Molecular detection of acaricide resistance in *Rhipicephalus microplus* in Engcobo Local Municipality, Eastern Cape, South Africa


By


Jaison Zanga

MSc Tropical Animal Health


2018


Supervisor: Dr. JE Crafford

Co-supervisor: Prof. C Maritz-Olivier

## Declaration

I Jaison Zanga declare that that this thesis which I submit to the University of Pretoria for the degree M. Sc (Tropical Animal Health) is an original report of my research, has been written by me and has not been submitted for any other degree or professional qualification.

Genomic DNA purification and polymerase chain reaction amplification of resistance associated genes were carried out by Anna-Mari Bosman at the Department of Veterinary Tropical Diseases Research and Training Laboratories. Other contributions have been referenced in the text.

Signature…………………………..  Date……………………………

# Acknowledgements

The successful completion of this research would not have been possible without:

# Table of Contents

# List of tables

# List of figures

## Abbreviations

AIT        Adult immersion test

AMR        Antimicrobial resistance

bp        base pairs

C        cytosine

CAHWs  Community Animal Health Workers

DDT        dichlorodiphenyltrichloroethane

DNA        Deoxyribonucleic acid

dNT        deoxynucleoside triphosphates

EDTA        ethlenediaminetetraacetic acid

ETS        external transcribed spacer

FAO        Food and Agricultural Organisation

G        guanine

GST        glutathione-S-transferase

HCl        hydrochloric acid

ITS1        first internal transcribed spacer

ITS2        second internal transcribed spacer

LM        Local municipality

OCT/Tyr Octopamine/tyramine

PCR  polymerase Chain reaction

PCR-RFLP Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

RAPD  Random Amplified Polymorphic DNA

RFLP  Restriction Fragment Length Polymorphism

RNA  Ribonucleic acid

SNPs  Single Nucleotide Polymorphisms

SP  synthetic pyrethroids

T  thymine

VGS  voltage-gated sodium channel

# Molecular detection of acaricide resistance in *Rhipicephalus microplus* in Engcobo Local Municipality, Eastern Cape, South Africa

By

Jaison Zanga

Degree: MSc Tropical Animal Health

Supervisor: Dr. JE Crafford

Co-supervisor: Prof. C Maritz-Olivier

## Abstract

*Rhipicephalus microplus* transmit two important diseases of livestock in South Africa, namely *Babesiosis* and *Anaplasmosis*. As cattle still play a key role in the livelihood of rural communities in the Eastern Cape province, animal health is of utmost importance to maintain and strengthen these communities. In most rural areas of the Eastern Cape, the South African government provides free dipping of cattle with amitraz. Deltamethrin, a synthetic pyrethroid (SP), has been used as pour-on dip in a few locations where there are no functional dips. Of concern, is the growing number of global reports on the increase of acaricide resistance. Routine screening for resistance to acaricides is therefore needed. In this study we investigated allele frequencies of acaricide resistance-associated single nucleotide polymorphisms (SNPs) in *R. microplus* ticks from the Engcobo Local Municipality. The estimated frequencies of amitraz resistance-associated SNPs were 0.58 (at locus 1) and 0.32 (at locus 3). Resistance against formamidines (amitraz) appears to be on the rise. The published mutation in the voltage gated sodium channel (VGS) receptor gene (domain II segment 4-5 region), known to confer resistance to synthetic pyrethroids, was not found. This could be attributed to the low selection pressure against pyrethroids in the study area.

# Chapter 1

## General introduction

### 1.1    Background

Ticks are haematophagous ecto-parasites of wild and domestic animals as well as humans. Ticks that belong to the family Ixodidae (also known as hard ticks) are important vectors of diseases of both animals and humans (Kiss, Cadar & Spinu 2012). Disease causing organisms transmitted by these ixodid ticks include protozoans, ricketsiae, spirochetes and viruses, most of which are of great economic importance worldwide (Jongejan & Uilenberg 2004, Rajput *et al.* 2006) The most important diseases transmitted to ruminants are babesiosis, theilerioses, anaplasmoses and cowdriosis (Jongejan & Uilenberg 2004), causing major drawbacks on livestock rearing in many parts of the world. Developing countries in the tropical and sub-tropical regions experience the most impact due to the high prevalence of tick vectors and suitable habitats.

Ticks have both direct and indirect effects on their hosts. Direct effects are mostly anemia and reduced weight gain (Jongejan & Uilenberg 2004, Rajput *et al.* 2006). Heavy infestation with *Rhipicephalus microplus* has even been shown to reduce the value of cattle hides by up to 20-30% (Kiss *et al.* 2012). Severe dermatitis can also result from heavy tick infestations (Rajput *et al.* 2006)**.** Indirectly, abscesses due to secondary bacterial infection form when ticks with large hypostomes (such as *Amblyoma* species) feed on animals (Jongejan & Uilenberg 2004). This may result in lameness or loss of teats depending on attachment site.

The burden of control and eradication of ticks is currently hinged on the use of acaricides which are considered to be cheap and quick in reducing tick numbers (Abbas *et al.* 2014). Some of the most predominant acaricides currently in use include formamidines (e.g. amitraz), organophosphates, cyclodienes (also known as organochlorides), macrocyclic lactones, carbamates and synthetic pyrethroids**.** Major setbacks have been reported

with long-term use of acaricides. These include selection for acaricide resistance in many tick species, environmental contamination and acaricide residue contamination of milk and meat (Taylor 2001). Tick resistance against a range of acaricides has become a concern globally (Dantas-Torres 2008) owing to the cost and prolonged period normally required to produce a new chemical product for the control of ticks (Graf *et al.* 2004).

Cattle dipping in the communal areas of the Eastern Cape Province is a free service provided by the South African government through the state veterinary services. Amitraz has been used almost exclusively for several years in the Eastern Cape province which could have resulted in the development of amitraz resistance, as reported globally for areas using a similar strategy of a single active ingredient over prolonged periods of time (Abbas *et al.* 2014). This situation is exacerbated by incorrect concentrations of the active ingredient being applied under field conditions, as it remains difficult to control. Under these conditions, the possible use of sub-therapeutic chemical concentrations could have selected for resistant ticks. Community animal health workers (CAHWs) employed under veterinary services in the province have the responsibility of mixing acaricides at every dipping, according to clause 7.6.2 of the dipping policy (Eastern Cape Province Department of Rural Development and Agrarian Reform 2013). However, there are not sufficient CAHWs for Engcobo Local Municipality, leaving the role of mixing of acaricides to dipping committee members, hence no records of accurate acaricide concentrations are available. The absence of a systematic way of recording dip strengths in the Eastern Cape has been reported in a previous study (Brown, Ainslie & Beinart 2013). Currently, there is a growing perception amongst local farmers that ticks do not die following plunge dipping of their cattle, which could be indicative of amitraz resistance development in the Eastern Cape (Brown *et al.* 2013).

The dipping policy of the Eastern Cape makes provision for a state veterinarian to check and monitor tick resistance to acaricides that are used. The last study conducted in 2008 pointed towards resistance to some acaricides in the Eastern Cape, based on the conventional larval bioassay methods (Ntondini, van Dalen, E. M. S. P. & Horak 2008). As with the latter case, most routine detection methods to date have relied on time-consuming resistant tests such as larval pocket tests that can take up to 3 months.

However, in the post-genomic era, screening for mutations associated with acaricide resistance offers an alternative, rapid test with a turn-around time of days.

## 1.2    Objectives of the study

This study focussed on the detection of SNPs associated with acaricide-resistant genes in *R. microplus* ticks from the Eastern Cape to provide updated information on the resistance status towards amitraz and pyrethroids, and to make recommendations to the current dipping practice(s).

# Chapter 2

## Literature review

### 2.1    Introduction

Ticks as well as mites belong to the order Acari with ticks further classified into the sub-order Ixodida, which encompasses three families of ticks namely the Ixodidae, Argasidae and Nuttalliellidae (Walker 2003). Ticks are invertebrates, and as such they possess an exoskeleton, which is a hard outer covering to which muscles attach. The exoskeleton contains and safeguards organs such as the salivary glands, gut and the reproductive organs.

Ticks are bilaterally symmetrical, segmented and have jointed legs (Sonenshine, Lane & Nicholson 2002). The structure of a tick basically consists of the capitulum (gnathosoma) and the body (idiosoma) to which the legs are attached (Sonenshine *et al.* 2002). The capitulum is composed of the *basis capituli* which articulates with the body, two four-segmented *palps*, two segmented tubular *chelicerae* located medially to the palps and the toothed hypostome (Anderson & Magnarelli 2008). The capitulum of ixodid ticks is located at the cranial end of the body. The chelicerae facilitate the attachment of ticks to the host by cutting in to tissues while the hypostome functions as a holdfast organ and food canal (Anderson & Magnarelli 2008). The body lies posterior to the capitulum. Jointed legs and the genital pore are situated on anterior part *podosoma* of the body and spiracles and anus at the posterior *opisthosoma* (Balashov 1972). In hard ticks, females have a hard cuticular surface or scutum on the anterior half of the dorsal body surface. In males the scutum occupies almost the entire dorsal surface. During feeding, the female engorges enormously because the cuticle, with the exception of the scutum, expands to accommodate the ingested blood meal (Anderson & Magnarelli 2008). Males engorge slightly upon feeding due to the scutum that limits expansion (Sonenshine *et al.* 2002).  The legs are basically used for locomotion. Larval ticks have six legs, while nymphs and adults have eight legs. The Haller's organ situated on the tarsus of the first leg detects temperature, air currents, odours, and chemicals (Anderson & Magnarelli

2008). Internal organs are bathed in haemolymph rich in salts, amino acids, soluble proteins and haemocytes (Sonenshine *et al.* 2002). Prominent internal organs include the midgut, salivary glands, reproductive organs and the central nervous system (CNS). The tracheal system connects to the exterior via the paired spiracles for respiration (Sonenshine *et al.* 2002). The central nervous system is composed of a fused structure called the synganglion located antero-ventrally above the genital pore. Through nerve innervation, the structure regulates the legs, palps, chelicerae, cuticular sensilla and internal organs (Sonenshine *et al.* 2002).

