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Immune response of *Bos indicus* cattle against the antigen Bm91 derived from local *Rhipicephalus (Boophilus) microplus* ticks and its effect on tick reproduction under natural infestation

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Meinen Eltern und
meiner Freundin

"Life is a journey, not a destination."

- Ralph Waldo Emerson

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Taxonomy

A. hebraeum	*Amblyomma hebraeum*
A. marginale	*Anaplasma marginale*
A. variegatum	*Amblyomma variegatum*
B. bovis	*Babesia bovis*
B. bigemina	*Babesia bigemina*
E. coli	*Escherichia coli*
H. anatolicum anatolicum	*Hyalomma anatolicum anatolicum*
P. pastoris	*Pichia pastoris*
R. appendiculatus	*Rhipicephalus appendiculatus*
R. (B.) annulatus	*Rhipicephalus (Boophilus) annulatus*
R. (B.) decoloratus	*Rhipicephalus (Boophilus) decoloratus*
R. (B.) microplus	*Rhipicephalus (Boophilus) microplus*
T. annulata	*Theileria annulata*
T. parva	*Theileria parva*

Units and other abbreviations

°C	degree Celsius
µg	microgram
µl	microliter
ADG	average daily gain
AIC	Akaike's information criterion
BoLA	bovine leukocyte antigens
bp	base pair
CBC	complete blood count
cDNA	complementary DNA
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DAB	diamino benzidine
dl	decilitre
DLD	Department of Livestock Development
DNA	deoxynucleic acid
dNTPS	deoxynucleotide triphosphates
EDTA	ethylene-diamine-tetraacetate
ELISA	enzyme-linked immunosorbent assay
fl	femtolitre
h	hour
h^2	heritability
Hb	haemoglobin
HCl	hydrochloric acid
IPM	integrated pest management
KCl	potassium chloride
kb	kilobase
kDa	kilo Dalton
kg	kilogram
MCHC	mean cell haemoglobin content
MCV	mean cell volume
$MgCl_2$	magnesium chloride
ml	millilitre
mm	millimetre

mM	millimolar
mRNA	messenger ribonucleic acid
nm	nanometre
OD	optical density
PCR	polymerase chain reaction
PBS	phosphate buffered saline
PBST	0.05% Tween-20 in PBS
PCV	packed cell volume
ρMol	pikomolar
ppi	post primary immunisation
PVDF	polyvinylidene difluoride
RBC	red blood cells
REI	reproductive efficiency index
RNA	ribonucleic acid
rpm	rounds per minute
RT	room temperature
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SD	standard deviation
SE	standard error
Taq	*Thermus aquaticus*
TBD	tick-borne diseases
TBE	Tris-(hydroxymethyl-)aminomethan-borate
Tris	Tris-(hydroxymethyl-)aminomethan
TTBS	50 mM Tris-HCl, 150 mM NaCl, pH 8.0, 0.05% TWEEN-20
TWEEN-20	polyoxyethylen$_{(20)}$-sorbitan-monolaurat
U	units of enzyme activity
UV	ultraviolet
V	volt
w/v	weight/volume
WBC	white blood cells

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1 SUMMARY

In the present study two immunisation trials with the anti-tick antigen Bm91 using *Bos indicus* cattle were conducted in Northern Thailand. The antigen was derived from a Thai *Rhipicephalus (Boophilus) microplus* strain. The trials were conducted at different locations with similar environmental conditions. At both locations the animals were raised in an extensive grazing system with natural exposure to ticks.

Trial 1 was conducted at RJ Ranch, a private breeding farm about 120 km south of Chiang Mai. On the one hand White Lamphun cattle as an example of indigenous Thai cattle which are highly adapted to the local environmental conditions and on the other hand Brahman cattle as an example of an exotic breed were used. Six adult females of each of this *Bos indicus* breeds were immunised with the antigen Bm91 and the other 6 animals remained as control animals. Three immunisations with a 3-week interval in-between were given. Weekly blood samples over a period of 3 months were collected to measure the anti-Bm91 antibody level by ELISA. Additionally, the health status was monitored by the complete blood count.

Trial 2 was conducted at the Department of Animal and Aquatic Science of the Chiang Mai University. Eighteen adult female White Lamphun cattle were divided into 3 treatment groups with 6 animals each. One of the groups was immunised with the same antigen formulation as in trial 1. The other groups received saline and adjuvant, respectively. The immunisation scheme included immunisations in week 0, 3, and 6. After 6 months a 4th immunisation dose was applied. The antibody level was monitored for the 3 months following the primary dose and additionally for 1 month after the 4th treatment (week 26 until week 30 ppi). The health status was monitored as in trial 1. Standard engorged female ticks (4.5 to 8 mm) were collected daily from week 6 until week 12 ppi and from week 27 until week 30. After the engorged tick weight was recorded ticks were reared in individual containers. The egg mass weight was measured to calculate the reproductive efficiency index (egg mass/tick weight). Furthermore, the egg viability was checked.

In both trials Bm91 evoked a strong and long-lasting antibody response. A significant difference between the indigenous and the exotic animals was not found in trial 1. At both locations the antibody level showed a characteristic course. After a strong response to the primary immunisation a further increase was observed after the 2 subsequent immunisations. A stable level was reached after the 3rd treatment. This level was maintained until week 12 ppi in both trials. Until the 4th immunisation in trial 2 (week 26) the anti-Bm91 ELISA values decreased insignificantly. After this booster dose a moderate increase of the antibody level was recognised. The Western blot analysis confirmed that recombinant Bm91 elicited antibodies that bind to *R. (B.) microplus* proteins. An effect of Bm91 on the parameters of the complete blood count was observed in none of the trials. The difference between Brahman and White Lamphun of several blood parameters was significant and indicated a superior health status in the indigenous animals.

Regarding the different tick parameters the number of ticks was highly variable within the groups (trial 2). A difference between the 3 treatment groups was not found. The tick weight was as well not different between the groups. A significant effect ($P < 0.05$) of Bm91 was recorded for the reproductive efficiency index and the egg viability which were reduced by 6 and 8%, respectively.

In summary, the recombinant antigen Bm91 from Thai *R. (B.) microplus* ticks induced substantial antibody responses independent of the *B. indicus* breed. However, the effect on the reproductive tick performance measured under natural tick infestation was low.

2 ZUSAMMENFASSUNG

In der vorliegenden Arbeit wurden zwei Immunisierungsversuche mit dem Zeckenantigen Bm91 an *Bos indicus* Rindern in Nordthailand durchgeführt. Das Antigen wurde aus einen thailändischen *Rhipicephalus (Boophilus) microplus* Stamm gewonnen. Die Versuche wurden an zwei unterschiedlichen Orten mit vergleichbaren Umweltbedingungen durchgeführt. An beiden Versuchsstandorten wurden die Rinder unter extensiven Weidebedingungen mit natürlicher Zeckeninfestation gehalten.

Versuch 1 wurde auf der RJ Ranch, einer privaten Rinderzuchtfarm, die sich ca. 120 km südlich von Chiang Mai befindet, durchgeführt. Auf der einen Seite wurden White Lamphun Rinder als Beispiel einer lokalen Rinderrasse benutzt, die an die lokalen Umweltbedingungen angepasst ist. Auf der anderen Seite wurden Brahman Rinder als Beispiel einer exotischen Rinderrasse verwendet. Sechs ausgewachsene weibliche Tiere von jeder dieser beiden *Bos indicus* Rassen wurden mit dem Antigen Bm91 immunisiert und weitere 6 Tiere jeder Rasse als Kontrolltiere behandelt. Es wurden 3 Immunisierungen in 3-wöchigen Abständen verabreicht. Über einen Zeitraum von 3 Monaten wurde den Rindern wöchentlich Blut abgenommen, um den anti-Bm91 Antikörperspiegel mit Hilfe der ELISA-Technik zu bestimmen. Zusätzlich wurde der Gesundheitsstatus der Tiere anhand des Blutbildes verfolgt.

Versuch 2 wurde am Department of Animal Science and Aquatic Science der Chiang Mai Universität durchgeführt. Achtzehn ausgewachsene weibliche White Lamphun Rinder wurden in 3 Versuchsgruppen aufgeteilt. Eine Gruppe wurde mit der gleichen Bm91-Antigenformulierung immunisiert wie in Versuch 1. Die beiden anderen Gruppen erhielten Injektionen mit physiologischer Kochsalzlösung bzw. Adjuvanz. Die Immunisierungen wurden in Woche 0, 3 und 6 vorgenommen. Eine zusätzliche Boosterdosis wurde nach 6 Monaten (Woche 26) verabreicht. Die anti-Bm91 Antikörperlevel wurde in den 3 Monaten nach der ersten Immunisierung bestimmt. Zudem wurde den Rindern nach der Boosterdosis in Woche 26 für einen weiteren Monat Blut abgenommen, um die Entwicklung der Antikörperspiegel verfolgen zu können. Der Gesundheitsstatus wurde ebenso wie in Versuch 1 bestimmt. Weibliche

Zecken mit einer Größe von 4,5 bis 8 mm ('standard engorged ticks') wurden von Woche 6 bis 12 und von Woche 27 bis 30 täglich gesammelt. Nach Bestimmung des Zeckengewichtes wurden die Zecken während der Eiablage in individuellen Plastikdosen aufbewahrt. Nach 3 Wochen wurde die Eimasse zur Berechnung des Reproduktions-Effizienz-Indexes (Eimasse/Zeckengewicht) bestimmt. Darüber hinaus wurde die Lebensfähigkeit der Eier ermittelt.

In beiden Versuchen löste Bm91 eine starke und langandauernde Antikörperproduktion aus. Die lokalen White Lamphun Rinder unterschieden sich dabei in Bezug auf die Antikörperantwort nicht signifikant von den exotischen Brahman Rindern (Versuch 1). An beiden Standorten wies der Verlauf der Antikörperlevel einen charakteristischen Verlauf auf. Dem starken Anstieg nach der Primärimmunisierung folgte eine weitere Erhöhung des Antikörperlevels nach den beiden folgenden Immunisierungen. Ein konstantes Level stellte sich nach der dritten Immunisierung (Woche 6) ein, welches bis Woche 12 unverändert blieb. Bis zur Boosterdosis in Woche 26 kam es nur zu einem geringen Absinken der anti-Bm91 Werte. Nach dieser Immunisierung wurde ein leichter Anstieg beobachtet. Durch ein Western Blot konnte bestätigt werden, dass die Antikörper gegen das rekombinante Bm91 Protein an *R. (B.) microplus* Proteine binden. Ein Effekt von Bm91 auf das Blutbild konnte in keinem der beiden Versuche beobachtet werden. Der signifikante Unterschied zwischen White Lamphun und Brahman Rindern in einigen Blutparametern deutete auf einen besseren Gesundheitsstatus der lokalen Rinder hin.

Bezüglich der unterschiedlichen Zeckenparameter (Versuch 2) wurde eine sehr hohe Variation in der Anzahl an Zecken innerhalb der Gruppen beobachtet. Eine Unterschied zwischen den 3 Gruppen konnte nicht festgestellt werden. Ebenso wurden keine Unterschiede im Zeckengewicht verzeichnet. Dahingegen hatte Bm91 einen signifikanten Effekt ($P < 0.05$) auf den Reproduktions-Effizienz-Index und auf die Lebensfähigkeit der Eier, die in der Bm91-Gruppe um 6 bzw. 8 % reduziert waren.

Zusammenfassend ist festzustellen, dass das rekombinante Antigen Bm91 von thailändischen *R. (B.) microplus* Zecken unabhängig von der *B. indicus* Rasse eine beachtliche Antikörperantwort hervorrief. Der Effekt auf die Zeckenreproduktion unter natürlicher Zeckeninfestation war jedoch gering.

3 INTRODUCTION

Agriculture is an integral part of Thailand's emerging economy, the 2nd largest in South East Asia. According to the 2003 Agricultural Census around 40 % of the labour force is employed in agriculture (NSO 2003). During the last years the agricultural sector contributed about 10 % to the gross domestic product of the country with 67 million inhabitants. While undergoing a substantial transformation, agriculture has been shifting towards high valued products. The proportion of livestock to the agricultural gross domestic product increased to more than 20 % (FAO 2005). In 2008 the Department of Livestock Development (DLD) amounted the cattle population in Thailand to 9.1 million head, whereof less than 500,000 head were dairy cattle (DLD 2009b). About 70 % of the cattle population are Thai indigenous cattle (DLD 2009a).

Serious problems to Thailand's livestock sector are caused by ectoparasite infestations. Like in tropical and subtropical countries generally, ticks are the most important cattle ectoparasites. The dominating species is the cattle tick *Rhipicephalus (Boophilus) microplus* which has a global importance due to its vast geographical distribution, its high reproductive capacity and its efficient transmission of tick borne pathogens. As the demand for meat and other animal products continues to increase with the growing economy, the importance of ticks and tick borne diseases (TBD) in Thailand will rise (Ahantarig et al. 2008; Chansiri 1997). One of the factors favouring tick infestations is the import of exotic cattle breeds especially of *Bos taurus* origin. These exotic breeds generally lack a natural immunity to tick infestations. Without this host resistance the cattle necessitate a more intensive use of acaricidal drugs for their survival when compared to *B. indicus* breeds (George et al. 2004). Beyond this, climate change will favour the expansion of tick-infested regions (Estrada-Peña 2001), and thus, the spread of TBD in Asia (Olwoch et al. 2007).

Currently, tick control relies largely on the use of acaricides. The high tick prevalence in most parts of Thailand requires intensive treatments with these drugs. However, a number of serious drawbacks are associated with this tick control method. These include the development of acaricide-resistant tick populations, environmental

contamination, contamination of animal products, high costs for farmers as well as expenses to develop new drugs. Therefore, new approaches to control *R. (B.) microplus* more sustainably are necessitated. Among the alternative tick control methods including host resistance to ticks, management strategies and biological control agents, anti-tick vaccines showed the most encouraging results. Two different vaccines based on the tick gut antigen Bm86 were developed in the 1990s in Australia and Cuba (Rodríguez et al. 1995a; Willadsen et al. 1995). An anti-tick vaccine targeting Thai *R. (B.) microplus* strains would constitute a major advance for Thailand's livestock sector. Nevertheless, the efficacy of these vaccines is still the reason for the limited application in practice. Beside the commercialised antigen other potential antigens have been isolated. One of these candidates is Bm91. The protein was isolated by Riding et al. (1994) and its efficacy against the cattle tick was shown when it was used combination with Bm86 (Willadsen et al. 1996). The results observed warrant the evaluation of Bm91 as a possible tool to control cattle ticks in Thailand in a more sustainable way. Therefore, Bm91 derived from a local *R. (B.) microplus* strain was tested in two immunisation trials under field conditions of natural tick infestation.

The specific objectives were

1. to evaluate the humoral immune response against the Bm91 antigen in *B. indicus* cattle,
2. to assess the efficacy of the Bm91 immunisation on the reproductive performance of *R. (B.) microplus* ticks under field conditions of natural infestation.

4 LITERATURE REVIEW

4.1 Taxonomy and life cycle of ticks

Ticks belong to the phylum Arthropoda and are related to crustaceans, insects, spiders, scorpions, and mites. As invertebrates they have an exoskeleton protecting the organs. They are further classified as members of the Arachnida class which can be distinguished from insects by the number of legs. While Arachnida have four pairs of legs, insects have three. Together with mites ticks share the sub-class Acari. The sub-order Ixodida (ticks) can be divided into the two main families Argasidae (soft ticks) and Ixodidae (hard ticks). The hard sclerotised shield, the scutum, which is found on the anterior dorsal surface of hard ticks, is absent in soft ticks. In soft ticks the mouthparts are located ventrally and in hard ticks anterior. The third family Nuttalliellidae is of minor importance and contains only one single species. Out of the 899 listed tick species by Barker & Murrell (2004) around 80 % belong to the Ixodidae and are divided into 12 genera.

After ticks crawl onto the host animal, they attach to the skin with their mouthparts. These consist of the chelicerae, the hypostome, and the palps. The chelicerae and the hypostome form a tube which penetrates the host's skin. Often cement is secreted with the tick saliva. This glues the palps to the outer epidermis and the rough chelicerae sheath and the toothed hypostome to the dermis. The chelicerae consist of moveable rods with sharp claws at the end. These cut a hole into the dermis and break the capillary blood vessels close to the surface of the skin. This forms the feeding lesion. The ticks feed on the blood and the lymph which is released into this lesion.

Ixodid ticks feed slow because the body wall has to grow before it can expand to take a very large blood meal. Larvae take typically 3 to 5 days to fully engorge with blood, nymphs 4 to 8 days, and females 5 to 20 days. After full engorgement with blood the ticks detach from the host's skin and drop to the ground. Males of most species feed enough for their reproductive organs to mature but do not expand like the females. Ticks find their hosts in several ways. Most commonly they live in open environments and crawl onto vegetation to wait for their host passing by. This behaviour is called

questing. Differentiated by the place where moulting takes place three different types of life cycle can be distinguished in ixodid ticks. Ticks moult either on the host or after detaching from the host on the ground. Each of the life cycles consists of the four stages egg, larva, nymph, and adult. Most common is the three-host life cycle, in which the larvae develop in the eggs and hatch usually within several weeks. After feeding once on the host, the larvae detach from the host and moult to nymphs on the ground. When the nymphs completed feeding on the host, they detach and moulting takes place on the ground. Females feed on a new host, detach when they are fully engorged, and lay one batch of eggs on the ground. The depleted female dies after egg laying is completed. The three-host life cycle is slow and takes from 6 months up to several years. Only the larvae and the nymphs feed on the same host, whereas the adults engorge on another host in the two-host life cycle. The one-host life cycle is less common but occurs in the most important sub-genus *Boophilus*. The larvae hatch after several weeks of development on the soil and crawl onto vegetation to quest for a host. After the larvae finished feeding, they stay on the host for moulting to nymphs, before they continue to feed on this host. Moulting to adults takes place on the same host. When the adults are partially fed, they mate, and thereafter fully engorge. The fully engorged females detach and lay a single batch of 2,000 to 20,000 eggs on the ground. This life cycle is usually rapid and takes around three weeks (Hitchcock 1955). Feedings at all stages of their live cycle are parasitic.

4.1.1 The cattle tick *Rhipicephalus (Boophilus) microplus*

Of all tick species *R. (B.) microplus* (Canestrini, 1887), also known as the cattle tick or the pan tropical blue tick, is the most important species for livestock production in the world. During the latter half of the 19th century it spread from its origin in South-East Asia throughout the tropics and subtropics including Australia, East and Southern Africa, and South and Central America (Jongejan & Uilenberg 2004; Labruna et al. 2009). Different strains with marked morphological differences resulted from evolutionary processes associated with habitat adaptation following biogeographical separation (García-García et al. 1999). Recent molecular and morphological studies revealed that the five species of *Boophilus* make the genus *Rhipicephalus* paraphylic

meaning that some species of the Rhipicephalinae are more closely related to *Boophilus* species than to other *Rhipicephalus* species (Murrell & Barker 2003). Therefore, *Boophilus* was synonymised with the Rhipicephalinae. This one-host tick completes its parasitic part of the life cycle in about three weeks and egg laying can be completed in about four weeks (Roberts 1968a).

In tropical climates *R. (B.) microplus* infestations occur throughout the whole year with peak infestations during the rainy season (Gomes et al. 1989; Lima et al. 2000; Turner & Short 1972). The cattle tick *R. (B.) microplus* is known to be highly reproductive and an efficient vector of the most important tick-borne parasites. Two characters which make this tick species a threat to livestock production in tropical and subtropical regions. As predicted with a simulation model for African tick species, climate change will favour the expansion of tick-infested regions, and thus, the spread of TBD (Olwoch et al., 2007). This is supported by observations made in different African countries where *R. (B.) microplus* is spreading fast and displaces other Rhipicephalinae species (Lynen et al. 2008; Madder et al. 2010). The higher reproductive performance together with the shorter generation interval compared to other tick species have been the most important factors favouring the efficient spread of the cattle tick.

4.1.2 Other important tick species

On the African continent *Amblyomma variegatum* and *A. hebraeum* are the most widely distributed and most important tick species for domestic livestock. The former is known as the tropical bont tick and is widespread throughout tropical Sub-Saharan Africa while the latter is known as the South African bont tick and inhabits the south-eastern part of the African continent. Another widespread species is *R. (B.) annulatus* which is present in the Mediterranean region, Southern Russia, the Near and Middle East, Western and Southern Africa, and Mexico. Confined to Africa the blue tick, *R. (B.) decoloratus*, is the most common one-host tick species in Africa. Spread in East and South Africa, *R. appendiculatus,* the brown ear tick, is the vector of East Coast Fever that is caused by an infection with *Theileria parva*, whereas *H. anatolicum anatolicum* is the main vector of the disease Tropical Theileriosis, a *T. annulata* infection in Southern Europe, and the Middle and Far East (Jongejan & Uilenberg 2004; 1994; Walker et al. 2003).

4.2 Impact of ticks on livestock

Ticks are among the most important ectoparasites of cattle in the tropics and subtropics and approximately 80 % of the world's cattle population is at risk from ticks and TBD (FAO 1984). Heavy tick infestations have adverse physiological effects on the host and result in decreased live weight gain (Jonsson 2006). Engorging ixodid ticks increase their live weight by 100 to 200 times (Kemp et al. 1982). The actual amount of blood ingested by the tick is even greater because the blood is concentrated and the remaining fluid is excreted in the saliva. Anaemia is a symptom of heavy infestations. It is characterised by a decrease of the packed cell volume (PCV) of the blood, of its haemoglobin content (Hb), and of the red blood cell count (RBC) (Riek 1957). Global losses of tick infestations and TBD have been estimated to be more than US$ 18 billion (de Castro 1997). In Brazil alone yearly losses caused by ticks and TBD have been estimated at US$ 2 billion (Grisi et al. 2002). Depending on location and farming system, costs for the chemical control of ticks have been estimated between US$ 2.50 and US$ 25.00 per animal per year (Pegram 2001). Prevalence studies undertaken in Thailand emphasised the importance of this ectoparasite for cattle, although a very limited number of cattle was observed (Changbunjong et al. 2009; Sarataphan et al.

1998). With the rising demand for meat and other animal products the importance of ticks and TBD in Thailand will further increase (Ahantarig et al. 2008; Chansiri 1997).

4.2.1 Direct effects of ticks

Direct effects of ticks are caused by the blood loss, tick burdens as well as toxicoses. Injurious tick bites can cause severe hide damage including abcessation and can be routes for secondary infections. Furthermore, crumbled ear pinnae, sloughed tits, missing tail tips, lameness, and foot rot can result from tick infestations (Holdsworth et al. 2006).

In Australia high-yielding Holstein-Friesian dairy cows with a low previous tick exposure were artificially infested weekly with an increasing number of tick larvae over a period of 12 weeks. By the end of the trial infested cows showed a 2.86 l/d reduced milk yield and a 10.6 kg reduced live weight when compared to non-infested cows (Jonsson et al. 1998).

In subsequent studies, Jonsson (2006) estimated that on average each engorging tick is responsible for the loss of more than 1 g of body weight. Differences between purebred *B. taurus* and crossbred *B. taurus* x *B. indicus* cattle were insignificant. In a previous study, Sutherst et al. (1983) amounted the loss of live weight gain to 0.6 to 1.5 g per engorging *R. (B.) microplus* tick. The magnitude of losses caused by ticks varies with the cattle genotype (Scholtz et al. 1991), the tick species (Norval et al. 1997a; Norval et al. 1997b) and the level of infestation (Sutherst et al. 1983).

4.2.2 Indirect effects of ticks

Beside these direct effects, ticks indirectly affect their hosts by transmitting a greater variety of pathogenic micro-organisms than any other arthropod vector group while taking their blood meal (Jongejan & Uilenberg 2004). Among the pathogens of economic importance for livestock are babesias, anaplasmas, theilerias, arboviruses, rickettsias, and tularaemia. These pathogens cause serious diseases responsible for high economical losses of livestock producers. *R. (B.) microplus* is the major vector of TBD in the tropics and subtropics and, among others, transmits the pathogens *Babesia bovis*,

B. bigemina, Anaplasma marginale, and *Theileria parva* and *T. annulata* (McCosker 1979).

4.2.2.1 Babesiosis

Babesiosis, commonly known as cattle or tick fever, is caused by the two protozoa *B. bigemina* and *B. bovis*. Babesiae, in general, are characterised by their asexual multiplication in erythrocytes of vertebrates, their sexual reproduction in the tick, and the production of sporozoites in the salivary gland of the tick. These two protozoa of major importance for livestock can be divided by the size of the merozoites[1] into large (*B. bigemina*) and small babesiae (*B. bovis*). The former is more widespread whereas the latter is more pathogenic (Homer et al. 2000). Clinical symptoms of *B. bovis* include fever followed by inappetence, depression, increased respiratory rate, weakness, and a reluctance to move. Due to the fact that haemoglobinuria is often present, the disease is also known as redwater (Bock et al. 2004). *B. bigemina* infections are characterised by erythrocyte destruction and haemoglobinuria is present earlier, whereas fever is less pronounced than in *B. bovis* infections (Bock et al. 2004; Riek 1964). In acute *B. bovis* and *B. bigemina* infections the percentage of infected erythrocytes in the circulating blood does not exceed 1 % (Levy & Ristic 1980). Compared to taurine breeds, zebus show milder clinical symptoms to primary infections. This is assumed to be the result of the evolutionary relationship between *B. indicus* cattle, ticks, and *Babesia* spp. (Bock et al. 1997). Live attenuated vaccines against both pathogens were developed (Callow 1977) and showed at least 90 % protection in vaccinated animals after challenge with field isolates in recent studies (Alvarez et al. 2004).

4.2.2.2 Anaplasmosis

On a global scale anaplasmosis is the most prevalent TBD of cattle caused by *A. marginale* (Ristic 1968). Its prevalence and incidence is highest in *R. (B.) microplus* endemic regions (Lincoln et al. 1987). The pathogen belongs to the order Rickettsiales

1 During the tick bite vertebrate hosts are infected by the injection of sporozoites with the tick saliva. These penetrate directly into the RBCs and the parasite produces two merozoites by binary fission of the sporozoite. After the lysis of the erythrocyte each merozoite invades a new erythrocyte (Chauvin et al. 2009).

and is an obligate intracellular bacterium found in membrane-bound vacuoles in the host cell cytoplasm. It develops persistent infections in mammalian and tick hosts which both serve as reservoirs for infection of susceptible hosts. Bovine erythrocytes are the only known site of replication and during acute infections up to 70 % of the erythrocytes may become infected (Richey 1981). When infected erythrocytes are taken up with the blood meal of the tick, tick gut cells become infected. Later the infection spreads to several other tick tissues including the salivary glands (Kocan 1992). The acute phase of the disease is characterised by weight loss, fever, abortion, and lowered milk production. Cattle often die after acute infections. As proposed by Kocan et al. (2004) immunising cattle with a combination of anti-tick antigens and *A. marginale*-derived proteins may provide an effective means of controlling infection and transmission. Vaccine formulations against a broad spectrum of strains has been developed, but provided only partial protection (Kocan et al. 2001).

