final report

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Prepared by: Prof David Emery
University of Sydney

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Immunisation against virulent genotypes of Theileria orientalis

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Abstract

Project P.PSH.1312 aimed to:

1. confirm that inoculation of naïve cattle with bovine blood infested with Theilerial genotypes (buffeli, ikeda, or chitose) will protect cattle from development of clinical theileriosis by significantly reducing the first peak of parasitaemia, preventing anaemia and death.

2. determine whether this blood immunisation will prevent the weight loss incurred when cattle arrive in an endemic region of virulent theileriosis and are immediately subjected to tick challenge.

3. integrate successful outcomes into control measures on-farm through communications to Industry.

Subcutaneous inoculation of blood containing *T.orientalis* ikeda or buffeli developed the "carrier state" in preinfected cattle after 4-6 weeks. In 2 field trials, when cattle were moved into a region of endemic theileriosis, the preinfection significantly reduced the first peak of ikeda parasitosis. Depending on the intensity of tick challenge, animals preinfected with ikeda blood fared better than those given buffeli blood. Significant reductions in weight loss in preinfected cattle also depended on the level of tick challenge and was only significant when high intensity challenge occurred where controls lost between 5-20kg around the first peak of parasitaemia at 6-9 weeks after arrival. Weight loss could be recovered by compensatory gain if nutrition was available. This result confirms speculation that carrier cattle possess some "premunity" that protects against the severity of repeated, seasonal tick challenges. It is noted that preinfection will not work for newborn calves in endemic areas as the virulent genotypes reach the first peak of parasitaemia by 6 weeks before any preinfection could induce protective coverage.

For management of theileriosis, the main priority is surviving the first peak of parasitaemia occurring around 6-8 weeks after arrival or birth. In the absence of registered chemical treatments and vaccines, the main aim of control measures is to reduce or slow the tick challenge. In addition to strategic acaricide treatments ("off label " for *H. longicornis*, bush tick), results within this project indicate that cooler months when tick activity is reduced is more suitable for calving and introducing susceptible cattle. The results indicated that a "lower and slower" tick infestation will delay and lower the first peak of parasitaemia allowing greater survival and less clinical disease. The same effect was found in an initial trial in neonatal claves where ear tags used to control buffalo fly were applied to calves within two days of birth.

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 (Perera 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. 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. However, 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). Carrier cattle calve successfully on their home farms.

In P.PSH 0832, we demonstrated that artificial establishment of the carrier state by inoculation of infected blood did not cause clinical disease. After challenge with infected ticks, the parasitaemia in "pre-infected" animals was significantly reduced through the first peak of the disease (Emery, 2020; Emery et al., 2021b). These results could NOT be extrapolated to young stock as infection with virulent theilerial genotypes occurred too quickly for any blood immunisation to generate protective efficacy.

This research project aimed to clarify and extend the "pre-infection" model for protection of introduced stock:

- whether mechanical infection with blood piroplasms could protect against sporozoite challenge in the field when "preinfected" cattle were moved to endemic zones in the field; 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").

This approach to diminish the impact of *T. orientalis* infection in naïve cattle was successful in significantly reducing the parasitaemias, anaemias and production losses of 5-20Kg in cattle which had been pre-infected with blood containing *T.orientalis* ikeda or buffeli genotypes. The ikeda genotype was more effective than buffeli blood when tick challenge was more intense. Weight losses could be recouped by compensatory gain if nutrition was adequate, but cattle introduced to

Dorrigo in autumn were deprived of feed over Winter and did not recoup fully, the 5-20Kg weight losses incurred around the first peak of parasitaemia in April. When pre-infected cattle were moved from Mudgee to Dorrigo in May 2022, infection rates were slower and lower due to the colder maxima (2-3C) during May- September. Consequently, all cattle did not become PCR-positive for Theileria until 12 weeks instead of four weeks, and there was no significant effect on weight gain. In addition, the first peak of parasitaemia was 100-1000-fold lower than that detected under “normal” levels of tick challenge, providing further evidence that reducing the rate and severity of tick challenge was a viable attenuation strategy to prevent losses in susceptible cattle and newborn calves. These results confirmed some previous studies from Japan and Korea, but also validated results from the single trial in P.PSH.0832. The procedure could be used for introduced stock that could be “preinfected” for six weeks before transport into endemic zones. It would not be suitable for calves born in endemic regions as the infestation with virulent Theilerial genotypes occurs too quickly. Introducing carrier animals to a region free from *T. orientalis*, but potentially harbouring a competent vector, could 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 (Stewart et al., 1996), the new syndrome was termed "**B**ovine **A**naemia ***T**heileria **o**rientalis* **g**enotype" (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 etal., 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.

The total cost of Theilerosis 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 (Perera 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 (Perera et al., 2014; Lawrence et al., 2019), this MLA-funded research over the past six years has been directed towards either blocking transmission of infection to naïve animals by pre-infecting naïve cattle to induce and mimic the protection afforded by the carrier state. We do not know how this works mechanistically, but the hypothesis was that this procedure would reduce the impact of tick challenge and minimise production losses and deaths. This was approach to "lower and slow" the theilerial parasitaemia also involves tick management and was 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 still being tested for management of 3 host ticks like *H. longicornis* which have multiple hosts other than cattle. There is a concerted effort for new acaricides in this area, especially the isoxazolines that are 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. The features of theilerial transmission and pathogenesis was presented and discussed in the final report of P.PSH.0832 (Emery 2020) and is not re-iterated here. Clinical signs of disease regularly present around 6-8 weeks after births of calves in endemic zones or after arrival of introduced cattle, well after ticks have fed and gone.

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). However, while infection may not be "abolished", the parasitaemia may be reduced. In contrast, a challenge of two ikeda carriers with 200 nymphal ticks infected the ikeda genotype in PPSH.0832, resulted in significant reduction in the first peak of parasitaemia (Emery et al., 2021b; noting that it did not prevent infection, but reduced the severity).

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 protozoan parasites in three-host ticks (including *H. longicornis*) 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. It is now established from this project and related studies that infected nymphs feeding on uninfected (or other unrelated second) hosts can retain infectivity through to the moult to the adult stage and infect cattle as adult ticks. Research 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 three days of feeding on uninfected cattle. This need for 3-4 days to mature or multiply up levels of sporozoites is reported for *Ixodes scapularis* (Eisen, 2018) and for *T. parva*, where infected ticks are fed for four days on rabbits to increase infection rates prior to stabilate production (Kimbata & Silayo, 1997; Konnai et al., 2007). This also means that an ability to kill ticks within three days of attachment may block sporozoite transfer, providing considerations of the "speed of kill" and its "persistence" when selecting acaricides.

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. Further research in this project indicated that nymphal ticks can remain alive and infective for more than six months; more than sufficient to over-winter and cause disease in Spring when moulted to adults. This also has implications for spelling pastures to reduce tick numbers.

1.1.3 Protection against *Theileria orientalis* virulent genotypes

Some form and level of immunity exists in cattle which have recovered from the "first wave" of parasitosis. This first peak can produce clinical disease, but it usually slowly resolves around 2-3 months after infection (Jenkins et al., 2015, Perera et al., 2014). Recovered animals enter a persistent carrier state with reduced parasitaemias (Hammer et al. 2016; Emery et al., 2021a;b). These cattle in endemic zones do not appear to suffer recrudescence upon further seasonal tick challenge, so some form of "premunity" (Neal et al., 1969) interferes with the severity of ongoing challenge infestations. Despite comparisons with *T. parva*, we do not know how this "premunity" operates to limit parasitaemias with *T. orientalis* ikeda and chitose genotypes. For *T. parva*, 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., 1975; Emery et al., 1982). For *T. orientalis*, a role for antibody appears less likely given that calves birthed from carrier dams develop clinical disease with six 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 x 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. This was considered due to the possible transmission of Enzootic Bovine Leukosis (EBL) and Pestiviruses. 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 Freunds 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 P.PSH.0832. After a successful first trial, the ability of preinfection with *T. orientalis* buffeli (and ikeda) was repeated several times in this project to validate the outcomes. 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 shown that immunisation with infected blood could protect against homologous or heterologous tick challenge. Inoculation of bovine blood containing *T. orientalis* ikeda induced a dose dependent parasitosis over time from multiplication of the parasite (Hammer et al., 2016) and this mode of immunisation did not cause clinical disease (Hammer et al., 2016). This lack of clinical disease after preinfection with blood has been confirmed in several trials since.

