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Research paper

A longitudinal study of the effect of *Theileria orientalis* Ikeda type infection on three New Zealand dairy farms naturally infected at pasture

K.E. Lawrence*, K. Gedye, W.E. Pomroy

School of Veterinary Science, Massey University, Palmerston North 4410, New Zealand

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ABSTRACT

The aims of this study were to monitor the change in *Theileria orientalis* Ikeda type infection intensity, haematocrit, milk production and reproduction on three New Zealand spring calving dairy herds, over the 2014–2015 milking season.

Three spring calving dairy farms, A, B and C, from high risk (endemically stable), low risk (endemically unstable), and zero risk (disease-free) tick areas respectively were followed through the 2014–2015 milking season. On Farms, A and B, 100 cows were randomly selected at the first visit, and the same cows blood sampled every month thereafter, whilst on Farm C, the whole herd was blood sampled bimonthly (140 cows). Blood samples were tested for haematocrit, by centrifugation, and Ikeda infection intensity, using qPCR. Animals that were Ikeda type PCR positive at the first sampling were described as prevalence cases and cows that were negative at the first sampling and became PCR positive during the sampling period were described as incidence cases. Production and reproduction data were accessed through LIC MINDA® and milk production data was standardised to energy corrected milk (ECM). In addition, the effect of buparvaquone (BPQ) treatment on milk production was estimated on Farm B.

The prevalence of infection at the first sampling was 100 % on Farm A, 57 % on Farm B and 26 % on Farm C. The incidence risk of infection over the sampling period on Farms B and C was 25 % and 2 % and the incident rate was 0.026 and 0.002 cases per cow-month respectively. The average infection intensity for prevalence cases on all farms was low throughout the milking season, < 7000 Ikeda organisms/μL however, cases of anaemia still occurred. There was no direct effect of infection intensity on milk production or from being a prevalence case compared to an uninfected cow on milk production, across all farms. However, on Farm B there was a loss of 266 kg (95 % CI 82- 450) ECM (~20 kg milk solids) for incidence cases and a loss of 458 kg (95 % CI 211- 710) of ECM for buparvaquone treated cows, compared to uninfected cows. No significant effect of Ikeda infection on reproduction could be shown for Farms B and C, reproductive data for Farm A was not available.

The effect of *T. orientalis* Ikeda type infection on production and reproduction appears to be minimal once animals have passed through the acute phase of infection and reached the chronic, asymptomatic carrier phase of infection.

1. Introduction

First diagnosed in 2012 (McFadden et al., 2013) bovine anaemia associated with *Theileria orientalis* Ikeda type infection (TABA) is now the principal cause of bovine anaemia in New Zealand. The spread of infection between cattle is via the tick *Haemaphysalis longicornis* (Heath, 2016) and the disease has rapidly reached endemic stability in the upper North Island of New Zealand, owing to both the favourable climate and habitats for tick infestation and the high density of cattle farms found there (Lawrence et al., 2017). Anecdotally, endemic stability has resulted in a drop in clinical disease in these areas. However, for much of the lower North Island and parts of the upper South Island, where climate and habitat are less favourable for ticks, the disease remains endemically unstable and *T. orientalis* Ikeda type infection continues to cause significant outbreaks of infectious anaemia and deaths on some dairy and beef farms in these areas. Parts of the North Island and much of the lower South Island remain disease free to this day.

Theileria orientalis comprise tick-borne obligate intracellular apicomplexan haemoprotozoan parasites of cattle around the world (Aktas et al., 2006) and are only rarely associated with severe disease outbreaks (Watts et al., 2016). To the best of our knowledge 4 of the 11 types of *T. orientalis* (Khukhuu et al., 2011) currently recognised, are

* Corresponding author.
E-mail address: K.Lawrence@massey.ac.nz (K.E. Lawrence).

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found in New Zealand cattle. These are Type 1 (Chitose), Type 2 (Ikeda), Type 3 (Buffeli) and Type 5 (Pulford et al., 2016). Of these the Ikeda type is the most pathogenic (Lawrence et al., 2016b) although the Chitose type has been sporadically associated with mild outbreaks of bovine anaemia in New Zealand since 1982 (James et al., 1984; Rawdon et al., 2006; McFadden et al., 2011).

There is only one competent arthropod vector for *T. orientalis* in New Zealand, the ixodid tick *Haemaphysalis longicornis*, and its lifecycle and distribution have been thoroughly reviewed (Heath, 2016). Non-tick associated, mechanical spread of infection is also described through biting flies, sucking lice or hypodermic needles (Hadi and Al-Amery, 2012; Fujusaki et al., 1993; Hammer et al., 2015, 2016) however this method of spread is not thought to be important in the wider epidemiology of *Theileria* associated bovine anaemia (TABA).

Cattle affected with *T. orientalis* Ikeda type usually show a combination of the following clinical signs; anaemia, lethargy, production drop, anorexia, ill-thrift, diarrhoea, constipation, jaundice, collapse, pale udders, possible haemoglobinuria, abortion and inappetance (Islam et al., 2011; Cufos, 2012; Lawrence et al., 2013). The morbidity rates reported in New Zealand and Japan for TABA are low, usually < 1 % (Shimizu et al., 1992; Vink et al., 2016) with mortality rates of 0.25 % (only recorded for New Zealand cattle; Vink et al., 2016). Post-mortem examination of dead animals often shows a massively enlarged "raspberry jam" spleen (the spleen removes parasitised RBCs from circulation) and a bronze coloured liver with generalised jaundice (Izzo et al., 2010; Gebrekidan et al., 2017).

Robust industry estimates of the cost of TABA for New Zealand don't exist, however within Australia, oriental theileriosis is estimated to cost the red meat industry $19.6 M per annum (Lane et al., 2015). Recent Australian and New Zealand studies have shown negative impacts of *T. orientalis* Ikeda type infection on milk production (Australia), on reproduction (New Zealand), on live weight gain in Friesian bulls (New Zealand) and on live weight gain in beef calves (New Zealand) (McDougall et al., 2014; Perera et al., 2014; Lawrence et al., 2018, 2019).

Overall, chemotherapy of TABA is considered unrewarding but one of the few drugs shown to have some efficacy against *T. orientalis* Ikeda type infection is buparvaquone (BPQ) given at a dose rate of 2.5 mg/kg (Carter, 2011). BPQ is a second-generation hydroxynaphthoquinone related to parvaquone (McHardy et al., 1985) and many New Zealand veterinarians consider its use as essential for the successful treatment of TABA (Theileria Handbook 2, 2015; Rankin, 2015). Buparvaquone (BPQ) works by causing progressive cytoplasmic vacuolation in *Theileria* spp. through inhibiting mitochondrial electron transport (Fry et al., 1984; McHardy et al., 1992). Industry-funded slaughter trials conducted in New Zealand in 2013 (McDougall et al., 2016) found concentrations of BPQ, above the limits of detection using liquid chromatography-mass spectrometry, up to 35 days in milk, up to 119 days at the injection site and up to 328 days in the liver post injection. These findings have led to the following legal requirements for milk and meat withhold periods for BPQ treated cattle in New Zealand, 43-day milk, 548-day meat. In addition, calves born to BPQ treated dams have a 9-month meat withhold, calves drinking milk from a BPQ treated cow have a 91-day meat withhold and offal from treated animals has a permanent withhold.

The New Zealand dairy cattle industry is highly seasonal and overwhelmingly based on cows calving from late winter to early spring i.e. between mid-July and early-October, with only a small proportion of herds or split calving herds calving in the autumn. The beef industry is also predominantly spring calving, although on average they calve about a month later than dairy herds (McFadden et al., 2005). The almost universal adoption of spring calving in New Zealand means that there is considerable synchronicity between questing *H. longicornis* nymphs, stressed periparturient cows and the birth of naïve calves.

The aims of this study were to document the change in infection status, infection intensity and HCT over a lactation on three spring calving dairy herds, the first from an endemically stable area, the second from an endemically unstable area and the third from a disease-free area (Theileria Handbook 2, 2015). Furthermore, the effects of infection and infection intensity on milk production and reproduction were estimated for each farm.

2. Materials and methods

The study was a prospective longitudinal observational study, in which a random sample of dairy cows on three spring calving New Zealand dairy farms were followed through the 2014–2015 milking season. The three dairy farms were purposively selected based on their location, TABA history and their willingness to participate in the study. Farm A was an 800-cow dairy farm in the Waikato region and a recent diversification for a large beef and sheep station. This farm had the misfortune to suffer a severe outbreak of TABA, with > 5 % mortality, in their first milking season in 2013. Farm B was an established 570-cow family dairy herd in the Manawatu-Wanganui region. This herd had also suffered a severe outbreak of TABA in 2013 with a total of 20 (3.5 %) cow deaths and 80 (14.0 %) clinically affected cows. Farm C was a 140-cow family dairy herd in the Wairarapa region, which had converted to the once-a-day milking system in 2013. Three clinical cases of TABA occurred in this herd at calving in late August 2014, with no associated deaths. Unlike Farms A and B, Farm C was first infected in 2014.

