Allele frequencies of acaricide resistance-associated specific nucleotide polymorphisms in *Rhipicephalus microplus* and *Rhipicephalus decoloratus* in Manicaland, Zimbabwe

By

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Declaration

I, *Spargo Reverend Moregood,* declare that the tick samples for this study were collected from cattle in the Mutate district of Manicaland province, Zimbabwe. Molecular work (genomic extraction and sequencing) was done at the Department of Veterinary Tropical Diseases molecular laboratory by Anna-Marie Bosman. Apart from the acknowledgements indicated and the advice and guidance by my supervisors, this dissertation is my own original work.

This full dissertation or any part of it has not been, is not being or will not be submitted for another degree at this or any other university.

This dissertation is presented in partial fulfilment for the requirements of a Master of Science Veterinary Tropical Diseases in the Department of Veterinary Tropical Diseases Faculty of Veterinary Science University of Pretoria.

Signed.....

Date……... 01 November 2018

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Abbreviations

AChE - acetylcholinesterase

AIT - adult immersion test

DDT - dichlorodiphenyltrichloroethane

DNA - deoxyribonucleic acid

dNTPs - deoxyribonucleotide triphosphates

ECF - East Coast Fever

EDTA - Ethylenediaminetetraacetic acid

FAO - Food and Agriculture Organization

GoZ - Government of Zimbabwe

GPS - Global Positioning System

LIT - larval immersion test

LPT - larval packet test

LTT - larval tarsal test

$MgCl_2$ – magnesium chloride

OCT/Tyr - octopamine/tyramine

PCR - polymerase chain reaction

RNA - ribonucleic acid

RR – homozygous resistant genotype

Rs – heterozygous genotype

SDS - Sodium Dodecyl Sulfate

SNPs - specific nucleotide polymorphisms

ss - homozygous susceptible genotype

TBDs -Tick-borne diseases

TE - Tris-EDTA

Tris - tris(hydroxymethyl)aminomethane

USD - United States America, dollar

VGS - voltage-gated sodium

WHO - World Health Organization

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Abstract

Cattle ticks from the genus *Rhipicephalus* are hematophagous ectoparasites of great veterinary and economic importance. Currently, the main method for managing ticks and tick-borne diseases is the application of chemical acaricides. Development of resistance to these acaricides however poses a threat to the livestock sector globally, including Zimbabwe. Surveillance for acaricide resistance is essential for controlling tick populations via early intervention with an active compound to which there is no resistance in the field. In this study the allele frequencies that occur in genes linked to pyrethroid and amitraz resistance, namely the voltage-gated sodium channel and the octopamine/tyramine receptor were investigated. Specific nucleotide polymorphisms (SNPs) of *Rhipicephalus* spp in these genes were analysed from 18 communal dip tanks in the eastern parts of Zimbabwe. No SNPs were identified in the domain II region of the voltage-gated sodium channel gene associated with pyrethroid resistance. Resistant alleles associated with amitraz resistance in both *R. decoloratus* and *R. microplus* tick populations were observed. The level of acaricide resistance was subsequently calculated and solutions for improved acaricide resistance control are discussed.

Key words: Acaricide resistance; Mutare; SNPs; *Rhipicephalus* spp

Chapter 1

1.0 General introduction

Livestock production in Zimbabwe is an important and integral part of the agricultural sector with livestock products contributing about 30% of the value of agricultural gross domestic product (Swanepoel et al. 2010). Livestock also provides a form of livelihood to a great portion of the population in Zimbabwe. As such, livestock makes a significant contribution to the national economy and to the nutritional and material wellbeing of the country's population. Ticks and tick-borne diseases adversely impact on 80% of the global cattle population and are extensively distributed throughout the world, especially in the tropic and sub-tropical regions (Karim et al. 2017), and as such they represent one of the major constraints to livestock production in the world (Jongejan and Uilenberg, 2004). Annually it is estimated that the world incurs economic losses over USD$ 18 billion due to ticks and tick-borne diseases (TBDs) (De Clercq et al., 2012). The losses are due to the direct effects of ticks such as weight loss and anaemia, and also via indirect effects such as secondary infections at feeding sites and the transmission of tick-borne diseases (TBDs). All of the latter lead to reduced livestock productivity and enhanced rates of mortality, notwithstanding the expenses associated with treatment and preventative measures (Minjauw and McLeod, 2003).

In Africa, TBDs are considered the most important livestock diseases (Muhanguzi et al. 2017). Zimbabwe is not an exception, with at least 60% mortalities in livestock attributed to ticks and tick-related diseases (Sungirai et. al, 2015). The livestock sector in Zimbabwe is broadly divided into two sub-sectors based on the reason for rearing livestock; commercial and communal farming. The commercial sub-sector is characterised by intensive production of livestock while the majority of cattle owners in

the communal sub-sector are the resource constrained communal farmers. In order to safeguard the livestock sector, especially cattle production, the Government of Zimbabwe initiated a tick control program for the communal farmers who hold more than 80% of cattle in Zimbabwe (Tavirimirwa et al., 2013). This tick program relies on dipping cattle regularly with acaricides. While commercial farmers are not part of the government initiated dipping program, they too make use of acaricides for tick control. This makes it the foremost tick control strategy being implemented in Zimbabwe with formamidines (amitraz) and synthetic pyrethroids (deltamethrin) being the mostly used acaricides (Sungirai et al, 2015). The main concern at this point is the declining efficacy of current acaricides due their repeated application, and possible development of acaricide resistance in the ticks and the selection of resistant ticks by incorrect use of acaricides (Shyma et.al, 2015). This makes it crucial to regularly carry out surveillance programmes on acaricide insensitivity (Ghosh et al., 2015).

1.1 Research question and hypothesis

The recent acaricide resistance status of tick species *R. microplus* and *R. decoloratus* against the two mostly used acaricides (amitraz and deltamethrin) is not known. The null hypothesis is that there are no amitraz and deltamethrin resistance-associated specific nucleotide polymorphisms (SNPs) in *R. decoloratus* and *R. microplus* from cattle in the Mutare district while alternative hypothesis is that there are amitraz and deltamethrin resistance-associated specific nucleotide polymorphisms (SNPs) in *R. decoloratus* and *R. microplus* from cattle in Manicaland Province of Zimbabwe.

1.2 Objectives

The main objective of the present study was to investigate allele frequencies for amitraz and deltamethrin resistance-associated specific nucleotide polymorphisms (SNPs) in *R. decoloratus* and *R. microplus* from Mutare district. Based on the findings, to proffer plausible strategies for acaricide management that may extend the lifespan of the currently available acaricides.

