UNIVERSITY OF CAPE COAST

TRYPANOSOME INFECTION AND GENETIC VARIATION IN MAJOR HISTOCOMPATIBILITY COMPLEX DRB3 GENE IN CATTLE IN GHANA

BY

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DECLARATION

Candidate's Declaration:

I hereby declare that this thesis is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere.

Candidate's Signature..... Date. 5/6/2014

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Supervisors' Declaration:

We hereby declare that the preparation and presentation of the thesis were supervised in accordance with guidelines on supervision of thesis laid down by the University of Cape Coast.

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ABSTRACT

The accurate diagnosis of trypanosome infection and control of trypanosomosis remain challenging problems to cattle production in Ghana and other countries in sub-Saharan Africa. In this study, two PCR methods, based on ITS primers and *Trypanosoma vivax*-specific primers, were evaluated for the diagnosis of trypanosome infection in 110 each of four breeds of cattle in Ghana, namely, N'Dama, Zebu, WASH and Sanga. Whereas ITS primers did not detect any infection in all 440 animals, *T. vivax*-specific primers detected 4 positives in WASH and 7 positives in N'Dama. Haematological studies showed that mean PCV values for *T. vivax*-infected WASH and N'Dama were lower in infected compared to uninfected animals with the difference being highly significant ($p < 0.01$) in N'Dama. Using HRM analysis followed by sequencing, MHC DRB3 gene in 51 N'Dama and 52 WASH were genotyped. Seventeen alleles were found in each breed, indicating that this gene was highly polymorphic in the two breeds. Alleles 2101 and 2002 were significantly associated (OR = 40.45, RR = 28.13, p = 0.02 and OR = 41.57, RR = 29.40, p = 0.03) with susceptibility to *T. vivax* infection in N'Dama and WASH, respectively. The study, using West African cattle breeds, demonstrated that ITS primers are less sensitive than *T. vivax*-specific primers in the diagnosis of trypanosome infection. This study is the first to use HRM analysis in genotyping MHC in cattle, from available literature. Also from available literature, this is the first study to determine MHC allelic variants associated with susceptibility to *T. vivax* infection in cattle.

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DEDICATION

To my grandfather Yekpormenawo Atsu-Hunor

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LIST OF ACRONYMS

Ag	Antigen
ANOVA	analysis of variance
AU	African Union
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
CBPP	contagious bovine pleuropneumonia
CD	cluster of differentiation
CI	confidence interval
CIRDES	Centre International de Recherche-Developpement sur l'Elevage en zone Subhumide
DDT	dichloro diphenyl trichloroethane
DFID	Department for International Development
DNA	deoxyribose nucleic acid
dNTPs	deoxynucleoside triphosphates
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organisation
fl	femtolitres
GIS	Geographic Information System
g/dl	grams per decilitre
GDP	gross domestic product
Hb	haemoglobin
HCl	hydrochloric acid

HRM	high resolution melting
IAEA	International Atomic Energy Agency
IFAD	International Fund for Agricultural Development
ILRAD	International Laboratory for Research on Animal Diseases
ILRI	International Livestock Research Institute
IPM	Integrated Pest Management
ISCTRC	International Scientific Council for Trypanosomosis Research and Control
ITC	International Trypanotolerance Centre
ITS	internal transcribed spacer
KNUST	Kwame Nkrumah University of Science and Technology
$MgCl_2$	magnesium chloride
MCH	mean cell haemoglobin
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
MHC	major histocompatibility complex
µL	microlitre
mL	millilitre
mM	millimolar
mg	milligram
mtDNA	mitochondria DNA
M	Molar
MOFA	Ministry of Food and Agriculture
NaCl	sodium chloride
NCBI	National Centre for Biotechnology Information

OAU	Organisation of African Unity
OLA	ovine lymphocyte antigen
OPEC	Organization of Petroleum Exporting Countries
OR	odds ratio
PAAT	Programme Against African Trypanosomosis
PAHO	Pan American Health Organisation
PATTEC	Pan African Tsetse and Trypanosomosis Eradication Campaign
PCV	packed cell volume
PCR	polymerase chain reaction
pg	picograms
RBC	red blood cell
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
RR	relative risk
RTTCP	Regional Tsetse and Trypanosomosis Control Programme
SD	standard deviation
SDS	sodium dodecyl sulphate
SSCP	single strand conformation polymorphism
TAE	tris-acetate EDTA
TE	tris-EDTA
TLU	Tropical Livestock Unit
Tm	melting temperature
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TTCU	Tsetse and Trypanosomosis Control Unit

UCC	University of Cape Coast
UV	ultraviolet
VAT	variable antigen type
VSG	variant surface glycoprotein
WASH	West African Shorthorn
WBC	white blood cell
WF	White Fulani
WHO	World Health Organisation

Bases

Code	Base
A	Adenine
G	Guanine
C	Cytosine
T	Thymine

Amino acids

single letter code	three letter code	amino acid
G	Gly	Glycine
A	Ala	Alanine
V	Val	Valine
L	Leu	Leucine
I	Ile	Isoleucine
M	Met	Methionine
F	Phe	Phenylalanine
W	Trp	Tryptophan
P	Pro	Proline

S	Ser	Serine
T	Thr	Threonine
Y	Tyr	Tyrosine
N	Asn	Asparagine
Q	Gln	Glutamine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
K	Lys	Lysine
R	Arg	Arginine
H	His	Histidine
C	Cys	Cysteine

CHAPTER ONE

INTRODUCTION

Background to the Study

Ghana has a diverse livestock sub-sector which is an important component of agriculture in the country even though it contributes about 9% to the agricultural GDP (World Bank, 1992). Prominent among the contributions the livestock sub-sector makes to the economy of the country is food security and the provision of animal protein to enhance nutritional adequacy in diets of people. The sub-sector also provides employment opportunities for a large part of the population, particularly, in the rural areas and offers considerable prospects for wealth creation, poverty reduction and improvement in rural livelihoods (MOFA, 2004). However, the slow pace of livestock development in the country has created a situation whereby scarce foreign exchange is used to import large numbers of ruminants, particularly cattle to slaughter, and large volumes of frozen meat and dairy products annually to meet the shortfall in demand (MOFA, 2004; MOFA/DFID, 2002).

The slow pace of growth and development of the livestock sub-sector in Ghana has been attributed largely to poor management practices, inadequate feed supply during the dry season, lack of access to farm credit and livestock diseases (Agyen-Frempong, 1988). Although some epidemic diseases such as anthrax and rinderpest have been controlled through rigorous annual vaccinations, parasitic diseases continue to cause significant but slow and

subtle harmful production losses (Mahama et al., 2003). One parasitic disease of major economic importance is animal trypanosomosis caused by protozoan parasites, trypanosomes, of the genus *Trypanosoma*, that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina sp.*) (Adam, Marcotty, Cecchi, Mahama, Solano, & Bengally, 2012; Mahama et al., 2003; Turkson, 1993).

Animal trypanosomosis is reportedly the most economically important disease constraining productivity of cattle in 37 countries in sub-Saharan Africa, including Ghana (Kristjanson, Swallow, Rowlands, Kruska, & de Leeuw, 1999; Murray & Dexter, 1988; Schofield & Kabayo, 2008; Steverding, 2008). The trypanosomosis-endemic region in sub-Saharan Africa covers an area of about 10 million square kilometres (Enyaru, Ouma, Malele, Matovu, & Masiga, 2010; Geerts, Holmes, Diall, & Esler, 2001; Itard, Cuisance, & Tacher, 2003; Rogers & Robinson, 2004; Swallow, 1998). Within this region, some 46-62 million heads of cattle and other livestock species are at risk of the disease (Swallow, 1998). The disease is also called Nagana, and the usual consequence of infection is anaemia which is followed by poor growth, weight loss, low milk yield, infertility, abortion and paralysis (Steverding, 2008; Trail et al., 1985). As the illness progresses, affected animals exhibit irreversible weakness and draught animals eventually become unfit for work; this condition aptly led to the name of the disease "Nagana" – a Zulu word meaning "powerless" or "useless" (Steverding, 2008). Death may result within a few weeks to several months after infection.

Gyening (1985) reported that the density of livestock, especially cattle,

in Ghana, is inversely proportional to the trypanosomosis-challenge and that in high challenge areas there is the virtual exclusion of the keeping of zebu cattle, horses and donkeys. Outbreaks of bovine trypanosomosis, leading to the decimation (in a matter of months) of entire immigrant herds brought from the tsetse-free areas of Accra Plains to the high trypanosome-challenge areas of Sene District in the Brong Ahafo Region, have been reported (Mahama et al., 2003).

Studies show that trypanosome-transmitting tsetse populations in Ghana include the following economically important species: *Glossina palpalis*, *G. tachinoides*, *G. morsitans* and *G. longipalpis* (Adam et al., 2012; Draeger, 1983; Mahama et al., 2004; Mahama et al., 2005; Nash, 1948; Simpson, 1914; TTCU, 1998) Another report (published by Offori, 1964) revealed the presence of the following species of tsetse flies in the country: *G. pallicera*, *G. nigrofusca*, *G. tabaniformis*, *G. longipalpis*, *G. fusca*, *G. caliginea* and *G. medicorum*.

The species of trypanosomes known to exist in Ghana, which are pathogenic to cattle, are *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei* (Pomeroy & Morris, 1932). Trypanosomosis surveys carried out, using the buffy coat technique, in various parts of the country between 1995 and 2001 showed the prevalence of the disease as ranging from 5% in the low risk areas, such as the Dangme West and Savelugu-Nanton Districts, to about 50% in the high risk areas, such as the West Mamprusi and Damango Districts (Mahama et al., 2003). An earlier survey in the Winneba District in the Coastal Savanna Zone, using the antigen ELISA test on 340 cattle samples, showed a prevalence of 39.0% comprising

50.4% *T. vivax*, 3.1% *T. congolense*, 14.5% *T. brucei* and 32.1% mixed infection (Turkson, 1993). A recent survey conducted by Adam and colleagues (Adam et al., 2012) in the Upper West Region of Ghana, involving 1800 cattle, using the buffy coat method, indicated a parasitological prevalence of 1.9% made up of 1.5% *T. vivax*, 0.4% *T. brucei* and 0% *T. congolense*. The studies by Turkson (1993) and Adam et al. (2012) revealed that *T. vivax* is the predominant trypanosome species in Ghana, agreeing with reports by Losos (1986) that *T. vivax* predominates in West Africa.

The accurate diagnosis of animal trypanosomosis and identification of trypanosome species and subspecies causing the disease have been a challenge in sub-Saharan Africa. Accurate identification of trypanosome species is essential for the collection of epidemiological data. With sound knowledge of the prevalence and diversity of trypanosomes, high risk areas can be identified and limited resources can be targeted efficiently (Gibson, 2007; Solano et al., 1999). Accurate identification is also necessary for the assessment of therapeutic efficiency, disease progression in infected animals (Clausen, Waiswa, Katunguka-Rwakishaya, Schares, & Mehlitz, 1999) and for finding out whether a control strategy works or not.

The traditional parasitological (Murray et al., 1977; Woo, 1971) and serological (Desquesnes, Bengaly, Millogo, Meme & Sakande, 2001; Luckins, 1977) methods for diagnosing the disease lack sensitivity and specificity (Authié, Muteti, & Williams, 1993; Nantulya, 1990; Solano et al., 1999; Vanhamme, Pays, McCulloch, & Barry, 2001). The polymerase chain reaction (PCR) technique has emerged as a highly sensitive and specific technique for the diagnosis of trypanosome infection based on the detection of trypanosomal

DNA in either the vector or the host (Desquesnes, McLaughlin, Zoungrana, & Davila, 2001; Masiga, Smyth, Hayes, Bromidge, & Gibson, 1992; Moser et al., 1989; Njiru et al., 2005).

In Ghana, pioneering work in trypanosomosis control mainly involved major programmes to control tsetse by bush-clearance to eliminate tsetse resting sites (Mahama et al., 2003). Such clearings were carried out on a massive scale in Nabogu Valley of the Northern Region (Stewart, 1937) and along River Kamba in the Upper West Region (Morris, 1947). Field trials were also carried out in the Upper West Region on the possible use of various trap designs to control tsetse flies (Morris & Morris, 1949). There were unpublished reports of limited game destruction to reduce the parasite reservoirs and host availability for tsetse (Mahama et al., 2003). These control methods were abandoned in the early 1950s due to their negative impact on the environment (Mahama et al., 2003). In 1996 a community-based tsetse control project which involved the use of deltamethrin-impregnated blue screens was carried out in the coastal savanna areas. About 600 deltamethrin-impregnated blue screens and 200 deltamethrin-impregnated traps were deployed in the Lower Volta river basin drainage network (TTCU, 1996). In the same exercise, about 500 cattle were topically treated with deltamethrin "pour on". Tsetse population was reduced by 98% within three months of the application of these methods. Although the exercise proved highly effective, it could not be sustained because community enthusiasm waned and this resulted in re-invasion (TTCU, 1996).

The use of prophylactic and curative drugs has remained the most popular method for the management of animal trypanosomosis in the country

from the late 1950s to date (TTCU, 1997). However, the continued use for more than half a century of a limited number of trypanocidal drugs that are closely related has led to the emergence of drug resistant trypanosomes (Sow et al., 2012). Thus, the traditional control measures including bush clearance, game culling, traps and targets and insecticides and drug use have not resulted in significant progress in controlling the disease in Ghana.

An alternative approach to the control of animal trypanosomosis could be the selection and use of resistant animals. Such a selection approach could be based on marker alleles associated with enhanced disease resistance as suggested by Bishop and Woolliams (2004). Possible genetic markers in this regard are genes of the major histocompatibility complex (MHC). Indeed, based on an MHC marker, Maillard and colleagues (2003) reported a successful selection procedure for dermatophilosis resistance in zebu Brahman cattle in Martinique. Here, over a period of five years, a marked reduction in disease prevalence from 0.76 to 0.02 was achieved through the elimination of only those individuals which were at the highest risk of contracting the disease.

MHC genes have been extensively studied as candidate genes for disease resistance in many domestic animal species because they play a central role in the immune system (Blattman et al., 1993; Davies et al., 1997; Lewin, Russel, & Glass, 1999; Xu, Van Eijk, Park, & Lewin, 1993). Genes within the MHC are highly polymorphic; that is, there are multiple variants of each gene within the population as a whole (Janeway, Travers, Walport, & Shlomchik, 2001). Each MHC allele may potentially bind and present to T-cells a different set of pathogen-derived peptides, and thus a population carrying a higher

number of MHC alleles may respond to a broader spectrum of pathogens (Babik, Kawałko, Wojcik, & Radwan, 2012; Parham & Ohta, 1996). This has led to suggestions that depletion of variation of the MHC genes may compromise the ability of populations to respond to pathogen assault and lead to an increased risk of extinction (Hedrick, 2001; Hughes, 1991; O'Brien & Evermann, 1988). In fact, MHC polymorphism has been associated with differences in susceptibility to diseases in man (Carrington et al., 1999; Hill et al., 1991; Thurz, Thomas, Greenwood, & Hill, 1997) and other vertebrates (Kaufman, Volk, & Wallny, 1995; Lamont, 1998).

Other studies showing the association between resistance or susceptibility of specific MHC alleles and parasite species have been carried out. In cattle, allelic variants of MHC genes were found to be associated with resistance to persistent lymphocytosis caused by bovine leukemia virus (Xu et al., 1993). Associations have also been observed for resistance to dermatophilosis in Brahman cattle of Martinique (Maillard, Martinez, & Bensaid, 1996); cystic ovarian disease and retained placenta (Sharif et al., 1998); as well as foot and mouth disease (Glass et al., 1991; Lewin et al., 1999). Alleles have also been associated with susceptibility to several infectious diseases such as mastitis (Duangjinda et al., 2009; Park et al., 2004; Rupp, Hernandez, & Mallard, 2007; Sharif, Maillard, & Sargeant, 2000; Sharif et al., 1998) and enzootic bovine leukemia (Juliarena et al., 2008). Studies relating cattle MHC genes and trypanosomosis are rare. Karimubo et al. (2011) showed that MHC alleles are associated with an increased risk of *T. brucei* and *T. congolense* infection in *Bos indicus* Masai and Boran cattle in Tanzania.

Virtually all the association studies reported for cattle have focused on the MHC class II DRB3 gene (Duangjinda et al., 2009; Glass et al., 1991; Juliarena et al., 2008; Kabeya et al., 1999; Lewin et al., 1999; Maillard et al., 1996; Park et al., 2004; Rupp et al., 2007; Sharif et al., 2000; Sharif et al., 1998; Xu et al., 1993). This gene is mostly chosen because it is the most polymorphic class II gene in cattle with over 90 different alleles detected (Takeshima, Ikegami, Morita, Nakai, & Aida, 2001), and much of the variation is in the functionally- important antigen-binding site (Ohta, 1998).

Methods for typing MHC genes include PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (Davies et al., 1994; Ellegren, Davies, & Anderson, 1993; Lewin et al., 1999; Van Eijk, Stewart, Haynes, & Lewin, 1992), PCR-single strand conformation polymorphism (PCR-SSCP) analysis (Orita, Iwahana, Kanazawa, Hayashi, & Sekiya, 1989; Pipalia, Josh, Rank, Brahmkshtr, & Solank, 2004; Sunnucks et al., 2000; Zhou, Hickford, Fang, & Byun, 2007) and sequencing. In recent times, high-resolution melting (HRM) analysis, a simple, fast, cost effective and highly sensitive closed-tube method, has been used for genotyping in human clinical studies (Andriantsoanirina et al., 2009; Areekit et al., 2009; Cheng et al., 2006; Nicolas, Milon, & Prina, 2002; Odell, Cloud, Seipp, & Wittwer, 2005; Waku-Kouomou et al., 2006) and veterinary science (Ghorashi, O'Rourke, Ignjatovic, & Noormohammadi, 2011; Pham et al., 2005), but is yet to be used for MHC genotyping.

Justification of the study

Ghana is a tsetse-infested and trypanosomosis-endemic country in sub-Saharan Africa (Adam et al., 2012; Bauer et al., 2011; Enyaru et al., 2010;

Geerts et al., 2001; Mahama et al., 2004; Mahama et al., 2005; Mahama et al., 2003; Turkson, 1993). The disease in cattle has, however, been kept from assuming alarming proportions as a result of the pattern of distribution of trypanotolerant and trypanosusceptible breeds countrywide (Mahama et al., 2003). In the relatively tsetse free areas of the Accra Plains, for example, there is a predominance of trypanosusceptible Zebu while in the high tsetse challenge areas, such as those along the Black Volta and around game parks, there is a predominance of the pure trypanotolerant West African Shorthorn (WASH) breed (Mahama et al., 2003). However, farmers are increasingly showing a preference for Zebu cattle which has a larger body size, higher milk yield and heavier live weight (World Bank, 1992) even in high tsetse challenge areas (Turkson, 1993) leading to reduction in the number of the trypanotolerant WASH in those areas (Ahunu & Boa-Amponsem, 2001). For example, the last Ghana Livestock Census published in 1997 indicated that the WASH constituted 70% of the cattle population in Ghana (Mahama et al., 2003); however, a later study gave a reduced WASH population of 47.5% (Ahunu & Boa-Amponsem, 2001). The larger bodied trypanosusceptible cattle breeds command higher prices compared to the smaller bodied WASH, but, unlike trypanotolerant cattle, trypanosusceptible cattle cannot survive in areas of high tsetse densities without veterinary intervention or other tsetse control strategies (Hanotte et al., 2002; Mahama et al., 2003; Turkson, 1993). Although trypanotolerant breeds are equally susceptible to trypanosome infection, they possess the ability to survive, reproduce and remain productive in areas of high tsetse challenge without the need for the use of chemicals to control the vector or drugs to control the parasite (Dayo et al., 2009; Rege,

Aboagye, & Tawah, 1994). Studies, however, show that in the face of increased tsetse challenge, there is fall in productivity of trypanotolerant cattle as a result of stunting, wasting, abortion and death (Trail, Murray, & Wissocq, 1984). Further, trypanotolerant cattle could serve as potential reservoirs of trypanosome parasites (Achukwi & Musongong, 2009).

Given the limitations of the traditional methods of controlling trypanosomosis, there is the need to look for alternative approaches in combating the disease in Ghana. One approach may be to identify MHC alleles that are resistant or susceptible to trypanosome infection as demonstrated in the control of dermatophilosis (Maillard et al., 2003). Once these alleles have been identified, carefully planned breeding programmes could be put in place to incorporate the resistant alleles in cattle herds or eliminate the susceptible alleles from the herds.

Objective of the Study

To determine MHC DRB3 alleles for controlling trypanosomosis in four breeds of cattle in Ghana

Specific objectives

(i) To determine the prevalence of trypanosome parasites in four breeds of cattle in Ghana using 2 tests based on internal transcribed spacer (ITS-1) region primers and *T. vivax* specific primers in PCR studies

(ii) To examine the level of polymorphism in MHC DRB3 gene in four breeds of cattle in Ghana using HRM analysis

(iii) To determine whether there is an association between resistance or susceptibility to *T. vivax* infection and allelic variants of MHC DRB3 gene in four breeds of cattle in Ghana

CHAPTER TWO

REVIEW OF RELATED LITERATURE

Causative Agents and Vectors of Trypanosomosis

The causative agents of the trypanosomosis are protozoan parasites referred to as trypanosomes, of the genus *Trypanosoma*, that live and multiply extracellularly in blood and tissue fluids of their mammalian hosts and are transmitted by the bite of infected tsetse flies (*Glossina sp.*) (Adam et al., 2012; Mahama et al., 2003; Turkson, 1993). The trypanosomes causing disease in cattle in Ghana, and other parts of sub-Saharan Africa, are *T. congolense*, *T. vivax* and *T. brucei brucei* (Adam et al., 2012; Darji et al., 1992; Mahama et al., 2003; Murray & Dexter, 1988; Pomeroy & Morris, 1932; Schofield & Kabayo, 2008; Turkson, 1993). Trypanosomosis occurs in Africa in relation to the distribution of tsetse flies and covers an area of 10 million km^2 between latitudes 14°N and 20°S (Steverding, 2008). Thirty-seven countries, including Ghana, lie within the tsetse belt (Enyaru et al., 2010; Geerts et al., 2001).

Adult tsetse – male and female – feed exclusively on vertebrate blood; the immature stages do not feed. Since tsetse larvae do not feed, but are nourished by the uterine glands of the mother, the entire nutritional intake of the flies is limited to vertebrate blood. Adult flies can become infected with trypanosomes by taking a blood meal from an infected host. Once the infection is established, the flies appear to remain infected for life. However,

cyclical development – the establishment and development of the trypanosomes in the tsetse vectors – is a complex process, involving a series of vector and parasite defence and counter-defence mechanisms (Welburn & Maudlin, 1999), and there is evidence that tsetse become harder to infect as they mature (Kuzoe & Schofield, 2004). Often therefore, natural populations of tsetse show relatively low infection rates – generally less than 0.1% in the case of human-infective forms, but often as high as 10-15% in the case of cattle-infective forms (Kuzoe & Schofield, 2004).

Under current classifications, the 31 currently recognized species and subspecies of *Glossina* are customarily placed into three species groups which are sometimes given subgeneric status: morsitans group (subgenus *Glossina*), fusca group (subgenus *Austenina*), and palpalis group (subgenus *Nemorhina*). (FAO, 1982; Haeselbarth, Segerman, & Zumpt, 1966). *Morsitans* are found in the African savanna (grassy woodland) and include *Glossina morsitans morsitans, G. morsitans submorsitans, G. morsitans centralis, G. longipalpis, G. pallidipes, G. austeni* and *G. swynnertoni* (Jordan, 1993). *Fusca* are found in the forest habitat and examples of species in this group are *Glossina fusca fusca, G. fusca congolensis, G. nigrofusca, G. medicorum, G. tabaniformis, G. vanhoofi, G. nashi, G. severini, G. schwetzi, G. longipennis, G. haningtoni and G. frezili* (Jordan, 1993). Riverine flies belong to the *Palpalis* group and include *Glossina palpalis palpalis, G. palpalis gambiensis, G. tachinoides, G. fuscipes fuscipes, G. fuscipes martini, G. fuscipes quanzensis, G. palllicera pallicera, G. pallicera newsteadi* and *G. caligenia* (Jordan, 1993). These groupings are based primarily on morphological features of the adult genitalia (Newstead, Evans, & Potts, 1924) although they also reflect differences in

distribution, habitat and behaviour (Jordan, 1993). These groupings can also be demonstrated by comparative gene sequence analysis and by geometric wing morphometry (Patterson & Schofield, 2004), and also coincide broadly with epidemiological significance (Kuzoe & Schofield, 2004).

The occurrence of tsetse species within different climatic zones and vegetation types in Ghana has been studied by various researchers. Earlier surveys by Nash (1948) indicated that *G. longipalpis* and *G. morsitans submorsitans* (morsitan group) were associated with game animals in open woodland savanna. Later surveys in northern Ghana (Mahama et al., 2003) reported the presence of *G. morsitans submorsitans* only in the Mole, Wahabu and Bui game parks and their immediate vicinity; in the Brong Ahafo Region, this tsetse species was found only in the game park of Sene District. Reid, Kruska, Deichman, Thornton and Leak (2000) suggested that the retreat of *G. morsitans submorsitans* into protected areas is associated with game destruction and agricultural expansion. With regard to the distribution of the *fusca* group in Ghana, Offori's (1964) map showed *G. longipalpis* as occurring in the transitional forest zone, while *G. fusca, G. nigrofusca, G. tabaniformis* and *G. medicorum* were shown as occurring in the forest zone. Surveys conducted by the Tsetse and Trypanosomosis Control Unit (TTCU, 1996, 1998, 2000) showed the presence of only *G. tachinoides* and *G. palpalis gambiensis* (riverine tsetse flies) in the vegetation on the fringes of water bodies in the coastal savanna and derived savanna zones. These surveys also showed that in areas where *G. palpalis* was found to coexist with *G. tachinoides*, the former occurred in much smaller numbers. A cross-sectional entomological survey conducted along the White Volta and its tributaries in

the Savelugu-Nanton and West Mamprusi Districts of Northern Ghana confirmed the presence of only *Glossina palpalis gambiensis* and *G. tachinoides* (Mahama et al., 2004). The study by Mahama et al. (2004) also showed that tsetse were absent in areas where fringing riverine vegetation had been completely destroyed, and this impacts on prevalence of trypanosomosis. For example, the parasitological prevalence of bovine trypanosomosis was lower in the Savelugu-Nanton District (8%) compared to 16% in the West Mamprusi District where land use in close proximity to riverine vegetation has completely destroyed tsetse habitat (Mahama et al., 2004). An earlier study reported by Mahama et al. (2003) indicated that the prevalence of the disease in the coastal savanna is higher in Dangme East than in Dangme West (where riverine vegetation is virtually non-existent). Animal trypanosomosis will continue to remain a problem in Ghana and other sub-Saharan African countries so long as environmental and climatic conditions that are conducive to the survival of tsetse continue to exist (Jordan, 1993).

The life cycle of tsetse is unusual since they do not lay eggs. Instead, an inseminated female develops the egg and young larva within her uterus, laying the mature (3rd instar) larva into shaded soil. The larva quickly burrows under the soil surface and pupates, and the adult emerges 20-45 days later depending on temperature (pupal development does not succeed below 17°C and above 32°C). Thus, each female produces only one offspring at a time, and can produce up to 12 offspring – at intervals of about 9-10 days – during her typical adult lifespan of 2-3 months. As a result, the intrinsic rate of tsetse population growth tends to be low, with the maximum rate of population increase estimated to be no more than 10-15 times per year (Hargrove, 1988).

For flight, adult tsetse rely on partial metabolism of proline, an amino acid derived from the bloodmeal, and when this is exhausted they must rest in order to reconstitute the limited proline reserve. One result is that tsetse are generally unable to fly for long periods, flying instead in short bursts, with a relatively low capacity for active dispersal (Kuzoe & Schofield, 2004).

A further consequence of the unusual life history of tsetse is their tendency to have low genetic variability within a given population (Gooding, 1984; Krafsur, 2003). This is partly a consequence of low dispersal rate, and partly due to the low reproductive rate, probably combined with selection for the most energetically-efficient individuals.

Cattle Host Preference of Tsetse

Although all cattle reared in tsetse endemic areas are prone to being fed on by tsetse leading to infection by trypanosomes, tsetse exhibit some preference when cattle of all age and sex categories are present in a herd (Rowlands et al., 2001; Simukoko, Marcotty, Phiri, Vercruysse, & Van den Bossche, 2007; Torr & Mangwiro, 2000; Torr, Maudlin, & Vale, 2007; Torr, Mwangwiro, & Hall, 2006; Trail, Wissocq, d'Ieteren, Kakiese, & Murray, 1994). Simukoko and colleagues (2007) found that the risk of infection relative to calves was about six times higher in oxen ($p < 0.001$) and about twice as high in cows ($p = 0.01$) as well as young bulls and heifers ($p = 0.05$). Other workers (Rowlands et al., 2001; Trail et al., 1994) also reported low infection rates for calves relative to older animals. These findings are supported by Torr et al., (2006) and Torr and Mangwiro (2000), who reported that tsetse flies are attracted significantly more by odour of large animals and those that showed less defensive behaviour and least by calves. The low

prevalence rate in calves has also been attributed to the protection offered by maternal antibodies (Fimmen, Mehilitz, Horchiners, & Korb, 1999).

Diagnosis of Trypanosome Infection

Parasitological diagnosis of trypanosome infection

Parasitological methods employ the use of microscopes, depend on actual detection of parasites and are referred to as direct methods (Uilenberg, 1998). Use of microscopes to examine wet blood, stained thick and thin blood smears is of very low sensitivity in cases of low parasitaemia (Uilenberg, 1998). In these circumstances, concentration methods consisting of microhaematocrit centrifugation technique, i.e. the Woo method (Woo, 1971) and buffy coat method (Murray et al., 1977) are necessary, as they increase the sensitivity of microscopic examination. However, the Woo and the buffy coat methods exhibit low sensitivity when applied during the chronic phase of the disease when parasitaemia is low (Desquesnes & Tresse, 1996; Solano et al., 1999) and are thus not reliable in field studies designed to examine the prevalence of trypanosome infection (Nantulya, 1990). Later studies (Picozzi et al., 2002) concluded that because of the often low parasitaemia, the buffy coat method has low sensitivity, and, hence, about 50% of cases are not diagnosed and a large proportion of infected animals remain untreated. In a survey conducted in the Upper West Region of Ghana, involving 1800 cattle of the West African Shorthorn, Sanga and Zebu, using the buffy coat method, Adam et al. (2012) reported an average parasitological prevalence of 1.9%. Such a result might not reflect the actual prevalence of trypanosomes due to the low sensitivity of the buffy coat method (Desquesnes & Tresse, 1996; Nantulya, 1990; Picozzi et al., 2002; Solano et al., 1999).

Serological diagnosis of trypanosome infection

Serological tests, also referred to as immunological methods, detect parasites indirectly from antibodies or antigens (Uilenberg, 1998). The antibody enzyme-linked immunosorbent assay (ELISA), first described by Luckins (1977) and later standardized by (Desquesnes et al., 2001), is capable of detecting trypanosome-specific antibodies in cattle (Luckins & Mehlitz, 1978) and was one of the first serological tests for diagnosing trypanosome infection in cattle (Luckins, 1977). Seropositivity must be interpreted with caution since antibodies can persist for several months following successful therapy or self-cure of infection, showing that the antibody ELISA test lacks specificity (Authié et al., 1993; Bocquention, Very, & Duvallet, 1990; Desquesnes et al., 1999; Desquesnes & Tresse, 1996; Luckins et al., 1979). Available serological tests detect antibodies after 3 to 4 weeks of infection; thus, the antibody ELISA test lacks sensitivity in the early stages of infection, before the rise of specific antibodies (Vanhamme et al., 2001).

