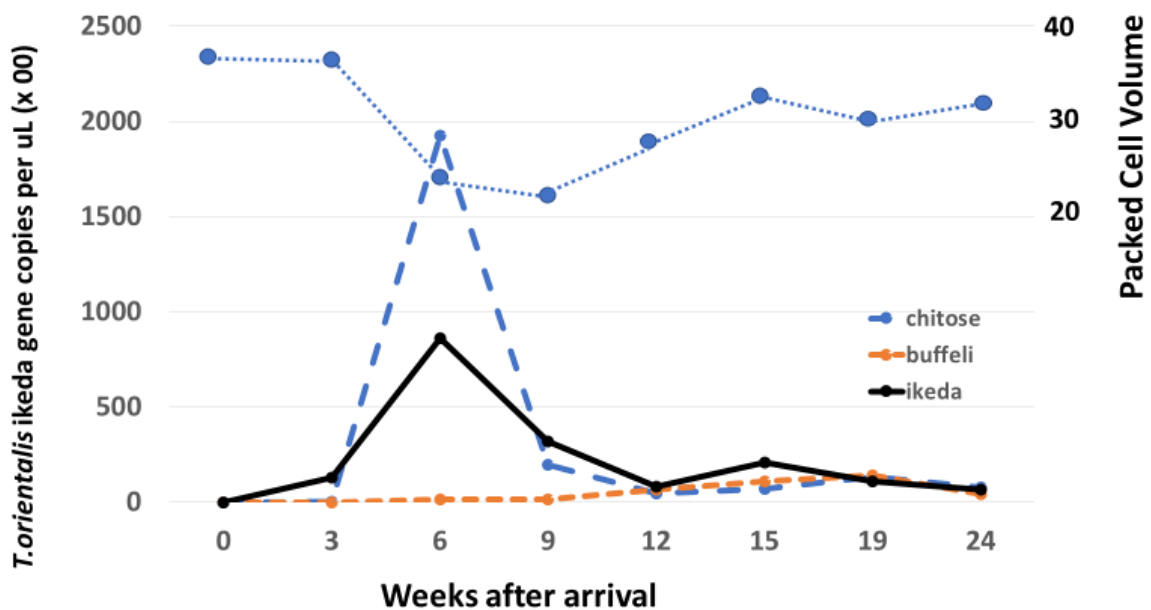


Figure 6. Kinetics of parasitosis by Theilerial genotypes ikeda (solid line), buffeli (short dashes) and chitose (long dashes), expressed as gene copies per µl blood in the first 6 months after 30 cattle were moved to Dorrigo in Feb 2020. The haematocrit (PCV; dotted lines) is also displayed. From Chantelle Loo, DVM3 research project 2020.

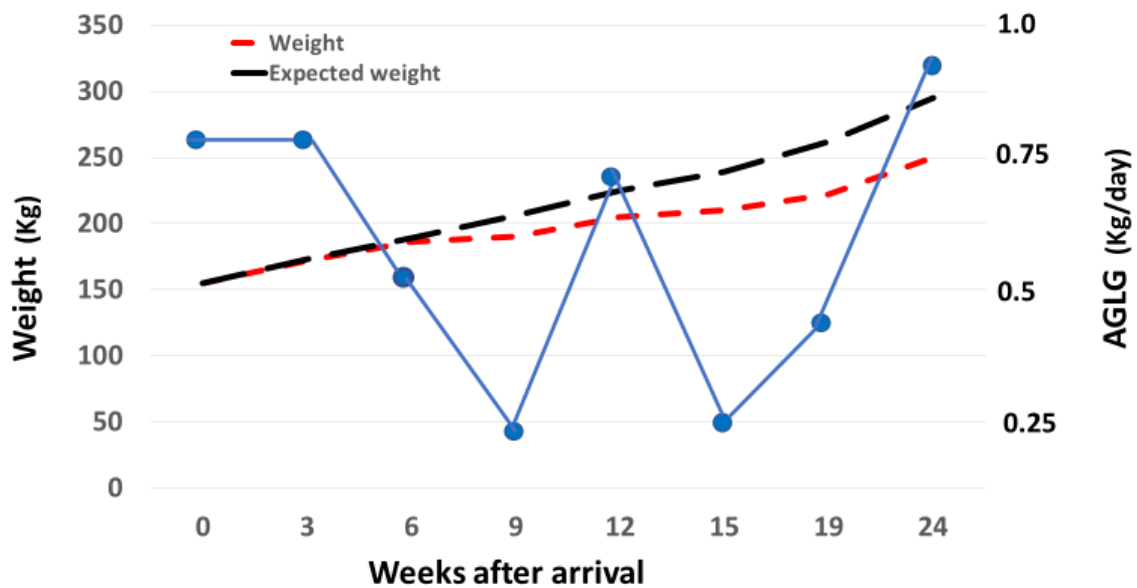


Figure 7. Average Daily Liveweight Gain (mean ADLG; solid line) compared with the progressive expected (long dashed line) and actual mean weights (lower, shorter, dashed line) over the first 6 months after 30 cattle were moved to Dorrigo in Feb 2020. From Chantelle Loo, DVM3 research project 2020.