4.2.2.3 Theileriosis

Protozoa of the genus *Theileria* are causative agents of a variety of disease symptoms in domestic and wild ruminants which are collectively responsible for economic losses amounting to hundreds of millions of dollars annually in Sub-Saharan Africa and Asia (Bishop et al. 2004). Globally the two most important species causing theileriosis in cattle are *T. parva* and *T. annulata*. Neither of the two protozoa has been found in Thailand (Ahantarig et al. 2008; Sarataphan et al. 1998). Nevertheless, benign *Theileria* which belongs to the *T. buffeli/orientalis/sergenti* group and causes mild disease, is highly prevalent in Thailand (Sarataphan et al. 1998).

4.2.2.4 Endemic stability of tick-borne diseases

Cattle breeds that are indigenous to regions where TBD are endemic often have a certain degree of natural resistance to these diseases and the consequences of infection are not as serious as when exotic, especially *B. taurus*, breeds are infected. This endemic stability is defined as 'a stable endemic situation with high prevalence of infection but no or little clinical disease in the target population caused by a high transmission rate of the parasites between vector tick and the vertebrate host' (Tatchell

1992). Passively acquired resistance from the colostrum lasts about 2 months when it is followed by an innate immunity from 3 to 9 months of age (Bock et al. 2004; Riek 1968). For *B. bovis* an inverse relationship between age and resistance with young animals being more resistant was found (Trueman & Blight 1978). Under conditions of endemic instability some animals will fail to become infected for a considerable period after birth and may therefore develop severe, life threatening symptoms when exposed to the protozoa later in life (Callow 1984). The import of exotic cattle, especially of taurine origin, from regions where ticks and TBD are non-endemic into tropical countries, as it is practised in Thailand, is accompanied by significant losses (Callow 1977). Prevalence studies have shown that up to 96.7 % of the cattle in Thailand are seropositive for *Babesia* spp. infections (Nishikawa et al. 1990). In a recent prevalence study by Iseki et al. (2010) the infection rate of cattle in the Northern regions of Thailand was around 70 % for both *B. bovis* and *B. bigemina* with more than half of the cattle being infected with both pathogens. Comparable values exist for Anaplasmataceae. In a survey of 7 provinces throughout Thailand 74 % of the calves were seropositive for *A. marginale* (Phrikanahok et al. 2000). Half of the beef cattle in Nan province, Northern Thailand, were found to be infected with benign *Theileria*, but no clinical signs of disease were observed (Kaewthamasorn & Wongsamee 2006). The specific tick species transmitting *Theileria* parasites in Thailand has not been identified yet (Ahantarig et al. 2008).

4.3 Immune response to tick infestation

The tick-host-pathogen interface is characterised by complex immunological interactions. Tick feeding stimulates the immune system of the vertebrate host and innate as well as specific acquired immune defences are involved in the responses of the host to the infestation. There is a dynamic balance between host responses and tick countermeasures against these mechanisms which is affecting the tick engorgement and the transmission of pathogens (Wikel 1996). After early studies focused mainly on the immunity to tick infestation directing at the development of anti-tick vaccines, later studies paid attention to the tick-host interaction with a particular view on how ticks can

modulate host immune mechanisms and facilitate the transmission of TBD (Wikel & Bergman 1997).

In order to obtain a blood meal successfully haematophagous arthropods generally must overcome host blood coagulation, platelet aggregation, and pain/itch responses. Therefore, the saliva contains a complex mixture of proteins with biological activity (Brossard & Wikel 2004). To counteract the host immune mechanisms, ticks have evolved numerous mechanisms (Ribeiro 1989; Ribeiro 1995). These complex interactions can be viewed as a balance between the different host defence mechanisms raised against the parasite and tick evasion strategies facilitating feeding and the transmission of pathogens. First of all, a local inflammatory response develops after the tick mouthparts penetrate the host skin. In this reaction host neutrophils as one of the first responders of the innate immune system participate (Brossard & Wikel 2004). The movement and activity of the neutrophils is controlled by the chemokine interleukin 8. Shown in *in vitro* experiments the salivary gland extract of several tick species inhibits the binding of interleukin 8 to its receptors (Hajnická et al. 2001). With this mechanism tick saliva is able to control the infiltration and activation of neutrophils at the attachment site. One of the tick salivary gland proteins that interacts with elements of the host immune system is calreticulin (Ferreira et al. 2002). This protein is expressed in all tissues and all developmental stages of ticks. It is a conserved calcium-binding protein with a diversity of biological functions. However, the specific roles of this molecule in tick-host interactions remain to be determined.

The complex of the host response to tick infestations was studied by Roberts (1968a). Eight days after a moderate *R. (B.) microplus* infestation resistance was acquired in cattle with no previous tick exposure. Between different animals a varying degree of resistance was observed. Contrary to non-exposed animals, those with previous tick exposure manifested their levels of resistance immediately after infestation. The author concluded that the degree of resistance exhibited by the cattle is the result of an immune response of the host. By transferring plasma from highly and lowly tick-resistant cattle and from tick-naive cattle to tick-unexposed calves, the involvement of a humoral component in tick resistance was demonstrated (Roberts & Kerr 1976). Plasma from

highly resistant cattle conferred some degree of resistance to unexposed calves, whereas the plasma of lowly resistant cattle had no significant effect.

Further studies on the immune response to tick infestation specified the complex array of host immune responses induced by tick feeding (Wikel 1996; 1982; Wikel, Ramachandra, & Bergman 1994). Acquired resistance of cattle after repeated tick infestations resulted in a diminished engorgement weight, increased duration of feeding, decreased numbers of ova, reduced viability of ova, blocked moulting, and death of engorging ticks (Wikel 1996). In the immune response antigen presenting cells, antibodies, T and B cells[2], cytokines, complement[3], basophils, eosinophils, as well as mast cells, and a number of bioactive molecules are involved. First of all, immune responses in the skin of tick-infested cattle evoked a stronger hypersensitivity reaction with increasing tick resistance in *B. taurus* cattle (Schleger et al. 1981) involving the infiltration of the two leukocyte fractions basophils (Askenase et al. 1978) and eosinophils (Schleger et al. 1976) at the tick attachment site. Further studies of the hypersensitivity reaction associated the bioactive molecule histamin with the expression of acquired resistance to tick feeding (Wikel 1982). It was suggested that histamine-binding proteins will be found in the majority of tick species (Brossard & Wikel 2004). In *R. (B.) microplus* infested cattle the release of histamin has been found to cause skin irritations and to result in increased host grooming (Koudstaal et al. 1978). Regarding the complement system, higher serum complement levels are associated with a higher host resistance (Wambura et al. 1998).

Wikel (1996) reported that tick feeding induces the production of immunoglobulin G antibodies against tick saliva antigens. Comparing antibody levels of tick-susceptible (Holstein-Friesian) and tick-resistant (Nelore) breeds after heavy tick infestations Kashino et al. (2005) found that tick infestations suppressed the immunoglobulin G antibody response in susceptible breeds.

2 T cells and B cells belong to the lymphocytes and have immunoregulatory and effector functions in the immunity to ticks (Wikel & Bergman 1997).

3 The complement system is a biochemical cascade supporting the antibodies to clear pathogens from the host.

4.4 Tick control methods

Since the development of cattle production in many tropical and subtropical countries in the middle of the 19th century, intense attention has been paid to the control of ticks and various control measures have been identified (Graf et al. 2004).

4.4.1 Anti-tick vaccines

Vaccination against ticks is a valuable tool for an effective tick control. As being cost-effective, reducing the environmental contamination, and the contamination of the animal products as well as preventing the selection of drug-resistant ticks, vaccination has several advantages over the use of chemicals (Willadsen 1997). Additionally, the inclusion of multiple antigens might target a broad range of tick species and may also prevent the transmission of TBD (Bastos et al. 2010; de la Fuente et al. 2007).

The development of a vaccine against the cattle tick *R. (B.) microplus* began with experiments of Agbede & Kemp (1986), Johnston et al. (1986), and Kemp et al. (1986). In these initial studies cattle were immunised with filtered homogenate of semi-engorged adult female ticks. High levels of protection against the cattle tick lasting about 3 months were observed. The protection was, first of all, expressed in a reduced number of engorging ticks. Beyond that, ticks suffered from leakage of gut contents into the haemocoel[4] following the ingestion of blood from immunised animals (Agbede & Kemp 1986; Kemp et al. 1986). *In vitro* studies confirmed the specific damage of the tick gut leading to the rupture of the gut wall (Kemp et al. 1986).

4.4.1.1 The anti-tick antigen Bm86

Following these initial experiments, the isolation of a single protective antigen from the extremely complex crude extracts by a series of fractionation, vaccination, and parasitic challenge experiments was the breakthrough for the development of an anti-tick vaccine. The identified gut-associated antigen was named Bm86 for the target tick species, formerly known as *B. microplus*, and the year of the first identification, 1986. It is an 89 kDa membrane-bound glycoprotein localised on the microvilli of the midgut digest cells (Gough & Kemp 1993). The functions of Bm86 have not been completely

4 Body cavity of ticks.

elucidated, but it has been shown to contain epidermal growth factor-like domains. These may be involved in blood coagulation and cell growth (Rand et al. 1989). After its isolation, it was cloned and expressed in *Escherichia coli* in Australia (Rand et al. 1989) and similarly in the yeast expression system *Pichia pastoris* in Cuba (Rodríguez et al. 1994).

Bm86 is a so-called 'concealed' antigen (Willadsen & Kemp 1988). Contrary to conventional or 'exposed' antigens which are secreted in the tick saliva and are transmitted to the host during tick attachment, 'concealed' antigens are hidden from the host. Due to this fact, the latter antigens do not induce an immune response during tick feeding and are only immunogenic when inoculated as tick tissue extracts. After immunisation these antigens induce the production of specific immunoglobulins which are then taken up by the tick with the blood meal. Using 'concealed' antigens has the drawback that antibody titres are not boosted by continuous tick infestation, but require continual booster injections. If directed against certain gut-derived antigens, the evoked antibodies interact with the antigen on the surface of the tick gut. This leads to the destruction of the digest cells, disruption of the gut wall, leakage of bovine blood into the haemocoel, and finally may cause the death of the tick (Rand et al. 1989).

The efficacy of immunising cattle against *R. (B.) microplus* can be evaluated as described by Willadsen et al. (1989). Cattle with no previous exposure to ticks were infested with 1,000 *R. (B.) microplus* larvae per day. Approximately 21 days later engorged adult female ticks were collected, counted, weighed, and their ability to lay eggs was estimated. Applying this artificial infestation, an effect of the vaccine was observed on the number of engorging ticks, their mean weight, and their ability to lay eggs. Additionally, the viability of the larvae after hatching was diminished. The most pronounced effect was seen on the weight and egg laying capacity of the engorged female ticks. According to Fragoso et al. (1998) the vaccine efficacy is calculated as the overall effect on the number of adult female ticks, the egg laying capacity, and on the egg viability. Hereby, stronger effects are seen in adult ticks. Due to the fact that Bm86 occurs in approximately the same amount in larvae as in adults, this is principally attributed to the volume of ingested blood, and hence the amount of antibodies (Willadsen et al. 1995).

The first results obtained for Bm86 in controlled pen trials by Tellam et al. (1992) showed a 20 to 30 % reduction in the number of engorging ticks, a 30 % reduction in the tick weight and a 60 to 80 % reduction in the egg weight laid per engorged female tick. Measured through its effect on the reproductive capacity of a single generation of ticks an overall vaccine efficacy of 90 % was calculated. Similar results were obtained in Cuba by immunising Holstein-Friesian cattle from tick-free areas (Rodríguez et al. 1994) and artificially infesting them with 1,000 *R. (B.) microplus* larvae on three consecutive days. The average weight of ticks collected from vaccinated animals and their reproductive capacity were significantly reduced by 50 and 70 %, respectively, when compared to control cattle.

Developed by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in collaboration with Biotech Australia Pty Limited (Bendigo, Australia), the Bm86 vaccine was released by Hoechst Animal Health in Australia during 1994 under the name TickGARD™ (Willadsen et al. 1995). Concordantly, it was marketed by Heber Biotec (Havana, Cuba) as Gavac™ in Cuba (Rodríguez et al. 1995a). The antigen of TickGARD™ is derived from the Australian Yeerongpilly strain of *R. (B.) microplus* and expressed in *E. coli*. For the Cuban vaccine formulation Bm86 is derived from the Cuban Camcord strain of *R. (B.) microplus* and *P. pastoris* is used as the expression system.

The first trial with Bm86 under field conditions was carried out in Australia using 10 to 12-months old Hereford steers with an extensive history of tick exposure (Willadsen et al. 1995). The previous tick exposure led to a naturally acquired immunity (Roberts 1968b). After an artificial infestation of 2 x 10,000 *R. (B.) microplus* larvae and counting the engorged adult females, the animals were allocated to a control and 3 groups with different immunisation schemes. Under natural tick infestation a significantly lower number of ticks was collected from immunised steers. Apart from the reduced tick infestation, the increased weight gain in immunised animals was an additional positive effect. Later, Jonsson et al. (2000a) immunised Holstein-Friesian lactating cows with the Australian vaccine formulation and artificially infested them repeatedly with 2,500 *R. (B.) microplus* larvae. Vaccination resulted in a 56 % reduction

of tick numbers and a 72 % reduction of tick reproductive performance accompanied by an 18.6 kg greater live weight gain over 6 months.

Similar experiments were undertaken in Mexico and South America using the Cuban vaccine formulation Gavac™. Early studies in Cuba, in which Holstein-Friesian crossbreds were immunised and artificially infested with approximately 100 *R. (B.) microplus* larvae, led to reductions of the tick number, the tick weight, and the egg laying capacity (Rodríguez et al. 1995b). Through means of the immunisation the number of tick generations decreased from 2 to 1.5 during the 33-week period. In Brazil, Gavac™ was tested on a different *R. (B.) microplus* strain under natural infestation. The infestation rate was reduced to the same degree than in the aforementioned study (Rodríguez et al. 1995a). The first study of the commercial field use of Gavac™ in Cuba was a retrospective analysis evaluating the cost-effectiveness of vaccination including data of more than 260,000 animals (de la Fuente et al. 1998). The analysis showed a 60 % reduction of the number of required acaricide treatments. Subsequently, Valle et al. (2004) conducted a similar analysis and assessed the field use of Gavac™ in Cuba including almost 600,000 vaccinated dairy cattle. Over a period of 8 years (1995 to 2003) the number of acaricide treatments for tick control was reduced by 87 %. An even greater vaccine efficacy of controlling nearly 100 % of *R. (B.) microplus* ticks under field conditions was reported from Mexico (Redondo et al. 1999).

Beside the cattle tick, Bm86 has been tested on a variety of other tick species. Fragoso et al. (1998) and Pipano et al. (2003) demonstrated that Bm86 is highly effective against *R. annulatus.* High efficacies were also found against the tick species *R. (B.) decoloratu*s, *H. anatolicum anatolicum,* and *H. dromedarii,* whereas an effect on *R. appendiculatus* and *A. variegatum* was not found (de Vos et al. 2001). The cross-reactivity is mainly explained by the partial conservation of tick antigens across tick species (de la Fuente et al. 2000).

After its release in Australia in 1994 sales of TickGARD™ grew rapidly and became the highest value tick treatment in the country. With an altered adjuvant formulation it was re-registered in Australia in 1996 as TickGARD$^{PLUS\ TM}$ by Intervet Australia Pty Limited (Bendigo, Australia). When a number of commercial factors arose the vaccine disappeared from the market. Several years later, TickGARD$^{PLUS\ TM}$ was re-introduced

by Intervet Australia Pty Limited, but targeted at the small Northern Australian dairy industry rather than the larger beef industry. At present the vaccine is only available in Australia through a producer organisation (de la Fuente et al. 2007).

4.4.1.2 The anti-tick antigen Bm91

The first Bm86 immunisation trials indicated that higher protection against tick infestations is achieved when other components in combination with Bm86 were used to vaccinate cattle (Willadsen et al. 1989). The search for other tick-protective antigens led to the isolation of a Bm86-similar glycoprotein in Australia (Riding et al. 1994). Analogous to Bm86, this low-abundance glycoprotein was named Bm91. Similar to the previously isolated protein, it exhibits a molecular weight of approximately 86 kDa. Together with several biochemical and enzymatic properties it shares 42 % of its amino acid sequence with the mammalian angiotensin-converting enzyme (Jarmey 1995). However, its natural substrate has not been identified. The angiotensin-converting enzyme is a carboxy-dipeptidase and plays a central role in mammals in the control of blood pressure, in fluid and electrolyte homoeostasis, and possibly in reproduction and immunity (Ehlers & Riordan 1989). Jarmey (1995) explained parallels between possible functions of the angiotensin-converting enzyme and Bm91 by the fact that the tick salivary gland is not only responsible for the secretion of substances needed for the establishment of the tick on the host, but also for the maintenance of the salt and water balance and the secretion of pharmacologically active compounds into the host (Binnington & Kemp 1980). The distinction of the two different tick-protective antigens was described by Riding et al. (1994). The removal of Bm86 from Bm91 preparations by polyclonal anti-Bm86 antibodies indicated the lack of a detectable immunologic cross-reaction between them. Furthermore, cattle vaccinated with Bm91 did not produce antibodies to Bm86. In addition, antibodies to native Bm91 did not react with tick-derived Bm86 on Western blots. Comparisons of the amino acid sequences did not show significant similarities. The presence of the soluble protein could not be demonstrated. This supports the fact that Bm91 is a 'concealed' antigen. Beyond this, cattle exposed to ticks over an extended period of time did not develop antibodies to the protein in ELISA and Western blot analysis. At the same time, vaccinations with low amounts of the

protein induced antibody production. While high concentrations of Bm86 are present on the tick gut cells, immunoflourescence staining showed Bm91 to be present in relatively high concentrations in the salivary gland and in lower concentrations in the midgut of *R. (B.) microplus*.

Willadsen et al. (1996) evaluated the vaccination with Bm86 alone or in combination with Bm91. After immunising Hereford steers two times with 200 µg of Bm86 or with 100 µg of each Bm86 and Bm91, the cattle were challenged with *R. (B.) microplus* larvae for a period of 3 weeks. In conclusion, the presence of antibodies to Bm91 significantly contributed to the effect on the weight of eggs per tick. Additionally, the reproductive capacity of the ticks was lower in animals inoculated with both antigens. Despite these encouraging results and the interesting similarity to the human angiotensin-converting enzyme, Bm91 was neither evaluated as a stand alone antigen or in combination with other antigens.

4.4.1.3 Other potential vaccine antigens

In order to target other tick species than *R. (B.) microplus*, homologues of Bm86 were developed for *R. (B.) annulatus* and *R. (B.) decoloratus* (Canales et al. 2008). Against the former tick species the homologue reached a vaccine efficacy of more than 80 % (Canales et al. 2009). A lower value of 60 % was obtained for the antigen Haa86, the Bm86 homologue against *H. anatolicum anatolicum* (Azhahianambi et al. 2009). In a subsequent study, a vaccine efficacy as high as 80 % was observed (Jeyabal et al. 2010). Apart from the two aforementioned antigens Bm86 and Bm91, further potential antigens inducing protection against tick infestations have been isolated. As valid for tick control in general, most attention has been paid on the species *R. (B.) microplus*. García-García et al. (2000) isolated the antigen Bm95. Results of vaccination trials suggested that this tick gut antigen could be a more universal antigen than Bm86. In these studies it protected cattle against infestations with the cattle tick from different geographical regions. From India a Bm95-efficacy of more than 80 % was reported (Kumar et al. 2009). In Thailand the antigen Bm95 was derived from a local *R. (B.) microplus* strain (Jittapalapong et al. 2008) and induced a promising antibody response in Holstein-Friesian steers (Jittapalapong et al. 2010). A further molecule expressed

from indigenous Thai *R. (B.) microplus* is calreticulin (Kaewhom et al. 2008). Like Bm91, this protein is found in the salivary glands and in the saliva. Through anti-thrombotic and complement-inhibition activities, it might facilitate tick feeding and pathogen transmission. This warrants its evaluation as an anti-tick vaccine candidate. Though the expression was described by the aforementioned authors, immunisation trials assessing the efficacy of this calcium-binding protein are not found in the literature.

One synthetic peptide (Sbm7462) derived from the Bm86 glycoprotein has shown its efficacy against *R. (B.) microplus* (Patarroyo et al. 2002). The advantages of synthetic peptides are their high purity, their complete chemical characterisation, their suitability for large-scale production and their high storage stability. Likewise immunised as synthetic peptides, trypsin inhibitors effectively interfered tick feeding on treated cattle (Andreotti et al. 2002).

The range of antigens has been expanded to other tick species. Recently subolesin, a highly conserved protein involved in the modulation of tick feeding and reproduction, has shown its suitability as tick-protective antigen against the tick species *Ixodes scapularis* (Almazán et al. 2003; Almazán et al. 2010). Another interesting antigen is 64P which is an 'exposed' antigen originally isolated from *R. appendiculatus* (Trimnell 2002). In addition to the protection against the tick species it was isolated from, 64P effectively controlled *Ixodes ricinus* in guinea pigs and rabbits (Trimnel et al. 2005).

Due to the fact that very few of the identified antigens achieved a degree of efficacy suggesting them as candidates for single-antigen vaccines, their use in multi-antigen or 'cocktail' vaccines was proposed by a number of researchers (Ghosh et al. 2006; Willadsen 2008). Though in most studies Bm86 was used as a stand alone antigen.

4.4.2 Acaricides

Despite the intensive search for anti-tick vaccines, the main achievements in tick control were made through the development of synthetic chemicals, namely acaricides. Until the middle of the 20th century means for tick control were limited and the major products used were arsenic derivates, characterised by their low efficacy, residual effects, and high toxicity for cattle (Graf et al. 2004). The development of resistant tick

populations demanded novel acaricides with new modes of action (Wharton & Roulston 1970). After organochlorines, such as DDT[5], were used in the mid-1940s and early-1950s for controlling ticks, organophosphates, such as diazinon and coumaphos, were chosen from the early 1960s onward, before carbamates and amidines, e.g. amitraz, were available. Synthetic pyrethroids (flumethrin, cypermethrin, and deltamethrin) appeared on the market, when amidine resistance developed in the late-1970s. The newest products are macrocyclic lactones[6], which appeared on the market during the last 2 to 3 decades and focused mainly on the control of *R. (B.) microplus.* Ivermectin, abamectin, doramectin, eprinomectin, milbemycins, phenylpyrazoles, insect growth regulators and naturalytes belong to this group (Graf et al. 2004; Taylor 2001). Due to the fact that ivermectin proved to be effective against endo- and ectoparasites, it was labelled 'endectocide'. Currently, it is widely used against a wide range of nematodes and arthropods (Campbell et al. 1983; Davey & George 2002; Omura 2008; Taylor 2001).

The application of chemicals to control ticks is associated with a number of serious drawbacks. With the first application of acaricides the development of resistant ticks began. Hereby, the history of resistant ticks parallels the introduction of new acaricidal products with a delay of relatively few years (Willadsen 1997b). To all main classes of acaricides - arsenic derivates, organochlorines, organophosphates, carbamates, amidines, and synthetic pyrethroids - ticks developed resistances (Baxter et al. 1999; Davey et al. 2006; Roulston et al. 1981). Even to ivermectin, the relatively new macrocyclic lactone, resistant *R. (B.) microplus* populations were reported from Brazil and Mexico (Klafke 2006; Martins & Furlong 2001; Perez-Cogollo et al. 2010). Although the development of new synthetic products has kept pace with the constantly evolving tick resistance to these drugs up to now, new approaches are necessary to overcome problems which arise when acaricides are the only tick control method. Apart from high costs of novel drug developments, other important restraints of these drugs are chemical

5 Dichlorodiphenyltrichloroethane.

6 Macrocyclic lactones are chemical derivates of soil micro-organisms belonging to the genus *Streptomyces*. They have a potent and broad antiparasitic spectrum at low dose levels (Burg et al. 1979).

residues in milk, meat, hides, and skins, environmental contamination, and high costs for farmers (Graf et al. 2004).

4.4.3 Host resistance to ticks and tick-borne diseases

Beside anti-tick vaccines, naturally acquired host resistance is another tick control method based on host immunity. It has been proposed as a viable cattle tick control method due to the potential reduction in expenditures on acaricides and husbandry practices which are associated with chemical control (Frisch 1999). This host immunity to ticks leads to a reduced number of ticks feeding to engorgement, a reduced egg production, a reduced egg viability (Wikel 1996), and additionally, to a reduced susceptibility to TBD (Bock et al. 1999ab). As defined by Mattioli et al. (2000) host resistance, in general, is the 'ability of certain genotypes of domestic ruminants to limit parasite burdens and their pathological consequences'. Host resistance to ticks is the ability of a host to induce an immune response to components of the tick saliva. Genetic host resistance is life-long and heritable and varies both between breeds and between individuals (Seifert 1971). The latter within-breed variation in genetic resistance to ticks can be used to breed for resistance (de Castro 1997). Selection for host resistance is a low cost and permanent solution of tick control with the majority of the resistance found in different breeds as the result of natural and not deliberate selection (Frisch 1999). It has been postulated that the immune mechanisms modulating host resistance to ticks developed through continuous and prolonged contact between host and parasite (Seifert 1971). Utech et al. (1978) developed a method to quantify the tick resistance by artificially infesting cattle with approximately 20,000 *R. (B.) microplus* larvae, equalling 1 g of eggs. Resistance was calculated as the mean percentage of larval ticks that failed to mature as engorged females, assuming a sex ratio among the applied larvae of 1:1. According to this approach, cattle with more than 98 % resistance are considered as highly resistant, with 95 to 98 % as moderate, with 90 to 95 % as low and cattle with less than 90 % resistance are considered as very low resistant.

4.4.3.1 Selection between breeds

Innate and specific acquired immune responses elicited by tick feeding depend on both the species of tick and host. Variations in host genetic composition are well recognised as factors determining immune responses to tick infestation and the selection of resistant cattle has resulted in the development of tick-resistant cattle breeds in Australia, Brazil, and in some regions of Africa (de Castro, J, & Newson 1993). It is well-known that breeds evolving in the presence of continuous tick challenge possess a high average resistance to these tick species (Frisch 1999). Scholtz et al. (1991) found that the breed of cattle has a major effect on the level of tick infestation. The Asian and African *B. indicus* breeds, African sanga breeds, West African *B. taurus* breeds, and the South American criollo breeds (*B. taurus*) have been described as a rich source from which specific breeds with a high average resistance to a particular tick species can be identified (Frisch 1999). The most pronounced difference in host resistance to ticks is between *B. indicus* and *B. taurus* cattle with the former being more resistant than the taurine breeds (Seifert 1971). The superior tick resistance of zebu cattle was confirmed by numerous studies, in which the proportion of *B. indicus* genes was proportionally related to the *R. (B.) microplus* resistance. Furthermore, these breeds acquired resistance more effectively than *B. taurus* breeds (Brizuela et al. 1996; Mwangi et al. 1998; Utech et al. 1978). A number of studies from Australia, South America, and Africa reported that Brahman, Nelore, and other indigenous *B. indicus* breeds are highly resistant to *R. (B.) microplus* (Frisch & O'Neill 1998; Gomes et al. 1989; Utech et al. 1978), *R. (B.) decoloratus* (Mwangi et al. 1998; Rechav & Kostrzewski 1991), *R. appendiculatus* (Mwangi et al. 1998; Rechav 1987), and *Amblyomma hebraeum* (Mwangi et al. 1998; Rechav 1987; Rechav et al. 1991). Indian zebu breeds may have high resistance to all *R. (Boophilus)* spp. (Frisch 1999). The Nelore breed in Brazil has such a high *R. (B.) microplus* resistance (> 99 %) that acaricide treatments are only necessary under distinct conditions (Gomes et al. 1989).