1.1.4 Overarching aims of the Project

As presented below, the principal aims of P.PSH.1312 were to reduce the severity of tick infection with virulent genotypes by preinfection of susceptible cattle with blood stages of *T. orientalis*.

2 Project objectives

2.1 Aims of P.PSH.1312

The research in project P.PSH.1312 were to:

- confirm that inoculation of naïve cattle with bovine blood infested with Theilerial genotypes (buffeli, ikeda, or chitose) will protect cattle from development of clinical theileriosis, preventing anaemia and death.
- determine whether this blood immunisation will prevent the weight loss incurred when cattle arrive in an endemic region of virulent theileriosis and are immediately subjected to tick challenge.
- Integrate successful outcomes into control measures on-farm through communications to Industry.

All studies in the research project were conducted in accordance with approvals from the University of Sydney Animal Ethics Committee permits 2021/1974.

3 Methodology

3.1 Studies on preinfection

3.1.1 Cattle trials, preinfection and movements

Three trials were conducted:

3.1.1.1 Trial 1. A total of 36 mixed breed beef cattle aged 10-12 months were randomly allocated into 3 treatment groups (12 per group).

- Group 1; infected subcutaneously with 1ml bovine blood containing 2 x 10^8 merozoites (blood stages) of *T. orientalis* buffeli genotype;
- Group 2; infected subcutaneously with 1ml bovine blood containing 2 x 10^8 merozoites (blood stages) of *T. orientalis* ikeda genotype;
- Group 3; uninfected controls.

The cattle blood was provided by the Tick Fever Centre (TFC; Wacol, Qld). No untoward reactions were observed following infection and cattle were allowed back onto pasture next morning. At Dorrigo, cattle were weighed for ADLG and bled for estimation of both parasitaemia and PCV every 3 weeks after arrival for 4.5 months.

3.1.1.2. Trial 2A. In September 2021, a total of 400 mixed breed beef cattle aged 10-12 months were infected with 1ml bovine blood subcutaneously containing 2 x 10^8 merozoites (blood stages) of *T. orientalis* ikeda genotype. There were 30 uninfected controls.
The cattle blood was provided by the TFC (Wacol, Qld). No adverse reactions were observed following infection and cattle were allowed back onto pasture next morning.
However, despite the sampled cattle testing positive by PCR 4 weeks later, the cattle became infested with Bathurst burr and were not moved to Dorrigo.

3.1.1.3. Trial 2B. In late March 2022, approximately 150ml of theileria-positive blood was sourced from two separate *T. orientalis* ikeda and mixed ikeda-buffeli positive Holstein donor calves located at John-Bruce Pye farm, Sydney. (The TFC was no longer supplying stabilate). The blood was collected and contained within Acid Citrate Dextrose (ACD) vacutainers (McFarlane Medical supplies, Melbourne). The donor blood was analysed by PCR and the number of *T. orientalis* Ikeda and buffeli gene copies per milliliter of blood (GC/ ml) in each of the donor cattle were 2 x 10^6 and 25 x10^6, respectively. The preinfected cattle were subdivided into two further groups, with one receiving 2ml of the ikeda-infected blood (n = 18) and the other receiving 2ml of the mixed ikeda-buffeli blood (n = 18) by subcutaneous inoculation. The control group (n = 36) was not inoculated. Four weeks after inoculation with the donor blood, 10 cattle from the pre-infected groups were bled and blood was analysed using the Theileria-PCR assay protocol (VPDS, Ausdiagnostics) to determine the parasitaemia prior to their transport to Dorrigo in mid-May 2022. All were positive for their respective genotypes.

3.1.2 Samples, assays and production parameters

Prior to infection, all animals in Trial 1 and 10 from each of Trials 2A & B were bled into EDTA-vacutainers and tested negative for theilerial infection by PCR using the Ausdiagnostics ™ kit. This assay kit detects the theilerial genotypes ikeda, chitose, buffeli and type-5. Results were expressed as “cycle take-off”(Ct) which is interpreted as lower Ct values indicated higher levels of parasite DNA (higher parasitaemia). Ct values of <15 indicated high parasitaemia, Ct values between 15-25 indicated moderate to low parasitaemia, while Ct values >30 were deemed negative.
Four weeks after infection, cattle were bled and tested for appropriate infection levels in the respective groups.
For Trial 1 after arrival at Dorrigo, all cattle were weighed for ADLG and 15 controls and 15 preinfected animals were bled for estimation of both parasitaemia and PCV every three weeks for 4.5 months.
For Trial 2B after arrival at Dorrigo, 20 preinfected cattle and 10 control animals were bled and all 72 were weighed at three weekly intervals for 15 weeks to determine parasitaemia, haematocrit and average daily liveweight gain (ADLG).

3.1.3 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 ul blood using spiked samples and sample volumes in the assays.

3.2 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 (GC/ul) 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 pre-infected cattle after arrival

4.1.1. P.PSH.0832. Experimental preinfection with *T. orientalis* buffeli (a recap of results as a foundation for validation in the field).

Since the current research aimed to validate the pre-infection established in the final aim of P.PSH.0832, It was deemed useful to present these results from the pen trial for comparison with the field trials conducted in project P.PSH.1312. In this trial 15 calves were allocated to four treatment groups: 1. Uninfected controls (three calves); two preinfected with buffeli blood (five calves) ; 3, infected with ikeda blood (five calves) and 4, control calves that were found carriers of the ikeda genotype (2).

Both intravenous (iv) and subcutaneous (sc) inoculation of bovine blood infected with *T.orientalis* buffeli produced parasitosis detectable by PCR within 4 weeks Inoculation. When challenged at 6 weeks by the application of the 200 infected *H.longicornis* nymphs, all 15 calves because positive for *T.orientali*s ikeda within 12 days after infestation (dai). The parasitosis in the control groups followed a typical pattern in peaking around 5 weeks (39 dai) after infestation at a mean GC/ul of 69734 before declining to <2000 GC/ul blood by 62 dai (Fig.1 Table 1). The parasitosis in the three

treatment groups were significantly reduced between 30 and 85% during the first wave of parasitaemia from 25-39 dai (Fig. 1, Table 1). 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 and remaining significantly lower than that group up to 62 dai (not shown; see Emery et al., 2021b).

Table 1. 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
Uninfected 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 (ikeda carriers) (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/ul blood +/- SE.

