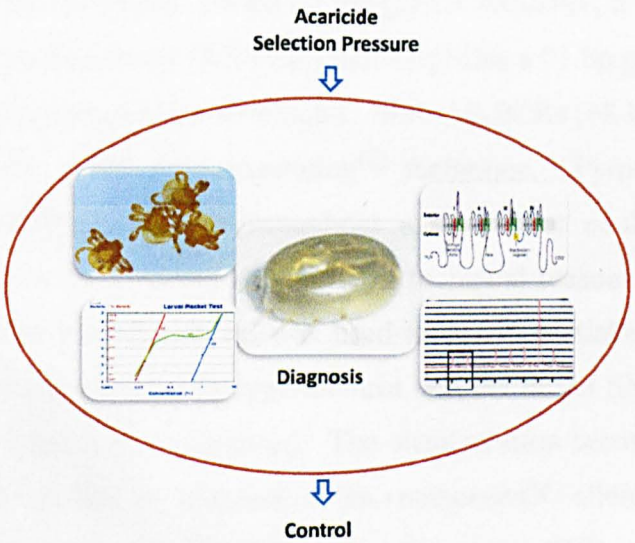
UNIVERSITY OF
LIVERPOOL

FACULTY OF VETERINARY SCIENCE

DEPARMENT OF VETERINARY PATHOLOGY

The effect of selection pressure on the genotype and phenotype of acaricide resistance in *Rhipicephalus* (*Boophilus*) *microplus*



Roger Iván Rodríguez-Vivas, DVM, MVSc

2008

This dissertation has been submitted in partial fulfilment of the requirements for the award of Doctor in Philosophy

ABSTRACT

The effect of selection pressure on the genotype and phenotype of acaricide resistance in *Rhipicephalus* (*Boophilus*) *microplus*

Roger Iván Rodríguez-Vivas

Acaricides have played a pivotal role in the control of the tick *Rhipicephalus* (*Boophilus*) *microplus*. As a consequence of extensive use, *R.* (*B.*) *microplus* has developed resistance to all major classes of acaricides, especially to pyrethroids. The thesis describes field and laboratory research undertaken in Yucatan, Mexico and Liverpool, UK to investigate pyrethroid resistance (PR) in *R.* (*B.*) *microplus*. For the first time gDNA of *R.* (*B.*) *microplus* was sequenced around the target single nucleotide polymorphism (SNP) in the sodium channel gene associated with PR and three new SNPs were identified. Based on the gDNA sequence, a new allele specific polymerase chain reaction assay (AS-PCR) that amplifies a 91 bp product and a novel Pyrosequencing™ technique were developed. Both AS-PCRs (68 bp and 91 bp) were validated against the novel Pyrosequencing™ technique. Pyrosequencing™ was shown to be a reliable and high-throughput method that could be used as an alternative method for genotyping; however, for technical reasons in field studies in Mexico the validated AS-PCR-68 bp was used to genotype ticks. The association between larval survival exposed to cypermethrin and the target SNP associated with PR in *R.* (*B.*) *microplus* was investigated. The clear relation between larval survival in the larval packet test and the presence of the resistance (R) allele (dead larvae 27.3 % vs. survivor larvae 78.3 %) suggested that the target SNP is one of the most important mechanisms that confer PR in *R.* (*B.*) *microplus* populations. The AS-PCR was used with the larval packet test to determine the prevalence of PR genotype and phenotype respectively in 49 field populations of *R.* (*B.*) *microplus* in Yucatan. The prevalence of susceptible, tolerant and resistant populations to cypermethrin was 26.5 %, 40.8 % and 32.6 % respectively. Furthermore, a clear correlation between the resistance factor (RF) and the frequency of the R allele was found, confirming that the target SNP is one of the most important mechanisms that confer PR in *R.* (*B.*) *microplus* populations. Having established the SNP as a reliable genotypic marker for

PR, a prospective interventional study was undertaken over two years in Yucatan to measure the evolution of resistance phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *R.* (*B.*) *microplus.* This novel experiment demonstrated the rapid evolution of resistance marked by significant increases in RFs and the proportion of the population carrying the SNP for PR (from 5.9-46.7 % to 66-95 %). In control populations where amitraz was substituted for pyrethroids there was no change in phenotype and genotype, showing that resistance phenotypes and genotypes were stable. To investigate if reversion to susceptibility could be produced by tactical management, a pyrethroid-susceptible *R.* (*B.*) *microplus* population was introduced into a pyrethroid-resistant *R.* (*B.*) *microplus* population over 33 months. This tactic led to a reduction of RF and frequency of the R allele in the resistant tick population.

Key words: *Rhipicephalus* (*Boophilus*) *microplus*, Pyrethroid Resistance, Genotype, Phenotype, AS-PCR, Pyrosequencing, Larval Packet Pest.

ACKNOWLEDGEMTS

I would like to express my gratitude to Prof. Alexander J. Trees for accepting to be my supervisor for second time, and for his guidance, constructive criticism of this work and for taking time from his tight schedule. I huge thank to my supervisor, Dr. Jane E. Hodgkinson, for her endless encouragement, excellent advice, patience and friendship. I am thankful to Dr. Sarah Lake for her patience in teaching me Pyrosequencing and Catherine Guy for her assistance with cloning, facilities in the laboratory of molecular biology and friendship. Also, to Nicola J. Beesley for her help in the laboratory in U.K.

My appreciation also to the head of Veterinary Parasitology group (University of Liverpool, UK), Dr. Diana Williams and everyone in the Department for being so kind and friendly with me (Catherine McCann, Rowena Barty, John McGarry, Paul Gilmore, Benjamin Makepeace and Samantha Dowdall).

I am grateful to Sandra Villegas-Pérez for her excellent technical assistance in the molecular biology work carried out in Mexico. Special thank to Alberto Rosado-Aguilar for his technical assistance with bioassays and his friendship. In addition thanks must go to members of Veterinary Parasitology Department in the Universidad Autónoma de Yucatán (Campus de Ciencias Biológicas y Agropecuarias) in Mexico, Genny Ramirez-Cruz and Ligia Cob-Galera for their support and friendship. Also, I would like to thank my under and postgraduate students who were involved in this work: Carolina Díaz-Medina, Virgen Castillo-Morales and Darwin Cabrera-Jiménez. I appreciate the generosity, technical assistance and friendship of Gertrudis Basto-Estrella.

I would like to thanks PROMEP-SEP-Mexico for providing the grant to study my PhD in the University of Liverpool, U.K. (UADY-135). Complementary support was received by CONACY-Complementario (No 52612), Bayer HealthCare, Animal Health, U.K and Laboratorio Lapisa, S.A. I am very grateful to Ford-Dodge laboratory in Mexico who kindly provided the technical grade of cypermethrin for this work. Thanks are also due to the farmers who generously gave their time and hospitality during this work.

Special thanks to my University, Universidad Autónoma de Yucatán (Campus de Ciencias Biológicas y Agropecuarias), for giving me the opportunity to work in, and the facilities received during my PhD programme.

I owe very special thanks to my wife Rossana and my children Lizette and Iván for their love, patience, encouragement and support during my long absence from home. I am equally grateful for all the support and encouragement I received from my Mum Fina, brothers (Carlos and Freddy), sisters (Leticia, Blanca, Josefina, Rosalinda and Ileana), Blanquita and all my family.

DEDICATION

This work is dedicated to Rossana, Lizette and Iván for their love, encouragement and patience

LIST OF ABREVIATIONS

A	Adenine
A. marginale	*Anaplasma marginale*
Ach	Acetylcholine
AChE	Acetylcholinesterase
A.I.	Active ingredient
AIT	Adult immersion tests
An.	*Anopheles*
AS-PCR-68 bp	Allele specific polymerase chain reaction 68 base pair
AS-PCR-91 bp	Allele specific polymerase chain reaction 91 base pair
ATP	Adenosine 5′-triphosphate
B. indicus	*Bos indicus*
B. taurus	*Bos taurus*
BHC	Benzene hexachloride
Bm86	Antigen in the TickGARD vaccine
bp	base pair
C → A	Cysteine → arginine
C	Cytosine
Ca2+	Calcium
CCBA-UADY	Campus de Ciencias Biológicas y Agropecuarias de la Universidad Autónoma de Yucatán
cDNA	Complementary deoxyribonucleic acid
CI	Confidence Interval
Cl-	Chlorine
Cz	Coatzacoalcos strain of *Rhipicephalus* (*Boophilus*) *microplus*
CzEST9	Esterase in the Coatzacoalcos strain of *Rhipicephalus* (*Boophilus*) *microplus*
Da	Daltons
dATP	Deoxyadenosine triphosphate
dATP-α-S	Deoxyadenisine α-thiotriphosphate
dCTP	Deoxycytidine triphosphate

DDD	Dicofol, methoxychlor
DDE	Dichloro-diphenyldichloroethane
ddH_2O	Double distilled water
DDT	(1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane).
dGTP	Deoxguanosine triphosphate
DI	Domain 1
DII	Domain 2
DIII	Domain 3
DIV	Domain 4
DNA	Deoxyribonucleic acid
DSC1	*Drosophila* sodium channel resistance
dTTP	Deoxythymidine triphosphate
E → K	Aspartic acid → glycine, glutamic acid → lysine, cysteine → arginine, and proline → leucine
F → I	Phenylalanine → isoleucine
FAO	Food and Agriculture Organisation
FG-221	Forward primer for ASPCR-68 bp (susceptible-specific sense)
FG-222	Forward primer for ASPCR-68 bp (resistant-specific sense)
FG-227	Reverse primer for ASPCR-68 bp (downstream non specific), amplification, cloning and sequencing a conserve region
g	Grams
G	Guanine
GABA	γ-amino butyric acid
Gavac®	A commercial anti-tick vaccine
gDNA	Genomic deoxyribonucleic acid
GST	Glutathione S-Transferases
h	Hours
H. armigera	*Helicoverpa armigera*
h^2	Heritability
H_2O	Water
IPM	Integrated pest management
IRV-F	Forward primer for ASPCR-91 bp (Upstream non specific)

IRV-R-Res	Reverse primer for ASPCR-91 bp (resistant-specific antisense)
IRV-R-Sus	Reverse primer for ASPCR-91 bp (susceptible-specific antisense)
IVR-seq	Sequencing primer to allow extension of directly sequenced DNA
K^+	Potassium
KCl	Potassium chloride
kdr	Knockdown resistance
L → F	Leucine → phenylalanine
L → F/H/S	Leucine → phenylalanine/histidine/serine
L → P	Leucine → proline
L	Litres
LC	Lethal concentration
LC_{50}	Lethal concentration 50 %
LC_{99}	Lethal concentration 99 %
LIT	Larval immersion test
LPT	Larval packet test
M → I	Methionine → isoleucine
M → T	Methionine → threonine
mg	Milligram
$MgCl_2$	Magnesium chloride
min	Minutes
MLs	Macrocyclic lactones
mM	Millimolar
Na^+	Sodium
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium Hydroxide
NBF	Forward primer for cloning a partial sodium channel gene of *Rhipicephalus* (*Boophilus*) *microplus*
NB-F	Forward primer to amplify, clone and sequence a conserved region of the sodium channel gene of *Rhipicephalus* (*Boophilus*) *microplus*

OC	Organophosphate
°C	Degrees Centigrade
OP	Organophosphate
Para	Paralytic resistance
PBO	Piperonyl butoxide
PCR	Polymerase Chain Reaction
pH	Negative logarithm of the hydrogen ion concentration
Phe→Ile	Phenylalanine → Isoleucine
pp	Pages
PPi	Pyrophosphate
PSQ	Pyrosequencing™
PyroFG-227	Reverse primer to amplify a region for Pyrosequencing™
PyroIRV-F	Forward primer to amplify a region for Pyrosequencing™
R	Resistant allele
R. (*B.*) *microplus*	*Riphicephalus* (*Boophilus*) *microplus*
r^2	Correlation Coefficient
RF	Resistance factor
RH	Relative humidity
rpm	Revolution per minutes
RR	Homozygous resistant genotype
RS	Heterozygous genotype
S	Susceptible allele
S1	Segment one
S2	Segment two
S3	Segment three
S4	Segment four
S5	Segment five
S6	Segment six
Seq-Ivan-1d	Sequencing primer in pyrosequencing reaction
SNP	Single Nucleotide Polymorphism
SP	Synthetic pyrethroids

SS	Homozygous susceptible genotype
super-kdr	Super-knockdown resistance
T → I/C/V	Threonine → isoleucine/cysteine/valine
T	Thiamine
TAE	Tris-acetate EDTA
Tick-Guard$^{plus®}$	A commercial anti-tick vaccine
UK	United Kingdom
UV	Ultraviolet
V → M	Valine → methionine
WHO	World Health Organisation
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
µg	Microgram
µl	Microlitre
%	Percent

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CHAPTER 1

INTRODUCTION

1.1. *Rhipicephalus* (*Boophilus*) *microplus*

Ticks are haematophagous arthropods belonging to the class arachnids. Ticks are obligate, blood-feeding ectoparasites of vertebrates, particularly mammals and birds. They are relatively large and stages are long-lived, feeding periodically, taking large blood meals. Tick bites may be directly damaging to animals, causing irritation, inflammation or hypersensitivity, and, when present in large numbers, anaemia and production losses. The salivary secretions of some ticks may cause toxicosis and paralysis; however, more importantly, when they attach and feed they are capable of transmitting a number of pathogenic viral, bacterial, rickettsial and protozoal diseases (Taylor et al., 2007).

The cattle tick *Boophilus microplus* (Canestrini) is a one host tick. There is evidence in support of the change of the generic name *Boophilus* to *Rhipicephalus* (Murrell et al., 2000; Beati and Keirans, 2001). For this reason in the present thesis I will refer this tick as *Rhipicephalus* (*Boophilus*) *microplus* (*R.* (*B.*) *microplus*). The life cycle of *R.* (*B.*) *microplus* can be described as made up of both free-living and parasitic cycles (**Figure 1.1.**). The larva, nymph and adult all attach to, and develop on, a single host. The engorged female tick drops off the host and lays between 2000 and 3500 eggs over a period of 4-44 days (Cen et al., 1998). Typically, these eggs are placed in crevices or debris, or under stones. The female tick dies after ovipositing. The larvae hatch after 14-146 days depending on temperature and relative humidity (Taylor et al., 2007). It has been shown that the free-living life cycle can last on average 164 days in the laboratory and 168.6 days in the field (Nuñez et al., 1985). The free-living larvae attempt to attach to a cattle host by climbing to the top of vegetation. The larvae then attach to the host, feed and moult to nymphs, these also engorge and emerge as either males or females. A female tick is fertilised by a male during engorgement. Males do not engorge but have the ability to attach and detach from the host at will, thus allowing fertilisation of many females (Nuñez et al., 1985). The time taken from attachment to engorgement of the adult female is three weeks. In optimal condition, the entire life cycle can be completed within two months, although unfed larvae survive for up to 20 weeks before attachment to the host (Taylor et al., 2007).

