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# CHANGES IN THE LEVELS OF *THEILERIA ORIENTALIS* IKEDA TYPE INFECTION IN *HAEMAPHYSALIS LONGICORNIS* NYMPHS OVER A SIX-MONTH PERIOD

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**KEY WORDS**

Theileriosis
Cattle
Ear bags
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*Theileria orientalis*
Ikeda
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**ABSTRACT**

This study aimed to investigate whether the infection intensity of *Theileria orientalis* Ikeda type organisms within *Haemaphysalis longicornis* larvae and nymph stages fluctuated over 6 mo after feeding as larvae on infected calves in the field. Naïve larvae, hatched from eggs, were fed on infected calves for 5 days while contained within cotton socks glued over the calves' ears. Larvae were first sampled immediately post-feeding and then sampled every 3 wk for 23 wk in total, after molting to nymphs. All larvae and nymphs were tested for *T. orientalis* Ikeda organisms using quantitative PCR. The qPCR results showed that the infection intensity of *Haemaphysalis longicornis* larvae and nymphs was not constant over the sampling period, and after initially dropping after molting to nymphs, it then rose with fasting to a maximum at 17 and 23 wk post-feeding. The significant rise in *T. orientalis* Ikeda organisms observed at 23 wk postfeeding may explain why more severe clinical cases of bovine theileriosis in New Zealand are seen in the spring when nymphs are the predominant instar questing.

In August 2012, an epidemic of bovine theileriosis, caused by the apicomplexan parasite *Theileria orientalis* Ikeda type, was first diagnosed in New Zealand (Lawrence et al., 2016). *Theileria orientalis* Ikeda belongs to a branch of the *Theileria* genus known as the non-transforming *Theileria* species (Watts et al., 2016). Sequencing of the highly diverse major piroplasm surface protein (*MPSP*) gene has identified 11 *T. orientalis* types, namely, Type 1 (Chitose), Type 2 (Ikeda), Type 3 (Buffeli), Types 4–8, and Types N1–N3 (Khukhuu et al., 2011; Cufos et al., 2012). The *MPSP* gene is abundantly expressed in the merozoite (Kawazu et al., 1992; Shiels et al., 1995) and sporozoite stages (Sako et al., 1999).

Although the non-transforming *Theileria* species are generally considered benign, causing few clinical signs in infected cattle, the Ikeda type has been more often associated with severe outbreaks of bovine anemia, loss of production, abortion, and death (Lawrence et al., 2016; Watts et al., 2016). The morbidity and mortality rates associated with Ikeda infection in New Zealand are low, with only 1% of cattle in infected herds showing clinical signs and only 0.25% dying (Vink et al., 2016). However, the subclinical effects are more extensive, with on average 26% of cows in recently infected dairy herds developing anemia (hematocrit [HCT] $\leq$ 0.24) (Lawrence et al., 2018b), non-clinical infected dairy cows having reduced milk production (Lawrence et al., 2019a), and infected beef calves and bulls having reduced live weight gains (Lawrence et al., 2018a, 2019b).

The life cycle of *T. orientalis* Ikeda is similar to all *Theileria* species (Shaw, 2002), although the schizont stage is only transiently observed in lymph nodes (Hagiwara et al., 1997; Sugimoto and Fujisaki, 2002), unlike the highly pathogenic transforming *Theileria* species, *Theileria annulata* and *Theileria parva*, where the schizont stage is seen in circulating lymphocytes.

Within New Zealand, the life cycle is spread between its 2 hosts: cattle and the tick *Haemaphysalis longicornis* (Heath, 2016). The life cycle of *T. orientalis* is possibly not as well understood as the transforming *Theileria* species, but, briefly, it begins its life cycle within the tick when it is ingested as part of the tick's blood meal from an infected bovine. The parasite is ingested in the form of a piroplasm within a red blood cell. Once inside the tick's midgut, the *Theileria* piroplasms migrate out of the midgut lumen and into the cells lining the midgut, where they develop into gamonts (Higuchi, 1987). The gamonts inside the tick's midgut cells release gametocytes, which recombine sexually to form zygotes. Within the tick gut cells, the zygotes of *Theileria* species develop into motile kinetes; these kinetes migrate through the hemolymph of the tick and invade the salivary glands (Takahashi et al., 1993). The trigger for the transformation of the zygote into the kinete appears to be tick molting (Fujisaki et al., 1993).