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

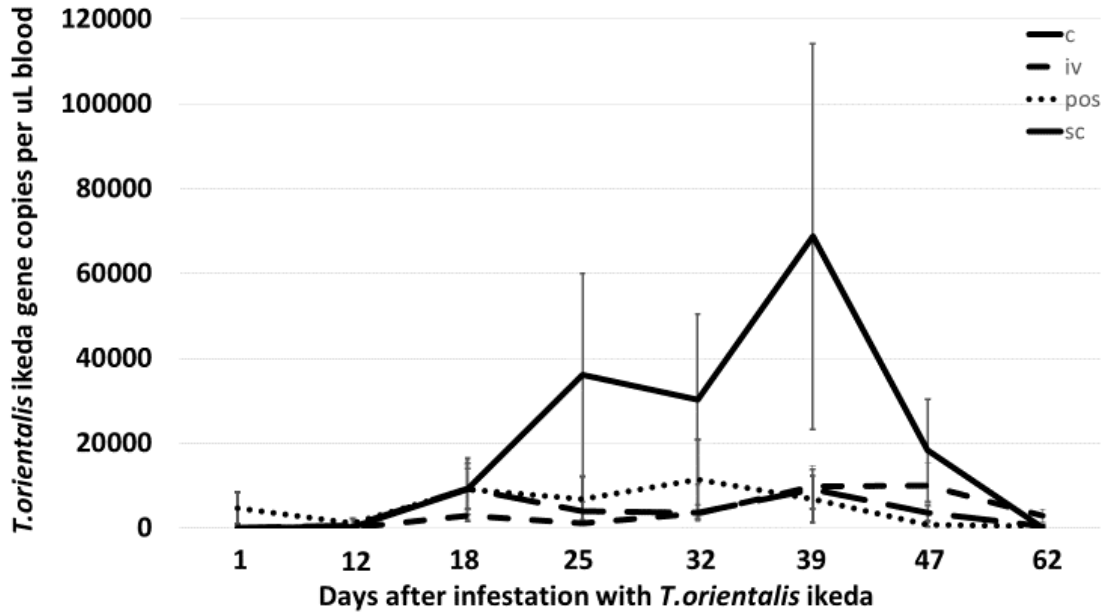

Figure 1. Group mean parasitoses for *T.orientalis* ikeda (GC/ per ul blood +/- SD) following infestation with 200 *H.longicornis* nymphs (see M&M, section 2.3.3). Group comparisons include: uninfected controls, (3 calves, solid line); previously infected controls (2 calves, dotted line); groups of 5 calves immunised with *T.orientalis* buffeli either IV (short dashes) or SC (long dash).

4.1.2 P.PSH.1312. Trial 1. Theilerial parasitaemias in preinfected cattle imported from Mudgee to Dorrigo.

Following the pre-infection at Mudgee, all inoculated cattle tested positive for the respective buffeli or ikeda genotype four weeks after their injections (data not shown). None of the cattle developed any clinical signs of theileriosis at Mudgee during the 6 weeks after inoculation and before they were moved to Dorrigo. Following transport and arrival at Dorrigo, none of the pre-infected cattle developed clinical disease in the first three weeks after arrival.

4.1.2.1 Parasitaemias of *T. orientalis* buffeli in treatment groups after arrival

Parasitaemias in the buffeli group (Group 1) which were pre-infected the *T. orientalis* buffeli were significantly lower ($P<0.05$) than those in the ikeda and control groups (2 & 3) in the period of 6-9 weeks after arrival (Figure 2). Subsequently, all cattle carried similar levels of parasitism with the buffeli genotype.

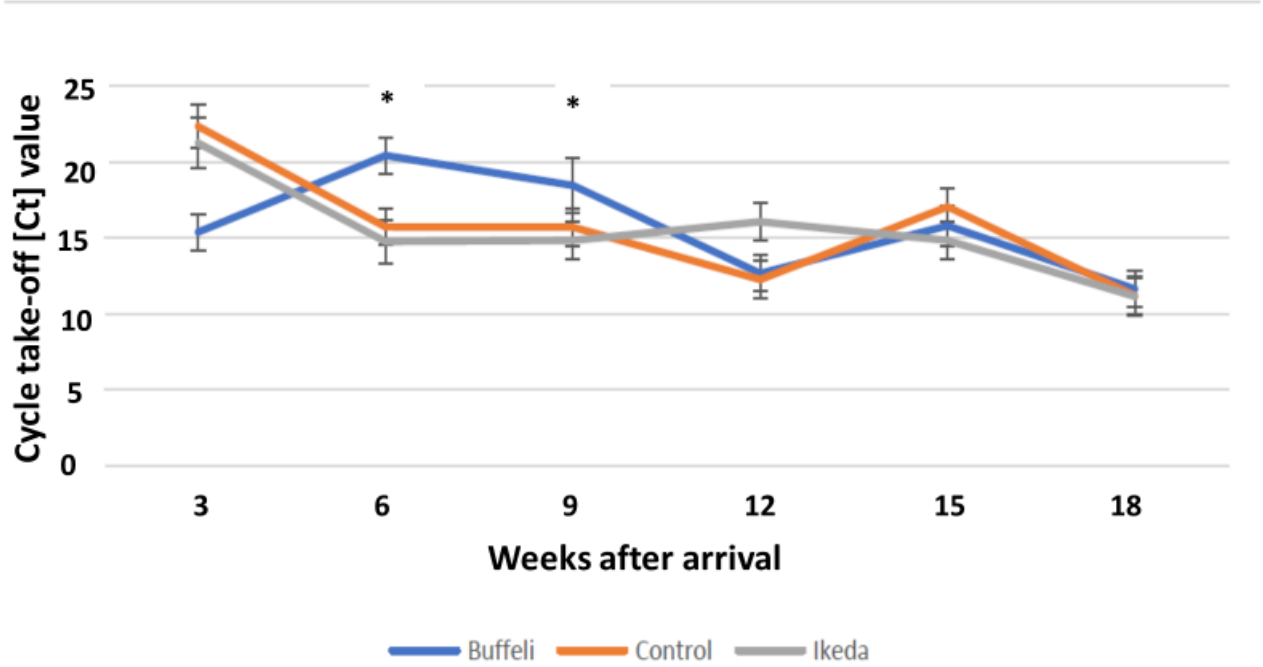

Figure 2. Parasitaemias with *T. orientalis* **buffeli** measured as " cycle take-off " (Ct values; lower values are higher parasitaemia) in the three treatment groups after arrival at Dorrigo. Significant reductions in parasitaemia (higher Ct values) for Group 2 (pre-infected with *T. orientalis* ikeda) are designated as; ** p<0.01; *, p,0.05.

- **4.1.2.2 Parasitaemias of *T. orientalis* ikeda in treatment groups after arrival**

The parasitaemias in Group 2 which were pre-infected the *T. orientalis* ikeda were significantly lower (P<0.05) than those in the buffeli and control groups (2 & 3) in the period of 6-12 weeks after arrival (Figure 3). This period coincided with the subclinical signs of theilerosis in the control group with reduced haematocrit (packed cell volume) PCV (Figure 4) and average daily liveweight gain (ADLG; Figure 5). After 12 weeks, the parasitaemias in the more affected Groups 1 & 3 declined to the lower levels of Group2 at Ct values around 12-15 reflecting the change to the carrier state (Figure 3).

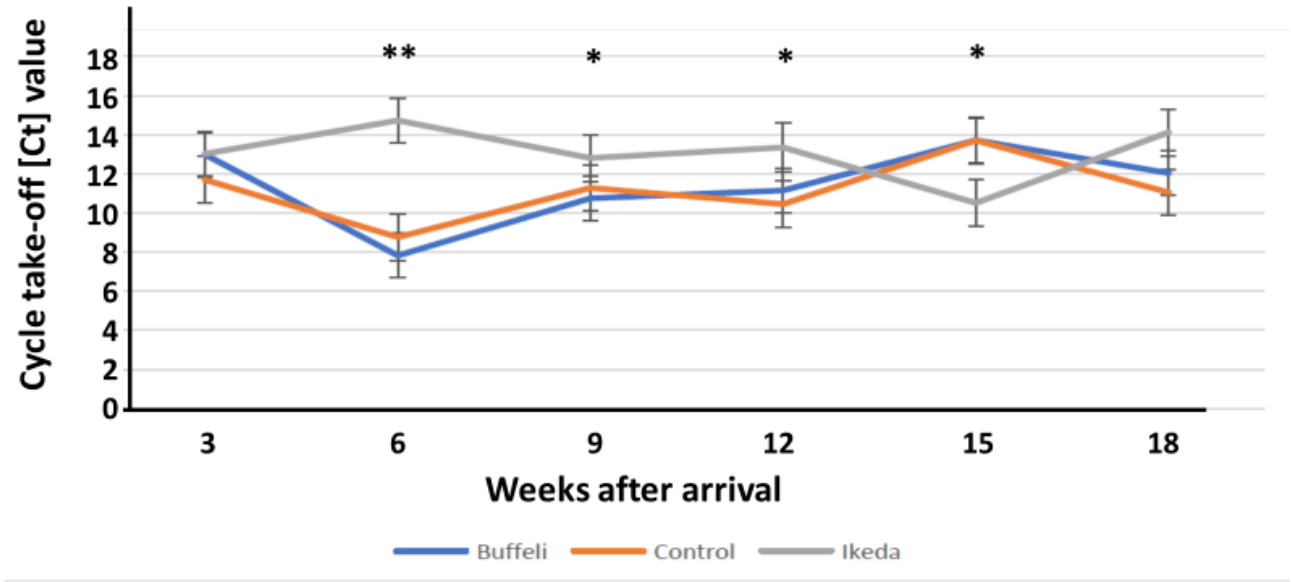

Figure 3. Parasitaemias with *T. orientalis* **ikeda** measured as " cycle take-off " (Ct values; lower values are higher parasitaemia) in the 3 treatment groups after arrival at Dorrigo. Significant reductions in parasitaemia (higher Ct values) for Group 2 (pre-infected with *T. orientalis* ikeda) are designated as; ** p<0.01; *, p,0.05.