1.3 Potential benefits of the research findings

The study improves the knowledge of tick acaricide resistance in Zimbabwe and informs policy makers on the efficacy of the current tick control strategy. The presence of amitraz resistant *Rhipicephalus microplus* and *R. decoloratus* tick populations observed in this study is not surprising, as amitraz has been in use for over 30 years in Zimbabwe (Sungirai et al. 2016). This indicates that using amitraz for the control of *Rhipicephalus* ticks should be carefully reconsidered to lessen the selection pressure and spread of resistant ticks in the future. By reducing the use of amitraz and increasing the use of an alternative active ingredients (such as a pyrethroid or macrocyclic lactones) may offer a solution to the increase in the number of amitraz resistant ticks (especially in *R. microplus*).

Pyrethroid resistance was not observed in the study hence the continued use of pyrethroids remains a viable, cost-effective control strategy for *R. microplus* and *R. decoloratus*.

Strict adherence to the livestock movement regulations, to allow only tick-free cattle to move will help prevent spread of resistant tick populations. There is also a need to educate farmers on the use of acaricides and integrated control strategies to avoid

development of acaricide resistance. All this will have the overall impact of improving the livestock sector in Zimbabwe.

Chapter 2

2.0 Literature Review

2.1 Introduction to the *Rhipicephalus* genus

The *Boophilus* genus, which has been renamed to *Rhipicephalus* contains six species of ticks that predominantly parasitize ungulates: *Rhipicephalus annulatus, R. australis, R. decoloratus, R. geigyi, R. kohlsi, and R. microplus* (Wang et al. 2017). The change in nomenclature from *Boophilus* to *Rhipicephalus* was as a result of molecular and morphological evidence supporting the *Rhipicephalus* genus as paraphyletic to the *Boophilus* genus (Barker & Murrell, 2004).

Rhipicephalus ticks are multivoltine (more than one generation per annum) and monoxenous (larvae, nymphs, and adults all feed and develop on a single host) that is followed by oviposition, incubation, and larval life in off-host habitats (Nicholson et al. 2009). These one-host ticks spend about three weeks on the host animal resulting in a longer exposure time to acaricides. During frequent application of acaricides, or in cases where the effective concentration of the acaricide is exceeded, the selection pressure for acaricide resistance is enhanced. Prolonged exposure to an acaricide results in removal of the susceptible individuals of the population with a resultant increase in the proportion of the resistant strains (Abbas et. al. 2014). Therefore, one-host ticks typically tend to develop acaricide insensitivity faster than multi-host ticks where the smaller animal host species are not routinely exposed to acaricides (such as mice or rabbits) (Mekonnen et. al. 2002).

Rhipicephalus decoloratus and *R. microplus* are among the most important tick species in Zimbabwe. The Animal Health Act (Cattle Cleansing) regulations of the Government of Zimbabwe (GoZ) (1993), classifies *Amblyomma*, *Rhipicephalus* and the *Rhipicephalus* (*Boophilus*) group as specified pests. This is because of the physical damage they cause to cattle, blood loss and irritation as they feed. They are also vectors of important livestock diseases that are associated with severe economic loss (Vudriko et. al., 2016).

2.2 *Rhipicephalus microplus*

Rhipicephalus microplus, also known as the Asian blue tick, is extensively distributed throughout the tropical and subtropical areas of the world (Guerrero et. al., 2014). *R. microplus* may have been introduced into Eastern and Southern Africa from Madagascar, where it had initially arrived with cattle from southern Asia (Walker and Olwage, 1987). In Zimbabwe, its distribution is limited to the Eastern highlands, northern, north-eastern and the north-western regions of Zimbabwe which are characterised by warm and humid climatic conditions (Katsande et al. 1996). *R. microplus* has been collected from low-lying areas of Zimbabwe were climatic conditions are too hot and dry for its proliferation. However, during the past few years' movement of cattle from different regions of the country has been attributed to the expansion in distribution of *R. microplus* (Sungirai et al. 2015). The tick is predominately found on cattle and can also occur on sheep, goats and horses (Norval, 1984). *R. microplus* transmits bovine babesiosis *(Babesia bovis and B. bigemina)*, anaplasmosis *(Anaplasma marginale),* spirochaetosis *(Borrelia theileri)* and *Babesia berbera* in cattle (Kocan et. al., 2004). It was also shown that *R. microplus* can compete and eventually replace *R. decolaratus* in warm and humid climatic conditions (Walker and Olwage, 1987).

2.3 *Rhipicephalus decoloratus*

R. decoloratus has a widespread distribution in Zimbabwe. It predominately parasitizes cattle, sheep and goats. It has a much wider host range compared to *R. microplus* and can be found on wild ungulates such as kudu (*Tragelaphus strepsiceros)* and sable *(Hippotragus niger)* (Horak, 1982). Where *R. microplus and R. decolaratus* co-exist*, R. decoloratus* is likely to be displaced by the former (Sungirai et. al., 2018). In cattle *R. decoloratus* is responsible for the transmission of babesiosis, a protozoal disease caused by *Babesia bovis* and *B. bigemina.* It also transmits anaplasmosis (*Anaplasma marginale)*, *Borrelia theileri* and *Pseudomonas aeruginosa* (Balashov, 1972).

2.4 Chemical control of ticks in Zimbabwe

The first dip tank in Zimbabwe, then Rhodesia was constructed in the year 1910 and by 1912 a total of 215 dip tanks were operational. This was necessitated after the introduction of East Coast Fever (ECF), a devastating lymphoproliferative tick–borne disease of cattle, imported with cattle from Eastern Africa. Mandatory dipping of cattle in acaricide was made into law in 1914 (Norval 1979) mainly for the control of ECF transmitted by the tick vector, *R. appendiculatus*. The cattle dipping program was a resounding success resulting in the eradication of ECF by the mid-1950s and control of other TBDs (Lawrence et al. 1980; Norval et al. 1992).

During this period however, challenges were faced with the choice of acaricide, mainly due to the toxicity of acaricides to cattle and the development of acaricide resistance by the ticks (Young *et al.* 1988). The dipping program continued with different classes of acaricides being used up to the early 1970s, when it was disrupted by the liberation war (Lawrence et al. 1980; Norval 1983b). Disruption of dipping services was also partly due

to the reluctance of communal farmers to pay cattle dipping fees, their lack of buy-in to mandatory dipping programs and the apparent lack of disease threat (Norval,1979). Because of the compulsory intensive dipping of cattle, the national herd had no herd immunity against TBDs, and the collapse of the dipping program led to the death of an estimated one million cattle from TBDs (Lawrence et al. 1980).

