final report

Vectors and epidemiology of Theileria orientalis on the Northern Tablelands

Executive summary

Bovine theileriosis caused by the *Theileria orientalis* complex is a tick-borne disease of red blood cells causing a mild persistent infection with severity dependent on the infecting genotype and host exposure status. Clinical theileriosis was first reported on the Northern Tablelands of NSW in 2009 and a high prevalence of infection reported in a 2013 study, but the parasite was not genotyped. The study also found no association between parasite prevalence and presence of ticks on properties as reported by the owners. In response to ongoing clinical cases, a cross-sectional study was conducted in the area around Armidale NSW between December 2017 and April 2018 with the aim to identify *Theileria* genotypes present in the region and potential vectors involved. Results based on multiplex qPCR of 90 blood samples from eight farms revealed 100% prevalence of *Theileria* in individual cattle with prevalence of the different genotypes being Ikeda (89%), Chitose (91%) and Buffeli (89%). The prevalence of Ikeda differed between farms; however, the level of parasitemia was not affected by genotypes. The high prevalence of co-infection with all three *T. orientalis* genotypes (73% of cattle) indicates the endemic status of the parasite in the region and confirms the lack of cross-protection among genotypes.

Sampling for questing ticks on pasture was done between November 2017 and May 2019 on six of the eight farms using a flannel dragging method. A total of 358 ticks were collected on only one of six farms and all ticks were morphologically identified as *Haemaphysalis bancrofti*. Counts were greatest for larvae followed by nymphs and adults. *Theileria orientalis* genotypes (Ikeda and Buffeli) but not Chitose were detected only in the nymphal stages. This is the first detection of *T. orientalis* in questing *H. bancrofti* ticks and together with previous transmission work with this parasite indicate that it is likely to be an important vector for *T. orientalis* in this region. However, the high prevalence of bovine infection is at odds with absence of captured ticks or owner-reported tick presence on 5 of the 6 farms raises the likelihood that other vectors or transmission pathways are important.

Among these is mechanical transmission by haematophagous insects. Previous studies have shown that inoculation of as little as 100µl of blood from parasitemic animals is able to establish infection in recipient calves. Considering that the average blood meal size of tabanids ranges from 20µl to 680µl, investigations into the seasonal and spatial distribution of various biting insects on the Northern Tablelands was conducted between December 2017 and May 2019 on the same six farms used for the seasonal tick study. In addition to the trapped biting flies, the role of sucking lice and stored specimens of *Culicoides* biting midge species feeding on cattle (*C. brevitarsis, C. dycei, C. nattaiensis, C. victoriae, C. marksi and C. bundyensis*) collected in the New England region of NSW were screened for *T. orientalis* using qPCR. A total of 431 biting flies of eight genera comprising eleven species were collected using unbaited Nzi traps. The tabanid species were present in all farms with *Dasybasis oculata* and *D. circumdata* being the most abundant and widespread species. The diversity and abundance of species in summer and autumn months were higher than spring. The fly activity pattern for *D. oculata* reached peaks in March, November and January and for *D. circumdata* March and November. The sucking lice (*Linognathus vituli* and *Haematopinus eurysternus*) and biting louse (*Bovicola bovis*) lice were also collected. A wide range of hematophagous insects or insect pools were subjected to PCR with *T. orientalis* only detected in the sucking lice. The

detection of the parasite in lice is of significance as it is in agreement with another Australian study and mechanical transmission of T. orientalis by sucking lice has been reported in Japan.

The 100% prevalence of *Theileria* in the present study in the absence of clinical disease is suggestive of the achievement of enzootic stability in the study area. However, the project has also confirmed the lack of association between tick presence and *Theileria* on the Northern Tablelands and it would appear that the situation of enzootic stability may be achieved by a combination of vertical transmission as demonstrated in previous studies, and lateral transmission by either or both of a biological tick vector and a mechanical insect vector, most likely sucking lice.

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1 Background

1.1 An overview of *Theileria orientalis* in Australia

Among tick-borne diseases, theileriosis caused by the haemoprotozoan parasite, *Theileria* (Apicomplexa: Piroplasmorida) is an economically important disease of ruminants mainly in tropical and sub-tropical countries (Aktas et al., 2006, Gebrekidan et al., 2016, Taylor et al., 2016). *Theileria parva* and *T. annulata* are the most pathogenic species causing East Coast fever and tropical theileriosis respectively (Salih et al., 2007, Yusufmia et al., 2010). Though these species have not been reported from Australia and New Zealand, Theileria-associated bovine anaemia (TABA) caused by *T. orientalis* has become increasingly important in clinical outbreaks (Izzo et al., 2010, Islam et al., 2011, Eamens et al., 2013a).