The outbreak of TABA on Farm A was linked to mixing of cattle from different herds in 2013, in order to establish the new herd, which unfortunately included the purchase of two persistently infected (PI) BVD cows. At the time of the outbreak in 2013 it was hypothesised that the presence of the two BVD PIs had suppressed herd immunity leading to a more severe outbreak of TABA than usual. The outbreak on Farm B was linked to wintering cows on a tick-infested coastal run-off, close to the main milking platform, from which the older cows were moved back to the milking platform just prior to calving in 2013, and on Farm C all three clinical cases were amongst 39 cows recently returned from winter grazing in the Hawke's Bay region.

The three selected farms were situated in three different *Haemaphysalis longicornis* risk areas (Heath, 2016), Farm A was in a high risk (endemically stable) area, Farm B was in a low risk (endemically unstable) area and Farm C was in a zero risk (disease-free) area. Farms A and B were visited every month from August to drying off, which was April 2015 for Farm A (9 visits) and May 2015 for Farm B (10 visits). On each of these two farms 100 cows were selected at the first farm visit in August 2014 using systematic random sampling, with every N^{th} cow on the rotary platform selected, where N = total cows in-milk/100, rounded to the nearest integer. These same 100 cows, on Farms A and B, were then repeatedly blood sampled at each visit through the milking season. In contrast, Farm C was visited only 4 times (bimonthly) through the season starting in September 2014 and the whole herd, 140 cows, blood sampled at each visit. Blood sampling on each farm commenced at the same time on each sampling visit. This was at the AM milking on Herds A and B and in the early afternoon on Herd C (the cows had already been milked in the morning). For Farm A the planned start of calving was mid-July 2014, for Farm B was the third week of July 2014 and for Farm C was the first week of August 2014.

Farm A dried off one month earlier than expected, in April 2015, owing to the effects of severe sporidesmin toxicity (facial eczema), Farm B fed turnips from mid-February through to the end of March 2015 and Farm C suffered a feed pinch in late summer 2015. Farm A ran two milking herds, normal and light, with herd membership based on body condition score, BCS ≥ 4 or BCS < 4 respectively, Farm B ran two milking herds partitioned on age and Farm C just ran one herd.

2.1. Haematocrit

At each sampling visit blood samples were collected from the tail

vein into 10 ml EDTA tubes and after measuring the haematocrit were stored at −20 °C until molecular testing was carried out. The haematocrit was measured by reading micro-capillary tubes centrifuged for 5 min at 10,000*g*, using a Heraeus Pico 17 (Thermo Fisher Scientific, Langenselbold, Germany), with the average of two samples recorded for each animal. If the haematocrit for paired samples differed by greater than 2 % then a repeat centrifugation and reading were done. The sampled animals were classed as anaemic if the haematocrit (HCT) ≤ 0.24 l/l (Riond et al., 2008).

2.2. Milk production data

Farm A had 3 Livestock Improvement Corporation (LIC) herd tests (the 4th LIC herd test was cancelled due to early dry-off), Farm B had 5 LIC herd tests, and Farm C had 4 LIC herd tests. Most New Zealand dairy farms that use the LIC herd testing scheme, have 4 herd tests a year in which 24 -h test-day milk data is recorded for each lactating cow in the herd. The main aim of herd testing in New Zealand is the genetic evaluation of sires and cows for lactation yield (Johnson, 1996; Harris et al., 2006). The LIC herd test results were accessed through MINDA® (LIC, Hamilton, New Zealand). MINDA® is a web-based software program that allows farmers to access and manage their herd production data, including herd test results, with the aim of making better on-farm management decisions.

The outcome measure used for the milk production analyses was 24 -h test-day energy-corrected milk (ECM; kg) production per cow at each herd test. This was calculated using the following formula (Santschi et al., 2011):

$$\text{ECM (kg/d)} = 12.55 \times \text{fat (kg/d)} + 7.39 \times \text{protein (kg/d)} + 0.2595 \times \text{milk yield (kg/d)}$$

New Zealand herd test data is recorded as volume and not as weight. The density of milk is estimated at 1.03 kg/l and although using volume instead of weight introduces a small bias, the calculation of ECM was not altered and remains consistent with the approach used by Mason et al. (2012).

2.3. Reproduction data

Reproduction data, where available, was also accessed through MINDA®.

2.4. DNA extraction

DNA was extracted from 250 µl of cow blood collected from the trial herds. DNA was extracted with 250 µl of an SDS lysis buffer (20 mM EDTA-Na2.2H2O, 60 mM Tris HCl, 1 % sodium dodecyl sulphate, 1 % Triton X-100, pH 8.0), and Proteinase K at 0.5 mgml-1 (Roche Diagnostics, Switzerland). The slurry was incubated at 56 °C for 30 min and equal volumes of phenol:chloroform:iso-amyl alcohol (25:24:1, Sigma, MO, USA) were added. Aqueous and organic phases were separated by centrifugation at 13,400xg for 15 min at 4 °C. DNA was precipitated using a 10:1 solution of ice cold 100 % ethanol and 3 M sodium acetate (Sigma, MO, USA). The DNA was washed twice with 70 % ethanol, allowed to air dry and resuspended in R40 (40µgml-1 RNaseA [Sigma, MO, USA] in TE [10 mM Tris, 1 mM EDTA, pH 8.0]) overnight at 4 °C. Extracted DNA was quantified with a Nanodrop (Thermo Scientific, MA, USA) and stored at -20 °C.

2.5. Quantitative PCR

Quantitative PCR (qPCR) on the extracted DNA was performed using the primers and probe previously described for *T. orientalis* Ikeda type by Pulford et al. (2016), all primers and probes were sourced from Integrated DNA Technologies (Iowa, USA). qPCR was performed with the following conditions; 1 x Platinum™ Quantitative PCR SuperMix-UDG (Invitrogen, CA, USA), 0.5 µM of each primer, 0.4 µM of the probe and 5 µL of template DNA in a final volume of 20 µL. Thermalcycling was performed in a Rotor-Gene Q thermalcycler (Qiagen, Hilden, Germany). Thermalcycling conditions were as follows; 95 °C for 2 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, with fluorescence capture on the 60 °C step. Standard curves were produced utilizing a serial dilution of a plasmid from a previously produced clone of a section of the *T. orientalis* Ikeda MPSP gene (provided by K. Gedye) and ranged from 100 pg to 10 fg/µL of the plasmid. Runs were analysed using the Rotor-Gene Q software and exported for further statistical analysis. The standards were also the positive controls, with negative controls being PCR grade water and the DNA extracted from naïve cattle.

Because of the large number of samples and financial constraints only one PCR molecular test was completed per animal. The individual cow PCR test was only repeated if the test gave an unexpected result compared to previous and succeeding test results.

2.6. Gene copies and validation of assay performance

The DNA extracted from 250 µl of whole blood was eluted in 50 µl and then 5 µl was pipetted for the PCR analysis. A serial 10-fold dilution of 5 Ikeda plasmid DNA standards (10^1 to 10^5 fg/µl) was used to quantify the gene-copies of Ikeda MPSP found in bovine blood sample. This was accomplished by multiplying the molarity of the *Theileria* DNA in the blood sample, as calculated from the standard curve using the Cq value for the PCR amplification, by Avogadro's number (6.022×10^{23}).

Two measures were used to assess the performance of the molecular assay, the limits of quantification (LOQ), which is defined as the lowest amount of analyte that can be accurately quantified to an acceptable level of precision, but may still indicate the animal is infected, and the cut point, which is defined as the quantity of analyte above which a sample can be categorised as positive (= animal is infected). For the LOQ, the lower limit of the Ikeda target gene DNA standards serial dilutions was extended to include 10^{-3}, 10^{-2}, 10^{-1} and 10^0 fg/ µl, and the assay was repeated with 3 replicates for each dilution. The linearity and R^2 of these extended standard curves were assessed and the PCR efficiency calculated. PCR efficiency (E) was calculated using the following formula; $E = 10\text{-}(1/\beta) - 1$ where β is the slope of the linear regression of Cq on $\log_{10}$ of the standard concentration. Acceptable target values for R^2 are > 0.98 and for PCR efficiency are 0.90–1.05 (Johnson et al., 2013). For estimating the cut point ROC curves were constructed using the qPCR results from the first sampling at Farm C where *a priori* we believed that the only infected cows were the ones returning from winter grazing in the Hawke's Bay Region. A ROC curve is a plot of sensitivity against 1 - specificity for a set of known positive and negative samples. Where sensitivity (Se) is the proportion of infected cattle diagnosed infected at a cut point and specificity (Sp) is the proportion of uninfected cattle diagnosed uninfected at the same cut point. The cut point is identified as that value which maximises the Youden index (J) where J = Se + Sp-1.