Once inside the cells of the tick's salivary glands, the motile kinetes develop into primary, secondary, and tertiary sporoblasts, with each stage budding multiple copies of the next (Higuchi,



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1986). A period of exposure to 20 to 25 C temperature is required for the sporoblasts to be sufficiently developed for sporogony to proceed and generate sporozoites from the tertiary sporoblasts (Fujisaki and Kamio, 1988). The process of sporogony is believed to be triggered by the release of salivary-gland growth hormones at the commencement of tick feeding. However, there is some evidence that sporogony can also be triggered by keeping ticks at a constant high temperature (Young and Leitch, 1981; Bishop et al., 2004), which likely mimics conditions in nature. Sporogony is the final multiplicative stage occurring within the tick, completed after sporoblast budding, and it results in approximately 40,000 sporozoites being produced per salivary acinar cell (Takahashi et al., 1993). Maximum production of sporozoites occurs 3 to 5 days post-commencement of tick feeding (Purnell and Joyner, 1968). This results in the release of thousands of sporozoites from the sporoblasts into the tick's saliva (Young et al., 1984). If the infected tick feeds on an uninfected naïve bovine, then this will propagate the infection.

Although several studies have estimated the infection level of ticks by examining the salivary glands (Fujisaki and Kamio, 1988; Kamio et al., 1990), only 1 study could be identified that has measured the *T. orientalis* Ikeda infection intensity of ticks using quantitative PCR (Dinkel et al., 2021). This investigation aimed to measure the change in *T. orientalis* Ikeda infection intensity in tick nymphs using qPCR, where the nymphs had been fed as naïve larvae on naturally infected calves.

## MATERIALS AND METHODS

### Larvae

Larval ticks were obtained by sourcing gravid *H. longicornis* female ticks from a farm 13 km south of Port Waikato, New Zealand (37°49′S, 174°77′E). Gravid female ticks were collected by the farm manager from dairy cows at the morning milking and transported to the laboratory at Massey University (Manawatu, New Zealand). Upon arrival, the viable gravid female ticks were sorted into small mesh-top containers. The ticks were held at 24 C, 95% humidity, in constant darkness, with 4 females per container. Temperature and humidity regulation was achieved by placing the mesh-top containers inside an airtight container, lined with wet paper towels, which was then placed in a Contherm Polar 1000C incubator (Contherm Scientific Ltd., Lower Hutt 5012, New Zealand) set at 24 C.

Once egg-laying had finished (the process taking on average a week), any dead or dying female ticks were removed from the mesh-top containers and discarded. This was to prevent any fungal growth from the dead or dying female ticks contaminating the eggs. Eggs were then held at the same temperature until they had hatched into larvae, whereupon the temperature setting of the incubator was lowered to 20 C. The tick larvae continued to be regularly checked for fungal infection and water condensation.

### Ear bags

The ear bag technique used was an adaptation of the method described by Ghosh and Azhahianambi (2007), using size 10 white cotton rich "Business Socks" (Kmart, New Zealand) and Kamar® adhesive (Kamar Products, Inc., Zionsville, Indiana) instead of cotton bags and ties. Before the tick-feeding experiment, a pilot study was conducted to study the effect of ear bags and Kamar® adhesive on calf behavior and welfare. This study was completed on a small farm 20 km north of Palmerston North (40°16′28.2″S, 175°25′49.7″E), and it conclusively showed that the Kamar® adhesive and ear bags did not negatively impact the welfare of calves (data on file).

