



Faculty of Sciences

Department of Biological Sciences and Biotechnology

ASSESSMENT OF *AMBLYOMMA HEBRAEUM* FOR ACARICIDE RESISTANCE IN THE
HEARTWATER-ENDEMIC SOUTHERN DISTRICT OF BOTSWANA

By

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BSc (Animal Sciences) (BUAN)

A Thesis Submitted to the Faculty of Sciences in Partial Fulfilment of the Requirements for the
Award of the Degree of Master of Science in Biological Sciences and Biotechnology of BIUST

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December, 2022

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LIST OF ABBREVIATIONS

AChE	Acetylcholinesterase
A	Adenosine
AIT	Adult immersion test
AITDD	Adult immersion test with a discriminating dose
bp	base pairs
C	Cytosine
DDT	Dichlorodiphenyltrichloroethane
DNA	Deoxyribonucleic acid
FAO	Food and agriculture organisation
G	Guanine
gDNA	genomic Deoxyribonucleic acid
LPT	Larval Packet Test
OCT/Tyr	Octopamine/tyramine
PCR	Polymerase chain reaction
SNPs	Single nucleotide polymorphisms
SP	Synthetic pyrethroids
VGS	Voltage-gated sodium channel
TBD	Ticks and tick-borne diseases

DECLARATION REGARDING THE WORK AND COPYRIGHT

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Thesis Titled: ASSESSMENT OF *AMBLYOMMA HEBRAEUM* FOR ACARICIDE RESISTANCE IN
THE HEARTWATER-ENDEMIC SOUTHERN DISTRICT OF BOTSWANA

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I, the Candidate's Primary Supervisor, hereby confirm that I have inspected the above titled thesis, and to the best of my knowledge, it is based on the original work of the candidate.

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Date: 13 December 2022

ACKNOWLEDGEMENTS

This research is successfully completed because of the following:

- The grant allocated to me by the University (Botswana International University of Science and Technology).
- The expertise and invaluable support of my supervisor Dr Lebani.
- The Veterinary Personnel's assistance in tick collection.
- The Farmers' participation in the study.
- The moral support, encouragement and love from my wife.

Thank you!

ABSTRACT

Cattle tick *Amblyomma hebraeum* has become a common problem for cattle herders in Botswana for many years, despite the availability of many different acaricides products. The aim of this research was to assess the resistance status of *A. hebraeum* to deltamethrin and amitraz at recommended doses using AIT. A cross sectional questionnaire survey was conducted on the 18th-23rd of November 2019 on four farms where farmers had reported acaricides failure before. Engorged adult female *Amblyomma hebraeum* ticks were collected and sent to Botswana international university of Science and technology laboratory in Palatye. The Adult immersion test was used to assess the resistance-susceptibility level of the *Amblyomma* tick population collected to amitraz and deltamethrin. The results showed slight resistance to deltamethrin I Lotlhakane and Pitseng and 100% susceptibility for Good hope. Ticks from all villages were susceptible to amitraz. DNA was extracted from ticks amplified and sequenced to confirm that there was no underlying genetic basis for deltamethrin resistance. No polymorphisms were identified in the loci associated with resistance to deltamethrin. The mechanism for deltamethrin tolerance used by *Amblyomma hebraeum* in the Southern District of Botswana remains unknown and needs further investigation. This study provides baseline information on the effectiveness of acaricides against *Amblyomma hebraeum*, infection level of the tick species with ehrlichia ruminantum and possible polymorphisms responsible for resistance to occur in the Southern District of Botswana, where heartwater continues to be a tick-borne livestock disease of concern.

CHAPTER ONE

INTRODUCTION

Ticks are blood-sucking ectoparasites in wild and domestic animals and humans. Some hard tick species (of the Ixodidae family) found in Southern Africa belong to the genera *Rhipicephalus*, *Hyalomma*, *Hemaphysalis* *Ixodes species*, and *Amblyomma*. These tick species are important vectors of animal diseases, and they transmit protozoans, rickettsia, spirochetes, and viruses that cause economically important diseases, such as babesiosis, heartwater, anaplasmoses, and theileriosis. Tick vectors can significantly affect developing countries in tropical and sub-tropical regions if there is a lack of human and financial resources to implement good tick control strategies. Ticks either directly or indirectly affect livestock. They directly suck blood from animals, causing anemia and reduced weight. Heavy tick infestation can also result in dermatitis. During feeding, ticks transfer disease-causing microorganisms to the host animals, which can lead to inflammation. Animal lameness or loss of teats can result from sustained or untreated inflammation. Ticks and tick-borne diseases (TBD) cause an outstanding monetary decline in the agricultural sector, particularly with respect to livestock production in tropical and sub-tropical regions where tick infestations are high [1,2].

Not all ticks in a given area are infected with pathogens; it is only during attachment and long feeding sessions that pathogens can parasitize ticks that they later pass to the next host [3]. These pathogens cannot pass from adult ticks to eggs in the ovary but can only be transmitted horizontally from one host to another [3, 4]. Therefore, tick bites cannot lead to infections, especially if ticks can be removed before 36 h elapsed while attached to the host [5]. Farmers, therefore, use acaricides as a mainstay in preventing TBD because they have been shown to be more effective as a tick control method than other methods, such as rotational grazing and physical killing of ticks [6].

Ticks are controlled by various acaricide classes, including macrocyclic lactones, organochlorines, organophosphates, carbamates, amidines, and synthetic pyrethroids [7]. However, the extensive use of acaricides has led to the alarming development of acaricide

resistance among tick populations, resulting in the failure of some tick control programs [8,9,10]. The three main forms of acaricide resistance are well known. (i) Metabolic tolerance is achieved through acaricidal detoxification via the metabolic activity of enzymes such as cytochrome P-450 (CYP), esterase, and glutathione-S-transferase (GST). (ii) Resistance resulting from structural changes in neuronal enzymes and receptors at target sites of acaricides leads to impaired interaction between the drug and the target. (iii) The reduction in acaricidal access to the internal environment due to changes in the outer layer of the tick (exoskeleton) is referred to as reduced penetration resistance [9].

1.1 Problem statement

Botswana does not have any policy that guides tick management and the use of acaricides. The consequence of the lack of such a policy is that individual farmers are responsible for their own tick control and acaricide use practices. When such practices are poor, they can lead to establishment of acaricide resistant tick populations that affect farmers and their livestock over large areas that are able to support tick expansion. However, there is a paucity of information on acaricides resistance and farmers knowledge on these issues. To address this gap a study was undertaken to study farmers perception on acaricides resistance, infection status of *Amblyomma* ticks with *Ehrlichia* as well as attempting to identify SNPs associated with resistance in *Amblyomma* ticks.

1.2 Significance of the study

Ticks can play a role in determining the success of many livestock projects as they can transmit pathogens that cause important livestock diseases. Acaricides resistance can also increase cost for production as well as livestock loss due to tick borne diseases. It is therefore relevant to regularly monitor acaricides resistance status of ticks farmers are fighting in their farms. The results of this research will help veterinary department and individual farmers in such a way that:

Reporting evidence or lack thereof of acaricide resistance can inform whether the status quo in terms of a lack of a tick control policy should remain or if there is need for development of a tick control policy.

