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Anaemia in East African short-horn Zebu calves: Field diagnosis, infectious causes and pathogen interactions

by

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SUMMARY

This study formed part of the collaborative IDEAL (Infectious Diseases of East African Livestock) project, which focused on the sedentary mixed crop-livestock smallholding system in Western Kenya. This was a longitudinal study where calves were recruited at birth and followed at 5-weekly intervals until 51 weeks of age. The main aim was to investigate the total disease burden of cattle in the study area for the first year of life.

The main objectives of the study concerned anaemia as a syndrome in the calves. Anaemia can provisionally be diagnosed based on clinical signs, but a confirmatory diagnosis is based on measuring of red blood cell parameters, such as packed cell volume (PCV), red cell counts (RCC) or haemoglobin (HGB). The FAMACHA© score card, a field diagnostic test developed to detect anaemia and haemonchosis in sheep, was designed to test pallor by measuring the colour of the ocular mucosa against a colour chart. The FAMACHA© as a field diagnostic tool was validated for use in East African short-horn Zebus, using PCV as the gold standard. The red cell parameters and indices as well as white cell and platelet parameters and indices of the East African short-horn Zebu were measured by a Sysmex® automated cell-analyzer.

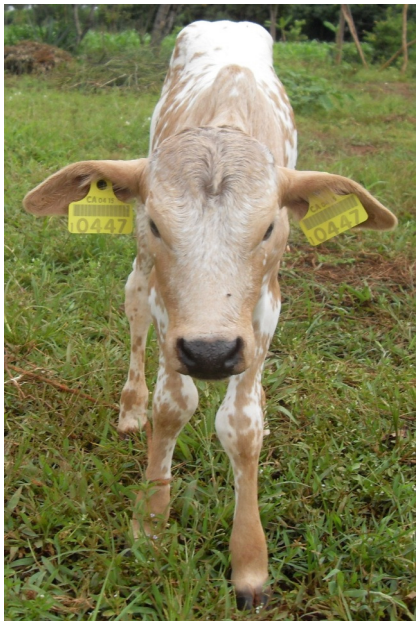
The age-related changes in the haematological profile of East African short-horn Zebu were investigated. The haematological profile of the study population, particularly during the neonatal period, differed from reference ranges for European breeds, both in levels and age-related trends. These differences could not be explained by what is known about the physiology for other cattle breeds. Anaemia was a significant syndrome in the general study population based on the high incidence of anaemic episodes and the longitudinal trend in the general study population towards an anaemic state.

In a tropical environment calves are exposed to a myriad of pathogens, even from early calfhood, many of which potentially cause anaemia. The prevalence and cumulative incidence of pathogens, in particular tick-borne parasites, trypanosomes and intestinal parasites, was investigated. The variation in prevalence with age has allowed the identification of high-risk periods. The prevalence of co-infection of pathogens was also found to be considerable, in particular pathogens known to cause anaemia.

The relative contribution of different pathogens to the development of anaemia was investigated through their impact on the haematological profiles of calves infected by specific

pathogens. Strongyle-type nematodes and trypanosomosis were found to be the major causes of anaemia in the calves during their first year of life.

Co-infections with pathogens were shown to have a significant impact on the haematological profile of calves. In many cases the cumulative effect of, or interactions between concomitant pathogens affected the severity of clinical symptoms, such anaemia, and in turn affected the prognosis of such calves. The impact of concomitant infections also complicates any diagnostic, treatment or intervention programmes in livestock and should be considered in any study on epidemiology in livestock kept under field conditions.



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LIST OF ABBREVIATIONS

AEZagro-ecological zone
AICAkaike's information criteria
APCantigen presenting cells
AUCarea under the curve
BICBayesian Information Criteria
CIcumulative incidence
CVcoefficient of variation
DCdendritic cells
DGdark ground buffy coat count technique
DVTDDepartment of Veterinary Tropical Diseases, University of Pretoria
ECFEast Coast fever
Eosabsolute eosinophil count
ELISAenzyme-linked immunosorbent assay
EPGegg per gram faeces
Hb Aadult haemoglobin
Hb Ffoetal haemoglobin
HCThaematocrit
HGBhaemoglobin concentration
ICCintraclass correlation
Ig A/E/Gimmunoglobulin A/E/G
IDEALInfectious diseases of East African livestock
ILinterleukin
ILRIInternational Livestock Research Institute
LR+positive likelihood ratio
Lymphabsolute lymphocyte count
MCHCmean corpuscular haemoglobin concentration
MCVmean corpuscular volume
mcrmicroscopy
MHCmajor histocompatability complex
Monoabsolute monocyte count
MPVmean platelet volume
Neutabsolute neutrophil count
ODoptical density
OPGoocysts per gram faeces
PCRpolymerase chain reaction

PCVpacked cell volume
PDWplatelet distribution width
Pltplatelet count
PP valuepercentage positivity
PV+positive predictive value
PV-negative predictive value
RBCred blood cells
RCCred cell count
RDWred cell distribution width
RLBTreverse line blot technique
ROCreceiver operating characteristic curve
SDstandard deviation
Sesensitivity
SEstandard error
SHZEast African short-horn Zebu
Spspecificity
TBDtick-borne diseases
Th-cellsT-helper lymphocytes
TSPtotal serum protein
varvariance
VATvariable antigen types in trypanosomes
VSGvariable surface glycoprotein in trypanosomes
WBCwhite blood cells
WCCwhite cell count

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

The sedentary mixed crop-livestock smallholding system encompasses >50% of poor people resident in East Africa. In Kenya, a total of 67.7% of the total population of 38.6 million people live in rural areas (KNBS 2010). Livestock are one of the principle capital assets of these resource-poor farmers. Indigenous breeds, of which the small East African Short-horn Zebu is the most common, are regionally very important and constitute up to 77% of the total Kenyan cattle population of 17,4 million (Rege, Kahi, Okomo-Adhiambo, Mwacharo & Hanotte 2001; Mwacharo, Okeyo, Kamande & Rege 2006; KNBS 2010). Farmers prefer indigenous breeds because of their adaptability in terms of disease resistance, heat tolerance, and water and food requirements (Mwacharo & Drucker 2005).

The tropical climate in western Kenya is conducive for the survival of many infectious pathogens and vectors. The most economically important diseases of livestock in sub-Saharan Africa are tick-borne diseases, especially East Coast fever (ECF), heartwater, anaplasmosis and babesiosis, and also trypanosomosis (Uilenberg 1995; Minjauw & McLeod 2003; Maudlin 2006) and helminthosis (Gray, Connell & Phimphachanhvongsod 2012). Small-holder farmers are particularly vulnerable to the economic impact of infectious diseases of livestock. Generally tick-borne infections do not affect trade in livestock, but they are a significant cause of production losses (Perry & Young 1995). Losses include lowered production rates, mortalities, decreased reproduction rates and costs of treatment and control measures. These diseases also indirectly constrain livestock production through limiting the use of the highly susceptible improved breeds of livestock which are used in other countries to improve livestock productivity (Perry & Young 1995).

2. LITERATURE REVIEW

Anaemia is a common clinical sign of many of the tick-borne diseases, trypanosomosis and helminthosis. Anaemia is defined as an erythrocyte count, haemoglobin concentration or a packed cell volume (PCV) that is below what is considered as reference values for the species (Jain 1993). Anaemia is the most common abnormality of blood (Anon. 2009a) and

can be grouped into three classes, namely *haemolysis* (erythrocyte destruction); *haemorrhage* (loss of erythrocytes) and *dyshaemopoiesis* (ineffective erythrocyte production) (Jain 1993; Anon. 2009a). It is not unexpected that haemoparasitic infections can cause anaemia, since their life cycles are closely linked to the circulatory system of their hosts. Apart from their effect on erythrocytes, these infections can also bring about changes in the white blood cells and thrombocytes. The cells are affected either directly by the infecting parasites, or indirectly by the host's response to the infection.

2.1 Infectious causes of anaemia

2.1.1 Tick-borne diseases

Anaplasmosis

Anaplasmosis is predominantly caused by *Anaplasma marginale*, an arthropod-borne parasite. Also known as gall-sickness, clinical disease is generally associated with fever, progressive anaemia and icterus (Potgieter & Stoltsz 2004). Another species, *Anaplasma centrale*, is less virulent and seldom causes clinical disease. The principal vectors of this parasite are Ixodid ticks, in particular *Rhipicephalus* (*Boophilus*) *decoloratus*, but other ticks that can potentially act as vectors are *Rhipicephalus* (*Boophilus*) *microplus, Rhipicephalus simus, Rhipicephalus evertsi evertsi* and *Hyalomma marginatum rufipes* (Potgieter & Stoltsz 2004). Mechanical transmission of anaplasmosis by blood-sucking arthropods has also been described (Potgieter & Stoltsz 2004), as well as transplacental transfer (Aubry & Geale 2010).

Anaplasma spp. are intra-erythrocytic parasites. Acute disease is associated predominantly with anaemia, which is caused by extensive erythrophagocytosis. The animal presents with pallor of mucous membranes, inappetance, general weakness and fever. As the disease progresses clinical signs such as constipation, rumen stasis, icterus and weight loss become apparent. As the parasite damages erythrocytes, the cells are removed by the reticuloendothelial system in the spleen, lungs, liver and associated lymph nodes. Anti-erythrocytic antibodies also damage the non-parasitised red blood cells. Macroscopic pathology is typical for erythrophagocytosis and includes severe anaemia, icterus, splenomegaly and hepatomegaly (Potgieter & Stoltsz 2004).

Calves have an age-related resistance to severe clinical disease up to the age of 6 months (Potgieter & Stoltsz 2004). Calves that are born in endemic areas and are exposed during this period of resistance will develop immunity to the disease before the age when clinical effects become severe. Clinical disease usually develops only in older animals that have not

been exposed during calfhood (Gale, Leatch, De Vos & Jorgensen 1996b). Older animals infected with *A. marginale* develop more severe reactions with higher parasitaemias and percentage reductions in PCV than younger animals (Parker, Shephard, Trueman, Jones, Kent & Polkinghorne 1985; Aubry & Geale 2010). Although the prepatent period in anaplasmosis is inversely related to the infective dose, the clinical outcome, namely anaemia, does not appear to be correlated to the infective dose (Gale *et al*. 1996b). Once animals have recovered from anaplasmosis, they remain life-long carriers of the parasite (Potgieter & Stoltsz 2004; Aubry & Geale 2010).

Babesiosis

Bovine babesiosis is caused by *Babesia bovis* and *Babesia bigemina*, respectively known as Asiatic and African redwater. The only known vector of *B. bovis* in southern Africa is *R. microplus*. Transmission in the vector is transovarial (De Vos, De Waal & Jackson 2004). *Babesia bigemina* has several tick vectors, including *R. microplus, R. decoloratus* and *R. evertsi evertsi*. Transovarial transmission as well as transstadial transmission of the parasite occurs in *Rhipicephalus* (*Boophilus*) spp. Both nymphal and adult stages thus transmit the parasite to the host. Only the nymphal stages of *R. evertsi evertsi* can infect the host (De Vos *et al*. 2004). *Babesia* spp. are intracellular organisms but only erythrocytes are involved in the development of the parasite. Once the infective sporozoites enter the erythrocyte, they develop into trophozoites which in turn develop into two new merozoites which are infective to the tick vector (De Vos *et al*. 2004).

During the first two months of life, calves are protected from clinical babesiosis by passive transfer of immunity from resistant dams (Bock, Jackson, De Vos & Jorgensen 2004; De Vos *et al*. 2004; Magona, Walubengo, Olaho-Mukani, Jonsson, Welburn & Eisler 2008a). After two months of age calves have a natural resistance to babesiosis which lasts for 6 to 9 months, irrespective of the immune status of their dams (De Vos *et al.* 2004; Mahoney, Wright & Mirre 1973). Animals that have recovered from infection remain latent carriers for variable periods, after which they lose the infection (De Vos *et al.* 2004). Recovery is followed by a solid, long-lasting immunity, even in the absence of re-infection. In cattle that have recovered from *B. bigemina* there is a level of cross-immunity against *B. bovis* (Callow, McGregor, Parker & Dalgliesh 1974; Bock *et al.* 2004). The reverse is not true, however.

The clinical signs associated with *B. bigemina* infections are the result of severe intravascular haemolysis. Animals present with high fever, pallor, haemoglobinuria, icterus, inappetance and lethargy. Clinical signs associated with *B. bovis* infections are similar but more severe with the development of a hypotensive shock syndrome. Disseminated

intravascular coagulation results in generalized organ failure. Cerebral involvement is manifested by clinical signs of hyperaesthesia, circling, nystagmus, head pressing and aggression, and without intervention is almost invariably fatal. As in anaplasmosis, the initial infective dose in babesiosis does not correlate with the clinical outcome or time to recovery (Allred 2007; Gale *et al.* 1996b).

Ehrlichiosis

Ehrlichia ruminantium causes heartwater in cattle, small stock and some wild ruminants. Heartwater is characterised by fever, neurological signs, hydropericardium, hydrothorax and lung oedema. The vector for this parasite in Kenya is *Amblyomma variegatum*. Transstadial transmission occurs in this three-host tick. Young calves have an age-related resistance to disease which lasts about four weeks from birth and is independent of the immune status of the dam. As in other tick-borne disease, there is also a difference in susceptibility between breeds. Zebu cattle are regarded as more resistant, and although infection can still be established, severe clinical signs are lesssevere (Allsopp, Bezuidenhout & Prozesky 2004).

After infection of the host, the parasite invades the endothelial cells of blood vessels where they multiply. Effusion into body cavities and tissue oedema develops due to increased vascular permeability. The extensive effusion leads to a reduction in blood volume. *Ehrlichia ruminantium* parasites can be demonstrated in the cytoplasm of endothelial cells of blood vessels of the brain by light microscopy during a post mortem examination. Infection of the endothelial cells in the brain and the development of brain oedema cause neurological signs (Allsopp *et al*. 2004).

Bovine ehrlichiosis, caused by *Anaplasma* (*Ehrlichia) bovis*, and Ondiri disease, caused by *Cytoecetes ondiri*, are lesser known rickettsial diseases but have been associated with disease in cattle in Kenya (Sumption & Scott 2004).

Although it is expected that the distribution of *A. bovis* is widespread, few studies have been done to confirm this (Sumption & Scott 2004). In a study done in Uganda close to Lake Victoria, 63% of cross-bred cattle and 23% of indigenous cattle sampled were positive for *A. bovis*. This suggests that *A. bovis* may potentially be a significant cause of disease in the area (Oura, Bishop, Wampande, Lubega & Tait 2004). The epidemiology of bovine ehrlichiosis has not been fully described yet, but it is suggested that *R. appendiculatus* is the vector in East Africa. Although rare, overt disease presents with clinical signs of fever, lymphadenopathy, depression and weight loss (Sumption & Scott 2004). Neurological signs are sometimes seen in acute primary infection and can readily be confused with heartwater

(Stewart 1992). Exotic breeds or naïve indigenous cattle are particularly susceptible to disease. In contrast to heartwater, haematological changes on bloodsmears can aid in diagnoses. Monocytes typically show vacuolisation which may contain some rickettsias. The nuclear membrane is also distorted. Monocytosis with eosinopenia is suggestive of bovine ehrlichiosis (Stewart 1992). Thrombocytopenia is also present (Sumption & Scott 2004).

Ondiri disease, also known as bovine petechial fever, has a very restricted distribution in the East African highlands. The vector is still unknown. Clinical signs associated with Ondiri disease include fluctuating fever, lowered milk yield, and generalized petechiation of mucous membranes. Case-mortality rates of up to 50% have been described. Infected granulocytes on blood smears can aid in diagnosis. Ondiri disease is not regarded as an important disease in Kenya (Sumption & Scott 2004).

Theileriosis

Four *Theileria* species have been reported from Kenya, namely *Theileria parva, Theileria mutans, Theileria taurotragi* and *Theileria velifera* (Kariuki 1990). Two types of *T. parva* are found in Kenya. Buffalo-derived *T. parva* strains are maintained within African buffalo populations and are the cause of Corridor disease in cattle. The disease is acute and usually fatal and cattle are regarded as dead-end hosts for the parasite (Lawrence, Perry & Williamson 2004b).

Cattle-derived *Theileria parva* strains cause ECF in cattle, which is regarded as a major disease of cattle in East Africa (Young & Mutugi 1990b). Classical ECF (Lawrence, Perry & Williamson 2004a) is fatal in European cattle breeds, but Zebu cattle raised in endemic areas suffer a less severe form of the disease or remain unaffected. The principal vector is the brown ear tick, *Rhipicephalus appendiculatus* (Young & Mutugi 1990b). This is a three-host tick. Transmission of *T. parva* is trans-stadially between larval and nymphal stages, as well as between the nymphal and adult stages (Young, Leitch, Newson & Cunningham 1986). In East Africa all the instars of the tick occur on cattle throughout the year (Young & Mutugi 1990b).

Two cell lines of the host are intimately involved in the intracellular proliferation of *Theileria* spp., namely lymphocytes and erythrocytes. After infection the sporozoites enter the lymphocytes of the host and develop into schizonts, which stimulate the lymphocytes to transform into lymphoblasts after which the schizonts divide. These infected lymphoblasts proliferate exponentially and spread throughout the lymphoid tissue as well as metastasising to non-lymphoid tissues. Later still, merozoites are liberated from a generation of

microschizonts, which then enter erythrocytes and thus complete their life cycle (Lawrence *et al.* 2004a).

East Coast fever is characterised by lymphoid proliferation followed by lymphoid destruction. Animals present with fever, enlargement of superficial lymphnodes and severe pulmonary oedema. Panleukopenia coincides with the onset of pyrexia (Lawrence *et al*. 2004a). A non-regenerative anaemia has been described but is not a consistent feature of the disease (Mbassa, Balemba, Maselle & Mwaga 1994; Fanduma, Marcotty, Brandt, Duchaleau, Speybroeck, Dolan & Berkvens 2007). As opposed to babesiosis and anaplasmosis, the initial infective dose does affect the clinical outcome and severity of disease (Koch, Kambeva, Norval, Ocama, Masaka, Munatswa, Honhold & Irvin 1990).

Zebu cattle that are raised in ECF-endemic areas exhibit a low innate susceptibility (Perry & Young 1995). Mortalities in these Zebu calves are well below 10% (Moll, Lohding, Young & Leitch 1986). Indigenous breeds outside endemic areas and introduced breeds are highly susceptible and ECF is frequently fatal.

When calves recover after infection they can become asymptomatic carriers of the parasite and thus remain a source of infection to ticks. The persistence of infection in the host is due to division and persistence of the schizont stage of the parasite (Young, Mutugi, Kariuki, Maritim, Linyonyi, Mining, Kwena, Ngumi, Ndungu, Lesan, Lampard, Awich, Stagg, Leitch, Williamson & Grootenhuis 1990a). There is, however, limited cross-protection between different strains of *T. parva* (Morrison 2009). It is postulated that for endemic stability to develop, calves in endemic areas must become sequentially infected with several field strains (antigenic variants) of *T. parva* and thus become immune but also carriers of all the different strains in the field (Moll *et al*. 1986; Young *et al*. 1990a). In the Trans-Mara division of the Narok district, 100% of the calf population became infected by three months of age with at least three incidents of clinical ECF (Moll, Lohding & Young 1984; Moll *et al*. 1986). Where calves do not become infected during calfhood, either due to the unsuitability of the environment for the tick vector or due to too rigorous tick control, the population immunity against *T. parva* will be low and high mortality rates can occur (Perry & Young 1995).

Theileria mutans, T. taurotragi and *T. velifera* are considered less pathogenic than *T. parva*. *Theileria mutans* has been described as a cause of low growth rates in calves (Moll *et al*. 1984) and clinical cases are typically associated with mild transient fever and anaemia (Kariuki 1990). *Theileria mutans* and *T. velifera* are transmitted by *Amblyomma* ticks, whereas *T. taurotragi* is also transmitted by *R. appendiculatus* (Moll *et al.* 1984). Mixed

infections of different *Theileria* species occur and can complicate the clinical presentation of disease as well as diagnostic test results.

2.1.2 Tsetse-borne pathogens

Trypanosomosis

Trypanosomosis, caused by protozoan parasites of the genus *Trypanosoma,* has been one of the most important diseases in livestock in sub-Saharan cattle since before colonial times. It remains a serious constraint to economic development in the region (Maudlin 2006). The main vector is tsetse flies, *Glossina* spp., although other biting flies and mechanical transmission are implicated in the transmission of certain species (Connor & Van den Bossche 2004).

Of the tsetse-transmitted trypanosomes, *Trypanosoma congolense, Trypanosoma vivax* and *Trypanosoma brucei* are of particular importance in cattle. *Trypanosoma theileri*, a stercorarian parasite, is transmitted by tabanid flies. It is regarded as non-pathogenic, but has been associated with disease in certain circumstances (Connor & Van den Bossche 2004). These trypanosomes are blood-borne, with *T. congolense* confined to the circulatory system of the host. However, *T. vivax* and *T. brucei* also invade the host's tissues (Murray & Dexter 1988).

Anaemia is a well recognized consequence of trypanosomosis. In fact, PCV is used as an indicator for trypanosomosis on a herd-level, even when trypanosomes are not detected by parasitological diagnostics (Van den Bossche, Mudenge, Mubanga & Norval 1999; Van den Bossche & Rowlands 2001b). Murray & Dexter (1988) divided the progression of anaemia in trypanosomosis into two phases. During the first, acute phase of anaemia the PCV falls at the same time that the first wave of parasitaemia develops. Anaemia is indirectly caused by infection through the activation of mononuclear phagocytosis which results in massive erythrophagocytosis. At the same time, trypanosome haemolysins and proteases also induce haemolysis (Murray & Dexter 1988). There is an indication of increased haemopoiesis to compensate for the massive erythrophagocytosis. The second, chronic phase can overlap with the first phase, but is associated with low transient parasitaemias. Erythrophagocytosis is ongoing but there are now indications of dyshaemopoiesis. The synthesis of red blood cells is insufficient to compensate for the degree of anaemia seen (Dargie, Murray, Murray, Grimshaw & McIntyre 1979; Murray & Dexter 1988). This indicates a degree of bone marrow dysfunction. The affected animal can show either spontaneous recovery, survive this phase with persistent low-grade anaemia or die. The response to treatment during the acute phase is rapid, compared to the chronic phase where treatment is

often ineffective (Urquhart & Holmes 1987). An acute haemorrhagic syndrome has been described in *T. vivax* (Magona, Walubengo & Odimin 2008b).

Pancytopenia develops in the host during the first wave of the parasitaemia. Apart from anaemia, thrombocytopenia and leukopenia are also constant findings in trypanosomosis (Wellde, Kovatch, Chumo & Wykoff 1978; Murray & Dexter 1988). The decrease in thrombocytes, lymphocytes and neutrophils correlate with the onset, severity and prevalence of trypanosomes in the host's blood. The development of disseminated intravascular coagulation in end-stage trypanosomosis adds to the loss of thrombocytes and is reflected by generalized petechiae and ecchymoses seen at post mortem examinations (Murray & Dexter 1988).

Immunosuppression in trypanosomosis is also well recognized (Holmes, Mammo, Thomson, Knight, Lucken, Murray, Murray, Jennings & Urquhart 1974; Mackenzie, Boyt, Emslie, Lander & Swanepoel 1975; Askonas 1984; Urquhart & Holmes 1987, Uilenberg 1998). Although the host exhibits an active lymphoid response, an immune response to other antigens is almost completely lacking, in particular antigens that stimulate an antibody response (Urquhart & Holmes 1987).

The long-term effects of trypanosomosis in cattle are emaciation, reduced growth rates, reduced reproductive rates, abortions and reduced milk yields (Trail, D'Ieteren, Murray, Ordner, Yangari, Collardelle, Sauveroche, Maille & Viviani 1993). Exotic breeds of cattle are more susceptible than local breeds, such as N'dama cattle (Connor & Van den Bossche 2004).

2.1.3 Gastrointestinal parasites

The most significant impact of helminthosis in cattle in Africa is through the erosive effect of reduced weight gain. Often these sub-clinical production losses largely go unnoticed (Waller 1997; Waruiru, Weda, Otieno & Ngotho 2002). Gastrointestinal helminths can be a significant cause of anaemia in ruminants, however, in particular haemonchosis and fasciolosis (Kaufmann, Dwinger, Hallebeek, Van Dijk & Pfister 1992). *Haemonchus* is considered as one of the most pathogenic parasites of ruminants (Kaufmann1996) and is consistently reported as the most prevalent helminth species in cattle in Kenya (Moll *et al.* 1984; Latif, Rowlands, Punyua, Hassan & Capstick 1995; Waruiru, Thamsborg, Nansen, Kyvsgaard, Bogh, Munyua & Gathuma 2001; Waruiru *et al.* 2002). The pathogenesis is that of a haemorrhagic anaemia (Kaufmann *et al.* 1992). During extremely high parasite burdens the animal will die due to haemorrhage from the abomasum. In chronic cases animals

develop a steady drop in PCV and serum albumin which results in emaciation of the animal. If the animal survives, the compensatory erythropoiesis will eventually deplete iron reserves.

Fasciolosis is an important disease in areas where the fluke's intermediate host, *Lymnaea* spp. snails, occur (Kaufmann 1996). Acute death due to liver fluke occurs rarely in cattle. Chronic infestation is more common and the animal presents with gradual wasting, severe anaemia, ascites and oedema and high faecal fluke egg counts. In massive infections, the fluke's migration through the liver of the host can cause acute anaemia. However, anaemia is mainly caused by the consumption of blood by the flukes when they arrive in the bile ducts. Initially the host exhibits compensatory erythropoiesis but eventually an iron deficiency develops which contributes to the level of anaemia. There is also a marked eosinophilia (Kaufmann 1996).

Prevalence of helminth infections appears to have a seasonal pattern, with faecal egg outputs reported to follow rainfall patterns (Fall, Diack, Diaté, Seye & d'Ieteren 1999; Waruiru *et al.* 2002). Where climatic conditions in parts of Kenya are favourable, however, hypobiosis does not appear to be important in the epidemiology of certain nematode species and animals remain at risk of infection throughout the year, be that at a lower level during the dry season (Waruiru *et al.* 2001). There also appears to be an age-related susceptibility, with calves harbouring higher burdens than adult cattle (Latif *et al.* 1995; Fall *et al.* 1999). This is possibly due to an acquired resistance that develops within five to eight months after weaning (Latif *et al.* 1995).

Coccidiosis is another potential cause of haemorrhagic diarrhoea and anaemia in cattle (Stewart & Penzhorn 2004). It primarily affects calves from three weeks to six months of age. Clinical coccidiosis in cattle is most commonly caused by *Eimeria zuernii* and *Eimeria bovis* (Kaufmann 1996). The clinical course of infection is dose-dependent, with overt disease only developing in animals that have ingested a large number of oocysts (Stewart & Penzhorn 2004).

2.1.4 Non-infectious causes of anaemia

There are also non-infectious causes of anaemia, such as nutritional deficiencies, traumatic blood loss and physiological anaemias in neonates. Nutritional deficiencies that result in anaemia include mineral deficiencies of iron, copper, cobalt and selenium, and certain vitamins (Jain 1993). Nutritional deficiencies are generally associated with dyshaematoopoiesis and cause normocytic, normochromic non-regenerative anaemia, suggesting that the bone marrow response is ineffective (Jain 1993). Iron deficiencies cause

microcytic hypochromic anaemia due to impaired synthesis of haemoglobin (Duncan, Prasse & Mahaffey 1994). Poor nutrition and resultant nutritional deficiencies will exacerbate anaemia caused by infectious agents and inhibit the host's response to treatment (Waller 1997; Waruiru *et al.* 2002; Swai, Karimuribo, Kambarage & Moshy 2009).

In several ruminant species the red cell parameters in neonates has been shown to be below reference ranges described for adult animals (Karesh, Janssen & Oosterhuis 1986; Harvey 1989; Parsons, Penzhorn, Reyers, Steyl & Becker 2006). Physiological changes soon after birth generally cause a significant decrease in haematocrit which is followed by a gradual increase to adult levels (Harvey 1989; Roberts 2011). Factors thought to contribute to these changes include: 1) an increase in plasma volume due to the osmotic effect of absorbed colostral proteins; 2) a decrease in the production of red blood cell early in the neonatal period; 3) red blood cells formed *in utero* have a decreased life span; 4) total plasma volume expansion occurring more rapidly than red cell numbers resulting in haemodilution; and 5) a temporary decrease in erythropoeitin production in certain species (Harvey 1989). A delayed switching from foetal haemoglobin to adult haemoglobin has been described in several ruminant species, including Mouflon sheep (Hawkey, Hart & Fitzgerald 1984) and roan antelope (*Hippotragus equinus*) (Parsons *et al*. 2006). This delay results in a decrease in total haemoglobin, and thus causes anaemia in neonatal animals. A possible link between this neonatal anaemia in roan antelope and an increased susceptibility to theileriosis has been suggested (Parsons *et al*. 2006).

2.2 From infection to disease

2.2.1 Concepts and definitions

Clinical disease does not invariably result when a pathogen infects a host. Several factors associated with the pathogen, the vector, the host, as well as the environment interact and as a whole determine the outcome of infection. A few relevant concepts and definitions will be briefly discussed.

Definitions

- *Immunodepression* is the lowering of an immune response or its complete abrogation (Cox 1987).
- *Non-specific immunity* is the protective immune response against subsequent infections not directed at a specific antigen (Cox 1987).

- *Heterologous immunity* is the immunity induced by a related or unrelated infectious agent, also called *cross-protection* (Cox 1987; Clark 2001).
- *Concomitant immunity:* The host has acquired immunity against reinfecting or clinical disease, yet remain persistently infected (Cox 1993; Brown, Norimine, Knowles & Goff 2006).
- *Premunity:* recovered animals remain latently infected without showing clinical signs (De Vos *et al.* 2004).

Endemic stability

There are situations where, without human interventions, the pathogen, vector, and host co-exist in an environment without any significant effect on animal production and few if any clinical cases are reported. Such a situation is referred to as endemic stability (Perry & Young 1995; De Vos *et al.* 2004).

Endemic stability of ECF is reported from calves in areas of Kenya (Young *et al.* 1986) and Uganda (Okello-Onen, Heuer, Perry, Tukahirwa, Senyonga, Heinonen & Bode 1995). According to Perry and Young (1995) the low incidence of clinical ECF in endemically stable areas can be attributed to: 1) the Zebu cattle that are raised in endemic ECF areas that show a *high innate resistance* to ECF; 2) the acquired immunity to infection develops rapidly and effectively due to a *low but continuous exposure to ticks*; and 3) because of low parasitaemias in immune carrier animals there are *low infection rates in ticks*. In these situations, there are few clinical cases of ECF and mortality is limited to young animals. There is a strong correlation between endemic stability and high antibody prevalence (Perry & Young 1995). Typically, high antibody prevalence rates for *T. parva* (70%) occur in animals over six months of age (Okello-Onen *et al.* 1995). The high antibody prevalence is possibly due to persistence of infection, re-infection or superinfection (Young *et al.* 1986). The age of first exposure is also an important factor in establishment of endemic stability of *Babesia* (De Vos *et al.* 2004). If calves are infected with *Babesia* during the period of age-related resistance early in life, a solid, long-lasting immunity will develop. In *Babesia* infections premunity is not considered to be important. After recovery from infection, cattle develop a lasting immunity even in the absence of infection (De Vos *et al.* 2004).

High antigenic diversity of *T. parva* as well as a lack of cross-immunity between strains is well-described. The implication of this is that for endemic stability to occur calves must be infected with the whole spectrum of prevailing antigenic strains over time (6 months) and thus undergo several clinical episodes of *T. parva* infections (Young *et al.* 1986). This period

coincides with the period of passively derived maternal antibody protection (Okello-Onen *et al*. 1995).

Endemic stability can develop for babesiosis as well. During the first two months calves are protected after birth by colostral antibodies. An innate immunity against babesiosis exists in calves from three to nine months of age. When calves are infected during this period, they rarely develop clinical disease but they do develop a solid long-lasting immunity. When primary infection only occurs after this innate immunity has passed, animals will develop severe clinical signs, which is typical under endemically unstable conditions (Bock *et al*. 2004).

The frequency at which transmission of the pathogen occurs is also important. *Babesia bigemina* has higher transmission rates in its tick vector compared to *B. bovis.* Because of this, *B. bigemina* has a higher prevalence than *B. bovis* in areas where both species occur. Endemic stability is therefore more likely to develop to *B. bigemina* than to *B. bovis* (De Vos *et al.* 2004).

In endemic *Trypanosoma* areas where cattle serve as the main reservoir of trypanosomes a stable situation can also potentially develop. In these areas there is a selection pressure against strains of trypanosomes that are very pathogenic or lethal, thus these strains become rare in the herd of cattle. Under these circumstances cattle are able to develop a non-sterile immunity which contributes to the development of an endemic state of trypanosomosis where its effect on herd production is relatively low, such as in Petauke District of eastern Zambia (Van den Bossche 2001a). In areas where tsetse flies rely heavily on wildlife as a source of blood meals, there is no selection pressure against the low pathogenic strains of trypanosomes and disease occurs in epidemics that are unpredictable. In these situations the impact of trypanosomosis on the production of cattle can be considerable (Van den Bossche 2001a).

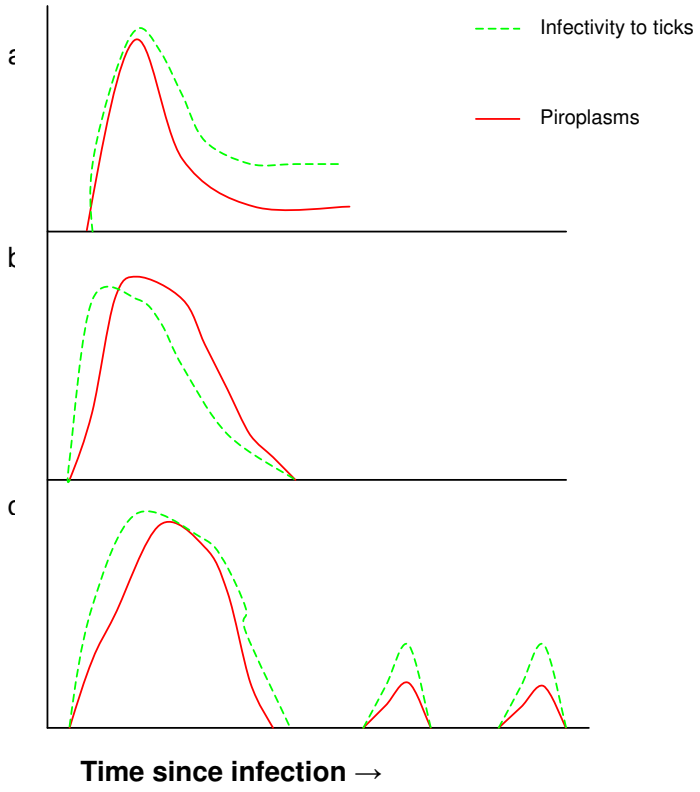
Carrier state

A carrier state has been defined as “the ability of parasites present in an infected and recovered (and therefore asymptomatic) host to infect ticks which are then able to transmit the parasites to a susceptible host” (Bishop, Sohanpal, Kariuki, Young, Nene, Baylis, Allsopp, Spooner, Dolan & Morzaria 1992).

Three types of carrier states in *T. parva*-infected hosts are described by Young, Mutugi, Maritim & Linyonyi (1990c). Cattle can act as initial carriers after which they clear the

infection, or they can act as either sporadic or continual carriers. This will depend on whether there are gametocytes infective to ticks present in the blood intermittently or continuously (Figure 1.1).

Figure 1.1 Types of carrier states (from Young *et al*. 1990c): a. Persistent carrier; b. Initial carrier; and c. Sporadic carrier



Carrier state in *Babesia* is seldom life long. Cattle infected with *B. bigemina* remain infective for ticks for a few weeks only and lose the infection in less than a year. Zebu cattle will lose an infection of *B. bovis* within two years. European breeds can remain infected with *B. bovis* for life, but only remain infective for ticks for up to two years (De Vos *et al.* 2004). Animals recovered from *Anaplasma* infections, however, remain infected for life (Potgieter & Stoltsz 2004).

The carrier state in animals can revert to clinical disease if the immune system of the animal is compromised to the extent that it cannot contain the infection. An example would be animals that are latently infected with *Anaplasma* that develop anaemia after they are superinfected with *T. parva* which causes immunosuppression (Moll *et al.* 1984).

Breed tolerance and susceptibility

The potential exploitation of possible genetic resistance to disease has been the subject of much investigation, especially with regard to resistance to trypanosomosis (Murray, Morrison & Whitelaw 1982). The term "trypanotolerant" is used in literature in different ways. Naessens, Teale & Sileghem (2002) used the term to describe animals that can remain productive while infected with trypanosomes. Murray *et al.* (1982) feel it more appropriate to use tolerance to describe animals that exhibit a greater degree of resistance to the disease. Trypanotolerance has also been associated with traits such as weight gain, an ability to control anaemia and efficient clearance of parasites (Van der Waaij, Hanotte, van Arendonk, Kemp, Kennedy, Gibson & Teale 2003).

The N'Dama breed of West Africa and West African shorthorn cattle are considered to be trypanotolerant. Zebu cattle in East Africa are more susceptible, but in some areas have developed a degree of trypanotolerance compared to European exotic cattle breeds (Murray *et al.* 1982). Indigenous wildlife are highly resistant or completely refractory to infection by trypanosomes and often serve as reservoirs of disease in certain areas (Murray *et al.* 1982, Van den Bossche 2001a).

Trypanotolerant cattle have a superior immune response against infection, thus develop lower parasitaemias, and also exhibit an ability to control the level of anaemia better, as compared to less tolerant breeds (Naessens *et al.* 2002). Tolerance is relative, however. The severity of clinical symptoms is related to infective dose, and when the challenge is high enough, even trypanotolerant breeds will develop disease (Murray *et al.* 1982).

Differences in breed susceptibility have also been described for other diseases. Taurine breeds are also considered to be more susceptible to a variety of tick species, ECF, babesiosis and anaplasmosis compared to Zebu and Sanga breeds (Norval, Perry & Young 1992; Perry & Young 1995; De Vos *et al.* 2004; Potgieter & Stoltsz 2004). However, one should bear in mind that even indigenous breeds that are immunologically naive will potentially develop severe disease when introduced into endemic areas (Perry & Young 1995).

2.2.2 Epidemiological states of vector-borne diseases in Kenya

Several studies have investigated the prevalence and epidemiological states of tick-borne diseases and trypanosomosis in cattle populations at a range of study sites throughout Kenya, including in the Coastal Province (Deem, Perry, Matende, McDermott, Mahan, Maloo, Marzaria, Musoke & Rowlands 1993; Maloo, Thorpe, Kioo, Ngumi, Rowlands & Perry

2001b), the Eastern Province (Gachohi, Ngumi, Kitala & Skilton 2010), in the Rift Valley (Moll *et al.* 1984; Moll *et al*. 1986); western Kenya (Okuthe & Buyu 2006); and the Central Province (Gitau, Perry, Katende, McDermott, Morzaria & Young 1997; Gitau, Perry & McDermott 1999; Gitau, McDermott, Katende, O'Callaghan, Brown & Perry 2000), as well as the neighbouring countries Tanzania (Swai, French, Beauchamp, Fitzpatrick, Bryant, Kambarage & Ogden 2005a; Swai, French, Karimuribo, Fitzpatrick, Bryant, Browne & Ogden 2005b) and Uganda (Rubaire-Akiiki, Okello-Onen, Musunga, Kabagambe, Vaarst, Okello, Opolot, Bisagaya, Okori, Bisagati, Ongyera & Mwayi 2006). In almost all of these areas ECF was recognized as the most important infectious disease in terms of morbidity, mortality and low production in cattle populations in Kenya (Uilenberg 1995; Phiri, Benschop & French 2010). Other vector-borne diseases that contributed considerably to morbidity in cattle populations, depending on endemic state of the causative pathogen, were anaplasmosis and trypanosomosis. Several risk factors have been identified by these studies that are associated with both spatial and temporal variation in the epidemiological states of vector-borne pathogens, and thus their association with disease.

Spatial distribution of pathogens

The endemic stability of the various pathogens varied considerably over the various study sites. The main attribute that affected the spatial variation in seroprevalence was agro-ecological zone (AEZ). Agro-ecological zone is a classification of smaller units of land based on soil type, landform, climatic conditions and land cover (FAO 1996). These characteristics determine the main land-use practices, e.g. mixed crop-livestock farming or dairy, and thus the predominant breed of cattle farmed and grazing systems used by farmers in such a zone (Gitau *et al*. 1997). These geophysical characteristics, together with the management practices employed by farmers in turn determine the ecological suitability for the vectors of these diseases (Deem *et al*. 1993).

Altitude is an important determinant of vector distribution. The prevalence and disease incidence of ECF in Uganda was reportedly higher at lower elevation (Rubaire-Akiiki *et al*. 2006). The calves in the lowland zone had a 2.6 times higher risk of seroconversion to ECF than calves in the upland zone due to a higher number of *R. appendiculatus* found in the lowland AEZ (altitude of 428-1275m). The highest number of *R.* (*B.) decoloratus* was in the upland AEZ (altitude of 1575-4368m). A similar pattern was described in the Murang'a District in Central Kenya (Gitau *et al*. 1997) where seroprevalence of *T. parva* was higher in lowland zones, compared to higher seroprevalence of *B. bigemina* in highland zones, which correlated with the abundance of the respective tick vectors in the two AEZ.

Different grazing systems affect the prevalence of TBD in cattle by influencing the levels of exposure cattle have to ticks. Calves in the same lowland AEZ in Uganda in a communal grazing system had a 10 times higher risk of seroconversion to ECF than zero-grazed calves (Rubaire-Akiiki *et al*. 2006). Cattle in an open-grazing, communal system are at a higher risk of exposure to ticks and contracting TBD (Gitau *et al.* 1999). It is expected that livestock in zero-grazing systems, especially with apparent stringent acaricide use would have no tick challenge, and thus a low prevalence or incidence of TBD (Maloo, Rowlands, Thorpe, Gettinby & Perry 2001a; Maloo *et al*. 2001b). However, cut forage is often a source of ticks, which are maintained by pastoral livestock. Thus, zero-grazing reduces, but does not prevent exposure to ticks (Swai *et al*. 2005b). Anaplasmosis was not necessarily found to be associated with grazing practices as stall-fed animals had similar incidence rates of anaplasmosis as grazed animals (Gitau *et al*. 1997). This is because *A. marginale* is also transmitted by biting flies (Gitau *et al.* 1997; Aubry & Geale 2010). Swai *et al*. (2009) on the other hand found that nutritional and environmental stress in pastoral grazing systems in Tanzania caused a higher mortality due to anaplasmosis.

Okuthe & Buyu (2006) reported a higher incidence and prevalence of theileriosis, anaplasmosis and babesiosis in peri-urban areas than in rural areas in the highlands of western Kenya. They attributed this to three reasons: a) more irregular tick control in peri-urban areas; b) rural people prioritising livestock rearing more highly; and c) the difference in grazing practices. Cattle in rural areas were kept in smaller groups and had more pasture available to them compared to peri-urban areas. Swai *et al.* (2005b) on the other hand found that cattle in rural areas were up to four times more likely to seroconvert to *T. parva* than cattle kept around town areas.

Temporal distribution of pathogens and incidence of disease

Seasonal variation in vector-borne parasites correlates with the seasonal occurrence of their respective vectors (Latif *et al*. 1995). In ECF endemically stable areas, the favourable climatic conditions allow for all instars of *R. appendiculatus* to occur simultaneously throughout the year (Deem *et al*. 1993). Therefore a good level of herd immunity in calves develops due to sufficient challenge by *T. parva* parasites. Seroprevalence in the calves is typically lower in areas where climatic conditions are less favourable and tick seasonality is more pronounced. In these endemically unstable conditions, herd immunity against *T. parva* is insufficient, which renders many adult cattle susceptible to ECF.

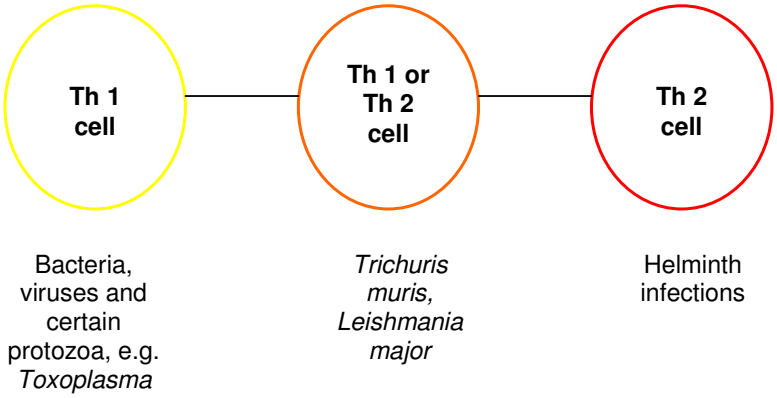
Seasonal fluctuations in rainfall also dictate the availability of forage to livestock. In Nigeria, nutritional stress during the dry season was thought to compound the effect of haemoparasites (Kamani, Sannusi, Egwu, Dogo, Tanko, Kemza, Tafarki & Gbise 2010).

