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The effects of *Theileria orientalis* Ikeda on bull fertility and
libido

A dissertation presented in partial fulfilment of the requirements for
the degree of

Master of Science

At Massey University, Palmerston North

New Zealand

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2017

Abstract

Theileria orientalis is a blood-borne parasite that is prevalent in New Zealand and other countries. The recent emergence (2012) of Ikeda-type *orientalis* has resulted in an epidemic of bovine anaemia in both dairy and beef cattle herds. The disease is spread by *Haemaphysalis longicornis* which is prevalent in the majority of the North Island.

The Ikeda type has been found to be more pathogenic than previously discovered types such as Chitose and Buffeli. Little is known about how Ikeda-type affects the reproductive performance of bulls. The aim of this experiment was to examine the effects of *Theileria* Ikeda on the fertility and libido of bulls. A group of 17 bulls were used in the experiment with 10 being infused with *Theileria* Ikeda-infected blood from two donor cows and the remaining 7 bulls used as controls. All 10 of the treatment bulls were successfully infected with *Theileria* and became clinically anaemic (Haematocrit below 24) between days 47 and 84 post transfusion. Semen and libido was tested every 2 weeks throughout the experiment. There was no observed change in wave motion score of semen between infected (7.51 ± 0.18) and control (7.08 ± 0.35) treatment groups ($P=0.2935$) along with no change in forward motion between infected (7.82 ± 0.16) and control (7.64 ± 0.2610) treatment groups ($P=0.5579$). The percentage of normal sperm ($P=0.0032$) was lower in the infected bulls (91.9 ± 0.05) compared to the control group (94.25 ± 0.06) although the density of sperm in an ejaculate ($P=0.0044$) was higher in infected bulls ($1.45 \times 10^{10} \pm 6.88 \times 10^6$ sperm per mL) compared to control bulls ($1.14 \times 10^{10} \pm 9.82 \times 10^6$ sperm per ml). Time to first mount ($P=0.7374$) and gap between first and second mount ($P=0.2204$) was not significantly different between infected and control groups. The number of mounts was similar between infected (2.33 ± 0.28) and control (2.36 ± 0.17) treatment groups ($P=0.9269$) and there was no interaction with time ($P=0.2221$). However, there was a significant effect of treatment on order of service with infected bulls coming in to the yard later in the herd on day 55 and was statistically significant ($P=0.02$). In conclusion, changes in fertility occurred in infected bulls but were not drastic enough to indicate a decrease in overall fertility. The only measure of libido affected was order and it is unknown how this would affect pregnancy rates in a herd situation.

Acknowledgments

I would firstly like to thank my supervisors Dr Rebecca Hickson and Dr Bill Pomroy for the opportunity to be a part of this research project along with their support and guidance throughout my time as a Masters student.

Secondly, I would like to thank Kevin Lawrence, Kristene Gedye, Anne Tunnicliffe, Barbara Adlington, Stefan Smith from IVABS, Jono Brophy from Tuapaka farm and Robyn Howe, Guy Haynes, Courtney Moffat from Tararua Breeding Centre for their help and expertise during the experiment and completing my thesis.

Thirdly I would like to thank Massey University and Beef + Lamb New Zealand for funding the experiment, and the Johannes August Anderson postgraduate scholarship, Taranaki Dairy Farmers Conference Scholarship and the Hurley Fraser Postgraduate Scholarship for their generous financial support in completing my masters.

And lastly I would like to thank my friends and family especially my parents for supporting and believing in me through the highs and lows of my university studies.

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List of abbreviations

TABA- Theileria associated bovine anaemia

MPSP- Major piroplasm surface protein

qPCR- Quantitative polymerase chain reaction

FANI card- Field anaemia nearest indicator card

HCT- Haematocrit

LH- Luteinizing hormone

GnRH- Gonadotrophin releasing hormone

FSH- Follicle stimulating hormone

ABP- Androgen binding hormone

HPT- Hypothalamo-pituitary- testicular axis

ASA- Antisperm antibodies

DNA- Deoxyribonucleic acid

1.0 Introduction

Theileria orientalis has been present in New Zealand for many years with the number of cases of death being minimal. However, in 2012 the emergence of a new type of *T. orientalis* called Ikeda that has a higher pathogenicity caused an outbreak of *Theileria* associated bovine anaemia, resulting in losses in production in herds and even cases of deaths. Common signs of *Theileria* are anaemia, inappetence, increased body temperature, lethargy and pale mucous membranes. Little is known about how *T. orientalis* Ikeda affects the cow and how the infection affects bull fertility and libido. With animals becoming anaemic and lethargic it is possible that this could affect bull libido. Anaemia and increased body temperature also have the potential to cause a decrease in fertility. If this is true farmers with newly infected bulls could see a reduction in pregnancy rates and herd production.

The aim of this experiment was to assess the effects of *Theileria orientalis* Ikeda on fertility and libido in bulls.

2.0 Literature Review

2.1 *Theileria*

Theileria orientalis is a blood-borne parasite that is found across New Zealand cattle herds although prevalent in the North Island. This species is recognised as a complex of 11 different types and these are of varying pathogenicity (Watts *et al.*, 2016). The first identification of theileriosis in New Zealand were reported in the early 1980s (James *et al.*, 1984) but after a short period of time there were no further reports of clinical disease except for two separate outbreaks in dairy cattle many years later (McFadden *et al.*, 2011, Rawdon *et al.*, 2006). However, in 2012 further cases were reported which investigation revealed were caused by the emergence of *T. orientalis* Ikeda type and this has caused an increase in the number of *Theileria*-associated bovine anaemia (TABA) cases in New Zealand with the majority of cases being in the upper North Island. The Ikeda type has a higher pathogenicity than the previous types found in New Zealand which have retrospectively been shown to be caused by *T. orientalis* Chitose and Buffeli types (McFadden *et al.*, 2013). The Ikeda type is able to cause mild to severe anaemia. The result of the infection has seen farms have reductions in production and fatalities in the herd.

Primary industries make up a large section of the New Zealand economy. Farming profits rely on the ability to produce offspring to sell and for milk production. In beef cattle, pregnancy rates are highly dependent on the ability of bulls to serve the cows in the herd as natural mating is the most common mating method used. In the last few years a decrease in bull reproductive success has been observed on some farms, which has been thought to be due to the outbreak of *T. orientalis* Ikeda.

2.1.1 History

Theileria is a tick-borne protozoal piroplasm that infects the red blood cells of animals (Hornok *et al.*, 2015). There are many species of *Theileria* present around the world; however, only one is currently present in New Zealand, which is *Theileria orientalis*. The *T. orientalis* species is only found in cattle and buffaloes whereas other genotypes can be found in different animals such as horses, *T. equi* and sheep, *T. lestoquardi* (Watts *et al.*, 2016). The *T. orientalis* species can be further divided into different genotypes based on the major piroplasm surface

protein sequences (MPSPs) present (Watts *et al.*, 2016). Of these, four are found in New Zealand. These are type 1 (Chitose), type 2 (Ikeda), type 3 (Buffeli) and type 5 (unnamed) (Pulford *et al.*, 2016a, Vink *et al.*, 2016). Chitose and Buffeli were the first types isolated in New Zealand and although they have been associated with anaemia, they are believed to be largely non-pathogenic (McFadden *et al.*, 2011).

The Ikeda-type is thought to have emerged in Japan by a silent mutation in the Buffeli strain that resulted in for a higher pathogenicity (Kamau *et al.*, 2011). The Ikeda-type has been associated with severe anaemia, losses of production and even death (Hammer *et al.*, 2016, Lawrence *et al.*, 2016b). It is common for the Chitose strain to occur with the Ikeda type as a mixed infection in both New Zealand and Australia (Eamens *et al.*, 2013, Pulford *et al.*, 2016a) , although it is unknown how this combination of types affects the severity of disease in clinical cases (Jenkins *et al.*, 2015). The Ikeda type is also found in Japan, eastern Asia, and China (Hammer *et al.*, 2016, Watts *et al.*, 2016). In 2012, the first New Zealand outbreaks of bovine anaemia associated with *T. orientalis* (Ikeda) were observed in dairy cattle in Waikato and Northland regions of New Zealand (Pulford *et al.*, 2016a)

Transmission of *T. orientalis* infection between cattle in New Zealand and parts of Australia is by the tick, *Haemaphysalis longicornis* (Hammer *et al.*, 2016, Heath, 2016). Naïve ticks become infected by feeding on infected cows and can then pass on the infection to naïve cattle when they feed again after moulting (Heath, 2016). Since 2012, the epidemic of bovine anaemia has rapidly dispersed throughout the North island with cases recorded in the top of the South Island from 2014 onwards. The rate at which the disease has spread was dramatic with the number of farms with cases of TABA increasing from 22 at the start of 2013 to 263 by October 2013 (Vink *et al.*, 2016). The rapid spread of disease is likely due to the combination of moving infected cattle into naïve tick populations causing establishment of infection and the movement of infected ticks by animal vectors onto previously naïve farms (Lawrence *et al.*, 2016b). This trend is seen in Figure 2.1 where there was a significant increase in the number of cases from period one to period three and the distribution of cases spread further around the upper North Island (Lawrence *et al.*, 2016b).

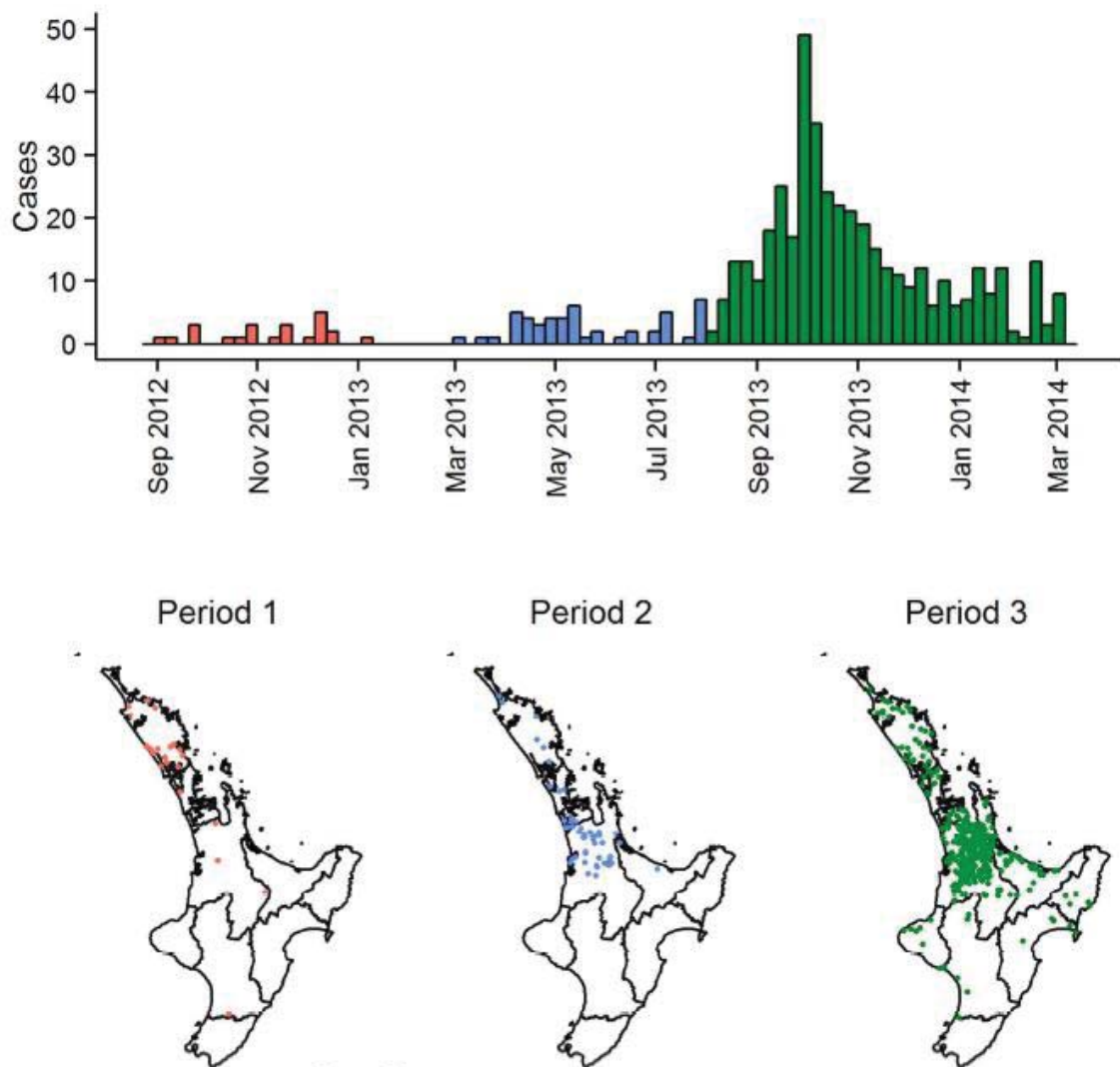


Figure 2.1: Number of herds with cases of anaemia associated to Ikeda type diagnosed between the 1st of August 2012 and the 30th of March 2014 as well as the spatial distribution of the case herds in the North Island of New Zealand. Period 1 (red) was from 30th August 2012 to 28th of February 2013), period 2 (blue) was from the 1st of March 2013 until the 31st of July 2013 and period 3 (green) was from 1st August 2013 until the 4th of March 2014 (Lawrence *et al.*, 2016b).

2.1.2 Mechanism of infection

The spread of *T. orientalis* is considered to be mostly through the active injection of saliva when a tick feeds although passive transfer of blood on mouth parts of various biting insects may result in mechanical spread (Hammer *et al.*, 2016). The only known competent vector for *T. orientalis* in New Zealand is the cattle tick, *H. longicornis* (Heath, 2016). *Theileria orientalis* will undergo development through to infective sporozoites in the tick, although the specific

nature of this process is not yet fully understood for this particular species of *Theileria* (Vink *et al.*, 2016). After a tick has fed on a cow it drops off to moult in to its next phase.

After ingestion the erythrocytes move into the *H.longicornis* lumen, where they are digested and lysed causing the release of the piroplasms (Shaw, 2002, Watts *et al.*, 2016) Once released, the piroplasms develop into male and female gametes and undergo sexual fertilisation to form a zygote. The zygote moves into the gut epithelial cell where it undergoes meiosis to divide into the motile kinete stage. This stage allows the kinete to move out of the gut cells and into the haemocoel during the tick's moulting phase. (Watts *et al.*, 2016). From here, it migrates to the salivary glands and into specific cells (Hammer *et al.*, 2016, Shaw, 2002). Each kinete then develops into a multicellular sporont (Watts *et al.*, 2016). Each sporont then multiplies asexually and develops into sporozites which is the infective stage (Hammer *et al.*, 2016).

As the tick feeds on the animal it introduces sporozites into the host where they invade leukocytes. As in the tick the progress of infection is poorly understood. The following describes the likely sequence. Once in the host cell the parasite moves out of the host membrane and lies in the cytoplasm of the cell and associates with an array of host cell derived microtubules. The parasite grows and the nucleus divides to make a multinuclear syncytium called a schizont (Shaw, 2002). The schizonts have rarely been observed for this species but are likely to be observed in places that are important in the lymph system such as lymph nodes, spleen and liver. Schizonts are not commonly found in the blood stream (Watts *et al.*, 2016). Asexual reproduction in the schizonts occurs in the leukocytes and merozoites are formed and released, infecting the bovine red blood cells (Mans *et al.*, 2015). This process is summarized in Figure 2.2 showing the lifecycle of *T. parva* including the stages that occur in the tick and in the host. It is likely there are many similarities for *T. orientalis* but also many differences. The mechanism by which piroplasms enter red blood cells is not fully understood. Infection into erythrocytes is likely to be through the use of MPSPs on the piroplasms that interact with heparin-like compounds on the host's cell surface. Immunoglobulins in the newly infected cow recognise the MPSP as non-self and mount an immune response (Jenkins and Bogema, 2016). A key question that remains largely unanswered is whether piroplasms can multiply within red blood cells. Epidemiologically it is important as it is a key factor as to whether iatrogenic transfer of small volumes of whole blood can transfer an infection to a

different animal. Mehlhorn and Schein (1985) describes binary fission of piroplasms within red blood cells forming the classic “Maltese cross” formation of four merozoites within one red blood cell. However, other reports seemingly never mention these forms within red blood cells for *T. orientalis*.

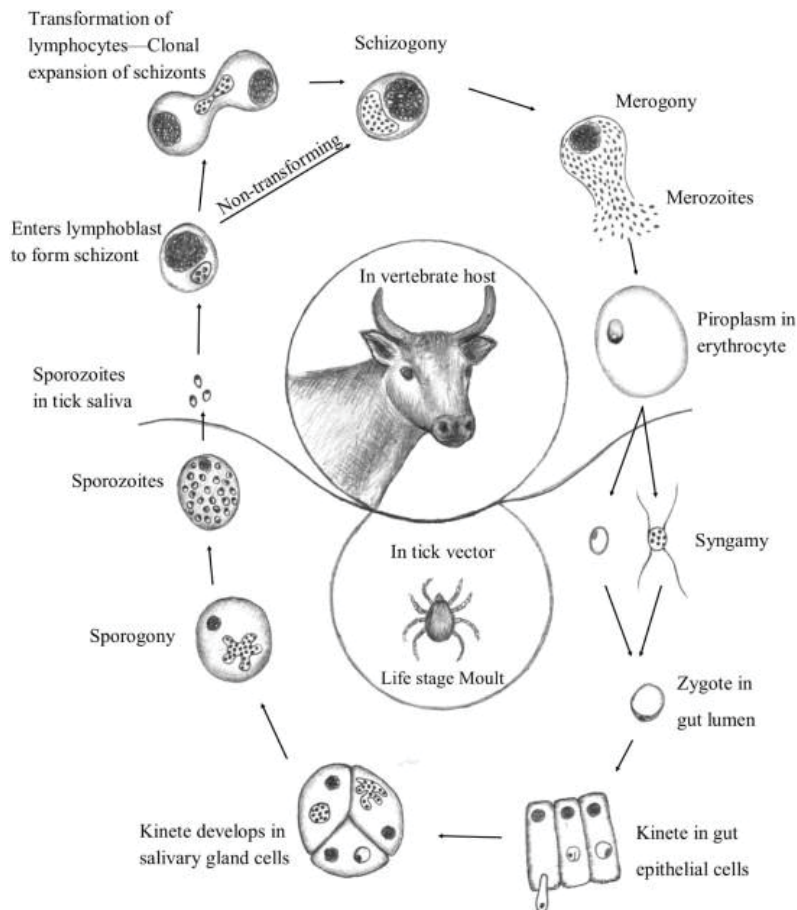


Figure 2.2: A generalized lifecycle of *Theileria parva* (Mans *et al.*, 2015).

In areas where ticks are common, animals are likely to be infected at an early age and have developed immunity prior to 6 months of age (Hewitt, 2017). It is less likely that the future production potential of these animals will be affected and the future chance of mortality is low. In other areas, where ticks are less prevalent, adult cattle may be naïve to the disease so are more susceptible to clinical infection (Vink *et al.*, 2016).

Mechanical spread of infection has been described in biting flies (horse fly and *Stomoxys calcitrans*), by lice (*Linognathis vituli*) and by needles (Fujusaki *et al.*, 1993, Hadi and Al-Amery, 2012). In Australia *L. vituli* has been found to carry the Ikeda-type but not the Chitose or Buffeli types (Hammer *et al.*, 2016). For mechanical spread, the chance of infection depends

on the level of parasite burden present in the previous cow and the volume of blood shared to the naïve cow, which depends on the size of the mouthparts of the fly or louse (Hammer *et al.*, 2016). If *T. orientalis* does not divide within red blood cells, then these insects must be transmitting developing schizonts within circulating leukocytes. Studies have demonstrated that even small volumes of blood (0.1 mL) from an infected calf are able to transfer infection (Hammer *et al.*, 2016) but does still not answer the question as to the ability of piroplasms to multiply. This study does demonstrate the possibility of either insect or iatrogenic transmission through husbandry practices.

This experimental transfer (Hammer *et al.*, 2016) also demonstrates that cattle can be artificially infected with *Theileria* by blood inoculation from an infected donor. In that study eight out of nine calves divided in to 3 treatment groups were successfully infected with 10 mL of cryopreserved blood, 1 mL of fresh blood or 0.1 mL of cryopreserved blood (Hammer *et al.*, 2016). The calf that was not successfully infected, came from the 10 mL of cryopreserved blood treatment group and had a anaphylactic reaction to infusion. All blood was harvested from one *Theileria* positive steer. The time taken until detection of disease was observed using molecular diagnosis using quantitative polymerase chain reactions (qPCR) was dependent on the initial infective dose. The higher the dose of *Theileria* given, the shorter the period between inoculation and detection of the disease (Hammer *et al.*, 2016). To measure the concentration of *Theileria* in the infective dose PCR was used (Hammer *et al.*, 2016). The doses of *Theileria* piroplasms were approximately 1.25×10^9 parasites in high dose group, 1.5×10^8 parasites in the medium dose group and 1.25×10^7 parasites in the low dose group.

Although *Theileria* DNA has been found in the colostrum of recently calved infected dams, studies that administered infected colostrum to naive calves found that no calves became infected through this process (Hammer *et al.*, 2016). This shows that infection cannot be transferred through milk and is likely to occur only through blood or tick transfer. However, other studies using *T. parva* have demonstrated the possibility of macroschizonts in cultured lymphoblasts being able to infect the lymphocytes of a recipient and have shown that it is possible that calves could be infected with this related species through colostrum (Emery *et al.*, 1982). Information on this mechanism is limited, but could suggest that the same could occur with *T. orientalis* (Hammer *et al.*, 2016). However, anti-theilerial antibodies are present in colostrum from infected dams and may limit the infectivity of infected colostrum either

directly or by passively immunising the calf. No research has been carried to see if the immunity can be helpful in the calves' resistance to the disease to date.

The evidence for vertical transfer from dam to foetus across the placenta is also contradictory. Experimental evidence from Korea appears to show the potential for transplacental infection (Baek *et al.*, 2003) whereas observational studies from Australia and New Zealand do not support this finding in the field (Hammer *et al.*, 2016, Lawrence *et al.*, 2016a), at least with chronically infected cattle. It is suggested that the cotyledonary placenta of the cow prevents maternal and foetal blood from crossing over during pregnancy so no piroplasms or infected leukocytes can be transferred (Hammer *et al.*, 2016). Even if this is not the case, transplacental transfer of *Theileria* infection is not important in the epidemiology of TABA.

2.1.3 Distribution of *Haemaphysalis longicornis*

The abiotic requirements of *H. longicornis* are well-researched (Heath, 1981, Yano *et al.*, 1985, Heath, 2016) These studies show that *H. longicornis* prefer a warm, wet humid climate, which could explain why they are more prevalent in the upper regions of the North Island.



Figure 2.3: The distribution of *H. longicornis* in New Zealand. Black regions are where the tick is the most common and the grey regions correspond with areas where the tick is less common (Heath, 2016).

Figure 2.3 shows how the *H. longicornis* tick is distributed through New Zealand. The upper regions of the North Island provide the ideal environment whilst the lower regions and most of the South Island are often too cold or too dry for the tick to establish. Small areas at the top of the South Island, such as Nelson and the Tasman district, do have the necessary climate

to support populations of ticks. There was a strong correlation between this map and the occurrence of TABA outbreaks, with the upper regions of New Zealand having a higher occurrence of disease compared to the rest of New Zealand (Heath, 2016).

Haemaphysalis longicornis is a three-host tick, requiring a new host for each of the three independent feeding stages, and has four stages in its life cycle; egg, six-legged larva, eight-legged nymph, and eight-legged adult (Heath, 2016). This can be seen in Figure 2.4. In New Zealand the *H. longicornis* life cycle is usually completed within 12 months, with overwintering nymphs mainly engorging from July to September, adults from November to December and larvae from February to April (Heath, 2016). This is summarised in Figure 2.5. All stages of *H. longicornis* are haemophagous although it is believed the larvae hatch uninfected (Higuchi, 1985). The adult tick feeds on the animal for around 7 days and, if not removed, will drop off. In late spring to mid-summer the female tick will lay around 2000 eggs that take 60-90 days to hatch, based on environmental factors such as temperature and humidity (Hammer *et al.*, 2015).