In Australia, infections of cattle with *T. orientalis* complex have been recognized since 1910 and considered benign (Callow, 1984, Seddon, 1952) with the exception of clinical cases in Queensland in the 1960s due to *T. buffeli* ("Warwick" strain) (Rogers and Callow, 1966). However, since 2006 pathogenic genotypes were identified, coincident with a large number of outbreaks in both beef and dairy cattle in all states except Tasmania (Islam et al., 2011, Eamens et al., 2013b, Perera et al., 2014). Based on sequence variations in the major piroplasm surface protein (MPSP) gene, four *T. orientalis* genotypes: type 1 (Chitose), type 2 (Ikeda), type 3 (Buffeli) and types 5 have been identified in Australia (Kamau et al., 2011b, Islam et al., 2011, Eamens, 2012; Eamens et al., 2013a, Perera et al., 2013, Cufos et al., 2012). However, the most important genotypes associated with clinical cases are Ikeda and Chitose (McFadden et al., 2011, Perera et al., 2013, Aparna et al., 2011, Izzo et al., 2010). Recently, a phylogenetic subgroup of Chitose (Chitose A) was found to be strongly associated with clinical cases in Australia in mixed infections with Ikeda (Jenkins et al., 2015; Jenkins, 2016).

A previous study of *T. orientalis* conducted on 46 farms located in the eastern parts of the Northern Tablelands Local Land Service areas (LLS) reported a herd prevalence of 72% and individual cattle prevalence (22%) based on detection in blood smears (Biddle et al., 2013). Of farms reporting the presence of ticks on their property, 16/22 (73%) were positive for *Theileria* while on farms with no reported presence of ticks, 17/24 (71%) were positive indicating a lack of association with tick presence. Given that blood smears are known to be a relatively insensitive method for *T. orientalis* detection, the project undertook a study of bovine theileriosis on 8 farms situated in the Glen Innes, Walcha and Armidale areas using more sensitive qPCR techniques that also allow for differentiation of the different genotypes of *T. orientalis*. (Bogema et al., 2015a). To further investigate the discrepancy between ticks and *Theileria* a parallel study into potential biological and mechanical vectors and their spatial and temporal distribution was also undertaken.

1.2 Tick vectors

The prevalence of various forms of theileriosis in different parts of the world is dependent on the presence of suitable tick vectors (Jabbar et al., 2015). Ticks are biological hosts for *Theileria* species and become infected with piroplasms upon taking a blood meal from infected host/s. Different *T. orientalis* genotypes have been reported; types 1 (Chitose) and 2

(Ikeda) in *Haemaphysalis megaspinosa*, *H. douglasi*, *I. persulcatus* and *I. ovatus* in Japan (Yokoyama et al., 2012) and types 1, 5 and N-3 in *Dermacentor nuttalli* in Mongolia (Altangerel et al., 2011). In contrast, a study from east Gippsland, Victoria reported that among the different genera of ticks collected (*Haemaphysalis*, *Bothriocroton* and *Ixodes*), the genotypes (types 1, 2 and 3) were detected only from *H. longicornis* (Hammer et al., 2015; Emery, 2016). Australian research prior to the clinical outbreaks in 2006 considered the *Boophilus microplus* (syn. *Rh. australis*) and *H. bispinosa* (syn. *H. longicornis*) as vectors (Seddon, 1952). Later on, *H. bancrofti* and *H. humerosa* were found to be competent and efficient vectors for *T. orientalis* as compared to *H. longicornis* under experimental conditions (Stewart et al., 1987c, Stewart et al., 1989, Stewart et al., 1987a). The *H. humerosa* used in that experiment was later re-classified as *H. bremneri* (Forshaw et al., 1985). In separate experiments, the native Australian wallaby tick, *H. bancrofti* was shown to transmit both *T. orientalis* Ikeda and a strain of *T. orientalis* sourced from Queensland, Buffeli type ("Warwick" strain) (Stewart et al., 1989). Interestingly, the extent of spread of clinical theileriosis in Australia due to *T. orientalis* Ikeda corresponds with the known range of *H. longicornis* rather than to that of *H. bancrofti* or *H. humerosa* (Jenkins, 2018). On the other hand, *T. orientalis* genotypes Buffeli and Chitose occur in areas outside the known range of *H. longicornis* which might indicate that different tick species transmit different genotypes or transmit them with differing efficiency. In Queensland, *H. bancrofti* and *H. humerosa* were shown to be vectors (Stewart et al., 1987b) with disjunct populations of the former reported to occur in southern NSW and Victoria (Laan et al., 2011). Within the lifecycle of the arthropod vector, inheritance of *T. orientalis* infection is transstadial but not transovarian (Higuchi, 1985, Stewart et al., 1987c) so larvae always hatch naïve. Few infected ticks, possibly just one, are required to infect an individual cow and infection of cattle with *T. orientalis* is considered to be lifelong (Sugimoto and Fujisaki, 2002). A complication of studies based on qPCR detection of *T. orientalis* in ticks collected from hosts is that they do not clearly differentiate between active infection of the tick and passive contamination via ingested host blood.