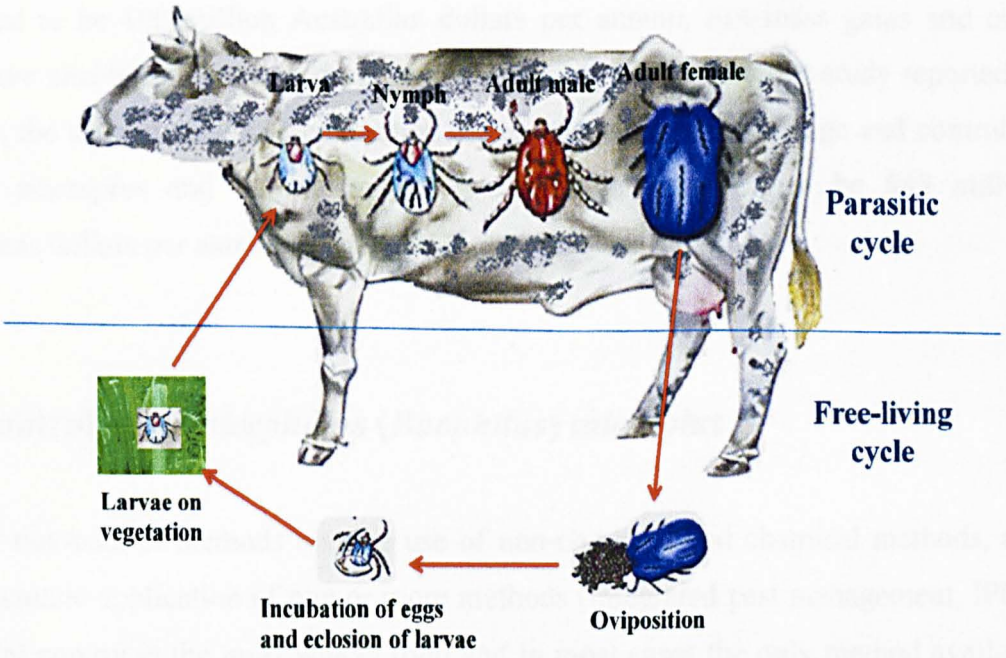

Figure 1.1. Diagram of the life cycle of *Rhipicephalus* (*Boophilus*) *microplus*.

1.2. Effect of *Rhipicephalus* (*Boophilus*) *microplus* on Cattle Production

Approximately 1 billion cattle, most of which are in the tropics, are at risk from various tick species or tick-borne diseases (Pegram et al., 1993), causing significant production losses. *Rhipicephalus* (*B.*) *microplus* is an endemic pest of cattle in tropical and subtropical regions of the world, causing major economic losses to cattle producers through direct physical effects on the parasitized animal and indirectly through disease transmission of infectious agents such as *Babesia bovis, B. bigemina* and *Anaplasma marginale* (Solorio-Rivera et al., 1999; Rodriguez-Vivas et al., 2004; 2005a). In addition to the costs of chemicals, labor, equipment and production losses associated with treatment, the cost of maintaining tick boundaries is highly expensive (Nari et al., 2000).

Each engorged female tick has been shown to reduce weight gain by 0.6 g in beef cattle (Sutherst et al., 1983) of which 65 % was attributed to tick infestation (stress and anorexia from the irritation cause by the ticks) and 35 % by loss of blood taken by the

ticks (Seebeck et al., 1971). In Australia, losses caused by *R.* (*B.*) *microplus* are estimated to be 100 million Australian dollars per annum, live-mass gains and milk yield have also been known to drop (Norval et al., 1988). In the last study reported in Mexico, the estimated cost of production losses, mortality, hide damage and control of *R.* (*B.*) *microplus* and its transmitted diseases was estimated to be $48 million Americans dollars per annum (Vega,1991).

1.3. Control of *Rhipicephalus* (*Boophilus*) *microplus*

Current tick-control methods involve use of non-chemical and chemical methods, and the systematic application of two or more methods (integrated pest management, IPM). Chemical control is the most widely used and in most cases the only method available to the producer. Although emphasis in this thesis is primarily on chemical control (especially on synthetic pyrethroids, SPs), non-chemical control and IPM will be discussed.

1.3.1. Non-Chemical Control

Several methods of non-chemical control of cattle ticks have been examined. These include: breeding resistant cattle, biological control, generating sterile hybrids of *R.* (*B.*) *microplus* x *R.* (*B.*) *decoloratus*, use of plant species that are unfavourable to cattle tick larvae, biological control, vaccination, geostatistics and remote sensing.

Breeding resistance: Differences in the ability of cattle to become resistant to ticks, whether *Bos indicus* or *B. taurus* or within the *B. taurus* breed, have long been recognized, as has the fact that the ability to acquire resistance is heritable (Utech et al., 1978). In zebu cross cattle for example, heritability for numbers of *R.* (*B.*) *microplus* is high (h^2= 0.34, the proportion of the total phenotypic variation due to additive gene effects) (Mackinnon et al., 1991). Resistance to cattle tick infestation varies among individuals and breeds of cattle. Furthermore, it has been shown that *Bos indicus* or crossbred cattle are more able to survive babesiosis (a tick borne-disease transmitted by *B. bovis* and *B. bigemina* in Mexico) than *B. taurus* animals (Bock et al., 1997).

Resistance is manifested by reduction in attachment and engorgement of ticks. If *B. indicus* cattle and their crossbreds carry less engorged ticks than *B. taurus* cattle, they would require less treatment with acaricide to evoid loss of productividity (Utech et al., 1978).

Pasture spelling: Pasture spelling is effective for controlling ticks, especially in relative hot, dry climates. Wharton et al. (1969) found that pasture spealling and planned dipping resulted in increased efficiency of tick control. Compared with British herds under conventional control which required dipping on 19 and 20 occasions, herds managed by pasture spelling were dipped only on seven occasions and showed a mean reduction of 81 % in tick burden.

Biological control: In practice, ticks are controlled at present mostly by chemical acaricides. However, biological control is becoming an increasingly attractive approach to tick management because of: (a) increasing concerns about environmental safety and human health (i.e. the gradual increase in use of chemical insecticides in several countries is stimulating the growing market of organic food), (b) the increasing cost of chemical control, and (c) the increasing resistance of ticks to acaricides. To date, biocontrol has been targeted largely at pests of plants, with only a few efforts to introduce biocontrol agents for the control of ticks (Samish et al., 2004). There are numerous potential tick biocontrol agents, including pathogens, nematodes, parasitoids and predators of ticks.

Some species of ants, including *Pheidole megacephala* may have an effect on tick populations in some areas of Australia, particularly in areas of friable, red volcanic soils (Wilkinson, 1970). Biological control based on entomopathogenic fungi is one of the most promising options to control ticks (Polar et al., 2005). The fungi *Metarhizium anisopliae* and *Beauveria bassiana* have been studied as a key regulatory organism for biocontrol (Dutra et al., 2004). *M. anisopliae* invades *R.* (*B.*) *microplus* by a process which involves the adhesion of conidia to the cuticle, conidia germination, formation of appressoria and penetration through the cuticle, observing a massive penetration 72 h post-inoculation (Arruda et al., 2005). Recently, Alonso-Diaz et al. (2007) found good

efficacy (40-91 %) of *M. anisopliae* to control *R.* (*B.*) *microplus* in cattle infested naturally in the Mexican tropics.

Release of sterile male hybrids: It has been shown that *R.* (*B.*) *annulatus* x *R.* (*B.*) *microplus* mating produce fertile females and sterile males (Osburn and Knippling, 1982). Backcrossing of the fertile female progeny also produce sterile males and fertile females through three to six generations. To be successful, release of hybrid ticks must be into small populations, for example where there is a new outbreak, or where there is already a high degree of control by other means (Hillburn et al., 1991). Problems with this method of control include the cost of production of hybrids, the effects of moderate infestations of hybrids over the period of eradication, and the risk of an extended range of hybrid or *R.* (*B.*) *annulatus* ticks (Jonsson, 1997).

Plant species that are unfavourable to ticks: Some plants have been shown to act as attractants for ticks; *Stylosanthes scabra* is a tropical legume which can trap larvae in the sticky exudate of glandular trichomes on stems and leaves. The plant collects between 12 % and 27 % of tick larvae (Wilson et al., 1989). Its effectiveness for tick control is limited by the proportion of this plant in pastures, physiological state of the plant, and by the modest percentage of larvae trapped. Furthermore, the African shrub *Acalypha fruticosa* is able to attract larvae of *R. appendiculatus*, which lie quiescent on the underside of the leaf (Hassan et al., 1994). Beside the modest effect of these plants, farmers in Australia and Africa do not use these plants as a routine method of tick control.

Vaccination: Vaccination is the most promising and well developed non-chemical control of cattle tick. A commercial vaccine, Tick-Guard$^{plus®}$ has been available in Australia since 1994 (Willadsen et al., 1995). It contains a recombinant Bm86 antigen preparation, derived from a glycoprotein within the tick gut. The vaccine, which causes leakage of gut content into the haemocoele of ticks, acts to reduce the number of females engorging, their mean weight and fecundity, and the viability of the eggs produced. Thus the vaccine effect is the reduction of larval infestations in subsequent generations. Another commercial vaccine containing a recombinant Bm86 antigen (Gavac®) was released in Mexico in 1997. Controlled pen and field trials in Mexico

provided evidence of the effect of recombinant Bm86 vaccination for the control of *R. (B.) microplus* and *R. (B.) annulatus* infestations (Redondo et al., 1999; de la Fuente et al., 1998, 1999, 2007a). Nevertheless, the tick vaccine had a limited use in Mexico due to difficulties associated with its commercialization and the lack of efficacy of Bm86 vaccination against *Amblyomma cajennense*, a tick that occurs concurrently with *R. (B.) microplus* and *R. (B.) annulatus* in some regions (de la Fuente et al., 2007a). However, despite these difficulties, tick vaccines have been an important tool for integrated control of tick infestations in Mexico due to the major growing problem of resistance to acaricides (Rodriguez-Vivas et al., 2006a, b).

Geostatistics and remote sensing: Estrada-Peña (1999) and Estrada-Peña et al. (2001, 2006) have used geostatistics and remote sensing to determine distribution of *R. (B.) microplus* to allow control methods to become targeted, help set up new eradication campaigns or to make predictions on distribution of *R. (B.) microplus* on a global scale.

1.3.2. Chemical Control

Available chemicals used in the treatment of ectoparasites of veterinary importance act either systemically, following uptake of the compound from host tissues, or by direct contact with the target parasites following external application. Virtually all ectoparasiticides are neurotoxins, exerting their effect on the ectoparasite nervous system (Taylor, 2001). Traditional methods for the delivery of an acaricide treatment to cattle to control ticks required formulations of the acaricide into a form such as an emulsifiable concentrate, wettable powder or flowable products that could be diluted in water and applied to cattle by a hand sprayer, spray race or through immersion of animals in a dipping vat. More recently, treatment possibilities include the use of pour-on products, injectables, an intraruminal bolus, acaricide-impregnated ear tag and pheromone-acaricide-impregnated devices attached in different ways to the host (George et al., 2004).

Many drug classes have been and are used as acaricides to treat cattle ticks, this include arsenicals, organochlorides (OCs), organophosphates (OPs), carbamates, amidines,

phenylpyrazoles, insect growth regulators, macrocyclic lactones (MLs) and SPs (Aguilar-Tipacamu and Rodriguez-Vivas, 2003; George et al., 2004).

In Mexico, OPs have been used to control cattle ticks since 1963, and during the national campaign of *Rhipicephalus* (*Boophilus*) tick eradication (1974–1984), coumaphos (OP compound) was the only authorized acaricide (Aguirre et al., 1991). In 1995, Rodriguez-Vivas et al. (2005b) reported that 65 % of the cattle industry of Yucatan, Mexico used SPs to control ticks. After 11 years of intensive use of SPs in the same region, Rodriguez-Vivas et al. (2006a) reported that use of SPs decreased to 21 % (cypermethrin 14 %, deltamethrin 6 % and flumethrin 1 %). This reduction in SP treatments has been due to the high cost of SPs in the market and low efficacy caused by resistance in Yucatan, Mexico (Rodriguez-Vivas et al., 2006a). However, SPs have showed excellent efficacy (> 98% of efficacy) to control ticks and flies (i.e. *Haematobia irritans* and *Stomoxys calcitrans*) (George et al., 2004) and a major imperative in tick control is the need to conserve and use SPs such a way that they are retained for effective use when necessary. For that reason, the present work will be focus on SPs to control ticks. Although emphasis in this thesis is primarily on SPs, the other families of acaricide will be discussed.

1.3.2.1. Synthetic Pyrethroids

Description. The term "pyrethroid" is commonly used to designate a synthetic insecticide that is derived structurally from the natural pyrethrins, the six insecticidal constituents of pyrethrum extract of *Chrysanthemum* species (pyrethrin I and II, cinerin I and II, jasmolin I and II). The principal drawback of pyrethrum as an insecticide is its instability in light and air, which limits its effectiveness in crop protection and other insect control contexts in which residual activity is essential. The development of SPs is the result of efforts to modify the structure of the natural pyrethrins in order to increase photostability while retaining the potent and rapid insecticidal activity and relatively low acute mammalian toxicity of pyrethrum (Soderlund et al., 2002).

Most SPs were discovered by the sequential replacement of structural elements of the pyrethrins with novel structural moieties (**Figure 1.2.**) that were selected to conserve the

molecular shape and physical properties of the template structure. Because the pyrethrins are esters of a cyclopropanecarboxylic acid and a cyclopentenolone alcohol, synthetic modifications typically held one of these major domains of the molecule constant while introducing new structural features in the other (Soderlund et al., 2002).

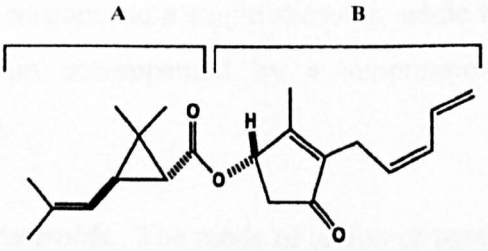

Figure 1.2. Structure of the natural pyrethrin I (A: acid moiety, B: alcohol moiety). Modified from Sumano and Ocampo (2006).