5 Discussion

5.1 Pathogenesis and transmission of *Theileria orientalis*.

5.1.1 *H. longicornis* as a vector

These results confirm that *H. longicornis* is a competent vector and final host for *T. orientalis* in Australia. This is consistent with the range of the tick in Australia, NZ and Asia and its likely introduction from Asia (Fig 8; Raghavan et al., 2019). Although histology was equivocal, it was gratifying that dissected salivary glands were both PCR-positive and infective *in vivo*, giving greater credence to the outcome by avoiding any contamination with erythrocytic stages leading to mechanical transfer. However, the entire process of stabilate production through tick feeding and quality control is unsuited to widespread field vaccination trials. This has been the experience with Infect and Treat vaccination (I&T) for *T. parva*, with vaccine production unable to keep pace with demand. For *T. parva*, infective doses of GUTS stabilate are equivalent to 10 ticks (Kimbata & Silayo, 1997), whereas stabilate used for this study was around 30 ticks per dose.

The lack of infection in newly moulted nymphs (unfed adults) was completely unexpected, but likely resulting from subliminal level of parasite DNA following the moult. Subsequent success with 3day and 5day -fed adults, together with the establishment of infection in the host steers at TFRC is consistent with many accounts of tick-borne disease transmission. In these instances, where vectored pathogens are transmitted trans-stadially, feeding of newly moulted arachnids for several days is required to mature protozoal parasites and transmit disease (Eisen, 2018; Schorderet-Weber

et al., 2017). Results in this study also indicated that 200 infected nymphs and 50 adult ticks were quite capable of transmitting *T. orientalis* ikeda, with infection detected by PCR within 10-12 days in recipient cattle. It is worth noting that while *Babesia bovis* and *B. bigemina* parasites appear adapted to the 1-host tick *Rhipicephalus australis* (ex *Boophilus microplus*) and infection is transmitted transovarially, Theilerial parasites utilise 3-host ticks as final hosts. This would indicate that protozoa in combined tick fever vaccines would not persist in the absence of the respective final tick host, outcomes noted following outbreaks of Theileriosis in introduced cattle at Narrabri and Terry Hei Hei in 2016.

In meeting the **project objective 1**, two interesting observations arose from the stabilate production. The first arises from the inability to quantify sporozoite infection rates in salivary gland acini of *H. longicornis*, indicating that by comparison with *T. parva* in *R. appendiculatus*, infection rates are very low. This accords with the time to parasitosis after stabilate (>21 days) using the equivalent of 30 *H. longicornis* ticks. By comparison, inoculation of *T. parva* GUTS stabilate equivalent to 10 ticks induces detectable parasitosis within 4-5 days (DE experience, 1978-81). Extrapolating to Japanese field studies where clinical *T. orientalis* can occur within 9-12 days after infestation (also seen in our experimental trials), then the infestation rates of infected ticks must be very high or hosts completely naïve. Where naïve cattle are introduced to endemic Theilerial zones in NSW or Victoria, or in newborn calves, clinical disease usually occurs from around 6 weeks later (Swilks et al., 2017; Hammer, Shirley, *pers comms*, Fig. 4), much longer than seen with *T. parva* (2-3 weeks). This was also observed in clinical infestations with experimental trials in the project where calves became PCR-positive within 12 days after tick application. However, clinical symptoms of decreased PCR and “clinical “parasite load detectable in blood smears did not occur until around 3 weeks later. This is consistent with a longer lead time to accumulate a sufficient quantum of parasites to cause clinical signs, ostensibly from lower infection rates in feeding ticks, reduced virulence of *T. orientalis* compared to *T. parva*, or simply the pathogenicity of the earlier schizont stage for *T. parva*. Recrudescence of clinical disease in Theilerial carriers can occur after transport (esp. if pregnant), although carriers appear to calve without problems in the home farm environment. One salutary reminder from *T. parva* studies in Africa is the apparent ability of ticks to become infected from “carriers” which have tested PCR-negative (Olds et al., 2018).

The second observation arising from stabilate generation is that the host steer used at TFRC for feeding our 3000-odd infected adult ticks did NOT develop clinical Theileriosis over the subsequent 2.5 months before it was sold. This calf was a splenectomised (SplX) Murray-Grey cross (not *Bos indicus*) and the experience at the TFRC, Wacol, is that that their SplX animals can control the initial peak of parasitosis (up to 10%) without reductions in haematocrit. In Queensland, the vast majority of clinical theileriosis occurs in NSW immigrants or *Bos taurus* cattle in South-east Queensland (Eamens et al., 2011, Kamau et al., 2013, P. Carter *pers comm*). This contrasts with NSW and the reasons are speculative, which is why a *T. orientalis* buffeli protection trial was undertaken.