Seasonal patterns in Western Kenya are associated with rainfall rather than daily temperature. The annual rainfall in western Kenya is bimodal, with two rainy seasons from April-July (long rains) and September-November (short rains) (Anon. 2009b). The climate is equatorial with little difference in hours of daylight between months of the year. An increase in cattle diseases was observed during the long rainy season in Busia, but this seasonal pattern was not consistent between years (Thuranira-McKeever, Shaw, Machila, Eisler, Welburn & Maudlin 2010).

2.2.3 Host immune mechanisms against pathogens

There are two arms to the immune cascade with one represented by the Th1-cell cytokine secretion profile and the other by the Th2-cell profile. During infectious disease, depending on the antigen present, polarization towards either a Th1-cell response or a Th2-cell response occurs (Figure 1.2) (Jankovic, Liu & Gause 2001).

Figure 1.2 The polarization of immune responses towards Th1-cell or Th2-cell responses (from Jankovic, Liu & Gause 2001)



The Th1- and Th2 cell have common T-helper (Th) cell precursors and the direction taken by the immune response is only determined at the level where the antigen interacts with antigen-presenting cells (APC) and dendritic cells (DCs). In the spleen and the T-cell-rich regions of lymphnodes antigen is presented to naïve T-helper cells by DCs. Dendritic cells express recognition receptors that will bind to molecular structures which are common to a

group of pathogens. From here the DCs express signals that will direct the effector Th cells towards either the Th1- or the Th2-cell cascade, depending on the antigen encountered. In both the Th1- and the Th2-cell cascades humoral and cell-mediated immune responses play a role, but different types of effector cells and antibody isotypes are involved (Jankovic *et al.* 2001).

Very briefly, the Th1-cells produce interferon-gamma and tumor necrosis factor which will activate macrophages and induce the production of immunoglobulin G (IgG) that supports opsonisation and phagocytosis. Also, Th-1 cells support antiviral effector T-cells. Th2-cells secrete interleukin-4 (IL-4), IL-5, IL-9, IL-10 and IL-13, which stimulate eosinophils and mast cells are stimulated to differentiate, aided by IgE (Jankovic *et al.* 2001).

2.2.3a Immunity against bovine protozoan infections

Babesiosis

Immune responses against *Babesia* infections involve both humoral and cell-mediated mechanisms. Calves have a natural resistance to babesiosis after two months of age, which lasts for 6 to 9 months, irrespective of the immune status of their dams (De Vos *et al.* 2004; Mahoney *et al.* 1973). This innate immunity is mainly cell mediated and the spleen has been shown to play a major role in this non-specific immunity (Bock *et al*. 2004). Monocytes, macrophages and neutrophils, together with the resultant cascade of antimicrobial agents, cytokines and enzymes, including nitrous oxide (NO), are responsible for phagocytosis of the pathogens.

Acquired immunity against re-infection in recovered animals or in vaccinated animals is strong and potentially life-long. Some bovines can become latent carriers of the parasite after recovery from disease without the development of clinical symptoms. Premunity is not required to maintain protection against re-infection with *Babesia* spp. (Callow & Dalgliesh 1982). The involvement of antibodies in immunity against babesiosis is through their role as opsonins which mediate phagocytosis during cell-mediated cytotoxicity. In response to re-infection, Th-1 (mainly CD4+) cells proliferate, which in turn promotes phagocytic cells, as well as promoting immunoglobulin production by autologues B- lymphocytes. The strain-specific antibodies are of mostly IgM and also IgG1 and IgG2 type and are directed against antigens on the surface coat of infected erythrocytes as well as the parasites (Bock *et al*. 2004; De Vos, Dalgliesh & Callow 1987, De Vos *et al*. 2004). Immunity against the sporozoite stage is an antibody response, but it is not known if this response is effective against re-infection. The response against the erythrocyte stage appears, in part, to be

antibody mediated, and prevents the invasion of red blood cells (Cox 1993). The antibody levels are, however, not correlated with the level of immunity in the animal.

Theileriosis

Immunity against *Theileria* infection is thought to be primarily cell-mediated and directed against the schizont stage. Cytotoxic T-lymphocytes (CD8+) recognize parasite- and strain-specific surface antigens on schizont-infected lymphoblasts in a classical MHC (major histocompatability complex) class I restricted reaction (Cox 1993; McKeever 2006; Morrison 2009). Other cellular responses, such as provided by CD4+ helper T cells, are secondary to and play a supportive role in the cytotoxic T-lymphocyte reaction (Morrison, Taracha & McKeever 1995). Memory T-cells are activated in re-exposed animals, which in turn stimulate the activation of the cytotoxic T-lymphocytes. The cytotoxic T lymphocytes target the schizont-infected lymphocytes, which allow the persistence of piroplasma-infected red blood cells (McKeever 2006). Recovered cattle have immunity against re-infection by homologous *T. parva* strains but cross-immunity against heterologous strains is poor (Irvin & Morrison 1987).

Infected animals elicit a humoral response, with antibody against all stages of *Theileria* parasites (Norval *et al.* 1992). The antibody response against sporozoites is directed against a cluster of surface antigens which neutralize infectivity *in vitro*, is less strain-specific than antibody against schizont stages, and is thought to play a protective role in subsequent infections. Antibody responses against schizonts are not considered to be important in acquired immunity (Lawrence *et al*. 2004a), but are exploited in the field of diagnostics. In haemoproliferative *Theileria* species, such as *T. mutans*, antibody against the piroplasm stage has proved to be protective (Irvin 1985).

Trypanosomosis

Trypanosomes are extracellular parasites. In contrast to *Babesia* and *Theileria* species, it appears that immunity to trypanosomes is primarily antibody mediated. The main target for IgM antibodies is the surface coat of trypanosomes which consists of glycoprotein. The antibody response agglutinates trypanosomes and initiates complement-mediated lysis. Before the infection is completely cleared, however, some of the parasites switch to a gene that codes for a different protein, namely a variable surface glycoprotein (VSG), which is not recognized by the initial antibody response. Infection with trypanosomes is characterized by successive waves of parasitaemia. Each parasitaemic peak consists of parasites in which a different VSG predominates. This VSG has enormous capacity for antigenic variation, called

variable antigen types (VAT), and is a very effective mechanism for evasion of the host's immune responses (Connor & Van den Bossche 2004; Mansfield & Paulnock 2005),.

Eventually, if the animal survives, it will have built up a degree of immunity against all major variants prevalent in the field. However, repeated immune responses to changing targets (VSGs) leads to gross immunopathological changes, such as auto-anitbodies against the host red blood cells and platelets, and prolonged immunosupression (Cox 1993).

Immunosupression due to trypanosome infections is well-known, although the aetiology is not yet fully understood. Several factors have been implicated, including blocking of the release of antibody by plasma cells, impaired T-helper function, B-cell mitogens and depletion of lymphoid cells. Disintegrating trypanosomes initiate the release of toxic products, as well as the release of cytokines by cells of the mononuclear phagocytic system (MPS). Both probably contribute to the immunosupression (Connor & Van den Bossche 2004).

2.2.3b Host immune response against helminth infections

Helminths are typically long-lived and are associated with chronic infections. In order to survive, these parasites need to modulate the immune response by their host, be that by suppressing the parasite-antigen-specific response or general immunosuppression. Helminth infections are typically associated with polarization towards a Th2-type response, as indicated by increased peripheral eosinophil levels and mast cells, as well as parasite-specific IgE levels (Maizels & Yazdanbakhsh 2003). Peripheral eosinophilia can be of value clinically as a marker for parasitic infection (Bejon, Mwangi, Lowe, Peshu, Hill & Marsh 2008). Experimental trials have shown that the peak of peripheral eosinophilia is associated with expulsion of worms, as indicated by a drop in faecal output of worm eggs in infected lambs (Buddle, Jowett, Green, Douch & Risdon 1992) and adult sheep (Doligalska, Moskwa & Stear 1999). Eosinophil counts can be used as a measure of the Th-2 immune-mediated response to infestation (Dawkins, Windon & Eaglesons 1989; Bejon *et al*. 2008; Jolles, Ezenwa, Etienne, Turner & Olff 2008) and has been used to monitor patients' recovery from helminth infection (Loutfy, Wilson, Keystone & Kain 2002).

Cytokines associated with Th2-response down-regulate potentially pathogenic Th1-cell-mediated inflammation, thus ensuring the host's own survival and ultimately the parasites' survival. This suppression is dependent on high parasite intensities (Maizels & Yazdankakhsh 2003) with a stimulatory effect seen in low infestation burdens (Kamal & El Sayed Khalifa 2006). These modulations affect immune responses against co-infecting

pathogens and the outcome can be either beneficial or detrimental for the host. The beneficial effect of the modulatory effect in allergic conditions in persons infected with helminths is well documented (Maizels & Yazdanbakhsh 2003; Kamal & El Sayed Khalifa 2006). On the other hand, helminth-infected hosts might have an impaired immune response to other pathogens where Th1-cell responses are required to limit the progression of infection.

2.3 Interactions between concomitant pathogens

Traditionally studies on infectious diseases have focused on single pathogens, often based on experimental conditions. In the field, however, it is impossible to study a disease complex in isolation without reference to other causes of disease (Moll *et al.* 1984). This is because animals in the field are exposed to a variety of pathogens that occur in the same environment (Petney & Andrews 1998). In fact, humans and animals living under field conditions are more likely to suffer from concomitant infections than single infections (Cox 2001; Telfer, Birtles, Bennett, Lambin, Paterson & Begon 2008). The higher the prevalence of each pathogen, the more likely a host will harbour co-infections of the various pathogens (Petney & Andrews 1998). The same risk factors that predispose a host to become infected with one pathogen might also increase the risk of infection with another pathogen. Swai *et al*. (2005b) found a correlation between tick-borne diseases in Tanzania in that cattle that were seropositive to one tick-borne pathogen were more likely to be seropositive to another tick-borne pathogen.

Several studies have reported concomitant infections of trypanosomes, *Anaplasma, Babesia, Theileria* and helminth species in cattle in Africa (Magona & Mayende 2002; Swai *et al*. 2005b; Kamani *et al*. 2010; Marufu, Chimonyo, Dzama & Mapiye 2010), and particularly Kenya (Moll *et al*. 1984; Maloo *et al*. 2001b; Muraguri, McLeod, McDermott & Taylor 2005).

2.3.1 Pathogen interactions

It is easy to conceive that pathogens that concomitantly infect a host would directly interact with each other, particularly pathogens that occupy the same niche in the host, e.g. the abomasum or red blood cells. These pathogens compete for the same resources, such as nutrients and attachment sites. The outcome is often a limitation on population size in either or both of the implicated pathogens (Petney & Andrews 1998).

Pathogens can also interact indirectly, by modifying the host's resistance or susceptibility to other infections. The immune modulation by helminth parasites has already been discussed

(see 3.3.2). Through polarization of the T-cell response towards the Th2- type response, the helminth parasite facilitates the establishment of the protozoal parasite, for instance by impairing the host's response against the super-infecting protozoal parasite.

Many pathogens are associated with immunosuppression of the host, e.g. trypanosomes (Holmes *et al*. 1974; Mackenzie *et al*. 1975; Askonas 1984; Urquhart & Holmes 1987); and ECF (Moll *et al*. 1986). When one pathogen causes immunosuppression in the host, it will lead to the recrudescence of other infections. Immunosupression can be caused directly by the implicated pathogen by depleting the host's lymphoid cells, or by impairing its cellular functions. By-products of ongoing protective immune or allergic reactions in the host might also result in immunodepression.

The outcome and type of interaction between pathogens are in part determined by the timing of each infection. Non-specific immune reactions against the first pathogen might impair the establishment of the second pathogen. Heterologous immunity, or cross-protection, induced by the first pathogen can in some cases be either partly or completely protective against a second pathogen (Clark 2001). This, in turn, will alter the clinical course of infection of the second pathogen.

The sequence in which the host acquires different infections also affects the clinical outcome and prognosis of disease. The interaction between *Trypanosoma congolense* and *Haemonchus contortus* in Djallonké sheep (Goossens, Osaer, Kora, Jaitner, Ndao & Geerts 1997) and in N'Dama cattle (Kaufmann *et al.* 1992) was investigated. Both breeds are regarded as *Trypanosoma* resistant. Sheep that were initially infected with *Haemonchus* larvae and subsequently super-infected with *T. congolense* developed less severe chronic anaemia. When sheep were first infected with *T. congolense* and subsequently infected with *Haemonchus* larvae, however, the sheep developed acute signs of trypanosomosis with severe drops in PCV (Goossens *et al.* 1997). Cattle with a primary *T. congolense* infection that were super-infected with *H. contortus* had a reduced prepatent period of *H. contortus* infection and showed increased pathogenicity as compared to controls. When a patent *H. contortus* infection was followed by a *T. congolense* infection, the disease process was even more aggravated (Kaufmann *et al.* 1992).

2.3.2 Clinical implications of concurrent infections

Each pathogen contributes to the clinical outcome and prognosis of infection in the host. Interactions between co-infecting pathogens can influence the course of the resultant infection (Petney & Andrews 1998). These interactions can potentially be synergistic,

neutral, as well as antagonistic from the point of view of the host (Cox 1987; Petney & Andrews 1998). Petney & Andrews (1998) summarise how the interaction between two pathogens can affect the pathogenicity to the host (see Table 1). The more pathogens that concurrently infect a host, the more outcomes are possible.

Table 1 Possible outcomes of a two-species parasite infection on the pathogenicity to the host (from Petney & Andrews 1998)

Influence of parasite 1 on parasite 2	Influence of parasite 2 on parasite 1	Relationship to pathogenicity
+	+	Each species increases the pathogenicity of the other
+	0	One species shows increased pathogenicity, the other its usual pathogenicity
+	-	One species shows increased pathogenicity, the other reduced pathogenicity
0	0	Neither species affects the other
0	-	One species shows its usual pathogenicity, the other reduced pathogenicity
-	-	Both species show reduced pathogenicity

Interaction between pathogens can result in reduced prepatent periods (Kaufmann *et al.* 1992), increased pathogenicity (Kaufmann *et al.* 1992; Goossens *et al.* 1997; Petney & Andrews 1998), reduced immune reactions (Holmes *et al*. 1974; Mackenzie *et al*. 1975; Urquhart & Holmes 1987; Kaufmann *et al*. 1992) and increased susceptibility (Holmes *et al*. 1974; Mackenzie *et al*. 1975).

Immunosuppression that is caused by certain pathogens is an important sequel to infection. Anaemia in field cases of ECF is thought to develop from co-infections with latent pathogens such as *T. mutans* or *A. marginale* in immunocompromised ECF cases (Moll *et al*. 1986). Immunosuppression in animals that suffer from trypanosomosis predisposes the host to secondary infections (Holmes *et al*. 1974; Mackenzie *et al*. 1975). This immunosupression also interferes with the effectiveness of vaccines against other diseases. In cattle infected with *T. congolense* that were vaccinated with mixed clostridial vaccines, foot-and-mouth disease vaccines and *Brucella abortus* vaccines showed reduced antibody responses (Urquhart & Holmes 1987). This interaction is of particular importance when designing control programmes for the various diseases (Howard, Donnelly & Chan 2001).

Interactions between pathogens that are of benefit to the host also occur. Earlier infection with *Oestrus ovis* decreased the level of eggs in faeces, worm fecundity and burdens of *Trichostrongylus colubriformis* in sheep compared to control groups (Yacob, Terefe, Jacquiet, Hoste, Grisez, Prevot, Bergeaud & Dorchies 2006). An increased resistance against *A. marginale* in *Theileria buffeli*-carrier cattle has been reported and is ascribed to a non-specific cell-mediated immunity. The *Theileria*-carrier animals had lower parasitaemias and increased time to maximum parasitaemias compared to controls (Gale, Leatch, Dimmock & Gartside 1997).

It is clear that co-infection of pathogens and interactions between them do occur. The relationship between pathogens is complicated and the level and type of interaction are determined by many factors, such as timing and order of infection. Pathogen interactions alter the outcome of infection (Cox 1987) and are of practical significance when clinical presentation of disease is unusual. This in turn can complicate diagnoses and treatment efforts. When designing control programmes, such as vaccination campaigns, one should take note of the diseases prevalent in the population (Cox 1987; Petney & Andrews 1998; Howard *et al*. 2001).

3. STUDY OBJECTIVES

This project formed part of the IDEAL (Infectious Diseases of East African Livestock) project, which is a collaborative effort between the Department of Veterinary Tropical Diseases, University of Pretoria (DVTD), the University of Edinburgh, the University of Nottingham and the International Livestock Research Institute (ILRI), Nairobi. The focus of the IDEAL project was on the sedentary mixed crop-livestock smallholding system. The study was set in Western Kenya which was considered representative of smallholder livestock farmers in East Africa. The main aim of the IDEAL project was to investigate the total disease burden of endemic cattle in the study area during their first year of life.

The main objectives of this particular study revolved around anaemia as a syndrome in the indigenous calves in the study area. Aspects investigated include diagnostic methods to identify animals with anaemia; longitudinal trends in baseline values of the haematological parameters of the calves; as well as the main infectious causes of anaemia and their contribution, either singly or in combination with other pathogens, towards the level of anaemia in the calves.

The general study design, including site selection, population description, sampling procedures, and general statistical methods used, are discussed in Chapter 2. Under each subsequent chapter, the methodology used to investigate the specific objectives of that chapter is discussed in more detail.

Anaemia in animals can be provisionally diagnosed based on clinical signs, such as pallor of mucous membranes, but a confirmatory diagnosis is based on measuring of red blood cell parameters, such as PCV, red cell counts (RCC) or haemoglobin (HGB). The FAMACHA® score card is a field diagnostic test developed to detect anaemia and haemonchosis in sheep. Its use and validity as a field diagnostic tool in East African short-horn Zebus, as measured against PCV, is evaluated and validated in Chapter 3. The red cell parameters and indices, as well as white cell and platelet parameters and indices of the East African short-horn Zebu were measured by a Sysmex® automated cell-analyzer. Prior to its use in the field, the Sysmex® performance was verified against an established automated cell-analyser with known precision and accuracy. These results are also presented in Chapter 3.

It is known that there are differences between breeds in the baseline ranges of haematological parameters (Jain 1993). There are no published values for these parameters in East African short-horn Zebu calves, thus, in Chapter 4, an attempt was made to define baseline values for this breed, as measured by the Sysmex® and manual PCV. The population means for each parameter were calculated at different ages and trends over time were then compared to published reference ranges for other breeds. In addition, in the absence of published reference ranges for this breed, a subgroup of calves was selected based on a set of criteria to identify relatively healthy calves. Using the data on healthy calves as a reference sample, the significance of anaemia in the general study population was further investigated.

In a tropical environment calves are exposed to a myriad of pathogens, even from early calfhood. Many of these pathogens potentially cause anaemia in the infected host. The prevalence and incidence of pathogens was investigated in Chapter 5 to get a better understanding of the disease burden of calves in the study area. The variation in prevalence with age has allowed the identification of high risk periods. The prevalence of co-infection of pathogens was also considered, with particular reference to pathogens known to cause anaemia.

The East African short-horn Zebu breed is considered to be less tolerant to trypanosomosis than West African breeds, yet more resistant against certain tick-borne disease, such as East Coast Fever, than European breeds and even West African breeds (Murray *et al*. 1982). In order to determine the relative contribution of different pathogens to the development of anaemia in the study population, the haematological profiles over age of calves infected by specific pathogens were investigated and the results are presented in Chapter 6. The development of other syndromes, such as thrombocytopenia and pancytopenia are also discussed.

Interaction between pathogens and its effect on the clinical course of disease has been investigated in several studies (Goossens *et al*. 1997; Kaufmann *et al*. 1992). These studies have focused mainly on single pairs of pathogens, often in controlled environments. However, controlled environments are not truly representative of the challenges cattle face in the field where cattle are exposed to multiple infections. It is very difficult to study animals in their natural environment due to the interplay of many risk factors that affect the incidence of infection and disease. Apart from a few studies on anaemia, little work has been done on how interactions between pathogens bring about changes in kinetics of white blood cells and thrombocytes. In Chapter 7 the cumulative impact of co-infecting pathogens and their interactions on several haematological parameters of calves were investigated.

An IDEAL calf at 51 weeks of age.
The calf is just over 1meter in length from snout to tail root.

CHAPTER 2

METHODOLOGY AND MATERIALS

1. PROJECT DESIGN

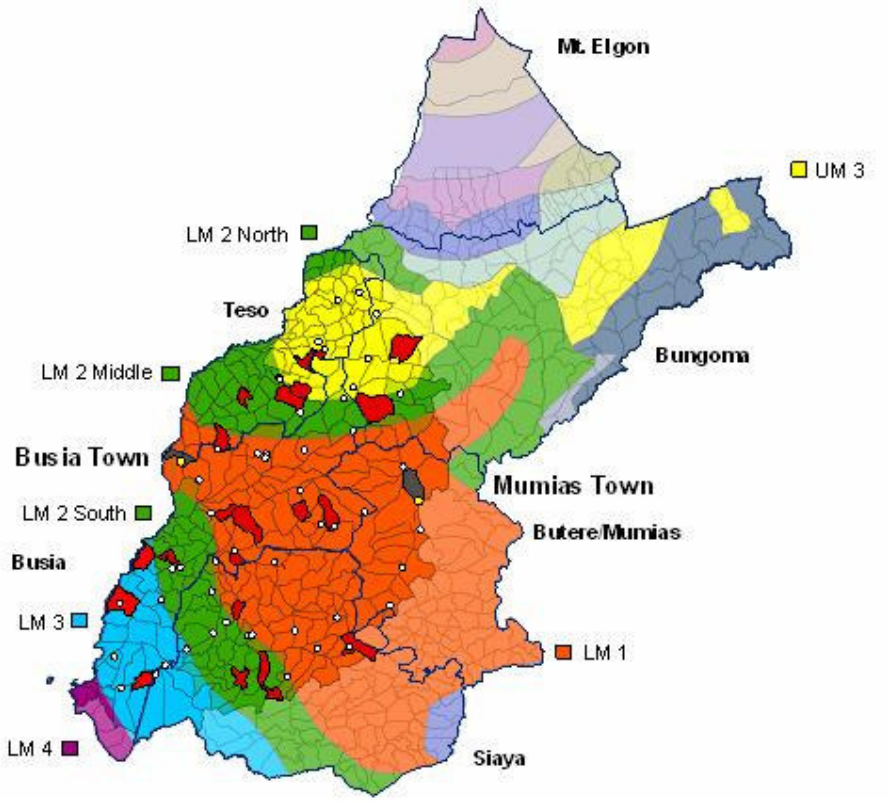
1.1 Study site (from IDEAL Protocols Version 17, 2008)

The study site selected is an area of Western Kenya which was considered representative of smallholder livestock farming in East Africa. The focus of the study was on the sedentary mixed crop-livestock smallholding system which encompasses >50% of poor people resident in East Africa. The area was approximately 45 x 90km and covered some or all of Busia (95.9%), Teso (96.3%), Siaya (55.5%), Butere/Mumias (26.9%) and Bungoma (20.4%) sub-locations. A sub-location is the smallest administrative unit and is typically about 10 km across and contains ~ 80–90 households / km^2 with plots 1-5 ha in size, and with ~ 60% households owning cattle. A typical household might own 3 cattle – one cow, one follower and one calf – although the range may be from 1 to 10, and the animals would normally be grazed communally. The main land use in the area is cultivation of maize, sugarcane, cotton, pigeon-peas and sisal. Cattle are used for animal traction, milk and meat, and the dung for fertilizer. In a survey on animal husbandry done in Busia, the main grazing system (60.4% of farmers) in the district was restricted grazing by tethering and the majority of farmers (79.9%) kept their cattle in an animal house at night (Jaetzold & Schmidt 1983).

1.2 Survey design (from IDEAL Protocols version 17, 2008)

The survey design can be described as a “stratified 2-stage cluster sample”. Stratification was by AEZ. Cattle density data were believed to be aggregated at a stratum one level up from the sub-location stratum. Agro-ecological zones that were represented in the study area were Lower midlands 1 (LM1), LM2 (with a northern, middle and southern section), LM3, LM4 and Upper midlands 3 (UM3) (Figure 2.1). It was anticipated that the random cluster sampling would ensure that there was reasonable representation of farmers with varying herd sizes and management systems. The 1^{st} stage cluster (sub-location selection) was selected using stratified-random sampling with replacement. The 2nd stage (calf selection) used ordinary random sampling without replacement, with a total sample size of 548.

Figure 2 Proportional random sample of sub-locations within AEZs [Total = 20; Random sample with replacement].



Trading/market centres are represented by white dots [n = 50]. Townships are represented by yellow dots [n = 2]. Excluded sub-locations [those containing Busia and Mumias townships] are shown in grey. Randomly selected sub-locations are shown in red.

Selection of sub-locations (from IDEAL Protocols version 17, 2008)
Sub-locations within each AEZ were selected using random sampling, with replacement. The proportion of sub-locations sampled in each AEZ was equal to the proportion of sub-locations of each AEZ in the total survey area. The minimum sample size within a sub-location was set at 3 (see Table 2.1).

The criteria to include a sub-location within the data frame for subsequent random selection (see Table 2.2) was that its geographical centre lay within 45 km of Busia town [n = 287 out of 306 sub-locations]. Mjini and Matungu sub-locations in LM1 were in Busia and Mumias townships respectively and were excluded. LM4 and LM2 north were not included as both would have resulted in a sub-location sample selection of <3.

Table 2.1 Weighted selections of sub-locations within AEZ

AEZ	Number of sub-locations in the AEZ*	Proportion of sub-locations in AEZ	Number of sub-locations to sample
LM1	[116 – 2 excluded sub-locations] = 114	0.40	8
LM2	86 [5 north; 37 middle; 44 south]	0.30 [0.02 north; 0.13 middle; 0.15 south]	6 [north not included; 3 middle; 3 south]
LM3	28	0.10	3
LM4	4	0.01	Not included
UM3	53	0.19	3
Totals	287– 2 = 285		20

* AEZ Sub-locations within 45 km of Busia

Selection of calves (from IDEAL Protocols version 17, 2008)
After receiving reports of newly born calves from farmers and local animal-health technicians, farmers/calves were randomly selected. All farmers in a sub-location were initially eligible for recruitment, on condition that the household was prepared to allow close monitoring of the calf. Once a calf was recruited from a farmer, the farmer was not eligible for selection in subsequent recruitment periods. That implied sampling without replacement. However, the population was large enough that the exclusion of previous recruited farmers/calves had little effect on selection probability or sampling variability.

Exclusion criteria for calves (from IDEAL Protocols version 17, 2008)
The traditional smallholder livestock-keeping farmers of South Western Kenya keep indigenous Zebu cattle. Calves needed to be representative of the area, and needed to have been exposed to the infectious pathogens normally occurring in the area.
A calf was not recruited in the study if:

- The dam had spent <12 months within the interest herd, regardless of its previous origin.
- The calf was born from artificial insemination.
- The calf was born with a congenital disorder.
- The farmer practised stall feeding only. It was expected that exclusion of such farmers would result in the exclusion of the vast majority of ‘purely exotic’ animals in the study.

Table 2.2. List and details of 20 randomly selected sub-locations

Data Source: last 'Kenya Official Map of Political Boundaries [1999]' & last 'Kenya Official Human Population Census [1999]'.

AEZ	Sub-location	Location	Division	District	Number of households	Area [km^2]
UM3	East Siboti	Siboti	Bumula	Bungoma	1245	15.8
	Kokare	Kokare	Amagoro	Teso	325	8.29
	Kidera	Kotur	Amukura	Teso	314	7.36
LM1	Yiro West	South Ugenya	Ugunja	Siaya	1361	13.7
	Simur East	Ukwala	Ukwala	Siaya	415	4.32
	Igero	Lwanya	Matayos	Busia	532	5.60
	Bumala 'A'	Bumala	Butula	Busia	724	4.38
	Ikonzo	Bujumba	Butula	Busia	1421	16.4
	Bulwani	Elugulu	Butula	Busia	478	6.87
	Bukati	Elukhari	Butula	Busia	993	11.2
	Otimong	Ochude	Chakol	Teso	506	8.66
LM2 MIDDLE	South Myanga	Kimatuni	Bumula	Bungoma	1575	22.5
	Kamunuoit	Kaujakito	Amukura	Teso	556	11.0
	Karisa	Apegei	Chakol	Teso	292	4.63
LM2 SOUTH	Kondieri	West Alego	Uranga	Siaya	630	6.38
	Namboboto	Namboboto Central	Funyula	Busia	351	4.46
	Ojwando 'B'	Alego	Boro	Siaya	832	12.6
LM3	Luanda	Namboboto	Funyula	Busia	726	9.76
	Bujwanga	Nanguba	Funyula Central	Busia	1025	16.7
	Magombe	East Bunyala	Budalangi	Busia	578	7.67

2. MONITORING AND SAMPLING OF CALVES

2.1 Recruitment of calves

Calves were recruited to the study starting October 2007 and ending September 2009. Farmers reported births of calves to either village elders or sub-chiefs in each sub-location who then reported back to the IDEAL Office, Busia. IDEAL technicians then randomly selected the required number of calves from the total number of calves reported in that week for each sub-location. The sample from which the random selection was made only included calves reported 3-7 days before the due day of the visit. Therefore, the calves had to be no less than 3 days and no more than 7 days old when first visited.

The farmer was interviewed and the protocol of the IDEAL project was explained. A signed contract agreement was reached and the farmer was paid a market-related price for the calf.

At recruitment a thorough herd history was taken and a clinical examination was done on the calf, including weight. The calf and dam were both ear-tagged with a unique barcode that had reference to the calf identification, sub-location and AEZ. Data were recorded on a visit record form as well as electronically in a personal digital assistant (PDA). Routine samples were taken (described under Sampling). Samples of the dam and photographs of both dam and calf were taken for morphometrics (not part of this study).

2.2 Monitoring

Calves were routinely visited by the IDEAL technicians, starting from week 1 (recruitment), every 5 weeks, up to 51 weeks of age. This implies that a calf that survived to 51 weeks of age, was routinely visited 11 times in total over its follow-up period. At each visit a clinical examination of the calf was performed, including a FAMACHA© score, and samples (see 2.3) were collected. Clinical episodes outside the frame of 5-weekly monitoring were reported by either the farmer or the local government Animal Health Technician. The IDEAL team then visited the calf the following day. Monitoring of calves started 12 November 2007 and ended 30 August 2010 when the last recruited calves were 51 weeks old.

2.3 Sampling

(Test methodology is discussed under Diagnostics methods)

Routine sampling occurred at recruitment of the calf and then every five weeks up to 51 weeks of age. That implied sampling each calf 11 times. Samples taken at routine visits, including recruitment visits, were the following:

- Two thin peripheral blood smears (MNS) collected from the marginal ear vein for manual haematology and screening of blood-borne protozoa. Blood was collected with a micro-capillary tube and decanted onto a glass slide.
- Two thick peripheral blood smears (MKS) collected from the marginal ear vein for screening of blood-borne protozoa. Blood was collected with a micro-capillary tube and decanted onto a glass slide.
- Jugular blood in one 10 ml plain vacutainer tube (RPL) for the separation of serum. At the 51-week visit two RPL samples were taken.
- Jugular blood in two 5ml EDTA plastic tubes (RED) for haematology, screening of trypanosomes and molecular diagnostics.
- Faecal samples (FCS) from the rectum for screening for helminths and protozoa.
- Tick species observed to be present on the calf were reported on the visit form.

Calves were visited outside the frame of routine visits whenever a clinical episode was reported. In the case of clinical episodes, whether it coincided with routine visits or not, the same samples were taken as above, as well as the following:

- Jugular blood in one 10 ml heparin tubes (SHB) for molecular diagnostics, virology or toxicology (not part of this study).
- Additional samples as needed, such as fine-needle aspirates of enlarged lymph nodes.

3. DIAGNOSTIC PROCEDURES

3.1 Sample processing and testing

Initial sample processing occurred at the local ILRI laboratory, Busia. Where samples from calves were taken in duplicate, one sample was processed further as described hereafter, and one sample was stored in the biobank at ILRI, Nairobi.

- One set of MNS and MKS was stained with Giemsa 10% for 30 minutes for microscopy for blood-borne protozoa.
- RPL samples were spun down and decanted. Two aliquots were made from serum (four aliquots for 51 week visits). One aliquot of 100 µL was used to measure total serum protein (TSP) with a refractometer (model RHC-200ATC, Westover Scientific). Aliquots were then stored at -20˚C prior to shipment to ILRI for serology.
- One of each RED set was stored at -80˚C prior to shipment to ILRI for PCR and RLBT. Aliquots were stored in the Biobank. One of each RED was processed at

Busia as follows: Haematology was done using the automated blood cell analyser, pocH-100iV Diff (Sysmex© Europe GMBH); two thin (BNS) and two thick blood smears (BKS) were made to check for blood-borne parasites; and two heparinised microcapillary tubes were filled and centrifuged. The microhaematocrit was read using the microcapillary tubes with a Hawksley haematocrit reader and the sample was screened for trypanosomes (HCT) after which the buffy coat was decanted onto a glass slide for dark ground examination (DG).

- Faecal samples were tested with the McMasters test for egg counts and oocyst counts, direct Baermans test for *Dictyocaulus* spp., and sedimentation for trematodes. If the McMasters test was negative, faecal flotation was also done to confirm a negative result. If either the McMasters test or the flotation technique was positive, larval culture for nematodes was then done.

After shipment to ILRI, Nairobi, the samples were either put in storage in the biobank or subjected to further diagnostic testing, including serology for specific tick-borne diseases and polymerase chain reaction (PCR) for trypanosomes,

Duplicates of BNS were shipped to DVTD, South Africa, for manual differential counts of blood cells for all visits.

An aliquot of the DNA extracted for the trypanosome PCR were also shipped to South Africa for use in the reverse line blot technique (RLBT) for several tick-borne diseases.

3.2 Diagnostic tests

Haematology

- Manual PCV was measured by filling a microcapillary tube with blood, centrifuging at 50 000 rpm for 5 minutes and reading the haematocrit with a Hawksley microhaematocrit reader (Jain 1993). A PCV <25% was considered anaemic.
- Differential cell counts
  Automated blood cell analysis was done with pocH-100iV Diff (Sysmex© Europe GMBH). The following parameters and units were measured by the analyser (pocH-100iV Diff User's Manual (Sysmex© Europe GMBH) 2006):
  - White blood cell count ($x10^3/\mu L$)
  - Red blood cell count ($x10^3/\mu L$)
  - Haemoglobin concentration (g/dL)

- Haematocrit (%)
- Mean corpuscular volume (MCV) (fL)
- Mean corpuscular haemoglobin (MCH) (pg)
- Mean corpuscular haemoglobin concentration (MCHC) (g/dL)
- Platelet count ($x10^3$/µL)
- Mean platelet volume (fL)
- Percentage: Lymphocytes (%)
- Percentage: Other white blood cell types (%)
- Absolute lymphocyte count ($x10^3$/µL)
- Absolute other white cells count ($x10^3$/µL)

In addition, manual differential counts were done on BNS samples to calculate absolute lymphocyte-, absolute eosinophil-, absolute neutrophil- and absolute monocyte counts.

Helminthology

- McMasters method (Kaufmann 1996) was used to calculate the number of nematode eggs (EPG) and number of coccidial oocysts (OPG) per gram faeces. Eggs were reported as either “strongyle” or “strongyloides” eggs. Where it was possible to identify eggs to the genus level, such as *Trichuris* sp.*, Capillaria* sp. and *Nematodirus* sp., this was reported.
- Faecal flotation (Kaufmann 1996) was done if the McMasters test was negative. Results were reported as either negative or positive.
- Direct Baermann test (Kaufmann 1996) was used to identify the larvae of lungworms (*Dictyocaulus* spp.). Results were reported as either positive or negative.
- Sedimentation of faeces (Kaufmann 1996) in water was used to identify eggs of trematodes. After sedimentation the supernatant was drawn off and the sediment stained with methylene blue (5%). The eggs appear brown or yellow against a pale blue background.
- Larval culture was done as described in Kaufmann (1996). Incubation was at room temperature for 14 days after which harvesting was done using a Baermann apparatus. Identification of third-stage larvae of nematodes was done as described in Reinecke (1983). Third-stage larvae were reported as percentages of the total larvae recovered per sample.

Microscopy

Thin blood smears (MNS and BNS) were air-dried, fixed in absolute alcohol and stained for 30 minutes with Giemsa 10% dilution. Thick blood smears (MKS and BKS) were air-dried, and stained as above. Smears were examined for blood-borne parasites with a standard light microscope under 10-100x magnification.

Quantification of parasitaemia on MNS and BNS was as follows:

- 1 = one organism found in every >10 fields;
- 2 = one organism found in every >1 field and <10 fields;
- 3 = one or more organisms found per field.

Trypanosome parasite concentration techniques

- Microhaematrocrit centrifugation technique (HCT), also called the Woo method (OIE 2005) was done using the microhaematocrit tubes that were used to read the PCV. Results were reported as either negative or positive.
- Dark-ground/phase-contrast buffy coat technique (DG) (OIE 2005) followed the HCT. Where species identification was possible based on morphology and movement of the parasites, it was reported as such.

Serology

Indirect enzyme-linked immunosorbent assays (ELISA) were used to evaluate the level of antibodies to *T. parva, T. mutans, A. marginale, B. bovis,* and *B. bigemina* (Katende, Goddeeris, Morzaria, Nkonge & Musoke 1990; Katende, Morzaria, Toye, Skilton, Nene, Nkonge & Musoke 1998). The results were expressed as percent positivity (PP) values of optical density (O.D.), relative to those of a strong positive control (Wright, Nilsson, Van Rooij, Lelenta & Jeggo 1993). A PP cut-off of 20 was considered positive for *T. parva* and *T. mutans*, and a PP cut-off of 15 was considered positive for *A. marginale* and *B. bigemina*.

Molecular diagnostics

- Trypanosome PCR

  A multi-species un-nested PCR was used to test for trypanosomes *(T. brucei; T. theileri; T. congolense* savannah*; T. c.* forest*; T. c.* kilifi*; T. c.* tsavo*;* and *T. vivax)* (Thumbi, McOdimbam, Mosi & Jung'a 2008). This PCR was done on the 51-week samples, as well as samples from individual visits where a calf tested positive for trypanosomes on microscopy (HCT and/or DG).

- RLBT
  RLBT was done at 51 weeks as a screening test for *T. parva, T. mutans, T. taurotragi, T. velifera, T. buffeli, Theileria* sp.*, A. centrale, A. marginale, B. bovis, B. bigemina, A. (E.) bovis,* and *E. ruminantium* (Gubbels, De Vos, Van der Weide, Viseras, Schouls, De Vries & Jongejan 1999; Bekker, De Vos, Taoufik, Sparangano & Jongejan 2002).

4. DATA ANALYSIS

The data analysis techniques and their applications specific for that chapter are discussed in more detail in the relevant chapters. The general techniques are discussed here. The computation of the results and the production of the graphs were done using R 2.8.1 (Ihaka & Gentleman 1996). All plots, except Kaplan-Meier curves, were drawn with R package *ggplots* 0.8.9 (Wickham 2009). The *Survival* package 2.36-9 in R (Lumley 2007) was used to calculate life tables and Kaplan-Meier plots where applicable.

4.1 Descriptive statistics

Descriptive statistics used in either one or more of the results chapters included the mean and the standard error (SE). The 95% confidence intervals (95%CI) for the means were calculated as follows:

$$\text{mean} - \text{t.value}*\text{SE to mean} + \text{t.value}*\text{SE},$$

where t.value was read from the table of t distribution probability and SE was calculated as the square root of variance (var) divided by the sample size (n) (Downie & Heath 1970). Differences in means were calculated, as appropriate, with either the Student's t-test (using R function "t.test(x)"), or where assumptions of normality was not met, the Mann-Whitney Test (using R function "wilcox.test(y ~ x)"). A p-value <0.05 was considered as significant (Crawley 2007). The 95% CI was calculated as follows:

$$(\text{difference in mean}) - 1.96*\text{SE to (difference in mean)} + 1.96*\text{SE},$$

where SE is square root of the sum of $\text{var}(x_1)^2/n(x_1)$ and $\text{var}(x_2)^2/n(x_2)$.

The 95% confidence intervals for proportions (p), such as prevalences, were calculated as follows:

$$p - (1.96*\text{SE}) \text{ to } p + (1.96*\text{SE}),$$

where p is a percentage and SE is the square root of (p*(100-p)/n) (Downie & Heath 1970).

4.2 Evaluation of diagnostic tests

In Chapter 3 the test performance of the FAMACHA© score test was evaluated using the *ROCR* (1.0-4) package from R (Sing, Sander, Beerenwinkel & Lengauer 2005). The function "prediction(x)" calculated the values of a 2x2 contingency table as a first step. The function "performance(x)" was then used to calculate all of the following measures using the contingency table values: "sens": Sensitivity (Se); "spes": Specificity (Sp); "ppv": postitive predictive value (PPV+); "npv": negative predictive values (PPV-); and "auc": area under the curve (AUC). The AUC is equal to the value of the Wilcoxon-Mann-Whitney test statistic (Greiner, Pfeiffer & Smith 2000). The 95% confidence intervals for the AUC could thus be calculated by rank correlation for censored data using the Hmisc (3.8-3) package with the function "rcorr.cens" (x). The output value "C Index" was then used to calculate the confidence interval as follows:

"C Index" – 1.96*SE to "C Index" + 1.96*SE.

4.3 Statistical modelling

Longitudinal studies allow one to investigate within-calf variation over time as well as between-calf variation. The assumption of independence of data was not met, however, since these were repeated measures of individual calves within a longitudinal study which implied clustering within calves (Finucane, Samet & Horton 2007).

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Generalized mixed-effect (GME) models were used to investigate the association between pathogens and the haematological measure of interest. The advantage of using mixed-effect models is that it takes into account the problem of repeated measures and allows for data to be unbalanced and/or incomplete (missing values for some time points). By distinguishing between parameters that affect the population equally (fixed effects) and factors that affect the individual calf differently (random effects), the mixed-effect models can distinguish within-calf from between-calf sources of variation (Finucane *et al*. 2007).

Generalized additive mixed models (GAMM) are an extension of generalized mixed-effect models and allow the use of the non-parametric smoothers in addition to parametric components where applicable (Wood 2006). Non-parametric smoothers were used to account for the non-linear effect of age on the distribution of the haematological parameters. The smoother function is denoted in the model by "s(x)", which produces isotropic smooths of multiple predictors (Wood 2006). Modelling of GAMM was done in R using the package

mgcv (1.7-0). In mgcv, generalized cross validation (GCV) criterion or unbiased risk estimator (UBRE) are used to estimate the smoothing parameters (Crawley 2007). Models are fitted using penalized likelihood maximization (Crawley 2007). The model was fitted by the following formula, using the "gamm" function:

```
model <- gamm(fixed=response~covariates, correlation=corAR1(), random=~covariates);
```

where the "corAR1" function allowed for autocorrelation nested within calves.

Calf number (CalfID) and sublocation (SL) were included as random effects. The consistent inclusion of SL conditions the model for environment and nutrition-related factors and differences in exposure levels to pathogens (Van der Waaij *et al*. 2003). Varying slopes and intercepts models were used and temporal autocorrelation within calves was accounted for in the model specifications. The cutoff for statistical significance was set to $p < 0.05$.

Model checking was done by inspecting the fit of the model by testing for heteroscedasticity by plotting the residuals against fitted values, and testing for non-normality of errors by plotting the residuals against the standard normal deviances (Crawley 2007). Akaike's information criteria (AIC) were also used as a measure of model fit. The model with the lowest AIC was chosen as a better fit.

CHAPTER 3

ANAEMIA IN EAST AFRICAN SHORT-HORN ZEBU CALVES: EVALUATION OF THE FIELD PERFORMANCE OF FAMACHA© COLOUR CHART IN DETECTING ANAEMIA AND THE PERFORMANCE OF THE SYSMEX© AUTOMATED ANALYZER FOR LABORATORY DIAGNOSIS OF ANAEMIA

1. INTRODUCTION

Anaemia is a common clinical sign of many of the tick-borne diseases, trypanosomosis and helminthosis. Anaemia is defined as an erythrocyte count, haemoglobin concentration or a packed cell volume that is below what is considered as reference values for the species (Jain 1993).