1.3 Aim

The aim of the research was to assess the infection status of ticks by *Erlichia ruminantium* and their resistance to used acaricides in the Southern district of Botswana.

1.4 Specific objectives

- Determine infection status of *Amblyomma* ticks with *Erlichia ruminantium*.
- Identify SNPs associated with acaricide resistance in *A. hebraeum* tick populations collected from the southern district of Botswana.
- To determine the resistance status of *A. hebraeum* to deltamethrin and amitraz at recommended doses using AIT.

CHAPTER TWO

LITERATURE REVIEW

Acaricides are a major method for tick control and management; however, this method is slowly becoming unsustainable as populations of acaricide resistant ticks are being selected by acaricides that are already in use. Acaricide resistance in ticks is a shift or change in the susceptibility of the target species to a drug, but it is generally viewed as a failure by a prescribed chemical's lethal dose to eliminate pest control [11]. The continued use of the same acaricides help select for resistance genes in the next generation through chromosomal DNA from survivors of acaricide treatment. Initially, rare mutations may persist over time within a population, as acaricide application favors these genes over susceptible ones [12]. Farmers, therefore, resort to high acaricide doses that often remain ineffective and leave residues in milk and meat, as well as affecting bees and human health [13]. Mitigation of acaricide resistance can be achieved through the rotation of acaricides and synergistic combinations of acaricides.

2.1 *Amblyomma hebraeum*

Amblyomma hebraeum is also known as the South African bont tick [15]. It is in the phylum *Arthropoda* and order *Acarina*. The genus *Amblyomma* comprises over 130 species [15]. In Africa, acaricide resistance research is mostly concentrated on *Amblyomma gemma*, *Amblyomma variegatum*, and *Amblyomma hebraeum*, which spread heartwater [15, 16].

Amblyomma hebraeum is a hard-bodied tick, with males having a rigid chitinous scutum covering the entire dorsal body surface [15]. The scutum in females is small, allowing swelling of the abdomen during feeding [3]. Female ticks also have a pink to orange enamel orientation, with pale rings on their legs. The body of *Amblyomma hebraeum* has three main regions: the gnathosoma, which includes the mouth parts consisting of paired chelicerae, segmented palps, and denticulate hypostome. All these structures constitute the capitulum [17]. The male *A. hebraeum* tick is oval, measuring 4.2–5.7 mm long, while the females are 5 mm long; engorged females grow up to 20 mm long, with a rectangular basis, convex lateral margins, and slightly noticeable posterolateral angles. The representation in

Figure 1 shows a smooth and convex scutum of females with fine black background and males with brown spotted and striped pale greenish white background. The legs are dark brown, moderately stout, with apical yellow banding at the distal end of each segment. These features were then used to identify *A. hebraeum*. The representation also shows a labeled capitulum on the female tick



Figure 1: Schematic representation of male and female *Amblyomma hebraeum* [18]

Amblyomma hebraeum exhibits chelicerae that penetrate the epidermis of the host animal and insert the hypostome into the wound [2]. The cement secreted by the salivary glands of the tick and its hypostome teeth aids its attachment to the host [18,19]. The eyes are located on the lateral margins of the scutum. The legs, genital pores, and spiracles are present in idiosoma [20]. The outside cuticle expands to accommodate the ingested volume of blood. Pathogenic microorganisms are transmitted through these blood vessels. In heartwater-positive animals, *Ehrlichia ruminantium* can parasitize ticks during blood meals [14]. This makes *A. hebraeum* an important heartwater vector, and thus, its control is pertinent to mitigating disease transmission.

2.2 Geographical Distribution of Amblyomma species in Southern Africa

Amblyomma hebraeum distribution extends from South Africa, Swaziland, Southern and Northern, eastern Botswana, Zambia, some parts of Zimbabwe, Mozambique, and Angola (Fig. 2) [16, 21,22]. *A. hebraeum* adapts mainly to areas with rainfall between 300 and 600 mm and mixed bushvelds of *Combretum* and *Acacia* species [23]. This cover provides a microclimate for the survival of its eggs [24].

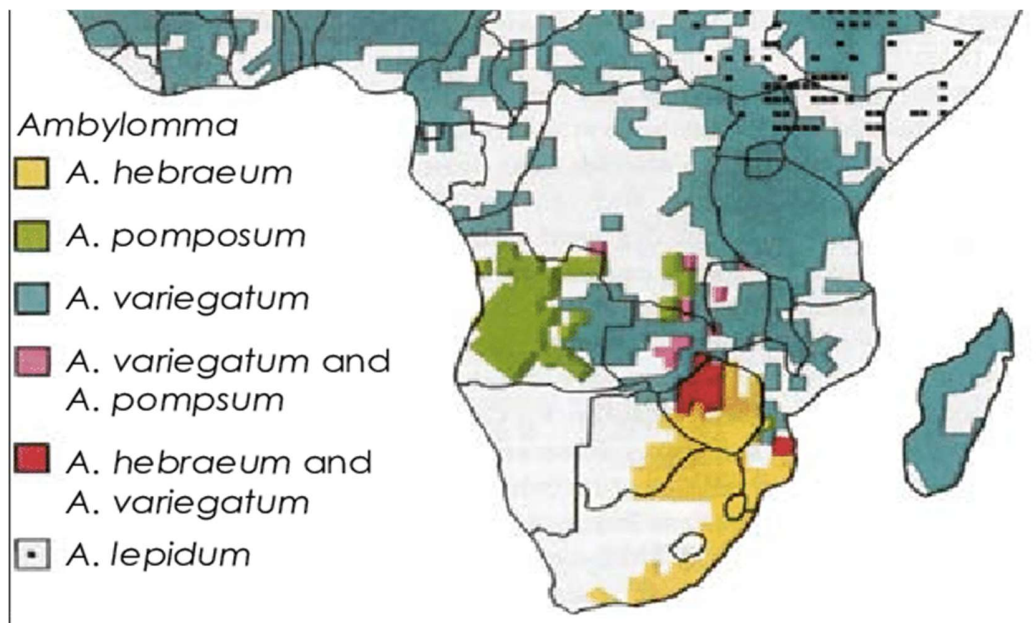


Figure 2: Map of Southern Africa showing *Amblyomma* species distribution in heartwater endemic areas [48]

The distribution of the heartwater vector extends from South Africa, Swaziland, southeastern and northeastern Botswana, Zambia, most parts of Zimbabwe, and some parts of Mozambique.

2.3 *Amblyomma hebraeum* life cycle

The life cycle of *Amblyomma hebraeum* consists of four developmental stages: eggs, larvae, nymphs, and adults (Fig. 3). *Amblyomma hebraeum* are classified as three host ticks, as they require three hosts to complete their life cycle [22]. Fully incubated eggs hatch into larvae that feed on the first host before molting to become nymphs [25, 26, and

27]. The nymph feeds on the second host and molts into adult ticks. Adult ticks feed on a third host and become engorged. Female ticks mate while still feeding, then continue feeding to become fully engorged, and later drop off the host to lay a batch of eggs [19, 2]. This makes the collection of female ticks important because feeding provides a chance for parasitism with or through transmission of *Ehrlichia ruminantium*.

