4 MATERIALS AND METHODS

4.1 Description of the study area

The cross-sectional study was conducted within the 6 divisions (Butula, Funyula, Budalang'i, Nambale, Matayos and Township) of Busia District (Figure 1). Following the findings from the cross-sectional survey, the longitudinal study subsequently was undertaken in Budalang'i and Funyula divisions. Busia District lies between latitudes 0°1'36" South and 0°33'North and longitudes 33°54'32" and 34°25'24" East. The District is bordered by the Butere-Mumias District to the East, Bungoma to the North East, Teso District to the North, Siaya to the South East, Bondo District to the South, and Lake Victoria and the Republic of Uganda to West and covers a total area of 3,072 km^2. The District lies within the Low Midland (LM) zone. It has an altitude ranging between 1,130-1,375 meters above sea level. The long rains are experienced between March and May while the short rains start in late August and continue into October. The mean annual rainfall is 1,500mm with most parts of the district receiving between 1,270mm and 1,790mm (Ministry of Finance and Planning, GoK, 2002).

The total human population of Busia District is 370,608 (Ministry of Finance and Planning, GoK, 1999; FITCA-K, 2001). The different types of domestic animals kept are cattle, sheep, goats, donkeys, pigs, rabbits, cats and dogs. Poultry production is also an important engagement for the farmers. Recent surveys indicate that there are 74,818 cattle, 28,194 sheep, 50,141 goats, 2,118 donkeys, 21,280 pigs and 16,814 rabbits (FITCA-K, 2001).