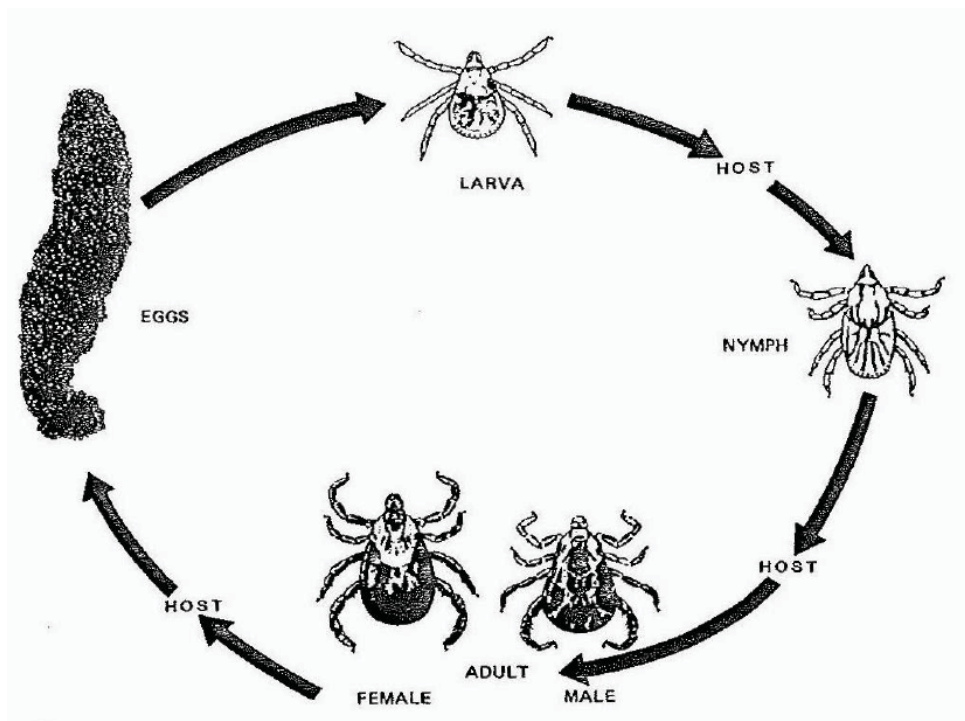


Figure 2.4: Hard tick lifecycle with the egg, larve, nymph and adult stages shown. The Figure also shows the stages that require a host (Kaye, 1974).

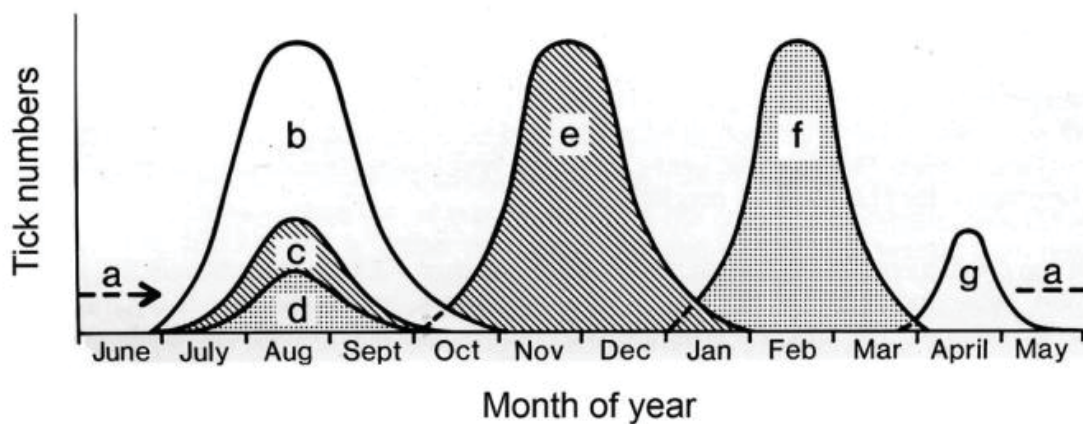


Figure 2.5: The seasonal pattern of *H. Longicornis* activity under optimum conditions. Where (a) is overwintering eggs, larvae, nymphs and adults, (b) is the nymphal peak caused by unfed nymphs from winter, (c) early adult activity from engorged nymphs that were dormant over winter, (d) is early larval activity from unfed larvae and eggs that have overwintered: these cause the rise in active nymphs in late spring to early summer, (e) is the main adult activity period caused by the nymphal peak (b), this causes the main larval activity period (f), and (g) is the smaller peak in nymphal activity from late larval feeding and moulting in mid-summer which causes the nymphal peak (c). Since nymphs and adults are the only stages that spread *Theileria*. Therefore, peak infection times would be in peaks (e) and (f) and to a lesser extent in peak (c) and (d) (Heath, 2016).

Cases of theileriosis in the South Island have occurred in areas thought to be free of ticks, it is possible that these were caused by mechanical spread of infection from infected cattle from the North Island being relocated to the South Island rather than the spread from ticks. It is also possible that these farms have very small or localised populations of ticks that have not been observed by the farmer. The lack of exposure to *Theileria* in the South Island could have disastrous effects if the tick gains the ability to establish either through climate change or irrigation (Heath, 2016).

Knowing the distribution of the tick, and *Theileria* itself, becomes important as it may affect the decision to move naïve animals into infected areas (McFadden and Marchant, 2014). Farmers in infected areas should consider this when purchasing cattle that are known to be naïve as it could result in clinical cases especially in cows that are in the late stages of pregnancy so will already have a compromised immune system. The same considerations need to be made for movements in the reverse direction. If an infected cow is moved in to a naïve herd, and there are ticks present on that property, then that infected cow will infect the

tick population. Alternatively, the infected cow could also carry infected ticks with it. Both methods would inevitably lead to the spread of disease in the resident herd. Moving cows within high risk areas is not likely to cause many problems as most cows are likely to have been exposed to *Theileria* at some stage in their life. However, movement of cows to and from the South Island could potentially have significant effects on herd or individual cow health as the majority of cows born in that area are likely to be naïve.

Moving infected animals to areas where *Theileria*-associated anaemia has been reported increases the risk of spreading the disease to naïve regions. The risk is higher if infected ticks are on the animals that are being moved and the climate is appropriate for ticks to survive when they arrive at their destination. This should be taken into consideration when moving stock and cattle should be checked for ticks and treated with a pour-on tickicide if required prior to leaving the farm (McFadden and Marchant, 2014).

The distribution of disease will also affect decisions around purchasing bulls. At present there is no evidence that bulls that become infected with *Theileria* exhibit signs of reduced fertility, either temporarily or permanently, although this possibility should not be ruled out. If *Theileria* affects bull fertility, farmers in infected areas should either purchase bulls already exposed to *Theileria* or purchase bulls earlier in the year to ensure they have sufficient time for their fertility to recover by the start of mating.

The distribution of diseases is also important for dairy calves raised on run-offs away from the main farm, especially if the risk of disease is different from the main farm and the run-off e.g. the run-off doesn't have ticks. When they come back to an infected farm they will be naïve to the disease and are likely to be pregnant. This increases the chance of the cows developing clinical theileriosis. Instead farmers may choose to send their calves to areas that have high numbers of infected ticks allowing time for them to be exposed prior to returning to the farm for their first calving in spring (McFadden and Marchant, 2014). Cows that are acutely infected, may also be at risk of clinical signs after transport due to the stress of being moved (Gebrekidan *et al.*, 2016).

2.1.4 Pathophysiology and Immunological effects leading to anaemia

The pathophysiology associated with the development of anaemia with TABA is not well understood. Antibodies can act on infected red blood cells causing oxidative damage. This is

combined with an increase in both Th1 and Th2 cytokines involved in a cell mediated immune response (Tagawa *et al.*, 2013). The end result is that infected erythrocytes are thought to be destroyed by the hosts immune system in an attempt to remove the infection. Destruction of erythrocytes results in a decrease in the number of red blood cells in the blood causing anaemia to develop (Tagawa *et al.*, 2013).

Some studies have suggested that immunoglobulins are not involved in destroying infected erythrocytes and instead, erythrocytes are damaged by increased amounts of methaemoglobin that causes oxidation of haemoglobin (Watts *et al.*, 2016). This causes oxidative damage to the erythrocytes causing them to be removed by the macrophages of the immune system (Watts *et al.*, 2016).

2.1.5 Clinical signs

The main clinical signs of the disease are from the anaemia resulting from the removal of erythrocytes (Watts *et al.*, 2016). These clinical signs include lethargy, inappetence, pale gums, pale mucous membranes and udders, exercise intolerance, abortion and stillbirth (McFadden *et al.*, 2013). The onset of clinical infection has been shown to result in an increase in body temperature in some animals (Aparna *et al.*, 2011). Animals which die will also demonstrate an enlarged spleen and jaundice, often with an enlarged liver.

2.1.6 Diagnosis of infection

Typically, *Theileria* infections are detected by observing stages in red blood cells on blood smears. However, technical issues with preparation of smears can cause a decrease in the sensitivity of detecting *Theileria* along with *Theileria* carriers having a low number of piroplasms that could not be detected in one smear (Noaman, 2014, Mans *et al.*, 2015). A study by Noaman (2014) found that using blood smears to detect *Theileria* had a sensitivity of 57% compared to PCR processes that have a sensitivity of 100%. Blood smears are still used as a fast detection of *Theileria* however, PCR has become a more common means of accurately identifying *Theileria* infection (Noaman, 2014). Detection of antibodies can be carried out using an indirect fluorescent antibody test or an enzyme-linked immunosorbent assay to show if an animal has been exposed and developed immunity to *Theileria* (Sato *et al.*, 1993).

Farmers can assess the degree of anaemia by looking at the colour of the vulval membranes which has a direct correlation with the severity of anaemia. Animals that show pale or yellow membranes are likely to be anaemic (Perera *et al.*, 2014b). A guide has been created to help farmers diagnose the severity of anaemia using a field anaemia nearest indicator card (FANI card) based on the colour of a cows vulva with a picture on the card to give a rough estimate of the Haematocrit (HCT).

2.1.7 Effects on production

Possible production effects of infection with *T. orientalis* Ikeda include reduced growth rates in young cattle, decreased fertility in both cows and bulls and decreased milk production (Bogema *et al.*, 2015). Milk yield can decrease by about 624 litres per head in severely infected cows and 115 litres in mildly infected cows during a 305-day lactation (Perera *et al.*, 2014a). A significant decrease in milk solids is also seen (Perera *et al.*, 2014b). Reproductive effects in clinically affected dairy cows have also been reported (Izzo *et al.*, 2010). Clinical cases are more likely in times of stress particularly with stress associated with transportation, or pregnancy.

Aggression has also been noted in some cases and is likely to be due to changes in mental activity from a lack of oxygenated blood to the brain (Izzo *et al.*, 2010). This is due to the anaemia causing a reduction in the number of red blood cells present to carry oxygen (Izzo *et al.*, 2010).

Morbidity and mortality rates are low with only 1% of cows in a recently infected herd developing clinical signs and 0.23% dying (Vink *et al.*, 2016). Australian studies have found that calves and pregnant heifers tend to be more at risk of dying from clinical theileriosis than empty adult cattle (Jenkins and Bogema, 2016), whereas within New Zealand young beef calves seem most at risk of disease followed by recently calved mature dairy cows (Lawrence *et al.*, 2016b). Some infected farms show higher mortality rates than others and it is not fully understood why this is the case but is likely to be due to farmers ability to identify sick cows and the stress placed on the cows (Vink *et al.*, 2016)

If an infected cow is necropsied, it will show signs of jaundice and blood will be watery (Perera *et al.*, 2015). Animals that present with clinical signs should be further tested using a HCT test from a fresh blood sample. A HCT measures the proportion of red blood cells in blood by

volume. The threshold for anaemia in cows is a proportion of red blood cells less than 0.24 in a blood sample (Jenkins and Bogema, 2016, Vink *et al.*, 2016). *Theileria* is often diagnosed by severe anaemia that cannot be explained by any other cause.

Some infected cattle show an increase in the size of the parotid and lateral retropharyngeal lymph nodes closest to where the tick infected the animal (Sato *et al.*, 1993). The lymph nodes become irregularly shaped due to the increased size and number of lymphocyte cells. The increase in the size of the lymphocyte cells is thought to be due to the cells being parasitized which also results in an increase in cell proliferation (Sato *et al.*, 1993). However, other studies suggest that the increase in cell numbers is simply a response to infection and that the cells are not parasitized (DeMartini and Moulton, 1973). Macrophages, including giant cells are seen in the lymphatic sinuses. These are also observed in the liver and spleen of the animal (Sato *et al.*, 1993).

Blood smears are often used for diagnosing theileriosis. A thin peripheral blood smear stained with Giemsa stain shows the *Theileria* piroplasms. Piroplasms are rod or ring shaped in *Theileria*-infected erythrocytes. However, differentiation between species of *Theileria* cannot be accomplished using the stain alone as morphological differences cannot be seen (Mans *et al.*, 2015).

Diagnosis of the genotype is carried out using quantitative polymerase chain reaction (qPCR) (Hammer *et al.*, 2016). The qPCR is used to detect MPSPs using probes which are specific to the Ikeda type. Each MPSP gene that is detected in the qPCR equates to one *Theileria* parasite due to the piroplasm being haploid and there being only one copy of this gene per organism (Hammer *et al.*, 2016, Perera *et al.*, 2015).

2.1.8 Treatment

Although animals can survive acute infection, it is likely that the animal remains chronically infected for life (Onuma *et al.*, 1998). Treatment may be needed if a cow shows clinical signs during the acute phase. Severe anaemia (HCT < 0.12) is treated with a blood transfusion. This is not always a practical treatment as it is expensive, and is used more commonly in high value cattle (Gharbi and Darghouth, 2015).

Oxytetracycline has been used as a treatment for *T. orientalis* Ikeda in the past. It comes in both a long acting and short acting dose. For a long acting oxytetracycline it should be administered at 20 mg/kg every second day for three doses and short acting oxytetracycline should be administered at 10 mg/kg daily for five days (McFadden and Marchant, 2014). However, the evidence that this treatment is effective is weak and the evidence that it doesn't work is stronger (McFadden and Marchant, 2014). The only effective treatment for *Theileria* is considered to be buparvaquone (2.5 mg/kg) and it is only effective during early stages of infection. However, the treatment has a long withholding period for meat (18 Months) and milk (43 days) making it impractical on many farms. Studies on *T. parva* found that over 90% of *Theileria* cases could be cured by buparvaquone (Mbwambo *et al.*, 2006). It is also not known whether buparvaquone will totally remove the infection from the animals or just reduce it whilst the animal develops an immune response. The consequence for the second is that the animal likely still remains chronically infected.

Stress can be reduced in infected dairy cows by reducing milking frequency to once a day milking to allow more energy to go in to recovery and less into milk production and walking to the shed. Reducing the amount of handling and stress the cattle undergo is beneficial. (McFadden and Marchant, 2014).

2.1.9 Prevention

Studies in Japan by Oura *et al.* (2004) with *T. sergenti* found that inoculation of piroplasms in calves could induce protective immunity. However, the use of live vaccine is restricted due to the chance of transmitting other infectious agents e.g. zoonotic bovine leukaemia. Issues with vaccination also arose, with the dose considered for the vaccine being lethal in some animals, and the antibodies produced by the calf in response to the vaccine not being protective against future infections (Onuma *et al.*, 1998). The use of vaccines against the Ikeda type has not been investigated but may be a potential preventative measure in the future.

A potential way of reducing the effects of *Theileria* is through the use of infect then treat protocols. This involves infecting animals at a young age, ideally before they are nine months old, then treating with buparvaquone. This is thought to induce endemic stability (Jonsson *et al.*, 2012). Alternatively, calves can be infected at a time when there are few other stresses for them and allowed to recover naturally. Calves are challenged by the infection and will

become immune by the time they are an adult resulting in a decrease in the number of clinical cases. Neither of these approaches have yet been studied with the Ikeda variety and would require more research before it could become a recommended strategy for reducing the effects of *T. orientalis* Ikeda in New Zealand

A reduction in the number of cows infected could potentially be achieved by using pour-on topical acaricide containing flumethrin e.g. Bayticol. The hypothesis is that by reducing the tick population in a herd the chances of a cow becoming infected is reduced, however ticks spend 90% of their lifecycles off animals which could limit the effectiveness of the strategy. Historically, in Japan methods overcame this by spraying the pastures with acaricides in order to kill ticks (Shimizu *et al.*, 2000). As stated earlier, acaricides can also be applied to cattle; however, this is costly and requires at least two treatments from July through the calving period (McFadden and Marchant, 2014). The application of flumethrin pour-on on cattle reduces the number of ticks present in the pasture and on the cattle. The ideal treatment plan for flumethrin is every 23 days when tick numbers are at their highest (Shimizu *et al.*, 2000). This can only be used as a preventative treatment, as once cows are showing clinical signs they will have already been exposed to *Theileria* around 6 weeks prior.

The disease is now widespread throughout the North Island, with a handful of cases being reported in the upper South Island. Many farms are still naïve to infection and are at risk of an outbreak if exposed to infected animals or ticks. Little can be done to prevent or treat infection, so farmers may rely on controlling when their cows get infected so that it does not occur during times where they are most vulnerable such as parturition.

There is no evidence that *Theileria* affects bulls fertility or libido. However, with infected animals becoming lethargic it is possible that the libido of bulls could decrease. Increased body temperatures along with anaemia, changes in hormone concentrations and semen production could be affected by infection. If this is true, infection of naïve bulls will need to be managed to prevent bulls becoming ill during mating as it could result in a decrease in herd conception rates. This may be by introducing bulls to infection prior to mating to ensure they have recovered in time. This would also have a significant effect on where bulls are brought from as naïve bulls are at risk of infection if moving on to infected farms.

2.2 Libido

2.2.1 Importance

Libido is defined by Chenoweth (1981) as the willingness and eagerness of a male animal to mount and attempt to mate a female. A bull's success in getting cows pregnant is dependent on his ability to identify cows in heat, and serve them. Bull libido plays an important role on farms that use natural mating. The effect of libido tends to be more obvious in short mating periods rather than long periods because the likelihood of an individual cow being detected in heat is decreased. This is due to an increase in the chance of cows not being in heat in the short mating period so could be missed in the first cycle (Parkinson, 2004). Bulls with higher libido will get more cows pregnant earlier in the breeding season resulting in calves being born earlier in the season and a longer period between calving and weaning allowing for higher weaning weights (Blockey, 1978, Smith *et al.*, 1981). High pregnancy rates tend to be observed in bulls that are classed as having a high serving capacity. Serving capacity is measured by the number of services completed by a bull per test period. The length of the test period is dependent on whether testing occurs in yards or in a pasture environment (Blockey, 1976a). Serving capacity is directly correlated with the number of oestrus heifers a group of bulls will serve in a mating period (Blockey, 1976a). Cows served by higher serving capacity bulls tend to calve earlier in the season compared to cows served by medium serving capacity bulls, even when pregnancy rates were the same over a 6 week period between the two groups (Smith *et al.*, 1981). When high and medium serving capacity bulls were compared in a libido test, bulls with a high serving capacity tended to have a lower number of mounts but a shorter reaction time to service leading to a higher serving capacity score, compared to medium serving capacity bulls. Therefore libido trait are an important indicator of serving capacity (Chenoweth *et al.*, 1988).

2.2.2 Social hierarchy

Hierarchies in bull herds tend to have older bulls at the top of the social order and younger bulls at the bottom. As older bulls are removed from the herd younger bulls tend to move up the ranks. Cow herds that are introduced to groups of bulls that are of mixed age during the mating season tend to show a higher pregnancy rate to the older bulls. This shows that the presence of older bulls tends to inhibit the mating behaviour of younger bulls. A study by

López *et al.* (1999) demonstrated this idea by libido testing young bulls in the presence and absence of a dominant bull. Sexual behaviour was observed and recorded during the testing period. This included visual, auditory and olfactory contact with the cow. The study found that when the mature bull was allowed to be in contact with the observed bull, the observed bull spent less time around the cow. Bulls that were 3-6 years old were more affected by the dominant bull than younger bulls. Part of this is likely to be due to a learned behaviour by older bulls to avoid bulls that are more dominant, that is not yet learned by younger bulls (López *et al.*, 1999).

Social hierarchy can also have an effect on the herd fertility. If dominant bulls serve the majority of a cow herd but are infertile, the number of cows pregnant at the end of mating could drastically decrease (McDiarmid, 1984). The social ranking of bulls in a herd should be taken in to account when observing and scoring libido especially in younger bulls that are likely to be submissive to older bulls (McDiarmid, 1984).

Social interactions during the early years of a bull's life can cause a change in the number of mounts a bull will achieve during a libido test. Bulls that are not kept in groups had a higher serving capacity compared to bulls that were ran as a herd (Lane *et al.*, 1983). Bulls that were run alone tended to be more sexually aggressive when tested beside other bulls. There was no difference if the bull had been around heifers or not on the libido of the bull (Lane *et al.*, 1983).

Bulls are found to work best when they have a low workload, i.e. high bull to cow ratio and have an established hierarchy in the herd. An established hierarchy can be achieved by ensuring that all bulls are run together prior to the start of mating (Parkinson, 2004). The serving activity of bulls tends to be dependent on the proportion of oestrous cows present and is likely to be contributed to by bulls typically only serving a cow once. A ratio of one bull per 20 to 30 cows tends to ensure that the majority of cows are mounted when in oestrus. Low bull to cow ratios tend to show a decrease in the proportion of cows mounted (Blockey, 1976a). Testing bulls individually allows for the effect of dominance to be taken out of testing and show a bull's true libido. Order through the gate is a possible indicator of libido and bulls make a conscious choice on whether to stand at the gate or hang back in the yard. Bulls that are first through the gate to be libido tested are likely to have a higher libido than bulls that

move through the gate later on. This is also likely to be an indicator of social hierarchy within the herd. This has not been previously examined so cannot be found in literature.

2.2.3 Soundness

The soundness of the bull can play a role in how much interest a bull will show towards a cow. Bulls that are in pain are likely to exhibit a lower libido. A study by Laflin *et al.* (2004) found that bulls that were in pain due to lumbar lesions showed a decrease in libido. Bulls had lower number of mounts and required more encouragement to mount (Laflin *et al.*, 2004). It is possible that pain could be a negative reinforcement for a bull's libido and cause a decrease in the number of mounts and bull success in the herd.

2.2.4 Physiology

Hormones play an important role in determining the expression of a bull's sexual behaviour. A lack of male sex hormones and other related hormones can result in a lack of observed libido.

The correlation between testosterone and libido has been debatable. Some studies claim that there is no effect of the concentration of plasma testosterone and libido (Foote *et al.*, 1976). However, a study by Smith *et al.* (1981) found a correlation of 0.44 between peripheral testosterone concentrations and libido. The differences in studies may be due to the time of day the testosterone was measured, as bulls' testosterone concentrations fluctuate during the day, which may affect the results observed in different studies. Other studies suggest that the concentration of testosterone has less of an effect on libido but rather the sensitivity of the tissues that the testosterone acts on (Galloway, 1978, Smith *et al.*, 1981). Although testosterone does not have a direct effect on libido, bulls that have low concentrations tend to show low libido which suggests that there is a minimum amount of testosterone required in order for libido to be maximised. Above this level, higher amounts of testosterone will have no effect (Galloway, 1978, Smith *et al.*, 1981, Parkinson, 2004)

Prolactin is a hormone secreted by the pituitary gland and regulated by the hypothalamus. It acts to increase the effect of luteinizing hormone (LH) on Leydig cells (Charreau *et al.*, 1977). This regulates the secretion of testosterone from the sertoli cells. Therefore, prolactin is thought to have a possible effect on libido in males (Charreau *et al.*, 1977). Studies in mice

have found that high concentrations of prolactin present in a male resulted in a decrease in libido. This is likely to be due to the prolactin causing a change in dopamine action in the brain. Increases in prolactin can be observed as sexual anticipation occurs, which drops back down to normal approximately 30 minutes after ejaculation. Prolactin is therefore thought to be associated with the anticipation of copulation rather than from copulation alone (Henney *et al.*, 1990).

The ratio of oestrogen to testosterone (E:T) tends to be higher in low libido bulls and is an indicator that testosterone production is not high. A high E:T ratio is often seen in bulls that have high fat deposits as testosterone is converted to oestradiol by adipose tissue (Henney *et al.*, 1990).

The changes in concentration of LH and testosterone due to sexual stimulation are highly debated, with some studies reporting no change in LH and testosterone whilst others claim significant changes (Lunstra *et al.*, 1989). A study by Gombe *et al.* (1973) compared the plasma LH concentrations in bulls prior to and after exposure to sexual stimulation. The results showed an age effect where older animals showed an increase in initial LH concentrations. There was no change in LH concentrations 3 hours after sexual excitement (Gombe *et al.*, 1973). Others have found the introduction of a female displaying oestrus caused bulls to have an increase in LH and testosterone concentrations (Lunstra *et al.*, 1989).

A better hormone predictor of libido is the effect gonadotrophin releasing hormone (GnRH) has on testosterone levels. Animals that showed a higher production of testosterone when injected with GnRH tended to have a higher libido (Post *et al.*, 1987). It was suggested by Post *et al.* (1987) that this way was more accurate than tests such as semen quality, scrotal circumference and service testing and allowed for bulls to be ranked in an order that was not subjective based on who is observing the semen or the bull (Post *et al.*, 1987).

Bulls that have chronic inflammation of the testis have been observed to show a reduction in libido. If bulls are not treated they will continue to show a lack of libido and therefore low pregnancy rates. Studies have suggested that infections in the testes can cause a decrease in the production of testosterone by Leydig cells (Sekoni *et al.*, 1992). Testosterone is thought to be an important determinant of libido as demonstrated by Post *et al.* (1987), so a decrease in the hormone is likely to cause a decrease in libido (Sekoni *et al.*, 1992)

Other illnesses that cause anaemia and pyrexia can cause the bull to become lethargic and have a lower libido (Sekoni *et al.*, 1992). Similar results would be expected in bulls that were lethargic either due to exercise or due to illness causing lethargy. *Trypanosoma vivax*-infected Zebu bulls showed a decrease in libido compared to uninfected bulls in a trial by Bittar *et al.*, (2015). Infected bulls had a libido that was classified as questionable to good compared to controls that had a libido score that was considered to be very good to excellent (Bittar *et al.*, 2015). Since trypanosomiasis causes similar effects to *Theileria* such as anaemia, fever and lethargy, it is possible that the changes in libido in *Trypanosoma*-infected bulls could occur in bulls infected with *Theileria*.