Early in the 1980s, after the attainment of independence, the compulsory intensive dipping program was re-introduced by the Government (Norval et al. 1992). This was against recommendations to rather introduce a new integrated tick control strategy. Arguments for integrated control strategies in the control of ticks and TBDs were presented, which showed that in the long term, an intensive dipping program was economically unsustainable, and it reduced the national herd's enzootic stability to TBDs (Norval 1983b; Norval et al. 1992).

Presently, Zimbabwe has adopted an integrated tick and tick-borne disease control strategy. This approach uses a combination of indigenous tick-resistant cattle, vaccination against some TBDs and strategic dipping to control tick vectors (Peter *et al.* 1998). Cattle dipping are done weekly in the rainy season of December to April when tick activity is at its peak. During the dry season, which is the rest of the year, fortnightly to monthly dipping is done guided by tick infestation levels at communal dip tanks (Ndhlovu *et al.* 2009). The current dipping fees are pegged at USD$2 per animal annually. The dipping program is supported by legislation, the Animal Health (Cattle Cleansing) Regulations of 1993, Government of Zimbabwe (GoZ) (1993) that makes it compulsory for livestock owners to bring their cattle for dipping as well as to pay for the dipping fees.

The Department of Veterinary Services is mandated by the Animal Health Act (Cattle Cleansing) regulations GoZ (1993) to run the dipping program with over 2000 dip tanks

and 100 spray-races across the country. Over 80% of the dip tanks use amitraz as the acaricide of choice with the remaining dip tanks and spray races using deltamethrin. The choice of acaricide is dependent on availability of dipping infrastructure and presence of tsetse fly in the area. In tsetse fly infested areas, deltamethrin is the acaricide of choice as it is effective in controlling ticks as well as tsetse flies (Shereni et al., 2016). Also, in areas where dip tanks have not yet been constructed or are damaged and where water to fill dip tanks is scarce, deltamethrin pour-on is being used as the acaricide of choice. The pour-on approach, applied monthly, is more flexible and adaptable in more remote regions (Swallow et al., 1995). However, this pour on method is relatively expensive. The lower cost of the dip tanks and spray races make them a very cost-effective measure to ticks and tick-borne diseases (Chadenga, 1992).

For the past 3 decades amitraz and deltamethrin have been in continual use in Zimbabwe for the control of ticks, tsetse-flies and the diseases they transmit (Pilossof, 2016). This over-reliance is a risk factor for the emergence of resistant strains. The rotation of different acaricide classes will however lower the selection pressure for resistance to any one acaricide class, but this will require knowledge-based selection of a suitable acaricide (Abbas *et al.* 2014).

2.5 Resistance development in ticks

For arthropods, resistance can be defined as an inherited characteristic that imparts an increased tolerance to an acaricide, such that the resistant individuals survive a concentration of the compound that would normally be lethal to the species (WHO,1992). Genetic changes that lead to structural changes of the target site, enhanced detoxification of the acaricide and impaired penetration of the acaricide through the tick's cuticle contribute to the emergence of resistance (Guerrero, et. al., 2012).

The most frequently noted resistance mechanism is target site mutation. It arises when an allele of the gene coding for the site of action for the acaricide has a nucleotide substitution due to mutation that render ticks insensitive to the active compound (Foil et. al., 2004). As an example, organophosphate and carbamate acaricides inhibit acetylcholinesterase (AChE). Tick populations became resistant to these acaricides when members within the population develop a structurally modified AChE enzyme that allowed them to survive exposure to organophosphate and carbamate acaricides (Cossio-Bayugar, et. al., 2018).

Metabolic resistance develops as a result of genetic changes that lead to increased metabolic detoxification of acaricides by enzymes such as esterases, cytochrome P450s, multifunction oxidases and glutathione transferases (Yessinou et. al., 2018). One of the most common types of metabolic mediated resistance in ticks is the enhanced activity of esterases which results in resistance to both carbamates and organophosphates (Fukoto, 1990).

2.6 Resistance against Amitraz (amidines)

Amitraz, is a formamidine compound that has been in use for over three decades (Jonsson and Hope, 2007). Amitraz resistance was first reported in *R. microplus* in Australia in 1980 spreading slowly in comparison to resistance against synthetic pyrethroids. The mode of action of amitraz involves the interaction with the neuromodulator, octopamine resulting in increased nervous activity of ticks (Chevillon et al., 2007). The octopaminergic receptors have been grouped into three classes, namely β-adrenergic-like (βOCT), α-adrenergic-like (αOCT) and octopamine/tyramine (OCT/Tyr) (Chevillon et al. 2007). More research has been done for *R. microplus* acaricide resistance mechanisms compared to *R. decoloratus* ticks where little data is available

(Guerrero et al., 2012). *R. microplus* resistance to amitraz has been reported globally in multiple tick populations (Chevillon et al., 2007; Mendes et al., 2013) and SNPs in the octopamine receptor (Baron et al., 2015) or glutathione-S-transferase mediated metabolic detoxification (Guerrero et al., 2012) have been suggested as possible resistance mechanism. Recent reports have also suggested the involvement of ATP-binding cassette transporters in the detoxification of formamidines in *R. microplus* (Koh-Tan et al., 2016). A recent study to evaluate amitraz resistance mechanism in *R. decoloratus* using RNA sequencing showed that amitraz resistant *R. decoloratus* ticks probably employ a rescue mechanism involving ionotropic glutamate receptors to enhance synaptic transmission and plasticity in the presence of neurosteroids (Baron et al. 2018)

2.7 Resistance against Deltamethrin (pyrethroids)

Deltamethrin is a synthetic acaricide structurally based on natural pyrethrins, which rapidly paralyze the arachnids' nervous system giving a quick knock-down effect (Haug and Hoffman, 1990). It acts on voltage-gated sodium (VGS) channels, causing nerve excitation due to changes in nerve membrane permeability to sodium and potassium ions, making it a potent neurotoxin (Weston et al. 2013). Genetic changes in the protein coding sequences of the voltage-gated sodium channels can result in structural changes leading to target site insensitivity (Soderlund and Knipple, 2003). Tick resistance to synthetic pyrethroids is linked with non-synonymous mutations in domains I, II, III, and IV of VGS channel genes (Stone et al. 2014). In *R. microplus* the specific VGS channel associated with synthetic pyrethroid resistance is the *para*-sodium channel, and resistance mechanisms in domains II and III of this gene have been reported in *R.*

microplus ticks. A domain II mutation (C190A) has been reported in *R. microplus* populations in Africa, Australia and South America (Li et al. 2008)

Metabolic mediated resistance to pyrethroids was also noted in many tick species. Cytochrome P450s, esterases and glutathione S-transferases all being involved (Guerrero et al., 2000; Guerrero et al., 2002; Hernandez et al., 2002; Baffi et al., 2007; Li et al., 2013; Chevillon et al., 2007).