Permethrin (**Figure 1.3.A**) proved to be the first SP with sufficient photostability for agricultural use. This compound contains structural replacements in both the alcohol moiety (3-phenoxybenzyl for 5-benzyl-3-furylmethyl) and the acid moiety (chlorines for methyl groups) that confers enhanced photostability without loss of insecticidal activity. Inclusion of a α-cyano substituent in the 3-phenoxybenzyl alcohol moiety, as in deltamethrin (**Figure 1.3.B**), produced compounds with much greater insecticidal potency than permethrin but with similar photostability. Synthetic pyrethroids related in structure to permethrin and deltamethrin, which constitute the largest chemical subfamily of SPs in current use, include cypermethrin (**Figure 1.3.C**), cyfluthrin, cyhalothrin, fenpropathrin and tralomethrin. These compounds exhibit structural features that confer an expanded range of insecticidal activity, enhanced overall insecticidal potency, modified photostability, or other desirable properties when compared to pyrethrum or earlier SPs (Soderlund et al., 2002).

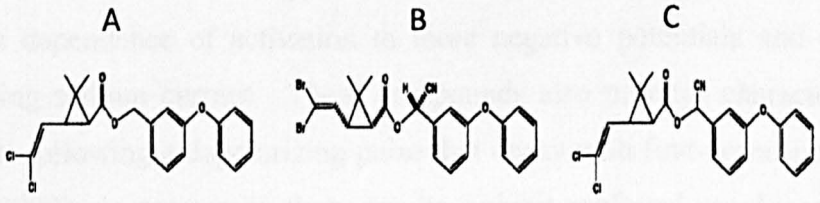

Figure 1.3. Structures of the synthetic pyrethroids: A: permethrin, B: deltamethrin and C: cypermethrin. Modified from Sumano and Ocampo (2006).

Pyrethroids are grouped into two categories (type I and type II) based on their distinct poisoning symptoms, effects on nerve preparations, and their chemical structures (Narahashi, 1986). Type I pyrethroids lack an α-cyano group which is present at the phenylbenzyl alcohol position of type II pyrethroids. Type I pyrethroids cause repetitive discharges in response to a single stimulus, while type II pyrethroids caused a membrane depolarization accompanied by a suppression of the action potential (Soderlund et al., 2002).

Mode of Action of Pyrethroids. The mode of action of pyrethroids has been conducted using vertebrate and non-insect invertebrate nerve preparations. Collectively, these studies show that pyrethroids cause prolonged opening of sodium channels in nerve, muscle and other excitable cells (Catterall, 2001), primarily by inhibiting channel deactivation and stabilizing the open configuration of the sodium channel (Catterall, 2001; Soderlund et al., 2002; Raymond-Delpech et al., 2005). The prolonged channel opening is evidenced by a large tail current associated with repolarization under voltage-clamp conditions. Furthermore, voltage-clamp experiments showed that type II pyrethroids inhibit the deactivation of sodium channels to a greater extent than type I pyrethroids. The decay of tail currents induced by type II pyrethroids is at least one order of magnitude slower than those induced by type I pyrethroids. These quantitative differences in tail-current decay kinetics between type I and type II pyrethroids may account for their different actions on the nervous system (Dong, 2007).

Recent studies on the mechanism of action of pyrethroids on insect sodium channels expressed in oocytes and the molecular mechanism of *kdr* confirmed that sodium channels are the target of SP insecticides (Dong, 2007). Type I pyrethroids (i.e. cismethrin and permethrin) appear to bind to resting or inactivated channels, shifting the voltage dependence of activation to more negative potentials and causing a slowly-activating sodium current. These compounds also produce characteristic sodium tail currents following a depolarizing pulse that decay with first-order time constants (Zhao et al., 2000). In contrast to these results, exhibit profound use-dependent modification of sodium currents, which implies that these compounds bind preferentially to activate sodium channel states (Smith et al., 1998; Vais et al., 2000; Tan et al., 2002). Similar

effects were also observed on house fly and cockroach sodium channels (Tan et al., 2005). Furthermore, these studies also demonstrated that the effects of type I and type II pyrethroids on insect sodium channels are similar to those observed from electrophysiological studies using non-insect nerve preparations. The lethal activity of SP seems to involve action on both peripheral and central neurones, while the knock-down effect, is probably produced by peripheral neuronal effects only (Casida et al., 1983).

Efficacy of Pyrethroids on Ticks: Cross-resistance to DDT (dichlorodiphenyl-trichloroethane), precluded or abbreviated the use of permethrin and fenvalerate in countries such as Australia and South Africa where DDT resistance had been diagnosed in *Rhipicephalus* (*Boophilus*) ticks (Kunz and Kemp, 1994). Cypermethrin, deltamethrin and cyhalothrin are examples of SPs that are effective on ticks (> 98% of efficacy). Flumethrin was designed for application to cattle as pour-on, but there is also an emulsifiable concentrate formulation that can be applied as a dip or spray. The active ingredient in the pour-on has a remarkable capacity for spreading rapidly on the skin and hair from points of application along the dorsal line of an animal to all areas of the body. The residual effect of treatment with flumethrin is extended if the pour-on formulation is applied (George et al., 2004). Flumethrin for the control of both one-host and multi-host ticks species on cattle is effective at relatively low concentrations compared to other SPs (Stendel, 1985). Flumethrin is approximately 50 times more toxic to *R.* (*B.*) *microplus* than the other most-toxic SPs, cypermethrin and deltamethrin (Schinitzerlin et al., 1989).

Voltage-Gated Sodium Channels: Sodium channels are the target site of a great variety of neurotoxins, such as tetrodotoxin, scorpion toxins, and batrachotoxin, which are produced by plants and animals for defense or predation (Wang and Wang, 2003). Insecticidal pyrethrins, found in extracts of the flowers of *Chrysanthemum* species, also act on sodium channels (Narahashi, 1986). Sodium channels are also the primary target of DDT and modern SPs, which are structural derivatives of the naturally occurring pyrethrins (Narahashi, 1986). Furthermore, recent evidence indicates that a new class of pyrazoline-like insecticides, oxadiazines, also target sodium channels (Wing et al., 2005).

Extensive molecular analysis of mammalian sodium (Na^+) channels in the last two decades has generated comprehensive views into the structure and function of voltage-gated sodium channels (Catterall, 2000; Yu and Catterall, 2003; Dong, 2007). Mammalian sodium channels consist of a large pore-forming transmembrane α-subunit and several small auxiliary β-subunits. The α-subunit contains four homologous domains (named I–IV), each having six membrane spanning segments (named S1–S6) connected by intracellular or extracellular loops of amino acid sequences (**Figure 1.4.**). The selectivity filter and pore are formed by transmembrane segments S5 and S6 together with the membrane-reentrant segments that are part of the loop connecting S5 and S6 of each domain (Dong, 2007).

Voltage-gated Na^+ channels are responsible for the rapidly rising phase of action potentials, and they are critical for electrical signaling in most excitable cells. In response to membrane depolarization, sodium channels open (activate) and allow sodium ions to flow into the cell, thereby depolarizing the membrane potential (Catterall, 2001).

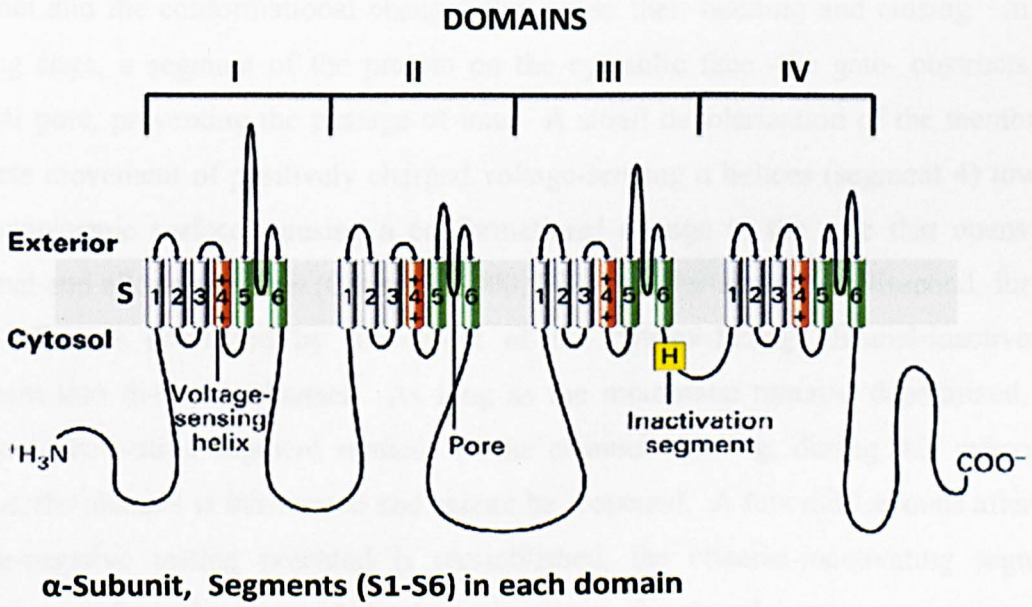

Figure 1.4. Schematic depictions of the secondary structures of voltage-gated Na^+ channel (Adapted from Lodish et al., 2004). It contains amino acids organized into four transmembrane domains (I-IV). The single channel-inactivating segment, located in the cytosol between domains III and IV, contains a conserved hydrophobic motif (H).

As Na^+ ions flow inward through opened channels, the excess positive changes on the cytosolic face and negative charges of the exoplasmic face diffuse a short distance away from the initial site of depolarization. This passive spread of positive and negative charges depolarizes (makes inside less negative) adjacent segments of the plasma membrane causing opening for additional voltage-gated Na^+ channel in these segments and an increase in Na^+ influx. As more Na^+ ions enter the cell, the inside of the cell membrane becomes more depolarized, causing the opening of yet more voltage-gated Na^+ channels and even more membrane depolarization, setting into motion an explosive entry of Na^+ ions. For a fraction of a millisecond, the permeability of this region of the membrane to Na^+ becomes vastly greater than for K^+, and the membrane potential approaches E_{na}, the equilibrium potential for a membrane permeable only to Na^+ ions. As the membrane potential approaches E_{na}, however, further net inward movement of Na^+ ions ceases, since the concentration gradient of Na^+ ions (outside > inside) is now offset by the inside-positive membrane potential E_{na} (Lodish et al., 2004).

Figure 1.5. schematically depicts the critical structural features of voltage-gated Na^+ channel and the conformational changes that cause their opening and closing. In the resting state, a segment of the protein on the cytosolic face -the gate- obstructs the central pore, preventing the passage of ions. A small depolarization of the membrane triggers movement of positively charged voltage-sensing α helices (segment 4) toward the exoplasmic surface, causing a conformational change in the gate that opens the channel and allows ion flow (Catterall, 2000, 2001). After about 1 millisecond, further Na^+ influx is prevented by movement of the cytosol-facing channel-inactivating segment into the open channel. As long as the membrane remains depolarized, the channel-inactivating segment remains in the channel opening; during this refractory period, the channel is inactivated and cannot be reopened. A few milliseconds after the inside-negative resting potential is reestablished, the channel-inactivating segment swings away from the pore and the channel return to the closed resting state, once again able to be opened by depolarization (Lodish et al., 2004).

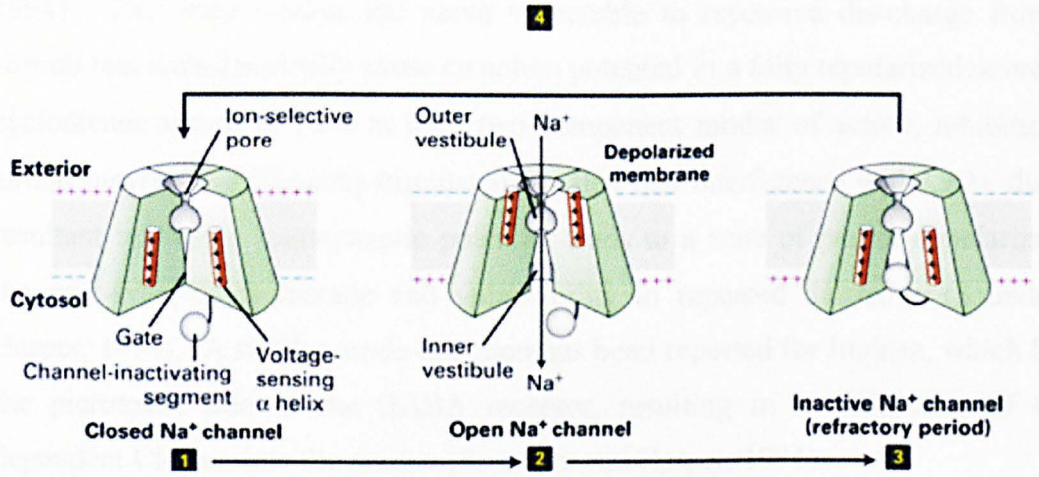

Figure 1.5. Operational model of the voltage-gated sodium channel (From Lodish et al., 2004). 1. In the closed, resting state, the voltage-sensing α helices, which have positively charged side chains every third residue, are attracted to the negative charges on the cytosolic side of the resting membrane. This keeps the gate segment in a position that blocks the channel, preventing entry of Na^+ ions. 2. In response to a small depolarization, the voltage-sensing helices rotate in a screwlike manner toward the outer membrane surface, causing an immediate conformational change in the gate segment that opens the channel. 3. The voltage-sensing helices rapidly return to the resting position and the channel-inactivating segment moves into the open channel, preventing passage of further ions. 4. Once the membrane is depolarized, the channel-inactivating segment is displaced from the channel opening and the gate closes; the protein reverts to the closed, resting state and can be opened again by depolarization.

1.3.2.2. Organochlorines

The popularity and use of OCs has declined due to environmental and persistence concerns but several compounds are still available in some countries. OCs fall into three main groups: (a) chlorinated ethane derivatives such as DDT, DDE (dichloro-diphenyldichloroethane) and DDD (dicofol, methoxychlor); (b) the cyclodienes that include chlordane, aldrin, dieldrin, hepatochlor, endrin, toxaphene; (c) the hexachlorocyclohexanes (HCH) such as benzene hexachloride (BHC) which includes the γ-isomer, lindane (Taylor, 2001). Chlorinated ethanes cause inhibition of sodium conductance along sensory and motor nerve fibres by holding sodium channels open, resulting in delayed repolarization of the axonal membrane (Saunders and Harper,

1994). This state renders the nerve vulnerable to repetitive dis-charge from small stimuli that would normally cause an action potential in a fully repolarized neuron. The cyclodienes appear to have at least two component modes of action; inhibition of γ-amino butyric acid (GABA) stimulated Cl- flux and interference with Ca2+ flux. The resultant inhibitory post-synaptic potential leads to a state of partial depolarization of the post-synaptic membrane and vulnerability to repeated discharge (Saunders and Harper, 1994). A similar mode of action has been reported for lindane, which binds to the picrotoxin side of the GABA receptor, resulting in an inhibition of GABA-dependent Cl- flux into the neuron (Saunders and Harper, 1994).