In an attempt to overcome the limitations of lack of specificity and sensitivity of the ELISA method based on antibody detection, Nantulya and Lindqvist (1989) developed a test to detect circulating antigens released by parasites in the blood of infected animals rather than antibodies produced against them. In this test, circulating antigens were detected as early as 10-12 days in sera of cattle experimentally infected with *T. vivax* and *T. congolense*, and 8-14 days in *T. brucei*-challenged cattle (Nantulya & Lindqvist, 1989). Furthermore, the antigen levels increased and were persistently present in circulation even on occasions where parasitaemia was not detectable by the buffy coat technique (Nantulya & Lindqvist, 1989). Following treatment with

Berenil, *T. vivax* and *T. congolense* antigens were cleared from circulation within 2 weeks. The rate of removal was slower but variable in *T. brucei* infections. Evaluation of the test in French Guyana with sheep experimentally infected with a local isolate of *T. vivax* demonstrated a lack of specificity and sensitivity (Desquesnes, 1996). Further re-evaluation by infecting calves at ILRAD, Nairobi, Kenya, with isolates of *T. vivax, T. brucei* and *T. congolense* from French Guyana confirmed the lack of sensitivity and specificity for the Ag-ELISA test (Desquesnes, 1996). In this re-evaluation procedure, detection rates of 3.8% for *T. vivax*, 4.4% for *T. brucei* and 3.1% for *T. congolense* were obtained using the Ag-ELISA. In the same procedure, the percentage of positive result obtained for *T. vivax* using the buffy coat method was 66% (Desquesnes, 1996), showing low sensitivity of the Ag-ELISA test compared to a parasitological technique. Furthermore, blood smears showed only *T. vivax,* and this suggested low specificity of the test for *T. brucei* and *T. congolense* (Desquesnes, 1996).

Polymerase chain reaction as a diagnostic tool for trypanosome infection

PCR has emerged as one of the most sensitive and specific molecular methods for the diagnosis of trypanosome infection in either the vector or the host (Cox et al., 2005; Majiwa et al., 1994; Masake et al., 1997; Masiga et al., 1992; Moser et al., 1989; Njiru et al., 2005; Solano et al., 1999). The first PCR-based studies for trypanosome detection used pairs of primers designed to amplify highly repetitive satellite DNA sequences specific to each species (Moser et al., 1989; Masiga et al., 1992; Majiwa et al., 1994; Solano et al., 1995; Reifenberg et al., 1997; Lefrançois et al., 1999). Identification is made by the presence of a PCR product of specific size on an agarose gel

(Lefrançois et al., 1999; Majiwa et al., 1994; Masiga et al., 1992; Moser et al., 1989; Reifenberg et al., 1997; Solano, Argiro, Reifenberg, Yao, & Duvallet, 1995). The application of species-specific tests to field-collected material led to the discovery of many novel species and strains (Adams & Hamilton, 2008). These are recognized when a DNA sample tests negative with tests for all known species (Adams & Hamilton, 2008). Species-specific PCR tests have been developed to differentiate *T. vivax, T. brucei brucei* and *T. congolense* (Majiwa, Maina, Waitumbi, Mihok, & Zweygarth, 1993; Masiga et al., 1992; McNamara, Mohammed, & Gibson, 1994; Moser et al., 1989). Specific PCR primers are now available to recognize three different *T. congolense* subspecies: the Savannah type (Majiwa & Otieno, 1990), the Riverine-Forest type (Masiga et al., 1992) and the Kilifi type (Masiga et al., 1992). The application of species-specific PCR tests to field surveys revealed that mixed infections of two, or even three or four, different trypanosomes were relatively common (Lefrançois et al., 1999; Lehane, Msangi, Whitaker, & Lehane, 2000; Majiwa & Otieno, 1990; Masiga, McNamara, Laveissière, Truc, & Gibson, 1996; McNamara, Dukes, Snow, & Gibson, 1989; McNamara, Laveissiere, & Masiga, 1995; Morlais et al., 1998; Solano et al., 1995; Solano et al., 1996; Woolhouse, McNamara, Hargrove, & Bealby, 1996). Field studies in Burkina Faso (Bengaly, Kasbari, Desquesnes, & Sidibé, 2001; Solano et al., 1999) and the Gambia (Pereira De Almeida, Ndao, Goossens, & Osaer, 1998) indicated higher prevalence of trypanosomosis (two to three times higher) for the species-specific PCR compared to parasitological techniques.

Although studies using species-specific tests to field-collected samples

have transformed understanding of trypanosomosis epidemiology, the method has severe limitations. First, the number of species-specific PCR reactions required for each DNA sample can make this method time-consuming and expensive (Adams & Hamilton, 2008). One pair of primers is required to detect each subgenus, species or type, leading in some cases to the processing of three to five different PCRs per sample (Desquesnes et al., 2001). Also, the method permits identification only of trypanosomes for which there are species-specific primers, leaving a substantial proportion of infections unidentified (Malele et al., 2003).

Consequently, an identification system using generic primers to amplify the internal transcribed spacer (ITS-1) region of the ribosomal RNA gene locus was proposed to replace the multiple species-specific tests for diagnosis of African trypanosome species infection (Cupolillo, Grimaldi, Momen, & Beverley, 1995; Desquesnes et al., 2001; McLaughlin et al., 1996). There is sufficient interspecies length variation in the ITS region to enable identification of trypanosome species by the size of the PCR amplified product using agarose gel electrophoresis (Desquesnes et al., 2001). This method is quicker and cheaper than the species-specific technique because the number of PCRs required per sample is greatly reduced, and, therefore, larger numbers of samples can be processed (Adams & Hamilton, 2008). It is also able to detect mixed infections in a single PCR, which can be identified by the presence of multiple bands (Adams & Hamilton, 2008). New species could potentially be identified if their PCR-amplified ITS-1 region differs in size from known trypanosomes (Desquesnes et al., 2001). Samples which were reported negative in a previous species-specific PCR study by Solano et al. (1999) were

re-tested with ITS primers by Desquesnes et al. (2001) and shown to be positive. Later studies by Njiru et al. (2005) comparing ITS and species-specific primers on cattle and camel blood samples in Kenya also showed higher trypanosome prevalence with ITS primers. Further research (Gonzales, Loza, & Chacon, 2006), however, yielded results contrary to those of Desquesnes et al. (2001) and Njiru et al. (2005). Gonzales et al. (2006) evaluated the sensitivity of a species-specific primer set and an ITS primer pair on blood samples of a 2-month old male sheep inoculated intravenously with *T. vivax* isolate obtained from an infected bovine from the Bolivian Pantanal. They found that the sensitivity of the ITS primers was less than that of the species-specific primers. The lower sensitivity of ITS primers compared to species-specific primers could be attributed to the fact that species-specific primers target satellite DNA sequences which are present in high copy number of between 10,000 to 20,000, whereas ITS primers target sequences which are only repeated 100-200 times (Desquesnes & Davilla, 2002). Due to the limited repetitiveness of ITS target sequences, it has been suggested that new ITS primers should be designed to reach 100% homology with all *Trypanosoma* species of veterinary interest, especially for *T. vivax* (Desquesnes & Davilla, 2002). Although PCR is so far the most sensitive and specific method compared to other methods for diagnosis of trypanosomosis, limited studies comparing the two main PCR techniques have produced conflicting results, which need to be resolved by further research (Desquesnes & Davilla, 2002).

Haematological diagnosis of trypanosome infection

Haematology is globally becoming an increasingly important tool in the diagnosis, treatment and prognosis of many diseases encountered by

veterinary medical practitioners (Fry, 2011; Ohaeri & Eluwa, 2011; Ramin et al., 2011). Indeed, it helps in distinguishing the normal state from the state of stress, arising from nutritional, environmental or physical factors (Aderemi, 2004). For a particular species, haematological values are affected by breed, sex, age, nutrition, illness, stress, exercise, transport and seasonal variations (Jain, 1998). For haematology to serve as a useful tool in diagnosing an underlying disease in cattle, haematological data obtained from the laboratory must be compared with normal reference values such as the readily available one compiled by Jain (1993) for clinically healthy animals. A number of studies have been carried out in many countries with a view to providing some baseline data to help in the interpretation of haematological values of various cattle breeds under trypanosomosis challenge (Bekele & Nasir, 2011; Mbanasor et al., 2003; Ohaeri & Eluwa, 2011; Silva et al., 1999).

The haematological parameters that are determined routinely are erythrocyte parameters comprising total red blood cell count (RBC), packed cell volume (PCV), haemoglobin concentration (Hb), mean cell volume (MCV), mean cell haemoglobin (MCH) and mean cell haemoglobin concentration (MCHC) as well as leucocyte parameters made up of total white blood cell (WBC) counts and WBC differential counts of lymphocytes, neutrophils, eosinophils, monocytes and basophils (Jain, 1986). In practice, a reduction in total red blood cell count, packed cell volume or haemoglobin concentration indicates anaemia (Birchard & Sherding, 2006; Davidson, Else, & Lumsden, 1998; Schalm, Jain, & Carroll, 1975). Anaemia may develop when there is blood loss through haemorrhage, blood sucking parasites, accelerated erythrocyte destruction and reduced or defective

erythropoiesis (Birchard & Sherding, 2006; Davidson et al., 1998; Schalm et al., 1975).

Studies show that anaemia is a well recognised and inevitable consequence of trypanosome infections in domestic animals in general and cattle in particular (Murray & Dexter, 1988; Murray, Trail, Davis, & Black, 1984). Silva et al. (1999) reported that in natural cases of bovine trypanosomosis due to *Trypanosoma vivax* in beef and dairy cattle from Bolivian wetlands and the Brazilian Pantanal, a main haematologic change produced by *T. vivax* infection was anaemia. Sharma, Chauham, Saxena and Agrawal (2000) studied haematological changes due to experimental *T. evansi* infection in Barbari goats and noted that animals exposed to the parasites had significant ($p < 0.01$) decline in PCV, Hb and RBC compared to the control group. A survey conducted by Bekele and Nasir (2011) involving 384 randomly selected cattle in western Ethiopia revealed that the mean packed cell volume of infected animals due to *T. congolense*, *T. vivax* and *T. brucei* was lower (20.8% ± 3.2) compared to non-infected animals (24.9% ± 3.8), and the difference was statistically significant ($p < 0.05$). Of 1309 Ankole longhorn cattle breed investigated from 10 different localities in Uganda, 6.4% (5.6% and 7.3% from each of two of the localities) tested positive for trypanosomes. The mean PCV for the trypanosome-positive animals was lower than that for the trypanosome-negative animals, whether the cattle considered were all those investigated (22.3% *v.* 29.0%; $p < 0.001$) or just those from each of the two localities (22.8% *v.* 28.2% or 21.5% *v.* 29.7%) where trypanosomes were detected (Waiswa & Katunguka-Rwakishaya, 2004). In Nigeria, Ohaeri and Eluwa (2011) produced evidence that domestic

ruminants, including cattle, that were naturally infected with trypanosomes had significantly lower ($p < 0.05$) packed cell volume and RBC count compared to uninfected animals. Furthermore, lower herd average PCVs for trypanosome-positive cattle compared to trypanosome-negative cattle have been reported from Zambia (Marcotty et al., 2008; Van den Bossche & Rowlands, 2001) and Cameroon (Achukwi & Musongong, 2009). However, in a typical trypanotolerance phenomenon, pathogenic *Trypanosoma* species infection does not usually result in anemia (Murray & Dexter, 1988; Murray, Morrison, & Whitelaw, 1982). For example, Mbanasor et al. (2003) observed that the mean RBC, Hb and PCV values in natural *T. vivax* infected and uninfected trypanotolerant Muturu cattle in Nigeria were well within the accepted normal values reported in many breeds of cattle by Schalm et al. (1975).

The effect of trypanosome infection on WBC parameters also features prominently in literature. For example, in Nigeria, Ohaeri and Eluwa (2011) reported that natural trypanosome infection of sheep, goats and cattle induced increased levels of mean total WBC counts (leucocytosis) with neutropenia (decrease in mean neutrophil count) and lymphocytosis; however, there was no significant difference ($p > 0.05$) between the eosinophil, basophil and monocyte counts of infected and uninfected animals. Silva et al. (1999) reported that the main haematologic changes produced by *T. vivax* infections in cattle in the Bolivian Wetlands and Brazilian Pantanal were anaemia and severe leucopenia (decrease in the mean total white blood cell counts); the leucocyte changes were characterized by relative lymphocytosis and monocytosis and decrease in the neutrophil counts. Additionally, the natural

occurrence of *Trypanosoma vivax* in cattle in Mosul, Iraq resulted in leucopenia due to lymphopenia and neutropenia in comparison with normal range for cattle (Rhaymah & Al-Badrani, 2012). Other studies, under experimental conditions, showing how *T. vivax* infection influences WBC parameters in cattle and other animals have also been published. For example, Esievo and Saror (1983) infected eight Zebu cattle with *T. vivax* stock Y58, while eight served as uninfected controls, and noted that the infected animals developed early leucopenia due to concomitant lymphopenia and neutropenia. Leucopenia, as well as monocytosis and eosinopenia, have been reported in *T. brucei* infection of mice and *T. vivax* of sheep (Anosa & Kaneko, 1983). Jenkins and Facer (1985) reported an initial leucopenia over the first 3 weeks of *T. vivax* infection in cattle, with values subsequently rising above the pre-infection levels. Anosa, Logan-Henfrey and Shaw (1992) analyzed eleven 6-month-old calves challenged with a stock of *T. vivax* (IL 2337) and observed an initial leucopenia which was followed at a later stage of the infection by leucocytosis associated with lymphocytosis and neutropenia.

The severe leucopenia observed in trypanosome-infected animals could cause immunosuppression; in other words, severe leucopenia could compromise the immune response (Silva et al., 1999). Ilemobade, Adegboye, Onoviran and Chima (1982) infected groups of cattle singly with *T. vivax* or *T. congolense* and with a combination of *T. vivax* and *T. congolense*, after the cattle had been vaccinated against contagious bovine pleuropneumonia (CBPP), and suggested that the protective immunity to CBPP engendered by vaccination was impaired during infection with trypanosomes. High levels of neutrophils and eosinophils may indicate an active infection; low counts may

indicate a compromised immune system (Davidson et al., 1998; Spivak, 1984). Ohaeri and Eluwa (2011) explained that the neutropenia they observed in trypanosome-infected cattle might be as a result of excessive utilization of neutrophils following inflammation process caused by trypanosome infection. Elevated levels of lymphocytes (lymphocytosis) may indicate an active infection and a depressed level may indicate an exhausted immune system or, if the neutrophils are elevated, an active infection (Davidson et al., 1998). An active infection causes monocyte level to rise; low levels are not clinically significant (Davidson et al., 1998). The function of basophils is unknown; they are the least numerous of the WBCs and in many samples none are present (Birchard & Sherding, 2006; Davidson et al., 1998; Schalm et al., 1975). The trypanotolerance phenomenon also enables trypanosome-infected animals to maintain WBC values as noted by Mbanasor et al. (2003) who reported similar values (within normal range) for all WBC parameters in trypanotolerant-infected and uninfected Muturu cattle in Nigeria.

A summary of haematological data reported by various workers for trypanosome-infected and uninfected cattle under natural conditions together with normal haematological values for cattle is shown in Table 1. In Ghana, very limited study has been carried out to provide baseline data to help in the interpretation of haematological values of the various cattle breeds in the country with regard to trypanosomosis. Adam et al. (2012) in a random sample of 1800 cattle of the West African Shorthorn (814), Sanga (904) and Zebu (82) breeds showed that although the parasitological prevalence of trypanosomal infections was highest in the Sanga breed, the average PCVs of the WASH, the Sanga and Zebu cattle (both trypanosome-positive and trypanosome-

negative) were 30%, 31% and 32%, respectively. Also, the PCV did not differ significantly between parasitologically positive and negative animals.

Control of Trypanosomosis

Drug use in the control of animal trypanosomosis

Several drugs have been used for chemotherapy of animal trypanosomosis in sub-Saharan Africa. These include the phenanthridine derivatives homidium bromide (Ethidium®, Novidium®) and isometamidium chloride (Samorin®, Trypamidium®); the aminoquinaldine derivative quinapyramine (Anthrycid®) and the aromatic diamidine derivative diminazene aceturate (Berenil®) (Kinabo, 1993; Mahama et al., 2003; Turkson, 1993). Trypanocidal drugs are expensive for African farmers (Matovu, Seebeck, Enyaru, & Kaminsky, 2001; Ross & Sutherland, 1997) and problems of toxic side effects in animals have been reported (Croft, 1997; Fairlamb, 2003). These drugs have been marketed for more than half a century and cases of drug resistance have emerged in 18 countries of sub-Saharan Africa (Delespaux, Geysen, Van den Bossche, & Geerts, 2008) and more recently in Benin, Ghana and Togo (Sow et al., 2012). The widespread distribution of resistance is attributed to a range of factors such as the long-term use of the same molecules, the misuse of the drugs and the often low quality of drugs available on the local markets (Geerts et al., 2001). Recent studies on the quality of the trypanocides sold in sub-Saharan Africa showed that a great majority of these products do not meet the standards established by the original producers (Schad, Allanson, Mackay, Cannavan, & Tettey, 2008; Tettey, Astriku, Chizyuka, & Slingenbergh, 2002).

Tsetse vector control

Due to the frustration posed to the control of trypanosomosis of

livestock in sub-Saharan Africa by the continued spread of resistance to trypanocidal drugs, a lot of resources and efforts have been channelled towards controlling the disease by attacking its insect vectors, the tsetse flies (*Glossina spp.*). Different options are available for tsetse control in sub-Saharan Africa. These include bush-clearance designed to eliminate the shaded places where tsetse rest and lay their larvae; wild game destruction designed to reduce the parasite reservoirs and eliminate the wild blood sources available for tsetse; insecticidal spraying of tsetse resting sites (Allsopp & Hursey, 2004) and application of sterile insect technique (Feldmann, 2004; Vreysen et al., 2000); insecticide-treated livestock (Hargrove, 2003; Torr et al., 2007) and insecticide-treated targets (Vale & Torr, 2004).

The complete clearing of bush, which entails the removal of all woody vegetation for a mile or two on each side of river-crossings, watering points and main roads, was successfully employed to control tsetse flies (Hocking, Lamerton, & Lewis, 1963). In Ghana, such clearings were carried out on a massive scale against the riverine species of tsetse in the Nabogu Valley of the Northern Region and along the River Kamba at Lawra in the Upper West Region (Mahama et al., 2003). In Kenya, protective barriers of between 8-10 miles (12-16 km) long were created in the Lake Victoria region to prevent re-invasion of areas cleared of *G. palpalis* (Whitside, 1958). Also, clearings along main roads to provide a safeguard against accelerated fly advance, and as a convenient line of defence, had been used against *G. morsitans* and *G. swynnertoni* in Tanzania (Hocking et al., 1963). Although complete bush-clearance had proved effective in the eradication of tetse flies, the method is expensive, laborious and time consuming with high speed of

Table 1: Effect of natural trypanosome infection on haematological parameters of cattle (mean ± SD)

Parameters	Infected beef cattle (Zebu)[a]	Infected dairy cattle (Holstein-Zebu crossbreed)[a]	Infected Muturu cattle[b]	Uninfected Muturu cattle[b]	Reference range[c] (average)
PCV (%)	20.93 ± 7.16	29.60 ± 4.43	28.50 ± 4.15	29.23 ± 4.12	24.0 - 46.0 (35.0)
Hb (g/dl)	6.30 ± 2.61	7.74 ± 1.41	11.64 ± 1.57	11.22 ± 1.57	8.0 - 15.0 (11.0)
RBC ($x10^6mm^3$)	2.35 ± 0.55	3.11 ± 1.12	7.23 ± 1.72	7.69 ± 1.67	5.0 - 10.0 (7.0)
MCV (fl)	109.04 ± 0.38	98.07 ± 6.60	40.64 ± 6.32	38.96 ± 5.98	40.0 - 60.0 (52.0)
MCH (pg)	30.93 ± 18.50	27.04 ± 7.23	16.83 ± 4.04	15.03 ± 2.94	11.0 - 17.0 (14.0)
MCHC (g/dl)	30.96 ± 15.98	27.36 ± 3.22	41.27 ± 6.47	38.60 ± 4.02	30.0 - 36.0 (32.7)
WBC ($x10^3mm^3$)	1.26 ± 0.61	1.54 ± 0.81	18.63 ± 6.17	18.77 ± 6.27	4.0 - 12.0 (8.0)
Neutrophil (%)	33.43 ± 9.55	35.62 ± 0.44	21.71 ± 8.12	19.96 ± 7.45	15.0 - 47.0 (28.0)
Lymphocyte (%)	50.06 ± 9.02	44.43 ± 0.85	68.64 ± 9.43	69.09 ± 8.92	45.0 - 75.0 (58.0)
Eosinophil (%)	6.91 ± 2.45	7.5 ± 4.58	6.50 ± 2.81	7.35 ± 4.21	0.0 - 20.0 (9.0)
Monocyte (%)	9.43 ± 2.49	11.87 ± 4.61	3.00 ± 2.02	3.51 ± 2.06	2.0 - 7.0 (4.0)
Basophil (%)	0.56 ± 1.50	0.56 ± 1.50	0.14 ± 0.37	0.08 ± 0.29	0.0 - 2.0 (0.5)

[a]Silva et al., 1999
[b]Mbanasor et al., 2003
[c]Jain, 1993

re-invasion (Hocking et al., 1963). Other arguments against complete bush clearance are that it can encourage soil erosion, decrease soil fertility and adversely affect water supplies (Hocking et al., 1963). It is against these drawbacks that the concept of discriminative bush-clearance has been advocated (Hocking et al., 1963). This concept is based on the outcome of studies which showed that tsetse fly population tends to concentrate periodically in certain identifiable plant communities that comprise a comparatively small proportion of the bush or woodland as a whole. This tendency is influenced by the fact that climatic conditions during the hot, dry seasons are unfavourable to wide dispersal throughout the woodland. Under such conditions, the fly population retreats to the cooler, shaded shelter of the type of vegetation which provides a micro-climate more suitable to their survival. At the onset of more favourable conditions generally, the flies again disperse widely and infest large areas of the woodland (Hocking et al., 1963). Thus, for this method to be effective it must be preceded by extensive and accurate surveys of the vegetation types most favourable as refugee or 'concentration site' (Hocking et al., 1963), which could make this method also expensive, laborious and time consuming where a large area of infested bush is involved (Hocking et al., 1963).

Many species of wildlife living in tsetse-infested areas carry trypanosome infections, and so act as important reservoir hosts, but generally show no obvious ill-effects (Mulla & Rickman, 1988). The main game animal hosts of tsetse comprise warthog, bushpig, porcupine, bushbuck, kudu, oryx, eland, waterbuck, buffalo, baboon, duiker and lion (Gates & Williamson, 1984; Mulla & Rickman, 1988). Game destruction had been used as a routine

method of tsetse control in East and South Africa (Hocking et al., 1963). For example, a controlled experiment at Shinyanga in Tanganyika in 1952 demonstrated clearly that game elimination resulted in the complete disappearance of *G. morsitans, G. swynnertoni* and *G. pallidipes* from an area of 1500 km^2 (Hocking et al., 1963). Later researchers are, however, of the view that decrease in the number of wildlife, a usual consequence of human encroachment, might increase the probability of domestic animals being fed on. An example of this was observed in the cultivated areas of the Eastern Plateau of Zambia where *G. m. morsitans* took 75.1% of its meals from cattle (Van den Bossche & Staak, 1997) even when other domestic animals (mainly goats, pigs and dogs) were present. In contrast, an earlier study by Sehof (1975) showed that in the Luangwa Valley in the Eastern Province of Zambia, where fauna was not affected by human interference, *Glossina morsitans morsitans* took the majority of its bloodmeals from wild suidae. Another example occurred with *G. m. morsitans* taking 91% of its meals from cattle in the cultivated areas of Nyanga District of eastern Zimbabwe (Robertson, 1983). However, where settlements were established close to wildlife habitats, the proportion of blood meals taken from cattle was often low since game animals were abundant there. This situation was demonstrated in the low proportion (34%) of cattle with anti-trypanosomal antibodies along the tsetse-infested edge of the Vwaza Game Reserve areas in Malawi (Van den Bossche, Shumba, & Makhambera, 2000) compared to 78% in Petauke District of the Eastern Plateau, Zambia, where cattle were kept in a tsetse-infested area where game animals were scarce. Thus, although game animals are the preferred host of tsetse, their elimination has the tendency to make cattle the major host,

thereby increasing the prevalence of trypanosomosis in cattle herds (Van den Bossche et al., 2000; Van den Bossche & Staak, 1997).

Current tsetse control methods

Current vector control strategies involve the use of insecticides (through the sequential aerosol spraying technique and bait systems) and the sterile insect technique (Vale, 2009). The sequential aerosol technique involves 4-5 aerial sprayings of low concentration, non-residual insecticide in the cool of the night to facilitate descent of droplets (Allsopp & Hursey, 2004; Barret, 1997). Excellent results are possible, especially with the newly improved methods of aircraft guidance, and large areas can be cleared of tsetse in a relatively short time (Kgori, Modo, & Torr, 2006). However, the aircraft must fly low at night, which precludes application in mountainous terrain. In most instances, eradication was not achieved because of re-infestation of cleared areas (Vale, 2009). Furthermore, the technique is expensive and requires substantial economic and infrastructure support so that operations covering an area of less than about 1000 km^2 are uneconomic (Vale, 2009). There are also concerns about the negative impact on the environment as was the case with the aerial and ground insecticide-spraying techniques that were in vogue from the 1950s to the 1980s, particularly in southern and eastern Africa, with the use of persistent organochlorine insecticides such as DDT and dieldrin, although recently replaced by low residual insecticides such as endosulphan (Barret, 1997).

Two bait systems, where flies are attracted to point sources and killed, are also currently relied on to control tsetse. The first is referred to as stationary artificial baits (Vale & Torr, 2004), which consist of traps and targets. Mono-

and biconical traps create visual stimulus to which tsetse respond by flying into them. Targets are made of fabric sheets treated with insecticide which kills tsetse on contact. Traps are costlier than targets and less efficient, but since they retain the flies they are useful for surveys (Vale, 2009). With the savannah species of tsetse, the performance of traps and targets is enhanced many times by adding odour attractants identified from cattle, but the riverine species respond poorly to odours (Vale, 2009). Theft, fire and the presence of new fly species reduce the certainty of traps and targets being effective (Itty & Swallow, 1993). Although targets generally provide control with negligible side effects, target-related mortalities of tabanids, which are responsible for pollinating plants fed on by fruit-eating and nectarivorous bird species in Kasungu National Park in Malawi, were speculated to have caused a statistically significant decline in the numbers of these bird species in target compared to control areas (De Garine-Wichatitsky, Cheke, & Lazaro, 2001).

The second of the bait systems is insecticide-treated livestock (Vale, 2009). Pyrethroids, insecticides of low mammalian toxicity, have been applied to cattle and the wanderings of such insecticide-treated cattle enhance fly-bait contact (Vale, 2009). Pyrethroids were initially used in cattle dips to protect cattle from tsetse, ticks and other biting flies. Animal health companies later introduced "pour-on" formulations to increase the flexibility in cattle treatments. Deltamethrin, a pyrethroid, has been successfully used to control bovine trypanosomosis in different parts of Africa; for example, as a pour-on insecticide in Burkina Faso (Bauer, Amsler-Delafosse, Clausen, Kabore, & Petrich-Bauer, 1995), Kenya (LoÈhr et al., 1991) and Uganda (Okello-Onen et al., 1994); and as a dip in Tanzania (Fox, Mmbando, Fox, & Wilson, 1993)

and Zimbabwe (Thomson & Wilson, 1992). Other workers (Codjia et al., 1993; Mulugeta et al., 1997) also applied deltamethrin as pour-on to cattle in the Ghibe valley, south-west Ethiopia, and recorded reductions of 95% in mean tsestse apparent density and 65% trypanosome prevalence. These reductions in tsetse apparent density and trypanosome prevalence have been maintained for 6 years. However, an attempt to use treated cattle as a barrier to tsetse re-invasion from Mozambique into north-eastern Zambia was unsuccessful as reported by Warnes et al. (1999) who treated 5400 cattle at two weekly intervals in an area of 428 km^2 and found that the area was rapidly invaded and there was serious deterioration in the disease situation. Baylis and Stevenson (1998) also found that, relative to a control area, the application of 50,000 doses of "Spot On" (1% deltamethrin) to cattle at Galana Ranch, Dakabuku, Kenya, produced little effect on the apparent densities of two species of tsetse. Mortality of dung beetles has been associated with pyrethroid residues in dung; dung beetles play the crucial role of burying fresh dung for the maintenance of soil structure, water holding capacity and fertility in semi arid regions (Kruger, Scholtz, & Reinhardt, 1998). As a result of the application of pyrethroids to cattle, ticks may develop insecticide resistance, young cattle may not have the opportunity to acquire resistance to tick-borne disease, and residual amounts of the insecticides may appear in cow milk (FAO, 2003). By and large, the bait techniques, which, unlike spraying campaigns, are potentially suitable for implementation by farmers, are now the methods of choice in the control of tsetse (Dransfeld, Brightwell, Kyorku, & Williams, 1990; Laveissiere, Hervouet, Couret, Ecouzan, & Merouze, 1985; Leak, Mulatu, Rowlands, & d'Ieteren, 1995; Leak, Peregrine, Mulatu,

Rowlands, & d'Ieteren, 1996). The relative low cost and simplicity of the traps or targets recommend them for use by local communities, but they are applied on a scale so small that control efforts are bound to be frustrated by re-invasion (Warnes et al., 1999).

The sterile insect technique involves the release of laboratory-reared and sterilised males to compete with wild males so that females inseminated by them produce no offspring (Feldmann, 2004). The technique demands the costly and lengthy establishment of large and complex rearing facilities, and requires insecticidal campaigns to suppress the wild tsetse population prior to sterile male release (Vale, 2009). Moreover, control by reducing the breeding of the long-lived tsetse is inherently much slower than killing the pest (Vale & Torr, 2005). The feasibility of this costly approach in large areas where multiple species are present remains doubtful (Bhalla, 2002; Enserink, 2007).

Vaccine development

Currently, there is no vaccine for trypanosomosis and attempts to develop it are hampered by the phenomenon of antigenic variation of trypanosomes (Nyame, Kawar, & Cummings, 2004). Each bloodstream form trypanosome cell is completely enwrapped in a monomolecular layer of a single species of glycoprotein, the variant surface glycoprotein (VSG) that determines its variable antigen type (VAT) (Turner, 1999). The density of VSG molecules is sufficiently high for the VSG layer to be visible by electron microscopy as a 12 nm thick surface coat (Vickerman, 1969) that comprises approximately 107 VSG molecules per cell and occupies a surface area of approximately 180 mm^2 (Jackson, Owen, & Voorheis, 1985). The surface coat

acts as a physical barrier, preventing access to the underlying plasma membrane by components of nonspecific immune responses (Ferrante & Allison, 1983). Protection of the trypanosome against VSG-specific immune responses is through antigenic variation, the switching off of expression between immunologically distinct VSGs (Turner, 1999). Each VSG is encoded by a separate gene, of which there are several hundred in the parasite genome (Marcello & Barry, 2007). Only one of these many VSGs is expressed at a time, and is transcribed from one of approximately 15 telomeric VSG ES (expression site) transcription units (Hertz-Fowler et al., 2008). The process of antigenic variation ensures the long-term survival of trypanosome parasites, allows them to maintain chronic infection within the host and has frustrated attempts to develop a vaccine (Nyame et al., 2004).

Economic Impacts of Animal Trypanosomosis

The direct and indirect impacts of animal trypanosomosis on agriculture have adverse effects on the economic development of tsetse fly infested areas of Africa (Holmes, 1997; Swallow, 1999). According to the WHO Programme Against African Trypanosomosis (PAAT), the problem of trypanosomosis, affecting both human and animal health, "lies at the heart of Africa's struggle against poverty" and dealing with this disease has the potential to impact on all eight Millennium Development Goals in the 37 countries with tsetse infestations, 21 of which are among the world's 25 poorest (PAAT, 2008). Direct impacts of trypanosomosis on livestock productivity include mortality, morbidity and impaired fertility; reduction of productivity in terms of reduced milk, meat and draught power; and the costs of implementing and maintaining tsetse fly and trypanosomosis control

operations. Indirect impacts arise from farmers' responses to the perceived risk of the disease, including the reduction and, in some cases, the exclusion of livestock from tsetse-infested grazing lands, and reduced crop production due to insufficient animal draft power (ILRAD, 1993; Shaw, 2004). The annual loss directly attributed to trypanosomosis, in terms of reduced meat and milk production and in terms of the costs related to treating the disease or controlling the vector, was estimated at US $1.2 billion. This figure could have risen to over US $4.5 billion per year, if losses in potential crop and livestock production attributable to the disease were considered and excluded the losses attributable to the effects of sleeping sickness in humans (Kabayo, 2002).