1.3   Potential mechanical vectors

In addition to cyclical transmission by ticks, mechanical transmission by sucking lice (*Linognathus vituli*) has been demonstrated (Fujisaki et al., 1993, Hammer et al., 2016) and is also probable for other hematophagous insects including mosquitoes (Hammer et al., 2015, de Marco et al., 2016), tabanids (Jirapattharasate et al., 2018) and Stomoxys (Changbunjong et al., 2016, Hadi and Al-Amery, 2012). Experimental inoculation with as little as 100 µl of blood from a highly parasitemic animal transmitted infection to recipient calves in an Australian study (Hammer et al., 2016).

Blood feeding female tabanids are a serious pest to livestock (Scoles et al., 2008, Baldacchino et al., 2014) especially during summer where a landing rates of up to 1000 on horses (Foil and Foil, 1988, Foil and Hogsette, 1994) and blood loss of up to 200 ml/animal/day have been reported (Hollander and Wright, 1980, Baldacchino et al., 2014). Moreover, the average blood meal size of tabanids ranges from 20µl to 680µl (Hollander and Wright, 1980) and a fully engorged medium-sized *T. pallipennis* could take approximately 40 µl of blood (Muzari et al., 2010a). A recent attempt in the detection of *T. orientalis* in tabanids (*Dasybasis spp*) collected within the premises of outbreak farms in the east Gippsland region of Victoria, Australia returned a negative result though the small number of flies and collection from a single farm

makes inference difficult (Hammer et al., 2015). Thus, further investigations on the spatial distribution and seasonal dynamics of potential biting insects is needed to understand their role in the epidemiology of the bovine theileriosis in Australia.

Stomoxyini flies (Diptera: Muscidae) are obligate hematophagous insects classified into the tribe Stomoxyini in the subfamily Muscinae with more than 50 species in 10 genera being recorded (Crosskey, 1993). The *Stomoxys* or stable flies contains 18 described species (Zumpt, 1973) of which 17 have a tropical distribution with the exception of the cosmopolitan species, *Stomoxys calcitrans* (Masmeatathip et al., 2006). Both male and female stable flies can consume an average of 11-15μL of blood per meal (Schowalter and Klowden, 1979). They are often aggressive and persistent feeders as they can even attack humans in the absence of preferred hosts (Baldacchino et al., 2013). Stable flies can cause severe problems in dairies and feedlots with a reductions of 19% in weight gain and 40-60% in milk yields have been reported (Campbell et al., 2001). In addition to *S. calcitrans*, several other stomoxyine flies readily attack domestic animals, including *S. niger*, *S. sitiens*, and *S. indicus*. Apart from the direct effects (annoyance, toxic effects of saliva, blood loss), a high number of stable flies biting cattle have been implicated as mechanical vectors of pathogens, including viruses, bacteria, protozoa, and helminths (Baldacchino et al., 2013). The buffalo fly, *Haematobia irritans exigua*, is one of the Stomoxyini flies measuring 3.5-4mm which were accidentally introduced into northern Australia from Asia (Seddon 1967). Since then it has spread through northern Western Australia, Northern Territory, Queensland and by 1978 to north-eastern NSW from there slowly spread south. Buffalo flies feed 10-40 times a day (Williams et al., 1985) making them an ideal candidate for mechanical transmission of *T. orientalis* as the parasite has been detected in their close relatives *Stomoxys* (Changbunjong et al., 2016). The distribution of buffalo flies within NSW varies from season to season but they are encountered on a seasonal basis on the New England Tablelands.

Biting midges, *Culicoides* (Diptera: Ceratopogonidae) are among the smallest hematophagous flies measuring 1-5mm in length with only females seeking blood for egg development (Mellor et al., 2000, Yu et al., 2005). They cause annoyance with a high biting intensity which makes them a good candidate for mechanical transmission (Garrett-Jones et al., 1964). They are well known as biological vectors for viruses pathogenic to animals and the most important of these are African horse sickness virus, bluetongue virus, epizootic haemorrhagic disease virus, equine encephalitis virus, Akabane virus and the Palyam viruses (Mellor et al., 2000). In Australia, the *Culicoides* species namely, *C. brevitarsis, C. marksi, C. dycei, C. victoriae, C. schultzei* and *C. peregrinus* are considered the most important species feeding on cattle and buffalo (Standfast and Dyce, 1972). Of special importance is *C. brevitarsis* which has been previously reported to have a seasonal movement from the northern/mid coastal areas of NSW to the inland parts (Bishop et al., 1995, Bishop et al., 1996, Bishop et al., 2000). Studies on the role of these midges in the epidemiology of bovine theileriosis has not been investigated and remains unclear.