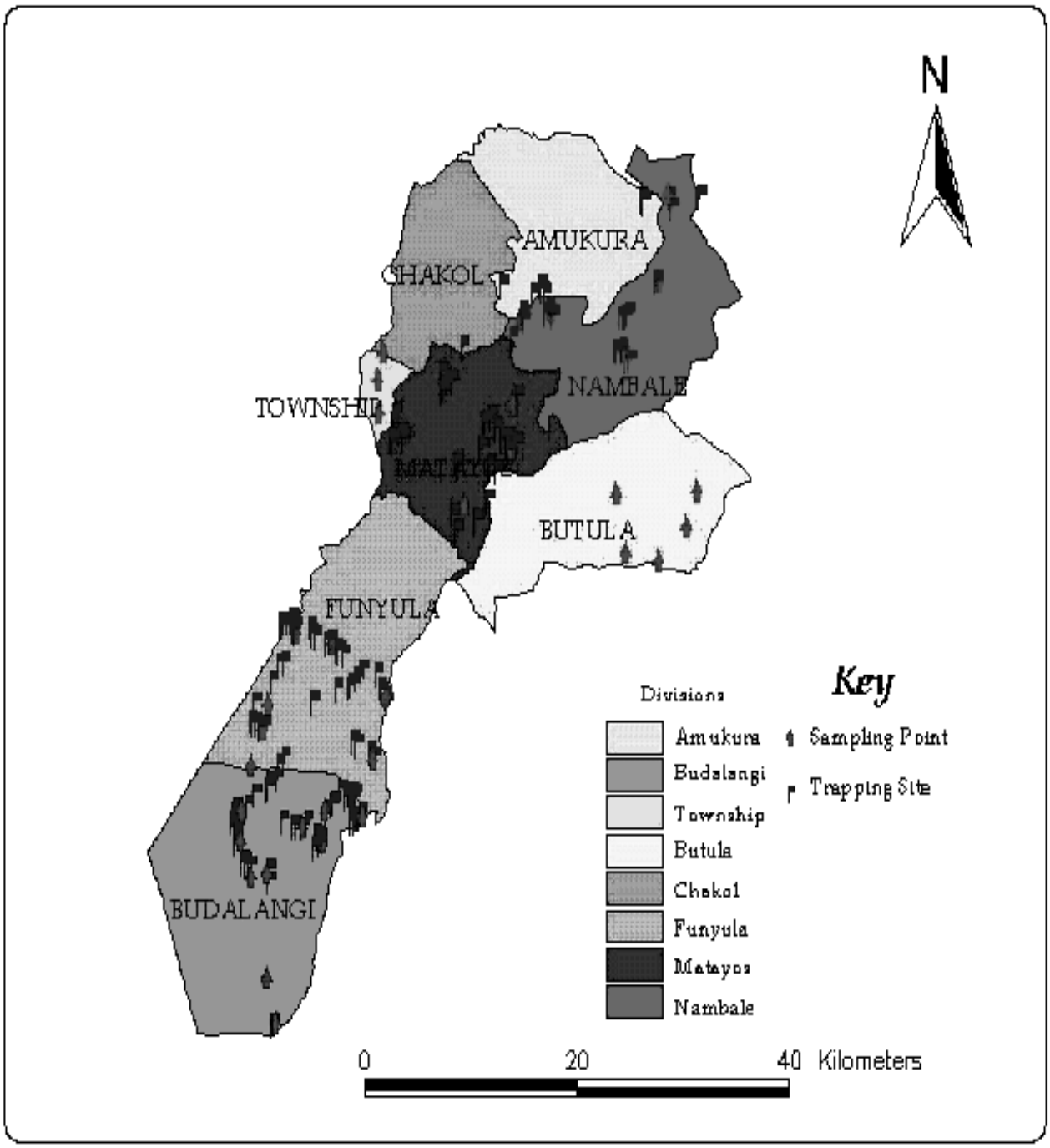

Figure 1. Map of Busia District (Kenya) indicating the animal sampling-points and tsetse fly-trapping sites during the cross-sectional study (2002)

4.2 Cross-sectional survey

4.2.1 Study animals

From approximately 39 grids (10 x 10 km^2 grid each) in Busia District, 20 grids representing 35 villages were randomly selected for cattle sampling. Most farmers owned between 1-6 head of cattle. From each farmer, a maximum number of three animals were sampled when herd sizes were larger than three animals per herd to give almost equal distribution of sampled subjects. For herd sizes with three animals and less, all of the animals were sampled. From each sampling point, 140 cattle were sampled and stratified by sex and age. In total, 2827 cattle were examined.

4.2.2 Animal sampling and examination

Sampling was undertaken during the month of November 2002. During sampling, animals were restrained in a crush pen, sexed and placed into three age categories (calves, young stock and adults). Animals aged up to one year were classified as calves while those aged up to three years were categorized as young stock and the ones aged three years and above were categorized as adults.

Approximately 70µl of blood was collected by venipuncture of the ear vein into two heparinised microhaematocrit capillary tubes for parasitological examination and determination of PCV (see 4.2.5). Lymph node aspirates were also collected for preparation of thin smears (see 4.2.6). Faecal samples were taken from calves for the estimation of helminth faecal egg counts (FEC). The species of ticks infesting cattle were recorded following examination on half-body basis. Abundance of tick infestation was recorded on a four-point scale (0 = none, low = < 20, moderate = 20-50 and high = >50 ticks) as described in Muraguri (2000).

4.2.3 Data collection

4.2.3.1 Questionnaire survey

During the survey, 91 standardized and pre-tested questionnaires were administered to farmers in October 2002 in order to collect information on family structure, herd structure and management, socio-economic activities and disease management. Major emphasis was laid on the importance and management of cattle diseases (i.e. trypanosomosis, helminthosis, anaplasmosis, babesiosis and ECF). The extent of drug usage with regard to source, frequency of treatments, dosage rates and costs was also addressed (Questionnaire, Annex 1).

4.2.3.2 Determination of Packed Cell Volume (PCV)

Microhaematocrit capillary tubes containing blood were centrifuged at 8000g for 5 minutes. The percentage PCV was then read with the help of a Hawksley microhaematocrit reader (Hawksley, Lancing, United Kingdom).