2.7. Interpretation of molecular results

Animals with an infection intensity > 300,000 Ikeda organisms/µL were considered to be acutely infected, animals with a moderate infection intensity, > 15,000 but ≤300,000 organisms/µL, were considered to be convalescing and animals with a low infection intensity, ≤15,000 organisms/µL, were presumed to be chronically infected (Bogema et al., 2015).

Using the cut point, previously established, and the pattern of results over the season for each individual cow on their respective farm, each sampled animal was categorised as either a prevalence case, an incidence case or uninfected. A cow was categorised as a prevalence case if they had an estimated infection intensity above the cut point at

their first sampling and for many of their subsequent samplings. A cow was classed as an incidence case if they had previously tested below the cut point for a number of samplings and then tested above the cut point at the next sampling, and a cow was classed as uninfected animal if they tested below the cut point for all their samplings. Some incidence cases had missing samples before testing positive or only had one previous negative test before testing positive, for these animals an acute infection intensity > 300 000 organisms/μL or a precipitate fall in the haematocrit in either the same month or at the next month after testing positive were used to support the diagnosis of incidence case.

Some prevalence cases had almost all positive results except for one or two tests where the estimated infection intensity was below the cut point, these results were called false negatives and were presumed to reflect the normal variability in infection intensity for chronically infected animals. Alternatively, some uninfected animals had all negative samples, i.e. estimated infection intensity below the cut point, except for a small number (1 or 2 maximum) of isolated positive samples, with no corresponding change in HCT at the current or subsequent sampling. These were called false positives and their occurrence recorded and analysed as such.

Once an animal was categorised as a prevalence or incidence case the LOQ was the lower limit to which the infection intensity could be accurately estimated, and these values were then included in all statistical analyses. Three measures of disease frequency were calculated for each farm (where appropriate), the prevalence, defined as the proportion of *T. orientalis* Ikeda type infected cows at a particular time, the incidence risk, defined as the proportion of the uninfected cows contracting *T. orientalis* Ikeda infection over the study period and the incidence rate defined as the incidence risk standardised by the number of cow-months the uninfected cows were at risk of contracting *T. orientalis* Ikeda infection over the study period.

2.8. Statistical analysis

Two additional continuous variables were calculated from the data, days in milk (DIM) was the time in days from calving to each of the LIC herd tests and days to sampling (DTS) was the time in days from calving to each sampling visit. The infection intensity data was normalised using a $\log_{10}$ transformation and age of cow was categorised by quartiles into four age groups: Age_Group 1 = cows aged 2 or 3 years; Age_Group 2 = cows aged 4 or 5 years; Age_Group 3 = cows aged 6 or 7 years; and Age_Group 4 = cows aged 8 years and over.

The data structure itself was hierarchical and had 2 levels of information and potential variability, the 1 st level was the repeated herd tests and sampling tests on individual cows, the 2nd level was the individual cows within each herd. It is reasonable to presume that measurements made on the same cow will be more similar than those made on different cows in the same herd. To account for this dependency in the data, a linear mixed effects model was built using the LMER package in R (Bates et al., 2014). A linear mixed effects model is one that has both fixed and random effects. Fixed effects are constant across individuals and estimate the mean effect whereas random effects vary across data levels and estimate the variance.

The equation for the multilevel mixed effects models used in this analysis was

$$Y_{ij} = b_{0j} + b_{1j}X_{ij} + \varepsilon_{ij}$$

$$b_{0j} = b_0 + u_{0j}$$

$$b_{1j} = b_1 + u_{1j}$$

Where Y_{ij} is the HCT or ECM or infection intensity for the i^{th} sampling in the jth cow, b_{0j} is the random intercept for the j^{th} cow which is broken down into the average model intercept b_0 and an error term for each cow, u_{0j}, b_{1j} is the random slope for j^{th} cow which is broken down into the average model slope b_1 and an error term for each cow's slope u_{1j}. ε_{ij} is the error term for the i^{th} sampling in the j^{th} cow. The two error terms u_{0j} and u_{1j} are not independent and have a covariance structure, whereas ε_{ij} is normally distributed with a mean of zero and is independent. The family wise error rate for multiple post-hoc comparisons was controlled using Tukey's HSD.

The fixed effects tested in the mixed effects models were age, DIM, DTS and Farm, a random intercept was fitted for cow and a random slope fitted for DIM or DTS. Polynomials and biologically plausible interactions were also tested in the model and all significant coefficients were retained in the final model at $p < 0.05$ using the log likelihood test. Model fit was assessed by graphing the residuals. Only data from prevalence or incidence cases where the infection intensity was above the LOQ were included in the mixed effect models and where possible, and appropriate, the data from more than one farm was combined in the same model with Farm fitted as a fixed effect. For Farm B, where several cows with suspected clinical TABA were treated with Buparvaquone, a mixed effects model was built to estimate the effect of BPQ treatment on ECM production compared to uninfected cows.

When only small numbers of cows were available for analysis, matched data sets were prepared using propensity scoring (Randolph and Falbe, 2014). Matching case and control animals on baseline data can improve study efficiency by improving precision in the analysis (Pearce, 2016).

Survival analysis was used to analyse the reproductive data, using a Cox proportional hazards regression model (Mills, 2010), with days from planned start of mating to conception used as the outcome variable. Empty cows for each farm were right censored on the last day of mating for that farm. Model fit for the Cox proportional hazards model (CPHM) was assessed through plotting the Cox-Snell residuals and testing the proportional hazards assumption by estimating the Schoenfeld residuals (Mills, 2010).

3. Results

Farm A was located 13 km south of Port Waikato (latitude 37°49′S, longitude 174°77′E), Farm B was located 20 km west of Whanganui (latitude 39°83′S, longitude 174°83′E), and Farm C was located 13 km south-west of Pahiatua (latitude 40°55′S, longitude 175°75′E). The sampling dates and LIC herd test dates are shown for the three study farms in Table 1. For Farm A, except for the November herd test, there is reasonable temporal proximity between LIC herd test dates and sample dates. For Farm B the temporal proximity is again good except for the September herd test and for Farm C the proximity is poor with only the December sample and LIC herd test dates occurring within the same calendar month.

3.1. Limits of quantification and cut point results

The LOQ plot was approximately linear to 100 ag (0.1 fg)/μL, Supplementary material, Fig. S.1, with an R^2 of 0.89 and a PCR amplification efficiency of 91 %, for target DNA dilutions down to this point. At 100 ag (0.1 fg)/μL the LOQ was equivalent to 22 Ikeda organisms per μL.

ROC curve analysis of the September sampling data from Farm C showed that the Youden index was maximised at a cut point of 5.2 fg/μL (1140 organisms/μL) with a sensitivity of 1.0 (95 % CI 0.91–1.00) and a specificity of 0.98 (95 % CI 0.94–1.00). At this cut point there were 2 false positive results and 0 false negative results.

3.2. Milk production

The average ECM milk production by farm and LIC herd test is shown in Fig. 1. Farm B achieved the highest ECM production, averaging 22.1 kg (95 % CI 21.6– 22.6) across 5 LIC herd tests, whilst production on Farm A was the poorest, averaging 15.4 kg (95 % CI 14.8–16.0) across 3 LIC herd tests. Production on Farm A dropped

Table 1
Dates of sampling and herd tests for three subject farms.

	Farm A		Farm B		Farm C	
Month	Sample	LIC Herd test	Sample	LIC Herd test	Sample	LIC Herd test
Aug	3/08/2014	—	11/08/2014	—	—	—
Sep	2/09/2014	—	9/09/2014	29/09/2014	22/09/2014	—
Oct	6/10/2014	1/10/2014	9/10/2014	—	—	19/10/2014
Nov	6/11/2014	27/11/2014	10/11/2014	12/11/2014	—	—
Dec	9/12/2014	—	10/12/2014	7/12/2014	15/12/2014	15/12/2014
Jan	13/01/2015	—	8/01/2015	—	—	—
Feb	11/02/2015	8/02/2015	8/02/2015	2/02/2015	—	8/02/2015
Mar	3/03/2015	—	9/03/2015	—	4/03/2015	—
Apr	16/04/2015	—	9/04/2015	1/04/2015	—	7/04/2015
May	—		19/05/2015	—	7/05/2015	—

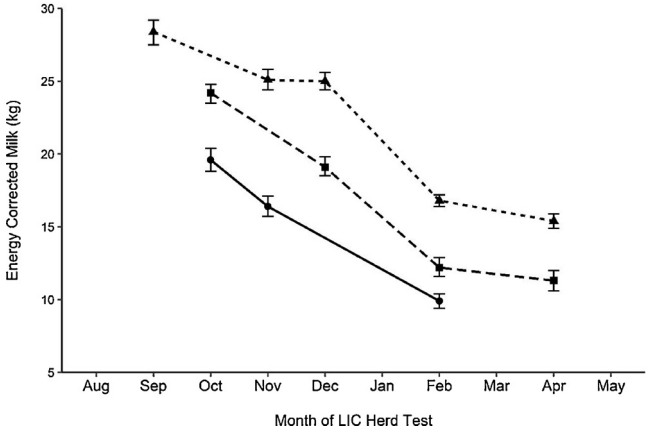

Fig. 1. Plot of average Energy Corrected Milk (kg) production, with 95 % confidence interval error bars, by month of LIC herd test for three study farms. The solid line with round points is Farm A, the dotted line with triangular points is Farm B, and the broken line with square points is Farm C.

dramatically in mid-summer due to sporodesmin toxicity, which triggered an early dry off. Farm C achieved good production, averaging 17.0 kg (955 CI 16.5– 17.6) across 4 LIC herd tests, for a once-a-day milking herd in only their second season after conversion from twice-a-day.