2.2.5 Measuring libido

The basis of libido testing comes from observations of bulls in a paddock environment. The frequency that a herd of bulls mounted the oestrous cows was recorded. The frequency a bull would mount a cow would determine the category of high, medium and low serving capacity bulls (Blockey, 1976a). Field studies take a large amount of time as bulls need to be observed for at least a day. If large numbers of bulls need to be libido tested, this is not a practical method.

Studies have shown that similar results to paddock testing can be replicated in yards using sedated and/or oestrous cows. How libido is measured differs between studies but is based on the idea that libido is the ratio of heifers mated to the number of heifers in oestrus (Smith *et al.*, 1981).

The technique of measuring a bull's libido and ability to mate relies largely on the immobilisation of the cow used. This has led to the development of service crates to immobilise the cow and reduce the weight placed on the cow when the bull mounts her (McDiarmid, 1984). The crate also ensures that bulls mount the cow in an effective manner reducing the likelihood of injury to the cow and bull (McDiarmid, 1984). Testing should also not be carried out in weather conditions such as high heat, or cold and rain. Adverse weather could lead to a decrease in libido or a higher risk of injuries occurring if the area being used becomes slippery (Chenoweth *et al.*, 1988).

The cow used is often on heat but studies have found that the use of a cow in standing heat does not affect the bulls libido however the cow should be properly restrained. Libido testing

should be carried out in a low stress environment where the bulls have not been placed under pressure prior to testing with procedures such as drenching or vaccination (Chenoweth *et al.*, 1988).

Bulls that have higher growth rates are assumed to have higher testosterone concentrations compared to slower growing bulls of the same age (Post *et al.*, 1987). This may not be entirely accurate as many factors have an interaction with growth rate. Nutrition and disease status have a large effect on the growth of young bulls and should be taken into account when selecting bulls for high libido (Post *et al.*, 1987).

The environment that a bull is tested in will also have an effect on the expression of libido. Bulls that are injured during libido testing may demonstrate a lack of libido in following tests. This could be due to slippery floor or incorrectly restrained cows. Some bulls dislike serving cows on a concrete floor due to the fear of falling over and will show greater expression of libido on rubber floors or soft ground (Kerruish, 1955).

Bulls may also be deterred by cows that are not fully immobilised which can occur when cows were not in standing heat. Although studies such as Kerruish (1955) suggest that cows used do not need to be in standing heat, they must have limited movement and may need to be sedated. Steers under sedation with limited movement can also be used since the cows in standing heat is not required. Bulls that are repeatedly tested for libido or collected from for AI purposes can show a decrease in interest and libido as time goes on. A decrease in anticipation and vigour may be observed along with the bull moving away from the teaser after service (Kerruish, 1955).

Bulls that are not experienced with mating should be given a longer period of time for the first libido test. This allows them multiple attempts at the cows as it is common for virgin bulls to not serve the cow on the first mating. The results from the first test should not be used to select bulls but rather a further one or two tests should be given (Blockey, 1976b).

An issue that arises from libido testing is the lack of repeatability. This is due to changes in the environment that the bull is tested in such as weather, cow, health status and feed allocation. The effect of environment on libido is difficult to quantify so to get a more accurate indication libido testing is repeated to take out the variance of environment. Libido testing and serving

capacity tests give an indication of libido in a paddock, but it is hard to know how accurately they reflect natural paddock mating. Bulls that do not display high libido in yard tests may have a higher paddock libido than bulls that do well in a yard testing situation. This means that libido testing should not be the only selection criteria used to select bulls (Chenoweth *et al.*, 1979).

2.3 Fertility

2.3.1 Introduction

The fertility of a male is the ability for him to cause pregnancy in a fertile female (Smith *et al.*, 1981). Bull fertility plays an important role in reproduction especially in farming. If low fertility sires are used during the mating period, low conception rates are common and the proportion of calves born to calves mated decreases. Soundness checks are used to remove the males that are unable to serve females so infertile sires can either be treated or replaced prior to mating. Semen quality has an important role in determining conception rates in a farm as a sire may be able to serve a female, but a lack of viable sperm will result in no fertilization of the egg. Semen samples can be collected and observed under a microscope to determine whether the male is producing viable sperm.

2.3.2 Spermatogenesis

Spermatogenesis is the process of cell differentiation to produce spermatozoa in the seminiferous tubules. This process takes approximately 61 days and is controlled by hormone concentrations that are signalled between the brain and the testis. The process starts at puberty with spermatogonia that develop from stem cells. Mitosis and meiosis follow to produce spermatids that have the correct number of chromosomes but are immotile. Spermatids then undergo maturation to become sperm that are motile and able to fertilise an egg. This is detailed in Figure 2.6.

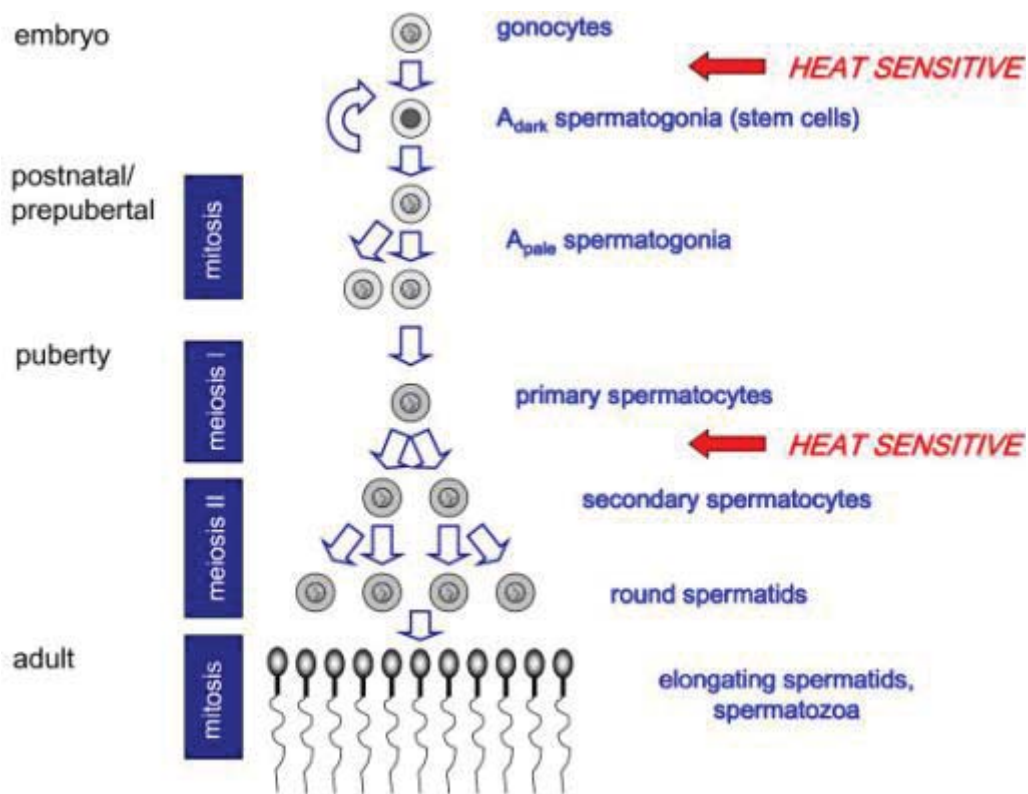


Figure 2.6 The process of spermatogenesis along with heat sensitive stages (Ivell, 2007)

2.3.3 Hormonal control

Gonadotrophin releasing hormone (GnRH) is secreted from the hypothalamus to act on the gonadotrophin cells in the pituitary. Gonadotrophin cells secrete follicle stimulating hormone (FSH) and LH in response to GnRH in the pituitary gland (Ramaswamy and Weinbauer, 2014). Exocytosis then transports the hormones to the peripheral circulation. Testosterone production is stimulated by LH binding to the receptors and leydig cells causing cholesterol to be converted to testosterone (Ramaswamy and Weinbauer, 2014). Follicle stimulating hormone and testosterone work together to stimulate spermatogenesis by stimulating androgen binding protein (ABP) secretion from sertoli cells. The sertoli cells then metabolise testosterone to oestrogen and dihydrotestosterone (DHT). The DHT then binds to ABP to keep the concentration high. Early sperm development of spermatogonia and complete maturation of sperm occurs by growth hormone acting directly on the testis (Magon *et al.*, 2011).

2.3.4 Spermatogenesis

Dihydrotestosterone binds to the androgen receptors of sertoli cells causing the promotion of spermatogenesis. Spermatogenic cells line the epithelial walls in the seminiferous tubules.

The outermost tubule cells are stem cells developed as spermatogonia which divide constantly until puberty. Type A spermatogonia undergo two rounds of mitosis with half of the daughter cells being kept as A₀ (stem cells) and the other half becoming A₁ cells. This process starts at puberty due to the increase in FSH. Cells undergo six rounds of mitosis to produce A₁, A₂, A₃, A₄, Intermediate, B₁ and B₂ spermatogonia. All spermatogonia have diploid number of chromosomes. B type spermatogonia pass through tight junctions between sertoli cells, known as the blood:testis barrier which separates mitotic and meiotic cells. Once the spermatogonia pass through the barrier they are known as primary spermatocytes and undergo meiosis I resulting in the formation of daughter cells called secondary spermatocytes that have a haploid number of chromosomes (Klug *et al.*, 2012). Secondary spermatocytes differentiate further in meiosis II to become early spermatids. Early spermatids carry haploid number of chromosomes and differentiate to become late spermatids and then spermatozoa. Spermatozoa are expelled out of the sertoli crypts into the lumen of the seminiferous tubules where they will be transported to the epididymis to mature (Klug *et al.*, 2012).

2.3.5 Sperm differentiation

The naïve sperm cell develops an acrosome, which is derived from the golgi apparatus. Enzymes on the acrosome allow penetration of the sperm head into the egg for fertilisation. The acrosome continues to develop at the anterior end whilst the centriole forms at the opposite end of the nucleus (Reece *et al.*, 2014). From here microtubules develop to form the flagellum used to allow the sperm to move around, mitochondria then develop around the proximal end of the flagellum and is used as a source of energy to power sperm movement. Excess cytoplasm is then sloughed off and the immature sperm is released from a sustentacular cell (Reece *et al.*, 2014).

2.3.6 Disease affecting spermatogenesis

Infection is a common reason for poor semen quality and can be localised to the testis and epididymis or be generalised to the whole animal resulting in changes in hormone secretion and pyrexia. Damage to the male reproductive tract, along with an immune response and change in hormone concentrations causes a decrease in viable sperm and spermatogenesis. As a result of this, affected males may be infertile with effects being temporary or permanent depending on the severity and length of infection.

Poor semen quality can also be caused by stressors such as transport and high temperatures during summer. Other factors include cryptorchidism and high scrotal insulation due to high amounts of fat in the testis region. These changes in semen quality are usually reversible and last 3-8 weeks due to the turnover rate of sperm if the condition is corrected. However, correction of the condition in the case of cryptorchidism is not always reversible (Bagley, 1997).

Sperm production only includes the development of the sperm cell itself. After the sperm cell is fully developed, seminal fluids from the prostate are added to the sperm to make it into semen. The onset of sperm production occurs at puberty with semen quality improving in the first few months after the onset of puberty. Semen quality then decreases in old age (Foote, 1978). Environment during the prenatal and prepubertal period of a bull's life can cause temporary and even permanent effects on bull fertility. The environment in gestation with a female twin can have an effect on bulls. Although this is limited compared to the impact on the female, but poor sperm quality can be seen in some cases (Foote, 1978).

Nutrition and the ingestion of toxins can cause a temporary decline in fertility in bulls. The male reproductive tract is highly regenerative and can overcome the damage caused by poor nutrition and toxins. However, in severe conditions for prolonged periods of time, the effects can be permanent (Foote, 1978).

There is also a genetic component to fertility where testicular defects, abnormal spermatogenesis and sperm morphology can be inherited from single gene pairs. Fertility has a low heritability so changes through genetic selection would be slow and take many generations. Testicular size has a direct effect on sperm production and is an inherited trait. Testicular size affects the number of sperm a bull can produce in a given time (Foote, 1978).

Extreme environmental temperatures can also cause changes in sperm viability. High temperatures can have the same effect as cryptorchidism or inflammation. Cold temperatures do not have detrimental effects on fertility unless damage is caused to the scrotum from freezing of tissues and skin.

A full cycle of spermatogenesis in a bull takes around 64 days so there will be a lag in abnormal sperm appearing in semen samples (Foote, 1978) as well as a lag in recovery after some short-term reason for a bull having poor semen.

Quality of semen is also dependent on the way the sample is collected. Bulls that ejaculate often tend to have samples of dilute and immature semen while bulls that do not ejaculate often will have samples that are more concentrated and contain mature sperm. When bulls are being collected artificially sperm output can be increased by sexual preparation. This is carried out by false-mounts in bulls where ejaculation is not allowed to occur to increase the volume and density of the ejaculate. Hafs *et al.* (1962) showed that three false mounts increased the number of sperm per ejaculate from 7 billion in bulls with no false mounts to 13.5 billion in bulls that had 3 false mounts. Bulls that had longer planned preparation also had a higher number of sperm per ejaculate (Hafs *et al.*, 1962). It is possible that the anticipation of mating with a cow, whilst a bull is in a yard can have a similar effect in increasing the density of the ejaculate the bull produces.

2.3.8 Hormonal level

The formation of lesions on the testes due to infection can cause a change in hormone concentrations and a decrease in plasma testosterone concentrations. This is likely due to a decrease in the number of interstitial cells as they are damaged by the infection. However, if the infection is only in one testis there will be a compensatory effect from the other testis to maintain normal hormonal concentrations to maintain spermatogenesis (Ahmad and Noakes, 1995).

Another possible reason for decreases in testosterone is thought to be pyrexia causing inhibition of GnRH. This is a dysfunction in the hypothalamo-pituitary- testicular (HPT) axis. In some infections changes in the HPT axis can be caused by damage to the pituitary resulting in a decrease in hormone sensitivity in the gland (Al-Qarawi *et al.*, 2004).

Changes in GnRH secretion can be exacerbated by the decrease in plasma cortisol levels observed with infection causing a further reduction in secretion of GnRH (Abebe *et al.*, 1993). A reduction in GnRH concentration reduces the secretion of FSH and LH. Luteinizing hormone stimulates the production of testosterone in leydig cells therefore testosterone concentrations will also decrease. Since testosterone is one of the main hormones involved

in the spermatogenesis, a decrease in the secretion of the hormone results in a degenerative change in spermatozoa production and maturation (Mutayoba *et al.*, 1994).

The mean cortisol profile of Boran cattle after corticotrophin-releasing hormone (CRH) challenge was observed in a study by Abebe *et al.* (1993). The results are summarised as Figure 2.7. The top line is the challenge pre infection and the bottom line is the challenge during an infection with *Trypanosoma congolense*. The mean cortisol concentrations during infection were lower than pre-infection. This shows that the infection caused a decrease in pituitary function (Abebe *et al.*, 1993). If a decrease in pituitary function causes a decrease in FSH and LH secretion, it is possible that a decrease in fertility would be observed. If this also occurs in *Theileria* infections, it is possible that infected bulls will have a decrease in fertility.

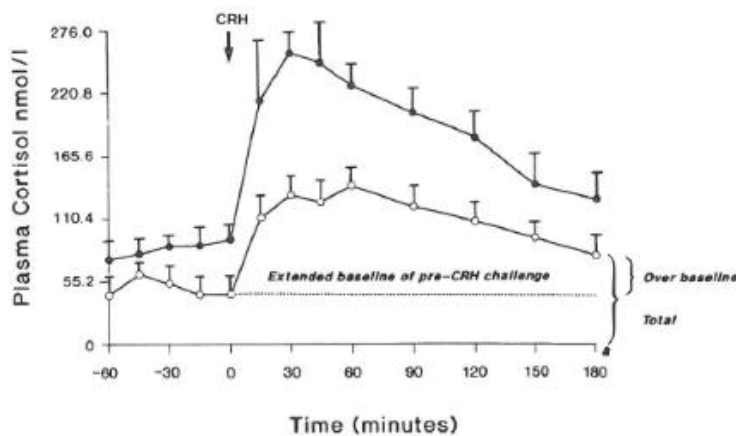


Figure 2.7 Mean cortisol concentrations between pre-infection and infection in animals infected with *Trypanosoma congolense* (Abebe *et al.*, 1993).

Other theories have looked at the effect of growth hormone (GH) in regards to control of spermatogenesis. During an immune response the secretion of hormones involved in the immunity such as tumour necrosis factor, cause inhibition of the secretion of hormones from the pituitary such as GH (Abebe *et al.*, 1993). Growth hormone acts directly on the testis in order to promote the early development of spermatogonia and complete maturation (Magon *et al.*, 2011). Studies looking at deficiencies in GH have found it causes a reduction in the concentration and number of viable sperm. The lack of GH has an effect in multiple regions of the epididymis as the developing sperm move through resulting in underdeveloped sperm at the end product of spermatogenesis that are not viable for fertilization (Gravance *et al.*,

1997). Underdeveloped sperm are likely to have small heads, cytoplasmic droplets and round cells.

If epithelial rupture occurs either by physical trauma or lesions in the epithelium, spermatozoa are leaked into the interstitium of the epididymis and peripheral smooth muscles of the vas deferens. The extravasted spermatozoa cause an immune response due to the exposure to immunocompetent cells resulting in inflammation of the epididymal tail and vas deferens causing fibrosis with masses of granulated tissue containing spermatozoa to form called a granuloma (Jessop and Ladds, 1995). Nests of macrophages and plasma cells are present due to the immune response (Ahmad and Noakes, 1995). Accumulation of cells involved in the immune response will phagocytose nearby spermatozoa. An increase in the number of lymphocytes is seen around blood vessels peripheral to the lesions on the testes from the infection (Jessop and Ladds, 1995). Increases in lymphocytes that are present in the development of new spermatic granuloma can have a cytotoxic effect and cause destruction of sperm. Neutrophils in the spermatic granulomas can immunoadhere to the sperm to prevent their movement. This is then followed up by intracellular digestion of the sperm by the neutrophils (Jessop and Ladds, 1995).

The formation of granulomas causes the production of antisperm antibodies (ASA) (Jessop and Ladds, 1995). Antisperm antibodies works on sperm by altering the DNA in the sperm. The antibody acts to delete sections of DNA. This causes disruptions in the sperm membrane resulting in a lack of protamine proteins that allow oxidative degradation of DNA. This overall affects the quality of the sperm and results in a decrease in viable sperm (Evans *et al.*, 1999).

Increases in the concentrations in plasma oestradiol-17 β secreted from the sertoli cells can also be seen in the event of infection. This is a result of sertoli cell dysfunction caused by immune complexes under the seminiferous tubules in infected animals. Oestrogen in the male causes negative feedback to the hypothalamus causing inhibition of GnRH secretion resulting in a decrease in LH secretion and therefore a decrease in testosterone secretion from the leydig cells. This results in the impairment of division and differentiation of germ cells to produce spermatozoa (Al-Qarawi *et al.*, 2004).

2.3.9 Factors affecting spermatogenesis

Spermatogenesis is affected by an increase in body temperature such as occurs when the animal is pyrexic. This causes the degradation of proteins in the sperm, affecting both morphology and viability of the sperm during development. Infection also causes activation of an immune response to form spermatocytic granulomas and an increase in the production of immune cells. A change in hormone secretion is also seen due to degradation of epithelial cells and changes in the activity of Sertoli and Leydig cells. Infection causes changes in hormone signalling from the hypothalamic-pituitary axis, resulting in a decrease in the secretion of luteinizing hormone and follicle stimulating hormone from the pituitary gland. Effects on the animal can be permanent due to scarring and degradation of tissues.

Parasitic infections commonly cause pyrexia. Infected testes can have an increased local temperature of 0.6-3.1°C (Rhodes, 1976). This has been shown to cause a change in semen morphology and the reproductive tract (Ogundele *et al.*, 2016). Stages most at risk of heat damage are meiosis and spermiogenesis where spermatocytes and spermatids are present. Intermediate and B-type spermatogonia are also affected but stages in the epididymis (spermatozoa) are unaffected by heat.

A study by Sekoni *et al.* (2004) found that bulls infected with 2 different *Trypanosoma* species had a reduction in fertility. Infected animals had an increase in rectal temperature from 38.3°C at pre-infection testing to a mean of 40.5-41.1°C when infected. The study found decreases in semen volume and sperm concentration compared to pre-infection samples. The percentage of morphologically abnormal sperm increased to 100% at week 7 of testing whilst the control bulls mean was around 5%.

Trypanosoma infections show similar clinical signs as *Theileria* with infected cattle showing signs of pyrexia, anaemia, lethargy and weight loss (Ogundele *et al.*, 2016). The increase in body temperature during a *Trypanosoma* infection is likely to have a similar effect on semen quality to what would be observed if pyrexia occurs in a *Theileria* infection. Changes in semen quality is likely due to the stress placed on the infected bull and damage to the testes. Damage to genitalia can cause loss of sperm reserves in the epididymis which causes a decrease in semen concentration (Sekoni *et al.*, 2004, Ogundele *et al.*, 2016). The depletion of spermatogenic cells can cause a reduction in semen production and in severe cases can cause

the complete cessation of spermatogenesis. Adamu *et al.* (2007) suggested that the effects of the disease may not be long-lived. It is possible that the destruction of leydig cells would result in a decrease in testosterone production. The decrease in testosterone results in testicular degeneration (Adamu *et al.*, 2007).

Heat stress increases the number of sperm abnormalities in the ejaculate and can result in the failure to fertilise or embryonic death due to disruption in testicular thermoregulation. Increased environmental temperatures causes testicular degeneration and a decrease in sperm output along with an increase in DNA fragmentation in spermatozoa (Rahman *et al.*, 2011). The increase in heat increases the metabolic demands of the tissues in the testis that cannot be kept up with normal blood flow. This leads to tissue hypoxia and oxidative stress causing damage to spermatogenic cells. Chromatin protamination becomes defective and cell death can occur with DNA being degraded (Rahman *et al.*, 2011). The effects of increased scrotal temperature have been artificially stimulated by insulating testicles with sacs to increase local temperature. A study by Vogler *et al.* (1993) found that using this method for 2 days resulted in changes in semen morphology from around Day 12. The abnormalities observed were tailless spermatozoa, diadem defects, vacuolated sperm heads, pyriform-shaped heads and knobbed acrosomes. The defects seen and the time taken for abnormalities to occur suggests that the effect of temperature occurs on the spermatogenic processes as it takes 8 to 11 days for epididymal transport to occur (Vogler *et al.*, 1993).

Testicular degeneration is breakdown of the testis and epididymis causing infertility and the potential for sterility. Testicular degeneration is a broad term that can be caused by a variety of diseases and illnesses such as *Trypanosoma*, hormonal imbalance and high scrotal temperatures.

Testicular degeneration in cases of *Trypanosoma* infections are thought to have been caused by pyrexia and anaemia (Ogundele *et al.*, 2016). Infected bulls show a breakdown of germinal epithelial cells in tubules due to the degradation of spermatogenic cells. As the disease progressed, the number of epithelial layers decreased and signs of necrosis in the seminiferous tubules becoming more severe. In the end spermatogenic and sertoli cells were completely depleted and a small number of leydig cells were left. Epididymal sperm reserves contained a higher proportion of abnormal sperm as the disease progressed. As testicular

degeneration progressed further, epididymal sperm stores were completely empty due to the cessation of spermatogenesis. This is seen in Figure 2.8 and 2.9 comparing normal seminiferous tubules (Figure 2.8) against the testis of a bull infected with *Trypanosoma Vivax*.

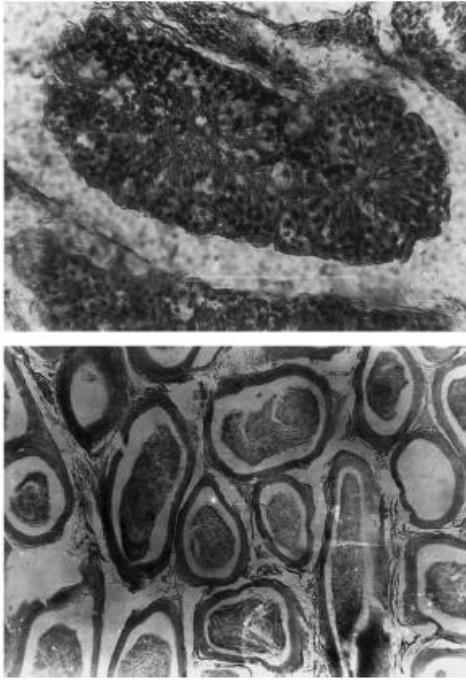


Figure 2.8 Histological image of a bull with normal seminiferous tubules filled with spermatogenic cells (top picture) and an image of the epididymis showing normally formed tubules with sperm reserves (Adamu *et al.*, 2007).