2.8 Acaricide resistance in Zimbabwe

The development of acaricide resistance has with time limited the use of different classes of acaricides worldwide (Bell-Sakyi et al., 2004). In Zimbabwe the first report of development of resistance of *R. decolaratus* to arsenic was in 1963. Resistance in *R. appendiculatus* followed this to cyclodienes and toxaphenes in 1966 and in *R. decolaratus* in 1967 (George *et al*. 2004). The progressive evolution of acaricide resistant ticks to almost all of the available acaricides emphasizes the importance of surveillance for acaricide resistance (Nari and Hansen, 1999).

A national survey was carried out in 1995 to assess acaricide resistance in *R. decoloratus*, *Amblyomma hebraeum* and *R. appendiculatus* in the communal and commercial areas of Zimbabwe to organochlorine (DDT), amidine (amitraz) organophosphate (dioxathion) and pyrethroids (cypermethrin, flumethrin and deltamethrin). The survey used the Larval Packet Test (LPT) (Stone and Haydock, 1962), and reported resistance to dioxathion, flumethrin, cypermethrin and deltamethrin in many samples of *R. decoloratus* while most samples of *Amblyomma haebreum* and *R. appendiculatus* were susceptible to all six acaricides (Mazhowu, 1995).

A study carried out in 2014 to evaluate the sensitivity of *R. appendiculatus* and *R. microplus* populations from Mazowe district in Zimbabwe using the LPT found no

evidence of resistance to amitraz (Gono et al., 2014). Recent genotyping of *R. microplus* tick populations using SNPs linked with resistance to organophosphates, amitraz and pyrethroids showed a high frequency (0.55) of mutant alleles for the octopamine/tyramine receptor marker linked with amitraz resistance and a low mutant frequency (0.052) for the carboxylesterase marker that has been linked with resistance to pyrethroids and organophosphates (Sungirai et al., 2018).

2.9 Diagnosis of resistance in ticks

The continued emergence of acaricide resistant tick populations make regular acaricide resistance testing an important component of any tick and tick-borne disease control strategy (Chapman, 1992). The ideal diagnostic method for acaricide resistance should possess the following qualities;

- provide a rapid and reliable result
- suitable for standardization among laboratories in many countries
- simple and inexpensive to carry out
- comprehensive, covering the full range of acaricide classes that are in use.

These qualities are essential to identify resistance early in its emergence. The fact that there are several tests in use for the diagnosis of acaricide resistance in ticks serves to indicate that none of the tests are perfect in all circumstances (Kemp et al., 1999).

An array of bioassay methods has been established for evaluating the sensitivity of ticks to acaricides. The principles of these bioassays involve the exposure of larva or adult ticks to acaricides and checking the mortality counts. Bioassays detect the phenotypic resistance which can be defined as how tolerant or sensitive tick populations are to the effects of exposure to a particular acaricide. The Adult Immersion Test (AIT) and the Larval Packet Test (LPT) were recommended and standardised by the FAO for

assessing tick resistance. These standardised methods facilitate the comparison of tick acaricide resistance data between laboratories. The AIT, which was initially established by Drummond et al. (1973), exposes engorged adult female ticks to acaricides and evaluates oviposition (number of eggs laid) between the exposed ticks and the control ticks (Guerrero et al. 2014). The main advantage is that results can be read after one week of tick collection while the down side is that it requires a large number of engorged female ticks and cannot be used when the full dose–response mortality curve is required (Jonsson et al. 2007).

Larval bioassays need small numbers of engorged females making them suitable for generating full dose–response mortality curves. The LPT established by Stone and Haydock (1962) has been widely used in the diagnosis of tick acaricide resistance studies and characterization of organophosphate and synthetic pyrethroid resistance mechanisms in ticks. In the LPT, larvae are exposed to filter papers soaked in acaricides and mortality counts are done after a day to check for sensitivity (Abbas et. al., 2014). The major advantage being its high repeatability (Jonsson et al. 2007), however it is limited by the fact that it is laborious and requires about 5-6 weeks before results can be obtained (Guerrero et al. 2014). Other bioassays have been developed using the same principle of the LPT but reducing the amount of labour and time required to carry out the test. An example being the Larval Tarsal Test (LTT) developed by Lovis et al. (2011) to bypass the tedious handling of larvae and uses multi-well plates. In this test eggs are placed in well on a multi-well plate containing acaricides and placed in an incubator. Two weeks after hatching, larval mortality is evaluated under a microscope. This makes the LTT a time-effective system, enabling testing of large numbers of compounds and doses in a single test.

Although bioassays are indispensable because of low-cost and portability, they cannot diagnose resistance genotypes thus are not able to detect the early stages of resistance emergence (Abbas et. al., 2004). The resistance genotype reflect as resistance phenotype and the same resistance phenotype can arise from different genetic variants (Guerrero et al. 2014). Rapid and sensitive PCR-based assays have been developed as alternatives to bioassays for detection of tick acaricide resistance. These molecular techniques include allele-specific PCR, target gene amplification and probe-based quantitative PCR assays (Chen et al., 2007, Guerrero et al., 2001, Hernandez et al., 2002, Rodriguez-Vivas et. al., 2011, Wada and Iwamoto, 2009). The advantages of molecular bioassays include the ability to detect known and novel mutations in the same gene and availability of results within 48 hours (Robbertse et. al., 2016). The detection of SNPs in the genes known to contribute to acaricide resistance can assist to assess the level of acaricide resistance in the tick population, (Sangster et al. 2002). Potential limiting factors for using molecular techniques to assess acaricide resistance include:

- in-depth knowledge of the resistance mechanisms at the molecular level are needed
- the identified mechanism should be the predominant phenotype for resistance in the field
- the mechanism being evaluated may not represent all resistance mechanisms
- cannot test for resistance against a range of acaricides at once except if a battery of tests are conducted
- molecular techniques are relatively complex and expensive

Chapter 3

3.0 Materials and methods

3.1 Study area

This study was carried out in Mutare district of Manicaland province January to April 2018.The district lies in the east of the country represented by the agro-ecological zone 1, characterised by rainfall of more than 1000mm/year, mean maximum temperatures of 19 to 23 ºC, high altitude and steep slopes.

The farmers make use of communal grazing where animals are released during the day to fend for grazing and water and then rounded up and kraaled during the night for security purposes as well as production of manure. Each farmer kraals his herd of cattle near his homestead. Cattle from 18 randomly selected dip tanks (Table 1: Names and GPS coordinates of the selected dip-tanks in Mutare

participated in the study. The dip tanks are clearly indicated on the map of the study area below (Figure1).Method for tick control consisted of weekly acaricide dipping in wet months (December to April) and fortnightly to monthly dipping in dry months (May to November) with formamidine (amitraz) and pyrethroid based (deltamethrin) acaricides. All the selected dip tanks having been using amitraz since inception and are being run by the Department of Veterinary Services. According to the current cattle census records from the Department of Veterinary Services in Mutare, the cattle population in the district is 43 000 and the total number of cattle owners is 2 663.