4.2.3.3 Detection and identification of trypanosomes

Trypanosome infection in cattle was detected using the BCT (Murray *et al*., 1977). Following blood centrifugation, trypanosomes are concentrated mainly in the buffy coat zone. The capillary tube is then cut using a diamond pen 1 mm below the buffy coat to include the uppermost layer of the red blood cells and 3 cm above to include the plasma. The contents of the capillary tube are expressed onto a slide, mixed and covered with a cover slip (22 x 22 mm). The preparation is then examined using a condenser with x25 objective and x10 eyepiece. In addition, thin blood smears of samples positive for trypanosomes were stained using the Giemsa staining technique (Shute and Maryon, 1966) in order to confirm the species of trypanosomes infecting cattle. A blood smear was air dried and fixed in methanol for 10 minutes. It was then dried and immersed in Giemsa stain (10 % Giemsa in Weise buffer, pH 7.2) for 35-40 minutes. After washing with tap water and drying, the stained smears were examined under a microscope at x100 objective and x10 eyepiece and oil emulsion.

4.2.3.4 Detection of *Theileria, Anaplasma* and *Babesia* parasites

For ECF, lymph node aspirates were collected from the prescapular lymph nodes of cattle using a large bore needle (G 18'). The lymph node aspirates were expressed on to a slide to make a thin smear. The smears were air-dried, fixed in methanol for 10 minutes and then stained using Giemsa (see 4.2.5). The stained smears were examined under a microscope for the demonstration of schizonts. In the case of *Anaplasma* and *Babesia* parasites, thin blood smears were prepared and stained in Giemsa and later examined under a microscope at x100 objective and x10 eyepiece and oil emulsion.

4.2.4 Entomological survey

4.2.4.1 Sampling

To determine apparent tsetse densities, 20 bi-conical traps were deployed at each animal sampling-point. The bushes were cleared from the sites to give sufficient visibility of the traps before deployment. To protect flies from ants, all trap poles were coated with grease. For *G. pallidipes*, traps were baited using about 250 ml of acetone in a 300 ml bottle closed with a perforated stopper. The traps were emptied 24 hours after deployment. Flies were identified, counted and separated into their respective species and sexes. Age determination of tsetse flies was based on the wing fray method (Jackson, 1946).

4.2.4.2 Dissection of flies to determine infection rate

All live flies were dissected for determination of trypanosome infection rates. The mouthparts, salivary glands and the mid-gut were dissected by the method described by Lloyd and Johnson (1924). Adult tsetse flies were pinned using sharp pins, on their back to a dissection board on which a drop or two of normal saline had been added. Using a pair of fine forceps, the midgut, salivary glands and mouthparts (labrum and hypopharynx) were carefully removed from the fly and spread onto a microscope slide containing a drop of normal saline and then covered with a cover slip. The dissected parts were examined using a microscope at x25 objective and x10 eyepiece. The trypanosome-group infecting flies were identified according to their location

within the fly. Trypanosomes recovered from the mouthparts only, were identified as the V*ivax* (*Duttonella*) group. When both the mouthparts and the mid-gut were infected, the trypanosomes were identified as *Congolense* (*Nanomonas*) group. Presence of trypanosomes in the salivary glands implied the *Brucei* (*Trypanozoon*) group.

4.2.5 Faecal sampling and determination of FEC

Faecal samples were collected *per rectum,* using plastic gloves from about 20 calves at each sampling site, for determination of faecal egg counts (FEC). The samples were put into labelled plastic bags. The bags were then stored in a cool box until delivery to the laboratory. Faecal egg counts (FEC) were determined using the McMaster method (Ministry of Agriculture, Fisheries and Food, UK, 1986): An amount of 4g faeces was suspended in 30 ml of saturated sodium chloride solution. On mixing thoroughly, saturated salt solution was added to attain a volume of 54 ml and then sieved through a tea strainer. The suspension was then stirred to obtain a completely homogeneous distribution of the eggs in the liquid. Both compartments of a McMaster chamber were then filled with the suspension using a Pasteur pipette. After waiting for 5-10 minutes for the eggs to float to the cover slip, eggs were counted under low magnification (x10 objective and x10 eyepiece). The epg was then calculated as follows:

$$\text{EpG} = \frac{\text{egg count (N)} \cdot \text{amount of sodium chloride solution [ml]}}{\text{faecal sample size [g]} \cdot \text{McMaster chamber compartment volume [ml}^3\text{]} \cdot \text{number of compartments counted (X)}}$$