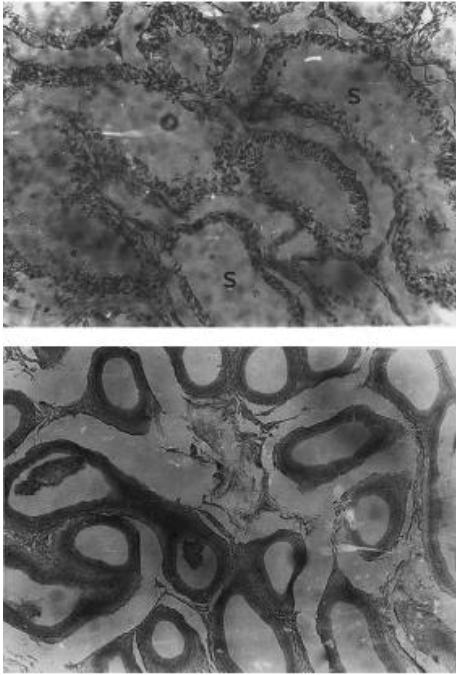


Figure 2.9: A histological image of the testis of a bull infected with *Trypanosoma vivax*. The top image shows severe degeneration of germinal epithelial cells in seminiferous tubes(s). The bottom image shows the epididymis of the same infected bull with the absence of epididymal sperm reserves (Adamu *et al.*, 2007).

The destruction of Leydig cells causes a decline in testicular steroidogenesis causing a degeneration in seminiferous tubules and spermatogenesis to be halted. It is possible that degeneration can be caused by anaemia and pyrexia that can cause the breakdown of organs (Adamu *et al.*, 2007)

Other studies have reported different species of *Trypanosoma* causing an increase in cell proliferation of Leydig cells in order to compensate for the damage caused by the disease. Leydig cell size was smaller with a decrease in mitochondria size and nuclear folding. This could be a cause for a decrease in testosterone secretion. Inflammation cells were found alongside the Leydig cells in the intertubular region (Anosa and Kaneko, 1984).

The effect on semen morphology is dependent on what stage of spermiogenesis the sperm cell is in at the time of increased temperature. This would mean that different abnormalities would be observed in semen samples in the time after exposure to high temperatures (Ogundele *et al.*, 2016).

The first abnormal sperm seen in semen samples will be tailless. This is due to high temperatures causing errors in spermatozoal implantation in spermiation. These are usually seen in the first 12 days after exposure to high temperatures. The next wave of sperm will have normal head shapes but will have vacuoles across the nuclear ring of the sperm head and is likely to have occurred from errors in DNA condensation during meiosis. This is seen 15 to 21 days after first exposure to temperature. Pyriform shaped heads also occur in this time and are a result of spermatozoa being affected when they undergo elongation from round cells to spermatids (Vogler *et al.*, 1993).

Animals that are clinically infected with *Theileria orientalis* Ikeda are likely to exhibit signs of anaemia, lethargy and increased body temperature. The effects of *Theileria* on sperm production could therefore be, similar to those of a *Trypanosoma* infection where sperm can be damaged or the production of sperm can be halted. With lethargic bulls showing a decrease in libido in a yard and paddock situation, clinically infected bulls are likely to exhibit a decrease in the willingness to serve cows.

The aim of this experiment was to test whether bulls clinically infected with *Theileria orientalis* Ikeda would exhibit a decrease in fertility and libido.

3.0 Materials and methods

This experiment was a prospective cohort study of the effect of *Theileria orientalis* Ikeda infection on the libido and fertility of naïve two-year-old Friesian bulls. The experiment was carried out at Massey University's Tuapaka farm with approval from the Massey University Animal Ethics Committee (MUAEC 16/55).

3.1 Animals/Management

3.1.1 Selection

To select the required number of bulls an initial group of 22 Friesian two-year-old bulls were tested with a PCR of whole blood for *T. orientalis* Ikeda. The bulls had a mean liveweight of 576 kg. Of these, 21 were negative and one was positive. The infected bull was excluded. The remaining 21 bulls were assessed for scrotal size, which resulted in exclusion of one further bull with a scrotal circumference less than 35 cm. Twenty bulls entered the pre-infection phase of the experiment. At the end of the pre-infection period, 1 bull was excluded for aggressive behaviour and 2 were excluded for low libido after being libido tested. This left 17 bulls to enter the infection phase of the experiment. These 17 bulls were randomly allocated to the infection (n=10) or control group (n=7).

3.1.2 Feeding and management

The bulls were set stocked at pasture, with supplementary big bale silage fed out through a ring feeder. Bulls were fed restricted rations from the start of the experiment until 70 days post-infection (D70) when they were offered unrestricted pasture allowance.

Bulls were treated with 50 mls of 2% flumethrin (Flumenil Pour On, Virbac NZ Ltd, Auckland New Zealand) topically down the midline every 4 weeks beginning day 7 to control any ticks.

The bulls were vaccinated for leptospirosis and clostridial diseases (Ultravac 7in1, Zoetis New Zealand Ltd, Auckland, New Zealand) and for BVD (Ultravac BVD, Zoetis New Zealand Ltd, Auckland, New Zealand), were well grown and appeared healthy in all other respects prior to infection.

3.2 Treatments

3.2.1 Infection

Donor blood was collected from 2 cows with confirmed TABA with 250ml of blood being collected from each cow using a CPDA blood bag. Bulls were infused with donor blood within 3 hours of collection from the donor farm. Bulls in the infected group were given 15 ml of blood from a clinically infected cow and 15ml from a subclinically infected cow by intravenous infusion via the jugular on Day 0. The clinically affected donor had a HCT of 9 and the subclinically infected donor had a HCT of 16. One bull showed an anaphylactic reaction to inoculation, with urticaria, muscle fasciculation and increased respiratory rates, and was treated by intramuscular injection with 1 mg/kg mepyramine (Antimine, 50 mg/ml mepyramine maleate, Ethical Agents International Ltd, Auckland, New Zealand).

3.3 Measurements

Prior to infection bulls were weighed and temperatures were recorded on Days -29, -15 and -1 and three times a week (Monday, Wednesday and Friday) for the first 12 weeks after infusion beginning on Day 3. Following this, weight and temperature were measured fortnightly from Day 91 to Day 138.

Temperature was taken rectally using a Digital Large Animal thermometer (Shoof International, Cambridge, New Zealand) and the weights measured using a Trutest EW7i weigh scales.

3.3.1 *Theileria*

Blood samples were collected on the same day as weights and temperatures. Blood was collected into BD Vacutainer EDTA tubes from the coccygeal vein. This gave a total of 49 blood samples per bull.

3.3.2 Libido

Libido and semen testing occurred every 2 weeks on Day -15, -1, 13, 27, 41, 55, 69, 83, 97, 111, 125 and 139. Prior to each libido test, 12 two-year-old heifers were injected intramuscularly with 500 µg cloprostenol (Ovuprost, 250 µg/ml sodium cloprostenol, Bayer New Zealand Ltd, Auckland, New Zealand) 72 hours before libido testing. At the same time as

the heifers were injected a 15 cm x 5 cm strip of Tell Tail aerosol paint (FIL Products, Mount Maunganui, New Zealand) was applied to the heifer's tail head for heat detection.

On the libido testing day, a heifer that was in standing heat was used as the mount. The mount heifer was restrained in the service bail. A diagram of the yard set up can be seen in Appendix 1. The same heifer was used for the duration of each service test except on Days 41, 125 and 139 when the original heifer became unsettled and was replaced part-way through the test. Replacements were made between bulls, not during an individual bull's test period. Each bull was individually let into the yard with the heifer and was free to move around the pen. Timing was started as the bull walked in the gate and the bull was allowed 5 minutes to make a first mount and then 3 minutes from his first mount to complete as many mounts as he could. A mount was considered to occur anytime the bull lifted both feet off the ground. Time of mount and whether ejaculation occurred was recorded. All ejaculates were collected using an artificial vagina (AV). The first ejaculate of each bull was collected in to a sterile AV. The second ejaculate was collected into a separate tube using an unsterile AV that was shared among bulls. Third and subsequent ejaculates from each bull were collected as a pooled sample using the non-sterile AV.

3.3.3 Semen evaluation

3.3.3.1 *Volume and colour*

Volume of each sample was recorded along with the colour of the sample. Colours were "watery", "watery-milky", "milky", "milky-creamy" and "creamy". The higher the concentration of sperm the creamier the semen appears. The densest looking sample was used to assess wave motion, forward motion, morphology and density.

Deviations of this colour such as pink or yellow tone were noted and were likely to have blood (pink colour) or urine and pus (yellow colour) (Parkinson and Bruère, 2007).

3.3.3.2 *Wave motion*

Wave motion was recorded by placing a drop of fresh semen (approximately 5 µl) on to a clean microscope slide on top of a heated microscope stage. Slides were observed on a warm-stage microscope at 200x magnification. Motion was scored out of 10 based on the speed the wave of semen moved with 10 being very fast and one having little to no movement

(Parkinson and Bruère, 2007). This technique was adapted from Chenoweth’s approach used for analysis of ram semen as seen in Table 3.1 (Chenoweth, 2015).

Table 3.1: Wave motion score chart based on microscope observations at 200x magnification (Chenoweth, 2015)

Score	Classification	Description
10	Very good	Dense, very rapidly moving waves, >90% motile
8	Good	Vigorous movement, 70-90% motile
6	Fair	Small, slow-moving waves. Individual sperm can be seen, 40-65% motile.
4	Poor	No wave motion but some movement visible, 20-40% motile
2	Very poor	<10% motile, possible slight flickering.
0	Dead	No movement apparent.

3.3.3.3 Forward motion

Forward motion was assessed based on the proportion of semen moving forward. This was assessed using the same drop of semen on a microscope slide used for wave motion but after adding a coverslip. This was again observed on a warm microscope stage with a magnification of 200x (Parkinson and Bruère, 2007). The proportion of semen moving was recorded and scored out of 10 with 0 indicating no semen moving forward and 10 indicating all semen moving forward. Semen that was clumped together (agglutinated) was not counted. Samples

that had low motility were checked for signs of agglutination. This could indicate infection or bacterial contamination.

3.3.3.4 Morphology

Morphology of semen was assessed by staining semen and smearing it on to a slide. The slide was then observed under a microscope at 1000x magnification. A total of 100 sperm were counted for each sample and categorised as normal or defective. The type of defect was recorded (Freneau *et al.*, 2010). A diagram of the possible defects can be seen in Appendix 2. This gave a proportion of normal with 70% normal considered to be acceptable (Parkinson and Bruère, 2007).

3.3.3.5 Density

Semen was diluted using a 50:50 mix of 2% formal saline and 2.9% trisodium citrate. A diluent of 1000 µl was added to 10 µl of semen in an 1.6 ml epindorph tube (Neptune, San Diego, CA). Semen density was then counted using a haemocytometer (Parkinson and Bruère, 2007).

3.3.4 Lab methods

3.3.4.1 Haematocrit

Blood was drawn up in to microhaematocrit tubes (Kimble Chase, Vineland, New Jersey, USA) the bottom plugged with putty and centrifuged at 17,000 g for 5 minutes using capillary tubes. The depth of the packed red cells was then measured as a proportion of the total depth to estimate HCT. Two recordings were taken per bull to get an average. Prior to Day 13, bloods were inadvertently spun at a lower force of 10,000 g for 5 minutes. All values were retained for analysis because the centrifugal force applied was the same for both treatment groups on any one day and comparisons were made between treatments and not across time prior to Day 13.

3.3.4.2 DNA extraction

DNA extraction was undertaken using a KingFisher Flex Nucleic Acid Extraction System (ThermoFisher, Carlsbad, CA, USA). Whole blood collected from the bulls was aliquoted into 96-well deep well plates. If DNA extraction did not occur immediately after being aliquoted, the plate was sealed and stored at -20°C. DNA was extracted from both the host and the parasite with the Nucleomag VET kit (Macherey-Nagel, Duren, Germany) with minor modifications. The first modification was to not add the carrier RNA, precluding the extraction

of RNA from these blood samples. The second modification, to allow the utilization of the KingFisher Flex Nucleic Acid Extraction System reduced the volume of blood from 200 µl to 50 µL. The extraction of DNA from the blood was performed using the KingFisher Flex system according to manufacturers instructions. A total of 50 µl of eluted genomic DNA was produced in a comparable 96 well plate, which was sealed and stored at -20°C until analysis.

Table 3.2 Polymerase chain reaction sequences for primers and probe (Pulford *et al.*, 2016b).

Reactant	Sequence
Forward primer	5'-AGTTAACGCCACCGCAGCCG-3'
Reverse primer	5'-ACGCGGTATCCCTCTTCGGCA-3'
Probe	[6FAM]-CGCCTCAAACGCCAACGACG-[BHQ1]

Made by Quantabio, Beverly, MA, USA

3.3.4.3 PCR analysis

The presence of *T. orientalis* was quantified in the DNA extracted from the bulls blood by using the Taqman qPCR protocol as developed by Pulford *et al* (2016b). The qPCR was performed in a MIC qPCR Cycler (Bio Molecular Systems, Upper Coomera, QLD, Australia) with the following cycling parameters; a hold activation step at 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds. Reactions were carried out in 20 µl volumes, containing 5 µl of DNA template, 0.5 µM of each Ikeda forward and reverse primers, 0.4 µM probe and 1x PerfeCTa qPCR ToughMix. Polymerase chain reaction sequences for primers and probes are summarized in Table 3.2. All qPCR runs contained positive, negative (water) and non-template controls. The non-template control was DNA extracted from an EDTA blood sample that tested negative for *T. orientalis* Ikeda. A standard curve for quantification was created in each run using previously cloned copies of the DNA target region. Concentrations of the standard curve were a 1:10 serial dilution from 0.5 ng to 50 fg. A threshold of 1 fg/µl was used to distinguish between positive and negative samples. This threshold was established using serial dilution of target gene standards to determine the limits of detection, and confirmed using a ROC curve based on samples collected after day 25 when infection was confidently established (Kevin Lawrence, unpublished data).

3.3.4.3 Haematological changes

On two occasions (Day 35 and 66) blood samples from all bulls were collected in duplicate and one set was submitted to a local veterinary laboratory (New Zealand Veterinary Pathology) for testing using a standard “sick cow” profile to measure the following parameters: Red blood cell count (RBC), haemoglobin, haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), platelets, White blood count (WBC), Seg nuet, lymphocytes, monocytes, eosinophils, fibrinogen and blood film.

3.4 Data handling

Bulls were allowed 5 minutes to perform the first mount. Bulls who did not mount in 5 minutes were considered censored at 300 seconds. Bulls were given 300 seconds for gap between first and second mount. Bulls who did not complete a second mount were considered censored at 300 seconds.

Semen traits were assessed for normality using the Kolmogorov-Smirnov test. Wave and forward motion were not normal and could not be normalised. Morphology was normalised using the transformation $\log(101 - \text{proportion normal})$. Density was normalised using a square root transformation.

3.5 Statistical analysis

Statistical analysis on blood parameters, temperature, weight and order was carried out using R v3.3.1 (R Development Core Team, 2016; R Foundation for Statistical Computing, Vienna, Austria). Temperature, HCT and weight were analysed using repeated measures random effects model with day and treatment fitted as fixed effects and bull as a random effect. Least square means for infected and control groups were calculated and tested for significance. Approximation of degrees of freedom were calculated using Satterwaite’s method and the multivariate t distribution was used to control for multiple post-hoc testing with significance set at $P < 0.05$. Least square means for control and infected bulls were regressed against day and the residuals tested for autocorrelation using the Durbin-Watson test. The sign binomial test was used to test whether there was an equal chance of the infected bulls having a greater and lesser average HCT, temperature and weight.

Statistical analysis for semen traits, gap between first and second mount and time to first mount was carried out using SAS (Statistical Analysis Software, version 9.4, SAS institute Inc, Carey, North Carolina, USA, 2014). Survival analysis (proc phreg) was used to analyse time to first mount and gap. The overall model considered the fixed effects of treatment and day and the random effects of bull. Survival curves for individual days were generated using the proc lifetest procedure, considering the effect of treatment.

Order of service was tested using Mann-Whitney-Wilcoxon test, a non-parametric test, to test whether the average order scores for the two treatment groups came from the same population or were distinct.

Wave and forward motion were analysed using a generalised model assuming a poisson distribution and allowing for repeated measures on each bull. The model included the fixed effects of treatment, day and their interaction. Transformed morphology and density were analysed using a mixed model for repeated measures on each bull. The model included the fixed effects of treatment, day and their interaction.

4.0 Results

4.1 Blood

4.1.1 PCR

Polymerase chain reaction results show that the ten bulls given a dose of blood infected with *Theileria* were successfully infected.

Figure 4.1 shows infected bulls had an increase in PCR from Day 12. It peaked between Day 40 and 50. The increase in PCR was followed by a decrease in HCT (Figure 4.2) which declined below that of the control bulls from Day 28 to reach a minimum on Day 60 before gradually recovering to pre-infection levels on Day 124. Of the 10 infected bulls 8 reached a stage where they became clinically infected (HCT below 25). The lowest HCT results were seen around Day 60 to 80. This means that there is a lag of approximately 20 days between peak PCR and minimum HCT. Throughout the experiment there was no time where bulls appeared unwell and there were no instances where the farm manager could identify a sick bull.

Small amounts of *Theileria* were detected in the control group from Day 30 and fluctuated throughout the rest of the experiment. At all times these values remained below the threshold considered positive (1fg/ μ l).

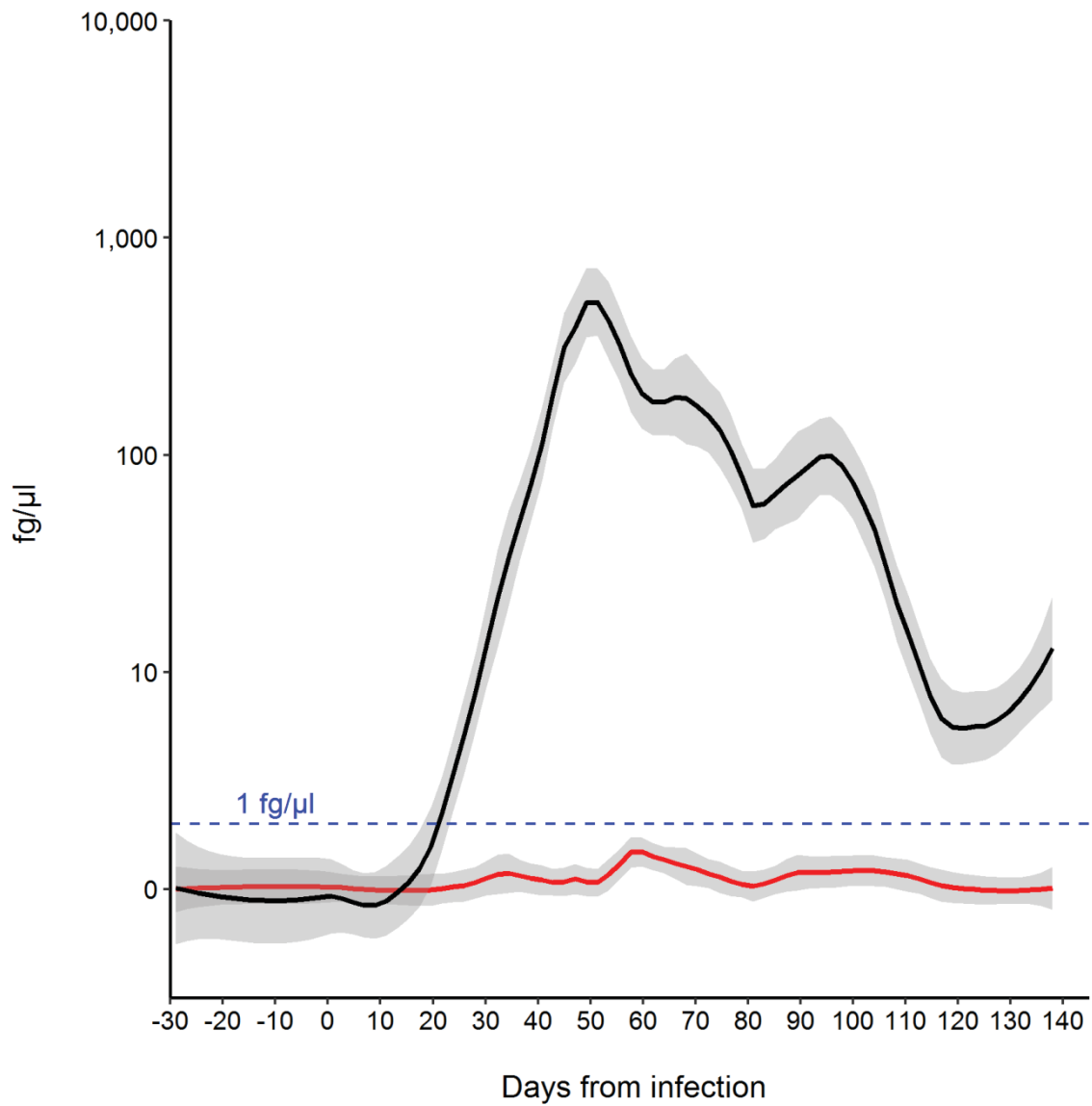


Figure 4.1 Loess smoothed regression line, span=0.2, count of organisms per mL against days from infection for control (red line) and infected bulls (black lines) with 95% confidence (grey area). The horizontal dotted line is the cut point of 1 fg/μl.

4.1.2 HCT

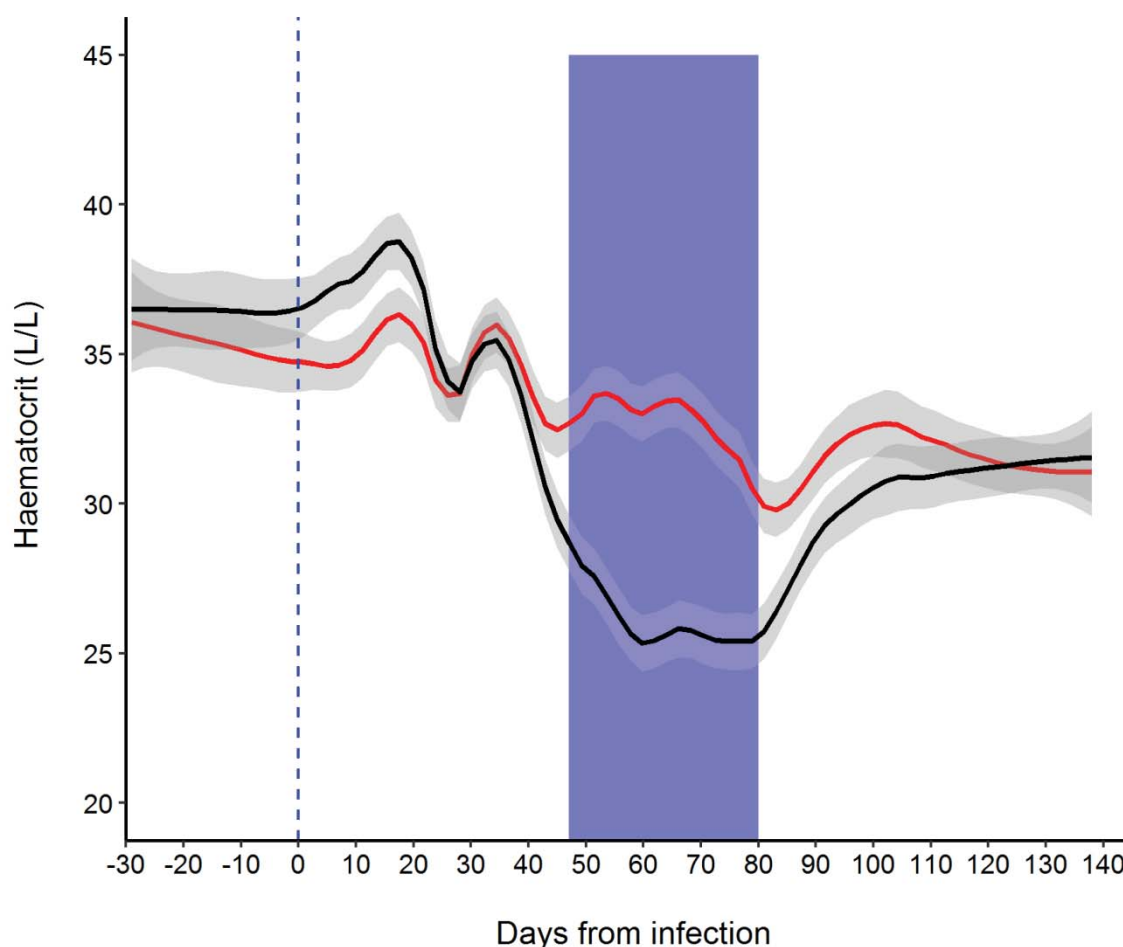


Figure 4.2 Loess smoothed regression line, span=0.2, of haematocrit against days from infection for control (red line) and infected bulls (black line) with 95% confidence (grey area). The vertical dotted line is the infection day and the blue box from Day 47 to Day 80 are the days when the HCT of the infected bulls was significantly lower (adjusted $P < 0.05$) than the control bulls.

4.2 Weight

The intention throughout the study was to keep the bulls on a maintenance diet throughout the trial to replicate the type of nutritional stress they would experience during a breeding season with bulls spending less time grazing. However, from Day 30 the bulls were fed a higher pasture intake to allow for weight gain. Consequently, the control bulls showed a steady weight increase from Day 70 that would reflect that the ultimate diet was above the estimated maintenance requirements. The total weight gain over the experiment is summarised in Table 4.1. Although the mean weight of the control bulls was higher than the

infected bulls this difference was not statistically significant (P=0.11). Figure 4.3 also shows this trend.