In the same manner as mentioned for the resistance of Brahman cattle against *R. (B.) microplus* in Australia (Utech et al. 1978), Rechav (1987), Rechav & Kostrzewski (1991), and Rechav et al. (1991) ascribed the superior tick resistance of the indigenous breeds in Africa to the co-evolution with the endemic tick species. Field infestations of

Brahman and the indigenous breeds Nguni[7], Bonsmara, and Africaner with the tick species *R. (B.) decoloratus* and *A. hebraeum* were compared. Accordingly, Wambura et al. (1998) reported that cattle indigenous to Africa are more resistant to tick infestations than their crosses.

In addition, it was demonstrated that the tick infestation significantly varied within the *B. indicus* breeds revealing that a genetic difference in natural or acquired resistance to ticks among seemingly homogeneous cattle within breeds exists. Imported Brahman cattle, resistant to *R. (B.) microplus*, were more susceptible to local *R. (B.) decoloratus* infestations than indigenous Nguni cattle (Rechav & Kostrzewski 1991). In South Africa the indigenous Nguni cattle has been recognised as a breed highly adapted to the local environment with a high resistance to ticks (Mattioli et al. 1993; Muchenje et al. 2008). Another example of the co-evolution of an indigenous cattle breed and a local tick species is the resistance of the African *B. taurus* breed N'Dama to the tick species *A. variegatum* (Mattioli et al. 1993).

The generally low tick resistance level of taurine breeds was confirmed by Jonsson et al. (2000b) applying the criteria of Utech et al. (1978). The majority of Holstein-Friesian cows was grouped as low or very low resistant. Cross-resistance among tick species seem to be low or even absent among different genera of ticks, but moderate among tick species within genera (Mattioli et al. 2000).

4.4.3.2 Selection within breeds

As mentioned above, breeds with high average tick resistance occur within several major tropical breed groups, but none of these breeds is totally resistant to ticks (Frisch 1999). Under favourable conditions for ticks the body weight gain of all tick-resistant breeds is reduced. The reduction is directly proportional to the number of engorging ticks across a wide range of cattle genotypes and tick species (Frisch & O'Neill 1998; Scholtz et al. 1991). Early studies of Wharton et al. (1970) and Hewetson (1972) showed that the tick resistance varies among animals within breeds. The heritability (h^2) of this trait ranged between 0.40 and 0.60. Other studies estimated the h^2 between 0.39

7 Nguni cattle are an ecotype of the sanga cattle which are the indigenous cattle of South Africa (Rechav & Kostrzewski 1991).

and 0.49 for taurine breeds (Wharton et al. 1970) and as high as 0.82 in crossbreds of zebu breeds (Africander and Brahman) (Seifert 1971). Mackinnon et al. (1991) rated the h^2 of *B. indicus* crosses for the number of *R. (B.) microplus* at 0.34. Reviewed by Davis (1993) the average h^2 rate of numerous studies for resistance to the cattle tick was 0.34 as well. Recently, Machado et al. (2010) found a h^2 of 0.21 in a F2 population of Gyr and Holstein crossbreds. In summary, all of these h^2 estimates are similar to values found for growth traits or milk yield in temperate environments (Mackinnon et al. 1991).

Resistance of cattle to *R. (B.) microplus* depends on various factors (Utech et al. 1978). Pregnancy and lactation reduced the resistance and a seasonal variation with lower resistance in winter was found. Young cows were more resistant than older cows and males were less resistant than females. Utech & Wharton (1982) described the theoretical potential of selecting for tick resistance in an Australian Illawara Shorthorn herd (*B. taurus*). By selection over 17 years the resistance increased by 10 % to 99 % of resistance. As claimed by a number of authors, culling the 10 % least resistant animals will result in a disproportionate reduction of tick populations between 18 and 22 % due to the skewed distribution of ticks on animals in a herd with only a few animals showing heavy tick infestations (Jonsson et al. 2000b; Utech et al. 1978).

Despite the successful selection against the tick species of greatest importance in Australia, namely *R. (B.) microplus*, results of a survey showed that a recommended control program was well regarded by farmers, but the adoption rate was very low (Jonsson & Matschoss 1998). Only 5 % of the dairy farmers in Queensland, Australia, used tick resistance as a criterion for culling decisions. This was mainly attributed to the difficulty of identifying highly resistant animals. Infestation levels that facilitate ranking of cattle for resistance are generally high with at least 100 ticks per animal (Wharton et al. 1970). However, the aforementioned survey showed that only 15 % of the dairy farmers would ever see more than 100 ticks on their cows at any time and 50 % never saw more than 10 ticks on their animals. Another limiting factor of the selection for tick resistance is the declining reliability of tick counts with age and lactation, meaning that the decision for selection should be made in non-lactating heifers (Stear et al. 1990). As the rate of genetic improvement in any single trait is reduced by the selection for

additional traits, unless those are positively correlated, and because the improvement in growth rate or milk yield is the foremost goal, the selection for tick resistance is rarely practised (Frisch 1999; Jonsson et al. 2000b). Nevertheless, a genetic correlation of zero between tick resistance and growth was reported by Mackinnon et al. (1991). In agreement, Jonsson et al. (2000b) described the potential of selecting Holstein-Friesian dairy cattle for improved tick resistance without necessarily selecting against high levels of milk production. Host resistance was neither negatively correlated to early pregnancy nor to milk yield.

4.4.3.3 Genetic markers for resistance

As noted previously, the difficulty of identifying highly or lowly resistant animals is a major limitation for genetic selection within breeds. The search for markers responsible for tick resistance is a promising tool to identify tick-resistant animals. Breeds that have experienced a long period of evolution in the presence of a particular tick species are likely to have accumulated a large number of genes each with minor effects on resistance to that specific tick species. Thus, the selection in moderately to highly resistant breeds will respond rapidly, whereas the selection in lowly resistant breeds, such as all temperate breeds, is impractical (Frisch 1999). However, a quantum increase can be achieved by the introgression of major resistance genes.

Major genes controlling tick resistance are more likely to be detected in populations that have only recently been subjected to intense selection for tick resistance, as it was the case for Belmont Adaptaur cattle (Kerr et al. 1994). This is a crossbred between the two originally highly susceptible *B. taurus* breeds Shorthorn and Hereford. Imported to Australia, the animals have only recently been exposed to *R. (B.) microplus.* Therefore, they did not have the opportunity to gradually accumulate polygenic resistance. The isolation of extremely resistant animals led to the demonstration of a putative major gene responsible for host resistance (Frisch 1999; Kerr et al. 1994). Carriers of the gene acquire resistance early in life and at a relatively low tick challenge. The resistance is stable, heritable, and lifelong. These characteristics differentiate this form of resistance from the resistance associated with extreme hypersensitivity reported from taurine breeds after repeated tick challenge. Total or near-total resistance is acquired by a

significant proportion of homozygous animals (Frisch 1994). In a genome-wide scan Machado et al. (2010) identified six quantitative trait loci (QTL) for tick resistance. Most of the different sets of genes were found to be involved in the resistance mechanisms varying between dry and rainy season.

Previously, studies of the bovine major histocompatibility (MHC) system associated the bovine leukocyte antigens (BoLA) with the number of female ticks (Stear et al. 1984; Stear et al. 1989). However, none of the antigens contributed to more than 2.1 percent of the variance. Although the potential of BoLA alleles as molecular markers for several health and production traits in cattle have been shown, weak associations and inconsistencies between studies do not indicate a great promise for the BoLA group as markers for tick resistance (Martinez et al. 2006; Stear et al. 1990).

4.4.3.4 Host resistance against tick-borne diseases

Breed differences in resistance to TBD are difficult to distinguish from resistance to ticks. They may just be a consequence of the tick infestation rate and the infection prevalence and intensity of TBD within ticks. Contrary to tick resistance, there is less evidence of breed differences in resistance to TBD (Minjauw & de Castro 1999).

Bock et al. (1997) studied the innate resistance of naive *B. taurus, B. indicus,* and its crossbreds to virulent *B. bovis, B. bigemina,* and *A. marginale* parasites. While pure zebu steers were significantly more resistant to *B. bovis* infection and did not require treatment, 20 to 30 % of the crossbreds required treatment. The pure taurine group showed a low resistance with 80 % of the cattle requiring treatment. In comparison, *B. bigemina* caused milder symptoms and treatment was not required in any group. However, pure *B. taurus* steers were significantly more affected. *A. marginale* caused a moderate effect in zebu animals with half of them requiring treatment, whereas all of the pure taurine animals required treatment. The conclusion that *B. indicus* cattle inhabit a higher resistance could be confirmed by subsequent studies (Bock et al. 1999ab). Despite these findings, Minjauw & de Castro (1999) proposed that controlling TBD first of all relies on controlling the vector.

4.4.4 Biological control

More than 100 species of pathogens, seven parasitic wasps, and nearly 150 predators have been identified as possible agents to control ticks (Samish & Rehacek 1999). The candidate agents range from ants (Wilkenson 1970), predatory mites (Holm & Wallace 1989), chickens (Hassan & Dipeolu 1993), parasitoid wasps (Mwangi & Kaaya 1997), entomopathogenic fungi (do Carmo Barcelos Correia et al. 1998) *Bacillus thuringiensis* (Zhioua et al. 1999), and entomopathogenic nematodes (Samish & Rehacek 1999) to oxpeckers (*Buphagus africanus*) (Grobler 1979). Commonly, the term 'biopesticides' is used to cover microbial agents and their secondary metabolites, entomopathogenic nematodes, plant-derived pesticides, and arthropod pheromones (Copping & Menn 2000). Despite the abundance of biocontrol agents targeting ticks, the reintroduction of oxpeckers in Zimbabwe remains the only known successful attempt of tick biocontrol (Grobler 1979). In recent studies it was demonstrated that the entomopathogenic fungi *Beauveria bassiana* (Campos et al. 2010), *Metarhizium flavoridae,* and *M. anisopliae* (do Carmo Barcelos Correia et al. 1998; Frazzon 2000; Onofre et al. 2001) are effective in the biological control of the cattle tick under field conditions. Results observed under laboratory conditions often cannot be achieved when applied under field conditions. The main problems include the manufacture, distribution, and stability of the living agents in the field and on the cattle (do Carmo Barcelos Correia et al. 1998; Willadsen 2006).

Apart from these agents, a number of plant species with a distinct potential to reduce populations of free-living ticks have been identified with *Stylosanthes* spp. showing the most promising results (Sutherst et al. 1982). Another method widespread in tropical regions to repel or kill ectoparasites is the application of plant extracts. Various of these extracts with an acaricidal effect have been identified (Borges et al. 2003; Broglio-Micheletti et al. 2010; Zahir et al. 2009). As valid for all the biological tick control agents, effects examined under laboratory conditions were either not tested under field conditions or showed limited effects when applied in the field. Furthermore, it might be impractical to fulfil the prerequisites. For example, *Stylosanthes* spp. have to comprise a large proportion of the pasture to affect the tick population, but this will have a negative effect on the productivity of the pasture.

4.4.5 Management strategies

Management strategies impairing tick infestations are well-known and are widely practised in Africa, America, and Australia. Nevertheless, the effect on the tick population varies among regions and tick species, the local geography, and with pasture and soil types. Although tick populations decrease after burning (Baars 1999), it is unlikely to be an efficient method of control as many tick stages miss the effects of fire while sheltering in the soil surface. Thus, ticks usually recolonise burnt areas rapidly (Young et al. 1988). Pasture spelling is a well-known management practice to reduce ticks. Though the application in developing countries where common pastures are an integral part of livestock grazing is limited (Brizuela et al. 1996; de Castro 1997; Utech et al. 1983). Tick populations can also be reduced by using livestock species which are not the natural host for a species, e.g. sheep with regard to *R. (B.) microplus* (de Castro 1997). As feedlots usually lack pasture cover and are therefore not suitable for the free-living stages of ticks, a positive effect on the tick infestation of cattle is expected when cattle are raised in feedlots (Jonsson et al. 2000b). Reducing the stocking rate of cattle can lower the tick population by limiting the overall host-finding rate of ticks (George 2000).

4.4.6 The choice of strategy

There is no single ideal solution for controlling ticks due to different problems which arise when only one single method is used for tick control. Main restrictions are the development of acaricide-resistant ticks, the difficulty of breeding tick-resistant cattle while maintaining desirable production characteristics, and the lacking efficacy of vaccines and biological agents (Willadsen 2006). Therefore, the approach of an integrated tick control strategy with the systematic application of two or more technologies is an option to achieve a more sustainable, environmental friendly, and cost-effective tick control (Bram 1994; de Castro 1997). The major aim of these integrated strategies is a reduced intensity of acaricide applications. Ever since tick control was practised, acaricides played the most important role and will continue to be necessary in tick control. Anti-tick vaccines are the most promising alternative to acaricides and constitute a tool to reduce the reliance on acaricides, especially when

highly producing, but lowly tick-resistant *B. taurus* breeds are used in areas with high tick prevalence (Frisch 1999). Already at the time when the anti-tick vaccine TickGARD^{TM} was commercialised, Willadsen et al. (1995) suggested the vaccine to be a component of an integrated pest management strategy providing immediate control by acaricides and long-term control by vaccination. Tick control programs with the strategic vaccination coupled with the use of acaricides for short-term tick control when tick infestations exceed certain levels were promoted. Through this management the drug application could be reduced substantially (de la Fuente et al. 1999; de la Fuente et al. 1998; Redondo et al. 1999; Rodríguez et al. 1995a)

Ideally, the use of acquired host resistance to ticks and TBD would be maximised in an integrated tick control strategy and modified to each individual country, region, or ecological zone (de Castro 1997). Apart from host resistance, the use of anti-tick vaccines in combination with the strategic use of acaricides will most likely constitute the main pillars of an integrated tick control. Nevertheless, farmers are unlikely to adopt this integrated strategies unless the benefits of adoption and the economic threshold of tick infestations can be quantified (Jonsson et al. 1998). The determination of economic thresholds demands accurate estimates of the production losses likely to be incurred from given numbers of ticks. In order to be an integral part of the tick control a high immunogenicity of anti-tick vaccines is necessitated. Re-immunisations every 6 months should be sufficient to maintain a constantly high antibody level. Furthermore, the effect on the tick reproduction should be considerable in order to decrease the tick population in the field.

5 MATERIALS AND METHODS

5.1 Study location and animals

5.1.1 Trial 1

The trial was conducted on the RJ Ranch in Doi Tao, Chiang Mai district, Northern Thailand, about 120 km south of Chiang Mai (17°57' N, 98°41'E; 575 m above sea level). It is a private cattle breeding farm owned by Mr. Changrung Chantaboon. The climate is tropical with a rainy (mid-May to mid-October), a winter (mid-October to mid-February), and a summer season (mid-February to mid-May). The average annual temperature and rainfall are 27 °C and 1,057 mm, respectively. The experiment lasted from April 2009 until July 2009.

For the study adult female cows of two different cattle breeds were used. On the one hand White Lamphun cattle as an example of indigenous Thai cattle which are highly adapted to the local environmental conditions and on the other hand Brahman cattle as an example of an exotic breed were used (Figure 1). In fact, White Lamphun is one of the four different ecotypes of Thai cattle. Nevertheless, it will be referred to as a breed in the following.

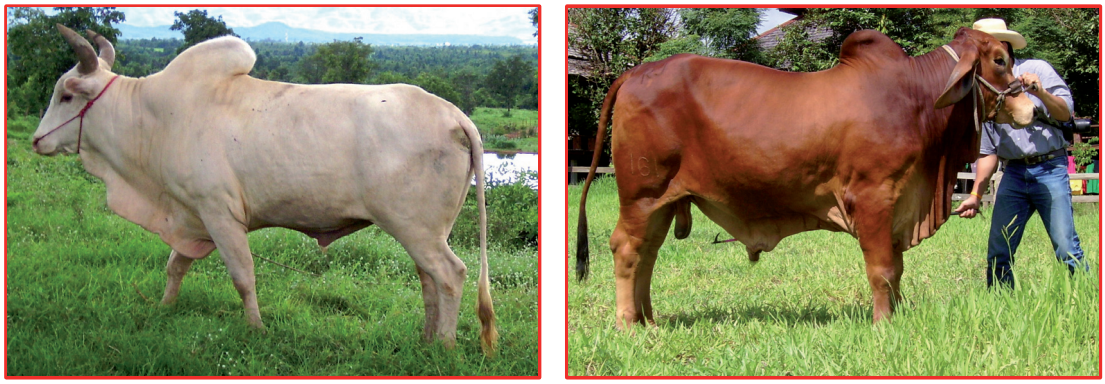

Figure 1: White Lamphun bull 'Inthanon' (left) and Brahman bull (right)

Mean live weights (± SD) at the start of the experiment were 178 (± 56) and 277 (± 80) kg for White Lamphun and Brahman, respectively. All animals were between 2 and 5 years of age. Twelve animals of each breed were allocated by live weight to control and immunised group with 6 animals each. During the preceding 6 months the animals had

not been treated with acaricides. Rectal temperature was measured weekly and the animals were weighed every 2 weeks.

5.1.2 Trial 2

The experiment was conducted at the Department of Animal and Aquatic Science of the Chiang Mai University, Thailand (18° 47' N, 98° 59' E; 312 m above sea level). The climate is similar to the one described above. Average daily temperature and rainfall are 25.9 °C and 1,197 mm. The experiment lasted from April 2009 until October 2009.
Eighteen female White Lamphun cattle with an average age of 3 years and 103 ± 17 kg live weight were used in this trial. The animals were randomly allocated to the 3 groups Bm91, control, and adjuvant with 6 animals each. Only non-pregnant animals without any prior calving were used for the study to reduce an error which may be introduced as a result of pregnancy status. During the preceding 6 months the animals had not been treated with acaricides. Rectal temperature and body weight were recorded as mentioned above for trial 1.

5.2 Farming system

The animals in both trials were raised in an extensive free-grazing system with the pasture consisting mainly of guinea grass *(Panicum maximum)*, ruzi grass *(Brachia ruziziensis),* napier grass *(Pennisetum purpureum)*, para grass *(Brachiaria mutica)*, and the forage tree legume *Leucaena leucocephala*. Grazing was allowed at all times, concentrate was not supplemented, and water was available *ad libitum*.

5.3 Immunisations

The two Bm91-immunised groups of trial 1 (White Lamphun and Brahman) as well as the Bm91-immunised group of trial 2 (White Lamphun) were inoculated with the same vaccine formulation based on the recombinant Bm91 antigen, which was derived from a Thai strain of *R. (B.) microplus*. The antigen was produced at the Centre for Agricultural Biotechnology, Kasetsart University, Thailand, by recombinant DNA technology as previously described by Kaewhom et al. (2008a). Messenger ribonucleic acid (mRNA) was isolated from salivary glands of *R. (B.) microplus* ticks indigenous to Thailand by

acid phenol extraction (Chomczynski & Sacchi 1987). Complementary deoxynucleic acid (cDNA) encoding for the Bm91 sequence was synthesised by reverse transcriptase polymerase chain reaction (PCR) using an automated thermocycler (Invitrogen, Carlsbad, CA, USA). The oligonucleotide primers containing T7 promotor sequences at the 5'-end for *in vitro* transcription and synthesis of double-stranded RNA were used to PCR-amplify the cDNA encoding Bm91. The sequences of the primers were 5'-GTA ATA CGA CTC ACT ATA GGC CAA CAT CAC (GC) GA (GT) TAC AAC (forward) and 5'-GTA ATA CGA CTC ACT ATA GGG (AT) GAC GCT GCT TC (GA) TTG G (reverse). The PCR reaction mixture (25 µl) consisted of 10 x PCR buffer (10 mM Tris, 50 mM KCl), 2.5 mM $MgCl_2$, 0.3 mM deoxynucleotide triphosphates (dNTPs, Promega Corporation, Madison, WI, USA), 2 µl cDNA, 2 U Platinum™ *Thermus aquaticus (Taq)* DNA polymerase (Invitrogen), 0.4 µM of the primers, and distilled water. Reactions were performed in a thermocycler (MyCycler™, Bio-Rad Laboratories, Veenendaal, The Netherlands) with the following conditions. After 5 min. with 95 °C and before 10 min. with 74 °C, 35 cycles of 95 °C for 1 min., 55 °C for 1 min., and 74 °C for 3 min. were run.

PCR amplicons were separated by electrophoresis on 1.5 % agarose gels and visualised under UV light in order to confirm the sequence of the target amplicon by comparison to a DNA molecular weight marker (1 kilobase (kb) Plus DNA Ladder, Promega). cDNA encoding the Thai strain Bm91 was transferred to the *P. pastoris* plasmid vector pPICZαA (Invitrogen). The expression constructs were sequenced at both ends and recombinant Bm91 plasmids were cloned into *E. coli* DH5-α competent cells. Plasmid DNA was extracted and sequenced by the dye terminator cycle sequencing method. The analysed Bm91 nucleotide sequences showed open reading frames of 1,893 base pairs (bp) encoding proteins of 631 amino acids.

For the immunisations doses of 2 ml containing 1 ml of the Bm91 protein at a final concentration of 200 µg/ml were prepared. The antigen was adjuvated with 1 ml of Montanide™ ISA 50V containing 10 % anhydromannitoletheroctodecenoate in mineral oil (Seppic, Paris, France). Doses were prepared with an Ultra-Turrax T8 high-speed homogeniser (IKA Labortechnik, Staufen, Germany) one day prior to use. The control groups received 2 ml doses containing phosphate buffered saline (PBS, pH 8.0).

Animals of the adjuvant group (trial 2) received injections of the adjuvant Montanide™ ISA 50V with the same volume of 2 ml. The animals were intramuscularly inoculated with a primary dose (week 0) followed by 2 booster doses at 3-week intervals (week 3 and 6). In trial 2 an additional 4th immunisation dose 26 weeks post primary immunisation (ppi) was given.

5.4 Blood sampling

Blood was collected weekly from the jugular vein into 3 different Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) for a period of 3 months ppi. In trial 2 the animals were sampled additionally for 1 month after the 4th immunisation dose (week 26 to week 30). Tubes for serological analysis contained clot activator. One day after collection serum was separated and stored at -20 °C until further analysis. For haematological studies and PCR analysis tubes with K_3-EDTA (ethylene-diamine-tetraacetate) as anticoagulant and citric acid were used, respectively. The latter tubes were stored at -20 °C until PCR analysis. All blood samples were collected in the morning in order to standardise time related variables which might influence certain blood components.

5.5 Anti-Bm91 antibody ELISA

The antibody level was determined by indirect enzyme-linked immunosorbent assay (ELISA) according to Jittapalapong et al. (2000) using the Thai recombinant Bm91 protein as antigen. Microtitre plates were coated overnight at 4 °C with 3 µg/well of the antigen diluted in 0.1 M carbonate coating buffer (pH 9.6). After washing five times with PBST (0.05 % (w/v) Tween-20[8] in PBS), the free binding sites on the plates were blocked with 0.1 % bovine serum albumin in PBS (100 µl per well) for 2 h at room temperature (RT). Once the plates were washed again with PBST, 100 µl of sample sera in a 1:100 dilution were added to each well and incubated for 2 h at RT. After washing as described above, 100 µl of peroxidase-conjugated goat immunoglobulins against bovine immunoglobulins (ICN, Aurora, USA) diluted 1:5,000 were added to each well. The plates were incubated for another 2 h at RT and another washing step followed. The

8 Polyoxyethylen$_{(20)}$-sorbitan-monolaurat is used to prevent non-specific antibody binding.

antibody-antigen complexes were visualised with 100 µl per well of 0.05 % 2,2'-azino-di-[3-thylbenzthiazoline sulfonate] (ICN, Aurora, USA) and 0.03 % hydrogen peroxide. Reactions were stopped after 7 min. by adding hydrochloric acid (HCl). Optical density (OD) measurements were performed using a TECAN Sunrise™ ELISA reader (TECAN Trading AG, Grodig, Switzerland) at a wave length of 450 nm. Mean OD values were calculated from duplicates of each sample. Positive and negative controls without antigen, primary antibody, secondary antibody, and substrate, respectively, were simultaneously measured to ensure that the colorimetric reaction was due to the formation of the antigen-antibody complex and not to non-specific reactions.

5.6 Antigen detection

In order to confirm that antibodies invoked by Bm91 bind to *R. (B.) microplus* proteins, the multiscreen Western blot analysis was performed for the sera obtained from one immunised animal of trial 2. A Mini-PROTEAN II multiscreen apparatus (Bio-Rad, Hercules, CA, USA) was used. For the multiscreen Western blot analysis, the Bm91 antigen preparation which was used for the immunisations was subjected to 10 % sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were denatured for 4 min. at 95 °C in loading buffer and resolved at 200 V for 40 min. under reducing conditions. Afterwards, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane and incubated in the multiscreen apparatus with the weekly taken serum samples diluted 1:100 at 100 V for 1.5 h. After blocking with TTBS (50 mM Tris-HCl, 150 mM NaCl, pH 8.0 containing 0.05 % (w/v) TWEEN-20), the blot was incubated overnight at RT. Prior to the incubation with peroxidase-conjugated anti-bovine immunoglobulins (Amersham, USA) at RT, the blot was washed three times with PBST. Finally, the blot was incubated with anti-bovine conjugate as secondary antibody diluted 1:5,000 in TTBS for 1 h at RT, after it was washed as described for the preliminary incubation step. After incubating with the secondary antibody, three washing steps with PBST followed, before the positive signals were visualised by adding DAB (diamino benzidine, Sigma, USA) substrate. Protein standards represented 15 to 250 kDa (Bio-Rad, Hercules, CA, USA).

5.7 Haematology

The haematological analysis was carried out by the Veterinary Diagnostic Laboratory, Small Animal Teaching Hospital, Faculty of Veterinary Medicine, Chiang Mai University, Thailand, using routine laboratory methods (Kenneth et al. 2003). The complete blood count included the following parameters: PCV (%), Hb (g/dl), mean cell volume (MCV, fl), mean cell haemoglobin concentration (MCHC, g/dl), RBC (10^6 cells/µl), white blood cell count (WBC, 10^3 cells/µl), differential WBC count of segmented neutrophils (10^3 cells/µl), lymphocytes (10^3 cells/µl), monocytes (10^3 cells/µl), eosinophils (10^3 cells/µl) and basophils (10^3 cells/µl), total plasma protein (g/dl), and platelet count (10^3 cells/µl). In the following neutrophils will be used instead of segmented neutrophils and protein instead of total plasma protein. For week 11 ppi no haematological analysis was done and protein was only obtained until week 12 ppi in trial 2.