The compartment volume of a standard McMaster chamber is 0.15 ml^3

Therefore: $$\frac{N \times 54}{4 \times 0.15 \times 2} = 45\ N$$

The lowest detection rate is one egg in one chamber (N = 1) corresponding to 45 epg.

For the interpretation of egg counts, the guide by Skerman and Hillard (1966) was used whereby, more than 500 epg were considered as heavy infection while less than 500 epg was considered light to moderate infection.

4.3 Longitudinal study

4.3.1 Study area

The longitudinal survey was undertaken in Budalang'i and Funyula divisions (Figure 2) of Busia District within a 9-month period (April to December 2003). The choice of these divisions was based on disease prevalence determined from the cross-sectional disease study and accessibility. In addition, presence of organized groups like community based organizations (CBOs) and community cattle crush pens were also considered important.

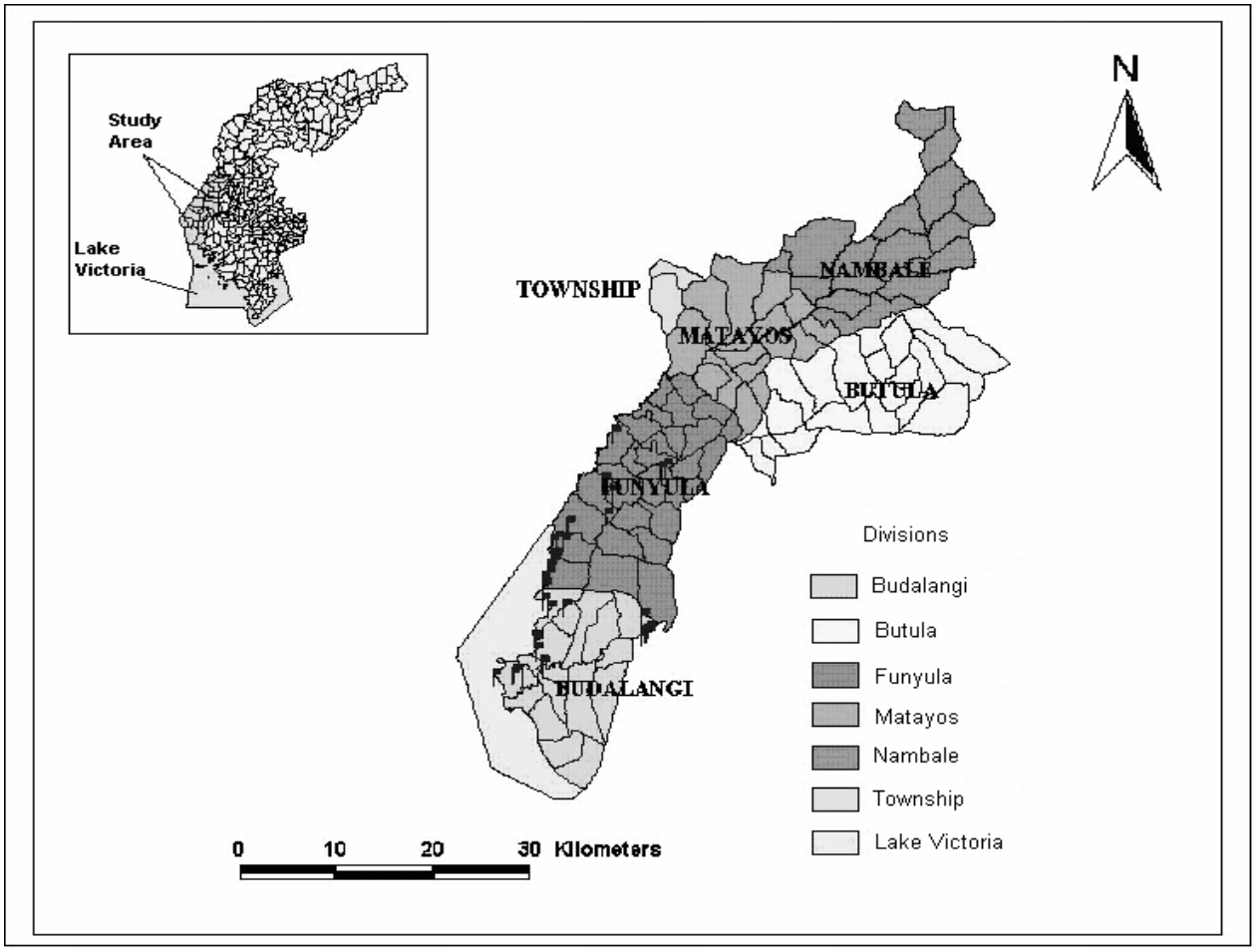

Figure 2. Map of Busia District (Kenya) indicating the tsetse fly-trapping sites in Budalang'i and Funyula divisions, during the longitudinal study (April-December 2003)

4.3.2 Study design

4.3.2.1 Study population and sampling

During the study, 200 ear-tagged local Zebu and exotic/crossbred cattle, stratified by both breed and age, were sampled from each division. Consequently, a total of 400 head of cattle (120 heifers, 120 calves and 160 adults) were selected through purposive sampling for this prospective study. The experimental design was a replication with respect to sample size, age and breed stratification and the treatment trial. The animals were randomly assigned into 4 treatment trial groups (Group I, II, III and IV) consisting of 50 animals per group, in each division (Table 3). Each treatment group consisted of 20 adults, 15 heifers and 15 calves.

1. **Group I:** Control group (untreated control animals)