Table 4.1 Total weight gain and average daily gain of infected and control bulls.

Treatment	Average total weight gain
Infected	82.0
Control	98.6
P-value	0.11

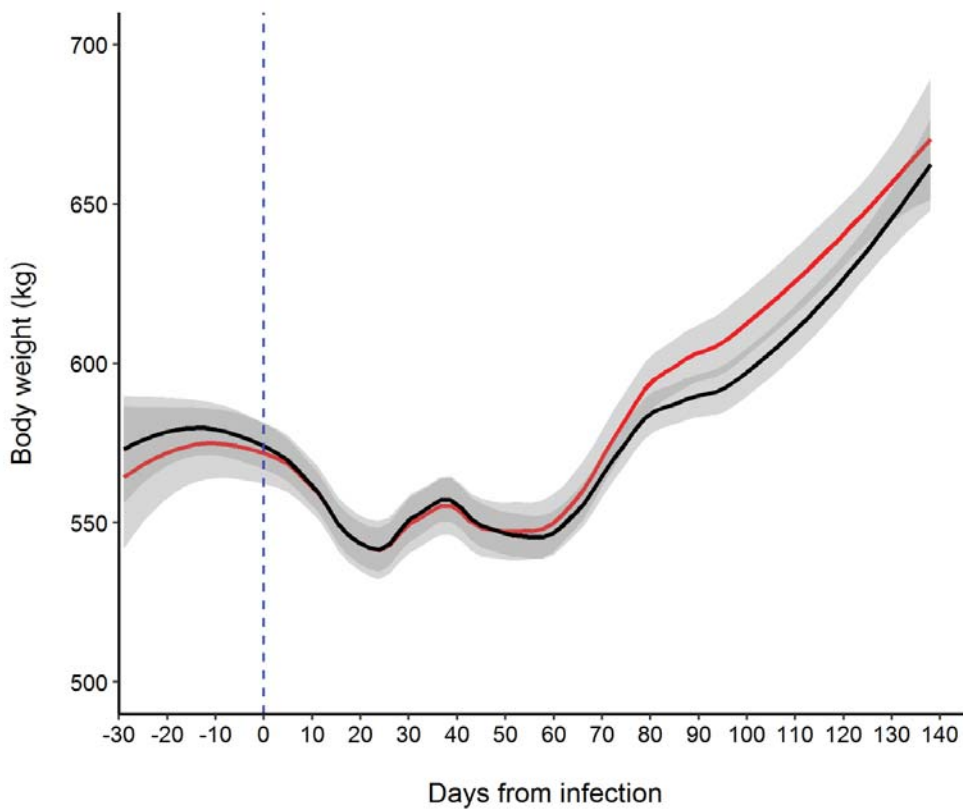


Figure 4.3 Loess smoothed regression line, span=0.4, of body weight against days from infection for control (red line) and infected bulls (black line) with 95% confidence (grey area). The vertical dotted line is the infection day.

4.3 Temperature

Rectal temperature was recorded at the same time weights were collected. Maximum differences in temperature occurred on Day 28 which infected bulls had a rectal temperature 0.33°C (CI 0.015-0.65°C) lower than control bulls (P= 0.04). This can be seen in Figure 4.4 represented by the blue line showing Day 28 having the largest separation between treatment groups. This significant change coincides with the qPCR results indicating a rise in the level of parasitaemia and the HCT of the infected group beginning to drop below the control group.

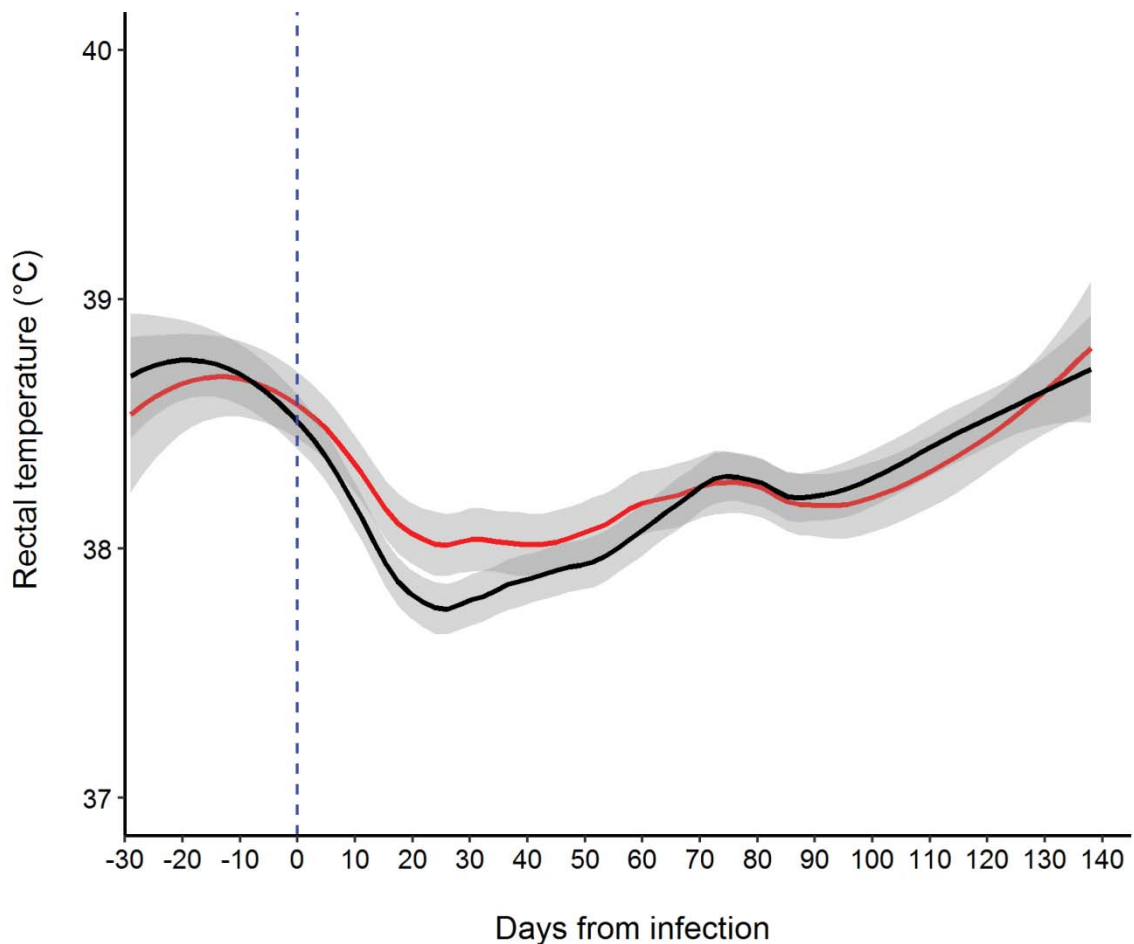


Figure 4.4 Loess smoothed regression line, span=0.4, of rectal temperature against days from infection for control (red line) and infected bulls (black line) with 95% confidence (grey area). The vertical dotted line is the infection day.

4.4 Clinical Pathology

Bloods were collected and sent to New Zealand Veterinary Pathology on Days 35 and 66. On Day 35, there was no significant differences in any of the haematology results between infected and control bulls. The results of Day 35 are summarised in Table 4.2. On Day 66, some significant changes in haematology results were observed (Table 4.3 and 4.4). The red blood cell count, haemoglobin, haematocrit and fibrinogen were all significantly lower in infected bulls ($P < 0.05$). This is indicative of anaemia in the infected group as is also shown with the drop in HCT in the infected bulls.

Table 4.2 Haematology results for uninfected and infected samples taken on 35 days post infection

Parameter ^a	Uninfected Mean (S.E.)	Infected Mean (S.E.)	P-value	Reference interval
RBC	8.24 (0.22)	8.47 (0.21)	0.47	5.0 – 7.7 x10 ¹² /L
Haemoglobin	127.71 (3.89)	128.9 (2.6)	0.8	85 – 130 g/L
HCT	0.37 (0.01)	0.38 (0.01)	0.68	0.24 – 0.40 L/L
MCV	45.57 (0.78)	44.8 (1.47)	0.65	38 – 56 fL
MCH	15.57 (0.3)	15.4 (0.4)	0.74	14 – 20 pg
MCHC	341.57 (3.6)	341.6 (2.45)	0.99	320 – 400 g/L
Reticulocytes	0 (0)	0 (0)	---	0 – 1 x10 ⁹ /L
Platelets	381* (---)	327.67 (38.52)	---	220 – 640 x10 ⁹ /L
WBC	7.93 (0.66)	7.88 (0.25)	0.95	3.8 – 11.0 x10 ⁹ /L
Seg. Neutrophils	39.71 (5.53)	31.4 (3.93)	0.24	0.7 – 4.9 x10 ⁹ /L
Lymphocytes	50 (4.43)	56.5 (4.47)	0.32	1.0 – 5.8 x10 ⁹ /L
Monocytes	4.14 (0.83)	3.9 (0.64)	0.82	0.0 – 0.9 x10 ⁹ /L
Eosinophils	4.14 (0.83)	3.9 (0.64)	0.82	0.0 – 1.9 x10 ⁹ /L
Fibrinogen	4.69 (0.51)	4.68 (0.44)	0.99	2.0 – 7.0 g/L

* Clumping of platelets meant only one sample had platelet counts performed

^a RBC= Red blood cell count, HCT= Haematocrit, MCV= Mean corpuscular volume, MCH= Mean corpuscular haemoglobin, MCHC= Mean corpuscular haemoglobin concentration, WBC= White blood count.

Table 4.3 Haematology results for infected and control bulls on Day 66 with P-values. Values with statistically significant values are highlighted in bold.

Parameter ^a	Uninfected Mean (S.E.)	Infected Mean (S.E.)	P-value	Reference interval
RBC	7.39 (0.29)	4.92 (0.33)	<0.01	5.0 – 7.7 x10 ¹² /L
Haemoglobin	115.86 (4.71)	86.1 (2.73)	<0.01	85 – 130 g/L
HCT	0.34 (0.01)	0.26 (0.01)	<0.01	0.24 – 0.40 L/L
MCV	45.86 (0.86)	53.5 (2.9)	0.03	38 – 56 fL
MCH	15.71 (0.36)	17.9 (0.85)	0.04	14 – 20 pg
MCHC	343.71 (2.57)	337.6 (2.63)	0.12	320 – 400 g/L
Reticulocytes	0 (0)	3.4 (2.2)	0.16	0 – 1 x10 ⁹ /L
Platelets	417.6 (49.49)	342.38 (27.06)	0.23	220 – 640 x10 ⁹ /L
WBC	7.13 (0.45)	6.07 (0.4)	0.10	3.8 – 11.0 x10 ⁹ /L
Seg. Neutrophils	3 (0.4)	2.11 (0.32)	0.11	0.7 – 4.9 x10 ⁹ /L
Lymphocytes	3.5 (0.58)	3.47 (0.17)	0.96	1.0 – 5.8 x10 ⁹ /L
Monocytes	0.21 (0.03)	0.27 (0.05)	0.33	0.0 – 0.9 x10 ⁹ /L
Eosinophils	0.39 (0.13)	0.21 (0.07)	0.26	0.0 – 1.9 x10 ⁹ /L
Fibrinogen	5.31 (0.13)	4.43 (0.17)	<0.01	2.0 – 7.0 g/L

^a RBC= Red blood cell count, HCT=Haematocrit, MCV= Mean corpuscular volume, MCH= Mean corpuscular haemoglobin, MCHC= Mean corpuscular haemoglobin concentration, WBC= White blood count.

Table 4.4 Additional biochemistry results for infected and control bulls on Day 66 with P-values. Values with statistically significant values are highlighted in bold.

Parameter ^b	Uninfected Mean (S.E.)	Infected Mean (S.E.)	P-value	Reference interval
Sodium	137.29 (0.42)	136.6 (0.34)	0.23	132 – 152 mmol/L
Potassium	4.73 (0.13)	4.58 (0.1)	0.38	3.9 – 5.8 mmol/L
Chloride	95.57 (0.69)	95.2 (0.79)	0.73	96 – 104 mmol/L
Creatinine	115.57 (5.15)	123.5 (5.31)	0.30	55 – 130 mmol/L
Urea	5.39 (0.44)	6.18 (0.34)	0.18	2.7 – 12.3 mmol/L
Phosphate	2.11 (0.07)	1.89 (0.1)	0.10	1.1 – 2.8 mmol/L
Total Protein	71.86 (1.06)	74.3 (0.91)	0.10	60 – 86 g/L
Albumin (A)	29.71 (0.68)	28.7 (0.68)	0.31	25 – 40 g/L
Globulin (G)	42.29 (1.38)	45.6 (1.33)	0.11	28 – 53 g/L
A/G Ratio	0.71 (0.04)	0.64 (0.03)	0.18	0.5 – 1.2 ratio
Calcium	2.17 (0.02)	2.22 (0.01)	0.09	2.0 – 2.6 mmol/L
Magnesium	0.75 (0.02)	0.74 (0.02)	0.59	0.49 – 1.15 mmol/L
Bilirubin	4.29 (0.18)	7.6 (0.91)	0.01	0 – 13 µmol/L
GLDH	29.29 (6.51)	12.4 (4.98)	0.06	8 – 41 IU/L 37°C
B.OHB	0.27 (0.03)	0.3 (0.05)	0.63	0.2 – 1.0 mmol/L
AST	116 (20.54)	76.4 (6.35)	0.11	0 – 179 IU/L 37°C
CPK	176 (6.66)	232.8 (42.33)	0.22	0 – 578 IU/L 37°C
GGT	20.29 (1.15)	16.2 (1)	0.02	0 – 36 IU/L 37°C

^b GLDH= Glutamate dehydrogenase, B.OHB= Beta hydroxybutyrate, AST= Aspartate aminotransferase, CPK= Creatine phosphokinase, GGT= Gamma-gutamyl transferase.

4.5 Libido

4.5.1 Time to first mount

Figure 4.5 shows little difference in time to first mount from pre-infection averages for each group until Day 69 and also little difference between treatment groups. Over time controls had a slower time to first mount than infected bulls with a maximum difference between treatment groups at Day 83. However, at Day 97 the infected bulls had a much slower time to first mount compared to the control bulls. This trend swapped back again on Day 111 with the median time to first mount for control bulls being much slower with a median value of 12 seconds compared to the infected group that had a median of 1.25 seconds. There was no significant effect of treatment ($P=0.7374$), day ($P=0.5060$) and bull ($P=0.1341$). The interaction of day and treatment had a tendency to have a significant effect ($P=0.0528$). Figure 4.6 and Table 4.5 show that days 83 and 111 had a significant difference between control and infected groups.

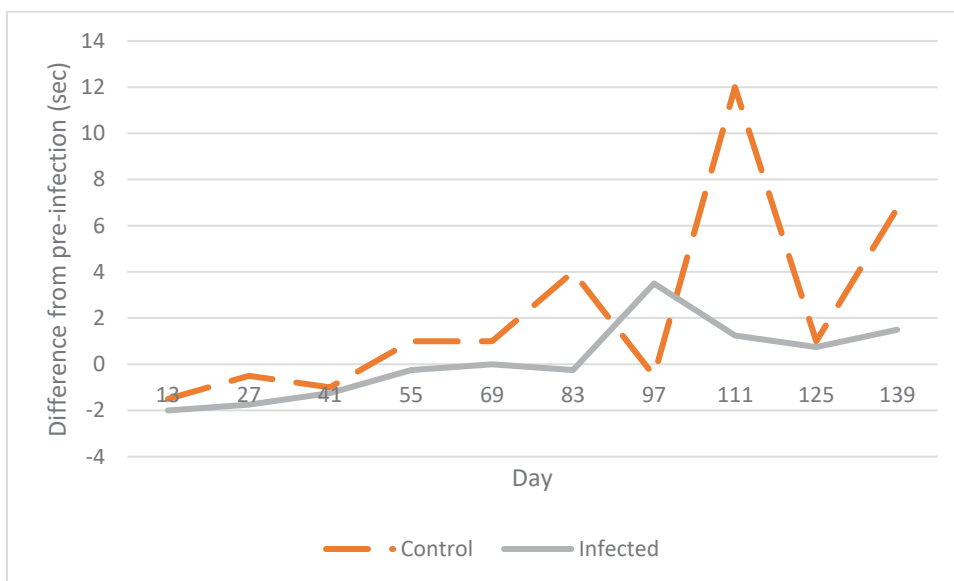
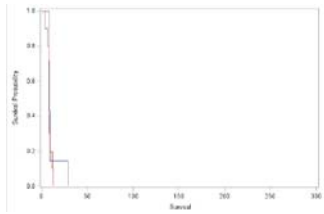


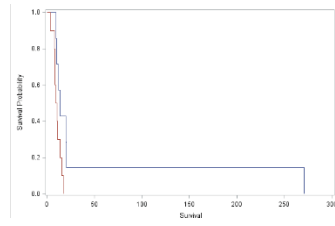
Figure 4.5 The median deviation time to first mount relative to pre-infection measures of time to first mount for bulls infected with *Theileria* infected (—, n=10) or uninfected control bulls (---, n=7) at fortnightly libido tests after infection.

Table 4.5 Summary for test of equality over strata for each day post infection for time to first mount

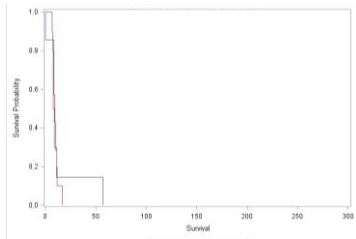
	Log-Rank	Wilcoxon	-2Log(LR)
Day 13	0.3008	0.2431	0.5215
Day 27	0.5388	0.5297	0.3324
Day 41	0.6676	0.9218	0.9726
Day 55	0.2906	0.4923	0.7411
Day 69	0.6749	0.7704	0.7070
Day 83	0.0411	0.0546	0.0014
Day 97	0.6480	0.6519	0.2983
Day 111	0.0302	0.1419	0.0467
Day 125	0.2578	0.5571	0.6856
Day 139	0.1213	0.3588	0.9021



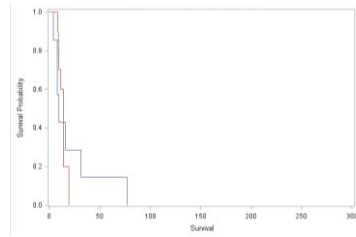
Day 13



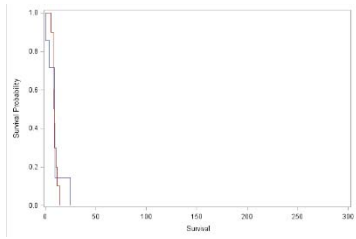
Day 83



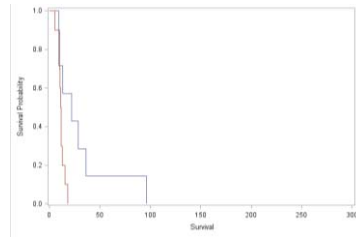
Day 27



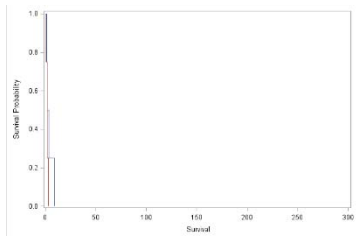
Day 97



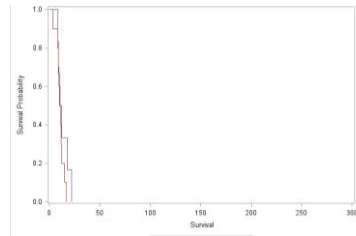
Day 41



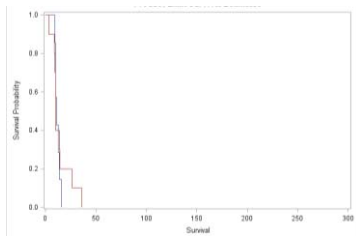
Day 111



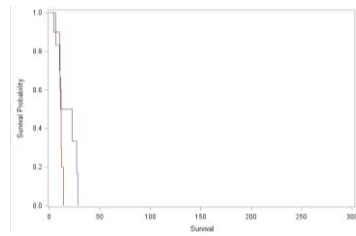
Day 55



Day 125



Day 69



Day 139

Figure 4.6- Product-limit survival estimates for each libido testing day post-infection for time to first mount

4.5.2 Gap from first to second mount

Figure 4.7 shows the gap between first and second mounts on each of the service testing days. The overall trend shows that the gap between first and second mounts increases for both treatment groups over time. Table 4.6 shows that the difference between days was not significant. There was a tendency for treatment to have an effect ($P=0.0640$). A bull effect was also significant reflecting the differences in libido in different bulls ($P=0.0003$). There was no evidence for an interaction ($P=0.0748$) between treatment and day. This is also seen in Figure 4.8 and Table 4.6 where there was no significant difference in control and infected groups for gap from first and second mount on each day.

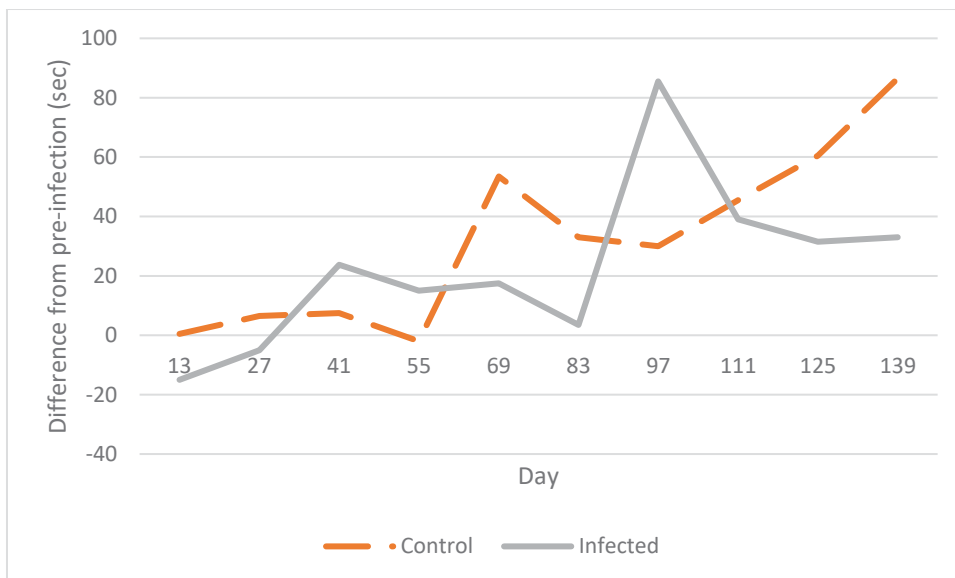
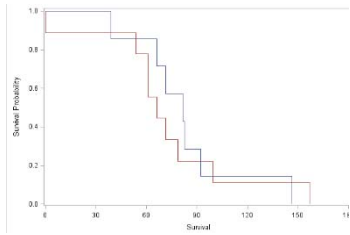


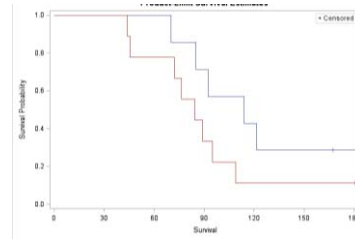
Figure 4.7 The median deviation time between gap from first and second mount relative to pre-infection measures of gap for bulls infected with *Theileria* infected (—, n=10) or uninfected control bulls (---, n=7) at fortnightly libido tests after infection.

Table 4.6 Summary of test of equality over strata for each day post-infection for gap between first and second mount.