Clinical diagnosis of anaemia in the field can be based on the presence of pale mucous membranes. The FAMACHA© eye colour chart was developed for use in small-stock farming in South Africa where helminthosis, in particular wireworm *Haemonchus contortus*, is a major cause of production losses (Anon. 2002b). The system is based on the assessment of colour changes of the mucous membranes as an indication of the development of anaemia in small stock infected with wireworm. The animals are classified into five categories by comparing the ocular mucous membrane with a laminated colour chart with categories ranging from red to pink to white, which represent increasing levels in severity of anaemia. The FAMACHA© has been tested in various countries and is considered a cheap pen-side test, that is easy to learn even by illiterate individuals, and can be a valuable tool in an integrated worm-control programme in sheep (Anon. 2002b; Kaplan, Burke, Terrill, Miller, Getz, Mobini, Valencia, Williams, Williamson, Larsen & Vatta 2004; Depner, Gavi, Cecim, Rocha & Molento 2007) and goats (Kaplan *et al*. 2004; Ejlertsen, Githigia, Otieno, & Thamsborg 2006; Scheuerle, Mahling, Muntwyler & Pfister 2010). This test has also been evaluated in West Africa as a possible tool in the diagnosis and control of trypanosomosis in cattle based on the presence of pale mucous membranes in infected animals (Grace, Himstedt, Sidibe, Randolph & Clausen 2007). The performance of the FAMACHA© eye colour chart as a field diagnostic tool in detecting anaemia in East African short-horn Zebu cattle was evaluated in this study.

The clinical diagnosis of anaemia is subjective and it is also difficult to detect the presence of subclinical cases. Therefore, a clinical diagnosis of anaemia needs to be confirmed by laboratory diagnosis. This is done by measuring the PCV or HCT, HGB and RCC of the animal. Automated cell analyzers such as the Sysmex© poch-100iV (Sysmex South Africa) allow for accurate measurements of various blood cell components in a short time. The performance of the Sysmex analyzer was assessed prior to its use in the field laboratory for this study.

2. MATERIALS AND METHODS

*General methodology (description of study site and sampling population) is discussed in Chapter 2.

2.1 Evaluation of the field performance of the FAMACHA© eye colour chart

The relationship between FAMACHA© score and PCV (%) was initially assessed using all observations of all the calves. Packed cell volume (%) was used as the reference test for anaemia, with PCV<25% considered as anaemic. The effect of age on the field performance of the FAMACHA© test was assessed by comparing the linear relationship between FAMACHA© and PCV (%) for three age groups: 6, 21 and 51 weeks. To exclude the possible effect of repeated measures of the calves as well as the effect of age, the field performance of the FAMACHA© test was then assessed using only data from the 21-week age group. The field performance of the test was measured by the sensitivity (Se) and specificity (Sp), positive (PV+) and negative predictive values (PV-), likelihood ratios (LR+/-) and receiver operating characteristic curves (ROC). A positive likelihood ratio is the odds that an animal is truly diseased when tested as positive. Receiver operating characteristic curves are a measure of accuracy of the test, given the anaemic status of the calf as either positive (PCV<25%) or negative (PCV≥25%). The area under the curve (AUC) of the ROC is an indication of what the probability is that a randomly selected animal with a PCV<25% had a higher FAMACHA© score than a randomly selected animal with a PCV≥25%. An AUC=0.5 can be considered as non-informative, $0.5 < AUC \leq 0.7$ as less accurate, $0.7 < AUC \leq 0.9$ as moderately accurate, $0.9 < AUC \leq 1$ as highly accurate, and AUC = 1 as a perfectly accurate test (Greiner *et al*. 2000). The accuracy of the FAMACHA© test was also investigated with a two-graph ROC plot method (Greiner *et al*. 2000; Reynecke, Van Wyk, Gummow, Dorny & Boomker 2011). On the two-graph ROC plot method the Se and Sp are plotted individually as a function of the FAMACHA© cut-points. To exclude the effect of age on the PCV, only observations at 21 weeks of age were used in the assessment of test performance using different cut-points.

Inter-rater performance of the FAMACHA© eye colour chart (using a FAMACHA© cut-off of 4) was also measured by the Se, Sp, PV+, PV- and ROC. The effect of rater experience on the accuracy of the test was measured by comparing the performance of raters from the first half of the study [Feb 2008 (when the first calves were 21 weeks old) – Sept 2009] to the second half of the study period [Oct 2009 – May 2010 (when the last calves were 21 weeks old)], also only using data from the 21-week visits. Raters scored the subjects only once per visit; therefore no intra-rater performance could be measured.

2.2 Evaluation of the performance of the Sysmex

The laboratory performance of the Sysmex poch-100iV Diff automated analyzer was evaluated before it was used under field laboratory conditions. For this purpose whole blood, using EDTA vacutainer tubes, from 78 clinically healthy bovines was analyzed at the Clinical Pathology laboratory, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria. The performance of the Sysmex was compared to an established automated analyzer, the Cell-Dyn® 3700 (Abbott, South Africa) that hasbeen shown to have adequate accuracy and precision with bovine samples. The Sysmex performance under field conditions was also assessed by a comparison between manual PCV and HCT as measured by the Sysmex. The samples from the IDEAL calves were used for this purpose.

The precision reported for the haemogram parameters for the Cell-Dyn®, given as coefficient of variation (CV) with a 95% confidence limit, were as follows: for white blood cell count (WCC) the CV≤2.5%; for red cell counts (RCC) the CV≤2.8%; for haemoglobin concentration (HGB) the CV≤1.2%; for mean corpuscular volume (MCV) the CV≤1.0%; and for platelet counts (Plt) the CV≤5.0%. The precision for the lymphocyte counts (%) was given as a ±2.6 difference from the mean of determinants with a 95% confidence limit (Anon. 2000a). The accuracy of haemogram parameters for the Cell-Dyn®, reported as correlation coefficients (CC) for WCC was CC≥0.99; for RCC the CC≥0.98; for HGB the CC≥0.98; for MCV the CC≥0.98; for Plt the CC≥0.98; and for lymphocyte counts (%) the CC≥0.94 (Anon. 2000a).

Limits of agreement plots (Bland & Altman 1986) were used to indicate the range of differences between the results of the Sysmex and the Cell-Dyn®, and the Sysmex HCT and manual PCV. In these graphs the difference (Δ) between values for each sample was plotted against the average of the values of the two test methods. Intraclass correlations (ICC) were used to assess what proportion of the total variance was accounted for by within-test (test

method, e.g. Sysmex) variation. An ICC close to 1 indicated that within group (test method) variation was small relative to variation between the two test methods.

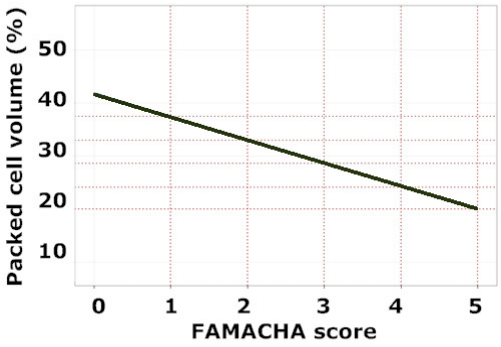
3. RESULTS

3.1 Evaluation of the field performance of the FAMACHA©

3.1.1 The relationship between FAMACHA© and PCV

All observations (n=5637) of all the calves were initially used to analyse the relation between FAMACHA© score and PCV (Fig. 3.1). There was linear relationship between the FAMACHA© score and PCV, with a mean PCV of 37.5%, 33%, 28.6%, 24.1%, and 20% for FAMACHA© score of 1, 2, 3, 4 and 5 respectively.

Figure 3.1 The relation between FAMACHA© score and average PCV (%) (n=5637)



3.1.2 The distribution of FAMACHA© at different age-groups

The distribution of PCV (%) for three age groups (6 weeks, 21 weeks and 51 weeks) is illustrated in Figure 3.2. There were marked differences between the 6-week age group compared to the other two groups, with a mean PCV = 36.12% for the 6-week age group (n=496), mean PCV = 28.94% for the 21-week age group (n=485), and mean PCV = 26.95% for the 51-week age group (n=453).

The frequency distributions of FAMACHA© scores for the three age groups are illustrated in Fig. 3.3a-c. Most calves had a FAMACHA© score of 2 at 6 weeks of age, whereas the majority of calves had a FAMACHA© score of 3 at both 21 and 51 weeks.

Figure 3.2 The distribution of PCV (%) for age groups 6, 21 and 51 weeks

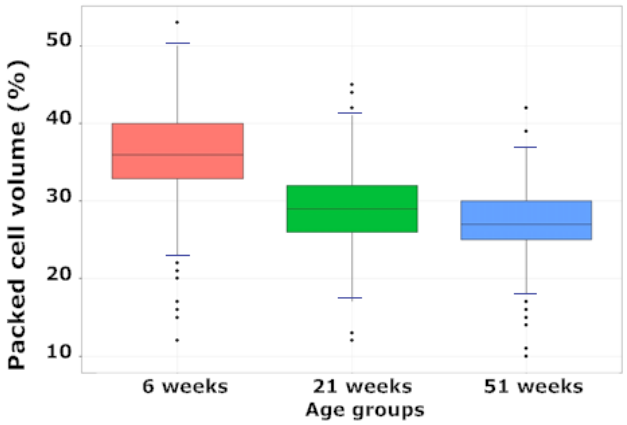


Figure 3.3a The frequency distribution of FAMACHA© scores for the 6-weeks age groups

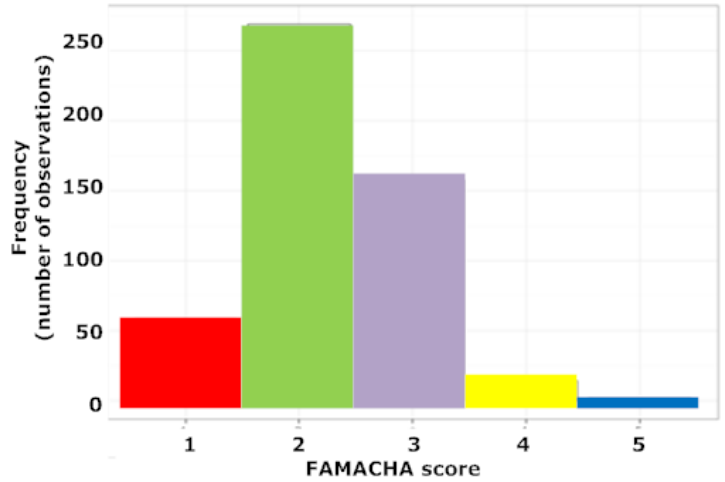


Figure 3.3b The frequency distribution of FAMACHA© scores for the 21-weeks age groups

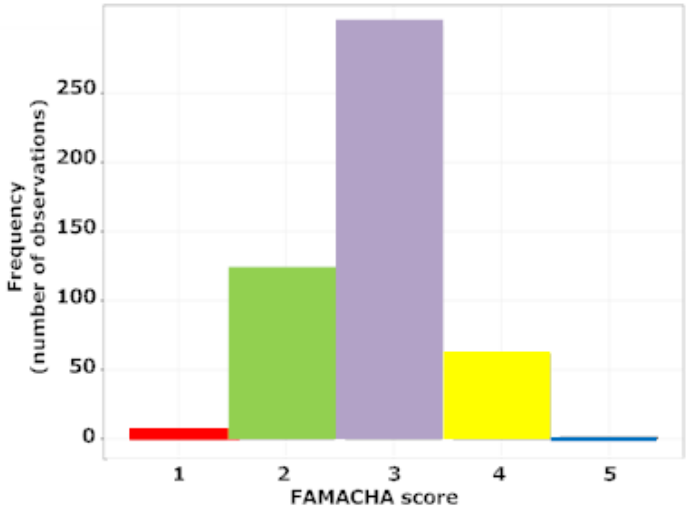
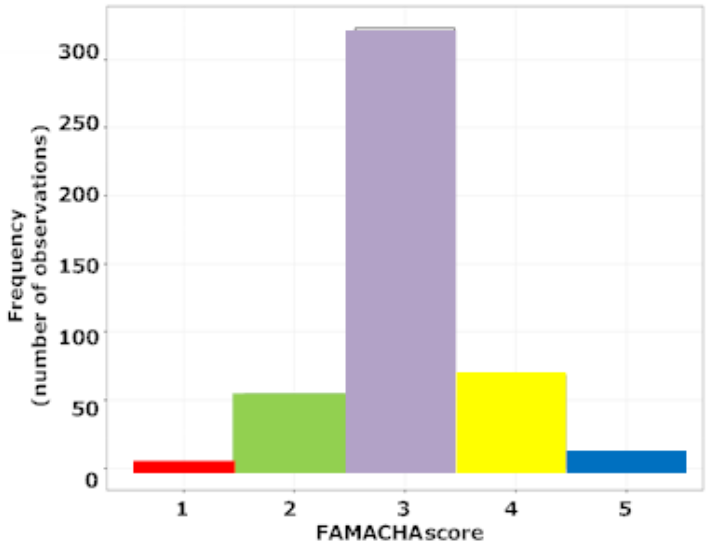
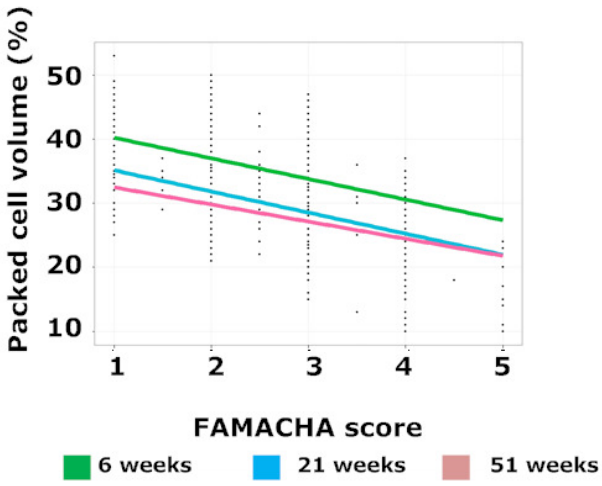


Figure 3.3c The frequency distribution of FAMACHA© scores for the 51-weeks age groups



The linear relationship between FAMACHA© score and PCV for each of the three age groups is given in Figure 3.4. The predicted PCV for each FAMACHA© score was consistently higher for the 6-week age group compared to the other two age groups. The predicted PCV (%) for calves with a FAMACHA© score of 2 for the 6, 21, and 51-week age groups respectively were 42.47%; 31.74% and 29.76%. The predicted PCV (%) for calves with a FAMACHA© score of 3 for the 6-, 21-, and 51-week age groups were 33.82%; 28.52% and 27.12%, respectively.

Figure 3.4 The linear relationship between FAMACHA© score and PCV (%) for the age groups 6 weeks, 21 weeks and 51 weeks



3.1.3 The performance of FAMACHA© using different chart cut-off points

Table 3.1 depicts how the sensitivity and specificity of the FAMACHA© test to detect anaemia (PCV<25%) changed with the use of different FAMACHA© cut-off values. The majority of cases were classified correctly, be that either as anaemic or not anaemic, when using a FAMACHA© score cut-off of 5.

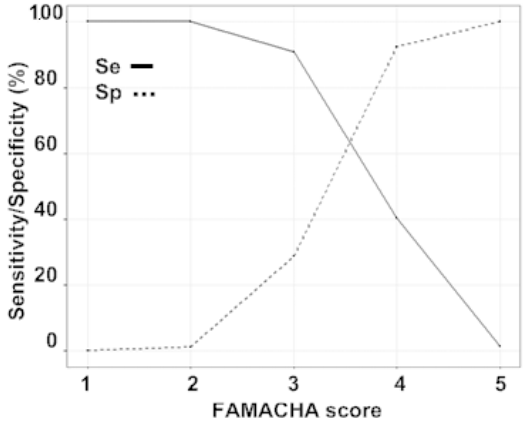
Table 3.1 Sensitivity and Specificity of FAMACHA© at different cut-off points (n=485)

Cut-off point	Se[1] (%)	Sp[2] (%)	Correctly classified (%)	LR+[3]	LR-[4]
1	100	0	15.9	1.00	Inf
2	100	1	16.7	1.01	0.0
3	90.9	28.7	38.6	1.27	0.312
4	40.3	92.4	84.1	5.30	0.646
5	13	100	84.3	Inf	0.99

[1] Se = sensitivity; [2] Sp = specificity; [3] LR+ = positive likelihood ratio; [4] LR- = negative likelihood ratio

The maximum test accuracy can be read off the two-graph ROC plot (Fig. 3.5) at the intercept of the Se and Sp curves (Reynecke *et al*. 2011). The Se-Sp intercept falls between a FAMACHA© score of 3 and 4. The LR+ at a FAMACHA© score of 4 (5.30) was considerably higher than at a score of 3 (1.27). It means that the odds of the animal being truly anaemic was over four times higher when a FAMACHA© cut-off of 4 was used

Figure 3.5 The two-graph receiver operating characteristic curve plot for the FAMACHA© test using a PCV<25% cut-off (n=485)



compared to a cut-off of 3. The Se at a FAMACHA© score of 4 (Se=40.3 %) was, however, considerably lower than at a FAMACHA© score of 3 (Se=90.9 %). Sensitivity <50% implies that an anaemic animal (PCV<25%) is more likely to be missed than diagnosed as positive.

3.1.4 The performance of FAMACHA© using different PCV cut-off points

In Table 3.2 the Se and Sp of the FAMACHA© test in detecting anaemia using a cut-off of PCV<25% is compared to Se and Sp of the test in detecting anaemia using a cut-off of PCV<21%. Using the FAMACHA© cut-off of 4 to detect anaemia with a cut-off of PCV<21% resulted in a higher Se but slightly lower Sp than when using a cut-off of PCV<25% anaemia. The PV+ and PV- for detecting PCV<21% were lower than in detecting PCV<25%, but the results were affected by the lower prevalence of cases with PCV<21%. The accuracy of the FAMACHA©, as measured from the two-graph ROC curve plot (Fig. 3.6), when used to detect PCV<21% was similar to the accuracy of the test when used to detect PCV<25%, as indicated by the intercept of the Se and Sp curves that fell between a score of 3 and 4.

Table 3.2 Comparison of the performance of FAMACHA© (cut-off = 4) using different PCV cut-off points (PCV<25% vs. PCV<21%)

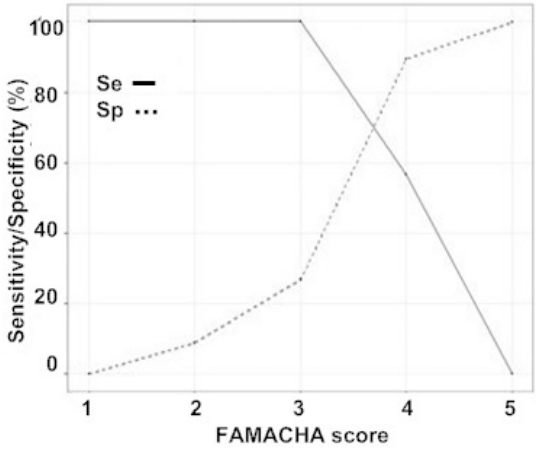
	PCV<25%		**PCV<21%**	
	Mean	**95%CI**	**Mean**	**95%CI**
Prevalence (%) of cases with PCV below cut-off points at 21 weeks age	15.88 ($n_{pcv<25}$=77/485)	12.61 to 19.14	4.74 ($n_{pcv<21}$=23/485)	2.84 to 6.64
Sensitivity (%)	40.50	33.13 to 44.87	56.52	52.11 to 60.93
Specificity (%)	92.4	90.04 to 94.76	89.39	86.65 to 92.13
PV+ (%)[1]	50	45.55 to 54.45	20.97	17.35 to 24.59
PV- (%)[2]	89.1	86.32 to 91.87	97.64	95.48 to 98.52

[1] PV+ = positive predictive value; [2] PV- = negative predictive value

3.1.5 Inter-rater performance of the FAMACHA® scoring system

The inter-rater performance of the FAMACHA® is illustrated in Table 3.3 and Figure 3.7. There was considerable variation between the performances of the different raters. Rater E showed the highest Se of 66.67%. Rater A had the highest Sp at 100%. Rater D correctly classified the most calves (88.24%). All raters had an AUC > 0.5 which implies that raters were able to discriminate between anaemic and non-anaemic animals using the

Figure 3.6 The two-graph receiver operating characteristic curve plot for a cut-off PCV<21% (n=485)



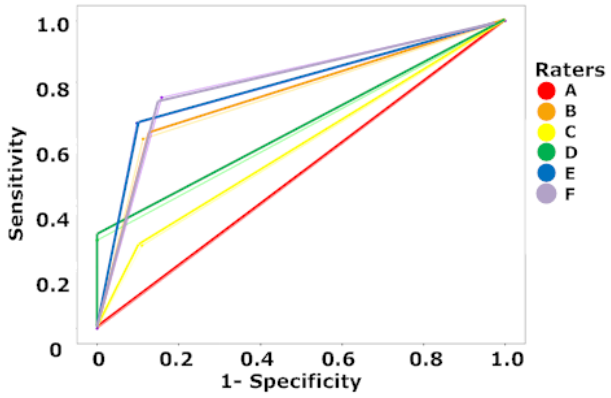
FAMACHA© test. The rater accuracy was low (AUC ≤ 7) for raters C and D, while the other raters were only moderately accurate (0.7 < AUC ≤ 0.9). Rater F was the most accurate with the highest AUC = 0.796.

Table 3.3 Inter-rater performance of the FAMACHA© scoring system

Rater	n	Se[1] (%)	Sp[2] (%)	Correctly classified (%)	PV+ [3] (%)	PV- [4] (%)	ROC area		
							AUC[5]	95%CI	
A	34	100	100	100	100	100	1	1	1
B	111	61.54	88.78	85.59	42.11	94.57	0.752	0.616	0.887
C	174	27.03	89.05	75.86	40	81.88	0.58	0.504	0.657
D	85	28.57	100	88.24	100	87.65	0.643	0.525	0.761
E	57	66.67	90.2	87.72	44.44	95.83	0.784	0.591	0.977
F	23	75	84.21	82.61	50	94.12	0.796	0.569	1.00

[1] Se = sensitivity; [2] Sp = specificity; [3] PV+ = positive predictive value; [4] PV- = negative predictive value; [5] AUC = area under curve

Figure 3.7 Receiver operating characteristic (ROC) curves of the various FAMACHA© score -raters



3.1.6 Comparison between FAMACHA© score-rater performances at different levels of experience

Rater A only made observations during the second year of the study and was thus excluded from this analysis. The accuracy of the raters B, E and F, as measured by the AUC of the ROC area, increased remarkably from the first half of the study to the second half, when they were more experienced. The accuracy of raters C and D did not change significantly and remained low (AUC < 0.7). Raters E and F improved their accuracy from moderate (0.7 < AUC ≤ 0.9) to highly accurate (AUC > 0.9). The Se of all the raters decreased from the first year to the second year, except for rater D who maintained a Se=100%. The Sp of all raters increased, except rater D who showed a decrease in Sp from 30% to 25% (Table 3.4).

3.2 Evaluation of the performance of the Sysmex automated analyzer

3.2.1 Comparison between the Sysmex and the Cell-Dyn® 3700

When comparing the mean differences of each parameter the Sysmex had higher readings (mean $\Delta<0$) than the Cell-Dyn® for lymphocyte counts, total WCC and MCHC; and lower readings (mean $\Delta>0$) than the Cell-Dyn® for other WCC counts,. RCC, HGB, HCT, MCV and Plt counts (Table 3.5). The limits of agreement for these parameters as measured by the Sysmex and the Cell-Dyn® are tabulated in Table 3.6. The intraclass correlation between these two analyzers is tabulated in Table 3.7.

Table 3.4 Comparison between FAMACHA© score-rater performances at different levels of experience

Rater	Year	n	Se[1] (%)	Sp[2] (%)	ROC[3] area		
					AUC[4]	95%CI	
B	1	74	95.31	50	0.727	0.567	0.884
	2	37	76.47	100	0.882	0.811	0.954
C	1	62	90.7	26.32	0.585	0.477	0.693
	2	112	88.3	27.78	0.58	0.472	0.689
D	1	52	100	30	0.65	0.508	0.792
	2	33	100	25	0.625	0.413	0.837
E	1	22	94.74	33.33	0.64	0.369	0.912
	2	35	87.5	100	0.938	0.88	0.995
F	1	11	87.50	66.67	0.771	0.481	1.00
	2	12	81.82	100	0.909	0.795	1.00

[1] Se = sensitivity; [2] Sp = specificity; [3]ROC = Receiver operating characteristic curve; [4]AUC = area under curve

Table 3.5 Within subject comparison of Cell-Dyn® and Sysmex

Parameter		Cell-Dyn®		Sysmex		Difference (Δ)*				
	n	Mean	SD	Mean	SD	Mean Δ	SD Δ	p-Value**	95% CI	
Lymph %	78	59.269	9.388	60.464	8.15	-1.195	3.167	0.0013	-1.909	-0.481
Other WBC %	78	40.731	9.388	39.536	8.15	1.195	3.167	0.0013	0.481	1.909
WCCx10^3/µL	77	11.6	3.56	11.523	3.29	0.077	0.606	0.266	-0.06	0.215
RCCx10^6/µL	78	8.446	0.793	8.432	0.77	0.014	0.142	0.391	-0.018	0.046
HGB g/dL	78	13.682	1.28	13.45	1.21	0.232	0.181	<0.001	0.191	0.273
HCT %	78	38.88	3.731	36.908	3.46	1.974	0.743	<0.001	1.807	2.142
MCV fL	78	46.118	3.11	43.88	3.21	2.238	0.704	<0.001	2.08	2.4
MCHC g/dL	78	35.245	0.732	36.463	0.9	-1.218	0.802	<0.001	-1.4	-1.04
Plt x 10^3/µL	78	484.11	164.107	432.35	157.38	51.76	49.572	<0.001	40.585	62.938

* Δ= CellDyn – Sysmex; **p-Value associated with Student's paired t-test

Table 3.6 Upper (UL) and lower limits (LL) of agreement between Cell-Dyn® and Sysmex

	Limits of agreement	
	LL=Mean(Δ) - 2SD*(Δ)	**UL=Mean(Δ) + 2SD(Δ)**
Lymph x 10^3/µL	-7.53	5.139
Other WBC x 10^3/µL	-5.139	7.53
WCC x 10^3/µL	-1.135	1.289
RCC x 10^6/µL	-0.27	0.298
HGB g/dL	-0.13	0.594
HCT %	0.488	3.46
MCV fL	0.83	3.646
MCHC g/dL	-2.822	0.386
Plt x 10^3/µL	-47.382	150.906

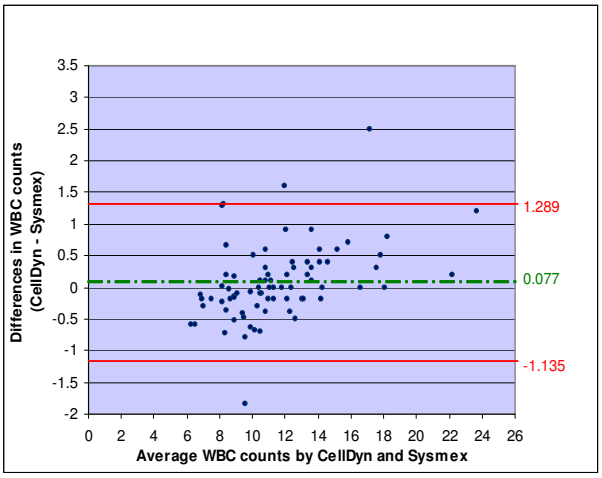
Table 3.7 Intraclasss correlation (ICC) between Cell-Dyn® and Sysmex

	ICC	**SE***	**95%CI**	
Lymph x 10^3/µL	0.927	0.016	0.896	0.958
Other WBC x 10^3/µL	0.927	0.016	0.896	0.958
WCC x 10^3/µL	0.984	0.003	0.977	0.991
RCC x 10^3/µL	0.984	0.004	0.976	0.99
HGB g/dL	0.972	0.006	0.96	0.985
HCT %	0.84	0.033	0.775	0.906
MCV fL	0.756	0.049	0.66	0.851
MCHC g/dL	0.00	0.11	0	0.22
Plt x 10^3/µL	0.904	0.02	0.863	0.945

*standard error of ICC

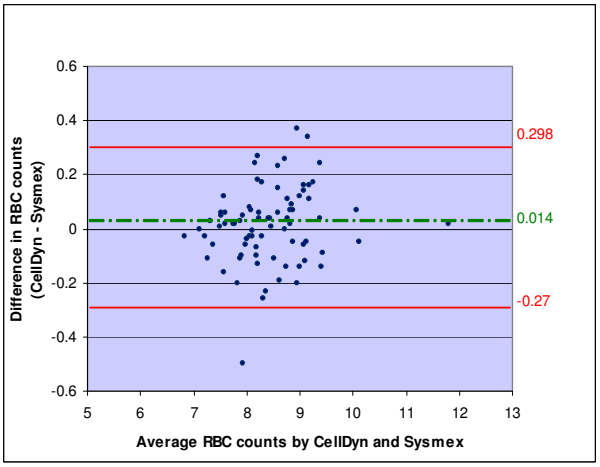
The mean Δ for both total WCC and RCC was not significant (P>0.1). The limits of agreement for WCC (-1.135; 1.289) x10^3/µL and RCC (-0.27; 0.298) x10^6/µL indicated that there was good agreement between the measurements using the Sysmex and Cell-Dyn® for these two parameters. The limits of agreement for WCC are illustrated in Figure 3.8 and for RCC in Figure 3.9.

Figure 3.8 Limits of agreement plots for white cell counts (x10^3/µL)



* mean Δ ; **UL & LL

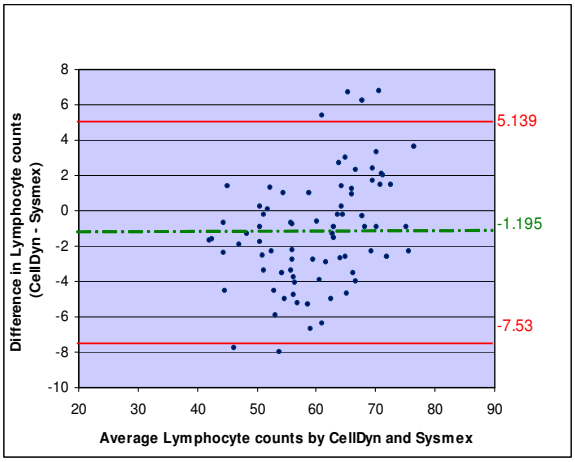
Figure 3.9 Limits of agreement plots for red cell counts (x10^6/µL)



* mean Δ ; **UL & LL

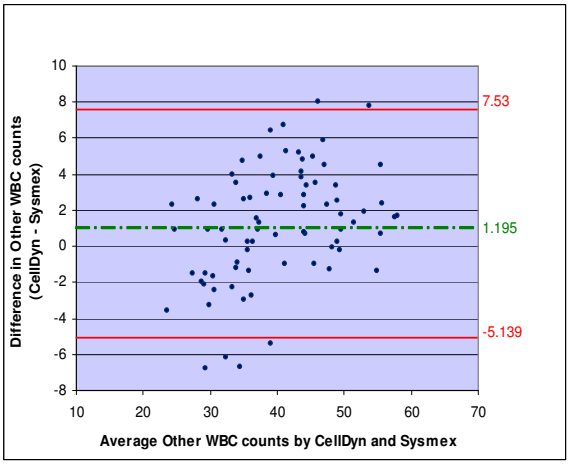
Differential WCC was reported by the Sysmex as either lymphocyte counts (% of total WCC) or other white blood cells (WBC) [WCC (100%) – lymphocyte (%)]. The limits of agreement for the two methods for lymphocyte counts (-7.53; 5.139) and for other WBC (-5.139; 7.53) indicated little agreement between the two methods. The Sysmex tended to give a higher reading than Cell-Dyn® for lymphocyte counts (between 1.909 and 0.481%) and a lower reading for other WBC (between 0.481 and 1.909%). The difference in means was significant (p=0.0013), but was of no clinical significance. The limits of agreement for lymphocyte counts are illustrated in Figure 3.10 and for other WBC in Figure 3.11.

Figure 3.10 Limits of agreement plots for lymphocyte relative counts (%)



* mean Δ; **UL & LL

Figure 3.11 Limits of agreement plots for other white cell relative counts (%)

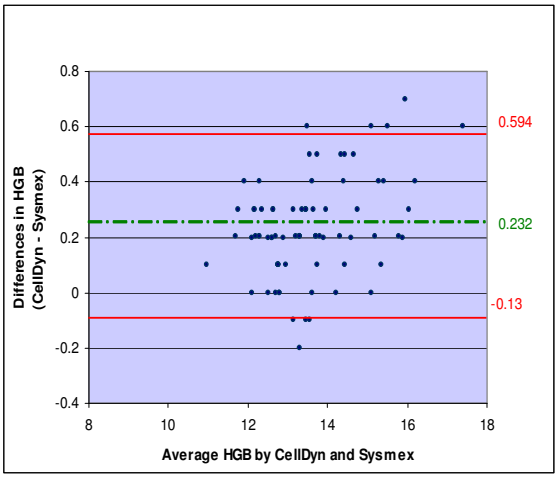


* mean Δ ; **UL & LL

There was good agreement between the two methods for HGB (limits of agreement = (-0.13; 0.594) g/dL) (Fig. 3.12). The difference in means between the two methods for HGB readings was significant ($p<0.001$), with the Sysmex consistently reading lower than the Cell-Dyn® by 0.191 and 0.273 g/dL. However, this was of no clinical significance.

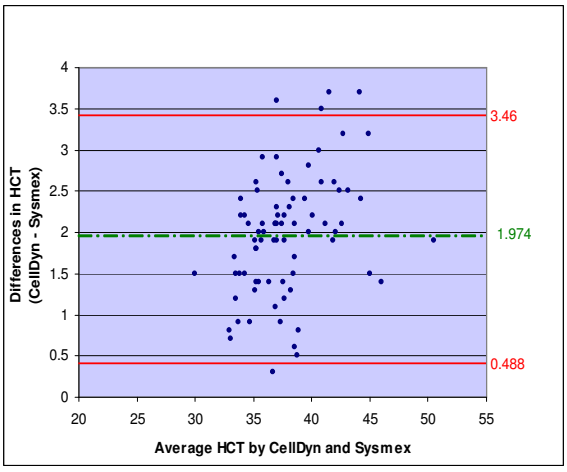
The Sysmex reading for HCT was lower than Cell-Dyn® (between 1.807 and 2.132%). The mean Δ was significant ($p=0$). The limits of agreement (0.488; 3.46) % were not clinically acceptable (Fig. 3.13). The ICC=0.84, indicating that some variation between the two sample sets was due to within-method variation as well as between-method variation.

Figure 3.12 Limits of agreement plots for haemoglobin concentration (g/dL**)**



* mean Δ ; **UL & LL

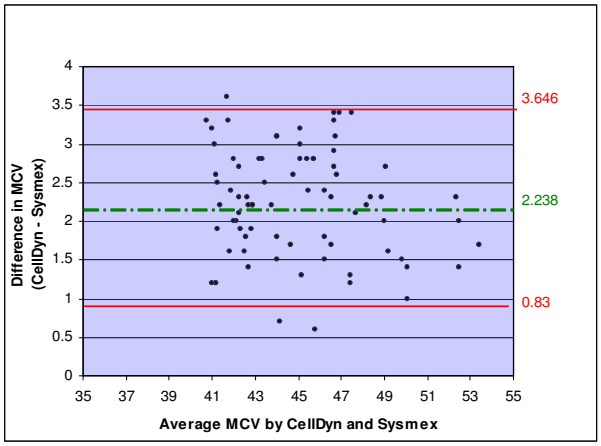
Figure 3.13 Limits of agreement plots for haematocrit (%)



* mean Δ ; **UL & LL

The mean Δ for MCV (2.238fL) was significant (p=0), with the Sysmex consistently reading lower than the Cell-Dyn® by between 2.08 and 2.4 fL. The limits of agreement (0.83; 3.646) fL indicated that there was no acceptable agreement between the two methods for this parameter (Fig. 3.14). Intraclass correlation (0.754) indicated that there was some variation between the two methods, as well as within the two sample sets. These results reflected the fact that MCV is a function of both HCT and RCC [MCV=HCTx10/RCC] and could thus have been affected by the readings for these two parameters.

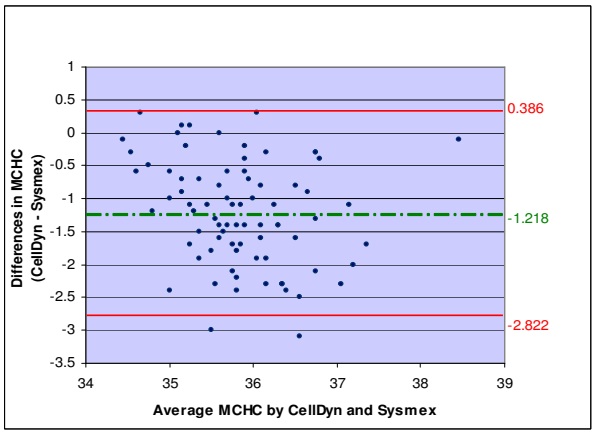
Figure 3.14 Limits of agreement plots for mean corpuscular volume (fL)



* mean Δ ; **UL & LL

Similarly, MCHC is a function of HGB and HCT [MCHC= (HGB x 100)/HCT] and can thus be affected by readings for both these parameters. The agreement was not good with limits of agreement of (-2.822; 0.386) (Fig. 3.15). The Sysmex reading was lower than the Cell-Dyn® reading by between 1.04 and 1.14 g/dL.

Figure 3.15 Limits of agreement plots for mean corpuscular haemoglobin concentrations (g/dL)

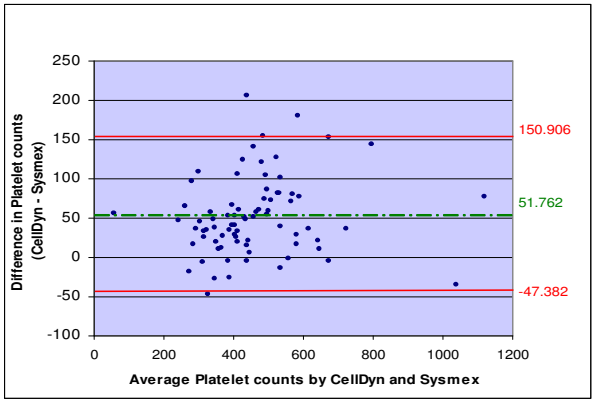


* mean Δ ; **UL & LL

The mean Δ in Plt counts between the two methods was significant ($p<0.001$), with the Sysmex reading lower than the Cell-Dyn® by between 40.59 and 62.9 x 10^3/µL. When evaluating the limits of agreement (-47.382; 150.906) x 10^3/µL, it indicated poor agreement between the two methods for this parameter (Fig. 3.16). The ICC also indicated that the variation was mainly due to inter-assay variation relative to intra-assay variation. However, the scale of the measurement should be considered here. Published reference values for Plt

counts (Jain 1993) give Plt values in units of 10^5/µL. If the Sysmex and Cell-Dyn® values are converted to units of 10^5/µL, the mean Δ =0.52 x 10^5/µL (95%CI = (0.41x10^5; 0.63x$10^{5)}$) and the limits of agreement of (-0.47 x 10^5; 1.5) x 10^5/µL, which indicates good agreement and is in accordance with published reference values for cattle (Jain 1993) and is clinically acceptable.

Figure 3.16 Limits of agreement plots for platelet counts (x10^3/µL)



* mean Δ ; **UL & LL

3.2.2 Comparison between the Sysmex HCT and manual PCV

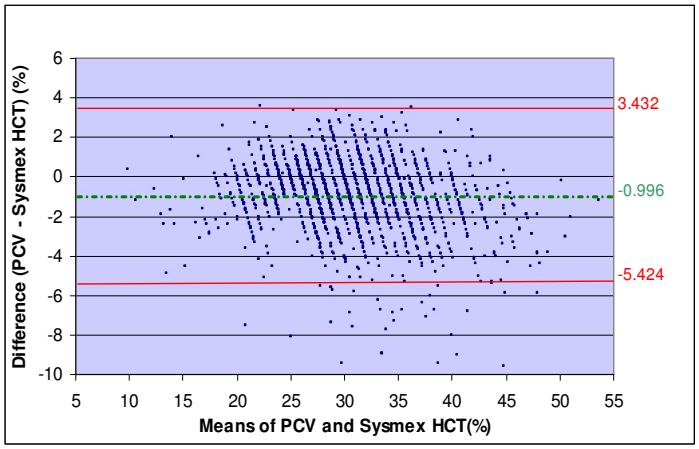
The Sysmex HCT generally tested higher (mean Δ = -0.996) than the manual PCV (Table 3.8). The agreement between PCV and HCT was not good, with limits of agreement of (-5.424; 3.432) (Fig. 3.17). The manufacturer's manual (Ginder 2007) recommends that a correction factor (CF) should be calculated to correct for a difference between PCV and HCT by calculating the ratio of PCV/HCT for each pair of values. The mean ratio (excluding values >mean+2SD and <mean-2SD) equalled the correction factor, which, for this data set, was measured as 0.987. There was improved agreement between PCV and the corrected HCT (Fig. 3.18). The mean Δ was insignificant (p=0.424).

Table 3.8 Within subject comparison of PCV and Sysmex HCT and PCV and the corrected HCT

	Mean	**Difference (Δ)***				
	(SD)	**Mean Δ**	**SD Δ**	**p-Value****	**95% CI**	
PCV (%)	30.19 (6.14)					
Sysmex HCT (%)	31.18 (6.73)	-0.996	2.214	<0.001	-1.436	-0.556
Corrected HCT (%)	30.34 (6.44)	-0.18	1.799	0.424	-0.601	0.255

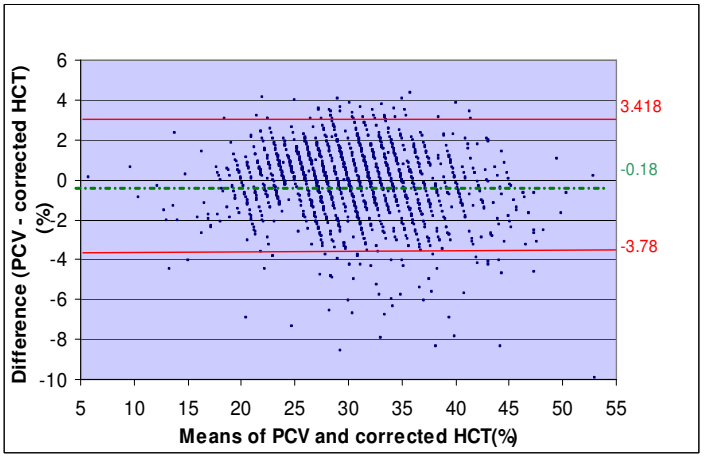
* Δ= PCV – HCT; **p-Value associated with Student's paired t-test

Figure 3.17 Limits of agreement plot for packed cell volume and Sysmex haematocrit



* mean Δ ; **UL & LL

Figure 3.18 Limits of agreement plot for packed cell volume and the corrected haematocrit



* mean Δ ; **UL & LL

4. DISCUSSION

4.1 Evaluation of the field performance of the FAMACHA©

The value of the FAMACHA® ocular mucosa chart lies in its simplicity and low cost, which both contribute to its value as a field screening-test that can be used by laymen. Two drawbacks to this ocular mucosa chart are its relatively low sensitivity and variation in the performance between raters. In order for a test to be considered a good screening test, it has to have a high sensitivity. The sensitivity can be increased by narrowing the inclusion criteria for anaemia, for example from PCV<25% to PCV<21%, but at this PCV the animal is already likely to be in critical need of intervention. The sensitivity can also be increased by

changing the cut-off of the FAMACHA® from 4 to 3. From the two-graph ROC plot the point of maximum accuracy of the test falls at a point between a score of 3 and 4, for both PCV cut-off points (<25% and <21%). A FAMACHA© score of 3 appears to be the optimal choice to maximize the accuracy as a FAMACHA© cut-off of 4 would reduce the Se of the test considerably and therefore a high number of positive cases would be misdiagnosed as negative. The accuracy of the scoring systems for most raters improved as they became more experienced. This came at a cost of a lower sensitivity in most raters. The age of the animal needs to be taken into account, however, as it was shown in this study that there was a difference in the PCV range corresponding to each FAMACHA® score between the different age groups. The younger age group had a higher mean PCV for each FAMACHA© score.

4.2 Evaluation of the performance of the Sysmex automated analyzer

Compared to the Cell-Dyn® 3700 automated analyzer, the Sysmex showed clinically acceptable agreement with regards to white blood cell parameters. This is in agreement with a similar study that compared the performance of the two analyzers, specifically using the optical channel method of the Cell-Dyn 3500 to measure bovine white cell parameters in bovines (Riond, Weissenbacher, Hofmann-Lehmann & Lutz (2011).

The two analyzers did not show good agreement on platelet counts in this study, but the difference was still within published reference ranges and was considered clinically acceptable. Riond *et al*. (2011) found a negative bias in PLT counts in bovine samples, but considered it to be not of any clinical importance as well. There was also good agreement between the two analyzers on RCC and HGB in this study. This too, is in agreement to what was found by Riond *et al*. (2011) for these two parameters in bovines.