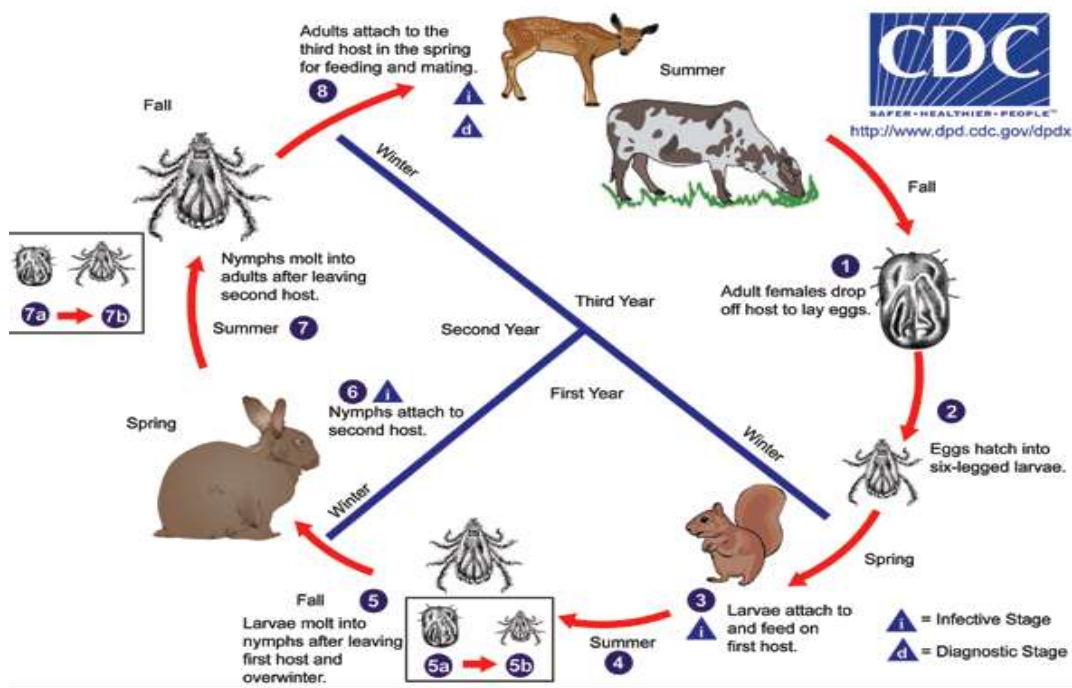


Figure 3: A schematic presentation of *Amblyomma hebraeum* three host tick life cycle [28]

The life cycle of *Amblyomma hebraeum* usually spans 3 years. Engorged female *Amblyomma hebraeum* drops off the host to lay eggs 1, and the eggs hatch 6-legged larvae (2) during winter. The larvae seek out a small rodent in spring to feed on (3) Engorged larvae then leave their first host in summer (4) and molt into nymph (5) in the fall. The nymph goes through winter during this stage. The nymphs parasitize the second host during spring (6). The nymph drops off immediately after completing the feeding stage in late summer and then molts into adults (7a-7b) towards the end of summer. Adults then seek

out and attach to a third host around a large herbivore. The adults feed, become engorged, and then drop off to lay eggs, and the cycle continues.

2.4 Tick control in Botswana

The development and use of acaricides date to 1895. Farmers used organophosphates, amidines, pyrethroids, phenylpyrazoles, cymtazoles, naturalytes, and macrocyclic lactones [9,29]. Different types of acaricides are usually applied by livestock dipping or spraying. The choice of acaricides has become a critical issue over the years, as there have been reports of resistance development in some parts of the world [30].

The Botswana government constructed tick control facilities on the back of an outbreak of East Coast fever spread by *Rhipicephalus appendiculatus*, which was imported from East African countries to the rest of Africa. This was a noteworthy action in disease control. The lack of commitment by farmers in maintaining dipping facilities due to the lack of tick-related livestock infections led to the collapse of community dipping facilities in Botswana. This meant that individual farmers had to develop their tick control practices for disease management. [31, 32, 33, 34]. This results in individual farmers deciding which acaricide to use. These choices may not necessarily be based on acaricide efficacy but rather on the affordability and availability of acaricides in their area. Tick control practices in Botswana remain effective and depends in the use of acaricides despite reports of acaricides failure by farmers in certain areas. This problem can also be compounded by inadequate application and/or inconsistent use of chemicals continuously, resulting in ineffective tick control and/or selection of acaricide resistant ticks [35,36].

2.5 Types of acaricides used in Botswana.

2.5.1 The amidines group

This group of acaricides inhibits monoamine oxidase, which is responsible for the metabolism of neurotransmitter amines present in the nervous system of susceptible ticks and mites [8,22,37]. It is a well-established acaricide that was developed 50 years ago [22]. It increases nervous activity by interacting with the octopamine receptor. Ticks treated with amitraz agitate detach their mouth parts in less than 60 minutes and begin moving rapidly

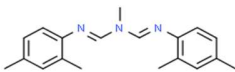
in a disoriented manner across the animal body [38]. This makes amidines the best detaching acaricides, as it does not kill ticks directly, and ticks that are treated cannot attach to the next animal. This acaricide is also environmentally friendly, as it is biodegradable, with no residue of the compound in the milk of treated animals [39,40].

2.5.2 The pyrethroids group

The members of this group include cypermethrin, flumethrin, deltamethrin, cyflumethrin, cyhalothrin, and alpermethrin]. These organic compounds are like natural pyrethrins, extracted from the pyrethrum flowers. They are generally harmless to humans at household concentrations; however, pyrethroids are also toxic to insects and ticks. Pyrethroids interact with sodium channels to excite them and cause them to open longer, thereby prolonging the inward sodium flow. They are axonic excitotoxins that prevent the closure of the voltage-gated sodium channel in the axonal membranes. The organism is then rendered paralyzed because the nerves cannot repolarize, leaving the axonal membrane permanently depolarized [41].

Acaricides are made specifically to control external parasites, and their composition differs depending on their chemical content and mode of action, as shown in Table 1.

Table 1. Commonly stocked and used acaricides in the Southern district of Botswana.

Formamidines	Active ingredients	Mode of action	Advice or Recommendation
Taktic	Amitraz 	Octopamine agonists	Use one acaricide at a time. Don't mix acaricides with crop pesticides.
Triatic			
Coopers Triarix			
Eraditic			

2.8 Strategies to mitigate acaricides resistance

Strategies to mitigate acaricide failure are ways to reduce the speed at which acaricide resistance can be established in a population of ticks in each area [23]. These strategies often slow down or prevent selection for resistance to newly introduced acaricides. Such strategies include a reduction in the frequency of acaricide application, rotation of acaricides, and use of appropriate concentrations of acaricides to reduce the chance and period of selection [23]. Rapid detection of acaricides resistance and regular monitoring are imperative as the early identification of resistance development can help in transitioning to a more effective acaricide of a different class toward vector control (15, 30)

Other tick control strategies may also be involved in developing and breeding tick-resistant animals. Animals may resist ticks by developing a thick skin coat, dense hair, and skin [46]. Biological control methods can be used, such as predators feeding on ticks as part of their food chain. Methods such as rotational grazing are also useful for breaking the life cycle of ticks [16, 31]. This results in the incomplete development of ticks in the area, as they need a source of blood (host animal) for their complete development.