Generally, the life cycle of ticks consists of four stages of development namely eggs, larvae, nymphs and adults (Figure 1). Larvae hatch from eggs and feed before they molt to become nymphs. Similarly, nymphs feed before molting to become adults. Fully engorged adult ticks mate and female ticks detach from host to lay a single batch of eggs (Walker 2003). Ixodid ticks can be classified as one-, two- or three-host ticks depending on the number of hosts they need to complete their life cycle. In the one-host tick cycle, larvae, nymphs and adults all attach to and develop on a single host. Larvae and nymphs feed on a single host and adults parasitize a second host in the two-host cycle. In the three-host cycle, larvae, nymphs and adults each parasitize a different host (Sonenshine *et al.* 2002) (Figure 2-1).
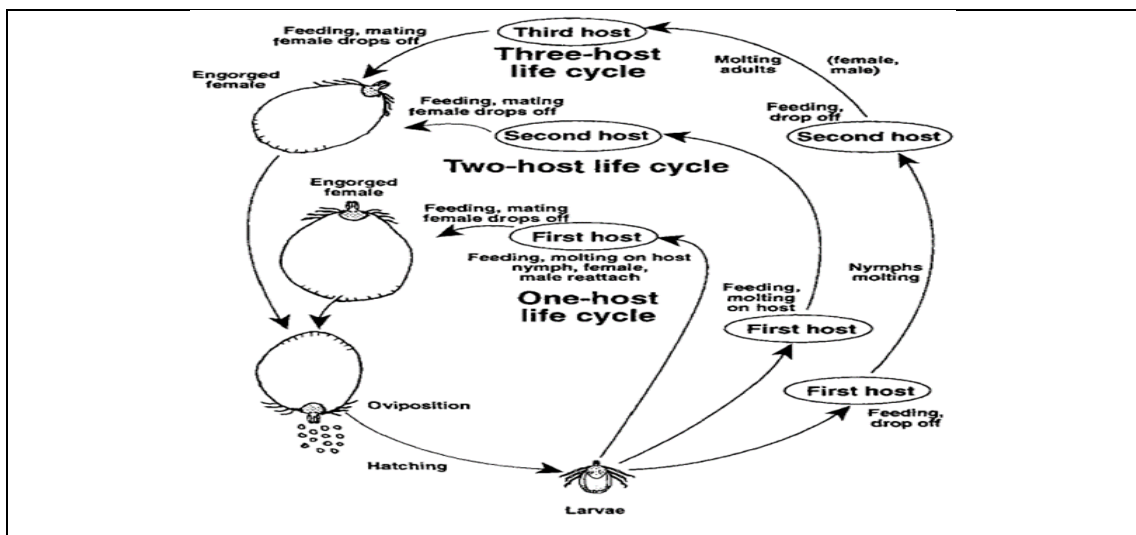


Figure 2-1: A schematic presentation of one- two and three-host tick lifecycles. Taken from *(Sonenshine* et al. *2002)*

Most tick species are adapted to seasonal variations in climate within a geographical area (Walker 2003). Generally ticks are more active during the rainy season when humidity is high (Sonenshine *et al.* 2002). Winter is generally dry and unconducive for most tick species to thrive, particularly for questing larvae which can succumb easily to drying out (Walker 2003). Diapause is a mechanism that allows ticks to survive through unfavourable environmental conditions until conditions become conducive. Ticks become inactive, reduce their metabolic rates and avoid feeding on hosts during this period (Sonenshine *et al.* 2002). An example is *R. appendiculatus* in southern Africa which enters diapause during the dry winter season as unfed adult ticks after they have hardened after emergence from the engorged nymphal stage (Randolph 2004).

## 2.2    Molecular/Genetic markers

Molecular markers also known as genetic markers are utilised in many genetics studies owing to their ability to reveal polymorphisms at the DNA level (Raza, Farooqi & Mubeen 2015). They are identifiable DNA sequences present at specific locations of the genome (Yang *et al.* 2013, Al-Samarai & Al-Kazaz 2015). Variations in DNA sequences may have the potential to cause a variation in phenotype (trait) under some conditions (Hayes *et al.* 2007, Al-Samarai & Al-Kazaz 2015). Examples of the later include changes in amino acid sequences (located in the protein coding DNA sequences), or changes in gene expression profile when mutations occurr in intron regions. These genetic markers can occur in both genomic and mitochondrial DNA and can be detected by a number of technologies. These include restricted fragment length polymorphism analyses (RFLPs), random amplified polymorphic DNA assays (RAPDs), amplified fragment length polymorphisms (AFLPs) and DNA sequencing to detect microsatellites and single nucleotide polymorphisms (SNPs) (Al-Samarai & Al-Kazaz 2015).

### 2.2.1    *Single Nucleotide Polymorphisms (SNPs)*

SNPs refer to a sequence polymorphism as a result of a single nucleotide mutation at a specific locus in the DNA sequence (Yang *et al.* 2013). SNPs are bi-allelic by nature, and can therefore yield homozygous and/or heterozygous individuals. Examples of SNPs

includes single base transitions, transversions, insertions and deletions of which transitions are the most common (Vignal *et al.* 2002). Transitions are switches of purine to purine (A to G, or vice versa) or pyrimidine to pyrimidine (C to T, or vice versa) whereas purine to pyrimidine switches are termed transversions (Vignal *et al.* 2002). SNPs are found in both coding and non-coding regions of genomes and contribute greatly to the process of creating population diversity and species evolution (Syvänen 2001). SNPs found in the coding regions of DNA can affect protein function directly if they are non-synonymous as this will cause a change in amino acid sequence (Koopaee & Koshkoiyeh 2014); whilst others are termed synonymous mutations, where the amino acid sequence is not altered. For this study, non-synonymous mutations are important as these have been shown to be linked to changes in protein targets of acaricides (such as the voltage-gated sodium channel for pyrethroids) (see section 2.3.2).

## 2.2.2    *Rhipicephalus cattle ticks*

*Rhipicephalus microplus* and *R. decoloratus* are one host species with similar life cycles completed entirely on the host (see Figure 2-1). They feed mainly on cattle but horses, sheep and goats may also serve as hosts (Sonenshine *et al.* 2002).

The parasitic life cycle of *R. decoloratus* can be completed in approximately 21 to 23 days with larva moulting after 6-8 days on the host and the succeeding nymph moulting after a further 7-9 days (Arthur & Londt 1973).  The parasitic life cycle of *R. microplus* can also be concluded in approximately 21 days with a majority of fully engorged females detaching 23–27 days after initial infestation (Londt & Arthur 1975). Once engorged and fertilized, adult female ticks drop off the host to oviposit (Cortinas & Jones 2006) with *R. microplus* female ticks laying slightly more eggs (3160 ± 73 eggs) on average than *R. decoloratus* female ticks (2974 ± 98 eggs) as demonstrated in a study using 20 engorged females (Spickett & Malan 1978).

```
┌─────────────────────────────────────────────┐
│                Host animal                   │
├─────────────────────────────────────────────┤
│   Larvae   moult Nymphs   moult    Adults    │
└─────────────────────────────────────────────┘
     ↑                                    ↓

Egg  ──→  Larvae                   Female  ──→  Egg
```
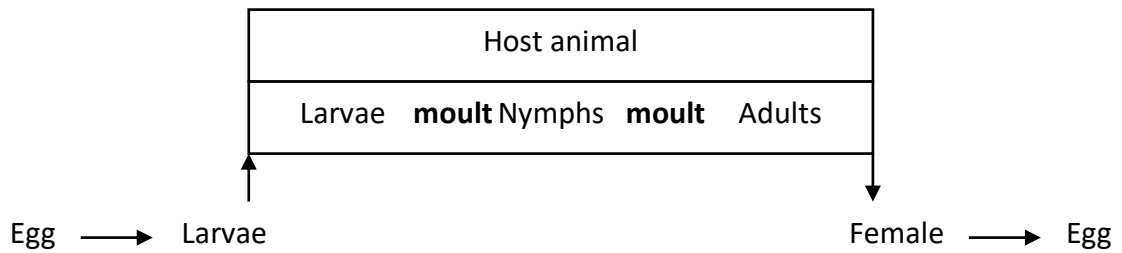
Figure 2-2: A schematic representation of a life cycle of one host ticks R. microplus and R. decoloratus.

The incubation period of the eggs depends to a greater extent on environmental temperature for both tick species. The period varies from between 30 and 140 days for *R. microplus*. The range is between 40 and 138 days for *R. decoloratus* (Spickett & Heyne 1990). Thus under conducive warm and humid conditions the whole life cycle can be completed in approximately 2 months for both spp., and more than one generation can be completed annually (Spickett & Heyne 1990).

