TICK CONTROL PRACTICES AMONG SMALL SCALE FARMERS IN ISOKA DISTRICT, ZAMBIA.

By

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A dissertation submitted to the University of Zambia in partial fulfillment of the requirements for the degree of Master of Science in Entomology

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DECLARATION

I, **Jackson Muyobela**, hereby declare that this dissertation represents my own work and that it has not previously been submitted for a degree at this or any other university.

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CERTIFICATE OF APPROVAL

This dissertation by **JACKSON MUYOBELA** is approved as fulfilling part of the requirements for the award of the degree of Master of Science in Entomology (Biological Sciences) University of Zambia.

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ABSTRACT

The traditional cattle sector in Isoka District has been experiencing an increase in the number of tick borne disease fatalities, despite the use of acaricides. Therefore, the objectives of the study were: to document the major acaricides in use, dosage and their method of application; to determine whether tick species in Isoka district are resistant to amitraz and cypermethrin; and to determine and compare the acaricidal properties of *Bobgunnia madagascariensis* Desv. and *Tephrosia vogelii* L. against ticks.

The prevailing chemical tick control practices in the study area were documented by administering a semi-structured questionnaire to 80 randomly selected resource poor small scale livestock farmers from four agricultural camps (Longwe, Kantenshya, Kapililonga and Ndeke) in Isoka District. Larval packet test bioassay experiments were used to determine the resistance status of *Rhipicephalus (Boophilus) microplus* (Canestrini), *Rhipicephalus appendiculatus* Neuman and *Amblyomma variegatum* (Fabricius) ticks against amitraz and cypermethrin acaricides. Free contact and topical application bioassay experiments were used to demonstrate and compare the acaricidal activity of the plant extracts of *T. vogelii* and *B. madagascariensis* against *A. variegatum* ticks.

Only 50% of respondents practiced chemical tick control with amitraz (27%) and cypermethrin (23%) being the acaricides in use, applied with knapsack sprayers. Farmers were able to follow the recommended dosage of 20 ml of acaricide to 10 litres of water for amitraz and 10 ml of acaricide to 10 litres of water for cypermethrin. On average, less than 2 litres of spray wash per animal was used which was considerably lower than the recommended delivery rate of 10 litres of spray wash per animal.

No significant susceptibility change to amitraz at 95% confidence level was observed in *R. appendiculatus* and *A. variegatum* against amitraz. However, a significant change in the susceptibility of *R.* (*Bo.*) *microplus* tested with amitraz was detected at 95% confidence. The test population had a lower susceptibility (LD_{50} 0.012 ml/l; LD_{90} 0.018 ml/l) than the reference population (LD_{50} 0.010 ml/l; LD_{90} 0.012 ml/l). The results indicated that resistance to amitraz was developing in *R.* (*Bo.*) *microplus*. For cypermethrin, no significant susceptibility change at 95% confidence was observed in any of the three species and thus resistance to this chemical was not observed.

Methanol extracts of the cortex (stem and branch) and leaf material of *T. vogelii* and methanol fruit extracts of *B. madagascariensis* produced 100% mortality of *A. variegatum* ticks in 24hr. The acaricidal activity of methanol leaf extracts of *T. vogelii* persisted for up to 8 days while that of fruit extracts of *B. madagascariensis* persisted for only 6 days. The toxicity of *T. vogelii* and *B. madagascariensis* extracts were found to be significantly different at 95% confidence level, with *B. madagascariensis* extracts (LD_{50} 0.031w/v) being more toxic than *T. vogelii* extracts (LD_{50} 0.585w/v).

It can be concluded that *R.* (*Bo.*) *microplus* is developing resistance to amitraz in Isoka District. Amitraz must therefore be used with caution, and with strict adherence to the recommended delivery rate. The study has also shown that plant extracts of *B. madagascariensis* and *T. vogelii* extracts have excellent acaricidal activity against ticks and can be considered as alternatives for tick control. Further research is however required in order to establish the required rate of application of *B. madagascariensis* and *T. vogelii* plant extracts, using water as an extraction solvent, for tick control.

DEDICATION

To my father, Ben Mwamba Muyobela who has understood the importance and value of education.

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This study would not have been possible without the support of many people. I would like to express my sincere thanks to my principal supervisor, Prof. P.O.Y. Nkunika for his guidance, direction and patience throughout the study. I am also grateful to my co-supervisor, Prof. E. T. Mwase for the many hours spent discussing various aspects of the study and for her support and guidance.

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ACRONYMS AND ABBREVIATIONS

AIT	..... Adult Immersion Test
BHC	..... Benezenehexachloride
CI	..... Confidence Interval
DDT	..... Dichloro Diphenyl Trichloroethane
DNA	..... Deoxyribonucleic Acid
DT	..... Drummond Test
ECF	..... East Coast Fever
FAO	..... Food and Agricultural Organization
LD	..... Lethal Dose
LDR	..... Lethal Dose Ratio
LIT	..... Larval Immersion Test
LPT	..... Larval Packet Test
TTBD	..... Tick and Tick Borne Diseases
TBD	..... Tick Borne Diseases

LIST OF SYMBOLS

g	..... Grams
ml	..... Milliliter
°C	..... Degrees Celsius
%	..... Percentage
P	..... Probability
w/v	..... Weight/Volume
No.	..... Number
ml/l	..... Millilitre/litre
H_O	..... Hypothesis
hr	..... Hours
µl	..... Micro litre

CHAPTER 1: INTRODUCTION

Ticks (Acari: Ixodidae) are by far the most important external parasites of economic importance that attack livestock. They have been implicated in heavy blood loss and low-quality hides (Jongejan and Uilenberg, 2004), secondary bacterial infections (Ambrose *et al.*, 1999), lowered productivity in terms of weight gain (Pegram and Oosterwijk, 1990) and milk yield, and increased mortality due to tick borne diseases (TBD) (Niyonzema and Kiltz, 1986). The most important tick species in the traditional livestock sector in Zambia are *Rhipicephalus appendiculatus* Neuman and *Rhipicephalus zambeziensis* Walker, Norval and Corwin, the vectors of *Theileria parva* (Theiler, 1904) which is the causative agent of Theileriosis (D'Haese *et al*., 1999). Other epidemiologically important tick species include *Amblyomma variegatum* (Fabricius), *Rhipicephalus (Boophilus) microplus* (Canestrini) and *Rhipicephalus (Boophilus) decoloratus* (Koch). In Isoka District, *Rhipicephalus appendiculatus*, *Amblyomma variegatum* and *Rhipicephalus (Boophilus) microplus* are known to occur on cattle (Luguru *et al.*, 1987).

The application of acaricides in dips and sprays to control cattle ticks has had a profound influence on livestock productivity through the significant reduction in the prevalence of tick infestations and tick borne diseases (Yilima *et al.*, 2001). However, the progressive evolution of resistance of ticks to almost every available class of acaricide continues to frustrate the efforts of cattle farmers (George *et al.*, 2004). Dimethoate resistance in *R. (Bo.) decoloratus* and *R. appendiculatus*, and dioxathion resistance in *A. variegatum* have been reported by Luguru *et al.* (1987) in Zambia. High levels of tick resistance to cypermethrin and amitraz on communal and commercial

farms in the Eastern Cape and Northwest Provinces of South Africa have also been reported (Mekonnen, 2002).

Acaricide resistance is an inherited phenomenon that leads to the survival and reproduction of ticks exposed to an acaricide. Resistance to a chemical stems from a directional selection pressure caused by the toxic effects of the chemical and results in the selection of a strain of individuals that possess specific genetically determined biochemical mechanisms (such as detoxifying enzymes) that enable them to survive treatment (Roush and McKenzie, 1987). Consequently, resistance leads to the failure of control programmes undertaken at recommended procedures (Kunz and Kemp, 1994). The development of resistance has been exacerbated by the misuse of drugs (Bianchi *et al*., 2003) and the use of incorrect doses (Dolan, 1999).

An analysis of the Northern Province Annual Veterinary Reports (2008 and 2009) indicated an increase in the number of tick borne disease fatalities in cattle in Isoka District. Tick borne disease fatalities have risen from 25 in 2005 to 157 in 2009. This 16% increase in fatalities may be an indication of the failure of chemical control strategies being applied in the area. At national level, tick transmitted diseases account for the highest cattle mortalities compared to other diseases in Zambia. Overall cattle deaths due to tick-borne disease are estimated to be between 2021 and 5834 annually (Makala *et al*, 2003). The high prevalence of tick infestations in Isoka has led to decreased meat and milk production, high mortalities and loss of draught power, despite the availability of adequate grazing land and water.

The general objective of the study was therefore, to assess the presence and magnitude of acaricide resistance against amitraz and cypermethrin in the common tick species

found in Isoka district and to test the possibility of using ethno-botanical pesticides as alternative control agents. The specific objectives of the study were;

i. To document the major acaricides in use, dosage and their method of application.

ii. To determine whether *R. appendiculatus*, *A. variegatum* and *R. (Bo.) microplus* tick species in Isoka district are resistant to amitraz and cypermethrin.

iii. To determine and compare the acaricidal properties of *B. madagscariensis* and *T. vogelii*.

The hypotheses to be tested were;

i. *R. appendiculatus*, *A. variegatum* and *R. (Bo.) microplus* ticks in Isoka had developed acaricide resistance to amitraz and cypermethrin.

ii. Ticks species in Isoka District are susceptible to the plant extracts of *T. vogelii* and *B. madagascariensis*.

CHAPTER 2: LITERATURE REVIEW

2.1 Taxonomy

Ticks are invertebrate animals that belong to phylum Arthropoda, class Arachinida order Acari. The Acari consists mostly of mites and ticks, which can be distinguished by their larger size and their exclusive parasitic mode of feeding (Walker *et al*., 2007). There are two main families of ticks which include the Argasidae (Argasids) and the Ixodidae (Ixodids). The Argasidae consist of soft ticks that lack hard plates on their bodies while the Ixodidae posses' hard plates and are termed hard ticks (Sonenshine, 1991 and Rajput *et al*., 2006). All cattle ticks belong to the family Ixodidae.

Currently, there are 867 described species of ticks in the world (Horak *et al*., 2002 and Horak, 2009) of which 48 species are important to the health of domestic animals in Africa (Walker *et al*., 2007). The most important Ixodid tick genera include *Amblyomma, Boophilus, Dermacenter, Hyalomma, Haemaphysalis, Ixodes,* and *Rhipicephalus* (Jongejan and Uilenberg, 2004), all of which have been reported in Zambia (Tandon, 1991). Recent molecular and morphological studies by Murrell *et al*. (2000), Beati and Keirans (2001) and Barker and Murrell (2002) concluded that the genus *Rhipicephalus* is paraphyletic with respect to the genus *Boophilus*. Consequently, Horak *et al*. (2002) and Horak (2009) reclassified this genus as a subgenera to the genus *Rhipicephalus* in their world list of valid tick names. Thus, the important tick species that infest cattle in Zambia are *Rhipicephalus (Boophilus) decoloratus* (Koch), *Rhipicephalus (Boophilus) microplus* (Canestrini), *Rhipicephalus appendiculatus* Neuman, *Rhipicephalus zambeziensis* Walker, Norval and Corwin, *Amblyomma*

variegatum (Fabricius), *Hyalomma trancatum* Koch and *Hyalomma mariginatum rufipes* Koch (Speybroeck *et al*., 2002 and Berkvens *et al*., 1998).

2.2 Geographical distribution

The specific geographical distribution of tick species is difficult to establish as the type of habitat in which species are found is widely distributed than their current geographical range (Walker *et al*., 2003 and Horak *et al*., 2009). As such, the distribution of ticks has been largely determined by livestock movements and species diversity is generally higher along popular livestock trading routes (Walker *et al*., 2007 and Lynen *et al*., 2008). In Zambia, it is known that there is significant variability in the species composition and relative abundance of ticks across the country with all major genera being reported in most parts (Speybroeck *et al*., 2002 and Berkvens *et al*., 1998). However, Tandon (1991) has provided a detailed account of the geographic distribution of ixodid ticks in each district of Zambia. Tandon reported the presence of *A. variegatum*, *R. (Bo.) microplus*, *H. truncatum*, and *R. appendiculatus* in Isoka District. Luguru *et al*. (1987) identified *A. variegatum*, *R. (Bo.) microplus* and *R. appendiculatus* to be the most important tick species in Isoka District.

2.3 Biology and life cycle

Mating of adult ixodid ticks occurs on the host except in the genus *Ixodes* where mating can occur while ticks are still on vegetation (Klompen *et al*., 1996 and Walker *et al*., 2003). Male ticks remain on the host and will mate with as many females as possible resulting in the transfer of spermatheca (sperm sac) to females. Females mate only once, fully engorge and detach from their host to lay between 2000 and 20000 eggs, in a single batch (Walker *et al*., 2007). Eggs are never laid on the host.

Three distinct life cycles occur among ixodid ticks. The commonest lifecycle is the three-host life cycle (Sonenshine, 1991 and Jongejan and Uilenberg, 2004) in which larvae, nymphs and adults attach to separate hosts (Figure 2.1). Upon hatching, larvae will attach and feed on a host, detach from it and hide in the soil or vegetation to moult into a nymph. Nymphs attach to another host, feed and detach to moult into a female or male tick. Females will feed once and lay a huge batch of eggs, after which the depleted females dies. The male will take several small feeds, mate and then die. The three-host life cycle is slow lasting from six months to several years (Oliver, 1989 and Walker *et al*., 2007).

The one-host life cycle (Figure 2.2) is less common but occurs in all *Boophilus* sub-genera of *Rhipicephalus* genus (Horak *et al*., 2002). Larvae hatch from eggs and crawl onto vegetation to wait for a host. This behaviour of waiting in ambush on vegetation is called questing (Sonenshine, 1991). When feeding is complete, they remain attached to the host and moult to nymphs. Nymphs feed on the same host and remain attached to moult into adults. Adults feed on the same host but may change position for mating purposes. Thus, all three feeding stages of an individual tick occur on the same host. The life cycle of a one-host tick is usually rapid and may take three weeks to complete feeding and two months for egg laying and larval development to complete (Oliver, 1989 and Cumming, 1999). The two-host life cycle is similar to the one-host tick with larvae and nymphs feeding on the same host while adults attach to another host (Mathysse *et al*., 1987 and Jongejan and Uilenberg, 2004). This life cycle is observed in *Hyalomma* species and *Rhipicephalus evertsi evertsi.* The life cycles of the important tick species in Isoka are detailed below.

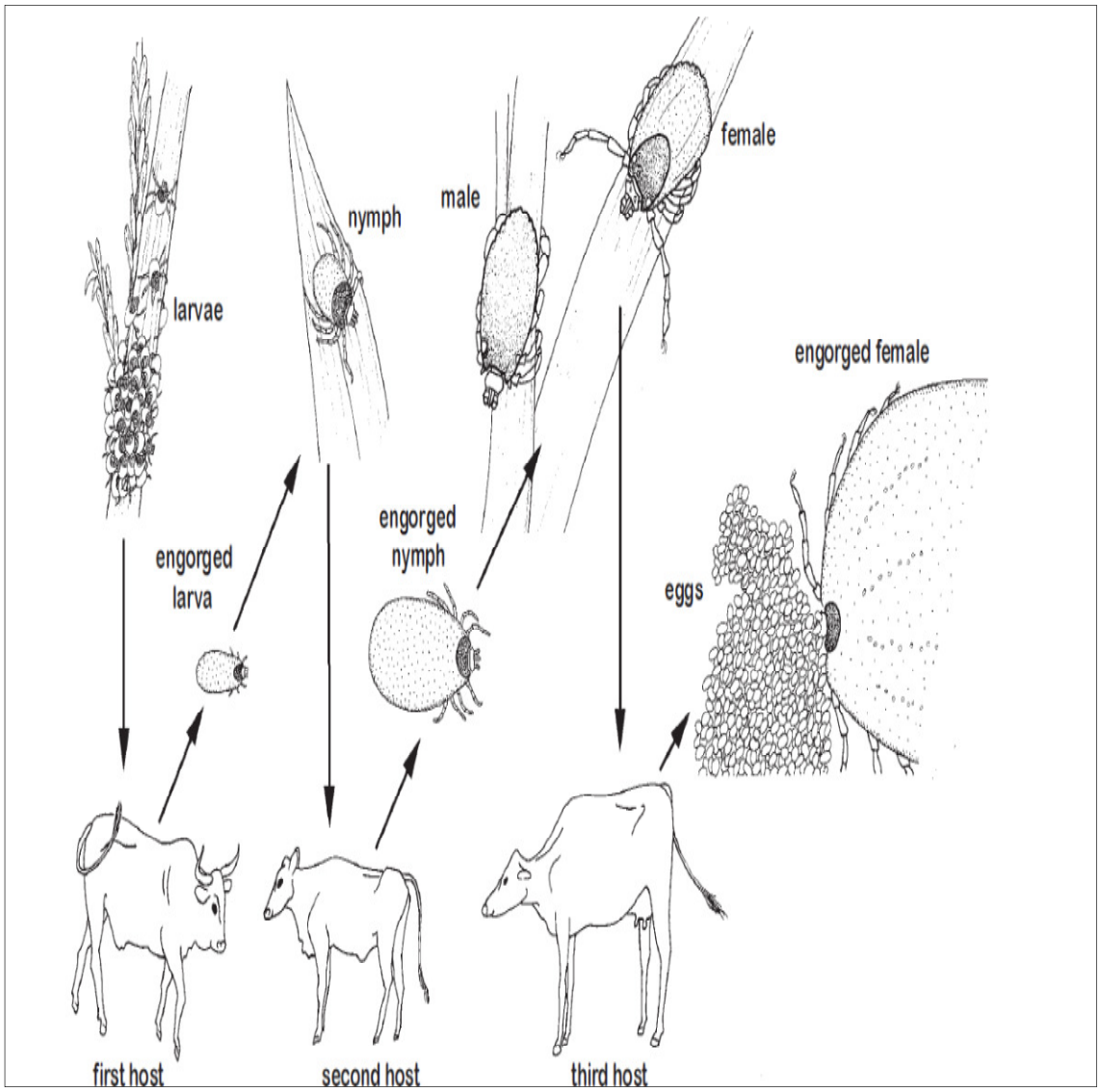

Figure 2.1 Three host tick life cycle (the examples being *Rhipicephalus appendiculatus* and *Amblyomma variegatum*). Source: Walker *et al*., 2007.