2.9 Assessment of acaricides resistance in ticks

The high profit losses incurred by farming communities because of the establishment of acaricide resistance calls for regular testing of acaricides to assess their effectiveness. This is important, as it can also help in decision-making regarding the best acaricides to use to mitigate acaricide resistance. It is important to have an informed approach to tick control as it can sustain the use of available acaricides. Producing new molecules that can exert efficient control as approved acaricides is expensive. *In vitro* bioassay methods are available for detecting the effectiveness of acaricides [23]. The methods of testing acaricides for effectiveness involve exposing ticks to concentrations of acaricides and then determining the proportions of dead and live ticks [34]. Testing for susceptibility to acaricides includes the Adult Immersion Test (AIT) and, more commonly, the Larval Packet Test (LPT). Both are recommended and standardized by FAO [46]. AIT is quick to undertake; it takes a few weeks to complete. However, its disadvantage over the LPT is

that it requires a large pool of engorged adult female ticks compared to the LPT, which only uses a few engorged ticks to lay eggs, and then the larvae are used to produce full dose-response curves [21]. Although bioassays are indispensable because of their low cost and ease of use, they are labor-intensive and provide long-term results. Therefore, there is a need to perform rapid and sensitive molecular-based assays that can detect resistance at the earliest stage as early as possible. Sequencing methods can be used to provide rapid tests for acaricide resistance at early stages and within a short period of time. Sequencing is used to identify genes with polymorphisms that facilitate acaricide resistance. Although this method is relatively quick, it requires technical training and has a significantly higher cost. [19].

CHAPTER THREE

MATERIAL AND METHODS

3.1 Study methodology

A sampling frame of the number of villages in the district and dip tanks were obtained from the veterinary department and selection of villages was done basing on the abundance of host animals and reported cases of resistance. Five infested (animals with ten or more ticks) cattle were selected for tick sampling at each crush. The developed villages like Moshupa, Kanye, and Jwaneng were excluded as little farming is practiced there. Only three villages

in the Southern District of Botswana, namely, Lotlhakane, Pitseng, and Good Hope, were chosen for sampling ticks basing on the fact that they had organized farms with proper animal handling facilities and accessible by roads for easy tick collection. The Southern district veterinary office also recommended these villages as they recorded highest number of heartwater infections and deaths in the previous years. All visibly engorged adult female ticks were collected from infested cattle herds by pulling with forceps. The oviposition inhibition effect and efficacy of acaricides were evaluated under a controlled experimental design in a laboratory at the Botswana International University of Science and Technology. A total of 25 pastoralists were selected from a pastoralists' list provided by the veterinary offices of each village. The selection was made by choosing a name of a pastoralist in the list, calling him and having his agreement for the survey. When the picked pastoralist did not agree, another one was chosen until the desired number for each area was achieved. The questionnaires contained quantitative and qualitative questions [Appendix 1] that helped assess acaricide use and effectiveness.

3.2 Study area

The study was conducted in four selected crushes ($n = 4$) in the Southern district of Botswana Fig.4 During the collection conducted on the 18th-23rd of November 2019; The southern district is home to many pastoralists with large privately owned ranges, and several governments run beef ranges which provide agricultural support to the local farmers. The ecological areas consist of *Acacia melifera*, *Acacia tortilis* and a wide range of grasses palatable to ruminants. The vegetation type is savannah, with tall grasses, bushes, and trees. The annual precipitation is around 500 – 650 mm per annum, most of which is received from November to May. The temperature ranges between 18°C and 38°C in summer and 6°C and 34°C in winter. The area has been reported to have *Amblyomma hebraeum* and *Rhipicephalus microplus* (36). The areas are densely populated with livestock

Figure 4: Map of Botswana showing the location of the Southern district and tick sampling crushes marked with a blue balloon.

Table 2: GPS coordinates of the sampling crushes

Sampling site	Latitude	Longitude
Good hope abattoir	25°26'40"S	25°22'56"E
Good hope bull ranch	25°26'38"S	25°28,16"E
Lotlhakane	24°55'25"S	25°24'15"E
Pitseng	24°49'44"S	25°15'57"E

3.3 Tick collection and questionnaire survey

Cattle were restrained using crushes before going out of the kraals. In total, 168 ticks were collected via whole-body collection. Engorged adult ticks were picked from relatively hairless parts of cattle: the ventral body surface, perineum, axillae, and underside of the tail. Ticks were stored in plastic containers closed with a metal gauze to facilitate air circulation and grass to maintain moisture. Each container was labeled with the collection date, predilection site on the cow, GPS coordinates (Table 2 above), and site name.

Twenty-five questionnaires prepared before the survey were administered through face-to-face interviews by veterinarians during tick collection periods to collect information related to the acaricides used. Cattle farmers were interviewed in the morning before animals could go out for grazing, as interviews and the collection of tick samples were merged in one visit. The Department of Veterinary Services personnel oversaw both activities.

During the survey, ticks were collected for later morphological and molecular identification, as well as for performance of acaricide resistance bioassays, determination of parasitism with *Ehrlichia ruminantium*, and sequencing of genomic material. Interviews and tick collection were conducted on the 18th-24th of November 2019.

3.4 Morphological identification of *Amblyomma hebraeum*

One hundred sixty-eight ticks were morphologically identified as *A. hebraeum* using standard taxonomic keys [19].

3.5 Assessing ticks for acaricides resistance using adult immersion test

The adult immersion test with a discriminating dose (AITDD) was performed as Drummond et al. (44) described to determine the resistance in engorged adult female ticks collected during the study. This method was selected because it is simple and it is the first step for other methods. It was observed to be significantly sensitive ($P < 0.001$) in detecting phenotypic acaricide resistance in *R. (B.) microplus* (56). The number of engorged ticks was limited therefore only single dose was used to run the experiment. The selected acaricides were diluted according to manufacturer-recommended doses using distilled water and olive oil. Using 50 mL distilled water as a diluent, Amitraz solution (0.125 g/mL) was prepared. The deltamethrin solution was prepared to a volume of 50 mL with olive oil, with a final volume of 0.124 g/mL. Testing of acaricides was performed using three replicates of 10 ticks for each acaricide/control. After immersing the ticks in 50 mL of each solution, they were dried on absorbent paper, stuck on a petri dish using sticky tape with enough space in between to avoid mixing of eggs and incubated for seven days at a temperature of 27°C. Observed with a magnifying glass, ticks that succumbed to acaricides were considered susceptible and ticks that survived and laid eggs after immersion in acaricides were considered acaricide-tolerant and possible resistance was investigated. Dead ticks were considered susceptible to the acaricide.

3.6 Genomic DNA extraction

Three ticks were used for species verification using molecular techniques. The ticks were quickly frozen in liquid nitrogen and crushed using a mortar and pestle. The pulp of the tick was placed in a 2 mL microcentrifuge tube, and DNA was extracted from individual ticks following the manufacturer's procedure for the DNeasy® Blood and Tissue kit (QIAGEN, Germany). Extracted genomic DNA was stored at -20°C.