*R. microplus* also known as the pan-tropical blue tick or the Asiatic blue tick has a wide geographical distribution (Horak Ivan G 2018). The tick originated from Asia but cattle transportation resulted in the spread of the tick and diseases transmitted by it to countries and regions such as Australia, Madagascar, South Africa, Latin America, Mexico and the United States (Hoogstraal 1956). *R. microplus* is also present in Swaziland, Zimbabwe, southern Mozambique (Horak, I. G. *et al.* 2009); Zambia and Madagascar (Berkvens *et al.* 1998).
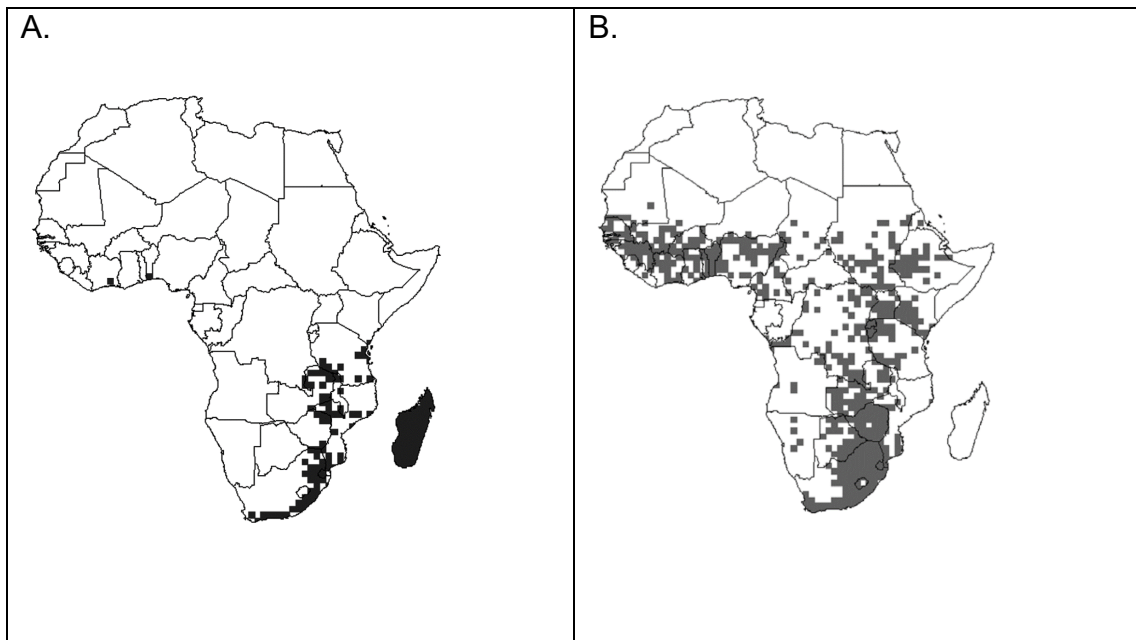
Figure 2-3: Distribution of R. microplus (A) and R. decoloratus (B) in Africa (Walker 2003)

*R. decoloratus* also known as the African blue tick is confined to Africa (Jongejan & Uilenberg 2004). It is one of the most known ticks in Africa (Horak Ivan G 2018). It ranges from southern Africa to the Sahara favouring tropical and subtropical grassland, savannah and shrubland ecosystems (Walker 2003). It is among the four most important indigenous tick species of cattle and other livestock in South Africa along with *Amblyoma hebraeum*, *R. appendiculatus and Ixodes rubicundus* (Horak, I. G. *et al.* 2009).

*R. decoloratus* and *R. microplus* are efficient vectors of *Babesia bigemina and A. marginale*, the causative organisms of African redwater and, anaplasmosis also known as gall sickness in cattle (Vos, Waal & Jackson 2004). *B. bigemina* can be transmitted transovarially by both *R. decoloratus* and *R. microplus* while *A. marginale* is transmitted transstadially by both tick spp. (Horak, Ivan G. *et al.* 2018). *R. microplus* is the only tick in southern Africa able to transmit the more virulent *B. bovis,* the causative agent for Asiatic redwater in cattle (Vos *et al.* 2004). Estimated economic losses of 70 to 200 million rands per annum were once attributed to *B. bovis, B. bigemina* and *A. marginale* infections in South Africa (Bigalke 1980). Both *R. decoloratus* and *R. microplus* also transmit *Borrelia theileri*, the causative agent of spirochaetosis in cattle (Horak, Ivan G. *et al.* 2018).

The invasive success of *R. microplus* has allowed it to displace *R. decoloratus* in certain localities in various countries including South Africa. This apparently has been attributed to the shorter life cycle of *R. microplus*, in addition to its tendency for positive associative mating and more efficient feeding on cattle (Tonnesen *et al.* 2004). Although the tick was not considered endemic in South Africa, it is now well established (Horak, I. G. *et al.* 2009).

Identification of *Rhipicephalus* ticks using conventional methods of comparing morphological characteristics is extremely cumbersome owing to the limited and variable differences among the species (Lempereur *et al.* 2010). A PCR-RFLP test that distinguishes species based on differences in nucleotide sequences on a part of the ITS2 segment was developed to alleviate the challenge (Lempereur *et al.* 2010, Barker 1998).

## 2.3    Use of acaricides in domestic animals

The use of chemical acaricides is the primary means of tick control in cattle (Abbas *et al.* 2014). The first acaricides were arsenic based, followed by organochlorines such as dichlorodiphenyltrichloroethane (DDT) and benzene hexachloride (Kunz & Kemp 1994). Organo-phosphates were then introduced to control organochlorine-resistant *Boophilus* (now *Rhipicephalus*) spp in the tropics and sub-tropics (Shaw, Thompson & Baker 1967). During the 1970's the formamidines were introduced which included Amitraz (Roy-Smith 1976). Pyrethroids were introduced around 1949 and permethrin and fenvelerate, the third generation pyrethroids soon became unpopular due to cross resistance with DDT (Nolan 1979, Coetzee, Stanford & Davis 1987). Cypermethrin and deltamethrin, the fourth generation pyrethroids are currently in use as acaricides among others. Acaricides are administered/applied to animals using various methods.

Plunge dipping entails wetting of animals in a dipping tub containing chemical solutions through immersion. Plunge dips or dipping vats or dipping tanks were already in use by 1893 in Australia, Africa and the United States of America (Matthewson, Wilson & Hammant 1976). This is a highly effective method of treatment of animals for tick control

provided careful management of the facility is in place to maintain proper acaricide concentration (Rajput *et al.* 2006).

The use of spray race systems is another method of applying chemical acaricides to animals. This is an alternative to plunge dipping suitable for large scale ectoparasite control. Cattle are treated individually by acaricides applied under pressure through a series of nozzles directed to all areas of the animal's body (Drummond 1983). However, wetting of some areas such as the ears and under the tail may not always be complete (Drummond, Whetstone & Ernst 1966). Hand spraying with a high pressure pump is commonly used in small-scale settings where the number of cattle is less than twenty (Drummond 1983).

Administration of pour-on formulations on the topline of an animal from poll to the base of the tail is also commonly practiced. Pour-ons are formulated to either penetrate the skin and act systemically or spread over the skin to get in contact with ectoparasites (Taylor 1997).

Other methods of administration of acaricides include the use of hand dressing to particular infested areas such as the ears and under the tail, injectables, an intra-ruminal bolus and acaricide-impregnated ear-tags among others (Drummond 1983, George, Pound & Davey 2004).

Virtually all ectoparasiticides are neurotoxic (Taylor 2001) broadly affecting action potential propagation or cholinergic synaptic transmission (Baynes 2017). An example is the inhibitory effect of organophosphates on acetylcholinesterase, the enzyme responsible for acetylcholine breakdown (Baynes 2017).

### 2.3.1    Mode of action of amidines (Formamidines)

Amitraz, a triazapentadiene compound is the main member of this group (Taylor 2001) used in veterinary medicine. It is available in various formulations for use in dogs, pigs and cattle for the treatment of ticks, lice and mites (Baynes 2017).

The mechanism of action of amitraz is not very clear (Baynes 2017). It is thought to target a receptor for the neuromodulator, octopamine found in the synganglion of ticks and other arthropods (Lees & Bowman 2007). There are three distinct types of octopamine receptors reported in arthropods namely α-adrenergic-like octopamine receptors (αAORs), octopamine/tyramine receptors and β-adrenergic like octopamine receptors (βAOR) (Corley *et al.* 2013). The octopaminegic effect of amitraz on susceptible insects and arthropods is thought to result in neuronal hyperexcitability and death (Nathanson 1985).

In other accounts, amitraz is thought to act as an inhibitor of mono-amine oxidase (MAO) which normally metabolizes neurotransmitter amines present in the CNS of ticks and mites (Baynes 2017). However, inhibition of MAO did not seem to be related to toxic action when Chlorodimeform, an amidine was used (Holden & Hadfield 1975).

## 2.3.2    Mode of action of synthetic pyrethroids (SPs)

Pyrethroids are synthetic derivatives of the natural pyrethrin molecule (Taylor 2001). Pyrethroids are more resistant to breakdown, which results in greater residual activity than the pyrethrins (Baynes 2017). Deltamethrin, cypermethrin and flumethrin are some of the most commonly used pyrethroids in veterinary medicine (Taylor 2001). They are available as pour-on, spot-on, spray and dip formulations for the control of flies, lice and ticks on domestic animals (Taylor 2001).

Synthetic pyrethroids target the voltage gated sodium channels (VGS) that control electrical signalling and regulate membrane excitability in the nervous system, particularly the axonal membrane (Dong *et al.* 2014). The VGS consists of four homologous repeats/domains (I-IV), each composed of six trans-membrane segments (S1-6) (Dong *et al.* 2014).