Table 1: Names and GPS coordinates of the selected dip-tanks in Mutare

Dip tank	**Latitude**	**Longitude**
Manyengava	-19.20943	32.60445
Chitora	-19.31901	32.60445
Bemhiwa	-19.28639	32.31755
Himalaya	-19.36473	32.76004
Mutore	-19.28184	32.68076
Takunda	-18.90321	32.26798
Tiverton	-18.85547	32.22527
Majuta	-18.81964	32.26537
Guvhu	-19.30391	32.14735
Rowa	-19.16679	32.61292
Heimat	-18.85849	32.28191
Chipendeke	-19.32773	32.71793
Musabayana	-19.27175	32.60464
Makomwe	-19.22072	32.27502
Dani	-19.14172	32.13807
Mhangaurwe	-19.10406	32.29798

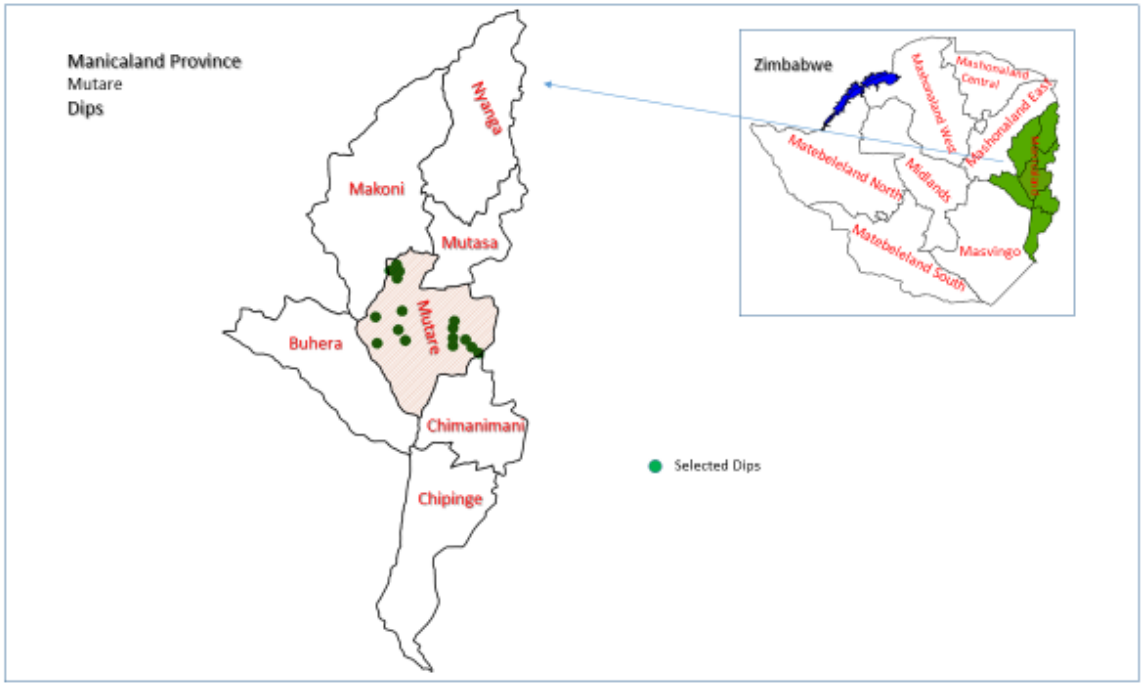

Figure 1: Map of study area showing the selected dip-tanks where samples were collected.

3.2 Study population

Cattle in the study population represented a mixture of both *Bos indicus* and *Bos taurus* breeds. Collection of tick samples took place during the months of January to April 2018 via random sampling from at least 10 cattle at the selected dip tanks. Animals were individually identified according to the stock number, name of owner, colour, sex and age as well as the name of the dip tank to which the animal belonged. Five randomly selected *R microplus* and/or *R decoloratus* ticks from each of the 18 dip tanks (where available) were subjected to SNP analysis in each of the Octopamine/tyramine (OCT/Tyr) receptor and voltage-gated sodium channel genes.

3.3 Tick identification

Ticks collected from the different dip tanks of Mutare district were identified to species level. Classification of collected ticks into their respective genera was performed according to previously described guidelines (Walker et al. 2003; Madder and Horak 2010). Distinguishing features were easily detectable for the three most common tick genera found in Zimbabwe namely; *Amblyomma, Hyalomma* and *Rhipicephalus*. To differentiate between *R. microplus* and *R. decoloratus* females, the hypostome dentition was examined along with the adanal spurs for the male ticks (Walker et al. 2003; Madder and Horak 2010).

3.4 Genomic DNA extraction

A modified salt-based extraction method published by Aljanabi et al. 1997 was used for genomic DNA isolation from whole adult ticks. Whole ticks were homogenized in 200 µl lysis buffer (0.5 M EDTA, 0.5% w/v Sodium lauroyl sarcosinate) using a mortar and pestle. The homogenized suspensions were placed into sterile 2 ml microcentrifuge tubes and an additional 400 µl of DNA extraction solution (0.4 M NaCl, 60mM Tris-HCl, 12 mM EDTA, 0.25% SDS, pH 8.0) was added to the samples along with 2 µl proteinase K (15 mg/ml). Samples were briefly vortexed and incubated overnight at 55°C.The following day samples were incubated for 20 min at 65°C to deactivate the proteinase K, after which 1 µl of RNase A (10 mg/ml) was added. Samples were vortexed briefly and incubated at 37°C for a further 15 min. Protein precipitation was done by adding 360 µl of 5 M NaCl, vortexing for 10 seconds, and incubation on ice for 5 min, followed by centrifugation at 25 500xg for 20 min at room temperature. The supernatants were transferred into sterile 2 ml microcentrifuge tubes while avoiding the white flocculants

that were present. An equal volume of isopropanol was added to the samples, briefly vortexed, followed by an incubation step of 1 hour at -20°C. Samples were then centrifuged for 20 min at 10 000xg and the supernatants discarded. DNA pellets were washed with 500 µl of 70% ethanol, centrifuged for 5 min at 10 000xg, and the supernatant discarded, followed by two more wash steps. The final DNA pellets were air dried in a flow cabinet for 20 min (or until ethanol was no longer visible at the bottom of the tube) and then re-suspended in 50 µl of 1 x TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH 7.0). Genomic DNA of whole adult ticks was visualized with 2% w/v agarose (Whitehead Scientific, SA) electrophoresis in TAE (40 mM Tris acetate, 1 mM EDTA, pH 8.0) containing ethidium bromide (1 µg/ml). Electrophoresis was performed in a Mini-Sub® Cell GT System (Bio-Rad Laboratories, SA) at a speed of 8 V/cm. DNA was visualized using the Gel Doc™XR+ System (Biorad, USA).