Impacts of trypanosomosis on herd productivity

The results of a number of studies on cattle (Agyemang et al., 1990; Agyemang, Little, Mattioli, Sonko, & Janneh, 1993; Feron et al., 1987; Thorpe et al., 1988; Trail et al., 1991) suggested that of all the productivity parameters, the largest and most consistent impacts of trypanosomosis were on birth rates and mortality of young animals. The general implication was that the incidence of trypanosomosis: (i) reduced calving rates by 1-12% in trypanotolerant breeds of cattle and by 11-20% in trypanosusceptible breeds; and (ii) increased calf mortality by 0-10% in trypanotolerant breeds of cattle and by 10-20% in trypanosusceptible breeds of cattle. Studies on trypanotolerant sheep and goats (Bealby, Connor, & Rowlands, 1996; ITC, 1997) also indicated that the main impacts of trypanosomosis were on lambing rates (reduced by 4-38%) and kidding rates (reduced by 37%). Kamuanga, Sigue, Kabore, Bauer and Swallow (1997) interviewed a sample of livestock

owners in the Yalé Province of Burkina Faso before and after tsetse control was implemented in their area. All of the livestock owners were Fulani pastoralists who had recently settled in the area with their Zebu cattle. The results showed that the majority (87%) of respondents recognised that there was a substantial reduction in the number of cattle deaths caused by trypanosomosis. The livestock owners estimated that the overall mortality rate in their herds was 63% in 1993/1994, before control, and 7% in 1996/1997, after control. Differences in mortality before and after tsetse control were similar for all age and sex groups of animals. The small standard deviations around the mean values indicated a great deal of consistency on opinion among respondents. Table 2 summarises the results of the study by Kamuanga et al. (1997). Brandl (1988) conducted an assessment of the potential benefits and costs of tsetse eradication through use of the sterile insect technique in the Sideradougou area of southern Burkina Faso. He predicted that the cattle population in the Sideradougou area would increase at about 0.9% per year without tsetse control and between 2 and 5% per year with tsetse control (depending upon the prior severity of trypanosomosis in the area). With that herd growth, it was estimated that trypanosomosis reduced milk offtake by 9 to 38% and animal offtake by 5 to 31%. Swallow (1999) gave an account of a study commissioned by the University of Berlin in 1993 to separately assess the benefits of tsetse control by traps on the two main production systems — sedentary and transhumance — in northern Cote d'Ivoire. The study concluded that without tsetse control the populations of sedentary and transhumance cattle in northern Cote d'Ivoire would increase at average annual rates of 10.4% and 9.2% respectively, while with tsetse control

those populations would increase at rates of 11.9% and 11.3%. With those assumptions, trypanosomosis reduced milk offtake by 12% in the sedentary herds and 8% in the transhumance herds; it would reduce animal offtake by 4% in sedentary herds and 10% in transhumance herds. Kristjanson, Swallow, Rowlands, Kruska and de Leeuw (1999) used a herd simulation model to estimate the milk offtake and meat offtake that were produced by cattle in tsetse-free areas compared to cattle kept in tsetse-infested areas. The results (summarised in Table 3) showed that the tsetse-free area produced 83% more milk and 97% more meat per unit land area than the tsetse-infested area. The reductions in milk and meat resulted in lower welfare for producers who earned less income from the production of meat and milk and consumers who consumed less of those products. Together, consumers and producers lost a total of $1,338 million per year (Kristjanson et al., 1999). Studies from The Gambia (Agyemang et al. 1990, 1993) indicated that trypanosomosis reduced milk offtake from trypanotolerant cattle by 10-26%. The major impacts of tsetse control on the productivity of cattle in the Ghibe Valley, southwest Ethiopia, were shown to be a reduction in calf mortality (including still births) and increases in adult body weight, particularly in males (Rowlands, Mulatu, Leak, Nagda, & d'leteren, 1999). In the same study, trypanosome prevalence in adult cattle was reduced from 41% to 16% during years of tsetse control (an absolute reduction of 25%) and the percentage of cattle requiring treatments with the trypanocidal drug diminazene aceturate declined from 42% to 21%. Annual growth of the herd increased from 7.6% to 13.3% per year (Rowlands et al., 1999). Seyoum, Terefe and Ashenafi (2013) reported a study they carried out from November 2011 to April 2012 to assess farmers' perception

on the presence, impact, management and the need of intervention programs of bovine trypanosomosis and tsetse fly in selected districts located in Baro-Akobo and Gojeb river basins in southwest Ethiopia. A standardized questionnaire survey was employed to collect the relevant information from the farmers. The result of this study showed that 94.1% of the respondents considered bovine trypanosomosis as an economically important cattle disease which accounted for 64.6% of the total annual deaths in the year 2011/2012. Estimated mean annual financial loss via mortality due to trypanosomosis was reported to be 3501 Ethiopian Birr (US$200)/household.

Impacts of trypanosomosis on animal traction productivity

Data collected by researchers in the Ghibe Valley of Ethiopia provided an opportunity to evaluate the effects of trypanosomosis incidence on the productivity of oxen used for traction. In 1995, data were collected from all 4,985 households resident in an area of successful tsetse control and from a sample of 191 households resident in nearby areas that had not had successful tsetse control. Tsetse control in this case resulted from the coordinated use of pour-ons by individual farmers (Leak, Mulatu, Rowlands, & d'Ieteren, 1995; Swallow, Mulatu, & Leak, 1995). Average area cultivated was plotted against number of oxen owned for three groups of households: households outside of the tsetse control area; households in the tsetse control area that did use pour-ons; households in the tsetse control area that did not use pour-ons. The results of the study showed that households outside of the control area that did not own oxen were not able to cultivate any land using animal traction. For each ox that households outside of the control area owned, they were able to cultivate 0.5 additional hectares. Households within the tsetse control area that

did not own oxen were able to cultivate up to a hectare of land, using either their other livestock (cows, bulls, heifers, donkeys) or their neighbours' animals. For each additional ox that they owned, they were able to cultivate an additional 0.8 hectares of land. The overall conclusion of the study was that oxen in the high risk area were 38% less efficient than oxen in the low risk area.

Impacts of trypanosomosis on trypanocidal drug use

It was estimated that about 35 million doses of trypanocidal drugs were administered each year in Africa (Geerts & Holmes, 1997). At an average purchase price of $1 per dose, this meant that African farmers were spending

Table 2: Percentage reduction in mortality due to trypanosomosis control as perceived by pastoralists in Burkina Faso

Age/sex category	1993/94 (s.d)	1996/97 (s.d)	% Reduction
Male calves (<1 year)	64.9 (20.7)	8.7 (8.4)	56.2
Female calves (<1 year)	63.3 (21.2)	7.3 (8.2)	55.9
Young males (1-2 years)	60.8 (18.5)	6.9 (8.9)	53.9
Young females (1-2 years)	58.1 (18.3)	6.1 (7.9)	52.0
Males (2-3 years)	60.6 (19.7)	6.7 (8.9)	54.0
Females (2-3 years)	58.1 (20.0)	5.9 (8.2)	52.2
Bulls (> 3 years)	69.9 (24.0)	7.2 (8.5)	62.7
Cows (> 3 years)	70.8 (21.4)	10.0 (7.5)	60.8
Oxen (> 3 years)	61.0 (34.4)	5.4 (8.6)	55.6
Overall	63.1	7.1	56.0

Source: Kamuanga et al. (1997)

Table 3: Annual meat and milk production in tsetse-infested areas of Africa

Region	Meat production ('000 tonnes)	Meat produced in tsetse-infected areas ('000 tonnes)	Milk production ('000 tonnes)	Milk produced in tsetse-infected areas ('000 tonnes)
Eastern Africa	1041	291	6577	1840
Western Africa	590	286	1203	583
Central Africa	146	112	230	176
Total sub-Saharan Africa	2845	772	11524	2872

Source: Kristjanson et al. (1999)

$35 million per year on trypanocidal drugs. The large majority of those treatments were likely given to cattle. Assuming that each animal treated was given 2 treatments per year, 17.5 million cattle were treated each year out of a total of 46 million cattle at risk. This implied that two-thirds of the cattle raised under trypanosomosis risk were not given treatments of trypanocidal drugs (Geerts & Holmes, 1997). Evidence from northern Cote d'Ivoire and southern Burkina Faso suggested that farmers' use of trypanocidal drugs depended most upon: (1) the breeds of cattle that they raised, (2) whether or not they practiced transhumance, (3) their knowledge of the disease and its treatment, and (4) their ability to pay (Swallow, 1999). A study conducted by ILRI and the Ministere de l'Agriculture et des Ressources Animales in Cote d'Ivoire showed that livestock owners' use of prophylactic treatments of trypanocidal drugs depended upon whether or not they took their animals on seasonal transhumance and the breed composition of their herds. All households that undertook seasonal transhumance into the forest zone reported using prophylactic treatments. Of households that did not undertake transhumance, 29% with only trypanotolerant cattle used prophylactic treatments, while 66% of households with some zebu used prophylactic treatments (Swallow, 1999).

Evidence from both West and Southern Africa showed that trypanosomosis risk was not an important determinant of the use of trypanocidal drugs. In the Yalé province of southern Burkina Faso, Kamuanga et al. (1997) found that livestock owners spent more money on trypanocidal drugs in 1997 than in 1994, despite a major decrease in trypanosomosis risk in the area. Two possible reasons for the low initial level of trypanocidal drug use were that the

pastoralists who settled in this area of high trypanosomosis risk were: (1) unfamiliar with the disease and (2) impoverished by the losses that the disease caused (Kamuanga et al., 1997). In eastern Zambia, Van den Bossche et al. (1999) cited by Swallow (1999) examined the use of trypanocidal drugs in two nearby areas, one with a moderate-to-high level of trypanosomosis risk and one with a very low level of risk. The researchers (Van den Bossche et al, 1999) found that livestock owners in both areas preferred to administer curative rather than prophylactic treatments of trypanocidal drugs, with most treatments given to oxen and cows. Most treatments were given to clinically sick animals, not necessarily to animals infected with trypanosomes. The average rate of treatment in both areas was 1.5 treatments per year.

Indirect impacts of farmers' perception of trypanosomosis risk on animal numbers

Mugalla, Swallow and Kamuanga (1997) conducted a study on the numbers of livestock held by households residing in adjoining areas of low, medium and high trypanosomosis risk in The Gambia. Table 4 gives a summary of results of the study. An observation from this study was that average herd size, expressed in terms of tropical livestock units, was lowest in the high risk area, twice as high in the medium risk areas, and four times as high in the low risk area. The numbers of herd cattle and horses follow similar trends across the study areas. The researchers found no statistical relationship between the numbers of draft cattle, donkeys, sheep and goats and the level of trypanosomosis risk. They attributed the differential effect of trypanosomosis risk on the different types of livestock to differences in animal management rather than degree of susceptibility to trypanosomosis. During the study

period, most draft cattle, sheep, goats, horses and donkeys were kept within the village areas, while herd cattle were grazed in closer proximity to the areas of highest tsetse challenge. Doran and Van den Bossche (1999) reported results of a similar study, in the southern Africa region, conducted in nearby areas with zero and medium-to-high trypanosomosis risk. There was a significant difference between the number of cattle per owner in the tsetse-free and tsetse-infested areas, with an average of 49.0 cattle per owner in the tsetse-free area and 32.4 cattle per owner in the tsetse-infested area. There were no significant differences between the two areas in the number of goats per owner. As in The Gambia, farmers in the tsetse-infested area owned a significantly higher proportion of oxen.

Studies of the relationship between tsetse infestation and cattle density were conducted for two of the African countries that had the largest cattle populations, Ethiopia and Nigeria (Bourn, 1978). The study showed that cattle density was positively related to human population density, rainfall, altitude and the presence or absence of tsetse. Tsetse-infested areas generally supported much lower cattle densities than non tsetse-infested areas. Wint (1998) did a follow up of Bourn's (1978) study of Ethiopia, with the results illustrated in Figure 1. Wint, in his study, identified areas whose livestock populations would increase between 1 and 50 head per square kilometre and demonstrated that the greatest increases were expected in areas adjacent to tsetse-free areas.

In studies at the continental level, Jahnke et al. (1988) compared the density of cattle in tsetse-infested and non-infested areas of the sub-humid and humid zones. In the sub-humid zone the average livestock density was 9.9 Tropical

Livestock Units (TLUs) per km^2 in the non-infested areas and 6.2 TLUs per km^2 in the infested areas (37% less). In the humid zone the average livestock density was 9.3 TLUs per km^2 in the non-infested areas and 2.8 TLUs / km^2 in the infested areas (70% less). Gilbert et al. (1999) also predicted increases in cattle density for the African continent as a whole using the coarse-resolution spatial data (pixels of approximately 5 square kilometres) available through the PAAT Information System. Geo-referenced data from 12,000 points located across the African continent were used to estimate statistical relationships that could be used to predict cattle density and cultivation intensity on the basis of a number of explanatory variables, especially rainfall, temperature, vapour pressure deficit, vegetation cover, elevation, potential evapotranspiration, length of growing period, human population, and the number of tsetse species present. Statistical relationships were estimated for a number of ecozones occurring in each country. The resulting equations were then used to predict cattle densities with and without the presence of tsetse. The results showed that there were about 172 million head of cattle kept in the 37 countries that had some level of tsetse infestation, of which 44.7 million were within the area of tsetse infestation. Without the presence of tsetse, there would be approximately 90 million more cattle. Effectively, therefore, they suggested a 200% increase in actual numbers. The greatest increase in numbers would be in Tanzania, Nigeria, Central African Republic, Ethiopia, Cameroon and Burkina Faso. Again, cattle densities were predicted to increase by between 1 and 50 head per square kilometre. Increases of 50 head or more were expected in areas adjacent to tsetse-free areas, for example in Tanzania, Ethiopia, Nigeria and Burkina Faso.

Table 4. Relationship between level of trypanosomosis risk and numbers of animals owned in the Central River Division of The Gambia

Type of animal	Levels of tsetse challenge				All areas
	Low risk (L. Fulladu West)	Medium risk (Bansang North Bank)	Medium risk (Bansang South Bank)	High risk (Niamina East)	
	Average number of animals per household (standard deviation)				
Herd cattle	27.3 (120.6)	9.9 (18.2)	10.3 (18.7)	3.0 (7.2)	13.7 (65.5)
Draft cattle	1.1 (1.8)	1.2 (1.8)	2.0 (2.3)	0.9 (1.3)	1.3 (1.9)
Horses	1.2 (1.4)	0.6 (1.1)	1.0 (1.6)	0.1 (0.3)	0.8 (1.3)
Donkeys	0.9 (1.0)	0.9 (1.1)	0.8 (1.2)	1.1 (1.1)	0.9 (1.1)
Sheep	5.1 (6.7)	4.8 (5.9)	5.1 (4.8)	4.6 (6.1)	4.9 (5.9)
Goats	5.9 (6.0)	5.6 (5.3)	5.7 (6.2)	4.9 (4.2)	5.6 (5.6)
Tropical Livestock Units	25.8 (97.7)	11.2 (16.1)	12.6 (17.3)	5.1 (6.8)	14.5 (53.4)

Note: 1 Tropical Livestock Unit = 10 sheep = 10 goats = 1.25 donkey = 1.25 bovine = 1 horse

Source: Mugalla et al. (1997)

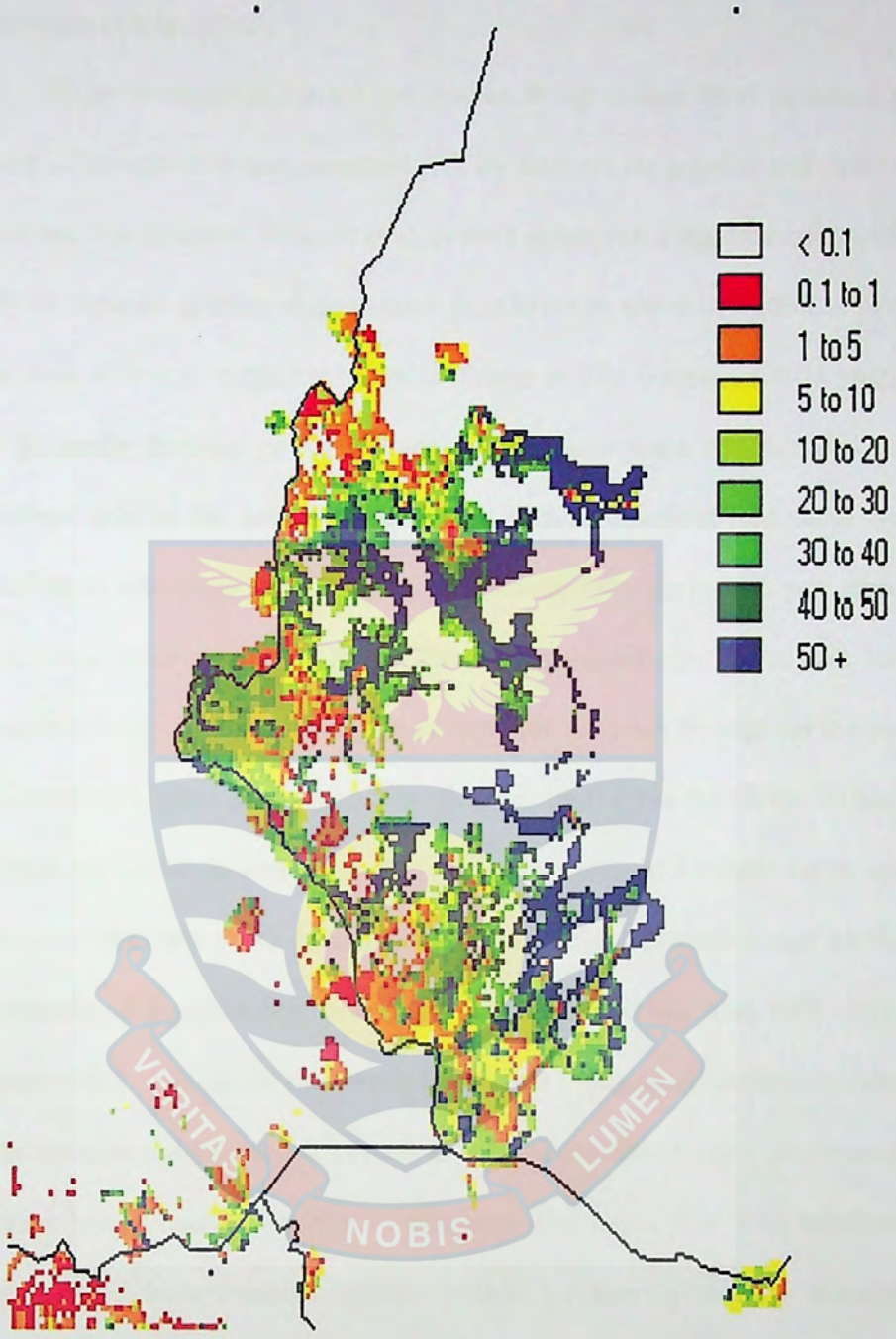


Figure 1. Predicted increase in cattle density with removal of economically-important species of tsetse in tsetse-infested areas of Ethiopia

Source: Wint (1999)

Indirect impacts of farmers' perception of trypanosomosis risk on grazing and seasonal migration

Many researchers carried out studies at the village level to assess the impact of perceived trypanosomosis risk by farmers on grazing and seasonal migration. For example, Wacher et al. (1994) undertook a detailed quantitative study of farmers' grazing management in relation to the distribution of tsetse in an area of low to moderate tsetse challenge in The Gambia. Cattle grazing was generally focused on the village, while tsetse were concentrated in a woodland area to the north of the village. It was observed that cattle were grazed in an area of relatively high tsetse density only during the wet season, when crops were planted in the fields around the village. There was large variation among herds in the degree of exposure to tsetse throughout the year. Swallow (1999) gave an account of work done by ILRI in the Ghibe Valley of Ethiopia that involved monitoring the grazing patterns of 11 cattle herds since 1993, less than two years after a tsetse control trial had started and within a few months of the time that tsetse were suppressed to less than 10% of their pre-trial level. The herds that were followed all began as collective village herds, with animals housed in corrals at each household at night, then brought together into collective herds each morning. The herds that were monitored were selected from adjacent villages within a relatively densely populated portion of the tsetse control area measuring about 50 km^2. Arndt (1995) and Arndt (1996) analysed the herd monitoring data collected by ILRI to evaluate how grazing patterns changed as the intensity of land use increased with tsetse control. GIS techniques were used to estimate the size and configuration of the grazing area of each herd. Additional information was obtained through 19

group interviews convened with groups of farmers in the 9 villages within the study area. The findings suggested that four related processes unfolded as a result of successful reduction of trypanosomiasis risk in the study area: (i) initial expansion of grazing areas toward the rivers due to the reduction in problems with tsetse, then reduction of grazing areas closer to villages as more and more of the land was brought into cultivation; (ii) splitting of collective herds as the intensification of cultivation made wet-season grazing more scarce and thus more difficult to manage large herds; (iii) individual farms began to put fences around the grazing areas on their own farms, especially in villages in which collective herds had been split; and (iv) some farmers began to send animals out of the area to more sparsely populated areas. Kamuanga et al. (1997) reported a 1995-6 study in southern Burkina Faso to evaluate the impacts that an experimental programme of tsetse control undertaken by CIRDES (Centre International de Recherche-Developpement sur l'Elevage en zone Subhumide) had on the local farmers. Households in two nearby areas, Satiri and Bekuy, were asked to describe their livestock enterprises and perceptions prior to 1991 and since 1991. Tsetse control began in the Satiri area in November 1987 and was extended to the Bekuy area in 1995, just prior to the survey. About 60% of respondents in the Satiri area said that it had become easier to access grazing areas near river valleys, swamps, in natural pastures and in gallery forests. Over 80% of the Bekuy respondents said that the current situation was about the same as it was before tsetse control began in Satiri (Kamuanga et al., 1999). National-level and regional-level studies were also done by researchers to assess the impact of perceived trypanosomosis risk by farmers on grazing and seasonal migration. Bassett

(1993) argued that seasonal fluctuations in the density of tsetse had important impacts on grazing patterns and consequently on land-use conflicts between Fulani herders and sedentary farmers in northern Cote d'Ivoire. The populations of the two dominant species of tsetse in the study area (*G. palpalis gambiensis* and *G. tachinoides* – riverine species) increased during the rainy season and declined during the dry season. At the height of the rainy season Fulani herders move their cattle away from the major rivers to the upland agricultural areas to avoid tsetse. This resulted in conflicts between pastoralists and crop farmers.

Indirect impacts of farmers' perception of trypanosomosis risk on crop agriculture

Changes in livestock productivity in terms of animal traction and migration were found to have impacts on crop agriculture. Studies conducted in the Ghibe Valley of Ethiopia and the Zambezi Valley of Zimbabwe suggested that households that migrated into an area of tsetse control could constitute a powerful force for change in agricultural production. The Ghibe Valley study found that the average migrant household owned 78% more oxen and 56% more cattle and cultivated 37% more land than the average long-term resident of the area. Migrants coming into the Ghibe Valley generally had no cattle when they arrived but built up their herds from year to year so that after 3-4 years they had as more cattle as the long-term residents (Kagwanja, Swallow, Reid, & Stevens, 1999). The study conducted in the Zambezi Valley of Zimbabwe also showed large differences between immigrants and people born in the study sites. In 1995, 61% of migrants owned cattle, while only 38% of the indigenous residents owned cattle. Forty-seven percent of migrants

owned ox teams, compared to 27% for indigenous residents. Migrants owned an average of Z$ 8467 worth of animal traction equipment, compared to Z$ 5015 for indigenous residents. With that extra capacity for animal traction, migrants planted more area to cotton, the main cash crop. While indigenous residents produced slightly higher yields of maize and cotton, they generated lower revenues from crops than migrants. Migrants and indigenous residents who used animal traction generated higher returns to land and labour than households that only used hoes. Migrants that used animal traction generated 45% more income per unit of land and 143% more income per unit of labour than migrants that cultivated with hoes. Indigenous residents that used animal traction generated 25% more income per unit of land and 140% more income per unit of labour than indigenous residents that cultivated with hoes (Govereh, 1999). The results obtained in the Ghibe Valley of Ethiopia and the Zambezi Valley of Zimbabwe were consistent with more general studies of the relationship between livestock and crop agriculture in sub-Saharan Africa. Steinfeld (1988) related the size of cattle holdings to maize area and maize yield for farms in the semi-arid and sub-humid zones of Zimbabwe. He found strong positive relationships between cattle holdings and area and between cattle holdings and yield. Similar conclusions were reached by Savadogo et al. (1994; 1996) who evaluated the productivity and price responsiveness of farmers that used animal traction, and those that did not use animal traction, in Burkina Faso.

Trypanosomosis Control Initiatives at the International Level

Although African trypanosomosis ranked high in the list of priorities of colonial powers (Lyons, 1992), the disease has been seriously neglected in

recent decades (Barrett, Boykin, Brun, & Tidwell, 2007; Smith, Pepin, & Stich, 1998; Veeken & Pecoul, 2000). During the colonial period, European powers made sustained efforts to control trypanosomosis out of concern for the epidemics of the human disease and the chronic loss of livestock impeding both transport and agriculture (Jordan, 1986). Their attempts – based largely on active detection and treatment of trypanosomosis cases, combined with major programmes to control tsetse by bush-clearance, wild game culling, and insecticidal spraying of tsetse resting sites – were largely successful. But following the wars of independence during the 1960s, and the progressive withdrawal of colonial infrastructure, case incidence of trypanosomosis began to rise and routine tsetse control activities were interrupted in many of the endemic countries (Schofield & Kabayo, 2008). However, important initiatives at the international level are presently trying to redress this, by setting ambitious targets for tsetse and trypanosomosis control at the continental and regional levels (Allsopp, 2001).

In 1974, the Food and Agriculture Organisation (FAO) was given a UN mandate to coordinate the eradication of tsetse and trypanosomosis from Africa in ten years. Unfortunately, the tools and infrastructure were not available and the mandate proved overambitious. It did, however, sow the seeds that led to the establishment of the Programme Against African Trypanosomosis (PAAT) (Allsopp, 2001).

In 1997, the 29th Conference of the FAO established PAAT (Resolution 5/1997), bringing together the efforts of FAO, the World Health Organisation (WHO), the International Atomic Energy Agency (IAEA) and the African Union (Allsopp, 2001). PAAT is an international advisory group that

facilitates data management and communication between most nations in sub-Saharan Africa (Allsopp, 2001). PAAT is closely associated with the AU International Scientific Council for Trypanosomosis Research and Control (ISCTRC), which has brought national control authorities and international scientists together for 50 years (Allsopp, 2001).

The PAAT Committee meeting of 1998 (PAAT, 1998) identified two main options for tsetse and trypanosomosis management: large-scale, essentially top-down operations, and small-scale, community based projects (Allsopp, 2001). An example of the large-scale operations was the systematic ground spraying operations which involved the clearing of 75,000 km^2 in Nigeria from 1970 to 1975. With some aerial spraying, the area cleared of tsetse increased to 196,000 km^2 by 1978, to 210,000 km^2 by 1981 (Bature, 1981). This progressive campaign could not be sustained and although human settlement had delayed reinvasion, tsetse had returned to many cleared areas (Ogunnusi, 1991). Similar operations were carried out in the Adamawa Region of Cameroun, where tsetse were cleared from 25,000 km^2 and trypanosomosis was controlled for 18 years by helicopter spraying. Disruption of annual operations in the late 1980s resulted in significant reinvasion. With the suspension of the aerial campaign in 1994, the situation proved difficult to maintain with barriers (Cuisance & Boutrais, 1995). The substantial gains made with ground spraying in Uganda were lost as a result of political unrest in the 1970s (Allsopp, 2001). In Botswana, annual aerial spraying operations from 1972 to 1991 reduced the distribution of tsetse from 20,000 km^2 to 5000 km^2, with the result that neither human nor animal trypanosomosis occurred from the early 1980s to 1999 (Allsopp, 2001). Aerial spraying operations were

also used between 1983 and 1988 in Somalia to successfully remove tsetse from 4500 km^2 along the Shebelle River (Clark, 1991). However, the deterioration of the security situation of the country resulted in curtailment of spraying operations leading to reinvasion (Allsopp, 2001). The Tsetse Control Branch of Zimbabwe did ground spraying of some 148,000 km^2, and, in the 1960s succeeded in pushing the tsetse distribution limits well back beyond the international borders. These extensive and effective annual campaigns were disrupted when the war of independence escalated in the 1970s, and much of this ground was reinvaded. After the war, aerial spraying was introduced to accelerate operations, and targets were used to mop up surviving populations. By the turn of the 20th century, tsetse distribution was reduced from 54,000 km^2 to 14,500 km^2. Efforts were made by the government of Zimbabwe to protect these cleared areas through operating barriers with targets and cattle dipping over 28,000 km^2 – mostly along its border with Mozambique (Lovemore, 1999).

Hargrove (1999) clearly identified the inherent difficulties of sustaining control achievements in small areas that remain vulnerable to reinvasion. Seven East African community-based projects, covering little more than 2000 km^2 in total, were investigated (Barrett & Okali, 1998) and, although community support was enthusiastic, the control achievements were difficult to sustain. According to Laveissiere and Meda (1992), sustainability can be a problem without outside help even in West Africa where there is more of a culture of community involvement. In Kenya, socioeconomic factors such as ownership of livestock and degree of exposure to trypanosomosis affected the willingness of communities and individuals to participate in control activities

(Kamara, Echessah, Curry, & Swallow, 1995). In the Congo, operational success was dependent on adaptation to local beliefs and mentalities (Leygues & Gouteux, 1989). In sum, despite the efforts of PAAT, the impact of trypanosomosis shows little sign of abatement (Allsopp, 2001).

The European Union founded the Regional Tsetse and Trypanosomosis Control Programme for southern Africa (RTTCP) to control tsetse based on the principle of Integrated Pest Management (IPM). The four participating countries — Malawi, Mozambique, Zambia and Zimbabwe — share a common tsetse belt and the original objective of the programme was to use a range of techniques in appropriate situations to eliminate the entire 320,000 km^2 distribution. In 1988, the RTTCP rejected large-scale chemical control methods and in doing so essentially discarded the area wide principle. Eradication from this area was no longer feasible with the remaining limited technical options and in 1995 it was abandoned as the programme re-focused on rural development. Having adjusted their control strategies and resources accordingly, the four national control authorities needed time to consolidate their positions, but the programme ended in 1991 with the participating governments, especially Zimbabwe, faced with the prospect of substantial and indefinite barrier maintenance (RTTCP, 1998).

Mehlitz et al. (1998) and Budd (1999) reviewed the impact of DFID support over a 20-year period and concluded that high quality research had resulted in the development of an extensive range of control methods. According to the authors, these methods, if implemented on a wide scale, would have a significant impact on the livelihoods of some 260 million Africans. The total cost of controlling tsetse throughout the continent was

estimated to be in the region of US$ 20 billion — but the benefit to agriculture would be in the region of US$ 50 billion within ten years. Unfortunately, cost, environmental impact and suitability for local community management have replaced technical effectiveness as the key determinants in project planning, and not all available techniques are being used in a concerted effort to reduce the tsetse problem (Mehlitz et al., 1998; Budd, 1999).

A characteristic feature of the externally-financed projects to control tsetse was that such projects were set up in countries of interest to external donor organisations. The projects were in administratively-defined areas and were run for an administratively-specified time. They were generally successful whilst in operation, but generally proved unsustainable once the formal project reached its endpoint, so many were ultimately judged to be failures (Schofield & Kabayo, 2008). Rather different was the tsetse eradication project on the Island of Zanzibar (Unguja). The campaign to eradicate *G. austeni* from the Island of Zanzibar (Unguja) was carried out partly for socioeconomic reasons – to improve livestock and agricultural productivity on the island – and partly as 'proof-of-principle' of an integrated approach to tsetse elimination using various means, involving the use of traps, targets, and/or sequential ultra-low-volume aerial spraying to reduce tsetse populations to very low levels, followed if necessary by sterile insect release to eliminate any remaining wild flies. The campaign was implemented during 1994-98 by the IAEA in partnership with the Governments of Zanzibar and Tanzania, with additional support from the International Fund for Agricultural Development (IFAD), the Organization of Petroleum Exporting Countries (OPEC) fund, and the Governments of Belgium, Canada, China, Netherlands, Sweden,

UK and USA (Dyck, 1998).