Binding of pyrethroids to their respective receptor sites on the VGS alters channel properties including ion conductance, ion selectivity and/or channel gating (i.e. opening and closing) (Dong *et al.* 2014). The nerve excitation due to changes in the permeability to sodium ($Na^+$) and potassium ($K^+$) ions has a knockdown effect on the ticks causing a

state of intoxication and partial paralysis which usually precedes death (Wickham, Chadwick & Stewart 1974).The VGS is also a primary target of synthetic compounds including insecticides, such as DDT and therapeutic medicines like local anaesthetics (Narahashi 1985).

## 2.4    Alternatives to use of acaricides

Resting of pastures from the end of season with adult tick abundance to the beginning of the next season has been reported to reduce significantly the number of adult ticks at the time of re-introducing cattle (De Deken *et al.* 2014). This only applies to tick species with life cycle lengths of one year. In addition, hosts of immature stages of the tick should not be allowed on the rested pastures for this method to be successful (De Deken *et al.* 2014).

Burning of pastures in spring as commonly practiced in southern Africa every two to three years can result in a short term reduction in tick abundance by destroying hatching *R. decoloratus* larvae (De Deken *et al.* 2014). This practice can unfortunately lead to the destruction of important vegetation types.

Immunization of hosts using vaccines composed of the recombinant *R. microplus* BM86 gut antigen resulted in reduced *R. microplus* infestations on cattle (Willadsen 1997). Such vaccines which were registered between 1993 and 1997 in Cuba and Australia under the names Gavac/Gavac Plus and TickGARD/TickGARD Plus respectively, were capable of reducing the number of engorging female ticks, their weight and reproductive capacity (Kiss *et al.* 2012). The high degree of immunity produced after immunization was however reported to be short-lived suggesting the need for repeated use throughout the year (De Deken *et al.* 2014).

## 2.5    Tick control in South Africa

When East Coast Fever was introduced in South Africa from the East Africa in the early 20th century, the government took various steps to control it. Plunge dipping of cattle was made compulsory by the state for all livestock farmers on a weekly basis to control

the tick *R. appendiculatus*. East Coast Fever was eradicated by 1960 but compulsory cattle dipping did not stop in the homelands (Hlatshwayo & Mbati 2005).

Since independence in 1994, provincial governments became responsible for overseeing state veterinary services. Cattle dipping prioritization varied from province to province. Generally, resource poor livestock farmers are prioritized where a province provides this service but not commercial farmers. However, some provinces like the North west and the Free State provinces stopped providing free dip to farmers due to financial constraints (Hlatshwayo & Mbati 2005). More intensive cattle dipping, sponsored by the province is practiced at wildlife-livestock interface in Limpopo and Mpumalanga provinces around the Kruger national park with the rest of the provinces receiving less attention. The Eastern Cape province provides the service based on a dipping policy.

The dipping policy of the Eastern Cape province signed in 2013 was crafted mainly to benefit resource poor farmers in order to promote rural development. Farmers are represented by dipping committees which work jointly with the department of rural development and agrarian reform (DRDAR) to implement the policy (Eastern Cape Province Department of Rural Development and Agrarian Reform 2013).

DRDAR selects, procures and provides acaricides. In addition, it also employs state veterinarians, animal health technicians and CAHWs. DRDAR should also train dipping committees on the management of dipping tanks. Facilities identified for use to perform a dipping activity on cattle are the plunge dip, spray race and a handling facility for pour on preparations (Eastern Cape Province Department of Rural Development and Agrarian Reform 2013).

State veterinarians are tasked with monitoring tick resistance to the acaricide in use and make recommendations in collaboration with other stakeholders. Animal health technicians (AHTs) should visit dipping stations in their respective areas once a month to ensure correct dipping management. Proper mixing of the dip at every dipping is done by the CAHW. The communities are responsible for the filling and replenishment of their

respective dip tanks. Dipping committees should assist CAHWs with dip mixing (Eastern Cape ProvinceDepartment of Rural Development and Agrarian Reform 2013).

The policy noted that dipping of livestock is vital for the control of tick borne diseases such as red water, gall sickness and heartwater and tick damage as supported by the Animal Health act, 2002 (Eastern Cape ProvinceDepartment of Rural Development and Agrarian Reform 2013).

Although the policy does not stipulate dipping frequencies, it recommends dipping at regular intervals during times of heavy tick challenges. Traditional leaders of respective communities are obliged to formulate by-laws to enforce farmers to comply with dipping schedules (Eastern Cape ProvinceDepartment of Rural Development and Agrarian Reform 2013).

There is a diversity in tick control practices in South Africa and discrepancies exist between policy and actual implementation on the ground. Farmers come up with their own initiatives for tick control to complement government dipping service or because government service is not available. The methods range from spraying with conventional acaricides, applying disinfectants, used oil, manual removal and use of pour-ons (Moyo & Masika 2009). These home-made formulations and practices can lead to a possible selection for resistance (Spickett & Fivaz 1992).

## 2.6    Acaricide resistance

Resistance in broad terms as defined by the World Health Organisation Scientific Group (1965) is "the ability of a parasite strain to survive and/or to multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject" (Abbas *et al.* 2014).

Individual cattle ticks can resist acaricide chemicals generally through target site modifications and increase in metabolic detoxification/excretion rate (Rosario-Cruz, Almazan *et al.* 2009). Target site modification involves an allele in the gene coding for a

target molecule for the acaricide resulting in mutation in the amino acid which in turn confers resistance to the acaricide. This mechanism has been extensively studied in pyrethroids. Metabolic detoxification of acaricides involves multigene- families of enzymes such as glutathione-S-transferases, esterases and mixed function oxidases (cytochrome P450) which are thought to enhance detoxification by increased hydrolysis or sequestration of respective acaricides (Rosario-Cruz *et al.* 2009). Extensive research on metabolic detoxification has been conducted in *R. microplus.*

In ticks, genetic resistance allows resistance to spread by reproduction of resistant individuals. Tick acaricide resistance is the ability of a strain of ticks to tolerate doses that would prove lethal to the majority of individuals in a normal population of the same species. Resistance genes in tick populations are present in very low proportions initially before the particular pesticide was ever used (Nolan 1987). The genes are thought to be found on chromosomal DNA. Resistance may be conferred by a single gene or may be polygenic and may involve one or more chromosomes.  Initially resistance genes are rare in a tick population but as selection by acaricides continues, the proportion of resistance genes increases and so does the proportion of resistant parasites (Rodriguez-Vivas *et al.* 2011). The tick population that originally is heterogeneous is transformed to a homogenous one by pesticide pressure (Rodriguez-Vivas *et al.* 2011).

Parasite genetic factors play a role in resistance genesis and these include dominance of resistance alleles, number of genes involved, initial proportion of resistance genes, genetic diversity of the tick population and potential for genetic recombination (Georghiou & Taylor 1977). The amount of time required for resistance genesis also depends on the mode of inheritance of the resistant allele (dominant, co-dominant or recessive), the frequency of treatment with acaricide and the proportion of the population in refugia. It is not easy to manage genetic factors as they are not under human control (Chevillon *et al.* 2013).

Tick acaricide resistance particularly with regards to *R. decoloratus* and *R. microplus* is costly and anticipation of such resistance within a few years of introduction of any new

chemical acaricide has been predicted (Abbas *et al.* 2014) unless control systems are reconsidered.

### 2.6.1    *Resistance against amitraz*

Mutations found in the octopamine/tyramine (OCT/Tyr) receptor gene of only amitraz-resistant *R. microplus* strains strongly suggest involvement of target site modification as one of the mechanisms of resistance in ticks (Abbas *et al.* 2014). Chen, He & Davey 2007 found that there were two SNPs in the OCT/Tyr receptor gene for resistant Brazilian and Mexican *R. microplus* strains which resulted in threonine to proline (T8P) and leucine to serine (L22S) amino acid changes respectively. Amitraz resistant *R. microplus* strains in South Africa possessed two SNPs in the OCT/Tyr receptor gene corresponding with the two SNPs published by Chen et al in 2007 (Baron *et al.* 2015).

Metabolic resistance has also been suggested following a study where up-regulation of glutathione-S-transferase was identified in a mildly resistant Mexican Pesqueria strain of *R. microplus* (Saldivar *et al.* 2008).

### 2.6.2    *Resistance against synthetic pyrethroids (SP)*

Studies have reported association between SNPs in the VGS channel gene and SP resistance in various insects, including fleas, flies, cockroaches, moths, aphids and mosquitoes among others. Most SNPs that render synthetic pyrethroid resistance in arthropods are located in the domain II S6 or in the linker between domain II S4-5 (Morin *et al.* 2002). Three SNPs in the sodium channel gene of *R. microplus* have been associated with resistance to synthetic pyrethroids.

The first SNP, reported in Mexico, was discovered in segment 6 (S6) of the Domain III leading to an amino acid change from phenylalanine to isoleucine in the VGS channel (He *et al.* 1999). The strain has since been reported to exist throughout Mexico (Rosario-Cruz, Guerrero *et al.* 2009) and also in Northern America (Miller, Davey & George 2007).