It is noted that for the execution of the project, tick challenge models are a tough gig! The painstaking and highly constructive collaborative efforts of the TFRC were instrumental to the ongoing success of this project.

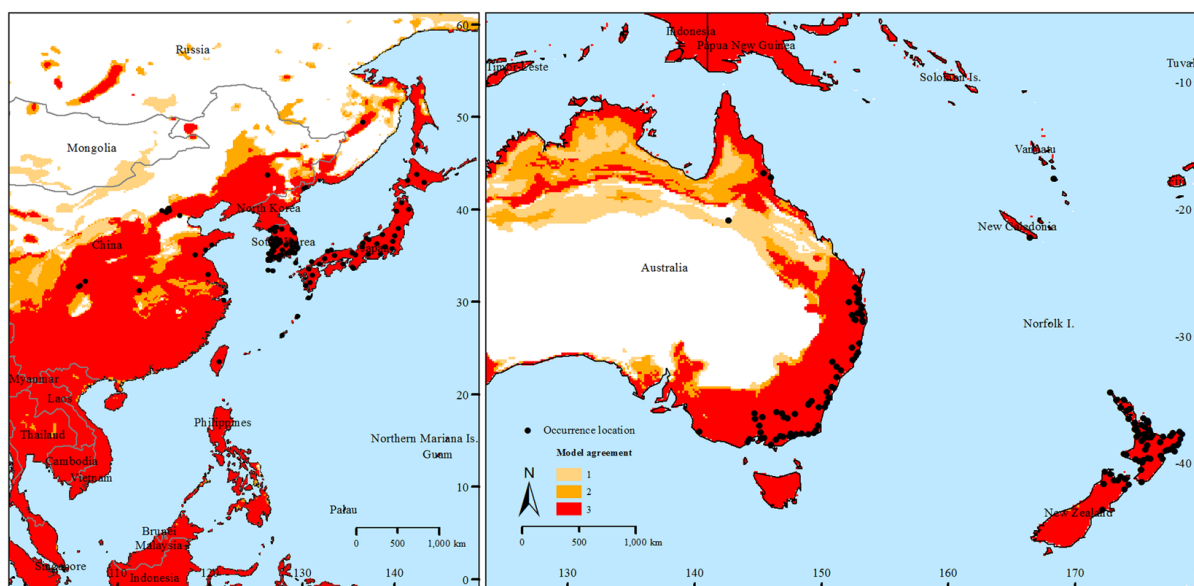


Fig. 8. Spatial distribution modelling for *Haemaphysalis longicornis* (from Raghavan et al., 2019 <https://www.nature.com/articles/s41598-018-37205-2>).

5.1.2 Chemotherapy of the carrier state

The drugs selected for the chemotherapy trial were based on the hypothesis that clinical disease from *T. orientalis* coincided with the appearance of piroplasms, fever and parasitaemia, and that these merozoitic stages could multiply in erythrocytes like Babesial and malarial parasites. This manifested as infections developing after mechanical transfer of infected blood. This reasoning also presumed that the schizont stages of *T. orientalis*, being difficult to observe in regional lymph node biopsies or failing to induce local node enlargement, were relatively less important for the development of clinical disease than the parasitic stages in red cells. Therefore, drugs with activity against other haemoprotozoa with important erythrocytic stages for asexual development were selected. To date, BPQ has not been registered for (minor) use in Australia. The main obstacles are the persistence of tissue residues (Bailey, 2013; B.AHE.0194) and so treated stock cannot enter the food chain or be sold. BPQ targets the schizont stage of the parasite- this being the stage associated with the clinical signs in East Coast fever (ECF) caused by *T. parva*. It also reduces numbers of *T. orientalis* ike da piroplasms in blood (B.AHE.0048) within 4 days, as it did in this trial.

In meeting **project objective 2 in the chemotherapy trial**, only BPQ (Osawa et al., 1988; Carter 2011, B.AHE.0048) suppressed parasitosis; Imidocarb, tulathromycin and oxytetracycline had no effect. Recent research in Australia has found that BPQ (2.5mg/kg) cures *T. orientalis* infections in splenectomised calves (MLA project B.AHE.0048). The treated calves had 89-95% reductions in parasitaemia by day 4 after treatment. Further field work would be needed to confirm BPQ's effectiveness in clinical outbreaks, although our experience from the Dorrigo studies and those of Jenkins et al (2015), indicates that clinical signs of reduced PCV and weight loss develop around 2 weeks after the peak of the first wave of parasitism (Fig. 4). This portion of the study discounted the use of oxytetracycline and imidocarb for treatment of *T.orientalis*, even though the former are effective against the schizonts of *T. annulata* and *T. parva* . They are used in the "infect & treat" vaccine regimen (Dolan, 1981), but must be given during the prepatent period. Imidocarb also failed

to reduce parasitaemias (blood smears) in 3 calves at Wacol (P.Carter pers. comm.), despite anecdotal evidence from Bega.