3.5 PCR amplification of resistance genes

For the Octopamine/tyramine (OCT/Tyr) receptor gene, primers published by Chen et al. (2007) were used for PCR amplification of a 417 bp fragment of the (OCT/Tyr); OAR-F171 (5'-GGT TCA CCC AAC CTC ATC TCTGAA- 3') and OAR-R587 (5'-GCA GAT GAC CAG CAC GTT ACC G-3') (Chen et al. 2007). Amplification was performed in a 25 µl reaction containing 200 ng of template DNA,10 pmol of each primer and EconoTaq® PLUS GREEN 2X Master Mix (Lucigen, USA) which contained the following: 1.25 U of EconoTaq DNA polymerase (0.1 units/µl), 200 µM dNTPs, 1.5 mM $MgCl_2$ and a proprietary PCR enhancer / stabilizer. Temperature cycles were as follows; 94°C for 4 min, 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 min with a final extension at 72°C for 7 min.

For the voltage-gated sodium channel gene, primers by Jonsson et al. (2010) were used to amplify a 167 bp fragment of the sodium channel (domain II segment 4-5 region); BmNaF5 (5'-TAC GTG TGT TCA AGC TAGC-3') and BmNaR5 (5'-ACT TTC GTA GTT CTT GC-3') (Jonsson et al. 2010). Amplification was carried out in a 25 µl reaction containing 200 ng of the template DNA, 10 pmol of each primer and KAPA2G™ Robust HotStart ReadyMix which contained 200 µM dNTPs, 2.0 mM $MgCl_2$ and the HotStart DNA polymerase in a propriety buffer, the cycling parameters were as follows; 94°C for 4 min, followed by 40 cycles of 94°C for 30 seconds, 51°C for 30 seconds and 72°C for 1 min with a final extension at 72°C for 7 min.

Purified PCR products were analyzed with DNA gel electrophoresis to determine if the concentration was sufficient for DNA sequencing. Concentrations were estimated by comparing the known concentrations of the molecular standard (1 kb DNA marker, Fermentas, Canada) with that of the unknown sample. All gene products were sequenced by Macrogen Inc. (Netherlands) in a 96-well plate according to the standard dye terminator sequencing strategy. Plate preparation required 5 µl of template (~ 50 ng/µl) along with 5 µl of primer (5 pmol) per well.

3.6 Sequence analysis

Sequences received from Macrogen were analyzed using Bio Edit sequence alignment editor version 7.2.0 (Hall 2007). Multiple alignments were performed using the online MAFFT program which is available at (http://mafft.cbrc.jp/alignment/software/) (Katoh and Standley 2013). Sequences were aligned with published NCBI sequences to determine if any SNPs were present in the gene. When novel SNPs were found, it was

determined if they were synonymous or non-synonymous mutations. This was done by comparing the amino acid sequences using Bio Edit.

3.7 Data analysis

Frequency counts were done for all the genotypes present for each molecular marker and these were expressed as a percentage of the total number of individual ticks which had positively amplified. This enabled calculation of the overall proportion of the genotypes as well as the proportion at the dip tank level.

Chapter 4

4.0 Results

4.1 Tick collection and identification.

In total 335 adult ticks were collected during the study period. A total of 8 tick species were identified from the 18 sampled dip tanks These included *Amblyomma hebraeum, R. microplus, R. decoloratus, Hyalomma truncatum, H. rufipes, R. punctatus, R. appendiculatus and R. evertsi.* (Table 2). At all dip tanks, *Rhipicephalus* ticks predominated. At only one dip tank (Himalaya) both *R. microplus* and *R. decoloratus* were found (Table 3).

Table 2: Summary of the tick species collected from the selected dip tanks.

Tick species	**Number of ticks collected**
Amblyomma hebraeum	*30*
Rhipicephalus evertsi	*65*
Rhipicephalus microplus	*41*
Rhipicephalus decoloratus	*119*
Rhipicephalus appendiculatus	*23*
Hyalomma truncatum	*18*
Hyalomma rufipes	*23*
Rhipicephalus (near) punctatus	*16*

Table 3: Summary of the number of *R. microplus* and *R. decoloratus* ticks collected from the selected dip tanks.

Name of dip tank	Tick spp.	No. of ticks
Manyengava	*R. decoloratus*	12
Chitora	*R. decoloratus*	10
Bemhiwa	*R. microplus*	3
	R. decoloratus	2
Himalaya	*R. decoloratus*	10
	R. microplus	10
Mutore	*R. microplus*	10
Takunda	*R. decoloratus*	10
Tiverton	*R. decoloratus*	10
Majuta	*R. decoloratus*	10
Guvhu	*R. microplus*	3
Rowa	*R. decoloratus*	15
Heimat	*R. decoloratus*	10
Chipendeke	*R. microplus*	15
Musabayana	*R. decoloratus*	15
Makomwe	*R. decoloratus*	3
Dani	*R. decoloratus*	7
Mhangaurwe	*R. decoloratus*	5

4.2 DNA sequencing, SNP identification and allele frequency analyses

Following DNA sequencing of the octopamine receptor and VGS gene, sequence analyses and alignments were done to identify SNPs as well as homozygous (ss or RR) or heterozygous (Rs) individuals (Figure 2). Indicated is locus 1, 2 and 3, which corresponds to the amitraz-associated SNPs at locus 1 and 3 (indicated with red arrows), as well as the SNPs, used for linkage equilibrium (described by Baron et al. 2016 and

indicated with a blue arrow). A number of heterozygous ticks for locus 1 and 3 were identified (Figure 2). Allele frequencies for all of the samples are indicated in Table 3. The data for *R. decoloratus* and *R. microplus*, respectively, is indicated in Tables 4 and 5. For *R. decoloratus*, the homozygous resistant genotype (RR) has been observed for 45% of the ticks at locus 1 and 8.2% at locus 3. It is higher for the *R. microplus* ticks, where 50% homozygous resistant ticks at locus 1 and 33% at locus 3 were identified (Table 5). When looking at the respective dip stations (Table 6), it is evident that amitraz resistance in *R. decoloratus* is already above 80% at Chitora, Heimat, Majuta, Mutore and Tiverton. With regards to *R. microplus*, 75% was observed at Mutore.