Two SNPs were located in the S4-5 linker of the Domain II of the sodium channel gene for *R. microplus*. The first SNP of the two resulted in an amino acid change from glycine

to valine (G72V) (Jonsson, Cutulle *et al.* 2010). An additional SNP at position 190 in the nucleotide sequence (C>A) confers moderate resistance to permethrin, cypermethrin and flumethrin due to an amino acid switch from leucine to isoleucine (Morgan *et al.* 2009). The same SNP reported by Morgan et al 2009 was also reported in South Africa for *R. microplus* population (Guerrero, Lovis & Martins 2012, van Wyk, Roelof D. J., Baron & Maritz-Olivier 2016)

Involvement of esterases has been reported as a metabolic resistance mechanism against synthetic pyrethroids in ticks and insects (Hernandez *et al.* 2002). A SNP in the carboxylesterase gene at position 1120 (G > A) for *R. microplus* results in an amino acid change from aspartate to asparagine (Hernandez *et al.* 2002). Enhanced sequestration and/or hydrolysis of the acaricide by these enzymes could possibly attribute to the resistance (Chevillon *et al.* 2007).

## 2.7    Acaricide resistance assessment

An ideal test/assay for tick acaricide resistance detection is expected to fulfil certain requirements. It should be able to detect resistance in its emergence phase, i.e. when the proportion of resistance genes to a particular chemical are still very low (FAO 2004). The test should be quick and able to give a reliable result. It should also at least be suitable for standardization among laboratories in many countries (George *et al.* 2004).

Numerous conventional bioassay methods have been used for acaricide resistance detection. The larval packet test (LPT) recommended by the Food and Agricultural Organization of the United Nations (FAO) for use as a standard tick bioassay method is used to conduct tests with organophosphates-carbamates and pyrethroids. Other methods not endorsed by FAO but in common use include the larval immersion test, adult immersion test (AIT) and the Drummond test  (FAO 2004, George *et al.* 2004, George *et al.* 2004).

Conventional bioassays fall short of the ideal requirements (FAO 2004). The methods are cumbersome, taking not less than 35 days to get results for the one-host-tick, such as *R. microplus* (George *et al.* 2004)*.* They involve complex steps from tick collection to

the provision of results. Regardless of the type of test to be used, engorged female ticks must be collected with bioassays. Storage conditions after collection are to be adhered to for bioassays such as the AIT to be successful. Adhering to prescribed moisture and aeration conditions is vital to maintain the health of ticks during transportation (FAO 2004).

At the laboratory, conditions for all tick stages should follow specific temperature and relative humidity requirements for them to stay healthy during the process. For ixodid ticks, the appropriate age of larvae for testing ranges from 7-14 days (George *et al.* 2004) and sometimes up to 21 days (FAO 2004). Some bioassays particularly the AIT require that engorged ticks be more than a certain threshold weight for them to qualify. The handling of acaricide-impregnated papers, their preparation for the introduction of ticks and the setting up of equipment at different stages of the process are quite involving (FAO 2004).

Molecular/genotypic techniques for the diagnoses of acaricide resistance are an alternative to bioassays. The scenario is to a greater extent similar to antimicrobial resistance (AMR) assessment where conventional phenotypic methods are compared to genotypic ones (Sundsfjord *et al.* 2004).

The conventional methods used to determine if a bacterial isolate is sensitive or resistant to different antibiotics are informative but time consuming (Anjum 2015). The phenotypic characterisation of AMR does not however identify defined resistance determinants (Sundsfjord *et al.* 2004). On the other hand, genotypic techniques are attractive in their ability to target specific resistance determinants (Sundsfjord *et al.* 2004). Resistance mechanisms involved in low-level resistance can be detected early with genotypic techniques.

Similarly, molecular methods for acaricide resistance detection are highly specific provided there is knowledge of mechanisms of acaricide resistance at molecular level and that the identified mechanism is the predominant one in the field (George *et al.*

2004). They are also quick, taking approximately two days for results to come out in some circumstances (George *et al.* 2004).

Molecular assessment of the frequency of tick acaricide resistance will allow for development of knowledge-based strategies for the control of resistant tick populations and could prolong the efficacy of currently available acaricides.

# Chapter 3

## Materials and methods

### 3.1    Study area

Engcobo LM is one of the 7 municipalities in the district of Chris Hani, Eastern Cape Province. It is entirely rural and covers an area of 2 258.78 square kilometers. Human population is estimated at 155 513 according to a census in 2011 (Statistics South Africa 2012). Cattle population is estimated at 49 000 (Based on annual anthrax vaccination records).

### 3.2    Study population

Forty-eight, mixed breed cattle, male or female, any age, tick infested, sourced from community farmers using local dip-tanks in the Engcobo LM in the Eastern Cape Province of South Africa was used.
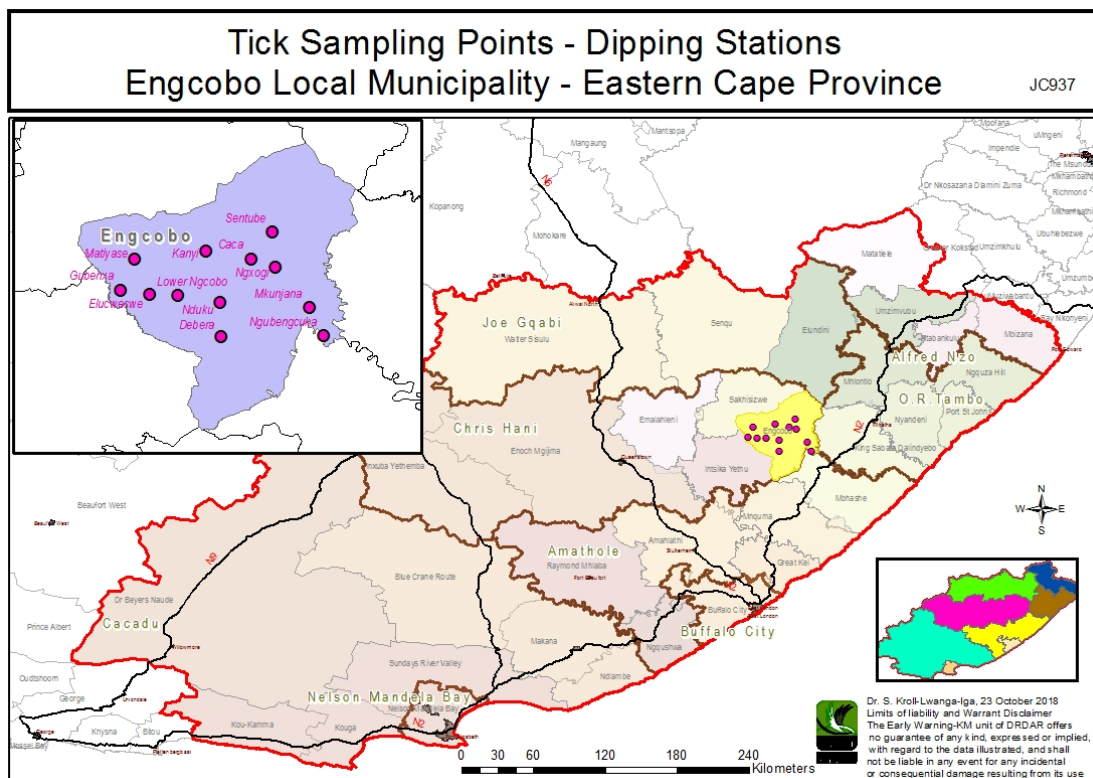


Figure 3-1: Tick sampling points-dipping stations, Engcobo LM

Table 3-1:GPS coordinates of dipping stations sampled, collection dates and the number of ticks collected.

| Collection Date | Dipping station | GPS coordinates | No. of ticks |
|---|---|---|---|
| 14-03-2018 | Debera | **E**:28°3'49" **S**:31°49'25" | approx. 40 |
| 15-03-2018 | Kanyi | **E**:28°1'43" **S**:31°37'25" | approx. 40 |
| 17-04-2018 | Ngxogi | **E**:28°11'30" **S**:31°39'35" | approx. 40 |
| 18-04-2018 | Gubenxa | **E**:27°49'39" **S**:31°42'53" | approx. 40 |
| 25-04-2017 | Mkunjana | **E**:28°16'14" **S**:31°45'22" | approx. 40 |
| 11-11-2017 | Lower Ngcobo | **E**:27°57'44" **S**:31°43'38" | approx. 40 |
| 28-04-2017 | Nduku | **E**:28°3'40" **S**:31°44'34" | approx. 40 |
| 13-04-2017 | Elucwecwe | **E**:27°53'46" **S**:31°43'29" | approx. 40 |
| 23-11-2016 | Matiyase | **E**:27°51'39" **S**:31°38'29" | approx. 40 |
| 03-05-2017 | Ngubengcuka | **E**:28°18'14" **S**:31°49'17" | approx. 40 |
| 21-04-2017 | Sentube | **E**:28°11'02" **S**:31°34'45" | approx. 40 |
| 04-05-2017 | Caca | **E**:28°08'01" **S**:31°38'28" | approx. 40 |

## 3.3    Study design and sampling

Preliminary investigations estimated the frequency of resistant SNPs to be 20% (Baron *et al.* 2015). We calculated the sample size by using a method for two-stage sampling for estimating herd-level prevalence with fixed herd sensitivity as described by Wagner 2004 (Wagner & Salman 2004). Calculations were performed using "EpiTools epidemiological calculators" (http://epitools.ausvet.com.au/content.php?page=2Stage FreedomSS_2)

The following input was used: Animal-level design prevalence 20%; Herd-level prevalence 50%; Test sensitivity 0.95; Test specificity 1; Herd sensitivity (SeH) 0.5; System sensitivity (SSe) 0.95; number of herds in population: 60. The number 60 also correlates with the total number of dipping stations in the local municipality. Twelve herds were selected in the LM and 4 randomly selected animals per herd was sampled giving a total of 48 animals. Ten ticks were collected from four randomly selected cattle that were visibly infested with blue ticks at twelve pre-selected dipping stations giving a total sample size of 480 ticks.