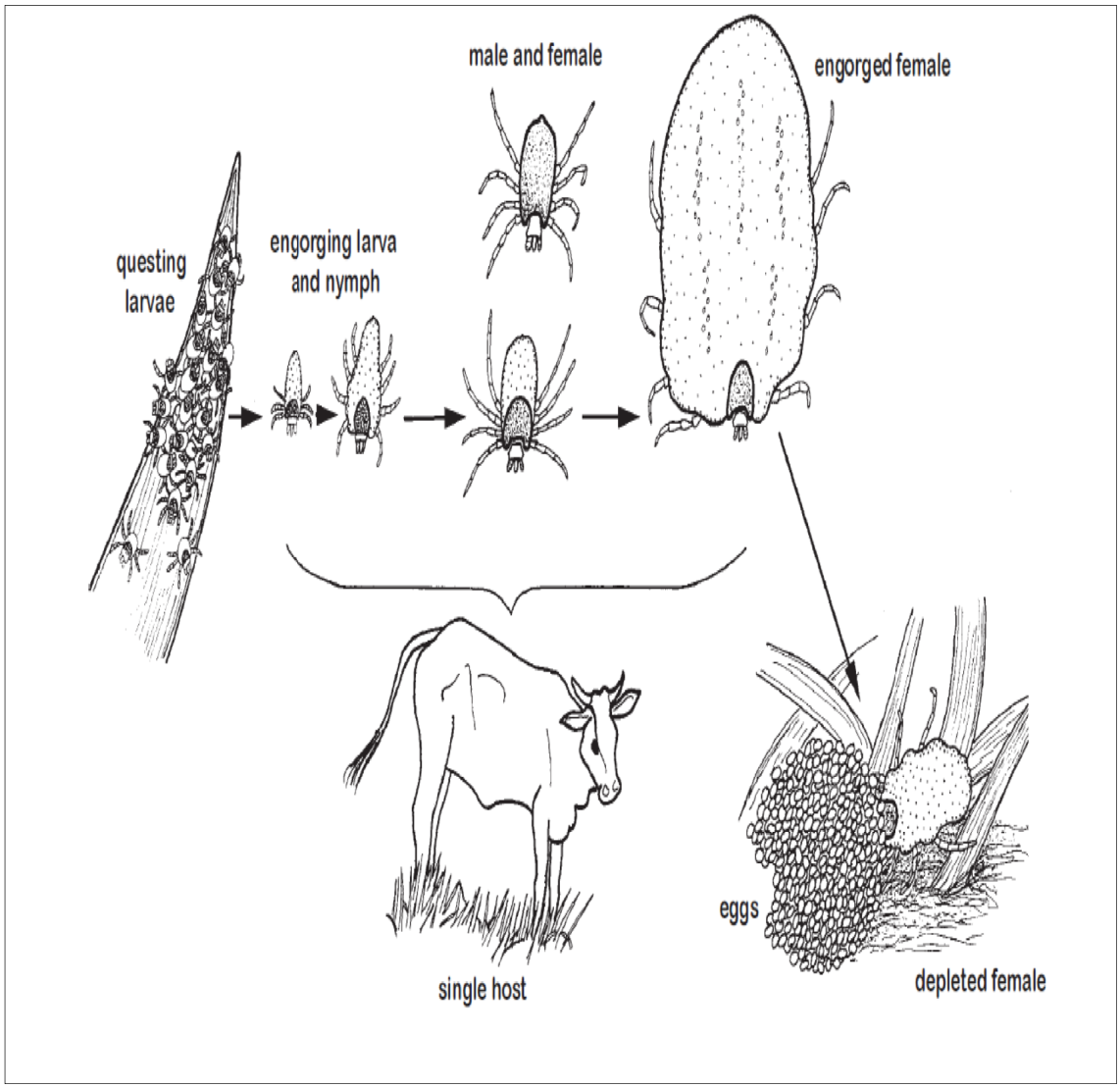

Figure 2.2 One host tick life cycle [the example is *Rhipicephalus (Boophilus) microplus*]. Source: Walker *et al*., 2007.

2.3.1 Biology of important tick species in Zambia

2.3.1.1 *Rhipicephalus (Boophilus) decoloratus*

This is the commonest, most widespread and frequently encountered one-host tick species in Africa (Walker *et al*., 2007). It is indigenous to Africa and has a monotrophic type of behaviour with cattle being the only maintenance host (Sonenshine, 1991 and Benitez *et al*., 2012). However, it may also be found on horses, donkeys, sheep, goats and wild ungulates. Engorged females lay 1000 to 2500 eggs which hatch about one week after detachment from the host (Sonenshine, 1991). Eggs hatch into larvae in 3 to 6 weeks after which they climb vegetation and attach to a host. Larval, nymph and adult stages spend a total of about 3 weeks on the same host. The entire life cycle, including the non-parasitic phase, can be completed in approximately two months, with more than one life cycle being completed annually (Walker *et al*., 2000 and Speybroeck *et al*., 2002). *R. (Bo.) decoloratus* has no strict seasonal occurrence in southern Africa, with adults occurring on host throughout the year (Walker *et al*., 2007). However, the highest numbers of adults occur on hosts from January to March and May to August.

2.3.1.2 *Rhipicephalus (Boophilus) microplus*

This is a one-host tick with a monotropic type of behaviour (Sonenshine, 1991 and Benitez *et al*., 2012). It infests cattle for a period of three weeks and egg laying can be completed in four weeks. *R. (Bo.) microplus* was introduced to the African continent from Madagascar after the rinderpest epidemic (Speybroeck *et al*., 2002 and Berkvens *et al*., 1998). It is postulated that where favourable moist and warm climatic conditions exist, *R. (Bo.) microplus* competes with and is able to displace the indigenous *R. (Bo.) decoloratus* due to its higher reproductive potential. Therefore, in Zambia, *R. (Bo.)*

decoloratus has been displaced in the warmer moist North Eastern regions by *R. (Bo.) microplus* (Berkvens *et al*., 1998). Cattle are probably the only effective host for this tick (De Vos *et al*., 2004 and Walker *et al*., 2007).

2.3.1.3 Rhipicephalus appendiculatus

R. appendiculatus is a three-host tick in which all developmental stages engorge within four to seven days (Mathysse *et al*., 1987 and Mtambo, 2008). Engorged females lay 3000 to 5000 eggs while hatching occurs in 20 to 90 days. Under favourable conditions, the life cycle can be completed in three months. However, this tick has a strict seasonal occurrence in southern Africa. Adults occur during the rainy season (December to March), larvae in the cooler winter months of April to August and nymph in the drier months of July to October (Okello-Onen *et al*., 1999a and Walker *et al*., 2007). This seasonal pattern of occurrence is regulated by unfed adults who enter diapause and do not engage into host-seeking until the onset of the rains (Sonenshine, 1991 and Madder *et al*., 1999). The main hosts are cattle, goats, buffaloes, eland, water buck, nyalas, greater kudus and sable antelopes (Walker *et al*., 2000 and Speybroeck *et al*., 2002). Dogs and sheep are also infested.

2.3.1.4 Amblyomma variegatum

This tick is a three-host tick that also has a clearly defined seasonal pattern of occurrence (Stachurski, 2006 and Walker *et al*., 2007). In Zambia, adults are most abundant in the wet season (October to February), larvae from March to May and Nymphs from May to September. Delayed development of females (Morphogenetic diapause) resulting in delayed oviposition is responsible for this pattern of seasonal abundance (Sonenshine, 1991 and Walker *et al*., 2007). All feeding stages of this tick

infest cattle, sheep and goats (Jongejan and Uilenberg, 2004 and Nyangiwe and Horak, 2007). Buffaloes and other large herbivores are also hosts.

2.4 Feeding behaviour and damage

All feeding stages of the tick life cycle are parasitic, feeding only on the blood of their hosts (Okello-onen *et al*., 1999b and Mtambo, 2008). Ticks attach to the skin of their hosts with their mouth parts which consist of the chelicerae, hypostome and palps. The chelicerae and hypostome form a feeding tube that penetrates the hosts' skin. Cement like material is often secreted in the saliva which glues the palps to the outer epidermis and the chelicerae sheath and toothed hypostome to the dermis (Klompen *et al*., 1996 and Camicas *et al*., 1998). Chelicerae possess moveable rods with sharp claws at the ends which cut a hole in the dermis, breaking capillary blood vessels close to the surface of the skin. This forms a feeding lesion into which blood and lymph are released and on which the tick feeds. The initial stages of ixodid tick feeding are slow as the body needs to grow before it can expand to take a very large meal. Larvae typically take three to five days, nymphs four to eight days and females 5 to 20 days to fully engorge with blood (Sonenshine, 1991 and Walker *et al*., 2000). Fully engorged ticks detach from the skin of the host and drop to the ground. Males do not expand like females and only feed enough for their reproductive organs to mature (Walker *et al*., 2003).

Host location in ixodid ticks varies (Walker *et al*., 2007). Some ticks live in open environments and crawl onto vegetation to quest for their host to pass by. The genera *Rhipicephalus*, *Haemaphyalis* and *Ixodes* exhibit this type of questing behaviour. These ticks grab onto their hosts with their front legs and then crawl over the skin to find a suitable place to attach and feed. Adults of *Amblyomma* and *Hyalomma* are active

hunters and run across the ground in search of nearby hosts (Sonenshine, 1991). The general behaviour of seeking hosts in an open environment is described as exophilic (Walker *et al*., 2007).

Tick feeding causes direct and indirect damage to their hosts. The long mouthparts of *A. variegatum* causes sores (Jongejan and Uilenberg, 2004, Peter *et al*., 2005 and Rajput *et al*., 2006) that may become infected with bacteria leading to abscessation (Ambrose *et al*., 1999 and Muchenje *et al*., 2008). Heavy infestations of *R. (Bo.) microplus* results in commercially important damage to hides by formation of scar tissue (granuloma) at the feeding site (Jongejan and Uilenberg, 2004 and Walker *et al*., 2007). Other direct damage as a result of tick feeding is reduction in live weight and live weight gain, and anemia in domestic animals (Pegram and Oosterwijk, 1990 and Estrada-Peńa and Salman 2013). Experiments have shown that there is a respective 0.6 g and 4.0 g loss of potential growth per every *R. (Bo.) microplus* and *R. appendiculatus* female that completes feeding (Camicas *et al*., 1998 and Jonsson, 2006). Ten engorging female *A. variegatum* ticks have been shown to decrease live weight by 20 Kg over a period of three months (Cumming, 1999 and Pegram *et al*., 2000).

The main indirect damage to hosts resulting from tick feeding is the transmission of diseases. *A. variegatum* transmits the Rickettsia *Ehrlichia ruminantum* formally *Cowdria ruminantum* which causes heart water in cattle, sheep and goats, and *Ehrlichia bovis* which causes bovine ehrlichiosis (Bekker *et al*., 2005 and Esemu *et al*., 2013). *R. (Bo.) microplus* transmits the protozoans *Babesia bovis* and *Babesia bigemina* causing bovine babesiosis (Red water) in cattle (Kaaya *et al*., 2000, Ravindran *et al*., 2006 and Rious-Tobion *et al*., 2014). This tick also transmits the Rickettsia *Anaplasma marginale*

which causes anaplasmosis or gall sickness in cattle (Coronado, 2001 and Rikhotso *et al*., 2005). *R. appendiculatus* transmits the protozoan *Theileria parva*, the causative agent of East Coast fever (ECF) in cattle (D'Haese *et al*, 1999, Makala *et al*., 2003 and Salih *et al*., 2007). Different strains of *T. parva* are responsible for Corridor or January disease (Billiouw *et al*., 2002 and Makala *et al*., 2003). Under heavy infestation, these ticks suppress the immunity of their hosts resulting in reemergence of tick borne diseases (Willadsen, 2006 and Walker *et al*., 2007).

T. parva accounts for the highest cattle mortalities compared to any other disease in Zambia (Makala *et al*., 2003). ECF epidemics strike with high case fatalities resulting in major cattle losses and significant social and economic distress to individual farmers (Billiouw *et al*., 1999 and Muleya *et al*., 2012). Total loss of an entire herd is common especially when no measures are taken to mitigate the deadly impact of an outbreak (Nambota *et al*., 1994, Bishop *et al*., 2004, Thompson *et al*., 2008). In Zambia, Cowdrosis, Basesiosis and Anaplasmosis cause low mortality in the traditional sector resulting in an endemically stable situation but become extremely important in the commercial sector whenever tick control is not optimal (Moorhouse and Snacken, 1984, Berkvens, 1991, D'Haese *et al*., 1999 and Makala *et al*., 2003).

2.5 Tick Control

2.5.1 Chemical control

Since their introduction into Southern Africa around 1890, acaricides have been applied to cattle using hand sprayers, spray races and in dipping vats (George *et al*., 2004) in order to control ticks and tick borne diseases (TTBD). Recent treatments have included pour-on products (Davey *et al*., 1998; Davey and George, 2002), injectibles (Remington

et al., 1997; Caproni *et al*., 1998), intra-ruminal boluses (Soll *et al*., 1990), and the use of acaricide impregnated ear tags and pheromone/acaricide impregnated devices attached to the host (Young *et al*, 1985).

The chemical classes of acaricides that have been used to control ticks on cattle include the arsenics, chlorinated hydrocarbons, organophosphates, carbamates, pyrethroids, formamidines and the macrocyclic lactones (George *et al*., 2004 and Abbas *et al*., 2014). Arsenic solutions were the first to be used for tick control (Angus, 1996) and were a key component in the successful eradication of *R. (Bo.) microplus* in the United States (George *et al*., 2004). The evolution of resistance to arsenic, its narrow limits between effective concentration for tick control and the toxic concentration to cattle, led to its replacement by synthetic organic compounds (George *et al*., 2004).

The organochlorine insecticides, DDT and benezenehexachloride (BHC) were the first synthetic organic insecticides marketed for tick control on cattle (Rajput *et al*., 2006 and Abbas *et al*., 2014). Dieldrin, aldrin and toxaphene have also been widely used for tick control. Resistance however, abbreviated the useful life of these chemicals and they are now unavailable or have been withdrawn from the market (Kunz and Kemp, 1994, Hope *et al*., 2010 and Corley *et al*., 2012).

Non-persistent organophosphates replaced the persistent organochlorines (Ware, 2000). Ethion, chlorpyrifos, chlorfenvinphos and coumaphos are four of the most widely used organophosphates for treatment of tick infested cattle (George *et al*., 2004 and Abbas *et al*., 2014). Carbamate acaricides (carbary and promacyl), like the organophosphates, function by inhibiting target cholinesterase, resulting in the overstimulation of the nervous system (Faza *et al*., 2013 and Temeyer *et al*., 2013). Since the early fifties,

cross-resistance of ticks to more than 30 organophosphates and carbamates, has eliminated or minimized their usefulness in over 40 countries (Kunz and Kemp, 1994, Van Leeuwen *et al*., 2010 and Arivalagan *et al*., 2013).

Among the foramidines, only amitraz has proven to be useful in controlling cattle ticks (Ware, 2000 and Jonsson and Hope, 2007). Successful test of amitraz to control *R. (Bo.) microplus* in Australia and subsequent commercial trails in Australia, the United States and South Africa provided evidence of the effectiveness of amitraz against, *R. (Bo.) decoloratus*, *R. appendiculatus*, *R. evertsi evertsi* and *A. hebraem* (George *et al*., 2004). Reports of amitraz resistance in literature are on the increase with confirmed cases of resistance being reported in Brazil (Furlong, 1999, Miller *et al*., 2002 and Mendes *et al*., 2013), Colombia (Benavides *et al*., 2000), Mexico (Soberanes *et al*., 2002) and South Africa (Mekonnen, 2002; Ntondini *et al*., 2008). The continued development of amitraz resistance is a major concern as this compound was developed to counteract organophosphate and pyrethroid resistance in ticks (Li *et al*., 2004 and Jonsson and Hope, 2007).