3.3. Farm A

Overall 865 blood samples from 106 different cows were taken during the 9 farm visits, the median number of samples per cow was 9 (range 1–9). The prevalence of infection on Farm A at the August sampling was 100 %, with all cows testing above the cut point at least once during the season. The infection intensity results for all samples were as follows, 550/865 (63.4 %) of samples returned an infection intensity above the cut point (1140 organisms/μL), 245/865 (28.3 %) of samples returned an infection intensity below the cut point but above the LOQ (22 organisms/μL) and 70/865 (8.1 %) of samples were below the LOQ. The median number of samples taken per cow was 8 (range 1–9), the median number of results with infection intensities below the cut point was 2 (range 0–8) and the median number of results below the LOQ per cow was 0 (range 0–3). Overall, the proportion of false negative results for Farm A was 315/865 (36.4 %).

The average $\log_{10}$ (infection intensity) was 3.66 (4500 organisms/μL), range (1.65 (45 organisms/μL) to 6.36 (2,280,000 organisms/μL)) and apart from the August sampling when the mean infection intensity was 22,000 organisms/μL, the average herd infection intensity by month was low, i.e. < 15,000 organisms/μL, Supplementary material, Fig. S.2a. Sixteen samples from 14 different cows (2 cows each had 2 samples > 300,000 organisms/μL) returned infection intensities equivalent to an acute infection. Of these, seven cows were recorded in August 2014, three in January 2015 and two in December 2014.

The average HCT peaked in April 2015 and was lowest in February 2015, Supplementary material, Fig. S.2b. Twenty-three samples from 17 different cows were anaemic (1 cow had 3 anaemic samples and 4 cows each had 2 anaemic samples). Proportionally cows with acute infections were more likely anaemic, 2/16 (12.5 %), both 2-year-old-cows from the thin herd sampled in August 2014, compared to 17/779 (2.2 %) of cows which didn't have acute infections ($p = 0.065$, Chi-squared test). The distribution of the anaemic cases over the sampling period is seen in Supplementary material, Fig. S. 2c, although the cases observed in the mid-summer to early autumn 2015 were potentially confounded by sporodesmin toxicity.

There was no difference between the light and normal herds in infection intensity ($p = 0.26$) and milk production ($p = 0.89$). However, the average HCT was slightly lower in the light herd, HCT = 0.300, compared to the normal herd, HCT = 0.306 ($p = 0.014$), and the average age was also younger 4.1 years compared to 4.9 years ($p < 0.0001$). The sample date and herd test dates were 5 days apart for October (2014-10-06), 21 days apart for November (2014-11-06) and 3 days for February (2015-02-11), Table 1.

Both the normal and light herds struggled to maintain body condition through the milking season and progressively lost BCS up to March 2015, Fig. 2, when the thinnest cows from both herds were dried off and the remaining cows combined into one herd. The BCS in April 2015 of the combined herd was 3.9.

3.4. Farm B

Overall there were 982 blood samples collected from 102 cows over 10 farm visits, the median number of samples per cow was 10 (range 2–10). The prevalence of *T. orientalis* Ikeda PCR positive cows at the August sampling was 58/102 (57 %). For the prevalence cases identified in August, 449/545 (82.4 %) samples returned an infection intensity above the cut point (1140 organisms/μL), 76/545 (13.9 %) samples returned an infection intensity below the cut point but above the LOQ (22 organisms/μL) and 20/545 (3.7 %) were below the LOQ. One prevalence case was culled after 2 months. There were 44/102 (43 %) uninfected cows identified at the August sampling, of which 11 became infected during the study giving an incidence risk of 11/44 (25 %) and an incidence rate of 0.026 cases per cow-month at risk. The other thirty-three cows diagnosed uninfected in August remained uninfected throughout the whole study.

The average $\log_{10}$ (infection intensity) of the prevalence and incidence cases was 3.84 (7000 organisms/μL) range (1.95 (88 organisms/μL)– 7.3 (20,500,000 organisms/μL)). Thirty-three samples returned infection intensities equivalent to an acute infection, i.e. > 300,000 organisms/μL, 22 samples were from 18 prevalence cows (1 cow had 3 samples and 2 cows each had 2 samples > 300,000 organisms/μL) and 11 samples were from 8 incidence cows (3 cows

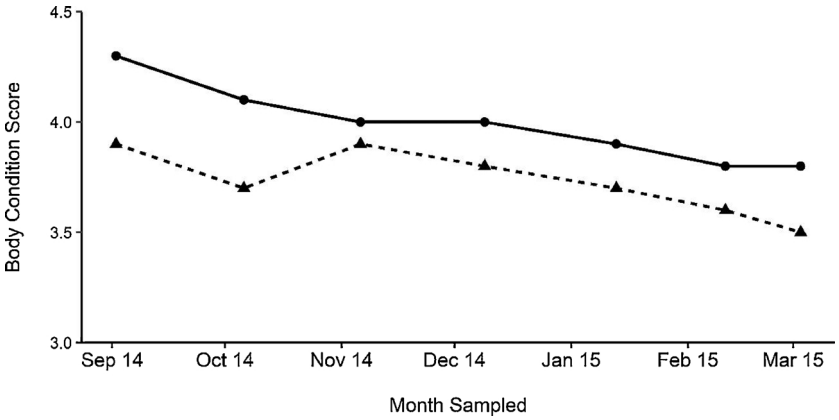

Fig. 2. Mean Body Condition Score (BCS) by month sampled for the light herd, broken line with triangular points, and the normal herd, solid line with round points, from Herd A. In New Zealand a BCS scale of 1 (thin) to 10 (fat) is used to assess dairy cow body condition.

each had 2 samples > 300,000 organisms/μL).

Forty two samples from 26 different cows were anaemic, 9 samples were from 7 uninfected cows (1 cow had 3 anaemic samples), 20 samples were from 10 prevalence cows (1 cow had 6 samples, 1 cow had 3 samples and 3 cows each had 2 samples ≤0.24 L/L) and 13 samples were from 9 incidence cows (1 cow had 3 samples and 2 cows each had 2 samples ≤0.24 L/L).

For the prevalence cases, infection intensities equivalent to an acute infection occurred throughout the lactation, although the highest number were seen in August (n = 8), whereas in the incidence cases they occurred close to when the cow first tested positive. The proportion of false positive sample results for the uninfected cows was 7/344 (2 %) and false negative sample results in the prevalence cases was 92/553 (16.6 %).

Ten incidence cases became infected in spring 2014, with six cows first testing positive by PCR in September, three in October, and one in November. A further incidence case occurred in January 2015, this latter cow was diagnosed severely anaemic in February 2015, HCT = 0.145, and dried-off soon after. The infection intensity, HCT and proportion anaemic for the prevalence and uninfected cows is shown in Supplementary material, Fig. S.3. The young herd had significantly lower ECM 20.2 kg (95 % CI 19.3–21.2) compared to the older herd 22.9 kg (95 % CI 22.3–23.6) ($p < 0.0001$) however, there was no significant difference in HCT between the two herds ($p = 0.32$). Except for September there was good temporal proximity between the blood sample dates and the LIC herd tests; 9th September 2014 (-20 days), 10th November 2014 (−2 days), 10th December 2014 (−1 day), 8th February 2015 (+6 days) and 9th April 2015 (+8 days), Table 1.

3.4.1. Prevalence cases, farm B

The average age of the prevalence cases on Farm B at the start of the lactation was 5.9 years old which was significantly higher than the average age of the uninfected cows which was 3.6 years ($p < 0.0001$). Further scrutiny of the data showed that 54/58 (93 %) prevalence cases came from the older herd and only 4/58 (7 %) from the younger herd ($p < 0.0001$ Chi-squared test). The infection intensity of the prevalence cases remained consistently below 15,000 organisms/μL over the whole sampling period, with the lowest average infection intensity reached in January 2015, 2200 organisms/μL, Supplementary material, Fig. S.3a. The prevalence cases also had a significantly lower HCT than the uninfected cows at the August sampling, 0.283 versus 0.330 ($p = 0.002$).