2. **Group II**: Treatment group: ISMM (treated prophylactically with isometamidium chloride (Trypamidium®, Sanofi Kenya Ltd) at 1 mg/kg bw intra-muscular (im) every three months for 9 months)
3. **Group III**: Treatment group: ALB (treated with albendazole (Gardal®, Hichem Kenya Ltd) at 10mg/kg bw *per os* (po) every three months for 9 months)
4. **Group IV**: Treatment group: ALB/ISMM (treated prophylactically with albendazole (Gardal®, Hichem Kenya Ltd) at 10mg/kg bw po every three months and isometamidium chloride (Trypamidium®, Sanofi Kenya Ltd) at 1 mg/kg bw im every three months for 9 months)

Table 3. Numbers of cattle sampled from any-one division (one replicate) during the longitudinal study (April-December 2003), and cross-tabulated by age, breed and treatment trial group

Category	Group I		Group II		Group III		Group IV	
	Local	Exotic	Local	Exotic	Local	Exotic	Local	Exotic
Adults	15	5	15	5	15	5	15	5
Heifers	10	5	10	5	10	5	10	5
Calves	10	5	10	5	10	5	10	5

Exotic/crossbred cattle: Friesian, Ayshire, Guernsey, Jersey or a cross breed any two; Local: East African Zebu

G I: Control group; G II: ISMM; G III: ALB; G IV: ALB/ISMM

At the start of the study (week -2) in April 2003, all animals were sampled and initially treated with diminazene aceturate (Veriben®, Sanofi Kenya Ltd) at 3.5 mg/kg bw im to clear any background trypanosome infections and ensure that animals entered into the study free of these infections. They were then randomly assigned to one of the four trial groups. Two weeks after the diminazene aceturate treatment (week 0), animals in group II and IV were treated with isometamidium chloride (Trypamidium®, Sanofi Kenya Ltd) at 1mg/kg bw im and the treatment repeated every 3 months during the 9-month study period. Animals in group III and IV were treated with an albendazole (Gardal®, Hichem Kenya Ltd) at 10mg/kg bw po every 3 months for 9 months. All animals were monitored on week 0, 2, and 4 after the initial treatment. During subsequent treatments, the animals were monitored and sampled every 4 weeks until 36 weeks.