There was poor agreement between the Sysmex and the Cell-Dyn® on HCT measurements. The difference was clinically significant. This poor agreement also led to poor agreement between the two analyzers on MCV and MCHC since both these parameters are not measured directly by the Sysmex but calculated from red blood cell indices (RCC, HCT and HGB) (Ginder 2007; Riond *et al*. 2011). There was also poor agreement between the Sysmex HCT and manual PCV, which was improved when a correction factor was taken into consideration. This difference is likely due a difference in methodology, as PCV was measured directly using the microhaematocrit method, whereas the HCT was calculated using the RCC pulse-height detection method by the Sysmex (Ginder 2007; Riond *et al*. 2011). The correction factor calculated could not be extrapolated for use in the comparison

to the Cell-Dyn®, however, since a different group of cattle were sampled. It would probably have been worthwhile to measure the manual PCV for this purpose and assess whether the corrected HCT would have improved the agreement between the Sysmex and Cell-Dyn® for MCV and MCHC as well.

5. CONCLUSIONS

The FAMACHA© test was designed for use in sheep. The reference ranges of PCV differ between species and this necessitates the calibration of the test for the species, such as cattle, before it is applied to that species. Moors & Gauly (2009) found that mucosa colour of different sheep breeds differ, and this is probably true for cattle breeds as well. They suggested adapting the FAMACHA© colour scales for different breeds to increase its validity. Such an adaptation for various species, breeds and age-groups within breeds might also be appropriate.

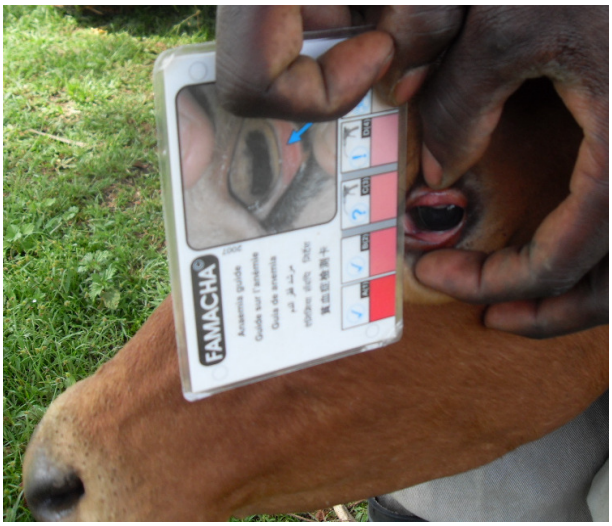
One has to weigh the benefits and costs of missing truly diseased animals by using a test with lower Se on the one hand and on the other hand the unnecessary treatment of false positive cases by using a test of high Se but low Sp. The unnecessary treatment of false positive cases increases the cost of treatment and drugs and can also contribute to the development of drug resistance. In the rural setting of Western Kenya, individual animals will most likely only be inspected for specific clinical signs, such as pale mucosa, once they are already suffering from more general signs such as ill-thrift or loss of condition. In such cases, criteria should be selected to optimize the sensitivity of the FAMACHA© test, such as using a cut-off of 3, in order to screen whether the animal actually requires treatment.

Only the FAMACHA© tests' validity in diagnosing cases of anaemia was investigated in this study and not its value in predicting specific causes of anaemia, such as helminthosis or trypanosomosis. Mixed infections are common in the tropics and many infections present with similar clinical signs. Focusing the FAMACHA© test on specific pathogens in this setting might lead the investigator to under-diagnose super-infections with other pathogens. Identifying individual animals with anaemia is only the initial step in managing the diseased animal. Treatment of such cases should not commence without further diagnostics into the specific underlying causes.

Overall the FAMACHA© was acceptable as a field test in detecting anaemia in East African short-horn Zebu calves (Fig. 3.19). Proper training of raters, being the farmer, veterinarian or

an animal health officer, is essential in optimizing the sensitivity and accuracy of the FAMACHA© chart in the field. If anything, the implementation of this scoring system forces the farmer to assess the health status of each animal individually from close-by and therefore promote earlier recognition of ill-health and initiation of intervention.

Figure 3.19 FAMACHA© scoring in an East African short-horn Zebu calf



CHAPTER 4

ANAEMIA IN EAST AFRICAN SHORT-HORN ZEBU CALVES: THE HAEMATOLOGICAL PROFILE FROM NEONATE TO 51 WEEKS

1. INTRODUCTION

Most reference ranges for the haematological parameters for cattle found in literature have been compiled for European breeds (Jain 1993; Knowles, Edwards, Bazeley, Brown, Butterworth & Wariss 2000; Brun-Hansen, Kampen & Lund 2006; Mohri, Sharifi & Eidi 2007). Studies on haematology of cattle in the tropics have indicated that there are differences in baseline values for the various breeds (Oduye & Okunaiya 1971). The haematological profile of the East African short-horn Zebu breed living in tropical field conditions in Kenya is described in this chapter, in particular the age-related changes in the blood parameters from neonate to 51 weeks.

The calves in this study were kept under field conditions and no controls in a disease-free environment were available. Therefore, in addition, a cohort of relatively healthy calves from the study population was selected, based on a set of criteria, to indicate the baseline values of the haematological parameters of healthy short-horn Zebu calves in the field. Using these data on healthy calves as a reference sample, the significance of anaemia in the general study population was further investigated.

2. MATERIALS AND METHODS

* General methodology (sampling and diagnostics) is discussed in Chapter 2

2.1 Age-related changes in the haematology of East African short-horn Zebu calves

The 5-weekly routine samples from October 2007 to September 2010 from calves of the IDEAL project were used to investigate the age-related changes in the haematological profile of East African short-horn Zebu calves. The haematological parameters investigated included: red cell count (RCC), haemoglobin concentration (HGB), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red cell distribution width (RDW), total white cell count (WCC), absolute lymphocyte count (Lymph), absolute eosinophil count (Eos), absolute total neutrophil count (Neut), absolute monocyte count (Mono), platelet count (Plt), mean platelet volume (MPV)

and total serum protein (TSP). Graphs are used to illustrate population median values and standard errors as they change over time for each parameter.

2.2 "Good" calves – haematology of relatively healthy calves

"Good" calves, representing relatively healthy, well-adapted calves that performed well under the environmental and nutritional constraints in the field, were identified by the following criteria:

- They survived to 51 weeks with a final weight above the median for the population at 51 weeks.
- No clinical episodes occurred during the 51-week follow-up period.
- No diarrhoea was present at any visit.
- FAMACHA© scores less than 4 (non-anaemic) at each visit.
- The calves tested negative for trypanosomosis on microscopy at each visit.
- No *Anaplasma* spp. or *Babesia* spp. were detected on microscopy at any visit.
- Eggs per gram faeces (EPG) < 1 000 for strongyle and strongyloides type eggs for all visits.
- Oocysts per gram faeces (OPG) < 1 000 for coccidian oocysts for all visits.
- Faecal samples tested negative for *Fasciola* spp. for all visits.

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Thirty-seven calves were identified retrospectively after they had finished their follow-up period of 51 weeks. Sampling was thus similar to the other calves in the study. The haematological parameters investigated include RCC, HGB, PCV, MCV, MCHC, RDW, WCC, Lymph, Eos, Neut, Mono, Plt and TSP. Graphs are used to illustrate the changes in the values for each parameter with age in comparison to the values for the total study population.

2.3 The level of anaemia in the population

"Good" calves were used as a reference sample to identify cases in the study population with anaemia. Conventionally, cut-off values are often determined by calculating the mean ± 2 standard deviation (SD) of a clinically healthy, disease-free reference population. However, the reference sample of "good" calves is not from a disease-free, nutritionally controlled environment. Therefore, cut-off values for PCV were measured both conservatively as the mean ± 1SD , as well conventionally as the mean ± 2SD to identify anaemic cases. These cut-off values were calculated for each 5-week age interval.

2.4 Statistical analysis

Descriptive statistics (mean and standard error) were used to describe the age-related changes in haematology parameters of the total calf population as well as the "good calf" subset. The changes with age are plotted in graphs.

Student's t-test was used to determine the significance ($p<0.05$) in the difference in means of the total calf population and the "good calf" subset for each haematological parameter at each sampling point (week 1 to week 51).

Cumulative hazard rates were plotted to illustrate the risk of a calf developing anaemia. Data from the routine visits as well as clinical visits were used to construct the graph. Censoring of the date was done to account for calves that died before the end of the follow-up period. Because the visits to the calves were not at the exact same age in days the graphs were constructed using intervals of 10 days.

3. RESULTS

3.1 Age-related changes in the haematology of East African short-horn Zebu calves

Figures 4.1-4.14 illustrate the changes for all parameters between week 1 and week 51 for the total calf population. Trendlines for European neonatal Holstein calves [adapted from Mohri *et al*. (2007) and Knowles *et al*. (2000)], are indicated where the trends in certain parameters for these breeds differ from the population in the present study.

The calves showed a significant increase in PCV, RCC and HGB from week 1 (means of 30 %, 7.73 $x10^6$/µL and 10g/dL) to week 6 (means of 36%, 10.23 $x10^6$/µL and 12 g/dL). From 6 weeks, all three parameters decreased gradually until 51 weeks. Red cell distribution width was relatively high between 1 and 6 weeks (mean 34, 9 and 35.9 fL for each point), indicating a significant variation in the size of red blood cells which may be suggestive of either the presence of foetal red blood cells, which are larger than adult-type red blood cells, or a high number of immature adult-type red blood cells. Between 6 and16 weeks RDW decreased significantly to where it remained between 31.2 and 31.8 fL up to 51 weeks. MCV was relatively high at week 1 (mean 39.8 fL), confirming the presence of a high number of larger red blood cells, but decreased gradually from week 1 up to week 21 (mean 33.6 fL), and then increased gradually until week 51 (mean 36.0 fL). The MCHC showed a similar but more gradual trend, where it decreased between week 1 (mean 32.2 g/dL) and week 11 (median 31.6 g/dL), and then increased up to 51 weeks (mean 33.05 g/dL).

The WCC and Lymph showed very gradual increases from week 1 (mean 9.00 x10^3/dL and 3.7 x10^3/µL, respectively) to week 51 (mean 11.40 x10^3/dL and 7.6 x10^3/µL, respectively). The Eos increased considerably from week 1 (mean 0.27 x10^3/µL) up to week 46 (mean 0.75 x10^3/µL). The Neut showed a gradual decrease from week 1 to week 16, after which they remained between 2.4-2.55 x10^3/µL (mean) until week 41 and increased again to week 51.

The Plt also showed a steady decrease from week 1 (mean 611 x10^3/µL) up to week 16 (mean 445 x10^3/µL) from where the decrease was more gradual up to week 51 (mean 385.5 x10^3/µL). The MPV was relatively high at week 1 (mean 6.5 fL), decreased gradually up to week 16 (mean 5.8fL) and increased up to week 51 (mean 6.2 fL).

The TSP was relatively high at week 1 (mean 9.8 g/dL), after which it decreased up to week 16 (mean 7.6 g/dL) but then remained between 7.6-8 g/dL (mean) up to week 51.

Figure 4.1 Age-related changes in the mean (SE) red cell counts of East African short-horn calves, from birth to 51-weeks of age (n=548)

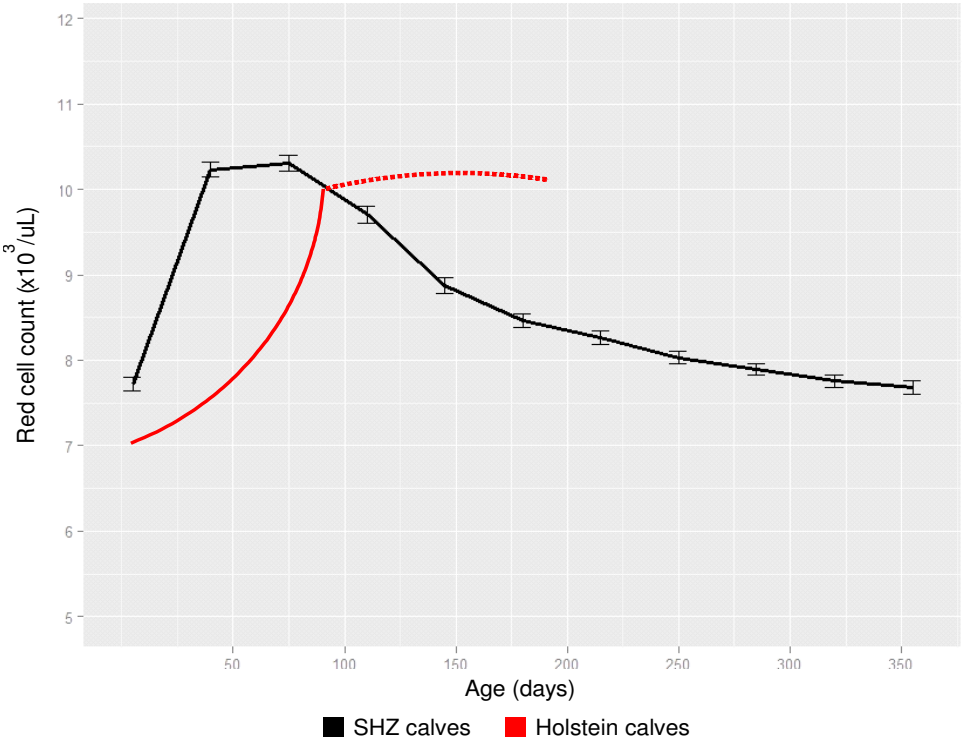


Figure 4.2 Age-related changes in the mean (SE) packed cell volume of East African short-horn calves, from birth to 51-weeks of age (n=548)

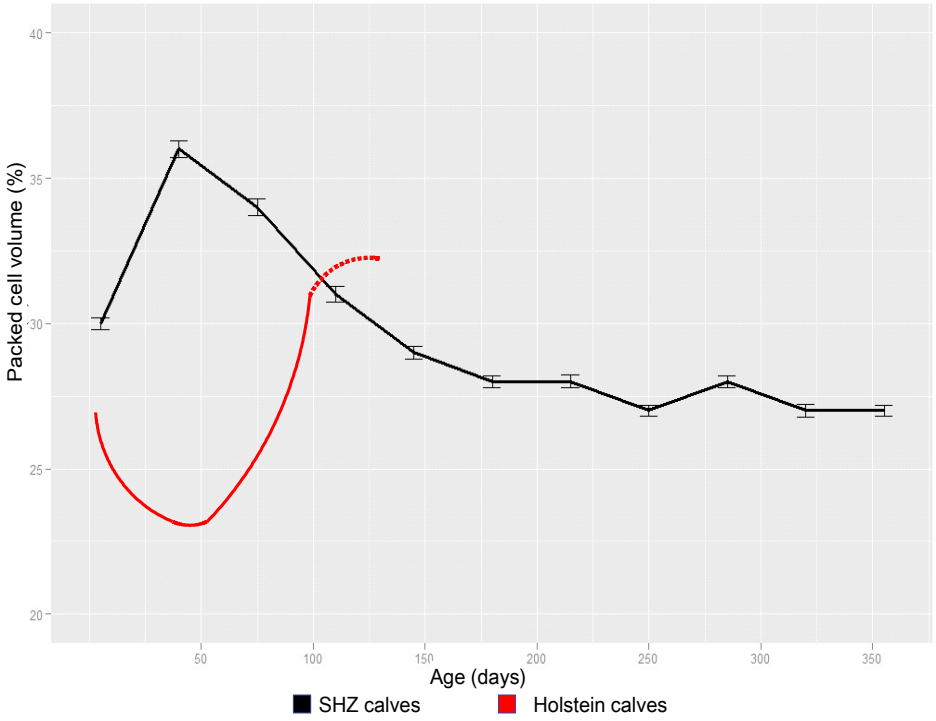


Figure 4.3 Age-related changes in the mean (SE) haemoglobin concentration of East African short-horn calves, from birth to 51-weeks of age (n=548)

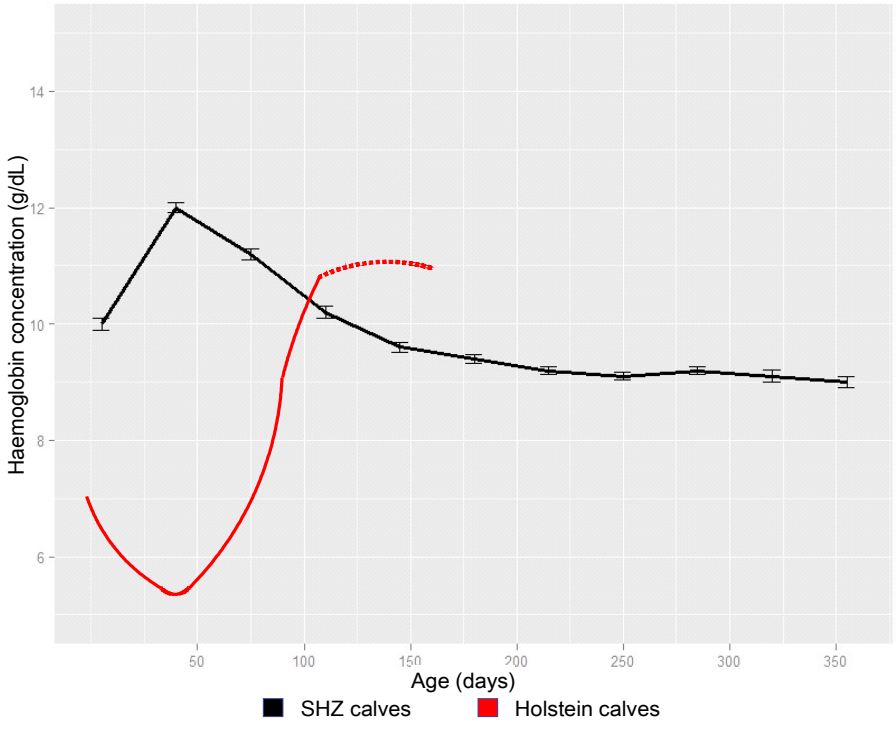


Figure 4.4 Age-related changes in the mean (SE) mean corpuscular volume of East African short-horn calves, from birth to 51-weeks of age (n=548)

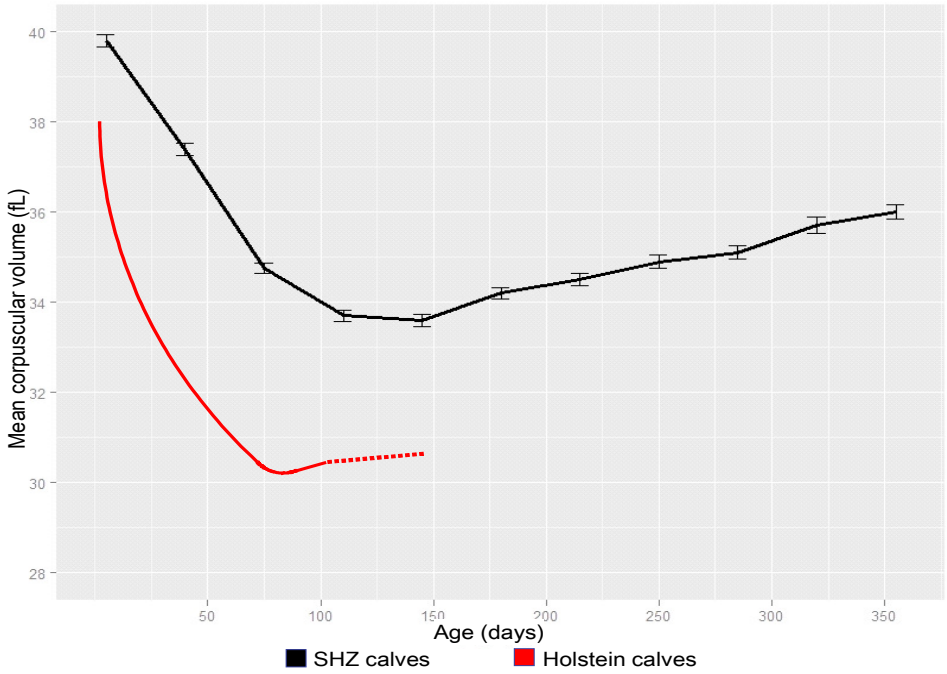


Figure 4.5 Age-related changes in the mean (SE) mean corpuscular haemoglobin concentration of East African short-horn calves, from birth to 51-weeks of age (n=548)

Figure 4.6 Age-related changes in the mean (SE) red cell distribution width of East African short-horn calves, from birth to 51-weeks of age (n=548)

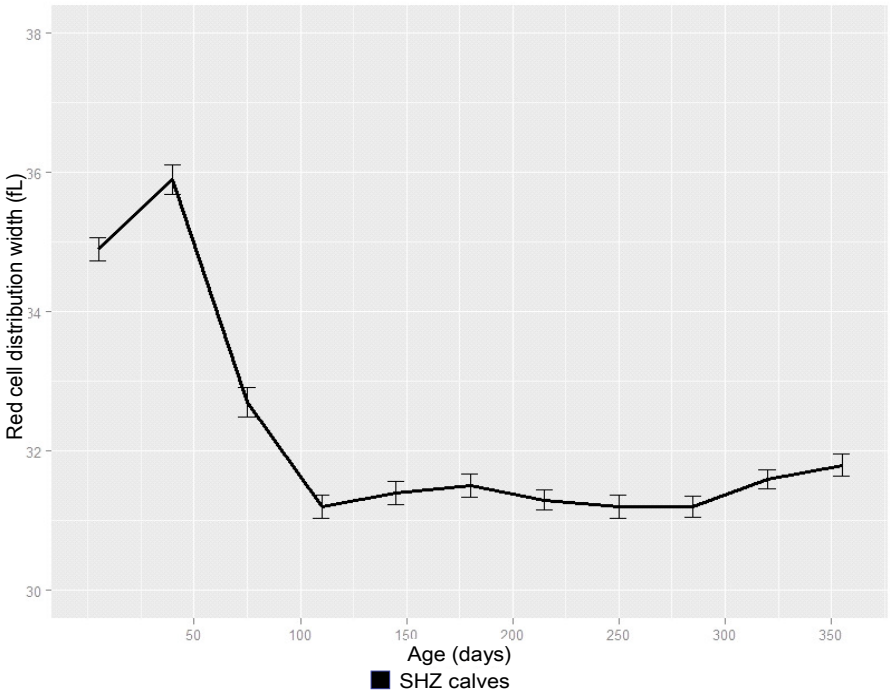


Figure 4.7 Age-related changes in the mean (SE) total white cell count of East African short-horn calves, from birth to 51-weeks of age (n=548)

Figure 4.8 Age-related changes in the mean (SE) absolute lymphocyte count of East African short-horn calves, from birth to 51-weeks of age (n=548)

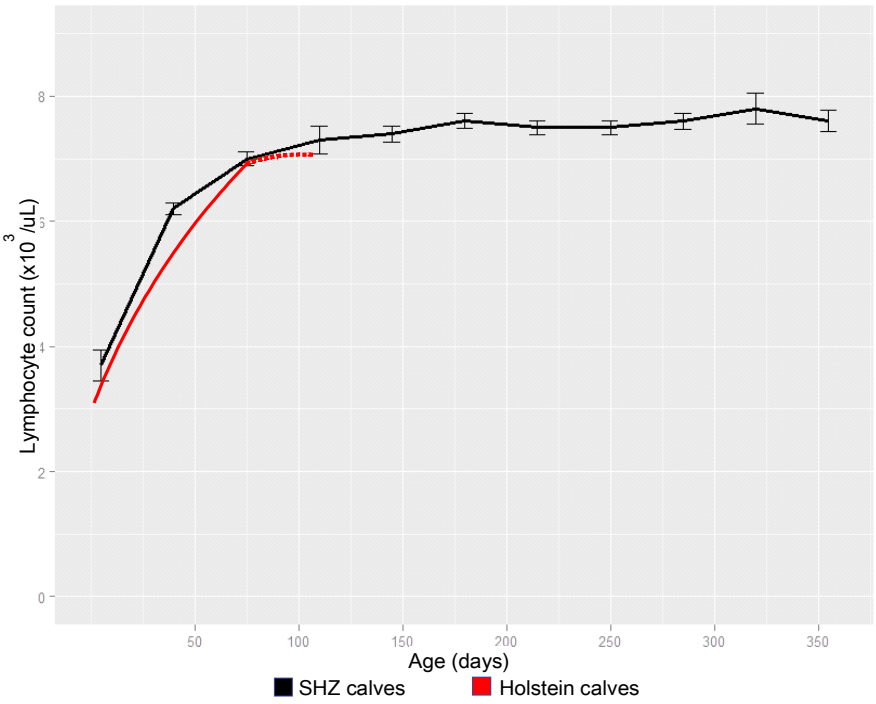


Figure 4.9 Age-related changes in the mean (SE) absolute eosinophil count of East African short-horn calves, from birth to 51-weeks of age (n=548)

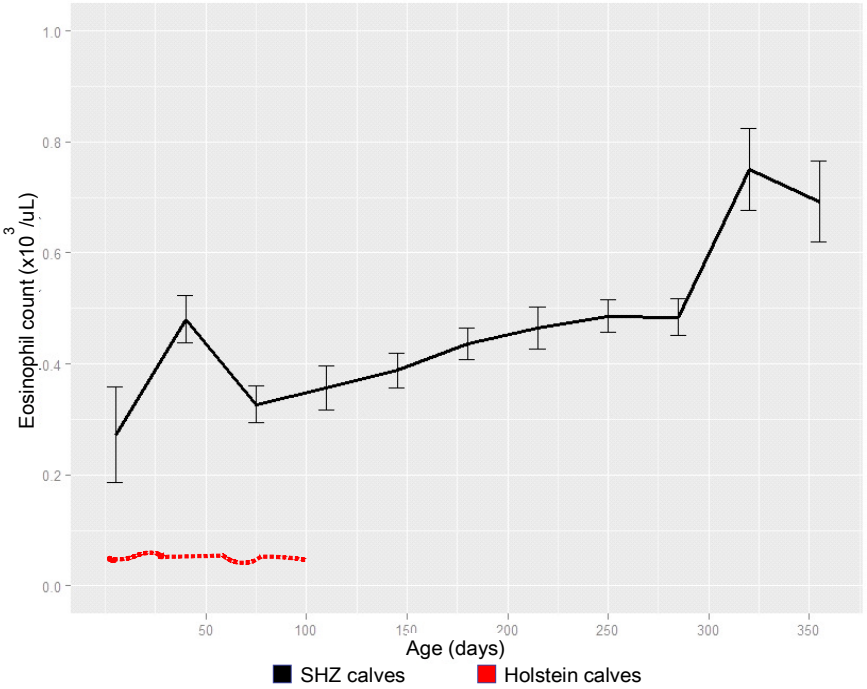


Figure 4.10 Age-related changes in the mean (SE) absolute neutrophil count of East African short-horn calves, from birth to 51-weeks of age (n=548)

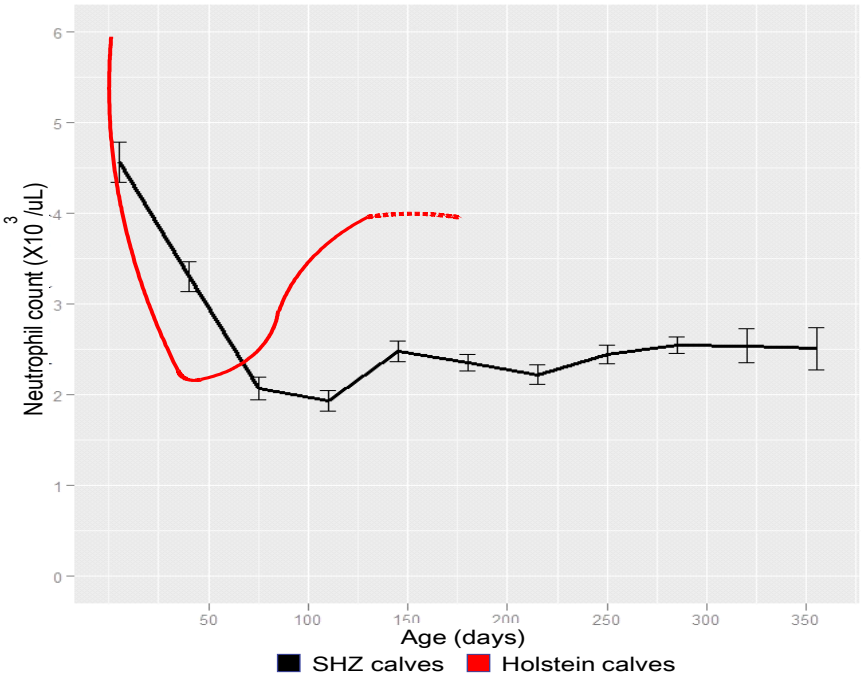


Figure 4.11 Age-related changes in the mean (SE) absolute monocyte count of East African short-horn calves, from birth to 51-weeks of age (n=548)

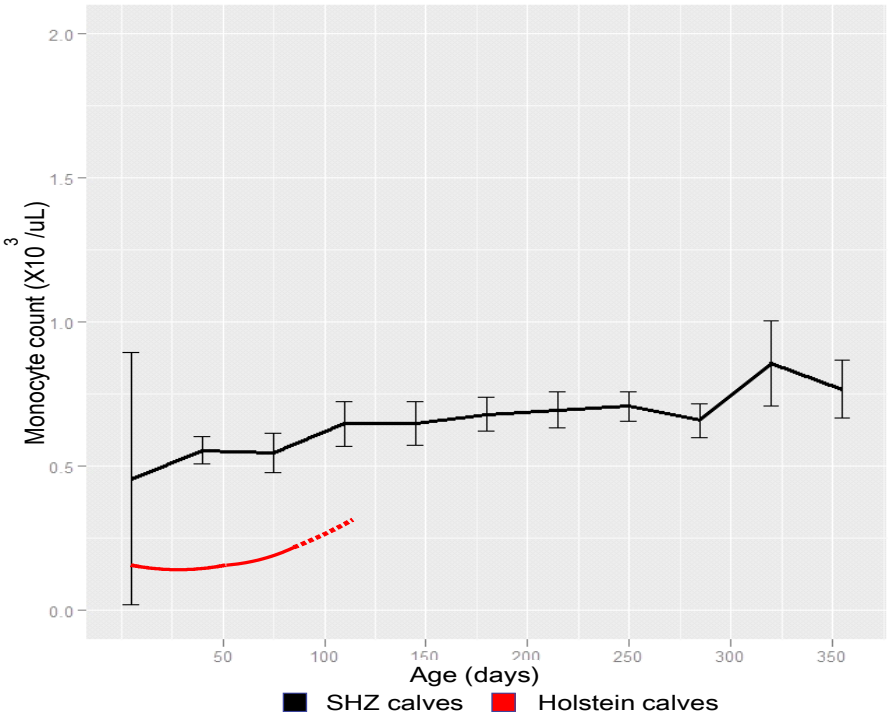


Figure 4.12 Age-related changes in the mean (SE) platelet count of East African short-horn calves, from birth to 51-weeks of age (n=548)

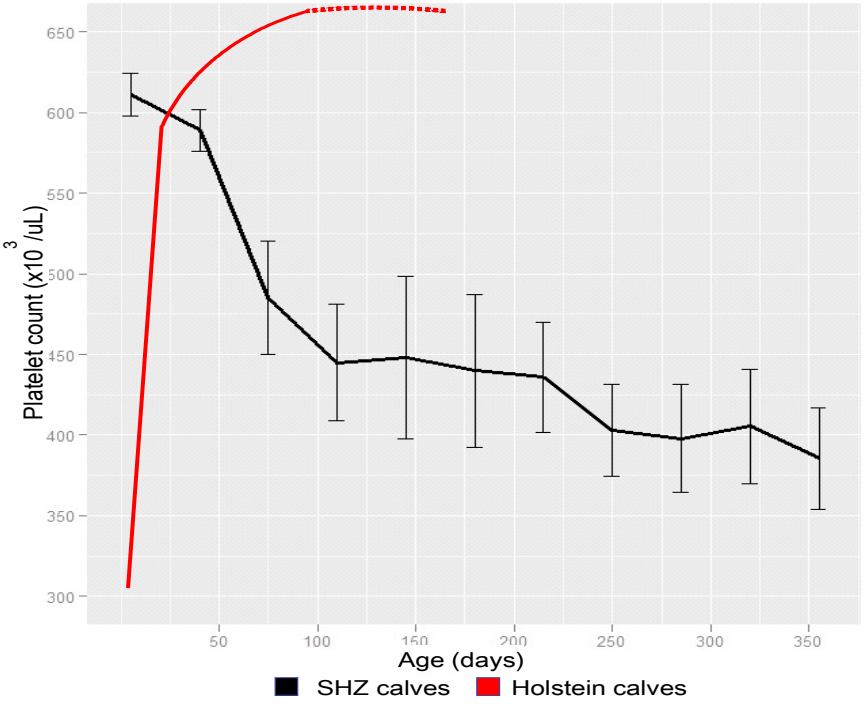


Figure 4.13 Age-related changes in the mean (SE) mean platelet volume levels of East African short-horn calves, from birth to 51-weeks of age (n=548)

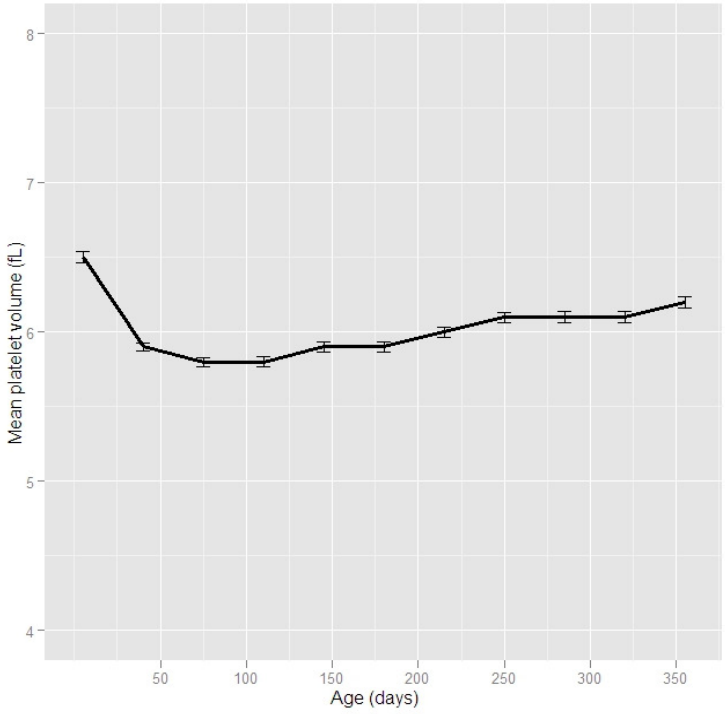
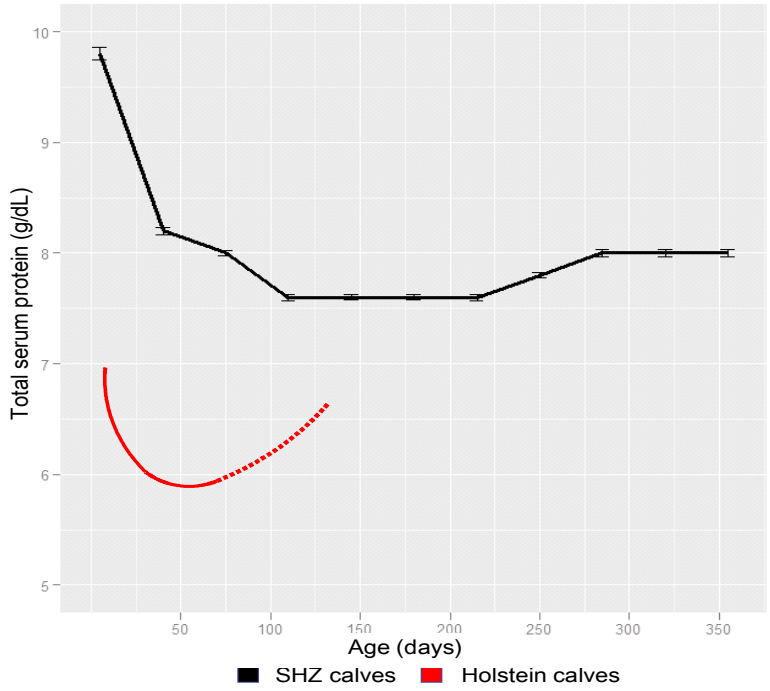


Figure 4.14 Age-related changes in the mean (SE) total serum protein levels of East African short-horn calves, from birth to 51-weeks of age (n=548)



3.2 "Good" calves – haematology in relatively healthy calves

Age-related changes for "good" calves are illustrated using graphs for each parameter (Fig. 4.15-4.23). "Good" calves had significantly ($p<0.05$) higher values than the total population for RCC, HGB and PCV from week 6 and lower values for RDW (weeks 6 and 21). The total population had higher MCV values than the "good" calves at all sampling points, with significant differences ($p<0.05$) at week 6, 16-21 and 36-51. The total population had significantly ($p<0.05$) lower MCHC than the "good" calves at all visits except week 11. The difference in means for all white blood cell parameters between "good" calves and the total population were generally not significant ($p>0.05$), except for WCC at week 6-36 where "good" calves had higher values and eosinophil counts at week 1 where "good" calves had significantly lower counts ($p<0.05$). There were significant ($p<0.05$) differences in Plt between the two groups from week 36 up to week 51 with "good" calves showing a lower Plt than the total population for that period. "Good" calves has significantly ($p<0.05$) lower TSP at week 1, 11 and 36-46.

Figure 4.15 Comparison between the age-related changes in mean (SE) red cell count of "good" calves (n=37) and the total study population (n=548)

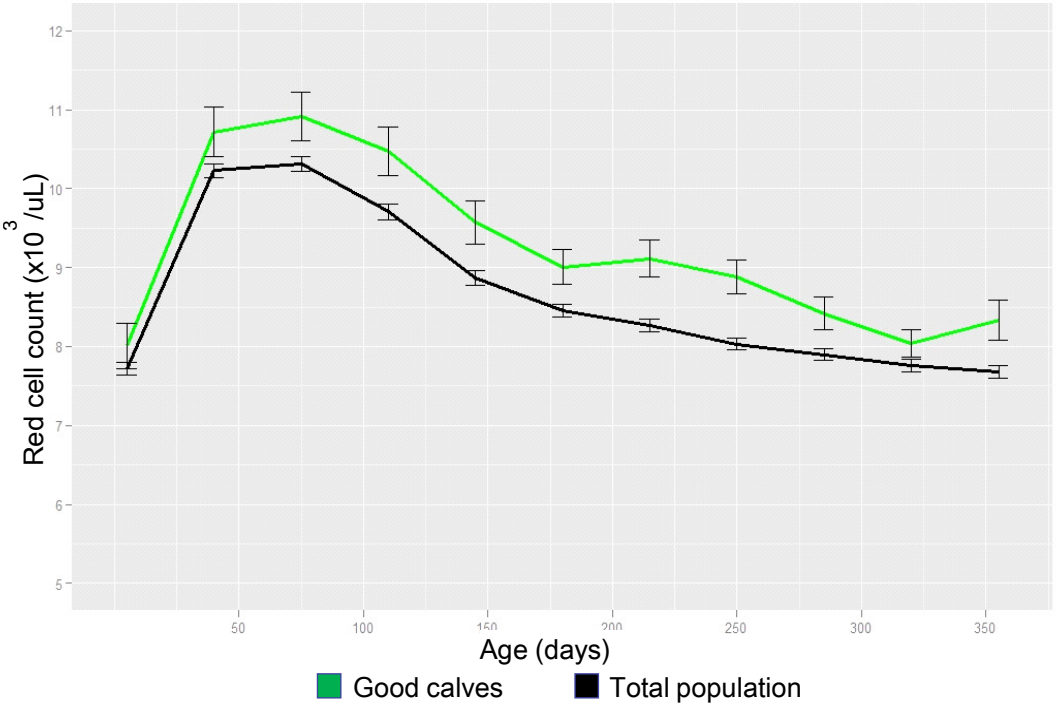


Figure 4.16 Comparison between the age-related changes in mean (SE) packed cell volume of "good" calves (n=37) and the total study population (n=548)

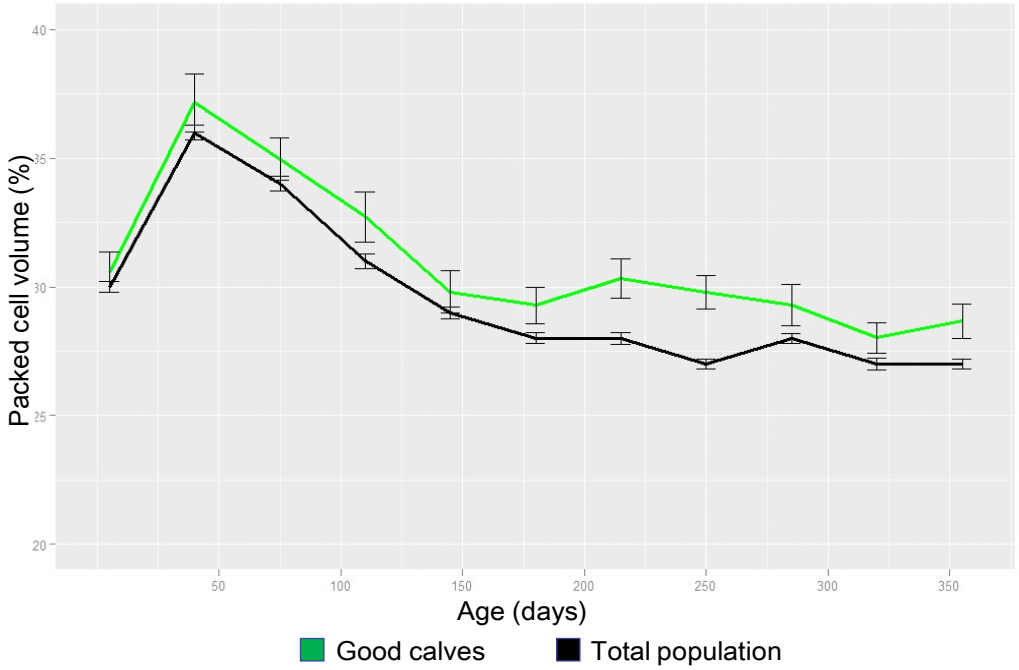


Figure 4.17 Comparison between the age-related changes in mean (SE) haemoglobin concentration of "good" calves (n=37) and the total study population (n=548)

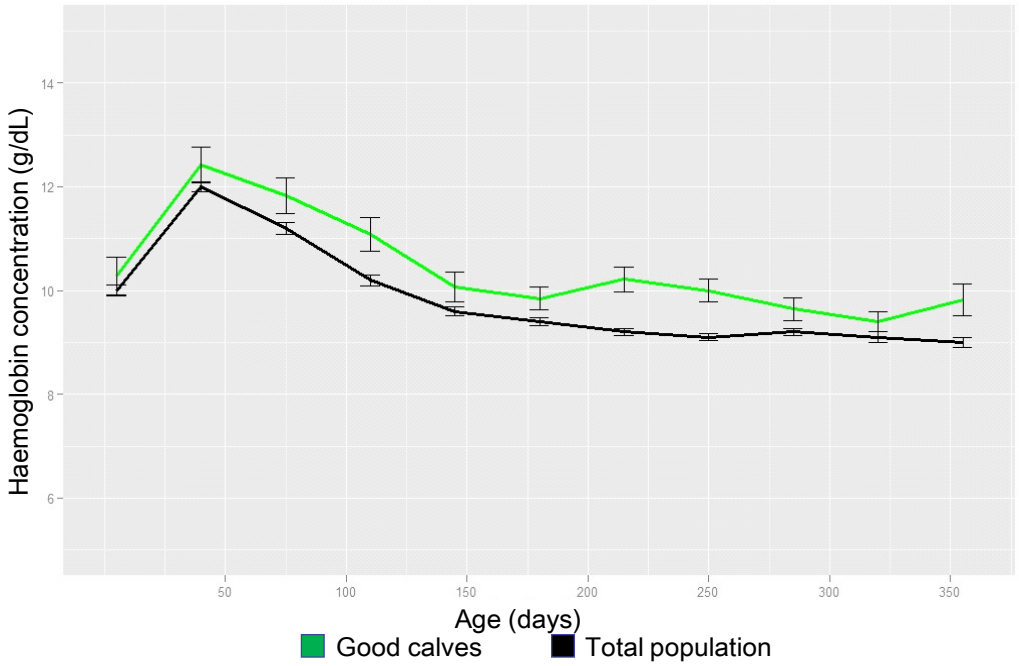


Figure 4.18 Comparison between the age-related changes in mean (SE) mean corpuscular volume of "good" calves (n=37) and the total study population (n=548)