3.4.2. Incidence cases, farm B

The eleven incidence cases found on Farm B were evenly spread across the two milking herds, with 6/11 (55 %) from the older herd and 5/11 (45 %) from the younger herd. The infection intensity, haematocrit and proportion anaemic relative to the month they tested positive is shown in the Supplementary material, Fig. S.4. The lowest HCT corresponded with the peak infection intensity for 7/11 incidence cases; for 5/7 the peak infection intensity and the lowest HCT were at the month the cows first tested positive (Month 1) and for 2/7 the peak infection intensity and the lowest HCT were measured the month after they first tested positive (Month 2). Six of these seven cows, where the lowest HCT corresponded with the peak infection intensity, were mildly anaemic and one was severely anaemic (HCT < 0.15), average HCT = 0.216. For the remaining 4/11 cows the infection intensity peaked later, in 2 cows the lowest HCT occurred one month after the infection intensity peaked and in the other two cows the lowest HCT occurred before the infection intensity peaked.

With only 5/11 incidence cows having peak infection intensities in the month they first tested positive this meant that overall the average $\log_{10}$ (infection intensity) for that month was only moderate, 4.96 (92,000 organisms/μL), and remained at a similar intensity, 4.87, for the month after (Supplementary material, Fig. S.4a). However, the average $\log_{10}$ (infection intensity) for the month when the infection intensity peaked for each incidence case was 5.99 (980,000 organisms/μL). In the same month the incidence cases first tested PCR positive, 45 % (95 % CI 17–77) were anaemic (HCT ≤ 0.24), 1 month later 40 % were still anaemic but by 2 months none were anaemic (Supplementary material Fig. S.4c).

3.4.3. Turnips, farm B

Turnips were fed on Farm B from mid-February through to the end of March 2015 and there was an unexpected effect on the HCT results observed for March and April, Supplementary material, Fig.S.3c. Whilst consuming the turnips and for the month after finishing, the HCT dropped across all cows but not uniformly. The prevalence cases tended to have a slightly higher HCT than the uninfected cows during this period. In March 2015, 3/56 (5.1 %) of the prevalence cows, 3/10 (30 %) of the incidence cases and 5/33 (15.1 %) of the uninfected cows were diagnosed anaemic ($p = 0.048$, Chi-squared test).

3.5. Farm C

There were 579 blood samples collected from 154 different cows over 4 farm visits, the median number of samples per cow was 4 (range 1–4). The prevalence of infection at the September visit was 40/154 (26 %), with 114/154 (74 %) uninfected. The average infection $\log_{10}$ intensity of the prevalence cases over the whole lactation was 3.76 (5800 organisms/μL), range (1.37 (22 organisms/μL) – 6.82 (6,550,000 organisms/μL)), although the average $\log_{10}$ infection intensity for September was much higher 5.06 (115,000 organisms/μL). The incidence risk of disease was low, only 2/114 (1.8 %) cases occurring, one

in December 2014 and one in May 2015, giving an incidence rate of 0.0021 cases per cow-month. The December incidence case developed an acute infection intensity of 490,000 Ikeda organisms per μL whereas the infection intensity for the May case was very low, 7100 Ikeda organisms per μL, neither of these incidence cases were diagnosed as anaemic.

Of the 39 cows from Farm C that went to the Hawkes Bay for winter grazing 38 were subsequently retained in the milking herd. All of these, as well as an additional 2 cows that did not travel to the Hawkes Bay, tested above the cut point at the September sampling visit. The average calving date for the cows that went to winter grazing was 18th September 2014 and for the cows that remained on farm was 19th August 2014. The $\log_{10}$ infection intensity of the 2 prevalence cases that did not travel to winter grazing was higher than those cows that went to winter grazing, 6.2 compared to 5, however the difference was not significant ($p = 0.3$). Twenty samples returned infection intensities equivalent to an acute infection, i.e. > 300,000 organisms/μL, with 19 recorded in September 2014 (prevalence cases) and one in December 2014 (incidence case). There were 13 samples classed as anaemic; 11 samples from prevalence cases in September and 2 samples from uninfected cows in December. Farm C had a similar false negative (FN) rate to Farm A, with 54/150 (36.0 %) of prevalence samples testing below the cut point. These FN results were not evenly distributed over the sampling year, 0/40 (0 %) in September 2014, 9/40 (22.5 %) in December 2014, 31/40 (77.5 %) in March 2015 and 14/30 (46.7 %) in May 2015. In contrast to Farm B there were fewer false positive results on Farm C, with only 2/421 (0.5 %), recorded in the uninfected cows over the whole 4 samplings.

At the September sampling 11/40 prevalence cases (27.5 %) were anaemic, this fell to 0 % by the December sampling (3 months later), although 2/110 (1.8 %) uninfected cows were found anaemic at the December sampling (Supplementary material, Fig. S.5c). The average HCT of the uninfected cattle in September 2014 was 0.320, which was significantly higher than that of the prevalence cases (HCT = 0.265; $p < 0.0001$; Supplementary material, Fig. S.5b). Similarly, the average HCT of the uninfected cattle in March 2015 was 0.324, which was significantly higher than the prevalence cases (HCT = 0.30; $p < 0.0001$). Interestingly, the associated average infection intensity of the prevalence cases at the March sampling was the lowest for the four samplings (Supplementary material, Fig. S.5a).

3.6. Prevalence cases, HCT and production effects across all farms

The average $\log_{10}$ (infection intensity) of the prevalence cases by month for each subject farm is shown in Fig. 3. The individual farm plots are not particularly similar although the infection intensity of all 3 farms is almost identical in December 2014 and on all 3 farms the infection intensity increases slightly in late autumn 2015, just before dry-off. The highest infection intensity was seen in September 2014 on Farm C, which was infected for the first time in 2014. The average infection intensities of the prevalence cases from the farms infected in 2013, A and B, were consistently below 15,000 Ikeda organisms/μL, except from August for Farm A.

A linear mixed effects model fitted to the prevalence case data for all 3 farms showed a significant interaction between Farm and infection intensity on HCT ($p < 0.0001$, R-squared = 0.43) in which the effect of infection intensity on HCT depended on the farm (Fig. 4). There was an apparent weaker association between infection intensity and HCT for farms A and B, than Farm C where the association appeared much stronger (Fig. 4c). No effect of age on infection intensity ($p = 0.23$) was found for the prevalence cases.

A matched data set was prepared, for the prevalence cases and uninfected cows from Farms B and C, with prevalence cases and uninfected cows matched 1:1 within Farm, on age and DIM. A linear mixed effects model fitted to the matched data set found no effect of being a prevalence case on ECM production compared to being uninfected, adjusting for age and DIM ($p = 0.92$).

For prevalence cases from Farms A and B there was no effect of infection intensity on ECM production either at the same month as LIC herd testing ($p = 0.30$) or lagged one month (infection intensity of previous month used as predictor of ECM at LIC herd test), ($p = 0.91$). However, there was a significant interaction between HCT and DIM on ECM for prevalence cases on Farms A and B adjusting for the effect of Farm ($p = 0.015$, R-squared = 0.92). Fig. 5 shows that at a low HCT, i.e. < 0.25, the ECM production decreases with decreasing HCT.

3.7. Incidence cases, production effects across farms

There were 11 incidence cases identified on Farm B and 2 on Farm C, however only incidence case data from Farm B were used in the analysis because the incidence cases on Farm C occurred too late, relative to the LIC herd tests. Each of the nine incidence cases from September and October on Farm B were matched 1:1 on age, DIM and HCT at the August sampling (i.e. prior to infection) to one of the 33 uninfected cows. A mixed effects model with ECM as the outcome variable, controlling for the effect of age and DIM, found that the incidence cases produced on average 2.6 kg/day (95 % CI 0.8– 4.3) ECM less at the September and November tests ($p = 0.02$) compared to the

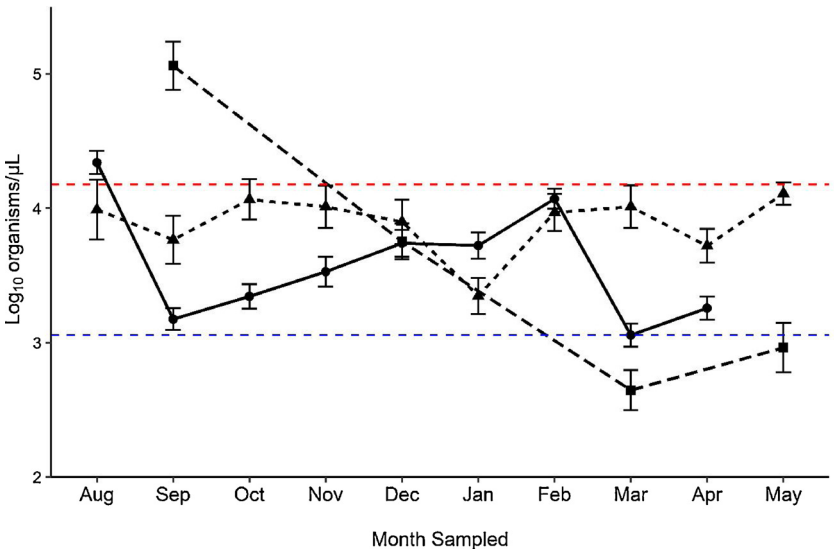

Fig. 3. Plot of average log10 infection intensity, with SE error bars, by month sampled for the prevalence cases from the three subject farms. The solid line with round points is Farm A, the dotted line with triangular points is Farm B, and the broken line with square points is Farm C. The broken red line is equivalent to a low infection intensity of 15,000 Ikeda organisms/μL (Bogema et al., 2015) and the broken blue line is equivalent to the cut point of 1140 Ikeda organisms/μL.