Project objective 2 was incomplete in that drugs used for treatment of canine babesiosis or human malaria (not registered for use in cattle anywhere), are expensive, lack residue depletion data, and are likely to have the same lack of efficacy. Diminazene (Berenil®) and Primaquine (Minami et al. 1985), are still possibilities, the latter (pamaquin and primaquine) being active against the piroplasms of *T. annulata* (Zhang, 1997; Luo and Lu, 1997), but unable to cure *T. orientalis buffeli* parasitosis (Stewart et al., 1996). However, due to residues and with-holding periods, these appear less important in the overall integrated management of Theileriosis and the “cure” is often administered too late if clinical signs are already apparent. Parenthetically, this “timing issue” gives rise to anecdotal chemotherapeutic “cures”, when drugs are administered after animals have passed the first peak of parasitosis and are already recovering.

In a separate trial, it was found that toltrazuril (Baycox®; known to be active against the schizont stages of *Eimeria* and *Isospora* spp., related to *Theileria*) did not prevent significantly, or ameliorate, parasitosis following challenge with 50 unfed adult *H. longicornis* which had been infected as nymphs with *T. orientalis ikeda* (Susan de Burgh, unpublished).

5.1.3 Pathogenesis of Theileriosis in introduced or naïve cattle

In both introduced naïve cattle and calves born into endemic regions of Theileriosis, infection occurs rapidly for *T. orientalis ikeda* and *chitose*, and much more slowly with *T. orientalis buffeli*. From PCR measures, gene copy numbers of *Theileria* below 15,000 are considered to indicate mild infection, 15,000-300,000 indicates moderate infection, while clinically significant parasitosis is considered when gene copies per μl exceed 300,000 (Bogema et al., 2015). In the current study, 3 animals died in the wider introduced cohort, and clinical signs of anaemia were readily apparent in 4 of the monitored 30 animals around 6 weeks after introduction. Around this period after arrival, these affected animals had parasitoses of 120000 - 360000 and 55000 – 150000 gene copies per μl blood for *T.orientalis chitose* and *ikeda* genotypes, respectively. The differences between levels in the two studies are likely to reside in the different diagnostic kits used, but closely approximate each other.

In 30 calves sampled from each of 2 Dorrigo farms in 2017 and 2019 where birth dates were available, results confirmed that calves were readily and heavily infected within 4-5 weeks of birth, consistent with results from Gloucester (Fig 9., from Swilks et al., 2017). Similarly, weaner calves introduced to Dorrigo in late February 2020 were PCR-positive within 3 weeks after introduction and exhibited clinical Theileriosis within 5-6 weeks after arrival. This was unexpected as the seasonal life cycle of *H. longicornis* would suggest that larval ticks would be present in late Summer and would need to feed on infected cattle to transfer the infestation as nymphs later in the year. However, not only did these cattle arrive at Dorrigo late February after a monthly rainfall of 580mm, but it would appear that clinical Theileriosis can occur throughout the year in Dorrigo.

The temporal kinetics of infection with *Theileria* genotypes in the 30 introductions were similar to those reported by Jenkins et al. (2015), where 10 cows introduced in to an endemic *Theileria* region were found sequentially infected with *T.orientalis ikeda*, then *chitose* and finally and to a lesser

extent, with *T.orientalis* buffeli over 2.5 months. One cow developed anaemia, but effects on productivity were not examined. However, in this study, 3 animals died within the larger bovine cohort (1% mortality), but the parasitosis with reduced PCV and ADLG incurred >20Kg productivity loss per animal over the first 3 months at Dorrigo (Fig. 7).

The reasons for changes in the population dynamics of Theilerial genotypes are speculative as the host immune response to *T. orientalis* infection and the genotypic interactions within the Theilerial merozoite populations in cattle are not completely understood. However, the development of a carrier state with low levels of genotypic GC/ μ l are attained whether host cattle are infested with single ikeda (Hammer et al., 2016) or multiple Theilerial genotypes (Jenkins et al., 2015; this study). Population changes during co-infection with multiple genotypes could mimic the strain-specific cell-mediated responses seen with *T.annulata* and *T.parva* (Machugh et al., 2008) or less likely, genetic recombination within the tick vector as seen in another apicomplexan, *Plasmodium falciparum*, within mosquitos (Masatani et al., 2016). In this case, genotypic changes establish a chronic parasitosis that impairs the establishment of subsequent infections and promote Plasmodial survival (Portugal et al., 2016). Thus older human children and adults have a lower incidence of clinical infection, often having asymptomatic infections with multiple parasite genotypes (Portugal et al., 2016). This scenario has distinct similarities to the lack of clinical disease in Theilerial carriers and does accord with the findings of Jenkins et al. (2015). Here, in 10 introduced cattle, a phylogenetic cluster of the *T.orientalis* chitose A genotype and the ikeda genotype were associated with clinical disease, with chitose the major genotype following the first wave of parasitosis (days 50 -75; Jenkins et al., 2015). The genotypic parasitoses in the Dorrigo cattle did not conform to the same pattern, but the 3 Theilerial genotypes (ikeda, chitose and buffeli) were each present at similar levels of GC/ μ l through the carrier state which established from 3-6 months after arrival. Whether this outcome resulted from a maturing host response or an inherent property of *T.orientalis* akin to helminth hypobiosis is not known. As Theileriosis can recrudescence in carrier cattle under stresses such as long distance transport, this situation strongly suggests an active component of host regulation on parasite populations, analogous to “arrested development” of nematodes.