### Tick-feeding study

The tick-feeding study was completed on a large dairy farm 24 km west of Palmerston North (40°12′07.8″S, 175°26′59.7″E). Twelve calves were randomly selected from a group of 30 6-mo-old calves for this study. A 10-ml blood sample was collected by jugular venipuncture into EDTA vacutainers from each selected calf. A pooled PCR test for *T. orientalis* Ikeda was carried out on the fresh blood samples by IDEXX Laboratories (Palmerston North, New Zealand) and confirmed that infection was present in the mob. The individual calf blood samples were then stored at –20 C until PCR testing was completed.

The left ear of each selected calf was lightly clipped, to reduce hair length, before a single white cotton sock containing the tick larvae was glued over the ear and attached to its base using Kamar® adhesive. Escape of larvae was prevented by having a small, lidded vial containing the larvae already attached to the inside of the sock using cyanoacrylate (Gorilla Glue Company™, Cincinnati, Ohio). Once the sock was securely fitted to the ear of the calf, the lid of the vial was manually removed through the sock, thus allowing the larvae to safely exit onto the calf's ear. Each vial contained approximately 500 to 700 live larvae.

Sock removal was planned for 5 days later, and the recovered fed larvae were to be maintained as before in mesh-top containers placed inside a larger air-tight container, lined with wet paper towels, and incubated at 20 C in constant darkness until they molted to nymphs. The ticks from each calf were maintained in separate repositories throughout the experiment. Two engorged larvae were initially sampled from each calf, at sock removal, to determine the average number of *T. orientalis* Ikeda organisms present. After molting to nymphs was completed, additional ticks were then sampled at 3-wk intervals from each calf's repository over 23 wk for the determination of numbers of *T. orientalis* Ikeda organisms.

### DNA extraction

For each date of tick sampling, DNA was extracted using the methodology outlined by Guerrero et al. (2001). Briefly, the sampled ticks, 2 per calf, were frozen in liquid nitrogen before being crushed inside a micro-tube using a micro-pestle. A volume of 100 µl of extraction solution (100 nm Tris HCl, 500 nm HCl) was then added to the crushed ticks, and the whole mixture was heated to 100 C for 3 min in a heating block. From this, a sample of 10 µl was taken and diluted in 100 µl of nuclease-free water to give a 1:10 dilution. Of this diluted solution, a 5-µl aliquot was then used in the qPCR process.

The first DNA extraction was on the day the ticks were removed from the calves. The next extraction was 2 mo after the first, to ensure all ticks had molted, and then every 3 wk after that, giving a total of 7 extractions, the last of which occurred 23 wk after feeding.

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### Quantitative PCR

The concentration of *T. orientalis* Ikeda organisms in the tick nymphs and the sampled calves was determined using a quantitative PCR (qPCR) assay as described previously (Lawrence et al., 2018a), utilizing the primers and hydrolysis probes for *T. orientalis* Ikeda type described by Pulford et al. (2016). The limit of quantification of this assay was previously established as 219 Ikeda copies per microliter (Lawrence et al., 2018a). The qPCR estimations were conducted 3 times for each composite tick sample and each calf blood sample, and the mean result was used for all statistical analyses.

### Statistical methods

Simple summary statistics were completed using a $\log_{10}$ transformation of the *T. orientalis* Ikeda infection levels. Analysis of variance (ANOVA) was used to compare the mean tick infection level across the 7 sampling points. The least-squares means (LS-means) differences (Lenth, 2016) between weeks were predicted from the ANOVA and tested for significance using Tukey's method to adjust the *P* value for multiple post hoc comparisons.

## RESULTS

On day 5, when the ear socks were initially removed from 5 calves, numerous larvae were observed still feeding on the ear margins. As a result, it was decided to leave the remaining 7 socks on for a further 2 days to allow more larvae to complete feeding and drop off. However, at sock removal 2 days later, all the larvae were found dead.

Approximately 80 live larvae were recovered from each sock of each of the 5 calves that were sampled on day 5, which probably represented 11–16% of the tick larvae released into each sock. Molting was confirmed to have occurred 6 wk after the engorged larvae had been collected from the calves, with the newly molted nymphs gathering around the lids of the mesh capped containers.

### Calf results

The infection level in the 5 calves on which the live engorged larvae fed ranged from 6,600 to 68,600 *T. orientalis* Ikeda organisms per microliter, with an average of 40,700 *T. orientalis* Ikeda organisms per microliter. The infection level of all 12 calves is shown in Figure 1.