With regards to pyrethroid resistance, no SNPs were identified in the domain II region of the voltage-gated sodium channel gene (indicated with red arrows in Figure 3). Based on this data, no resistance to pyrethroids can be ascribed at this time.

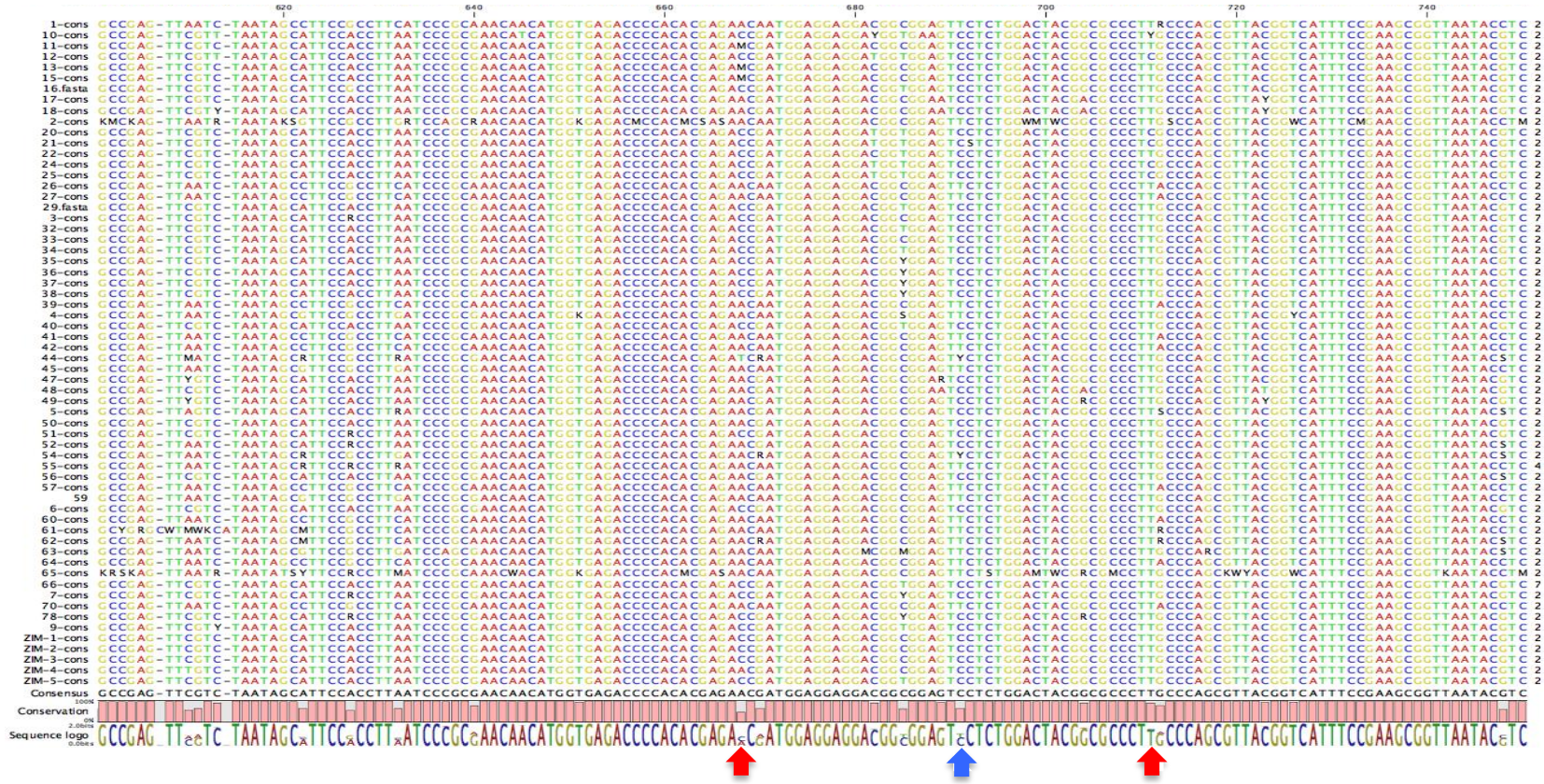

Figure 2: DNA sequence analyses of the octopamine receptor gene. Indicated are loci 1, 2 and 3, which corresponds to the amitraz-associated SNPs at locus 1 and 3 (indicated with red arrows), as well as the SNPs. used for linkage equilibrium (indicated with a blue arrow)

Table 4: Overall frequency of genotypes detected in *R. microplus* species collected at dip tanks in the Manicaland Province in Zimbabwe.

Molecular marker	Locus	Genotype		
		ss	Rs	RR
Octopamine/Tyramine receptor gene (Amitraz)	1	24/60, (40.0%)	3/60, (5.0%)	27/60, (45.0%)
Octopamine/Tyramine receptor gene (Amitraz)	2	39/59, (66.1%)	5/59, (8.5%)	15/59, (25.4%)
Octopamine/Tyramine receptor gene (Amitraz)	3	55/61, (90.2%)	1/61, (1.6%)	5/61, (8.2%)

Table 5: Overall frequency of genotypes detected in *R. decoloratus* species collected at dip tanks in the Manicaland Province in Zimbabwe.

Molecular marker	Locus	Genotype		
		ss	Rs	RR
Octopamine/Tyramine receptor gene (Amitraz)	1	6/12, (50.0%)	0	6/12, (50.0%)
Octopamine/Tyramine receptor gene (Amitraz)	2	6/12, (50.0%)	0	6/12, (50.0%)
Octopamine/Tyramine receptor gene (Amitraz)	3	8/12, (66.7%)	0	4/12, (33.0%)

Table 6: Frequency of genotypes detected in all *Rhipicephalus microplus* and *R. decoloratus* species collected at the respective dip-tanks in the Manicaland province in Zimbabwe