Subsequently, the parasitosis subsided into the carrier state after 3 months and remained relatively stable for the following 3 months to August. Over the same 3-month period, the PCV and ADLG recovered to pre-infection levels of around 32 and 0.8kg/day, respectively, but the initial weight loss caused by Theilerial infestation had not been recovered by the end of winter, some 6 months after arrival. These observations from 3-6 months (after recovery from the initial wave of Theilerial parasitosis) were consistent with the “normal” performance recorded for Theilerial carriers (Perera et al., 2014; Lawrence et al., 2019). The results also suggest that if the impact of this initial parasitosis can be more effectively managed or reduced, productivity losses may be mitigated to some extent (see below). In this “carrier” state, the concentrations of *T.orientalis* genotypes ikeda and chitose were reduced to similar levels exemplified by the buffeli genotype throughout the monitoring period. This result tantalisingly suggests some regulatory mechanism of this particular level of parasitosis and possibly explains why *T.orientalis* buffeli is considered “benign”.

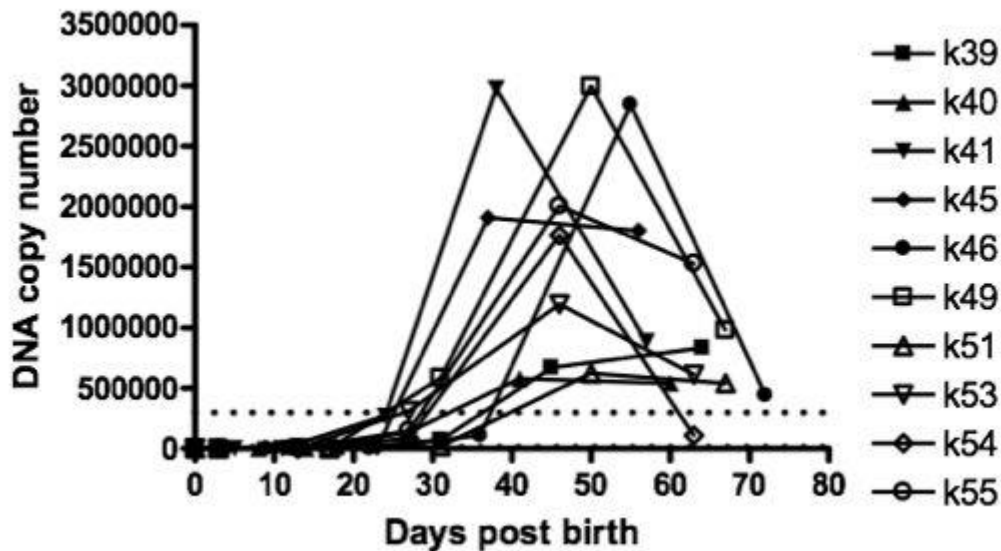


Fig. 9. The pathogenesis of Theilerial parasitosis in 10 calves born into an endemic area of *Theileria orientalis* at Gloucester NSW (from Swilks et al., 2017).

5.2 Immunisation of cattle with *Theileria buffeli*

5.2.1 Outcomes of immunisation

Both intravenous (iv) and subcutaneous (sc) inoculation of bovine blood infected with *T. orientalis buffeli* or *T. orientalis ikeda* each produce parasitosis detectable by PCR within 4 weeks, consistent with previous reports (Stewart et al., 1996; Hammer et al., 2016; Gibson, 2017). In each case the parasitosis appeared to peak around 6–8 weeks before stabilising at around 2,000–10,000 gene copies. As a warning, when Holstein blood was used for iv inoculation of Holstein calves from the local area (and possibly related to the blood donor), several animals suffered anaphylactic reactions, 2 of them twins. This also occurred with sc injection. Past experience with this procedure from earlier trials and from Queensland's Combivac 3 in1™ *Babesia* vaccine would indicate that this adverse event is very rare.