## 3.4    Tick collection

Tick collection was performed in standing animals while in the cattle race before dipping. Usually animals were sufficiently restrained during this time to facilitate collection of ticks. Only where necessary were the animals restrained. Lifting of the tail was used when necessary. In some instances, poles were inserted through the cattle race in front and behind the animal and a rope used in a halter configuration to secure the head of the animal to the side of the crush for the brief period while the ticks were collected. Informed consent was obtained from each cattle owner or their representative.

## 3.5    Tick identification

On collection an attempt was made to identify and collect to genus of interest, *Rhipicephalus* using the naked eye. This is supported by Walker (Walker 2003). A stereomicroscope further aided in morphological identification to genus level post-collection. A microscope was used to identify *Rhipicephalus* ticks at species level. Some of the important features to consider were be hypostomal dentition; shape of genital aperture posterior lips; spurs on coxae I, II, and III; ventral plates spurs in males among others (Walker 2003).

## 3.6    Genomic DNA purification.

The protocol for the purification of total DNA of ticks was done using the QIAamp DNA Mini kit.

Whole ticks of weight not exceeding 25 mg were added to 1,5 ml microcentrifuge tubes containing no more than 80 µl of PBS. Physical disruption of whole ticks was performed using forceps and a scalpel after which 100 µl Buffer ATL was added. This was followed by addition of 20 µl of proteinase K (600 mAU/ml, solution) and subsequent vortexing and incubation on a rocking platform at 56 °C until the tissues were completely lysed. The 1,5 µl microcentrifuge tubes were briefly centrifuged to remove drops from the inside of the lid.

To obtain RNA-free genomic DNA, 4 µl RNAase A (100 mg/ml) was added and mixed by pulse-vortexing for 15 s followed by a 2 min incubation at room temperature (15-25°C). A brief centrifuge of the 1,5 ml microcentrifuge tubes to remove drops from inside the lead was followed by the addition of 200 µl Buffer AL to the samples. Samples were mixed by pulse-vortexing for 15 sec before incubation at 70 °C for 10 min was conducted. The 1,5 ml microcentrifuge tubes were briefly centrifuged to remove drops from inside the lid. This was followed by the addition of 200 µl of ethanol (96-100%) and mixing for 15 sec by pulse-vortexing. A brief centrifugation of the 1,5 ml microcentrifuge tubes was performed to remove drops from inside the lid.

The mixture (including the precipitate) was carefully applied to the QIAamp Mini spin column in 2 ml collection tubes and centrifuged at 6000 x g (8000 rpm) for 1 min. Subsequently the QIAamp Mini spin columns were placed in clean 2 ml collection tubes and old collection tubes containing the filtrate discarded. A further 500 µl Buffer AW1 was added and centrifugation performed at 6000 x g (8000 rpm) for 1 minute. The QIAamp Mini spin columns were placed in clean 2 ml collection tubes and old collection tubes containing the filtrate discarded. To the QIAamp Mini spin columns, 500 µl of Buffer AW2 was added followed by centrifugation at full speed (20000 x g; 14000 rpm) for 3 min. To eliminate the chance of possible Buffer AW2 carryover, the QIAamp Mini spin columns were placed in new 2 ml collection tubes and centrifuged at full speed for 1 min.

The QIAamp Mini spin columns were further placed in clean 1,5 ml microcentrifuge tubes and the old collection tubes containing the filtrate were discarded. To the columns, 200 µl Buffer AE was added before incubation at room temperature for 1 minute and subsequent centrifugation at 6000 x g (8000 rpm) for 1 min were performed. The step was repeated once.

To visualize genomic DNA of individual adult ticks, electrophoresis was conducted with a 2% w/v agarose (Whitehead Scientific, SA) in TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). This was performed in a Mini-Sub® Cell

GT System (Bio-Rad Laboratories, SA) at a speed of 8 V/cm. The Gel Doc™XR+ System (Biorad, USA) was used for DNA visualization.

## 3.7 PCR amplification of resistance genes

PCR reactions were performed using the GeneAmp 9700 thermocycler (PE Applied Biosystems, USA).

### 3.7.1 Octopamine/tyramine receptor gene

Primers as published previously by (Baron *et al.* 2015) were used for OCT/Tyr receptor gene amplification. For *R. microplus* amplification of a 417-base pair (bp) fragment of concern was conducted using primers OAR-F172 (5'-AGC ATT CTG CGG TTT TCT AC-3') and OAR-R587 (5'-GCA GAT GAC CAG CAC GTT ACC G-3'). Amplification was conducted in a 25 μl final mixture consisting of 200 ng of template DNA, 10 pmol of each primer and EconoTaq® PLUS GREEN 2X Master Mix (Lucigen, USA) consisting of the following: 1.25 U of EconoTaq DNA polymerase (0.1 units/μl), 200μM dNTPs, 1.5 mM Magnesium chloride (MgCl$_2$) and a proprietary PCR enhancer/stabilizer. Reaction steps were as follows: denaturation at 94 °C for 4 min followed by 40 temperatures cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for one minute ending with an extension step at 72 °C for 7 min. The process described above was applicable for adult ticks. Larval DNA was amplified in the same way as described above except that 2-5 μl of the crude homogenate was added to the 25 μl reaction mixture.

### 3.7.2 VGS channel gene

Primers previously published by (Jonsson *et al.* 2010) for amplification of a 167 bp (domain II segment 4-5 region) segment were used. BmNaF5 (5'-TAC GTG TGT TCA AGC TAGC-3') and BmNaR5 (5'-ACT TTC TTC GTA GTT CTT GC-3') (Jonsson *et al.* 2010) were used as the forward and reverse primers respectively. The resultant composition of a 25 μl amplification reaction mixture was as follows: 200 ng of template DNA, 10pmol of each primer and KAPA2G™ Robust HotStart ReadyMix containing 200 μM dNTPs, 2.0 mM MgCl$_2$ and the HotStart DNA polymerase in a propriety buffer. Reaction steps were

as follows: initial denaturation for 4 minutes at 94 °C followed by 40 temperature cycles of 94 °C for 30 sec, 51 °C for 30 seconds and 72 °C for 1 min with a final extension step at 72 °C for 7 min.

## 3.8     PCR amplicon purification and gene sequencing

PCR products from 3.7.1 and 3.7.2 were analyzed on 2% w/v agarose gel. This was followed by purification of PCR amplicons in a 96-well plate using PureLink® Pro 96 PCR purification kit (Invitrogen™, Life Technologies, USA). DNA gel electrophoresis of purified PCR products was performed to determine if concentrations were sufficient for DNA sequencing. This was achieved by comparing known concentrations of the molecular standard (1kb DNA marker, Fermentas, Canada) with the unknown samples. Sequencing of gene products was conducted by Inqaba Biotechnical Industries (Pty) Ltd. (South Africa).

## 3.9     Sequence analysis

Nucleotide sequences for the OCT/Tyr receptor gene and the VGS channel receptor gene trace files were obtained from the sequencing laboratory. They were edited by combining the forward and reverse sequences of each individual tick in the case of the OCT/Tyr receptor gene, using Gap4 of the Staden Software package (Bonfield et al. 2002) to generate a consensus sequence. On the other hand, Trev also belonging to Staden Software package was utilized to view and edit forward sequences for the VGS channel receptor gene for each tick.

Multiple alignments of the consensus sequences for the OCT/Try receptor gene and edited sequences for the VGS channel receptor gene were carried out using CrustalX (Thompson et al. 1997). BioEdit (Hall 1999) was subsequently used to edit multiple alignments from ClustalX where sequence terminals were trimmed to produce sequences of desirable lengths for both genes.

Alignment of final edited sequences for the OCT/Tyr receptor gene to Rhipicephalus microplus susceptible Gonzalez strain (GenBank Accession: AJ010743.1) and resistant

Santa Luiza strain (GenBank Accession: EF490688.1) was performed to check for the presence of mutations using ClustalX. Likewise, final edited sample sequences for the VGS channel receptor were compared with nucleotide sequences of a portion of *R. microplus* putative sodium channel mRNA (GenBank Accession: AF134216.2).

Allele frequency was calculated as the relative abundance of an allele i.e. counting the number of that type of allele of the gene with reference to its other alleles i.e. total allele count. Genotype frequency was calculated as a relative proportion of a particular genotype for the loci of interest in processed ticks.

# Chapter 4

## Results

After initially collecting 480 tick specimens as per sample size calculations (refer to section 3.3), good quality genomic DNA was obtained from only thirty-six ticks. All the thirty-six were successfully used for amitraz associated resistance and twelve for pyrethroid associated resistance. This was due to limited funds as well as an unanticipated difficulty to extract from fully engorged ticks. Successful extraction was achieved with unfed ticks.

### 4.1    SNPs on the OCT/Tyr receptor gene

Sequencing analysis of the partial OCT/Tyr receptor gene of 36 *R. microplus* ticks was performed. A mutation at nucleotide position 220 (locus 1, A220T) was observed in 18 out of 36 ticks sampled. Another mutation at nucleotide position 263 (locus 3, T263C) was present in 9 out of the 36 ticks (see Figure 4-1 and Table 4-1).  The two nucleotide substitutions at loci 1 and 3 were situated at nucleotide positions 157 and 200 as published previously (Baron et al. 2015). The two are non-synonymous sites leading to a switch from threonine to proline at position 8 (T8P) and leucine to serine at position 22 (L22S) of the OCT/Tyr receptor protein respectively. The mutation at nucleotide position 237 (locus 2, C237T) (see Figure 4-1 & Table 4-1) reported to be involved in linkage-equilibrium with a SNP at position 65 (Baron et al. 2015) was present in 16 out of the 36 ticks sampled.