Dip Tank	Tick species	Locus 1			Locus 2			Locus 3		
		ss	Rs	RR	ss	Rs	RR	ss	Rs	RR
Chipendeke	*R. microplus*	4/4 (100.0%)	0	0	4/4 (100%)	0	0	4/4 (100%)	0	0
Chitora	*R. decoloratus*	0	0	4/4 (100%)	1/4 (25.0%)	1/4 (25.0%)	2/4 (50.0%)	3/4 (75.0%)	1/4 (25.0%)	0
Heimat	*R. decoloratus*	1/5 (20.0%)	0	4/5 (80.0%)	4/5 (80.0%)	0	1/5 (20.0%)	5/5 (100%)	0	0
Himalaya	*R. decoloratus*	0	3/4 (75.0%)	1/4 (25.0%)	3/4 (75.0%)	0	1/4 (25.0%)	3/4 (75.0%)	0	1/4 (25.0%)
Himalaya	*R. microplus*	2/4 (50.0%)	0	2/4 (50.0%)	2/4 (50.0%)	0	2/4 (50.0%)	3/4 (75.0%)	0	1/4 (25.0%)
Majuta	*R. decoloratus*	1/5 (20.0%)	0	4/5 (80.0%)	1/5 (20.0%)	3/5 (60.0%)	1/5 (20.0%)	5/5 (100%)	0	0
Manyengava	*R. decoloratus*	4/5 (80.0%)	0	1/5 (20.0%)	4/5 (80.0%)	1/5 (20.0%)	0	5/5 (100%)	0	0
Masikandoro	*R. decoloratus*	4/4 (100%)	0	0	4/4 (100%)	0	0	4/4 (100%)	0	0
Munyawiri	*R. decoloratus*	1/2 (50.0%)	0	1/2 (50.0%)	1/2 (50.0%)	0	1/2 (50.0%)	2/3 (100%)	0	0
Musabayana	*R. decoloratus*	3/4 (75.0%)	0	1/4 (25.0%)	4/4 (100.0%)	0	0	4/4 (100%)	0	0
Mutore	*R. microplus*	0	0	4/4 (100%)	0	0	4/4 (100%)	1/4 (25.0%)	0	3/4 (75.0%)
Mwenda	*R. decoloratus*	5/5 (110%)	0	0	4/5 (80.0%)	0	0	5/5 (100%)	0	0
Rowa	*R. decoloratus*	3/4 (75.0%)	0	0	4/4 (100%)	0	0	4/4 (100%)	0	0
Takunda	*R. decoloratus*	2/3 (66.7%)	0	1/3 (33.3%)	2/3 (66.7%0	0	1/3 (33.3%)	3/3 (100%)	0	0
Tiverton	*R. decoloratus*	0	0	4/4 (100%)	1/4 (25.0%)	1/4 (25.0%)	2/4 (50.0%)	4/4 (100%)	0	0

Figure 3: Sequence alignment of domain II of the voltage-gated sodium channel. Indicated are loci 1 and 2 where the pyrethroid-associated SNPs are located in resistant individuals.

Chapter 5

5.0 Discussion, conclusions and recommendations

In this study the two SNPs in the OCT/Tyr receptor associated with amitraz resistance were analysed. A high frequency of homozygous resistant alleles associated with amitraz resistance in both *R. decoloratus* and *R. microplus* tick populations were observed at locus 1 (Table 6). The frequency of these resistant alleles seem to be higher in *R. microplus* compared to *R. decoloratus* at locus 1 (45% versus 50%; Table 4 and 5) while it is 2.4 times higher at locus 3 in the *R. microplus* ticks. However, it must be noted that a very small sample number (n=5) was analysed in this study and that additional sampling and SNP analyses will be needed to statistically validate these findings. The frequency of homozygous resistant SNPs present in these amitraz-resistance associated alleles suggests that amitraz resistance is present in Zimbabwe. The latter support the findings of Sungurai et al. (2018) where he described 28.5-47.2% of the homozygous resistant genotype for this gene within a larger sample size (n=103). In his studies, the Hardy-Weinberg equilibrium analyses revealed selection pressure ($p<0.05$) for amitraz resistance while there was no selection pressure for organophosphates and pyrethroids using the carboxylesterase gene marker ($p>0.05$). Combined, findings for Zimbabwe indicate that using amitraz for the control of Rhipicephalus ticks should be carefully reconsidered to lessen the selection pressure and spread of resistant ticks in the future. By reducing the use of amitraz, and increasing the use of an alternative active ingredient (such as a pyrethroid or macrocyclic lactones) may offer a solution for hindering growth in the number of amitraz resistant ticks (especially in *R. microplus* which transmits Asiatic

babesiosis). This finding is not surprising, as amitraz has been in use for over 30 years in Zimbabwe (Sungirai et al. 2016).

With regard to pyrethroid resistance, no SNPs were identified in domain II of the voltage-gated sodium channel gene. This suggests that presently both species are susceptible to synthetic pyrethroids. However, it is important to note that resistance to pyrethroids can also be mediated by metabolic detoxification and this resistance mechanism cannot be detected by the PCR based assay carried out in this study. Rosario-Cruz et al. (2009) reported that enhanced hydrolysis of pyrethroids by ticks with an increased expression of carboxylesterases was another mechanism for resistance. However, based on the lack of SNPs in the voltage-gated channel combined with the Hardy-Weinberg equilibrium conducted by Sungirai et al. (2018), where no selection pressure for organophosphates and pyrethroids using the carboxylesterase gene marker ($p>0.05$) was described, the possibility of continued use of pyrethroids remains a viable, cost-effective control strategy for *R. microplus* and *R. decoloratus*.

Over reliance on amitraz which have been in use for over 30 decades is a risk factor that needs to be addressed. One way of addressing it is by reducing the frequency of dipping of cattle, but this is not a viable option in areas where high tick loads are present and where environmental factors support a growing population of ticks. A more realistic approach would be to make use of another chemical active, such as pyrethroids or macrocyclic lactones (as stated previously). Alternatively, the alternating or rotation of acaricides with different modes of action (i.e. chemical active ingredients) can be used to reduce the development of resistance (Rodriguez-Vivas et al. 2014b). Strict movement

control of cattle from this region does however need to be considered to prevent spread of amitraz-resistant alleles across the whole country. This was also suggested by Sungirai et al. (2018) that movement of cattle from different regions of Zimbabwe can be attributed to the expansion in distribution of *R. microplus*. Strict adherence to the livestock movement regulations, to allow only tick-free cattle to move will help prevent spread of resistant tick populations.

Over reliance on a single tick control strategy, such as chemical control, can be problematic and thus the need to implement a more integrated approach is always encouraged. Examples of the latter include a combination of breeding indigenous tick-resistant cattle, grazing rotation, strategic dipping and in future even using tick vaccines (Peter *et al.* 1998). However, with chemical control being the most cost-efficient method of tick control, more so in areas where grazing rotation is not feasible, regular surveillance for acaricide resistance is needed to be enable knowledge-based selection of active compounds and to lessen the development of resistance.

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Animal Ethics Approval

This project was approved under Amendment 1 Project number V029-17

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
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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

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Faculty of Veterinary Science
Animal Ethics Committee

Ref: V029-17 (Amendment 1)

31 August 2017

Dr Jannie Crafford
Department of Veterinary Tropical Diseases
University of Pretoria
(jannie.crafford@up.ac.za)

Dear Dr Crafford

Project V029-17 (Amendment 1): 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 and Mutare district, Zimbabwe

The project is approved on condition that we've receive a progress report on the current phase.

Yours sincerely

Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

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