5.8 PCR

PCR was used to detect the tick borne parasites *Babesia* spp. (Ano et al. 2001), *A. marginale,* and *Theileria orientalis* (Sarataphan et al. 1996), and additionally, to detect infections with *Trypanosoma evansi* (Artama et al. 1992). For PCR analysis the blood samples collected in the week of the primary immunisation (week 0) were used. The primers used are given in Table 1. For *Babesia* spp. the PCR analysis consisted of two successive runs (nested PCR).

DNA was isolated by phenol extraction and ethanol precipitation as described by Sambrook & Russel (2001). Briefly, 500 µl denaturing solution were added to 100 µl of blood and vortexed for 5 min. After adding chloroform (150 µl) and phenol (150 µl) the mixture was vortexed for another 10 min. The phases were separated by centrifuging at 13,000 rounds per minute (rpm) for 5 min. The supernatant (600 µl) was transferred to a clean microtube and chloroform and phenol were added in the same amounts as mentioned above. The vortex and centrifugation steps were repeated to clean the supernatant. DNA was precipitated by adding 1 ml of absolute ethanol (99.99 %) and storing at -20 °C overnight. Thereafter, the sample was centrifuged at 13,000 rpm for 10 min. The supernatant was removed and the DNA pellet was washed twice by adding

700 µl of 75 % ethanol and centrifuging at 13,000 rpm for 5 min. The air-dried pellet was dissolved in 20 µl TE buffer containing 10 mM Tris-(hydroxymethyl-)amino-methan-HCl (Tris-HCl, pH 8.0) and 1 mM EDTA. Until PCR analysis it was stored at -20 °C.

Table 1: Primers used to detect *Babesia* spp.*, Anaplasma marginale, Theileria annulata,* and *Trypanosoma evansi*

Parasite	Primer sequences (5' to 3')	PCR product (bp)
Babesia spp. 1st run	AGT CAT ATG CTT GTC TTA CCA TCA TTC CAA TTA CAA	500
Babesia spp. 2nd run	ATA ACC GTG CTA ATT GTA GG TGT TAT TTC TTG TCA CTA GG	327
Anaplasma marginale	ATT TCC ATA TAC TGT GCA G GAG CGC ATC TCT TGC CGC C	200
Theileria orientalis	CAC GCT ATG TTG TCC AAG AG CGA CGA AGT CAT AGA GGC AC	349
Trypanosoma evansi	CGA ATG AAT AAA CAA TGC GCA GT GCT TAC TTA TTT GTT ACG CGT CA	177

PCR amplifications were performed in 20 µl volumes with the mixtures as given in Table 2. Reactions were performed in an automated DNA thermocycler (MyCycler™, Bio-Rad Laboratories, Veenendaal, The Netherlands) programmed with the PCR conditions as summarised in Table 3. For both rounds of the nested PCR of *Babesia* spp. the same conditions were used.

Table 2: Reaction mixtures for PCR amplification of *Babesia* spp., *Anaplasma marginale, Theileria annulata,* and *Trypanosoma evansi*

Substance, µl	*Babesia* spp.	*Anaplasma marginale*	*Theileria orientalis*	*Trypanosoma evansi*
10 x PCR buffer[1]	2.00	2.00	2.00	1.10
$MgCl_2$	0.60	0.80	0.60	0.33
DNTPs[2], 10 mM/µl	0.40	0.40	0.40	0.88
Forward primer, 100 ρMol/ µl	0.20	2.00	0.40	0.55
Reverse primer, 100 ρMol/ µl	0.20	2.00	0.40	0.55
Taq-DNA polymerase, 5 U/µl	0.04	0.20	0.06	0.11
DW[3]	14.60	10.60	14.04	6.48
Total volume	18.00	18.00	18.00	10.00
DNA template	2.00	2.00	2.00	1.00

[1] Buffer contains 10 mM Tris and 50 mM Kcl.
[2] dNTPs, deoxynucleotide triphosphates.
[3] DW, distilled water.

Ten µl of the PCR-amplified solution was loaded with 2 µl of loading dye to a 1 % (w/v) agarose gel to check the size of the amplified fragments by comparison to a DNA molecular weight marker (1 kb Plus DNA Ladder, Promega). Electrophoresis was carried out in TBE buffer (90 mM Tris-borate, 2 mM EDTA) at a constant voltage of 100 V for 60 min. The gel was stained by ethidium bromide (1 µl/ml) for 5 min. and photographed under UV light (Sambrook & Russel 2001). For *Trypanosoma evansi* a 2 % agarose gel was used at a voltage of 120 V.

Table 3: PCR conditions for *Babesia* spp., *Anaplasma marginale, Theileria annulata,* and *Trypanosoma evansi*

	Babesia spp.		*Anaplasma marginale*		*Theileria annulata*		*Trypanosoma evansi*	
Number of cycles	30		35		40		30	
Step	Temp.	Time	Temp.	Time	Temp.	Time	Temp.	Time
Initial denaturation	90	120	95	300	94	240	94	60
Denaturation	90	30	94	60	94	35	94	30
Annealing	55	120	60	60	51	35	60	60
Extension	72	120	72	90	72	35	72	30
Final extension	72	300	72	240	72	600	72	120

5.9 Tick collection and tick reproductive performance

In both locations the animals were kept on tick-infested pastures. Thus, they were naturally infested with *R. (B.) microplus* larvae, nymphs, and adults. In order to evaluate the effect of the immunisation on the reproductive tick performance, standard female *R. (B.) microplus* ticks (4.5 to 8 mm) were collected daily after the 3rd immunisation from week 6 until week 12 ppi. In trial 2 ticks were also collected after the 4th treatment from week 26 until week 30. Standard female ticks are those that are destined to complete engorgement within the following 24 h (Wharton & Utech 1970). The collected ticks were rinsed in water and dried on tissue paper, before their weight was recorded. Thereafter, ticks were kept individually in tick rearing plastic tubes in a photoperiod with 12 h of light at 30 ± 5 °C and 70 to 80 % relative humidity. Approximately 3 weeks after tick collection when oviposition was completed, the egg mass oviposited by each female was weighed.

Dead female *R. (B.) microplus* were removed from the tubes to avoid fungal growth and contamination of the eggs. The weighed eggs were placed in a new plastic container covered with cloth in order to asses the egg viability. Hatching of the eggs was

evaluated 3 weeks after egg masses were weighed. If more than half of the tick larvae hatched during this period, the batch was recorded as viable. The reproductive efficiency index (REI) was calculated by using the following formula proposed by Bennett (1974):

$$\text{Reproductive efficiency index (REI)} = \frac{\text{Egg mass weight (mg)}}{\text{Tick weight (mg)}} \text{ x } 100$$

5.10 Statistical analysis

Data were statistically analysed with the statistical package SAS, version 9.2 (SAS Institute Inc. 2008). Statistical calculations for ELISA data, haematological parameters and body temperature were carried out using a repeated measures ANOVA over time (MIXED procedure).

For trial 1 data were analysed as a 2x2 factorial design with the following model:

$$Y_{ijkl} = \mu + T_i + B_j + W_k + (T_i \text{ x } B_j) + (T_i \text{ x } W_k) + (B_j \text{ x } W_k) + e_{ijkl}$$

Where,

Y_{ijkl} = observation

μ = population mean

T_i = effect of i^{th} treatment $(i = 1, 2 = \text{Bm91 , control})$

B_j = effect of j^{th} breed $(j = 1, 2 = \text{WhiteLamphun , Brahman})$

W_k = effect of k^{th} week $(k = 0 - 12)$

$(T_i \text{ x } B_j)$ = treatment x breed interaction

$(T_i \text{ x } W_k)$ = treatment x week interaction

$(B_j \text{ x } W_k)$ = breed x week interaction

e_{ijkl} = residual effect

The analysis provides *P* values for differences between breeds and treatments, differences over time, and all the two-way-interactions. An interaction between breed or treatment and week demonstrates that the rate of change differs between the breeds and treatments, respectively.

For trial 2, the ELISA data, haematological parameters, and body temperature were analysed with a one-way repeated measures ANOVA over time of the MIXED procedure. The following model was used:

$$Y_{ijk} = \mu + T_i + W_j + (T_i \text{ x } W_j) + e_{ijk}$$

Where,

Y_{ijk} = observation

μ = population mean

T_i = effect of i^{th} treatment $(i = 1...3 = \text{Bm91, control, adjuvant})$

W_j = effect of j^{th} week $(k = 0...12, 26...30)$

$(T_i \text{ x } W_j)$ = treatment x week interaction

e_{ijk} = residual effect

The analysis provides *P* values for differences between the treatments, differences over time, and the interaction between treatment and week.

Chosen by Akaike's information criterion (AIC)[9] the compound symmetry structure, which assumes that the observations of the same animal have homogeneous variances and covariances, was used. The approximation for the denominator degrees of freedom was done after Kenward & Roger (1997). The ELISA values were adjusted to an OD value of 0 at the onset of the study to exclude differences between the groups. Prior to analysis a log-transformation was done for WBC count and neutrophil:lymphocyte (N:L) ratio. The proportions of neutrophils, lymphocytes, monocytes, and eosinophils

9 The AIC is a measure of the goodness of fit of an estimated statistical model.

on the WBC count were analysed after √arcsin (*x)*-transformation. Results are presented as Least Squares Means (LSM) ± standard error (SE). Multiple comparisons were done with the Tukey test. Differences of $P < 0.05$ were considered as statistically significant.
The average daily gain was calculated by regression analysis using the REG procedure. Values were analysed by two-way ANOVA (GLM procedure) and compared with the Tukey test ($P < 0.05$). Data are presented as LSM ± pooled standard error (PSE). If the standard error differed between the groups, the highest standard error is given as PSE.
Due to the fact that the number of ticks was too low for a statistical analysis, only the tick parameters of trial 2 were analysed. Data of tick number, tick weight, and REI were analysed by one-way ANOVA (GLM procedure) with the treatment as fixed effect. Means were compared by the Tukey test ($P < 0.05$). Results of the tick number, the tick weight, and the REI are given as mean ± standard deviation (SD). To satisfy the assumptions of ANOVA, the tick weight was log-transformed and the REI was √arcsin (*x)*-transformed prior to analysis. The percentage of ticks ovipositing and the egg viability were compared by χ^2 test ($P < 0.05$) with Bonferroni adjustment.
For both trials correlations between the body temperature and the haematological parameters PCV, Hb, RBC, MCV, MCHC, WBC, neutrophils, lymphocytes, platelets, and protein were calculated (CORR procedure). Prior, WBC was log-transformed and the proportions of neutrophils and lymphocytes on the WBC count were analysed after √arcsin (*x)*-transformation.

6 RESULTS

6.1 Trial 1

6.1.1 Anti-Bm91 antibody level

The anti-Bm91 antibody level of both control groups did not vary significantly over time, whereas the antibody level in the immunised White Lamphun and Brahman group increased significantly from the 1st week ppi on (Figure 2). The antibody level of the 2 immunised groups showed a similar course. Post primary immunisation a rapid rise was detected. Both the 2nd and the 3rd treatment led to a further increase. From week 7 until week 12 ppi the anti-Bm91 ELISA values were maintained. Values of the Bm91 groups were about 2 OD units higher than of the control groups.

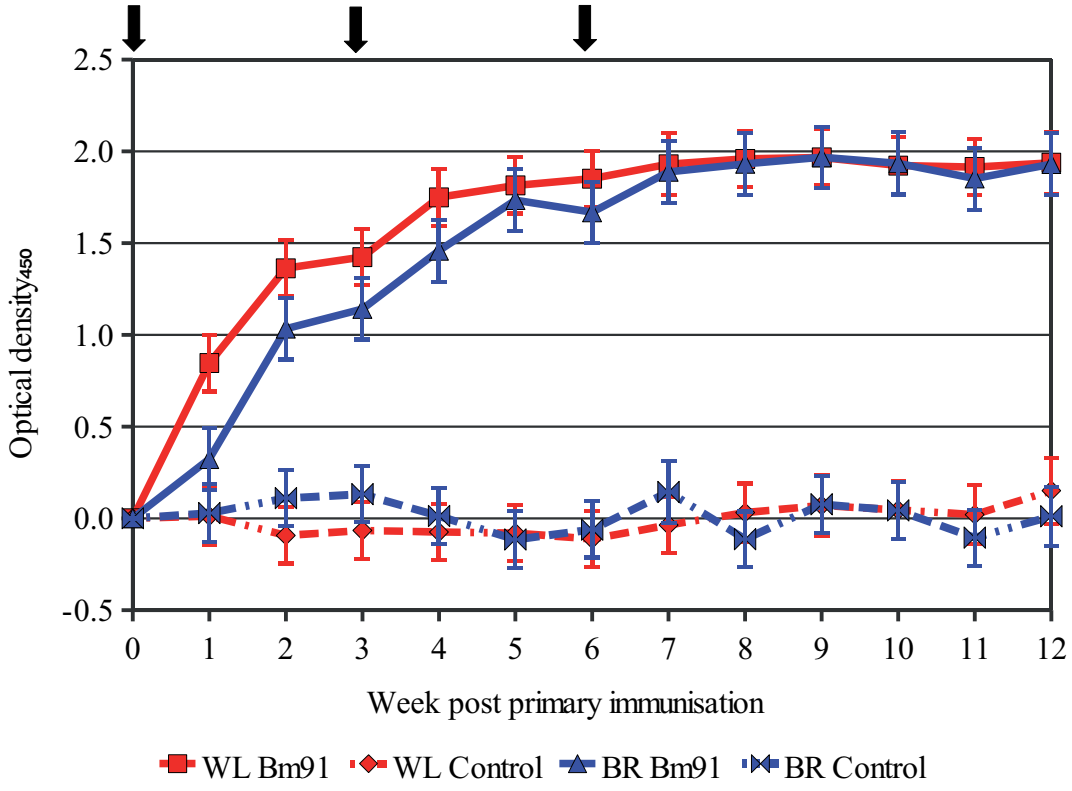

Figure 2: Anti-Bm91 antibody responses of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 (solid line) and injected with saline (Control, dashed line). Values were measured by ELISA and are expressed as OD_{450nm} value (LSM ± SE; N = 6 except N = 5 for the BR Bm91 group). Values are adjusted to week 0. Arrows indicate the week of immunisation

In order to evaluate the antibody response of the immunised White Lamphun and Brahman group, the statistical model was reduced to the effects of breed, week, and the breed x week interaction. Neither a significant breed effect ($P > 0.05$), nor a significant breed x week interaction ($P > 0.05$) resulted from the repeated measures ANOVA. Although not statistically significant, the antibody level increased more rapidly after the primary immunisation in the White Lamphun group. The difference to the Brahman animals was most pronounced in the first week ppi (Appendix 1).

6.1.2 Body temperature

During the experimental period the mean body temperature ranged between 38.0 and 39.6 °C (Figure 3). A significant week and breed x week interaction was calculated by repeated measures ANOVA (Table 4).

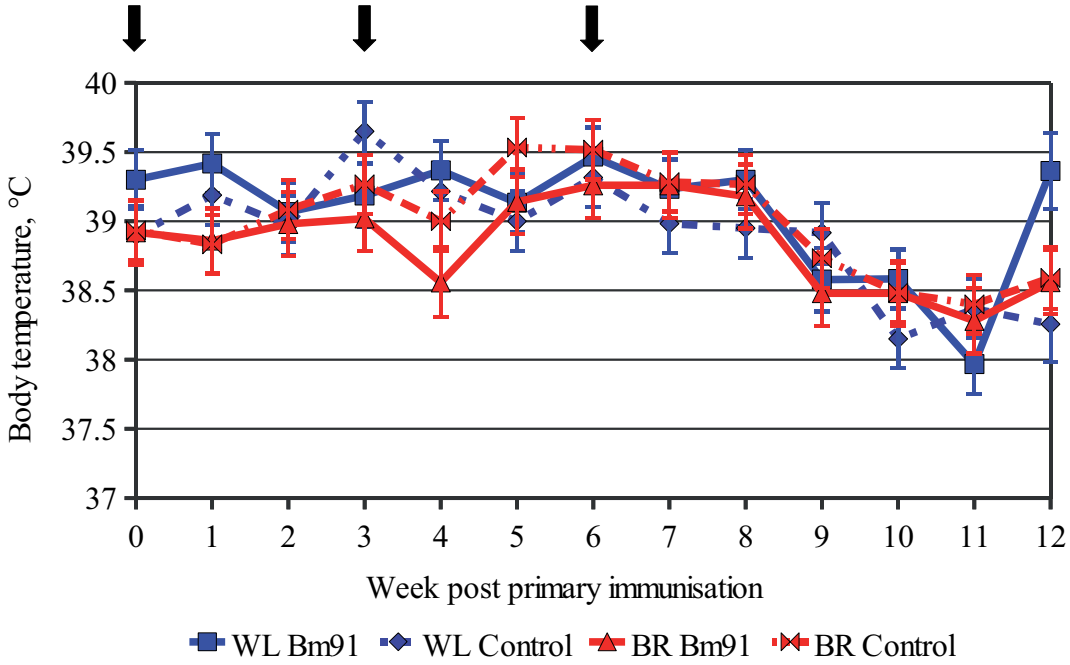

Figure 3: Body temperature of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 (solid line) and injected with saline (Control, dashed line) (LSM ± SE; N = 6 except N = 5 for the BR Bm91 group). Arrows indicate the week of immunisation

6.1.3 Haematology

The erythrocyte parameters of the complete blood count including PCV, Hb, and RBC are presented in Figures 4 to 7. Data for MCV, MCHC, protein, and platelet count are presented in Appendices 2 to 5. The different parameters varied within the reference intervals proposed by Kramer (2000) with the following exceptions. The PCV and Hb slightly fell below the reference value within two weeks ppi in the immunised Brahman group. With the exception of the White Lamphun control group, the MCV was below the reference interval during the first 4 weeks ppi. In addition, this parameter was lower than normal in the Brahman animals in the last week. The *P* values of the repeated measures ANOVA for the different erythrocyte parameters, protein, and platelet counts are given in Table 4. Significant breed, week, and breed x week interactions were calculated with the exception of the breed x week interaction for protein. White Lamphun animals had significantly higher PCV and Hb and lower MCV and MCHC when compared to Brahman animals ($P < 0.05$). A significance over time was calculated for all these parameters ($P < 0.0001$). The breed x week interaction reveals that the rate of the change in the respective parameter differed between the breeds with the Brahman showing a higher rate of change for PCV, Hb, MCHC, RBC, and protein. The platelet count varied around the lower reference value except for the 3 weeks following the 3rd immunisation. Apart from the week effect, the breed x week interaction was significant indicating a higher rate of change over the time for the two Brahman groups.

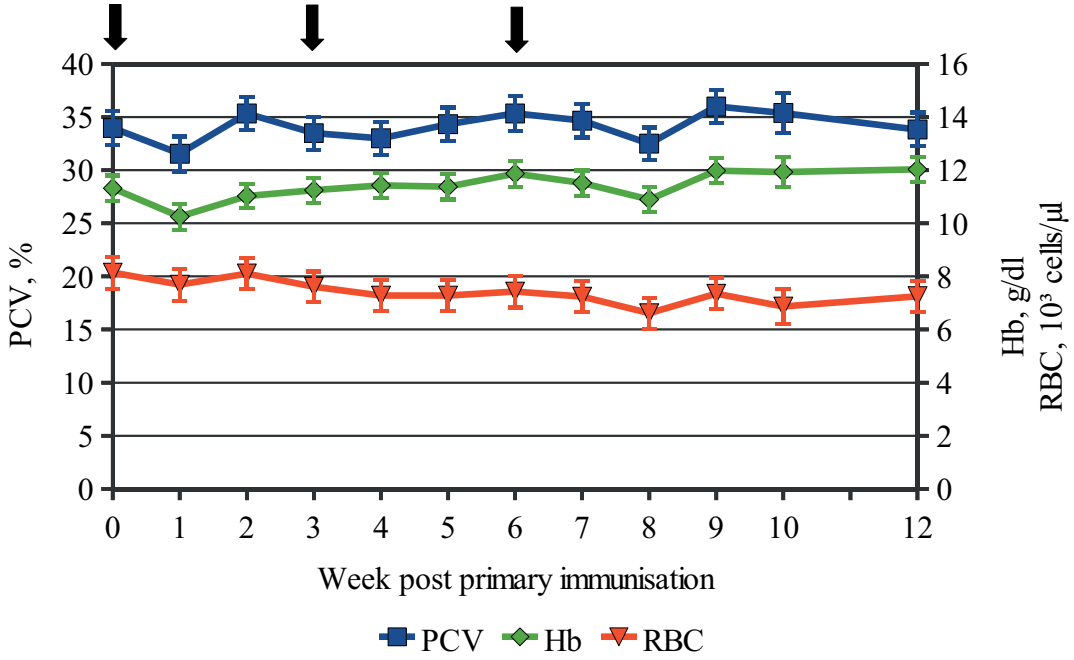

Figure 4: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of White Lamphun cattle immunised with the antigen Bm91 (LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

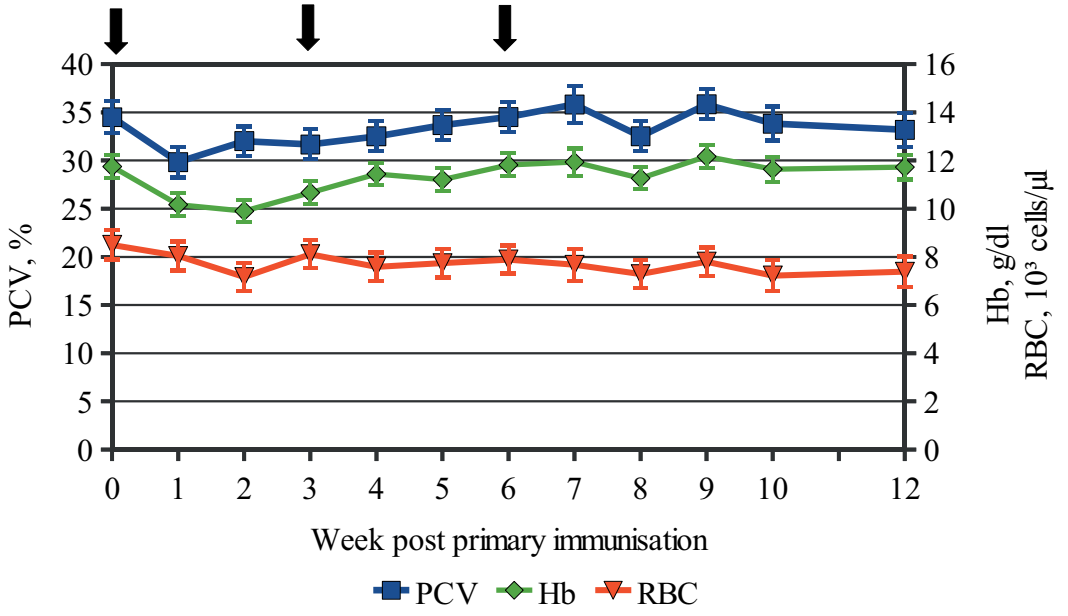

Figure 5: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of White Lamphun cattle injected with saline (Control, LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

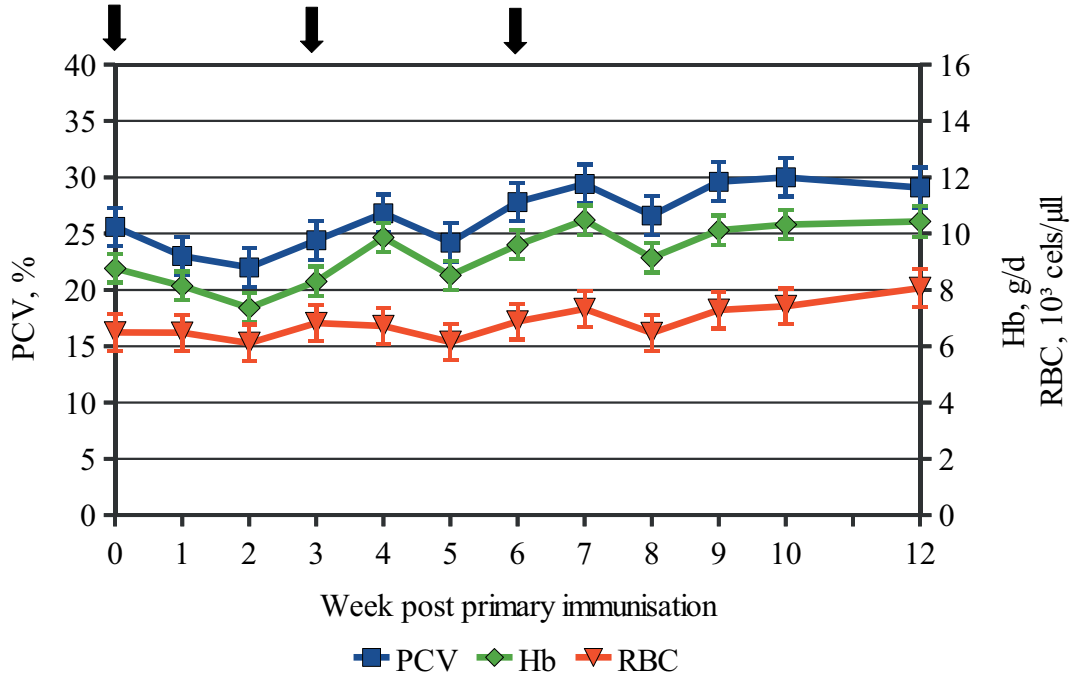

Figure 6: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of Brahman cattle immunised with the antigen Bm91 (LSM ± SE; N = 5). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

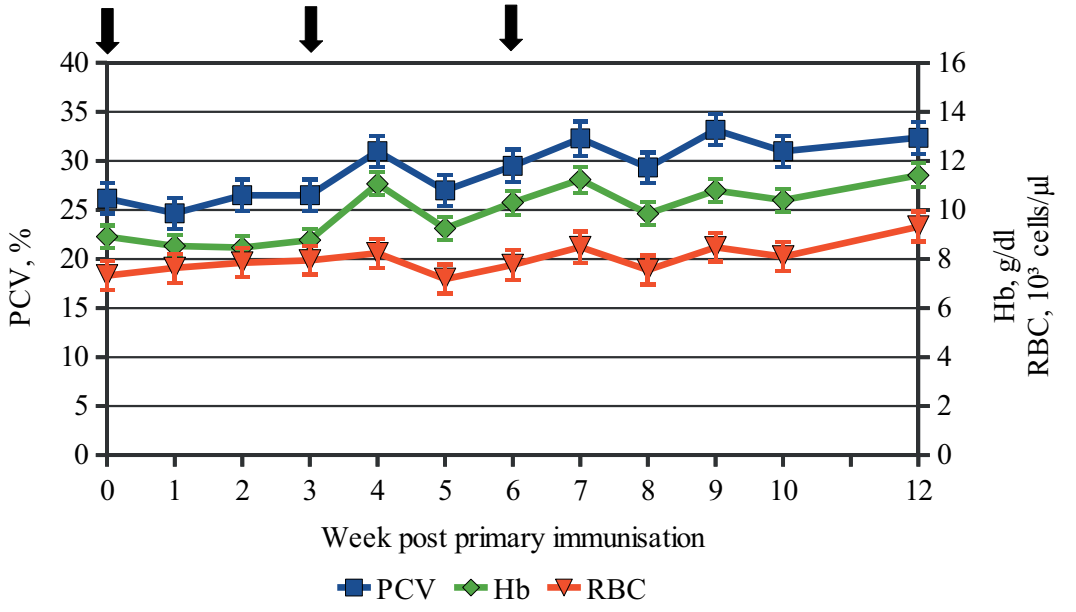

Figure 7: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of Brahman cattle injected with saline (Control, LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

Table 4: Probability values of breed (B), treatment (T), week (W), and all two-way-interactions of body temperature, erythrocyte parameters, protein, and platelet count calculated by repeated measures ANOVA

	Independent variable					
Dependent variable	Breed	Treatment	Week	B * T	B * W	T * W
Body temp., °C	0.5966	0.9933	< 0.0001	0.3200	0.0316	0.1000
PCV[1], %	0.0002	0.1998	< 0.0001	0.4851	< 0.0001	0.6439
Hb[2], g/dl	0.0001	0.3211	< 0.0001	0.4598	< 0.0001	0.4418
RBC[3], 10^6 cells/µl	0.8086	0.4379	0.0005	0.1886	< 0.0001	0.3085
MCV[4], fl	0.0017	0.6669	< 0.0001	0.1224	< 0.0001	0.8328
MCHC[5], g/dl	0.0252	0.0580	< 0.0001	0.8588	0.0012	0.9470
Protein, g/dl	0.0147	0.8941	< 0.0001	0.1111	0.0698	0.8985
Platelet, cells/µl	0.4807	0.8392	< 0.0001	0.5413	0.0044	0.0817

[1] PCV, packed cell volume.
[2] Hb, haemoglobin.
[3] RBC, red blood cell count.
[4] MCV, mean cell volume.
[5] MCHC, mean cell haemoglobin concentration.