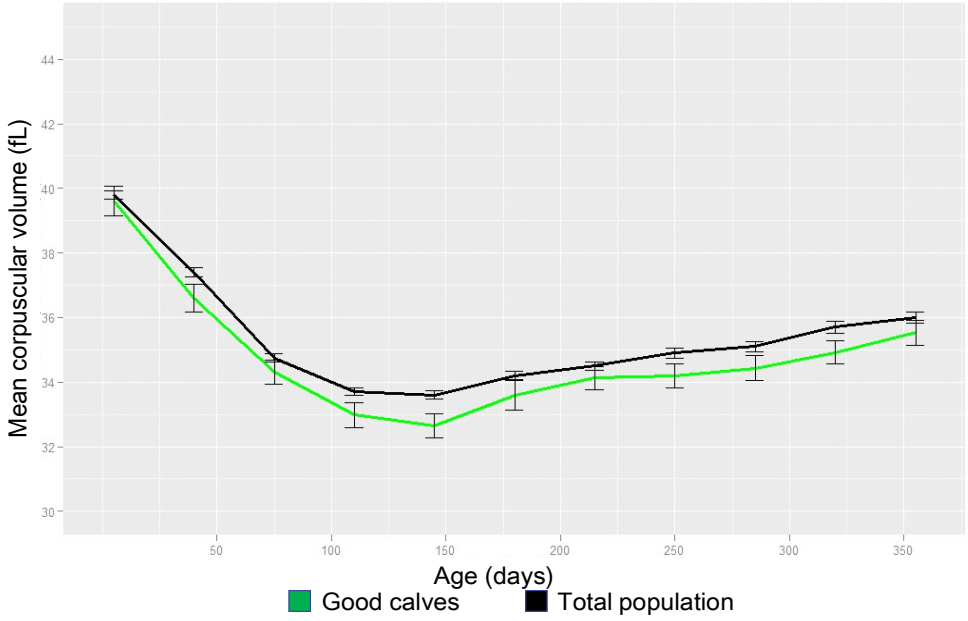


Figure 4.19 Comparison between the age-related changes in mean (SE) mean corpuscular haemoglobin concentration of "good" calves (n=37) and the total study population (n=548)

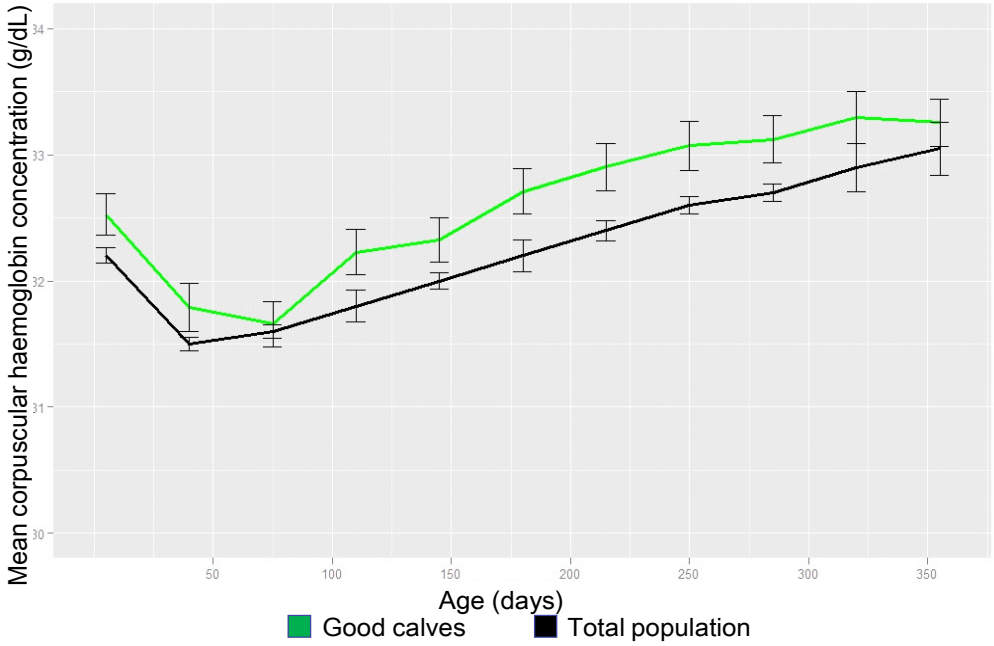


Figure 4.20 Comparison between the age-related changes in mean (SE) red cell distribution width of "good" calves (n=37) and the total study population (n=548)

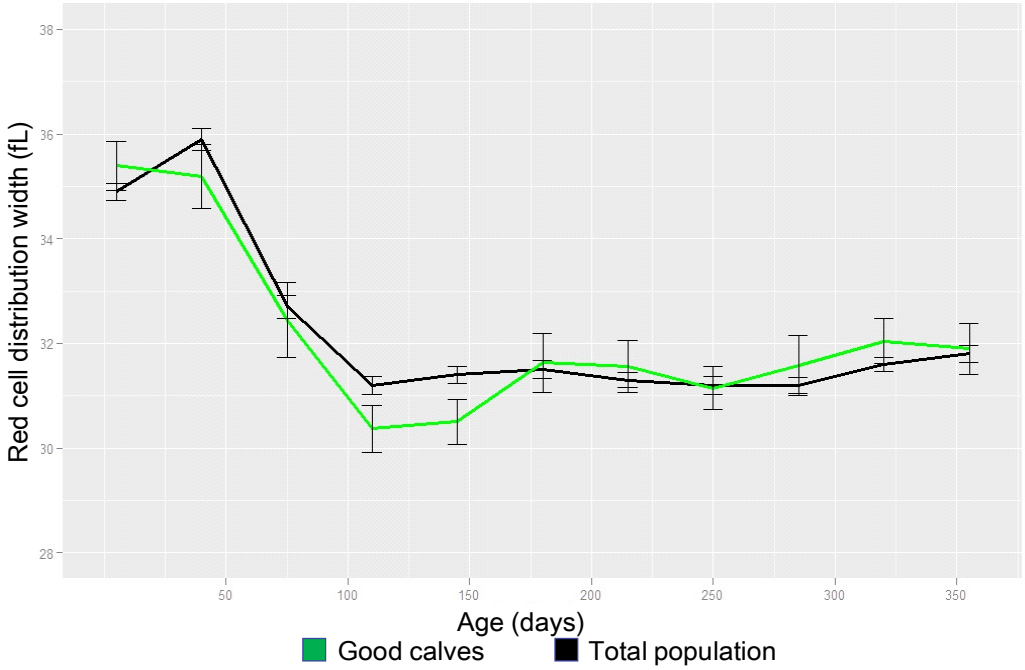


Figure 4.21 Comparison between the age-related changes in mean (SE) total white cell count of "good" calves (n=37) and the total study population (n=548)

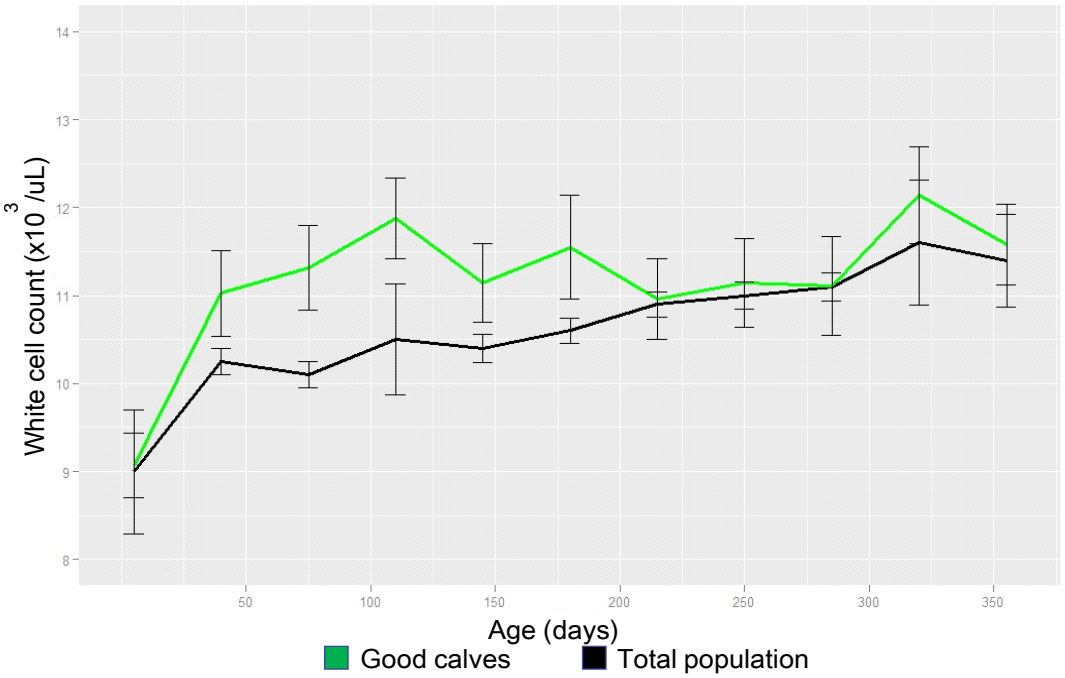


Figure 4.22 Comparison between the age-related changes in mean (SE) platelet counts of "good" calves (n=37) and the total study population (n=548)

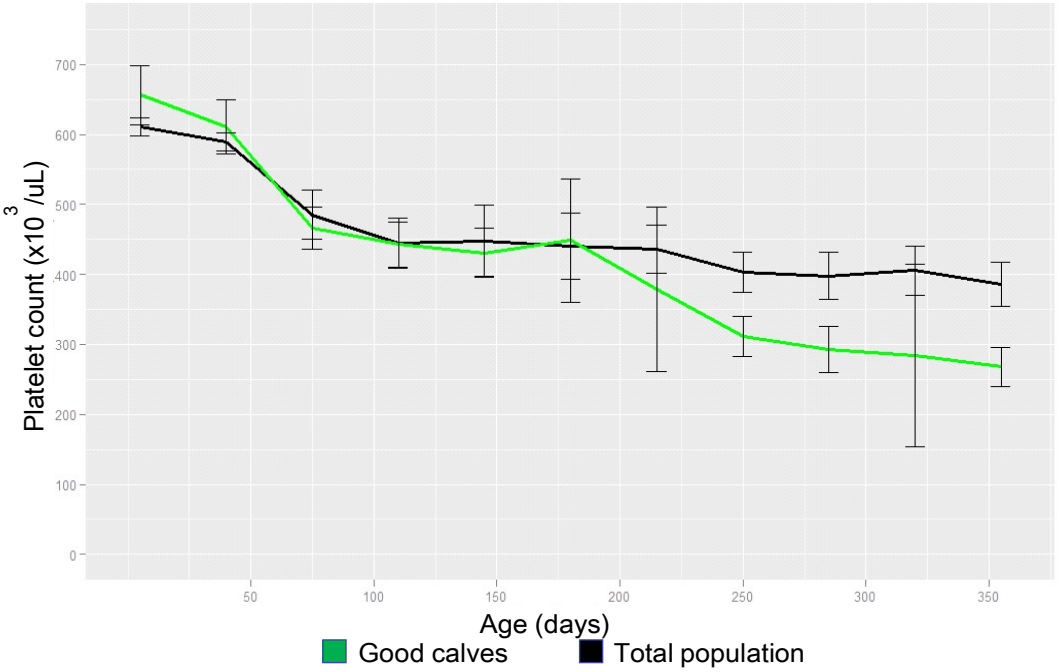
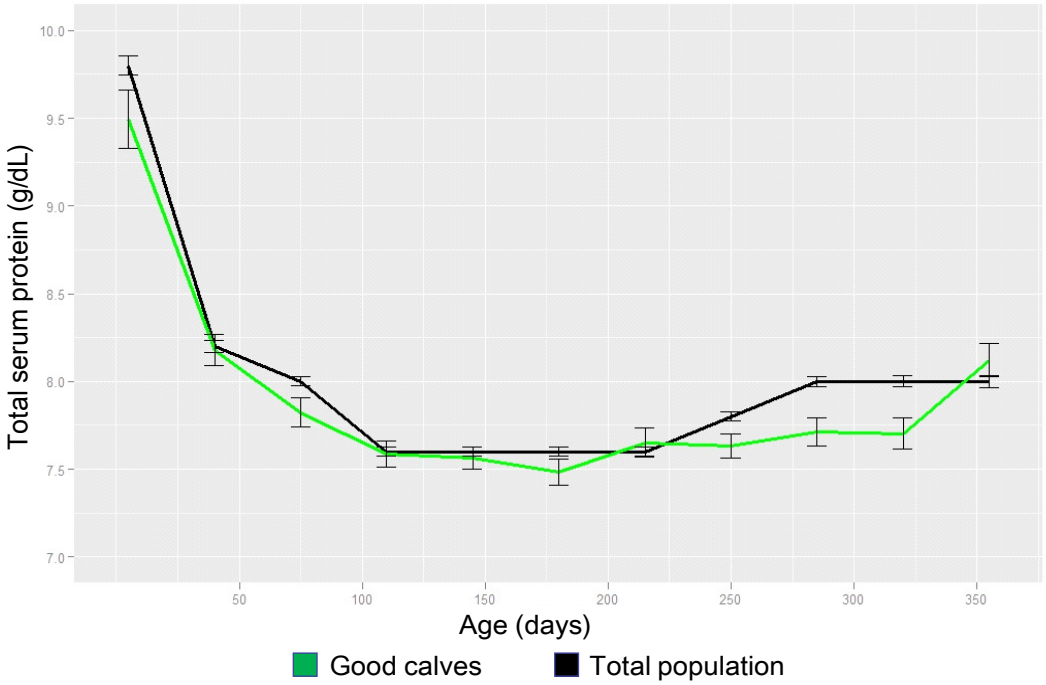


Figure 4.23 Comparison between the age-related changes in mean (SE) total serum protein of "good" calves (n=37) and the total study population (n=548)



3.3 The level of anaemia in the population

The cut-off values for PCV for each age group, as calculated using the "good" calves as a reference sample, are depicted in Table 4.1. From weeks 21 to 51 the values for (mean PCV – 1SD) and (mean PCV – 2SD) were very similar to what is considered anaemic (PCV<25%) and moderately anaemic (PCV<21%) in the literature for European breeds (Tvedten 2010).

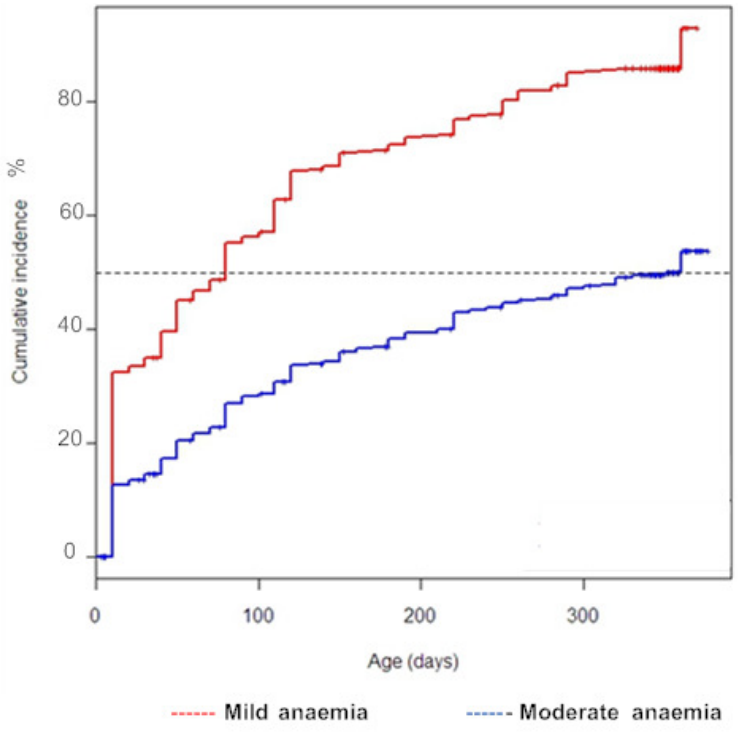
In Figure 4.24 the ages of first detection of an incident of anaemia according to the criteria above is illustrated. Of 548 calves in the study, 468 (85.56%) had at least one episode of anaemia (mean PCV - 1SD) of which 267 (48.81%) had at least one episode of moderate anaemia (mean PCV - 2SD). A total of 1 301 incidents of anaemia (mean PCV – 1 SD) and 372 incidents of moderate anaemia (mean PCV – 2SD) were recorded for the study population during the whole observation period.

More than 50% of calves had an incident of anaemia by the age of 80 days. Fifty percent of calves had an incident of moderate anaemia by the age of 360 days.

Table 4 Cut-off packed cell volume (PCV) values calculated from reference "good" calves sample

Age (weeks)	PCV (%) Mean – 1 SD	PCV (%) Mean – 2 SD
1	28.40	25.42
6	32.49	27.62
11	30.62	25.32
16	28.13	23.55
21	25.27	20.18
26	25.70	21.40
31	26.40	22.81
36	26.35	22.29
41	25.59	21.26
46	24.89	20.48
51	25.46	20.15

Figure 4.24 Cumulative incidences of mild anaemia and moderate anaemia in the calf population



4. DISCUSSION

4.1 Age-related changes in the haematology of East African short-horn Zebu calves

The most significant age-related changes in red blood cell parameters occurred between week 1 and week 16 for the calves in the present study. Concurrent low RCC and PCV with a relatively high or normal MCV and RDW in the first week suggest a macrocytic anaemia. A high RDW value is an indication of anisocytosis which can be either due to an increase in larger cells, typically reticulocytes, as part of a regenerative response, or due to an increase in small red blood cells, often found in cases of iron deficiency. Unfortunately reticulocyte counts were done only on cases with PCV<25%, which excludes almost all observations from week 1 to week 11. A high number of small red blood cells would however, result in a decreased MCV, which is in contrast to what was found in these calves.

When one compares the age-related changes in red blood cell parameters for the calves in this study to the reported values for European breeds (Knowles *et al*. 2000; Mohri *et al*. 2007), there are considerable differences in both the ranges for different age groups, as well as the trends in change over time (Fig. 4.1-14). The reference ranges for European breeds were established in cattle in controlled environments that controlled for disease and nutrition. For this reason a direct comparison to the short-horn Zebus in this study is problematic to interpret, but it is of value since the physiology of age-related changes in these breeds has been studied more extensively than in indigenous African breeds. Red blood cell indices for European breed calves during the neonatal period are much lower than for the short-horn Zebu calves in this study. This may possibly be partly due to haemoconcentration in the short-horn Zebu calves considering their high TSP at week 1 compared to the other breeds. Haemoconcentration falsely increases the values of other blood parameters since these are measured as concentrations per volume of blood. Whether this haemoconcentration is physiological and normal for the short-horn Zebu breed, or pathological, e.g. dehydration due to tropical environmental conditions, or husbandry practices such as tethering, which is a common practice, requires further investigation.

There also appeared to be differences in the trendlines of these parameters over time between the short-horn Zebus and European cattle breeds. In other ruminants, including European cattle breeds, there is a decrease in PCV, HGB and MCV in neonates up to 6 weeks that coincides with an increase in RCC, after which all three parameters increase up to adult levels (Karesh *et al.* 1986; Knowles *et al*. 2000; Mohri *et al*. 2007). These changes are considered physiological and are ascribed to a decline in foetal erythrocytes at a faster rate than the production of adult-type erythrocytes (Karesh *et al*. 1986). This also coincides

with the replacement of foetal haemoglobin by adult haemoglobin (Jain 1986; Mohri *et al*. 2007). Ruminants and primates have a distinct type of haemoglobin during foetal life. In these species embryonal haemoglobin is soon replaced by foetal haemoglobin (Hb F), which in turn is eventually replaced by adult haemoglobin (Hb A). Haemoglobin F has a higher affinity for oxygen than Hb A and its function is to maintain partial pressure of oxygen of foetal blood. The replacement of Hb F with Hb A, referred as "switching", occurs within the first few weeks after birth in ruminants (Boyd & Bolon 2010). Cattle do, however, retain the capacity to synthesize Hb F, and often do in response to anaemia (Boyd & Bolon 2010).

There was no initial decrease of HGB and PCV in the short-horn Zebu calves after birth as in other ruminants. However, there was a significant increase in PCV, HGB and RBC between week 1 and week 6 accompanied by a high RDW that remained relatively high up to 6 weeks which may be suggestive of increased erythropoiesis. The gradual decrease in MCV appeared to be at a slower rate with minimum levels only reached at 21 weeks compared to Holstein cattle which reach a minimum at 10 weeks (Fig. 4.4). It would be of future interest to investigate the switching from Hb F to Hb A in these short-horn Zebu calves. If Hb F replacement by Hb A is more rapid and efficient in reaching adequate Hb A levels before Hb F starts declining, or if there is a delay in the decline of Hb F while Hb A is produced by an increasing number of adult-type erythrocytes, such that the total HGB is maintained or even increased, it would be a possible explanation for the increase in HGB, PCV and RCC during the first weeks after birth.

Prolonged postnatal production of Hb F has been described in humans in certain haemoglobinopathies where production of Hb F continues into adulthood (Pasvol, Weatherall, Wilson, Smith & Gilles 1976; Nagel 1990). It is considered benign, but has been shown to be protective against the effects of malaria in early childhood by retarding the intraerythrocytic growth of *Plasmodium falciparum* (Pasvol *et al*. 1976; Nagel 1990). Other haemoglobinopathies, such as the Hb mutation that causes sickle cell anaemia, have been found to be protective against malaria in humans (L□pez, Saravia, Gomez, Hoebeke & Patarroyo 2010; Pishchany & Skaar 2012). In areas where malaria is endemic there is a high frequency in the gene for these haemoglobinopathies in the local human populations (L□pez *et al*. 2010). Prolonged production of Hb F in humans has also been described in neonates under hypoxic stress (Shiao & Ou 2006). The production of Hb F is also pharmacologically induced in the treatment of sickle-cell anaemia. It makes for interesting speculation whether this adaptive change is present in the short-horn Zebu cattle breeds and will require further diagnostic tests to distinguish between haemoglobin types. Such an adaptation would equip calves with increased tolerance to the effects of the endemic infectious causes of anaemia,

such as trypanosomosis, during the first few months after birth. If such calves become infected early in life they would develop mild symptoms of disease but develop an immunity that is protective into adulthood.

Neonatal anaemia due to delayed switching from Hb F to Hb A has been described in several ruminant species, including Mouflon sheep (Hawkey *et al*. 1984) and roan antelope (*Hippotragus equinus*) (Parsons *et al*. 2006). In these species there is a sharp decline in total haemoglobin due to the delay in the production of Hb A. A possible link between this neonatal anaemia in roan antelope and an increased susceptibility to theileriosis has been suggested (Parsons *et al*. 2006).

Certain blood-borne pathogens have been shown to vary in their affinity for red cells of different sizes. Fandamu *et al*. (2007) found that, in cattle infected experimentally with *T. parva*, parasitized red blood cells were significantly smaller that non-parasitized cells, and that the MCV in cattle with lethal reactions was significantly lower than cattle with non-lethal reactions. *Plasmodium vivax* has also been reported to have a tendency to parasitize smaller red cells, in contrast to *P. falciparum* that can infect red cells of any age (Ghosh & Ghosh 2007). A high number of large red cells during the first weeks of life, as indicated by the high RDW from week 1 to week 6, together with a high MCV, might also have a protective effect against blood-borne pathogens, including *T. parva*, during early calf-hood in the indigenous calves in the study area.

The high MCV with a concurrent decrease in MCHC between week 1 and week 6 could possibly be due to the increasing numbers of immature RBC. Immature RBC do not yet produce haemoglobin optimally since haemoglobin production per cell increases as red blood cells mature (Harvey 1989). Mohri *et al*. (2007) found that the size of erythrocytes decreased in neonatal Holstein calves up to the age of 3-4 months. In this study there was a decrease in PCV and HGB together with a continuing decrease in MCV and MCHC from week 6, while RCC started decreasing from week 11. This may be suggestive of the development of microcytic hypochromic anaemia. Mohri, Sarrafzadeh, Seifi & Farzaneh (2004) found that iron supplementation during the first four weeks after birth corrected the drop in HGB in dairy calves. The iron levels of calf serum were not evaluated in this study but could possibly have added value to explaining the results in this study.

The relatively low numbers of WCC and absolute lymphocyte counts and high absolute neutrophil count around birth are consistent with what is reported for other cattle breeds (Mohri *et al*. 2007; Knowles *et al*. 2000) and is due to perinatal stress and high levels of

cortisol during partus (Jain 1986). After the first week, levels in WCC, absolute lymphocyte- and eosinophil counts increase as the immune system matures and the animals become exposed to pathogens with resultant cellular responses. Compared to values for neonatal calves of European breeds, the absolute eosinophil and monocyte counts of the short-horn Zebu are considerably higher and possibly related to high parasite burdens in these calves.

The decrease in platelets from week 1 to week 11 is in contrast to what is described for European breeds. The reason for the high value at week 1 followed by the decrease is unclear. The MPV is relatively high at week 1. Together with a high RDW at week 1 this may be suggestive of a regenerative response by the bone marrow at the time around birth.

The initial high levels in TSP in the first week are likely due to the uptake of immunoglobulins from colostrum. The degradation of absorbed immunoglobulins contributes to the decreasing TSP at subsequent sampling points. Chronic blood loss, such as seen in chronic parasitic infections, including heavy helminth infestations, will also results in a decline in TSP over time. Over the whole follow-up period it appeared that the TSP in the short-horn Zebu was higher compared to what is expected in European cattle breeds. One possible reason, as discussed earlier, is their hydration status with possible haemoconcentration. Another possible explanation for the high TSP is high levels of antigenic stimulation resulting in high globulin levels. Exposure to pathogens, even from an early age, is considerable under field conditions in the tropical environment. Unfortunately only total serum proteins were investigated. Without distinguishing between different proteins, in particular albumin and the various immunoglobulins, it is difficult to come to any conclusions with regard to the levels and trends of TSP levels in the calves in this study.

4.2 "Good" calves – haematology in relatively healthy calves

There were no significant differences ($p>0.05$) between any of the haematology parameters between "good" calves and the rest of the population at week 1. As the calves aged, the difference in red blood cell parameters became more evident. There was an indication that the general population under study tended toward an anaemic state as time progressed. The mean MCV of the total population was higher than the mean MCV of the "good calves" from week 6. In contrast, the MCHC of the total population was lower than the "good calves". The higher MCV and lower MCHC of the total population is suggestive of macrocytic hypochromic anaemia. The high RDW for the total population suggests that many animals had anisocytosis, which is associated with a reticulocytosis and regeneration of red blood cells. The insignificant difference in the white blood cell responses could suggest that the "good" calves were exposed to pathogens at the same rate as the general population and

had similar cellular responses. The platelet count of the total population was higher than that of the “good calves” from week 31. Thrombocytosis develops in response to chronic blood loss, such as haemolysis or haemorrhage as a result of chronic parasitism. There was also no significant difference in TSP at any time-point between “good” calves and the total population. It is thus unlikely that their colostral intake or immunoglobulin responses differed significantly either. It therefore seems that “good” calves were not suffering to the same extent from the development of a progressive anaemia as the general population.

The control of anaemia has been described as a characteristic of trypanotolerance (Naessens *et al*. 2002). It can be speculated that this is true for other haemoparasitic infections as well, and may prove a worthwhile topic to investigate further.

4.3 The level of anaemia in the population

The cumulative hazard rates of anaemia illustrate that anaemia is a significant syndrome in this calf population. Since the calves were from an area with a high prevalence of tick-borne diseases and helminth infections one would expect a high incidence of anaemia. The level of anaemia in the population appeared to be increasing in severity, as shown by the downward trend in PCV for the population from week 6 to week 51 (Fig. 4.3). It would be interesting to investigate the PCV of calves past 51 weeks to see what their blood profile would be in adulthood.

Although most calves suffered from anaemia at least once during their follow-up period, the number of cases of moderate anaemia (PCV< mean - 2SD) was considerably low compared to the total number of cases of anaemia (PCV<mean – 1SD). This is possibly due to the inherent tolerance these calves have to the endemic diseases and their capacity to manage the progressive development of clinical signs. These diseases cause severe clinical signs in other less-tolerant exotic cattle breeds.

One should keep in mind that the reference sample of calves was not from a disease-free nutritionally controlled environment. These “good calves” were alsolikely to be infected with pathogens such as helminths, which are very prevalent in the population (see Chapter 5), and is suggested by the upward trends over time in their white blood cell and eosinophil counts. Although these “good” calves did not show clinical signs of anaemia, some may have had subclinical anaemia, which would have affected the calculated cut-off points.

5. CONCLUSION

Anaemia appears to be an important syndrome in the calf population under study as demonstrated by the high number of calves showing anaemic episodes. Whether these trends and levels in the haematological parameters of the short-horn Zebus play a role in the susceptibility or resistance to infectious disease would be an interesting topic for further investigation.

There is a need to establish breed-specific reference ranges for blood parameters for the East African short-horn Zebu. It is also clearly evident that baseline values differ with age in the neonate and need to be taken into consideration when assessing the health status of an animal. The changes in the red blood cell parameters of the calves under study, especially during the neonatal period, are not explained by what is known about the physiology for other cattle breeds. This warrants further research into the dynamics of blood cell parameters of the East African short-horn Zebu and probably other indigenous cattle breeds.

CHAPTER 5

ANAEMIA IN EAST AFRICAN SHORT-HORN ZEBU CALVES: THE DISTRIBUTION OF PATHOGENS IN THE POPULATION

1. INTRODUCTION

Animals that live in a tropical environment are exposed to a variety of pathogens, including tick-borne pathogens, tsetse-borne pathogens and intestinal parasites. The clinical significance of pathogens on a population basis is in part determined by the exposure rate of animals to these pathogens. If the prevalence of pathogens is significantly high, the risk of exposure is high and the potential that infectious loads are high enough to cause disease is increased.

In this chapter measuring the pathogen burden of calves in the study area during the first year of life is attempted. The frequency distributions of pathogens were calculated to determine which pathogens were the most prevalent in the study area. The variation in the pathogen burden with age was investigated through the prevalence and cumulative incidences of pathogens. These two indices were used to highlight periods of high infection risk.

The frequency of co-infections of pathogens was also investigated in this chapter, both at the visit-level (specific age groups) and the calf-level. Particular reference was made to pathogens that are known to cause anaemia, such as strongyle-type worms and *Trypanosoma* spp.

2. MATERIALS AND METHODS

* General methodology is discussed in Chapter 2.

2.1 Frequency distribution of pathogens

The samples from 5-weekly routine visits and the additional clinical visits from October 2007 to September 2010 from calves of the IDEAL project were used to describe the distribution of pathogens in the population at a calf level, as well as at a visit level. Results from the following diagnostic tests were used for this analysis: thin and thick peripheral blood smears, DG and HCT for trypanosomes, McMasters test, faecal flotation, direct Baermann test,

faecal sedimentation, faecal larval culture, and serology for tick-borne pathogens. Where diagnostic tests, e.g. RLBT, were only done on the 51-week samples, the prevalences of these pathogens were calculated only at this time-point. The pathogen variables were distinguished by the test used for diagnosis, e.g. serology or microscopy. At the calf level, a calf was considered positive if the relevant pathogen was detected at least once during its follow-up period.

2.2 Prevalence and cumulative incidences of pathogens

The point prevalence of a pathogen is the number of positive animals as a percentage of the animals sampled at that visit (age) (Dodge 2003). Comparing the point prevalences of pathogens at sequential time-points (age group) allows one to measure the variation in prevalence of pathogens with age. Infections of short duration are also highlighted since animals that have cleared themselves of infection will not be included at subsequent time points unless they are re-infected. Separate histograms were used to illustrate the point prevalences of intestinal parasites (helminths and coccidia) and the blood-borne pathogens (tick-borne pathogens and *Trypanosoma* spp.).

The incidence of a pathogen is the number of new infections during a specified time period as a percentage of the total number of calves at risk at the beginning of that time period. The cumulative incidence is the sum of the incidences of each subsequent time-point from the beginning of the study period. The cumulative incidence is in essence a measure of the total number of calves that become infected with specific pathogens over the follow-up period (Dodge 2003) and can be used to calculate the probability of infection at specific time-points. The cumulative incidences of intestinal parasites and tick-borne pathogens are illustrated with Kaplan-Meier graphs.

Serology results were used to calculate the cumulative incidence of seroconversion to the tick-borne pathogens (*Theileria parva, Theileria mutans, Anaplasma marginale* and *Babesia bigemina*). The cut-off values in percentage positivity (PP) values for specific pathogens were as follows:

1. PP value >20 for *T. parva* and *T. mutans* (Katende *et al*. 1998); and
2. PP value >15 for *A. marginale* and *B. bigemina* (Katende *et al*. 1990).

Calves were classed as seroconverted under the following criteria:

1. In calves under 10 weeks of age, if there was a rise in titre above the cut-off for the specific pathogen;

2. If antibody PP levels in calves that did not seroconvert before 10 weeks of age increased to levels above specific cut-offs for at least two consecutive visits; or
3. If, at the last visit of each calf that had not seroconverted previously, the PP value was above the cut-off.

2.3 Prevalence of co-infections

The level of co-infections of pathogens was investigated with the use of histograms which illustrate the frequency distribution of the number of co-infecting pathogens per visit as well as the number of infections per calf. The number of co-infections per visit is the number of infections that were detected at one visit. The number of infections per calf is the total number of pathogens the calf was infected with during its follow-up period, be that at the same time or at subsequent follow-up visits. Results from routine 5-weekly and clinical visits were used to calculate the number of co-infecting pathogens.

The frequency distribution of co-infections between pathogens that are known to cause anaemia were additionally analysed and illustrated by the histograms. The pathogens known to cause anaemia that were considered include*: T. mutans; B. bigemina; A. marginale; Trypanosoma* spp.*;* strongyle-type nematodes; and specifically *Haemonchus bedfordi;* coccidia and *Fasciola gigantica*.

2.4 Data analysis

Data analysis was done using R 2.8.1 (Ihaka & Gentleman 1996). Cumulative incidences and Kaplan-Meier plots were calculated with survival analysis and life tables using the Survival package (version 2.36-9) in R. All other graphs were drawn in gg-plots (Wickham 2009).

3. RESULTS

3.1 The frequency distribution of pathogens in the calf population

The frequency distributions of pathogens at calf and at visit level are shown in Table 5. The most common pathogen detected in the population was *Theileria* spp. with 97.63% of calves positive for piroplasms on microscopy at some time point during their follow-up period. A high number of calves were exposed to both *T. parva* and *T. mutans*, as reflected by seroconversion rates of over 70% for both pathogens. Just over 35% of calves seroconverted to *A. marginale* and fewer than 10% seroconverted to *B. bigemina*. There were even lower numbers of calves positive for these two pathogens on microscopy (fewer

Table 5 The frequency distribution of pathogens per calf and per visit

Pathogen		Number of calves positive for factor n=548		Number of visits positive for factor n=5602	
		n	%	n	%
Tick-borne pathogens					
Theileria spp.	Piroplasma (mcr)[1]	535	97.63	3260	58.19
T. parva	seroconverted	396	72.26	-	-
T. mutans	seroconverted	396	72.26	-	-
Anaplasma spp.	Mcr[1]	53	9.67	56	1.0
A. bovis	Positive[3]	207	38.12	-	-
A. marginale	seroconverted	197	35.95	-	-
Babesia spp.	Mcr[1]	4	0.73	4	0.07
B. bigemina	seroconverted	132	24.09	-	-
***Trypanosoma* spp.**					
Trypanosoma spp.	Mcr[1]	42	7.66	68	1.21
T. vivax	Pcr[2] at final visit	49	8.94	-	-
Intestinal pathogens					
Coccidia	Positive[3]	477	87.04	1442	25.74
	Av OPG > 1000[4]	112	20.44	-	-
Strongyle-type nematodes	Positive[3]	531	96.90	3555	63.46
	Av EPG >1 000[5]	117	21.35	-	-
	EPG > 1000[6]	-	-	725	12.94
Haemonchus bedfordi	Positive[3]	525	95.80	2857	51.0
Strongyloides spp.	Positive[3]	319	58.21	604	10.78
Fasciola gigantica	Positive[3]	88	16.06	119	2.12

[1] Mcr: microscopy. This indicates that microscopy was used to diagnose the pathogen.
[2] Pcr: polymerase chain reaction. This indicates that PCR were used to diagnose the pathogen.
[3] Positive. This indicates, at the calf-level, whether the calf was infected at least once, and at the visit-level, whether the pathogen was detected.
[4] Av OPG>1000. The average oocysts per gram faeces per calf was over 1000.
[5] Av EPG>1000. The average eggs per gram faeces per calf was over 1000.
[6] EPG>1000. This indicates that the eggs per gram faeces per visit was over 1000.

than 10% for *Anaplasma* spp. and less than 1% for *Babesia* spp.). Calves were only tested at their last visit for *Anaplasma bovis* using the RLBT with 38.12% of calves testing positive.

The PCR for *Trypanosoma* spp. was done only at visits where calves had tested positive by microscopy as well as at the last visit of each calf. The prevalence of *T. vivax* (PCR) was therefore only calculated on a calf level. More calves were positive for *T. vivax* on PCR at

the last visit than calves positive for *Trypanosoma* spp. on microscopy and it can be assumed that the *T. vivax* is the main trypanosome in the population.

The second most common pathogen type detected was strongyle-type nematodes with almost 97% of calves infected at least once. Over 20% of calves had an average EPG over 1000 and at almost 13% of visits the EPG was over 1000. Specifically, *Haemonchus* spp. were detected at least once in over 95% of calves. Coccidia was the second most common intestinal pathogen type and the third most common pathogen overall with over 87% of calves infected at least once. A high number of calves (20.44%) had an average OPG over 1000. Although almost 60% of calves were infected with *Strongyloides* spp. at least once, *Strongyloides* spp. were only detected at 10.8% of visits. A relatively low number of calves (16.1%) were affected by *Fasciola gigantica*.

3.2 The prevalence and cumulative incidences of pathogens

Intestinal parasites

The point prevalences of intestinal parasites, specifically strongyle worms, *Strongyloides* spp. worms, coccidia and *Fasciola gigantica* are illustrated in Figure 5.1. The cumulative incidences of intestinal parasites are illustrated in Figure 5.2.

Figure 5.1 The point prevalences of intestinal parasites

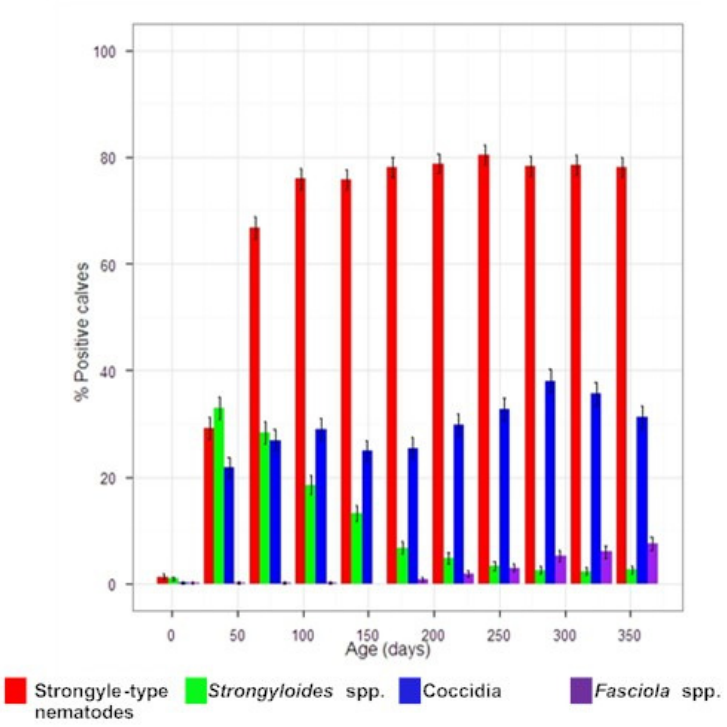
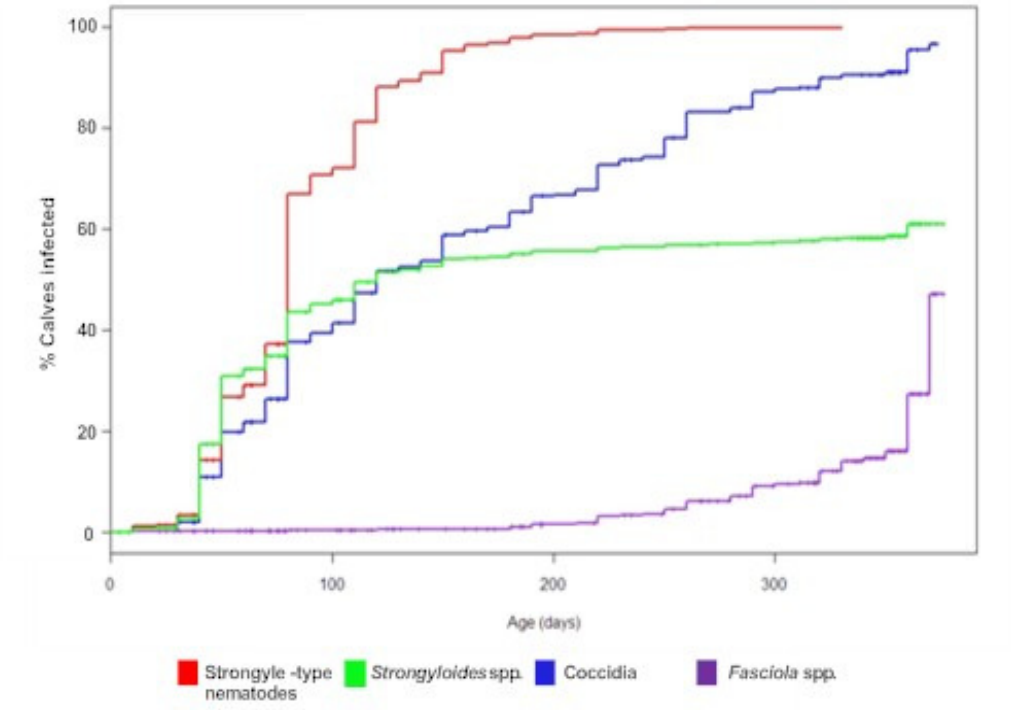


Figure 5.2 Cumulative incidences of intestinal parasites



Very few calves were infected with any helminth species at one week of age. Strongyle worms were the most prevalent of the helminth species at most time-points. The prevalence of strongyle worms increased with age with a prevalence that remained between 67% and 80% from 16 weeks (100 days) age. A relatively high number of new cases occurred between 6 and 11 weeks, with a 68% probability of becoming infected by 80 days. The probability of becoming infected by 150 days was 95%. The probability of becoming infected by 350 days was 99.8%.

Strongyloides spp, had the highest prevalence of the intestinal parasites at week 6 (33%), but the prevalence decreased with age and very few calves were infected with these worms by 51 weeks of age (2.6%). The probability of becoming infected by the end of the follow-up period was 61%. The highest number of new cases occurred between 40 and 80 days (218 new cases). Few calves acquired new *Strongyloides* infections after 180 days of age, with only 20 new cases between 180 days and the end of the follow-up period.

Coccidia, as the second most common intestinal parasites, had a prevalence above 20% from 6 weeks and older. The highest prevalence was at 41 weeks (38%). The probability of becoming infected by the end of the follow-up period was 97%. The number of new infections increased steadily over the study period. The total number of new infections by

100 days was 220, between 100 and 200 days a total of 129 new infections, and between 200 and 350 days a total of 119 new infections.

Fasciola gigantica showed an increase in prevalence with age with the highest prevalence of 7.5% at 51 weeks. Compared to other intestinal pathogens, the number of cases at any time point was relatively low, except when compared with *Strongyloides* that had a lower prevalence than *F. gigantica* after 250 days.

Blood-borne pathogens (microscopy)

The point prevalences of blood-borne pathogens identified by microscopy on the 5-week visits are illustrated in Figure 5.3.1. No distinction to species level was made on microscopy. *Theileria* spp. (piroplasms and/or schizonts) were the predominant parasites observed, with prevalence fluctuating around 80% of calves infected from 250 days and over. Figure 5.3.2 illustrates the blood-borne pathogens other than *Theileria* spp., as identified on microscopy. The number of calves infected with either *Anaplasma* spp., *Babesia* spp. or *Trypanosoma* spp. was considerably lower than the number infected with *Theileria* spp. *Anaplasma* spp. were identified in calves at all time points, although a higher number of older calves were positive. It was the most prevalent blood-borne pathogen, after *Theileria* spp., in neonatal calves. Trypanosomes were also indentified in calves of all age groups, with more calves infected in older age groups. Based on microscopy, *Trypanosoma* spp. was the most prevalent blood-borne pathogen at 51 weeks, after *Theileria* spp., with 2.4% of calves infected. Only three individual cases of *Babesia* spp. were detected on microscopy.