1.3.2.3. Organophosphates

Organophospates, i.e. coumaphos, and carbamates have a similar mode of action acting on acetylcholinesterase (AChE) (Corbett, 1974). AChE is one of the most efficient enzymes, being capable of an extremely rapid rate of hydrolysis of acetylcholine and generation of the active enzyme (Kwong, 2002). Organophosphates are neutral esters of phosphoric acid or its thio analogue and act by inhibiting the action of AchE at cholinergic synapses and at muscle end plates. The OP mimics the structure of acetylcholine (Ach) and when it binds to AChE it causes transphosphorylation of the enzyme. The transphosphorylated AchE is unable to breakdown accumulating Ach at the post-synaptic membrane leading to neuromuscular paralysis (Taylor, 2001). Organophosphates compounds can be extremely toxic in animals and humans causing an inhibition of AChE, and other AChEs. They are generally active against fly larvae, flies, lice, ticks and mites on domestic livestock and fleas and ticks on dogs and cats, although activity varies between compounds and differing formulations (MacDonald, 1995).

1.3.2.4. Phenylpyrazoles

Fipronil is a phenylpyrazole compound, which blocks transmission of signals by the inhibitory neurotransmitter, GABA, presents in insects (Raugh et al., 1990). The compound binds within the chloride channel and consequently inhibits the flux of Cl-ions into the nerve cell resulting in hyperexcitation of the insect nervous system (Postal

el al., 1995). Fipronil is used worldwide for the treatment and control of flea and tick infestations on cattle, cats and dogs (Taylor, 2001; George et al., 2004). Fipronil applied as a pour-on to cattle infested with *R. (B.) microplus* had a therapeutic efficacy greater than 99 % (Davey et al., 1998).

1.3.2.5. Insect Growth Regulators

They constitute a group of chemical compounds that do not kill the target parasite directly, but interfere with the growth and development. Insect growth regulators act mainly on immature stages of the parasites and as such are not usually suitable for the rapid control of established adult populations of parasites. Based on their mode of action they can be divided into: a) chitin synthesis inhibitors (benzoylphenyl ureas), b) chitin inhibitors (triazine/pyrimidine derivatives) and c) juvenile hormone analogues (Taylor, 2001). Fluazuron, a benzoyl phenyl urea, is efficacious against ticks and some mite species. The adverse consequences for ticks on cattle treated with a pour-on of this acaricide are the reduction of the fecundity and fertility of engorged females to near zero, and mortality of immature ticks because they unable to moult to the next instar (George et al., 2004).

1.3.2.6. Amidines

Amitraz is a formamidine-like acaricide. The main member of this group is amitraz, which acts at octopamine receptor sites in ectoparasites resulting in neuronal hyperexcitability and death (Evans and Gee, 1980). It is toxic against mites, lice and ticks in domestic livestock. In cattle, for example, amitraz has been widely used in dips, sprays or pour-on formulations for the control of single-host and multihost tick species (Taylor, 2001). Amitraz continues to be one of the most popular acaricides for the control of *R. (B.) microplus* in Australia, southern Africa and Latino America (Jonsson and Hope, 2007). Amitraz is unstable in dipping vats, but adding sufficient calcium hydroxide or hydrated lime to raise and maintain the pH of the vats to 12 insures the stability of the active ingredient (George et al., 2004).

1.3.2.7. Macrocyclic Lactones

Macrocyclic Lactones (MLs) act at glutamate-gated chloride channel receptors (Arena et al., 1995). The MLs are broad-spectrum antiparasitic drugs, extensively used in veterinary medicine. They are known as "endectocide" compounds based on their unique activity against endo-and ectoparasites (Shoop et al., 1995). The MLs include two chemical families: avermectins (i.e. abamectin, ivermectin, doramectin, eprinomectin and selamectin) and milbemycins (i.e. nemadectin, moxidectin), which are commercially available to use in livestock and pet animals as injectable, oral and/or pour-on formulations (McKellar and Benchaoui, 1996). The efficacy of ivermectin, doramectin and moxidectin for the control of *R. (B.) microplus* populations resistant to OPs, amidine and SPs has been demonstrated (Sibson, 1994; Aguilar-Tipacamu and Rodriguez-Vivas, 2003; Davey et al., 2005). In the Mexican tropics, moxidectin (1 %) has been shown to have an efficacy against natural infestation of *R. (B.) microplus* greater than 95 %, 28 days after application (Aguilar-Tipacamu and Rodriguez-Vivas, 2003). Recently, long acting moxidectin-10 % (1mg/kg) and ivermectin-3.15 % (0.63 mg/kg) have been shown to have an efficacy against natural infection of *R. (B.) microplus* greater than 95 %, 56 and 70 days after applications, respectively (Arieta-Roman et al., 2008).

1.3.2.8. Mixture of Acaricide Families

Several OPs would synergize the toxicity to *R. (B.) microplus* of cypermethrin and deltamethrin. The reduction in concentration of a relatively expensive SP that could be used with a relative cheap OP synergist provided an efficacious, inexpensive product for the control of OP-resistant tick populations (Schnitzerling et al., 1983). In Australia, the combination products of cypermethrin + chlorfenvinphos and deltamethrin + ethion remain on the market (George et al., 2004). Furlong (1999) listed products consisting of mixtures of cypermethrin + chlorfenvinphos and permethrin + dichlorvos among acaricides marketed in Brazil. In Mexico, mixtures of acaricides are available in the market and cymiazole + cypermethrin is one of the most used (Rodriguez-Vivas et al., 2006a). One value of these mixtures may be their possible use for the control of both ticks and the horn fly. Recently, significant synergism was observed when amitraz was

used as a synergist in deltamethrin bioassays (Barré et al., 2008). This finding may lead to the adoption of an acaricide mixture strategy for the control of pyrethroid-resistant *R. (B.) microplus.*

1.3.3. Integrated Pest Management

Integrated pest management (IPM) involves the systematic application of two or more technologies to control pest populations which adversely affect the host species. The ultimate aim is to achieve pest or parasite control in a more sustainable, environmentally compatible and cost-effective manner than is achievable with a single, stand alone technology (Willadsen, 2006).

In the development of approaches which allow effective management of tick populations, which minimise non-target effects and preserve the availability of our existing acaricides, it is essential to develop more fully the use of IPM. In such approaches, cascades of management tactics may be deployed as and when necessary, with acaricide available as just one component, to be used in appropriate circumstances (Peter et al., 2005; Wall, 2007). A wide range of new tools are becoming available to assist in this goal. These include molecular techniques, which are providing powerful new insights into diagnosis (Guerrero et al., 2001), spatial distribution of ticks and acaricide resistance of ticks (Rodriguez-Vivas et al., 2007), simulation modeling (Wall et al., 2002), satellite imagery (Estrada-Peña and Venzal, 2006), anti-tick vaccine (de la Fuente et al., 2007a) and biological control (Alonso et al., 2007).

In Mexico a combination of anti-tick vaccine (Gavac®) and acaricide treatments have been used to control *R. (B.) microplus* ticks. Redondo et al. (2004), using an integrated system employing vaccination with Gavac® and amidine treatments, under field conditions achieved nearly 100 % control of *R. (B.) microplus* populations resistant to SPs and OPs. This method effectively controls tick infestations while reducing the number of chemical acaricide treatments and consequently the rise of *R. (B.) microplus* populations resistant to acaricides. Furthermore, in a farm using this IPM for ten years, substantial reduction of acaricide treatments has been achieved (from 24 to 7-8 per year)

with consequent reduction in tick burden from 100 to 20 adult ticks per animal (de la Fuente et al., 2007a).

Bahiense et al. (2006) evaluated the association of deltamethrin and the entomopathogenic fungus *M. anisopliae* against *R.* (*B.*) *microplus* larvae resistant to pyrethroid. High mortality rates were observed when deltamethrin was associated with the entomopathogen. The authors concluded that this association can be used as a tool for integrated control of the tick *R.* (*B.*) *microplus*.

The use of tick-resistant cattle breeds (*B. indicus* and their crosses), host management (i.e. lowering the stocking rate), selection application of acaricide during annual season when they will be most effective and pasture spelling can be usefull components of an integrated tick management strategy (George, 1990). The general consensus is to reduce the frequency of acaricide use, but this is not always easy in cattle production in the tropics and subtropics (Kunz and Kemp, 1994).

1.4. Current Status of Acaricide Resistance of *Rhipicephalus* (*Boophilus*) *microplus*

Although the control of ticks relies heavily on the use of chemicals, the development of resistance to these compounds is a serious threat to the sustainability of this approach. The development of resistance in arthropods is dependent on the frequency of application of the insecticides, as well as the insects' life cycles. The single-host tick, *R.* (*B.*) *microplus*, has a short life cycle, and produces many young; whilst multi-host ticks have a longer life cycle. There has therefore been faster development of resistance in *R.* (*B.*) *microplus* worldwide (Peter et al., 2005).

1.4.1. Worldwide Acaricide Resistance in Boophilus Ticks

Since the first report of the development of resistance of *R.* (*B.*) *microplus* to arsenicals in Australia in 1937 (Newton, 1967), the progressive evolution of resistance in ticks affecting cattle to almost all of the available acaricide has frustrated the efforts of cattle

producers to manage ticks and tick-borne diseases affecting their animals. The history of the resistance of ticks to acaricides parallels, with a relative few years of delay, the introduction of new acaricide products representing several different classes of chemicals (George et al., 2004). Selected records of the geographic distribution and year of documentation of first report of acaricide resistance in *R.* (*B.*) *microplus* worldwide is presented in **Table 1.1.**

Table 1.1. An overview of occurrences of acaricide resistance in the cattle tick *Riphicehalus* (*Boophilus*) *microplus* (Adapted from George et al., 2004).

Chemical (~ date introduced)	Localization
Arsenic (1893)	Australia, 1936; Argentina, 1936; Brazil, 1948; Colombia, 1948; Uruguay, 1953; Venezuela, 1966
DDT (1946)	Argentina, 1953; Brazil, 1953; Australia, 1953; Venezuela, 1966
Organophosphate and Carbamates	Australia, 1963, Argentina, 1964; Brazil, 1963; Colombia, 1967; Venezuela, 1967; 1979; Uruguay, 1983; Mexico, 1986
Formamidines (1975)	Australia, 1978; Brazil, 1989; Mexico, 1994; Venezuela, 1995; Colombia, 1997; Argentina, 2000
Pyrethroids (1977)	Australia, 1981; Brazil, 1995; Colombia, 2000
Macrocyclic Lactones (1981)	Brazil, 2001

In recent years, resistance to amitraz was also found in *R.* (*B.*) *microplus* populations from Colombia (Benavides et al., 2000), Brazil (Furlong, 1999; Miller et al., 2002) and Mexico (Rodriguez-Vivas et al., 2006b, 2007). ML resistance of *R.* (*B.*) *microplus* in Brazil to doramectin and moxidectin was reported in ticks from one farm. Recently, six strains of *R.* (*B.*) *microplus* collected from northern Mexico were surveyed for resistance to ivermectin by the larval immersion test and none of the strains showed resistance to ivermectin (Miller et al., 2008b). The widespread use of MLs for parasite control (endo and ectoparasite) and limited choice of alternative acaricides has caused concern that ML resistance will become a major problem.

The emerging of resistance in *R.* (*B.*) *microplus* to OPs, SPs, amitraz and LM in Australia and Latino America does not mean that none of the products containing these kinds of active ingredients have any further value. Tick populations susceptible to a variety of acaricides exist and can be controlled, but it is more critical than ever to use existing and improved diagnostic tools to determine where products are still useful and

to employ tick control strategies that minimize the rate of selection for resistance (George et al., 2004).

1.4.2. Acaricide Resistance in Rhipicephalus (Boophilus) microplus in Mexico

Organophosphate acaricides were heavily used in the national tick eradication program between 1974 and 1984 in Mexico (Trapapa, 1989). The OPs used during that period include coumaphos, chlorpyriphos, chlorfenvinphos, diazinon and ethion. The first case of OP resistance was detected in *R. (B.) microplus* ticks from a farm in the southern part of Mexico (Tuxpan, Veracruz) in 1983. The tick strain established from this location demonstrated 10- to 14-fold (shows 10-14 fold resistance when compared with a susceptible reference strain) resistance to coumaphos, chlorpyriphos and ethion (Aguirre and Santamaria, 1986). Resistance to OPs soon became widespread in central, eastern and southern Mexico. Pyrethroid acaricides were then introduced into Mexico in 1986 in order to alleviate OP resistance problems. Resistance to SPs was first detected in 1993 and soon became extensive (Fragoso et al., 1995). The levels of resistance to SPs were generally in the range of 10- to 350-fold, with the exception of two tick populations in which more than 1000-fold resistance was detected (Miller et al., 1999). As a result of intense selection pressure from the use of OPs and SPs. *R. (B.) microplus* were found to have developed resistance to both classes of the acaricides in at least 15 states of Mexico (Santamaria et al., 1999).

In addition to the SPs, amitraz was also introduced in 1986, but its use was initially limited due to a higher cost. The use of amitraz became more frequent after 1993 when SP resistance problems started to hinder the tick control efforts in Mexico. The first case of amitraz resistance in Mexico was reported in 2002 (Soberanes et al., 2002). Recently, Rodriguez-Vivas et al. (2007) studied 217 field populations of *R. (B.) microplus* and determined the prevalence (measured by bioassays) of farms with resistance to SPs, OPs and amitraz in the southern Mexico, and they found that SP resistance such as deltamethrin, cypermethrin and flumethrin was one of the most serious problems in the Mexican tropics (from 66 % to 96 % farms showed resistance to SPs). Furthermore, Rodriguez-Vivas et al. (2006a) studied 98 field populations of *R. (B.) microplus* in Yucatan, Mexico and found that 63 %, 61% and 59 % of those tick

populations were resistant to flumethrin, deltamethrin and cypermethrin, respectively. The findings of *R. (B.) microplus* resistant to all three major classes of acaricides in Mexico underscores the seriousness of the resistance situation and the importance of having a resistance management strategy in Mexico (Rodriguez-Vivas et al., 2007).

1.5. Mechanisms of Acaricide Resistance in *Rhipicephalus (Boophilus) microplus*

Resistance is defined as having the ability to withstand doses of toxicant which would normally be lethal to most individuals in a typical population of the same species (WHO, 1957). Most resistance mechanisms in ticks can be divided into two groups, target site insensitivity (mutations in the sodium channel, acetylcholinesterase, γ-aminobutyric acid (GABA) and octopamine receptors genes) and metabolic (alterations in the level or activities of detoxification proteins) (Nolan, 1985; Chen et al., 2007). Alone and or in combination these mechanisms confer resistance to all of the available classes of acaricides. Although emphasis in this thesis is primarily on target site insensitivity (especially at the sodium channel of *R. (B.) microplus*), metabolic resistance mechanisms will be briefly discussed.