4.1.2.3 Clinical measures of Theileriosis in cattle after arrival (PCV and ADLG)

Previous studies on newborn calves and naïve cattle introduced into endemic regions for theileriosis indicated that the first wave of parasitaemia occurred 5-9 weeks after birth or arrival and was accompanied by anaemia and reduced weight gains (P.PSH.0832). These clinical changes were measured by reductions on PCV and ADLG.

In the current trial, PCVs declined from six weeks after arrival (Figure 4) concurrent with the first wave of parasitaemia but had recovered by 15 weeks. PCVS were significantly lower at six and nine weeks in the buffeli and control Groups (1 & 3) compared with the ikeda-inoculated Group 2 (Figure 4).

ADLG also declined from three weeks onwards coincident with the developing parasitaemia (Figure 5). While there was a trend for cattle in Group 2 (ikeda infected) to show a reduced decline, this effect was only significant at week 12. Once the first wave of parasitaemia passed, ADLG recovered by week 18, so that by six months, there was no significant difference between the average weights at cattle in each group (data not shown). The mean arrival weight of cattle in Dorrigo was 360 kg, and after 18 weeks, the final weight would ideally be expected to stand at 468.8 kg. However, the observed mean weight at this time was in fact 460.4 kg, providing a net loss of 8.4 kg over the monitoring period.

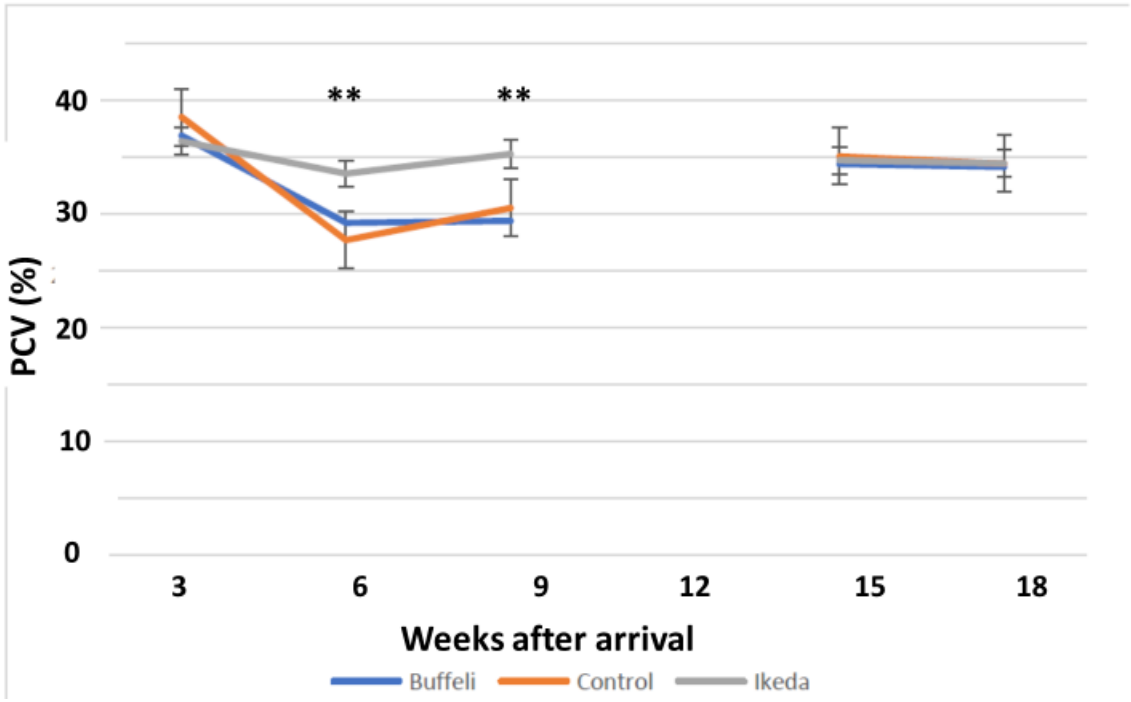

Figure 4. Packed red cell volumes (haematocrit) in the three treatment groups after arrival at Dorrigo. Significantly higher PCVs for Group 2 (pre-infected with *T. orientalis* ikeda) are designated as; ** p<0.01; *, p,0.05.
The 12-week blood sample was haemolysed due to a covid-induced delay in transport from Dorrigo.

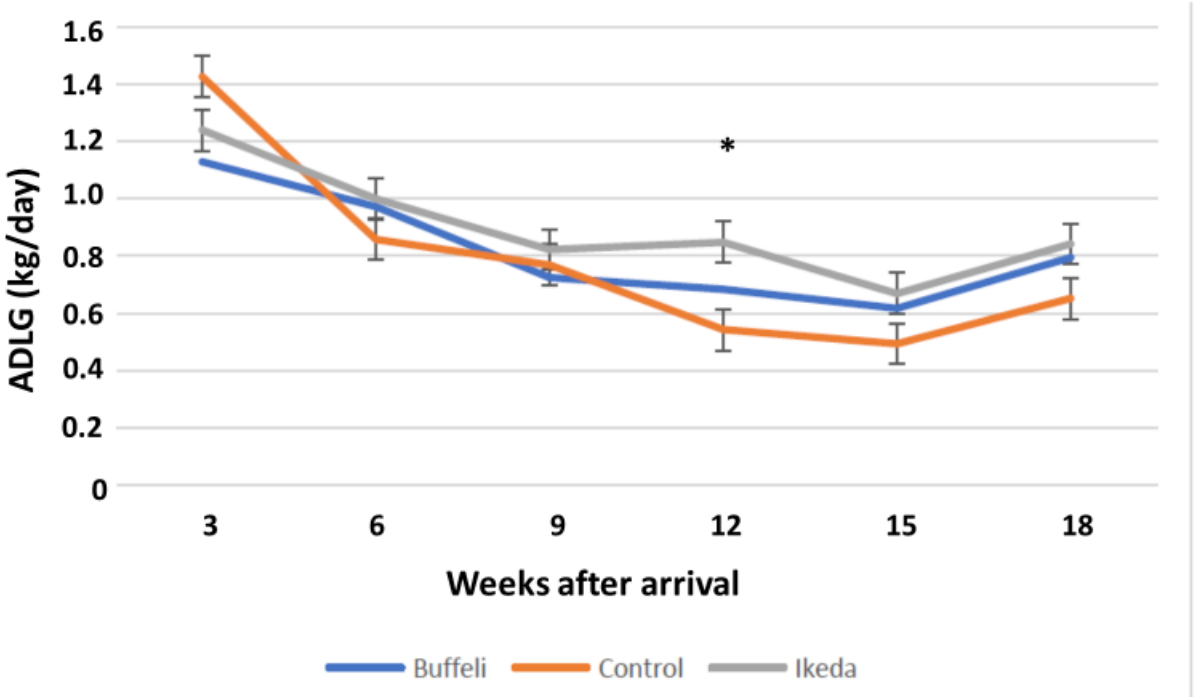

Figure 5. Average daily liveweight gains (ADLG) in the 3 treatment groups after arrival at Dorrigo. Significant differences for Group 2 (pre-infected with *T. orientalis* ikeda) are designated as; ** p<0.01; *, p,0.05.