The WBC counts exceeded the upper reference value of 12 x 10^3 cells/µl throughout the entire period (Figure 8). At the same time, the proportions of neutrophils, lymphocytes, monocytes, and eosinophils remained within the reference interval in all groups (Figures 9 to 12). The only exception was observed for the immunised White Lamphun group in the week following the 3rd immunisation. Here, a higher neutrophil and a lower than normal lymphocyte proportion was recorded. The N:L ratio is given in Appendix 6. For WBC a significant week and breed x week interaction was calculated, whereas for

the proportions of neutrophils and lymphocytes as well as for the N:L ratio the treatment and breed x treatment effect was significant ($P < 0.05$). Compared to Brahman the rate of change for WBC was lower in White Lamphun. Higher proportions of lymphocytes were observed in the indigenous breed than in the Brahman cattle. At the same time, the proportion of this cell type was higher in the immunised group of the exotic breed when compared to the control group of this breed. The N:L ratio was higher in immunised than in control animals. Beyond this, the difference between control and immunised animals was greater for White Lamphun.

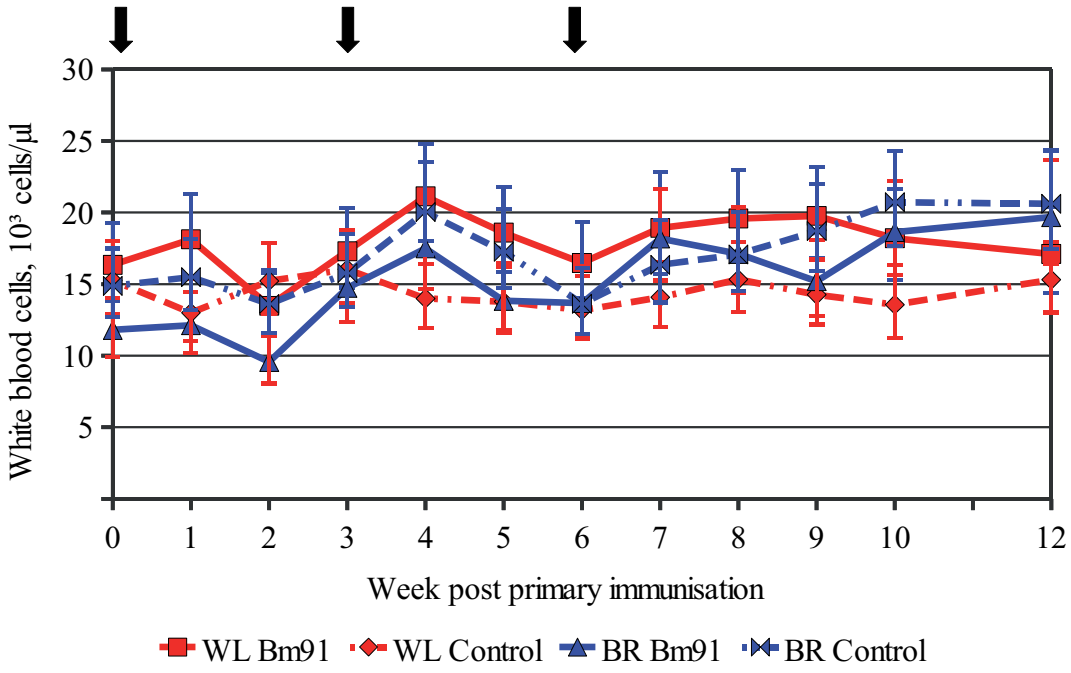

Figure 8: White blood cell count (WBC) of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 (solid line) and injected with saline (Control, dashed line) (LSM ± SE; N = 6 except N = 5 for the BR Bm91 group). Reference interval: 4 to 12 x 10^6 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

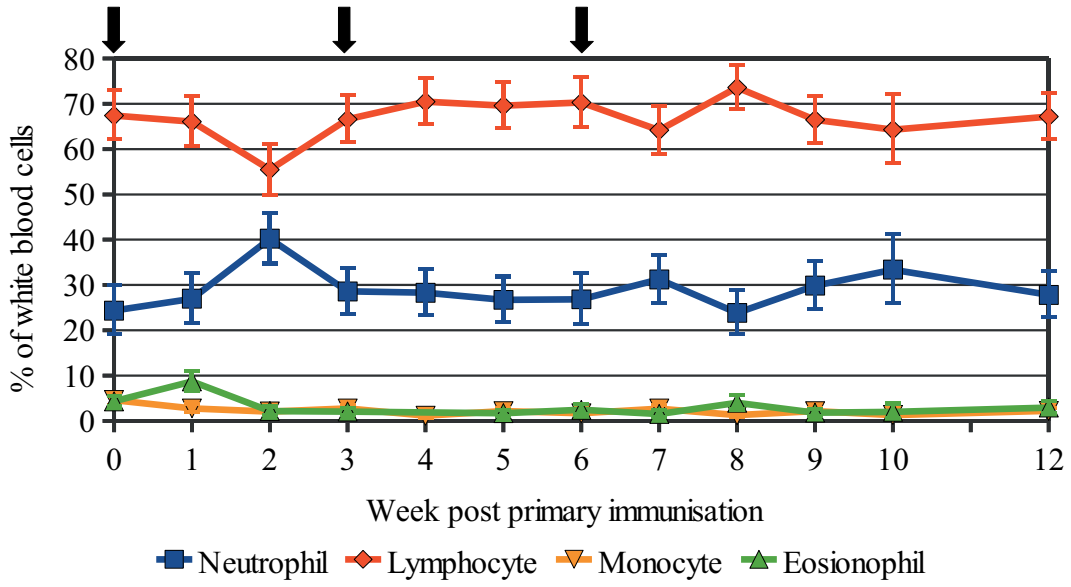

Figure 9: White blood cell differential of White Lamphun cattle immunised with the antigen Bm91 (LSM ± SE; N = 6). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

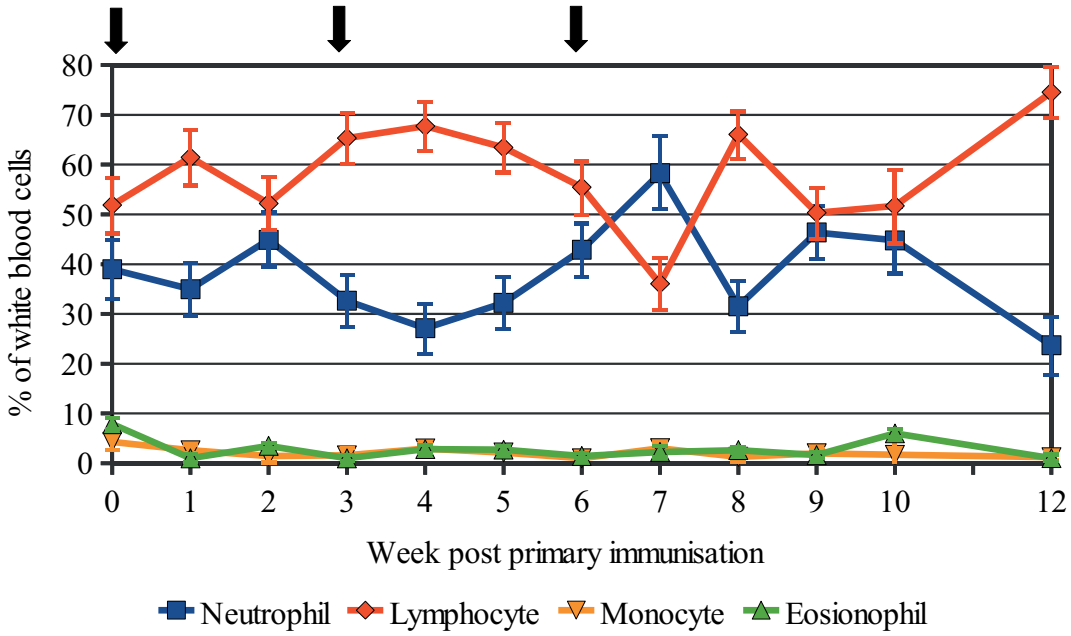

Figure 10: White blood cell differential of White Lamphun cattle injected with saline (Control, LSM ± SE; N = 6). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

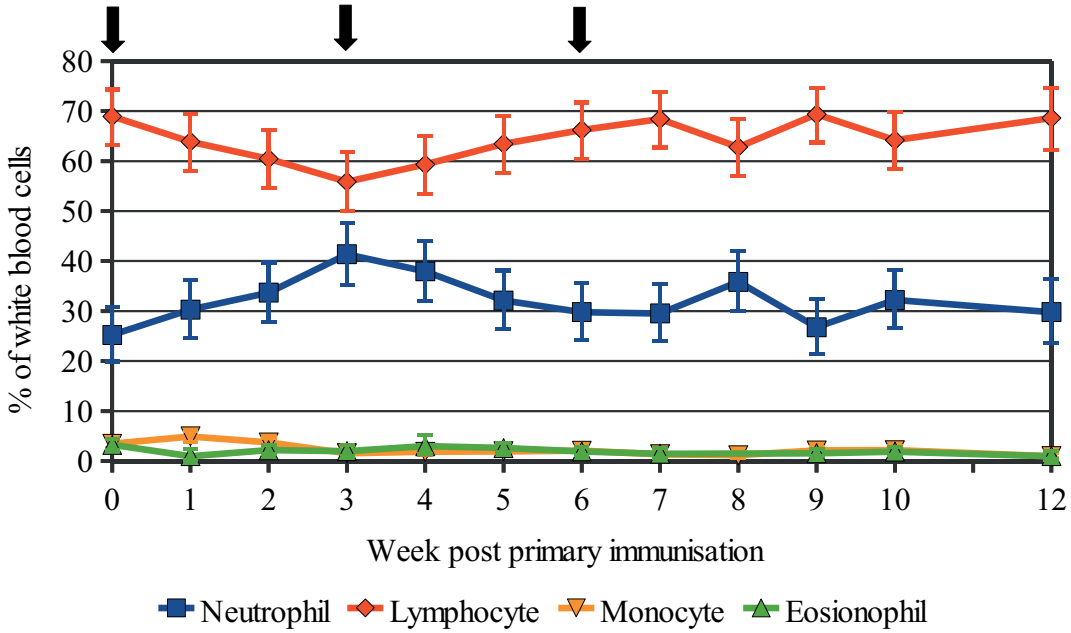

Figure 11: White blood cell differential of Brahman cattle immunised with the antigen Bm91 (LSM ± SE; N = 5). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

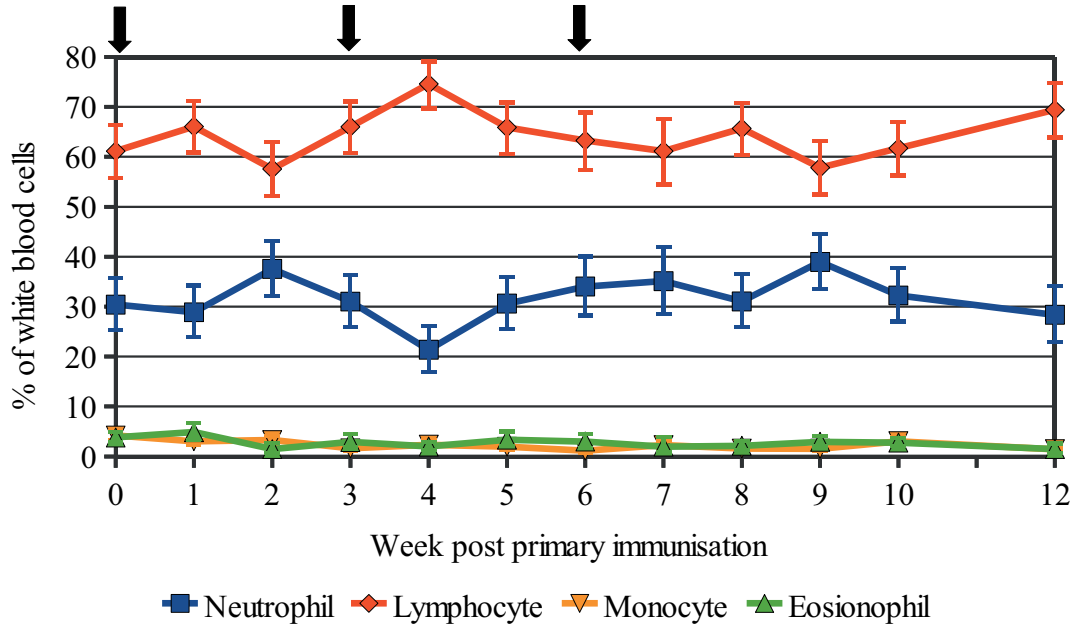

Figure 12: White blood cell differential of Brahman cattle injected with saline (Control, LSM ± SE; N = 6 except N = 5 for the BR Bm91 group). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

Table 5: Probability values of breed (B), treatment (T), week (W), and all two-way-interactions of the white blood cell differential calculated by repeated measures ANOVA

	Independent variable					
Dependent variable	Breed	Treatment	Week	B * T	B * W	T * W
WBC[1], 10^3 cells/µl	0.9200	0.7510	< 0.0001	0.2235	0.0466	0.1696
Neutrophil[2], %	0.2767	0.0062	0.1160	0.0134	0.7210	0.9915
Lymphocyte, %	0.2339	0.0118	0.0472	0.0115	0.6905	0.9722
Monocyte, %	0.5901	0.6016	< 0.0001	0.7331	0.2614	0.8971
Eosinophil, %	0.2542	0.1284	0.0131	0.2057	0.5942	0.0539
N:L ratio[3]	0.3541	0.0077	0.0858	0.0131	0.7452	0.9888

[1] WBC, white blood cell count analysed after log-transformation.
[2] Proportion of neutrophil, lymphocyte, monocyte, and eosinophil on WBC analysed after arcsin-transformation.
[3] N:L ratio, neutrophil:lymphocyte ratio analysed after log-transformation.

The correlations between body temperature and the haematological parameters are presented in Appendix 8. The WBC was positively correlated (0.48; $P < 0.0001$) with Hb in the White Lamphun control group. WBC was furthermore positively correlated ($P < 0.0001$) with PCV (0.54), Hb (0.51), and RBC (0.54), respectively, in the Brahman control group. In none of the other groups significant correlations for these parameters were found.

6.1.4 Tick-borne diseases

All animals were found to be infected with *Babesia* spp., whereas none of the blood samples was PCR positive for *A. marginale* or *Trypanosoma evansi*. One animal each in the White Lamphun control group and the two Brahman groups was found to be infected with *Theileria orientalis*.

6.1.5 Average daily weight gain

As shown in Table 6, the ADG ranged from 565 to 735 g, whereby Brahman showed a greater weight gain than White Lamphun ($P < 0.05$). Neither the treatment nor the breed x treatment interaction had a significant effect. Though, in tendency the weight gain of the immunised animals was lower than of the control animals.

Table 6: Least Squares Means (± SE) for average daily gain (ADG) of White Lamphun and Brahman cattle immunised with the tick antigen Bm91 and injected with saline (Control)

	White Lamphun		Brahman		
Treatment	Bm91 (N = 6)	Control (N = 6)	Bm91 (N = 5)	Control (N = 6)	PSE[1]
ADG, g/d	565	608	624	735	50

[1] PSE, pooled standard error.

6.2 Trial 2

6.2.1 Anti-Bm91 antibody level

The anti-Bm91 antibody level of the control and the adjuvant group did not vary significantly over time and remained on the pre-immunisation level throughout the entire observation period (Figure 13). In contrast, the antibody level of the Bm91 group increased rapidly after the primary immunisation. Both the 2nd and the 3rd treatment resulted in a further increase. A stable level was reached after the 3rd immunisation dose and was maintained until week 12 ppi. This level was about 2 OD units higher when compared to the control and adjuvant group, respectively. Until week 26 the anti-Bm91 ELISA values decreased slightly. After the 4th immunisation a moderate increase was recognised. Until the end of the trial in week 30 this level remained unaltered.

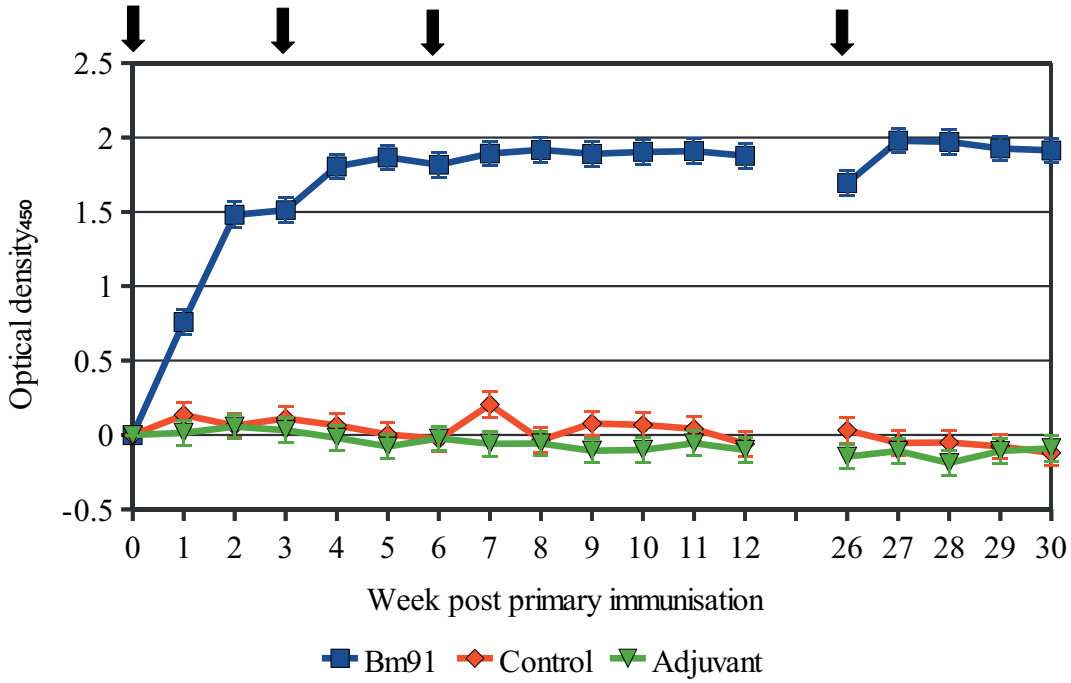

Figure 13: Anti-Bm91 antibody responses of White Lamphun cattle immunised with the antigen Bm91 and injected with saline (Control) and adjuvant, respectively. Values were measured by ELISA and are expressed as OD_{450nm} value (LSM ± SE; N = 6). Values are adjusted to week 0. Arrows indicate the week of immunisation

6.2.2 Western blot analysis

The Western blot analysis, which was exemplary done for one Bm91-immunised animal, demonstrated that the immune sera from this animal bound to the proteins which were used in the Bm91 antigen formulation. A band at a molecular weight of 86 kDa, which is consistent with the recombinant Bm91 protein from the Thai *R. (B.) microplus* strain, was visible (Figure 14). This 86 kDa band was recognised from week 3 ppi on until the end of the study. In week 26 the intensity of the reaction was reduced. Furthermore, the analysis indicated that in pre-immune sera from the same immunised animal this band at 86 kDa was not present.

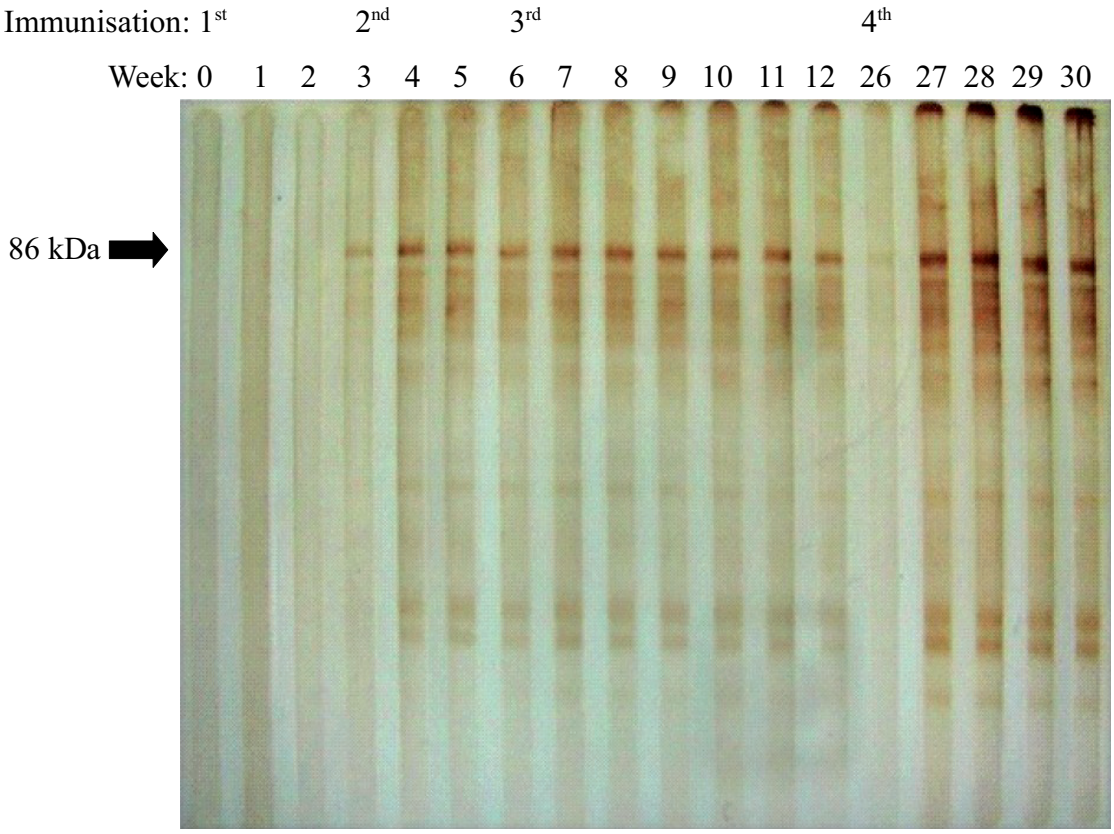

Figure 14: Multiscreen Western blot of sera from one Bm91-immunised animal using the Bm91 antigen formulation

6.2.3 Tick parameters

The different parameters recorded from the naturally infested *R. (B.) microplus* ticks are given in Table 7. The number of ticks collected did not differ significantly between Bm91, control, and adjuvant group. Within the 3 groups a high animal-to-animal variation was recorded. No significant group difference was noticed for the tick weight. In comparison to the control group the proportion of ovipositing ticks was reduced by 5 and 1 % in the adjuvant and Bm91 group, respectively ($P < 0.05$). The most pronounced effect of Bm91 was recorded for the REI and the egg viability. Compared to control animals, a 6 and 8 % reduction was observed for REI ($P < 0.05$) and egg viability ($P < 0.05$) in the Bm91 group, respectively.

Table 7: Number, weight, oviposition, and hatching of naturally infested *R. (B.) microplus* ticks collected from White Lamphun cattle immunised with the antigen Bm91 and injected with saline (Control) and adjuvant, respectively (N = 6)

Parameter	Control	Adjuvant (% Reduction)*	Bm91 (% Reduction)
Tick number (N, Mean ± SD)	90 ± 64^{NS}	62 ± 40	81 ± 37
Tick weight (mg, Mean ± SD)	84.0 ± 32.3^{NS}	80.4 ± 30.5 (4)	84.0 ± 30.2 (0)
Ticks ovipositing (%)	95.3^{a}**	91.7^{b} (5)	94.3^{a} (1)
REI (%)***	38.8 ± 9.7^{a}	38.5 ± 9.6^{a} (1)	36.6 ± 9.2^{b} (6)
Egg viability (%)	84.4^{a}**	84.7^{a} (-1)	77.8^{b} (8)

* The percent reduction was calculated with respect to the control group.
^{NS} Not significantly different ($P > 0.05$; Tukey test).
** Values within the same row differ significantly ($P < 0.05$; χ^2 test).
*** Reproductive efficiency index. Values within the same row differ significantly ($P < 0.05$; Tukey test).

6.2.4 Body temperature

The mean body temperature ranged between 37.8 and 39.0 °C (Figure 15). A significant effect of the treatment and the interaction between treatment and week, respectively, was not determined by repeated measures ANOVA (Table 8).