Unguja Island covers an area of 1650 km^2; it has a mixture of agro-pastoral land and forest, including primary forest in the southern part (Jozani), and some 46,000 cattle and 26,000 sheep and goats. Prior to the campaign, annual losses due to tsetse and trypanosomiasis were estimated at around US$ 2 million. The tsetse suppression phase made use of cattle treatment using pour-on pyrethroid insecticides, together with some insecticide-impregnated blue cotton targets in the agro-pastoral regions. In Jozani forest however, high densities of such targets had to be deployed, typically 40-70 targets/km^2. Monitoring throughout the campaign was carried out using blue/white sticky panel traps at fixed sampling sites. A total of 8.5 million laboratory-reared sterile male flies were released by aircraft on fixed GPS-guided routes over the island (at an average of about 30,000 flies per week) from June 1994 to December 1997, even though no wild flies have been captured on the island since July 1996 (Dyck, 1998; Vreysen et al., 2000). According to unpublished IAEA reports, the Zanzibar campaign continues to be monitored, and no wild tsetse have since been encountered. The full costs of the campaign are not yet available. IAEA direct investment for 1995-97 (excluding staff) was US$ 539,578 (Dyck, 1998), although estimates by Hargrove (2003) suggest a true cost nearer to US$ 850000 – equivalent to around US$ 500 per km^2. Against this can be put the economic benefits to the Zanzibar community, which has now become a nett exporter of meat and dairy products (Kuzoe & Schofield, 2004).

Those involved with the Zanzibar project were quick to realise the parallels with American trypanosomosis control and raised the question of

whether or not similar tsetse elimination might be possible on mainland Africa (Dias, Silveira, & Schofield, 2002; Schofield, Jannin, & Salvatella, 2006; Schofield & Kabayo, 2008). In both Africa and in Latin America there had been a series of control projects following administratively-defined boundaries (rather than covering biologically-relevant areas) that had been technically successful until being halted before a sustainable end-point had been reached. The Latin American response came through multinational initiatives designed to promote political continuity of action and to cover the biologically relevant area represented by the entire distribution of the target vector species. In Latin America, the first step had been scientific debate (Schofield & Dias, 1991), followed by the political mandate (Ministerial resolution number 04-3-CS in 1991), designation of coordinating body, Pan American Health Organisation (PAHO), and work with each country within the mandated framework.

In Africa, the persistence of this trans-boundary disease has certainly been enhanced by the lack of a concerted, multi-national approach (Adam et al., 2012). The idea of a Pan-African initiative against tsetse and trypanosomosis was discussed and recommended at the 25th ISCTRC (International Scientific Council for Trypanosomosis Research and Control) in Mombasa, Kenya, in October, 1999. The recommendation was presented to the 36th summit of the OAU (Organization of African Unity, now African Union) in Lomé, Togo, in July 2000. In response, the Heads of State and Government of the 36 member states of the OAU passed resolution AHG/Dec.156 XXXVI recognising the seriousness of the tsetse and trypanosomosis problem, and calling on member states "*to act collectively..... to render Africa tsetse-free within the shortest time possible*". With this

mandate, the OAU set up the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC), which is now an integral part of the AU Commission for Rural Development (Schofield & Kabayo, 2008).

PATTEC was formally launched at the 26th ISCTRC meeting in Ouagadougou in October 2001, and its plan of action endorsed by the OAU summits in Lusaka (OAU Decision AHG/Dec.169/XXXVII) and Durban (OAU/AU Decision CM/Dec.661/LXXVI.2002). The General Conferences of the FAO and IAEA adopted resolutions in support of the initiative (FAO 31st Gen.Conf. Res 4.2001; IAEA 45th Gen Conf. Res GC(45)/RES/12), as did the World Health Assembly of WHO (Res WHA56.7) (Schofield & Kabayo, 2008).

No specific technical package is defined, since elimination of each tsetse population will have its own technical requirements, and each target region has its own background, experience and practical capabilities. Thus each target area will derive its own technical package, drawing from the wide range of available control methods – including combinations of traps and insecticide-impregnated targets, insecticide-treated cattle, ultra-low dose aerial spraying or ground-spraying or fogging of tsetse resting sites, and sterile insect technique if feasible and necessary for definitive elimination of the target population. Much depends on the ability to define biologically feasible targets – i.e. tsetse populations that are sufficiently geographically discrete that reinvasion would be unlikely, or that occupy regions around which effective barriers could be maintained until neighbouring areas can be treated. To assist in this, PATTEC has set up a research network supported by the Leverhulme Trust (Schofield & Kabayo, 2008) that uses phenetic and genetic markers to

assess geographical structuring of target tsetse populations (Bouyer et al., 2007; Camara et al., 2006).

Although AU-PATTEC is still at an early stage, considerable progress has been made, most notably in reviving national programmes in a number of countries. Almost all the tsetse-endemic countries now have designated PATTEC focal points within the Ministry of Livestock and/or Ministry of Health, and a series of multinational interventions has begun with support from the national Governments. In addition, the African Development Bank has provided a series of loans and grants totalling some $72 million to support tsetse elimination activities in Ethiopia, Kenya, Uganda, Mali, Burkina Faso, and Ghana, with several other countries currently negotiating similar arrangements (Schofield & Kabayo, 2008).

On-going regional programmes include the following:

- The 'Cotton Belt' of Mali, Burkina Faso, and northern Ghana (main targets: *G. palpalis gambiensis, G. morsitans submorsitans, G. tachinoides*) which is planned to extend progressively also into Cote d'Ivoire, Guinea, and Senegal
- The Lake Victoria Basin, including parts of Kenya, Uganda, Tanzania, and Ruanda (main targets: *G. fuscipes fuscipes, G. morsitans submorsitans, G. pallidipes*)
- Southern Rift Valley – southern Ethiopia and neighbouring parts of Sudan (main target: *G. pallidipes*)
- The south-eastern tsetse pocket (*G. brevipalpis, G. austeni*) of southern Mozambique and north-eastern South Africa (KwaZulu Natal)
- The southern tsetse belt (*G. morsitans centralis*) of Botswana, Namibia, western Zambia and southern Angola (Schofield & Kabayo, 2008).

Figures 2 and 3 were generated by Adam et al. (2012) in a PATTEC funded study in Ghana to provide information on the prevalence of trypanosomes and its vector to guide decision making in project implementation, as well as to generate sound baseline data for monitoring and evaluation. In the study, three main breeds of cattle that were sampled for trypanosome detection were the West African Shorthorn (WASH), Zebu and Sanga. The study area, the Upper West Region, is located in the Volta basin, in the north-western corner of Ghana between latitude 9.62–11° N and longitude 1.40–2.76° W. The total surface area of the Upper West Region is approximately 18,000 km^2. In this region, suitable land cover for tsetse flies was represented by open woodland and rangeland, mostly clustered along the main rivers (Black Volta, Kulpawn and Sissili) and their tributaries. The region was chosen as project area for the first phase of the Ghana component of the PATTEC initiative based on economic (i.e. potential for high economic return; Shaw et al., 2006), entomological (i.e. proximity to the northern tsetse distribution limits) and strategic criteria referring to the geographic location of the three national projects in West Africa. The study area is bordered to the North and West by Burkina Faso (the Black Volta River represents the natural border with Burkina Faso) (Adam et al., 2012).

Many scientists are sceptical that the PATTEC project will succeed as similar eradication campaigns failed in the past because the tsetse fly infested areas could not be isolated (Bhalla, 2002). The huge costs associated with the eradication project are also a concern as most of the countries involved belong to the most heavily indebted poor countries in the world (Bhalla, 2002).

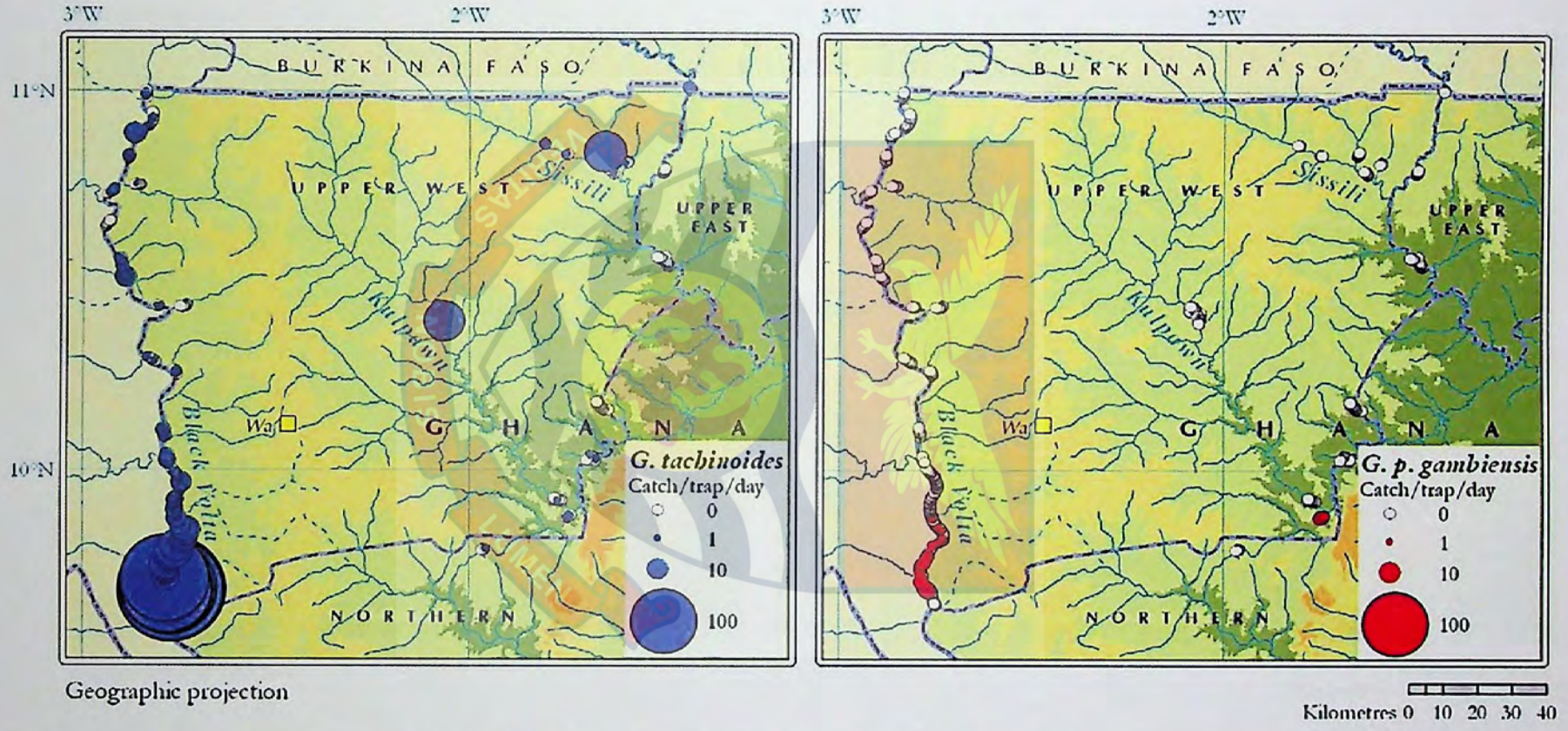


Figure 2. Distribution of *G. tachinoides* and *G. palpalis gambiensis* in the Upper West Region of Ghana

Source: Adam et al., 2012

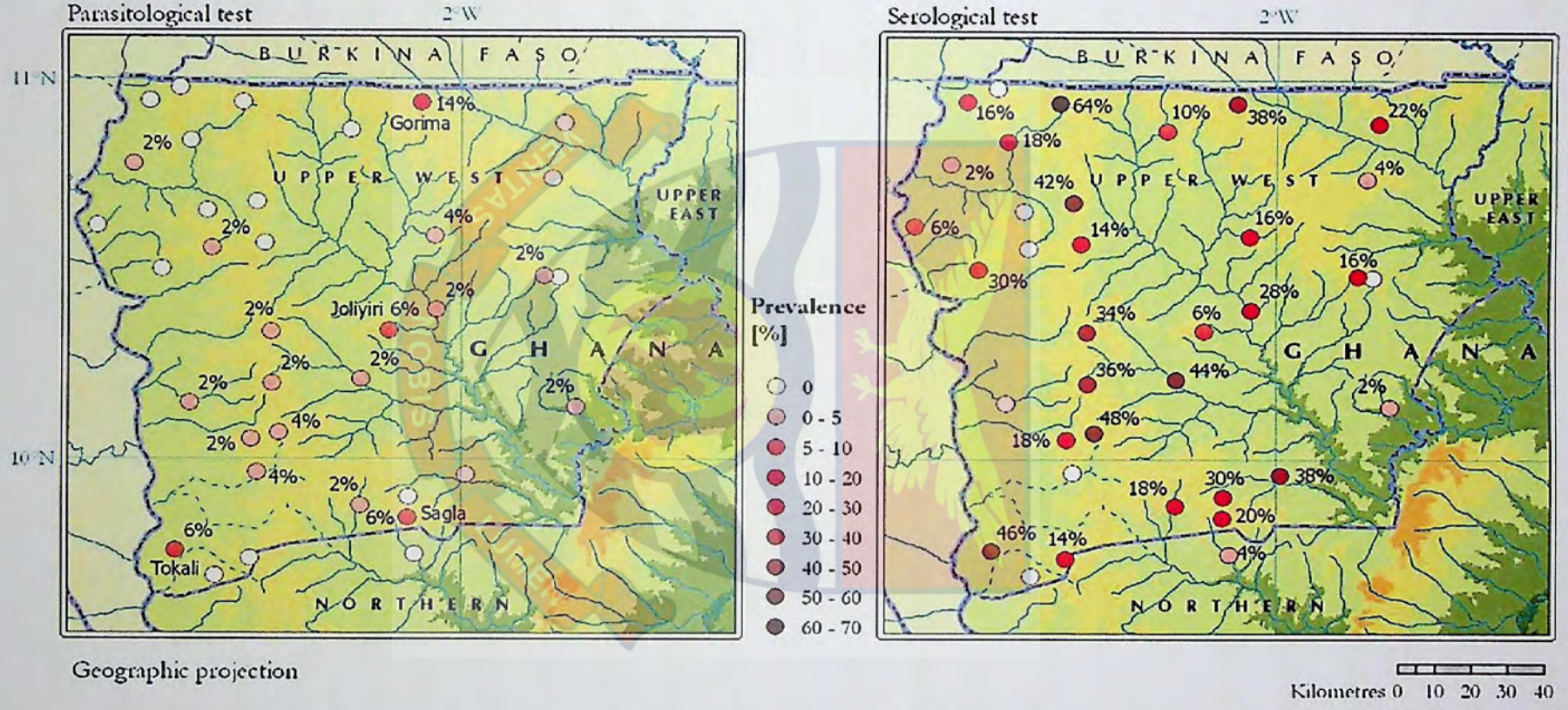


Figure 3. Parasitological and serological prevalence of livestock trypanosomosis in the Upper West Region of Ghana

Source: Adam et al., 2012

Use of Trypanotolerant Cattle in Trypanosomosis Control

Because of the limitations of the current methods for control and the likelihood that a vaccine will not become available in the foreseeable future, increasing consideration is now being given to the use of trypanotolerant cattle breeds as a sustainable, alternative option for the control of trypanosomosis in sub-Saharan Africa. Trypanotolerant breeds, although equally susceptible to the initial infection, possess the ability to survive, reproduce and remain productive in areas of high tsetse challenge without the need for the use of chemicals to control the vector or drugs to control the parasite (Dayo et al., 2009; Rege et al., 1994) where other breeds rapidly succumb to the disease (Murray et al., 1982). The trypanotolerant trait is generally attributed to the taurine breeds of cattle in West and Central Africa, namely, the N'Dama and the West African Shorthorn (Roelants, 1986).

It is thought on the basis of Egyptian tomb paintings of the XIIth Dynasty that the taurine Hamitic Longhorn breed, from which the N'Dama is descended, arrived in the Nile Delta from the Near East at about 5000 BC, whereas the taurine Shorthorn cattle were introduced into the same area between 2750 and 2500 BC (Epstein, 1971; Payne, 1964) . In contrast, *Bos indicus* cattle, which are the most prevalent cattle type in Africa, did not become numerous in Africa until after the Arab invasions of AD 669, although they were recognised on tomb paintings in Egypt between 2000 and 1500 BC. Apart from the Egyptian tomb findings, other researchers have presented archaeological evidence for the presence of domesticated cattle in North Africa during the early stages of the Neolithic period around 10,000 years ago (Carter & Clark, 1976; Gautier, 1987; Grigson, 1991; Wendorf & Schild,

1994). Recent mtDNA analysis (Bradley, MacHugh, Cunningham, & Loftus, 1996; Troy et al., 2001) has provided evidence for a Near Eastern origin for European taurine cattle and an African origin for taurine breeds found on the African continent, thus debunking the widely accepted assumption of a single common origin for African and European taurines in the early domestic centers of the Near East (Clutton-Brock, 1989; Epstein, 1971; Epstein & Mason, 1984; Payne, 1964). An earlier mtDNA study (Loftus, MacHugh, Bradley, Sharp, & Cunningham, 1994) confirms the Asian ancestry for Zebu cattle. It is worth recalling that wild Bovidae, which are extremely resistant to trypanosomosis (Murray, Clifford, Gettinby, Snow, & McIntyre, 1981), emerged in Africa some twenty to forty million years ago (Leaky & Lewin, 1977) and that tsetse probably originated even earlier (Ford, 1971). Thus, the trypanotolerant breeds have stayed for a longer period on the African continent and could have become locally adapted to trypanosome-challenge through natural selection (Ibeagha-Awemu, Jager, & Erhardt, 2004).

Major comparative investigations on the question of trypanotolerance have been carried out on cattle throughout Africa, notably in Nigeria (Desowitz, 1959; Roberts & Gray, 1973), Senegal (Toure, Gueye, Seye, Ba, & Mane, 1978), The Gambia (Murray, Morrison, & Whitelaw, 1982), Kenya (Ismael, Njogu, Gettinby, & Murray, 1985; Njogu, Dolan, Wilson, & Sayer, 1985) and Burkina Faso (Akol et al., 1986; Roelants, 1986). The main breeds studied included Ayrshire, Friesian, Holstein, Hereford and their crosses, as well as indigenous African breeds such as Zebu, Boran, West African Shorthorn and N'Dama. In these investigations, cattle were infected by syringe inoculation with bloodstream trypanosomes, exposed to field challenge,

laboratory challenge with wild caught tsetse or with tsetse that are experimentally infected. Irrespective of the mode of infection, the N'Dama (and the West African Shorthorn) were trypanotolerant (Akol et al., 1986; Desowitz, 1959; Ismael et al., 1985; Murray et al., 1982; Njogu et al., 1985; Roberts & Gray, 1973; Roelants, 1986; Toure et al., 1978). The studies further showed that the basis of this trait was associated with the capacity of these animals to develop less severe anaemia in the face of infection, as assessed by packed cell volume (Akol et al., 1986; Desowitz, 1959; Ismael et al., 1985; Murray et al., 1982; Njogu et al., 1985; Roberts & Gray, 1973; Roelants, 1986; Toure et al., 1978). This observation was corroborated in later studies by Achukwui and Musongong (2009), who, from a study of 296 trypanotolerant *Bos taurus* Doayo and trypanosusceptible *Bos indicus* White Fulani zebu cattle in Cameroun, produced evidence that trypanosome-infected Doayo and White Fulani zebu had lower PCV than uninfected animals. Although the infection rate was higher in the Doayo (16.4%) than WF zebu (11.9%), the PCV was higher in Doayo (both infected and uninfected) than WF zebu (infected and uninfected).

Anaemia is a well recognised and inevitable consequence of trypanosome infections in domestic animals in general and cattle in particular (Morrison, Murray, & McIntyre, 1981; Murray et al., 1984). Thus, an estimate of the ability of an infected animal to maintain PCV, following either experimental or field infection, could be used as a method for identifying trypanotolerant individuals (Murray et al., 1984).

Beyond trypanotolerance: Genetic management of trypanosomosis through selection of resistant cattle

It had been observed that diseases rarely occur in all members of animal populations exposed to pathogens (Caron, Malo, Schutta, Templeton, & Adams, 2004). Natural disease resistance refers to the inherent capacity of an animal to resist disease when exposed to pathogens, without prior exposure or immunization (Caron et al., 2004). Although some of the observed variation in natural resistance to infection is related to environmental factors, a significant component of variation appears to be heritable, and, therefore, stably passed from parent to offspring (Snowder, 2006). It is imperative to distinguish "resistance" which refers to the ability of the host to resist infection from "tolerance" which refers to a situation where the host is infected by the pathogen, but suffers little adverse effect (Bishop, de Jong & Gray, 1999). This distinction can be important in situations where the objective is to prevent the spread of the disease to other populations (as in the case of zoonotic diseases); in such instances, disease resistance rather than tolerance is required (FAO, 2007). Selection for disease resistance is much more complicated than selecting for production traits which can be measured directly or indirectly on each animal (Snowder, 2006).

MHC marker-assisted selection

The selection for breeding purposes of individuals that have high levels of disease resistance or tolerance can be facilitated if molecular genetic markers associated with these traits have been identified (Bishop & Woolliams, 2004; FAO, 2007). Obvious candidate genes affecting resistance traits in mammals are genes of the major histocompatibility complex (MHC)

(Andersson & Davies, 1994; Blattman et al., 1993; Davies et al., 1997; Klein, 1986). The MHC is the most important genetic component of the mammalian immune system and comprises a group of closely linked genes which play a fundamental role in the immune response against pathogens (Hedrick & Kim, 2000; Hill, 1996; Klein, 1986). MHC genes encode cell-surface glycoproteins that bind peptide fragments derived from pathogens and display them on the cell surface for recognition by the appropriate T-cells through T-cell receptors, thereby triggering an effective adaptive immune response (Babiuk et al., 2007; Janeway et al., 2001). There are two types of classical MHC genes: MHC class I and MHC class II (Babiuk et al., 2007; Janeway et al., 2001). MHC class I genes encode molecules that present endogenous peptides between 8-10 amino acids in length to CD8+ cytotoxic T-cells, whereas MHC class II genes encode molecules that present exogenous peptides between 12-15 amino acids in length to CD4+ helper T-cells (Babiuk et al., 2007; Janeway et al., 2001). The differences in antigen presentation by MHC class I and MHC class II molecules allow the immune system to differentiate intracellular and extracellular pathogens (Babuik et al., 2007). Because they play a central role in the immune system, MHC genes have been extensively studied as candidate genes for disease resistance in many domestic animal species (Blattman et al., 1993; Davies et al., 1997; Lewin et al., 1999; Xu et al., 1993). Genes within the MHC are highly polymorphic; that is, there are multiple variants of each gene within the population as a whole (Janeway et al., 2001). Each MHC allele may potentially bind and present to T-cells a different set of pathogen-derived peptides, and thus a population carrying a higher number of MHC alleles may respond to a broader spectrum of pathogens (Babik et al., 2012;

Parham & Ohta, 1996). This has led to suggestions that depletion of variation at the MHC genes may compromise the ability of populations to respond to pathogen assault and lead to an increased risk of extinction (Hedrick, 2001; Hughes, 1991; O'Brien & Evermann, 1988). In fact, MHC polymorphism has been associated with differences in susceptibility to diseases in man (Carrington et al., 1999; Hill et al., 1991; Thurz et al., 1997) and other vertebrates (Kaufman et al., 1995; Lamont, 1998).

Parasite-mediated selection and MHC evolution

The characteristically high levels of functional polymorphism of MHC genes is believed to be maintained by balancing selection operating through host-parasite coevolution, mediated either through heterozygote advantage or frequency-dependent selection (Bernatchez & Landry, 2003; Hedrick, 1999; Penn, Damjanovich, & Potts, 2002; Richman, 2000). The hypothesis of heterozygote advantage predicts that heterozygous individuals are fitter than individuals homozygous for an MHC allele because two different alleles will bind and present more parasite-derived peptides, thereby increasing the range of parasites recognized by the immune system, compared with one (Doherty & Zinkernagel, 1975; Hughes & Nei, 1989; Takahata & Nei, 1990). MHC heterozygote advantage was indicated in humans by a slower progression to AIDS after HIV infection (Carrington et al., 1999) and in a more effective clearance of hepatitis B viral infections (Thurz et al., 1997). In a study carried out in Japan using Holstein cattle, Takeshima et al. (2008) produced evidence that heterozygosity of an MHC gene was associated with resistance to mastitis progression, whereas homozygous genotypes promoted susceptibility to mastitis. In laboratory experiments, MHC heterozygous mice showed reduced

susceptibility to bacterial and viral infections (Chen, Cohen, Zaleski, & Albini, 1992), *Salmonella*, *Listeria* (Penn et al., 2002), *Salmonella enterica* as well as Theiler's virus (McClelland, Penn, & Potts, 2003). Such mice also exhibited increased T-cell-mediated immunity during lymphocytic choriomeningitis infection (Doherty & Zinkernagel, 1975), and they had a faster clearance rate of parasitic worms *Heligmosomoides polygyrus* (Behnke & Wahid, 1991) and *Schistosoma mansoni* (Sher, Hieny, & James, 1984) than the average homozygote. Tumor incidence was lower and regression faster in heterozygous rous sarcoma virus-infected chicken compared to the homozygote (Senseney, Briles, Abplanalp, & Taylor, 2000). MHC class IIB heterozygote captive-raised Chinook salmon (*Oncorhynchus tsawytscha*) infected by a haematopoietic necrosis virus had an increased survival rate compared with the homozygote (Arkush et al., 2002). Similarly, MHC class IIB heterozygote captive-raised Gila top minnows (*Poeciliopis o. occidentalis*) infected by the fluke (*Gyrodactilus turnbulli*) had a higher survival rate than the homozygote (Hedrick, Kim, & Parker, 2001).

In some cases, there is evidence that heterozygotes actually have reduced fitness relative to homozygotes (Gutierrez-Ezpeleta, Hedrick, Kalinowski, Garrigan, & Boyce, 2001; Ilmonen et al., 2007; Wedekind, Walker, & Little, 2005). Gutierrez-Ezpeleta et al. (2001) observed high levels of MHC DRB gene allelic variation and heterozygosity in bighorn sheep that had suffered a drastic population decline due to infectious disease. These researchers (Gutierrez-Ezpeleta et al., 2001) concluded that high genetic variation of an MHC gene was no safeguard against the detrimental effects of infectious disease. On the other hand, Mikko, Roed, Schmutz and Andersson

(1999) found that beaver, moose and roe deer, all of which have low MHC variation, showed dramatic population expansions in Sweden in the last century. A strong association of the extent of MHC variation and disease resistance does not appear universal and other factors, probably nongenetic, appear to be of overriding significance in determining dramatic changes in population numbers (Gutierrez-Ezpeleta et al., 2001).

The frequency-dependent selection hypothesis proposed there is strong selective pressure in favour of any pathogen that has mutated in such a way that it escapes presentation by the most common MHC alleles. This hypothesis is also described as '*rare-allele advantage hypothesis*', '*Red Queen hypothesis*' or '*moving-target hypothesis*' (Ebert & Hamilton, 1996; Jaenike, 1978; Ladle, 1992; Penn & Potts, 1999). New parasite variants will decrease the common host alleles and confer a selective advantage to individual hosts that have new, rare MHC alleles. As an MHC allele resistant to a particular parasite becomes more common in the host population, this creates increasing selective pressure on the parasite to adapt to the common MHC allele (Bernatchez & Landry, 2003; Janeway et al., 2001; Jeffery & Bangham, 2000; Lively & Dybdahl, 2000). Thus, the frequency-dependent selection hypothesis considers host-parasite interactions as a dynamic process or a coevolutionary "arms race", with MHC alleles favoured at low frequencies and rising in frequency, only to induce a corresponding shift in the genetic composition of the parasite population, which reduces the fitness of common host MHC alleles (Apanius, Penn, Slev, Ruff, & Potts, 1997; Takahata & Nei, 1990).

There is some evidence that rare alleles confer resistance to parasites. For example, an association between certain MHC alleles and disease

resistance or susceptibility was found in a free-ranging sheep population (Soay sheep) where MHC allelic variants appear to play a major role in protection against strongyle nematode invasion, the most prevalent gastrointestinal parasite found (Paterson, Wilson, & Pemberton, 1998). As expected by the assumptions of the '*frequency-dependent selection*' (Bodmer, 1972; Clarke & Kirby, 1996; Takahata & Nei, 1990), the most common alleles OLADRB 205 and 257 (allele frequencies: 0.21-0.24) were associated with decreased lamb or yearling survivorship, whereas the rarer OLADRB 263 allele (allele frequency: 0.13) was associated with increased yearling survival. Other workers have demonstrated that rare alleles confer resistance to HIV progression in a population of HIV-infected men (Trachtenberg et al., 2003), and are also associated with low nematode parasite burdens of free-ranging mouse lemurs in four littoral forest fragments in southeastern Madagascar (Schad, Ganzhom, & Sommer, 2005) and in a population of the striped mouse in the Southern Kalahari (Froeschke & Sommer, 2005).

On the other hand, other reports (Grimholt et al., 2003; Hill et al., 1991; McClelland et al., 2003; Thurz et al., 1997; Wu et al., 2004), have found no evidence of heterozygote advantage, but rather showed resistance that is attributable to single, specific MHC alleles (not rare alleles), which were considered to be more consistent with frequency-dependent selection. Studies showing association between resistance or susceptibility of specific alleles and parasite-species abound in the literature. For example, certain MHC alleles played a role in resistance or susceptibility to a fungal disease caused by *Cryptococcus neoformans* in mice (McClelland et al., 2003). MHC alleles were also associated with resistance or susceptibility to infections induced

with gastrointestinal nematodes *Trichinella spiralis* (Wassom, Brooks, Babisch, & David, 1983; Wassom, Krco, & David, 1987), *Nematospiroides dubius* (Enriquez, Brooks, Cypess, David, & Wassom, 1988) and *Trichuris muris* (Else, Wakelin, Wassom, & Hauda, 1990) in laboratory mice, and *Ostertagia circumcincta* in straightbred Scottish Blackface sheep (Buitkamp, Filmether, Stear, & Epplen, 1996; Schwaiger et al., 1995). Association between resistance and MHC alleles was found in chicken suffering from infection with Marek's disease (a tumour disease caused by a herpes virus) (Briles, Stone, & Cole, 1977). Experimental evidence for MHC-allele-specific resistance to *Aeromonas salmonicida* bacteria (Langefors, Lohm, Grahn, Andersen, & von Schantz, 2001; Lohm et al., 2002) and to the infectious salmon anaemia virus was found in captive-raised Atlantic salmon *Salmo salar* (Grimholt et al., 2003). In cattle, allelic variants of MHC genes were found to be associated with resistance to persistent lymphocytosis caused by bovine leukemia virus (Xu et al., 1993). Associations have also been observed for resistance to dermatophilosis in Brahman cattle of Martinique (Maillard et al., 1996); cystic ovarian disease and retained placenta (Sharif et al., 1998); as well as foot and mouth disease (Glass et al., 1991; Lewin et al., 1999). The alleles were also associated with susceptibility to several infectious diseases such as mastitis (Duangjinda et al., 2009; Park et al., 2004; Rupp et al., 2007; Sharif et al., 2000; Sharif et al., 1998). Juliarena et al. (2008) found that MHC alleles were associated with high and low proviral loads in bovine leukemia virus-infected cattle. However, studies relating cattle MHC genes and trypanosomosis are rare. Karimubo et al. (2011) showed that MHC alleles were associated with an increased risk of *T. brucei* and *T. congolense*

infection in *Bos indicus* Masai and Boran cattle in Tanzania.

Virtually all the association studies reported for cattle focused on the MHC class II DRB3 gene. This gene is mostly chosen because it is the most polymorphic class II gene in cattle with over 90 different alleles detected (Takeshima et al., 2001) and much of the variation is in the functionally-important antigen-binding site (Ohta, 1998).