2 Project objectives

The broad project objectives were to determine the following in the Armidale, Glen Innes and Walcha areas of the Northern Tablelands of NSW:

a) The prevalence of different *T. orientalis* genotype(s) in cattle using qPCR detection in blood

b) The likely vector(s) for transmission of *T. orientalis* using qPCR detection of trapped or recovered ticks and insects

c) Define the spatial and seasonal distributions of potential *T. orientalis* vectors

3 Methodology

3.1 Collection and identification of samples

3.1.1 Cattle blood

The study was approved by the Animal Ethics Committee of the University of New England (Authority No: AEC18-015). Consent from property owners/managers was obtained before samples were collected. A total of 90 blood samples from cattle on 8 farms (10/farm on seven farms, 20 on one farm) were collected from the coccygeal vein using an 18-gauge needle into vacutainer tubes containing ethylenediamine tetra acetic acid (EDTA). The samples were stored at -20^0C until DNA extraction.

3.1.2 Questing ticks

Questing ticks were collected on monthly basis between November 2017 and May 2019 on six selected farms; where *T. orientalis* presence was confirmed, using an optimized drag-sampling method (Spickett et al., 1991, Curtis Russell and Jain-Sheehan, 2015). Owners reported prior observation of ticks on cattle on only one of the six farms. The pasture was divided into two vegetation transects: semi-open woodland/ scrub and open grazing land to include a full range of habitats and feeding hosts' preferences of ticks. At the end of each drag, ticks were placed in a 50ml screw-capped plastic bottles containing absolute ethanol, speciated under a stereomicroscope using the morphological identification keys of Barker and Walker (2014) and photographed.

3.1.3 Hematophagous insects

Biting flies were collected between December 2017 and May 2019 on same farms used for tick survey using a non-baited Nzi traps constructed from locally available fabric and netting materials as described by Mihok (2002). Two traps were deployed for approximately 48hrs (Bawm et al., 2015) on each farm which were placed at least 60m apart (Muzari et al., 2010b, Van Hennekeler et al., 2011). Identifications of pinned specimens were done using identification keys of Mackerras (1971), (Mackerras, 1961, Mackerras, 1960), Lessard and Yeates (2013) and comparisons with reference collections at the Australian National Insect Collection (CSIRO Entomology, Canberra).

Similarly, ten cattle from each farm were inspected for lice with great attention laid to predilection sites of both chewing and sucking lice as described by Bailey (2015). After removal of the lice using a flea comb/forceps, were placed in vials containing absolute

ethanol. Finally, morphological identifications were done using morphological characteristics as was described by Bailey (2015). Attempts were also made to collect buffalo flies using sweeping nets on cattle.

Culicoides are one of the smallest hematophagous flies measuring 1-5mm in size with only females seeking blood for egg development. To investigate the potential role of these biting midges in *Theileria* transmission, samples and data from 26 years of *Culicoides* trapping on the Northern Tablelands were made available from the National Arbovirus Monitoring Program (NAMP). The project investigated species composition and spatio-temporal distribution of *Culicoides* trapped at 13 sites in the New England region of NSW, Australia between 1990 and 2018 using automated light traps. Trapping locations were divided into three subregions (tablelands, slopes and plains). Data on the few trapping events in winter months were excluded.

3.2   Detection of *T. orientalis* in cattle blood and potential vectors

For the extraction of *T. orientalis* DNA from thawed whole blood samples, the detergent-proteinase K (DPK) method (Bogema et al. 2015b; Jenkins, 2016) was used. As there was a need to screen farms for *T. orientalis* presence, blood samples were pooled as described by Gebrekidan et al. (2017). The quantity and purity of DNA extracted was measured using a spectrophotometer (NanoDrop® ND-8000 UV-Vis, Thermo Fisher Scientific, Australia) and extracts were stored at -20^0C until used.

The questing ticks were dissected to remove the bulk of the digestive tract, and were then pooled based on developmental stages. Dissection involved the removal and collection of the capitulum and the anterior portion of the scutum (Hammer et al., 2015) between the second and third coxa to include the majority of the salivary glands (Edwards et al., 2009). The pooled tissue samples were homogenised using a TissueLyser II (Qiagen, Hilden, Germany) before DNA extraction. Finally, DNA was extracted from tick specimens using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

Prior to DNA extraction, individual tabanids, *Stomoxys* and buffalo flies were longitudinally sectioned and tissues from the head, abdomen and thorax were used as described previously by Hammer et al. (2015). However, for the lice and *Culicoides* species of cattle (*C. brevitarsis, C. dycei, C. nattaiensis, C. victoriae, C. marksi* and *C. bundyensis*)*,* a pool made of half-sections from 5 lice (Kumsa et al., 2012) and a pool of 10 *Culicoides* was used (Hammer et al., 2016). Tissues were homogenized before extraction using the the DNeasy Tissue kit (Qiagen, Hilden, Germany).