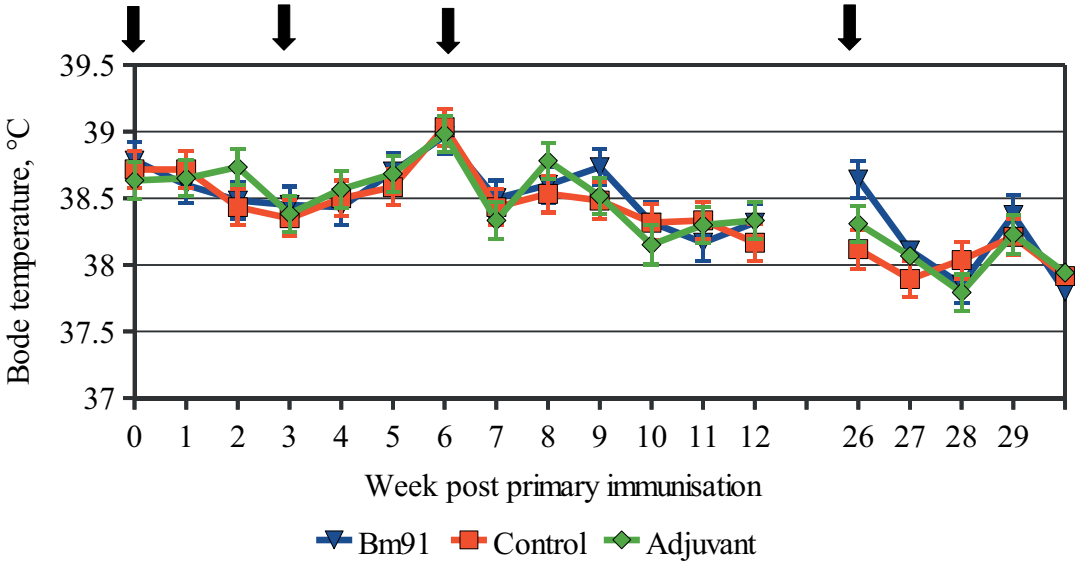

Figure 15: Body temperature of White Lamphun cattle immunised with the antigen Bm91 and injected with saline (Control) and adjuvant, respectively. (LSM ± SE; N = 6). Arrows indicate the week of immunisation

6.2.5 Haematology

The course of the PCV, Hb, and RBC is given in Figures 16 to 18. MCV, MCHC, protein, and platelet are presented in Appendices 8 to 11. All three former parameters of the erythrocytes section varied within the reference intervals proposed by Kramer (2000). After the 4th treatment (week 26) higher values were observed than during the 3 months ppi. The *P* values calculated by repeated measures ANOVA are given in Table 8. For none of the variables a significant treatment effect was observed. Neither for PCV, Hb, and RBC nor for the platelet count a significant treatment x week interaction was noticed, whereas the interaction was significant for MCV, MCHC, and protein ($P < 0.05$). The week effect was significant for all variables ($P < 0.0001$).

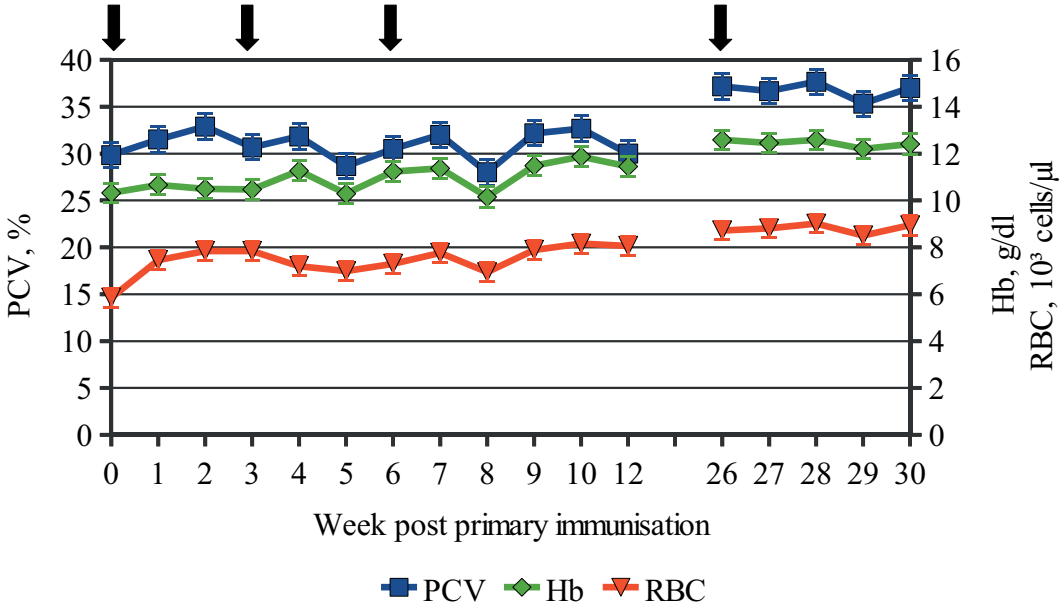

Figure 16: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of White Lamphun cattle immunised with the antigen Bm91 (LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

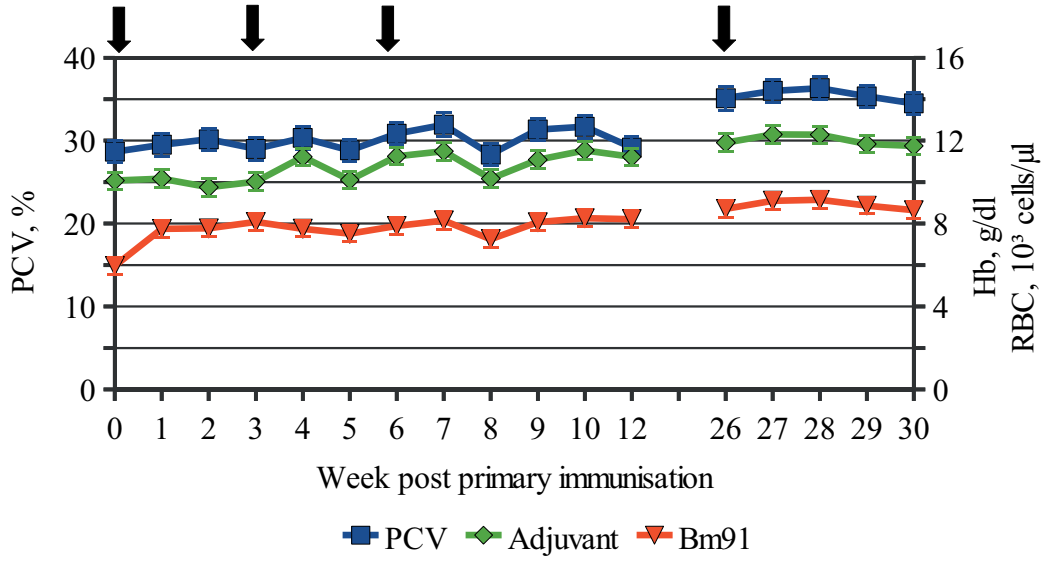

Figure 17: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of White Lamphun cattle injected with saline (LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

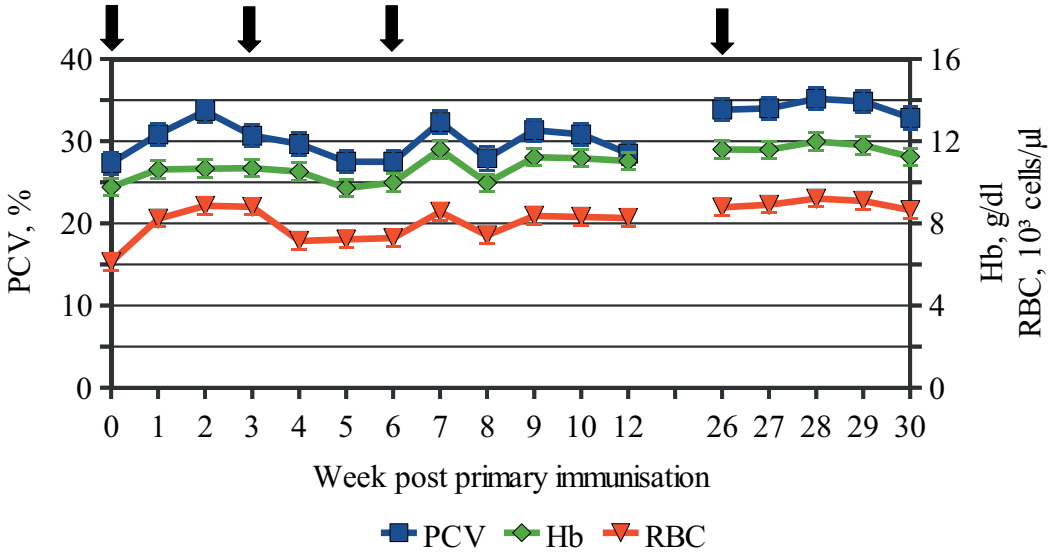

Figure 18: Packed cell volume (PCV), haemoglobin (Hb), and red blood cell count (RBC) of White Lamphun cattle injected with adjuvant (LSM ± SE; N = 6). Reference intervals: PCV 24 to 46 %, Hb 8 to 15 g/dl, RBC 5 to 10 x 10^3 cells/µl (Kramer 2000). Arrows indicate the week of immunisation

Table 8: Probability values of treatment (T), week (W), and the interaction of body temperature, erythrocyte parameters, protein, and platelet count calculated by repeated measures ANOVA

	Independent variable		
Dependent variable	Treatment	Week	T * W
Body temp., °C	0.8176	< 0.0001	0.7926
PCV[1], %	0.5406	< 0.0001	0.5684
Hb[2], g/dl	0.5314	< 0.0001	0.5262
RBC[3], 10^6 cells/µl	0.7364	< 0.0001	0.5348
MCV[4], fl	0.0544	< 0.0001	0.0298
MCHC[5], g/dl	0.8939	< 0.0001	0.0319
Protein, g/dl	0.2037	< 0.0001	0.0154
Platelet, cells/µl	0.7464	< 0.0001	0.2271

[1] PCV, packed cell volume.
[2] Hb, haemoglobin.
[3] RBC, red blood cell count.
[4] MCV, mean cell volume.
[5] MCHC, mean cell haemoglobin concentration.

The WBC counts ranged around the upper reference level (Figure 19). No abnormalities were observed for the proportions of neutrophils, lymphocytes, monocytes, and eosinophils with the exception of week 26 (Figures 20 to 22). Here, a decreased percentage of lymphocytes and an increased percentage of neutrophils was observed in all three treatment groups. The change of the proportions led to a higher than normal N:L ratio in the immunised group (Appendix 12). Neither for WBC nor for the proportions of lymphocytes and neutrophils the treatment and the treatment x week interaction, respectively, was significant (Table 9).

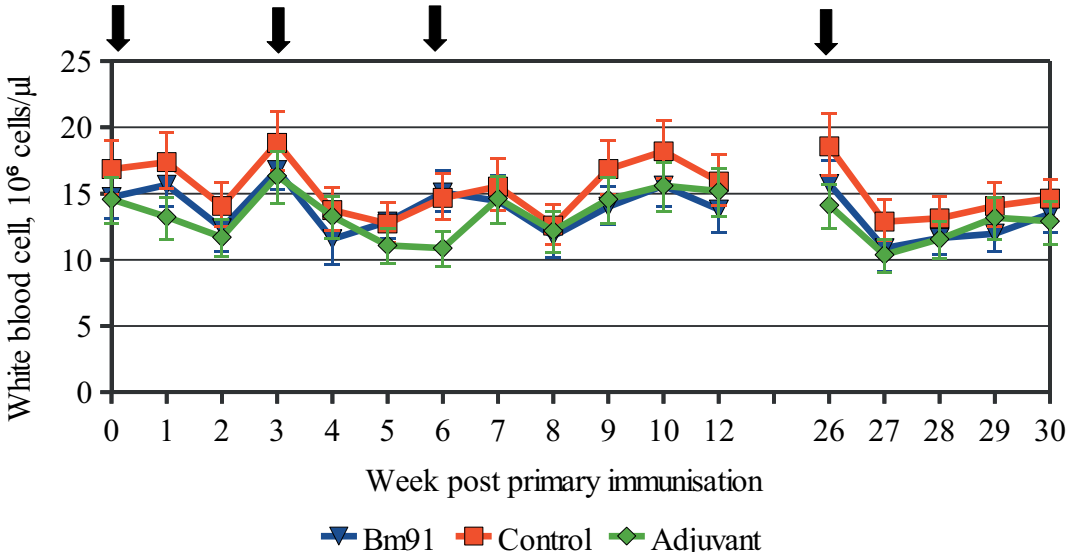

Figure 19: White blood cell count (WBC) of White Lamphun cattle immunised with the antigen Bm91 and injected with saline (Control) and adjuvant, respectively (LSM ± SE; N = 6). Arrows indicate the week of immunisation

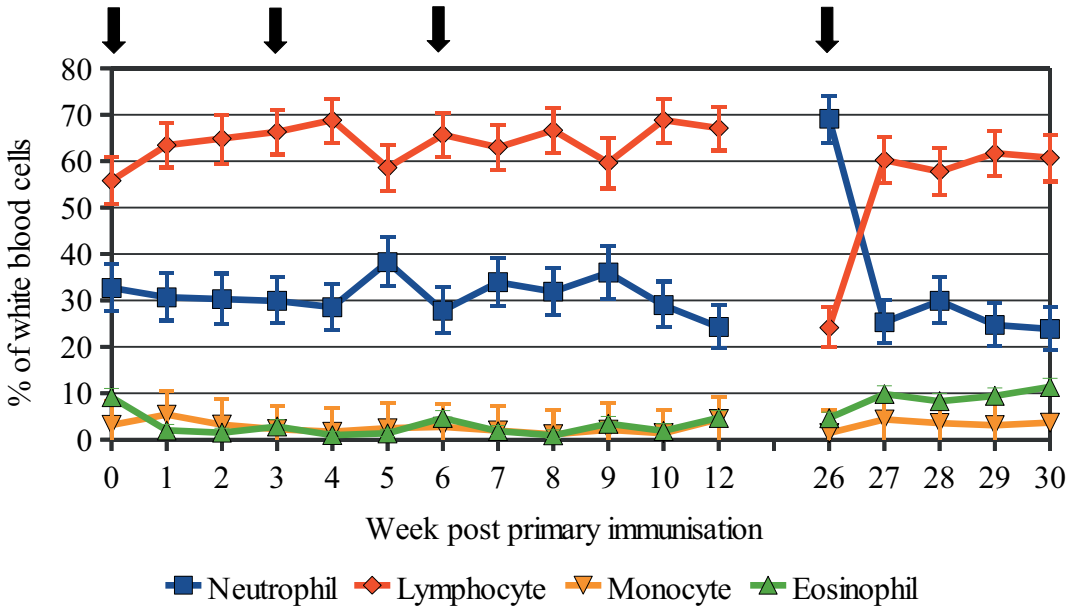

Figure 20: White blood cell differential of White Lamphun cattle immunised with the antigen Bm91 (LSM ± SE; N = 6). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

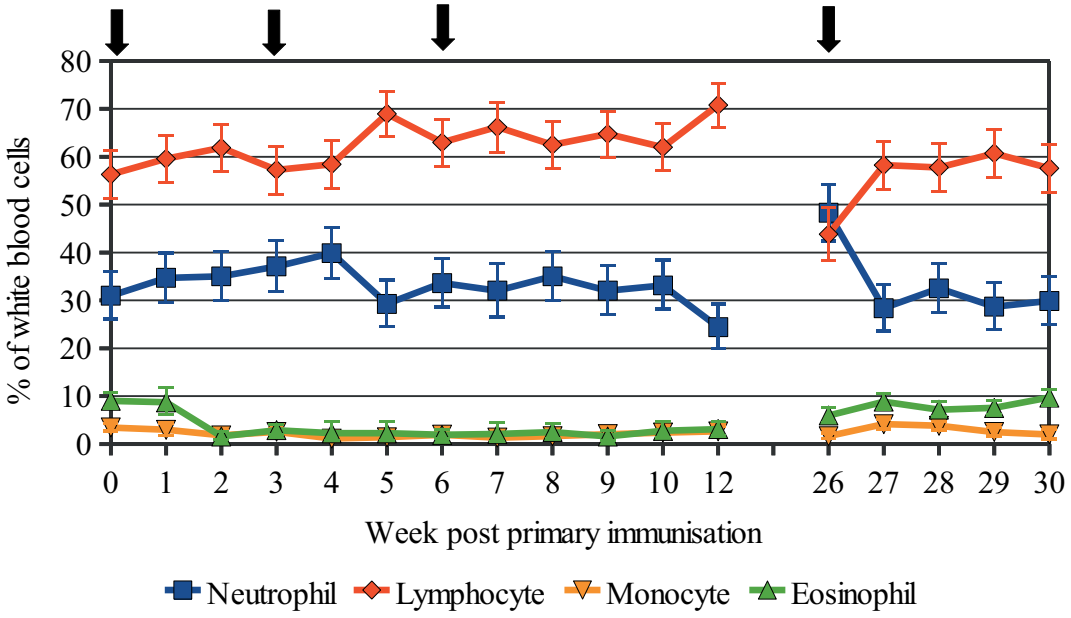

Figure 21: White blood cell differential of White Lamphun cattle injected with saline (Control) (LSM ± SE; N = 6). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

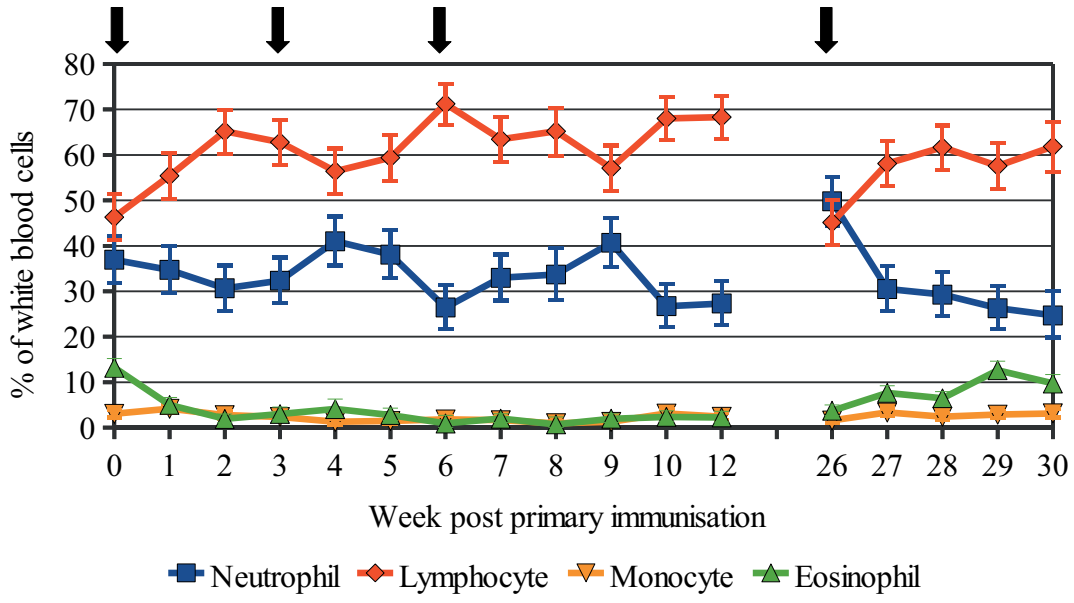

Figure 22: White blood cell differential of White Lamphun cattle injected with adjuvant (LSM ± SE; N = 6). Reference intervals: neutrophil 15 to 45 %, lymphocyte 45 to 75 %, monocyte 2 to 7 %, eosinophil 0 to 20 % (Kramer 2000). Arrows indicate the week of immunisation

Table 9: Probability values of treatment (T), week (W), and the interaction of the white blood cell differential calculated by repeated measures ANOVA

	Independent variable		
Dependent variable	Treatment	Week	T * W
WBC[1], 10^3 cells/µl	0.4716	< 0.0001	< 0.9862
Neutrophil[2], %	0.8097	< 0.0001	0.8606
Lymphocyte, %	0.9642	< 0.0001	0.5351
Monocyte, %	0.0524	< 0.0001	< 0.0001
Eosinophil, %	0.2542	< 0.0001	0.9830
N:L ratio[3]	0.9059	< 0.0001	0.7059

[1] WBC, white blood cell count analysed after log-transformation.
[2] Proportion of neutrophil, lymphocyte, monocyte, and eosinophil on WBC analysed after arcsin-transformation.
[3] N:L ratio, neutrophil:lymphocyte ratio analysed after log-transformation.

The correlations of the body temperature and the haematological parameters are given in Appendix 13. A correlation of around -0.30 ($P < 0.01$) between body temperature and PCV, Hb, and RBC was found in all three treatment groups. The correlation between WBC and RBC was negative (-0.20, $P < 0.05$) in the control and positive (0.22 and 0.21, $P < 0.05$) in the Bm91 and adjuvant group, respectively. Positive correlations between WBC and Hb were detected in the Bm91 (0.38; $P < 0.01$) and the adjuvant group (0.21; $P < 0.05$). In addition, the leukocyte count was positively correlated with PCV in the Bm91 group (0.22; $P < 0.05$).

6.2.6 Tick-borne diseases

All animals were detected to be infected with *Babesia* spp., whereas none of the blood samples was PCR positive for *A. marginale* or *Trypanosoma evansi*. In both the control and the adjuvant group one cattle was found to be infected with *Theileria orientalis*.

6.2.7 Average daily weight gain

The average daily weight gain was 203, 174, and 175 g/d (PSE = 11) for the control, the adjuvant, and the Bm91 group, respectively. Differences between the groups were not significant ($P > 0.05$).

7 DISCUSSION

7.1 Study design

The aim of the present study was to evaluate the humoral immune response against the Bm91 antigen in *B. indicus* cattle and to assess the efficacy of the immunisation on the reproductive performance of *R. (B.) microplus* under field conditions of natural infestation. Therefore, two immunisation trials were conducted in Northern Thailand. The region is characterised by a tropical climate and the onset of the trials was timed at the later part of the dry season which is followed by the rainy season.

Trial 1 was conducted on the RJ Ranch (Owner: Mr. Changrung Chantaboon) in Doi Tao, Chiang Mai district, under practical conditions. Two *B. indicus* breeds were used. On the one hand White Lamphun cattle as an example of indigenous Thai cattle which are highly adapted to the local environmental conditions and on the other hand Brahman cattle as an example of an exotic breed were included into the trial. Six animals of each breed were immunised with the antigen Bm91 and the other 6 animals remained as control animals. The antigen was derived from a Thai *R. (B.) microplus* strain. Three immunisations with a 3-week interval in-between were given. Weekly blood samples were taken for 3 months to measure the anti-Bm91 antibody level by indirect ELISA.

Trial 2 was conducted at the Department of Animal and Aquatic Science of the Chiang Mai University. Eighteen female White Lamphun cattle were randomly allocated to 3 groups of 6 animals each. The Bm91 group was immunised with the same antigen formulation as in trial 1 and the two other groups received saline and adjuvant, respectively. Three immunisations with a 3-week interval in-between were followed by a 4th immunisation after 6 months (week 26 ppi). The antibody level was monitored for 3 months following the primary dose and for 1 month following the 4th immunisation (week 26 until week 30 ppi). Standard engorged female ticks (4.5 to 8 mm) were collected daily after the 3rd and the 4th immunisation dose for a total period of 10 weeks. The reproductive capacity was evaluated by recording the egg mass and the egg viability.

7.2 Cattle breeds

The two breeds used in the present study contribute an important number of animals to the livestock population in Thailand. During the last decade the livestock production increased rapidly and the cattle population in Thailand grew from 4.6 to 9.1 million head (DLD 2009a). About 70 % of the population are Thai indigenous cattle (DLD 2009b). These indigenous cattle can be categorised by region into Northern, North-eastern, Southern and Central ecotype (DAD-IS 2010). As one of the four Thai ecotypes, White Lamphun are indigenous to Northern Thailand. The animals have been selected over generations for survival under stressful environmental conditions, especially heat stress and endo- and ectoparasite infections (Intharatham, 2002). Results of this selection are a relatively high fertility and a high ability to use low quality roughage. Like all zebu breeds the animals have a hump over the top of the shoulder and neck. Characteristics of White Lamphun are the white coat colour, their light hoofs and horns, and their pink-coloured nose. Initiated by governmental activities the crossbreeding of indigenous cattle with exotic breeds is widely practised. Among the exotic breeds imported to Thailand the most important ones are the *B. indicus* breeds Brahman and the *B. taurus* breeds Charolais, Simmental, and Holstein-Friesian (Na-Chiangmai, 2002).

In contrast to White Lamphun, Brahman is a breed intensively selected for high beef production. Through centuries of exposure to harsh environmental conditions including feed quantity and quality, insect pests, parasites, diseases, and weather extremes in India - its country of origin - the breed developed disease and parasite resistance as well as heat tolerance. Other characteristics of Brahman are the long ears and loose skin at the dewlap. Compared to taurine breeds the cattle have highly developed sweat glands. Their disease and parasite resistance as well as their heat tolerance emphasise the suitability of this breed for the environmental conditions of Thailand. The Brahman animals of the present study were descendants of animals which were imported from Australia about 7 years prior to the study. Thus, they can be considered as exotic to the local environmental conditions. In conclusion, a minor adaptation was expected for Brahman when compared to White Lamphun. Taking the superior tick resistance of *B. indicus* into consideration (Seifert 1971; Utech et al. 1978), this would be expressed in a

greater tick resistance of White Lamphun. Therefore, a more distinctive immune response of the indigenous breed and as a consequence a greater effect on the reproductive tick performance was assumed. A direct comparison of the immune response to anti-tick antigens and its efficacy between cattle breeds is not found in the literature. Likewise, studies assessing the vaccine efficacy against *R. (B.) microplus* under field conditions of natural infestation are very limited. Furthermore, immunisation trials found in the literature are usually conducted with *B. taurus* breeds. Though, the high proportion of *B. indicus* in Thailand and in many other tropical and subtropical countries warrants the inclusion of these breeds into immunisation trials. Particularly in countries with large zebu populations the application of vaccines is a valuable tool for a more sustainable tick control strategy. In combination with the relatively high host resistance of *B. indicus*, chemical treatments might be reduced substantially. Similar effects are expected when *B. indicus* x *B. taurus* crossbreds are used.

7.3 Sequence variations between *R. (B.) microplus* strains

Whenever tick-protective antigens are evaluated the sequence variation of *R. (B.) microplus* strains has to be taken into consideration. In dependence of the tick strain the antigen is derived of, the effect on the tick reproduction may differ substantially. This relationship is well described for the tick gut antigen Bm86 which is used in the commercial vaccines TickGARD$^{PLUS\ TM}$ and Gavac^{TM} (García-García et al. 1999). The authors studied the divergence of the amino acid sequence between the recombinant Bm86 protein used in the vaccine formulations and native Bm86 expressed in ticks from different geographical regions of the world. Based on the calculation of the mutation fixation index, a tendency of an inverse correlation between the vaccine efficacy and the sequence variations in the antigen locus was found. Hereby, the mutation fixation index was applied to the Bm86 sequence containing the amino acids 539 to 573. The study included cattle tick strains from Australia, Mexico, Cuba, Argentina, and Venezuela. Between the strains from the different locations the sequence of the 35 amino acid fragment diverged from 5.7 to 8.6 %. It was concluded that the sensitivity to the vaccination decreases, if the amino acid sequence divergence is greater than 2.8 %.

Analysing the Bm86 and the Bm95 gene of 30 strains from various geographic regions of Brazil, Argentina, Uruguay, Venezuela, and Colombia variations of 1.8 to 3.7 and of 3.4 to 6.1 % were detected in the nucleotide and amino acid sequences of Bm86, respectively (Sossai 2005). For the Bm95 sequences of the different tick strains slightly lower values were found. The suggestion that a geographic variation between *R. (B.) microplus* strains exists was recently confirmed by the study of Bastos et al. (2010). The amino acid sequence of Bm86 from cattle ticks collected in South Texas, USA, was compared to the Bm86 sequence from the Cuban Camcord and the Australian Yeeronpilly strain. From this two strains the Bm86 antigen of the commercial vaccines TickGARD$^{PLUS\ TM}$ and Gavac^{TM} is derived. Between the isolates from the USA and Australia a divergence of 8.3 % was found.