MHC Genotyping Methods

Since MHC genes are highly polymorphic, it has been difficult to establish a genotyping approach to define the functionally relevant allelic variations (Orita et al., 1989; Pipalia et al., 2004; Slade, Moritz, Heideman, & Hale, 1993; Zhou & Hickford, 2004). Previous methods for typing MHC genes include PCR-restriction fragment length polymorphism (PCR-RFLP) analysis (Davies et al., 1994; Ellegren et al., 1993; Lewin et al., 1999; Van Eijk et al., 1992), PCR-single strand conformation polymorphism (PCR-SSCP) analysis (Orita et al., 1989; Pipalia et al., 2004; Sunnucks et al., 2000; Zhou et al., 2007) and sequencing (Ripoli, Peral-Garcıa, Dulout, & Giovambattista, 2004; Snibson, Maddox, Fabb, & Brandon, 1998; Zhou & Hickford, 2004).

PCR-RFLP analysis involves the use of restriction enzymes and requires prior knowledge of recognition sequence (Orita et al., 1989). PCR-RFLP analysis has been very useful for distinguishing alleles at chromosomal loci and has been used extensively for MHC polymorphism studies in livestock (Amills, Francino, & Sanchez, 1995; Amills, Francino, & Sanchez, 1996; Behl, Verma, Behl, Mukesh, & Ahlawat, 2007; Blattman et al., 1993; Dongxiao & Yuan, 2004; Duangjinda et al., 2009; Escayg, Hickford,

Montgomery, Dodds, & Bullock, 1996; Escayg, Montgomery, Hickford, & Bullock, 1993; Grain et al., 1993; Juliarena et al., 2008; Maillard et al., 2003; Maillard et al., 1996; Rupp et al., 2007; Sharif et al., 2000; Sharif et al., 1998). However, it has the limitation of not being able to differentiate distinct alleles differing only by few base pairs that are not present in the recognition sequence for the corresponding restriction endonucleases, thus scoring distinct alleles as being the same, which may lead to underestimation of polymorphism in a particular gene (Orita et al., 1989; Pipalia et al., 2004; Slade et al., 1993; Zhou & Hickford, 2004).

Unlike PCR-RFLP, PCR-SSCP technique neither involves the use of restriction enzymes nor requires any prior knowledge of recognition sequence, and has been used successfully to explore polymorphism, with marked improvement over PCR-PFLP analysis (De, Singh, & Butchaiah, 2002; Girman, 1996). The method relies on variable electrophoretic mobility of secondary structures formed by single-stranded DNA fragments of different primary structure (i.e. nucleotide sequence) (Nguyen et al., 2006; Orita et al., 1989; Sunnucks et al., 2000). PCR products are denatured by heat (95°C) and immediately placed on ice. Under these conditions the single strands will not anneal to their complements. Instead the strands will fold upon themselves into stable conformations according to base pairing rules (G-C and A-T). Sequence differences cause different conformations that are detected by electrophoresis (Nguyen et al., 2006; Orita et al., 1989; Sunnucks et al., 2000).

Both PCR-RFLP and PCR-SSCP methods require separation of PCR products by agarose gel electrophoresis, and, for large-scale detection of mutations, are expensive, laborious and time-consuming (da Mota, Martinez,

& Coutinho, 2004; Lo et al., 2009; Millat, Chanavat, Rodriguez-Lafrasse, & Rousson, 2009; Wang et al., 2011). Furthermore, since these methods are open tube assays, the process in transferring PCR products for further analyses creates a high risk of contamination (Nettuwakul, Sawasdee, & Yenchitsomanus, 2010).

Genotyping large numbers of animals is generally a prerequisite for studies investigating associations of gene variants with complex phenotypic traits such as immune responsiveness or disease resistance (Baxter, Hastings, Law, & Glass, 2008). Sequencing is the most reliable and efficient genotyping method (Baxter et al., 2008; da Mota, Gabriel, Martinez, & Cutinho, 2002; Gonzalez-Cadavid, Zhou, Battifora, Bar-Eli, & Cline, 1989). However, it is very laborious and time consuming (Pipalia et al., 2004) and requires expensive equipment in specialized facilities (Fitzgerald et al., 2001). While obtaining DNA sequence has become less expensive and time-consuming, it is still a limiting step in most projects (Sunnucks et al., 2000). In the light of the limitations of PCR-RFLP and PCR-SSCP, another method, high resolution melting analysis (HRM), has been proposed to facilitate genotyping of DNA samples (Wittwer, Reed, Gundry, Vandersteen, & Pryor, 2003).

High resolution melting analysis

High resolution melting analysis is a simple, fast, cost effective and highly sensitive closed-tube method that can scan or detect single nucleotide polymorphic loci and unknown sequence variants without the need to sequence individual genotypes or to use restriction enzymes or gel separation (Tindall, Petersen, Woodbridge, Schipany, & Hayes, 2009; Vaughn & Elenitoba-Johnson, 2004; Wittwer et al., 2003; Wu, Wirthensohn, Hunt,

Gibson, & Sedgley, 2008). The HRM procedure begins with DNA amplification by PCR in the presence of double-stranded DNA-binding dye which has higher level of fluorescence when bound to double stranded DNA than in single stranded DNA bound or in unbound state (Wittwer et al., 2003). PCR products (amplicons) are slowly heated until they fully dissociate. The result of HRM is a melting curve that can be generated if the fluorescence is continuously monitored during the heating of a sample through its dissociation temperature (melting temperature, Tm).

The melting temperature of a double stranded DNA fragment is defined as the temperature where 50% of the DNA is single stranded (SantaLucia Jr, 1998). DNA secondary structure, the double helix, is held together by hydrogen bonds between base pairs. Specifically, adenine bases pair with thymine bases and guanine bases pair with cytosine bases. Heating a DNA sample disrupts these hydrogen bonds, thus "unwinding" the double helix and denaturing the DNA. Because three hydrogen bonds form between guanine/cytosine base pairs and two hydrogen bonds form between adenine/thymine base pairs, more energy is required to denature the former. If an allele has a higher proportion of CG pairs than another allele, the allele shows a higher melting temperature. Melting temperature is one of the characteristics of DNA and can be used in describing the difference (polymorphism) among alleles (Howell, Jobs, & Brookes, 2002; Wittwer et al., 2003).

After HRM, amplicons containing the same sequence variants are identified as groups that exhibit similar melting profiles (Montgomery, Wittwer, Palais, & Zhou, 2007). However, since HRM cannot identify the

actual nucleotide changes in an amplicon, it does not replace sequencing, but could represent a good screening method for detecting whether or not DNA fragments are identical in sequence, thereby greatly reducing the amount of sequencing necessary (Bennett et al., 2003). The HRM analysis has mostly been applied in human clinical studies for differentiating bacterial species (Cheng et al., 2006; Odell et al., 2005); for genotyping measles virus isolates (Waku-Kouomou et al., 2006); for detection of point mutations in *Plasmodium falciparum* genes associated with anti-malarial drug resistance (Andriantsoanirina et al., 2009); for differentiation of old world Leishmania species (Nicolas et al., 2002); and for rapid discrimination of two closely-related nematodes, *Brugia malayi* and *Brugia pahangi* (Areekit et al., 2009). In veterinary science, HRM analysis has been used to detect viral strains in bird samples (Ghorashi et al., 2011; Pham et al., 2005); for population studies of *Fascioloides magna*, the giant liver fluke of ruminants (Radvansky, Bazsalovicsova, Kralova-Hromadova, Minarik, & Kadasi, 2011); for classification of fowl adenoviruses (Marek, Gunes, Schulz, & Hess, 2010); and for rapid identification and differentiation of *Mycobacterium avium* subspecies *paratuberculosis* types (Castellanos, Aranaz, & de Buck, 2010).

CHAPTER THREE

METHODOLOGY

Cattle Breeds

One hundred and ten animals each were sampled from four cattle breeds in Ghana, namely, N'Dama, WASH, Zebu and Sanga (Figures 4, 5, 6, and 7). The 110 sample size for each cattle breed was based on convenience sampling.

Figure 4. N'Dama cattle

Source: Fieldwork, 2011

Figure 5. West African Shorthorn cattle

Source: Fieldwork, 2011

Figure 6. Zebu cattle

Source: Fieldwork, 2011

Figure 7. Sanga cattle

Source: Fieldwork, 2011

Study Sites

The N'Dama herds were maintained by the Teaching and Research Farm at the University of Cape Coast, Cape Coast (latitude 5°7'23"N and longitude 1°4'14"W), and Kwame Nkrumah University of Science and Technology, Kumasi (latitude 6°41'15"N and longitude 1°37'53"W). The WASH were kept at the Ministry of Food and Agriculture Breeding Station located at Pong Tamale (latitude 9°42'43"N and longitude 0°5'4"W) in the Savelugu-Nanton District and the Animal Research Institute Breeding Station at Chegbani (latitude 9°34'45"N and longitude 0°5'54"E) in the Saboba-

Chereponi District, both in the Northern Region. The Zebu were kept by Fulani livestock keepers at Pong Tamale (latitude 9°42'43"N and longitude 0°5'4"W) and Ying (latitude 9°41'8"N and longitude 0°43'12"W) in the Savelugu-Nanton District of the Northern Region. Sanga cattle (a stabilised interbred population of Zebu and WASH) were maintained at Aveyime Cattle Ranch Limited, at Aveyime (latitude 6°1'50"N and longitude 0°22'6"E) in the North Tongu District of the Volta Region. Other Sanga were maintained at Amrahia Dairy Farm, a Ministry of Food and Agriculture facility located at Amrahia (latitude 5°46'41"N and longitude 0°9'20"E) in the Adenta Municipality of the Greater Accra Region. Figure 8 shows the geographical locations of the sampling sites. These sites were chosen because pure breeds of cattle were kept there.

The agroecological zones

A brief account of each of the agroecological zones in which the sampling sites are located is given below:

The Guinea Savanna Zone: Pong Tamale, Ying and Chegbani

This agroecological zone covers most parts of the Upper East Region, all of the Upper West, Northern, most parts of Brong-Ahafo and parts of the Eastern and Volta regions. It comprises a continuous cover of tall grasses like *Andropogon, Pennisetum* and *Panicum* interspersed with fire-resistant trees, typically *Parkia spp., Jacandra spp* and *Butyrospermum parkii.* These areas are subject to annual burning to remove the thick undergrowth and to free the land surface for the fresh and tender regrowth of grass for grazing by livestock. The area has a single rainy season from April to October, and an annual rainfall of between 1,000 and 1,200 mm. Temperatures are usually

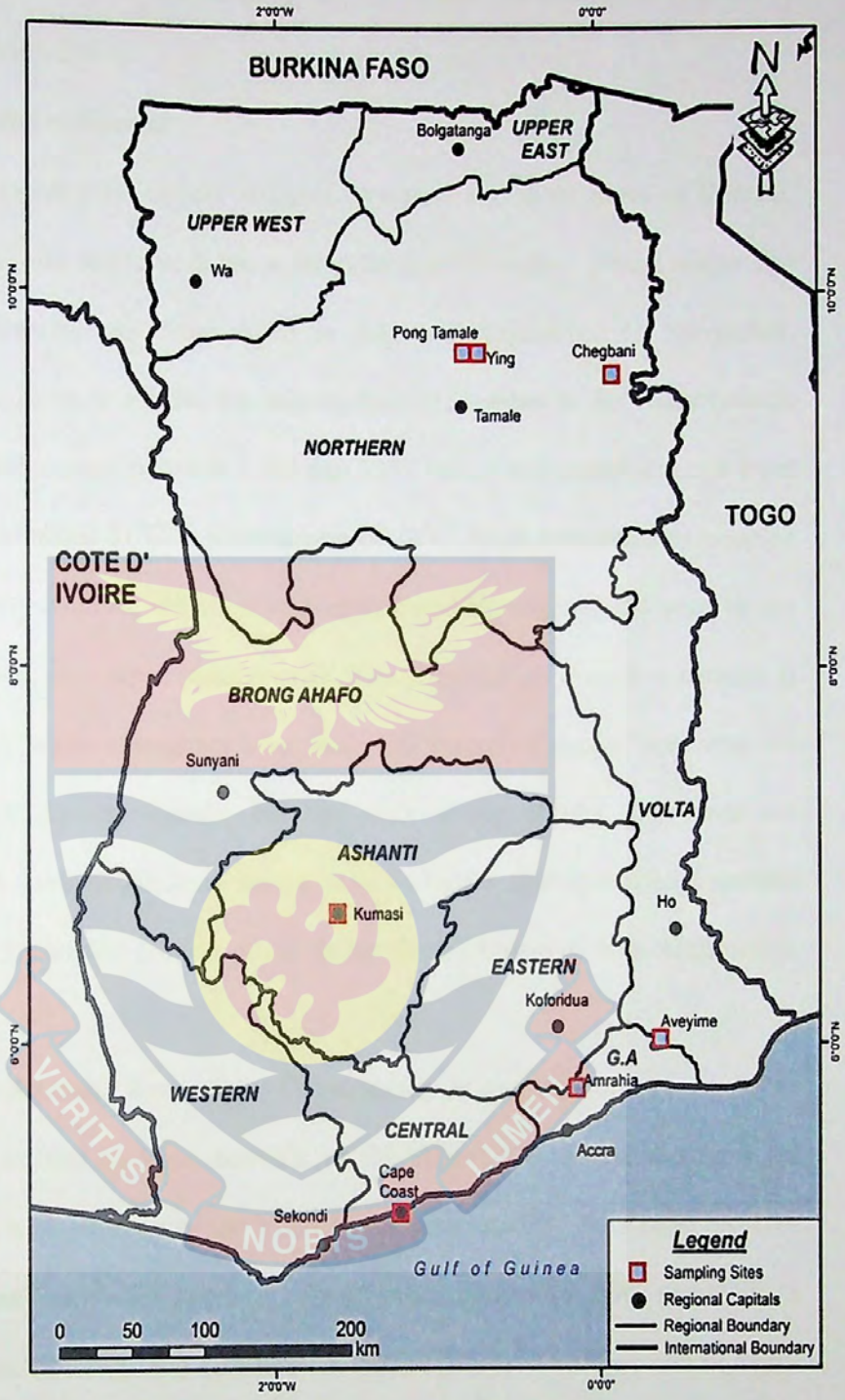


Figure 8. A map of Ghana showing the geographical locations of sites where cattle were sampled

Source: GIS/Remote Sensing Section, Department of Geography and Regional Planning, University of Cape Coast, Cape Coast, GHANA.

high, with mean annual maximum of 34.5°C in the extreme north (Ahunu & Boa-Amponsem, 2001).

The Forest Zone: Kumasi

The forest zone covers Ashanti, Western and most parts of Central, Eastern and Volta Regions. It has a bimodal rainfall pattern giving major and minor growing seasons from April to July and September to November, respectively. There is a main dry season from November to February-March. Annual rainfall ranges between 1210 and 3312 mm. Temperatures range from a minimum of about 21°C to a maximum of 30°C. High temperatures coupled with the heavy rainfall, which is well distributed throughout the year in the zone, promotes very rapid plant growth. The zone has an even tree canopy at 30-40 metres while emergents may attain 60 metres. Canopy trees may be deciduous in the dry season but the understorey shrubs and trees are evergreen. A herbaceous layer which may include a few specialized grasses occurs over a variable portion of the forest floor (Ahunu & Boa-Amponsem, 2001).

The Coastal Savanna Zone: Cape Coast, Amrahia and Aveyime

The coastal savanna consists of the Accra Plains and the Ho-Keta Plains lying west and east of the Volta River, respectively. This zone receives the minimum rain with average annual precipitation of 800 mm with a bimodal distribution giving rise to 3 seasons as follows: major rains (April-July), minor rains (August-November) and dry season (December-March). Daily temperatures are high (32°C) with high relative humidity of 70%. The dominant grass species here are *Andropogon, Brachiara* and *Hyperrhenia*

interspersed with tree species like *Azadirachta indica, Leucaena leucocephala, Gliricidia spp* and other shrubs (Ahunu & Boa-Amponsem, 2001).

Husbandry Practices at Sampling Sites

Cattle were reared under extensive practice which allowed free grazing. Animals were housed in kraals in the night. They were taken out for grazing by herdsmen early in the morning and brought back to be kraaled at night. All categories of cattle were herded together. No supplementation was given during the study period.

Study Design

The four cattle breeds were sampled from May to July, 2011 in a cross-sectional study.

Sampling of Cattle

At each site, the animals were stratified according to age and sex classes/categories. Weighted random sampling was then used to select samples according to age and sex structure in the herd at the sampling site. A total of 440 animals were sampled as follows: 43 bull calves and 43 heifer calves (<1 year old), 74 heifers and 64 young males (between 1 and 3 years of age), 149 cows and 67 breeding bulls (>3 years). Table 5 shows the class and age composition of the animals sampled, on the basis of breed.

Blood Collection

From each animal, about 4 ml of blood was collected from the jugular vein by a veterinary assistant and transferred into three separate vacutainer tubes containing EDTA as anticoagulant. The vacutainer tubes were then placed in a coolbox containing ice packs and later refrigerated at -4°C for subsequent analysis.

Table 5: Composition of cattle breeds of different ages and sex at sampling sites in Ghana

Class	Age	Total	N'Dama		WASH		Sanga		Zebu	
			UCC	KNUST	Pong Tamale	Chegbani	Aveyime	Amrahia	Pong Tamale	Ying
Bull calf	< 1 year old	43	5	5	5	5	10	5	0	11
Heifer calf	< 1 year old	43	5	3	5	6	10	5	5	5
Heifer	Between 1 and 3 years	74	14	7	9	11	5	19	2	6
Young bull	Between 1 and 3 years	64	9	8	6	6	5	3	5	17
Cow	>3 years old	149	19	29	20	22	21	20	5	14
Breeding bull	>3 years old	67	3	3	10	5	4	3	21	19
Total		440	55	55	55	55	55	55	38	72

Source: Fieldwork, 2011

Haematological Studies

The haematological studies were carried out to provide some baseline data to support the molecular work, which was the focus of this study. The haematological parameters determined were packed cell volume and haemoglobin concentration; total red blood cell and white blood cell counts; and differential white blood cell count. Red blood cell indices were calculated using standard formulae.

Packed cell volume determination

Packed cell volume (PCV), a measure of the proportion of the volume of the whole blood that is occupied by red blood cells, was determined by the microhaematocrit centrifugation technique (Jain, 1986). Blood in a sample vacutainer tube was mixed by gently inverting the tube about 20 times. The blood was drawn three-quarters of the way up a 75 mm x 1.0 mm microhaematocrit capillary tube. Blood was wiped off the tip of the capillary tube, and the end of the capillary tube was carefully plugged with plasticine. The capillary tube was placed, with the closed end outwards, in a microhaematocrit centrifuge (Hawksley and Sons Limited, England) and spun at 12,000 rpm for 5 min. The capillary tube was removed from the centrifuge, placed on a haematocrit tube reader and the PCV was read.

Haemoglobin concentration determination

Haemoglobin concentration was measured spectrophotometrically by the cyanmethaemoglobin method (Jain, 1986). Blood in a sample vacutainer tube was mixed gently by inverting about 20 times. Twenty microlitres of blood was added to 5 ml of Drabkin's solution (which contains potassium ferricyanide and potassium cyanide) in a test tube. In the Drabkin's solution,

the red blood cells were haemolysed and the haemoglobin was oxidised by the ferricyanide to methaemoglobin. The methaemoglobin was then converted by the cyanide to stable cyanmethaemoglobin. The mixture was allowed to stand for 15 min. One millilitre of the mixture was pipetted into a cuvette. The cuvette was placed in a spectrophotometer (Jenway, England, Model: Genova MK3) set at 540 nm, and the absorbance of the cyanmethaemoglobin solution was read after zeroing the spectrophotometer using neutral Drabkin's solution. Haemoglobin concentration of the blood sample was calculated by dividing the absorbance value by the slope obtained from a calibration graph.

To obtain the calibration graph, a standard blood sample (of known haemoglobin concentration) was diluted 5 in 0, 4 in 1, 3 in 2, 2 in 3 and 1 in 4 with Drabkin's solution. The absorbance of each of the five solutions was read in the spectrophotometer after the spectrophotometer was zeroed using neutral Drabkin's solution. A graph of absorbance for each of the five solutions was plotted against the corresponding haemoglobin concentration, and the slope of the graph was determined. The haemoglobin concentration of each of the five solutions was obtained by multiplying the proportion of standard haemoglobin in that solution with the haemoglobin value of the standard.

Total red blood cell count determination

Total red blood cell (RBC) count was done manually using a haemocytometer according to the procedure outlined in Merck Veterinary Manual (1986). Counting was preceded with the preparation of RBC dilution. Blood in a sample vacutainer tube was mixed gently by inverting gently about 20 times. Twenty microlitres of blood was added to 4 ml of RBC diluting fluid

in a test tube, and the contents of the tube were mixed thoroughly. The dilution of blood in the tube was 1:200.

The haemocytometer and cover glass were thoroughly cleaned, ensuring that both were free of grease. The haemocytometer was placed flat on the working bench and, using firm pressure, the cover glass was slid into position over the ruled counting areas. After mixing the RBC dilution by inverting 10-20 times, a portion was taken up in a pipette. The counting chamber of the haemocytometer was filled by holding the pipette at an angle of 45° to lightly touch the tip against the edge of the chamber and cover glass. It was ensured that too much fluid was not introduced into the chamber as this may raise the cover glass, causing a variation in the depth and resulting in gross errors.

The counting chamber was placed on a microscope stage and, in order to allow the RBCs to settle, 2 min was allowed to elapse before the commencement of counting. The x100 high dry objective and the x10 eyepiece of the microscope were used to focus the centre square millimetre of the ruled area of counting chamber; and all the cells contained in 80 of the 400 small squares, i.e. five groups of 16 small squares, one at each corner and one in the middle, were counted. Total red blood cell was then calculated as follows:

Total RBCs = Number of cells counted x depth of counting chamber x dilution x area of counting chamber

Total white blood cell count determination

Total white blood cell (WBC) count was also done manually using a haemocytometer according to the procedure outlined in the

Merck Veterinary Manual (1986). Counting was preceded with the preparation of WBC dilution. Blood in a sample vacutainer tube was mixed gently by inverting gently about 20 times. Fifty microlitres of blood was added to 0.95 ml of WBC diluting fluid in a test tube, and the contents of the tube were mixed thoroughly. The dilution of blood in the tube was 1:20.

The haemocytometer and cover glass were thoroughly cleaned, ensuring that both were free of grease. The haemocytometer was placed flat on the working bench, and, using firm pressure, the cover glass was slid into position over the ruled counting areas. After mixing the WBC dilution by inverting 10-20 times, a portion was taken up in a pipette. The counting chamber of the haemocytometer was filled by holding the pipette at an angle of 45° to lightly touch the tip against the edge of the chamber and cover glass. It was ensured that too much fluid was not introduced into the chamber as this may raise the cover glass, causing a variation in the depth and resulting in gross errors.

The counting chamber was placed on a microscope stage, and, in order to allow the WBCs to settle, 2 min was allowed to elapse before the commencement of counting. The x100 high dry objective and the x10 eyepiece of the microscope were used to focus the centre square millimetre of the ruled area of counting chamber, and, unlike in RBC counting, only the four corner 1 mm squares were counted. Total white cell was then calculated as:

Total WBC = Average number of cells counted per square mm x depth of counting chamber x dilution

Differential white blood cell count determination

A drop of well-mixed blood was placed in the centre line of a clean,

dry, grease free slide about 1 cm from one end. A spreader was immediately placed in front of the drop at an angle of about 45° to the slide. The spreader was moved backwards to make contact with the drop. With a steady movement of the hand, the drop of blood was spread along the slide, resulting in a film of blood. After the film had been made, it was rapidly dried by waving the slide in the air. To preserve the morphology of the cells, the films were fixed soon after they were dried by placing them in a tray containing absolute methanol for 3 min. The slides were removed, and the films were stained with Giemsa stain for 10 min in a staining rack. The Giemsa stain had been diluted 1:3 with water buffered at pH 7.2. The stain was washed off by dipping the slide in buffered distilled water at pH 7.2. The slides were then placed in a vertical position in a draining rack for the film to dry.

After this step, the battlement method (Merck Veterinary Manual, 1986) was employed in the counting of the different white blood cells. By this method, the film was examined systematically by being traversed three fields along the edge, two fields up, two fields along, two fields down, starting at the thin end of the smear. This sequence was continued until a minimum of 200 cells were counted. Within these 200 cells, the five different white blood cells were identified and the number and proportion of each were recorded. The different white blood cells identified, based on appearance and staining reactions, were lymphocytes, monocytes, neutrophils, eosinophils and basophils (Merck Veterinary Manual, 1986).

Calculation of RBC indices

The RBC indices that were calculated were mean cell volume (MCV)

(the average size of each red blood cell); mean cell haemoglobin (MCH) (the average amount of haemoglobin present in each red blood cell); and mean cell haemoglobin concentration (MCHC) (the average proportion of each red blood cell taken up by haemoglobin or the average percentage of haemoglobin in each red blood cell).

To calculate the MCV, expressed in femtolitres (fl), the following formula was used:

$$\text{MCV} = \frac{\text{PCV (\%) x 10}}{\text{RBC count (millions/mm}^3\text{ blood)}}$$

To calculate the MCH, expressed as picograms (pg), the following formula was used:

$$\text{MCH} = \frac{\text{haemoglobin (g/100 ml) x10}}{\text{RBC count (millions/mm}^3\text{ blood)}}$$

To calculate the MCHC, expressed as grams of haemoglobin per 100 ml packed cells (g/dl), the following formula was used:

$$\text{MCHC} = \frac{\text{haemoglobin (g/100 ml) x100}}{\text{PCV (\%)}}$$

Molecular Studies

Red blood cell lysis and DNA extraction

To lyse the red blood cells, 200 µL of blood was added to 1800 µL of lysis solution in a 2 mL Eppendorf tube. After gentle mixing, the mixture was allowed to stand for 10 min at room temperature and spun at 4000 rpm for 15 min. The supernatant was removed, leaving a whitish pellet at the bottom of the tube. One ml of 95% ethanol was added to the pellet, followed by storage in a -20°C freezer for subsequent DNA extraction (Biéler et al., 2012).

DNA was extracted from the stored pellet according to the ammonium

acetate precipitation protocol for DNA extraction from bird blood or animal tissue (Bruford, Hanotte, Brookfield, & Burke, 1998). Based on this protocol, the pellet stored in 95% ethanol, following red blood cell lysis, was centrifuged twice for 1 min at 13000 rpm. Ethanol was pipetted off, and 250 µL of cell lysis Digsol buffer (20 mM EDTA, 120 mM NaCl, 50 mM Tris HCl, 1% SDS, pH 8.0) and 10 mg/mL of proteinase K were added to the pellet. The mixture was vortexed for 30 sec and incubated at 37°C overnight (12-14 hrs). After the incubation, vortexing was done for 30 sec, then 300 µL of 4 M ammonium acetate was added and the mixture vortexed again for 30 sec. This was followed with an incubation step at room temperature for 15 min while vortexing for 30 sec. The mixture was then centrifuged at 15,000 rpm for 15 min and the supernatant transferred into clean and sterile 1.5 mL Eppendorf tubes where 1 mL of absolute ethanol was added. The tube was inverted 10-20 times, gently, and centrifugation done again at 15,000 rpm for 15 min to precipitate the DNA. The ethanol was poured off with care taken not to lose the pellet. Immediately, 900 µL of 70% cold ethanol (stored at -20°C) was added and the tube inverted gently 10-20 times to rinse the DNA pellet. Centrifugation was done at 15,000 rpm for 5 min and the ethanol poured off. A second wash with cold 70% ethanol was done, followed by centrifugation at 15,000 rpm for 5 min to ensure that the DNA pellet stuck at the bottom of the tube and was not lost during the pouring off of ethanol. After ethanol was poured off following the final wash, the tubes were allowed to stand at room temperature under a lamp for 30 min to dry the pellet. Once fully dry, the DNA pellet was resuspended by adding 50 µL of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 7.4). The tubes were gently flicked to bring the pellet into

solution. If the pellet was too dry, the tubes were placed in a water bath set at 37°C for 30 min; during this period, the tubes were flicked after every 10 min. The resuspended solution, which contained DNA, was stored at -20°C for later use in PCR; for long term usage, storage was done at -80°C.

All the centrifugation steps were performed in BIOFUGE fresco centrifuge (DJB Lab care Ltd, UK).

DNA amplification by polymerase chain reaction using ITS CF and BR primers

Amplifications were carried out for the internal transcribed spacer region (ITS) of the trypanosomes using the ITS primers i.e. ITS CF (5'-CCGGAAGTTCACCGATATTG-3') and ITS BR (5'-TTGCTGCGTTCTTCAACGAA-3') (Gonzales et al., 2006). PCR was done in a 20 µL reaction volume containing 1 µL 10 pmoles of each primer (ITS1 forward primer and ITS1 reverse primer), 4.0 µL 5X HF Buffer (Finnzymes), 0.4 µL 10 mM dNTPs, 0.2 µL 1 unit Taq polymerase (Finnzymes) and 1.0 µL DNA template. Cycling conditions for the PCR were accomplished in a 96-well thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Gaithersburg) as follows: initial denaturation at 98°C for 2 min, followed by 35 cycles of denaturation at 98°C for 10 sec; annealing at 64°C for 30 sec, primer elongation at 72°C for 30 sec, and a final extension at 72°C for 7 min.

DNA amplification by polymerase chain reaction using *T. vivax* (West Africa) specific primers

Amplifications were carried out targeting the 170-base pairs (bp)

satellite DNA monomer of *T. vivax*. The PCR was carried out in a total volume of 20 µL containing 1.0 µL 10 pmoles of each primer i.e. TVW_A (5'-GTGCTCCATGTGCCACGTTG-3') and TVW_B (5'-CATATGGTCTGGGAGCGGGT-3') (Masiga et al., 1996), 4.0 µL 5X HF Buffer (Finnzymes), 0.4 µL 10mM dNTPs, 0.2 µL 1 unit Taq polymerase (Finnzymes) and 1 µL of DNA template. Cycling conditions for the PCR were accomplished in a 96-well thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Gaithersburg) as follows: initial denaturation at 98°C for 30 sec, followed by 35 cycles of denaturation at 98°C for 10 sec, annealing at 68°C for 30 sec, primer elongation at 72°C for 15 sec, and a final extension at 72°C for 7 min.

Agarose gel electrophoresis

A 1.5% agarose gel was prepared by dissolving 1.5 gm of agarose powder in 100 ml of 1 X TAE buffer. The mixture was stirred, boiled in a microwave and ethidium bromide (50 mg/µl) was added. The mixture was allowed to cool before being poured into the casting tray to polymerize. The PCR product (7 µL) was mixed with the loading dye and samples were loaded into the gel alongside a molecular weight DNA marker. Electrophoresis was set at 75 volts for 1 hr 20 min, followed by visualization of the DNA under UV-illumination. The gel photo was analyzed and documented using the software: KODAK Gel Logic 200 Imaging System (Raytest GmbH, Straubenhardt).

Genotyping of MHC class II DRB3 gene in N'Dama and WASH using real-time PCR and HRM analysis

The Sanga and Zebu were not genotyped because they did not record

any trypanosome infection and were, therefore, not relevant to the main focus of the study, which was to determine MHC DRB3 alleles for controlling trypanosome infection.

From each N'Dama and WASH sample, a 319-base pairs (bp) fragment of the Bovine MHC DRB3 gene was amplified using a Rotor-Gene Q 5-plex High Resolution Melting (HRM)-capable thermocycler (Qiagen; Hilden, Germany). This was done in a total volume of 20 µL containing 1 µL 10 pmoles of each primer i.e. DRB3 Fw (5'-CGCTCYTGTGAYCAGATCTATCC-3') and DRB3 Rv (5'-CACCCCCGCGCTCACC-3') (Miltiadou, Law, & Russel, 2003; however, the forward primer of Miltiadou et al., 2003 was modified based on multiple alignments of sequences in GenBank), 4 µL 5X Hot Firepol® Blend master mix (10 mM $MgCl_2$, 2 mM dNTP's, BSA, 5X Blend Master Mix Buffer, proofreading enzyme, Hot Firepol® DNA polymerase) (Solis BioDyne) and 1 µL of DNA template. The amplification conditions were as follows: initial denaturation at 95°C for 14 min, followed by 35 cycles of denaturation at 95°C for 20 sec; annealing at 61°C for 40 sec, and primer elongation at 72°C for 1 min followed by a final extension at 72°C for 10 min. The amplified products were melted from 70°C to 90°C with fluorescent readings taken at 0.1°C increments to generate HRM curves (Figure 9). Resultant melt profiles were compared, and homozygous and heterozygous genotypes were distinguished as single peaks and double peaks, respectively (Figure 10). HRM analysis to determine amplicon product melting temperature (Figure 11) was performed using the Rotor-Gene Q software (Qiagen; Hilden, Germany).