Detection, differentiation and quantification of *T. orientalis* genotypes was done using a validated quantitative PCR (qPCR) assay described previously by Bogema et al. (2015a). If pooled blood samples were positive, individual animal samples were then subjected to two qPCR tests to detect and enumerate the *T. orientalis* genotypes Ikeda (I), Chitose (C) and Buffeli using the multiplex UIC and singleplex buffeli qPCR assay of Bogema et al. (2015a).

4 Results

4.1 Cattle blood qPCR

The pooled subsamples from each property were positive for *T. orientalis* indicating a 100% herd prevalence. The overall individual animal (n=90) prevalence of Ikeda, Chitose and Buffeli genotypes were 89% (80/90), 91% (82/90) and 89% (80/90) respectively. The presence of mixed infections with *T. orientalis* genotypes was typical with 73% (66/90) having a mixed infection with the three genotypes (Ikeda, Chitose and Buffeli), 22% (20/90) with two and 4% (4/90) with only one genotype.

The parasitaemia level in cattle revealed a significant difference in farms within location and between locations but not for *Theileria* genotypes. The Chitose phylogenotype was determined on a subsample of 17 samples on 6 farms with moderate to high levels of infection with Chitose. This revealed that 5.9% (1/17) were infected with Chitose A only, 70.6% (12/17) were infected with B only and 23.5% (4/17) were coinfected with both A and B.

Details of results can be found in the submitted scientific paper in Appendix 1.

4.2 Seasonal abundance of ticks and biting flies

A total of 358 questing ticks of different developmental stages were collected. These were collected on only 1 of the 6 farms, the farm that had reported prior evidence of tick presence on cattle. They were all morphologically identified as *H. bancrofti*. Larvae accounted for 59%, followed by nymphs (34%) and adults (7%) of the total. The phenology of tick capture revealed that the majority of larvae (90.5%) were collected in autumn, nymphs (89.4%) in spring and adults (84%) in summer. No ticks of any lifecycle stage were detected during the winter months. Details are provided in the paper in Appendix 1.

Similarly, a total of 431 biting flies were collected during the seasonal study that were identified to eight genera comprising of eleven species. Tabanids in the tribe Diachlorini (subfamily: Tabaninae) were the most abundant (81.0%) followed by Bouvieromyiini (Chrysopinae) (12.1%) with tabanids in the Scionini (Pangoniinae) were the least (2.32%), yet contained four of the species reported. Overall, the most abundant species were *D. oculata* (Ricardo) (43.6%) and *D. circumdata* (Walker) (27.6%), with Stomoxyini flies being caught in small numbers. The diversity and abundance of species in summer (38.1%; n=8) and autumn (36.7%; n=7) months were higher compared to spring (25.3%%; n=6). The fly activity pattern for *D. oculata* reached peaks in November, January and March as compared to March and November for *D. circumdata*. In April 2019 buffalo flies (*H. irritans exigua*) landing on cattle were collected using sweep nets in one of the farm situated in the Wollomombi area.

The abundance and diversity of biting fly species was the highest on farms clustered in the Wollomombi (n=205, N=11) than Walcha (n=173, N=5) and Guyra (n=53, N=4) locations. The rare tabanid species; *M. bancrofti*, *S. brevirostris*, *C. subappendiculata, S. abdominalis* and *P. mackerrasi* were collected only in the Wollomombi area.

Short-nosed (*Haematopinus eurysternus*) and long-nosed (*Linognathus vituli*) sucking, and chewing (*Bovicola bovis*) cattle lice were present in all farms with the later only collected from one. However, their presence was limited to the cooler wniter and spring months (July to November 2018) of the year.

Details of the insects collected ny NZI trapping and their distributions are provided in the paper at Appendix 2.

Nineteen species of *Culicoides* were identified from the NAMP dataset between 1991 and 2018. *Culicoides marksi* and *C. austropalpalis* were the most abundant and widespread species while eight of the species caught made up 99.2% of the total counts. *Culicoides brevitarsis*, the principal vector of livestock diseases in NSW comprised 2.9% of the total catch and was detected in 12 of the 13 locations in the study. Abundance as determined by Log_{10} *Culicoides* count per trapping event for the eight most abundant species did not vary significantly with season (spring, summer, autumn) but trended towards higher counts in summer for *C. marksi* (P=0.09) and *C. austropalpalis* (P=0.05). Significant geographic variation in abundance was observed for *C. marksi, C. austropalpalis* and *C. dycei* with counts decreasing with increasing altitude from the plains to the slopes and tablelands. *C. victoriae* exhibited the reverse trend in abundance (P=0.08). Greater abundance during the warmer seasons and at lower altitudes for *C. marksi* and *C. austropalpalis* was indicative of temperature and rainfall dependence in this region with moderate summer dominance in rainfall. The Shannon-Wiener diversity index of species was higher on the tablelands (H=1.59) than the slopes (H=1.33) and plains (H=1.08) with evenness indices of 0.62, 0.46 and 0.39 respectively. *Culicoides* species on the tablelands were more diverse than on the slopes and plains where *C. marksi* and *C. austropalpalis* dominated. The temporal and spatial variation in abundance, diversity and evenness of species reported in this diverse region of Australia provides additional insight into *Culicoides* as pests and disease vectors and may contribute to future modelling studies.