7.4 Bm91 antigen

In early studies it was hypothesised that vaccine formulations with other antigens than Bm86 might enhance the efficacy against tick infestations (Willadsen et al. 1988; Willadsen et al. 1989). A number of tick-protective antigens were isolated afterwards. One of these antigens is Bm91. It was isolated in Australia and has distinct similarities with the previously isolated Bm86 (Riding et al. 1994). Exhibiting a molecular weight of approximately 86 kDa, it shares several biochemical and enzymatic properties with the mammalian angiotensin-converting enzyme (Jarmey 1995). Forty-two percent of the amino acid sequence are equal between Bm91 and the mammalian enzyme. This enzyme plays a central role in mammals in the control of blood pressure, in fluid and electrolyte homeostasis, and possibly in reproduction and immunity (Ehlers & Riordan 1989). The natural substrate of the carboxy-dipeptidase has not been identified yet. Parallels between possible functions are explained by the fact that the tick salivary gland is not only responsible for the secretion of substances needed for the establishment of the tick on the host, but also for the maintenance of the salt and water balance and the secretion of pharmacologically active compounds in the host (Binnington & Kemp 1980; Jarmey 1995).

Bm91 can be distinguished from Bm86 by a number of factors (Riding et al. 1994). The removal of Bm86 from Bm91 preparations by polyclonal anti-Bm86 antibodies

indicated the lack of a detectable immunologic cross-reaction between them. Furthermore, cattle vaccinated with Bm91 did not produce antibodies to Bm86. In addition, antibodies to native Bm91 did not react with tick-derived Bm86 on Western blots. Similarities between the amino acid sequences were not found. The presence of soluble Bm91 could not be demonstrated. This confirms that Bm91 is a 'concealed' antigen. Beyond this, cattle exposed to ticks over an extended period of time did not develop antibodies to the protein in ELISA and Western blot analysis. At the same time, vaccinations with low amounts of the protein induced anti-Bm91 antibody production. Immunoflourescence staining showed that high concentrations of Bm86 are present on the tick gut cells, while Bm91 is present in relatively high concentrations in the salivary gland and in lower concentrations on the gut cells of *R. (B.) microplus*. Due to the fact that Bm91 is present in the salivary glands as well as in the saliva a further mode of action on the ticks is possible beside the midgut antigen Bm86.

Bm91 was tested in an immunisation trial in combination with Bm86 (Willadsen et al. 1996). The authors immunised Hereford steers two times with 200 µg of Bm86 or with 100 µg of each Bm86 and Bm91. Thereafter the cattle were challenged with *R. (B.) microplus* larvae for a period of 3 weeks. The results showed that the presence of antibodies to Bm91 significantly contributed to the effect on the weight of eggs per tick. When inoculated with both antigens the reproductive capacity of the ticks was lower. Even though not studied intensively after this encouraging results, Bm91 is a candidate for controlling *R. (B.) microplus* in Thailand. It was cloned from a *R. (B.) microplus* strain indigenous to Thailand (Kaewhom et al. 2008a). By using this antigen in the present trials, a divergence between the recombinant protein of the vaccine and the native antigen of the tick could be excluded.

7.5 Humoral immune response

The antibody titre was demonstrated to be the major determinant of the effect of anti-tick vaccines (Willadsen et al. 1995). A broad correlation was found between the titre of antibodies to Bm86 and the efficacy of the vaccination (de la Fuente et al. 1998). This positive correlation of the antibody titre with the vaccine efficacy could be confirmed not only for Bm86 (Almazán et al. 2010; Andreotti 2006), but also for Bm91 (Willadsen

et al. 1996), and Bm95 (Kumar et al. 2009). In conclusion it permits the evaluation of the vaccine efficacy through the measurement of antibody titres in immunised animals (de la Fuente et al. 1999). Given the primary immunisation followed by 2 booster doses with 3 weeks in-between, the time scheme of the present trials is consistent with other vaccination trials. The schedule with 3 initial doses followed by booster doses in 6 month intervals is furthermore in agreement with the recommendations of the manufacturers of the commercial vaccines.

In both trials the immunised animals developed a strong and specific humoral immune response expressed by high anti-Bm91 antibody levels. The Bm91 groups of both trials responded immediately after the primary immunisation. This response was followed by a further increase of the antibody level after the 2 subsequent immunisation doses. Thereafter, a stable level which was maintained until week 12 ppi was reached. In trial 2 the antibody level decreased only slightly until week 26 ppi. After the 4th infection the antibody level increased moderately and the highest level was measured during the weeks 27 to 30 ppi. In both trials the anti-Bm91 level was about 2 OD units higher in the immunised groups than in the control and adjuvant groups. The variation within the Bm91 groups was higher in trial 1 than in the other trial. A significant difference between the indigenous and the exotic breed was not found in trial 1.

Contrary to the results of the present trials, the most pronounced rise in anti-Bm86 and anti-Bm95 titres was not measured after the 1st but after the 2nd immunisation in the study of García-García et al. (2000). As well not in agreement with the Bm91 course of the present study, is the steady decrease of the Bm86 and Bm95 titres after their peaks 2 weeks after the 2nd immunisation. Using synthetic peptides derived from Bm86 resulted in antibody levels which were consistent with the aforementioned study (Patarroyo et al. 2002). As observed in the previously mentioned study of García-García et al. (2000), immediately after the peak of the antibody level a decline was noticed. Low responses to the primary immunisation with TickGARD$^{PLUS\ TM}$ and Gavac^{TM} were also measured by Andreotti (2006). In agreement with the aforementioned Bm86 and Bm95 studies the antibody level peaked 2 weeks after the 2nd immunisation. A distinct decrease followed after the peak. A low response to the first two immunisations (week 0 and 4) was as well shown in the retrospective analysis of the use of Gavac^{TM} in the field (Valle et al. 2004).

As observed under controlled conditions by Rodríguez et al. (1995b), a strong increase of the antibody level was apparent after the 3rd Gavac™ treatment (week 7). The peak 2 weeks after this dose was followed by a strong decline. Anti-Bm95 antibody levels showed a similar course with a strong rise following the 3rd immunisation in the study of Kumar et al. (2009). The short duration of 'protective' titres in some animals in the trial of Jonsson et al. (2000a) is not in agreement with the immunised groups of this study. Contrarily, the anti-Bm91 antibodies of the White Lamphun and Brahman animals were maintained at a high level throughout the observation period in both trials. Particularly the high level observed prior to the 4th immunisation after 6 months contrasts other studies. Within 6 months antibody levels decreased to pre-immunisation levels in the study of García-García et al. (2000). A similar decline was described by Rodríguez et al. (1995b), Valle et al. (2004), and Andreotti (2006). In broad agreement with the course of the anti-Bm91 level measured here, are the ones for Bm95 and Bm86 obtained by Jittapalapong et al. (2010).

The insignificant difference between White Lamphun and Brahman shows that the intensity of the immune response was not influenced by the assumed difference in the adaptability to the local environmental conditions. However, it has to be mentioned that both breeds were of *B. indicus* origin. Hence, the difference in tick resistance might have been too low to induce a significant difference in the immune response. The comparison with the anti-Bm95 level of *B. taurus* animals (Jittapalapong et al. 2010) does not imply that a difference between zebu and taurine breeds exists. In the mentioned study Holstein-Friesian steers were immunised with Bm95 derived from a local *R. (B.) microplus* strain. The resulting antibody level showed a similar course than observed here for White Lamphun and Brahman.

The low variation between the immunised White Lamphun of trial 2 and the slightly higher variation in White Lamphun and Brahman in trial 1 indicates that the Thai Bm91 antigen is strongly immunogenic and induces a strong and long-lasting antibody response in cattle. The higher variation within the groups after the primary immunisation reveals that not all animals responded immediately to the 1st injection. In fact, in one animal of the immunised White Lamphun and in two animals of the immunised Brahman group of trial 1 an increase after the 1st immunisation was not

visible. However, this finding is not uncommon. Rodríguez et al. (1995a) stated that 6 % of the animals failed to respond properly to Bm86. One out of 16 animals reacted poorly to both Bm91 and Bm86 in the study of Willadsen et al. (1996). In general, higher variations within immunised groups than found here are usually reported in the literature. For example, this was described for the anti-Bm86 titres of Holstein-Friesian dairy cows in the study of Jonsson et al. (2000a). Similarly, the anti-Bm86 antibody level to both commercial vaccine formulations was more variable than reported here (Andreotti 2006).

Comparing 3 immunisation schemes with Gavac™ vaccinations (week 0, 4, and 7 vs. week 0 and 4 vs. week 0 and 7), the number of vaccinations and the interval between the treatments was not found to affect the antibody level (Vargas et al. 2010). This suggests the possibility of reducing the number of immunisations. Though not significantly different to the two other schemes, the scheme with 3 immunisations induced the highest antibody level which was maintained over at least 6 months. Therefore, constantly high antibody levels can be expected when animals are re-immunised every 6 months. However, studies monitoring the antibody level over more than 8 months are not found in the literature. In conclusion, it can be stated that independent of the cattle breed Thai Bm91 induced a strong and long-lasting humoral immune response in cattle.

7.6 Western blot analysis

The ELISA results could be confirmed by the Western blot analysis. The 86 kDa band which is consistent with recombinant Bm91 from the Thai *R. (B.) microplus* strain was recognised from the 3rd week ppi onwards. Hence, the glycoprotein elicited specific antibodies that bind to *R. (B.) microplus* proteins. Riding et al. (1994) showed that antibodies against native Bm91 do not react with tick-derived Bm86 on Western blots. This proved that there is no detectable immunologic cross-reaction between these two antigens. Cross-reactive epitopes between the Bm86 and its orthologues Bd86 and Ba86 were shown by Canales et al. (2008). Sera of cattle which were immunised with synthetic Bm86 peptides (Patarroyo et al. 2002) or with glycoproteins isolated from *R.*

(B.) microplus and *H. anatolicum anatolicum* (Ghosh et al. 2005) revealed similar Western blot results than found here.

7.7 Tick infestation

In both trials the animals were raised in an extensive grazing system. This system is commonly practised for beef cattle farming in Thailand. Studying the immunisation against ticks under these conditions has the advantage that the actual effect on the natural tick infestation can be assessed. With artificial infestations this effect can only be simulated. A drawback of artificial infestations is that the animals have to be separated from each other to prevent grooming. Therefore, the vaccine efficacy antigens have shown against artificially infested ticks must also be confirmed under field conditions. The natural tick population depends on a variety of factors with rainfall determined as the main cause of the variation complicating the prediction of the tick population size (Lima et al. 2000). Jonsson et al. (2000a) highlighted the difficulty of conducting field trials that are dependent on field tick infestations. In the study of Willadsen et al. (1995), which was carried out with natural infestations, the tick infestation reached unethically high levels. To avoid this in the present study, solely animals of *B. indicus* origin were used. Raising highly tick-susceptible taurine breeds in free-grazing systems in most parts of Thailand is impractical because of the high exposure to tick infestations. In spite of the superior host resistance of zebu breeds, tick infestations in free-grazing systems are commonly observed in Thailand (Changbunjong et al. 2009; Saratapan et al. 1998). However, zebu breeds only require chemical tick control for a distinct period of the year when tick loads are exceeding distinct levels. Though, in tropical and subtropical regions cattle are infested by ticks year-round (Walker et al. 2003). This particularly occurs where the highly productive *R. (B.) microplus* cattle ticks are endemic (Madder et al. 2010). It is the case in South-East Asia where this tick species originates from. Albeit, peak infestations usually occur during the rainy season (Turner & Short 1972). Gomes et al. (1989) observed the *R. (B.) microplus* infestation of indigenous as well as of crossbreds between indigenous and exotic breeds in Brazil over 39 months. Independent of the genotype the animals were

infested during the whole period. Beside this, a seasonal pattern with the infestation peaking in the rainy season was described.

In order to schedule the tick collection at the expected peak infestation during the rainy season, and to ensure that the antibody level has reached a stable level until the peak infestation, the primary immunisation was given in the late dry season. In spite of the high tick infestation expected, the number of ticks collected after the 3rd immunisation in trial 1 was extremely low. Therefore, the effect of the immunisation on the reproductive tick performance in trial 1 could not be statistically analysed. After a regular course of the summer season, the rainy season started after the 2nd treatment. Thus, the low tick population may be only partly been explained by climatic factors. Another factor which has to be taken into account is the low cattle density at the private breeding farm of trial 1. This might have reduced the tick population because the ticks could not find enough hosts to sustain the population size during the summer season and to increase it after the onset of the rainy season.

7.8 Tick reproductive performance

In contrast to trial 1 the tick infestation was higher in trial 2. However, it has to be mentioned that most of the ticks were collected during the period from week 27 to 30 ppi. As the ELISA results demonstrated, all ticks that attached to an Bm91 immunised cattle were subjected to the ingestion of anti-Bm91 antibodies with the blood meal. It is widely described in other studies that the ingestion of blood containing antibodies to Bm86 causes the lysis of the digest cells (Rand et al. 1989). This results in leakage of blood into the haemocoel. Finally, the tick may die from the ingestion of the antibodies. Similar mechanisms are ascribed to the ingestion of Bm91 antibodies (Riding et al. 1994). Varying efficacies of tick-protective antigens on the number of adult female ticks, the egg laying capacity, and the egg viability are reported in the literature. An overall vaccine efficacy of 90 % was calculated when Bm86 was tested the first time under controlled conditions with artificial *R. (B.) microplus* infestation (Tellam et al. 1992). The number of engorging ticks and their weight was reduced by 20 to 30 %, whereas ticks collected from immunised animals showed a 60 to 80 % reduced egg viability. Rodríguez et al. (1994) treated cattle with the same antigen and found

reductions of 50 and 70 % in the average weight of ticks and their reproductive capacity, respectively. After these initial studies a number of other immunisation trials with Bm86 as well as with several other antigens were conducted. Willadsen et al. (1995) found a significantly lower number of ticks on Bm86 immunised steers under field conditions. Under artificial infestation the same Bm86 formulation reduced the number of ticks and the reproductive performance by 56 and 72 %, respectively (Jonsson et al. 2000a). Using both commercial vaccines Andreotti (2006) found a protection efficacy of 49 and 46 % for Gavac™ and TickGARD™, respectively. García-García et al. (2000) vaccinated cattle with Bm86 and Bm95 derived from Cuban tick strains. Thereafter, the animals were challenged with *R. (B.) microplus* larvae of either the Cuban Camcord strain or the Australian Yeerongpilly strain. Against the Cuban tick strain efficacies of 84 and 89 % were calculated for Bm86 and Bm95, respectively. Contrarily, the protection against the Australian strain was 0 and 54 % for Bm86 and Bm95, respectively. In a recent study, Almazán et al. (2010) found Bm86 to reduce the number of ticks, the egg weight, the oviposition, and the egg fertility by 51, 5, 14, and 6 %, respectively. Similar vaccine efficacies were also reported for the Bm86 homologues Ba86, Bd86, and Haa86 against *R. (B.) annulatus* (Canales et al. 2008), *R. (B.) decoloratus* (Canales et al. 2009; Canales et al. 2008), and *H. anatolicum anatolicum* (Azhahianambi et al. 2009; Jeyabal et al. 2010), respectively. Among the other potential candidates the most promising results were achieved with the synthetic peptide Sbm7462 (Patarroyo et al. 2002), subolesin (Almazán et al. 2010), and 64P (Trimnel et al. 2005).

Concordant values for Bm91 are not found in the literature because this antigen was not tested as a stand-alone antigen in immunisation trials. The effect was only estimated when the antigen of the present study was used in combination with Bm86 (Willadsen et al. 1996). For each 10-fold increase in antibody titre to Bm86 the weight of eggs laid per tick decreased by 24 mg, while a 10-fold increase in anti-Bm91 titre reduced the egg weight by 7.2 mg.

In conclusion, the most pronounced effect of anti-tick antigens is rather seen in the reproductive tick performance than in the direct reduction of engorging ticks. Nevertheless, the effect varies between the different parameters describing the

reproductive tick performance. This emphasises the prophylactical use of vaccines with the greatest effect seen in a gradual reduction of the tick population.

In comparison to other reports, the effect of Bm91 on the tick parameters, particularly on the REI and the egg viability, found in the present study was low. Regarding the number of ticks, it has to be taken into account that the animals were naturally infested with *R. (B.) microplus* ticks. The collection of standard engorged ticks includes the risk that not all ticks can be collected. Ticks may be removed by grooming or mechanically when the animals are grazing. The high variation of tick counts between animals indicates the skewed distribution of ticks in a herd as explained previously (Jonsson et al. 2000b; Utech et al. 1978). The study of Jittapalapong et al. (2004) was conducted under comparable conditions to the present study. Nevertheless, the tick infestation was considerably higher. Even though the reduction of engorging ticks is not the major effect of anti-tick immunisations, the tick infestation of immunised animals is usually reduced. This could not be observed in the present study. Likewise, an effect of Bm91 on the tick weight and the percentage of ticks ovipositing was not recorded. Similarly, Almazán et al. (2010) did not find a reduction in tick weight and the percentage of ovipositing ticks for subolesin, although this antigen showed a 51 % vaccine efficacy. However, the 6 and 8 % reduction of the REI and the egg viability, respectively, indicates that Bm91 affected the reproductive tick performance negatively. But these effects are considerably lower than found in the above mentioned studies. Despite the significantly reduced REI and the egg viability, distinctly greater effects on the tick reproduction are necessitated in order to control the highly reproductive *R. (B.) microplus* ticks by immunisation.

The inclusion of the adjuvant group in trial 2 was due to observations made in a preliminary study (Jittapalapong et al. 2004). In this study the injection of the adjuvant alone had an effect on the tick parameters. As observed here, the proportion of ovipositing ticks was slightly reduced in this group. However, the inclusion of one control group either injected with saline or with adjuvant is recommended for further immunisation experiments.

7.9 Tick-borne diseases

The high infection rate of the experimental animals of both trials with *Babesia* spp. indicates the endemic stability of these protozoa in Thailand. Comparable prevalences were found in the studies of Nishikawa et al. (1990) and Iseki et al. (2010). In contrast, the high prevalence of *A. marginale* found in a survey of seven provinces throughout the country could not be confirmed by PCR analysis (Phrikanahok et al. 2000). Due to the endemic stability, no clinical disease was caused by *Babesia* spp. This was supported by the haematological parameters. Neither anaemia nor high MCV values indicated clinical *Babesia* spp. outbreaks. Both are signs for blood loss caused by haemoparasites (Schalm et al. 1975).

Despite the infection with *Babesia* spp., the animals developed a strong humoral immune response against Bm91. Therefore, in regions with endemic stability of *Babesia* spp., cattle can be inoculated with tick-protective antigens without considering the *Babesia* spp. infection. This is particularly important because under practical conditions the vaccine is applied to animals which are commonly infected with TBD. The impact of tick vaccines on the transmission of TBD can only be assumed (de la Fuente & Kocan 2007). Initial trials using Bm86 vaccines resulted in a reduction of the incidence of babesiosis (de la Fuente et al. 1998). However, a reduced transmission of *A. marginale* was not observed. Recently, Bastos et al. (2010) recommended the use of Bm86 to control tick infestations particularly in *B. bovis* endemic areas. Gene silencing of Bm86[10] decreased the number and the survival of engorged female ticks and the weight of the egg masses. Contrarily, the efficiency of transovarial transmission of *B. bovis* from surviving female ticks to their larval offspring was not affected by the gene silencing. These initial studies indicate the effect of tick-protective antigens on the transmission of TBD. This is particularly important because these diseases will probably be controlled more efficiently by anti-tick vaccines than by TBD vaccines.

10 In the study silencing of the Bm86 gene was done by injecting double stranded RNA identical to the *R. (B.) microplus* Bm86 gene into freshly moulted unfed female ticks. The ticks were then fed on cattle during acute *B. bovis* infection.

7.10 Haematological parameters

The CBC is a valuable tool for screening the general health and nutritional status. Beyond this, it indicates the adaptability to adverse environmental conditions (Barger 2003; Ndlovu et al. 2007). The evaluation can be broken down into the three sections erythrocytes, leukocytes, and platelets. The different parameters are influenced by several factors including breed, age, nutritional status, diseases, and stress (Kramer 2000). The fact that trial 1 ended well before the calving season and the animals were not pregnant in trial 2 minimises the error which might be introduced as a result of the pregnancy status. In order to evaluate the observed haematological parameters, they were compared to the reference intervals proposed for adult cattle by Kramer (2000). However, it should be mentioned that the experimental animals in the present study were of *B. indicus* origin, whereas the reference intervals were derived from *B. taurus* animals. Howes et al. (1957) observed greater values for PCV, Hb, MCV, RBC, and WBC counts in Brahman when comparing them to Hereford cattle. The greater values of *B. indicus* than of *B. taurus* breeds were confirmed by several other studies (Kirk & Davis 1970; Thahar et al. 1983).

The erythrocytes section includes values such as the PCV, Hb, RBC, MCV, and the MCHC. A decreased PCV, Hb, and RBC indicates anaemia. In generative anaemias which are caused by RBC destruction, elevated MCV values, and at the same time decreased MCHC values are commonly found. The total plasma protein can further assist the interpretation of the PCV. Elevated protein levels are associated with dehydration or inflammation while a decreased level in an anaemic animal could indicate blood loss. Anaemia is an inevitable consequence of heavy infestations with blood-feeding parasites. It is a common symptom of heavy *R. (B.) microplus* infestations (Jonsson 2006; Riek 1957). Evaluation of the leukocytes involves the interpretation of the WBC together with the WBC differential count. An elevated WBC is called leukocytosis. Inflammation is associated with leukocytosis including neutrophilia[11]. Due to the fact that the WBC count is a reflection of the numbers of cells in the circulating pool, leukocytosis is seen in excited animals. This leukocytosis is caused by an increasing number of lymphocytes and neutrophils. An elevated number of

11 An elevated neutrophil count is called neutrophilia.

eosinophils, called eosinophilia, is observed in hypersensitivity responses, e.g. in allergic or parasitic syndromes. Thrombocytosis - an elevated platelet count - may be caused by acute or chronic inflammation.

Taking all CBC parameters into account, the White Lamphun animals showed a superior health status to the Brahman animals in trial 1. Concerning the environmental conditions, the course of the haematological parameters of both breeds can be considered as normal. Regarding the erythrocyte parameters, values for White Lamphun were greater than for Brahman. This is consistent with results of other Thai studies conducted under similar climatic and environmental conditions. Dongpalee et al. (2007) studied the haematology of adult female White Lamphun cattle and observed values of 32 %, 9.2 g/dl, and 8.0 g/dl for PCV, Hb, and protein, respectively. A superior health status of indigenous cattle was also reported by Boonprong et al. (2007). In this study the blood biochemical profiles of Thai indigenous cattle and Simmental x Brahman crossbreds were analysed. Glucose, urea, protein, and different liver enzyme levels were higher in the animals originating from Thailand when compared to the other genotype. The differences between the two breeds found in the present study are as well consistent with the results of Kuha et al. (2009). The authors compared the CBC of healthy Nan cattle which are indigenous to Northern Thailand and their crossbreds with Brahman. Values of all erythrocyte parameters were greater for the indigenous animals. Nevertheless, differences between adult cows of both genotypes were not significant. As found in the present study, WBC counts of both genotypes were elevated. In fact, WBC counts were greater in Brahman (13.3 x 10^3 cells/µl) than in Nan cows (11.3 x 10^3 cells/µl), whereas the proportions of neutrophils and lymphocytes were 33 vs. 39 and 60 vs. 51 % for Brahman and Nan cattle, respectively. Considering the values observed for the other Thai indigenous cattle, the WBC counts of the present trials can be interpreted as a normal WBC differential. This could be confirmed by the correlations between WBC and erythrocyte parameters. In none of the groups an indication was found that a high WBC was associated with lower PCV, Hb, or RBC values. Albeit, the high WBC counts might be a reflection of inflammation and stress caused by the dry season. However, this was not reflected by the total plasma protein level which is associated with the evaluation of hydration status or possible haemorrhage (Stockham & Scott

2002). As a useful marker for acute and chronic active inflammation, the plasma protein profile of a particular individual is relatively constant over a considerable length of time (Coppo 2004). This constancy of the protein level is apparently controlled genetically. During the entire observation period, no abnormalities of the protein level were noted in any of the two trials.

Due to the fact that the experiment started in the later part of the dry season, the quantity and quality of the feed improved in the course of the observation period. While the first part of the dry season is characterised by feed shortage with low quality of the feed, the later part is the most critical with limited quantity and quality of feed (Boonprong et al. 2007). Reflecting the better adaptation of the indigenous animals to the environmental stress during the dry season, the difference in PCV and Hb between the White Lamphun and Brahman animals of trial 1 was most pronounced during the first weeks ppi. During this time the animals were still exposed to harsh conditions including feed shortage. The course of the body temperature reveals the exposure of the animals to the environmental conditions. The decreasing body temperature noticed in both trials reflected the increasing health status during the study. The lowest temperatures were recorded during the last part of trial 2 (week 26 to 30 ppi). Apart from the increasing health status the course of the body temperature decreased. Beside the improving health status, this may be due to the decreasing ambient temperature after the dry season. The breed x week interaction of trial 1 with the rate of change being higher for Brahman than for White Lamphun implies that the health status of the exotic animals is fluctuating to a higher degree between the different seasons.

Although the immunisations were timed at the later part of the dry season with a limited feed quality and quantity, the treatment did not influence the health status of the animals in both trials. During the dry season the animals are exposed to the greatest environmental stress, mainly due to feed shortage and low quality of the feed. Nevertheless, the health status of the animals was not influenced by the immunisation. In conclusion the animals can be immunised independent of the season. To gain the greatest effect the immunisation is recommended before the onset of the rainy season to ensure that the antibody level has reached a stable level during the rainy season when the highest tick infestation is usually observed.

7.11 Average daily gain

The weight gains observed in the present study reflect the influence of the season. Particularly the values of the indigenous breed of trial 1 can be considered as high. Though, data of comparable White Lamphun cows raised under similar conditions are not available in the literature.

With the duration of the study, the feed quality and quantity improved. Therefore, the compensatory weight gain following the dry season is suggested to be largely responsible for the weight gains. The difference between Brahman and White Lamphun is mainly attributed to the higher body weight of the former breed. Considering the body weight of the two breeds at the start of the study a higher difference in ADG between Brahman and White Lamphun was expected. This points to a higher compensatory weight gain in the White Lamphun animals. Given the adult body weight of 200 to 270 kg of White Lamphun cows (Wannapat 2004), the animals of trial 1 were within this range. Taking the age of three years in trial 2 into account the body weights were lower than reported in the above mentioned study. Due to the low tick infestation in the present study, an effect of the immunisation on the weight gain could not be expected. The positive effect of the immunisation on the body weight recorded elsewhere (Jonsson et al. 2000a) has to be substantially associated to the lower tick infestation on immunised animals over an extended period of time.