Allele frequency calculations at locus 1 and locus 3 for 36 ticks (72 alleles per each respective position) were as follows:

For A allele at locus 1 (30/72) = 0.416

For C allele at locus 1 (42/72) = 0.583

For T allele at locus 3 (49/72) = 0.681

For C allele at locus 3 (23/72) = 0.319

The proportion of homozygous resistant genotype (RR) was highest at locus 1 (see Table 4-1). There were also heterozygote ticks in all three loci. Diverse genotypes observed at the two SNP positions (see Table 4-2) correspond with findings reported in a previous study (Sungirai et al. 2018).

At dip station level, the proportion of RR ticks was 60% or more for Debera, Gubenxa, Kanyi and Nduku. The range of proportions for RR ticks was between 40-60 % for Elucwecwe Matiyase, Mkunjana (see Table 4-3 and Figure 4-2).
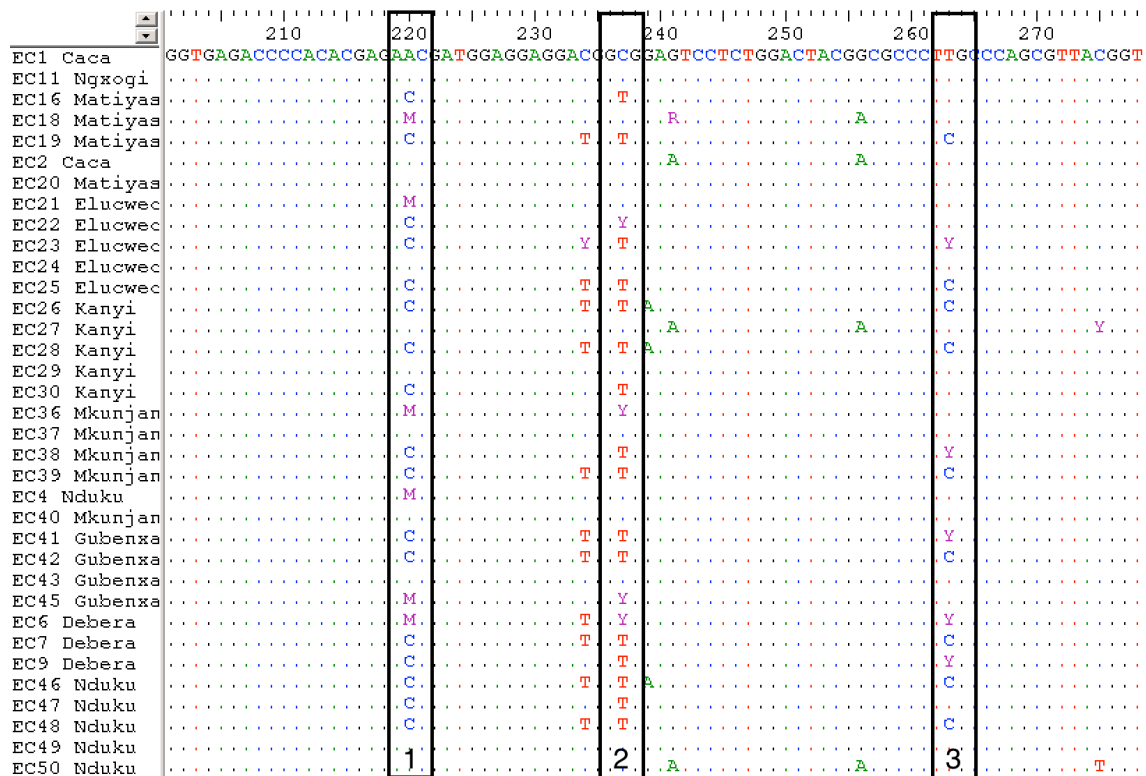
Figure 4-1: Alignment of the OCT/Tyr gene sequences in the partial segment of the open reading frame indicating loci 1-3. Tick sample sequences all start with EC. R. microplus (Gonzalez) (GenBank Accession: AJ010743.1) was used as the reference sequence alongside with the Santa Luiza resistant strain (GenBank Accession: EF490688.1).

Table 4-1: Genotypes of *R. microplus* at loci 1, 2, and 3 of the OCT/Tyr receptor gene. Another locus 1 is for the VGS channel gene receptor associated with resistance to pyrethroids SS is the homozygous wild susceptible type, RR homozygous resistant and RS heterozygous.

| Molecular marker | Locus | Genotype | | |
|---|---|---|---|---|
| | | ss | Rs | RR |
| Octopamine/Tyramine receptor gene (Amitraz) | 1 | 12/36, (33.3%) | 6/36, (16.7%) | 18/36, (50.0%) |
| | 2 | 15/36, (41.7%) | 5/36, (13.9%) | 16/36, (44.4%) |
| | 3 | 22/36, (61.1%) | 5/36, (13.9%) | 9/36, (25.0%) |
| Voltage-gated sodium channel receptor gene (Pyrethroids) | 1 | 12/12, (100%) | 0 | 0 |

Table 4-2 Genotypes of samples of *R. microplus* ticks at locus 1/locus 3 per dip station sampled.

| Dip station | Sample | Genotype | Dip station | Sample | Genotype |
|---|---|---|---|---|---|
| Caca | EC1 | AA/TT | Kanyi | EC28 | CC/CC |
| Caca | EC2 | AA/TT | Kanyi | EC29 | AA/TT |
| Caca | EC4 | AC/TT | Kanyi | EC30 | CC/TT |
| Debera | EC6 | AC/TC | Matiyase | EC16 | CC/TT |
| Debera | EC7 | CC/CC | Matiyase | EC18 | AC/TT |
| Debera | EC9 | CC/TC | Matiyase | EC19 | CC/CC |
| Elucwecwe | EC21 | AC/TT | Matiyase | EC20 | AA/TT |
| Elucwecwe | EC22 | CC/TT | Mkunjana | EC36 | AC/TT |
| Elucwecwe | EC23 | CC/TC | Mkunjana | EC37 | AA/TT |
| Elucwecwe | EC24 | AA/TT | Mkunjana | EC38 | CC/TC |
| Elucwecwe | EC25 | CC/CC | Mkunjana | EC39 | CC/CC |
| Gubenxa | EC41 | CC/TC | Mkunjana | EC40 | AA/TT |
| Gubenxa | EC42 | CC/CC | Nduku | EC46 | CC/CC |
| Gubenxa | EC43 | AA/TT | Nduku | EC47 | CC/TT |
| Gubenxa | EC44 | CC/TT | Nduku | EC48 | CC/CC |
| Gubenxa | EC45 | AC/TT | Nduku | EC49 | AA/TT |
| Kanyi | EC26 | CC/CC | Nduku | EC50 | AA/TT |
| Kanyi | EC27 | AA/TT | Ngxogi | EC11 | AA/TT |

Table 4-3: Dip station level genotype proportions at loci 1, 2, and 3 of the OCT/Tyr receptor gene.