Full details of the results of this study can be found in the paper at Appendix 3.

4.3   qPCR detection of *T. orientalis* in hematophagous arthropods

Theileria orientalis was detected in 3 of 4 pools made from *H. bancrofti* nymphs, but not in 4 pools made of adult questing ticks or larvae. The genotypes Ikeda and Buffeli were each detected in one of the four pools made from nymphs with no detections of Chitose. These are the first reports of detection of *T. orientalis* in questing *H. bancrofti* ticks in the field. Full details are provided in the paper in Appendix 1.

Biting insects of the different genera were screened for presence of *T. orientalis* using the T. orientalis generic PCR (detects all genotypes). *T. orientalis* was detetected in 2 of 4 pools made from the long nosed sucking louse *Linognathus vituli* and in 2 of two pools made from the short nosed sucking louse *Haematopinus eurysternus*. This is the first report of detection of *T. orientalis* in the latter species. No detections were made from pools made for all the other biting flies (Tabanids, Stomoxys, Hematobia and *Culicoides*). Full details are provided in the paper in Appendix 2.

5 Discussion

5.1 Prevalence of *T. orientalis* genotypes in cattle

There are previous reports on the presence of *T. orientalis* in the eastern parts of the Northern Tablelands (Biddle et al., 2013) and the North Coast (Proctor et al., 2016), and results from this project clearly demonstrate the widespread ingress of the parasite to this region with very cold winters where ticks are not prevalent. The herd and animal prevalence of *T. orientalis* infection in apparently healthy cattle and herds determined using qPCR was 100% indicating endemicity of the parasite in the region. A prevalence study from the same region reported a herd and animal prevalence of 72% and 22% respectively based on blood smear examination (Biddle et al., 2013). The higher prevalence in the current study may reflect a true increase in prevalence or the higher sensitivity of the qPCR method used relative to blood smears (Altay et al., 2008, Naomi et al., 2009).

Infection of cattle with *T. orientalis* genotypes (Ikeda, Chitose and Buffeli) was common on the properties targeted for sampling in this study. The presence of mixed infections is also consistent with previous reports from eastern Australian states (Eamens et al., 2013a, Islam et al., 2011, Kamau et al., 2011a, Gebrekidan et al., 2015) and New Zealand (Perera et al., 2015). The high prevalence of the pathogenic Ikeda genotype in areas where ticks are absent is strongly suggestive of introduction via infected cattle and is at odds with the previous reported distribution of this genotype which had been reported to mirror the presence of *Haemaphysalis* species in Australia (Hammer et al., 2015). Maintenance of infection following introduction in farms without ticks would appear to due to some combination of vertical transmission (Jenkins, 2016; Swilks et al., 2017, Hammer et al., 2016, Mekata et al., 2018) and lateral transmission by mechanical means via sucking lice and/or husbandry practices (Hammer et al., 2016). However, the sexual reproduction that occurs after blood feeding in ticks is necessary to generate and maintain the virulence/genetic diversity of the parasite and its capacity to cause clinical disease and deaths. In the case of *T. orientalis*, mechanical transmission results in the direct transfer of haploid phase piroplasms, thereby bypassing the sexual phase of the lifecycle. The inability of the parasite to genetically recombine is expected to reduce overall diversity within parasite population. Thus, extensive mechanical transfer of the parasite would be expected to decrease the ability of the parasite to evade the host immune system and this method of spread is thought not to be important in the wider epidemiology of the disease (Lawrence et al., 2019). However repeated mechanical transmission on the Northern Tablelands, could possibly form part a component of enzootic stability, with reduced parasite genetic diversity and ability to evade host immune mechanisms.

The presence of mixed infection with the three genotypes was observed in 73% of cases and confirms the lack of cross immunity between species. The 100% prevalence in adult cattle also is consistent with lifelong infected, although lower levels of parasitaemia were seen in heifers than cows in the current study, suggestive of some immune modulation. Analysis of clinical case records of *T. orientalis* infections from the Northern Tablelands and North Coast regions since 2009 revealed that infections with pathogenic genotypes occurred concurrently but were strongly associated with cattle introductions on the Northern Tablelands before

2016 but not afterwards. The high herd and animal level prevalence of *T. orientalis* in cattle without clinical disease, in some cases with high levels of parasitaemia indicate the need for caution in using *Theileria*-positive PCR results to arrive at a definitive diagnosis of theileriosis, being rather an indication for inclusion in the differential diagnosis (Proctor et al., 2016).