Any animal found parasitaemic within one month of ISMM treatment was treated curatively with diminazene aceturate at 7.0 mg/kg bw im. New trypanosome infections detected in cattle two months after ISMM treatment were treated with diminazene aceturate at 3.5 mg/kg bw im. During the follow-up period, animals were examined for trypanosome infection, helminths, *Anaplasma*, *Babesia* and *Theileria* parasites. Monthly pregnancy diagnosis by palpation *per rectum* was undertaken in cows and heifers. The daily milk production per lactating cow was recorded (see 4.3.7). All the calves were weighed every month to determine weight changes (see 4.3.6). Monthly PCV profiles were monitored and recorded for all the trial animals.

4.3.3 Determination of trypanosome incidence density

The incidence density was determined by first defining a 'case' of trypanosomosis. In this prospective study, a *'case'* of trypanosomosis was defined as positive detection of trypanosomes (*T. vivax, T. congolense, T. brucei* or a mixed infection) in a buffy-coat smear using the BCT (Murray *et al.,* 1977). Any animal positive for trypanosome infection was recorded as a "failure". During the ensuing 2 weeks after diminazene treatment, the animal was considered "not at risk" of contracting trypanosomosis. To calculate the total animal-time at risk, the animal-weeks at risk were summed up, across the different strata (location, treatment trial groups, age-categories and breeds). The incidence density was calculated using the formula given by Dohoo *et al.* (2003) as:

$$\text{Incidence density} = \frac{\text{number of new cases of trypanosomosis within 36 weeks}}{\text{Total animal-weeks at risk during the 36 weeks}}$$

Lymph node aspirate smears (for ECF) and thin blood smears (anaplasmosis and babesiosis) were prepared from monthly collected samples and stained with Giemsa for the determination of the infection rates with TBDs.

4.3.4 Faecal sample examination

Faecal samples were collected *per rectum* from animals initially at week -2, 0, 2, and 4 using plastic gloves for the determination of faecal egg counts (FEC). Later, the samples were collected every 4 weeks until 36 weeks. The samples were put into clearly labelled plastic bags and stored in a cool box until delivery to the laboratory. Faecal egg counts were determined using the McMaster method (Ministry of Agriculture, Fisheries and Food, U.K, 1986) as described in section 4.2.5.

4.3.5 Examination for trematode eggs

In order to examine for trematode eggs, 10g of fresh faeces were mixed with water by means of a spatula in a 100 ml beaker. The suspension was strained through a fine sieve. The filtrate was left standing for one hour and the supernatant decanted. The sedimentation and decantation steps

were repeated twice. The sediment was stirred homogenously with a glass bar. A few drops (Thienpont *et al*., 1990) were applied on to a microscope slide by means of the glass bar, and a drop of 1% methylene blue solution in water added. The dye and the faecal suspension were mixed and spread over the object glass. The methylene blue stains the suspension whereas, the eggs remain yellowish brown and therefore are easily recognised. Two or three preparations were examined completely and systematically by means of a microscope at x10 objective and x10 eyepiece. The large size of the operculated eggs (approximately 90x150 µm), the yellow colouration and the fine granulation (cluster of vitelline cells) observed helped in partial identification of the trematode eggs. In addition, information on the condemnation of cattle livers at slaughter due to *Fasciola,* gathered from the former District Veterinary Officer, Dr Orot (personal communication), helped to corroborate our findings. Faecal samples with trematode eggs were labeled positive and hence the animals designated as infected. Data from the study animals were therefore dichotomised as being either positive or negative.