3.7 Molecular tick identification

Pair of primers was used to amplify a fragment of the mitochondrial cytochrome oxidase 1 gene (COI). A 710 bp amplicon was formed using the LCO149 (5'-GGTCAACAAATCATAAAGATATTGG-3') and HCO 2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') primers. Thermal cycling conditions were initial denaturation at 95°C for 2 min, followed by 30 cycles of 94°C for 30 s, annealing at 51°C for 30 s, and extension at 72°C for 35 s, followed by a final extension step at 72°C for 3 min and a 4°C hold. A 1% agarose gel stained with 10 µL ethidium bromide per 200 mL agarose gel was used to confirm the presence of the amplicons. The gel was visualized using a UV transilluminator (Bio-Rad, Gel doc XR+, USA). The observed bands of the PCR product were carefully cut from the agarose gel, purified using a QIAGEN gel purification kit (USA), and sent for sequencing at Inqaba Biotech Industries (Pretoria, South Africa).

3.8 *Ehrlichia ruminantium* molecular detection

The presence of *Ehrlichia ruminantium* was investigated in *Amblyomma hebraeum* nymphs and engorged female ticks using semi-nested PCR. Three primers, AB128 (5'-ACTAGTAGAAATTGCACA ATCTAT-3') as the internal forward primer, AB129 (5'-TGATAACTTGGTGCGGG AAATCCTT-3') as the internal and external reverse primer, and IT130 (5'-TCAATTGCTTAATGAAGCACTAACTCAC-3') as the external forward primer, were used for PCR. The initial PCR reaction was run using AB129 and IT130 primers with the following PCR conditions: A 25 µL reaction was made with 12.5 µL of Q5 high fidelity master mix (New England Biolabs, USA), 2 µL of 10 µM forward and reverse primers (Inqaba Biotech) respectively, and 2 µL of tick gDNA and 6.5 µL of nuclease free water and 12.5 µL of PCR mastermix. The thermal cycling conditions were as follows: initial denaturation at 95°C for 2 min, 40 cycles of 94°C for 30 s, annealing at 56°C for 60 seconds 72°C for 30 s, and a final extension for 10 min. The second round of amplification was carried out using 2 µL of AB128 and AB129 primers in a final reaction volume of 25 µL and the product of the first round as an amplicon. The thermal cycling conditions were as follows: 30 cycles of 94°C for 30 s, 68°C for annealing at 72°C for 30 s,

followed by a final extension for 10 min at 72°C, and a hold at 4 °C. A 1% agarose gel stained with 10 µL ethidium bromide per 200 mL agarose gel was used to confirm the presence of the amplicons. The gel was visualized using a UV transilluminator (Bio-Rad, Gel doc XR+, USA).

3.9 Assessment of deltamethrin target genes for SNPs

The presence or absence of nucleotide polymorphisms in voltage-gated sodium channel genes and carboxyl esterase genes was assessed using eight ticks that were found to be tolerant to different doses of deltamethrin. Eight ticks that were susceptible to the same acaricide and eight ticks from the control group were selected for comparison of nucleotide sequences. Ticks were crushed individually, and DNA was extracted following the manufacturer's procedure for the DNeasy® Blood and Tissue kit (QIAGEN, Germany). The gene of interest was amplified using PCR and a set of primers (Table 3).

Amplicons were visualized on a 1% agarose gel using a UV transilluminator (Bio-Rad, Gel doc XR+, USA). The observed bands of the PCR product were carefully cut from the agarose gel, purified using a QIAGEN gel purification kit (USA), and sent for sequencing at Inqaba Biotech Industries (Pretoria, South Africa).

Table 3: Primer sequences for PCR assay targeting single polymorphisms in the sodium channel gene and carboxyl esterase genes that confer synthetic pyrethroid resistance.

Gene	Primers
Voltage-gated sodium (VGS) channel gene	BmNaF5 (5'-TAC GTG TGT TCA AGC TAGC-3') BmNaR5 (5'-ACT TTC GTA GTT CTT GC-3')
Putative carboxyl esterase encoding gene	GS138BF (5'-AGCATCGACCTCTCGTCCAAC-3') GS139R, (5'-GTCGGCATACTTGTCTTCGATG-3')

3.10 Sequence analysis

DNA sequences were analyzed using Snap gene software version 5.2.5. All trimmed and edited sequence data were analyzed by multiple sequence alignments using the Clustal W algorithm in BioEdit package version: 7.2.5 to compare the VGS channel and putative carboxyl esterase genes of susceptible ticks with the resistant ticks.

3.11 Data management and analysis

Microsoft Excel 365 was used to record bioassay data from field and laboratory work. Then, the acaricidal efficacy was calculated according using the mean values of the test repetitions of the control and treated groups, resulting in the acaricidal efficacy in vitro using the formula: acaricidal efficacy (AE) = (mean survival control group – mean survival treated group)/mean survival control group $\times$ 100.

CHAPTER FOUR

RESULTS

4.1 Analysis of pastoralists responses to survey questions

The pastoralists (25/25) 100% who participated in the questionnaires reported that the highest tick infestations occur in summer and autumn (September to April); as a result, they rely on a list of acaricides to fight the ticks (Table 4)

Table 4: Commonly used acaricides, their mode of action, and application frequency

Village	Commonly used acaricides	Mode of application
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Pitsane	deltamethrin, amitraz, ivermectin	Spray, dipping, injectable, pour-on
Lotlhakane	deltamethrin, biological control	Rubbing, pour-on, dipping
Good hope	deltamethrin, amitraz	Pour-on, rubbing, Spray

In terms of acaricides used, (13/25) 52% of respondents reported that they used amitraz, which falls under the amidine class of acaricides, (10/25) 40% used deltamethrin, which falls under the pyrethroid class of acaricides; and 8 % (2/25) used engine oil and injectable ivermectin to control tick infestation. Regarding the frequency of acaricides used (13/25), 52% of respondents applied acaricides monthly (6/25), 24% applied acaricides fortnightly to keep their animals safe from tick infestation, and the remaining 24 % (6/25) dipped their animals only when animals were heavily infested. Regarding the efficiency of the acaricides used, 80% (20/25) of the farmers were content with the acaricides used, and the remaining 20% (5/25) reported failure of the acaricides to eliminate all ticks. They noticed re-infestation of the animals with ticks a few days after dipping. Most respondents (22/25), 88%, preferred using knapsack sprayers to apply acaricides, and (3/25), 12%, rubbed acaricides on the livestock body using cotton cloths, 100% of respondents were male and their age ranged between 35-65 years, All respondents admitted that, at some point, cattle were lost to heartwater, babesiosis, and/or anaplasmosis. All respondents could read instructions on the acaricides used as they have secondary education qualification and stated that *Amblyomma hebraeum* and *Rhipicephalus microplus* were the most problematic ticks in their areas.

4.2 Molecular identification of ticks collected.

A total of 168 fully engorged female *Amblyomma* ticks were collected, 58 ticks being collected from Lotlhakane, 71 ticks from Good hope and 47 ticks from Pitsane.

Table 5: Molecular identification of representative samples of collected ticks.