1.5.1. Target Site Insensitivity

Sodium Channel: Resistance to SPs was first observed in a DDT-resistant strain of the housefly, *Musca domestica*, and termed knockdown resistance or *kdr* (Milani, 1954). Subsequent analysis also identified a greatly enhanced type of SP-resistance called *super-kdr* (Sawicki, 1978). Linked genetically to the sodium channel gene locus, the molecular basis for *kdr* resistance has been investigated in many insects including ticks (Jamroz et al., 2000).

Knock down resistance mutations confer reduced neuronal sensitivity to SPs and DDT in insects (Soderlund and Bloomquist, 1990). *Kdr* mutations are linked to the *para*-homologous genes in several insect species (Soderlund and Knipple, 2003). The term *para* was first adopted to refer the paralytic resistance in *Drosophila melanogaster.*

There are multiple point mutations in the *para*-homologous genes that are associated with *kdr* and *kdr*-type resistance to SPs in many insects (Soderlund, 2005). Both common and unique mutations in sodium channel genes are found to be responsible for SP resistance in different insect and arachnid pest species. Up to date, ten sodium channel mutations have been confirmed to be responsible for *kdr* and *kdr*-type resistance in many arthropods (Dong, 2007; Soderlund, 2008):

1. valine → methionine (V → M) in the tobacco budworm, *Heliothis virescens.*
2. methionine → isoleucine (M → I) in the head lice, *Pediculus capitis.*
3. leucine → phenylalanine (L → F) in the head lice, *Pediculus capitis.*
4. leucine → phenylalanine/histidine/serine (L → F/H/S) in many insects.
5. phenylalanine → isoleucine (F → I) in the cattle tick *R. (B.) microplus.*
6. leucine → proline (L → P) in the honey bee mite, *Varroa destructor.*
7. threonine → isoleucine/cysteine/valine (T → I/C/V) in the diamondback moth, *Plutella xyllostella*; head lice, *Pediculus capitis;* western flower thrip, *Frankliniella occidentalis* and cat flea, *Ctenocephalides felis.*
8. methionine → threonine (M → T) in the house fly, *Musca domestica* and horn fly, *Haematobia irritans.*
9. cysteine → arginine (C → A) in the German cockroach, *Blattella germanica.*
10. aspartic acid → glycine, glutamic acid → lysine, cysteine → arginine, and proline → leucine (E → K) in the German cockroach, *Blattella germanica.*

Figure 1.6. shows the localization of *kdr* mutations that have been confirmed to reduce the sodium channel sensitivity to SPs in arthropods. More comprehensive information on *kdr* mutations that confer reduced neuronal sensitivity to SPs is found in Soderlund and Knipple (2003), Dong (2007) and Soderlund (2008). Dong (2007) mentioned that new *kdr* mutations will likely be identified in other agricultural and medically important arthropod pests as SPs continue to be used as a major pest control strategy.

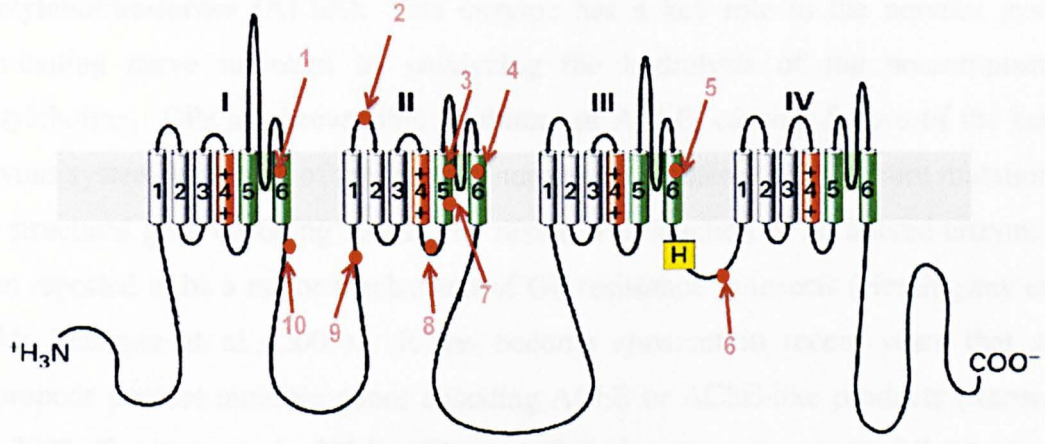

Figure 1.6. Knock down resistance (*kdr*) mutations in insect sodium channels. Only those *kdr* mutations that have been confirmed to reduce the sodium channel sensitivity to SPs are indicated (solid red dots). 1 (V → M, *H. virescens*), 2 (M → I, *P. capitis*), 3(L →F, *P. capitis*), 4 (L → F/HS, many insects), 5 (F → I, *B. R.* (*B.*) *microplus*), 6 (L → P, *V. destructor*), 7 (T → I/C/V, *P. xyllostella, P. capitis, F. occidentalis, C. felis*), 8 (M → T, *M. domestica, H. irritans*), 9 (C → A, *B. germanica*), 10 (E → K, *B. germanica*). Adapted from Lodish et al. (2004) and Dong (2007).

He et al. (1999a) investigated the molecular mechanism of resistance to SPs in *R.* (*B.*) *microplus* and obtained and sequenced a partial *para*-homologous sodium channel cDNA from susceptible and SP-resistance ticks strains. This partial sodium channel gene sequence is composed of 3599 bp (cDNA) (GenBank access number: AF134216, **see Appendix I**). A point mutation that results in an amino acid change F → I was identified in a highly conserved domain III segment 6 (IIIS6) of the homologous sodium channel from ticks that were highly resistant to SPs (**see Appendix II, Figure 1.6, no. 5**). As this amino acid substitution is caused by a change in one nucleotide from T → A (He et al., 1999a) it is known as a single nucleotide polymorphism (SNP). The nucleotide that is substituted is the first nucleotide of the amino acid codon (TTC → ATC) (He et al., 1999a). The authors concluded that IIIS6 of the sodium channel of *R.* (*B.*) *microplus* are target sites of SPs. Although other mutations exist, this point mutation seems to be the most important one associated with resistance to SPs in Mexico (Foil et al., 2004). In a study carried out in the Mexican tropics Rosario-Cruz et al. (2005) found that the presence of the F → I substitution in the sodium channel of *R.* (*B.*) *microplus* can be associated with resistance to flumethrin, deltamethrin and cypermethrin.

Acetylcholinesterase (AChE): This enzyme has a key role in the nervous system, terminating nerve impulses by catalyzing the hydrolysis of the neurotransmitter acetylcholine. OPs are irreversible inhibitors of AChE, causing failure of the central nervous system and death of the insect (Fournier and Mutero, 1994). Point mutations in the structural gene encoding AChE that result in production of an altered enzyme has been reported to be a major mechanism of OP resistance in insects (Hemingway et al., 2004; Temeyer et al., 2007). It has become apparent in recent years that some arthropods possess multiple genes encoding AChE or AChE-like products (Ranson et al., 2002; Temeyer et al., 2004). These AChE-like genes appear to fall into several homology groups (Weill et al., 2002). The role of these multiple genes remains unclear; however, a single gene has usually been associated with OP resistance in each organism, suggesting that it is the key target for OP inhibition and functional disruption of the nervous system. The gene associated with this neural role in different organisms may fall into different homology groups, so it is not possible to predict with certainty, which AChE plays the critical functional role in neural function (Weill et al., 2002).

Baxter and Barker (1998) isolated the first putative AChE gene (AChE1) in *R. (B.) microplus* larvae from Australia. This was the first report of alternative splicing at the 5′ end of the protein-coding region of an AChE gene and the first report of any type of alternative splicing in an AChE gene from *R. (B.) microplus*. Two other putative *R. (B.) microplus* AChE genes (AChE2 and AChE3) have since been discovered (Hernandez et al., 1999; Temeyer et al., 2004). Sequence analysis of these three putative *R. (B.) microplus* AChE genes failed to show any significant homology to one another suggesting that they were only distantly related (Temeyer et al., 2004). Unfortunately, no mutation that could have explained the genetic basis of the target site resistance mechanism has been found (Baxter and Barker, 1998).

γ-aminobutyric acid (GABA): In arthropods, GABA is an inhibitory neurotransmitter at neuromuscular junctions and synapses in the central nervous system. One of the main current agents in tick control is fipronil, an antagonist of GABA-gated chloride channels (Taylor, 2001). To date mutations of the GABA gene of *Drosophila melanogaster* have been reported (Hemingway et al., 2004); however, no GABA gene has been isolated in *R. (B.) microplus*.

Octopamine receptor: There is strong evidence that the octopamine receptor is the target site of formamidines (i.e. amitraz). The putative octopamine receptor was sequenced from two Australian *R.* (*B.*) *microplus* strains: an amitraz-susceptible and -resistant strain. Both of these sequences were identical (Baxter and Barker, 1999). Two possible explanations for this finding are that there may be more than one octopamine receptor gene in *R.* (*B.*) *microplus* or resistance to amitraz may be due to a metabolic process (Baxter and Barker, 1999; Jonsson and Hope, 2007). Recently, Chen et al. (2007) reported for the first time mutations in a putative octopamine receptor gene in amitraz-resistant *R.* (*B.*) *microplus*. Discovery of these mutations only in amitraz-resistant ticks provides the first evidence for the possibility of an altered pesticide target site as a mechanism of amitraz resistance in *R.* (*B.*) *microplus*.

1.5.2. Metabolic Resistance Mechanism

Carboxylesterases: These enzymes structurally belong to a superfamily of α/β-fold proteins, which consist of alternated α-helix and β-sheets connected by loops with a varying length (Oakesshott et al., 1999). These enzymes hydrolyze chemicals containing such functional groups as a carboxylic acid ester, amide, and thioester (Satoh and Hosakawa, 1998). Studies on carboxylesterases largely focus on detoxification of pesticide and metabolism of drugs and other xenobiotics (Satoh and Hosokawa, 1998). Carboxylesterases are structurally similar to acetylcholinesterase, a well established target of OPs and carbamate insecticides during acute exposure (Sussman et al., 1991). Binding of carboxylesterases to these insecticides, therefore, is considered as a detoxification pathway (Xie et al., 2002).

Riddles et al. (1983) described the partial purification of an *R.* (*B.*) *microplus* enzyme with carboxylesterase-like activity that could hydrolyze permethrin, providing early evidence that metabolic enzymes also can be involved in pyrethroids resistance. There are many reports of enhanced esterase activities in arthropods including mosquitoes (Hemminway et al., 2004). Enhanced carboxylesterase-mediated metabolic detoxification has been indicated in both OP and SP resistance in *R.* (*B.*) *microplus* ticks (Rosario-Cruz et al., 1997; Jamroz et al., 2000). Jamroz et al. (2000) identified a pyrethroid-resistant *R.* (*B.*) *microplus* strain (Cz) having high esterase-hydrolytic

activity (CzEST9) compared to a susceptible strain of *R. (B.) microplus*. The same high hydrolytic activity was found following purification of CzEST9 and therefore it was hypothesized CzEST9 is associated with permethrin resistance in the Cz strain (Pruett et al., 2002). In a pyrethroid-resistant *R. (B.) microplus* strain from Mexico a point mutation in an esterase gene was identified (Hernandez et al., 2000), but further research found that the occurrence of resistance was not associated with the presence of the mutation (Guerrero et al., 2002). Recently, Baffi et al. (2008) working with OP and SP resistant Brazilian strains of *R. (B.) microplus*, found that metabolic detoxification by two acetylcholinesterases contributed toward the development of resistance of these tick populations. However, Rosario-Cruz et al. (2005) working with nine field populations of *R. (B.) microplus* in Yucatan, Mexico, did not find positive correlations between esterase activity and larval survival exposed to cypermethrin, deltamethrin and flumethrin.

Recently, six strains of *R. (B.) microplus* collected from northern Mexico were found to be resistant to fipronil. Selection with fipronil for three generations produced a resistance ratio of 8.3 and 9.4 at the LC_{50} and LC_{99} estimates, respectively (Miller et al., 2008a). The authors concluded that resistance to fipronil seems to be due in part to elevated esterase activity (CZEST9) that was preselected in Mexico by the widespread use of permethrin in the 1980s. However more work needs to be completed in order to know the true mechanism(s) of fipronil resistance.

P450 monooxygenases: P450 enzymes (mixed functional oxidases, cytochrome P450 monooxygenase) are a complex family of heme containing enzymes found in most organisms. P450 enzymes bind molecular oxygen and receive electrons from NADPH (nicotinamide adenine dinocleotide phosphate) to introduce an oxygen atom to the substrate. In insects, the diverse functions of P450 enzymes range from the synthesis and degradation of ecdysteroids and juvenile hormones to the metabolism of xenobiotics (Feyereisen, 1999). P450 enzymes play important roles in adaptation of insects to toxic compound in their host plants and are involved in metabolism of all commonly used insecticides. P450 monoxygenase bioactivation of OP is a requisite to develop the highly toxic effect of OP upon its target acetylcholinesterase (Feyereisen, 1999; Sams et al., 2000). However, in general, P450 enzymes mediate metabolic

detoxification of their insecticides, particularly SPs (He et al., 2002). Diversity is conferred by the existence of multiple P450 isoforms, different expression pattern and wide substrate spectra (Scott and Wen, 2001). There are many reports demonstrating elevated P450 activities in insect-resistant mosquitoes, frequently in conjunction with altered activities of other enzymes (Hemingway et al., 2004).

Li et al. (2003) used synergist studies with coumaphos and piperonyl butoxide (PBO), an inhibitor of cytochrome P450 mixed function oxidase activity, to study mechanisms of OP resistance in both the Munoz (susceptible) and San Roman (resistant) strains of *R. (B.) microplus*. Those studies found that the toxicity of coumaphos in the presence of PBO was reduced 2-fold in the Munoz OP susceptible strain yet increased 3-fold in the San Roman OP resistant strain. In parallel studies with the OP diazinon, PBO again significantly reduced the toxicity of OP in the susceptible Munoz strain. However, in the OP resistant San Roman strain, the toxicity of diazinon was not affected by PBO, a contrast with the coumaphos results. Li et al. (2003) hypothesized that the activity of the cytochrome P450 responsible for bioactivation of either coumaphos or diazinon is adversely affected by PBO in all strains, leading to the decline in toxicity when either coumaphos or diazinon are applied with PBO. Recently, Miller et al. (2008b) found a Mexican *R. (B.) microplus* strain highly resistant to diazinon but not highly resistant to coumaphos. When exposed to coumaphos and PBO or triphenylphosphate (another inhibitor of cytochrome P450), the toxicity was reduced by 3.5- and 6.3-fold, respectively, suggesting that mono-oxygenases and/or esterases were involved in resistance to coumaphos. Another recent study, Cossio-Bayugar et al. (2008) showed a linkage between increased monooxygenase activities and pyrethroid acaricide resistance in *R. (B.) microplus* from Mexico.