4.1.3. P.PSH.1312. Trial 2B. Theilerial parasitaemias in preinfected cattle imported from Mudgee to Dorrigo.

The mean weight for all cattle upon arrival at Dorrigo (week-0) was 227.6 kg and upon conclusion of the trial (week-15) the mean weight was 275.2kg, giving a total average weight gain of 47.6kg across all cattle (Fig 6A). Over the course of the trial period, total body weight gain (in kilograms) was 47.5 ± 6.8 for the 'control' group; 54.7 ± 7.1 for the 'ikeda' group; and 54.2 ± 6.2 for the 'buffeli-ikeda' groups, respectively. The cumulative body weight gain for each over the trial period is shown in Figure 6A. Linear modeling of the total 12 week weight gain (TWG) did not reveal significant differences between any of the treatment groups tested.

The average daily liveweight gain (ADLG) was found to be 0.57 ± 0.01 in the 'control' group; 0.61 ± 0.03 in the 'ikeda' group; and 0.6 ± 0.3 in the 'buffeli-ikeda' group over the twelve-week period after arrival (Fig 6B). As with the TWG, a linear mixed model was utilised and there was found to be no statistically significant differences ($p > 0.05$) in ADLG between any of the treatment groups in the 12 period after arrival in Dorrigo.

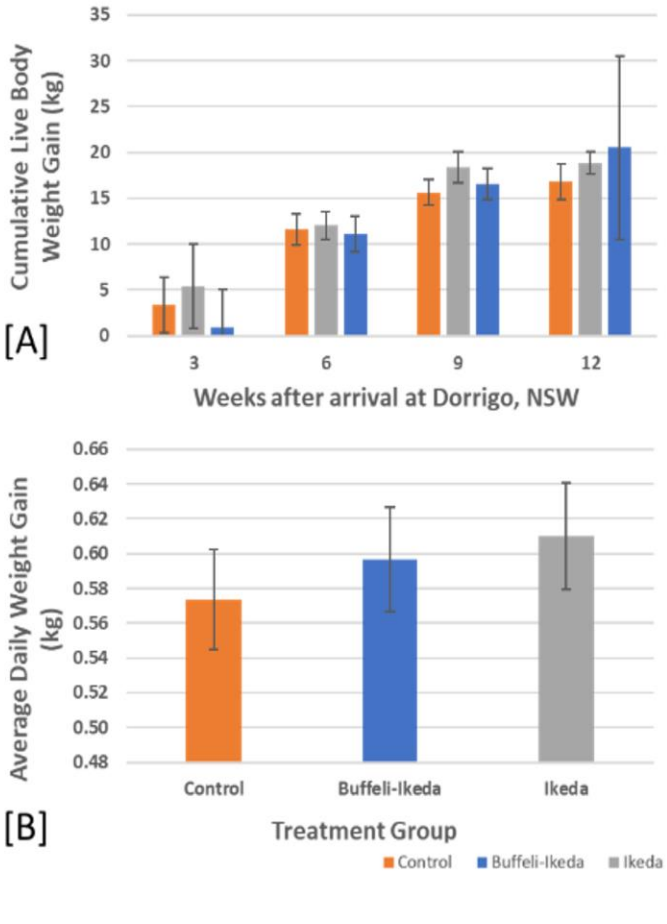

Figure 6. Cumulative weight gain (A) and average daily liveweight gain (ADLG; B) for the three groups of cattle in the first 12 weeks after arrival at Dorrigo. Comparisons include 36 controls (orange); 18 preinfected with a mix of *T. orientalis* buffeli and ikeda (blue); 18 cattle preinfected with the ikeda genotype (grey).

4.1.1 4.1.2 Parasitaemias of *T. orientalis* in treatment groups after arrival

After arrival, the parasitaemia in the control group peaked at the 12 week sampling period (Fig 7, blue). In pre-infected cattle, parasitaemias of the original genotypes buffeli and ikeda, declined modestly in the period following the respective peaks and remained at stable levels for the remainder of the trial period (Fig 7, grey and orange). Conversely, *T.orientlis* ikeda parasitaemias rose progressively throughout the trial period and reached the highest point in the control group at week 12, with a mean peak of 11,300 GC/ µL, and declined by 15 weeks. This peak of parasitaemia was around 100 to 1000-fold lower than usually observed around six weeks in susceptible groups of cattle at Dorrigo (see Emery,[2020] and Fig.1). This “slower and lower” development of parasitaemia was due to a slower rate of infection in all groups which were not 100% infected until 12 weeks after arrival (Fig. 8). For this comparison, the *T. orientalis* chitose genotype was monitored as one preinfected group received both ikeda and buffeli parasites.

All theilerial genotypes (ikeda, buffalo, and chitose) were significantly affected by time ($p < 0.001$). Furthermore, both the ikeda and buffeli groups reported a significant effect ($p < 0.001$) for the treatment and the interaction between treatment and time ($p < 0.001$).

However, from week 12, the levels of ikeda parasitaemia in the control group was significantly higher ($P>0.05$; $p<0.01$) that those apparent in the 2 preinfected groups (Fig 7).

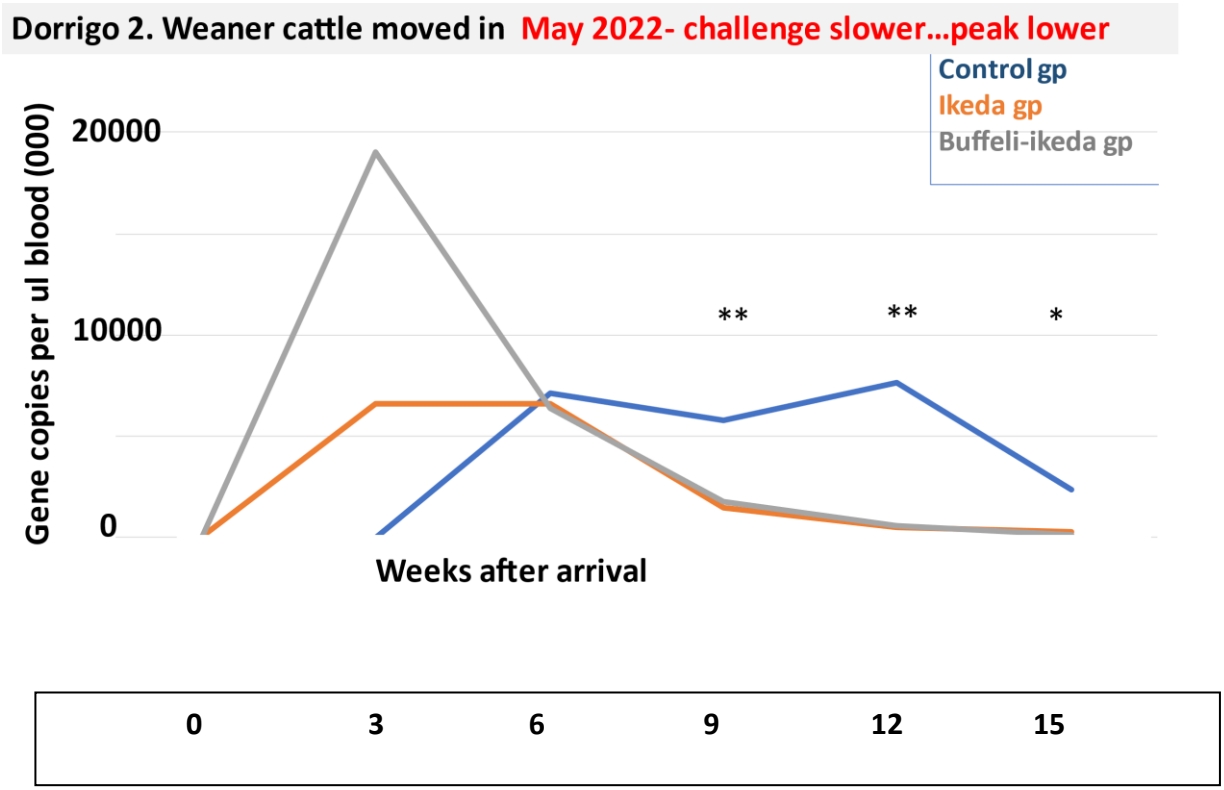

Figure 7. Trial 2B. Parasitaemias (gene copies per ul blood) in the control and preinfected cattle in the 15 weeks after movement to Dorrigo. Comparisons include 10 controls (blue); 10 preinfected with a mix of *T. orientalis* buffeli and ikeda (grey); 10 cattle preinfected with the ikeda genotype (orange).