Species Name	Accession Number	Identity (%)
<i>Amblyomma hebraeum</i>	MT549815	99.84

A 279 bp amplicon was generated using nested PCR in the presence of *Ehrlichia ruminantium*, as shown in Figure 5. The following positive results were recorded: Lotlhakane (13/58) 22%, Good Hope (25/71) 35%, and Pitsane (22/47) 46% were parasitized by *Ehrlichia ruminantium*.

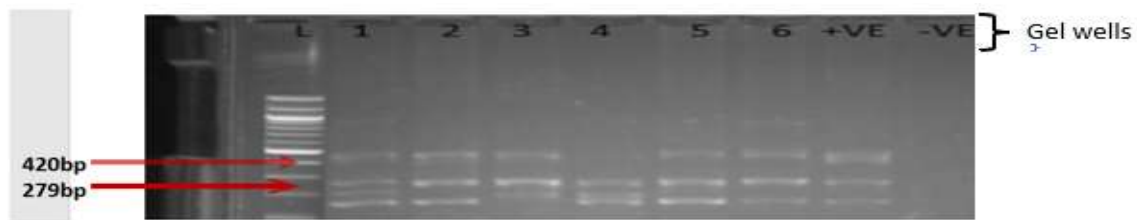


Figure 5: Analysis of PCR products of *E. ruminantium*. DNA from adult engorged *A. hebraeum*. DNA analyzed by agarose gel electrophoresis

L= 100 bp molecular marker, 1-6= selected positive tick samples, +ve= positive control (*E. ruminantium*-positive brain sample); -ve = negative control (water).

4.3 Assessment of ticks for possible resistance using adult immersion test

The survival rates of *Amblyomma hebraeum* ticks treated with the recommended dosages of amitraz and deltamethrin were 0% and 10%, respectively, as shown in Figures 4 and 5.

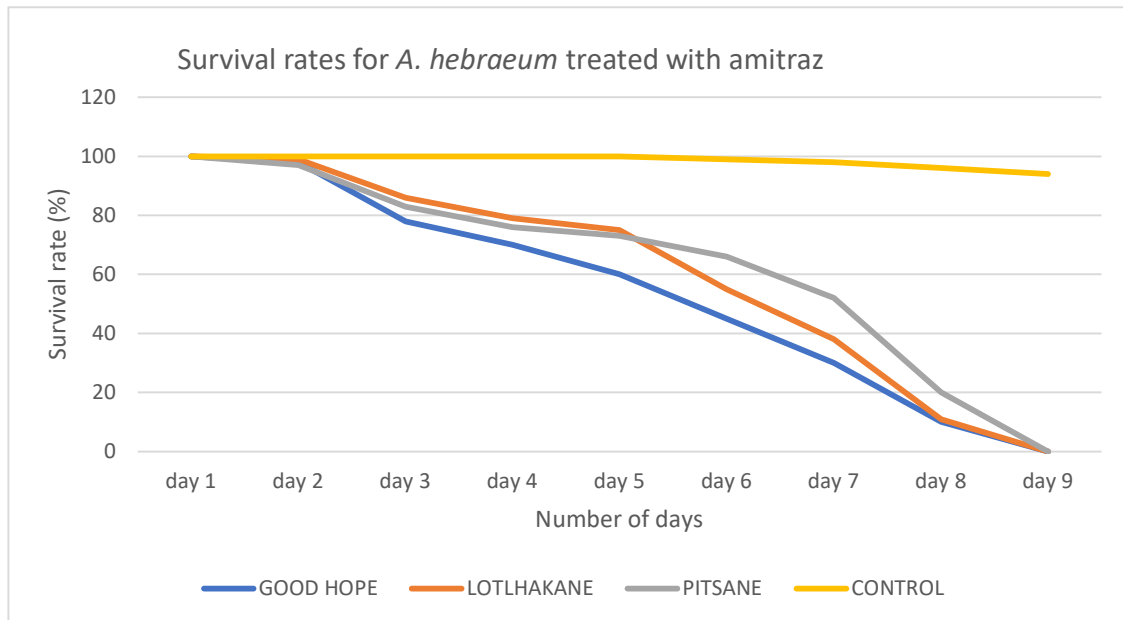


Figure 6: Survival of ticks immersed in discriminating dose of amitraz.

The number of live ticks exposed to the amitraz solution from all locations was reduced to zero by day 9.

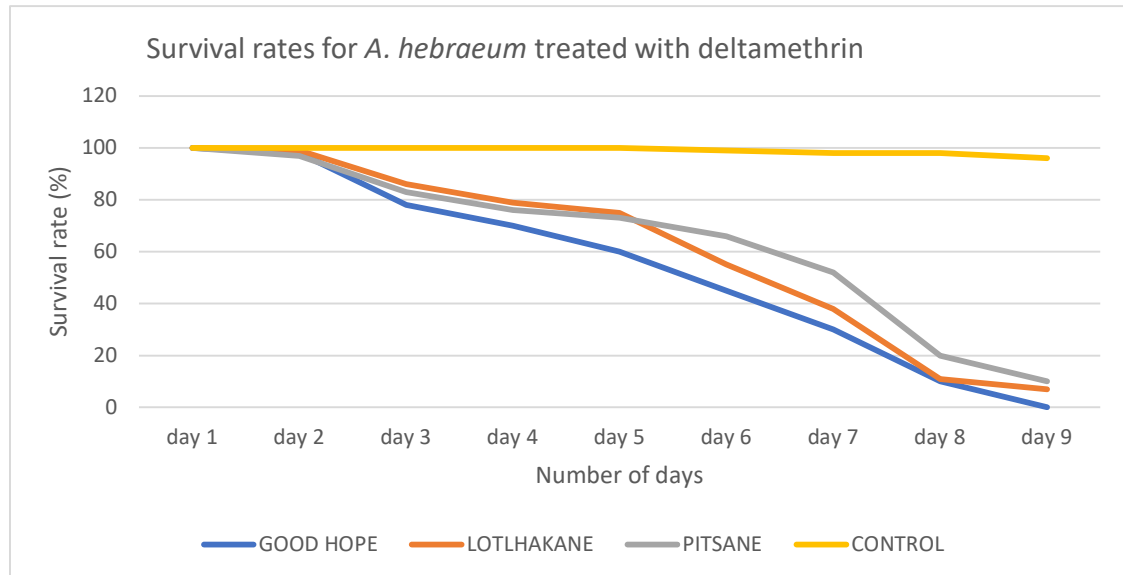


Figure 7: Survival of ticks immersed in discriminating dose of deltamethrin.

A 100% mortality rate was recorded for ticks collected from Good Hope, 93% for ticks collected from Lotlhakane, and 90% for those collected from Pitsane.

4.4 Testing hatched nymphs for resistance using larval packet test method.

Larval packet assays were performed to determine whether there was vertical transmission of deltamethrin resistance. *Amblyomma hebraeum* ticks that survived deltamethrin application and showed tolerance to acaricide had their larvae subjected to deltamethrin. There was 100% mortality in all larvae treated with deltamethrin.

4.5 Detection of deltamethrin and amitraz resistance in engorged female ticks DNA.

The amplified VGS genes and putative carboxyl esterase encoding genes of engorged ticks were separated using gel electrophoresis to yield 167 bp and 372 bp amplicons, respectively, as presented in Figures 7 and 8.