It was also noted when *H. bancrofti* ticks failed to feed and died, that *H. longicornis* nymphs failed to transmit *T. orientalis buffeli* to infect naïve calves at Camden for the second time. These results were consistent with previous vector studies indicating that *H. bancrofti* and *H. humerosa* were likely vectors for *T. orientalis buffeli* in Queensland (Stewart et al., 1987a,b). *H. longicornis* occurs in the coastal areas of Victoria and New South Wales and extends northwards as far as Gympie in Queensland but is absent from large areas of Northern Australia where *Theileria* sp (*T. orientalis buffeli*) is present (Riek, 1982). The results do not explain how *T. orientalis buffeli* is vectored around Dorrigo and Stroud in NSW. In these 2 locations, *H. longicornis* consistently ingest bovine blood from cattle multiply-infected with several Theilerial genotypes and subsequently appear able to re-infect naïve cattle with these genotypes, including with *T. orientalis buffeli*. However, as noted with the kinetics and age-related infections in naïve animals at Dorrigo, *T. orientalis buffeli* infestations are the slowest to develop.

This could arise for several reasons (also see Jenkins et al., 2015):

- *T. orientalis buffeli* may be selectively outcompeted for maturation in the tick (so less sporozoites inoculated); or,
- replicate more slowly in the host compared with the virulent genotypes; or,
- simply reflect that fact that *H. longicornis* does not transmit *T. orientalis buffeli* as readily or effectively as the *T. orientalis ikeda* and *chitose* genotypes.

To resolve these differences, development of *in vitro* cultivation of *T. orientalis* similar to that practised for *T. equi* (Alhasan et al., 2007) would be instructive and could enable drug screening and antigen preparation.

5.2.2 Effects of *T. orientalis buffeli* merozoites on subsequent *T. orientalis ikeda* infestation with *H. longicornis* ticks

Project objective 3 was completed. Prior inoculation of *T. orientalis buffeli*-infected blood containing between 6.5×10^6 (iv) or 4×10^9 (sc) piroplasms and allowed to “consolidate/incubate” for 13 and 4 weeks, respectively, before infected ticks were applied, significantly reduced by >80%, the initial parasitosis of *T. orientalis ikeda* from 18 DAI over the next 30 days. This outcome augmented reports from several historical Theilerial trials from Japan and Korea (Minami et al., 1981; Baek et al., 1992), ostensibly on *T. orientalis sergenti*, which has been confirmed as *T. orientalis* (Stewart et al., 1996). The outcome also fulfilled one the recommendations by de Vos (2011; B.AHE.0076), in that a tick challenge model was developed/ resuscitated to enable this approach and achieve the “anticipated” result.

Cattle recovering from the initial wave of parasitism after *T. orientalis* infection or following receipt of infected blood subsequently remain in a carrier state with parasitosis detectable by PCR in blood for greater than 30 months (Hammer et al., 2016, unpublished). Irrespective of the Theilerial genotype(s) present, this carrier state arising after natural tick-borne infection appears to prevent recurrence of clinical disease following seasonal tick challenge in endemic regions (Izzo et al., 2012; Kamau et al., 2011; Eamens et al., 2013). This has been witnessed by the progression of the epidemic curve through eastern and southern Australia and New Zealand, with mainly newborn calves and introduced cattle remaining susceptible to clinical disease in endemic regions while carriers appeared “unaffected” (Bailey 2012; Jenkins et al., 2015). Moreover, the widespread presence of *T. buffeli* carrier cattle in Queensland has been deemed at least partly responsible for the low prevalence of virulent/ clinical Theileriosis in that State (de Vos, 2011). Infestations of virulent Theilerial genotypes *ikeda* and *chitose* clearly outpaced the parasitosis of *T. orientalis buffeli* in susceptible cattle at both Gloucester and Dorrigo (Jenkins et al., 2015; this study), negating any opportunity for *T. orientalis buffeli* to induce “protection” before the establishment of the virulent genotypes. Leaving vector competence aside (discussed above), some type and level of immunity exists in carrier cattle which resembles “premunity” (Neal et al., 1969), interfering with the severity of subsequent challenge infestations. This effect has a long history in early “vaccinations” against Leishmania, Malaria, East Coast fever, Babesiosis and poultry Coccidiosis (“precocious strains”) (see McAllister, 2014; Masatani et al., 2016). This “premunity” can be negated by severe stress, resulting in recrudescence of clinical disease. But considering the circumstantial and historical evidence for *T.*

orientalis (Minami et al., 1981), it was prudent to examine whether immunisation with *T. orientalis buffeli* could reduce the severity of subsequent *T. orientalis ikeda* infestation.