4.3.6 Weighing of calves

Calves were properly restrained and weighed on week -2, 0, 2 and 4 and then later, once every 4 weeks until 36 weeks. During weighing, a calf was placed on a mat made out of a sack with two wooden handles firmly fastened on the two edges of the mat. A calf was then suspended on a weighing scale (0-200kg) with the use of two pieces of ropes tied onto the wooden handles. Once there was minimal movement of the animal was established, the weight was read. The weight of the weighing mat was also registered. The weight of the calf was therefore recorded in kilograms as the net of the total weight minus the weight of the weighing mat.

4.3.7 Production parameters

Daily milk production from each lactating cow was measured by each farmer involved in the study, using a standard 1-liter jar. Pregnancy diagnosis was performed monthly, through rectal palpation of all heifers and cows. Farmer-records of cows that had been served either by a bull (if available) or artificial insemination were considered. During pregnancy diagnosis, palpation of the ovaries of non-pregnant cows/heifers was carried out to determine whether an animal was

cycling or not. Reports of any births, stillbirths and abortions were also recorded from the farmers.

4.3.8 Entomological survey

To determine the apparent population dynamics of tsetse flies, 20 geo-referenced bi-conical traps were deployed in monitoring transects every 4 weeks for 36 weeks, starting from April 2003. All the other procedures involving fly collection, aging, and dissection for determination of trypanosome infection rates were carried out as described in the cross-sectional survey.

4.3.8.1 Collection and identification of bloodmeal samples

Tsetse blood meals were collected as described in the FAO training manual for tsetse control personnel (FAO, 1982). Using a pair of fine forceps, the gut and its contents were removed from the fly and spread out thinly and uniformly onto a section of filter paper (Whatman No. 1). Samples from eight individual flies were smeared onto each filter paper. To avoid contamination, the forceps were cleaned with 70% ethanol between subsequent samples. The filter papers were labelled, and an accompanying record of the sample with information on fly species, sex, date of preparation and location of trapping maintained. After air-drying, the filter papers were deep-frozen awaiting analysis. The samples were analysed by Wamwiri (2005) during her Master of Science (MSc) work entitled “ELISA for Identification of host-blood source in tsetse flies”.

4.4 Data management and analysis

4.4.1 Cross-sectional study

Data collected from farmers through questionnaires during the cross-sectional survey were coded and stored in the Microsoft Excel software. Analyses of household data, variables on animal husbandry and management practices, perceptions of the importance of different diseases and disease management were undertaken using descriptive statistical procedures.

Mean prevalences and the corresponding 95% confidence intervals (95% CI) for trypanosome-, *Anaplasma*-, *Babesia*- and *Theileria*-infections were calculated using the method described by Collett (2002). Chi square statistics were used to relate the disease prevalences (independent variable) with predictor variables at different levels i.e. sex, age, husbandry system and location. Comparisons of percentage mean packed cell volume (PCV %) in parasitaemic and aparasitaemic animals were also undertaken using 95% CI. The apparent tsetse densities were calculated as the number of flies per trap per day after biconical traps were left in place for a period of 24 hours. Estimated means for the various levels of main or interactive effects were deemed significantly different when the indicated probability for the test of equality was less than 5% ($p < 0.05$). The computer softwares used were Microsoft Excel 98 and Epi Info 2000 (version 1.0).

4.4.2 Longitudinal (prospective) study

Initially, all the animals were treated with diminazene aceturate on week -2 to clear any current trypanosome infection before the start of the study. Two weeks later (week 0), the animals were randomly assigned into 4 different treatment groups and sampled as per the protocol, signalling the starting point for the calculation of animal-time at risk. Animal-time at risk was recorded in weeks. During the 36-weeks follow-up period, an animal experiencing more than one case of trypanosomosis was designated as having had "multiple failures". The incidence density was calculated as described under section 4.3.3.

The risk of trypanosomosis in cattle was expressed as crude mean hazard rates (CHR), and the numbers of trypanosome infections per animal were expressed in terms of 52 animal-weeks-at risk. Hazard ratios were computed to compare the risk of new trypanosome infections between the control and the treated animals. This was determined as the reciprocal of the ratio of mean CHR in treated compared to control animals.