* Significant reduction in parasitaemia ($p<0.05$); ** significant reduction in parasitaemia ($p<0.01$)

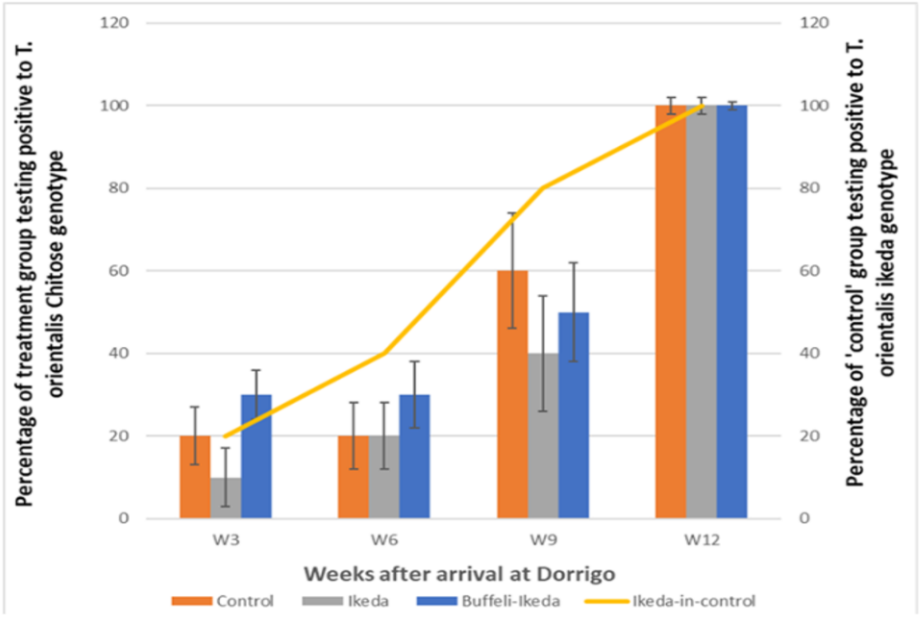

Figure 8. Cumulative percentage infection of treatment groups with *T. orientalis* chitose genotype over the 12 weeks after arrival. Comparisons in the bar chart include 10 controls (orange); 10 preinfected with a mix of *T. orientalis* buffeli and ikeda (blue); 10 cattle preinfected with the ikeda genotype (grey). The progressive infection rate for *T. orientalis* ikeda genotype in the control cattle is shown as the yellow line.

4.2 Communication and Technology transfer

A webinar by David Emery and Matt Playford was arranged and delivered through Paraboss on Tuesday April 11, 2023. The audience was mixed, with roughly equal numbers of producers and advisors. However, it was agreed by the Paraboss Technical Advisory Group, that due to biosecurity concerns regarding possible spread of Leucosis and Pestiviruses, that a certified source of infected blood needed to be established before the preinfection techniques could be endorsed and publicised for Industry. Previously, the Tick Fever Centre (TFC) supplied certified Theilerial stabilates raised in disease-free cattle. However, the TFC underwent a restructure and several experienced staff have left. The TFC no longer supplies stabilate, so any new supplied would have to be produced, stored and made available at and through, a facility such as the DPI NSW or Victoria. With this in mind, the preinfection results were not introduced to this webinar.

Therefore, the webinar discussed control measures to reduce or prolong the infection to successfully attain the carrier state. The priority was to attain a 'slower and lower' infection rate which generated a much lower first peak of parasitaemia, which also took longer to develop. This concentrated on an integrated control strategy using acaridicides and environmental measures (as outlined above) to reduce and avoid tick infestation and enable immunity to develop and the protective carrier state to be achieved with minimal production loss. This applies to both introduced cattle and calves born in endemic regions of theileriosis.

4.3 Initial approaches to control theileriosis in newborn calves

Although not part of Milestones and outcomes for P.PSH.1312, there are two populations of susceptible cattle for theileriosis. In the project, imported cattle were protected by preinfection before arrival. The other population is newborn calves in endemic zones. These cannot be protected by preinfection because they are infected with virulent genotypes from tick infestation soon after birth. The preinfection inoculum does not have sufficient time to induce the carrier state before the ikeda and chitose parasitaemias reach their peak around 6 weeks after birth (Emery, 2020; Emery et al., 2021a).

Consequently, the only methods available to reduce the parasitaemias in newborn calves is to reduce the level tick infestation which also decreases the quantum of theileria transmitted by bush ticks. This requires applications of acaricides as soon as possible after birth. Ms M. Shea offered to place Python® ear tags (containing synthetic pyrethroids) "off-label" onto calves within 2 days of birth at Milton. Subsequently, 10 of treated and 10 control calves were bled by a Veterinarian at 5 and 8 weeks for PCR. Parasitaemias after five and eight weeks in both groups are shown in Fig. 9.

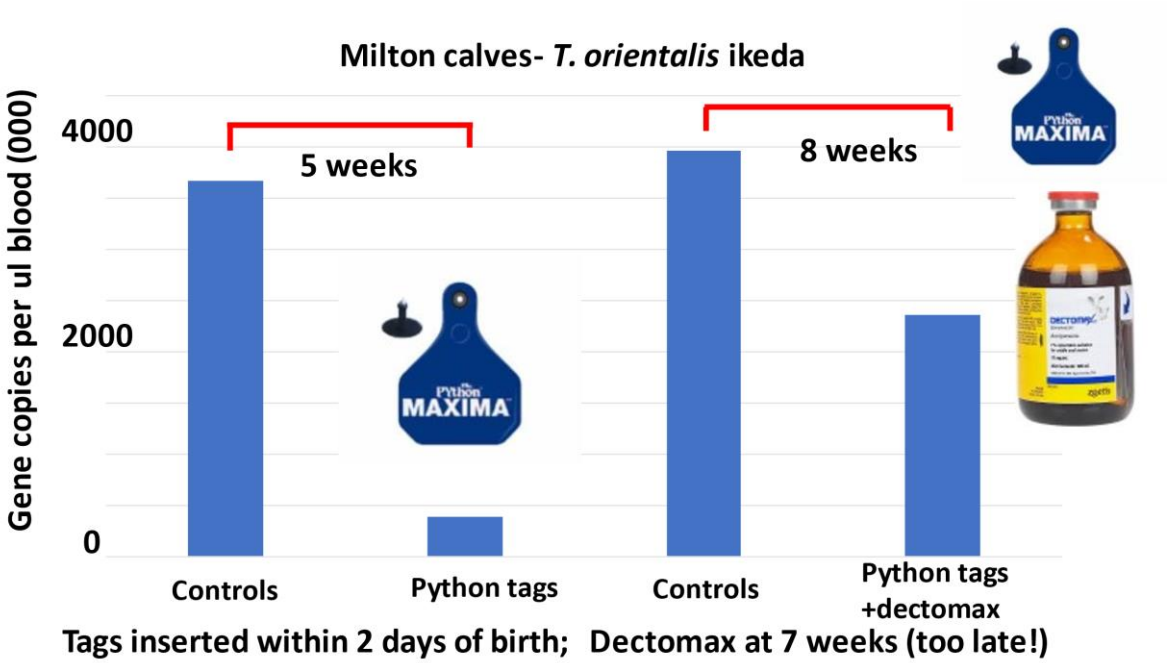

Figure 9. Comparisons of parasitaemias with *T. orientalis* ikeda in calves when a single ear tag was placed in 1 ear within two days of birth. Results from treated and control calves are shown at five and eight weeks of age. Dectomax was given intramuscularly at seven weeks of age (which is too late as the peak parasitaemia has been reached).