The main pyrethroid acaricides currently in use are the cyano-substituted pyrethroids such as cypermethrin, deltamethrin, cyhalothrin and flumethrin (Aguirre *et al*., 2000, George *et al*., 2004 and Rajput *et al*., 2006). Pyrethroid resistance has been reported by Caracostantogolo *et al*. (1996), who observed reduced efficacy of cypermethrin against *R. (Bo.) microplus* in the eastern part of Argentina. High levels of cypermethrin resistance in *R. (Bo.) decoloratus*, *R. appendiculatus* and *A. hebraem* have been reported from commercial and communal farms in South Africa (Mekonnen, 2002).

Recently, pyrethroid resistance in cattle ticks has been reported in Brazil (Mendes *et al*., 2007) and Iran (Enayati *et al*., 2010).

The macrocyclic lactones ivermectin, doramectin and moxidectin have been shown to have excellent acaricidal activity against *R. (Bo.) microplus* (Aguilar-Tipacamu and Rodriguez-Vivas, 2003 and Lopes *et al*., 2013). These chemicals act systemically and are generally administered as subcutaneous injections (Gonzales *et al*., 1993, Remington *et al*., 1997 and Caproni *et al*., 1998). Although macrocyclic lactones are efficacious, their high cost limits their use in tick control (Aguilar-Tipacamu and Rodriguez-Vivas, 2003 and George *et al*., 2004).

2.5.1.1 Acaricide resistance in ticks

Resistance can be defined as a genetic change in a population in response to selection pressure by a toxicant that impairs control in the field (Sawicki, 1987, Meyer *et al*., 2012 and Corley *et al*., 2013). It involves a significant genetically based shift in the molecular, biochemical or behavioural response against a stimulus that is applied to a population of a given species (Robertson *et al*., 2007). It is a heritable trait and the genes that confer resistance are likely to be present in the population at low frequencies before the introduction of a new chemical (Roush and Mckenzie, 1987 and Alonso-Diaz *et al*., 2013). The factors that influence the establishment and development of resistance in ticks include the frequency of the original gene mutation, mode of inheritance of the resistant allele, frequency of acaricide treatment, application of acaricides when free living refugia population is small, use of low doses and the use of poor quality acaricides (Bianchi *et al*., 2003, Shyma *et al*., 2012 and Bardosh *et al*., 2013). The mechanisms of acaricide resistance involves the action of detoxification enzymes or

mutations at the target site of the chemical (Foil *et al*., 2004 and Guerrero *et al*., 2012). Organophosphate resistance is largely determined by the action of cytochrome P450 monooxgenase (Li *et al*, 2003; 2004) and an insensitive acetylcholine esterase (Pruett, 2002, Van Leeuwen *et al*., 2009 and Lwande *et al*., 2012). Pyrethroid resistance is the result of elevated levels of CzEst9 esterase (Jamroz *et al*., 2000) and a mutation of sodium channels (Weston *et al*., 2013 and Frank *et al*., 2013). Mutations on the sodium channels confers cross resistance of pyrethroids with DDT (He *et al*., 1999) as the pharmacological effect of the two chemicals is similar; they cause persistent activation of sodium channels by delaying the normal voltage-dependent mechanism of inactivation (Hemingway and Ranson, 2000). The mechanism for Foramidine resistance is currently the least understood (Foil *et al*., 2004, Perez-Cogollo *et al*., 2010 and Lovis *et al*., 2013).

2.5.1.2 Diagnosis of acaricide resistance

Diagnosis of acaricide resistance is done by the use of various bioassay techniques. The Larval Packet Test (LPT) bioassay has been recommended by FAO as a standard tick bioassay method for organophosphate, carbamate and pyrethroid acaricides (Kemp *et al*., 1998, George *et al*., 2004 and Abbas *et al*., 2014). Analysis of LPT bioassay is done using probit regression, a model that assumes the normal distribution of errors, linearity, exogeneity and no multicollinearity among data (Robertson *et al*., 2007). For amitraz, a modified LPT that incubates filter papers for 48 hrs instead of 24 hrs has been successfully used to determine dose mortality relationships of ticks (Miller *et al*., 2002). Other bioassays techniques used for diagnosis of acaricide resistance include the Larval Immersion Test (LIT), Adult Immersion Test (AIT) and Drummond Test (DT) (Kemp

et al., 1998, George *et al*., 2004 and Abbas *et al*., 2014). The time lapse between identification of a resistance problem and the availability of results from bioassays is a major problem with most existing bioassays. With a one-host tick such as *R.* (*Bo.*) *microplus*, a minimum of 35 days is required from collection of engorged females to the availability of sufficient larvae of the appropriate age (7-14 days) (George *et al*., 2004). If multi-host tick species are involved, it may take much longer to obtain sufficient number of ticks of uniform age to do an analysis. Due to these limitations, a number of researchers have investigated the potential usefulness of using molecular methods to detect acaricide resistance (Sangster *et al*., 2002). Advances in this line of research has resulted in the development of PCR assays that detect target site resistance to pyrethroids and organophosphates (Guerro and Pruett, 2003, Miller *et al*., 2007 and Carvalho *et al*., 2013). However, for PCR assays to be practically useful, assays need to conduct a full array of molecular tests in order to detect all forms of acaricide resistance that may manifest in the field (Sangster *et al*., 2002). Such comprehensive PCR assays are currently unavailable for use (George *et al*., 2004, Guerrero *et al*., 2012 and Abbas *et al*., 2014).

2.5.1.3 Acaricide resistance management

The acaricide resistance management tactics that have been proposed so far include the use of a high dose tactic, rotation and the use of a mixture of acaricides (Roush, 1993, George *et al*., 2004 and Abbas *et al*., 2014). Though these tactics offer a theoretical advantage in models and laboratory experiments, the tactics have yet to be evaluated in field experiments so as to ascertain their value in mitigating resistance (Thullner *et al*., 2007 and Adakal *et al*., 2013).

Currently, the pyrethroids cypermethrin, deltamethrin and formamidine acaricides represent the most effective acaricides used to control cattle ticks (Chevillon *et al*., 2007). Therefore, recent reports from different parts of the world that resistance to these acaricide is developing (Miller, 2002; Soberanes *et al*., 2002; Mekonnen, 2002; Ntondini *et al*., 2008) is of great concern as the development of new chemical classes of acaricides has proven to be a slow and difficult process (Habeeb, 2010). Consequently, there is need to identify the areas where these chemical are still effective so that strategies based on an integrated approach may be employed in order to prolong their usefulness (George *et al*., 2004 and Abbas *et al*., 2014).

2.5.2 Use of botanical pesticides: Ethno-veterinary medicine

A considerable amount of research to find alternative, more sustainable cost effective methods to control cattle ticks is currently underway (Opiro *et al*., 2010 and Abbas *et al*., 2014). One alternative that has recently attracted a lot of research is based on the use of botanical pesticides (Habeeb, 2010, Babar *et al*., 2012 and Zaman *et al*., 2012). The pesticidal properties of many plants have been known for a long time and natural pesticides based on plant extracts such as rotenone, nicotine and pyrethrum have been commonly used in pest control (Kamanula *et al*., 2011; Nyirenda *et al*., 2011). Botanical acaricides provide a cheaper and safer alternative method to chemical tick control (Mwale *et al*., 2005 and Habeeb, 2010).

The opportunity to use local, readily available acaricidal plants to control ticks would be desirable among smallholder poor resource farmers (Madzimure *et al*., 2013). As such, a considerable number of extracts from various plants native to the tropics and subtropics have been tested so as to demonstrate their acaricidal activity against various

tick species (Jonsson and Piper, 2007, Opiro *et al*., 2010 and Habeeb, 2010). *Tephrosia vogelii* aqueous leaf extracts have been shown to have significant acaricidal activity when used as a livestock tick spray in Zambia (Kaposhi, 1992). Madzimure *et al*. (2013) recently demonstrated the acaricidal properties of aqueous fruit extracts of *Strychnos spinosa* (Lam.) and *Solanum incanum* L. against cattle ticks. Other plant extracts that have been reported to exhibit significant acaricidal activity against cattle ticks are custard seed oil (Kalakumar *et al*., 2000), *Stylosanthes scabra* (Kundrathula and Jagannath, 2000), *Tamaridus indicus* (Chungsamamyart and Jansawan, 2001), *Eucalyptus* spp. (Chagas *et al*., 2002), *Copaifera reticulate* (Fernades and Freitas, 2007), *Senna italica* (Magano *et al*., 2008) and *Lippia javanica* (Madzimure *et al*., 2011).

One of the commonly cited advantages of botanical acaricides for tick control is their biodegrability which makes them less toxic to the environment and to non-target species (Belmain and Stevenson, 2001 and Liang *et al*., 2003). Furthermore, their availability to local farming communities and ease of production and processing makes them an asset to traditional farmers in the poor regions of the world (Belmain *et al*., 2012 and Stevenson *et al*., 2012). Therefore, the evaluation of plants with acaricidal activity presents an opportunity of developing cheaper and safer alternative tick control measures that are indigenous to cattle producing area (Wanzala *et al*., 2005 and Opiro *et al*., 2010).

2.5.3 Anti-tick vaccine

Another alternative method to tick control is the use of tick vaccines. Commercially available tick vaccines currently on the market include Gavac® (Heber Biotec; Havana, Cuba), TickGARD (Hoechst Animal Heath; Australia) and TickGARDPLUS. These vaccines are based on a recombinant form of the antigen Bm86 which is isolated from the mid gut of *R. (Bo.) microplus* ticks (Freeman *et al*., 2010 and Abbas *et al*., 2014). These vaccines are primarily effective against *R. (Bo.) microplus* and there is also strong evidence of cross protection with *R. (Bo.) decoloratus* (Pipano *et al*., 2003 and Popara *et al*., 2013), *Hyalomma anatolicum* and *Hyalomma drommedarri* (Rodriguez-Vallele *et al*., 2012 and Nabian *et al*., 2013). This vaccine however, has little effect on *R. appendiculatus* and *A. variegatum* (Makala *et al*., 2003). In the field, Bm86 vaccine has been shown to induce over 60% reduction in tick numbers in one tick generation and about 72% reduction in the laboratory. The effect of the vaccine is reduction in the number of engorging female ticks, reduction in weight and impaired reproductive capacity, consequently reducing the larval infestation in subsequent tick generations (Pipano *et al*., 2003, Willadsen, 2006 and Popara *et al*., 2013). It is therefore recommended that the vaccine be used concurrently with limited acaricide application to enhance the efficacy of overall tick control. Although immunological protection of hosts offers a practical sustainable alternative tick control method, there is need to conduct research on developing a more effective vaccine that may confer protection against multi-host ticks that are more widespread in Zambia (Makala *et al*., 2003).

2.5.4 Host resistance

Cattle breeds with naturally acquired tick resistance (host immunity) have been found to have significantly lower tick burdens than breeds with no resistance (Mathioli *et al*., 2000 and Maryan *et al*., 2012). Frisch *et al*. (1997) classified *Bos indicus* cattle (African and Indian Zebu) as having high tick resistance, *Bos taurus* cattle of the Sanga group as having intermediate tick resistance, and *Bos taurus* of British and European origin as having low tick resistance. Host-resistance to ticks is a heritable trait which can be passed on over a long period of time in subsequent cattle generations (Willadsen, 2006, Ayres *et al*., 2013 and Rodriguez-Valle *et al*., 2013). Good nutrition has been found to be essential in the maintenance of high levels of immunity to tick bites (Tolleson *et al*., 2010 and Lictman, 2013). However, stresses such as lactation or sickness may on the contrary cause a drop in tick resistance. Tick resistant cattle in general, tend to carry fewer numbers of ticks compared to susceptible cattle and will tend to have fewer numbers of female ticks which reach engorgement (Willadsen, 2006 and Abbas *et al*., 2014).

2.5.5 Pasture spelling

Another alternative to tick control is reducing the number of larvae on pasture through rotational grazing areas and by using forage grass that does not favour the development of ticks. Rotational grazing keeps paddocks free of cattle until larvae die (Jonsson and Piper, 2007 and Stachurshi and Adakal, 2010). However, rotational grazing is not feasible on small farms because of inadequate land. The use of grass which is unfavourable to ticks can also be utilised in integrated tick control (Jonsson and Piper, 2007 and Soares *et al*., 2010). However, the use of tick repelling grass has a major

drawback in that such grass species tend to have very poor nutritional value for cattle grazing. *Stylosanths spp*., *Melinis minutriflora* and *Andropogon gayanus* are some of the known grasses with tick repelling properties (Jonsson and Piper, 2007 and Soares *et al*., 2010).

CHAPTER 3: MATERIALS AND METHODS

3.1 Study Area

Isoka District (10°2'4"S, 32°6'1"E) is located in the North Eastern part of Zambia, in the recently created Muchinga Province (Figure 3.1). It is bordered by Nakonde District to the North, Chinsali District to the West, Chama District to the South and Malawi to the East. Isoka District was chosen as the study site because it has been reporting consistently higher numbers of tick and tick borne infections with more fatalities than any other district in Northern and Muchinga Provinces (Veterinary Report, 2008 and 2009).

A tropical climate prevails which is characterized by high rain fall (1000 mm to 1500 mm annually) and is restricted to the period of November to April with temperatures ranging from 17 to 28 °C. May to July is cool and dry with temperatures ranging from 11 to 25 °C. August to October is hot and dry and temperatures range from 13 to 30 °C.

The predominant vegetation type in Isoka District is the Open forest or Savannah woodland which covers most parts of the District. These woodlands are mainly Miombo in nature and are characterized by *Brachystegia*, *Julbernardia* and *Isoberlinia* trees (Aregheore, 2009). The woodlands are generally two-storied with an open and evergreen canopy 10 to 20 m high (Davies, 1971). Grass lands occur mainly in poorly drained dambos and swamps which are common in the District. The dominant grasses are *Hyparrhenia* spp., *Themeda triandra* and *Heteropogon contortus*. Dambo areas constitute the major grazing areas for cattle especially in the hot dry season (August to November).

Map A.

Map B.

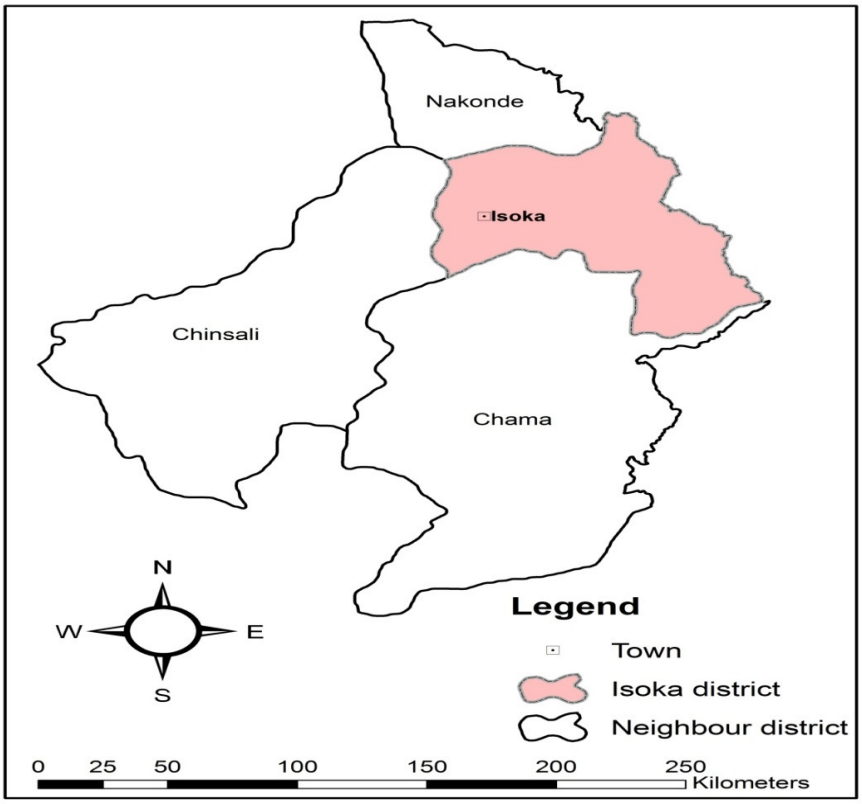

Figure 3. 1 Map showing location of Isoka District. Map A shows the location of Isoka District relative to the rest of Zambia. Map B shows the location of Isoka District relative to is neighboring districts. Source: Ministry of Agriculture and Livestock, Department of Veterinary Services, Data Management Unit.

3.2 Livestock farming practices in the study area

The 2010 census of population and housing preliminary report indicates that the population of Isoka district is 164,410 people, with over 80% of them engaged in small scale agriculture. The major crops cultivated include finger millet, cassava, beans, maize, groundnuts and rice. Apart from crop production, livestock farming is also undertaken. The major livestock reared include cattle, goats, poultry and sheep. Mixed crop and livestock production system is practiced which allows for diversification to mediate risks, efficient use of labour and recycling of crop residues and animal waste.