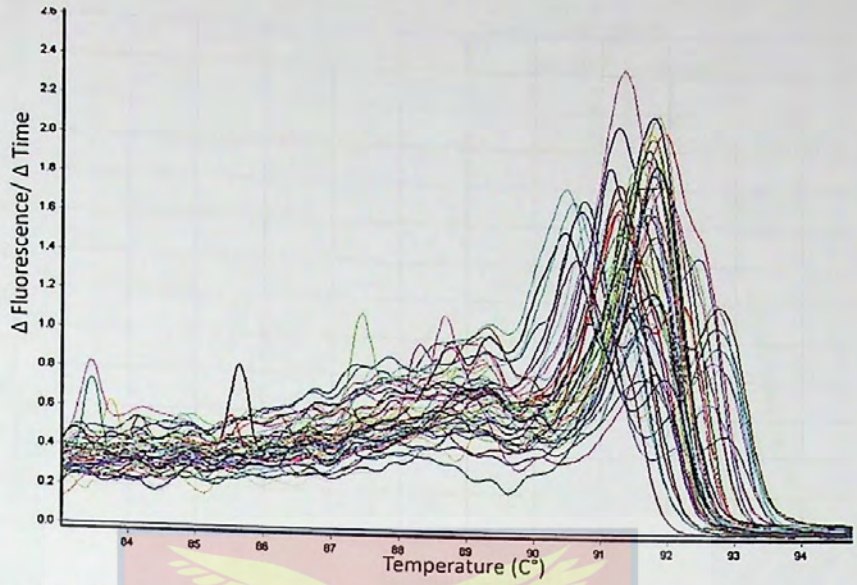


Figure 9. HRM curves generated from melting amplified products from 70°C to 90°C with fluorescent readings taken at 0.1°C

Source: Labwork, 2012

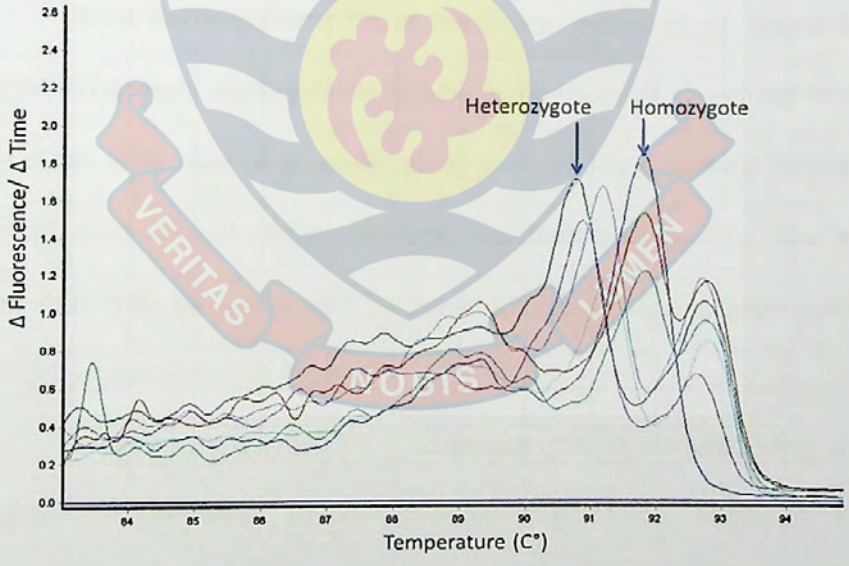


Figure 10. Resultant melt profiles showing homozygous and heterozygous genotypes distinguished as single peaks and double peaks, respectively

Source: Labwork, 2012

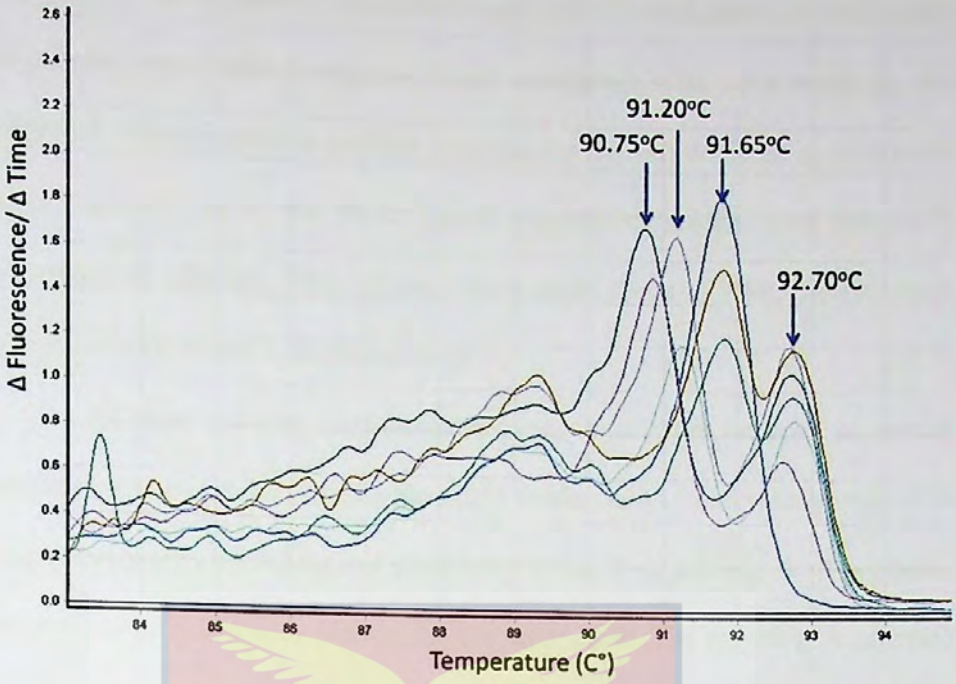


Figure 11. Resultant melt profiles with resolved melting temperatures

Source: Labwork, 2012

DNA sequencing and final scoring of MHC class II DRB3 alleles

Under the established HRM conditions, groups of 17 unique melting temperatures each were resolved for the N'Dama at UCC and the WASH at Chegbani, with one, or a combination of 2, of these melting temperatures (within each group) being observed for individual cattle. This was in agreement with the presence of homozygous or heterozygous genotypes at the DRB3 locus as shown in Figure 10.

To determine the nucleotide sequences (alleles) corresponding to HRM profiles of individual animals (Figure 12), DRB3 amplified product representatives of the resolved melting temperatures were selected for sequencing. Samples were purified using ExoSAP-IT prior to capillary sequencing at Macrogen in South Korea.

Nucleotide sequences were viewed using Geneious software

(version 6.0, developed by Biomatters Ltd). After trimming away low-quality sequences, nucleotide sequences were compared with sequences in the National Centre for Biotechnology Information (NCBI) database to determine matching hits, using the Basic Local Alignment Search Tool (BLAST) algorithm of Altschul, Gish, Miller, Myers and Lipman (1990). This allowed the alleles sequenced to be determined.

As most samples were heterozygotes, these were returned as mixed sequences (evident by double base-pair peaks in the chromatogram). The sequences were cleaned up and degenerate codes were entered at all positions where double peaks were seen in the chromatogram. The resulting degenerate sequences were then blasted to obtain putative alleles. The putative alleles were aligned with the degenerate sequences to create the hypothetical second sequence by using the opposite possible base. The hypothetical second sequence was then blasted to determine the second allele.

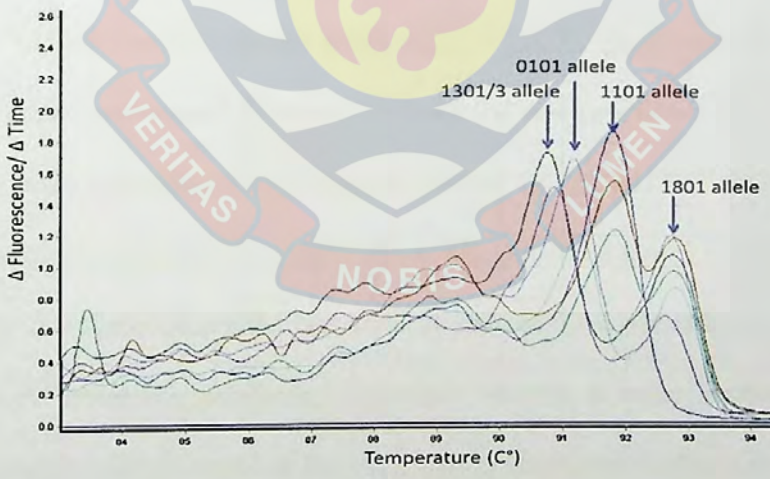


Figure 12. Allele scores/names, corresponding to HRM temperatures, assigned to heterozygous and homozygous samples after sequencing and blast search

Source: Labwork, 2012

Statistical and Sequence Analysis

The numbers of animals infected according to breed, sex and age were summarised as counts and percentages. Comparative analysis for these categorical variables was performed using the Fisher's exact test as incorporated in the R statistical software (Appendix 1-2). The haematological parameters analyzed were PCV, Hb, RBC, WBC, MCV, MCH, MCHC and counts of lymphocytes, neutrophils, monocytes, eosinophils, and basophils. These variables were summarised as means ± standard deviations. The one way analysis of variance (ANOVA) test was used to compare the means for the variables. An example is found in Appendix 3. Pairwise comparison of means for components of a categorical variable was further carried out in cases where ANOVA test showed significant differences among the components of such categorical variable. Data analysis was performed using the R statistical software version 2.3.7.1 (R Development Core Team, 2008). Tests of significance were done at $\alpha = 0.05$.

Allelic variants of the MHC class II DRB3 gene were counted and percentages determined. Odds ratios (OR) and relative risks (RR) for various alleles were calculated using MedCalc version 12.7.0 (2013). Examples of the output are in Appendix 4 - 7. An OR or RR value less than 1.0 indicated that having the allele decreased the odds or risk of *T. vivax* infection and was therefore protective, conferring resistance whereas a value greater than 1.0 indicated that the presence of the particular allele increased the odds or risk of infection. A value of 1.0 implied that there was no difference in risk or odds of infection for the particular allele.

Nucleotide sequences and deduced amino acid sequences of all the

alleles found in the study were fully aligned with the multiple sequence alignment tool, MAFFT, developed by Katoh and colleagues (Katoh, Misawa, Kuma, & Miyata, 2002). Phylogenetic trees to indicate genetic distances between alleles were constructed using the neighbour-joining method (Saitou & Nei, 1987).

CHAPTER FOUR

RESULTS

Prevalence of Trypanosome Infection in Four Breeds of Cattle in Ghana using ITS Primers

Using the ITS-1 generic primers, none of the 440 blood samples from N'Dama, WASH, Sanga and Zebu cattle breeds tested positive for trypanosome species.

Prevalence of Trypanosome Infection in Four Breeds of Cattle in Ghana using *T. vivax*-specific Primers

Using primers specific for the West African strain of *T. vivax*, 2.5% (11/440) of the animals were positive for trypanosome infection. None of the Zebu and Sanga cattle breeds sampled tested positive for *T. vivax*, while 7 (6.4%, n = 110) of N'Dama cattle and 4 (3.6%, n = 110) of WASH tested positive for *T. vivax* (Table 6). Figure 13 is a gel image of DNA samples screened using primers specific for the West African strain of *T. vivax*.

Table 6: Prevalence of *T. vivax* in four cattle breeds

Breed	Number Sampled	Number positive (%)
Zebu	110	0
N'Dama	110	7 (6.4)
Sanga	110	0
WASH	110	4 (3.6)

The 7 N'Dama cattle that tested positive for *T. vivax* were from the herd in UCC giving a herd prevalence of 12.7% (n = 55), while the 4 WASH cattle that tested positive for *T. vivax* were from Chegbani giving a herd prevalence of 7.3% (n = 55).

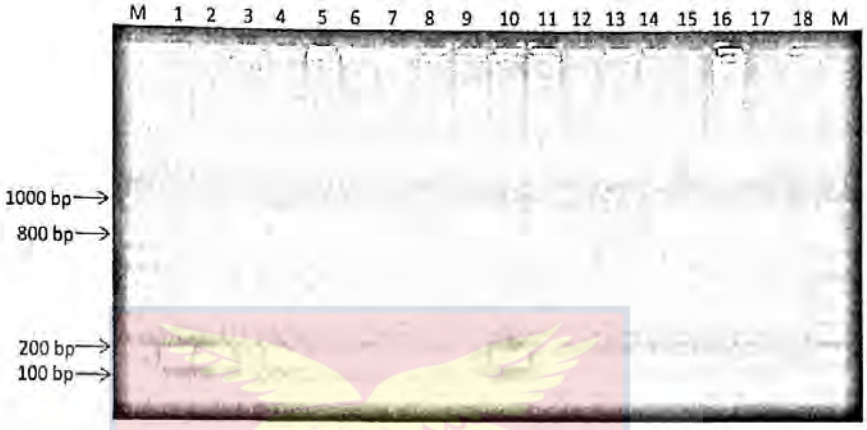


Figure 13. Gel electrophoresis of *T. vivax* DNA (in genomic cattle DNA samples) amplified with TVW_A and TVW_B primers. The lanes marked M contain a 100 bp DNA ladder (Fermentas) which was used as the molecular weight marker; and lane 18 contains water, which served as negative control. The other lanes contain genomic cattle DNA from WASH cows from Chegbani (lanes 1and 2), N'Dama cows from KNUST (lanes 3, 5, 9, 12 and 13), N'Dama cows from UCC (4, 6, 7, 14 and 15), N'Dama young bulls from UCC (lane 8 and 17), a WASH heifer and breeding bull from Chegbani (lanes 10 and 11, respectively), an N'Dama heifer calf from KNUST (lane 16).

Prevalence on basis of sex of cattle

There were more trypanosome-infected female N'Dama cattle than their male counterparts (5 compared to 2). This trend was the same for the WASH (3 compared to 1) (Table 7). However, there were no statistically significant differences in the seropositivity of males and females.

Table 7: Prevalence of *T. vivax* in N'Dama and WASH by sex

Breed	Sex	Number sampled	Number positive (%)
N'Dama	Male	17	2 (11.8)
	Female	38	5 (13.2)
WASH	Male	16	1 (6.3)
	Female	39	3 (7.7)
Both breeds	Male	33	3 (9.1)
	Female	77	8 (10.4)

Source: Labwork, 2012

Prevalence on the basis of category of cattle

Table 8 presents the prevalence of *T.vivax* infection on the basis of category of cattle.

Haematology of N'Dama, WASH, Zebu and Sanga Cattle

Table 9 summarizes the haematological parameters of all the four breeds sampled. The PCV, Hb and RBC values were significantly higher ($p < 0.001$) in the N'Dama and Sanga compared to the Zebu and WASH. No significant differences ($p > 0.05$) were found among the four breeds with respect to MCV, MCH and MCHC but the Zebu and Sanga showed highly significant difference ($p < 0.01$) in MCHC. With the exception of the basophils, significant differences were seen in counts for WBC, neutrophils, eosinophils, monocytes ($p < 0.001$) and lymphocytes ($p < 0.01$).

Table 8: Prevalence of *T. vivax* on basis of category of cattle

	WASH		N'Dama		Total	
Category	**n**	**Prevalence (%)**	**n**	**Prevalence (%)**	**n**	**Prevalence (%)**
Breeding bull	5	1 (20.0)	2	0	7	1 (14.3)
Young bull	6	0	10	2 (20.0)	16	2 (12.5)
Cow	22	2 (9.1)	19	5 (26.3)	41	7 (17.1)
Heifer	11	1 (9.1)	14	0	25	1 (4.0)
Calf	11	0	10	0	21	0

n represents number of samples in each category

Source: Labwork, 2012

Table 9: Haematological parameters of N'Dama, WASH, Zebu and Sanga cattle (mean ± SD)

Breed / Parameter	N'Dama	Sanga	WASH	Zebu	*p* value
	n=110	n=110	n=110	n=110	
PCV (%)	34.50 ± 4.37^{a}	34.39 ± 4.78^{a}	31.79 ± 4.73^{b}	30.30 ± 4.95^{b}	0.000***
Hb (g/dl)	12.81 ± 1.67^{a}	12.44 ± 1.88^{a}	11.79 ± 1.72^{b}	11.58 ± 1.96^{b}	0.000***
RBC (x10^{6}mm^{3})	6.76 ± 2.03^{a}	7.15 ± 2.41^{a}	5.87 ± 1.43^{b}	5.97 ± 1.38^{b}	0.000***
MCV (fl)	55.34 ± 17.14^{a}	54.16 ± 21.75^{a}	56.49 ± 12.58^{a}	52.66 ± 12.08^{a}	0.347
MCH (pg)	20.58 ± 6.57^{a}	19.56 ± 7.89^{a}	21.03 ± 5.00^{a}	20.20 ± 5.17^{a}	0.360
MCHC (g/dl)	37.31 ± 4.05^{ab}	36.30 ± 3.78^{a}	37.29 ± 3.88^{ab}	38.52 ± 5.33^{b}	0.002**
WBC (x10^{3}mm^{3})	8.60 ± 3.47^{a}	9.04 ± 3.25^{a}	10.18 ± 2.48^{b}	11.00 ± 2.40^{b}	0.000***
Neutrophil (%)	37.65 ± 12.55^{a}	36.93 ± 11.69^{a}	39.37 ± 13.76^{ab}	43.65 ± 12.49^{b}	0.000***
Lymphocyte (%)	43.25 ± 13.84^{a}	45.16 ± 13.45^{ab}	49.35 ± 16.00^{b}	47.03 ± 12.25^{ab}	0.010**
Eosinophil (%)	18.93 ± 10.40^{a}	17.65 ± 9.73^{a}	9.12 ± 6.20^{b}	7.86 ± 5.29^{b}	0.000***
Monocyte (%)	0.15 ± 0.49^{a}	0.05 ± 0.23^{a}	1.95 ± 2.75^{b}	0.88 ± 1.64^{c}	0.000***
Basophil (%)	0.05 ± 0.25^{a}	0.03 ± 0.16^{a}	0.02 ± 0.19^{a}	0.07 ± 0.26^{a}	0.268

n represents number of samples in each category; Means with different superscripts (a, b, c) across rows are highly significantly (**, $p < 0.01$ and ***, $p < 0.001$) different, whereas means with the same superscript are not significantly ($p > 0.05$) different

Source: Labwork, 2011

Effect of *T. vivax* infection on haematological parameters of infected and uninfected N'Dama at UCC

The mean haematological values for trypanosome positive and negative cattle at UCC are shown in Table 10. Significant differences were observed in PCV ($p < 0.01$), total RBC count, MCV and MCH ($p < 0.001$) between infected and uninfected cattle. PCV, MCV and MCH values were significantly ($p < 0.01$) higher in uninfected compared to infected cattle. The other parameters were similar for both groups (Table 10).

Table 10: Haematological parameters of *T. vivax* infected and uninfected N'Dama cattle at UCC (mean ± SD)

Parameter	Infected	Uninfected	*p* value
	n = 7	n = 48	
PCV (%)	30.71 ± 4.35	36.54 ± 4.02	0.023*
Hb (g/dl)	11.83 ± 1.89	13.05 ± 1.56	0.065
RBC ($x10^6mm^3$)	9.06 ± 2.14	6.94 ± 1.93	0.010 **
MCV (fl)	35.79 ± 11.02	53.07 ± 14.07	0.003 **
MCH (pg)	13.83 ± 4.45	20.02 ± 5.23	0.004 **
MCHC (g/dl)	38.64 ± 4.61	37.97 ± 4.40	0.710
WBC ($x10^3mm^3$)	6.95 ± 2.90	8.57 ± 3.40	0.238
Neutrophils (%)	38.71 ± 12.75	39.10 ± 13.56	0.943
Lymphocytes (%)	39.57 ± 15.60	40.56 ± 12.04	0.845
Eosinophils (%)	21.43 ± 13.66	20.00 ± 11.51	0.765
Monocytes (%)	0.29 ± 0.79	0.23 ± 0.63	0.829
Basophils (%)	0.00 ± 0.00	0.04 ± 0.20	0.590

n represents number of samples in each category
* Indicates level of significance which is at 5% level ($p < 0.05$)
** Indicates level of significance which is at 1% level ($p < 0.01$)

Source: Labwork, 2011

Effect of *T. vivax* infection on haematological parameters of WASH cattle at Chegbani

No significant differences ($p > 0.05$) were observed in the erythrocyte and leukocyte parameters of uninfected and infected WASH cattle at Chegbani. However, the PCV, Hb and RBC values for uninfected cattle were higher than those for infected cattle (Table 11).

Table 11: Haematological parameters of infected and uninfected WASH cattle at Chegbani (mean ± SD)

Parameter	Infected	Uninfected	*p* value
	n = 4	n = 51	
PCV (%)	28.25 ± 1.50	31.49 ± 5.14	0.218
Hb (g/dl)	11.46 ± 1.07	11.68 ± 2.04	0.834
RBC ($x10^6mm^3$)	5.48 ± 1.07	5.77 ± 1.33	0.671
MCV (fl)	53.38 ± 12.81	56.57 ± 11.79	0.606
MCH (pg)	21.54 ± 4.73	20.98 ± 4.73	0.821
MCHC (g/dl)	40.51 ± 1.98	37.29 ± 4.57	0.171
WBC ($x10^3mm^3$)	11.50 ± 1.36	10.46 ± 2.23	0.367
Neutrophils (%)	47.50 ± 19.54	45.04 ± 12.39	0.715
Lymphocytes (%)	47.50 ± 18.48	42.12 ± 13.48	0.456
Eosinophils (%)	4.75 ± 1.71	9.55 ± 5.10	0.069
Monocytes (%)	0.25 ± 0.50	2.86 ± 3.55	0.151
Basophils (%)	0.00 ± 0.00	0.04 ± 0.28	0.782

n represents number of animals in each category

Source: Labwork, 2011

Genotyping of N'Dama and WASH Cattle

Genetic polymorphism at MHC DRB3 gene in N'Dama cattle

Genotyping of 51 N'Dama cattle samples by HRM and nucleotide sequence analysis revealed 17 different alleles. The frequency distribution of the alleles is shown in Table 12. Alleles 1701 and 1801 were the most common with the highest frequency (Figure 14).

Table 12: Melting temperatures and frequencies of alleles of MHC DRB3 gene in 51 N'Dama cattle

Allele	HRM temperature (°C)	Number of alleles	Percentage (%)
1701	91.58	24	23.5
1801	92.70	24	23.5
0101	91.20	12	11.8
14	91.32	12	11.8
0902	91.25	6	5.9
1201	91.48	4	3.9
2201	92.35	3	2.9
2	91.28	3	2.9
0201	91.22	3	2.9
2101	90.60	2	2.0
1501	92.12	2	2.0
3202	91.35	2	2.0
1901	92.80	1	1.0
2705	91.05	1	1.0
8	92.42	1	1.0
1101	91.65	1	1.0
2802	90.90	1	1.0
Total		102	100

Source: Labwork, 2012

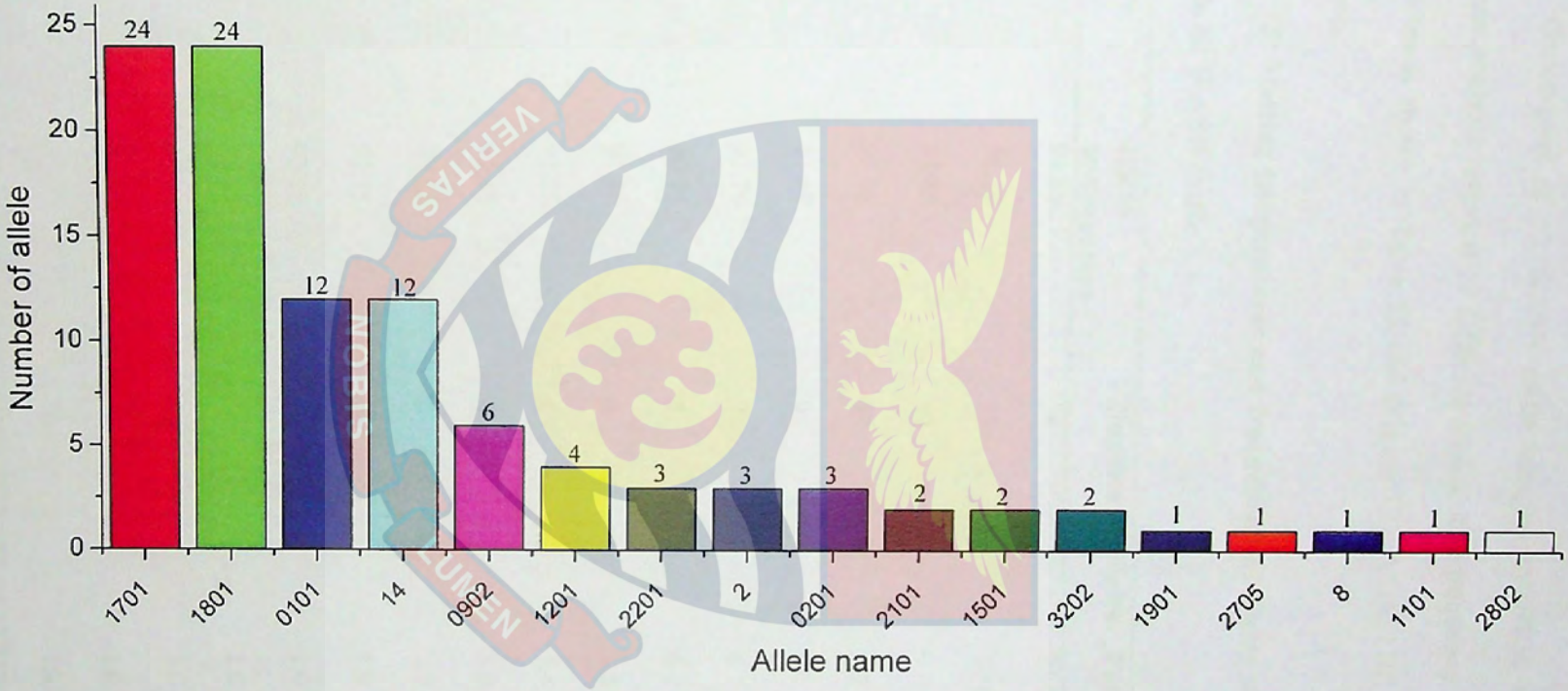


Figure 14. Allele frequencies of MHC DRB3 gene in 51 N'Dama cattle

Source: Labwork, 2012

Genetic polymorphism at MHC DRB3 gene in WASH cattle

Genotyping of 52 WASH cattle samples by HRM and nucleotide sequence analysis revealed 17 different alleles. The frequency distribution of the alleles is shown in Table 13 and (Figure 15). Allele 1101 was the most common.

Table 13: Melting temperatures and frequencies of alleles of MHC DRB3 gene in 52 WASH cattle

Allele	HRM temperature (°C)	Number of alleles	Percentage (%)
1101	91.65	26	25.0
2401	91.30	11	10.6
0801	91.80	10	9.6
3601	91.95	9	8.7
2101	91.60	9	8.7
1801	92.70	7	6.7
1301 or 1303	90.75	7	6.7
0101	91.20	6	5.8
2	91.28	6	5.8
2201	92.35	4	3.9
3021	90.60	3	2.9
4802	92.15	2	1.9
2001	92.50	1	1.0
2002	92.45	1	1.0
2710	91.75	1	1.0
1601	89.42	1	1.0
Total		104	100

Source: Labwork, 2012

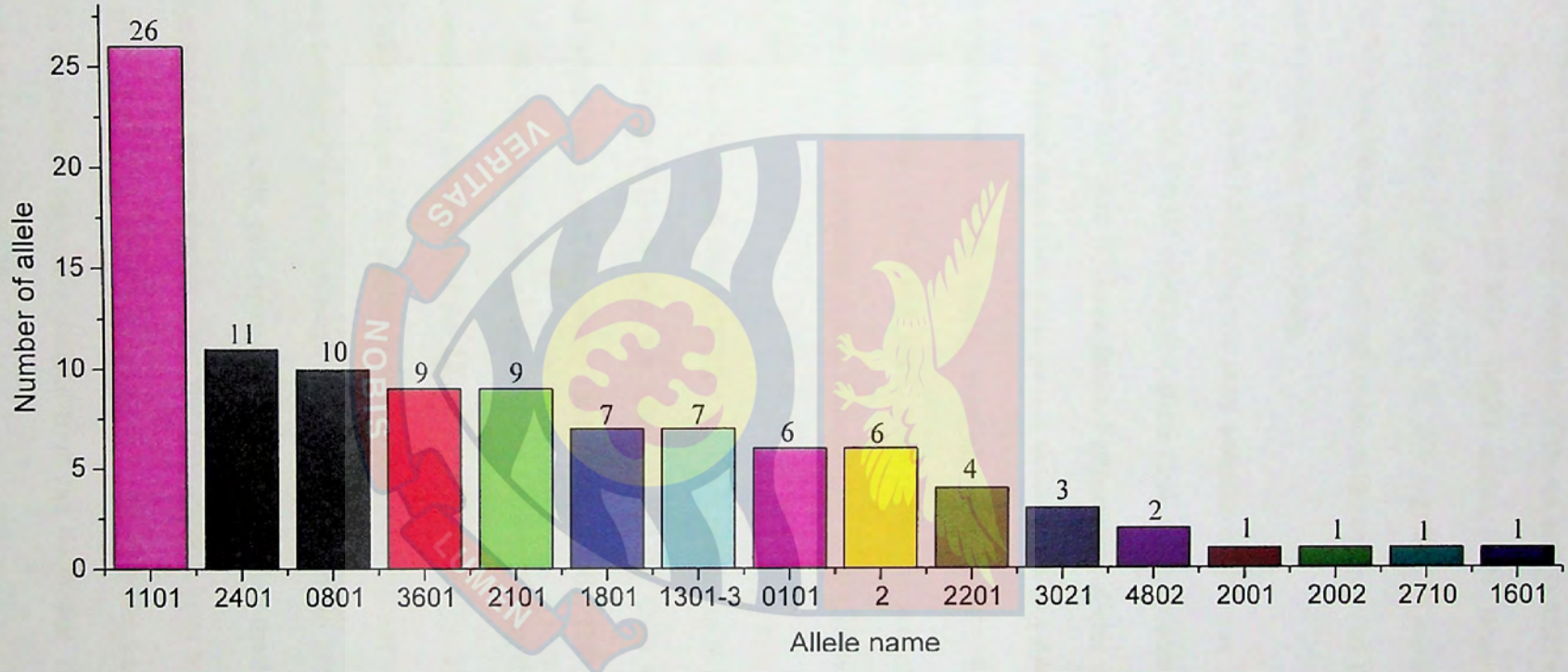


Figure 15. Allele frequencies of MHC DRB3 gene in 52 WASH cattle

Source: Labwork, 2012

Distribution of MHC DRB3 alleles in N'Dama and WASH cattle breeds

The distribution of MHC DRB3 alleles in N'Dama and WASH is compared in Table 14 and Figure 16. All together 28 alleles were found. Nucleotide sequences and deduced amino acid sequences of these alleles are in Figures 17 and 18, respectively.

In N'Dama, one of the two most common alleles was 1701 which was absent in WASH. On the other hand, allele 1101, the most common in WASH, was found in only one N'Dama cattle. Furthermore, alleles 14, 0902, 1201, 0201 in N'Dama, representing the second, third, fourth and fifth most frequent alleles, respectively in this breed, were absent in WASH. Similarly, alleles 2401, 0801, 3601, 1301-3 and 3021, representing the second, third, fourth, fifth and sixth most frequent alleles, respectively, in WASH were not detected in N'Dama.

Eleven alleles found in WASH were not present in N'Dama, while 11 alleles found in N'Dama were not found in WASH. Only six alleles were found in both breeds and these were 1801, 0101, 2, 2201, 2101 and 1101.