### Tick results

The average infection levels of the sampled larvae and nymphs across the 7 sampling weeks ranged from 1,400 to 80,900 *T. orientalis* Ikeda organisms per nymph, with an overall average of 27,100 *T. orientalis* Ikeda organisms per nymph.

The results of the ANOVA showed there was a significant effect of week of sampling on the average $\log_{10}$ *T. orientalis* Ikeda organisms per tick at the $p < 0.05$ level, $F_{6,14} = 116.9$, $p < 0.0001$. The estimated LS-means $\log_{10}$ *T. orientalis* Ikeda infection levels per tick by week of sampling and the post hoc comparisons using Tukey's method are shown in Figure 2.

The results presented in Figure 2 show that the *T. orientalis* Ikeda infection intensity was not constant within these juvenile ticks, and after initially rising post-feeding in the engorged larvae, it then dropped in the first sampling after molting to nymphs and then rose again with fasting to a maximum at 17 and 23 wk post-feeding, when the study was terminated. The *T. orientalis* Ikeda infection levels found at weeks 17 and 23 were significantly higher than those at other weeks, and the infection levels found at weeks 11 and 14 were significantly lower than those at other weeks, except between weeks 0 and 14. The *T. orientalis* Ikeda infection levels found at weeks 8 and 17 were similar and intermediate between these other weeks.

## DISCUSSION

In this study, an investigation into the change in the infection intensity of *T. orientalis* Ikeda type organisms in *H. longicornis* nymphs over 23 wk post-feeding was completed. The study clearly showed that the infection intensity of *T. orientalis* Ikeda type is not constant within unfed nymphs and that infection levels were significantly higher at the end of the study period, compared to immediately post-feeding, and significantly lower post-molt.

The most likely explanation for the change in *T. orientalis* Ikeda type infection levels over the study was the competing risks of destruction by the nymph's immune system and multiplication by sporoblasts budding through progressive stages before sporogony in the nymph's salivary gland. Ticks possess a strong humoral immune response (Hernandez et al., 2019), and the most vulnerable stage of *T. orientalis* is probably the motile kinete stage, when it migrates through the hemolymph. So, the drop in *T. orientalis* Ikeda type organisms during weeks 11 and 14 represents their destruction by the tick's immune system. It is also possible that the tick immune response targets infected cells, such as the acinar cells of the salivary gland. The destruction of infected salivary gland acinar cells and their contents by the tick immune system is another likely explanation for the fluctuations in tick *T. orientalis* Ikeda infection rates seen in this study and could explain the significant drop in *T. orientalis* Ikeda type organisms seen at week 20.

The large increases in the *T. orientalis* Ikeda population seen after week 14 are in strong agreement with the findings of Sasaki et al. (1990) and are most likely due to the triggering of temperature-dependent sporogony within the infected acinar cells of the salivary glands. Temperature-dependent sporogony may simulate what happens in the field in the spring, when the overwintering nymph comes out of diapause (Heath, 2016). It is likely that the increasing soil temperature, as spring advances, triggers salivary gland development and expansion, in preparation for questing and feeding, which in turn trigger sporogony. Studies suggest that sporogony can be triggered through the keeping of ticks at a constant high temperature of between 23 and 28 C (Young and Leitch, 1981; Young et al., 1984; Bishop et al., 2004). The juvenile ticks in this experiment were kept at a constant 20 C, which was lower than the cited literature, but it appears to have been sufficient to trigger sporogony in the *T. orientalis* Ikeda type sporoblasts.