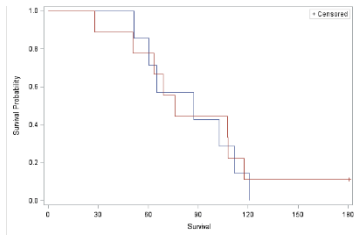
	Log-Rank	Wilcoxon	-2Log(LR)
Day 13	0.7367	0.3742	0.7826
Day 27	0.8246	0.9584	0.7645
Day 41	0.3438	0.2930	0.6543
Day 55	0.0897	0.1732	0.3425
Day 69	0.6190	0.2823	0.9309
Day 83	0.1541	0.1275	0.3276
Day 97	0.2861	0.1999	0.5386
Day 111	0.6990	0.7118	0.7221
Day 125	0.5958	0.5571	0.5447
Day 139	0.2620	0.3846	0.2387



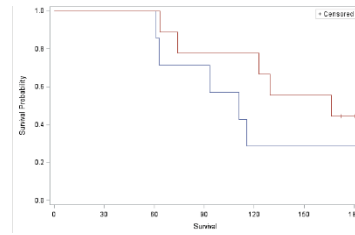
Day 13



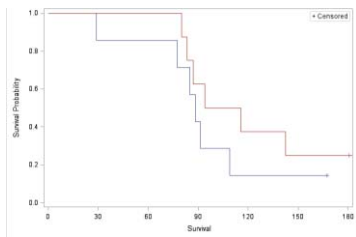
Day 83



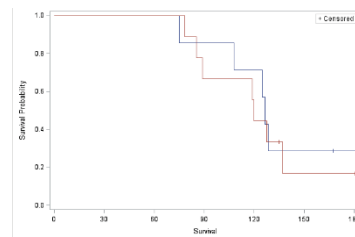
Day 27



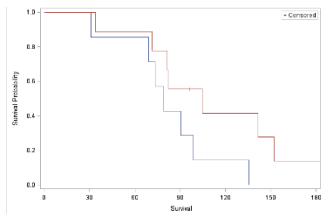
Day 97



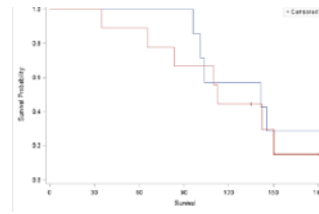
Day 41



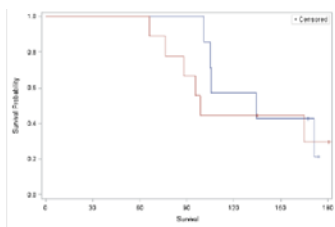
Day 111



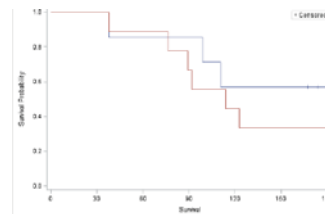
Day 55



Day 125



Day 69



Day 139

Figure 4.8 Product-limit survival estimates for each libido testing day post-infection for time to first mount

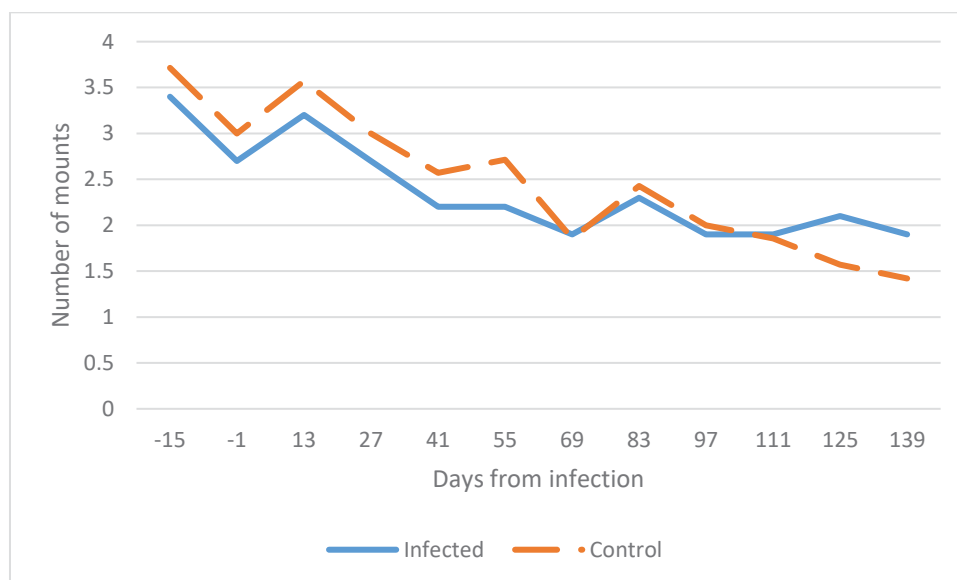
4.5.3 Number of mounts

Table 4.7 shows the average number of mounts over the experiment with no difference between the treatment groups ($P=0.9271$). Figure 4.9 shows no differences in the number of mounts between the infected and control groups. Also important to note is the apparent decrease in the number of mounts in both treatment groups over time.

Table 4.7 Mean number of mounts for each treatment group, standard error and P-value

Treatment	Mean	Standard error
Infected	2.33	0.2758
Control	2.36	0.1725
P-value	0.9269	

Figure 4.9 The average number of mounts for bulls infected with *Theileria* (—, n=10) or uninfected control bulls (---, n=7) at fortnightly libido tests after infection. There was no significant effect of treatment ($P=0.9269$), day ($P=0.2106$), on the number of mounts. There was no interaction ($P=0.2221$) between treatment and day.



4.5.4 Order

The average service order score for each group against days from infection was plotted (Figure 4.10). If 17 bulls randomly lined up, then the average position score should be 9 for each treatment (indicated by the blue line). Average order was similar ($P>0.05$) between control and infected bulls throughout the experiment, except on Day 55 when infected bulls were slower to line up for libido testing compared with control bulls ($P=0.02$). On Day 41, there was no significant difference in order among treatments ($P=0.16$), however, it would appear that the factors that caused the reduction in order that was present for the infected group on Day 55 may have been beginning to have an effect on Day 41.

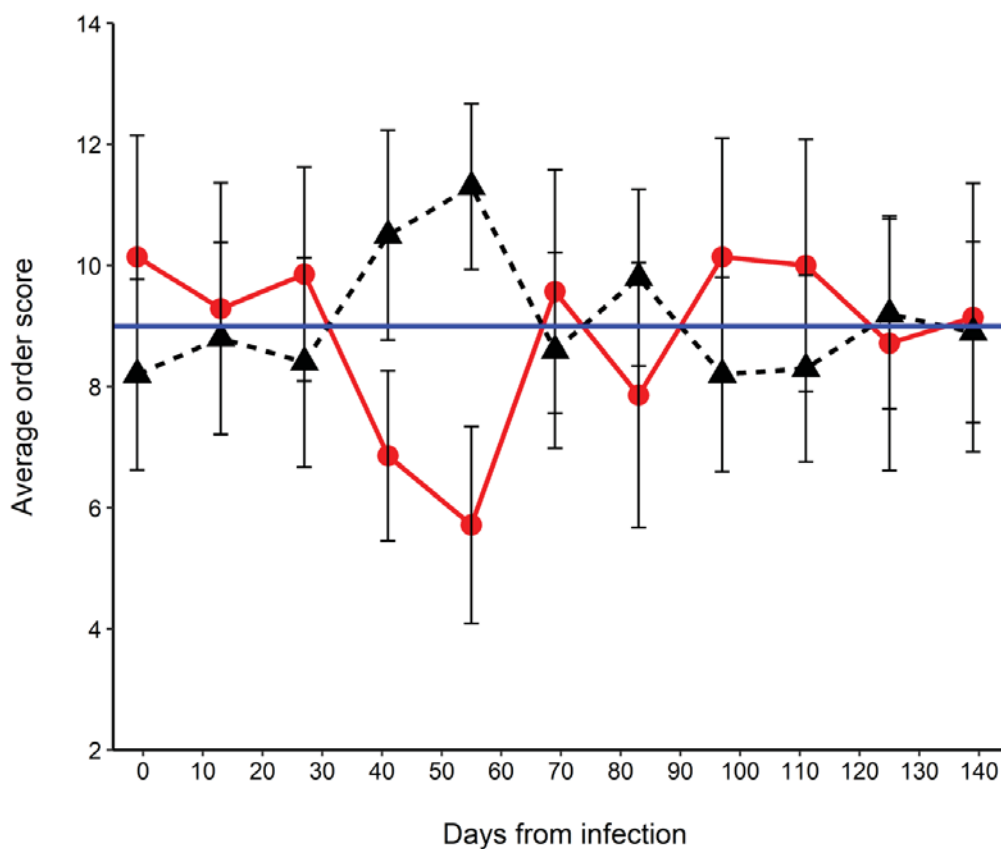


Figure 4.10 Plot of average service number, with error bars, for infected, ---, and control, —, bulls against days from infection. Blue line represents the expectation for random order of bulls.

4.6 Fertility

The results for forward motion, wave motion, density and morphology are summarised in Table 4.8.

Wave and forward motion were similar for control and infected groups ($P>0.05$). This is seen in Figure 4.11 and 4.12 where there was no significant separation of the control and infected groups average wave and forward motion and no interaction with time ($P>0.05$, Table 4.8).

Infected bulls had a significantly denser semen sample represented with a mean of 1.45×10^{10} sperm per ml compared to control bulls that had a mean density of 1.14×10^{10} sperm per ml ($P=0.0044$). There was no interaction of treatment with time ($P=0.36$, Table 3.10). This trend can be seen in Figure 4.13.

For morphology significant differences were observed between the two treatment groups with control bull semen having a mean morphology of 94.3% normal and infected bull semen having a mean morphology of 91.9% normal. This showed that infected bulls had poorer morphology over the whole experiment ($P=0.0032$). Day was statistically significant ($P<0.0001$), showing that the mean morphology value differed between days. There was no interaction of treatment with day ($P=0.5266$) indicating that this difference in morphology between infected and controls was not developing with advancing infection. This trend can be seen in Figure 4.14.

Table 4.8 Mean values for wave motion, forward motion, density and morphology with standard error and p-values.

	Infected	Control	P-value treatment	P-value Day	P-value treat*day
Wave Motion	7.51± 0.18	7.08 ±0.35	0.2935	0.2705	0.8679
Forward motion	7.82 ± 0.16	7.64± 0.26	0.5579	0.2041	0.3691
Density	1.45x10 ¹⁰ ± 6.88x10 ⁶	1.14x10 ¹⁰ ± 9.82 x10 ⁶	0.0044	<0.0001	0.3626
Morphology	91.9 ± 0.053	94.25 ± 0.065	0.0032	<0.0001	0.5266

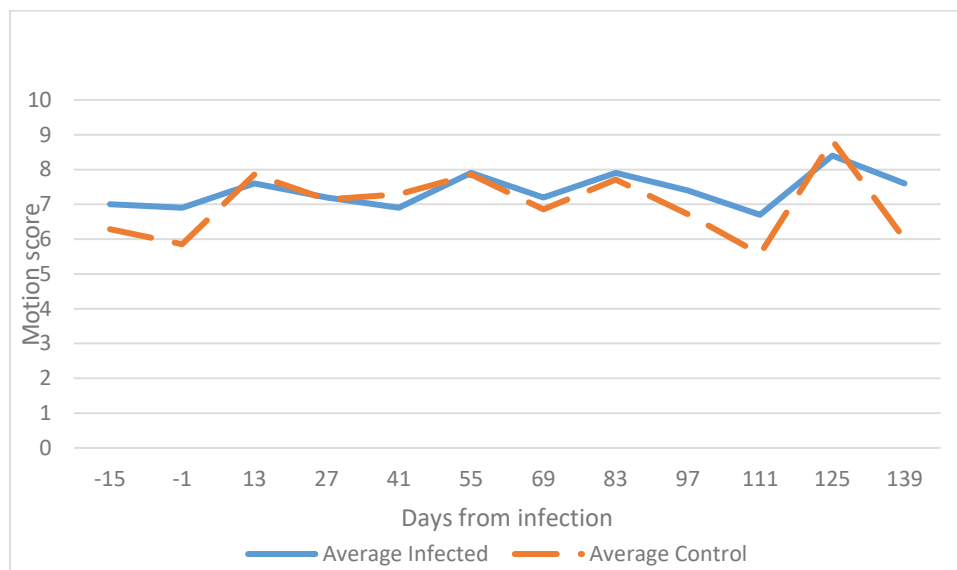


Figure 4.11 Average wave motion for bulls infected with *Theileria* infected (-, n=10) or uninfected control bulls (--, n=7) at fortnightly libido tests after infection.

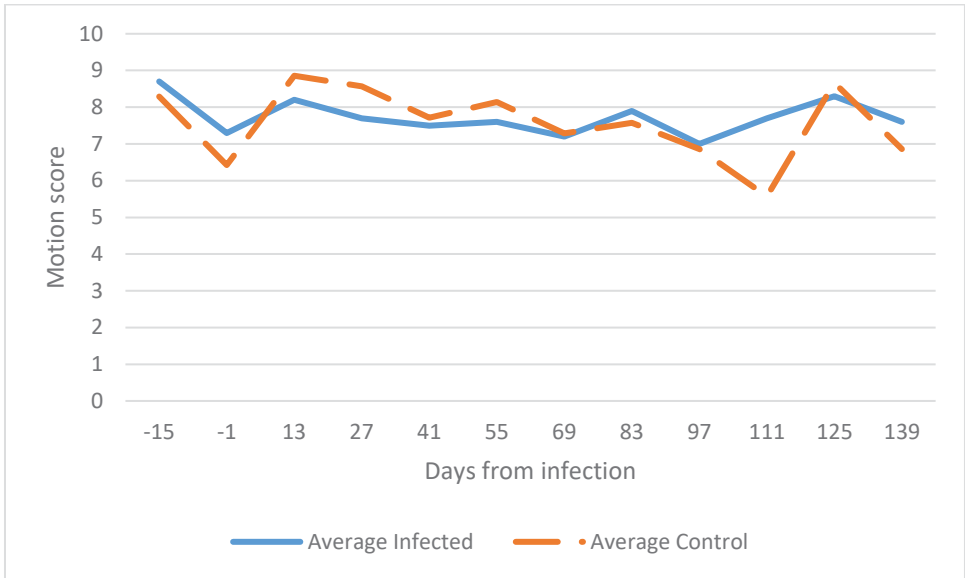


Figure 4.12 Forward motion for bulls infected with *Theileria* infected (-, n=10) or uninfected control bulls (--, n=7) at fortnightly libido tests after infection.

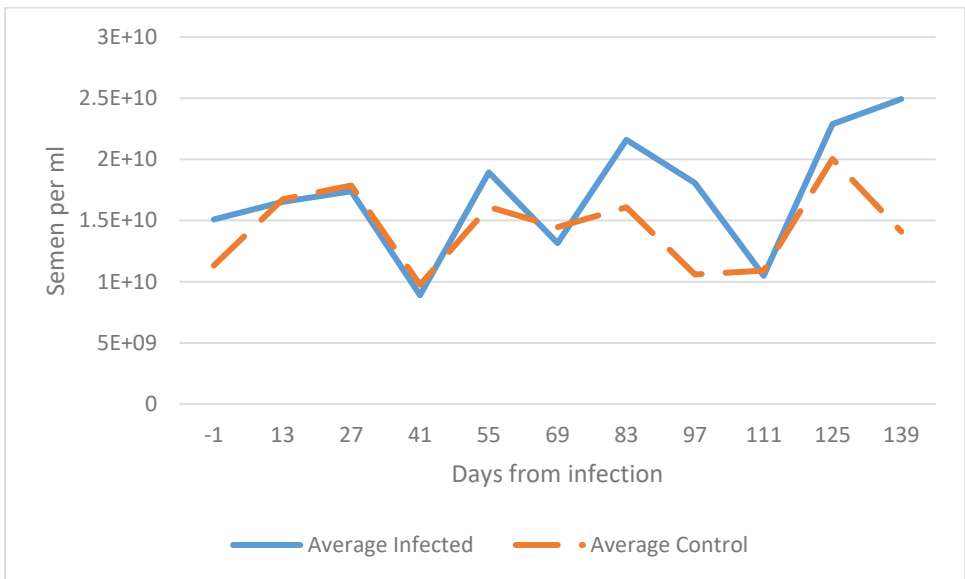


Figure 4.13 Average density for bulls infected with *Theileria* infected (-, n=10) or uninfected control bulls (--, n=7) at fortnightly libido tests after infection.

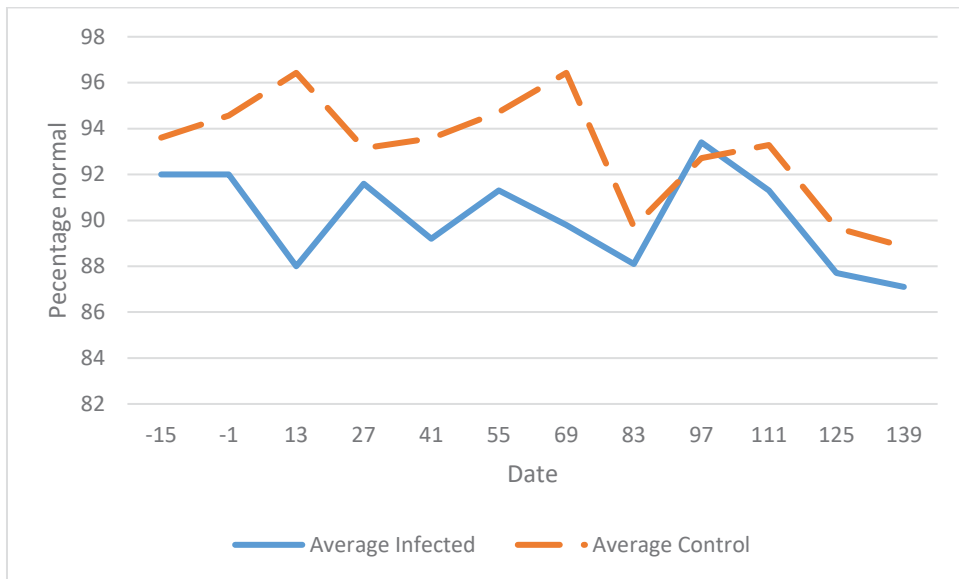


Figure 4.14 Average percentage of normal for semen samples collected from *Theileria* infected (—, n=10) or uninfected control bulls (---, n=7)

5.0 Discussion

The aim of this experiment was to determine the effects of *Theileria* Ikeda on the libido and fertility of bulls. The main interest of this study was to assess if infected bulls were likely to show a lack of libido and/or poor semen quality. All bulls in the treatment group were successfully infected with *Theileria* Ikeda with a peak DNA occurring between Days 40 and 50 and a minimum HCT occurring shortly after on Day 60. A decrease in libido was observed with infected bulls coming in the yard for service later than the controls. Time to first mount, gap between first and second mount, and the number of mounts were not affected by treatment. Infected bulls had a higher density but a lower number of normal sperm compared to the control groups however, wave motion and forward motion were not affected by treatment.

All ten bulls in the treatment group were successfully infected with *T. orientalis* Ikeda. The number infected was a little higher than Hammer *et al.* (2016) who successfully infected 8/9 calves by intravenous inoculation. Hammer *et al.* (2016) used three different volumes of infected blood 0.1 ml, 1 ml and 10 ml. The animal which failed to become infected reacted very badly to the 10ml transfusion and this was suggested as the reason for the transmission failure. The bull which had an anaphylactic reaction to transfusion in this study still became infected, although from the description this reaction was much milder than that seen in the Hammer *et al.* (2016) study. The current study used 30ml of blood from 2 different donor cows to compensate for the increased size of the two-year-old bulls compared to the calves in Hammer *et al.* (2016). However, the evidence from Hammer *et al.* (2016) would suggest lower doses would have sufficed although the time to PCR positive and peak *Theileria* DNA were much extended at the lower doses. The first positive detection of *Theileria* in the infected group was on Day 19 with all bulls being positive by Day 26. This was earlier than in the study by Hammer *et al.* (2016), where calves that were in the 10 ml dose group were first detected as positive after 4 weeks whilst being blood tested three times a week for 13 weeks post- infusion (Hammer *et al.*, 2016). The time taken after inoculation until the detection of *Theileria* could be due to differences in dosage and the effect in the animal.

The finding that the average HCT of the infected bulls was elevated compared to the control bulls for the first 20 days after infection was an unexpected finding. Haemoconcentration post infection has not been previously reported in the literature and could mean that the observed

change was not caused by the Ikeda infection but by the transfusion itself. It is also possible that the increase in haematocrit is not observed as the change occurred prior to infection being detectable using PCR, so would not be seen in field studies. Clearly the most likely cause of the elevated HCT was moderate dehydration and it is conceivable that either the Ikeda or the transfusion could have caused it but the former seems more likely as any effect of the transfusion would be short-lived and unlikely to persist beyond a few days at most. A third possibility is that the transfusion introduced an opportunist bacterial or viral infection. The haematology results for Day 35 post infection show no elevated white blood count or neutrophilia in the infected bulls so it is probable that the cause of the haemoconcentration was not a bacterial or viral infection introduced with the transfused blood.

Very small amounts of *Theileria* were detected in the control group from day 30 and fluctuated throughout the rest of the experiment. However, the values were below the cut point of 1 fg/ μ l so were considered negative. It is unsure whether this was a result of an infection with other types of *T. orientalis* or a lack of specificity of the PCR primers. The primers used in the experiment were considered specific to Ikeda, however, when the concentration of Chitose or Buffeli is above 10^5 to 10^6 genome copies the taqman probe becomes less specific to Ikeda (Pulford *et al.*, 2016b). All except 4 of the PCR results for the control bulls fell below the cut point of 1 fg/ μ l. Since these 4 individual false positive results were neither followed or preceded by a positive result it was assumed that they were probably the result of cross contamination prior to DNA extraction. This possibly occurred at blood collection where infected blood was mixed in to the controls blood sample possibly through the use of the same needle or a mislabelled tube. Crosscontamination could have also occurred when the extraction plate was set up with a blood fragment off a tube dropping in to the wrong well.

Rectal temperature was significantly different on Day 28 after infection. However, the change in rectal temperature was not what was suggested from the literature with the infected bull group having lower temperatures rather than elevated temperatures (Aparna *et al.*, 2011). Other studies such as Hammer *et al.* (2016) did not report a change in temperature as it was not measured. No studies reported a decrease in temperature as was observed in this study. A possible reason for lower temperature could be reduced activity of the bulls due to lethargy from infection. A decrease in activity would result in a decrease in metabolism and less heat

production. Also, the study was conducted in the spring when cold nights were still common. It is possible that either the reduced activity or the infection affected the thermogenic response to low temperatures causing the infected bulls' temperatures to fall relative to the control bulls.

Although over the experiment the control bulls gained more weight this difference was not significant. It appears that the control bulls did gain weight at a faster rate over the infected bulls, when the infected bulls were at their lowest HCT. However, this advantage was short-lived as the infected bulls showed compensatory growth towards the end of the trial and caught the control bulls up. Studies by Izzo *et al.* (2010) observed anorexia on two out of eight farms with cases of Theileriosis. Farms with severe cases of *Theileria* observed significant anorexia in animals that died. Some animals showed minor anorexia and lethargy but could be treated. None of the bulls in this study were severely enough affected to be at risk of death. This could mean that the small changes in weight compared to the controls reflects the mild nature of an infection that was not severe enough to induce the severe anorexia observed in fatal infections. This agrees with the observations of Hewitt (2017) where mild infections did not affect the growth rates of infected calves.

Changes in the average service order means that if one bull is later in the order compared to the previous test, another bull must have gone earlier. If the infected bulls' average order increases from one fertility test to the next, it must mean that the control bulls have gone earlier in the order. Infected bulls entered the yard later in the order than controls compared to pre-infection tests. The order at which the bulls enter the yard shows the enthusiasm that bulls place on serving the cow and although it has not been used previously, it could be a good measure of libido.

Service order reflects the social hierarchy within the bulls. In a paddock situation, dominant bulls will get the first chance to mate in-oestrus cows (Parkinson and Bruère, 2007). This was demonstrated in the yard testing where dominant bulls pushed to the gate into the yard containing the cow. If bulls are sick the social hierarchy can change in a herd. Dominant bulls becoming ill gave an opportunity for some of the less dominant controls to move up the social hierarchy. The change in service order did not persist and as the infected bulls recovered the earlier pre-infection social hierarchy was re-established. Changes in hierarchy could alter the

number of offspring born to each bull with an increase in the number calves born to bulls that were submissive prior to infection of dominant bulls. The effect of infection on social hierarchy would be dependent on what bulls were infected and to what degree they were affected.

With a change in order occurring that suggests a decrease in libido, it would be expected that there would be a change in time to first mount and the number of mounts. However, there was no significant difference in the time to first mount between infected and control bulls. It would be expected that sick animals would show less interest in mounting cows. The lack of difference may be due to the bulls poor health from the infection not being severe enough to overcome the drive to procreate. The lack of change in the average number of mounts may be due to bulls usually producing two mounts in the given time, this still occurred in infected bulls with an increase in the time between first and second mounts. Given that there was no effect in time to first mount, changes in the gap between the first and second mount was unexpected. It is possible that the reason for a potential effect of treatment on gap between the first and second mount is because the infected bulls have insufficient stamina to complete a second mount as quickly as the control bulls. This could be an indication that bulls would have poor performance in paddock mating as they may serve a lower proportion of cows that were on heat on any one day.

Changes in wave and forward motion of sperm were not observed. A decrease in wave and forward motion usually occurs due to an increase in abnormal sperm causing a decrease in the normal movement of sperm. The morphology of sperm collected from infected bulls had a very slight increase in the number of abnormal sperm but not enough to cause a significant change in wave and forward motion. It was expected that a decrease in wave motion and forward motion would be observed if infection had led to pyrexia as observed in a trypanosoma infection reported by Ogundele *et al.* (2016). *Trypanosoma* may cause similar effects to those expected with a *Theileria* infection including anaemia, increased body temperature and an increase in the number of abnormal sperm. Since no increase in body temperature was observed, it is a possible reason for no major effect on morphology and therefore no significant change in wave and forward motion.

Anaemia in trypanosoma infections also leads to hypoxia resulting in testicular degeneration resulting in a high number of abnormal sperm and cessation of spermatogenesis (Ogundele *et al.*, 2016). The anaemia observed in this trial did not reach a critical level so no changes in spermatogenesis as a result of hypoxia would be expected.

Density of semen collected from infected bulls increased and was higher than control bulls. This result is not supported by the literature where bulls with higher libido have a higher concentration of sperm in their semen. When boars became lethargic (Foote, 1978), a decrease in the number of sperm per sample was observed. This trend was expected in the current study. It is possible that the lack of lethargy for the first mount meant that density did not decrease, however this does not explain the increase in density that was observed in the infected bulls. A possible reason for an increase in density could be due to changes in order. Infected bulls were on average later in the yard. This means they would spend more time in the holding yard observing other bulls. This could work as a teasing stimulus. This would agree with the study by Hafs *et al.* (1962) where teasing in the form of false mounts caused an increase in sperm density.