Figure 5.3.1 Point prevalences of blood-borne pathogens identified by microscopy

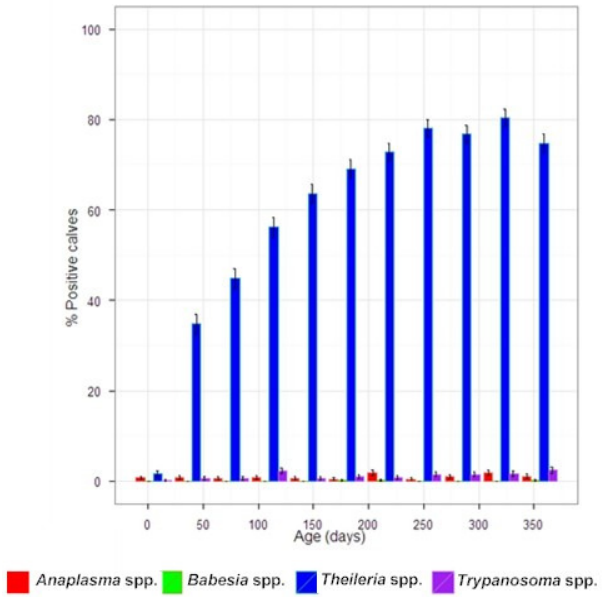
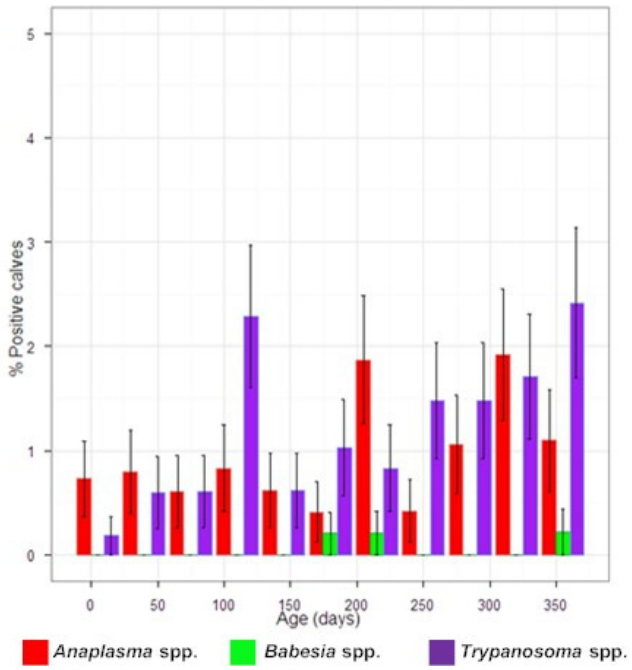


Figure 5.3.2 Point prevalences of blood-borne pathogens other than *Theileria* spp. identified by microscopy

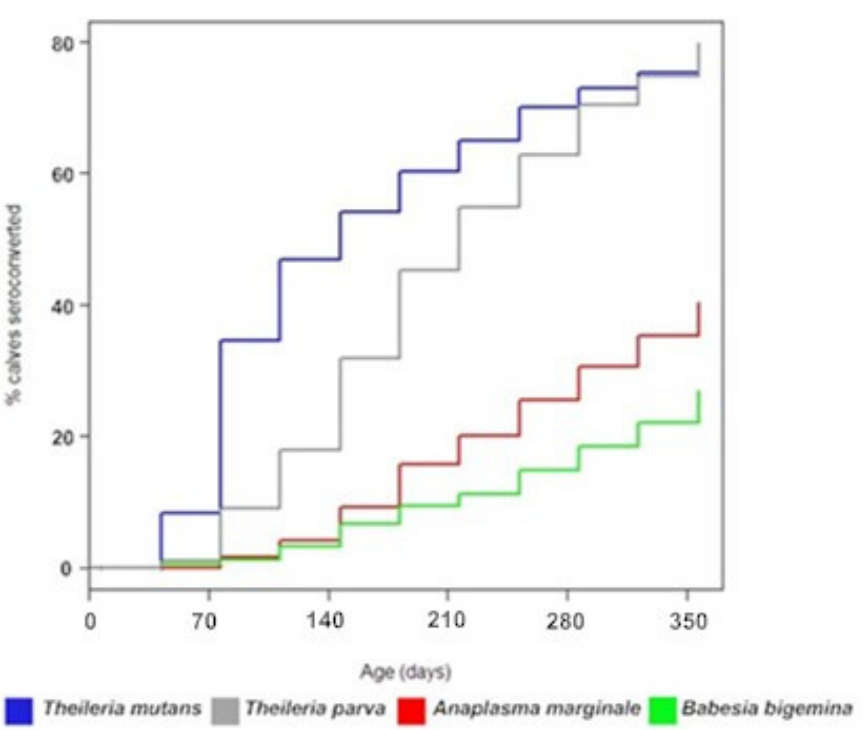


Seroconversion is a measure of exposure to pathogens. The cumulative incidences of seroconversion to the tick-borne pathogens *Theileria mutans, Theileria parva, Anaplasma marginale* and *Babesia bigemina* are illustrated in Figure 5.4.

Of the 548 recruited calves, 396 (72.3%) seroconverted to *T. parva*, 395 (72.1%) seroconverted to *T. mutans*, 197 (35.9%) seroconverted to *A. marginale* and 132 (24.1%) seroconverted to *B. bigemina*. The probability of seroconversion to *T. parva, T. mutans, A. marginale* and *B. bigemina* by 51 weeks of age was 89.8%, 87.6%, 40.2% and 26.9%, respectively.

There appeared to be a high risk period of seroconversion to *T. mutans* at 11 weeks, as indicated by the high probability (44.5%) of seroconversion, with a total of 138 new cases reported for this period. The number of calves that seroconverted to *T. parva* peaked a bit later, at 21 weeks, with a total of 71 new cases reported for this period. The number of new cases of seroconversion to *A. marginale* was consistent over time and no specific high risk-period could be identified. The picture for *B. bigemina* was similar, except for a slightly higher number of cases seroconverting at week 51.

Figure 5.4 Cumulative incidence of seroconversion of tick-borne disease



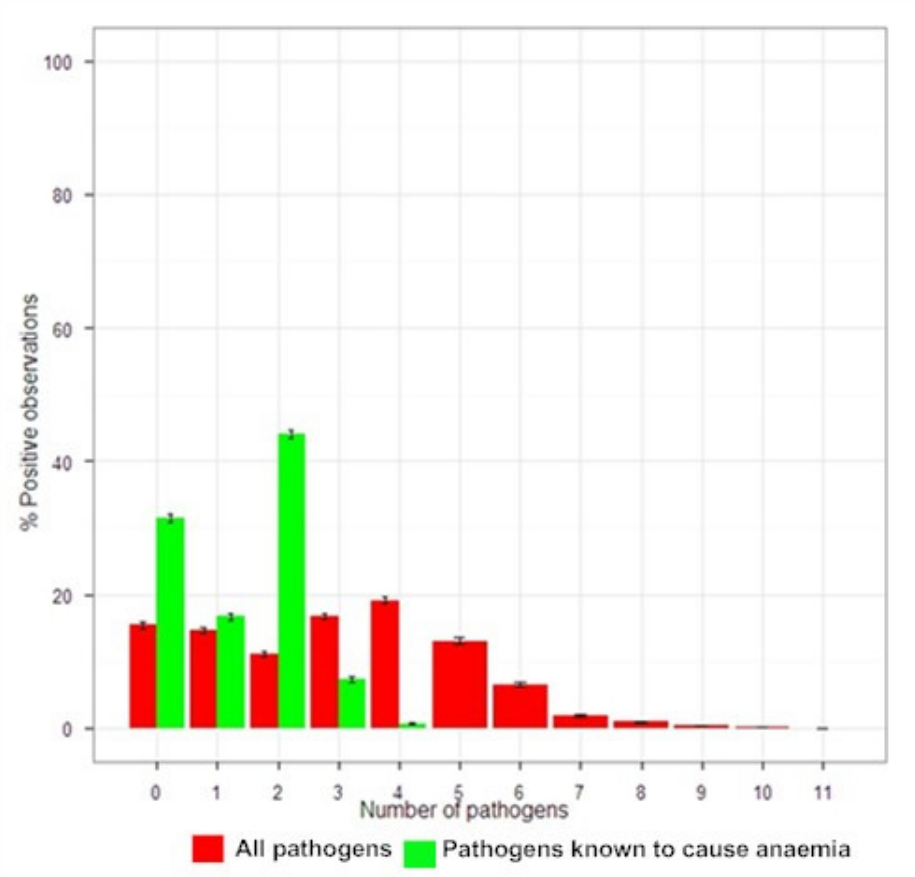
3.3 The level of co-infections of different pathogens in the study population

Co-infections per visit

The distribution of number of pathogens per visit detected is illustrated in Figure 5.5. No pathogens were detected at 15.4% (851) visits, the majority (508 visits) being calf recruitment visits. The maximum number of pathogens detected per single visit was 11 (two observations). At least four pathogens were detected in the majority (19.1%) of visits.

No pathogens known to cause anaemia were detected at 31.4% (1 742) visits, the majority of which were the calf recruitment visits. At over 16% of the visits at least one pathogen known to cause anaemia was detected. At 44% of visits at least two pathogens known to cause anaemia were detected. At least four pathogens known to cause anaemia were detected at 85 (1.5%) visits.

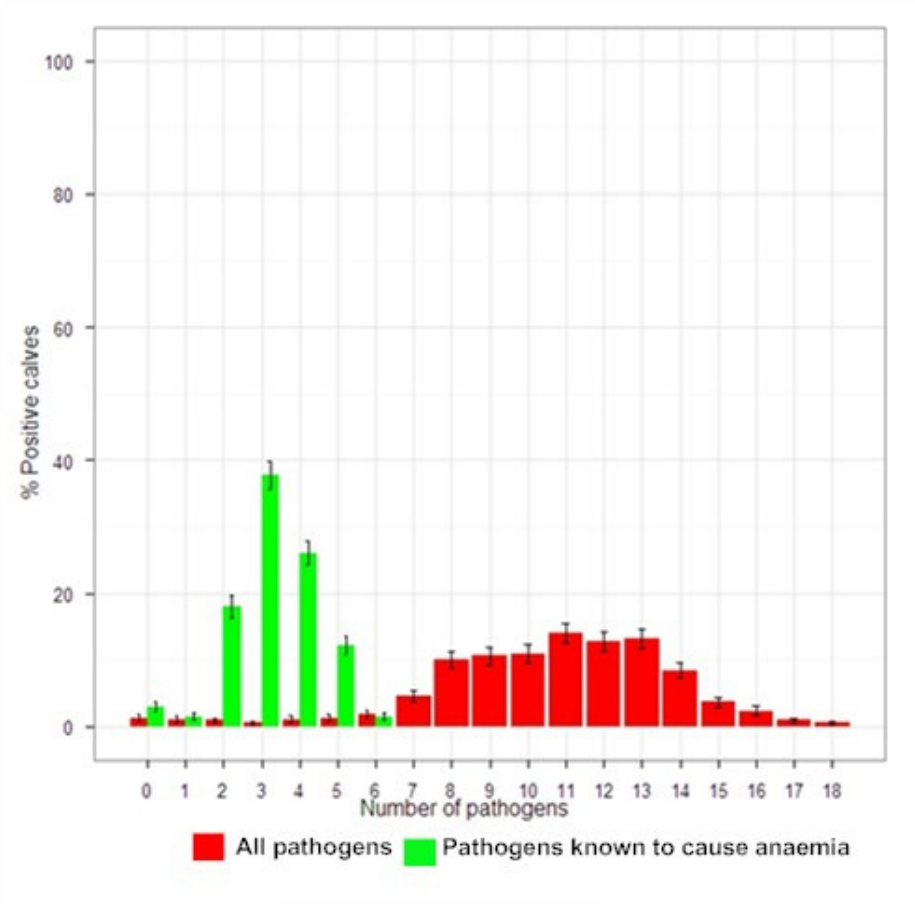
Figure 5.5 The frequency distribution of co-infections per visit



Co-infections per calf

The distribution of the number of pathogens per calf detected is illustrated in Figure 5.6. In seven calves, no pathogens were detected. These calves were only visited once because they died before the 6-week visit. More than 90% of calves were exposed to at least seven pathogens, and over 95% of calves were exposed to at least two pathogens known to cause anaemia in the first 51 weeks of their lives. Over 37% were exposed to at least three pathogens known to cause anaemia, with six calves exposed to six of these pathogens over the first year of life. In only 16 calves were none of these anaemia-causing pathogens detected.

Figure 5.6 The frequency distribution of co-infections per calf



4. DISCUSSION

Few pathogens were detected at recruitment visits. The reasons might be that the time between birth and recruitment was too short for exposure to have occurred. Also, for the calves that were exposed before the recruitment visit, it is possible that the incubation period before pathogens could be detected exceeded the period between exposure and sampling. Calves would not yet have grazed, which is the main route of infection for intestinal pathogens, as evidenced by the significant increase in prevalence of these parasites by the second visit. The pathogens that were detected at recruitment could be due to trans-placental infection, such as *Anaplasma* spp. (Aubry & Geale 2010), or through colostrum or milk, such as some *Strongyloides* species (Kaufmann 1996).

No distinction was made between *Theileria* species on microscopy and one can not rule out the presence of species other than *T. parva* and *T. mutans*. The high percentage of calves infected with *Theileria* spp. (mcr) is reflected in the high number of calves that seroconverted

to both *T. parva* and *T. mutans*. With over 70% of calves that were exposed to both these pathogens, co-infections with both were probably common. Both *T. parva* and *T. mutans* should be considered as very important pathogens in the calf population. The antibody prevalence rates for *T. parva* and *T. mutans* is an indication of endemic stability of these two pathogens in the study area (Okello-Onen *et al.* 1995; Perry & Young 1995). In a situation of endemic stability one would expect to see few clinical cases and these would be mainly seen in young calves (Perry & Young 1995).

Relatively few calves seroconverted to *A. marginale* and even fewer to *B. bigemina*. The discrepancy in the number of seroconversions to *A. marginale* and *B. bigemina* and the number of calves positive on microscopy is due to the difference in sensitivity between the two methods. Microscopy has a detection level of parasitaemias with a minimum of 1 parasite per 10^7 red blood cells (OIE 2005). Microscopy is mostly useful in detecting acute infections. In latent carrier animals the parasitaemia is often too low to detect via microscopy (Potgieter & Stoltsz 2004). Serology is an indirect measure of exposure and is useful in detecting carrier animals (Aubry & Geale 2010). It would seem that there are significant numbers of carrier animals of both *B. bigemina* and *A. marginale* in the population.

Interestingly, over 38% of calves were infected by *A. bovis* at their last visit. This is not a well-studied parasite but generally thought to be benign (Oura *et al*. 2004). It would be interesting to investigate its clinical significance in the study.

A relatively low number of calves tested positive for *Trypanosoma* spp., compared to other tick-borne diseases. The low number of visits where *Trypanosome* spp. were detected might be due to the fluctuating nature of parasitaemias (Connor & Van den Bossche 2004). The five-week intervals between visits probably caused an underestimate of the prevalence of this pathogen group. Trypanosomes were detected in calves at all time-points, with a few calves even testing positive at recruitment. Congenital transmission of *T. vivax* has been described, but is not common (Uilenberg 1998). It would be interesting to test these calves' dams for the presence of either the parasite or antibody against trypanosomes. Although *Trypanosoma* spp. did not appear to be common pathogens of very young calves, their prevalence increased with age and they are potentially of more clinical importance in adult animals in the study area. *Trypanosoma vivax,* which appeared to be the most prevalent trypanosome in the population, is generally considered less pathogenic than *T. congolense* (Uilenberg 1998). The clinical importance of *T. vivax* depends on the susceptibility of the animal, however, and will be investigated in the next chapter.

Intestinal parasites were some of the most common pathogens at all time-points and should be considered as important pathogens in the calf population. The probability of infection with strongyle-type nematodes by 51 weeks of life was almost 100%. Although strongyles are not considered to be as pathogenic in cattle as in sheep, the clinical significance of infection and the probability of causing clinical signs, such as anaemia, are dependent on the infective dose as well as the animal's susceptibility to infection (Kaufmann 1996). A high number of calves had significantly high EPGs at each of their visits, even from relatively young ages. Even infections with low EPG can potentially become clinically significant and should not be discounted. These infections seldom occur singly, and tend to occur in conjunction with multiple pathogens, some of which share the same niche in the host, namely the intestines (e.g. coccidia). The impact of these co-infections on the clinical course of infection will be investigated in Chapter 7.

S*trongyloides* worms were the predominant helminth at week 6. *Strongyloides* worms can infect calves before birth by crossing the placenta and are also passed to new-born calves in colostrum (Kaufmann 1996). Infection can also be through skin penetration (Kaufmann 1996). A relatively significant number of animals were infected by *Strongyloides* spp., but calves appeared to self-cure the infection and by 51 weeks very few calves were still infected. This could be due to premunity that develops in calves that were infected at early ages, or strongyle species which have high fecundity rates that out-compete the *Strongyloides* spp. for host resources.

When one considers the high rainfall and tropical conditions in the study area, the small number of calves infected with *Fasciola* spp. was perhaps a little surprising. The presence of *Fasciola* spp. infection was diagnosed through testing faecal samples for eggs by the sedimentation technique. The eggs of *Fasciola* spp. only appear in the faeces 10 weeks post-infection (Kaufmann 1996). This would explain in part why calves only tested positive late in the follow-up period. *Fasciola gigantica* infections did not appear to be significant in young calves but are likely to be more prevalent and of clinical relevance in older animals.

Co-infections

It is evident that the calves were suffering from the burden of multiple pathogens rather than single infections. Calves were infected with multiple pathogens at the majority of visits but also suffered from consecutive infections in following visits. Pathogens known to cause anaemia represented a significant number of these pathogens. The number of co-infections detected was limited by the number of tests done on samples from each visit. It is likely that

the infectious burdens of these calves were even higher, given that many more pathogens would probably have been detected if these calves were subjected to more tests.

5. CONCLUSIONS

It is clear that the calves in this study suffered from high burdens of multiple infectious agents from early calfhood. Many of the most common pathogens that infect these calves are known to cause anaemia. The pathogens with the most significant prevalences and cumulative incidence are the intestinal parasites, strongyle-type worms and coccidia, and the two tick-borne pathogens, *T. parva* and *T. mutans*. The calves had a high probability of getting infected by all of these pathogens by the first six month of life. At a population level, calves living with such an infectious burden should either have an innate resistance to these pathogens or tolerance to the clinical effects of disease for the population to survive into adulthood. It is probable that many of these infections are subclinical and many animals become latent carriers.

Despite the high infectious burden of calves in this study, the majority of calves survived to at least 51 weeks of age, which illustrates the resilience of the East African short-horn Zebu breed. The clinical significance of these infections is further investigated in the following two chapters.

CHAPTER 6

ANAEMIA IN EAST AFRICAN SHORT-HORN ZEBU CALVES: THE PATHOGENIC CAUSES OF ANAEMIA

1. INTRODUCTION

There are several pathogenic causes of anaemia, and the clinical course in many of infectious diseases has been well studied (Irvin 1983; Murray & Dexter 1988). Apart from anaemia, other haematological parameters are also affected during infection, be that directly by the pathogen and its by-products or by the host's response to infection. Fewer studies have investigated the effects of infection on the complete haematological profile of calves.

It is known that cattle breeds differ in their susceptibility to infection by pathogens and parasites (Perry & Young 1995) and their ability to control the development of clinical signs of infection (Murray *et al*. 1982). East African short-horn Zebu cattle are considered to be less trypanotolerant than West African cattle breeds (Murray *et al*. 1982), yet are considered to be more resistant against certain tick-born diseases, e.g. East Coast fever (Perry & Young 1995).

In this chapter the effect of certain pathogens on the haematological profile of the East African short-horn Zebu calves under field conditions was investigated. The aim was to describe the haematological outcome of infection with these pathogens and each pathogen's contribution towards the occurrence of anaemia in the study population. Pathogens investigated included the tick-borne parasites *Theileria parva, Theileria mutans, Anaplasma marginale* and *Babesia bigemina, Trypanosoma* spp. and specifically *T. vivax*, and intestinal parasites *Strongyloides* spp., strongyle-type nematodes, coccidia and *Fasciola gigantica.*

2. MATERIALS AND METHODS

* General methodology is discussed in Chapter 2

2.1 The haematological profile of calves infected with specific pathogens

The changes in the haematological profile over time due to infection were investigated for each pathogen. The haematological parameters investigated include: packed cell volume

(PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white cell counts (WCC), absolute lymphocyte counts (Lymph), absolute eosinophil counts (Eos), absolute monocyte counts (Mono), absolute neutrophil counts (Neut), platelet counts (Plt), platelet distribution width (PDW) and mean platelet volume (MPV). Pathogen infections were investigated in their singularity and co-infections with other pathogens were not considered. Co-infections are investigated in Chapter 7.

As the majority of calves became infected with *T. parva* and *T. mutans* during their follow-up period, no comparison was made between infected and non-infected calves. The calves were thus grouped into three groups based on their age of first seroconversion, namely Group 1 (1-16 weeks); Group2 (16-31 weeks); and Group 3 (>31 weeks). The changes in the haematological profile of the calves were compared between these various groups. Grouping of calves according to age of seroconversion to *A. marginale* and *B. bigemina* was based on the same age groups. A further group (Group 4) was included for calves that did not seroconvert during their follow-up period.

A distinction was made between *Trypanosoma* spp. diagnosed by microscopy (*Trypanosoma* spp. (mcr)) and *T. vivax*, as diagnosed by PCR since molecular diagnostics was not done at each visit. Calves infected with *Trypanosoma* spp. (mcr) were classed as either infected or not infected. No distinction was made between the *Trypanosoma* species based on microscopy. All calves that survived to 51 weeks were screened with species-specific PCR for *Trypanosoma* spp. The haematology in calves that tested positive for *Trypanosoma vivax* was investigated for this time-point only.

Roundworm eggs were identified as either strongyle-type nematodes or *Strongyloides* spp. The mean strongyle EPG per calf over its entire follow-up period was then calculated. The strongyle-infected calves were then grouped based on their mean EPG as high (mean EPG > 1000) or low (mean EPG ≤ 1000). Similarly coccidia infected calves were also grouped based on their mean OPG as high (mean OPG > 1000) or low (mean OPG ≤ 1000).

The haematological parameters in calves infected with *Strongyloides* spp. were only investigated at week 6 due to the low number of calves infected at other time points. Calves were either grouped as *Strongyloides* spp. infected or non-infected.

Calves infected with *Fasciola gigantica* were only investigated at week 51 due to the low numbers of calves infected at younger ages. The haematological parameters in *Fasciola gigantica* infected calves were compared to non-infected calves.

2.2 Data analysis

The significance of the difference in the means of haematological parameters at different time-points was calculated, be that between infected and non-infected calves, or between groups of infected calves, using either the Student's t-test, or where assumptions of normality were not met, the Mann-Whitney Test (see Chapter 2.4). A p-value <0.05 was considered as significant. All graphs were drawn in gg-plots (Wickham 2009) in R.

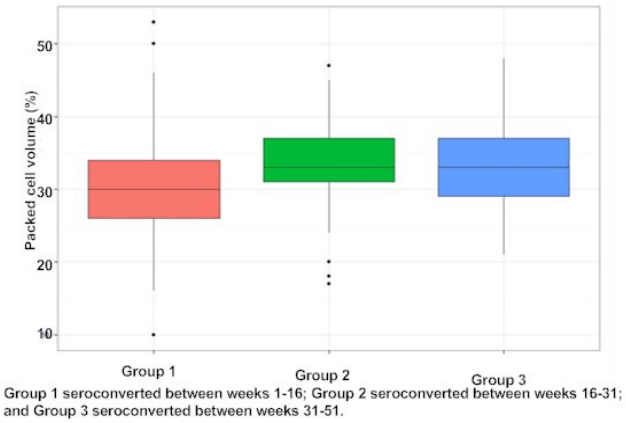
3. RESULTS

3.1 Infectious causes of anaemia: Blood-borne pathogens

Theileria mutans

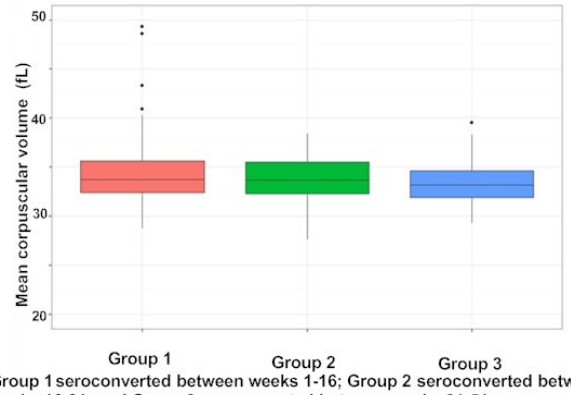
Calves that seroconverted to *T. mutans* by 16 weeks of age (Group 1, n=216) had a significantly lower PCV from week 6 to week 16 than calves that seroconverted later ($p<0.05$). There was no significant difference between the PCV of calves that seroconverted between 21 to 31 weeks (Group 2, n=79) and calves that seroconverted between 36 and 51 weeks (Group 3, n=54) ($p>0.05$). Group 1 had a significantly higher MCV than Group 3 from week 16 to 31 ($p<0.05$). Group 2 had a significantly higher MCV than Group 1 and 3 between weeks 26 to 31. The distribution of PCV and MCV of each group for Week 16 and Week 51 are illustrated in Figure 6.1.1-6.1.4. There were no significant differences in MCHC between the groups ($p>0.05$).

Figure 6.1.1 The distribution of packed cell volume at Week 16 in calves of different ages of seroconversion to *T. mutans*



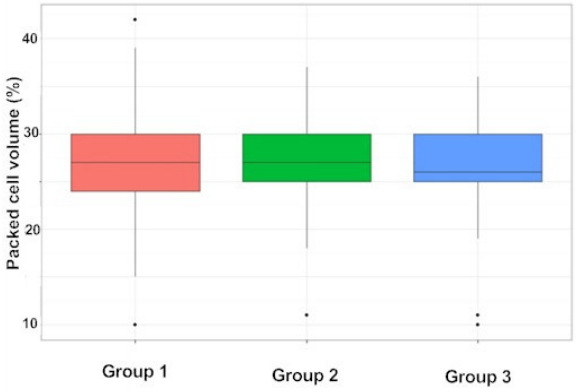
Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Figure 6.1.2 The distribution of mean corpuscular volume at Week 16 in calves of different ages of seroconversion to *T. mutans*



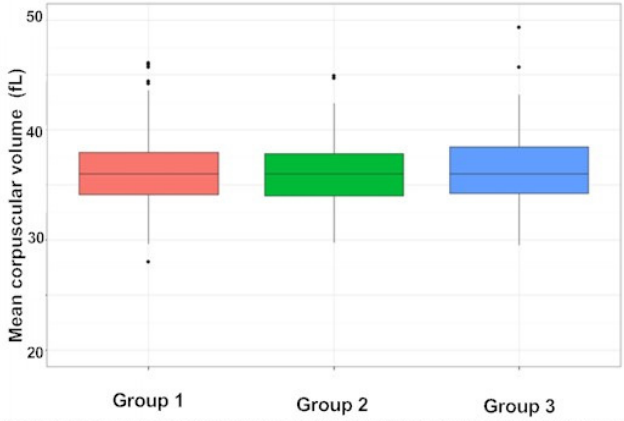
Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Figure 6.1.3 The distribution of packed cell volume at Week 51 in calves of different ages of seroconversion to *T. mutans*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Figure 6.1.4 The distribution of mean corpuscular volume at Week 51 in calves of different ages of seroconversion to *T. mutans*



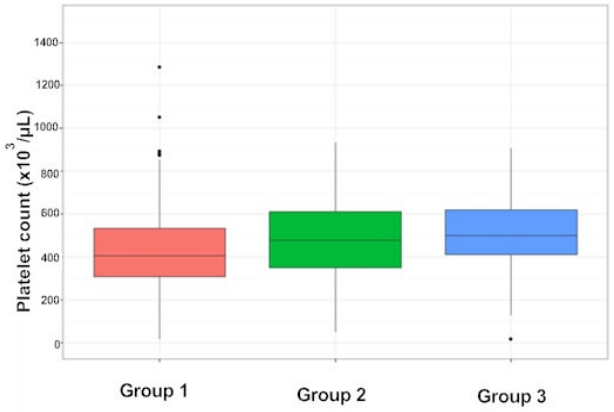
Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

The platelet counts were lower following seroconversion to *T. mutans* for all groups. There was a significant difference in platelet counts between calves in Group 1 and 2 at week 11 and 16, between Group 1 and 3 from week 11-31, and between Group 2 and 3 from week 21-31 ($p<0.05$). There was no significant difference between the three groups by week 36 onward when calves in Group 3 also seroconverted.

Figures 6.1.5-6.1.7 illustrate the distribution of platelet counts at week 16, week 31 and week 51 of the calves that have seroconverted to *T. mutans*. The median platelet count at week 16 of Group 1 was 405x10^3/µL, of Group 2 479x10^3/µL and Group 3 502x10^3/µL. The median platelet count at week 31 of Group 1 was 410x10^3/µL, of Group 2 456x10^3/uL and Group 3 559x10^3/µL. By week 51, the mean platelet counts of all three groups were below 400 x10^3/µL. No significant differences were found in MPV and PDW between any group at any time-point ($p>0.05$).

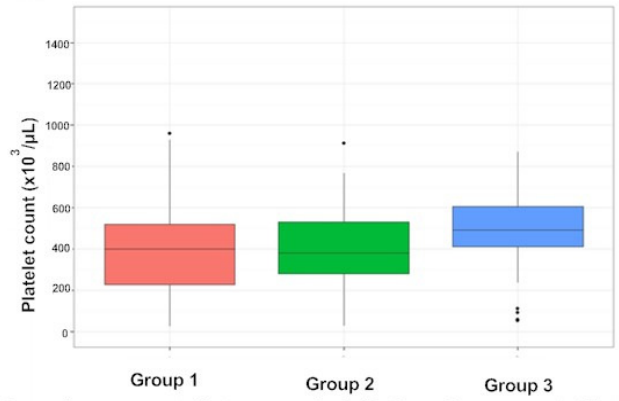
There were no significant differences in any white cell parameter between the *T. mutans*-seroconversion groups.

Figure 6.1.5 The distribution of platelet counts at Week 16 in calves of different ages of seroconversion to *T. mutans*



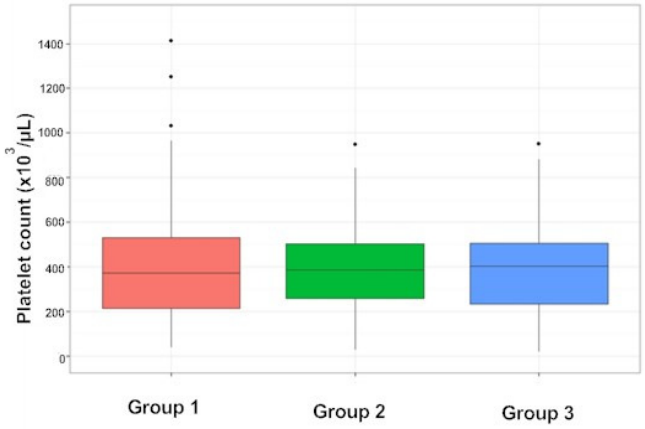
Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Figure 6.1.6 The distribution of platelet counts at Week 31 in calves of different ages of seroconversion to *T. mutans*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Figure 6.1.7 The distribution of platelet counts at Week 51 in calves of different ages of seroconversion to *T. mutans*



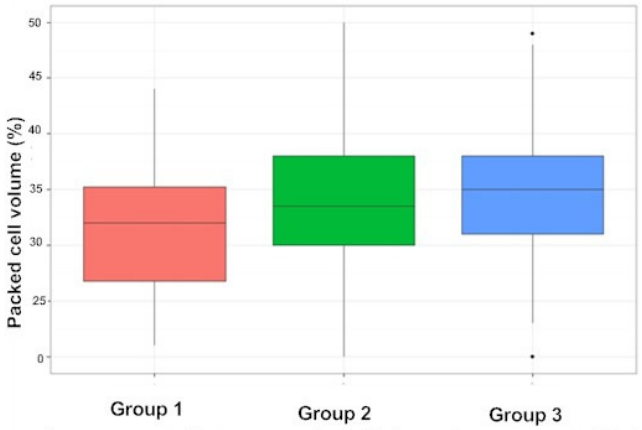
Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Theileria parva

The PCV of Group 1 (n=92) was significantly lower at week 6-11 than Group 2 (n=184) and lower than Group 3 (n=120) from week 6-11 ($p<0.05$). There were no other significant differences in PCV between groups at other time points. No significant differences in MCV or MCHC were measured at any time point in calves that seroconverted to *T. parva*. The distribution of PCV in the three groups at week 11 is illustrated in Figure 6.2.1.

Calves from Group1 had significantly lower WCC and Lymph than both Group 2 and 3 from week 6-16 ($p<0.05$). Group 1 also had significantly lower Eos, Mono and Neut than both Group2 and 3 at week 6. No significant differences were found in any white cell parameter

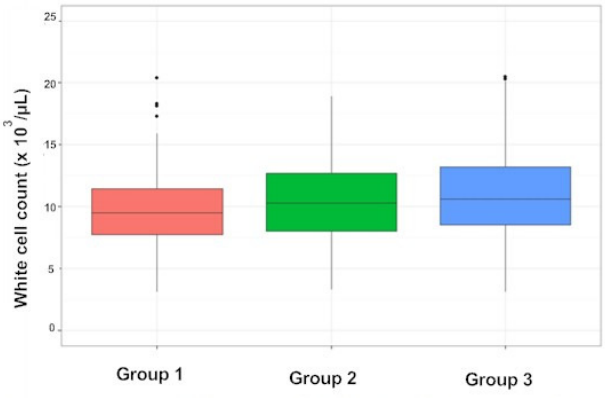
Figure 6.2.1 The distribution of packed cell volume at Week 11 in calves of different ages of seroconversion to *T. parva*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

between Group 2 and Group 3. The distribution of WCC at week 11 in calves in different groups that seroconverted to *T. parva* is illustrated in Figure 6.2.2.

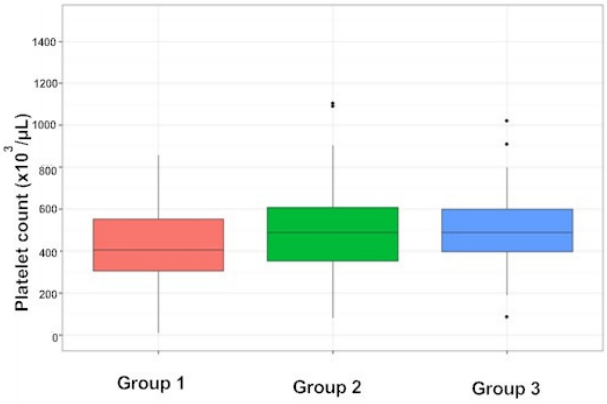
Figure 6.2.2 The distribution of white cell counts at Week 11 in calves of different ages of seroconversion to *T. parva*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Calves from Group 1 also had significantly lower platelet counts than Group 2 at week 11 and 31, and lower than Group 3 at week 6, 11 and 31. No differences in MPV and PDW were found between any groups at any time point. The distribution of Plt at week 11 in calves in different groups that seroconverted to *T. parva* is illustrated in Figure 6.2.3.

Figure 6.2.3 The distribution of platelet counts at Week 11 in calves of different ages of seroconversion to *T. parva*

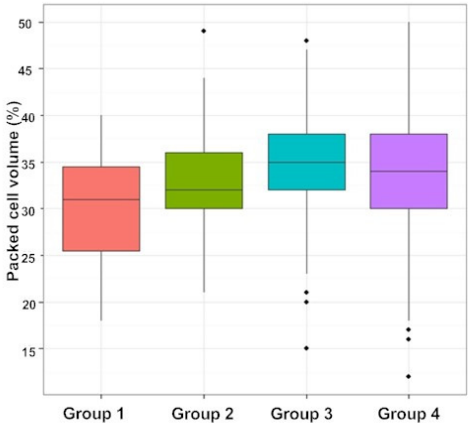


Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; and Group 3 seroconverted between weeks 31-51.

Anaplasma marginale

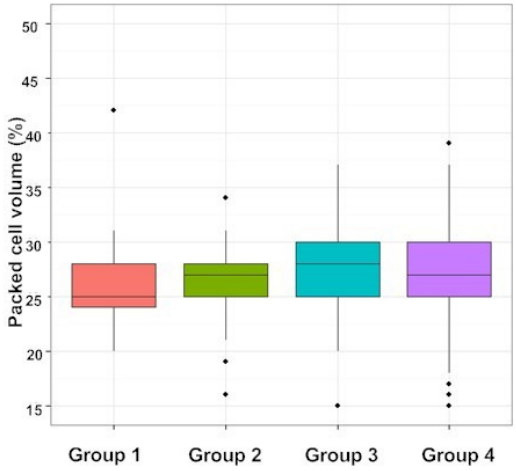
The PCV of Group 1 (n=22) was significantly lower than Group 3 (n=96) at week 6-16, and the PCV of Group 2 (n=79) was significantly lower than Group 3 at week 11-31 ($p<0.05$). There was a significant difference in PCV between Group1 and 4 (n=351) at week 11 ($p<0.05$). No other significant differences in PCV were found at other time points. The mean PCV at week 11 for Groups 1, 2, 3 and 4 was 29.8%, 33.1%, 34.5% and 33.8% respectively (Figure 6.3.1). At week 26 the mean PCV for Groups 1, 2, 3 and 4 was 28.2%, 27.7%, 29.6% and 28.1% respectively. At week 51 the mean PCV for Groups 1, 2, 3 and 4 was 26.6%, 25.9%, 27.4% and 27.1% (Figure 6.3.2). No significant differences were found in other haematological parameters at any time-point ($p>0.05$).

Figure 6.3.1 The distribution of packed cell volume at week 11 in calves of different ages of seroconversion to *A. marginale*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; Group 3 seroconverted between weeks 31-51; and Group 4 did not seroconvert by week 51.

Figure 6.3.2 The distribution of packed cell volume at week 51 in calves of different ages of seroconversion to *A. marginale*



Group 1 seroconverted between weeks 1-16; Group 2 seroconverted between weeks 16-31; Group 3 seroconverted between weeks 31-51; and Group 4 did not seroconvert by week 51.

Babesia bigemina

No significant differences were found in any haematological parameter between groups of calves that seroconverted to *B. bigemina* (Group 1: n=14; Group 2: n=40; Group 3: n=75) or between seroconverted and non-seroconverted (Group 4: n= 416) calves.

Trypanosoma spp. (mcr)

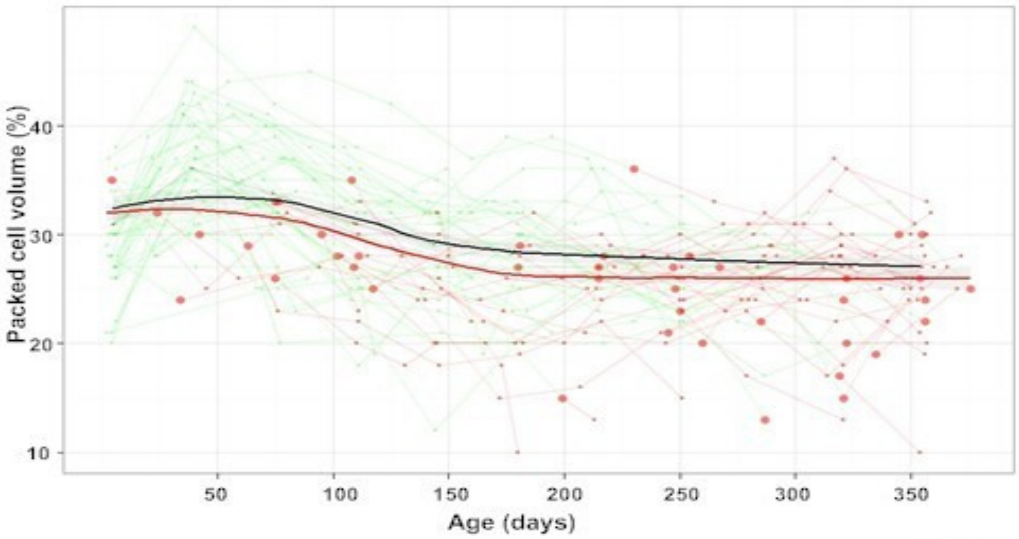
The PCV profile of *Trypanosoma*-infected calves, diagnosed by microscopy (n=42), is illustrated in Figure 6.4.1. The *Trypanosoma*-infected calves had a significantly lower PCV than the total population from week 26 to 36 ($p<0.05$). The calves that were infected at an early age (before 100 days) had a considerably lower PCV during early calf-hood. Towards 350 days there was significant variation in PCV with many of the individual infected calves recovering their PCV to similar levels as uninfected calves. Several calves positive for *Trypanosoma* spp. developed severe anaemia (PCV <15%) at various time points.

There was no significant difference in MCV and MCHC between the *Trypanosoma*- positive and negative calves at any time point.

The variation in WCC between individual positive calves was marked, with several calves displaying quite low WCC (< 5 x 10^3/µL) and several calves that developed relatively high WCC (> 20 x 10^3/µL). In contrast to the total WCC, calves infected with *Trypanosoma* spp. developed a lymphocytosis compared to the negative calves, which became significant from

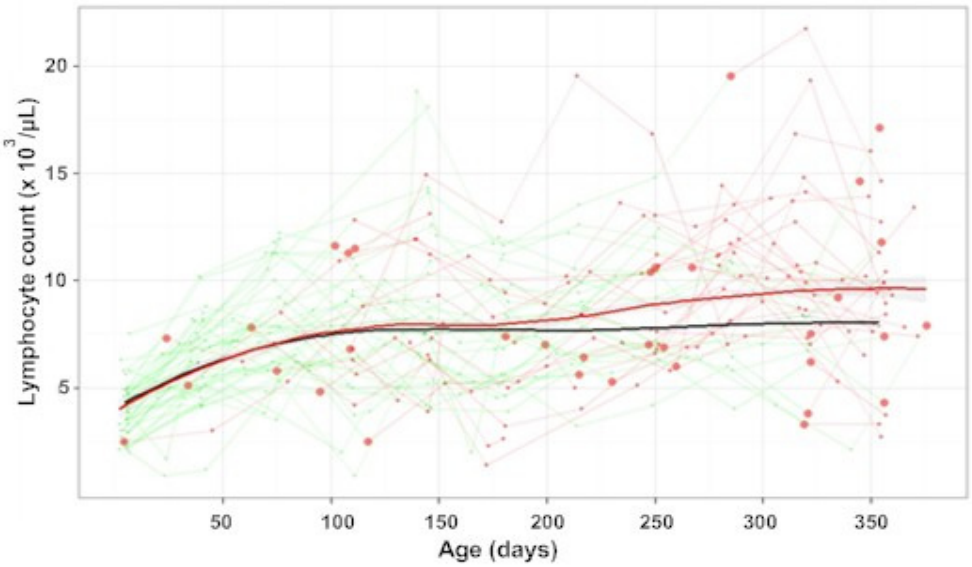
week 31 to week 51 ($p<0.05$). The Lymph profile of *Trypanosoma*-infected calves is depicted in Figure 6.4.2.

Figure 6.4.1 The packed cell volume profile in *Trypanosoma* spp. (mcr) infected calves



• The point of first infection for each calf . The profile line of each calf is green coloured (–) prior to this • point (negative/uninfected), after which the colouring changes to red (–) (positive/infected). The smoothed red line (—) is the mean of the infected calves over time. The mean for the total population is indicated in black (—).

Figure 6.4.2 The lymphocyte count profile in *Trypanosoma* spp. (mcr) infected calves



Figures 6.4.1-6.4.2 indicate the profile of individual calves infected with *Trypanosoma* spp. for the Lymph. The point of first infection for each calf is indicated by (•). The profile line of each calf is green coloured (—) prior to this • point negative/uninfected), after which the colouring changes to red (—) (positive/infected). The smoothed red line (—) is the mean of the infected calves over time. The mean for the total population is indicated in black (—).

No significant difference between positive and negative calves in any other white blood cell parameter was found at any time point.

The most significant difference in the profiles of *Trypanosoma*-infected calves compared to negative calves was the platelet counts, with most calves developing a very low platelet count from the point of infection (indicated with a red dot on the graph, Figure 6.4.3). The difference in mean platelet count between infected and non-infected calves was significant from week 21 to week 51 ($p<0.05$). Several infected calves appeared to recover from thrombocytopenia with several calves displaying a relatively high Plt at 350 days. There was no significant difference in MPV and PDW at any time point between the infected calves and negative calves.