Both intravenous (IV) and subcutaneous (SC) inoculation of *T. orientalis buffeli* produced detectable parasitosis (by PCR) within 4 weeks. Due to problems with tick vectors, parasitosis from the lower dose given by the IV route was patent for 13 weeks before challenge and induced more consistent protection than the SC vaccination (4 weeks and higher dose before challenge). As noted with temporal kinetics of *T. orientalis ikeda* infestation, the highest levels of the parasite occur around 4-6 weeks after tick challenge (Jenkins et al., 2015, Swilks et al., 2017, this study), such that the observed and significant, quantitative reduction in parasitosis over this period demonstrates the feasibility of this approach. However, as recommended by de Vos (2011), dose-response studies with single or mixed genotypes would need to be done to establish vaccination potential. It may be that prior immunisation with merozoites from any genotype(s) of *T. orientalis* may reduce the severity of parasitosis following challenge with virulent genotypes, as appears to occur in areas of endemic Theileriosis. However, this mode of protection will not work for calves in endemic zones as the virulent genotypes appear first. It would only work for proposed introductions that could be “immunised” before movement into endemic areas.

Field trials in endemic regions with high levels of tick infestation carrying multiple genotypes is vital to determine the robustness of the method; this is currently in progress at Dorrigo. Despite the difficulties of experimental tick infestation, these may be required to determine dose rates and genotypic combinations for establishing any reliable immunisation protocols. There may also be some synergy for a combination of “immunisation” before movement and application of long-acting effective acaricides on arrival to attenuate the tick challenge by also reducing the quantum of ticks infesting immigrant cattle. Given that ticks require 2-3 days of feeding to mature sporozoites before inoculation, acaricides for cattle, such as isoxazolines capable of killing ticks within 24h on companion animals and thereby preventing intoxicosis by *Ixodes holocyclus*, would be ideal.

6 Conclusions/recommendations

6.1 Control of Theileria and breaking transmission

6.1.1 Control of *H. longicornis*

Haemaphysalis longicornis has been confirmed to be a major vector and a definitive (final) host for *T. orientalis* ikeda and from studies at Dorrigo, is capable to transmit genotypes chitose, Type 5 and buffeli. For tick control, anecdotal evidence suggests that topical moxidectin has some effect at Dorrigo. However, dipping and integrated control measures for tick fever in Queensland has dealt with *Rhipicephalus australis*, a 1-host tick. Three host ticks feed for around 5-7 days at each stage, and may feed on alternative hosts to cattle and survive for extended periods off the host between moults. This makes control difficult, especially when only around 15 infested adult ticks can produce disease. We are currently examining whether *H. longicornis* infested during the larval feed can retain the ability to transmit Theilerial infection through to the adult stage when the nymph has engorged on an uninfected calf (second host).

Acaricide applications and field control measures for 3-host ticks have not been developed in Australia. In Africa, monthly dipping for *R. appendiculatus* does effect control of *T. parva*, so lessons may be learned for IPM here. Should development of isoxazoline compound preparations for use in cattle be forthcoming, these would be a substantial support for cattle producers and without doubt, marketing from the relevant companies would ensure widespread uptake, especially in Queensland for control of babesiosis and anaplasmosis (Tick Fever).

It is also considered that the seasonal distribution of *H. longicornis* be more definitively mapped to determine danger zones for cattle movements.

6.1.2 Chemotherapy of carrier cattle

Information from both overseas and recent MLA-funded trials (Bailey 2013, Carter, 2011), indicates that while buparvaquone (BPQ) reduces clinical parasitosis with Theilerial species, it does not eliminate the parasite. From the drugs examined in this study, other anti-protozoals and oxytetracycline did not exert any effect on the Theilerial parasitosis derived following inoculation of infected blood. In a separate trial, toltrazuril did not significantly alter the pathogenesis of *T. orientalis* ikeda infestation following tick challenge (Susan de Burgh, USyd, unpublished data). Several animals treated with tulathromycin for respiratory problems prior to challenge were equally susceptible. The widespread use of BPQ to treat clinical Theileriosis should be recommended for valuable animals especially bulls, which obviously then cannot be sold through the supply chain, but are still alive for use. There is no doubt from the Dorrigo cohorts and previous research (Eamens et al. 2013a,b; Jade Hammer, pers comm.), that mortality rates for seasonal Theileriosis can reach 30%, but are usually much lower. Animals in good condition mostly recover, but our experience at Dorrigo indicates that weight losses from the first wave of parasitosis are not recouped readily.