Glutathione S-Transferases (GSTs): GSTs are a group of enzymes that catalyze the conjugation between glutathione (GSH) and several molecules. These enzymes have a central role in detoxification of xenobiotic and endogenous compounds. In populations with a long history of chemical exposure, high GST activity is associated with resistance to insecticides (Ketterman et al., 2001). Resistance to OCs and OPs is specifically associated with increased GST activity (Vontas et al., 2001. 2002). These facts suggest that insecticide conjugation to glutathione, which is catalyzed by GST,

may be a detoxification mechanism in arthropods (Wei et al., 2001). He et al. (1999b) reported the purification, characterization, and molecular cloning of a larval *R.* (*B.*) *microplus* GST. Synergist bioassays on several amitraz-resistant strains from Mexico and one Brazilian strain of *R.* (*B.*) *microplus* indicated some involvement of esterase and glutathione S-transferase (Li et al., 2004).

1.6. Selection Pressure for Acaricide Resistance

Harris et al. (1988) conducted a study to generate resistance in *R.* (*B.*) *microplus* to OPs under laboratory conditions. The study consisted of selecting for resistance to coumaphos by dipping groups of engorged *R.* (*B.*) *microplus* females in serial dilutions (0.2, 0.1, 0.06, 0.03 and 0.01 % of active ingredient) prepared from a commercial 50 % flowable formulation of coumaphos. Surviving offspring from females treated with the most concentrated coumaphos dilutions were retained for reproduction. This method of selection was used for the three generations in the laboratory, then the authors changed to a technique in which larvae from a single female were selected and treated with coumaphos (0.1 % to 1 %). During this selection process (12 generations), the "Tuxpan" strain of *R.* (*B.*) *microplus* became 38 times more resistant than the "Escondido" strain to coumaphos. Working with a resistant strain ("Tuxpan"), Wright and Anrens (1989) made selection pressure in three generations by dipping groups of engorged females in dilutions of 42 % (active ingredient) flowable formulation of coumaphos. They found that Tuxpan strain became more resistance to coumaphos as the generation proceeded.

In another study conducted by Davey et al. (2003) larvae from F_1 generation and all subsequent generations up to the F_{14} generation were selectively exposed to coumaphos (0.2 % to 0.45 % active compound) to maintain or increase the amount of OP resistance in the strain. The F_2 resulted in an estimated lethal dose concentration for 50 % ($LC_{50\%}$) of 0.623, whereas ticks in the F_{14} generation, resulted in an estimated $LC_{50\%}$ of 0.688 %. Comparison of these results with the OP-susceptible reference strain revealed that the F_2 generation of OP-resistant ticks was approximately 12-times more resistant to coumaphos than the OP-susceptible strain, whereas the F_{14} generation was

approximately 13-times more resistant to coumaphos than the susceptible strain. Therefore, although the 12 successive generations of continuous selective exposure to coumaphos maintained resistance in the strain, it had little effect on substantially increasing the amount of resistance. Working with the same OP-resistant strain and making pressure with coumaphos treatments during virtually all subsequent generations (22 generations), Davey et al. (2004) found that the level of resistance did not significantly increase.

Li et al. (2004) developed a selection pressure using amitraz on larvae of a *R.* (*B.*) *microplus* train ("Santa Luizia"). The strain was challenged with various concentrations of amitraz after its establishment in the laboratory. The "Santa Luizia" responded to selection quickly, and the resistance factor (RF: level of resistance in relation to a susceptible reference strain) was elevated from 13.3 in F_1 to 154 in F_6. Although resistance decreased sharply without selection in the following generations (F_8= 68.72) and at low dose pressure of amitraz (F_9=50.7, F_{12}=49.43). Furthermore, in a field study conducted in the Mexican tropics, Rosado-Aguilar et al. (2008) treated three field populations of *R.* (*B.*) *microplus* with amitraz. After 15 months of amitraz selection pressure all populations increased their RFs (from 1 to 13, from 1 to 22 and from 2 to 6).

Rapid onset and development of SP resistance in a controlled field trials was observed by Coetzee et al. (1987) who reported that the development of resistance to fenvalerate in *B. decoloratus* occurred during an 18-month period (in 5-6 tick generations). Furthermore, in another controlled pen trial, Davey and George (1998) were able select a *R.* (*B.*) *microplus* strain for resistance to permethrin by treating larvae with increasing doses (range, 0.05%-0.35% active ingredient) through successive generations (generations F_2-F_7). At the beginning of the selection process (F_2), the SP resistant strain was 5.4 times more resistant to permethrin than the SP susceptible strain, and the level of resistance increased in each successive generation of the SP resistant strain, reaching a RF of 20.9 in the F_7 generation. The results demonstrated that under continuous selection pressure the tick population increased in resistance in a relatively short time. However, the development of acaricide resistance in populations under normal field conditions has not been reported to our knowledge.

1.7. Persistence of Insecticide Resistance in *Rhipicephalus* (*Boophilus*) *microplus*

Fitness costs associated with pesticide resistance have been documented in many pest species (Roush and McKenzie, 1987; Coustau et al., 2000; Oliveira et al., 2007). Resistant populations of *Helicoverpa armigera* may have reduced fitness, a suggestion supported by the observations that a homozygous resistant population from Australia showed a lower biotic potential in comparison with susceptible population (Bird and Akhust, 2004). If resistance genes have negative effects on fitness components, reduction in resistance levels could be expected without insecticide selection pressure (Razaq et al., 2007).

The reproductive fitness of *R.* (*B.*) *microplus* strains resistant to OPs, SPs or amitraz was compared to an acaricide-susceptible strain to determine whether the acquisition of resistance affected reproductive fitness in the resistant strains (Davey et al., 2006). The authors found that the OP-resistant strain produced 30 % fewer eggs than the susceptible strain indicating the acquisition of resistance placed the OP-resistant at a selective disadvantage relative to the susceptible strain. The fitness cost of amitraz and SP-resistant strains was not found. However, in Mexico, the level of resistance of *R.* (*B.*) *microplus* to amitraz in the San Alfonso strain decreased from 42-fold to 10-fold after six generations on laboratory colonization without selection (Soberanes et al., 2002). On field populations of *R.* (*B.*) *microplus*, Rodriguez-Vivas et al. (2005b) found persistent resistance to OP for more than four years. This type of resistance has been a major incentive to the development of alternative tick control measures.

Regeneration of tick populations susceptible to acaricides is difficult; however, in some insects, such as mosquitoes, mass release of susceptible males could dilute the resistance (May and Dobson, 1986). Migration can also greatly influence the reversion of pyrethroid-resistance level in the cotton bollworm, *H. armigera* (Daly and Fitt, 1990). In eastern Australia, high densities of pyrethroid-resistant pupae diapausing beneath cotton stubble during winter ensure effective carryover of resistance between

seasons. This effect is countered to some extent by spring immigration of susceptible moths from alternative hosts, causing a temporary reversion in resistance level (Daly and Fitt, 1990). Conversely, the wet weather during spring, which promotes emigration of resistance moths from cotton, has led to a gradual increase in resistance on unsprayed hosts (Gunning and Easton, 1989).

1.8. The Mode of Inheritance of Resistance to Pyrethroids

In most cases, it is likely that genes that confer resistance are already present at very low levels in the tick population before the introduction of a new acaricide. The rate at which a resistant allele becomes established in the population and the time it takes for the control of ticks to break down is dependent upon many factors. These include the frequency of the original mutation in the population before treatment, the mode of inheritance of the resistant allele, the frequency of acaricide treatment, the concentration gradient of the acaricide and the proportion of the total tick population that is not exposed to the acaricide defined as refugia (Nari et al., 2000).

The resistant phenotype of acaricides may be inherited as a recessive, partially dominant or dominant character (ffrench-Constant and Roush, 1990). The dominance of an insecticide-resistance gene is best described by the relative position of the mortality lines of heterozygotes compared to both susceptible and resistant homozygotes (Stone, 1968). Dominance level was initially determined by comparing the mortality curves of susceptible, resistant, and hybrid individuals (Milani, 1963). Resistance was qualitatively (and arbitrarily) classed as recessive or dominant according to whether the hybrid mortality curve was closer to the susceptible or resistant mortality curve, respectively. Resistance was considered codominant (or absence of dominance) if hybrid mortality curves were equidistant from those of homozygotes. A quantitative measure of dominance level (effective dominance) was then introduced by Stone (1968) and improved by Bourguet et al. (2000), to classify the resistance as complete recessive or complete dominance.

The mode of inheritance of resistance to pyrethroids has been identified in a wide range of insect species such as mosquitoes (Halliday and Georghiou, 1985), the horn fly *Haematobia irritans* (Roush et al., 1986), the diamondback moth *Plutella xylostella* (Tabashnik et al., 1992), and the codling moth *Cydia pomonella* (Bouvier et al., 2001). Conclusions on the mode of inheritance of the resistant trait were made on the basis of the phenotype as measured by toxicological bioassays.

Based on reciprocal crosses of a susceptible and a resistant *R.* (*B.*) *microplus* strain, Aguilar-Tipacamu et al. (2008) evaluated the inheritance of pyrethroid resistance using the "effective dominance of survival method" described by Bourguet et al., (2000). The authors found that pyrethroid resistance (deltamethrin, flumethrin and cypermethrin) is inherited as a partially dominant trait when the *R.* (*B.*) *microplus* female is resistant. However, when the male is resistant for deltamethrin and flumethrin the resistance is inherited as complete recessive (partially dominant for cypermethrin). The same authors found that one single mutation at the sodium channel can produce a multiple resistance to pyrethroids phenotype (deltamethrin, flumethrin and cypermethrin), since no mortality was produced in the resistant strain when increasing concentrations of the pyrethroids were used, probably due to the high frequency of homozygous resistant genotype. Based on phenotype results (larval mortality), Tapia-Pérez et al. (2003) have found that resistance to flumethrin in *R.* (*B.*) *microplus* may be controlled by more than one gene; however, further studies are needed to elucidate this statement.

1.9. Methods of Detecting Genotypic Resistance in *Rhipicephalus* (*Boophilus*) *microplus*

1.9.1. Allele Specific Polymerase Chain Reaction

Comparison of genomic DNA sequences in different individuals reveals some positions at which two, or in some cases more than two, bases can occur. These single nucleotide polymorphisms (SNPs) are highly abundant, and are estimated to occur at 1 out of every 1,000 bases in the human genome (Sachidanandam et al., 2001). Depending on where a SNP occurs, it might have different consequences at the phenotypic level. SNPs in the

coding regions of genes that alter the function or structure of the encoded proteins are a necessary and sufficient cause of most of the known recessively or dominantly inherited monogenic disorders. These SNPs are routinely analysed for diagnostic purposes (Syvänen, 2001). Based on a mutation in the sodium channel of *R.* (*B.*) *microplus* associated with SP resistance, Guerrero et al. (2001) developed an allele-specific polymerase chain reaction assay (AS-PCR) to genotype pyrethroid resistant strains of *R.* (*B.*) *microplus*. The SNP involved in SP resistance in *R.* (*B.*) *microplus* involves a T to A nucleotide substitution (He et al., 1999a). Therefore, the allele specific primers end in either T for susceptible (S) or an A for resistant (R) genotype detection. Following PCR, agarose gel (3-4 %) electrophoresis is used to identify products. If a product is found in only one PCR reaction then the individual is either homozygote resistant (RR) or susceptible (SS) whereas if a product is found in both PCR reactions the individual is heterozygous (RS). This AS-PCR produces a product of 68bp (Guerrero et al., 2001) and can be performed on any life stage of the tick, even samples preserved in alcohol or dry ice, with results available in a single day if necessary. The PCR assay can also be performed on a tick hemolymph sample drawn from a live tick, which can then be used for further studies or propagation if desired (Foil et al., 2004).

1.9.2. Pyrosequencing™

Pyrosequencing™ (PSQ) (Biotage, Uppsala, Sweden) is a real-time quantitative sequencing method based on the detection of pyrophosphate (PPi) release during the synthesis of the complementary strand PCR product (Ronaghi et al., 1996, 1998; Alderborn et al., 2000). Several reactions are taken in the PSQ assay that involves PCR products, primers, enzymes and nucleotides.

Reactions in the Pyrosequencing Assay: During DNA synthesis, one PPi is released per nucleotide incorporated (unlabeled natural nucleotides except for the deoxyadenisine α-thiotriphosphate (dATP-α-S) (**Figure 1.7.**). The PPi is converted to ATP that is catalyzed by ATP sulfurylase (a recombinant version from the yeast *Saccharomyces cerevisiae*). The ATP is, in turn, converted to light in a luciferase-catalyzed reaction (the luciferase is from the American firefly *Photinus pyralis*). The light intensity from each of the dispensed nucleotides is displayed in real time as a

pyrogram, representing the DNA sequence, with peak heights corresponding to the number of identical residues incorporated. The overall reaction from polymerization to light detection takes place within 3-4 sec at room temperature. One pmol of DNA in a PSQ reaction yields 6×10^{11} ATP molecules which, in turn, generates more than 6×10^{9} photons at a wavelength of 560 nanometers. This amount of light is easily detected by a photodiode, photomultiplier tube, or a charge-coupled device camera. Unincorporated nucleotides are degraded by apyrase before the next nucleotide is dispensed (Lavebratt and Sengul, 2006).

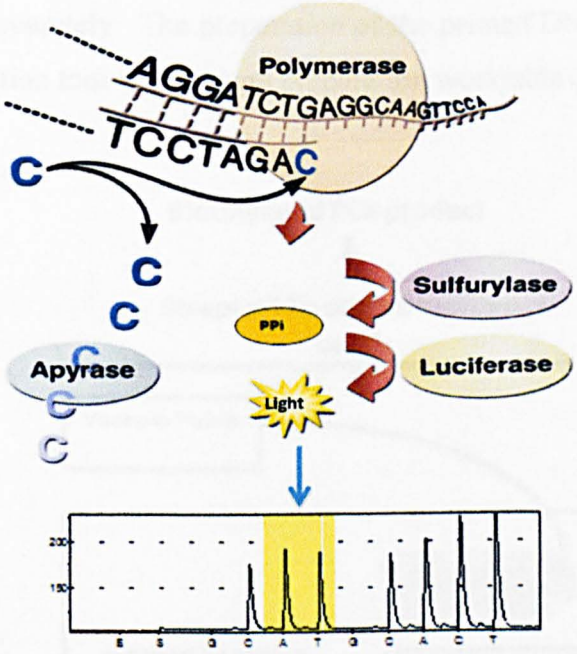

Figure 1.7. Schematic representation of the reactions in the Pyrosequencing™ method. Nucleotides (colored in blue) are sequentially added to form the complementary strand of the single-stranded PSQ template, to which a sequencing primer has been annealed. This is carried out in the presence of polymerase, sulfurylase, luciferase and apyrase enzymes. One molecule of pyrophosphate (PPi) is released for every nucleotide incorporated into the growing strand by the DNA polymerase, and is converted to ATP by sulfurylase. Visible light is produced from luciferin in a luciferase-catalyzed reaction that utilizes the ATP produced above, and unincorporated nucleotides are degraded by apyrase between each cycle. A pyrogram displays peaks representing the amount of generated light, which is proportional to the amount of incorporated nucleotides, at each nucleotide dispensation (Adapted from Lavebratt and Sengul, 2006).