The *T. orientalis* Ikeda type infection levels recorded in the nymphs after week 17, 2,200 to 81,000 Ikeda organisms/nymph, are similar to the range of infection levels found by Dinkel et al. (2021), who found 1,630 to 217,000 organisms. The Dinkel et al. (2021) estimates were made on salivary glands removed from 9 infected adult *H. longicornis* ticks that had fed for 4 days to stimulate salivary gland development and sporogony. We believe

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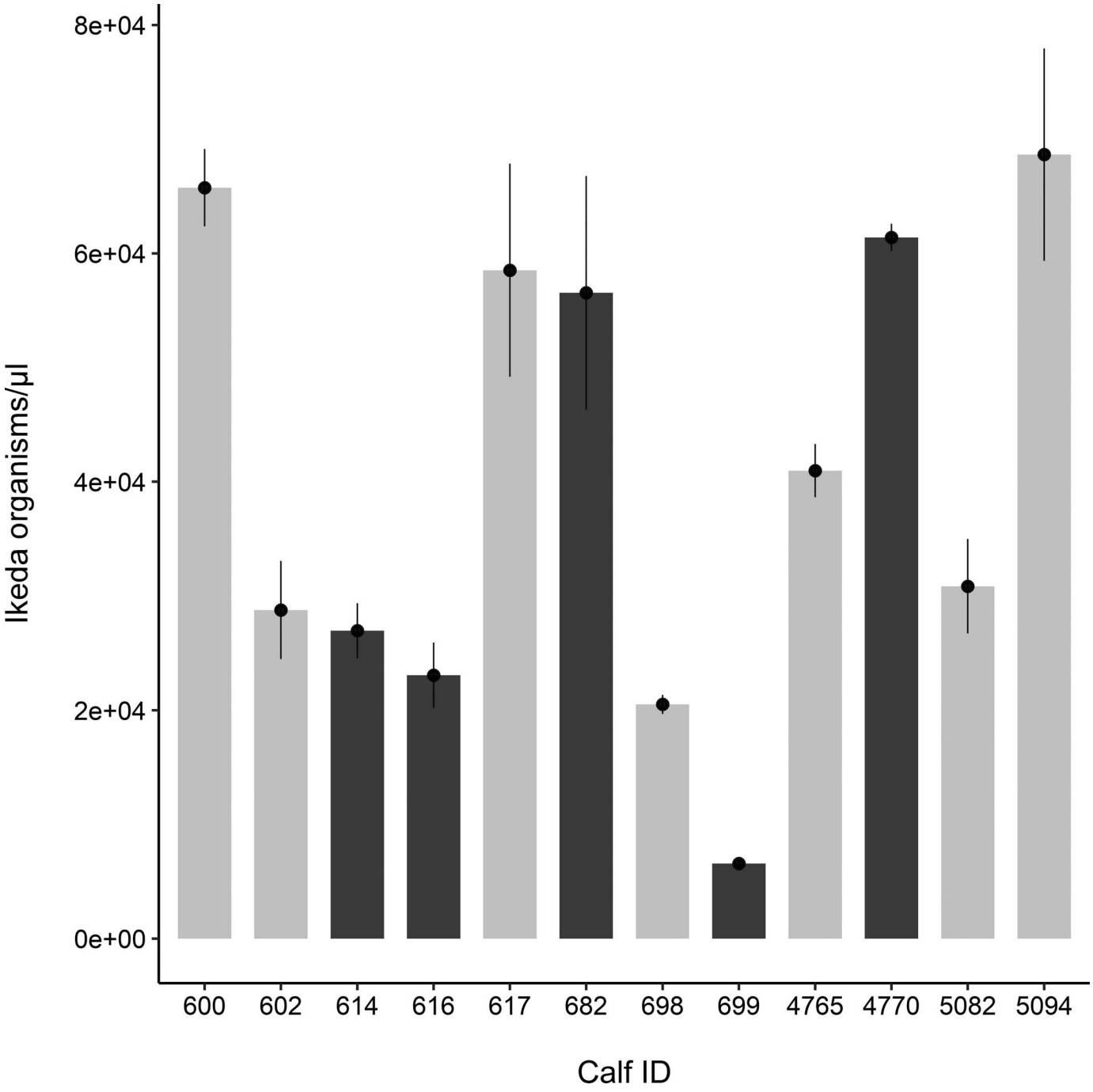


**Figure 1.** *Theileria orientalis* Ikeda infection levels based on qPCR analysis (organisms/µl, with standard error bars) for 12 calves initially selected for the tick-feeding experiment. The dark columns are those calves from which live larvae were recovered on day 5.

the Dinkel et al. (2021) findings validate the findings from our study and again suggest that sporogony had already started in the salivary glands of the study nymphs by week 17.