5.2   Potential vectors for *T. orientalis* on the Northern Tablelands

In the present study, only a single species of tick *H. bancrofti* (wallaby tick) was detected, a species for which cattle are not the preferred host. All developmental stages were collected and *T. orientalis* genotypes Ikeda and Buffeli but not Chitose, were detected in nymphs only. This is consistent with demonstration of trans-stadial transmission of *T. orientalis* Buffeli by *H. bancrofti* ticks in Queensland (Stewart et al., 1989). It is clear that *H. longicornis* is a biological host for *T orientalis* (Tsugihiko Kamio et al., 1990, Marendy et al., 2020, Hammer et al., 2015). However, in some of these studies e.g. Hammer et al. (2015) where adult ticks have been collected, it is not clear whether the tick is infected or just carrying infected blood. It is therefore important not to do PCR on adult engorged ticks. However, in questing nymphal stages of *H. longicornis* in Japan, masses of *T. sergenti* were detected in the salivary glands stained with methyl green-pyronin (Tsugihiko Kamio et al., 1990). Overall, the present findings confirm *H. bancrofti* as a likely vector for *T. orientalis* in Australia. However, ticks were detected on only one of 6 farms and the fact that 16 monthly collections on five farms that did not report tick presence yielded no ticks is strong evidence of there being no significant tick presence on these farms with alternative means of transmission of *Theileria* required to explain the high prevalence of the parasite.

Haemaphysalis bancrofti is a common parasite of marsupials, particularly wallabies in north-eastern Australia, with additional occurrences on bettongs, bandicoots, quolls, possums and koalas, and occasional collections from cattle, sheep, horses, feral pigs, deer and dogs as well as single records from a micro-bat and from a Pheasant Coucal *Centropus phasianus* (Roberts, 1970, Oakwood and Spratt, 2000, Brown and Copeman, 2003). As in the present study Laan et al. (2011) reported collection of all developmental stages of *H. bancrofti* from Raymond Island, Victoria, Australia using the dragging method and the presence of *H. bancrofti* in the Capoompeta National Park on the Northern Tablelands and in the Richmond Range National Park on the North Coast of NSW. The same authors state that *H. bancrofti* has disjunct populations in Australia as was the case in the east Gippsland region of Victoria, but are usually found in subcoastal areas of south eastern Queensland and northern NSW (McKenzie et al., 1985, Roberts, 1963, Speare et al., 1983, Greay et al., 2016) with a single record from southern NSW (Nowra) (Roberts, 1970) and the Northern Territory (Oakwood and Spratt, 2000). The absence of bush tick (*H. longicornis*) in the present study is not surprising as it was not detected from the environment and dogs from coastal areas of Sydney (Chandra and Šlapeta, 2020). Overall, its distribution has been reported to be limited to a narrow 100 km coastal strip with high rainfall from Gympie in Queensland to Wodonga-Tallangatta in south (Roberts, 1970, Dicker, 1978).

As to the seasonal dynamics of tick detection, a unimodal reproduction cycle was observed most likely due to the cold winter in the region. The adult stages were collected only from mid-spring (October) to summer (December-February) with a peak in December. Larvae were collected from mid-spring to autumn (March-May) with peak activity in March, and peak

activity of the nymphs was in spring (October and November). These are suggestive of peak adult egg laying in summer, producing an autumn peak of larvae that overwinter and develop into nymphs in spring. Prior studies showed that all stages of *H. bancrofti* are present all year round in the milder climates of southeast Queensland (Heath, 1986) and Raymond Island, Victoria (Laan et al., 2011).

Lice were collected in the cooler winter and spring months of the survey when they were most prevalent. The detection of *T. orientalis* in the sucking lice species *L. vituli* and *H. eurysternus* in the present study is consistent with an earlier Australian report of presence in *L. vituli* feeding on calves infected with *Theileria orientalis* in (Hammer et al., 2016). However our finding appears to be the first report of detection in *L vituli* in the field and the first detection in *H. eurysternus* the short-nosed cattle louse. The long-nosed cattle louse (*L. vituli*) has been shown to transmit *Theileria* to splenectomised calves when previously fed on infected cattle that had been inoculated with a sporozoite suspension (Fujisaki et al., 1993). The transmission in that study was shown to be mechanical via regurgitation and not biological.