Template Preparation for Pyrosequencing: To allow sequencing of a section of DNA, a biotinylated PCR product is immobilised on streptavidin-coated sepharose magnetic beads (Nyrén et al., 1993). A magnetic vacuum probe is used to pick the beads up. These are dipped into 70 % ethanol to remove salt, alakali denaturation (sodium hydroxide) to yield single-stranded DNA and finally washing buffer to re-establish the correct pH (tri-acetate buffer). The immobolized biotinylated strand is annealed to a specific sequencing primer and used as primed template for sequencing (Ronaghi et al., 1996), and allows stepwise elongation of the sequence by addition of each nucleotide separately. The preparation of the primed DNA template is done using a vacuum preparation tool and vacuum preparation worktable (**Figure 1.8.**).

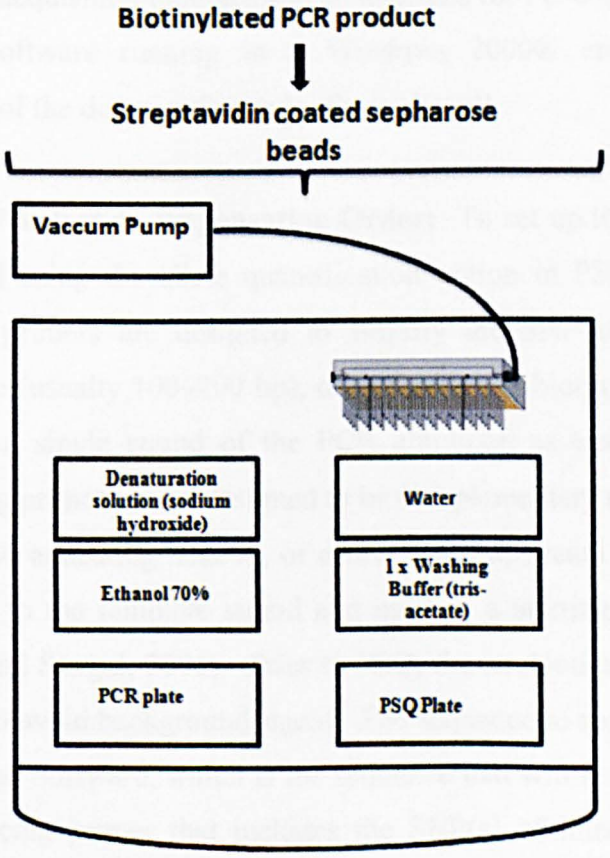

Figure 1.8. Vacuum preparation tool used to bind and immobilize the biotinylated PCR product to streptavidin coated sepharose beads and anneal the sequencing primer to the single stranded.

The multi-channel PSQ instrument utilizes 96-well plates. It uses an inkjet cartridge for precise pneumatic delivery of small volumes of deoxynucleotide triphosphate (dAPT, dCTP, dGTP, dTTP) into a temperature-controlled microtiter plate. The enzymes (polymerase, sulfurylase, luciferase and apyrase) and nucleotides are added in the inkjet cartridge in specific order determined by the company. The nucleotides are added one by one to the pysosequencing solution. The microtiter plate is continuously agitated during PSQ to increase the rate of the enzymatic reactions. A lens array focuses the light signal generated from each reaction well on the microtiter plate onto a specific locus of a CCD-camera. Nucleotides are dispensed into alternating wells with a pulse delay to minimize cross-talk of generated light between adjacent wells. A cooled high sensitivity CCD-camera images the plate every second to follow the progress of the PSQ reactions. Data acquisition modules and an interface for PC-connection are used in this instrument. Software running in a Windows 2000® environment enables experimental control of the dispensation order for each well.

Primer Design and Nucleotide Dispensation Order: To set up the PSQ assay, three primers are designed using the allele quantification option in PSQ™ Assay Design software: two PCR primers are designed to amplify the SNP to be analyzed and surrounding sequence (usually 100–200 bp), one of which is biotinylated at the 5′ end to allow capture of a single strand of the PCR amplicon as template for the PSQ reaction; a sequencing primer is also designed to be complementary to the PSQ template strand, with its 3′ end annealing next to, or a few bases upstream of, the SNP. This primer is hybridized to the template strand and used as a starting point for the PSQ reaction (Lavebratt and Sengul, 2006). Prior to PSQ, the nucleotide dispensation order has to be optimized to avoid background signal. The sequence to analyze is entered into the PSQ Assay Design Software, which is the sequence that will be elongated from the 3′end of the sequencing primer that includes the SNP(s) of interest. The software suggests a dispensation order corresponding to the sequence to analyze, but also adds two nucleotide dispensations that should not generate specific signal, one before and one after the SNP, in order to detect possible background noise and to provide an internal quality-control measure. Alternative dispensation orders can be made manually (Lavebratt and Sengul, 2006).

Pyrosequencing Applications: Several reports have been published addressing different applications of PSQ technique for SNP and mutation analyses. These include SNP discovery, genotyping, allele frequency in pooled samples (Gruber et al., 2002; Neve et al., 2002), methylation analyses (Uhlmann et al., 2002), molecular haplotyping (Ahmadian et al., 2000), identification of short DNA sequences in bacterias, virus and parasites (Gharizadeh et al., 2001, 2003; Hodgkinson et al., 2008). Direct sequencing of the SNP region with PSQ provides additional information about adjacent nucleotides in the DNA template. This technique has mostly been used for pharmacogenetics and association studies; however, there are a few reports using this technique for plant genetic studies as well. Gruber et al. (2002) have demonstrated detection of allele frequency differences of less than 2 % between pools, indicating that this method may be relatively sensitive for use in association studies involving complex diseases where a small difference in allele frequency between cases and controls is expected. In another study, Lavebratt and Sengul (2006) found that the difference between true allele frequencies and those estimated from pools of DNA by PSQ has been shown to vary by 1.1–6.5 % and the correlation between true and estimated allele frequencies was good ($r^2 = 0.92–0.99$). PSQ has the advantage of displaying a 50-100 bp sequence next to the SNP, which allows it to detect and quantify the allele frequency of a variety of sequence variation types - such as bi-, tri- and tetra-allelic SNPs, point mutations and insertion/deletions, as well as providing sequence and purity control of the PSQ template. Multiple closely located variations (4-5 variations) can be analyzed simultaneously in one reaction (Langaee and Ronaghi, 2005).

1.10. Methods of Detecting Phenotypic Resistance in *Rhipicephalus (Boophilus) microplus*

The standard bioassay recommended by the FAO for testing phenotypic resistance to acaricides in *R. (B.) microplus* is the larval packet test (LPT), originally described by Stone and Haydock (1962). Other tests have been used, including the larval immersion test (LIT) of Shaw (1966) and adult immersion tests (AIT) described by Drummond et al. (1973). The LPT is considered to be the most repeatable, although it is limited by the length of time that it takes (45 days). Hence it remains the test of choice for surveys

and for definitive confirmation of a diagnosis of resistance. In this test, tick larvae are exposed to chemically impregnated filter papers (a series of concentrations of the acaricide) and their subsequent mortality is quantified after 24 hours. Mortality results are plotted: percent concentration or dose (x-axis) by probit mortality (y-axis) using log/probit graph paper. Alternatively, the data can be submitted to *Polo-Plus* for analysis (LeOra, 2004).

An example of a dosage mortality test is showed in **Figure 1.9.** If the population is homogeneously susceptible (a) or homogeneously resistant (c), a straight line will be obtained. If, on the other hand, a line similar to (b) is obtained, it indicates that the population is a mixture of susceptible and resistant individuals. The horizontal portion of this line (b) will vary in position depending on the proportion of resistant ticks in the sample (McKenzie, 1996).

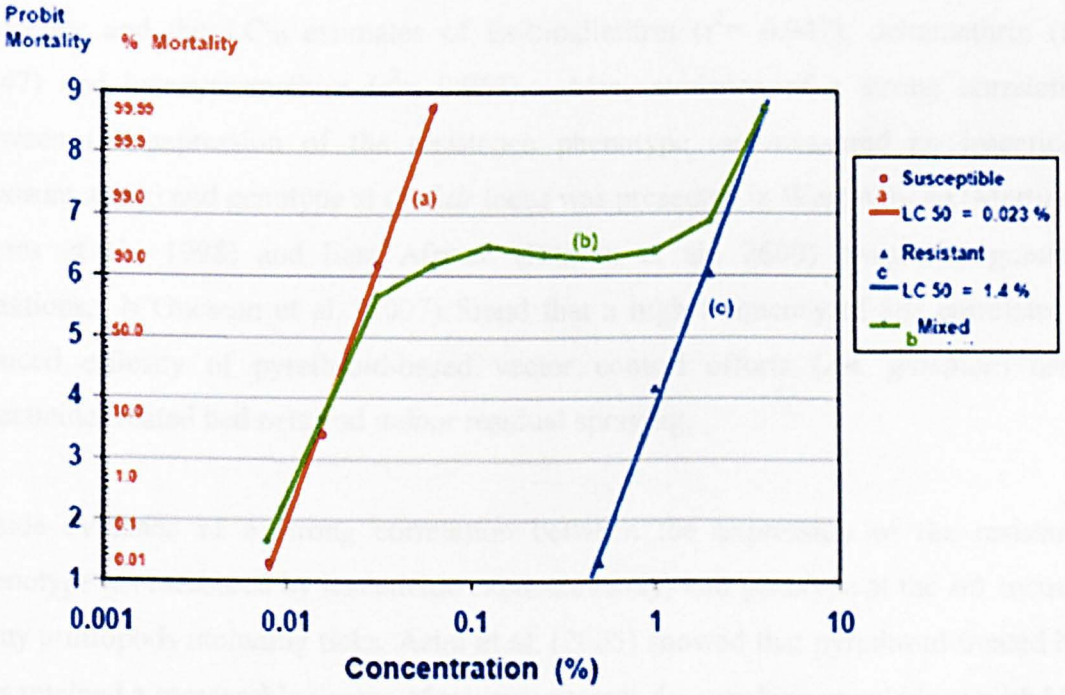

Figure 1.9. Examples of probit mortalities for samples of three populations (a, b and c) of *Rhipicephalus* (*Boophilus*) *microplus*, subjected to a complete dose-response test for acaricides (Adapted from McKenzie, 1996).

1.11. Correlation Between Phenotype and Genotype for Pyrethroid Resistance

Rosario-Cruz et al. (2005) working with nine Mexican populations of *R.* (*B.*) *microplus* found a positive correlation (deltamethrin r^2 = 0.887; cypermethrin r^2 = 0.856; and flumethrin r^2 = 0.849) between larval survival (using discriminating doses) and the percentage of the resistant allele of the sodium channel mutation known to be involved in pyrethroid resistance. Recently, Li et al. (2007) found a significant correlation (r^2= 0.827) between the permethrin resistance ratio and allele frequency of the sodium channel mutation in five laboratory strains of *R.* (*B.*) *microplus*. This significant correlation between mutated alleles and lethal concentration (LC) to pyrethroids has been found in other insects. Kwon et al. (2004) found in *Plutella xylostella* that the increasing presence of the T929I mutation correlated well with increased levels of resistance to both cypermethrin (r^2= 0.912) and fenvalerate (r^2: 0.810). Song et al. (2007) working with *C. pipiens pallens* found significant correlation between *kdr* allelic frequency and the LC_{50} estimates of Es-bioallethrin (r^2= 0.947), deltamethrin (r^2= 0.747) and betacypermethrin (r^2= 0.967). Also, evidence of a strong correlation between the expression of the resistance phenotype (as measured by insecticide exposure assay) and genotype at the *kdr* locus was presented in West African (Martinez-Torres et al., 1998) and East African (Ranson et al., 2000) *Anopheles gambiae* mutations. N'Guessan et al. (2007) found that a high frequency of *kdr* correlates to reduced efficacy of pyrethroid-based vector control efforts (*An. gambiae*) using insecticide-treated bed nets and indoor residual spraying.

Beside evidence of a strong correlation between the expression of the resistance phenotype (as measured by insecticide exposure assay) and genotype at the *kdr* locus in many arthropods including ticks, Asidi et al. (2005) showed that pyrethroid-treated bed nets retained a measurable degree of toxicity against *An. gambiae* populations with high frequencies of *kdr*. Henry et al. (2005) showed that sufficient use of pyrethroid-treated bed nets is able to reduce asymptomatic malaria infection in areas where the frequency of *kdr* is high in *An. gambiae*. Also, Matambo et al. (2007) and Abdalla et al. (2008) demonstrated the presence of *kdr* in *An. arabiensis* from Sudan. In both cases the

expression of DDT and pyrethroid resistance assorts independently of genotype at the *kdr* locus, implying that *kdr* is not associated with the expression of the resistance phenotype. Alternatively, Xu et al. (2006) proposed that *kdr* variation at the genomic DNA level may not be sufficient to produce a resistance phenotype unless it is transcribed into RNA variation at the RNA editing stage in *Culex quinquefasciatus.*

1.12. Implications for the Monitoring and Management of Knockdown Resistance

Historically, the detailed mechanistic characterization of insecticide resistance has occurred only after effective insect control has been compromised. However, the identification of resistance-conferring mutations offers the opportunity to design molecular diagnostic tools capable of detecting resistance-conferring alleles in individual insects, thereby facilitating the early detection and monitoring of resistance genes in populations under selection pressure (Soderlund and Knipple, 2003).

A central dogma of the knockdown resistance field is that this type of mutation confers global cross-resistance to pyrethroids as a class. However, this dogma is based principally on results of toxicity bioassays. With the aid of molecular biology, several point mutations associated with SP resistance have been identified (Dong, 2007). Thus the SP susceptibility status of target arthropod populations ought to be monitored as often as possible using a suitable array of methodologies. These would include biometric measurement of insecticide resistance phenotype (bioassays) in conjunction with molecular and biochemical techniques. Tailored resistance management strategies are best designed with this kind of data (Brooke, 2008).