There are no known estimates of the volume of blood ingested by *H. longicornis* larvae; however, there are estimates for the lone star tick, *Amblyomma americanum*, a similar-sized tick to *H. longicornis* (A. Heath, pers. comm.). Koch and Sauer (1984) estimated that *A. americanum* larvae ingest an average of 1.34 µl of blood when feeding on dogs. If we use that volume as an estimate for the volume that *H. longicornis* larvae may ingest from calves, then that would indicate that only about 5% of *T. orientalis* Ikeda type organisms survived the immediate effects of digestion in the tick (based on an average infection level in the 5 calves at sock application of 40,716 *T. orientalis* Ikeda organisms per microliter and an average infection level in the tick larvae measured immediately post-feeding of 2,852 *T. orientalis* Ikeda organisms). This is an intriguing discovery and possibly indicates that we cannot presume that every time naïve larvae feed on

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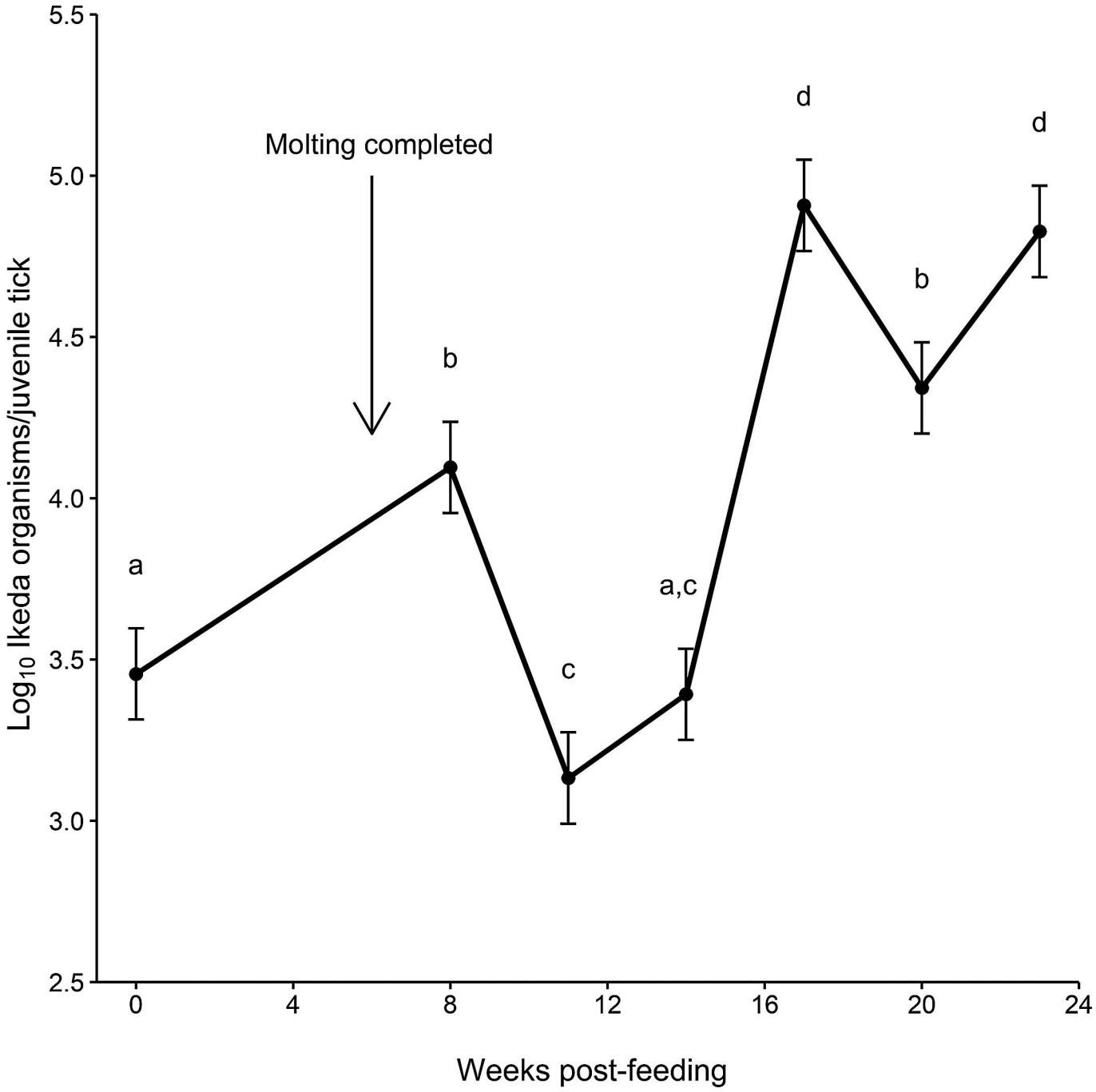