The Neighbour-Joining tree that was generated using the 319-base pair (bp) nucleotide sequences of the 28 alleles is in Figure 19. The tree revealed three main groups of the alleles; however, this grouping was not supported by strong bootstrap values. Although one of the three main groups had alleles found only in WASH, this group was not separated from the other two groups with strong bootstrap support. Thus, this Neighbour-Joining tree indicated that the alleles were not clustered according to breed. The Neighbour-Joining tree of the amino acid sequences derived from the nucleotide sequences of the alleles is found in Figure 20.

Table 14: MHC DRB3 allelic numbers, frequencies (%) and p values of allelic frequencies in N'Dama and WASH populations

Allele	N'Dama frequency (%)	WASH frequency (%)	*p* value
1701	24 (23.5)	0 (0)	<0.001
1801	24 (23.5)	7 (6.7)	0.001
0101	12 (11.8)	6 (5.8)	0.145
14	12 (11.8)	0 (0)	0.000
0902	6 (5.9)	0 (0)	0.014
2	3 (2.9)	6 (5.8)	0.498
2201	3 (2.9)	4 (3.9)	1.000
1201	4 (3.9)	0 (0)	0.058
0201	3 (2.9)	0 (0)	0.120
2101	2 (2.0)	9 (8.7)	0.059
1501	2 (2.0)	0 (0)	0.244
3202	2 (2.0)	0 (0)	0.244
1901	1 (1.0)	0 (0)	0.495
2705	1 (1.0)	0 (0)	0.495
8	1 (1.0)	0 (0)	0.495
1101	1 (1.0)	26 (25.0)	<0.001
2802	1 (1.0)	0 (0)	0.495
2401	0 (0)	11 (10.6)	0.001
0801	0 (0)	10 (9.6)	0.002
3601	0 (0)	9 (8.7)	0.003
1301-3	0 (0)	7 (6.7)	0.014
3021	0 (0)	3 (2.9)	0.246
4802	0 (0)	2 (1.9)	0.498
2001	0 (0)	1 (1.0)	1.000
2002	0 (0)	1 (1.0)	1.000
2710	0 (0)	1 (1.0)	1.000
1601	0 (0)	1 (1.0)	1.000

Source: Labwork, 2012

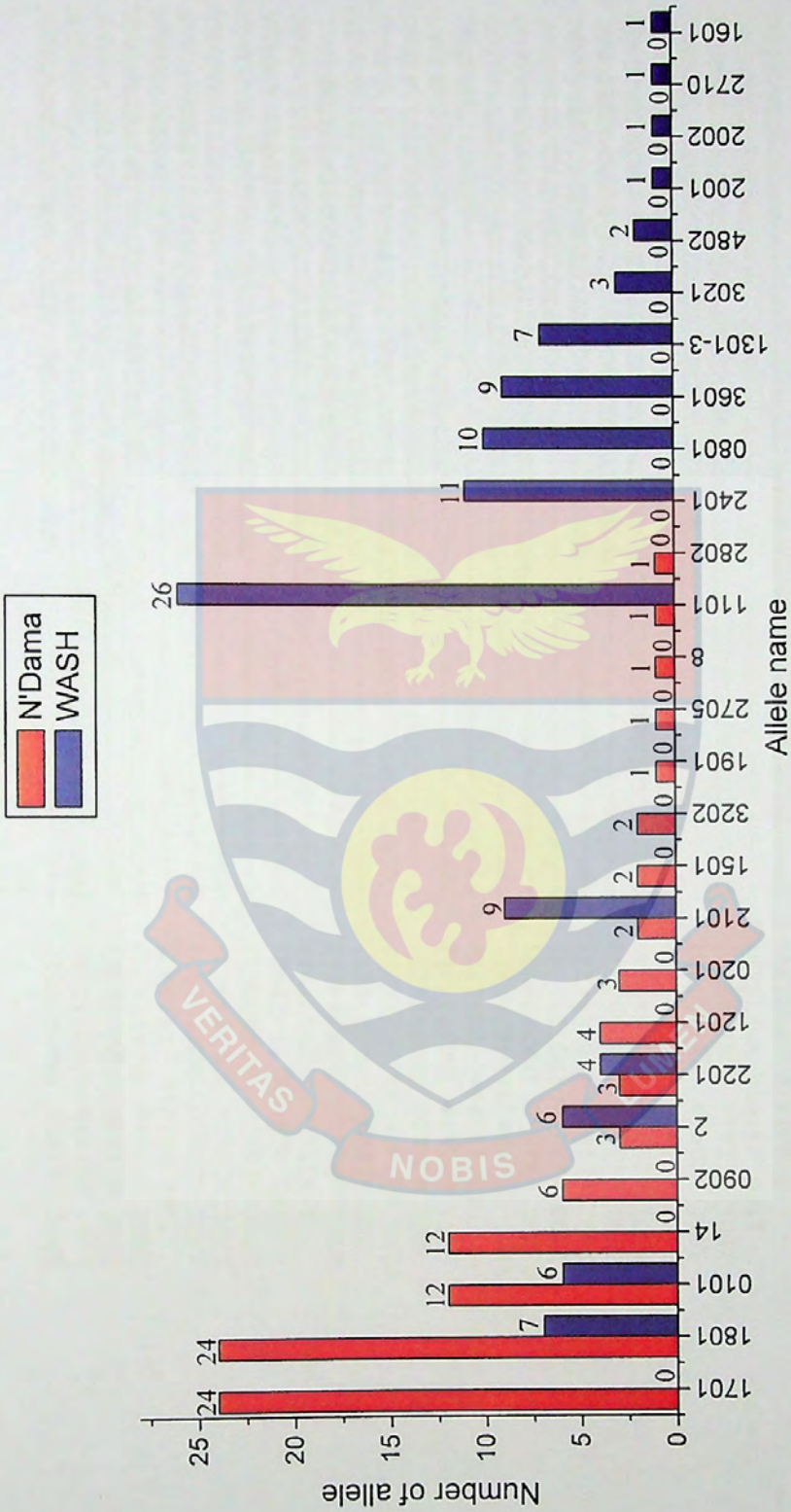
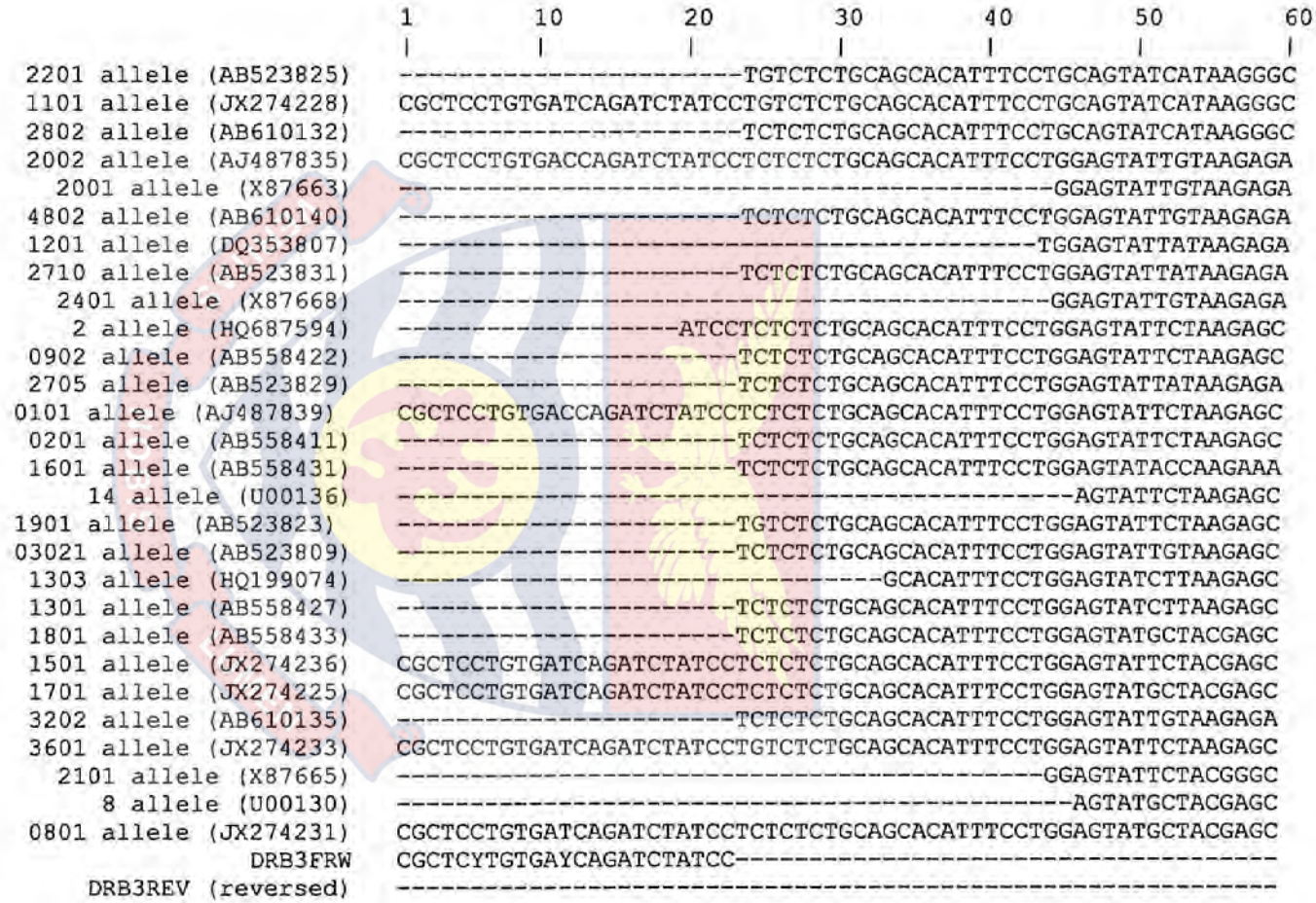
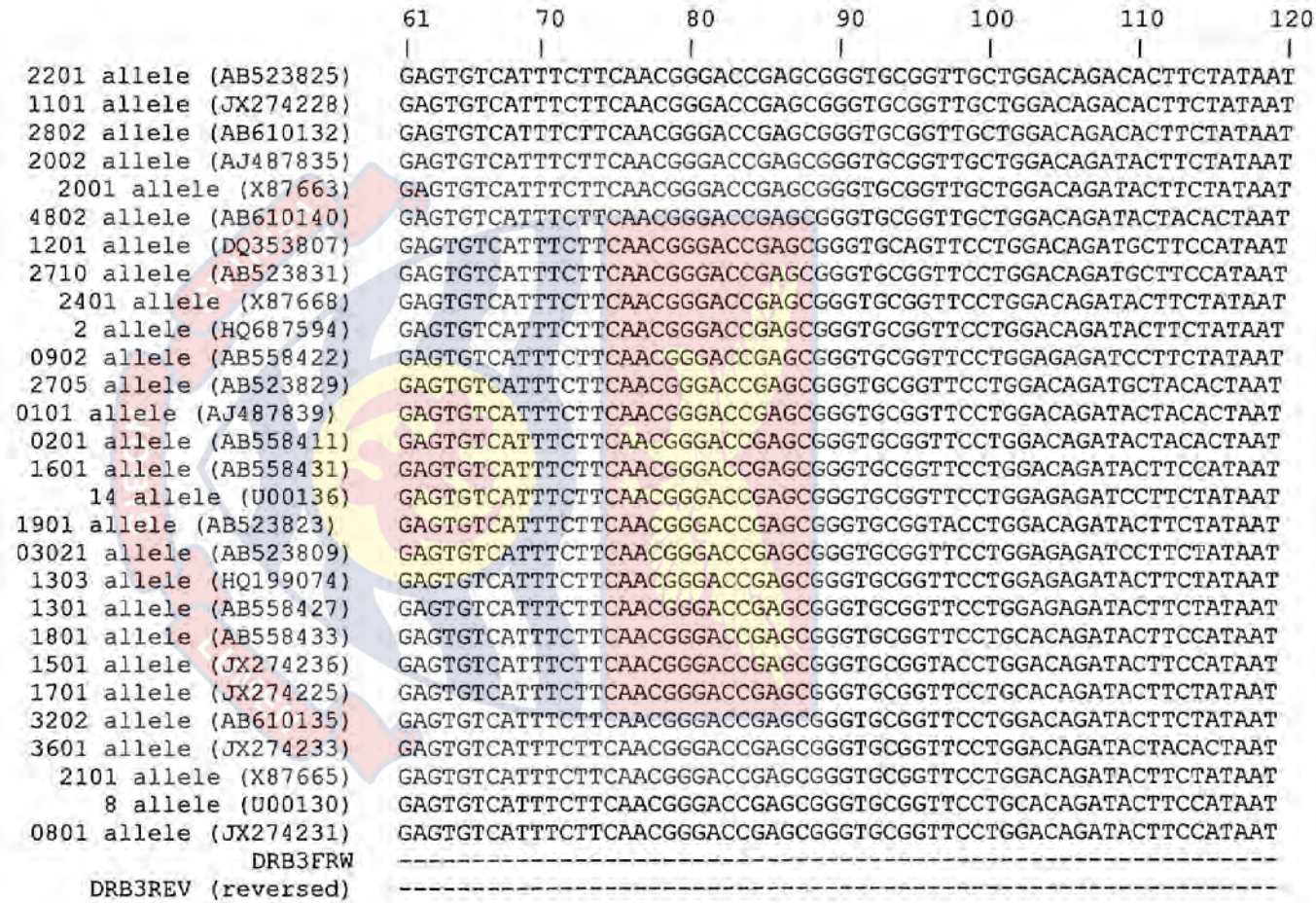
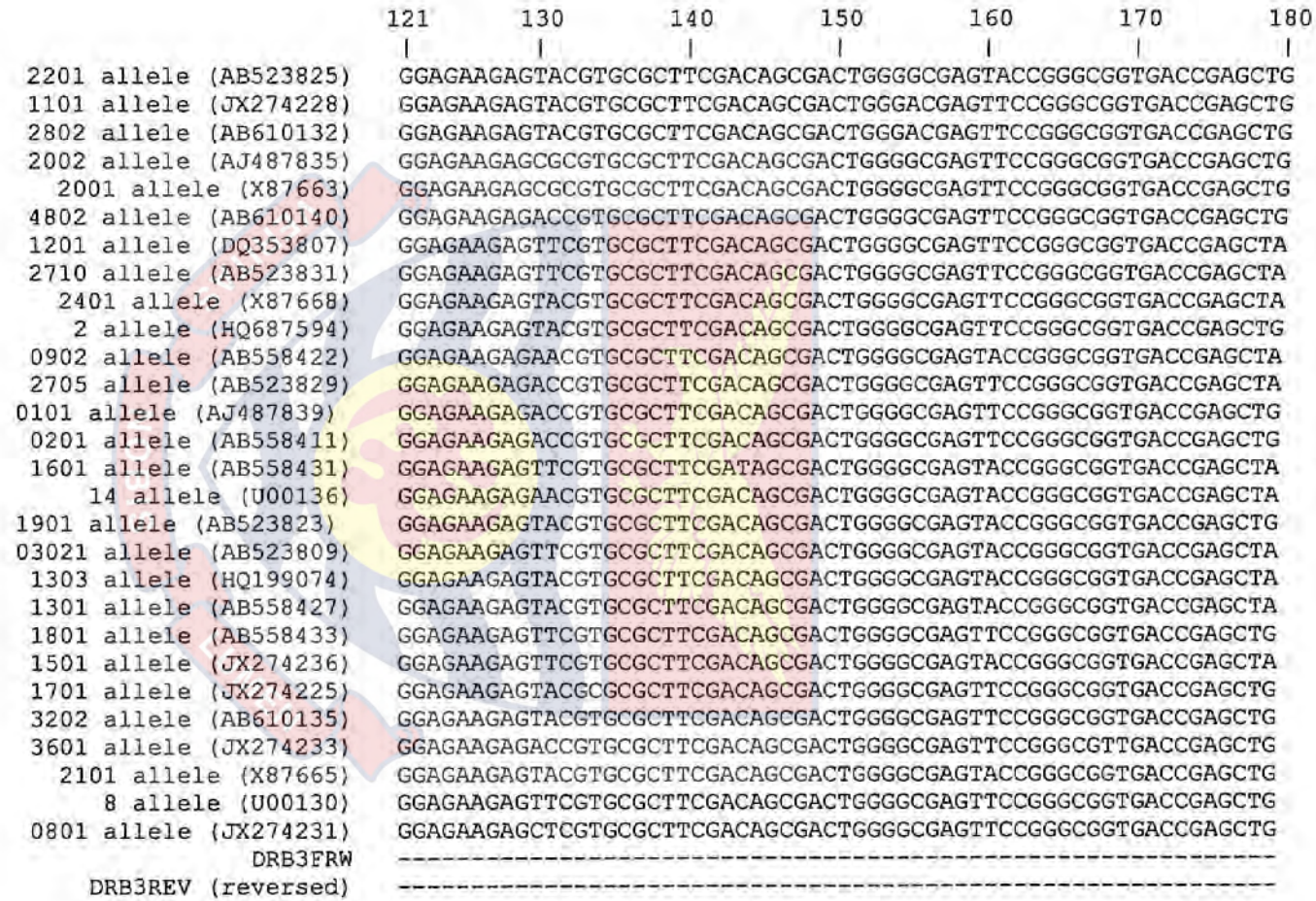


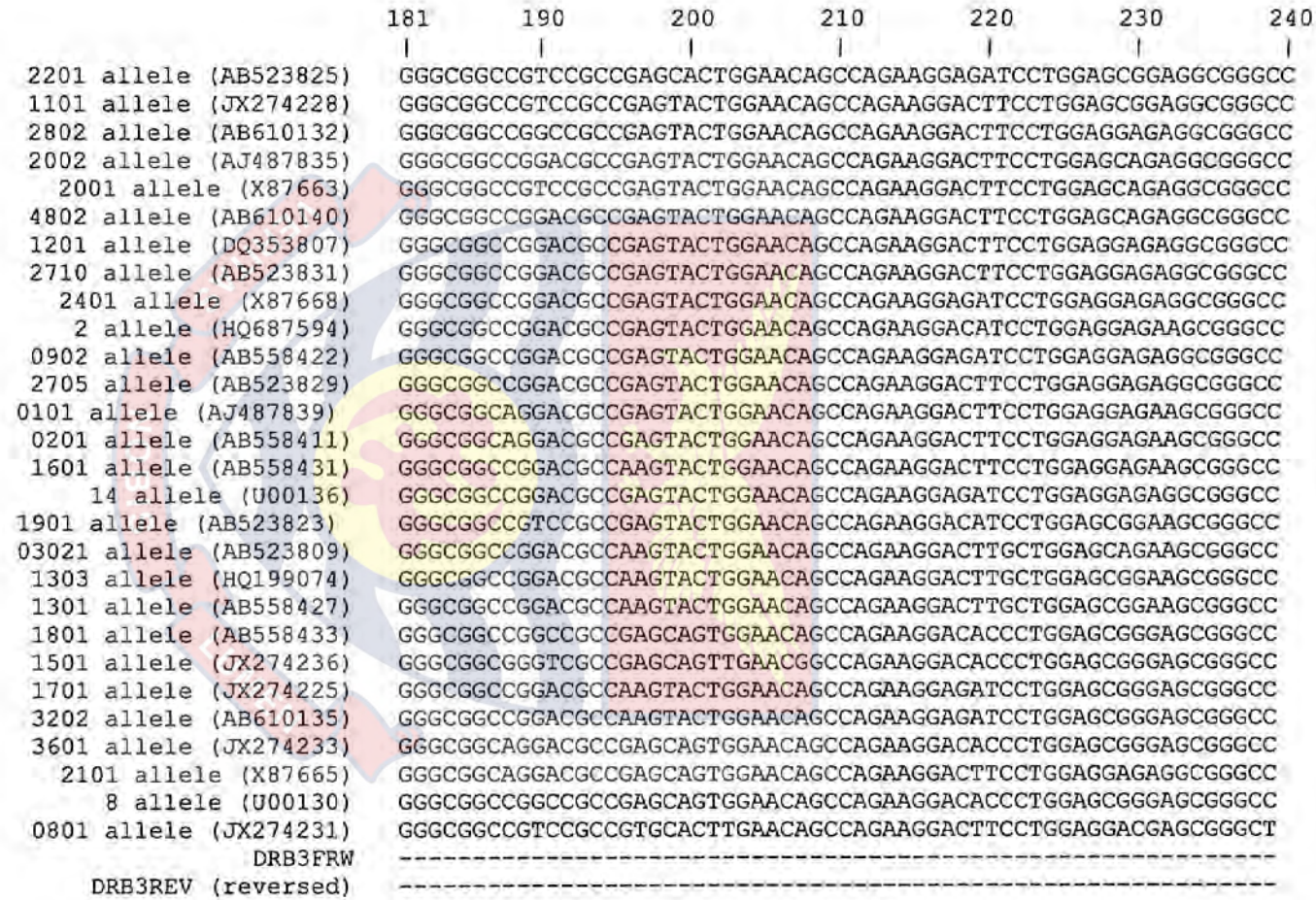
Figure 16. Distribution of MHC DRB3 alleles in N'Dama and WASH cattle breeds

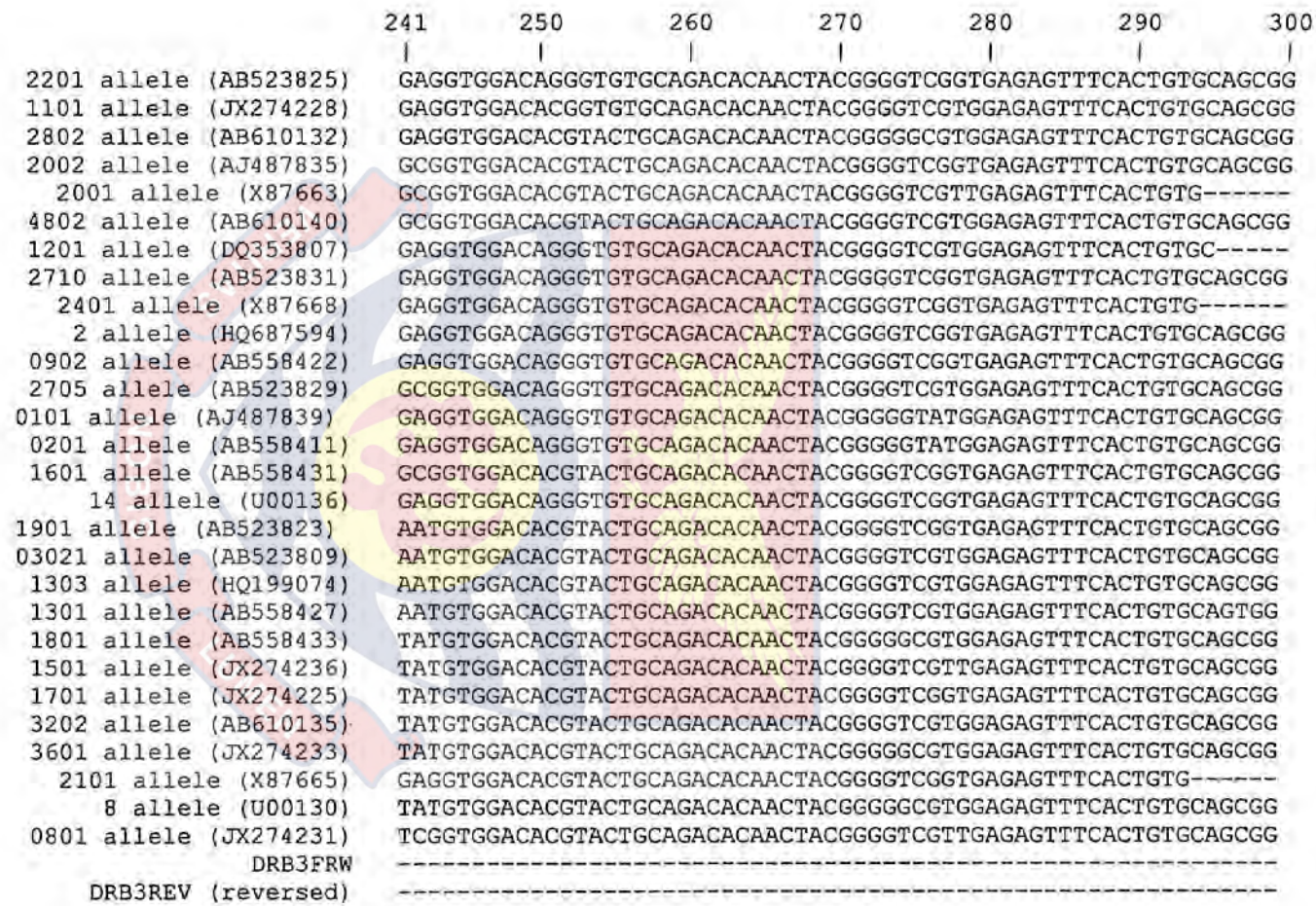
Source: Labwork, 2012











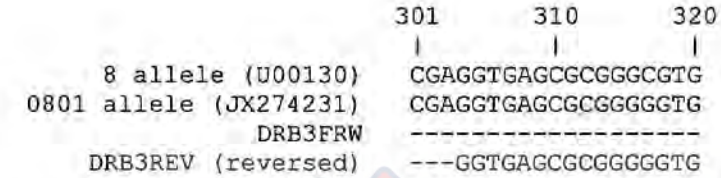
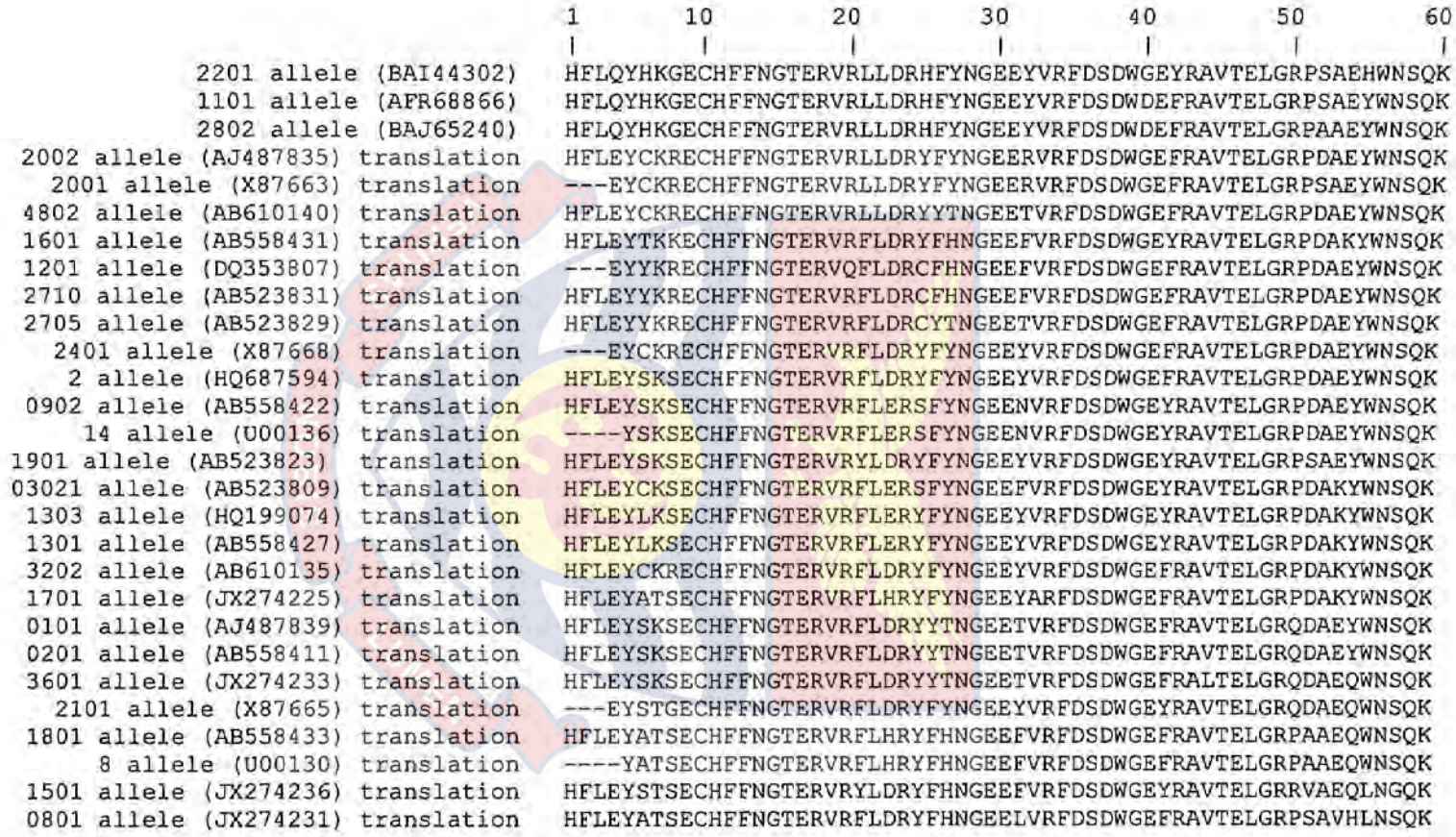
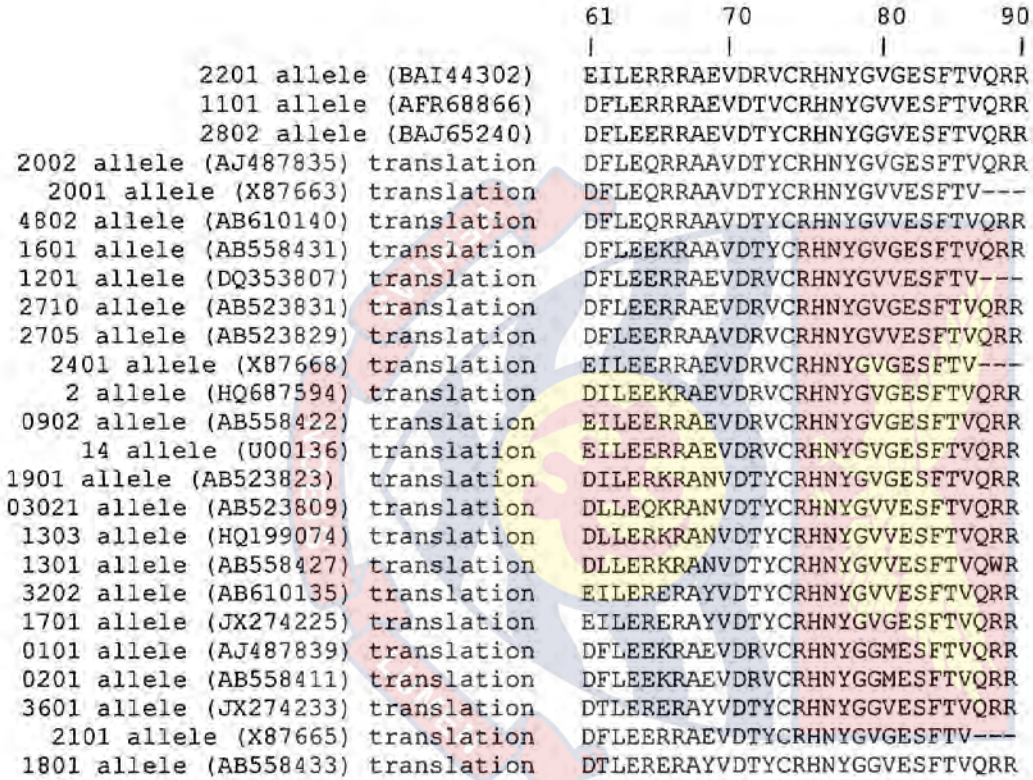


Figure 17. A 319-bp nucleotide sequence alignment of MHC-DRB3 alleles of N'Dama and WASH cattle

Source: Labwork, 2012





```
                                  61        70        80        90
                                  |         |         |         |
     8 allele (U00130) translation   DTLERERAYVDTYCRHNYGGVESFTVQRR
1501 allele (JX274236) translation   DTLERERAYVDTYCRHNYGVVESFTVQRR
0801 allele (JX274231) translation   DFLEDERASVDTYCRHNYGVVESFTVQRR
```

Figure 18. Amino acid sequence alignment of MHC-DRB3 alleles in N'Dama and WASH cattle