The cattle population of Isoka district is estimated at 18,017 which represent 18% of the total cattle population in Northern Province (Northern Province Veterinary Annual Report, 2010). The main breed kept by small-scale and emergent farmers is Angoni type, which is a short horned Zebu animal originating from the Eastern Province of Zambia (Yambayamba *et al*., 2003). Livestock farming in the district is done by the Namwanga speaking people in the west and the Tumbuka in the East. Cattle are kept for agricultural purposes to provide meat, milk, draft power, manure and hides to small-scale farmers. Animals are kraaled at night for protection and easy control and are released in the morning for communal grazing. The major disease threat to the cattle in Isoka district is tick borne diseases spread by various tick species. East Coast Fever, which entered the district from the Tanzanian frontier, is considered to be the most important disease (Starkey *et al*., 1991).

3.3 The study design

3.3.1 Acaricide questionnaire survey

A field survey of acaricide usage in the study area was conducted using a semi-structured questionnaire (Appendix A). Participants in the survey were randomly selected from a list of cattle farmers provided by the Isoka District Veterinary Office. Every tenth farmer on the list was included in a sample of 80 participants. This sample size was determined using the formula adopted from Campbell (2005); $n = \left(\frac{Z}{SE}\right)^2 (P)(1-P)$ where; n: sample size; Z: critical value for 95% confidence interval (1.96); SE: standard error, taken as 10%; P: expected proportion of farmers applying acaricides, taken to be 30%; and1-P: expected proportion of farmers not applying acaricides.

3.3.2 Acaricide resistance testing

3.3.2.1 Experimental cattle

The cattle selected for the study were all indigenous breeds predominantly of the Angoni type from four agricultural camps: Longwe, Kantenshya, Kapililonga and Ndeke. The camps were randomly allocated numbers one to 24, which were written on pieces of folded paper. The folded papers were then placed in a hat which was shaken vigorously before drawing out four papers representing the four camps. From the selected camps, a list of small holder cattle farmers who regularly applied acaricides was obtained from the Camp Extension Officers of each camp and every fifth farmer was selected in order to obtain sixteen farmers (four farmers per camp) whose cattle would be included in the sampling of ticks.

At the time of tick sampling, numbers one to the total number of cattle at each sampling site (kraal) were randomly allocated to cattle. Selection of the animals to be sampled was again done by selecting the first five numbered animal which corresponded to the first five numbers drawn from folded papers in a hat. This resulted in a total of 80 cattle being sampled during the study (Yilima *et al*., 2001). No consideration was given to the sex or age of cattle as treatments with acaricides was done indiscriminately. A similar sampling procedure was used by Yilima *et al*. (2001) who detected organophosphate resistance in *R. (Bo.) decoloratus* in Ethiopia.

3.3.2.2 Collection of engorged female ticks

A number of engorged female ticks were collected from animals secured in a crush pen during the peak occurrence period of adult ticks (January to March, 2012). These ticks were collected from the head, mid-section and the rear. This was done early in the morning before the animals were taken into pasture. Collected ticks were placed in plastic containers with perforated lids for air circulation. Freshly cut grass was added to the containers for the purpose of providing moisture. The time, date and place of collection were clearly labeled on containers. Test ticks were collected from each of the selected kraals from each camp. Reference or baseline ticks were collected from a kraal in Sansamwenge where acaricide usage was reported to be minimal or non-existent.

3.3.3 Acaricidal properties of *Tephrosia vogelii* and *Bobgunnia madagascariensis*

T. vogelii and *B. madagascariensis* plant extracts were tested for their acaricidal properties against adult *A. variegatum* ticks. This tick and tick stage were selected for testing because it was the most abundant tick and stage during the time the study was conducted. The testing procedure involved collection of plant material, preparation of

plant extracts, collection of ticks and the undertaking of free contact and topical bioassays.

3.3.3.1 Collection of plant material and pre-extraction procedure

Plant samples of *T. vogelii* and *B. madagascariensis* were collected by chopping off 30 cm branch ends with fruits from Longwe agricultural camp, located in the study area, in March 2012. These were brought to Isoka District Regional Veterinary laboratory for testing. Materials collected were bark, leaves, and fruits. The plant materials were then chopped into small pieces and were air dried at room temperature in a shade as described by Matovu and Olila (2007).

3.3.3.2 Preparation of crude extracts

The dried plant of each plant part was then crushed into finer particles using an iron pestle and mortar. The particles were then weighed into 5, 10 and 15 g portions which were placed in separate beakers. For each weight category of plant material, three different extracts were prepared using 50 ml of each of the following solvents; methanol, chloroform, and acetone to give crude mixtures. These mixtures were then allowed to stand overnight prior to filtration. 10 ml of the supernatant from each beaker were filtered out using Whatman no.1 filter paper into separate vials of known weights. For each solvent, a control was prepared by adding the same amount of solvent in each control vial. Solvents in both treatments and controls were then allowed to evaporate completely overnight in a fume chamber. After evaporation of the solvents, thin layers of residues were obtained in treatment vials. The weight of these residues were then determined by subtracting the weight of empty vials with vials with residues.

3.3.3.3 Collection of adult *Amblyomma variegatum* ticks

Adult ticks of the species *A. variegatum* were collected from Kantenshya Agricultural camp in a crush pen mainly from mid and rear sections of cattle from a selected kraal. This was done early in the morning before the animals were taken into pasture. Collected ticks were placed in plastic containers with perforated lids for air circulation. Freshly cut grass was added to the containers for the purpose of providing moisture. The time, date and place of collection were clearly labeled on containers. Collection of ticks was done as required at every stage of Bioassays.

3.4 Data collection

3.4.1 Acaricide questionnaire survey

Interviews with all 80 farmers were conducted using the semi-structured questionnaires and general conversation in November 2011 to January 2012 as described by Moyo and Masika (2009). The questionnaire documented the major diseases encountered, chemical types of acaricides in use (past and present), their dosage, and methods of application, average delivery rate and the proportion of cattle farmers that applied acaricides to their cattle.

3.4.2 Acaricide resistance testing

Upon arrival at Isoka District Regional Veterinary laboratory, engorged female ticks were washed in distilled water to remove eggs laid during transportation. Five clean engorged females of the species *R. (Bo.) microplus, R. appendiculatus and A. variegatum* (identified using the pictorial guide provided by Walker *et al*., 2007) were then placed in a 150 mm glass rearing tube, which were closed firmly with a ventilated stopper. This was done for each species collected. Rearing tubes were then incubated at

a temperature of 27 ± 1˚C and relative humidity of 85-95% (maintained with a saturated solution of potassium chloride) for egg laying and hatching of larvae. Bioassays were carried out on 14 to 21 day old larvae.

3.4.2.1 Larval packet test bioassay

The acaricides used in the study were amitraz 12.5% m/v (Milbitraz - Bayer Animal Heath Pty Ltd), registration number G2084 and Cypermethrin 15% m/v (Cydip-United Phosphorous Ltd), and registration number G505. Both were registered according to Act 36/1947, South Africa.

Olive oil (sterilized by heating to 110°C for 75min) and chloroform were mixed together in a 1:2 by volume ratio in order to make a stock solution. Then an appropriate volume of acaricide was added to this solution to make the highest concentration. Serial dilutions were done so as to come up with the following test concentrations; for amitraz, 0.00025, 0.0025, 0.0125, 0.015, 0.025 and 0.25 ml/l: and cypermethrin 0.0005, 0.005, 0.05, 0.1, 0.15 and 1.5 ml/l. A volume of 0.67 ml of these solutions were then pipetted on to 5 cm by 10 cm Whatman 541 filter papers. Control filter papers (stock solution without acaricide) were prepared first followed by the test papers in order of increasing concentration. Papers were then air dried for an hour to allow the chloroform to vaporize.

The rectangular filter papers were then folded horizontally (in half) with the label of the active ingredient and concentration being inside. Bulldog clips were then slide up on each of the short sides of the paper. The resulting filter paper packet was then placed on a stand.

A rearing tube was obtained directly from the incubator, placed in the rubber bung and stood in a small petri dish within a larger one forming a moat. The whole unit was then placed on a white enamel tray and water added to the moat so as to trap escaping larvae. A conical flask containing fine paint brushes and glass rods, two supports for the acaricide packets and two beakers of water (one containing detergent and cotton wool) were also placed on the tray.

Ticks were allowed to freely aggregate at the top rim of the rearing tubes. Using a fine brush, a small cluster of approximately 100 larvae was picked up from the open rim of the tube and eased into the filter paper packets with the aid of a glass rod, starting with control packets. Care was taken such that the brush and rod did not come in contact with the packets. The remaining tick larvae on the brush and rod were cleared by rubbing them on wetted cotton wool. Packets were then removed from the stand, closed by sliding a plastic clip on the open end and placed in a tray awaiting subsequent transfer to the incubator. Packets were then incubated for 24 hrs in cypermethrin and 48 hrs in amitraz tests at a temperature of 27 ± 1˚C and relative humidity of 85-95%. The bioassays in amitraz tests were incubated for 48 hrs as 24 hr incubation does not produce significant regression on Whatman 541 filter papers (FAO, 1998).

After the incubation period, the packets were examined in the same order in which they were prepared. The criterion for death was taken as the inability to walk even if larvae were able to move their legs. Packets were secured on polystyrene blocks. Live larvae were removed using a fine brush and immobilized on cotton wool moistened with water. The dead larvae were then counted with a tally counter and magnifying glass. All procedures described above were done for both reference and test populations for each

tick species. The above procedure was first described by Stone and Haydock (1962) and has been modified by FAO (1998).

3.4.3 Acaricidal properties of *Tephrosia vogelii* and *Bobgunnia madagascariensis*

The extracts obtained were then tested for anti-tick properties using the free contact and topical application. These bioassays have been successfully used by other researchers (Nchu *et al*., 2005; Magano *et al*., 2008) in screening plants for anti-tick properties.

3.4.3.1 Free contact assays

Ten adult ticks were introduced into each vial of the treatment and control groups. Vials were then closed with a mesh and rubber bands to prevent ticks from crawling out. Tick mortality was then recorded every after 24 hrs for 8 days. In order to assess the stability of the potent extracts (i.e. the extract that kills all ticks in 24 hrs) dead ticks were replaced by live ones. Extracts for topical application procedure were selected after 24 hrs.

3.4.3.2 Topical application assays

Only extracts that exhibited mortality in 24 hrs were used in this bioassay. Preparation was done as in the free contact assay. However, for each treatment, 10 ml of each solution was filtered out and the solvent allowed to evaporate completely. The residues obtained were weighed after which olive oil was added in order to obtain three different concentrations, following the initial weight categories and yields obtained. Each mixture was stirred gently for three minutes and left to stand overnight to ensure homogeneity. Live ticks were divided into treatment and controls, each with ten ticks. For each treatment group, 10 µl of a mixture of extract was topically applied onto the dorsal surface of the idiosoma using a micropipette. A similar procedure was carried out

for control ticks using oil only (control). Four replications were done for each concentration. Tick mortality was recorded after 24 hrs.

3.5 Statistical analysis

3.5.1 Acaricide questionnaire survey

The results were analyzed as descriptive statistics using the statistical package SPSS 16 and Microsoft Excel 2007 (Mugabi *et al*., 2010).

3.5.2 Acaricide resistance testing

3.5.2.1 Determination of tick mortality

Dead larvae were easily detected after exposure to cypermethrin as many were desiccated and obviously dead. For amitraz, mortality was more difficult to determine as many larvae did not desiccate after exposure and appeared to be alive. Therefore, we considered larvae that could walk across treated papers after incubation to be alive, but larvae that did not move or could only move legs without walking as dead (Miller et al., 2002).

Dead and live larvae were counted using a tally counter and magnifying lens. Mortality was computed using the formula as described by Yilima *et al*. (2001) as shown below:

$$\text{Mortality (\%)} = \frac{\text{Dead Tick larvae count}}{\text{Total Tick larvae count}} \times 100$$

In tests with control mortality between 0-5%, every mean mortality was corrected using Abott's formula:

$$\text{Corrected mortality (\%)} = \frac{\text{(\% test mortality - \% control mortality)}}{\text{100 - \% control mortality}} \times 100$$

The pooled mean mortality for each species was computed as;

$$\text{Pooled mean mortality (\%)} = \frac{\text{Mortality } i + \text{Mortality } ii \ldots + \text{Mortality } n}{N}$$

Where '*i*', '*ii*', ... '*n*' denote the 1st, 2nd, ... and the nth sample data, respectively, and '*N*' stands for the total sample size.

3.5.2.2 Probit analysis

The t-ratios for the slopes of each regression line were computed in order to determine the significance of regression in each bioassay experiment. In order to test how well the experimental data fitted the assumptions of the probit model used in the analysis, chi-square test for goodness of fit was conducted on the dose mortality data for each experiment. Lethal dose ratio estimates were determined and then used to compare the relative susceptibility between reference and test populations against amitraz and cypermethrin for each tick species. All calculations were done using Polo Plus Probit and Logit Analysis Software 2007 (Robertson *et al*., 2007).

3.5.3 Acaricidal properties of *Tephrosia vogelii* and *Bobgunnia madagascariensis*

Determination of mortality and probit analysis was conducted as shown in sections 3.5.2.1 and 3.5.2.2 except the lethal dose ratio estimates were then used to compare the relative toxicity of extracts from the two plants. All calculations were again done with Polo Plus Probit and Logit Analysis Software 2007 (Robertson *et al*., 2007)

CHAPTER 4: RESULTS

4.1 Acaricide questionnaire survey results

Only small holder traditional livestock farmers were found in the study area. These had an average of 16 cattle per respondent. The majority of livestock farmers were mostly males (Table 4.1) and the average age of respondents was 55 years. Table 4.1 further indicates that the educational back ground of respondents was such that the majority (44%) had primary education, 20% junior secondary and 25% attained senior secondary school education. Only 8% attained tertiary education and 2% had no formal education. All farmers interviewed were not in formal employment and farming (livestock and crop based) was their only form of income.

All respondents acknowledged that ticks were a major problem in their farming systems with East Coast fever being the most important disease affecting cattle. Farmers reported that the disease mostly affected calves below the age of 6 months and was predominantly encountered between late-January to mid-March. Mortalities associated with East Coast Fever were reported to be as high as 50%. Other important tick borne diseases included babesiosis (23%) and anaplasmosis (17%). Worm infestations (45%), Lumpy Skin Disease (10%) and Black Quarter (5%) were reported to be the most important non-tick borne diseases encountered by respondents. Farmers were able to describe these diseases using clinical signs exhibited by sick animals.

Table 4.1 Percentage of farmer gender, literacy level and occupation of respondents in the acaricide questionnaire survey. Most important livestock diseases also shown.

Background character	Description of group	Frequency	Percentage
Gender	Female	2	3
	Male	78	97
Literacy	No education	2	3
	Primary	36	45
	Junior secondary	16	20
	Senior secondary	20	25
	Tertiary	6	8
Occupation	Farming	80	100
	Formal employment	0	0
Disease situation	East Coast fever	80	100
	Anaplasmosis	14	17
	Babesiosis	18	23
	Lumpy Skin Disease	8	10
	Black Quarter	4	5
	Worm infestation	36	45

4.2 Tick control methods used by farmers in Isoka District

Fifty percent of respondents practiced chemical tick control, with amitraz (27%) and cypermethrin (23%) being the acaricides commonly used (Figure 4.1). All of these farmers applied acaricides to cattle using knapsack sprayers of various capacities (5 - 16 litres). Acaricides were generally applied to cattle at various intervals, with bi weekly application being the most common (35%). However, weekly, monthly, bi monthly, bi annual and annual applications were also practiced (Figure 4.2).

Farmers were able to follow the dosage recommended by the manufactures of acaricides with the aid of veterinary extension workers. For amitraz, the dosage was 20 ml of acaricide to 10 litres of water, whereas 10 ml to 10 litres of water was used for cypermethrin. However, farmers did not follow the recommended delivery rate of 10 litres of spray wash per animal. On average, less than 2 litres of spray wash per animal was used.

Ten percent of respondents conducting chemical tick control were able to control ticks successfully. These were mostly farmers that applied acaricides weekly and bi-weekly. Monthly, bi monthly, annual and bi annual applications had no effect on tick infestations.

The average annual cost of acaricide per farmer was found to be ZMK 43, 000.00 which was considerably low and was a further indication of poor delivery rate of spray wash onto animals. When recalculated at recommended practices, the average annual cost rose to ZMK 469, 000.00 per farmer, which was expensive for the farmers.

Other tick control methods used in the study area were the application of tick grease especially to the ears of cattle, the smearing of *Tephrosia vogelii* (ububa) leaves onto

the skin of animals and hand picking (Figure 4.3). Application of tick grease and the smearing of *T. vogelii* leaves reportedly had some effect but most farmers thought it to be less effective than conventional acaricide application. Although *B. madagascariensis* was present in the study area, farmers did not use it for tick control.

In light of the current problems facing farmers in the study area, 60% preferred government to subsidize tick control by providing acaricide and constructing dip tanks. The other 40% considered community run programmes where farmers would contribute money to purchase acaricides to be the most appropriate method of control.