Figure 6.4.3 The platelet count profile in *Trypanosoma* spp. (mcr) infected calves

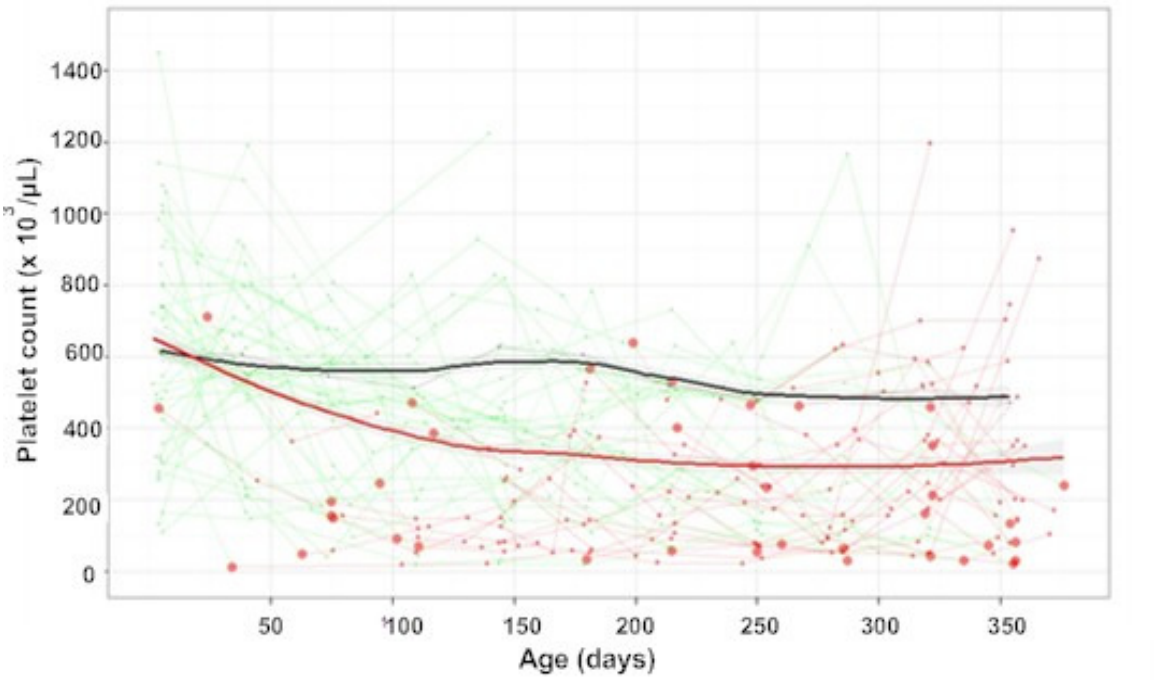


Figure 6.4.3 indicates the profile of individual calves infected with *Trypanosoma* spp. for the PLT. The point of first infection for each calf is indicated by (●). The profile line of each calf is green coloured (—) prior to this ● point negative/uninfected), after which the colouring changes to red (—) (positive/infected). The smoothed red line (—) is the mean of the infected calves over time. The mean for the total population is indicated in black (—).

Trypanosoma vivax (week 51)

Forty-nine calves were positive for *T. vivax* by PCR at week 51. The *T. vivax* positive calves had a significantly higher Lymph (p=0.023) and a significantly lower Plt (p=0.004) than negative calves. No significant differences were found between *T. vivax* positive and negative calves for any other haematological parameter, including PCV. Boxplots (Figures 6.5.1-6.5.2) are used to illustrate the difference in Lymph and Plt between calves that tested positive and negative, respectively, for *T. vivax* at the final visit.

Figure 6.5.1 The distribution of absolute lymphocyte counts in *T. vivax* calves at week 51

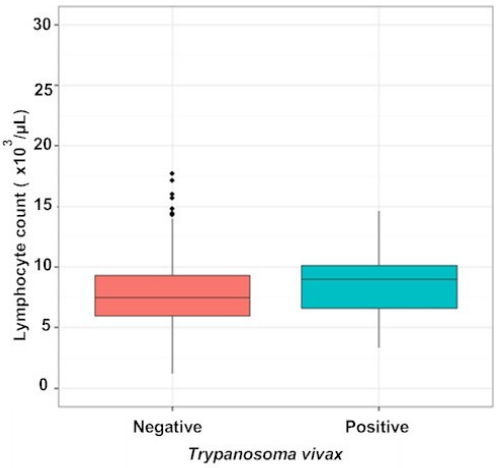
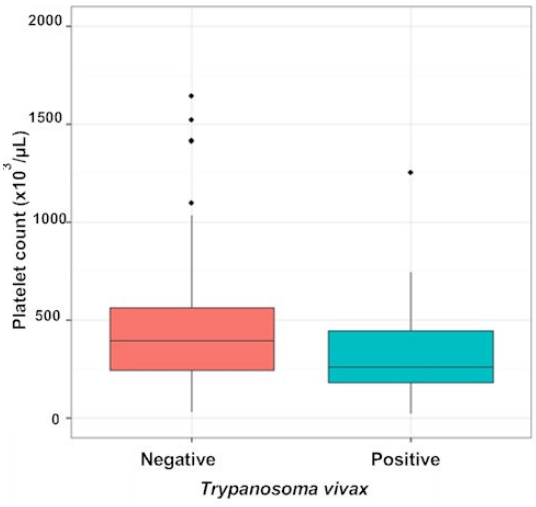


Figure 6.5.2 The distribution of platelet counts in *T. vivax* calves at week 51



3.2 Infectious causes of anaemia: Intestinal parasites

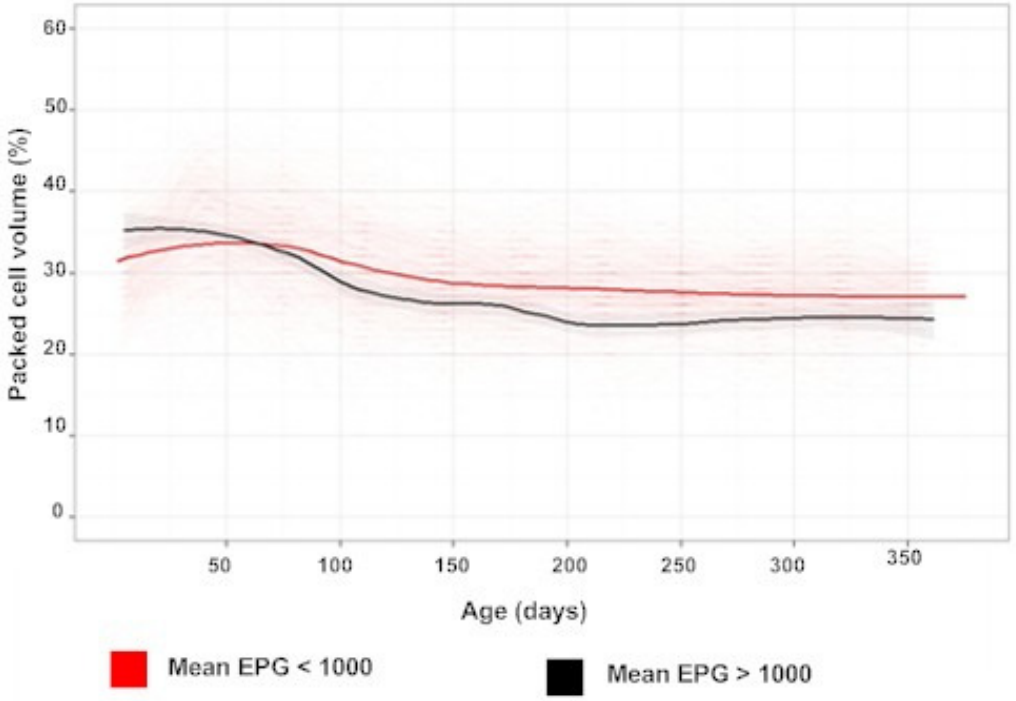
Strongyle-type nematodes

Calves with a high mean strongyle EPG had a significantly lower PCV ($p<0.05$) than calves with a low mean EPG from 100 days and older (Figure 6.6). The apparently higher PCV at week 1 in calves with a high mean EPG was due to high variance in PCV in calves at this time-point. The MCV in the calves became progressively higher in calves with a high mean EPG, with a significant difference from 250 days. There was no significant difference in MCHC between the two groups.

There were also differences in the WCC, Lymph and TSP between the calf groups, with the calves with a high mean EPG displaying a lower WCC, Lymph and TSP values than the calves with a low mean EPG. The differences were significant from 100 days to the end of the follow-up period. No differences were found in Eos, Mono and Neut between the two groups.

The calves suffering from a high mean EPG developed a mild thrombocytosis compared to the calves with lower mean EPG.

Figure 6.6 The packed cell volume in calves infected with strongyle-type nematodes with a high mean EPG compared to calves with a low mean EPG



Strongyloides spp.

There were no significant difference in any parameter measured between *Strongyloides*-infected calves and non-infected calves, as measured at the 6-week visit ($p>0.05$).

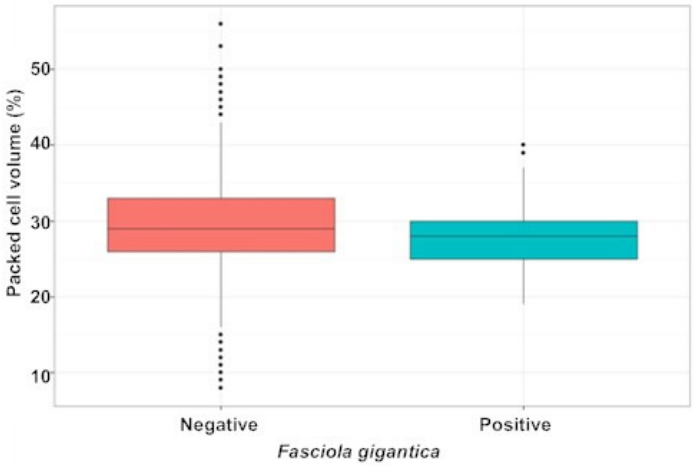
Coccidia

There were also no differences in any parameter measured between coccidia-infected calves with a high and low mean OPG at any time point ($p>0.05$).

Fasciola gigantica

There was a significant difference in PCV (%) ($p = 0.0002$) between calves infected with *F. gigantica* (n=34) compared to non-infected calves, as measured at 51 weeks of age (see Figure 6.7). There were no differences between the two groups in either MCV or MCHC. No difference could also be measured for any white blood cell parameter or platelet counts.

Figure 6.7 The distribution of packed cell volume in *Fasciola gigantica* infected and non-infected calves at the 51-week visit



4. DISCUSSION

4.1 Infectious causes of anaemia: Blood-borne parasites

Theileria mutans

Although usually considered as benign, *T. mutans* has been described as a cause of anaemia in cattle in Kenya (Young, Purnell, Payne, Brown & Kanhal 1978; Moll *et al*. 1986; Kariuki 1990). The calves in this study developed a lower PCV when they were exposed to *T. mutans* infection during early calfhood, compared to calves that were exposed at a later age. Anaemia is thought to be caused by the invasion of the parasite during intraerythrocytic

proliferation which results in erythrocyte destruction (Young *et al.* 1978, Fandamu *et al*. 2007). The prevalence of *T. mutans* in the study population is relatively high. Considering that there appears to be a high risk of exposure to *T. mutans* around 11 weeks of age and calves that get infected around this age tend to develop an anaemia compared to the rest of the calves, *T. mutans* is potentially a significant cause of anaemia during early calf-hood in the study population.

Theileria parva

In contrast to *Trypanosoma* spp., it appears that Zebu cattle are more resistant than taurine breeds to the clinical effects of East Coast fever (ECF) (Irvin 1983). The clinical course of the disease is, however, dependent on the infectious dose, the pathogen strain, and the animals' history of previous exposure (Koch *et al.* 1990). Anaemia is not consistently seen in cases of *T. parva* (Mbassa *et al*. 1994), but has been described in literature (Irvin 1983), and can occur as part of a pancytopenia syndrome (Maxie, Dolan, Jura, Tabel & Flowers 1982; Mbassa *et al*. 1994). No compensatory erythropoeisis occurs in clinical cases of ECF (Hill & Matson 1970; Maxie *et al*. 1982), which results in a normocytic normochromic anaemia (Fandamu *et al*. 2007). Hill & Matson (1970) reported reticulocytosis in subacute cases of experimentally infected animals. These animals recovered after infection. Field cases have been thought to be due to co-infection with other TBD such as *Anaplasma* spp*., Babesia* spp*.* or *T. mutans* (Moll *et al*. 1986).

In this study the age at infection with *T. parva* appeared to be of importance in determining whether a calf develops anaemia. Calves that were infected at a very early age had lower PCV, WCC and Plt counts than calves that were infected at a later age. A pancytopenia would suggest suppression of precursor cells in the bone marrow (Irvin 1983). The apparent pancytopenia was only temporary, however, for the haematological parameters did not differ between groups as the calves grew older. The haematological profile of the total calf population indicated that these calves are in a physiological state of cellular regeneration during early calfhood. It would seem that infection with *T. parva* during this period causes a slightly reduced rate of cellular regeneration, from which the calves in general were able to recover. The severity of infection would of course depend on the infectious load and strain of the parasite (Lawrence *et al*. 2004a). Although *T. parva* should be considered a very important pathogen in the study population, it was not an important cause of anaemia.

Anaplasma marginale & Babesia bigemina

Similar to *T. mutans* and *T. parva*, calves that seroconverted to *A. marginale* before 16 weeks of age displayed lower PCV around this period than calves that seroconverted later.

By 51 weeks there were no significant differences between calves that seroconverted to *A. marginale* at different ages. The PCV was not significantly lower in calves exposed to *B. bigemina* compared to unexposed calves. These two pathogens did not appear to be significant causes of anaemia in the population.

The relatively low prevalence and seroconversion rates for *A. marginale* and *B. bigemina* suggest a state of endemic instability and one would expect that infection with either pathogen would more likely result in clinical disease (Bock *et al*. 2004). One reason for the low incidence of clinical anaplasmosis and babesiosis might be the limited follow-up period of 51 weeks. Typically, clinical anaplasmosis usually develops only in adult animals that have not been exposed during calfhood (Gale, Dimmock, Gartsidet & Leatch 1996a). This is in part due to the age-related resistance against the disease in young animals. This is also true for babesiosis, where innate resistance lasts up to 9 months of age (Mahoney *et al*. 1973; De Vos *et al.* 2004). A low infective dose would not explain the low incidence of clinical disease since the outcome of infection and severity of disease does not depend on the infective dose in either parasite (Gale *et al.* 1996a; Allred 2007). The infected animals are likely to have become latent carriers, which is life-long in the case of *A. marginale* (Potgieter & Stoltsz 2004). There is still a risk of developing clinical disease when these carriers become immunocompromised to the extent that they can no longer contain these latent infections. Carrier animals have been reported to develop clinical anaplasmosis when immunosuppressed due to concurrent trypanosomosis (Magona & Mayende 2002).

Trypanosoma spp.
Trypanotolerance is defined as an ability to control parasitaemia as well as the ability to control the progressive development of anaemia (Murray & Dexter 1988). Zebu cattle, although indigenous, are not as tolerant to *Trypanosoma* spp. infection as N'dama or West African short-horn breeds, and do develop anaemia after infection (Murray & Dexter 1988). Calves appear to be more tolerant than adult animals due to a better erythropoietic response (Murray & Dexter 1988).

Trypanosoma vivax is thought to be less pathogenic than *T. congolense* and most animals develop a less severe anaemia with *T. vivax* infection than wtih *T. congolense* infection (Uilenberg 1998). However, a syndrome marked by acute death accompanied by massive non-specific haemorrhage has been described in *T. vivax* (Murray & Dexter 1988).

The Zebu calves in this study developed anaemia and showed only a slow recovery or partial recovery of anaemia after infection, with many calves surviving with persistent low-

grade anaemia. Compared to *Trypanosoma* spp. negative calves, there was no difference in the MCV and MCHC. Reticulocyte counts would need to be done to confirm this. This would suggest that the erythropoietic response by the bone-marrow is not increased, which would result in a progressive anaemia. The majority of infected calves did not develop a terminal anaemia, however, which suggests some level of trypanotolerance in this breed. The relatively low number of calves [42 (mcr) and 49 (PCR)] that were infected with *Trypanosoma* spp. implies that this parasite is not an important cause of anaemia in the population.

Thrombocytopenia is consistently found in *Trypanosoma* spp. infection in all species (Murray & Dexter 1988). Davis, Robbins, Weller & Braude (1974) found a direct response relationship between the degree of thrombocytopenia and the height of the parasitaemia. This response occurs irrespective of the *Trypanosoma* strain or species. The causes of thrombocytopenia in trypanosomosis are multifactorial. The first cause is parasite by-products that cause initial damage to these cells, while immunological reactions, such as antigen-antibody complexes and auto-antibodies to platelets maintain the thrombocytopenia (Murray & Dexter 1988). Fibrin deposits that form due to disseminated intravascular coagulation (DIC) further damage thrombocytes. The formation of platelet aggregations has been histologically shown in *T. vivax* infections (Murray & Dexter 1988) and *T. rhodesiense* (Davies *et al*. 1974), which indicates a consumptive loss of thrombocytes. These platelet aggregations are thought to be due to antibodies directed against platelets (Assoku & Gardiner 1989). Clumping of platelets can artificially cause a low PLT since such clumps would not be counted as platelets using an automated cell analyser.

There was a marked thrombocytopenia in calves infected with *Trypanosoma* spp. It is interesting that the PCV in some trypanosome-infected calves recovered after initial anaemia, yet many were unable to recover from the thrombocytopenia. This implies that there are continuing subclinical disease processes despite apparent recovery from clinical anaemia. The clinical significance of the thrombocytopenia would depend on the pathogenesis. The formation of platelet aggregations due to subclinical trypanosomosis might predispose calves affected by other infectious agents to succumb to terminal DIC and accelerated the time to death. DIC has been implicated in terminal disease in several infectious agents, including ECF (Maxie *et al*. 1982). Thrombocytopenia due to cellular destruction might also result in bleeding tendencies if severe enough (Assoku & Gardiner 1989) and would present clinically as multifocal petechiation and ecchymosis (Duncan *et al*. 1994).

4.2 Infectious causes of anaemia: Intestinal parasites

Strongyloides spp.

The prevalence of *Strongyloides* worms was only significant in very young calves. This parasite did not contribute significantly to the level of anaemia in the population at any time-point.

Strongyle-type nematodes

After 6 weeks strongyle worms became the predominant intestinal parasite with the majority of calves becoming infected by the end of their follow-up period. Infection occurs through ingestion of infective larvae and calves are increasingly exposed as they start grazing. Strongyles cause anaemia in animals through blood loss. *Haemonchus* spp., a strongyle-type nematode, is considered as one of the most pathogenic parasites of ruminants (Kaufmann1996) and is consistently reported as the most prevalent helminth species in cattle in Kenya (Moll *et al.* 1984; Latif *et al*. 1995; Waruiru *et al*. 2001; Waruiru *et al.* 2002). The pathogenesis is that of a haemorrhagic anaemia (Kaufmann *et al.* 1992) and presents as a hypochromic macrocytic anaemia. During extremely high parasite burdens the animal will die due to severe blood loss. In chronic cases animals develop a steady drop in PCV and serum albumin which results in emaciation of the animal. If the animal survives, the compensatory erythropoiesis will eventually deplete iron reserves (Kaufmann *et al*. 1992). This hypoferonaemia then presents as a normochromic microcytic anaemia (Duncan *et al.* 1994).

The development of anaemia appears to be dose-dependent, as only calves with a strongyle-associated EPG > 1000 developed anaemia. The increased MCV levels in the calves with a high strongyle EPG indicate a regenerative response. If the animals were treated with anthelmintics they were likely to have recovered and their PCV to return to pre-infection levels. Strongyle worms should be considered as a potentially important cause of anaemia in the population and due to the chronic nature of infection it is likely a very erosive disease.

Coccidia

Almost all calves were infected with coccidia at some point in their follow-up period. It did not appear to be an important cause of anaemia in the study, but the clinical significance of its presence would depend on the particular species involved (Kaufmann 1996) and might prove interesting to investigate further.

Fasciola gigantica

Fasciola gigantica infection is usually associated with chronic hepatic fibrosis and hyperplastic cholangitis in cattle (Kaufmann 1996). Cattle are less susceptible to infection than sheep, but in cases of high infectious load of metacercariae calves can develop acute symptoms such as anaemia, weight loss and hypoalbuminaemia. Anaemia develops due to blood consumption by adult flukes, depletion of iron stores from chronic bloodloss and haemolysis due to chronic inflammation and parasite by-products (Valero, Gironès, Garcia-Bodelón, Periago, Chico-Calero, Khoubbane, Fresno & Mas-Coma 2008). Fasciolosis appeared to become of more relevance in older calves. This was also reported to be the case in Uganda where adult cattle had higher prevalence of *F. gigantica* than calves (Magona & Mayende 2002). Although fasciolosis caused anaemia in the calves in this study, it is not an important cause of anaemia during the first year of life but may become more important in adult animals. The economic importance of fasciolosis lies in its erosive effect on the production of livestock through reduced weight gain or weight loss (Kaufmann 1996).

5. CONCLUSIONS

Strongyle-type nematodes were shown to be an important infectious cause of regenerative anaemia in the calf cohort while *T. mutans, T. parva, Trypanosoma* spp. and *Fasciola gigantica* caused non-regenerative anaemia in the calf cohort. The clinical outcome of strongyle infections, although a highly prevalent pathogen, depended on the infectious load of the pathogen and only caused a significant change in PCV at high doses.

In Chapter 4 the haematological profile of the East African short-horn Zebu calves was discussed. It was shown that these calves have a physiological rise in red cell parameters during early calfhood. It was speculated that this period of red cell generation buffered the effects of infection during early calf-hood and that it possibly formed part of their innate ability to control infection from developing into overt disease. There was a relative decrease, or a reduced physiological increase of PCV of calves that seroconverted to *T. mutans, T. parva* and *A. marginale* before 16 weeks at the time of seroconversion which was not found in calves that seroconverted at later ages. The immune system in calves is not completely mature by 16 weeks, and calves are still vulnerable to infection, despite their innate resistance to infection. These calves recovered, however, and PCV levels between early seroconverters and late seroconverters did not differ at later time points.

Other pathogens known to cause anaemia in cattle, e.g. *A. marginale* and *B. bigemina*, were shown not to contribute significantly to the level of anaemia in the calf population. This was possibly due to low prevalences, age-related resistance against infection, or the limited time-frame of the follow-up period. Although these latent infections appeared to be benign, they do pose a risk to these calves and can develop into clinical disease if the animal becomes immunocompromised (Potgieter & Stoltsz 2004).

Several pathogens did not cause acute decreases in PCV at infection, but due to the chronic nature of infection, were responsible for persistent low-grade anaemia in infected calves. The clinical significance of such infections, e.g. chronic strongyle infection and *Trypanosoma* spp., lies in the erosive effect on the production of the calf. There was also evidence of ongoing subclinical disease processes in animals that apparently recovered from certain overt clinical diseases, such as trypanosomosis. Similar to calves that suffer from low-grade progressive disease, calves with unapparent subclinical conditions are likely to suffer from ill-thrift and slow weight gain and are possibly more susceptible to super-infections with other pathogens or the effects of malnutrition.

In field conditions, where calves are more likely to be burdened by multi-pathogen infections, it is more relevant to consider the combined impact of pathogens on the clinical course of infection. The additive effects of multi-pathogen infections, even at low infectious loads or subclinical disease, might result in clinically significant changes in the haematological parameters of the infected calves. The effect on the haematological profile in multi-pathogen infections will be further investigated in Chapter 7.

CHAPTER 7

ANAEMIA IN EAST AFRICAN SHORT-HORN ZEBU CALVES: THE IMPACT OF CO-INFECTIONS ON THE HAEMATOLOGICAL PROFILE OF CALVES

1. INTRODUCTION

Under field conditions an animal is more likely to suffer from the burden of multiple infectious causes of disease rather than single infections (Petney & Andrews 1998). In Chapter 5 it was shown that this is true for the East African short-horn Zebu calves in western Kenya. These calves are infected with multiple pathogens from early calfhood, either at the same time point or in succession over their lifetime. Co-infections complicate the clinical presentation of disease as each pathogen is likely to contribute to the clinical outcome of infection in the host. Pathogens often share and compete for the same resources in the host. Interactions between such pathogens can alter the onset, duration and clinical course of disease, as well as the host's susceptibility or tolerance to other pathogens (Cox 1987; 2001).

It is therefore more relevant to study an infectious disease in context of the complete multi-pathogen burden the animal is suffering from (Moll *et al.* 1984). In Chapter 6 the impact of single pathogenic infections on the haematological profile of East African short-horn Zebu calves, in particular the PCV, was investigated. It was shown that several pathogens alter the haematological profile of the calves and that several of these pathogens cause anaemia in the population. Co-infections with other pathogens were not considered.

This chapter aims to investigate the impact of co-infections of the most prevalent pathogens in the study area on the haematological response of the population. This was achieved by modelling the infectious status of pathogens against specific haematological responses. The presence of significant interactions between pathogens was also investigated by the models. Finally, the predicted outcomes of these models were then used to illustrate the cumulative effect of co-infections between different pathogen pairs on each haematological parameter of the calves.

2. MATERIALS AND METHODS

* General methodology is discussed in Chapter 2

2.1 Univariate analysis: The impact of single pathogens

Univariate analysis of single pathogen infections was used as a preliminary screening tool for potential inclusion in the subsequent multivariable analysis. The haematological parameters, analysed as response variables, include packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), white cell counts (WCC), absolute lymphocyte counts (Lymph) and platelet counts (Plt).

Pathogens considered for analysis as independent variables included the tick-borne parasites *Theileria* spp., *Theileria mutans, Theileria parva, Anaplasma* spp., *Anaplasma marginale* and *Babesia bigemina; Trypanosoma* spp. and specifically *T. vivax;* and the intestinal parasites strongyle-type nematodes, strongyloides-type nematodes, coccidia and *Fasciola gigantica*.

Theileria spp. and *Anaplasma* spp. were diagnosed by microscopy and no species differentiation was made. There were only four visits where *Babesia* spp. was detected by microscopy and thus *Babesia* spp. was not included as a covariate.

Theileria parva, T. mutans, A. marginale and *B. bigemina* diagnosis was based on seroconversion. The time-point of seroconversion was firstly used as a covariate in the analysis to capture the acute response around the time of infection. Secondly, to capture the long-term impact of infection on the haematology, an indicator for seroconverted status was included as a covariate and for this purpose a calf was considered as positive (seroconverted) for all visits post seroconversion.

Trypanosoma-positive calves were deemed to be those calves that were diagnosed positive bymicroscopy (mcr) and no distinction between species was made. *Trypanosoma vivax*-positive calves were diagnosed by microscopy and speciation confirmed by PCR.

Strongyle worm species were considered as a categorical covariate at three levels, namely negative, positive with a high EPG of strongyle-type eggs (EPG> 1000) or positive with a low EPG (EPG<1000). The same classification was used for *Strongyloides* spp., as either negative, or positive with a high EPG (>1000) or low EPG (<1000) of strongyloides-type eggs. Similarly coccidia were analysed as a three-level categorical covariate as either

negative, or based on the OPG, as positive with either high OPG (>1000) or low OPG (<1000). *Fasciola gigantica* was only categorised as either present or absent.

Generalized mixed effect models were used for univariate models, and included single pathogens as fixed effects, with calf identification and sublocation as random effects. The significance of terms was determined by the *p*-value <0.1. A less restrictive *p*-value was chosen to increase the sensitivity of the univariable models a screening tool for identifying independent variables that will be used in further multivariable model building.

2.2 Multivariable analysis: The impact of interaction between co-infecting pathogens
Generalized additive mixed effect (GAMM) models were used in multivariable analysis, due to the non-linear distribution of the haematological parameters with age, as discussed in Chapter 2. Random effects included calf identification and sublocation, and fixed effects included age and pathogens, including interactions between age and pathogens and two-way interactions between pathogens.

Multivariable modelling started by including all covariates (pathogens) in the model that had a significant outcome based on univariate analysis. Two-way interaction terms were included in a step-up process and tested for both significance and model fit (reduction in residuals and AIC). If the interaction term was found significant ($p<0.05$), the term was left in the model and the next interaction term included. When found insignificant, the interaction terms were excluded. All covariates excluded based on univariate analysis were fitted into the multivariable model to check for significant interaction terms. The final minimal model thus only included significant terms of pathogens and age, where applicable, and interaction terms as fixed effects and calf identification and sublocation as random effects.

The estimated coefficient of each covariate included in the minimal model is interpreted as an increase in the response variable equal to the coefficient when the covariate (pathogen) is positive (two-level categorical covariates), or an increase equal to the value of the coefficient for an increase in one unit of the covariate (for three-level categorical covariates). A negative value of a coefficient should be interpreted as a decrease in the response variable, calculated in the same way as for positive coefficients.

To improve model fit, log transformation of PIt data was necessary. The model parameters reported represent the log-transformed data. The coefficient is interpreted as a percentage increase in the response variable equal to the value of the coefficient when positive, or a percentage decrease in the response variable when the coefficient is negative.

2.3 Pathogen interactions

Interaction between the co-infecting pathogens is said to occur when the outcome of infection, e.g. a reduction in PCV, during co-infections differs from the sum of the individual outcomes of the single infections. A positive interaction occurs when the outcome of infection is increased compared to the sum of the individual outcomes, e.g. a more severe reduction in PCV than what is expected to occur due to both pathogens combined. A negative interaction occurs when the outcome of infection is reduced compared to the sum of the individual outcomes, e.g. the reduction in PCV is less severe than what is expected from the combined effect of the individual pathogens.

For the purpose of this study, only two-way interactions between pathogens were considered.

2.4 Predicted outcomes of interactions between co-infecting pathogens

The predicted mean value of the response variables for each model was calculated for uninfected calves, calves positive for each single covariate, as well as two-way combinations between pathogen pairs. The model-predicted mean value was calculated from the sum of the intercept and the coefficients of the selected pathogens included in the minimal model. The 95% confidence intervals (95%CI) for the predicted mean values were calculated in the same way, using the standard error (SE) of each coefficient. To calculate the predicted mean Plt (pPlt), the model-predicted log interval (mean ±95%CI) was first calculated and then back transformed through exponentiation (10^x) by the calculated value (x).

3. RESULTS

3.1 Univariate analysis

The covariates found to significantly predict the various response variables in univariate analysis are listed in Table 7.1. These covariates were further used in model-building in multivariable models.

Table 7.1 The significance of *p*-values (p<0.1) obtained from univariate analysis of single covariates

Covariate		Response variables					
		PCV[6]	MCV[7]	MCHC[8]	WCC[9]	Lymph[10]	Plt[11]
Blood-borne pathogens							
Theileria spp. (mcr)[2]	Presence/absence	****	NS	*	NS	NS	****
T. parva	Positive	NS	*	**	**	****	****
	At seroconversion	NS	NS	NS	****	****	*
T. mutans	Positive	****	***	NS	NS	****	****
	At seroconversion	**	NS	NS	NS	NS	**
Anaplasma spp. (mcr)[2]	Presence/absence	NS	NS	NS	NS	*	NS
A. marginale	Positive	NS	*	NS	***	****	****
	At seroconversion	NS	NS	NS	NS	NS	**
B. bigemina	Positive	NS	****	NS	**	****	****
	At seroconversion	NS	NS	NS	NS	*	**
Trypanosoma spp. (mcr)[2]	Presence/abscence	****	NS	NS	NS	NS	****
T. vivax (PCR)[3]	Positive	****	NS	NS	NS	*	****
Intestinal pathogens							
Coccidia	Presence/absence	****	*	NS	**	*	NS
	OPG[4] > 1000	NS	NS	NS	NS	NS	NS
Strongyle-type species	Presence/absence	****	NS	*	*	****	****
	EPG[5] > 1000[7]	****	****	*	****	****	NS
Strongyloides spp.	Presence/absence	NS	NS	NS	NS	****	****
	EPG[5] > 1000	NS	NS	NS	NS	****	****
Fasciola gigantica	Presence/absence	NS	***	NS	NS	*	NS

**** p<0.0001, ***p<0.001, **p<0.01, *p<0.05, NS: non significant (p>0.0.5)
[1] s(): use smoothing function
[2] mcr: microscopy. This indicates the test used to diagnose the pathogen.
[3] PCR: polymerase chain reaction. This indicates the test used to diagnose the pathogen.
[4] OPG>1000: Oocysts per gram faeces per >1000.
[5] EPG>1000: Eggs per gram faeces >1000.
[6] PCV: packed cell volume
[7] MCV: mean corpuscular volume
[8] MCHC: mean corpuscular haemoglobin concentration
[9] WCC: white cell count
[10] Lymph: absolute lymphocyte count
[11] Plt: platelet count

3.2 Generalized additive mixed effect models

Packed cell volume

The coefficient estimates of the GAMM model for PCV are tabulated in Table 7.2. The effect of age on PCV was significant but nonlinear and was included in the model as a covariate with a smoothing function (estimated degrees of freedom (edf) = 7.698 and p<0.0001).

Pathogens that caused a significant decrease in PCV include *Theileria* spp. (mcr); *T. mutans; Trypanosoma* spp. (mcr); and strongyle worms (p<0.05). Strongyle species had a more severe effect on PCV when EPG was high. The only pathogen that caused a significant increase in PCV was coccidia (p<0.05). *Theileria mutans* caused a slightly more severe decrease in PCV at the time of seroconversion than at later time-points after seroconversion.

Table 7.2 Generalized mixed-effect model analysis of packed cell volume (n=3917)

Covariate	**Coeff** [1]	**SE** [2]	***p*** [3]
Theileria spp. (mcr)[4]	-2.147	0.321	<0.0001
T. mutans (at seroconversion)	-1.172	0.29	<0.0001
T. mutans (seroconverted)	-0.892	0.324	0.006
Trypanosoma spp. (mcr) [4]	-6.089	1.027	<0.0001
Strongyle-type species (EPG<1000) [5]	-0.76	0.258	0.003
Strongyle-type species (EPG>1000) [5]	-3.148	0.306	<0.0001
Coccidia (positive)	0.459	0.139	<0.001
T. mutans : *Theileria* spp.	0.709	0.326	0.03
T. parva (at seroconversion) : *Theileria* spp.	-0.889	0.279	0.001
Theileria spp. : age	0.007	0.002	<0.0001
Trypanosoma spp. : *Theileria* spp.	4.542	1.212	<0.001
Anaplasma marginale : *Strongyloides* spp. (EPG>1000)[5]	-5.006	2.376	0.035

[1] Coefficient
[2] Standard error of coefficient
[3] *p*-value indicate significance of coefficient
[4] mcr (microscopy)
[5] Eggs per gram faeces >1000

The impact of *Theileria* spp. on PCV became less severe as the calf age increased. Interactions were found between *T. mutans* and *Theileria* spp., as well as *T. parva* and *Theileria* spp. The decrease in PCV was 1.7 times more severe in calves when positive for both *Theileria* spp. (mcr) and *T. mutans* (seroconverted) than when only positive for *T. mutans* on serology only. *Theileria parva* had a significant impact on PCV only at the time of seroconversion, and only when seroconverted calves were also positive for *Theileria* spp. on microscopy.

A negative interaction was found between *Trypanosoma* spp. and *Theileria* spp. Although both pathogens caused a decrease in PCV by themselves, the total decrease in PCV during co-infections with *Trypanosoma* spp. and *Theileria* spp. (–3.696) was less than single infections with *Trypanosoma* spp. (-6.089).

An interaction between *A. marginale* and *Strongyloides* spp. was also found. Although neither pathogen had a significant impact on PCV by themselves, co-infection with these two pathogens caused a significant decrease (-5.006) in PCV.

Mean corpuscular volume

The effect of age on MCV was significant but nonlinear and was included in the model as a covariate with a smoothing function (edf = 8.067 and $p<0.0001$). The coefficient estimates of the model are depicted in Table 7.3. Pathogens that caused a significant increase in MCV include *B. bigemina, T. mutans, T. parva* and strongyle-type nematodes ($p<0.05$). The only pathogen that caused a statistically significant decrease in MCV was *A. marginale* ($p<0.05$). The impact of *A. marginale* and *T. parva* was very small, however, and dependent of the age of the calf.

A positive interaction was found between strongyle-type nematodes and *Fasciola* spp. The increase in MCV was 3.89 times higher in co-infection with *Fasciola* spp. than infection with strongyle-type nematodes alone. The interaction depended on the infectious load of the strongyles (EPG).

Negative interactions were found between *T. parva* and *Fasciola* spp. and *B. bigemina* and coccidia. *Fasciola* spp. had no significant impact on MCV by itself, but in *T. parva*-positive calves, *Fasciola* spp. infection caused a decrease in MCV of 1.178. The increase in MCV was 3.7 times less in co-infections with coccidia than in only *B. bigemina*-positive calves.

Table 7.3 Generalized additive mixed-effect model analysis of mean corpuscular volume (n=3904)

Covariate	**Coeff** [1]	**SE** [2]	***p*** [3]
Theileria mutans	0.479	0.117	<0.0001
Babesia bigemina	1.078	0.182	<0.0001
Anaplasma marginale : age	-0.001	0.0005	0.004
Theileria parva : age	0.001	0.0005	0.04
B. bigemina : Coccidia	-0.79	0.189	<0.0001
Strongyles (EPG>1000)[4]	0.591	0.127	<0.001
T. parva : *Fasciola* spp.	-1.178	0.246	<0.001
Strongyles (EPG>1000) [4] : *Fasciola* spp.	1.708	0.444	<0.001

[1] Coefficient
[2] Standard error of coefficient
[3] *p*-value indicate significance of coefficient
[4] EPG: eggs per gram faeces

Mean corpuscular haemoglobin concentration

The effect of age on MCHC was significant but nonlinear and was included in the model as a covariate with a smoothing function (edf = 8.138 and $p<0.0001$). Only two pathogens had an impact on MCHC, namely *T. parva* and *A. marginale.* There was an interaction between both pathogens and the age of the calf. The model coefficient estimates are depicted in Table 7.4. Very little of the variance of the model was explained by the presence of pathogens ($R^2=0.089$)

Table 7.4 Generalized additive mixed-effect model analysis of mean corpuscular haemoglobin concentration (n=5516)

Covariate	**Coeff** [1]	**SE** [2]	***p*** [3]
Theileria parva : age	-0.001	0,0002	0.017
Anaplasma marginale : age	0.001	0.0002	0.035

[1] Coefficient
[2] Standard error of coefficient
[3] *p*-value indicate significance of coefficient

White cell count

The effect of age on WCC was significant but nonlinear and was included in the model as a covariate with a smoothing function (edf = 1 and $p<0.001$). The coefficient estimates of the model are tabulated in Table 7.5.

Pathogens that had a negative impact on WCC include strongyle-type nematodes (EPG>1000) and *T. parva* (at the time of seroconversion). Pathogens that had a positive impact on WCC include *Trypanosoma* spp., *T. parva* (after seroconversion), and *B. bigemina*. The impact of *T. parva* (after seroconversion) and *B. bigemina* depended on the age of the calf.

Two negative interactions were found, between strongyle-type nematodes and coccidia, and between *Trypanosoma* spp. and coccidia. The decrease in WCC during co-infections with coccidia was four times less than in single infections with strongyles. By themselves *Trypanosoma* spp. caused an increase in WCC, but with co-infection with coccidia, a decrease in WCC was found.

Table 7.5 Generalized additive mixed-effect model analysis of mean white cell count (n=3906)

Covariate	**Coeff** [1]	**SE** [2]	***p*** [3]
Strongyle-type nematodes (EPG>1000) [4]	-0.931	0.156	<0.0001
Trypanosoma spp. (mcr)[5]	1.393	0.468	0.003
Theileria parva (at seroconversion)	-0.724	0.163	<0.0001
Theileria parva (after seroconversion) : age	0.002	0.0006	0.012
Babesia bigemina ; age	0.002	0.0007	0.02
Strongyle-type nematodes (EPG>1000) [4] : Coccidia	0.703	0.208	<0.001
Trypanosoma spp. : Coccidia	-2.154	0.865	0.013

[1] Coefficient
[2] Standard error of coefficient
[3] p-value indicate significance of coefficient
[4] Eggs per gram faeces > 1000
[5] Microscopy

Absolute lymphocyte count

The effect of age on Lymph was significant but nonlinear and was included in the model as a covariate with a smoothing function (edf = 6.016 and $p<0.0001$). The coefficient estimates of the model are tabulated in Table 7.6.

The only pathogens that caused a significant increase in Lymph were *B. bigemina* and *Trypanosoma* spp. ($p<0.05$). The impact *Trypanosoma* spp. had on Lymph was dependent on the age of the calf. Both strongyles and *T. parva* (at time of seroconversion) caused a decrease in Lymph ($p<0.05$).

Interaction between *T. parva* (after seroconversion) and strongyle-type nematodes (EPG>1000) resulted in a decrease in Lymph 1.47 times lower than in a strongyle (EPG>1000) infection alone. *Anaplasma marginale* caused an increase in Lymph only when calves were co-infected with coccidia (OPG>1000).

Table 7.6 Generalized additive mixed-effect model analysis of lymphocyte count (n=3856)

Covariate	**Coeff** [1]	**SE** [2]	***p*** [3]
Babesia bigemina : age	0.001	0.0005	0.0145
Theileria parva (at seroconversion)	-0.467	0.116	<0.0001
Trypanosoma spp. (mcr) [4] : age	0.003	0.001	0.016
Strongyle-type nematodes	-0.206	0.101	0.04
A. marginale : coccidia (OPG>1000) [5]	0.923	0.339	0.007
T. parva : Strongyles (EPG>1000) [6]	-0.193	0.074	0.009

[1] Coefficient
[2] Standard error of coefficient
[3] *p*-value indicate significance of coefficient
[4] Microscopy
[5] Oocysts per gram faeces > 1000
[6] Eggs per gram faeces > 1000

Platelet counts

The platelet counts were log transformed to allow for a better fit of the final model by normalizing the distribution of the residuals. The coefficient estimates of the model are listed in Table 7.7.

Table 7.7 Generalized additive mixed-effect model analysis of log-transformed platelet counts (n=3856)

Covariate	Coeff [1]	SE [2]	*p* [3]
Anaplasma marginale	-0.405	0.077	<0.0001
Babesia bigemina	-0.092	0.04	0.021
Theileria spp.	-0.096	0.033	<0.0001
T. mutans	-0.117	0.03	<0.001
T. parva	-0.307	0.076	<0.0001
Trypanosoma spp. (mcr) [4]	-1.647	0.326	<0.0001
T. vivax	-0.37	0.094	<0.0001
Strongyle spp.	-0.114	0.04	<0.01
Strongyloides spp.	0.122	0.036	<0.001
Theileria spp. : *A. marginale*	0.227	0.059	<0.001
T. parva : *Strongyle* spp.	0.197	0.075	0.009
T. parva : *Fasciola* spp.	0.142	0.066	0.03
T. mutans : *Strongyloides* spp.	-0.188	0.058	0.001
T. mutans : *Trypanosoma* spp.	0.416	0.167	0.013
Trypanosoma spp.: *Strongyle* spp.	0.854	0.325	0.009

[1] Coefficient
[2] Standard error of coefficient
[3] *p*-value indicate significance of coefficient
[4] Microscopy

The only pathogen that caused a significant increase in Plt was *Strongyloides* spp. Pathogens that caused a significant decrease in Plt included *A. marginale*, *B. bigemina, Theileria* spp., *T. mutans, T. parva, Trypanosoma* spp. (mcr), *T. vivax,* and *Strongyle* spp. *Trypanosoma* spp. caused the most severe decrease in Plt.

The decrease in Plt was 1.48 times more in *A. marginale*-positive calves than calves positive for both *A. marginale* and *Theileria* spp. The decrease in strongyle-positive animals was twice as low when also positive for *T. parva. Theileria parva* also interacted with *Fasciola* spp. On its own, *Fasciola* spp. had no significant impact on Plt, but in *T. parva*-positive calves caused a decrease of 16%.

There was also an interaction between *Trypanosoma* spp. and strongyl*e*s. Co-infection with these two pathogens resulted in a decrease in Plt almost eight times more than in strongyle infection alone. An interaction between *Trypanosoma* spp. and *T. mutans* was also detected. The decrease was ten times more than in *T. mutans*-positive calves that were not infected with *Trypanosoma* spp. as well.

3.3 Model-predicted mean of the response variables

Predicted packed cell volume

The model-predicted mean PCV (pPCV) at 150 days of age was calculated to illustrate the impact of the cumulative effect of pathogens on the PCV of the calves (Table 7.8). The pathogen-group that caused the most severe decrease in pPCV was *Trypanosoma* spp. (mcr). Strongyle-type helminths caused the second most severe decrease in pPCV, but the decrease was only clinically significant at a high EPG. Other pathogens that caused a decrease in mean pPCV were *T. mutans* and *Theileria* spp. but these were not clinically significant unless they occurred as part of multi-pathogen infections. Coccidia caused a slightly higher pPCV than in uninfected calves, but this was not clinically significant.

There was a cumulative decrease in pPCV in co-infections between *Trypanosoma* spp. and both strongyle-type helminths and *T. mutans*. The overall lowest mean pPCV was found in co-infections with *Trypanosoma* spp. and strongyle-type species (EPG>1000) (mean pPCV=21.38% at 150 days). Co-infection with coccidia only marginally improved the pPCV. The mean pPCV in concomitant infections between *Trypanosoma* spp. and strongyle-type species, *T. mutans*, *Theileria* spp. and coccidia is illustrated in Figure 7.1.