5.1 Future work

This study was undertaken in a yard-based test where bulls were only required to express libido for a short period of time. Since there was a potential change in libido due to infection, the next possible step would be to validate this finding in a paddock situation. One way this can be done would be to compare the percentage of cows pregnant to bulls that are in the acute stage of infection and ones that are not. Data from the treatment bulls in the mating season before infection would be required to demonstrate that there was a difference in pregnancy rates before and after infection. This would show changes in social dominance in the herd and also how the bulls' libido changes over time in a commercial environment. In this situation bulls will also be put under the stress of the breeding season where they should be more actively searching for cows in oestrus and spending less time grazing.

A possible study for the future will be to compare the precise timing of natural infection to the decrease in libido. Although this experiment looked at changes in libido, the effects of natural infection may be different due to how the bull becomes infected and the severity of infection.

5.2 Limitations

The bulls were not under the same stress as breeding season, so it is likely that bulls under stress are more susceptible to infection.

The study is limited by the relative small number of bulls tested. Although it gives us an indication of the possible effects, more bulls would be required to give a more accurate trend in the effects of *Theileria*.

Since none of the bulls had a severely low HCT it cannot be ruled out that there may be an effect on fertility if that had occurred and that the changes in libido would have been more drastic. The aim of this study was to look at bulls that would not be detected to be unwell as if the bulls had become obviously unwell, farmers would notice and be less likely to use the bulls for breeding purposes. The infection used was artificial, so it is possible that a natural infection may follow a different clinical expression and affect libido in some other way. Differences in how artificial and natural infection affects bulls could affect how fertility and libido change over the course of the acute stage.

6.0 Conclusion

6.1 Main findings

The results show that the treatment bulls were successfully infected with *T. orientalis* Ikeda. Infected bulls showed a maximum PCR result at a similar time to the lowest HCT. Infected bulls showed a small decrease in libido that has the potential to decrease the pregnancy rates of a herd. Semen quality was not affected by infection but should not be ruled out as a possible effect in severe infection cases.

6.2 Implications

From the observed results, it is possible that *Theileria* has an effect on bull libido. For the farmer this means that recently infected bulls may show a decrease in libido. Changes in libido may be small so may not be clinically detectable. If this is not compensated for by the farmer in some way, then it is possible that the percentage of cows pregnant could decrease. Farmers living in areas where *Theileria* is endemic should take this into account when purchasing bulls. Their options being to either buy bulls from infected areas to ensure that they have already been infected and recovered from this challenge or to buy bulls earlier in the season so that the bulls have time to become infected and overcome the infection prior to the start of mating. To know how early, the bulls would need to be introduced before mating, further research would be required using naturally infected bulls to track the changes in libido and the time for bulls to resume normal libido. The timing of the tick activity would also need to be considered in relation to mating. Farmers may also want to purchase an additional bull to compensate for infected bulls mating with less cows.

Bulls that have lower libido may either serve no cows at all or more likely, may not have the energy to serve multiple cows in a day. This means that out of the proportion of cows in heat in a day only a small number will be served. The number of times a cow is served when she is in standing heat may also decrease. If bulls are infected at mating, farmers may need to compensate for this issue by increasing the number of bulls in the herd or replace infected bulls with others.

7.0 References

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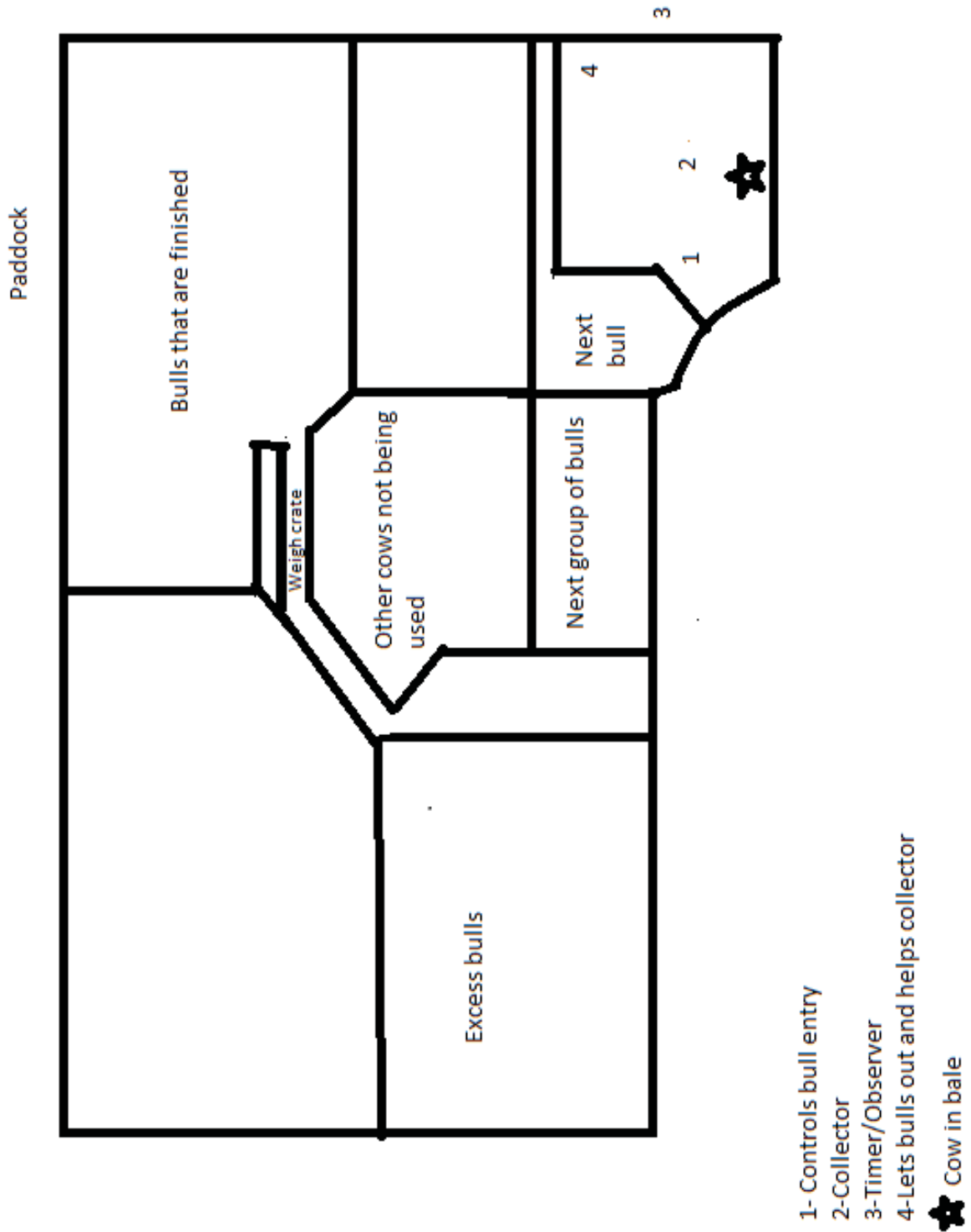
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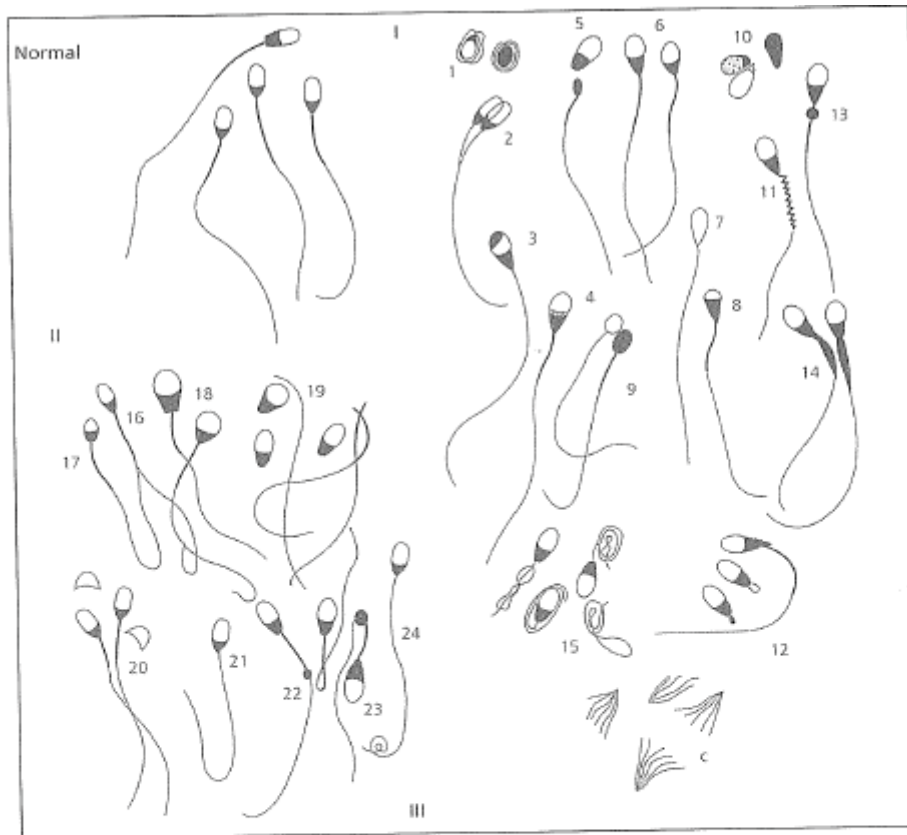
8.0 Appendices

8.1 Appendix 1 Yard plan for Tuapaka farm used for libido testing



8.2 Appendix 2 Semen abnormalities

Taken from (Parkinson and Bruère, 2007)



Major abnormalities		Minor abnormalities	
1	Underdeveloped cells	9	Small abnormal heads
2	Double forms	10	Detached abnormal heads
3	Acrosome (knobbed acrosome) defects	11	Corkscrew midpiece defect
4	Diadem defects	12	Other midpiece defects (incl. tail-stump defect)
5	Decapitated sperm (active tails)	13	Proximal cytoplasmic droplet
6	Pyriform or Pear-shaped head	14	Other thickened midpieces
7	Head narrow at the base	15	Coiled tails and Dag defect
8	Head abnormal contour		Looped tail, enclosed droplet (not illustrated)
c	Stellate forms		
		16	Narrow heads
		17	Small normal heads
		18	Giant or broad heads
		19	Detached normal heads
		20	Detached acrosomal membranes
		21	Abaxial tails
		22	Distal cytoplasmic droplets
		23	Simple bent tail
		24	Terminally looped tails

Redrawn from (Blom, 1983)

8.4 Appendix 4 SAS code for Libido

```

data frailty;
input Bull      treat $ Day      TTFM      TTFMensor      N_MTS      EJAC_MTS      GAP      GAPNA $
      GAPcensor      ORDER      Days      Diffttff      Diffgap;
cards;
551  I      2      8      1      5      100      37      37      1      5      -15      0
      0
553  I      2      7      1      4      75      37      37      1      10      -15      0
      0
554  C      2      9      1      3      100      95      95      1      4      -15      0
      0
555  I      2      6      1      5      100      25      25      1      9      -15      0
      0
557  C      2      19     1      5      80      33      33      1      2      -15      0
      0
558  C      2      14     1      3      100      48      48      1      8      -15      0
      0
560  I      2      18     1      4      100      120     120     1      1      -15      0
      0
561  I      2      2      1      1      100      180     NA      0      16     -15      0
      0
562  I      2      2      1      2      100      120     120     1      13     -15      0
      0
563  C      2      6      1      4      75      40      40      1      6      -15      0
      0
564  I      2      8      1      2      50      37      37      1      14     -15      0
      0
565  C      2      6      1      7      100      35      35      1      3      -15      0
      0
566  C      2      7      1      2      100      37      37      1      11     -15      0
      0
567  I      2      5      1      4      75      66      66      1      15     -15      0
      0
568  I      2      12     1      5      60      12      12      1      7      -15      0
      0
569  I      2      6      1      2      100      67      67      1      12     -15      0
      0
570  C      2      5      1      2      100      75      75      1      17     -15      0
      0
551  I      3      5      1      4      100      48      48      1      16     -1      6.5
      42.5
553  I      3      9      1      3      100      49      49      1      13     -1      8
      43
554  C      3      7      1      3      100      92      92      1      17     -1      8
      93.5
555  I      3      6      1      5      100      26      26      1      12     -1      6
      25.5
557  C      3      11     1      4      100      45      45      1      14     -1      15
      39
558  C      3      18     1      2      100      51      51      1      5      -1      16
      49.5
560  I      3      9      1      3      100      58      58      1      6      -1      13.5
      89
561  I      3      9      1      1      100      180     NA      0      1      -1      5.5
      180
562  I      3      9      1      2      100      132     132     1      4      -1      5.5
      126
563  C      3      3      1      3      66.66666667  56      56      1      8      -1
      4.5      48
564  I      3      12     1      2      100      124     124     1      7      -1      10
      80.5
565  C      3      5      1      4      75      46      46      1      15     -1      5.5
      40.5
566  C      3      8      1      3      100      48      48      1      3      -1      7.5
      42.5
567  I      3      7      1      3      100      67      67      1      2      -1      6
      66.5
568  I      3      5      1      2      100      136     136     1      11     -1      8.5
      74
569  I      3      11     1      2      100      59      59      1      10     -1      8.5
      63

```

570	C	3	5	1	2	100	99	99	1	9	-1	5
		87										
551	I	4	5	1	5	100	40	40	1	5	13	-1.5
		-2.5										
553	I	4	6	1	6	83.33333333	23	23	1	12	13	
		-2	-20									
554	C	4	6	1	2	100	159	159	1	17	13	-2
		65.5										
555	I	4	4	1	4	100	44	44	1	13	13	-2
		18.5										
557	C	4	14	1	4	75	50	50	1	1	13	-1
		11										
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		0.5										
560	I	4	7	1	3	100	74	74	1	3	13	-6.5
		-15										
561	I	4	7	1	1	100	180	NA	0	14	13	1.5
		0										
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		-81										
563	C	4	3	1	4	100	33	33	1	6	13	-1.5
		-15										
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		-9.5										
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		1.5										
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		-9.5										
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		-27.5										
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		-20										
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		76										
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		-42										
551	I	5	4	1	5	100	25	25	1	2	27	-2.5
		-17.5										
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		-5										
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		0	-29.5									
555	I	5	5	1	4	100	62	62	1	10	27	-1
		36.5										
557	C	5	16	1	3	100	70	70	1	7	27	1
		31										
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		-20.5										
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		-30										
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		0										
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		-53										
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		-16										
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		99.5										
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		-1.5	6.5									
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		-1	26.5									
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		-12										
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		27										
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		-1										
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		-1	86.5									

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		-10	7.5									
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		1.5	0									
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		.										
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		4										
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		99.5										
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		-3.5										
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		14.5	10.5									
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		13										
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		-1.5	107									
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		-52										
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		1										
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		54.5										
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		0.5										
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		-1	-12									
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		-7.5										
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		-47										
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		0										
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		15										
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		-2										
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		9.5										
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		-15										

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	99.5											
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	53.5											
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	106											
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	84											
570	C	8	11	1	1	100	180	NA	0	12	69	6
	93											
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	3.5											
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	8											
554	C	9	8	1	1	100	180	NA	0	16	83	0
	86.5											
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	-4.5											
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	-11											
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	130.5											
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	14											
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	-7											
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	12.5											
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	-18											
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	99.5											

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	34.5											
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	117											
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	-20											
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	-2.5											
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554	C	11	11	1	1	100	180	NA	0	6	111	3
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	4.5											
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	44											
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	130.5											
560	I	11	9	1	2	100	97	97	1	8	111	-4.5
	8											
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	0											
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	54											
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	27											
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	99.5											
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	-0.5	45.5										
566	C	11	26	1	2	100	90	90	1	14	111	18.5
	47.5											
567	I	11	7	1	2	100	113	113	1	1	111	1
	46.5											
568	I	11	11	1	2	100	130	130	1	11	111	2.5
	56											
569	I	11	9	1	2	100	102	102	1	7	111	0.5
	39											
570	C	11	17	1	2	100	81	81	1	16	111	12
	-6											
551	I	12	7	1	4	100	27	27	1	10	125	0.5
	-15.5											
553	I	12	9	1	2	100	112	112	1	4	125	1
	69											
554	C	12	8	1	2	100	158	158	1	5	125	0
	64.5											
555	I	12	8	1	4	100	28	28	1	2	125	2
	2.5											
557	C	12	14	1	1	100	180	NA	0	6	125	-1
	141											
558	C	12	18	1	2	100	110	110	1	11	125	2
	60.5											
560	I	12	7	1	2	100	118	118	1	3	125	-6.5
	29											
561	I	12	12	1	1	100	180	NA	0	16	125	6.5
	0											
562	I	12	7	1	1	100	180	NA	0	9	125	1.5
	54											
563	C	12	3	1	2	100	63	63	1	7	125	-1.5
	15											
564	I	12	15	1	1	100	180	NA	0	12	125	5
	99.5											
565	C	12	.	0	0	.	180	NA	0	1	125	.
	139.5											
566	C	12	15	1	2	50	65	65	1	14	125	7.5
	22.5											
567	I	12	5	1	2	100	98	98	1	8	125	-1
	31.5											
568	I	12	8	1	3	33.33333333	28	28	1	15	125	
	-0.5	-46										

569	I	12	7	1	2	100	124	124	1	13	125	-1.5
	61											
570	C	12	17	1	2	100	107	107	1	17	125	12
	20											
551	I	13	8	1	3	100	51	51	1	9	139	1.5
	8.5											
553	I	13	8	1	2	100	76	76	1	6	139	0
	33											
554	C	13	9	1	1	100	180	NA	0	11	139	1
	86.5											
555	I	13	6	1	3	100	21	21	1	1	139	0
	-4.5											
557	C	13	32	1	2	100	57	57	1	7	139	17
	18											
558	C	13	12	1	1	100	180	NA	0	17	139	-4
	130.5											
560	I	13	8	1	2	100	46	46	1	12	139	-5.5
	-43											
561	I	13	7	1	1	100	180	NA	0	5	139	1.5
	0											
562	I	13	7	1	2	100	168	168	1	14	139	1.5
	42											
563	C	13	5	1	2	50	5	5	1	3	139	0.5
	-43											
564	I	13	12	1	1	100	180	NA	0	4	139	2
	99.5											
565	C	13	.	0	0	.	180	NA	0	2	139	.
	139.5											
566	C	13	20	1	3	100	72	72	1	16	139	12.5
	29.5											
567	I	13	10	1	1	100	180	NA	0	10	139	4
	113.5											
568	I	13	12	1	2	100	177	177	1	13	139	3.5
	103											
569	I	13	9	1	2	100	74	74	1	15	139	0.5
	11											
570	C	13	23	1	1	100	180	NA	0	8	139	18
	93											

;

*TTFM;

Proc sort data=frailty;

By treat day;

Proc means median data=frailty;

Var diffttff ;

By treat day;

Run;

Quit;

PROC PHREG DATA = frailty ;

class treat Bull ;

MODEL Diffttff*TTFMcensor(0) = treat|day ;

random Bull/solution;

hazardratio "Frailty Model Analysis" treat ;

hazardratio treat / at (day=2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13) diff=ALL;

run;

data frailty;

set frailty;

diffttff=diffttff+10;

data _null_;

%let url = //support.sas.com/documentation/onlinedoc/stat/ex_code/132;

infile "http:&url/templft.html" device=url;

file 'macros.tmp';

retain pre 0;

input;

if index(_infile_, '</pre>') then pre = 0;

if pre then put _infile_;

if index(_infile_, '<pre>') then pre = 1;

run;

%inc 'macros.tmp' / nosource;

```

%ProvideSurvivalMacros

%let xOptions = label="Survival"
                linearopts=(viewmin=0 viewmax=300
                             tickvaluelist=(0 50 100 150 200 250 300));

%CompileSurvivalTemplates
data day13;
set frailty;
if day~=13 then delete;
run;
quit;
proc lifetest data=day13;
time diffttff*ttfmcensor(0);
strata treat;
run;
quit;

*Gap;
Data no561;
set frailty;
if Bull= 561 then delete;
Run;
Quit;

Proc sort data=no561;
By treat day;
Proc means median data=no561;
Var diffgap ;
By treat day;
Run;
Quit;
data no561;
set no561;
diffgap=diffgap+81;
run;
quit;

PROC PHREG DATA = no561 ;
class treat Bull ;
MODEL Diffgap*gapcensor(0) = treat|day ;
random Bull/solution;
hazardratio "Frailty Model Analysis" treat ;
hazardratio treat / at (day=2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13) diff=ALL;
run;

data _null_;
%let url = //support.sas.com/documentation/onlinedoc/stat/ex_code/132;
infile "http:&url/templft.html" device=url;
file 'macros.tmp';
retain pre 0;
input;
if index(_infile_, '</pre>') then pre = 0;
if pre then put _infile_;
if index(_infile_, '<pre>') then pre = 1;
run;

%inc 'macros.tmp' / nosource;

%ProvideSurvivalMacros

%let xOptions = label="Survival"
                linearopts=(viewmin=0 viewmax=180
                             tickvaluelist=(0 30 60 90 120 150 180));

%CompileSurvivalTemplates
data day13;
set no561;
if day~=13 then delete;
run;
quit;

```

```

proc lifetest data=day13;
time diffgap*gapcensor(0);
strata treat;
run;
quit;
*Number of mounts;
proc genmod data=Frailty;
title 'N_MTS';
class treat day bull;
model N_MTS= treat day treat*day/type3 dist=poisson;
repeated subject=bull /type=exch;
lsmeans treat treat*day/pdiff ilink;
run;
quit;

```

8.5 Appendix 5 SAS Code for Semen traits

```

data semen;
input Bull tmt Day Sample$ Wave Fwd Morphology Density;
Cards;
551 1 2 B 3 7 77 970000000.00
553 1 2 A 3 7 90 1220000000.00
554 2 2 C 3 7 94 1070000000.00
555 1 2 A 5 7 95 1440000000.00
557 2 2 A 9 10 . 1400000000.00
558 2 2 A 0 3 88 340000000.00
560 1 2 A 7 10 96 1310000000.00
561 1 2 A 10 10 93 1480000000.00
562 1 2 A 9 9 95 1690000000.00
563 2 2 A 6 10 98 1050000000.00
564 1 2 A 10 10 89 1540000000.00
565 2 2 A 8 9 93 1660000000.00
566 2 2 A 9 9 95 1040000000.00
567 1 2 A 4 8 96 680000000.00
568 1 2 A 9 9 96 1350000000.00
569 1 2 A 10 10 93 1480000000.00
570 2 2 A 9 10 . 880000000.00
551 1 3 C 7 7 77 8332500000.00
553 1 3 B 10 10 90 15352000000.00
554 2 3 C 8 8 94 11463500000.00
555 1 3 A 8 8 97 19190000000.00
557 2 3 C 9 9 91 8231500000.00
558 2 3 B 7 7 99 13130000000.00
560 1 3 C 10 10 94 16059000000.00
561 1 3 A 8 8 96 16160000000.00
562 1 3 B 8 8 84 9544500000.00
563 2 3 B 8 8 95 15200500000.00
564 1 3 B 10 10 98 18735500000.00
565 2 3 C 8 8 92 12473500000.00
566 2 3 A 8 8 94 12827000000.00
567 1 3 B 7 8 91 8837500000.00
568 1 3 B 8 8 97 21866500000.00
569 1 3 A 8 8 96 16766000000.00
570 2 3 B 6 8 97 5959000000.00
551 1 4 D 9 9 90 15301500000.00
553 1 4 B 6 8 86 14493500057.40
554 2 4 A 7 9 96 29239500231.60
555 1 4 B 5 8 94 11918000141.60
557 2 4 B 9 9 98 12776500202.40
558 2 4 C 8 9 92 18533500367.00
560 1 4 B 8 8 96 13988500332.40
561 1 4 A 7 9 99 18937500525.00
562 1 4 B 9 9 93 16463000521.60
563 2 4 B 9 9 97 11173125398.25
564 1 4 A 9 9 97 32320001280.00
565 2 4 A 5 8 97 16210500706.20
566 2 4 C 8 9 97 11463500544.80
567 1 4 B 6 7 57 10352500533.00
568 1 4 C 9 9 92 23331001293.60
569 1 4 B 8 6 76 8231500489.00
570 2 4 C 9 9 98 17826501129.60
551 1 5 A 8 7 88 16261000193.20
553 1 5 B 8 9 94 13483500160.20
554 2 5 A 9 10 96 24997500297.00