4.3 Acaricide resistance test

The values of the t-ratios of all slopes of the regression lines produced by reference and test populations for each of the three tick species tested for amitraz and cypermethrin resistance were greater than 1.96 (Table 4.2). This indicated that for each bioassay experiment, there was significant regression of log dose on probit mortality, at 95% confidence level ($P = 0.05$).

The heterogeneity factors (Chi square/degrees of freedom) produced for each bioassay were all less than 2.9 (Table 4.2). This indicated that the dose mortality data of each regression line fitted the assumptions of the probit model used to conduct the analysis at 95% confidence level ($P = 0.05$).

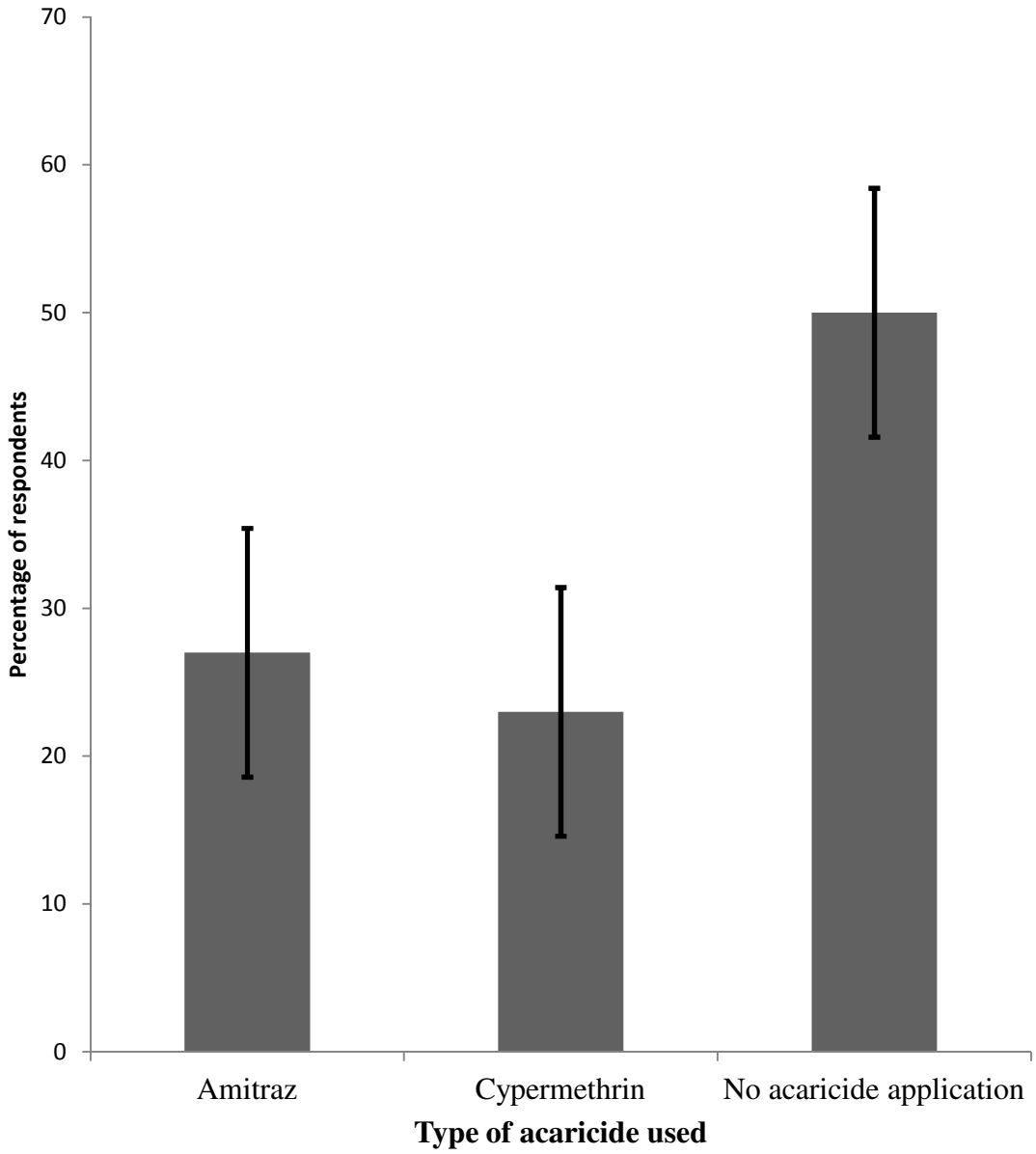

Figure 4. 1 Acaricides currently in use in Isoka District. Vertical bars show the standard deviation of the sample.

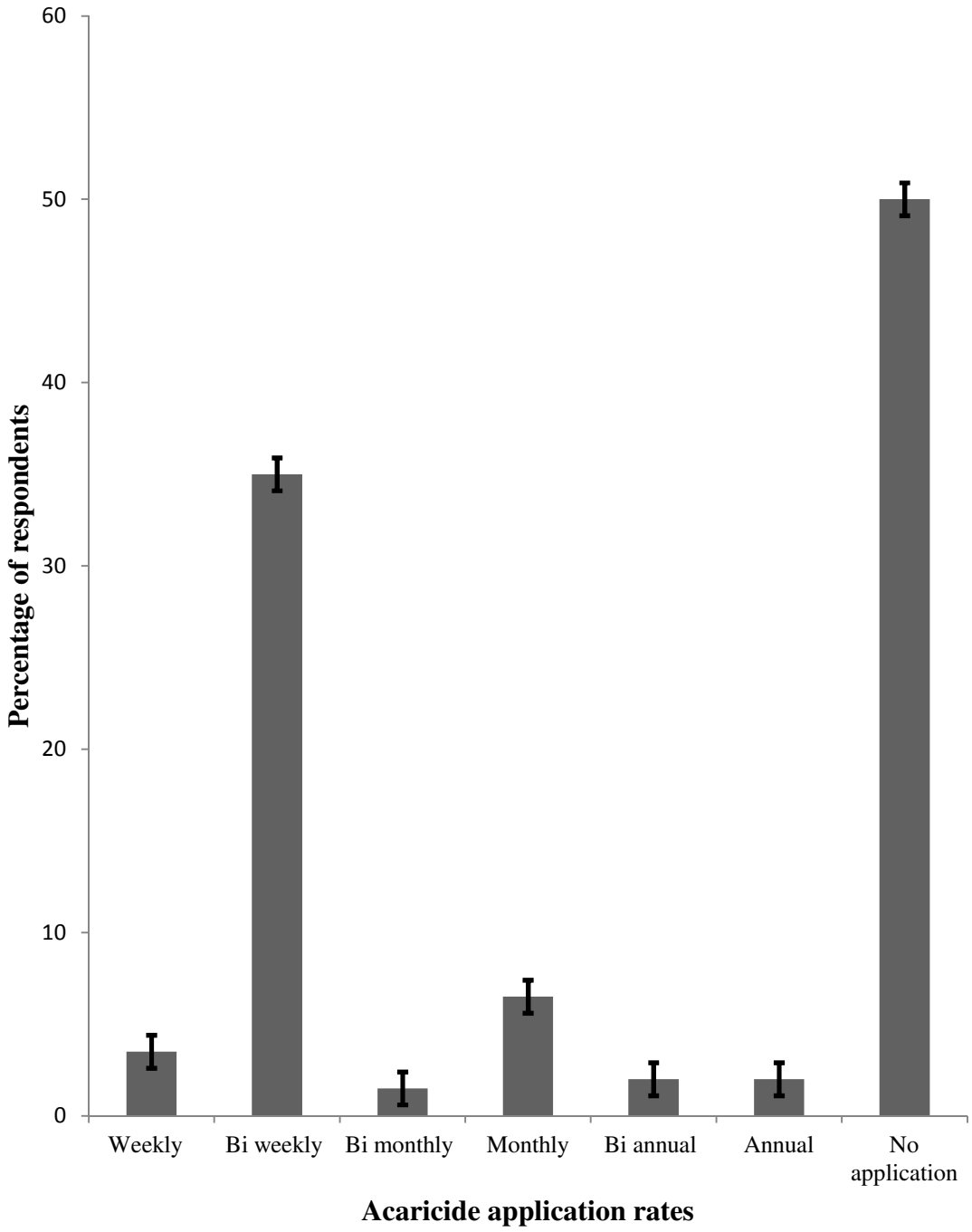

Figure 4. 2 Acaricide application rates used by small scale livestock farmers in Isoka District. Majority of farmers applied acaricides in the tick season; November to April. Vertical bars show the standard deviation of the sample.

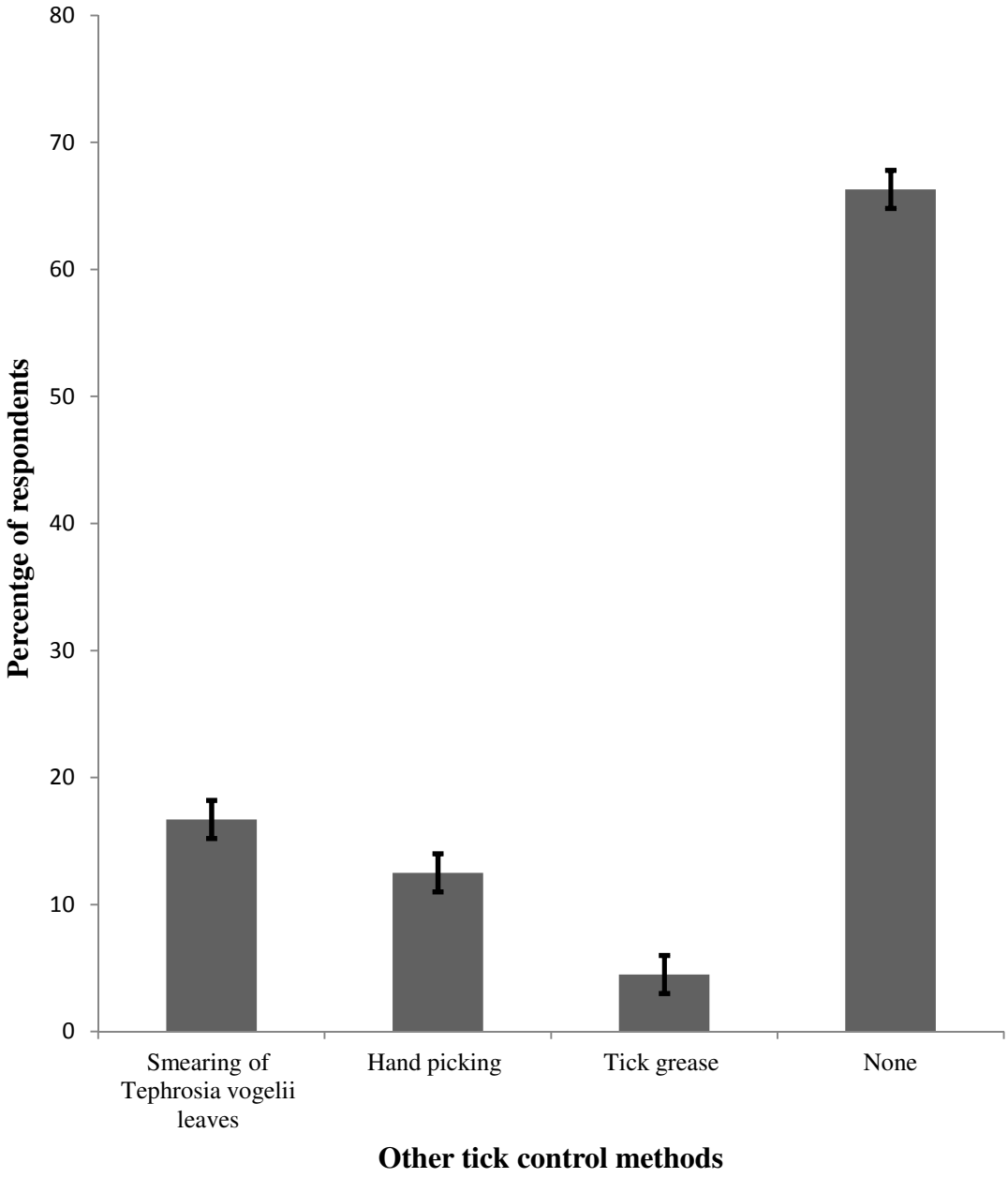

Figure 4. 3 Other tick control methods used by small scale farmers in Isoka District. These were mainly applied to young animals. Vertical bars show standard deviation of the sample.

Table 4.2 Significance of regression and assessment of goodness of fit of larval packet test bioassay data. Heterogeneity factors were all less than 2.9 indicating that data followed the probit model ($P < 0.05$).

Species	**Population**	**t-ratio of slope**	**Heterogeneity factor**
R. (*Bo.*) *microplus*			
Amitraz	Reference	6.40	1.82
	Test	8.65	1.77
Cypermethrin	Reference	9.56	0.84
	Test	6.36	2.01
R. appendiculatus			
Amitraz	Reference	6.84	1.77
	Test	8.40	0.18
Cypermethrin	Reference	9.79	2.29
	Test	10.57	2.54
A. variegatum			
Amitraz	Reference	6.34	1.87
	Test	6.67	2.69
Cypermethrin	Reference	9.88	2.58
	Test	9.61	1.73

4.3.1 Bioassay results for tick species tested for amitraz and cypermethrin susceptibility.

The LD_{50} and LD_{90} of *R. (Bo) microplus* reference population tested for amitraz resistance were estimated to be 0.010 (95% CI = 0.009 - 0.011) ml/l and 0.012 (95% CI = 0.011 - 0.015) ml/l (Table 4.3). The values for the test population were 0.012 (95% CI = 0.010 - 0.013) ml/l for LD_{50} and 0.018 (95% CI = 0.0155 - 0.026) ml/l for LD_{90}. The LD_{50} and LD_{90} ratio tests indicated that both LD_{50} and LD_{90} of the reference population were significantly lower than those of the test population as the 95% confidence interval of both LD_{50} and LD_{90} dose ratios did not include one (Table 4.3). The susceptibility of the two populations at 95% confidence level was significantly different, with test population having a lower susceptibility than the reference population ($P = 0.05$). The probit line of the test population shows a shift to the right of the reference population as a result of higher values of LD_{50} and LD_{90} in the test population (Figure 4.4).

The reference population of *R. (Bo) microplus* tested for cypermethrin resistance gave LD_{50} and LD_{90} estimates of 0.020 (95% CI = 0.014 - 0.025) ml/l and 0.076 (95% CI = 0.059 - 0.103) ml/l respectively (Table 3). The values for the test population were estimated to be 0.032 (95% CI = 0.006 - 0.055) ml/l for LD_{50} and 0.111 (95% CI = 0.068 - 0.285) ml/l for LD_{90}. The LD_{50} ratio test indicated that the reference population had a lower susceptibility than the test population as the 95% confidence interval did not include one (Table 4.3). However, the 95% confidence interval for LD_{90} ratio included one which indicated that the susceptibility of the two populations was not significantly different.

Table 4.3 Comparison of the susceptibility of reference and test populations against amitraz and cypermethrin. Lethal dose estimates are presented as ml/l of the active ingredient. Lethal dose ratios (LDR) are relative to the reference strain.