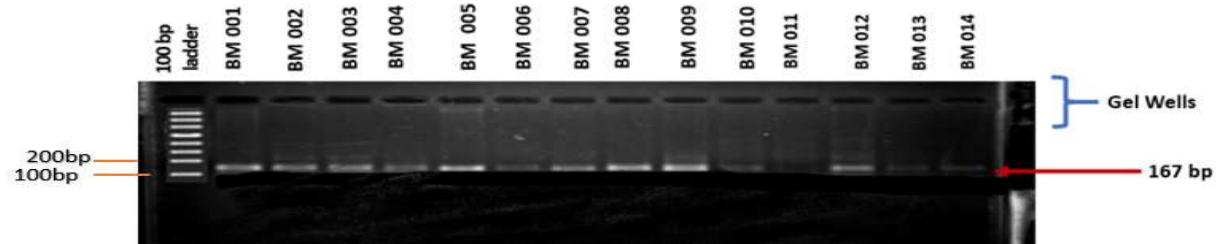


Figure 8: Analysis of PCR products of the amplified voltage-gated sodium channel gene from adult engorged *A. hebraeum* DNA analyzed by agarose gel electrophoresis.

100 bp , molecular marker; BM001-BMO14 = selected tick sample identifiers.

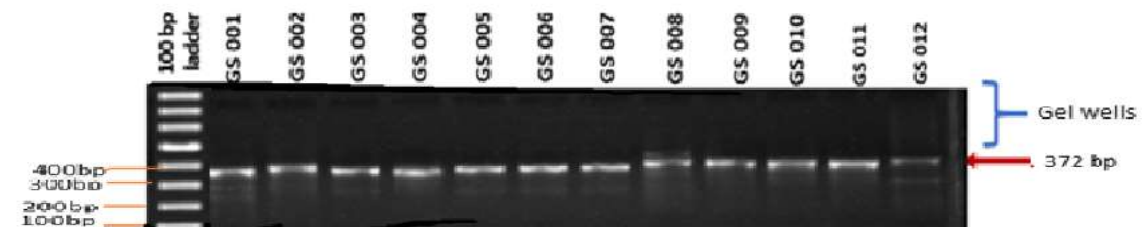


Figure 9: Analysis of PCR products of the amplified putative carboxyl esterase encoding gene from adult engorged *A. hebraeum* DNA analyzed by agarose gel electrophoresis.

100 bp = molecular marker; GS001-GSO12 = selected tick sample identifiers.

The results of multiple aligned sequences as indicated in Figures 9 and 10 show no alterations to the target sites that affect sensitivity to deltamethrin and amitraz.

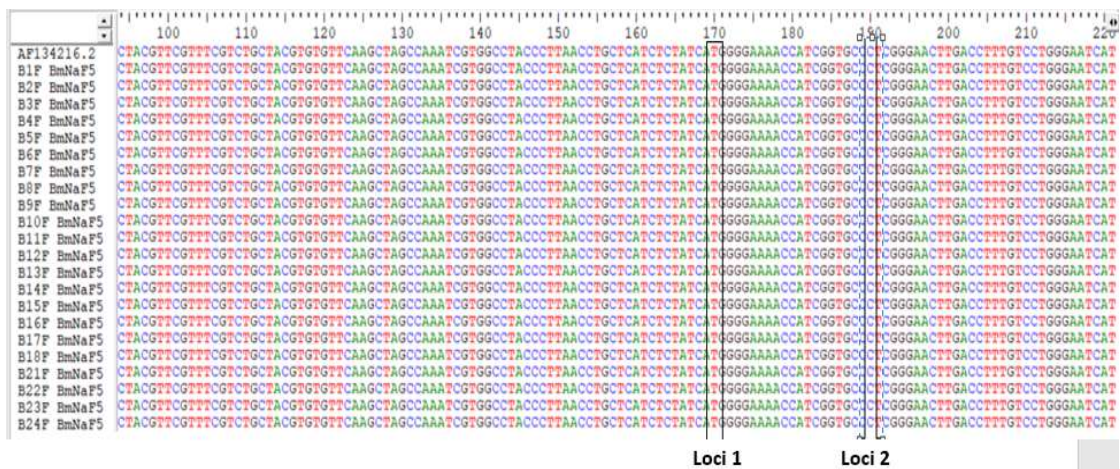


Figure 10: Sequence alignments of partial segments of the voltage-gated sodium channel gene

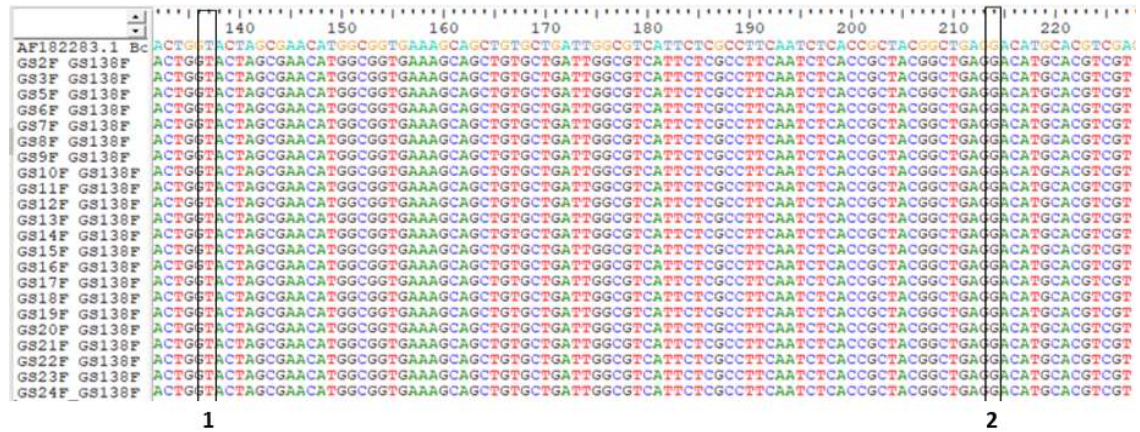


Figure 11: Sequence alignments of partial segments of the carboxyl esterase gene

CHAPTER FIVE

DISCUSSION

This study indicated that among the pastoralists in the study area, males remain livestock owners more than women. These findings are supported by previous reports by Yawa et al (65) that livestock are usually owned by males as they are traditionally thought to better manage and face challenges such as feeding, dipping, milking, handling and treating of the animals. All respondents were above 35 years and seem to depend on farming as source of income and these findings can be compared with those of Scholtz et al (66) who reported that elderly are found in rural areas while young people populate cities in search for job opportunities. Tick control in the Southern district of Botswana was determined to entirely dependent on acaricides, in which 92% of farmers preferred dipping using amitraz and deltamethrin. This finding echoes the reports of Ramotadima et al (36) and Masika et al (58), both reported the wide use of plunge dipping in communal areas. The use of amitraz and deltamethrin has been observed in the neighboring South Africa (25) and Zimbabwe (29). Dipping is the easiest and cheapest method to use as the entire animal body is completely covered by dipping solution. However, dipping as a tick control strategy is inherently fragile because dipping is not effective against ticks in protected areas such as under the toes, ears or tail. Therefore, when farmers found ticks actively growing on livestock after dipping, they had to supplement dipping with chemical acaricidal sprays, pour-ons and injectables. However, the use of acaricide sprays is considered one practice that promotes acaricide resistance (31). Therefore dipping must be used strategically for maximum effectiveness. The popularity of amitraz among farmers may be due to its relatively cheaper price and rapid onset effect compared to other acaricides (21). Respondents applied acaricides weekly or bi-weekly based on tick abundance and this alone increases the probability of resistance development to acaricides. Acaricide resistance is generally rare in multi-host ticks because ticks have a wide range of hosts from domestic animals to wild animals. Additionally, three host ticks tend to have a longer life cycle and the parasitic phase is often shorter than the treatment intervals used by most farmers (52).