**Figure 2.** LS-means $\log_{10}$ *Theileria orientalis* Ikeda infection levels based on qPCR analysis, with standard error bars, in juvenile ticks by week since feeding on *Theileria orientalis* Ikeda–infected calves as larvae. Mismatching letters (a, b, c, d) denote statistically significant differences between samples.

infected cattle they necessarily become infected. Since none of the ticks of this study was individually qPCR tested, we cannot be certain that all 10 ticks at each sampling point were infected, but in a similar study, conducted on sheep, all the individually qPCR tested nymphs were found to be infected (Lawrence et al., 2021). However, in the previously mentioned study of Dinkel et al. (2021), only 9/35 (26%) of naïve nymphs, having previously fed on 3 *T. orientalis* Ikeda–infected calves, were positively identified as infected using qPCR testing. It is likely that the infection rate of feeding ticks in the field may vary, as seen in Japanese and American studies, where large proportions of field-collected nymphs were found to be uninfected (Kamio et al., 1990; Thompson et al., 2020).

*Theileria orientalis*–associated bovine anemia disease outbreaks in New Zealand show strong seasonality (Lawrence et al., 2017), with the greatest incidence and severity of cases being seen in the spring, when nymphs are the most numerous instar present (Heath, 2016). These nymphs will have overwintered unfed, having last fed as larvae in the previous autumn. A fast of 17 wk or more, as used in this study, approximates the winter period in

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New Zealand that unfed nymphs must survive until they resume questing in July or August. Sporogony is usually triggered by the onset of feeding, which means that maximum numbers of mature sporozoites do not appear in the tick saliva until at least 3 to 5 days after attachment (Purnell and Joyner, 1968). However, if temperature-dependent sporogony has already occurred, as in overwintered nymphs, we hypothesize that there will be a reduced or absent delay, and large numbers of mature sporozoites could be injected from the first day of nymph attachment. If there is no delay to maximum sporozoite production, then we further hypothesize that over the whole period of tick attachment, a much higher total infectious dose could be delivered by a tick that has experienced temperature-dependent sporogony compared to one that has not. It is possible that the higher total infectious doses delivered to cattle by overwintered nymphs (which have experienced temperature-dependent sporogony) may explain why more severe clinical cases of bovine theileriosis are seen in the spring in New Zealand, when overwintered nymphs are predominantly questing, than in the summer, when non-overwintered adults are predominantly questing.

Again, it is important to note that the infection intensities estimated in these nymphs reflect the levels of infection in the nymph before feeding. At feeding, sporozoite production increases dramatically (Young et al., 1984), and we have no way of establishing if these organism counts estimated pre-feeding correlate with organism counts while feeding, but it would seem likely.

In conclusion, the infection level of *T. orientalis* Ikeda type organisms in *H. longicornis* nymphs fluctuated significantly over a period of 23 wk, and this may have been a result of the competing effects of the tick's immune response and temperature-dependent sporogony.

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*Ethical approval:* This experiment was performed under the approval of Massey University Animal Ethics Committee (MUAEC), Protocol 18/93.

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