6.2 Control of *Theileria* by pre-immunisation

Given that pre-immunisation of experimental calves by inoculation of *T. orientalis* buffeli either intravenously (IV) or subcutaneously (SC) could reduce the initial parasitosis around 1-2 months after tick challenge, this warrants further investigation. Some points to consider and suggested recommendations from the initial study are:

- IV performed better than SC in the current trial, even though the dose of the latter was larger. This may be a prepatent period issue where the initial infecting dose needs a certain minimal or optimal time to establish as the tick challenge occurred 13 weeks after the IV and 4 weeks after the SC vaccinations. As one indication, Combivac 3-in-1 recommend 8 weeks freedom from stress to allow for effective vaccine take.
- In the current trial, 200 nymphal *H. longicornis* infected as larvae with *T. orientalis* ikeda were applied. We have no idea how this level of challenge reflects the vagaries of differing field challenges in endemic regions. Animals in this trial did develop high levels of parasite DNA and 2 controls exhibited a decline in PCV. It was noted that only 1/10 naïve cows introduced into an established endemic area of coastal NSW developed anaemia (Jenkins et al., 2015), but 5/30 of introductions in the Dorrigo study had PCVs of <20 by 6 weeks after arrival.
- As recommended by de Vos (2011), both dose response trials (for marketing) and field trials (to determine efficacy and validation) would be needed to mitigate failure. The ability to vaccinate with blood infested with ikeda or buffeli genotypes and then transport recipients without causing clinical disease is currently being investigated. It may be that prior immunisation with merozoites from any genotype(s) of *T. orientalis* may reduce the severity of parasitosis following challenge with virulent genotypes, as appears to occur in areas of endemic Theileriosis.
- Field studies, apart from determining whether the carrier state does protect against the initial parasitosis, would also determine any mitigation against production loss (weight gain)
- This mode of protection will NOT work for calves in endemic zones as the virulent genotypes appear first. It would only work for proposed introductions that could be “immunised” before movement into endemic areas.

6.3 Additional comments and acknowledgements

This project and associated research involved 9 DVM3 student research projects and a current MPhil degree by research. It was a wonderful learning experience for all of us which will hopefully see some students become engaged in livestock research.

The temporal kinetics and monitoring of the Dorrigo calves and introduced cattle will be published with Dr. Chris Shirley as one of the authors. I acknowledge Chris’s invaluable support and enthusiasm throughout this project, arising from his concern to do something tangible to alleviate the ravages of *Theileria* on his clients and their cattle.

The Buffeli immunisation study will be submitted for publication to “advertise” the findings; it forms part of the MPhil Thesis work of Susan de Burgh at Sydney University, together with a DVM3 research project of Therese Hoang Hieu Hanh Dinh. Ongoing field studies will be proposed with Dr Chris Shirley and an Industry Partner.

I also wish to recognise those invigorating, valued and productive discussions with Drs Peter Rolls and Phil Carter at TFRC, Wacol and with Dr Johann Schröder at MLA, throughout the course of the

project. These provided the historical foundations for the research questions and clarified the existing dogma. Without the crucial collaboration with TFRC and with Cath Covacin and Ralph Sutchbury at BSL in Brisbane and their expertise to produce and infect ticks, this research would not have been possible.

7 Key messages

7.1 *H. longicornis*.

The 3-host tick is a proven biological vector and final host for transmission of *Theileria orientalis* ikeda. It would appear capable of effective transmission for *T. orientalis* chitose, but is confirmed as a poor vector for *T. orientalis* buffeli. In endemic Theilerial zones in NSW and southern states, this host-parasite relationship means that the virulent genotypes appear first after tick infestation, effectively negating any ability of *T. orientalis* buffeli to generate any effective immunity unless this is deliberately undertaken in non-endemic zones prior to introduction.

7.2 Blocking transmission and parasitosis

7.2.1 Eliminating the carrier state

The carrier state with Theileriosis is apparently irreversible. Even BPQ does not eliminate the parasitism, as found overseas with *T. parva*. Toltrazuril (Baycox) and tulathromycin (Draxxin) do not prevent or reduce infestation. It is arguable that if the carrier state does not jeopardise productivity (Perera et al, 2014; Lawrence et al., 2019), then it may actually be beneficial in preventing re-infestation in endemic zones. Carrier animals may recrudescence with significant transport (>200Km), but this could be managed. In the absence of the vector, (*H. longicornis*), any recrudescence of disease after introduction of carrier cattle into non-endemic zones would die out (and has done at Narrabri and Terry Hei Hei).

A carrier state with minimal effect on productivity would certainly be advantageous to the parasite, allowing uptake by 3 host-ticks which have to renew infestation in each generation before sexual reproduction and transmission of *Theileria* can re-occur.

7.2.2 Pre-immunisation with *Theileria*-infested blood

This study is the first confirmation of speculations that the carrier state remaining in cattle recovered from the initial parasitosis from Theilerial genotypes, establishes some type of “resistance” to subsequent seasonal tick challenge. Prior immunisation with infected Theilerial blood did not cause clinical disease and significantly reduced the severity of the first wave of parasitosis arising from a subsequent tick challenge with virulent *T. orientalis* ikeda. This result requires further field investigation to determine the parameters where it will work reliably and the amount of production loss that can be recovered in introduced cattle.

It may be that prior inoculation with any genotype of *T. orientalis* may confer similar levels of “protection” against challenge with virulent genotypes.

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