Species	**Population**	**LD_{50} ml/l**	**LD_{90} ml/l**	**LDR_{50} (95% CI)**	**LDR_{90} (95% CI)**
R. (*Bo.*) *microplus*					
Amitraz	Reference	0.010	0.012	0.866 (0.812 - 0.923)	0.662 (0.596 - 0.742
	Test	0.012	0.018		
Cypermethrin	Reference	0.020	0.076	0.612 (0.386 - 0.971)	0.683 (0.466 - 1.009)
	Test	0.032	0.111		
R. appendiculatus					
Amitraz	Reference	0.010	0.013	0.979 (0.904 - 1.060)	0.905 (0.815 - 1.004)
	Test	0.010	0.015		
Cypermethrin	Reference	0.019	0.093	0.759 (0.536 - 1.073)	0.820 (0.53 - 1.269)
	Test	0.759	0.820		
A. variegatum					
Amitraz	Reference	0.009	0.013	0.901 (0.817 - 1.192)	0.896 (0.805 - 1.398)
	Test	0.010	0.014		
Cypermethrin	Reference	0.015	0.072	0.851 (0.585 - 1.238)	0.930 (0.599 - 1.245)
	Test	0.018	0.078		

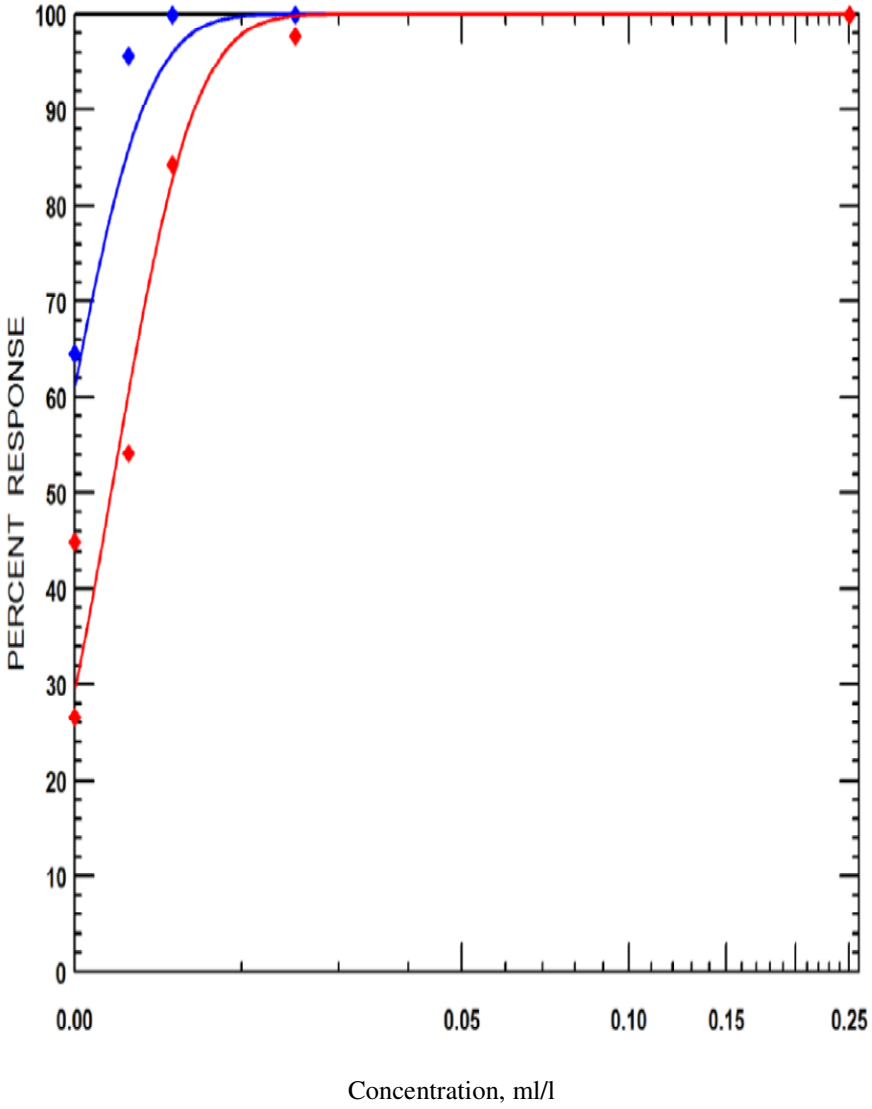

Figure 4. 4 Dose-mortality curve for reference (left) and test (right) populations of *Rhipicephalus (Boophilus) microplus* against amitraz. The equations of the regression lines were Y = 35.89 + 17.96X (reference population) and Y = 13.17 + 6.81X (test population).

The LD_{50} and LD_{90} of the reference population of *R. appendiculatus* tested for amitraz resistance were estimated to be 0.010 (95% CI = 0.008 - 0.011) ml/l and 0.013 (95% CI = 0.012 - 0.016) ml/l respectively (Table 4.3). For the test population, the values were estimated to be 0.010 (95% CI = 0.010 - 0.011) ml/l for LD_{50} and 0.015 (95% CI = 0.014 - 0.016) ml/l for LD_{90}. Both LD_{50} and LD_{90} ratios 95% confidence interval included one which indicated that the susceptibility of reference and test populations against amitraz was not significantly different (Table 4.3).

With regards to *R. appendiculatus* tested for cypermethrin resistance, the LD_{50} and LD_{90} of the reference population were estimated to be 0.019 (95% CI = 0.009 - 0.032) ml/l and 0.093 (95% CI = 0.055 - 0.248) ml/l respectively. The values in the test population were estimated to be 0.759 (95% CI = 0.536 - 1.073) ml/l for LD_{50} and 0.820 (95% CI = 0.530 - 1.269) ml/l for LD_{90}. Both LD_{50} and LD_{90} ratios 95% confidence interval included one which indicated that the susceptibility of reference and test populations against cypermethrin was not significantly different (Table 4.3).

The LD_{50} and LD_{90} of the reference population of *A. variegatum* tested for amitraz resistance were estimated to be 0.009 (95% CI = 0.007 - 0.010) ml/l and 0.013 (95% CI = 0.012 - 0.017) ml/l respectively (Table 4.3). For the test population, the values were estimated to be 0.010 (95% CI = 0.007 - 0.011) ml/l for LD_{50} and 0.014 (95% CI = 0.013 - 0.024) ml/l for LD_{90}. Both LD_{50} and LD_{90} ratios 95% confidence interval included one which indicated that the susceptibility of reference and test populations against amitraz was not significantly different (Table 4.3).

In the case of *A. variegatum* tested for cypermethrin resistance, the LD_{50} and LD_{90} of the reference population were estimated to be 0.015 (95% CI = 0.008 - 0.023) ml/l and

0.072 (95% CI = 0.040 - 0.157) ml/l respectively. The values in the test population were estimated to be 0.018 (95% CI = 0.009 - 0.027) ml/l for LD_{50} and 0.078 (95% CI = 0.049 - 0.165) ml/l for LD_{90} at 95% confidence. Both 95% confidence interval for LD_{50} and LD_{90} ratios included one which indicated that the susceptibility of reference and test populations against cypermethrin was not significantly different (Table 4.3). The POLOPLUS outputs for the above results are shown in appendix B and C.

4.4 Acaricidal properties of *Tephrosia vogelii* and *Bobgunnia madagascariensis*

4.4.1 Extraction yields

The mean weights of the plant extracts of *T. vogelii* and *B. madagascariensis* extracted with methanol, acetone and chloroform are shown in Table 4.4 Methanol extracts of *T. vogelii* gave an average yield of 0.10 g, 0.14 g and 0.03 g per one gram of bark, leaf and fruit material respectively. Chloroform extracts of this plant yielded 0.016 g, 0.024 g and 0.0026 g, while acetone gave 0.014 g, 0.016 g and 0.001 g per one gram of cortex, leaf and fruit material respectively. The highest mean weight of extracts for *T. vogelii* were obtained from leaf material using methanol as an extraction solvent.

With regards to *B. madagascariensis*, methanol extracts gave an average of 0.0013 g, 0.0023 g and 0.0062 g per one gram of bark, leaf and fruit material respectively. Chloroform extracts of this plant yielded 0.0013 g, 0.0017 g and 0.0037 g, while acetone gave 0.00093 g, 0.00097 g and 0.0013 g per one gram of bark, leaf and fruit material. Consequently, the highest mean weight of extracts for *B. madagascariensis* were obtained from fruit material using methanol as an extraction solvent.

Table 4.4 Mean yield (g) of extracts for each extraction solvent per gram of plant material. Mean weights shown with their 95% standard deviation.

Species	**Weight (g) of extract of**		
	Bark	**Leaves**	**Fruit**
Tephrosia vogelii			
Methanol	0.10 ± 0.05	0.014 ± 0.07	0.03 ± 0.02
Chloroform	0.016 ± 0.008	0.024 ± 0.012	0.0026 ± 0.0013
Acetone	0.014 ± 0.007	0.016 ± 0.008	0.001 ± 0.0005
Bobgunnia madagascariensis			
Methanol	0.0013 ± 0.0006	0.0023 ± 0.0015	0.0062 ± 0.0025
Chloroform	0.0013 ± 0.0006	0.0017 ± 0.0006	0.0037 ± 0.0015
Acetone	0.00093 ± 0.00012	0.00097 ± 0.00006	0.0013 ± 0.0006

4.4.2 Free contact bioassays

Only methanol extracts of bark and leaf material of *T. vogelii* and methanol fruit extracts of *B. madagascariensis* obtained from 15 g/50 ml crude mixture produced 100% mortality of ticks in 24 hrs (Table 4.5). Residues obtained from other solvents showed no anti-tick activity. Methanol leaf extracts of *T. vogelii* and methanol fruit extracts of *B. madagascariensis* were selected to be used in topical assays to compare the acaricidal properties of the two plants. As shown in Table 4.5, the acaricidal activity of methanol leaf extracts of *T. vogelii* persisted for up to 8 days while that of fruit extracts of *B. madagascariensis* persisted for only 6 days.

4.4.3 Topical application bioassays

The t-ratios of the slopes of the regression lines for *A. variegatum* ticks tested with methanol leaf extracts of *T. vogelii* (3.03) and methanol fruit extracts of *B. madagascariensis* (3.35) were both greater than 1.96. This indicated significant regression of extracts on the mortality of ticks at 95% confidence level.

The Chi-square test of goodness of fit for both *T. vogelii* and *B. madagascariensis* gave heterogeneity values of 0.142 and 0.007, respectively. These values were much less than 2.9 which indicated that the dose mortality data obtained from both plant extracts fitted the assumptions of the probit model used to analyse the data.

The hypothesis of equality of the two regression lines was rejected ($P < 0.05$) while that of parallelism was accepted ($P > 0.05$). This indicated that the regression lines obtained from *T. vogelii* and *B. madagascariensis* extracts were not equal, but were parallel at 95% confidence level; the slopes were not significantly different ($P > 0.05$).

Table 4.5 Percent mortality of *Amblyomma variegatum* ticks caused by crude methanol extracts in contact bioassay. Mortality was taken every 24 hr and free ticks introduced. The longer the extracts remained active, the more stable the extracts were.

Plant crude mixture	**24hr**		**48hr**		**72hr**		**96hr**		**120hr**		**144hr**		**168 hr**		**192hr**	
	C	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**	**C**	**T**
T. vogelii (leaf)	0	100	0	100	0	100	0	100	0	90	0	80	0	80	0	50
B. madagscariensis (fruit)	0	100	0	90	0	70	0	50	0	20	0	10	0	0	0	0

C = Control; T = Treatment.

The LD_{50} of *B. madagascariensis* was 0.03 (95% CI = 0.020 - 0.045) w/v while that of *T. vogelii* was 0.585 (95% CI = 0.327 - 0.989) w/v. The LD_{50} dose ratio of *B. madagascariensis* and *T. vogelii* was 0.052 (95% CI = 0.030 to 0.090). Since the 95% confidence interval of LD_{50} dose ratio did not include one, the relative toxicity of the two plants extracts was significantly different, with *B. madagascariensis* extracts being more toxic than *T. vogelii*. The regression lines of the two plant extracts are shown in Figure 4.5.

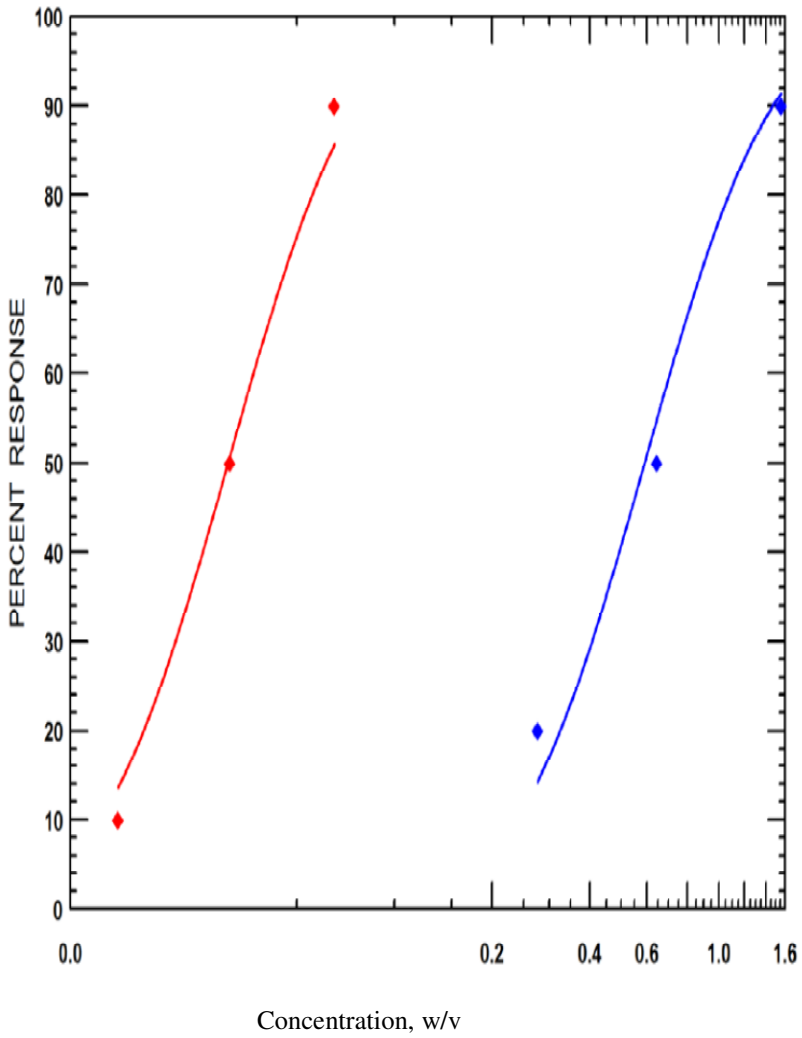

Figure 4. 5 Dose-mortality curves for *T. vogelii* (left) and *B. madagascariensis* (right) methanol extracts tested against adult Amblyomma variegatum ticks. The equations of the regression lines were Y = 0.65 + 2.80X (*T. vogelii*) and Y = 5.82 + 3.35X (*B. madagascariensis*). X = slope of the probit line.

CHAPTER 5: DISCUSSION

5.1 Acaricide questionnaire survey

It was widely acknowledged by farmers (100%) and livestock extension workers that ticks were the most problematic external parasites that affected cattle in the study area, with tick borne diseases constituting the major animal health problem. Farmers reported high incidence of tick related problems with East Coast fever, babesiosis and anaplasmosis being the most common tick borne diseases. Since bovine babesiosis and anaplasmosis are strongly associated with *R. (Bo.) microplus* (Jonejan *et al*., 1988, Kaaya *et al*., 2000 and Makala *et al*., 2003) and ECF with *R. appendiculatus* (D' Haese *et al*., 1999, Konnai *et al*., 2006 and Muleya *et al*., 2012) in this region of Zambia, these ticks were the most important in terms of disease transmission in the study area.

Farmers indicated that mortalities as a result of diseases such as ECF were as high as 50% during outbreak years. Similar observations were reported by Billiouw *et al*. (1999 and 2002) in the Eastern Province of Zambia, total loss of susceptible herds infected by ECF was not uncommon. Nambota *et al*. (1994) reported country wide losses to ECF to be about10, 000 cattle annually.

5.2 Tick control methods used by farmers in Isoka District

In an effort to control cattle ticks, 50% of livestock farmers practiced conventional chemical tick control which was low. This observation could be attributed to the fact that cattle rearing was not a major source of livelihood for farmers in the study area. Rather, cattle are kept mainly as a capital asset which were converted to liquid cash and barter items, when the need arose; for instance, if they required money for fertilizers, school fees or marriages. Tick control among these farmers was undertaken when they

observed high tick burdens on their animals. Similar observations have been reported in Soroti District in Uganda by Ocaido *et al*. (2005 and 2009).

All farmers that practiced chemical tick control used knapsack hand sprayers to apply acaricides to cattle. This was attributed to the fact that spraying equipment was much cheaper than the construction of a dip tank and only small amounts of acaricides were required per spray application (Makala *et al*., 2003, George *et al*., 2004 and Rajput *et al*., 2006). However, only 10% of farmers reported successful control of ticks. This observation could be attributed to inadequate delivery of dip wash onto cattle, as less than 2 litres of dip wash was delivered onto cattle by all farmers interviewed. Similar observations were reported by Ocaido *et al*. (2005), Mugisha *et al*. (2005) and Mugabi *et al*. (2010) who found smallholder livestock farmers using less than 3 litres of dip wash per animal. In order to achieve thorough wetting of the animal for effective tick control, FAO (1998) recommended using at least 10 litres of dip wash per head of cattle. Farmers considered such applications to be too extravagant arguing that they could not afford to use acaricides in this manner as the cost would be too high.

The most common acaricide used in the study area was amitraz marketed either as Triatix® or Milbitraz®. The choice of this acaricide seemed to have been influenced by the fact that this cyclic amidine is relatively cheaper than most compounds (Mugabi *et al*., 2010 and Brito *et al*., 2011). Cypermethrin was the other acaricide in use. Cypermethrin based acaricides are relatively more expensive than the cyclic amides (Mugisha *et al*., 2008). Makala *et al*. (2003) reported the use of several organophosphorous compounds for tick control in Zambia. However, no organophosphorous acaricides were currently in use for tick control in the study area.

Hand picking was another tick control method that was observed among farmers in the study area (12.5%). This technique was generally applied to young animals when tick infestations were high. Manual removal of ticks is widely practiced in many smallholder livestock farming systems of the developing world. Masika *et al*. (1997) reported that 10% of the livestock owners in the central region of the Eastern Cape Province of South Africa either cut ticks off with blades, scissors or pulled them from animals; the latter being the method of choice in the study area. The pulling of ticks from cattle has also been observed by Chamboko *et al*., (1999) who reported that 6.9% of Lowveld smallholder farmers and 28.3% of Highveld farmers in Zimbabwe removed ticks by hand. Manual removal of ticks could be an alternative to complement the main tick control method especially where herds are small. However, manual removal is a laborious task and the pulling of ticks' damages animal tissues which may lead to secondary bacterial infections (Ambrose *et al*., 1999, Rajput *et al*., 2006 and Muchenje *et al*., 2008).