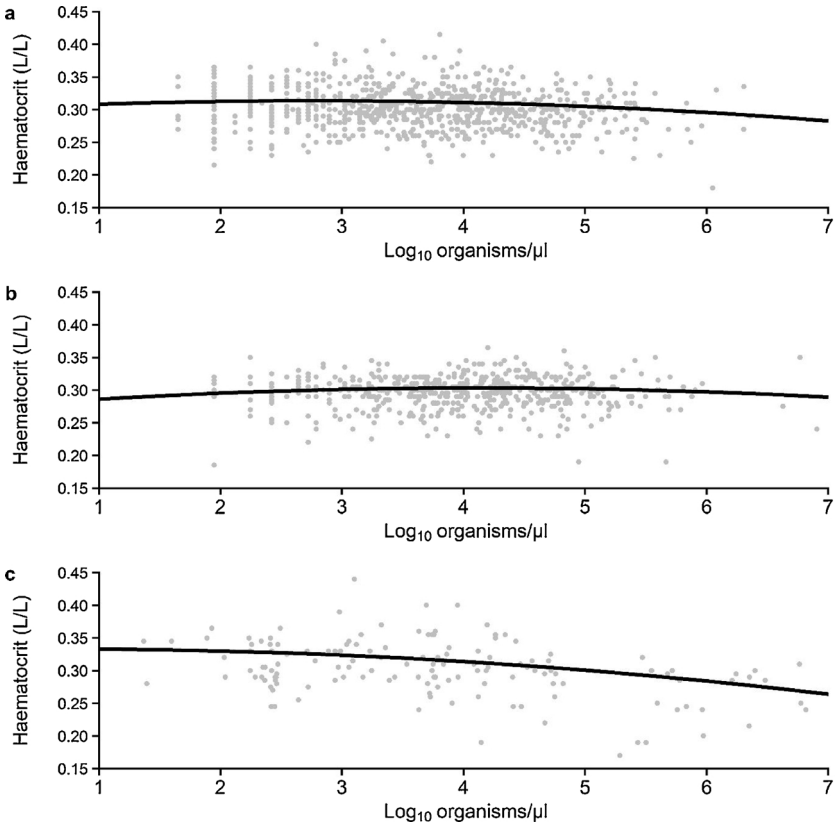

Fig. 4. Plot of the estimated effect of infection intensity of prevalence cases on haematocrit for 3 subject farms, Fig. 4a = Farm A, Fig. 4b = Farm B and Fig. 4c = Farm C.

uninfected cows (Fig. 6a). However, the effect was no longer significant by December ($p = 0.08$), or over the 5 herd tests ($p = 0.25$). The average days in milk at the November herd test for the incidence cases was 104 days which represents an average total loss of 266 kg (95 % CI 82– 450) of ECM, equivalent to 19.8 kg (95 % CI 5.7– 33.9) of milk solids, up to this point in lactation compared to uninfected cows.

3.8. Buparvaquone treatment and production effects

During August, September and early October 2014, 48/573 (8.4 %) cows from Farm B were treated with buparvaquone (BPQ) by the farm's private veterinarian (Fig. 7). Selection for BPQ treatment was based on severity of clinical signs and vulval mucous membrane colour, using the FANI card (Vink et al., 2013). Two cows, 2/48 (4 %), treated with BPQ subsequently died. The remaining 46 BPQ treated cows included 3 cows selected for the trial, one prevalence case (treated 18/08/2014), one September incidence case (treated 30/09/2014) and one uninfected cow (treated 30/09/2014). Interestingly, the uninfected cow was moderately anaemic when sampled in October 2014, HCT = 0.225, which may explain how it was erroneously selected for treatment based

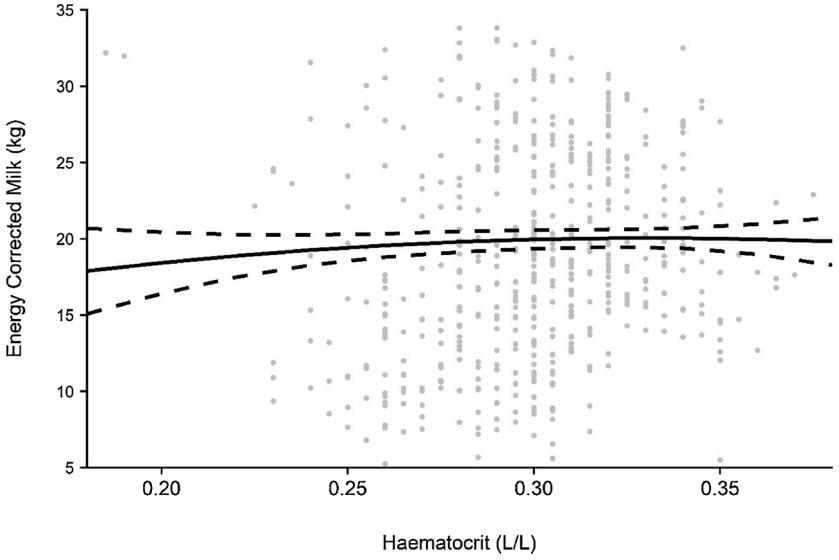

Fig. 5. Plot of the effect of haematocrit on Energy Corrected Milk production (kg) for prevalence cases across Farms A and B, adjusting for the effect of Farm and DIM.

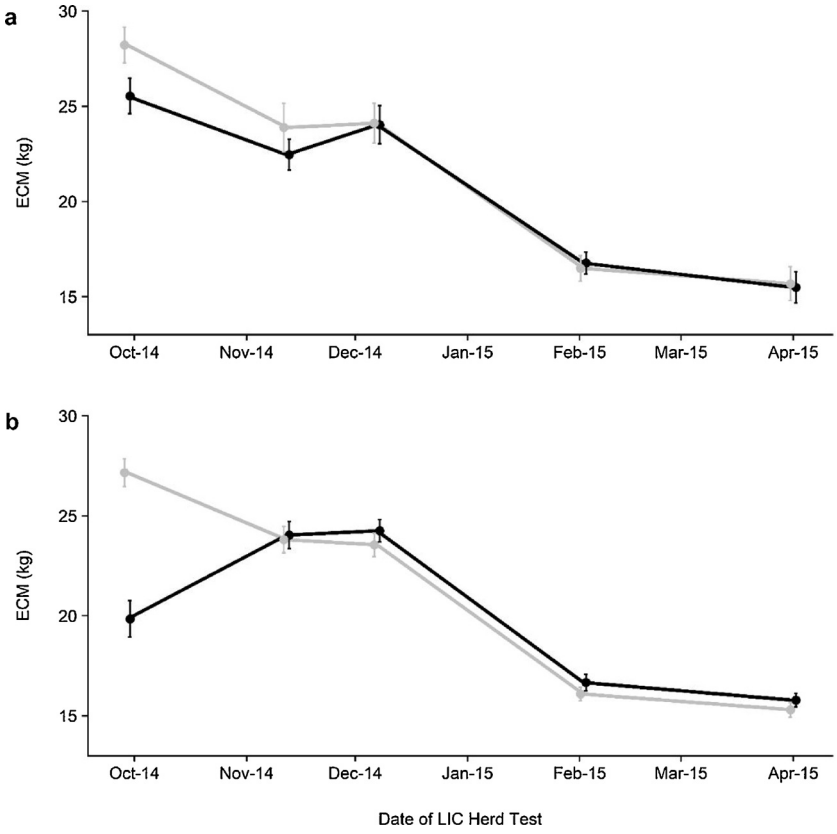

Fig. 6. (a) Plot from Farm B showing the mean Energy Corrected Milk (ECM) production (kg) with standard error bars by herd test for incidence cases, black line and points, and uninfected matched controls, grey line and points. Fig. 6(b). Plot from Farm B showing the mean Energy Corrected Milk (ECM) production (kg) with standard error bars by herd test for Buparvaquone treated cases, black line and points, and uninfected untreated controls, grey line and points.