It is apparent that like the cooler weather delaying infection because of reduced tick activity, theoretically decreasing the tick numbers by the ear tags (ticks were NOT counted) has a similar effect. Both measures appear to slow the development of the parasitaemia and likely the peak levels (around the eight-week bleed in Fig 9) as well.

5 Discussion

5.1 The Efficacy of pre-infection

5.1.1 Background comments and overall results

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), and the fact that subcutaneous inoculation of blood stages of the parasite does not appear to cause clinical disease (Hammer et al., 2016; Lawrence et al., 2019), studies in P.PSH.0832 found that preinfection with *T. orientalis* buffeli could reduce the severity of subsequent *T. orientalis* ikeda infestation. In the same trial, it was found that 2 calves which were carriers of *T. orientalis* ikeda (likely infected through blood in milk after birth) also showed a significant reduction in the peak ikeda parasitaemia after tick challenge (Emery et al., 2021b). This mimicked the effect of the carrier state and validation of this effect in the field was the aim of P.PSH.1312.

Previous studies reported that the carrier state did not compromise productivity (Lawrence et al, 2019; Perera et al, 2014). This was also the case when around 400 yearlings were preinfected at Mudgee but were not moved to Dorrigo due to Bathurst burr infestation. Cumulative weight gains of preinfected animals and control cattle did not differ significantly over the subsequent eight months before cattle were sold. So, at Mudgee in Trial 1, both ikeda and buffeli genotypes were compared for protective capacity after movement of 10-month-old, preinfected cattle in August (early spring) to Dorrigo, a region of heavy tick challenge (Emery et al., 2021a). In addition, previous monitoring of weaner calves from Mudgee after arrival at Dorrigo in February, found that the tick infestation

inflicted a 1% mortality and 20kg loss in weight gains over the first three months after arrival (Emery et al., 2021b).

Since it was appreciated that clinical theileriosis could be induced in infected cattle by transport stress, we examined whether overnight travel around 500km from Mudgee to Dorrigo would induce any relapse in preinfected cattle. This did not occur, either in the six weeks after pre-infection at Mudgee or in the first three weeks after arrival at Dorrigo from four such movements. The effects of tick challenge were becoming apparent within 4 weeks after arrival as the theilerial parasitaemia increased.

Both Trials 1 and 2B examined the level of protection afforded by the preinfection at Mudgee. It was apparent that the level of tick challenge at Dorrigo was able to overcome any protection afforded *by T. orientalis* buffeli. In contrast, cattle, pre-infected with *T. orientalis* ikeda at Mudgee were significantly protected against tick challenge as evidenced by lower parasitaemias with the ikeda genotype and the associated clinical effects of red blood cell and weight losses. This validated the preinfection methodology and rationale for adoption in the field. However, a certified source of blood stabilate is needed to negate any viral transmission from indiscriminate injections.

Previous studies on newborn calves and naïve cattle introduced into endemic regions for theileriosis indicated that the first wave of parasitaemia occurred 5-9 weeks after birth or arrival and was accompanied by anaemia and reduced weight gains (P.PSH.0832). However, in Trial 2B the infection rate was delayed and prolonged (Figs 7, 8). Using the infection rates with *T. orientalis* chitose as an indication of infection, the endemic *H. longicornis* took 12 weeks to infect all arrivals. As discussed previously, this appeared correlated with lower than normal maximum temperatures over the trial period from May to September when the Dorrigo property was sold. The parasitaemia with *T.orientalis* ikeda reached a lower peak at 12 weeks before declining, whereas in previous trials, this had occurred by week 9 before diminishing.

5.1.2 Effects of pre-infection on weight gains after arrival into endemic zones

In both Trials 1 and 2B, there was a trend for higher ADLG in cattle pre-infected with *T. orientalis* ikeda which was only significant around the peak of parasitaemia in Trial 1 and not significant following the delayed challenge in Trial 2B. Weaner cattle previously moved to Dorrigo in Autumn lost around 20Kg from expected weight gains to September, likely due to diminishing feed availability in winter when survivors had fully recovered from the first wave of theileriosis in April-May. In contrast, in Trial 1 yearling cattle moved to Dorrigo in September had ample pasture available in late spring after recovery from the initial effects of theileriosis. These cattle were able to recover the 5kg weight losses by five months after arrival. However, in Trial 2B when cattle were moved to Dorrigo in late Autumn, infection rates and parasitaemias were too slow and low, respectively, to have any significant effect on weight gains. So, it is apparent that both feed availability and the severity of tick challenge will determine how much compensatory gain can recover any weight lost during the first peak of parasitaemia caused by the virulent theilerial

genotypes, ikeda and chitose. Reducing the quantum of challenge by controlling tick levels will have the same effect by diminishing the peak parasitaemia.

5.2 Preinfection of cattle with *Theileria* ikeda or buffeli

5.2.1 Effects of preinfection

Subcutaneous (sc) inoculation of bovine blood infected with *T. orientalis* buffeli or 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 per ul blood. Preinfection significantly reduced the first peak of parasitaemia by *T.orientalis* ikeda . 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.

5.2.2 Neonatal calves.

Preinfection will not work for newborn calves in endemic zones because they are infected with virulent genotypes from tick infestation soon after birth. The preinfection inoculum does not have sufficient time to induce the carrier state before the ikeda and chitose parasitaemias reach their peak around 6 weeks after birth (Emery, 2020; Emery et al., 2021a). Therefore, measures to protect calves should involve environmental avoidance of 'ticky' areas, early calving in late winter when tick mobility is reduced and effective treatment as soon as possible after birth to reduce tick infestation.

In this project, we made initial attempt to see whether acaricides could slow the acquisition of theileria. Python® ear tags, which have been applied to reduce tick paralysis of cattle in Queensland, were inserted into the ear of calves within two days of birth (1 tag per calf). It was apparent that like the cooler weather delaying infection from reduced tick activity, theoretically decreasing the tick numbers by the ear tags also reduces and slows the development of the parasitaemia and likely the peak levels (around the 8 week bleed) as well. More research is needed in this area.

6 Conclusions/recommendations

6.1 Control of Theileria and breaking or reducing transmission

Given that there are currently no vaccines or chemicals registered for the prevention or treatment of *T. orientalis* in Australia, therapy is limited to 'tender loving care'. Under normal conditions for tick challenge in endemic regions, the danger period for susceptible groups of cattle is six weeks after birth or introduction when the first peak of parasitaemia usually occurs. Subsequent anaemia from the asexual reproduction of virulent genotypes of the parasite causes anaemia from which all other clinical manifestations ensue. To manage the parasitaemia requires control of the vector tick to reduce the rate or quantum of challenge. This can be achieved by reducing of tick numbers or activity and

concomitantly, the rate of infection OR as shown from this project research for imported susceptible cattle, preinfection for 4-6 week before entry.

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 ikeda, chitose and type 5. From experimental studies, it appears to be a poor final host and vector for the buffeli genotype, which is transmitted by *H. bancrofti* and *H. humerosa* (Stewart et al. 1987a,b; 1989). For protecting susceptible imported cattle, there would be some synergy from a combination of preinfection 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 would be ideal. Isoxazlines (Bravecto, Credelio, Nexguard and Simparica) have prevented intoxicosis by *Ixodes holocyclus*, and this tick also takes around 3 days of feeding to progressively increase toxin production.