With regards to the use of ethno-veterinary methods, the only plant used for tick control in the study area was *T. vogelii* (16.7%). This plant is widely distributed in Zambia and has been used for tick control in some communities (Kaposhi, 1992). However, the crushing of leaves and then smearing them on the skin of the animal, as observed in the study area, is an inefficient method of exposing acaricidal leaf extracts to ticks. Soaking of leaves in water for 24 hrs and then spraying extracts on the skin of the animal has been reported to be much more potent (Kaposhi, 1992 and Habeeb, 2010). Despite the presence of *B. madagascariensis* in the study area, a plant that is known to have significant pesticidal properties (Stevenson *et al*., 2010, Nyahangare *et al*., 2012 and

Sola *et al*., 2014), it was not used for tick control. This was attributed to the fact that farmers were ignorant of the acaricidal properties of this plant.

5.3 Acaricide resistance

All larval packet test bioassay experiments had significant regression of log dose on probit mortality ($P = 0.05$). This indicated that the observed mortality in the bioassays was due to the toxic effects of the acaricides used in the bioassays. Insignificant regression in bioassay experiments indicates that treatments have no effects and data collected from such experiment must be excluded from any further analysis (Robertson *et al*., 2007).

The results of the chi square tests for goodness of fit for each bioassay indicated that the dose mortality data of each regression line produced fitted the assumptions of the probit model used to conduct the analysis ($P = 0.05$). All inferences and conclusions drawn from these data were valid under the assumptions of the probit model (Robertson *et al*., 2007).

The LD_{50} and LD_{90} of the reference population of *R (Bo.) microplus* tested for amitraz resistance were significantly lower than those of the test population ($P = 0.05$). This result indicated a significant difference in the susceptibility of the two populations with the test population having a lower susceptibility than the reference population. A higher dose or concentration of amitraz was required in the test population in order to illicit a similar level of response as in the reference population. Significant increases in the LD estimates especially the LD_{90} are indicative of a reduction in susceptibility of an arthropod population (Yilima, 2001; Robertson *et al*., 2007; Heong *et al*., 2010) which is characteristic of resistance development. However, the resistance ratios 1.15 (95% CI

= 1.08 -1.23) and 1.51 (95% CI = 1.35 - 1.69), indicated by the inverse values of LD_{50} and LD_{90} ratios 0.866 (95% CI = 0.812 - 0.923) and 0.662 (95% CI = 0.591 - 0.742), were low at *P = 0.05*, respectively. Miller *et al*. (2002) reported the resistance ratio of amitraz resistant in *R. (Bo.) microplus* to be as high as 26.3 (95% CI = 25.7 - 26.8) while Cutullé *et al*. (2012) reported the resistance ratios of two field isolates of the same tick from Argentina to be 32.5 and 57.0 respectively. Clearly, the low resistance ratios reported in this study indicated that amitraz resistance in *R (Bo.) microplus* populations in Isoka District was in the initial phase (FAO, 1998).

The detection of emerging amitraz resistance in *R (Bo.) microplus* in the present study is similar to the finding of Ntondini *et al*. (2008) who detected emerging amitraz resistance in this tick on communally grazed cattle, in the eastern region of the Eastern Cape Province of South Africa. In fact, amitraz tick resistance appears to be on the increase as high levels of amitraz resistance have been reported by Mekonnen (2002), Miller *et al*. (2002), Li *et al*. (2004) and Cutullé *et al*. (2012) in various parts of the world.

The LD_{50} and LD_{90} of the reference of *R (Bo.) microplus* tested for cypermethrin resistance were found not to be significantly different from those of the test population (*P = 0.05*). This indicated that cypermethrin resistance in *R (Bo.) microplus* had not yet developed in the study area. The detection of vigour tolerance against cypermethrin may be an indication of the first steps in susceptibility change in this tick against cypermethrin, as Hoskins and Gordon (1956) pointed out that the development of vigour tolerance precedes resistance development. The none detection of cypermethrin resistance in *R (Bo.) microplus* is in contrast to the results reported by

Caracostantogolo *et al*. (1996) who reported high levels of cypermethrin resistance in *R. (Bo.) microplus* in the eastern part of Argentina. Cypermethrin resistance has also been reported by Mekonnen (2002) who detected it in *R. (Bo.) decoloratus* on communal and commercial farms in South Africa.

No resistance to either amitraz or cypermethrin acaricides was detected in *R. appendiculatus* as indicated by the fact that the LD_{50} and LD_{90} of reference and test populations were not significantly different ($P > 0.05$). The result agrees with the findings of Ntondini *et al*. (2008) who reported fully susceptible populations of *R. appendiculatus* against amitraz. However, emerging resistance to cypermethrin was observed in this tick in the same study. Similarly, no resistance to either amitraz or cypermethrin acaricides was detected in *A. variegatum* as LD_{50} and LD_{90} of reference and test populations were not significantly different ($P = 0.05$).

The results of the present study support the general notion that resistance development in ticks is not a universal phenomenon but is more common in the one-host tick species (Kunz and Kemp, 1994, Abdullah *et al*., 2012 and Abbas *et al*., 2014). This can be explained by the fact that multi-host ticks develop resistance more slowly as these ticks have longer generation times, less acaricidal exposure of immature stages and an availability of alternative hosts that reduces their overall exposure to acaricides (Kunz and Kemp, 1994, Jonejan and Uilenberg, 2004 and Walker *et al*., 2007). On the other hand one-host ticks are subjected to considerably high selection pressure at all parasitic stages, even in poorly implemented acaricide treatment regimes (Kunz and Kemp, 1994 and Abdullah *et al*., 2012).

From the results of the acaricide survey, the development of amitraz resistance in *R (Bo.) microplus* in the study area could be attributed to inadequate delivery of dip wash to cattle (Less than 2 litres instead of the recommended 10 litres of dip wash per animal) and to rampant erratic treatment regimes. Studies by Spickett and Fivaz (1992) and Mekonnen (2002) support this assertion as they reported the highest percentage of confirmed resistance among farmers that delivered inadequate amounts of acaricide spray washes in South Africa. Brito *et al*. (2011) also attributed the reduction in efficacy of several synthetic pyrethroid and amidine acaricides to inadequate spraying and under dosage.

The detection of the initial phase of amitraz resistance in *R (Bo.) microplus* in the study area implies that continued application of amitraz at practices reported by this study will lead to the further selection of resistant individuals, leading to a population with an increased resistance ratio (Roush and Mckenzie, 1987, Kunz and Kemp, 1994 and Brito *et al*., 2011). Such populations are difficult to control and the numbers of individuals surviving treatment are high enough to transmit diseases and significantly reduce productivity of cattle. Use of higher doses of amitraz may serve to eliminate heterozygous resistance individuals (Roush, 1993, Thullner *et al*., 2007 and Adakal *et al*., 2013) but will further increase the cost of cattle tick control. Ultimately, higher levels of resistance will require amitraz to be replaced by another chemical (George *et al*., 2004 and Abbas *et al*., 2014).

Previous studies by Luguru *et al*. (1987) detected dimethoate resistance in *R. (Bo.) decoloratus* and *R. appendiculatus*, and dioxathion resistance in *A. variegatum* in the Northern Province of Zambia, including Isoka District. As indicated by the results,

these organophosphate acaricides are no longer used for tick control in the study area, a fact that can be attributed to resistance development. Numerous examples of acaricide resistance rendering the use of various chemical acaricides ineffective due to resistance development occur in literature (George *et al*., 2004, Mendes *et al*., 2007, Enayati *et al*., 2010, Perez-Cogollo *et al*., 2010 and Abbas *et al*., 2014).

5.4 Acaricidal Properties of *Tephrosia vogelii* and *Bobgunnia madagascariensis*

The data collected from the plant extraction experiments indicated that large amounts of active ingredients were extracted from the leaf and bark material of *T. vogelii* than from the fruit material. These findings support the observations made by Matovu and Olila (2007) who tested the acaricidal activity of *T. vogelii* extracts on nymph and adult ticks in Busimbi Sub Country in Uganda. These researchers reported that the leaves and bark of this plant accumulate relatively high amounts of active ingredients than the roots. This is the reason why the leaves are preferred by many local farming communities who use *T. vogelii* for pest control (Gadzirayi *et al*., 2009 and Noudogbessi *et al*., 2012). The utilization of the leaves not only maintains the life of the plant but also ensures a sustainable harvest especially by farmers who propagate this plant (Ismail *et al*., 2002 and Dzenda *et al*., 2007).

With regards to *B. madagascariensis*, the plant extraction experiments showed that large amounts of active ingredients were extracted from the fruit than from the leaf and bark material. This finding supports those of Magalhaes *et al*. (2003) who reported molluscicidal activity only in the fruit and seed extracts of this plant thus demonstrating its pesticidal activity. This suggests that the fruits of these plant accumulate relatively higher amounts of active ingredient than the leaves and bark.

Free contact bioassays of both plants revealed that only methanol extracts of the leaves of *T. vogelii* and the fruits of *B. madagascariensis* produced a mortality of 100% in 24 hrs. These results corresponded with the highest levels methanol extracts from experimental acaricidal compared to the other two solvents. This observation was in agreement with Matovu and Olila (2007) who observed high acaricidal activity in methanol extracts of *T. vogelii* leaves. A study by Noudogbessi *et al*. (2012) also reported insecticidal activity in ethanol extracts of *T. vogelii* leaves. The results of this study suggest that the active ingredients of *T. vogelii* and *B. madagascariensis* were more soluble in the polar solvent methanol. This conclusion is supported by results presented by Mi-Kyeong *et al*. (2004) who found that the polar extracts of 28 medicinal plants were more effective against larvae of *Attagenus unicolor japonicus* (Japanese black carpet bettle) than less polar extracts of the same plants. It is for this reason that methanol extracts of these plant parts were selected for the testing and comparison of the acaricidal activity of the two plants in the topical assays in the present study.

In the topical assays, the values of the t-ratios for plants indicated significant regression at 95% confidence. It was observed that the mortality of adult *A. variegatum* ticks increased significantly with an increase in the concentration of methanol extracts of both *T. vogelii* and *B. madagascariensis*. This observation was consolidated by the fact that the heterogeneity factors obtained for the chi-square test of goodness of fit for the two plants (0.142 for *T. vogelii* and 0.007 for *B. madagascariensis*), indicated extremely good fit and was evidence of the tick genetic homogeneous susceptible response to the extracts of the two plants (Robertson *et al*., 2007 and Heong *et al*., 2010).

The likelihood ratio tests of equality and parallelism indicated that the regression lines obtained from *T. vogelii* and *B. madagascariensis* extracts were not equal and but were parallel. The slopes of the two lines were not significantly different at 95% confidence level. Since the slope of a probit regression line estimates the change in activity per unit change in dose or concentration (Robertson *et al*., 2007 and Heong *et al*., 2010), the obtained parallel lines suggest that the relative potency of the two extracts tested in the study was not significantly different (Robertson and Rappaport, 1979). The LD_{50} dose ratio of *T. vogelii* and *B. madagascariensis* at 95% confidence, confirmed this observation.

The LD_{50} of the two plant extracts were found to be significantly different with LD_{50} of *T. vogelii* (0.585) being higher than that of *B. madagascariensis* (0.031). This indicated that the toxicity of *B. madagascariensis* was higher than that of *T. vogelii*. Robertson *et al*. (2007) attributed to lower LD_{50} values to higher toxicity which means that the fruit extracts of *B. madagascariensis* have acceptable acaricidal activity.

The acaricidal activity of *T. vogelii* plant extract has also been demonstrated by the works of various researchers which include Kaposhi (1992), Matovu and Olila (2007) and Gadzirayi *et al*. (2009). The last named researchers went further to compare the effectiveness of *T. vogelii* with that of the conventional Triatix dip in the control of ticks on dairy animals among small scale dairy farmers in Mashonaland Central Province in Zimbabwe. The results of their study indicated that there was no significant difference in the effectiveness of *T. vogelii* and Triatix dip in controlling ticks (Gadzirayi *et al*., 2009). Adedote *et al*. (2011) reported larvicidal activity of the stem bark of *B.*

madagascariensis against *Culex quinquefasciatus* Say mosquitoes, which provided further evidence of the pesticidal properties of this plant against arthropods.

Results of this study and others (Opiro *et al*., 2010, Babar *et al*., 2012, Zaman *et al*., 2012 and Sola *et al*., 2014) indicate that a considerable amount of research has been undertaken to find alternative, more sustainable cost effective methods to control cattle ticks. Much of the research has focused on documenting and evaluating the acaricidal properties of various plants species (Habeeb, 2010, Abbas *et al*., 2014 and Sola *et al*., 2014), resulting in a vast number of plants being demonstrated to have significant acaricidal properties. However, as indicated by the results of this study, only 16.7% of farmers used plant material (*T. vogelii* in this case) to control cattle ticks. Moyo and Masika (2008) and Hlatshwago and Mbati (2005) found 6.8% and 0% of smallholder cattle farmers using plants to control ticks in the Easter Cape Province of South Africa. Surveys conducted in Malawi and Zambia (Kamanula *et al*., 2011; Nyrienda *et al*., 2011) indicated that although farmers were knowledgeable about plant materials used for pest control, their relative safety and cost effectiveness compared to synthetic pesticides, less than half of the small-scale farmers in Malawi and less than 20% in Zambia actually used them. This indicates that while information on the types of plants that can be used to control tick infestation on cattle is increasing, the adoption levels of ethno-tick-control method has currently remained low. There is need therefore, to enhance research on the optimal use of such plant materials as pesticides focusing on field efficacy, propagation, cultivation, processing and utilization especially among small scale farmers (Stevenson *et al*., 2012 and Sola *et al*., 2014).

There is also a need for the development of extension packages for veterinary extension workers and farmers on the use of various local plants for ethno-botanical tick control alternative strategies, such as the use of farmer field schools (Feder *et al*., 2004). In this approach, farmers learn the benefits of new technologies through self-discovery by field testing and participatory learning after which they decide what is the best alternative for their particular circumstance (Feder *et al*., 2004). It is hope that this learning by doing approach may enhance adoption of ethno-botanical alternative for tick control.

A counter argument to the use of indigenous plants in tick control being cost effective, easy and environmentally friendly, is that information about their efficacy and safety has not been adequately investigated (Stevenson *et al*., 2012; Belmain *et al*., 2012). In fact, some reports on certain pesticidal plants have actually shown acute mammalian toxicity (Nyahangare *et al*., 2012). Acute oral toxicities of *T. vogelii* leaves (Dzenda *et al*., 2007) and *B. madagascariensis* fruits (Nyahangare *et al*., 2012) have been reported. In both studies, very high dosages were employed compared to those recommended for effective tick control on cattle (Madzimure *et al*., 2013). This finding indicats that typical use of *T. vogelii* and *B. madagascariensis* is unlikely to expose farmers and their animals to toxic levels of these plant extracts. However, advice on the safe use of these plants should still accompany their promotion extension packages.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The major acaricides that were used by small holder farmers in the study area were amitraz marketed either as Triatix or Milbitraz and/or cypermethrin marketed as Cypdip. The dosages used by farmers were 20 ml of acaricide to 10 litres of spray wash for amitraz and 10 ml of acaricide to 10 litres of spray wash for cypermethrin, which were the recommended manufactures dosages. Less than 3 litres of spray wash was applied per animal which was considerably less than the recommended.

Acaricide resistance was detected in *Rhipicephalus* (*Boophilus*) *microplus* tested for amitraz resistance. However, the LD_{50} and LD_{90} resistance ratios were very low which indicated that resistance to amitraz in this tick was in its initial or emerging phase. No resistance to cypermethrin was detected in *Rhipicephalus* (*Boophilus*) *microplus*.

Acaricide resistance was not detected in either *Rhipicephalus appendiculatus* or *Amblyomma variegatum* against amitraz or cypermethrin. These ticks were found to be fully susceptible to both acaricides at recommended manufacturers' doses.

The plant extracts of both *Tephrosia vogelii* and *Bobgunnia madagascariensis* were observed to exhibit significant acaricidal activities against adult *Amblyomma variegatum* ticks. The relative toxicity of methanol leaf extracts of *Tephrosia vogelii* and of the fruit extracts of *Bobgunnia madagascariensis* were significantly different at 95% confidence level. Therefore, these plants provide a potentially suitable alternative to chemical tick control in the study area.

6.2 Recommendations

i. Farmers in the study area should use FAO recommended delivery rates of 10 litres of spray wash per animal and should strictly adhere to instructions on acaricide labels.

ii. The acaricide amitraz can still be used to control ticks in the study area. However, a slightly higher dose, 25 ml in 10 litres of water should be used in order to kill heterozygote resistance *Rhipicephalus* (*Boophilus*) *microplus* ticks. For cypermethrin, this acaricide can still be used at the manufacturers' recommended dose of 10 ml in 10 litres of spray wash.

iii. *Tephrosia vogelii* and *Bobgunnia madagascariensis* leaf and fruit extracts be recommended for inclusion in future tick control programmes.