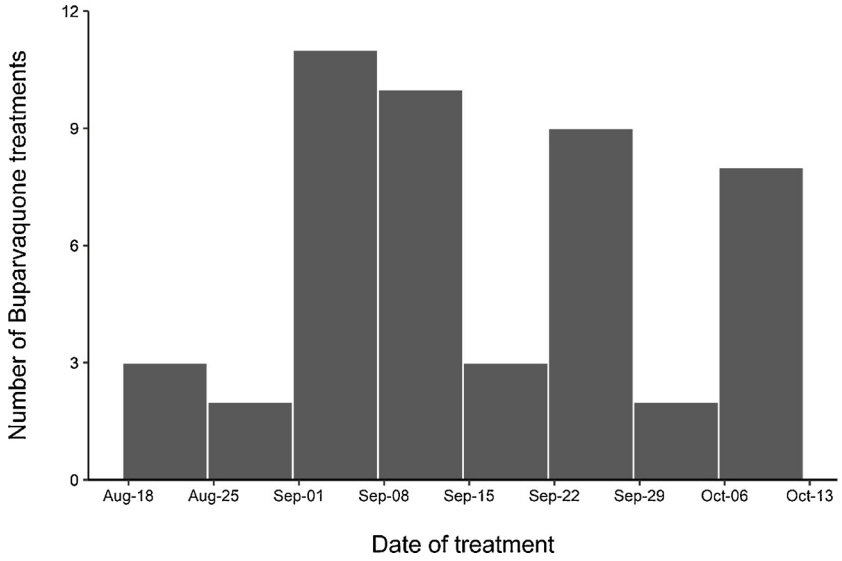

Fig. 7. Count of Buparvaquone (BPQ) treatments given to suspected Theileria Associated Bovine Anaemia clinical cases on Farm B by week, for spring 2014.

on mucous membrane colour. For the single prevalence case treated with BPQ, the infection intensity dropped from 7,660,000 organisms/μL on 11/8/2014 (7 days before treatment) to 71,000 organisms/μL on the 9/9/2014 (equivalent to a 99 % reduction in infection intensity by 22 days after treatment), with a corresponding increase in the HCT from 0.125 to 0.24 L/L.

A subset of the full Farm B data set was prepared, henceforth referred to as the BPQ data set, consisting of 45 BPQ treated cows (minus 2 cows which died) and 32 uninfected cows (minus one uninfected cow that was BPQ treated). A mixed effects model fitted to the BPQ data set, adjusting for DIM and age, showed that over the 5 herd tests, the BPQ treated cows produced on average 1.75 kg/day (95 % CI 0.8– 2.7) less ECM milk than the uninfected cows. The average DTS at the May sampling test, completed just before the herd was dried off, was 262 days for the BPQ treated cows which represents a total loss of approximately 458 kg (95 % CI 211– 710) of ECM per BPQ treated cow, compared to uninfected cows, over the whole lactation. As Fig. 6b shows, all the lost milk production was in the period up to the second LIC herd test. This amount is the gross reduction in ECM and makes no allowance for additional milk losses due to the statutory WHP of 43 days.

3.9. Reproduction effects across farms

The reproductive data for Farm A was not available, for Farm B the overall empty rate was 61/557, 10.9 %, but amongst the subject cows the empty rate was much lower 4/102, 3.9 % (1/33 uninfected, 0/11 incidence and 3/58 prevalence cows), and for Farm C the empty rate was 9/152 (5.9 %). Survival analysis using Cox Proportional hazard on the days to conception, with data right censored at 31/12/2014 on Farm B and at 20/1/2015 on Farm C, showed that on Farm B there was a non-significant reduction in the hazard of conceiving for incidence cases, HR = 0.61 (95 % CI 0.30–1.2) and for prevalence cases HR = 0.83 (95 % CI 0.53–1.3) compared to uninfected cows and on Farm C there was a similar non-significant reduction in the hazard of pregnancy for prevalence cases, HR = 0.88 (95 % CI 0.61–1.3) compared to uninfected cows found. For both models the proportional hazards assumption was met.

4. Discussion

The change in infection status, infection intensity and HCT over the 2014–2015 milking season on three spring calving New Zealand dairy herds has been presented. The effects of infection status and infection intensity on milk production, haematocrit and reproduction were estimated for each farm. Previously there have been a number of studies which have repeatedly sampled individual *T. orientalis* Ikeda type infected cattle over an extended period (Jenkins and Bogema, 2016: McFadden et al., 2017; Lawrence et al., 2018, 2019). However, the study presented here is the first to follow so many adult cattle through both the acute and chronic asymptomatic stages of infection.

The results for Farm A, from an endemically stable area, and for Farm B from an endemically unstable area show that on average the prevalence cases maintained a low infection intensity, < 15,000 Ikeda organisms/μL, throughout almost all their lactation and yet some cases of anaemia still occurred. In contrast the prevalence cases from Farm C, once they were past the acute phase of infection, returned the lowest average infection intensities found across all the subject farms. The infection intensity results also showed that a significant proportion of samples from the prevalence cases on each farm, 36 % from Farm A, 17 % from Farm B and 36 % from Farm C returned a false negative (FN) result. The difference in FN rates between the three farms is difficult to explain but clearly the continued exposure of cows to infected ticks and how recently the prevalence cases were infected has a huge impact on the FN rate. The variable prevalence of FN results in infected herds, depending on whether they are from endemically stable, endemically unstable or disease-free areas, and whether they are in the acute or chronic asymptomatic phase of infection, could have important consequences when testing cattle to establish freedom from disease.

For Farms B and C there were no significant difference in ECM production over the lactation between prevalence cases and uninfected cows. This finding is in complete agreement with Perera et al. (2014) who also found no difference between non-clinical Ikeda infected cows and uninfected cows at 100 days and 305 days in milk. This is a welcome result for producers and should mean that established dairy farms within endemic stable areas will have little ongoing production losses, once their infected cows have entered the chronic asymptomatic state.

Milk production on Farm A was poor and was likely the result of under-nutrition and the serious outbreak of sporodesmin toxicity from mid-summer 2015. Yet despite this highly stressful environment the infection intensity of the Farm A cows remained low throughout the whole milking season. This clearly suggests that the physiological responses to stress do not impact the infection intensity of *T. orientalis* Ikeda type in individual animals. Although this appears counter-intuitive it makes some sense from an evolutionary standpoint, if the *Theileria* infection capitalised on the host's ill health or poor nutrition by increasing schizogony then this could potentially contribute to the death of the host and limit opportunities for disease transmission. It may even be possible that during stressful periods, when the physiological effects of stress may be expected to confound the effect of infection intensity on the haematocrit or production (such that the same infection intensity has a greater negative effect on HCT or ECM), that schizogony may actually decrease to prevent this occurring. This latter supposition is partly supported by an observation made on Farm C during a late summer feed pinch, where the HCT of the prevalence cases dropped relative to the uninfected cows in March 2015, even though the infection intensity was at its lowest for the sampling period.

Although no direct effect of infection intensity on milk production for prevalence cases was shown, there was a weak effect of infection intensity on HCT and a weak association between HCT and ECM production for HCT < 0.25 L/L. Logically this should mean that some indirect effects of chronic infection on production through HCT could occur. However, this study would suggest that these indirect effects in chronically infected animals are both complex and small and are of minor importance compared to the direct effects of under-nutrition or disease.

On Farm B the average age of the prevalence cases was much higher than the uninfected cases, owing to most prevalence cases being present in the older herd. This was expected since in the previous year the TABA disease outbreak mostly occurred in the older herd. The older herd having been moved back to the coastal run-off in the lead up to the 2013 calving period, due to a feed shortage on the main milking platform, whereas the younger herd stayed on the main milking platform.

The estimated production loss for incident cases from Farm B in September and October was 266 kg ECM at 104 DIM, again this result is in close agreement with Perera et al. (2014) who found that infected cows with mild clinical signs, produced on average 274 kg ECM (153 L, 13.6 kg fat, 8.6 kg protein) less milk compared to uninfected cows at 100 DIM. The Fonterra pay out for 2014 – 2015season, including dividend, was $4.65 per kg MS which would represent a loss of around $92 (95 % CI $26.5– $157.6) per incidence case on Farm B.

The prevalence of infection on Farm B was 57 % at the beginning of the testing season, which would categorise the farm as a high incidence, endemically unstable herd (L'Hostis and Seegers, 2002). This occurs when the herd prevalence of infected cows is between 10 and 75 % of cows and results in a high incidence of clinical disease and a moderate case fatality rate. This classification seems appropriate for Farm B with 8.4 % of cows being treated with buparvaquone for suspected clinical TABA in the 2014–2015season.

Perera et al. (2014) found that untreated clinical TABA cows produced on average 892 kg ECM (624 L, 42.9 kg fat, 26.0 kg protein) less milk over a 305-day lactation compared to uninfected cows. In contrast, BPQ treated cows on Farm B produced on average 458 kg (95 % CI 211–710) less ECM compared to the uninfected cows over their lactation, although this figure does not include discarded milk through the statutory WHP. The additional milk production lost, discarded during the 43 days of the WHP, above the 1.75 kg/day already accounted for, would be about 875 kg ECM. The lactation length for Farm B was shorter than Perera et al. (2014) and when corrected up, the results from Farm B equate to a loss in production of around 503 kg (95 % CI 246– 827) over 305 days. Potentially this could mean that treatment with BPQ has reduced the milk production loss associated with TABA from 892 kg to 503 kg i.e. by 44 %. In addition, a reduction in the case fatality rate was also possibly observed in BPQ treated cows from Farm B. The case fatality rate for clinical TABA in New Zealand has previously been established as 25 % (Vink et al., 2016), the case fatality rate for the BPQ treated cows from Farm B was 4 %, which is significantly lower ($p = 0.04$, Fisher exact test).