In order to estimate associations of different risk factors with recurrence of trypanosomosis, six variables were considered: location (2-levels), treatment group (4-levels), animal identification (1-level), age (3-levels), breed (2-levels) and time (weeks). Clustering was important at the

animal identification-level because of repeated measurements on the same individual. Multiple failure-time data (same endpoints and ordered as described by Dohoo *et al*., 2003) was then modelled using survival analysis. The variables included in the model were location, treatment group, animal id, age, breed and time of observation. The data were entered and set for analysis in Stata/SE 8.0 (Stata Corporation, College Station, TX, USA, 2004) as described by Cleves (1999). The marginal risk set model referred to as the Wei, Lin and Weissfeld-Model (Wei *et al.,* 1989), was used for analysis. In this approach, the dependencies between the failure times are not included in the model. Instead, the covariance matrix of the estimators is adjusted to account for the additional correlation due to repeated measures on the same individual over time. Such a model is described as a "variance corrected" model. In addition, the "robust" command for the robust variance estimation was used. Application of this "robust approach" over-estimates the standard errors of the coefficient (resulting in wider confidence intervals) than would be expected in usual variance estimates (Dohoo *et al,* 2003). This was deemed important for the clustered data obtained in this study since it relaxes the level of assumption of independence to the required "independence of observations" only among clusters and not within clusters. Unlike in the incidence density calculation where hazard rates and ratios were determined, in the multivariate analysis, the exponentiated coefficients were estimated. However, this option affects only how results are displayed and not how they were estimated (Stata Reference Manual release 7, 1985-2001).

The number of flies caught per trap per day (apparent tsetse density) was recorded. The arithmetic mean catches-per-trap per day once a month over a period of 9 months using 20 geo-referenced traps was used to determine the monthly apparent tsetse density. The monthly tsetse dynamics were presented as mean apparent tsetse density plots together with corresponding monthly rainfall data. The proportions of tsetse bloodmeals taken from ruminants were also calculated. In addition, the trypanosome infection rates in tsetse were calculated as:-

$$\text{Tsetse infection rate} = \frac{\text{Total number of tsetse flies with mature trypanosome infection}}{\text{Total number of dissected flies}}$$

Tsetse challenge was estimated using the method described by Leak *et al.* (1993) as: the product of apparent tsetse density (flies/trap/day) and the mature trypanosome infection rate in dissected

flies. Challenge was adjusted to include the proportion of bloodmeals taken from cattle and then summed-up for the number of fly-species in the area. In this study, challenge was estimated for domestic ruminants (cattle, sheep and goats) since the bloodmeal identification was carried out to the ruminant-level. The challenge then was expressed as an index.

Descriptive statistics were used to summarize the FEC data using the mean, median, range and standard deviation. A distribution was then fitted on the data. Due to the heavy skewing of the data to the right, mean FEC were transformed using ln(n+45). However, this transformation could not normalise the data and therefore, the data was modelled using the negative binomial distribution. This type of modelling was used to estimate the strengths of associations between the predictor variables (location, trial group, husbandry system, animal age, breed and time) and FEC. "Robust standard errors" were estimated for use in correcting auto-correlation present in the data.

Temporal variations in shedding of strongyle and ascarid eggs by calves were plotted for each of trial group. Further, faecal egg count reduction (FECR) was determined using the method described by Coles *et al.* (1992) as: FECR% = 100 x (1-T/C), where T and C are the arithmetic means of the number of eggs per gram of faeces in the treated and controlled trial groups, respectively. This analysis was performed on the data collected 2 weeks after the initial anthelmintic treatment.

Monthly weight changes in calves were recorded and plotted. Due to animal-level clustering as a result of repeated measures on the same individual, absolute group mean weights were plotted with 95% CI every month. The resultant monthly comparison at every sampling-time data point and the corresponding 95% CIs helped to correct for auto-correlation.