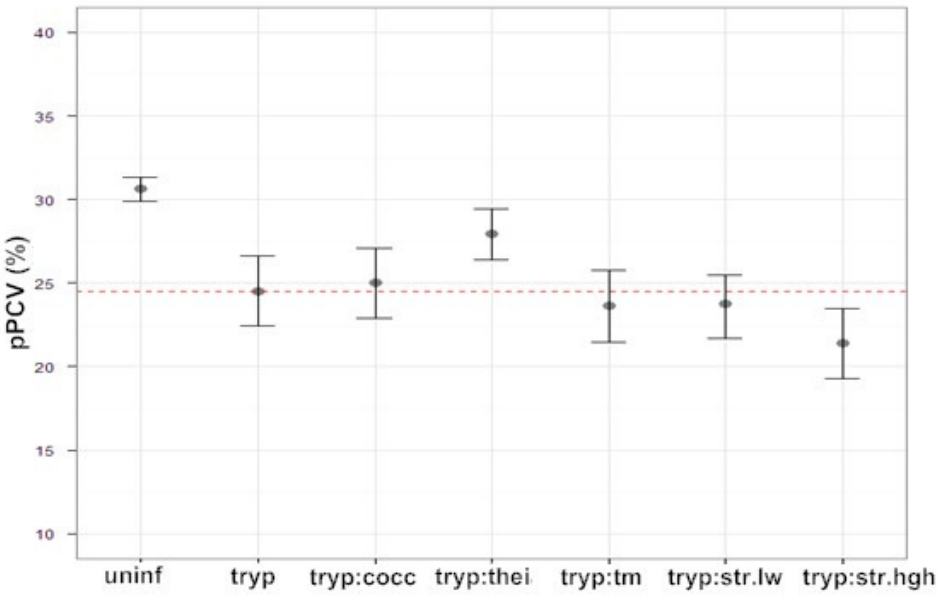
The decrease in pPCV in *T. mutans*-positive calves was slightly more significant when the calf was also positive for *Theileria* spp. on microscopy. The impact of *Theileria* spp. on pPCV was dependent on the age of the calf, and this was also reflected in the impact of *T. mutans* on pPCV (Figure 7.2). At 300 days of age, the pPCV of *T. mutans*-positive calves (28.05 (27.34-28.76) %) was not markedly different than in uninfected calves (pPCV=28.38 (27.51-29.25) %).

Table 7.8 The GAMM-predicted mean packed cell volume (%) and 95% confidence intervals in co-infections with pathogen pairs at 150 days

Covariate	Uninfected	*Theileria* spp,	*T. mutans*	*T. parva*	*Trypanosoma* spp.	Strongyle–type species (EPG<1000)	Strongyle-type species (EPG>1000) [1]	Coccidia
Uninfected	30.62 (29.91-31.32)	-	-	-	-	-	-	-
Theileria spp. (mcr)[1]	29.47 (28.77-30.17)	29.29 (28.58-30.00)	28.58 (27.73-29.44)	**27.92 (26.39-29.46)**	28.71 (28.20-29.22)	26.32 (25.73-26.92)	29.93 (29.24-30.62)	
Theileria mutans		29.73 (28.94-30.52)	29.73 (28.94-30.52)	**23.64 (21.49-25.78)**	28.97 (28.32-29.61)	26.58 (25.85-27.30)	30.19 (29.41-30.97)	
Theileria parva (at seroconversion)			30.62 (29.91-31.32)■	**24.53 (22.43-26.63)**	29.86 (29.33-30.39)	27.47 (26.86-28.08)	31.08 (30.38-31.77)	
Trypanosoma spp. (mcr) [1]				**24.53 (22.43-26.63)**	**23.77 (21.71-25.83)**	**21.38 (19.30-23.46)**	**24.99 (22.89-27.09)**	
Strongyle-type species (EPG<1000) [2]					29.86 (29.33-30.39)	NA	30.32 (29.76-30.87)	
Strongyle-type species (EPG>1000) [2]						27.47 (26.86-28.08)	27.93 (27.30-28.55)	
Coccidia							31.08 (30.38-31.77)	

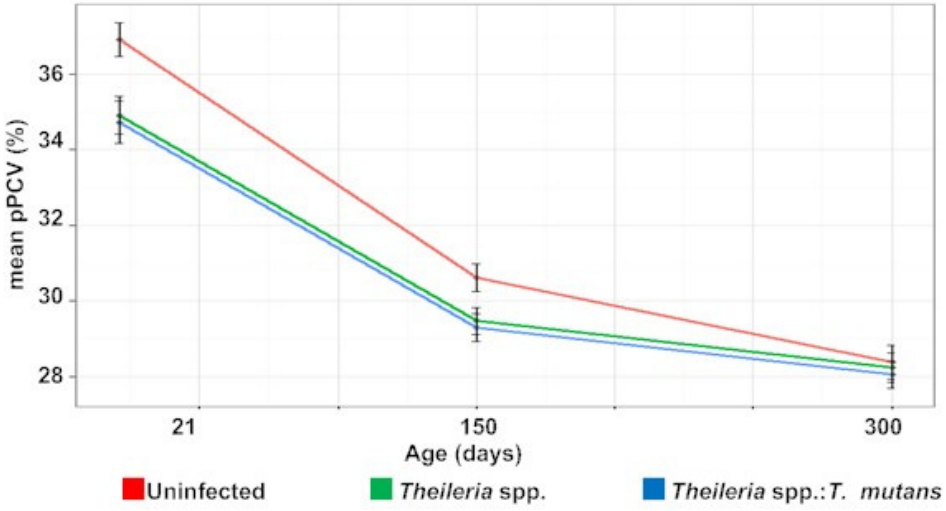
■ pPCV not significantly different than pPCV of uninfected calves.
■ Co-infections with *Trypanosoma* spp. (mcr).
[1] mcr: microscopy
[2] EPG: eggs per gram faeces.

Figure 7.1 The distribution of the GAMM-predicted mean packed cell volume in co-infections associated with *Trypanosoma* spp.-positive calves at 150 days of age



pPCV- model-predicted packed cell volume; a:b denotes co-infection between pathogen a and b; uninf- uninfected; tryp- *Trypanosoma* spp.; cocc- Coccidia; thei- *Theileria* spp.; tm- *Theileria mutans*; str.lw- *Strongyle* spp. (EPG<1000); str.hgh- *Strongyle* spp. (EPG>1000); ----- mean pPCV of *Trypanosoma* spp. -positive calves.

Figure 7.2 The distribution of the GAMM-predicted mean packed cell volume in *Theileria mutans*-positive calves at 150 days of age



pPCV- model-predicted mean packed cell volume; •*Theileria* spp.: *T. mutans*-calves are positive for both *Theileria mutans* on serology and *Theileria* spp. on microscopy.

Predicted mean corpuscular volume

The model-predicted mean MCV (pMCV) for co-infections with pathogen pairs was calculated to illustrate what the impact of the cumulative effect and interaction between pathogen was on MCV.

Overall there was very little difference between pMCV in uninfected calves and calves infected with various pathogens. Both *T. mutans* (34.19 (33.91-34.84) fL at 150 days) and strongyle-type nematodes (34.14 (33.82-34.45) fL at 150 days) caused an increase in pMCV compared to uninfected calves (33.76 (33.48-34.03) fL at 150 days). The lowest pMCV (32.51 fL at 150 days) was found in calves positive for both *T. parva* and *Fasciola* spp. The highest pMCV (36.15 fL at 150 days) was found in calves infected with both strongyle-type worms and *Fasciola* spp.

Mean corpuscular haemoglobin concentration

The model-predicted mean MCHC (pMCHC) at three time points was calculated to illustrate the impact of the cumulative effect of pathogens on the MCHC in calves. The pMCHC of uninfected calves at 21 days of age was 31.86 (31.75-31.97) g/dL. *Theileria parva*- and *A. marginale*-positive calves had a pMCHC 31.85 (31.74-31.96) g/dL and 31.87 (31.76-31.98) g/dL respectively at 21 days of age. In calves positive for both pathogens at 21 days the pMCHC was 31.86 (31.75-31.98) g/dL.

At 150 days of age uninfected calves had a pMCHC 31.92 (31.8-32.04) g/dL; *T. parva*-positive calves 31.84 (31.71-31.66) g/dL, and *A. marginale*-positive calves 31.99 (31.86-32.13) g/dL. Calves positive for both pathogens had a pMCHC of 31.91 (31.77-32.05) g/dL.

At 300 days of age there was still very little difference between uninfected and infected calves. The pMCHC of uninfected calves at this age was 32.79 (32.63-32.94) g/dL. The pMCHC at 300 days of age in *T. parva*-positive calves was 32.62 (32.49-32.76) g/dL, in *A. marginale*-positive calves 32.94 (32.75-33.13) g/dL, and in calves positive for both pathogens 32.77 (32.62-32.93) g/dL.

White cell count

The model-predicted mean WCC (pWCC) at 150 days of age was calculated to illustrate the impact of the cumulative effect and interaction between pathogens on WCC. The results are tabulated in Table 7.9.

The pathogen with the most significant impact on pWCC was *Trypanosoma* spp. This pathogen caused an increase of 1.39 x 10^3/µL WCC in pWCC which was not dependent on age. The highest pWCC was in co-infection between *B. bigemina* and *Trypanosoma* spp. (pWCC = 13.28 x10^3/µL at 350 days). Co-infections between *Trypanosoma* spp. and either strongyle-type nematodes or coccidia resulted in a lower pWCC than infections with only *Trypanosoma* spp. The pWCC in calves infected with both *Trypanosoma* spp. and coccidia was even lower than in uninfected calves. Strongyles caused a decrease in pWCC but only when the EPG>1000. With co-infections with coccidia the difference in pWCC between strongyle-infected and uninfected calves became even less. This decrease was, however, clinically insignificant.

Table 7.9 The GAMM-predicted mean white cell count (x10^3/µL) and 95% confidence intervals at age 150 days for co-infections with pathogen pairs

Covariate	Uninfected	*B. bigemina*	*T. parva*	*Tryapnosoma* spp.	Strongyle-type nematodes (EPG>1000)	Coccidia
Uninfected	10.77 (10.54-11.00)	-		-	-	-
Babesia bigemina		11.03 (10.72-11.34)	11.26 (10.95-11.56)	12.42 (11.46-13.39)	10.1 (9.69-10.51)	11.03 (10.72-11.34)
Theileria parva			11.00 (10.76-11.24)	12.39 (11.45-13.33)	10.07 (9.70-10.43)	11.00 (10.76-11.24)
Trypanosoma spp.				12.16 (11.23-13.1)	11.23 (10.26-12.21)	10.01 (8.52-11.5)
Strongyle-type nematodes (EPG>1000)					9.84 (9.48-10.2)	10.54 (10.15-10.93)
Coccidia						10.77 (10.54-11.00)■

■ pWCC not significantly different than pWCC of uninfected calves

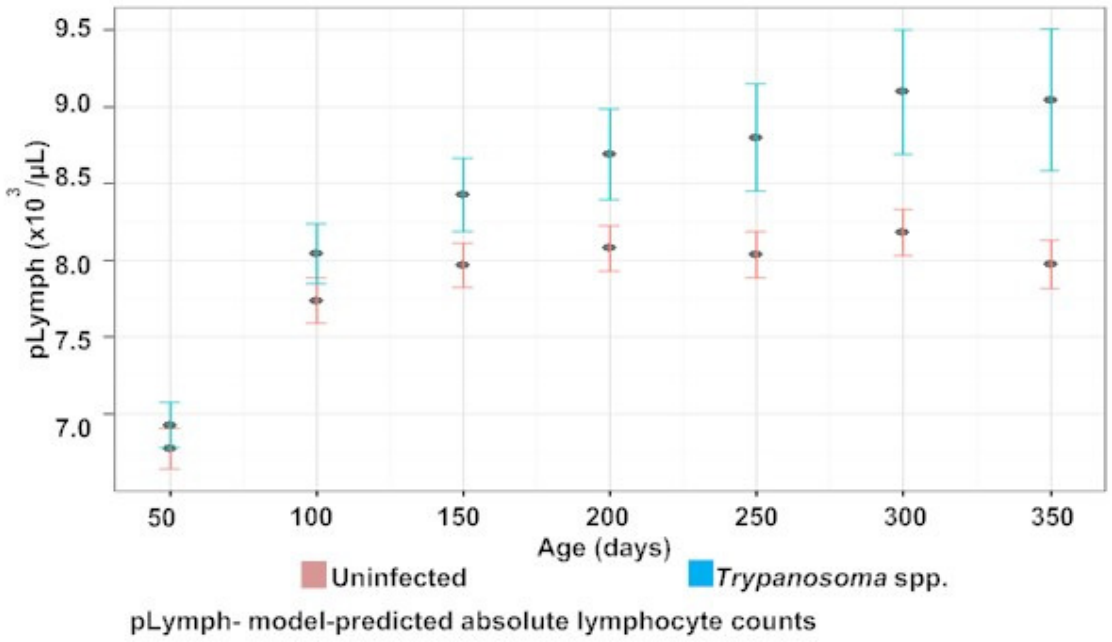
The impact of *B. bigemina* and *T. parva* on pWCC was age-dependent and the predicted mean value increased as the calves grew older. Infection with these two pathogens therefore increased the pWCC in co-infections with other pathogens as well.

Predicted absolute lymphocyte counts

The model-predicted mean absolute lymphocyte counts (pLymph) at 150 days of age associated with co-infection with different pathogen pairs was calculated to illustrate the impact of co-infections on Lymph of calves (Table 7.10).

The highest pLymph (8.89 x10^3/µL) at 150 days was in calves positive for both *A. marginale* and coccidia. The lowest pLymph (7.55 x10^3/µL) at 150 days was in infections with strongyles with a high EPG. The pLymph for *Trypanosoma* spp. (mcr) increased with age (Fig. 7.3). At 350 days of age the pLymph was 9.044 (8.14-9.95 x10^3/µL), which is 1.13 times higher than in uninfected calves.

Figure 7.3 The GAMM-predicted mean absolute lymphocyte counts for *Trypanosoma* spp. (mcr) positive calves at different ages



pLymph- model-predicted absolute lymphocyte counts

Table 7.10 The GAMM-predicted mean absolute lymphocyte counts (x10^3/µL) and 95% confidence intervals at 150 days for co-infections with pathogen pairs

Covariate	Uninfected	*A. marginale*	*B. bigemina*	*T. parva*	*Trypansoma* spp.	Strongyle-type nematodes.	Strongyles (EPG>1000)	Coccidia (OPG>1000)
Uninfected	7.97 (7.68-8.25)	-	-	-	-	-	-	-
Anaplasma marginale		7.97 (7.68-8.25)■	8.15 (7.83-8.47)	7.97 (7.68-8.25)	8.43 (7.96-8.9)	7.76 (7.55-7.97)	7.55 (7.26-7.84)	8.89 (8,17-9.61)
Babesia bigemina			8.15 (7.83-8.47)	8.15 (7.83-8.47)	8.61 (8,12-9.11)	7.95 (7.7-8.2)	7.74 (7.42-8.06)	8.15 (7.83-8.47)
Theileria parva				7.97 (7.68-8.25)■	8.43 (7.96-8.9)	7.76 (7.55-7.97)	7.36 (7.12-7.61)	7.97 (7.68-8.25)■
Trypanosoma spp. (mcr)					8.43 (7.96-8.9)	8.22 (7.79-7.97)	8.014 (7.55-8.48)	8.43 (7.96-8.9)
Strongyle-type nematodes						7.76 (7.55-7.97)	NA	7.76 (7.55-7.97)
Strongyle-type nematodes. (EPG>1000)[1]							7.55 (7.26-7.84)	7.55 (7.26-7.84)
Coccidia (OPG>1000) [2]								7.97 (7.68-8.25)■

■ pLymph not significantly different than pLymph of uninfected calves
[1] EPG: eggs per gram faeces
[2] OPG: oocyts per gram faeces

Predicted platelet counts

The model-predicted mean platelet counts (pPlt) associated with co-infection with different pathogen pairs was calculated to illustrate the impact of co-infections of pathogens on the Plt of calves (Table 7.11).

Table 7.11 The back-transformed GAMM-predicted mean platelet counts (x10^3/µL) and 95% confidence intervals for co-infections with pathogen pairs

Covariate	Uninfected	*A. marginale*	*B. bigemina*	*Theileria* spp.	*T. mutans*	*T. parva*	*Trypanosoma* spp.	*T. vivax*	Strongyle-type nematodes	*Strongyloides* spp.
Uninfected	1657 (1368-2006)	-	-	-	-	-	-	-	-	-
A. marginale		651 (444-957)	527 (346-805)	882 (625-1245)	648 (447-943)	417 (272-641)	**15 (3-67)**	278 (160-484)	502 (356-707)	862 (586-1269)
B. bigemina			1341 (1032-1742)	1076 (827-1400)	1025 (780-1348)	661 (458-954)	**30 (6-134)**	572 (351-934)	1032 (834-1278)	1775 (1376-2291)
Theileria spp.				1329 (1094-1616)	1015 (821-1257)	655 (474-907)	**30 (7-130)**	567 (356-904)	1023 (907-1155)	1760 (1455-2128)
Theileria mutans					1266 (1018-1575)	625 (451-864)	**74 (16-347)**	540 (337-868)	975 (837-1136)	1088 (827-1430)
Theileria parva						817 (589-1133)	**18 (4-81)**	349 (204-595)	990 (848-1157)	1082 (768-1523)
Trypanosoma spp. (mcr)[1]							**37 (8-162**	**16 (4-68)**	**205 (113-375)**	**49 (11-216)**
Trypanosoma vivax								707 (445-1125)	544 (351-845)	936 (591-1482)
Strongyle-type nematodes									1275 (1126-1444)	1688 (1421-2006)
Strongyloides spp.										2193 (1832-2625)

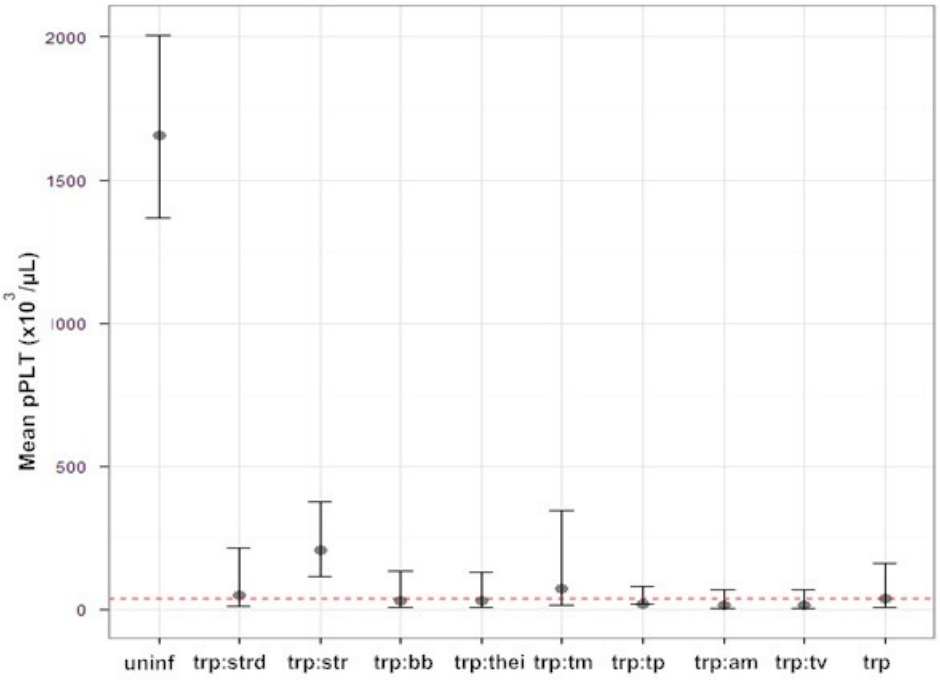
[1] mcr: Microscopy; ■ *Trypanosoma* spp.-associated co-infections

The pathogen that had the most significant impact on pPlt was *Trypanosoma* spp. (mcr). The mean pPlt for *Trypanosoma*-infected calves was less than 3% of the mean in uninfected calves. The mean pPlt in calves that were positive for both *Trypanosoma* spp. (mcr) and *T. vivax* (PCR) was half (16 x10^3/µL) of the pPlt in calves only positive on microscopy. The distribution of pPlt for various co-infections associated with *Trypanosoma* spp. is illustrated in Figure 7.4.

Tick-borne diseases all caused a significant drop in pPlt, although not as severe as the trypanosomes. *Anaplasma marginale* had the lowest pPlt of the tick-borne pathogens, with a mean pPlt of 651x10^3/µL. The lowest pPlt for tick-borne disease occurred in concomitant infections with *A. marginale* and *T. parva*. Co-infections between *T. parva* and the other tick-borne parasites also resulted in decreased pPlt. The distribution of pPlt for the various co-infections with tick-borne parasites is illustrated in Figure 7.5.

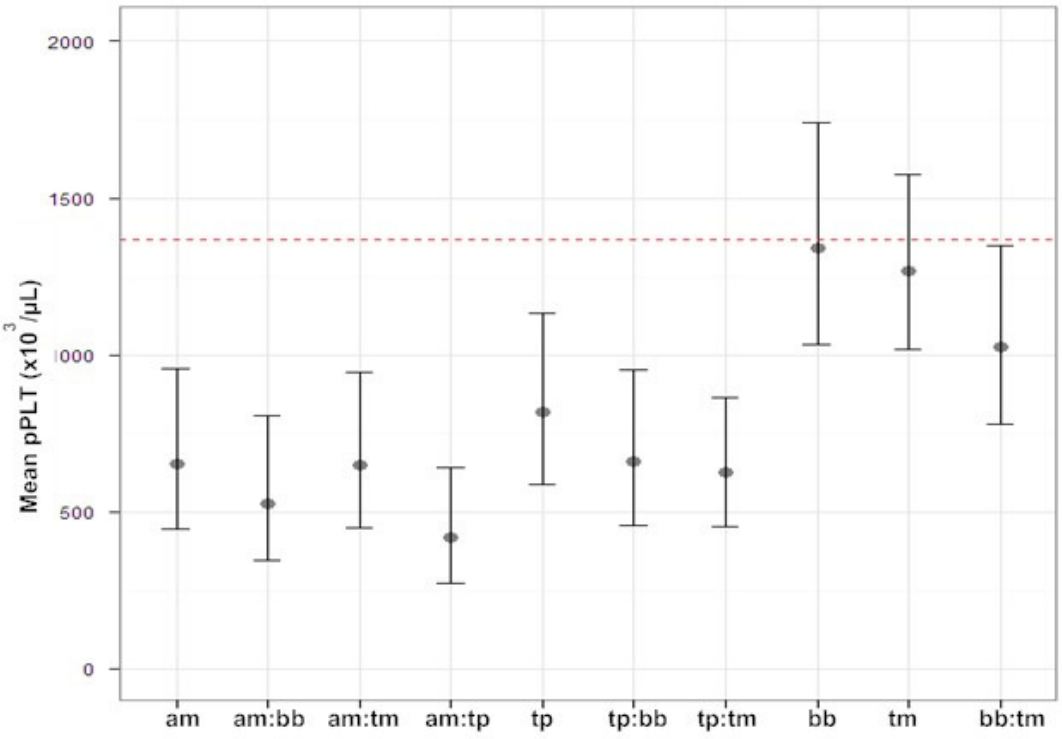
The only pathogen that caused an increase in pPlt was *Strongyloides* spp.

Figure 7.4 The GAMM-predicted mean platelet counts for co-infections associated with *Trypanosoma* spp. infections



pPLT- model-predicted platelet count; a:b denotes co-infection between pathogen a and b; uninf- uninfected; trp- *Trypanosoma* spp.; strd- Strongyloides-type worms; str- Strongyle-type worms; bb- *Babesia bigemina*; thei- *Theileria* spp.; tm- *T. mutans*; tp- *T. parva*; am- *Anaplasma marginale*; tv- *T. vivax*; ---- mean pPLT of *Trypanosoma* spp.-positive calves.

Figure 7.5 The GAMM-predicted mean platelet counts for co-infections associated with tick-borne pathogens



pPLT- model-predicted platelet count; a:b denotes co-infection between pathogen a and b;
am- *Anaplasma marginale;* bb- *Babesia bigemina*; tm- *T. mutans*; tp- *T. parva*;
---- mean pPLT of uninfected calves.

4. DISCUSSION

Packed cell volume

Trypanosoma spp., followed by strongyle-type nematodes, should be considered as the two major pathogenic causes of anaemia in the population. Although the pPCV in all co-infections involving *Trypanosoma* spp. were well below the pPCV of uninfected calves, interactions with other pathogens, apart from strongyle-type nematodes, reduced the final pPCV only marginally compared to single infections with *Trypanosoma* spp. The pathogenicity of *Trypanosoma* spp. partially depends on the intensity of the parasitaemia. The parasitic wave during trypanosome infections coincides with the drop in PCV (Murray & Dexter 1988). Due to its relatively low sensitivity compared to molecular diagnostic tests, microscopy is more likely to detect the peak of such parasitic waves (Uilenberg 1998). This

would explain why *Trypanosoma* spp. (mcr) caused a reduced PCV whereas this was not the case with *T. vivax*, which was diagnosed by PCR.

Interaction between strongyle-type nematodes and other pathogens, apart from coccidia, also resulted in a reduced PCV compared to single infections with strongyles. The pathogenicity of strongyle-type nematodes depended on its infectious load, with a more significant decrease in PCV as the parasitic load (EPG) increased. Co-infection with strongyle-type nematodes at high EPG and *Trypanosoma* spp. caused the most severe decrease in PCV compared to uninfected animals. The cumulative impact of these two pathogens can potentially result in anaemias severe enough to cause mortality.

The pathogenicity of coccidia is dependent on the species involved. Clinical coccidiosis in calves is most commonly caused by *Eimeria zuerni* and *E. bovis* (Kaufmann 1996). Although coccidiosis can result in haemorrhagic diarrhoea, it is not consistently found (Kaufmann 1996). The slight increase in PCV due to coccidia in this study was not clinically significant and was possibly due to a level of dehydration due to diarrhoea.

The total decrease in PCV caused by *T. mutans* was of clinical significance, particularly in young calves. This decrease in PCV became more significant if the calf also had piroplasms on bloodsmears as indicated by a positive *Theileria* spp. status. It is possible for a calf to be positive for antibodies against *T. mutans* in the absence of a parasitaemia. This would imply that the calf has either cleared the infection or it has become a latent carrier. In latent carriers the parasitaemia is often too low to be detected by microscopy (Young *et al*. 1990c). The detection of parasitaemia on microscopy would thus indicate a higher parasitic load which is reflected in the lower PCV. Speciation of *Theileria* spp. was, however, not done on microscopy. One can therefore, not rule out that co-infection between *T. mutans* and other *Theileria* species was the reason for the additional decrease in PCV. By one year of age the decrease in PCV caused by *T. mutans* was not clinically significant. This age-related tolerance is likely related to the development of immunity in the calf.

Mean corpuscular volume

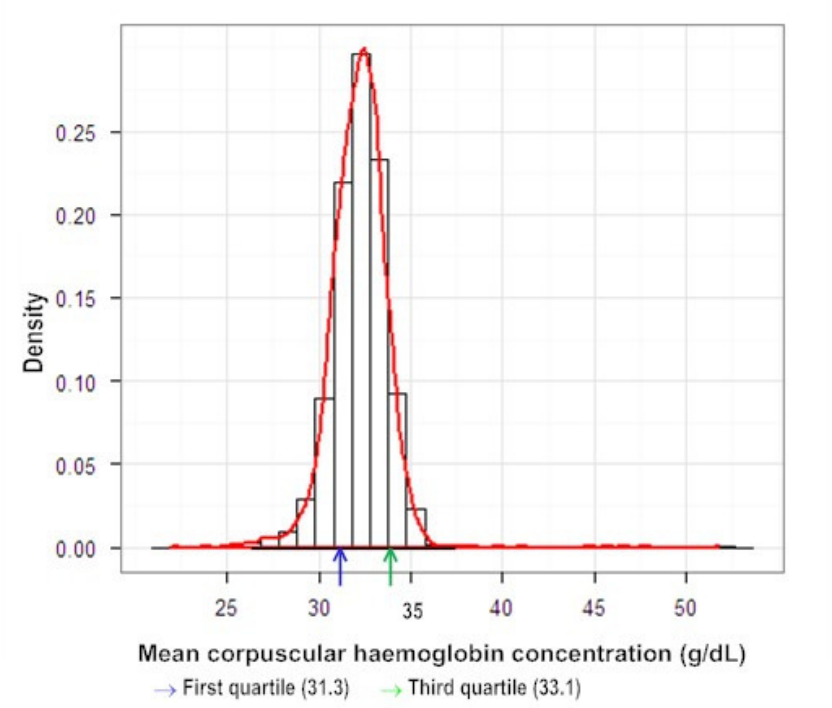
Both strongyle-type nematodes, at EPG>1000, and *T. mutans* infections resulted in an increase in MCV. This is expected in light of the decrease in PCV that is caused by both pathogens. An increase in MCV indicates that the infected calf elicits a regenerative red blood cell response to compensate for the developing anaemia. Interaction between strongyle-type nematodes and *T. mutans* both resulted in an increase in MCV equal to the sum of the response of infection with each pathogen.

Trypanosoma spp. did not have a significant effect on pMCV. The pMCV in concomitant infections between this pathogen and either strongyle-type nematodes or *T. mutans* was not high enough to compensate for the decrease in pPCV that was seen in such infections. This would imply that that the calf will not be able to compensate for the anaemia caused by *Trypanosoma* spp. and in time possibly develop a severe progressive anaemia. Reticulocyte counts are a more accurate measure of red cell regeneration, however, and will need to be done to confirm this statement.

Mean corpuscular haemoglobin concentration

Only two pathogens had a statistically significant impact on MCHC, namely *A. marginale* and *T. parva*. The impact on MCHC was not clinically significant in either of these two pathogens. The range of MCHC of all observations over the age range of 51 weeks is very limited. The frequency distribution of MCHC for all observations is illustrated in Figure 7.6. It is possible that the number of reticulocytes during anaemia caused by the pathogens is too low to significantly decrease the MCHC.

Figure 7.6 The frequency distribution of mean corpuscular haemoglobin concentration of all observations (n=5516)



White cell count and absolute lymphocyte counts

The pathogen with the most significant impact on WCC was *Trypanosoma* spp. Acute trypanosomosis typically causes a leucopenia which coincides with the first parasitic wave. This leucopenia is followed by a leucocytosis (Murray & Dexter 1988). The long interval between observation points probably does not reflect the acute change in WCC in trypanosome-infected calves in this study.

Theileria parva and *B. bigemina* caused an increase in WCC, which was dependent on age of the calf. There was possibly a confounding effect between these two pathogens and age, since the likelihood of seroconversion increased with age. The WCC can be used as an indicator of prognosis in cases of ECF (Irvin 1983). Chronic ECF is usually associated with a leucopenia (Maxie *et al*. 1982; Irvin 1983). Animals that maintain their WCC are more likely to recover from ECF (Irvin 1983).

The pWCC of uninfected calves also increased with age. This is probably due to challenge by pathogens other than what is accounted for in the model.

Strongyle-type nematodes interacted with blood-borne pathogens by decreasing the WCC in co-infections. This interaction was only significant in infections of high intensity (EPG>1000). Helminth infections are typically associated with polarization of the host's immune reaction towards the Th2 (T-helper lymphocytes) –type response as indicated by increased peripheral eosinophil levels and mast cells, as well as parasite-specific IgE levels (Maizels & Yazdanbakhsh 2003). Cytokines associated with Th2-cell response down-regulate the Th1-cell response which is associated with a potentially pathogenic cell-mediated inflammatory response, thus ensuring the host's own survival and ultimately the parasites' survival. Bacteria, viruses and certain protozoa are also associated with Th-1 responses (Jankovic *et al*. 2001; Fenton, Lamb & Graham 2008). This suppression of Th-1 cell response by the Th2-response is dependent on high parasite intensities (Maizels & Yazdankakhsh 2003) with a stimulatory effect seen in low infection burdens (Kamal & El Sayed Khalifa 2006). This modulation of the immunity can possibly explain the reduced WCC responses against co-infections that are associated with strongyle-type nematodes. To confirm this one will have to investigate further including cytokine and antibody levels (Bradley & Jackson 2008).

Platelet count

Trypanosoma spp. caused the most significant decrease in platelet counts. The pPlt of *Trypanosoma* spp. was 40 times lower than in uninfected calves. Co-infections with other pathogens decreased the pPlt even more, although not considerably. Platelet counts as low

as those seen in infections associated with *Trypanosoma* spp. cause coagulopathies, and clinically present as generalized petechiation and ecchymosis. This was confirmed by post mortem examinations of calves that died during the course of this study (data not shown here).

All four tick-borne infections caused a reduction in Plt, particularly *A. marginale* and *T. parva*. Co-infections between all pairs of tick-borne diseases resulted in a cumulative decrease of platelets. The pathogenesis of the reduced Plt in all four infections is multifactorial, and includes a combination of factors form reduced platelet production, increased consumption to immune-mediated platelet destruction (Pantanowitz 2003). Splenomegaly is a common symptom of many tick-borne diseases, and also contributes to low Plt due to sequestration of platelets and destruction by macorphages in the spleen (Pantanowitz 2003). There is thus a cumulative reduction in Plt during co-infections with tick-borne diseases.

5. CONCLUSIONS

Traditionally studies on infectious diseases have focused on single pathogens, often based on experimental conditions. Animals in the field are exposed to a variety of pathogens that occur in the animal's environment (Petney & Andrews 1998), particularly in a tropical environment such as western Kenya. It is therefore impossible under field conditions to study a disease in isolation without reference to other causes of disease (Moll *et al.* 1984).

It is evident that co-infections between pathogens complicate the clinical picture of disease (Cox 2001). The animal might present with clinical signs that can not be explained by the disease that was diagnosed. For example, classical ECF is not associated with anaemia, but concomitant infections with strongyle worms or trypanosomes can result in clinical anaemia. If diagnosis are based solely on clinical signs, co-infections could be overlooked if two or more pathogens cause the same presenting signs in a host.Co-infections could lead to a missed diagnosis when, in the case of concomitant infections with pathogens that present with similar clinical signs, and the diagnosis was solely based on clinical presentation. Such a patient might not respond to treatment as expected and this should prompt the investigator to consider further diagnostic procedures.

Co-infection between pathogens also affects the prognosis of a disease state (Cox 2001). Each concomitant pathogen contributes to the clinical outcome of infection (Petney & Andrews 1998). Even if the contribution of the pathogen in itself was clinically insignificant, the cumulative effect of the various co-infecting pathogens could potentially shift the host

from a state of apparent health into a state of clinical disease. *Theileria mutans* caused a statistically significant, yet clinically insignificant decrease in PCV. Such a small decrease in PCV would not present as overt clinical symptoms, e.g. pallor of mucous membranes, but such subclinical disease processes would erode the overall health status of the calf.

Immunosupression caused by certain pathogens, such as *Trypanosoma* spp. or *T. parva* (Holmes *et al* 1974; Askonas 1984; Moll *et al*. 1986), or immune-modulation, as seen in helminth infections at times (Maizels & Yazdankakhsh 2003), could undermine the host's response to other pathogens which in turn increase the host's susceptibility to infection or impede its ability to resolve such an infection. Premune latent carrier animals have been reported to develop clinical anaplasmosis after superinfection due to *Trypanosoma vivax* and *T. congolense* (Magona & Mayende 2002).

Not all pathogens caused clinical disease in the calf population. Clinical anaplasmosis and babesiosis in cattle are classically associated with anaemia (De Vos *et al*. 2004; Potgieter & Stoltsz 2004). However, neither *A. marginale* nor *B. bigemina* appear to be significant causes of anaemia in the study population. This implies that the majority of calves infected with either of these pathogens are probably latent carriers. There is evidence that both pathogens cause subclinical disease processes, however, particularly in association with multi-pathogen infections. The significant thrombocytopenia that was found in *A. marginale*-positive calves may be an example of such a process.

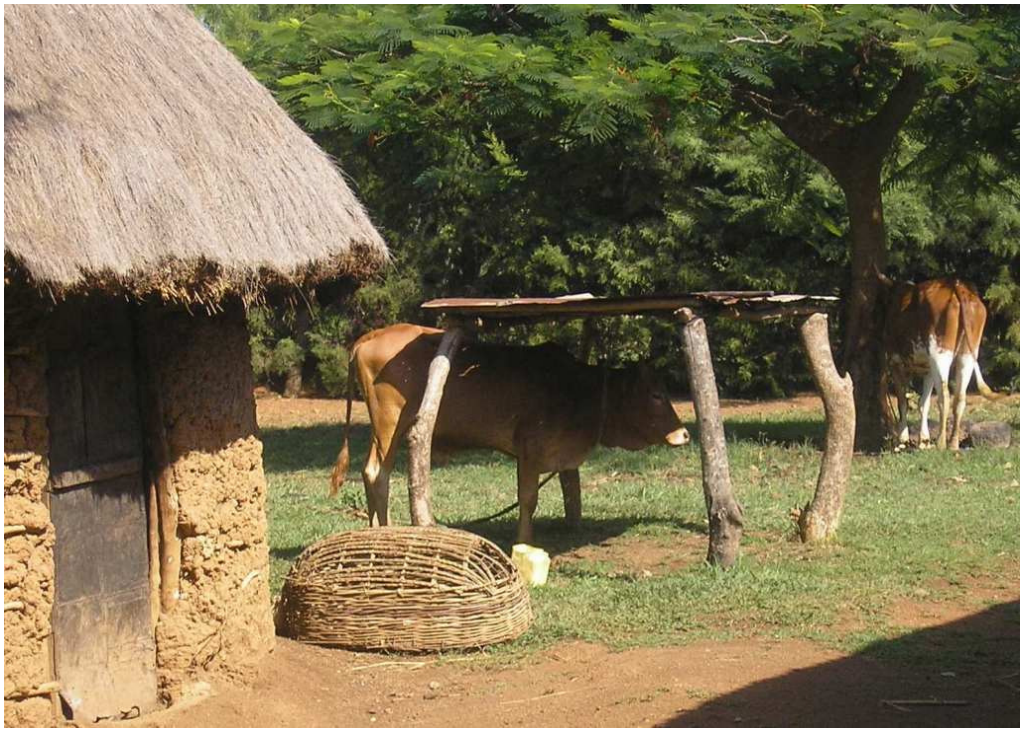
The impact many pathogens and concomitant infections had on the haematological parameters was dependent, at least partially, on the infectious load of the pathogen, e.g. strongyle-type nematodes and trypanosomes. This is known to be true for *T. parva* as well (Koch *et al*. 1990). The diagnosis of tick-borne diseases in this study was based on serology, which is a measure of exposure to the pathogen. The antibody response does not necessarily correlate to the infectious load of the pathogen. The pathogen load and possibly parasite strain of both of these parasites are likely to affect the clinical presentation of infection. Using quantitative antigen-based diagnostic tests for tick-borne parasites, in particular *T. parva* and *T. mutans*, would therefore have added some dimension to this study. Both pathogens had a high prevalence in the population and ECF is considered as a major disease in the area.

Interactions between pathogens are not limited to pairs of pathogens, however, and to get a complete understanding of disease processes one would have to consider the whole pathogenic community of the host. Many other infectious agents, such as bacteria and

viruses, circulating in the calf population were tested for (results beyond the scope of this study) and probably many more present for that were not tested for, that could have impacted on the haematological parameters of the calves. In addition there are several pathogen-related factors that dictate the pathogenicity of the infection, including infectious load, pathogen strain, and virulence types within strains. Several inherent characteristics of the host, such as breed and age, determine the susceptibility of the animal to infection, whereas other acquired attributes such as maternal antibody, premunity due to prior infection, or nutritional state could alter the animals' ability to respond to infection.

It is evident that interactions between concomitant pathogens complicates the clinical picture in infected calves and should be taken into consideration in any study that investigates disease under the field conditions.

Small-holding homestead with East African short-horn cattle



CHAPTER 8

GENERAL CONCLUSIONS

Livestock production in Western Kenya is constrained by harsh environmental conditions as well as the impact of infectious disease (Uilenberg 1995). Livestock-dependent and mixed crop-livestock small-holder farmers prefer to keep indigenous livestock breeds (Minjauw & McLeod 2003), due to their adaptability to the environmental constraints and disease tolerance. It is evident from this study that calves are exposed to significantly high infectious burdens from early calfhood. The economically most important diseases in the area include the tick-borne diseases, e.g. ECF, anaplasmosis, and babesiosis, trypanosomosis and helminthosis.

Anaemia was shown to be an important syndrome in cattle in Western Kenya. The FAMACHA© scoring test is a field test that is used to diagnose anaemia in animals (Anon. 2002b). It meets the criteria of being cheap, simple and easy to use, and with proper training, farmers should be able to perform the test themselves. Although the test is not necessarily useful in the diagnosis of specific infectious disease, it does serve as a useful early screening tool to identify animals that require intervention. There is a real need for robust pen-side diagnostic tests that are affordable and suitable for use under the challenging field conditions in rural Africa. In human medicine several point of care rapid tests have been developed to diagnose anaemia, mainly based on haemoglobin levels in patients, such as Hemocue (Hemocue AB®, Sweden) and WHO haemoglobin colour scale (Stott & Lewis 1995). The costs and availability of such tests constrain the uptake of this technology in veterinary medicine (Magona, Walubengo, Anderson, Olaho-Mukani, Jonsson & Eisler 2004), yet these tests can potentially be very valuable as pen-side tests in animal health in settings where access to diagnostic laboratories is limited.

After pen-side diagnostics, the next level in an animal health care system is local field laboratories. The Sysmex© automated analyser was also validated for use in the field laboratory in Busia, Western Kenya, and was proved to be useful in monitoring the clinical profile of calves throughout the study. Automated analyzers are easy to use and results are available within minutes, although some technical training is required for interpretation of the results and maintainance of the analyzer. Such technology is ideal for veterinarians working in remote areas, where maintenance of a cold chain for sample storage and transport to regional laboratories, mostly located in larger towns, is not practical.

Any diagnostic test or analyser first needs to be validated for the specific population on which it will be used. For this purpose, baseline values need to be available in order to accurately interpret the diagnostic test. In this study it was shown that the East African short-horn calf has a unique haematological profile. Despite the amount of research done on the infectious diseases of these indigenous cattle, for example ECF and trypanosomosis, data on the baseline physiological parameters, such as haematological reference values, on indigenous breeds in the literature is scant. Many studies used extrapolated data, from other breeds, particularly more exotic breeds, e.g. PCV cut-offs as a measure of anaemia (Dargie *et al*. 1979; Fanduma *et al*. 2007) and this practice may prove to be inappropriate. The age-related changes in red cell parameters of the Zebu calves in this study, particularly during the first 12 weeks of life, do not correlate with what is described in literature for exotic breeds. There is a need to determine breed-specific baseline reference levels for indigenous livestock breeds.

Helminths, particularly strongyle-type nematodes, and trypanosomes were the two most important infectious causes of anaemia in the population. Although almost all calves became infected with strongyles during the first year of life, clinical anaemia only developed in cases where the helminth burden was quite high.

These calves also had a high rate of seroconversion to tick-borne pathogens. Despite the high prevalence of tick-borne pathogens, relatively few infections, apart from ECF, actually resulted in clinical disease in the study population. East Coast fever was found to be a significant disease in the study area, but was not an important cause of anaemia. Neither anaplasmosis nor babesiosis, both diseases typically associated with the development of anaemia, resulted in a significant decrease in PCV. This might be due to the calves' innate age-related resistance to these two diseases or possibly breed-related tolerance to the diseases (De Vos *et al*. 2004; Potgieter & Stoltsz 2004). Indigenous cattle, such as the East African short-horn Zebu, that are raised in areas where tick-borne disease is endemic, over many generations develop a level of tolerance to endemic pathogens (Norval *et al.*1992; Perry & Young 1995; De Vos *et al.* 2004; Potgieter & Stoltsz 2004).

Although many infections never developed into overt clinical disease, there was evidence of ongoing subclinical disease processes in many infected calves. Even in infections with pathogens considered to be benign, such as *T. mutans*, calves developed mild thrombocytopenias. Over the long term, these subclinical disease processes will have an erosive effect on the overall health status of the population, as is illustrated by the progressive decline in PCV as the calf population ages.

Co-infections between pathogens were also shown the have a significant impact on the haematological profile of infected calves. In many cases the cumulative effect of or interactions between concomitant pathogens affected the severity of clinical signs, such as anaemia, and in turn affected the prognosis of such calves. Considering the high prevalence of concomitant infections, in particular pathogens that cause anaemia, any individual, be that a researcher, a clinician or a farmer, who is investigating a disease condition in livestock under field conditions, should not lose sight of the fact that the clinical presentation of the animal is likely to be complicated by several super-infecting pathogens. Diagnostic approaches should be thorough and treatment and vaccination regimens should be thoughtfully planned.

The majority of the economically important pathogens that burden cattle in Western Kenya are either preventable or treatable. Therefore, the production losses of livestock incurred through these pathogens should, at least in theory, be manageable. One constraint in disease control in the developing world is the development of drug resistance against many anthelminthic (Gray *et al*. 2012) and trypanocides (Connor & Van Den Bossche 2004), two of the most important causes of anaemia in the population in this study. Strategic treatment has been proposed in commercial farming systems to curb the development of drug resistance. In this system only animals with high parasite loads or clinical signs are treated and by doing so the high costs of pharmacological treatments are reduced.

Another option to lessen the burden of livestock diseases is farm with breeds that are more resistant to infectious agents. Herein lays the value of indigenous breeds. The East African short-horn Zebu breed is known to be adapted to the infectious burdens it faces under field conditions (Perry & Young 1995). They survive under conditions where other breeds, such as improved European cattle breeds, do not.

The last recruited IDEAL calf

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