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APPENDICES

Appendix A: Questionnaire

ACARICIDE USE IN TICK CONTROL SURVEY FORM

Preamble

The purpose of this survey is to assess the importance of ticks to the livestock sector in this region. The results of the survey will complement the findings of a larger project on the resistance status of ticks to the commonly used acaricides in Isoka District.

Your participation in this survey is extremely important because your information will provide a basis, if any, to look for alternative and sustainable strategies for tick and tick borne disease control in Isoka District.

Thank you for taking the time to participate in this survey.

Date.....

1.0 Identification details

District.....
Camp.....
Village.....
Reporting officer.....

2.0 Farmer details

Name (optional).....
Sex.....
Age.....
Level of education.....

3.0 Cattle population

Number of cows.....
Number of bulls.....
Number of young.....
Total number of cattle.....

4.0 Disease situation

4.1 What are the major cattle diseases that you face (list in order of importance)?

.....
.....
.....
.....

4.2 Do you think that ticks are a major problem in your farming operation (tick where appropriate)

a. Yes
b. No

5. 0 Tick control

5.1 Do you apply acaricides to control cattle ticks?

a. Yes
b. No

If yes, what is the name of the acaricide that you currently apply to your cattle?

.....

5.2 Have you used any other acaricides before?

a. Yes
b. No

If yes, name the acaricides below:

.....
.....
.....
.....

5.3 Do you follow the mixing instructions as recommended by the manufacturer?

a. Yes
b. No

If no, give reasons:.....
..
.....
.....
.....
.....

5.4 How often do you apply the acaricide?.....

How do you apply the chemical?

a. Spray
b. Dipping
c. Others
(specify).....
.....
.....

5.5 From your experience, are you succeeding in controlling ticks with the above method?

a. Yes
b. No

If no, why?.....
.....
.....

5.6 How much does tick control by acaricides cost you per year?.....

5.7 Is the cost of acaricide fair?

a. Yes
b. No

5.8 What other tick control methods do you undertake if any? (Elaborate).....

.....

5.9 Do you believe that this method is effective?

a. Yes
b. No

5.10 In your view, how should tick control be done in Isoka district? (Explain)

.....
.....
.....

THANK YOU FOR YOUR TIME

Appendix B: POLOPLUS 2.0 Outputs for Amitraz Resistance Tests

(a) *Rhipicephalus (Boophilus) microplus* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference population
Estimating natural response

	parameter	standard error	t ratio
Baseline	35.882	5.575	6.436
NATURAL	0.111	0.033	3.354
SLOPE	17.963	2.808	6.398

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	100.	45.	53.98	-8.978	0.540	-1.801
	0.010	85.	55.	45.88	9.119	0.540	1.984
	0.013	70.	67.	67.20	-0.204	0.960	-0.125
	0.015	90.	90.	89.93	0.073	0.999	0.270
	0.025	80.	80.	80.00	0.000	1.000	0.000
	0.250	70.	70.	70.00	0.000	1.000	0.000
NATURAL		90.	10.	10.01	-0.014	0.111	-0.005

chi-square: 7.2710 degrees of freedom: 4 heterogeneity: 1.8177

Effective Doses

		dose	limits	0.95
LD50	Baseline	0.010	lower	0.009
			upper	0.011
LD90	Baseline	0.012	lower	0.011
			upper	0.015

Intercepts and slopes unconstrained. Preparation is (2) Test population
Estimating natural response

	parameter	standard error	t ratio
Test	13.186	1.505	8.762
NATURAL	0.033	0.019	1.764
SLOPE	6.815	0.788	8.652

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	75.	20.	26.31	-6.313	0.351	-1.527
	0.010	60.	27.	21.05	5.950	0.351	1.610
	0.013	70.	38.	41.97	-3.970	0.600	-0.968
	0.015	90.	76.	70.45	5.548	0.783	1.418
	0.025	88.	86.	87.01	-1.009	0.989	-1.019
	0.250	78.	78.	78.00	0.000	1.000	0.000
NATURAL		90.	3.	2.95	0.052	0.033	0.031

chi-square: 8.9129 degrees of freedom: 4 heterogeneity: 2.2282

Effective Doses

		dose	limits	0.95
LD50	Test	0.012	lower	0.010
			upper	0.013
LD90	Test	0.018	lower	0.015
			upper	0.026

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): REJECTED (P<0.05)
(chi-square: 64.33, degrees of freedom: 2, tail probability: 0.000)

HYPOTHESIS OF PARALLELISM (equal slopes): REJECTED (P<0.05)
(chi-square: 23.58, degrees of freedom: 1, tail probability: 0.000)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.866	lower	0.812
		upper	0.923

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.662	lower	0.591
		upper	0.742

(b) *Rhipicephalus appendiculatus* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference Population
Estimating natural response

	parameter	standard error	t ratio
Baseline	21.628	3.073	7.037
NATURAL	0.158	0.044	3.614
SLOPE	10.845	1.586	6.838

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	60.	27.	33.49	-6.485	0.558	-1.686
	0.010	55.	38.	30.69	7.305	0.558	1.983
	0.013	100.	85.	86.41	-1.408	0.864	-0.411
	0.015	95.	93.	92.41	0.588	0.973	0.371
	0.025	70.	70.	70.00	0.001	1.000	0.025
	0.250	80.	80.	80.00	0.000	1.000	0.000
NATURAL		70.	11.	11.07	-0.070	0.158	-0.023

chi-square: 7.0836 degrees of freedom: 4 heterogeneity: 1.7709

Effective Doses

		dose	limits	0.95
LD50	Baseline	0.010	lower	0.008
			upper	0.011
LD90	Baseline	0.013	lower	0.012
			upper	0.016

Intercepts and slopes unconstrained. Preparation is (2) Test Population
Estimating natural response

	parameter	standard error	t ratio
Test	16.675	2.242	7.438
NATURAL	0.050	0.024	2.053
SLOPE	8.400	1.152	7.293

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	85.	38.	40.59	-2.592	0.478	-0.563
	0.010	90.	45.	42.98	2.021	0.478	0.426
	0.013	100.	78.	76.68	1.325	0.767	0.313
	0.015	75.	68.	68.74	-0.738	0.917	-0.308
	0.025	50.	50.	49.97	0.031	0.999	0.175
	0.250	65.	65.	65.00	0.000	1.000	0.000
NATURAL		80.	4.	3.99	0.012	0.050	0.006

chi-square: 0.722 degrees of freedom: 4 heterogeneity: 0.181

Effective Doses

		dose	limits	0.95
LD50	Test	0.010	lower	0.010
			upper	0.011
LD90	Test	0.015	lower	0.014
			upper	0.016

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): NOT REJECTED (P>0.05) (chi-square: 4.23, degrees of freedom: 2, tail probability: 0.121)

HYPOTHESIS OF PARALLELISM (equal slopes): NOT REJECTED (P>0.05)
(chi-square: 1.63, degrees of freedom: 1, tail probability: 0.202)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.979	lower	0.904
		upper	1.060

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.905	lower	0.815
		upper	1.004

(c) *Amblyomma variegatum* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference Population
Estimating natural response

	parameter	standard error	t ratio
Baseline	17.686	2.683	6.593
NATURAL	0.043	0.024	1.770
SLOPE	8.674	1.369	6.336

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	80.	44.	51.83	-7.830	0.648	-1.833
	0.010	90.	67.	58.31	8.691	0.648	1.918
	0.013	100.	87.	88.57	-1.572	0.886	-0.494
	0.015	90.	88.	87.32	0.679	0.970	0.421
	0.025	80.	80.	79.99	0.006	1.000	0.076
	0.250	70.	70.	70.00	0.000	1.000	0.000
NATURAL		70.	3.	3.01	-0.011	0.043	-0.007

chi-square: 7.4658 degrees of freedom: 4 heterogeneity: 1.8664

Effective Doses

		dose	limits	0.95
LD50	Baseline	0.009	lower	0.007
			upper	0.010
LD90	Baseline	0.013	lower	0.012
			upper	0.017

Intercepts and slopes unconstrained. Preparation is (2) Test population
Estimating natural response

	parameter	standard error	t ratio
Test	17.052	2.492	6.844
NATURAL	0.100	0.034	2.983
SLOPE	8.554	1.283	6.668

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	75.	30.	39.73	-9.729	0.530	-2.251
	0.010	70.	47.	37.08	9.919	0.530	2.375
	0.013	90.	72.	72.18	-0.185	0.802	-0.049
	0.015	75.	70.	70.03	-0.034	0.934	-0.016
	0.025	80.	80.	79.97	0.029	1.000	0.171
	0.250	70.	70.	70.00	0.000	1.000	0.000
NATURAL		80.	8.	8.01	-0.012	0.100	-0.005

chi-square: 10.741 degrees of freedom: 4 heterogeneity: 2.6852

Effective Doses

		dose	limits	0.95
LD50	Test	0.010	lower	0.007
			upper	0.011
LD90	Test	0.014	lower	0.013
			upper	0.024

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): REJECTED (P<0.05)
(chi-square: 11.26, degrees of freedom: 2, tail probability: 0.003)

HYPOTHESIS OF PARALLELISM (equal slopes): NOT REJECTED (P>0.05)
(chi-square: 0.00, degrees of freedom: 1, tail probability: 0.949)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.901	lower	0.817
		upper	0.992

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.896	lower	0.805
		upper	0.998

Appendix C: POLOPLUS 2.0 Outputs for Cypermethrin Resistance Tests

(a) *Rhipicephalus (Boophilus) microplus* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference population
Estimating natural response

	parameter	standard error	t ratio
Baseline	3.715	0.340	10.929
NATURAL	0.120	0.043	2.813
SLOPE	2.176	0.228	9.562

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	60.	20.	21.06	-1.065	0.351	-0.288
	0.010	85.	32.	29.84	2.158	0.351	0.490
	0.050	90.	74.	75.11	-1.106	0.835	-0.314
	0.100	75.	69.	70.92	-1.922	0.946	-0.979
	0.150	80.	80.	78.08	1.917	0.976	1.402
	1.500	70.	70.	70.00	0.001	1.000	0.036
NATURAL		60.	7.	7.21	-0.207	0.120	-0.082

chi-square: 3.353 degrees of freedom: 4 heterogeneity: 0.838

Effective Doses

		dose	limits	0.95
LD50	Baseline	0.020	lower	0.014
			upper	0.025
LD90	Baseline	0.076	lower	0.059
			upper	0.103

Intercepts and slopes unconstrained. Preparation is (2) Test population
Estimating natural response

	parameter	standard error	t ratio
Test	3.548	0.434	8.178
NATURAL	0.147	0.048	3.036
SLOPE	2.374	0.373	6.363

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	70.	19.	17.18	1.824	0.245	0.507
	0.010	60.	16.	14.72	1.278	0.245	0.383
	0.050	70.	48.	50.73	-2.726	0.725	-0.729
	0.100	60.	50.	53.85	-3.852	0.898	-1.640
	0.150	85.	85.	80.96	4.035	0.953	2.058
	1.500	80.	80.	80.00	0.002	1.000	0.050
NATURAL		70.	9.	10.30	-1.298	0.147	-0.438

chi-square: 8.0554 degrees of freedom: 4 heterogeneity: 2.01

Effective Doses

		dose	limits	0.95
LD50	Test	0.032	lower	0.006
			upper	0.055
LD90	Test	0.111	lower	0.068
			upper	0.285

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): REJECTED (P<0.05)
(chi-square: 10.25, degrees of freedom: 2, tail probability: 0.006)

HYPOTHESIS OF PARALLELISM (equal slopes): NOT REJECTED (P>0.05)
(chi-square: 0.35, degrees of freedom: 1, tail probability: 0.555)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.612	lower	0.386
		upper	0.971

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.686	lower	0.466
		upper	1.009

(b) *Rhipicephalus appendiculatus* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference population
Estimating natural response

	parameter	standard error	t ratio
Baseline	3.202	0.293	10.910
NATURAL	0.105	0.033	3.147
SLOPE	1.865	0.191	9.789

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	90.	32.	33.51	-1.512	0.372	-0.330
	0.010	75.	33.	27.93	5.073	0.372	1.212
	0.050	80.	58.	64.32	-6.316	0.804	-1.779
	0.100	70.	63.	64.32	-1.321	0.919	-0.578
	0.150	90.	90.	86.14	3.861	0.957	2.009
	1.500	80.	80.	79.99	0.015	1.000	0.122
NATURAL		90.	9.	9.45	-0.454	0.105	-0.156

chi-square: 9.1487 degrees of freedom: 4 heterogeneity: 2.2872

Effective Doses

		dose	limits	0.95
LD50	Baseline	0.019	lower	0.009
			upper	0.032
LD90	Baseline	0.093	lower	0.055
			upper	0.248

Intercepts and slopes unconstrained. Preparation is (2) Test Population
Estimating natural response

	parameter	standard error	t ratio
Test	3.132	0.284	11.013
NATURAL	0.032	0.022	1.428
SLOPE	1.962	0.186	10.565

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	60.	12.	14.36	-2.363	0.239	-0.715
	0.010	90.	27.	21.54	5.455	0.239	1.348
	0.050	80.	52.	58.24	-6.241	0.728	-1.568
	0.100	60.	51.	52.98	-1.978	0.883	-0.795
	0.150	70.	70.	65.61	4.388	0.937	2.164
	1.500	85.	85.	84.98	0.021	1.000	0.144
NATURAL		70.	2.	2.22	-0.215	0.032	-0.147

chi-square: 10.142 degrees of freedom: 4 heterogeneity: 2.5355

Effective Doses

	dose	limits	0.95
LD50 Test	0.025	lower	0.014
		upper	0.040
LD90 Test	0.114	lower	0.067
		upper	0.321

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): NOT REJECTED (P>0.05) (chi-square: 3.53, degrees of freedom: 2, tail probability: 0.171)

HYPOTHESIS OF PARALLELISM (equal slopes): NOT REJECTED (P>0.05)
(chi-square: 0.15, degrees of freedom: 1, tail probability: 0.703)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.759	lower	0.536
		upper	1.073

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.820	lower	0.530
		upper	1.269

(c) *Amblyomma variegatum* larval packet test bioassay.

Intercepts and slopes unconstrained. Preparation is (1) Reference population
Estimating natural response

	parameter	standard error	t ratio
Baseline	3.422	0.332	10.296
NATURAL	0.102	0.032	3.158
SLOPE	1.877	0.200	9.393

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Baseline	0.010	90.	32.	39.11	-7.108	0.435	-1.511
	0.010	80.	44.	34.76	9.237	0.435	2.083
	0.050	75.	60.	63.99	-3.995	0.853	-1.304
	0.100	70.	66.	66.16	-0.159	0.945	-0.083
	0.150	70.	70.	68.09	1.906	0.973	1.400
	1.500	80.	80.	79.99	0.006	1.000	0.079
NATURAL		90.	9.	9.21	-0.206	0.102	-0.072

chi-square: 10.303 degrees of freedom: 4 heterogeneity: 2.5757

Effective Doses

	dose	limits	0.90	0.95	0.99
LD50	Baseline	0.015	lower	0.008	0.007
		upper	0.023	0.025	
LD90	Baseline	0.072	lower	0.046	0.041
		upper	0.157	0.234	

Intercepts and slopes unconstrained. Preparation is (2) Test popuation
Estimating natural response

	parameter	standard error	t ratio
Test	3.490	0.325	10.739
NATURAL	0.125	0.035	3.537
SLOPE	1.991	0.207	9.610

Chi-squared goodness of fit test

prep	dose	n	r	expected	residual	probab	std resid
Test	0.010	80.	28.	31.79	-3.787	0.397	-0.865
	0.010	75.	35.	29.80	5.200	0.397	1.227
	0.050	90.	74.	75.50	-1.495	0.839	-0.429
	0.100	60.	54.	56.48	-2.484	0.941	-1.366
	0.150	90.	90.	87.46	2.536	0.972	1.615
	1.500	80.	80.	80.00	0.004	1.000	0.066
NATURAL		90.	11.	11.22	-0.218	0.125	-0.070

chi-square: 6.9216 degrees of freedom: 4 heterogeneity: 1.7304

Effective Doses

		dose	limits	0.95
LD50	Test	0.018	lower	0.009
			upper	0.027
LD90	Test	0.078	lower	0.049
			upper	0.165

HYPOTHESIS OF EQUALITY (equal slopes, equal intercepts): NOT REJECTED (P>0.05) (chi-square: 1.02, degrees of freedom: 2, tail probability: 0.599)

HYPOTHESIS OF PARALLELISM (equal slopes): NOT REJECTED (P>0.05)
(chi-square: 0.17, degrees of freedom: 1, tail probability: 0.682)

Lethal dose ratio (LD50)

	ratio	limits	0.95
Test	0.851	lower	0.585
		upper	1.238

Lethal dose ratio (LD90)

	ratio	limits	0.95
Test	0.930	lower	0.599
		upper	1.445