Source: Labwork, 2012

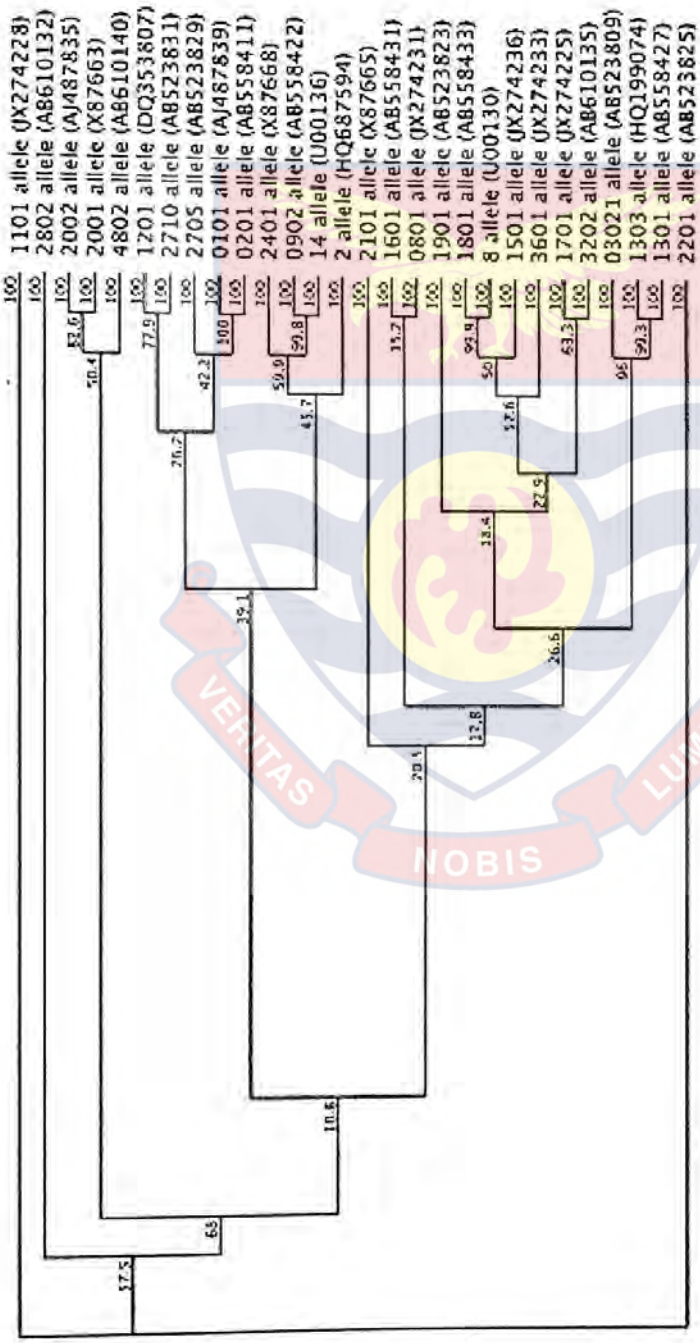


Figure 19. Neighbour-Joining tree constructed from 319-bp nucleotide sequences of MHC-DRB3 alleles found in N'Dama and WASH cattle breeds (Numbers are bootstrap percentages that support each node. Bootstrapping was performed with 10, 000 replicates to assess the reliability of individual branches)

Source: Labwork 2012

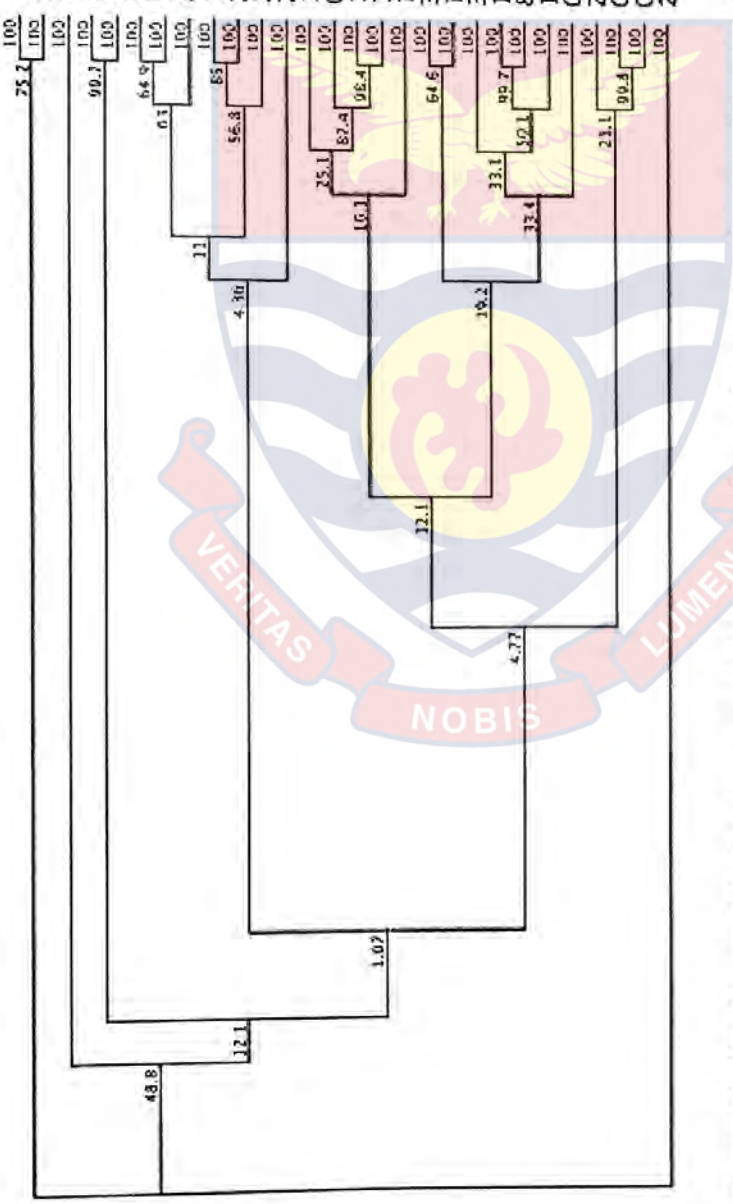


Figure 20. Neighbour-Joining tree constructed from amino acid sequences deduced from 319-bp nucleotide sequences of MHC-DRB3 alleles found in N'Dama and WASH cattle breeds (Numbers are bootstrap percentages that support each node. Bootstrapping was performed with 10, 000 replicates to assess the reliability of individual branches)

Source: Labwork, 2012

Association between MHC DRB3 alleles and *T.vivax* infection in N'Dama cattle

Table 15 presents odds ratios and relative risks of *T. vivax* infection with respect to MHC DRB3 alleles in N'Dama cattle. Allele 2101 had an OR and RR of 40.5 and 28.1, respectively, with a significant p-value of 0.02. Other alleles that were associated with increased odds or risk of *T. vivax* infection (susceptible alleles) but having ORs and RRs not significant were alleles 1701, 1801, 0101, 1501, 3202, 1901, 2705, 8, 1101 and 2802. On the other hand, alleles 14, 0902, 1201, 2201, 2 and 0201 were associated with decreased odds or risks of *T. vivax* infection and could be considered resistant alleles, although they had ORs and RRs that were not significant.

Association between MHC DRB3 alleles and *T.vivax* infection in WASH cattle

Odds ratios and relative risks of *T. vivax* infection with respect to MHC DRB3 alleles in WASH cattle are shown in Table 16. Allele 2002 had significant ($p < 0.05$) OR and RR of 41.6 and 29.4 respectively, indicating it was associated with increased odds or risk of *T. vivax* infection. Also, alleles 1101, 0801, 3601, 2, 2201, 3021, 4802, 2001, 2710 and 1601 had ORs and RRs indicating association with susceptibility to *T.vivax* infection, but the ORs and RRs were not significant. To the contrary, alleles 2401, 2101, 1801, 1301/1303 and 0101 had ORs and RRs indicating association with decreased risk of *T. vivax* infection, although the values were not significant.

Table 15: Odds ratios and relative risks of alleles in N'Dama

Allele	Odds ratio		Relative risk		
	Value	CI	Value	CI	*p* value
1701	1.93	0.38 - 9.67	1.40	0.67 - 2.91	0.40
1801	1.93	0.38 - 9.67	1.40	0.67 - 2.91	0.40
0101	2.11	0.34 - 13.15	1.80	0.46 - 6.96	0.42
14	0.88	0.09 - 8.49	0.90	0.13 - 6.23	0.91
0902	0.48	0.02 - 9.60	0.51	0.03 - 8.37	0.63
1201	0.79	0.04 - 16.92	0.80	0.05 - 14.12	0.88
2201	0.79	0.04 - 16.92	0.80	0.05 - 14.12	0.88
2	0.79	0.04 - 16.92	0.80	0.05 - 14.12	0.88
0201	0.79	0.04 - 16.92	0.80	0.05 - 14.12	0.88
2101	40.45	1.71 - 956.41	28.13	1.48 - 532.97	0.02*
1501	7.17	0.39 - 130.31	6.29	0.44 - 89.38	0.17
3202	1.13	0.05 - 26.03	1.13	0.06 - 21.32	0.94
1901	1.93	0.07 - 52.06	1.88	0.08 - 42.08	0.69
2705	20.54	0.75 - 559.52	16.88	0.75 - 3.78	0.08
8	1.93	0.07 - 52.06	1.88	0.08 - 42.08	0.69
1101	1.93	0.07 - 52.06	1.88	0.08 - 42.08	0.69
2802	1.93	0.07 - 52.06	1.88	0.08 - 42.08	0.69

CI is Confidence Interval
*significant

Source: Labwork, 2012

Table 16: Odds ratios and relative risks of alleles in WASH

	Odds ratio		Relative risk		
Allele	**Value**	**CI**	**Value**	**CI**	***p* value**
1101	1.82	0.24 - 14.13	1.41	0.49 - 4.04	0.57
2401	0.46	0.02 - 9.34	0.52	0.04 - 7.61	0.63
0801	5.86	0.70 - 48.68	3.43	1.04 - 11.33	0.10
2101	0.53	0.03 - 10.78	0.58	0.04 - 8.58	0.69
3601	1.67	0.15 - 18.14	1.50	0.25 - 9.18	0.66
1801	0.61	0.03 - 12.64	0.65	0.04 - 9.83	0.76
2	3.67	0.31 - 43.96	3.00	0.43 - 20.87	0.27
1301/1303	0.73	0.03 - 15.13	0.75	0.05 - 11.50	0.84
0101	0.73	0.03 - 15.13	0.75	0.05 - 11.50	0.84
2201	3.52	0.12 - 99.64	3.27	0.15 - 70.06	0.46
3021	7.67	0.53 - 110.66	6.00	0.68 - 52.71	0.11
4802	2.07	0.09 - 50.04	1.96	0.11 - 35.40	0.66
2001	3.52	0.12 - 99.64	3.27	0.15 - 70.06	0.45
2002	41.57	1.42 - 122.38	29.40	1.37 - 630.51	0.03*
2710	3.52	0.12 - 99.64	3.27	0.15 - 70.06	0.46
1601	3.52	0.12 - 99.64	3.27	0.15 - 70.06	0.46

CI is Confidence Interval
*significant

Source: Labwork, 2012

CHAPTER FIVE

DISCUSSION

Prevalence of Trypanosome Infection in Cattle Breeds using ITS Primers and *T. vivax*-specific Primers

None of the 440 blood samples from N'Dama, WASH, Sanga and Zebu cattle tested positive for trypanosome species when ITS-1 generic primers were used. However, 11 out of the 440 animals tested positive when primers specific for the West African strain of *T. vivax* were used. The study demonstrated that ITS primers are less sensitive than species-specific primers in detecting *T. vivax* infection in West African cattle breeds. The lower sensitivity of the ITS primers compared to the *T. vivax*-specific primers could be attributed to the fact that species-specific primers target satellite DNA sequences which are present in high copy number of between 10,000 to 20,000 whereas ITS primers target sequences which are only repeated 100-200 times (Desquesnes & Davila, 2002).

The finding in this study agrees with that of Gonzales et al. (2006), who on evaluating the sensitivity of a *T. vivax*-specific primer set and an ITS primer pair on blood samples of a 2-month old male sheep inoculated intravenously with *T. vivax* isolate obtained from an infected bovine, found that the sensitivity of the ITS primers was less than that of the *T.vivax*-specific primers. To the contrary, studies by Njiru et al. (2005) comparing ITS and

species-specific primers on cattle and camel blood samples in Kenya showed higher trypanosome prevalence with ITS primers.

The trypanosome diagnostic procedure using generic primers to amplify the internal transcribed spacer (ITS-1) region of the ribosomal RNA gene locus was a quicker and cheaper technique that was developed to replace the multiple species-specific tests for diagnosis of African trypanosome species infection (Adams & Hamilton, 2008; Cupolillo et al., 1995; Desquesnes et al, 2001; McLaughlin et al., 1996). However, as shown by this study, ITS primers may not be sensitive and therefore should be used with caution in Ghana for the diagnosis and surveillance of the West African strain of *T. vivax*. Further work in this area is needed. Indeed, due to the limited repetitiveness of ITS target sequences, Desquesnes and Davila (2002) suggested that new ITS primers should be designed to reach 100% homology with all *Trypanosoma* species of veterinary interest.

In this study, *T. vivax* infections were recorded in N'Dama and WASH cattle but not in Zebu and Sanga cattle. It is known that N'Dama and WASH are trypanotolerant breeds whereas the Zebu is a trypanosusceptible breed (Rege et al., 1994). Trypanotolerant breeds, although equally susceptible to initial infection, possess the ability to survive, reproduce and remain productive in areas of high tsetse challenge without the need for the use of chemicals to control the vector or drugs to control the parasite (Dayo et al., 2009; Rege et al., 1994). On the other hand, trypanosusceptible breeds rapidly succumb to the disease (Murray et al., 1982). The trypanotolerant trait is generally attributed to the taurine breeds of cattle in West and Central Africa, namely, the N'Dama and the West African Shorthorn (Roelants, 1986).

The finding in this study that some of the trypanotolerant N'Dama and WASH used in this study tested positive for *T. vivax* whereas none of the trypanosusceptible Zebu and Sanga tested positive is consistent with that of Achukwi and Musongong (2009). In a survey of a total of 296 trypanotolerant *Bos taurus* Doayo and trypanosusceptible *Bos indicus* White Fulani zebu cattle in Cameroun, they found that more Doayo cattle had detectable trypanosome infections than the White Fulani. According to these researchers (Achukwi & Musongong, 2009), none of the sampled Doayo cattle breeders used trypanocides on their farms while the owners of the White Fulani zebus frequently treated sick animals with trypanocides. In the present study, informal information revealed that livestock keepers who keep trypanosusceptible breeds use trypanocidal drugs regularly or strategically to control the infection. This could explain why the N'Dama and WASH recorded some positives for *T. vivax* while all the Zebu and Sanga sampled were negative for *T. vivax*.

Haematology of N'dama, WASH, Zebu and Sanga Cattle

Among the four cattle breeds sampled, the N'Dama had the highest overall PCV value (34.50 ± 4.37%) compared to the Sanga (34.39 ± 4.78%), WASH (31.79 ± 4.73%) and Zebu (30.30 ± 4.95%). The fact that the N'Dama also had the highest *T. vivax* infection rate of 6.4%, against 0% for the Sanga, 3.63% for the WASH and 0% for the Zebu, could be a reflection of the trypanotolerant attribute of the N'Dama. The PCV values for the various breeds were within the normal range as discussed elsewhere (Jain, 1993). Our findings agree with those of Adam et al. (2012) who reported that the average PCVs of WASH, Sanga and Zebu cattle (both trypanosome-positive and

trypanosome-negative) sampled in Ghana were 30%, 31% and 32%, respectively.

Haematology of normal and *T. vivax* infected N'Dama and WASH Cattle

The mean PCV, Hb and RBC values observed in both infected and uninfected N'Dama and WASH cattle studied were within the normal values reported by Jain (1993). The presence of *T. vivax* parasites in the blood of cattle in this study was expected to cause a reduction in the mean red blood cell values below the normal range, an indication of anaemia (Morrison et al., 1981; Murray & Dexter, 1988; Murray et al., 1984; Silva et al., 1999). Both infected and uninfected cattle in the present study had mean red blood cell values within the normal range and this could be attributed to the trypanotolerant nature of the N'Dama cattle and WASH. As noted by Murray et al. (1982) and Murray and Dexter (1988), in a typical trypanotolerance phenomenon, pathogenic *Trypanosoma* species infection does not usually result in anaemia.

The finding in this present study compares favourably with the observation by Mbanasor et al. (2003) who found the mean RBC, Hb and PCV values in natural *T. vivax* infected and uninfected trypanotolerant Muturu cattle in Nigeria to be well within the accepted normal values reported in many breeds of cattle by Schalm et al. (1975).

The mean PCV for infected animals in this study was lower but not significantly different than that for uninfected cattle at the location where *T .vivax* was detected for a particular breed (28.25 ± 1.50% vrs 31.49 ± 5.14% for WASH at Chegbani; 30.71 ± 4.35 vrs 34.54 ± 4.02 for N'Dama at UCC). This data agrees with that of Waiswa and Katunguka–Rwakishaya (2004) who

reported that the mean PCV for the trypanosome-positive animal was lower than that for the trypanosome-negative animal.

A survey conducted by Bekele and Nasir (2011) in cattle in western Ethiopia revealed that the mean packed cell volume of animals infected with *T. congolense*, *T. vivax* and *T. brucei* was significantly lower (20.8 ± 3.2 %) compared to non-infected animals (24.9 ±3.8 %). In Nigeria, Ohaeri and Eluwa (2011) reported that domestic ruminants, including cattle, that were naturally infected with trypanosomes had significantly lower ($p < 0.05$) PCV and RBC count compared to uninfected animals. Lower herd average PCVs for trypanosome-positive cattle compared to trypanosome-negative cattle have been also reported from Zambia (Marcotty et al., 2008; Van den Bossche & Rowlands, 2001) and Cameroon (Achukwui & Musongong, 2009).

The mean WBC counts for the N'Dama and WASH in the present study were within the normal values reported by Jain (1993) but were lower than those reported for *T. vivax* infected and normal Muturu cattle in Nigeria (Mbanassor et al., 2003).

Polymorphism at the MHC DRB3 Gene in N'Dama and WASH Cattle using HRM Analysis

MHC genotyping is usually done using PCR-RFLP (Duangjinda et al., 2009; Van Eijk et al., 1992) or PCR-SSCP (Orita et al., 1989; Pipalia et al., 2004; Zhou et al., 2007) whose limitations have been noted in the literature review. An evaluation of MHC DRB3 polymorphism by HRM analysis in this study showed that HRM is a suitable technique for MHC genotyping. From available literature, this is the first study to use HRM for MHC genotyping in cattle.

The study revealed high levels of MHC DRB3 genetic variation in N'Dama and WASH as shown by the number of alleles, 17 in each breed. The number of alleles observed in this study is similar to the number of DRB3 alleles detected in Jersey cattle (Sharif et al., 1998) and bighorn sheep (Gutierrez-Ezpeleta et al., 2001). It is, however, lower than the 40 alleles reported at the DRB3 locus in Holstein x Zebu crossbred cattle (Duangjinda et al., 2009). The higher number of DRB3 alleles found in the Holstein x Zebu dairy cows was attributed to the allelic combination between taurine and zebu cattle. In the present study, the animals used were taurine purebreds. In all 28 composite alleles were observed for the N'Dama and WASH in this study.

The high degree of polymorphism noted for each of the two breeds in this study implies the existence of many alleles that may potentially bind and present to T-cells a different set of pathogen-derived peptides, thus enabling the animals to respond to a broader spectrum of pathogens (Babik et al., 2012; Parham & Ohta, 1996). This could possibly explain the low levels of *T. vivax* infection in the two breeds. It has been suggested that depletion of variation at the MHC genes may compromise the ability of populations to respond to pathogen assault and lead to an increased risk of extinction (Hedrick, 2001; Hughes, 1991; O'Brien & Evermann, 1988).

The frequency distribution of the MHC DRB3 alleles was different in the N'Dama and WASH populations studied. This finding reveals that in terms of their allelic variations in the DRB3 gene, the N'Dama and WASH are distinctively different. Sharif et al. (1998) observed that the frequency distribution of MHC DRB3 alleles was different in Holstein and Jersey populations. The difference in founder populations, selection pressures and

sample size were reasons given for the different DRB3 allelic frequencies observed in the Holstein and Jersey cattle (Sharif et al., 1998). In the present study, the sample size of the N'Dama was similar to that of WASH; hence, the difference in allelic frequencies observed could be attributed to difference in founder populations and selection pressures.

Association between MHC DRB3 Alleles and *T.vivax* Infection in N'Dama and WASH

This study examined potential associations between MHC DRB3 alleles and resistance or susceptibility to *T. vivax* infection in N'Dama and WASH cattle in Ghana. From available literature, this is the first study to determine MHC allelic variants associated with susceptibility or resistance to *T. vivax* infection in cattle.

In the N'Dama, alleles 14, 0902, 1201, 2 and 0201 were found to be associated with resistance to *T.vivax* infection, whereas alleles 1701, 1801, 0101, 1501, 2101, 3202, 1901, 2705, 8, 1101 and 2802 were susceptible alleles. In WASH, alleles 2401, 2101, 1801, 1301/1303 and 0101 were found to be associated with resistance to *T. vivax* infection, whereas alleles 1101, 0801, 3601, 2, 2201, 3021, 4802, 2001, 2002, 2710 and 1601 were susceptible alleles. Further work using a larger population of each breed is needed to reduce the chance of spurious associations (Juliarena et al., 2008), and to ascertain the result obtained in this study.

The association of MHC DRB3 alleles with susceptibility or resistance to some infectious diseases in cattle has been reported in a number of studies (Duangjinda et al., 2009; Glass et al., 1991; Juliarena et al., 2008; Lewin et al., 1999; Maillard et al., 2003; Maillard et al., 1996; Park et al., 2004; Rupp et al.,

2007; Sharif et al., 2000; Sharif et al., 1998; Xu et al., 1993). Allelic variants of MHC genes were found to be associated with resistance to persistent lymphocytosis caused by bovine leukemia virus (Xu et al., 1993). Associations have also been observed for resistance to dermatophilosis in Brahman cattle of Martinique (Maillard et al., 1996); cystic ovarian disease and retained placenta (Sharif et al., 1998); as well as foot and mouth disease (Glass et al., 1991; Lewin et al., 1999). Alleles have also been associated with susceptibility to other infectious diseases such as mastitis (Duangjinda et al., 2009; Park et al., 2004; Rupp et al., 2007; Sharif et al., 2000; Sharif et al., 1998) and enzootic bovine leukemia (Juliarena et al., 2008).

The MHC DRB3 resistant and susceptible alleles determined in this study could greatly facilitate selection for *T. vivax* resistant N'Dama and WASH through a carefully planned breeding programme. For example, by eliminating animals with alleles susceptible to dermatophilosis, disease prevalence in Brahman cattle in Martinique was reduced from 0.76 to 0.02 over 5 years (Maillard et al., 2003).

Use of OR and RR in Reporting Association

In this study, we calculated both odds ratio (OR) and relative risk (RR) to determine statistical associations between resistance or susceptibility and allelic variants of cattle MHC DRB3 gene. Odds ratio is a powerful tool that has long been used in epidemiological and medical research to show statistical associations (Holcomb, Chaiworapongsa, Luke, & Burgdorf, 2001; Osborne, 2006). It is a statistical term used to describe the odds of a certain event happening in one group versus another (Osborne, 2006). However, as pointed out by researchers (Davies, Crombie, & Tavakoli, 1998; Holcomb et al., 2001;

Pedhazur, 1997), correctly interpreting odds ratios for a scientific or practitioner audience is particularly challenging, and often done incorrectly. For example, Holcomb et al. (2001) reported that in a survey of high quality medical journals, 26% of authors explicitly misinterpreted odds ratios as relative risks.

Relative risk is a statistical term used to describe the risk of a certain event happening in one group versus another. Relative risk is intuitive (compared to odds ratio), yet this statistic is rarely the one reported in research (Osborne, 2006). For example, in their studies on associations between certain health outcomes and cattle MHC DRB3 allelic variants, Sharif et al (1998) and Juliarena et al. (2008) explicitly misinterpreted odds ratios as relative risk. One can report odds ratios as long as an accurate interpretation of the OR is provided (Osborne, 2006). However, odds are not intuitive as risks are, and the language needed to technically describe an odds ratio can be quite convoluted (Osborne, 2006).

While the OR and RR will be in the same direction, ORs can illegitimately inflate the effect size substantially (Davies et al., 1998). This was demonstrated by data generated in the present study.

CHAPTER SIX

SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

Summary

The accurate diagnosis of trypanosome infection and control of trypanosomosis remain challenging problems to cattle production in Ghana and other countries in sub-Saharan Africa. PCR, based on ITS primers and species specific primers, has emerged as the most sensitive and specific technique for diagnosing infection. This study sought to resolve contradictions with regard to sensitivity in results from limited research that compared ITS primers and species-specific primers. The study found species-specific primers to be more sensitive. Given the limitations of the traditional methods of controlling trypanosomosis, the study proposed a genetic approach to control the disease through determining MHC DRB3 allelic variants associated with susceptibility or resistance to disease. The study found specific alleles whose elimination from cattle populations could result in control of trypanosomosis.

Conclusions

The PCR technique based on ITS primers did not detect any trypanosome species in all 440 animals tested while the PCR based on *T. vivax* specific primers identified some positives. The study suggests that ITS primers are less sensitive than species-specific primers in detecting *T. vivax* infection in the cattle used for this study.

Although infection of cattle with *T. vivax* causes reduction in mean

PCV, N'Dama cattle maintained the highest mean PCV value among the four cattle breeds studied, despite recording the highest *T. vivax* infection rate.

This study identified alleles at the MHC DRB3 locus using HRM. The study showed high level of polymorphism of the MHC DRB3 gene in WASH and N'Dama at Chegbani and UCC, respectively, and that a high level of genetic variability existed in the WASH and N'dama. The most common alleles found in N'Dama were 1701 and 1801, whereas in WASH allele 1101 was the most prevalent. Alleles 1701, 1801, 0101, 1501, 2101, 3202, 1901, 2705, 8, 1101 and 2802 were associated with susceptibility to *T. vivax* infection while alleles 14, 0902, 1201, 2 and 0201 were associated with resistance to *T. vivax* infection in N'Dama cattle sampled at UCC. Alleles 1101, 0801, 3601, 2, 2201, 3021, 4802, 2001, 2002, 2710 and 1601 and 3021 were associated with susceptibility whereas alleles 2401, 2101, 1801, 1301/1303 and 0101 were associated with resistance to *T. vivax* infection in WASH at Chegbani.

Recommendations

The study showed that *T. vivax*-specific primers were more sensitive than ITS primers in the diagnosis of trypanosome infection in West African cattle breeds. The *T. vivax*-specific based protocol used in this study is, therefore, recommended for Ghana.

Although the species of trypanosomes known to exist in Ghana, which are pathogenic to cattle are *T. congolense*, *T. vivax* and *T. brucei brucei*, this study was limited to the use of *T. vivax*-specific primers only for the diagnosis of trypanosome infection in cattle in Ghana. Further studies using primers

specific for *T. congolense* (forest and savanna types) and *T. brucei* are suggested.

An evaluation of MHC DRB3 polymorphism by HRM analysis in this study showed that HRM is a suitable technique for MHC genotyping and is therefore recommended for Ghana and elsewhere.

This study determined resistant and susceptible MHC DRB3 alleles which could form the basis for planned breeding programmes to control trypanosomosis in cattle in Ghana. It is recommended that further studies involving larger numbers of animals be done with the view to eliminating spurious associations. Such studies would form the basis of carefully planned breeding programmes to determine *T. vivax* resistant N'Dama and WASH cattle breeds for crossbreeding programmes by Ghanaian cattle farmers under the auspices of stakeholders such as the Animal Production Directorate of the Ministry of Food and Agriculture and cattle breeders associations.

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APPENDIX: STATISTICAL ANALYSIS OUTPUT

1: Fisher's exact test for proportion of male and female N'Dama cattle positive for *T. vivax* at UCC

```
> NDama <- read.table ("C:/Users/Ganyo/Desktop/NDama.txt",
header=TRUE, sep="",
+    na.strings="NA", dec=".", strip.white=TRUE)
> library (abind, pos=4)
> .Table <- xtabs (~Sex+T.vivax, data=NDama)
> .Table
          T.vivax
Sex       Negative  Positive
 Female         33         5
 Male           15         2
> fisher.test (.Table)

        Fisher's Exact Test for Count Data

data:  .Table
p-value = 1
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
 0.07577916  6.18338881
sample estimates:
odds ratio
 0.8819943
> remove (.Table)
```

2: Fisher's exact test for proportion of male and female WASH cattle positive for *T. vivax* at Chegbani

```
> WASH <- read.table("C:/Users/Ganyo/Desktop/WASH.txt", header=TRUE,
sep="",
+  na.strings="NA", dec=".", strip.white=TRUE)
> library (abind, pos=4)
> .Table <- xtabs (~Sex+T.vivax, data=WASH)
> .Table
        T.vivax
Sex      Negative  Positive
 Female        36         3
 Male          15         1
> fisher.test (.Table)

        Fisher's Exact Test for Count Data

data:  .Table
p-value = 1
alternative hypothesis: true odds ratio is not equal to 1
95 percent confidence interval:
0.01430343  10.96137333
sample estimates:
odds ratio
0.8031347
> remove (.Table)
```

3: ANOVA for difference between *T. vivax*-infected and uninfected N'Dama at UCC

```
> UCCNdamaInfection <-
+   read.table ("C:/Users/Ganyo/Desktop/UCCNdamaInection.txt",
header=TRUE,
+   sep="", na.strings="NA", dec=".", strip.white=TRUE)
> library (multcomp, pos=4)
> library (abind, pos=4)
> AnovaModel.1 <- aov(PCV ~ T.vivax, data=UCCNdamaInfection)
> summary (AnovaModel.1)
             Df  Sum Sq  Mean Sq  F value  Pr(>F)
T.vivax      1   90.5    90.47    5.503    0.0228 *
Residuals    53  871.2   16.44
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
> numSummary (UCCNdamaInfection$PCV ,
groups=UCCNdamaInfection$T.vivax,
+   statistics=c ("mean", "sd"))
              mean       sd       data:n
Negative    34.56250  4.015429    48
Positive    30.71429  4.347961    7
```

4: Odds ratio for association between allele 2101 and susceptibility to *T. vivax* infection in N'Dama cattle at UCC (using MedCalc)

Cases with positive outcome

Number in 1st group: a= 2

Number in 2nd group: b= 5

Cases with negative outcome

Number in 1st group: c= 0

Number in 2nd outcome: d= 44

Test

Results

Odds ratio	40.4545
95 % CI	1.7112 to 956.4135
z statistic	2.293
	p = 0.0219

5: Relative risk for association between allele 2101 and susceptibility to *T. vivax* infection in N'Dama cattle at UCC (using MedCalc)

Exposed group

Number with positive outcome: a= 2

Number with negative outcome: b= 5

Control group

Number with positive outcome: c= 0

Number with negative outcome: d= 44

Test

Results

Relative risk	28.1250
95 % CI	1.4842 to 532.9743
z statistic	2.223
	p = 0.0262

6: Odds ratio for association between allele 2002 and susceptibility to *T. vivax* infection in WASH cattle at Chegbani (using MedCalc)

Cases with positive outcome

Number in 1st group: a= 1

Number in 2nd group: b= 3

Cases with negative outcome

Number in 1st group: c= 0

Number in 2nd outcome: d= 48

Test

Results

Odds ratio	41.5714
95 % CI	1.4161 to 1220.3844
z statistic	2.162
	p = 0.0306

7: Relative risk for association between allele 2002 and susceptibility to *T. vivax* infection in WASH cattle at Chegbani (using MedCalc)

Exposed group

Number with positive outcome: a= 1

Number with negative outcome: b= 3

Control group

Number with positive outcome: c= 0

Number with negative outcome: d= 48

Results

	Test
Relative risk	29.4000
95 % CI	1.3709 to 630.5147
z statistic	2.162
	p = 0.0306