Resistance against amitraz was not detected in this study. High susceptibility of ticks to amitraz was partly unexpected result since farmers in the Southern district had reported acaricide failure but the result was also unsurprising acaricide resistance is uncommon in three-host ticks. The lack of amitraz resistance however was similarly reported Makuvadze et al who found that *Amblyomma hebraeum* ticks were susceptible to amitraz in Domboshawa, Zimbabwe (62). Two villages (Pitseng and Lotlhakane) included in the current study had slight resistance to deltamethrin. The low tolerance of ticks to deltamethrin presents a likelihood for farmers in Lotlhakane and Pitseng using deltamethrin as an acaricide to have challenges with controlling *Amblyomma hebraeum* and heartwater. Essentially, this suggests future consideration of rotation of other classes of acaricides for the control of deltamethrin resistant ticks in the area. This is the first time deltamethrin resistance is being reported in *Amblyomma hebraeum* ticks in Botswana.

A larval test using resistant adult tick larvae showed sensitivity to deltamethrin with 100% mortality. This result indicates that there was no vertical transmission of resistance. Tolerance to the acaricide was therefore not inherited. Penetration resistance in the case of resistance to pyrethroid-based acaricides could be the most likely reason for the emergence of deltamethrin resistance but this assertion requires further investigation (32).

In this study, two target sites associated with pyrethroids resistance were analyzed. Resistance to deltamethrin develops through mutations of the carboxyl esterase gene (59,62) and the voltage gated sodium channel (31). Not all domains of the VGS channel gene were investigated for nucleotide substitutions associated with deltamethrin resistance. The difference between the results of this study and those previously reported Sichibalo et al (63); Sungirai et al., (64) could be due to the difference in the ticks under investigation – three-host vs one-host ticks - and ticks possible use of different mechanisms of resistance as previously asserted. Other mechanisms of resistance against pyrethroid compounds have duly been proposed by Jonsson et al. (56)..

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Amblyomma hebraeum in the Southern District of Botswana has a low level of deltamethrin tolerance. However, the mechanism underlying this tolerance remains unknown. An exhaustive investigation of the possible genetic underpinning of acaricide resistance needs to be undertaken together with investigations of alternate mechanisms for tolerance.

6.2 Recommendations

The education of farmers and data-driven advice from veterinary services are critical components for a successful strategy to reverse or delay the establishment of acaricide resistance. An effective tick management approach is required to prevent the development of tick resistance to acaricides. This plan should involve farmer education on tick control malpractices as well as regular monitoring of acari. Further research into the underlying pathways enabling *Amblyomma hebraeum* resistance to the acaricides tested in this work is advised.

6.3 Limitations & future work

Tick collection for the present study occurred during a narrow sampling window and within a few sites. This reduced the number of ticks collected. Broadening the sample collection window and increasing the number of sites would increase tick numbers and this would be beneficial for ascertaining the true prevalence of deltamethrin tolerance. Future studies will expand the sampling area to improve the utility of generated data.

Several polymorphic sites can result in acaricide resistance. Not all the sites were investigated because of budgetary constraints. Future studies could target all known sites associated with acaricide resistance in the relevant acaricide classes. Next generation

sequencing of the entire genome could also be explored. Investigation of alternative methods of acaricide resistance, such as penetration resistance, can also be considered.

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APPENDIX

APPENDIX 1: QUESTIONNAIRE

ASSESSMENT OF *AMBLYOMMA HEBRAEUM* FOR ACARICIDES RESISTANCE IN THE HEARTWATER-ENDEMIC SOUTHERN DISTRICT OF BOTSWANA

Household Questionnaire

Respondent name(s):	Surname: _____
	First name: _____
Date : _____ / _____ / _____	
Interviewer Name:	_____ ID _____
	Interviewer

	Sub-district: _____
Location ID :	Village/ Town: _____
	GPS Coordinates _____

1.1	What is your gender? 1= Male, 2= Female
1.2	What is your age range? 1=18- 29 years, 2=30-39 years, 3=40- 49 years, 4= 50- 59 years, 5= ≥60 years
1.3	What is your marital status? 1= Married, 2= Divorced, 3= Widowed, 4= single (never married), 5= Other (specify).....
1.4	Are you disabled? 1= Yes, 2= No, 3= Prefer not to say
1.5	Are you literate? 1= Yes, 2= No, 3= Prefer not to say
1.6	What is your highest level of education?

	1= None, 2= Primary, 3= JC, 4= Form 4-5 (senior), 4= vocational, 5= Tertiary, 6= Prefer not to say, 7= Other (Specify).....
1.7	What is your profession? 1= Farmer, 2= Self-employed, 3= Entrepreneur, 4= Employee, 5= Not working, 6= Other
1.8	How do you access information about ticks and acaricides? (May select more than one). 1= Audio/visual source (Radio/TV), 2= veterinary professionals, 3=Print media (newspapers and others), 4= Electronic sources (Cellphone, computers and others) 5= Friends/ family/ relatives, 6 = Own experience, 7 = Other (Specify).....
1.9	How many animals are you keeping?
1.10	Give example of parasites that affect your animal most. 1=ticks, 2= mites, 3= mosquitoes, 4 = lice (Specify).....
1.11	If your answer is 1 in 1.10, explain the kind of tick that mostly infest your animals
1.12	Do you have a dipping structure? 1= Yes, 2= No
1.13	If yes to 1.12, How often do you dip your animals?

	
1.14	Name the acaricides you often use.
1.15	When you change acaricides where do you throw the contents from the dip pit
1.16	What other methods do you use to eradicate ticks?
1.17	How many types of ticks do you know?
1.18	Do you think they are dangerous?
1.19	

2.0	Which tick species are affecting your animals?
2.1	How do you identify them?

2.2	Apart from dipping which other methods do you use to apply acaricides to animals?
2.3	Are the tick control measures efficient in controlling ticks?
2.4	If yes how long does it take before you can re-apply the same acaricides?
2.5	If no, what other strategies do you use to cope with acaricides failure?
2.6	Do you know ticks go through different developmental stages?
2.7	At which stage do you think ticks can be easily eradicated?
2.8	Do your animals interact with your neighbors' animals?
2.9	What measures do you put in place to ensure animal and worker safety?
2.10	Would you say that you are competent in mixing and applying acaricides?

	
2.11	Are there any reports of acaricides failure from your neighbors?