| | Locus 1 | | | Locus 2 | | | Locus 3 | | |
|---|---|---|---|---|---|---|---|---|---|
| | SS | SR | RR | SS | SR | RR | SS | SR | RR |
| Caca | 2/3 (66.7%) | 1/3 (33.3%) | 0 | 3/3 (100%) | 0 | 0 | 3/3 (100%) | 0 | 0 |
| Debera | 0 | 1/3 (33.3%) | 2/3 (66.7%) | 0 | 1/3 (33.3%) | 2/3 (66.7%) | 0 | 2/3 (66.7%) | 1/3 (33.3%) |
| Elucwecwe | 1/5 (20.0%) | 1/5 (20.0%) | 3/5 (40.0%) | 2/5 (40.0%) | 1/5 (20.0%) | 2/5 (40.0%) | 3/5 (60.0%) | 1/5 (20.0%) | 1/5 (20.0%) |
| Gubenxa | 1/5 (20.0%) | 1/5 (20.0%) | 3/5 (60.0%) | 1/5 (20.0%) | 2/5 (40.0%) | 2/5 (40.0%) | 3/5 (60.0%) | 1/5 (20.0%) | 1/5 (20.0%) |
| Kanyi | 2/5 (40.0%) | 0 | 3/5 (60.0%) | 2/5 (40%) | 0 | 3/5 (60.0%) | 3/5 (60.0%) | 0 | 2/5 (40.0%) |
| Matiyase | 1/4 (25.0%) | 1/4 (25.0%) | 2/4 (50.0%) | 2/4 (50.0%) | 0 | 2/4 (50.0%) | 3/4 (75.0%) | 0 | 1/4 (25.0%) |
| Mkunjana | 2/5 (40.0%) | 1/5 (20.0%) | 2/5 (40.0%) | 2/5 (40.0%) | 1/5 (20.0%) | 2/5 (40.0%) | 3/5 (60.0%) | 1/5 (20.0%) | 1/5 (20.0%) |
| Nduku | 2/5 (40.0%) | 0 | 3/5 (60.0%) | 2/5 (40%) | 0 | 3/5 (60.0%) | 3/5 (60.0%) | 0 | 2/5 (40.0%) |
| Ngubengcuka | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ngxogi | 1/1 (100%) | 0 | 0 | 1/1 (100%) | 0 | 0 | 1/1 (100%) | 0 | 0 |
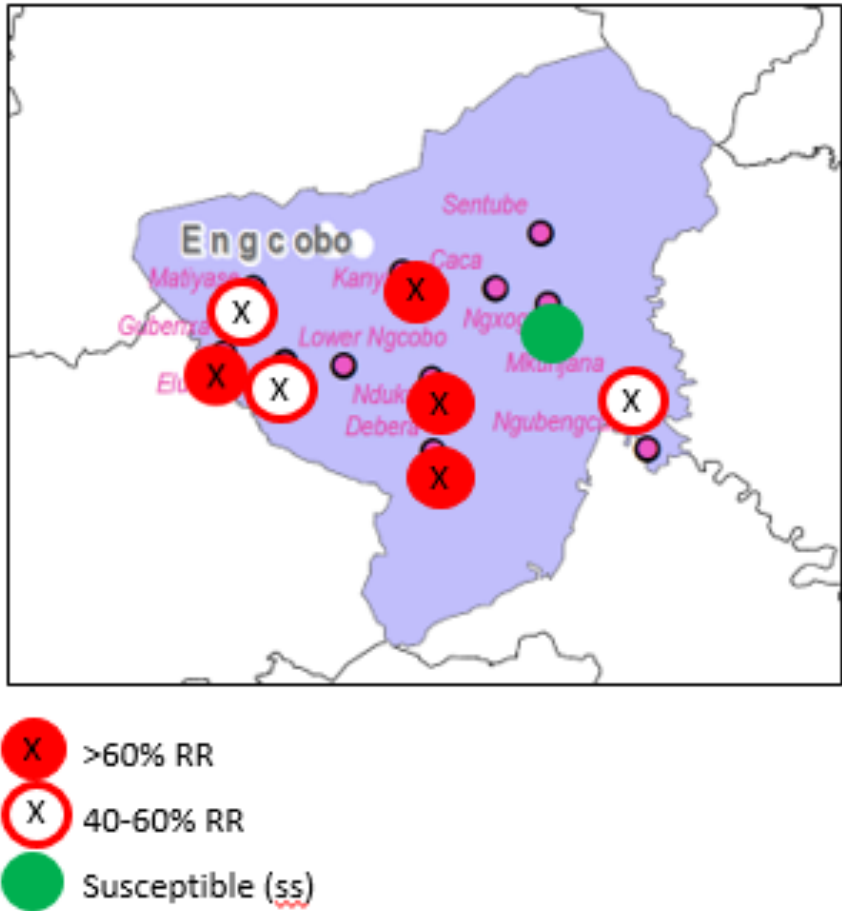
Figure 4-2: Genotypic proportions and distribution of the OCT/Tyr resistance alleles in Engcobo LM

## 4.2 Voltage-gated sodium channel gene

Sequence analysis of the VGS channel receptor gene (domain II segment 4-5 region) in twelve *R. microplus* ticks did not show mutations i.e. there was no pyrethroid resistance. Sample sequences were compared with nucleotide sequences of a portion of *R. microplus* putative sodium channel mRNA (GenBank Accession: AF134216.2).
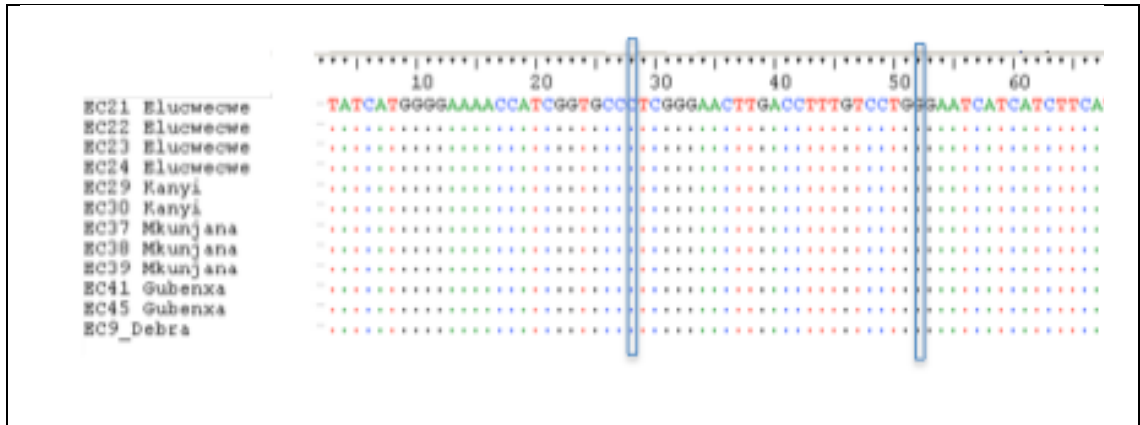
Figure 4-3: Alignment of the partial segment VGS gene sequences showing no resistance associated mutations.

# Chapter 5

## Discussion

In this study two target-site-resistant alleles associated with amitraz and pyrethroid resistance (Chen *et al.* 2007, Baron *et al.* 2015) were analised. Amitraz resistence develops through mutations at position 220 (locus 1, A220T) and position 263 (locus 3, T263C) of the OCT/Tyr receptor gene. Resistance against formamidines (amitraz) appears to be on the rise. Four dipping stations reported 60% or more homozygous resistant ticks. This is congruent with the almost exclusive use of amitraz by the government over the years in the area.

The presence of heterozygous genotypes (AC/TC) at resistance-associated loci 1 and 3 of the OCT/Tyr receptor gene supports previously published findings  (Baron *et al.* 2015, Sungirai *et al.* 2018). It is believed that heterozygous genotypes are susceptible to amitraz (Baron *et al.* 2015), however, the detection of heterozygosity at resistance associated loci, is an important finding. Reproduction of heterozygous (Rs) ticks can potentially produce about 25% fully resistant (RR) ticks in the second generation by merely applying Mendel's laws of inheritance. Continued expansion of resistant (RR) ticks is probable in subsequent generations simply due to mating (see Figure 5-1) and this can be exacerbated by the continuous utilization of amitraz to control such a tick population.

|   | R | s |
|---|---|---|
| R | RR (25%) | Rs |
| s | Rs | ss |

Second generation

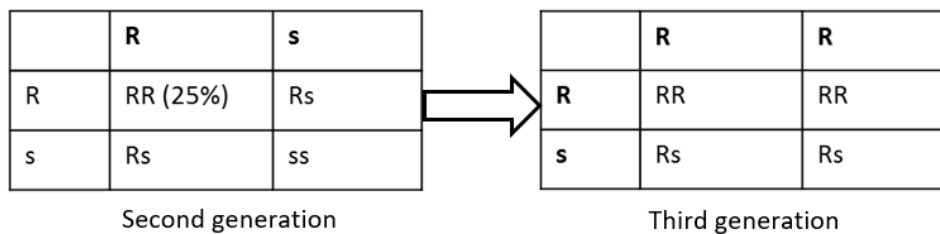|   | R | R |
|---|---|---|
| R | RR | RR |
| s | Rs | Rs |

Third generation

Figure 5-1: Mating of heterozygote ticks to give about 25% resistant genotypes (RR) in second generation. Subsequently about 50% RR ticks can be produced in the third generation if resistant ticks in the second generation mate with heterozygous ticks.

The fact that mutant alleles known to confer resistance to synthetic pyrethroids in the VGS channel receptor (domain II segment 4-5 region) were not detected in this study, supports the data published by van Wyk *et al*. in 2016, who reported a large proportion of homozygous susceptible *R. microplus* ticks in the Eastern Cape.

This study was based on a small sample size and results should be treated as preliminary. It will be of value to sample more ticks from this population to monitor the development of resistence in future.

A valuable lesson learnt in this study was to avoid collecting engorged ticks as these did not yield good quality genomic DNA.

An acaricide rotation strategy is recommended to reduce the number of resistant ticks. This will also help to reduce ticks that are heterozygous for amitraz resistance. Pyrethroids can be a good choice as a substitute for amitraz. This should increase the frequency of non-resistant ticks and re-establish the previous susceptibility levels of the population (Belinato & Martins 2016).

The findings in this study can play a crucial role in raising awareness in livestock farmers on the judicious use of acaricides. This should also be extended to rural communities through targeting the dipping committees as well as State Veterinary services.

References

Appendix 1. Animal Ethics Committee certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

## Animal Ethics Committee

| PROJECT TITLE | Allele frequencies of acaricide resistance-associated SNPs in *Rhipicephalus microplus* and *Rhipicephalus decoloratus* ticks in Ngcoba Local Municipality, Eastern Cape Province, Republic of South Africa |
|---|---|
| PROJECT NUMBER | V029-17 |
| RESEARCHER/PRINCIPAL INVESTIGATOR | Dr. J Zanga |

| STUDENT NUMBER (where applicable) | U_16391196 |
|---|---|
| DISSERTATION/THESIS SUBMITTED FOR | MSc |

Change ICF to "Ticks will be collected to see if the dip used is working in this area"

| ANIMAL SPECIES | n/a | |
|---|---|---|
| NUMBER OF ANIMALS | n/a | |
| Approval period to use animals for research/testing purposes | | April 2017-April 2018 |
| SUPERVISOR | Dr. J Crafford | |

KINDLY NOTE:

Should there be a change in the species or number of animal/s required, or the experimental procedure/s – please submit an amendment form to the UP Animal Ethics Committee for approval before commencing with the experiment

| APPROVED | Date | 9 May 2017 |
|---|---|---|
| CHAIRMAN: UP Animal Ethics Committee | Signature | |

S4285-15