The trapping of the large biting flies over 18 months yielded total of eight genera comprising eleven species were trapped of which the tabanid flies *Dasybasis oculata* and *D. circumdata* were the most abundant and widespread species. Abundance and diversity of biting fly species was highest in summer followed by autumn and spring, and with no detection in winter. PCR screening for the presence of *T. orientalis* of seven species from this study produced no positive results. The negative findings are significant given the 100% prevalence of *T. orientalis* in cattle on the farms where the flies were trapped. Similar negative findings for *T. orientalis* presence were reported for *Dasybasis* spp. collected from one locality in the east Gippsland region of Victoria, Australia (Hammer et al., 2015). The screening of tabanids from a wider area in present study further supports their unlikely role in the transmission of *T. orientalis*. Nevertheless, *T. orientalis* DNA has recently been detected in tabanids and *Stomoxys* in Thailand (Changbunjong et al., 2016, Jirapattharasate et al., 2018) in mosquitoes in the United Kingdom (de Marco et al., 2016) and Australia (Hammer et al., 2015) and in *Stomoxys calcitrans* (99% positive) in Hungary (Hornok et al., 2020). The differences in results may reflect differences in host density as suggested by Hornok et al. (2020) with this affecting the level of bovine blood meal in the insects tested.

Biting midges, *Culicoides* (Diptera: Ceratopogonidae) are among the smallest hematophagous flies measuring 1-5 mm in length with only females seeking blood for egg development (Mellor et al., 2000, Yu et al., 2005). They cause annoyance with a high biting intensity with the average size of a bloodmeal mentioned by (Venter et al., 2003) ranging between 0.01 μl and 0.06 μl which makes them a good candidate for mechanical transmission (Garrett-Jones et al., 1964). In Australia, the *Culicoides* species namely, *C. brevitarsis, C. marksi, C. dycei, C. victoriae, C. schultzei* and *C. peregrinus* are considered the most important species feeding on cattle and buffalo (Standfast and Dyce, 1972). Of special importance is *C. brevitarsis* which has been previously reported to have a seasonal movement from the northern/mid coastal areas of NSW to the inland parts (Bishop et al., 1995, Bishop et al., 1996, Bishop et al., 2000). In the present study *Theileria* DNA was unable to be detected from pools of *C. brevitarsis, C. marksi, C. dycei, C. victoriae, C. nattaiensis* or *C. bundyensis* that have cattle as a host and were trapped on the Northern Tablelands. This is the first attempt at detecting *Theileria* in these insects and the lack of detection may reflect their small meal size of fact that *Theileria*

reside inside erythrocytes and *Culicoides* are known pool feeders which do not directly take blood from blood vessels unlike mosquitoes (Venter et al., 2003).

6 Conclusions and recommendations

The main conclusions that can be drawn from the project results are as follows.

a) *T. orientalis* infection is ubiquitous in the sampled area (100% prevalence) with a high prevalence of Ikeda (89%) Chitose (91%) and Buffeli (89%) genotypes.

b) Mixed infections with all three genotypes were present in 73% of cases indicative of high prevalence and lack of cross protection between genotypes. This is supportive of the proposition that they are probably separate species.

c) The absence of overt clinical disease on the properties despite the ubiquitous presence of the parasite is indicative of the area achieving enzootic stability with a low proportion of susceptible animals.

d) This status appears not be associated with widespread presence of a biological tick vector. The only tick vector identified in the study was the ixodid three-host tick, *H. bancrofti* but it had a very limited distribution being present on only one of the six sampled farms. This is consistent with the earlier finding by Biddle et al. (2013) of no connection between infection in cattle and tick presence.

e) Several biting flies and midge species were present and trapped in the regions with varying abundance and seasonality, but none tested positive for *T. orientalis* by qPCR suggestive of a lack of likely role in the epidemiology of the disease in the area.

f) On the hand two species of sucking lice were positive of presence of *T. orientalis.* This, coupled with previous evidence of mechanical transmission of *T. orientalis* by sucking lice, is suggestive of a significant role in the epidemiology *Theileria* infection in the area.

On balance it would appear that the status of enzootic stability has been able to be to achieved and maintained following *Theileria* introduction by a combination of vertical transmission by colostral (Hammer et al., 2016; Mekata et al., 2018) or transplacental transfer (Lawrence et al., 2016; Swilks et al., 2017) and mechanical transmission by lice or through husbandry practices involving potential transfer of blood. Repeated mechanical transmission without passage through ticks removing the ability of the parasite to genetically recombine can be expected to reduce overall diversity within parasite population and decrease the ability of the parasite to evade the host immune system thus reducing virulence potential (Lawrence et al., 2019).

Recommendations for further research arising from this study include the following

a) In the present study, the level of anaemia present in sampled animals was not determined and association with parasitaemia ascertained. It would be useful to determine whether the parasite is causing significant subclinical anaemia under conditions of enzootic stability.

b) Improved understanding on the role of lice as *Theileria* transmitting agents is warranted. This could include work on the rate of transfer of lice between hosts, particularly to young calves and estimation of the rate of natural transfer of *Theileria* by lice by mixing infested cattle with those not infested and measuring the rate of lateral transfer.

c) Further studies on the role of repeated mechanical transmission on attenuation of the parasite and converse studies on reversion when passaged back through ticks would be useful not only with regard to potential vaccine development, but also as a guide to the

likely epidemiological situation with the disease when either biological or mechanical transmission predominate.

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