7.12 Conclusions

The present study consisting of two immunisation trials with the antigen Bm91 derived from local *Rhipicephalus (Boophilus) microplus* ticks was conducted under field conditions. Thereby, the effect of the immunisation on naturally infested *R. (B.) microplus* ticks could be assessed. The two *B. indicus* breeds used - on the one hand local White Lamphun and on the other hand exotic Brahman cattle - constitute a major proportion of Thailand's cattle population.

Based on the two immunisation trials the following conclusions can be drawn:

1. The anti-Bm91 ELISA and the Western blot analysis demonstrated that recombinant Bm91 derived from a Thai *R. (B.) microplus* strain induces strong

and long-lasting antibody responses independent of the *Bos indicus* breed. All immunised cattle responded to Bm91 to such an extent that the studied immunisation scheme with 3 primary immunisations and booster doses every 6 months can be recommended as appropriate in order to maintain adequate antibody levels.

2. The Bm91 immunisation does not affect the body temperature and the different parameters of the complete blood count, and thus does not influence the health status of the animals. Furthermore, the infection with the tick-borne disease *Babesia* spp. did not affect the vaccine response. Therefore, the immunisation with Bm91 can be applied to animals during periods of extensive stress, e.g. heat stress and reduced feed availability, and to animals in *Babesia* spp. endemic regions.
3. The reduced reproduction efficiency index and the reduced egg viability of the naturally infested *R. (B.) microplus* ticks collected from Bm91-immunised cattle in comparison to ticks collected from non-immunised cattle indicate that the antigen Bm91 has only a limited effect on the tick reproduction.

The use of anti-tick vaccines as part of an integrated tick control strategy in combination with acaricides for short-term control constitute the most promising method of a more sustainable tick control. Despite the limited effect of Bm91 on the tick reproduction, the high immunogenicity of the antigen under field conditions shown here warrants its further evaluation. In order to use Bm91 as part of the integrated tick control strategy more pronounced effects on the tick reproduction are necessitated. However, it is unlikely that a substantial effect on ticks can be achieved when Bm91 is used as a stand-alone antigen. Therefore, the evaluation of Bm91 under field conditions of natural *R. (B.) microplus* infestation in combination with other antigens, which have shown their efficacy under artificial *R. (B.) microplus* infestations, is recommended.

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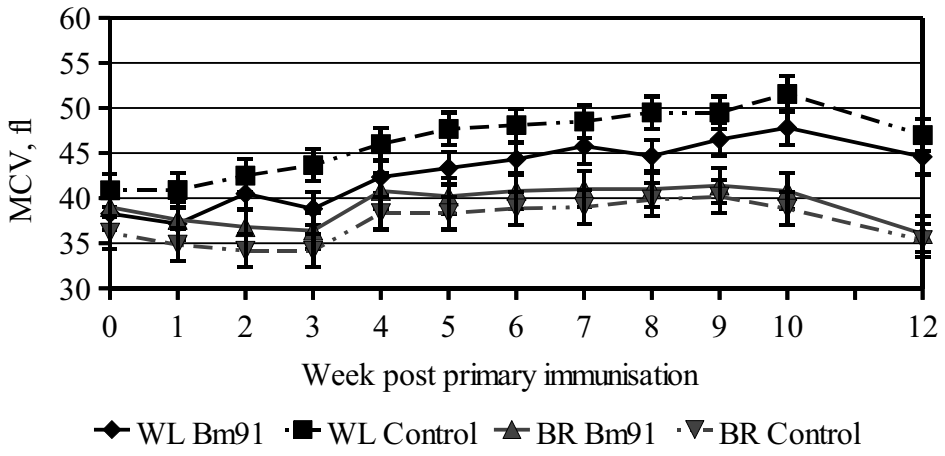
9 APPENDICES

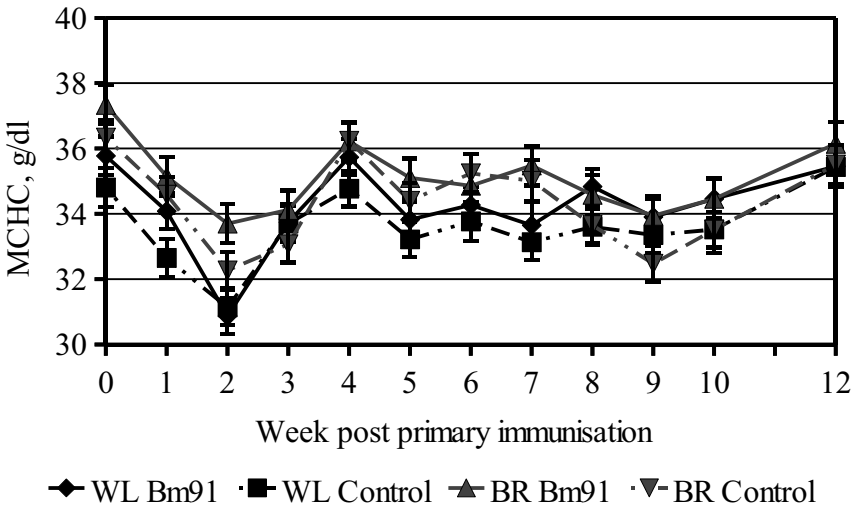
Trial 1

Appendix 1: Antibody level of White Lamphun and Brahman cattle immunised with Bm91 in week 0, 3, and 6. The values were determined by ELISA and are expressed as OD_{450nm} value (LS Means ± SE). The *P* value was derived from the repeated measures ANOVA

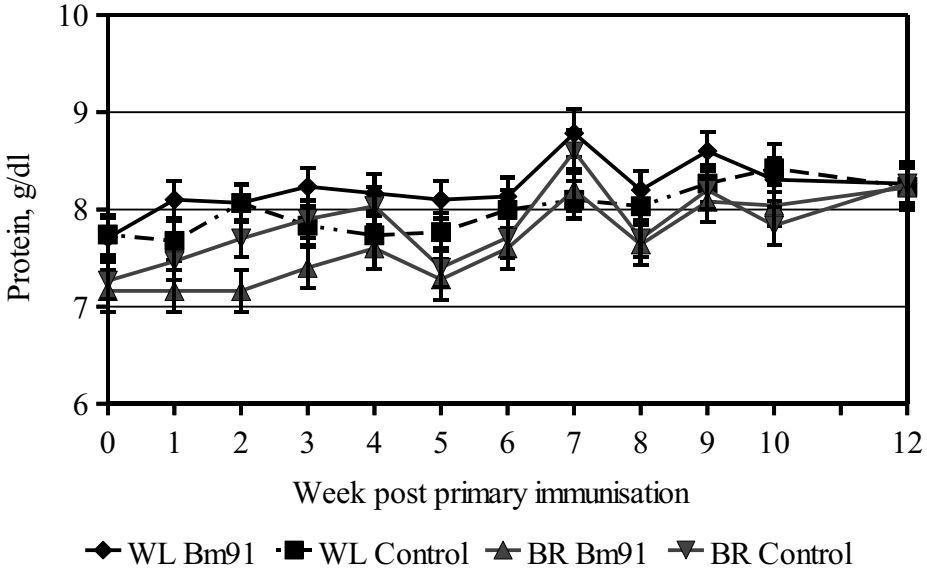
	White Lamphun (N = 6)		Brahman (N = 5)		
Week	LS Means	SE	LS Means	SE	*P* value
0	0.00	0.00	0.00	0.00	-
1	0.85	0.20	0.32	0.22	0.0977
2	1.36	0.20	1.03	0.22	0.2844
3	1.42	0.20	1.14	0.22	0.3551
4	1.75	0.20	1.46	0.22	0.3425
5	1.81	0.20	1.73	0.22	0.7921
6	1.85	0.20	1.67	0.22	0.5464
7	1.93	0.22	1.89	0.22	0.8860
8	1.95	0.20	1.93	0.22	0.9301
9	1.97	0.20	1.97	0.22	0.9976
10	1.92	0.20	1.93	0.22	0.9692
11	1.91	0.20	1.85	0.22	0.8342
12	1.94	0.22	1.93	0.22	0.0000



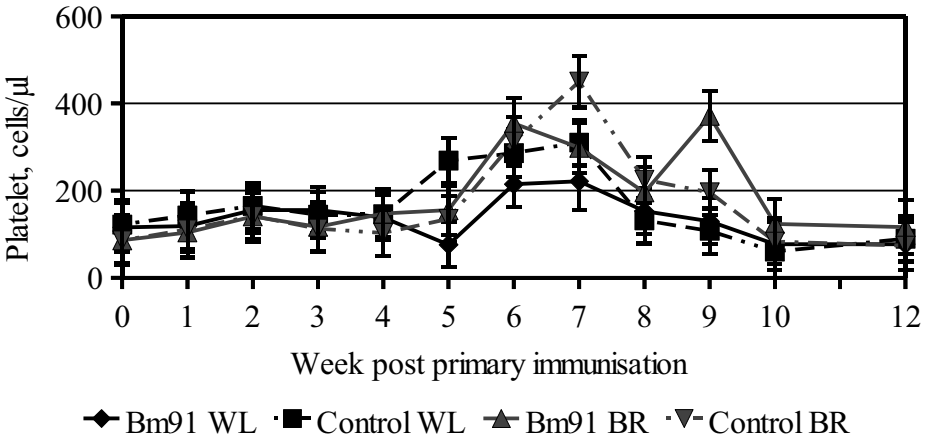
Appendix 2: MCV of White Lamphun and Brahman cattle immunised with the antigen Bm91 and injected with saline (Control) (LSM ± SE, N = 6; except for N = 5 for the BR Bm91 group). Reference interval: 40 to 60 fl (Kramer, 2000)



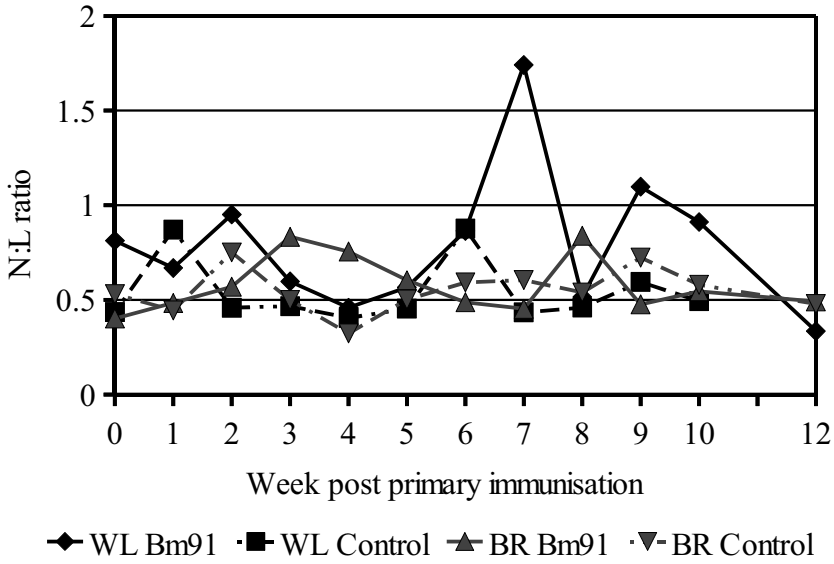
Appendix 3: MCHC of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 and injected with saline (Control) (LSM ± SE, N = 6; except for N = 5 for the BR Bm91 group. Reference interval: 30 to 36 g/dl (Kramer 2000)



Appendix 4: Protein of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 and injected with saline (Control) (LSM ± SE, N = 6; except for N = 5 for the BR Bm91 group. Reference interval: 7.0 to 8.5 g/dl (Kramer 2000)



Appendix 5: Platelet count of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 and injected with saline (Control) (LSM ± SE, N = 6; except for N = 5 for the BR Bm91 group. Reference interval: 100 to 800 cells/µl (Kramer 2000)



Appendix 6: Neutrophil:lymphocyte ratio of White Lamphun (WL) and Brahman (BR) cattle immunised with the antigen Bm91 and injected with saline (Control) (LSM ± SE, N = 6; except for N = 5 for the BR Bm91 group

Appendix 7: Correlations between body temperature and haematological parameters.

a) Group: White Lamphun, immunised

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	ns	ns	ns	-0.36**	ns	ns	ns	ns	0.28*	ns
PCV		0.89***	0.41**	0.38**	ns	ns	ns	ns	ns	0.30*
Hb			0.43**	0.37**	0.39**	ns	ns	ns	ns	0.31*
RBC				-0.47***	ns	ns	ns	ns	ns	ns
MCV					ns	ns	ns	ns	ns	0.40**
MCHC						ns	ns	ns	ns	ns
WBC							ns	ns	ns	ns
NEU								-0.96***	ns	ns
LYM									ns	ns
PLAT										ns

[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

b) Group: White Lamphun, Control

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PCV		0.89***	0.42**	0.31**	0.51***	ns	ns	ns	ns	0.40**
Hb			0.24*	0.39**	ns	0.48***	ns	ns	ns	0.44**
RBC				-0.69***	-0.25	ns	ns	ns	ns	-0.28**
MCV					ns	0.31**	ns	ns	ns	0.61***
MCHC						ns	ns	ns	-0.27**	ns
WBC							ns	ns	ns	0.31**
NEU								-0.95**	ns	0.28**
LYM									ns	-0.25*
PLAT										ns

[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

c) Group: Brahman, immunised

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	ns	ns	ns	ns	ns	ns	ns	ns	0.37**	ns
PCV		0.97***	0.89***	ns	-0.31*	ns	ns	ns	0.56***	0.46**
Hb			0.85***	ns	ns	ns	ns	ns	0.53***	0.51***
RBC				-0.61***	-0.37**	-0.33*	ns	ns	0.47**	0.33*
MCV					ns	0.54***	ns	ns	ns	ns
MCHC						ns	ns	ns	-0.34**	ns
WBC							ns	ns	-0.31*	
NEU								-0.98***	ns	ns
LYM									ns	ns
PLAT										ns

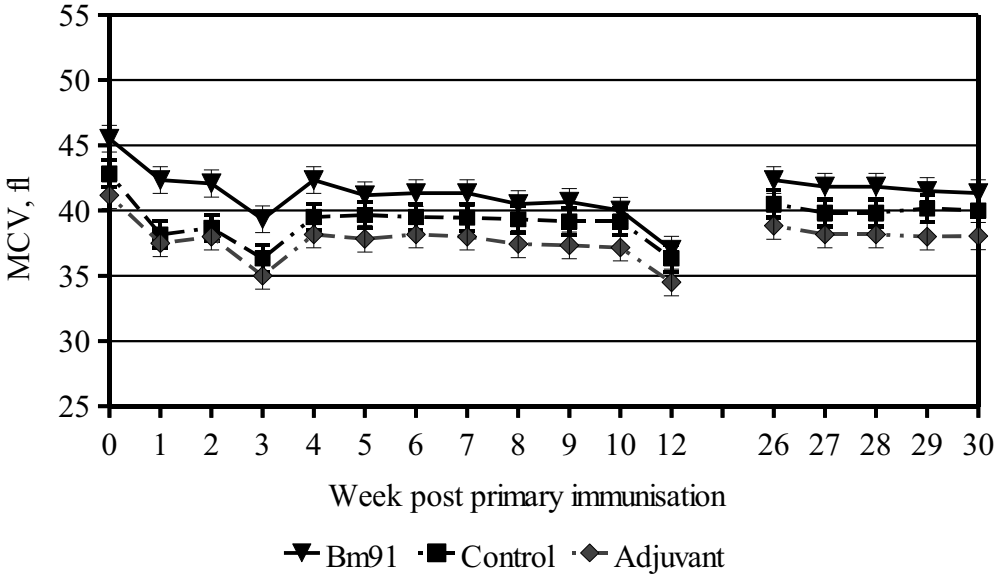
[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

d) Group, Brahman, Control

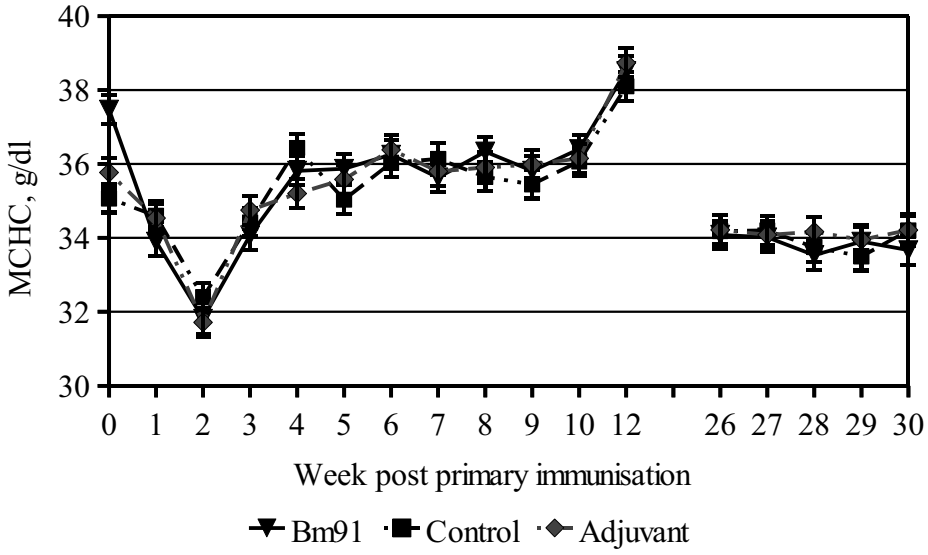
	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	0.32**	0.24*	0.46***	-0.40**	-0.33**	ns	ns	ns	ns	-0.26*
PCV		0.95***	0.83***	ns	-0.44**	0.54***	ns	ns	ns	ns
Hb			0.71***	ns	ns	0.51***	ns	ns	ns	ns
RBC				0.67***	-0.55***	0.54***	ns	ns	ns	ns
MCV					0.39**	-0.29*	ns	ns	0.28*	0.35**
MCHC						-0.30*	-0.24*	ns	ns	ns
WBC							ns	ns	-0.42**	0.25*
NEU								-0.95***	ns	ns
LYM									ns	ns
PLAT										ns

[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

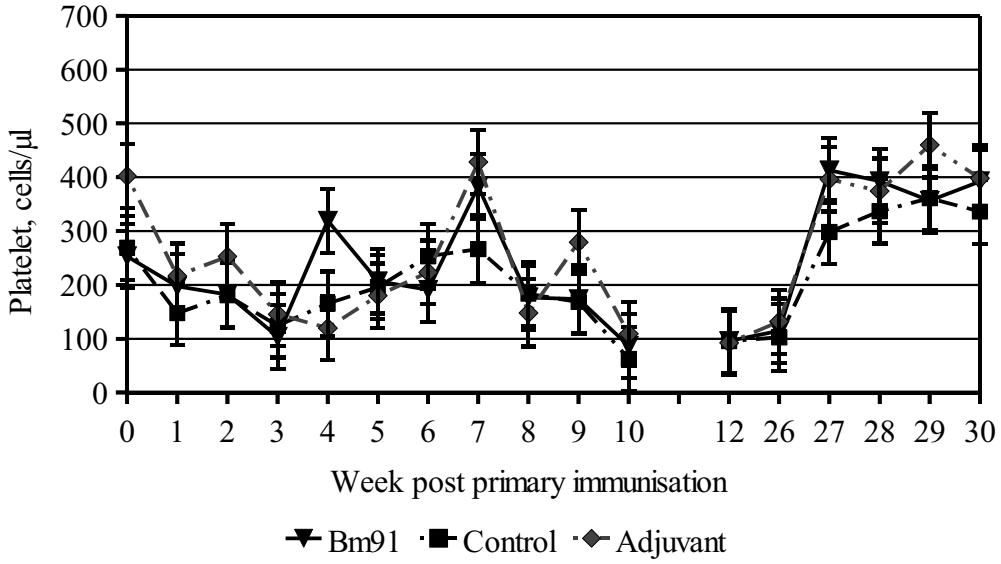
Trial 2



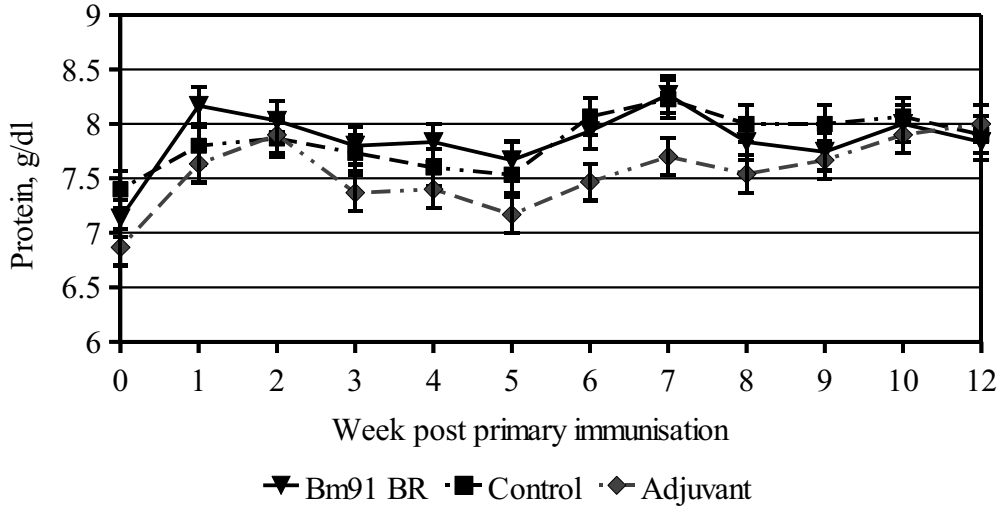
Appendix 8: Mean cell volume (MCV) of White Lamphun cattle immunised with the antigen Bm91 and injected with saline and adjuvant, respectively (LSM ± SE, N = 6). Reference interval: 40 to 60 fl (Kramer, 2000)



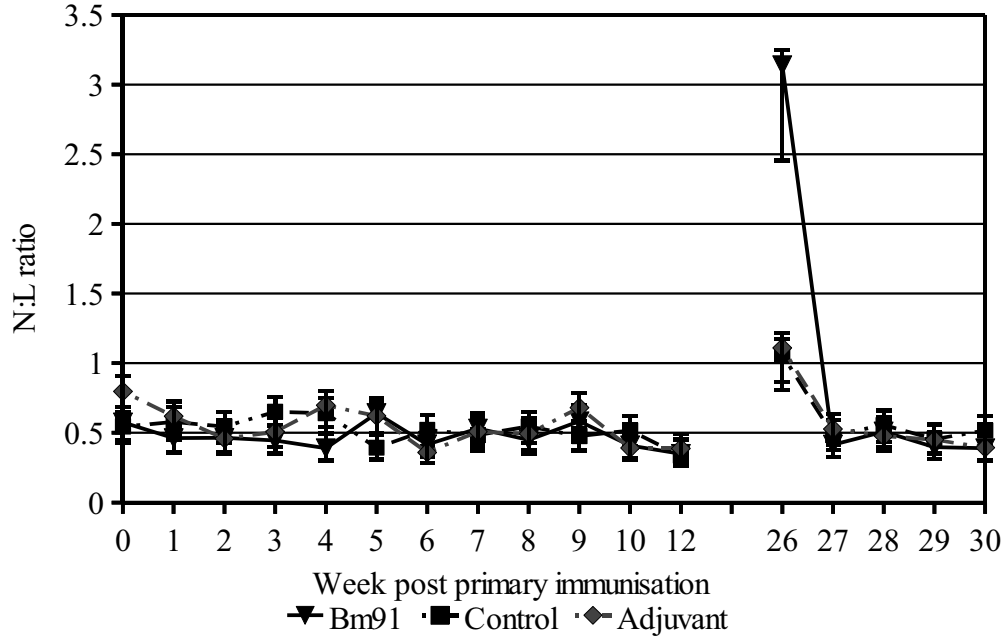
Appendix 9: Mean cell haemoglobin concentration (MCHC) of White Lamphun cattle immunised with the antigen Bm91 and injected with saline and adjuvant, respectively (LSM ± SE, N = 6). Reference interval: 30 to 36 g/dl (Kramer 2000)



Appendix 10: Platelet count of White Lamphun cattle immunised with the antigen Bm91 and injected with saline and adjuvant, respectively (LSM ± SE, N = 6). Reference interval: 100 to 800 cells/µl (Kramer 2000)



Appendix 11: Protein level of White Lamphun cattle immunised with the antigen Bm91 and injected with saline and adjuvant, respectively (LSM ± SE, N = 6). Reference interval: 7.0 to 8.5 g/dl (Kramer 2000)



Appendix 12: Neutrophil:Lymphocyte ratio of White Lamphun cattle immunised with the antigen Bm91 and injected with saline and adjuvant, respectively (LSM ± SE, N = 6)

Appendix 13: Correlations between body temperature and haematological parameters

a) Group: Immunised

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	-0.39***	-0.41***	-0.34**	ns	ns	ns	ns	ns	-0.27**	ns
PCV		0.92***	0.85***	ns	-0.56***	0.22*	ns	ns	0.32**	0.52***
Hb			0.83***	ns	0.21*	0.38**	ns	ns	0.24*	0.54***
RBC				-0.45***	-0.38***	0.22*	ns	ns	ns	0.53***
MCV					ns	ns	ns	ns	0.39***	ns
MCHC						0.21*	ns	ns	-0.31**	ns
WBC							ns	ns	ns	0.24*
NEU								-0.89***	ns	ns
LYM									ns	ns
PLAT										ns

[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

b) Group: Control

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	-0.27**	-0.30**	0.28**	ns	ns	ns	ns	ns	ns	ns
PCV		0.90***	0.77***	ns	-0.38**	ns	ns	-0.24*	0.48***	ns
Hb			0.76***	ns	ns	ns	ns	ns	0.34**	0.23*
RBC				-0.59***	ns	-0.20*	ns	-0.21*	0.34**	0.44***
MCV					-0.24*	0.32**	ns	ns	ns	-0.47***
MCHC						ns	ns	0.23*	-0.35**	ns
WBC							ns	ns	-0.43***	ns
NEU								-0.90***	ns	ns
LYM									ns	ns
PLAT										ns

1 NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

c) Group: Adjuvant

	PCV	Hb	RBC	MCV	MCHC	WBC	NEU[1]	LYM	PLAT	PROT
Temp	-0.31**	-0.30*	-0.31**	ns	ns	ns	ns	ns	-0.21*	ns
PCV		0.93***	0.90***	0.21*	-0.54***	ns	ns	ns	ns	0.48***
Hb			0.88***	ns	-0.29**	0.21*	ns	ns	ns	0.48***
RBC				-0.21*	-0.49***	0.21*	ns	ns	ns	0.46***
MCV					ns	ns	ns	ns	0.41***	ns
MCHC						ns	ns	ns	ns	-0.24*
WBC							ns	ns	-0.48***	ns
NEU								-0.91***	ns	ns
LYM									ns	ns
PLAT										ns

[1] NEU, neutrophils (%); LYM, lymphocytes (%); PLAT, platelet (cells/µl) ; PROT, protein (g/dl).
*** $P < 0.0001$; ** $P < 0.01$; * $P < 0.05$.

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