For tick control, Paraboss webinars provided a table for acaricides which can be used ***off label*** to reduce tick numbers and infestations (Table 2 below, courtesy of Dr Matt Playford). Most were developed for cattle tick (*Rhipicephalus australis*), a 1-host tick which remains and is restricted to a cattle host for 3-5 weeks, so is readily treated. The problem for three-host ticks is that the bush tick can develop readily on non-livestock hosts and each stage only feeds for 5-7 days to engorge before dropping off to moult. This would require either repeated application or a persistent chemical. We have also found that nymphs can remain infective for at least 6 months, which enables easy overwintering and they are resistant to spelling in the cooler months. This means that products with a short **persistence period** will require repeated treatment to reduce tick numbers. The other feature of acaricides is the **speed of kill** to prevent pathogen transmission (Schorderet-Weber et al., 2017; Eisen, 2018)**.** Since *H. longicornis* requires 2-3 days of feeding to mature theilerial sporozoites prior to inoculation in saliva, products which kill ticks within 24-48h of attachment will block transmission of theileria.

We have also found that infected larvae of *H. longicornis* can carry theilerial parasites through 2 moults and does not need reinforcement of the infection at the nymphal stage if feeding on a non-infected second host (cattle or wildlife). This also complicates pasture control, as does the fortitude of infected nymphal ticks during colder months. However, as Trial 2B indicated, cooler weather results in reduced tick activity and infection, enabling a slower and lower first peak of parasitaemia. This is instructive for both calving and introductions in that one can try to:

- introduce susceptible cattle to endemic zones in cooler months when challenge is " lower and slower"; and,
- in endemic regions, try to calve earlier, moving to late winter and keep calving paddocks away from bush or where kangaroos are numerous.

The seasonal occurrence of clinical theileriosis is related to temperatures and the availability of susceptible cattle as the vast majority, if not all cattle in endemic zones have recovered from

infection and are carriers, likely for life. This protects them from seasonal disease with tick challenge unless this is exceptionally intense or animals are severely stressed. So in endemic regions, ticks are always present, and a lack of theileriosis does not means the area is tick-free for imported cattle.

Table 2. a list of current acaricides with claims for bush tick (*H. longicornis*) control (courtesy of Dr Matt Playford, Paraboss).

Class of chemical	Product (examples)	Active ingredient(s)	Comments
Synthetic Pyrethroid (SPs)	Bayticol cattle dip and spray Arrest Fly & Tick Dip & Spray	Flumethrin Deltamethrin	✓✓ Bush tick claim Knockdown- up to 10d
Organophosphates (OPs)	Barricade S	Chlorphenvinphos (+ cypermethrin)	✓✓ Bush tick claim Knockdown- up to 14d
Amitraz	Taktic cattle dip & spray	Amitraz (in EC or WP)	✓✓ Bush tick claim Knockdown- 1d
Macrocyclic Lactone (MLs, mectins)	Cydectin Pour-on Dectomax injection (off-label use)	Moxidectin Doramectin	No claim for bush tick Selects for resistance in internal parasites
Isoxazolines	Simparica, Credelio, Bravecto, NexGard	Sarolaner, Lotilaner, Fluralaner, Afoxolaner	Only registered for dogs (Also Advantix)

As a final note on acaricides, should development of isoxazoline preparations (red in Table 2) 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. This is especially the case for the fringe regions such as the New England areas in NSW and in the South Australian borderlands and in Tasmania.

6.1.2 Control of Theileria by preinfection of imported animals

From the confirmation in this project, of early finding in P.PSH.0832, preinfection of susceptible cattle for 4-6 weeks, by inoculation of blood containing theilerial merozoites significantly reduces the dangerous first peak of parasitaemia after tick challenge with virulent genotypes. From experimental trials, this method operates in carrier cattle; they do exhibit an increase on parasitaemia, but the peak is significant reduced (Emery et al., 2020b). Overall, preinfection with the ikeda genotype was more consistently protective in this project. As indicated from the webinar detailed above (section 4.2), a certified virus-free stabilate needs to be developed and stored to prevent viral spread of EBL and pestivirus. These were available through the Tick Fever Centre, but no longer. Perhaps DPI-NSW could provide such a service and recoup a price? The preinfecting dose appears to generate PCR-positivity and works after an initial dose ranging from 5×10^7 to 2×10^8 parasites per ml, and animals are held for 4-6 weeks; the longer likely the better. 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 preinfected before movement into endemic areas. Protection of newborn calves requires a different strategy as outlined above.

6.2 Additional comments and acknowledgements

This project and associated research involved 4 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.

I acknowledge Chris Shirley and Wade Timms' invaluable support and enthusiasm throughout this project, arising from concerns to do something tangible to alleviate the ravages of Theileria on clients, producers and their cattle.

I also recognise those valued and productive discussions with Drs Peter Rolls and Phil Carter at TFC, Wacol and with Dr Johann Schroder at MLA, throughout the major part of this project. These provided the historical foundations for the research questions and clarified the existing dogma. Without the crucial collaboration with TFC 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 three-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 or blood inoculation to generate any effective immunity unless this is deliberately undertaken in non-endemic zones prior to introduction. The tick also has the advantage of carrying theileria through 2 moults. If infected as larvae, the nymph does not require a boost to infection on the second host, whether this is an uninfected cow, a dog or a wildlife host. Similarly, it must be appreciated that the nymphal stage is the usual stage to over-winter, and this stage can remain infective for at least 6 months. Therefore, killing ticks in coastal

areas by paddock spelling is difficult due to favourable climatic conditions and the presence of alternative hosts such a kangaroos, to transport and re-contaminate paddocks.

7.2 Blocking transmission and parasitosis

7.2.1 Protection is enabled by the carrier state

The carrier state with theileriosis is apparently irreversible (fortunately) and therefore its protective capacity ("premunity") is vital to the control of theileriosis in endemic regions (Emery, 2021). The mechanism remains elusive, but is likely immunologically based as a lead time after blood inoculation is required for detectable infection and protection to develop. It does require active infection (like Babesial vaccines) to generate a sufficient parasitaemia which closely resembles the levels in the blood of carrier cattle. It is arguable that if the carrier state does not jeopardise productivity (Perera et al, 2014; Lawrence et al., 2019), then it is beneficial in preventing re-infestation in endemic zones and protecting imported animals through the first wave of parasitaemia. 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 three-host ticks which have to renew infestation in each generation before sexual reproduction and transmission of Theileria can re-occur. However, as has been shown, production losses and deaths are minimal.

The carrier state can be induced by the deliberate inoculation of infected blood. After establishing the carrier state over the ensuing 4-6 weeks, these preinfected cattle can be moved into an endemic region. The anticipated significant reduction in the first peak of parasitaemia reduces the chance of anaemia, clinical disease and weight loss. Prior to widespread adoption, a source of certified disease-free blood stabiliate is needed. Since the tick Fever Centre (TFC) no longer supplies theilerial stabilate (as cases are low due to the prevalence of *T. orientalis* buffeli and lack of a tick vector), a new centre needs to be established in the endemic regions.

7.1.2 **Reducing tick challenge.**

This was not part of the current project, but results from Trial 2B revealed that when cooler temperatures prevail and likely reduced tick activity, the development of theilerial parasitaemias is delayed and reduced. This effect enables planning for both calving times and the introduction of susceptible stock, or even those which are preinfected, increasing their chances of survival and development of a protective carrier state if the advantages of cooler weather are utilised.

8 Bibliography

8.1 Publications from the project

Emery, D. 2021. Approaches to Integrated Parasite Management (IPM) for *Theileria orientalis* with an emphasis on Immunity. Pathogens, 10(9), 1153. https://doi.org/10.3390/pathogens10091153

Emery, D., Zhang, S., Loo, C., Shirley, C. 2021a. A longitudinal study of infection with genotypes of *Theileria orientalis* in calves and introduced cattle at Dorrigo, New South Wales, and the effect on weight gains. Veterinary Parasitology, 296, 1-7. https://doi.org/10.1016/j.vetpar.2021.109487

Emery, D.L.; deBurgh, S.; Dinh, T.H.H.H.; Rolls, P.; Carter, P. 2021b. Merozoites of *Theileria orientalis* buffeli reduce the parasitaemia of *T. orientalis* ikeda following tick challenge. Veterinary Parasitology, 298, 109532, https://doi.org/10.1016/j.vetpar.2021.109532 .

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