Taken together these two results suggest that BPQ treatment of acute clinical TABA could reduce deaths by around 80 % and production loss by around 40 %, however it cannot be certain that all cows treated with BPQ on Farm B had TABA. In another herd outbreak of TABA, on a similar sized dairy farm in the same area, the same veterinarian correctly identified 38/46 (83 %) cows as infected with *T.*

orientalis Ikeda type, with an average HCT = 0.18 (K. Lawrence, data on file). Clearly if the BPQ treated cows on Farm B were ill from non-Ikeda associated diseases, then BPQ treatment would not be expected to have any effect on production, in which case the true reduction in lost production attributable to BPQ treatment could be greater. Although the corresponding reduction in case fatality rate would be lower.

The prevalence cases from both Farms A and B had a similar average HCT at their first samplings in August 2014 and on Farms B and C the prevalence cases at their first samplings in August and September 2014 respectively had significantly lower average HCTs than the uninfected cows sampled in the same month. Taken together these results suggest that the HCT of prevalence cases falls relative to uninfected animals during the dry period. Unfortunately, without PCR and HCT results for June and July, it cannot be ascertained if an increased infection intensity during the dry period preceded this lower HCT in early spring.

It is highly likely that on Farm A, and to a much lesser extent on Farm B, cows were seasonally exposed to hundreds of infected tick bites throughout the 2014/2015 grazing season and yet the infection intensities for the prevalence cases on both these Farms did not alter greatly. On Farm A the infection intensity was highest in August and in February and this likely corresponds to the seasonal peak activity of over-wintering infected nymphs and infected adults in July and December respectively (allowing for a lag of 4–6 weeks from infected tick bite to peak parasitaemia). It is possible that this continued exposure to infection helped maintain the infection intensity in prevalence cases at around 4500 to 7000 organisms/μL on Farms A and B. On Farm C, where ticks were absent, it was presumed that the prevalence cows were not challenged by further infected ticks and the infection intensity dropped dramatically throughout the season to extremely low levels just before dry-off, < 1000 organisms/μL.

Theoretically genetic recombination in the sexual phase of the *T. orientalis* life cycle, conducted during the tick phase of the life cycle, should produce a genetically diverse population of Ikeda sporozoites which could then superinfect already infected animals and overwhelm their immune system. The evidence from Farms A, where tick challenge was observably high (ticks were frequently observed feeding on the udder escutcheon at blood sampling), does not support this supposition since the change in infection intensity on average was slight and only a small number of animals achieved infection intensities equivalent to acute infection i.e. > 300,000 organisms/μL. It is likely that, as with all Apicomplexan infections, the host/pathogen/environment/vector interactions for *T. orientalis* Ikeda type are complex, for example in malaria (*Plasmodium*), current infections have been shown to inhibit the establishment of future infections so that concurrent infections with different genotypes of *Plasmodium* are maintained as low asymptomatic parasitaemias (Portugal et al., 2011). This is thought to have evolved to prevent life-threatening parasitaemias, since a dead host does not ensure the survival of an infectious agent.

The results from this study clearly indicate that anaemia in dairy cows can be a consequence of both acute and chronic Ikeda infection, although more often seen in acute infections and recently infected cases as evidenced by the prevalence of anaemia for incidence and prevalence cases from Farms B and C respectively. However, chronically infected prevalence cases on Farms A and B also became anaemic, and as was clearly shown on these same farms it is important for New Zealand veterinarians to discriminate between infectious, dietary and toxic causes of anaemia. Both brassica feeding (Farm B) and sporodesmin toxicity (Farm A) are significant causes of bovine anaemia in New Zealand cattle and practitioners need to differentiate and apportion causality when encountering an outbreak of anaemia. Facial eczema (Lawrence et al., 2016a) and hypophosphataemia (G. Orbell pers. comm.) have previously been misdiagnosed as oriental theileriosis outbreaks within New Zealand.

An important limitation of the study was that cows were not followed through the dry period, and as the findings from Farms A and B would suggest there are significant changes in the HCT of infected cattle from dry-off to calving. Future work would need to establish the change in HCT of infected and uninfected dairy cows through the dry period and early lactation, with a view to finding methods to potentially support the HCT of infected cattle. Since a low periparturient HCT in infected cows may be associated with lower production and may predispose them to post-parturient health problems. A further limitation was the lack of repetitions for the molecular assay, more usually in these types of studies three PCR replicates for each sample are carried out and the average used to estimate infection intensity (Lawrence et al., 2016b, 2018). However, the additional expense involved in doing 3 repetitions for such a large sample size meant that at the time this study was completed it was not economically possible. It is likely with 3 replicates the number of reported false negative and false positive results would have dropped.

There was no significant effect of acute infection, i.e. incidence cases, on reproduction found on Farm B, this result was not surprising given the small number of affected animals (n = 11). McDougall et al. (2014) found a similar, but significant, reduction in the hazard of conception for 30/446 cows diagnosed as anaemic and recently infected, HR = 0.63 (95 % CI 0.42–0.95). No effect of prevalence cases on days to conception for Farms B and C was found, this was also expected since it would be highly unusual in a New Zealand context to find a negative effect on reproduction without a corresponding negative effect on milk production.

The finding on Farm B that uninfected cattle fed on turnips had a lower HCT and were more likely anaemic than prevalence cases was surprising. Turnips can contain s-methyl cysteine sulphoxide (SMCO) which causes intravascular haemolytic anaemia and haemoglobinuria, and yet being infected with *T. orientalis* Ikeda type appears in this case to be protective. A likely mechanism for this is that in the prevalence cases erythropoiesis was already increased allowing them to better cope with the effects of SMCO toxicity. In contrast the higher anaemia rate in the incidence cases suggests that for these animals' erythropoiesis had not recovered sufficiently to cope with the SMCO toxicity.

The prevalence of anaemia in the prevalence cases identified in August on Farm C and the recovery of anaemic animals from Farms B and C was similar to the findings by Lawrence et al. (2018) that the prevalence of anaemia in recently infected herds is 26 % and that the herd prevalence of anaemia returns to around zero after about 80 days. The prevalence of anaemia in the incidence cases on Farm B was higher at 45 % however the 95 % confidence intervals for this estimate overlapped 26 %.

The LOQ for the molecular assay used in this study was 0.1 fg/μL (22 organisms/μL) and the cut point was 5.2 fg/μL (1140 organisms/μL) for whole blood. Using an alternative qPCR assay Lawrence et al. (2018) measured the LOQ as 1 fg/μL (219 organisms/μL) and the cut point as 1.9 fg/μL (415 organisms/μL) for whole blood. These results are not as different as they first appear, since 0.1 fg/μL is only one serial dilution lower than 1 fg/μL, however it does suggest that the molecular assay used in this study was less sensitive but more specific. It is also important to note that as well as using different molecular assays the two studies were also conducted on different populations of cattle, under different experimental conditions. The current study was an observational study of naturally infected dairy cows whereas Lawrence et al. (2018) was an experimental study of artificially infected Friesian bulls.

At the September testing on Farm C all the cows that over-wintered in the Hawkes Bay, for their dry period, were infected. A further 4 cows that remained on the farm also became infected, 2 prevalence cases identified at the September testing, one incidence case in December and one incidence case in May. It is likely that either infected ticks were carried back to the home farm, to infect other cows domiciled on Farm C, or else non-tick associated spread of disease occurred, since ticks had never been observed on Farm C. The May incidence case is suggestive of non-tick associated spread of infection, given the low infection intensity measured and how late in the year it occurred. May is a period when

ticks are unlikely to be active in New Zealand. Possible routes of infection for non-tick associated spread of disease at this time of year could include iatrogenic through routine vaccinations, or mechanical through stable flies or lice (Fujusaki et al., 1993; Hadi and Al-Amery, 2012; Hammer et al., 2016).

5. Conclusion

For chronically infected dairy herds, from endemically stable areas, the effect of *T. orientalis* Ikeda type infection on production are likely to be less important than the effects of poor-nutrition or other endemic diseases. However, managers of farms in these regions should be aware that sporadic cases of *Theileria* associated anaemia will still occur and may not necessarily be associated with stressful conditions. The affected animals will need to be promptly identified and appropriately treated. In herds from endemically unstable areas *T. orientalis* Ikeda type infection will continue to cause sporadic clinical disease, and production losses of around 300 kg ECM can be expected for non-clinical incidence cases infected in the spring. This study provides some evidence that if farmers are willing to accept the long withdrawal periods for meat and milk, then buparvaquone treatment may be useful in the control of clinical disease on dairy farms experiencing severe outbreaks of TABA.

Ethical approval

This experiment was performed under the approval of Massey University Animal Ethics Committee, Protocol 14/54

Declaration of Competing Interest

The authors declare there is no conflict of interest with any other parties for this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.108977.

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