```

555	1	5	A	6	9	97	15806500187.80
557	2	5	B	8	8	90	14342000170.40
558	2	5	B	8	8	94	17776000211.20
560	1	5	B	7	6	90	17675000210.00
561	1	5	A	8	8	94	14190500168.60
562	1	5	A	8	8	95	34592500411.00
563	2	5	B	7	8	95	18483000219.60
564	1	5	A	5	7	98	14544000172.80
565	2	5	B	6	8	93	16412500195.00
566	2	5	A	6	9	96	18533500220.20
567	1	5	B	6	6	81	12625000150.00
568	1	5	B	8	9	92	16867000200.40
569	1	5	B	8	8	87	17877000212.40
570	2	5	A	6	9	88	14493500172.20
551	1	6	B	7	8	96	9645500114.60
553	1	6	A	5	7	90	8837500105.00
554	2	6	A	5	6	90	2525000030.00
555	1	6	B	8	8	90	6767000080.40
557	2	6	B	9	9	91	13231000157.20
558	2	6	B	7	8	90	8938500106.20
560	1	6	B	5	6	82	7019500083.40
561	1	6	A	6	7	100	11362500135.00
562	1	6	A	8	8	84	7676000091.20
563	2	6	B	6	6	97	17473000207.60
564	1	6	A	5	7	91	4141000049.20
565	2	6	B	9	9	95	7777000092.40
566	2	6	C	8	8	96	6363000075.60
567	1	6	B	8	8	79	16513500196.20
568	1	6	B	9	9	94	12574500149.40
569	1	6	B	8	7	86	4444000052.80
570	2	6	A	7	8	96	11968500142.20
551	1	7	B	7	8	90	17927500213.00
553	1	7	B	8	8	88	11362500135.00
554	2	7	B	6	7	94	5151000061.20
555	1	7	B	8	8	89	9645500114.60
557	2	7	A	9	9	93	18382000218.40
558	2	7	A	9	9	94	21311000253.20
560	1	7	A	8	8	95	23179500275.40
561	1	7	A	9	8	92	23356250277.50
562	1	7	B	8	7	90	23381500277.80
563	2	7	B	8	8	100	28633500340.20
564	1	7	B	7	6	92	22573500268.20
565	2	7	B	9	9	96	21765500258.60
566	2	7	B	8	8	94	10807000128.40
567	1	7	A	7	7	93	15604500185.40
568	1	7	B	9	9	95	21412000254.40
569	1	7	A	8	7	89	20806000247.20
570	2	7	B	6	7	92	6666000079.20
551	1	8	C	9	8	94	19216438164.00
553	1	8	B	8	8	80	13586301234.00
554	2	8	A	6	6	96	6963926871.00
555	1	8	C	8	8	91	17888584296.00
557	2	8	A	9	10	97	18334246392.00
558	2	8	B	9	9	93	16335616275.00
560	1	8	B	7	7	96	9675798990.00
561	1	8	A	5	6	94	12114155130.00
562	1	8	A	9	9	94	24412785144.00
563	2	8	B	7	7	99	21119634492.00
564	1	8	A	5	5	97	5433789900.00
565	2	8	B	8	9	94	12507305811.00
566	2	8	B	8	8	98	20876712120.00
567	1	8	B	7	7	78	.
568	1	8	A	6	7	86	16296803490.00
569	1	8	B	8	7	88	12978995304.00
570	2	8	A	1	2	98	5063013648.00
551	1	9	A	8	8	94	27981800000.00
553	1	9	B	8	8	86	16141400000.00
554	2	9	A	8	8	97	32738200000.00
555	1	9	A	9	9	85	18216000000.00
557	2	9	B	7	7	88	8703200000.00
558	2	9	A	9	8	96	28892600000.00
560	1	9	B	9	9	94	22517000000.00
561	1	9	A	9	9	95	18317200000.00
562	1	9	A	7	7	92	19430400000.00
563	2	9	A	7	5	93	6982800000.00
564	1	9	A	9	9	96	33649000000.00
565	2	9	B	7	7	69	14572800000.00

566	2	9	B	9	9	92	8652600000.00
567	1	9	A	5	5	77	15736600000.00
568	1	9	A	10	10	93	31979200000.00
569	1	9	B	5	5	69	11992200000.00
570	2	9	A	7	8	93	11992200000.00
551	1	10	C	8	8	98	12245200000.00
553	1	10	B	8	7	86	14016200000.00
554	2	10	A	5	6	77	2985400000.00
555	1	10	B	9	9	97	13054800000.00
557	2	10	A	9	9	98	19531600000.00
558	2	10	A	9	9	95	16799200000.00
560	1	10	A	8	7	94	29803400000.00
561	1	10	A	7	7	95	24743400000.00
562	1	10	A	8	8	94	18114800000.00
563	2	10	C	8	8	96	14522200000.00
564	1	10	A	9	9	99	15028200000.00
565	2	10	A	4	4	95	1720400000.00
566	2	10	A	5	5	97	7387600000.00
567	1	10	A	8	7	95	28892600000.00
568	1	10	A	6	6	95	4351600000.00
569	1	10	A	3	2	81	20493000000.00
570	2	10	B	7	7	91	11182600000.00
551	1	11	C	8	9	95	11334400000.00
553	1	11	B	9	8	91	8652600000.00
554	2	11	A	1	3	89	1922800000.00
555	1	11	B	8	9	95	12599400000.00
557	2	11	B	10	9	91	41340200000.00
558	2	11	A	1	0	98	6325000000.00
560	1	11	A	6	8	96	14016200000.00
561	1	11	A	5	7	92	15736600000.00
562	1	11	A	8	9	96	3166900000.00
563	2	11	B	9	9	97	14724600000.00
564	1	11	A	5	6	76	2884200000.00
565	2	11	B	8	8	96	8855000000.00
566	2	11	B	1	2	93	354200000.00
567	1	11	B	1	4	85	12548800000.00
568	1	11	B	9	9	92	11941600000.00
569	1	11	A	8	8	95	12042800000.00
570	2	11	B	9	8	89	2810500000.00
551	1	12	B	8	8	81	10170600000.00
553	1	12	B	10	10	91	21150800000.00
554	2	12	A	9	9	85	22314600000.00
555	1	12	B	8	8	94	17153400000.00
557	2	12	A	9	8	85	16748600000.00
558	2	12	B	9	9	90	21909800000.00
560	1	12	A	8	8	91	24844600000.00
561	1	12	A	7	7	88	23984400000.00
562	1	12	A	9	9	84	36330800000.00
563	2	12	A	10	10	95	27273400000.00
564	1	12	A	8	8	91	27526400000.00
565	2	12
566	2	12	A	9	9	93	22972400000.00
567	1	12	B	9	8	85	25957800000.00
568	1	12	B	8	8	90	19582200000.00
569	1	12	B	9	9	82	21960400000.00
570	2	12	B	7	7	90	8956200000.00
551	1	13	C	8	8	87	16698000000.00
553	1	13	B	8	8	84	22871200000.00
554	2	13	A	9	9	86	34306800000.00
555	1	13	A	8	8	91	21555600000.00
557	2	13	A	8	8	86	27425200000.00
558	2	13	A	1	1	88	1973400000.00
560	1	13	A	6	6	93	15686000000.00
561	1	13	A	8	8	79	15382400000.00
562	1	13	A	8	8	79	38607800000.00
563	2	13	A	7	7	88	16647400000.00
564	1	13	A	9	9	94	39265600000.00
565	2	13	.	.	.	85	4301000000.00
566	2	13	B	8	8	94	12093400000.00
567	1	13	A	6	6	85	22264000000.00
568	1	13	A	7	7	94	39113800000.00
569	1	13	A	8	8	85	17760600000.00
570	2	13	A	2	8	95	1973400000.00

;

```
proc means mean min max n median data=semen;
run;
```

```

quit;

*Wave motion;
proc genmod data=semen;
title 'wave motion';
class tmt day bull;
model wave= tmt day tmt*day /type3 dist=poisson;
repeated subject=bull ;
lsmeans tmt tmt*day/pdiff ilink;
run;
quit;

*Forward motion;
proc genmod data=semen;
title 'Forward motion';
class tmt day bull;
model Fwd= tmt day tmt*day/type3 dist=poisson;
repeated subject=bull ;
lsmeans tmt tmt*day/pdiff ilink;
run;
quit;

*morphology;
data semen;
set semen;
logmorph=log(101-morphology);
run;
quit;

proc mixed data=semen;
title 'Morphology';
class tmt day bull;
model logmorph= tmt day tmt*day/solution;
repeated /subject=bull group=day type=ar(1);
lsmeans tmt*day/cl pdiff;
lsmeans tmt day /cl ;
run;
quit;

*Density;
data semen;
set semen;
sqrtdens=sqrt(density);
run;
quit;

proc mixed data=semen;
title 'Density';
class tmt day bull;
model sqrtdens= tmt day tmt*day/solution;
repeated /subject=bull group=day type=ar(1);
lsmeans tmt*day/cl pdiff;
lsmeans tmt day /cl ;
run;
quit;

```

8.6 Appendix 6 R code for service order, Haematocrit, PCR and order

R code for plots and service order

```
library(plyr); library(doBy); library(ggplot2); library(grid)

library(zoo);library(lattice);library(data.table)

dat <- read.table("masterkl.csv", header = TRUE, sep = ",", na.strings="NA")

names(dat)

dim(dat)

head(dat)

dat$Diseased <- ifelse(dat$infected==1, "Infected", "Control")
```

Haematocrit plot

```
p <- ggplot(dat, aes(x=days, y= avePCV,group=Diseased,color=Diseased))+
geom_rect(mapping=aes(xmin=47, xmax=80, ymin=15,ymax=45),fill="blue",linetype="blank",alpha=0.002)
d<- p + stat_smooth(method="loess",span=0.2)+
coord_cartesian(ylim = c(20,45))+
scale_x_continuous(expand = c(0,0),limits = c(-30,145),breaks=seq(-30,140,10))+
theme(panel.grid.major = element_blank(),
      panel.grid.minor = element_blank()+ xlab("\n Days from infection")+
      ylab(" Haematocrit (L/L)\n")+
      scale_color_manual(values=c('red','black'))+
      theme(axis.title.x = element_text( family = "Times", colour = "black", size = 12))+
      theme(axis.title.y = element_text(family = "Times", colour = "black", size = 12))+
      theme(axis.text.x = element_text(family = "Times", colour = "black", size = 10))+
      theme(axis.text.y = element_text(family = "Times", colour = "black", size = 10))+
      theme(panel.background = element_blank(),axis.line=element_line(colour="black"))+
      theme(legend.position = "none")

d

e<-d + theme(axis.line.x = element_line(color="black", size = 0.6),
            axis.line.y = element_line(color="black", size = 0.6))+
geom_vline(xintercept=0, color="blue",linetype=2)
tiff("MGPCV.tiff", width = 6, height = 5, units = 'in', res = 300)

e

dev.off()
```


Temperature plot

```
p <- ggplot(dat, aes(x=days, y= temp,group=Diseased,color=Diseased))
d<- p + stat_smooth(method="loess",span=0.4)+
coord_cartesian(ylim = c(37,40))+
scale_x_continuous(expand = c(0,0),limits = c(-30,145),breaks=seq(-30,140,10))+
theme(panel.grid.major = element_blank(),
       panel.grid.minor = element_blank()+ xlab("\n Days from infection")+
       ylab(" Rectal temperature (°C)\n")+
       scale_color_manual(values=c('red','black'))+
       theme(axis.title.x = element_text( family = "Times", colour = "black", size = 12))+
       theme(axis.title.y = element_text(family = "Times", colour = "black", size = 12))+
       theme(axis.text.x = element_text(family = "Times", colour = "black", size = 10))+
       theme(axis.text.y = element_text(family = "Times", colour = "black", size = 10))+
       theme(panel.background = element_blank(),axis.line=element_line(colour="black"))+
       theme(legend.position = "none")

e<-d + theme(axis.line.x = element_line(color="black", size = 0.6),
            axis.line.y = element_line(color="black", size = 0.6))

e
f<-e +
geom_vline(xintercept=0, color="blue",linetype=2)

f
tiff("MGtemp.tiff", width = 6, height = 5, units = 'in', res = 300)

f
dev.off()
```

Weight plot

```
p <- ggplot(dat, aes(x=days, y= weight,group=Diseased,color=Diseased))
d<- p + stat_smooth(method="loess",span=.4)+
coord_cartesian(ylim = c(500,700))+
scale_x_continuous(expand = c(0,0),limits = c(-30,145),breaks=seq(-30,140,10))+
theme(panel.grid.major = element_blank(),
       panel.grid.minor = element_blank()+ xlab("\n Days from infection")+
```

```

ylab(" Body weight (kg) \n")+
scale_color_manual(values=c('red','black'))+
theme(axis.title.x = element_text( family = "Times", colour = "black", size = 12))+
theme(axis.title.y = element_text(family = "Times", colour = "black", size = 12))+
theme(axis.text.x = element_text(family = "Times", colour = "black", size = 10))+
theme(axis.text.y = element_text(family = "Times", colour = "black", size = 10))+
theme(panel.background = element_blank(),axis.line=element_line(colour="black"))+
theme(legend.position = "none")

```

```

e<-d + theme(axis.line.x = element_line(color="black", size = 0.6),
axis.line.y = element_line(color="black", size = 0.6))+
geom_vline(xintercept=0, color="blue",linetype=2)

```

e

```
tiff("MGweight.tiff", width = 6, height = 5, units = 'in', res = 300)
```

e

```
dev.off()
```

PCR plot and cutpoint

```

dat$avePCR1<- rowMeans(dat[c('PCR1', 'PCR2')], na.rm=TRUE)
dat$avePCRμl<-dat$avePCR1/5
dat$logPCR<-log10(dat$avePCRμl+1)
dat$avecopies<-dat$avePCRμl*219.0216403
dat$logcopy<-log10(dat$avecopies+1)
summary(dat$logPCR)
summary(dat$logcopy)
p <- ggplot(dat, aes(x=days, y= logPCR,group=Diseased,color=Diseased))
d<- p + stat_smooth(method="loess",span=.22)+
scale_y_continuous(expand = c(0,0),limits = c(-.5,4),breaks=seq(0,4,1),labels=c("0", "10", "100","1,000","10,000"))+
scale_x_continuous(expand = c(0,0),limits = c(-30,145),breaks=seq(-30,140,10))+
theme(panel.grid.major = element_blank(),
panel.grid.minor = element_blank())+ xlab("\n Days from infection")+
ylab("fg/μl \n")+

```

```

scale_color_manual(values=c('red','black'))+
theme(axis.title.x = element_text( family = "Times", colour = "black", size = 12))+
theme(axis.title.y = element_text(family = "Times", colour = "black", size = 12))+
theme(axis.text.x = element_text(family = "Times", colour = "black", size = 10))+
theme(axis.text.y = element_text(family = "Times", colour = "black", size = 10))+
theme(panel.background = element_blank(),axis.line=element_line(colour="black")) +
  theme(legend.position = "none")
e<-d + theme(axis.line.x = element_line(color="black", size = 0.6),
  axis.line.y = element_line(color="black", size = 0.6)) +
geom_hline(yintercept=0.3, color="blue",linetype=2) +
annotate("text", x = -10, y=0.4, label = "1 fg/ $\mu$ l",color="blue")
e
tiff("MGPCR.tiff", width = 6, height = 6, units = 'in', res = 300)
e
dev.off()

```

Service position plot and statistics

```

dat <- read.table("bullmountcens.csv", header = TRUE, sep = ",", na.strings="NA")
cdata <- ddply(dat, c("treat","days"), summarise,
  N = sum(!is.na(ORDER)),
  mean = mean(ORDER, na.rm=TRUE),
  sd = sd(ORDER, na.rm=TRUE),
  se = sd / sqrt(N)
)
cdata
dat1<-subset(cdata, days>-2)
limits <- aes(ymax = dat1$mean + dat1$se, ymin = dat1$mean - dat1$se)
d<-ggplot(data = dat1, aes(x=days, y=mean)) + geom_line(aes(linetype = treat, color=treat),size=1) +
  geom_point(aes(shape=treat, color=treat), size=4)+ geom_errorbar(limits, width = 2.5,
  position=position_dodge(20))+
  scale_y_continuous(expand = c(0,0),limits = c(2,14),breaks=seq(2,14,2))+
  scale_x_continuous(expand = c(0,0),limits = c(-5,145),breaks=seq(0,140,10))+
  theme(panel.grid.major = element_blank(),
  panel.grid.minor = element_blank()+ xlab("\n Days from infection")+

```

```

ylab("Average order score\n")+
scale_color_manual(values=c('red','black'))+
theme(axis.title.x = element_text( family = "Times", colour = "black", size = 12))+
theme(axis.title.y = element_text(family = "Times", colour = "black", size = 12))+
theme(axis.text.x = element_text(family = "Times", colour = "black", size = 10))+
theme(axis.text.y = element_text(family = "Times", colour = "black", size = 10))+
theme(panel.background = element_blank(),axis.line=element_line(colour="black"))+
  theme(legend.position = "none")
e<-d + theme(axis.line.x = element_line(color="black", size = 0.6),
  axis.line.y = element_line(color="black", size = 0.6))+
  geom_hline(yintercept = 9, colour="blue",lwd=1)
e
tiff("bullorder.tiff", width = 6, height = 5, units = 'in', res = 600)
e
dev.off()
sum(1:17)/17
Non-parametric statistical test
mean(1:17)
wilcox.test(dat$ORDER[dat$days==41]~dat$treat[dat$days==41])
wilcox.test(dat$ORDER[dat$days==55]~dat$treat[dat$days==55])

```

8.7 Appendix 7 Standard operating procedure for semen analysis

Standard Operating Procedure # 1

Date: 31/07/2017

Revision # Original

Massey University
Institute of Veterinary, Animal and Biomedical Sciences
Diagnostic Services

Title: *Semen analysis of bulls*

1. Purpose

This SOP describes the procedure for performing analysis on bull semen in order to evaluate the fertility of the bull.

2. Responsibility

This SOP must be followed by all participants in the bull *Theileria* study.

3. Materials/ Equipment list

- Warm stage
- Pippettes (1000 μ l, 100 μ l)
- Frosted microscope slides (brand)
- Cover slips

- Microscope slides with bevelled edges
- Paper towels
- Centrifuge tubes (15ml?)
- Diluent- Citric acid (2.9% pH=7) and Formal saline (10%) 1:1 ratio
- Morphology stain- Giesma
- Slide holder trays
- Microscope

4. Safety

Good hygiene is required and disposable gloves should be worn in order to prevent cross contamination and zoonosis.

5. Definitions

AV= artificial vagina

PCR= Polymerase Chain Reaction

6. Procedure

Semen should be collected using a sterile AV and kept warm until the wave and forward motion has been assessed. Each collection from the bulls should be looked at before decided on the sample to use.

1. Colour

Look at semen once it has been collected in the 15ml tube and class as either watery, milky or creamy. This should be carried out for all samples for each bulls.

2. Volume

Measure the volume of the semen in each tube using the lines on the outside of the tube.

3. Wave motion

Select the best tube of semen using the densest sample.

Place a drop of semen on a microscope slide and examine under a heated stage microscope.

Rate motility out of 10 based on the speed the semen is moving with 10 being the fastest and 1 being no movement.

If poor movement is seen, look at the next best sample to see if it is better. If it is continue with this sample.

4. Forward motion

Place coverslide on to drop of semen used for wave motion. Assess the motion of the semen and score out of 10 based on the proportion of semen moving forward.

5. Morphology

Pipette a drop of Giesma stain onto a microscope slide

Dip a fresh pipette into the semen bottle. Blow a bubble with the end of the pipette and pop it on to the stain. Mix well. Push a clean slide onto the side of the stain for the stain to move across the edge of the slide. Holding a clean slide, Smear the slide with a bit of stain across the clean slide with constant pressure. Examine the smeared slide under a microscope to see if the smear is of good quality. I.e a good distribution of semen and the stain is not too thick.

Using 1000x magnification and oil emersion, count 100 semen recording whether it is normal or abnormal. If it is abnormal, record what abnormality is present. Calculate the percentage of normal semen using the following equation.

Percentage of normal semen= (Number of normal semen/ total semen counted) * 100

6. Density

Prepare 10mls of 2%formal saline with 10ml of sodium citrate to become the diluent for density reading.

Add 1000 μ l of diluent into a 1.5ml epindorph tube. Add 10 μ l of undiluted semen into the tube. Write on tube the bulls ear tag number and date.

Add one drop of the diluted solution under the coverslip of a haemocytometer ensuring that the liquid moves through the whole way.

Count the semen in the 4 corner squares and the centre square with the haemocytometer under a microscope. Average the number of semen in the 5 squares and times by 25.

Multiply this value by 1/10000 and then multiply by the dilution factor (101 fold).

7. PCR

Label and store the remainder of the semen in a freezer in case of use in PCR.

7. Notes

Used to aid understanding of the reasoning behind an activity, i.e. background information.

8. History of this SOP

Author: Michaela Gibson

8.8 Appendix 8- Standard operating procedure for quantification of *Theileria Ikeda*

Standard Operating Procedure # 1

Date: 31/07/2017

Revision # Original

Massey University
Institute of Veterinary, Animal and Biomedical Sciences
Diagnostic Services

Title: *Quantification of Theileria orientalis Ikeda*

1. Purpose

This SOP describes the procedure for performing DNA extraction and detection of *Theileria orientalis* Ikeda using qPCR.

2. Responsibility

This SOP must be followed by all participants in the bull *Theileria* study.

3. Materials/ Equipment list

- DNA extraction
- ❖ Nucleomag Vet viral RNA/DNA isolation kit (Macherey-Nagel, Duren, Germany)
- ❖ 96 samples of blood or semen for extraction.
- ❖ Kingfisher ® Flex Nucleic Acid Extraction System (ThermoFisher, Carlsbad, CA, USA)
 - qPCR
- ❖ MIC qPCR Cycler (Bio Molecular Systems, Upper Coomera, QLD, Australia)
- ❖ PerfeCTa qPCR ToughMix
- ❖ Ikeda forward primer
- ❖ Ikeda reverse primer
- ❖ Ikeda Probe
- ❖ Wildtype DNA (non-template control of DNA extracted from an EDTA blood sample that tested negative for *T.orientalis* Ikeda.
- ❖ Standards using a 1:10 serial dilution from 0.5ng to 50fg.

4. Safety

Good hygiene is required and disposable gloves should be worn in order to prevent cross contamination and zoonosis.

5. Definitions

6. Procedure

- DNA extraction
 1. Aliquot 50µl of whole blood samples in to a 96- well deep well plate (lysis plate).
 2. Mix together 18ml of VL1 with 2ml of proteinase K
 3. Add 200µl of the proteinase K mix to each well in the lysis plate.
 4. Fill 600µl of VEW1 to each well of an empty Thermo 96-well deep well plate (VEW1 plate)

5. Fill 600µl of VEW2 to each well of an empty Thermo 96-well deep well plate (VEW2 plate)
 6. Fill 600µl of 80% ethanol to each well of an empty Thermo 96-well deep well plate (Ethanol plate)
 7. Fill 100µl of VEL to each well of an empty Thermo 200µl 96-well plate (elution plate)
 8. Add 20µl of B-Beads and 600µl of VEB buffer to each well of the sample lysis plate.
 9. Place plates in to Kingfisher and set to mn_vet_flex program.
 10. Cover plate and store at -20°C until PCR
- qPCR
 1. Make a stock solution of PCR with 10µl of PerfeCTa tough mix, 1 µl of forward primer, 1 µl of reverse primer, 0.8 µl of probe and 2.2 µl of water per sample (plus 7 for standards).
 2. Aliquot 15 µl of stock in to each of the microPCR tubes
 3. Add 5 µl of DNA in each tube
 4. Set up standards using samples of 5 µl of 100pg, 10pg, 1pg, 100fg, 10fg. Wildtype and water.
 5. Run PCR using Mic qPCR cycler using the cycling parameters of; a hold activation step at 95°C for 2 minutes followed by 45 cycles of 95°C for 15 seconds and 60°C for 30 seconds.

7. Notes

If DNA extraction or qPCR doesn't occur immediately after being aliquoted, the plate should be sealed and stored at -20°C

8. History of this SOP

Author: Michaela Gibson (2017)

Version 1

9. Appendices

Polymerase chain reaction sequences for primers and probe

Reactant	Sequence
Forward primer	5'-AGTTAACGCCACCGCAGCCG-3'
Reverse primer	5'-ACGCGGTATCCCTCTTCGGCA-3'
Probe	[6FAM]-CGCCTCAAACGCCAACGACG-[BHQ1]

Quantabio, Beverly, MA, USA

Date: 31/07/2017

8.9 Appendix 9- Standard operating procedure for libido testing of bulls

Standard Operating Procedure # 1

Date: 31/072017

Revision # Original

Massey University
Institute of Veterinary, Animal and Biomedical Sciences
Diagnostic Services

Title: *Libido testing of bulls*

1. Purpose

This SOP describes the procedure for performing analysis on bull libido in order to get an indication of bull performance in a paddock situation.

2. Responsibility

This SOP must be followed by all participants in the bull *Theileria* study.

3. Materials/ Equipment list

- Cattle bail
- Artificial Vagina (AV)
- AV cone
- AV liner

- Lubricant
- Timer
- Recording sheet
- Halter
- Rope
- Cow/ Steer

4. Safety

Good hygiene is required and disposable gloves should be worn in order to prevent cross contamination and zoonosis. Covered boots and overalls should also be worn at all times.

5. Definitions

AV= artificial vagina

6. Procedure

1. Place heifer in to bail and restrain using halter and rope to tie to the fence. If using heifer that is not in standing heat or a steer, a sedative should be administered.
2. Let one bull in to the yard and start timer as shoulder passes through gateway.
3. Record the time of the first mount and score based on whether the penis was not present (N), Penis was present but no ejaculation (P) or Ejaculation occurred (E).

Note: If no mount occurs in the first 5 minutes of the bull entering the yard, the bull should be removed from the yard.

4. Record subsequent mounts

5. After 3 minutes from the first mount, the test is finished and the bull should be let out of the yard.

7. Notes

Used to aid understanding of the reasoning behind an activity, i.e. background information.

8. History of this SOP

Author: Michaela Gibson (2017)

Version 1

Date: 31/07/2017