The conclusion of this literature review is that acaricides have played a pivotal role in the control of the tick *R. (B.) microplus* and as a consequence of extensive use this tick specie has developed resistance to all major classes of acaricides, especially to pyrethroids. In this thesis, we evaluated the effect of selection pressure on the genotype and phenotype of acaricide resistance in *R. (B.) microplus*

1.13. Aims of the study

To evaluate the effect of selection pressure on the genotype and phynotype of acaricide resistance in *R.* (*B.*) *microplus* the following aims were approached:

1) To modify the AS-PCR and develop a novel Pyrosequencing™ method to detect the presence of a target SNP associated with pyrethroid resistance in *R.* (*B.*) *microplus.*
2) To validate the ability of the AS-PCR to detect the presence of a target SNP associated with pyrethroid resistance in *R.* (*B.*) *microplus* using Pyrosequencing™ method as a gold standard.
3) To determine the association between survival of larvae exposed to cypermethrin and pyrethroid resistance genotypes in populations of *R.* (*B.*) *microplus.*
4) To determine the prevalence of pyrethroid resistance phenotype and genotype on field populations of *R.* (*B.*) *microplus* using a cross sectional study in Yucatan, Mexico.
5) To measure the evolution of resistance phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *R.* (*B.*) *microplus.*
6) To evaluate a tactical management strategy to introduce a pyrethroid susceptible *R.* (*B.*) *microplus* population into a pyrethroid-resistant *R.* (*B.*) *microplus* population.

CHAPTER 2

MATERIALS AND METHODS

2.1. General Laboratory, Chemicals and Reagents

Chemicals for the preparation of solutions and general laboratory use were purchased from different companies. For the AS-PCR-68 bp assay general reagents were purchased from QIAGEN (USA), dNTP from ROCHE (USA) and primers from SIGMA (USA). For the AS-PCR-91 bp assay general reagents were purchased from QIAGEN (UK), dNTP from ABgene (UK), and primers from SIGMA (UK). For the PCR-pyrosequencing general reagents were purchased from ABgene (UK) and primers from SIGMA (UK) and biotinylated primers from MWG (UK). The reagent for DNA visualization was purchased from Invitrogen (UK). All reagents for Pyrosequencing™ were purchased from Biotage AB (Sweden). Alcohols for general laboratory use and molecular biology work were purchased from BDH Laboratory Supplies (UK). For bioassays, reagents were purchased from SIGMA (USA) and technical grade cypermethrin was donated from Fort-Dodge Animal Health, Mexico.

2.2. Standard Laboratory Procedures

Solutions were made using deionised, sterile water (18.2 MOhms MilliQ water, Millipore, U.K.) in clean glass or plastic and were stored at room temperature unless stated otherwise.

Tris-acetate EDTA (TAE) agarose gel running buffer: A 10 X stock solution of 400 mM Tris-acetated and 10 mM EDTA (pH 8.0) in sterile water was prepared. A work solution of 1 X TAE was created by 1 in 10 dilution of the stock in sterile water.

Cypermethrin dissolvent: A mixture of trichloethylene and olive oil was prepared in a ratio of 2:1 to dissolve technical grade cypermethrin and treat filter papers.

2.3. The Field Study Site

Four field studies were carried out in this thesis (**see chapters 4-7**). All field studies were carried out in the state of Yucatan, Mexico (**Figure 2.1.**). The state is located between 19° 30' and 21° 35' north latitude and 90° 24' west longitude of the Greenwich meridian. The climate of the state is sub-humid tropical with a summer rainy season. The monthly maximum temperature varies from 35°C to 40°C (mean 26.6°C). The relative humidity (RH) varies from 65 to 100 % (mean 80 %) and the annual rainfall varies from 415 mm to 1290 mm depending on the area. There are two different seasons: rainy (June to October) and dry (November to May) (INEGI, 2002).

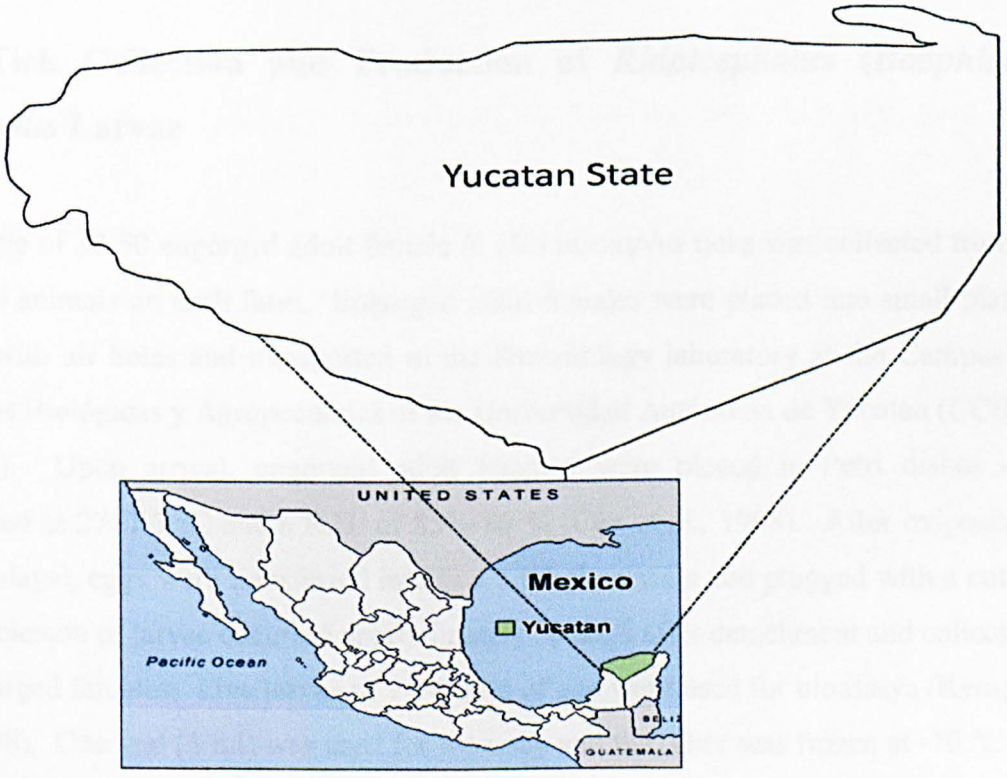

Figure 2.1. Locality of the state of Yucatan, Mexico where field studies were carried out.

According to the type of soil, vegetation and agricultural development, the state of Yucatan is classified into three zones: the sisal zone (centre-north), the agricultural zone (southern) and livestock zone (eastern). The state has a cattle farm population of 4629

with 624,488 head of cattle (INEGI, 2002). Sixty-five percent of the cattle population in the state of Yucatan is concentrated in the livestock zone where the present study was carried out (Tizimin, Panaba and Sucila areas) (INEGI, 2002). The predominant livestock-production system is semi-intensive (beef farms), based mainly on year-round grazing on improved pastures i.e. Guinea grass (*Panicum maximum*) and Star grass (*Cynodon plectostachyus*), with supplementary feeding during the dry season. The use of acaricides to control ticks is a common practice in Yucatan, Mexico (Solorio-Rivera et al., 1999). Twenty one percent of the farms in Yucatan state use pyrethroids to control ticks and forty-two percent of the farms applied acaricides >12 times/year (Rodriguez-Vivas et al., 2006b).

2.4. Tick Collection and Production of *Rhipicephalus* (*Boophilus*) *microplus* Larvae

A sample of 30-50 engorged adult female *R.* (*B.*) *microplus* ticks was collected from at least 10 animals on each farm. Engorged adult females were placed into small plastic boxes with air holes and transported to the Parasitology laboratory at the Campus de Ciencias Biológicas y Agropecuarias of the Universidad Autónoma de Yucatán (CCBA-UADY). Upon arrival, engorged adult females were placed in Petri dishes and incubated at 27±1.5 °C and a R.H. of 85%-86 % (Cen et al., 1998). After oviposition (14-18 days), eggs were transferred into two 3 ml glass vials and plugged with a cotton cap. Eclosion of larvae occurred approximately 30 days after detachment and collection of engorged females. Live larvae of 7-14 days of age were used for bioassays (Kemp et al., 1998). One vial (3 ml) was used for bioassay and the other was frozen at -70 °C for DNA isolation and PCR.

2.5. Phenotypic Analysis by Dose-Response Bioassays

The modified larval packet test (Stone and Haydock, 1962) was used to test cypermethrin resistance of *R.* (*B.*) *microplus* at the phenotypic level. Dose-response

bioassays were carried out using different dilutions of technical grade cypermethrin (2.0-0.007 %) to test the susceptibility of larvae. Cypermethrin dissolved in a mixture of trichloroethylene and olive oil (2:1 ratio) was used to treat filter papers that were then set for 2 h in a fume hood to allow the trichloroethylene to evaporate before being folded into packets using bulldog clips. Approximately 100 *R.* (*B.*) *microplus* larvae were added to the treated filter paper packets, which was then sealed with additional bulldog clips and placed in an incubator (27 °C and 85-86 % RH). Three replicates of the acaricide-treated and a control (papers treated with dilutants only) were used. The treated larvae were exposed to cypermethrin for 24 h and the numbers of live and dead larvae (i.e. those that could walk) were counted to calculate the percentage of larval mortality. Live but inactive larvae were stimulated to move.

2.6. Genotype Analysis by Allele-Specific Polymerase Chain Reaction

The AS-PCR-68 bp to detect the presence of the SNP target in the sodium channel gene (**see Appendix III**) was carried out to determine the genotype of *R.* (*B.*) *microplus* exposed to cypermethrin.

2.6.1. Deoxyribonucleic Acid Extraction

Genomic DNA was isolated from individual larvae as previously described by Guerrero et al. (2001) (**see Appendix III**). Briefly, individual frozen larvae were transferred onto a Petri dish on dry ice and placed in individual 1.5 ml microcentrifuge tubes also kept on dry ice. Twenty microlitres of sample buffer (100 mM Tris, pH 8.3; 500 mM KCl) were added to the tube and a disposable pellet pestle was used to crush and grind the larva against the tube wall for 20 seconds until close visual inspection ensured that the larva was broken into several fragments. The tube was briefly microcentrifuged and placed in a boiling water bath for 3 min. After cooling, 2 µl of this DNA solution was used in each PCR reaction.

2.6.2. Polymerase Chain Reaction Conditions

PCR is a method for the selective amplification of DNA sequences. When a DNA duplex is heated, the strands separate. If the single-strand sequences can be copied by a DNA polymerase, the original DNA sequence is effectively duplicated. If the process is repeated many times, there is an exponential increase in the number of copies of the starting sequence. The length of the fragment is defined by the 5`ends of the primers, which helps to ensure that a homogeneous population of DNA molecules is produced. Thus, after relative few cycles, the target sequence (PCR product or amplicon) becomes greatly amplified. Gel electrophoresis and stain of PCR products allow visualization of a disgnosis band (Nicholl, 2008). For the detection of a point mutation of the sodium channel de *R. (B.) microplus*, the method described by Guerrero et al. (2001) was slightly modified. Briefly, PCR was performed in thin-walled 0.5 ml microcentrifuge tubes using 20 µl reaction volumes. Final optimized PCR reaction conditions used 2 µl of genomic DNA, 20 pmol of each primer (QIAGEN) (**see Table 2.1.**), 10 nM Tris (hydroxymethyl) aminomethane hydrochloride pH= 8.3, 50 nM KCl (QIAGEN), 0.25 nM each dNTP (ROCHE), 1.75 nM $MgCl_2$ (QIAGEN), and 0.25 µl of hotStartTaq DNA polymerase (5 U/ µl stock) (QIAGEN). Amplification was carried out using a DNA engine programmed for 96 °C for 2 min followed by 42 cycles, each consisting of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The program also included a final extension step at 72 °C for 7 min. PCR products of 68 bp were separated on 3.0 % NuSieve agarose TBE gels and DNA visualized by staining with GelStar DNA staining dye, ethidium bromide and UV illumination. The primers used are presented in **Table 2.1.** Primer FG-227 was designed to anneal to both the wild type and the resistant alleles down-steam of the nucleotide substitution site, and primers FG-221 and FG-222 were designed as diagnostic primers to detect the identity of the variant nucleotide by hybridizing to the antisense strand of the wild type and resistant alleles, respectively. Genomic DNAs from *R. (B.) microplus* adults were extracted and genotyped by Pyrosequencing™ and used as controls. Initially, for a proportion of PCR reactions, three positive controls (larvae of known genotype) were used (genotypes A/A, A/T and T/T). A negative master-mix control was used for each PCR reaction.

2.6.3. Genotyping of larvae

Genotype interpretation of each PCR assay using gel analysis was based on whether or not the diagnostic product (68 bp) for the target SNP was detectably amplified in only the susceptible (S) reaction, only the resistance (R) reaction, or in both the S and R reactions. Detectable amplification of the 68 bp diagnostic product in only the S reaction indicates a susceptible homozygote genotype. A 68 bp product in only the R reaction indicates a sodium channel mutation resistant homozygote and a 68 bp product in both the S and R reactions indicates a heterozygous genotype (Guerrero et al., 2001).

Alleles were considered as R for resistant and S for susceptible. Based on this nomenclature, genotypes were RR for homozygous resistant, RS heterozygous and SS homozygous susceptible. Allele amplification by PCR was interpreted as the presence or absence of either or both alleles. Todate only one sodium clannel gene of *R. (B.) microplus* has been reported by He et al. (1999). For that reason the frequency of the R allele was determined as follows: percentage of the muted sodium channel allele (R) in the total number of alleles assayed (assuming two alleles per individual).

Table 2.1. Details of primers used in the AS-PCR-68 bp to detect target SNP in the sodium channel gene of *Rhipicephalus* (*Boophilus*) *microplus*.

Primer ID	Primer Description	Primer sequence 5' → 3'
FG221*	Forward AS-PCR-68 bp primer (wild type-specific sense). Susceptible	TTA-TCT-TCG-GCT-CCT-TCT
FG222*	Forward AS-PCR-68 bp primer (mutant-specific sense resistant). Resistant	TTA-TCT-TCG-GCT-CCT-TCA
FG227*	Antisense AS-PCR-68 bp primer (non-specific-antisense).	TTG-TTC-ATT-GAA-ATT-GTC-GA

* Guerrero et al. (2001)

2.7. Probit Analysis

Probit analysis was performed on dose-response bioassay results using *Polo-Plus* (LeOra Software, 2003). A probit is the number of standard deviations from the mean (of assumed normal distribution of individual lethal concentration/susceptibilities) plus 5; 5 was added to allow toxicologists to avoid having to deal with negative numbers (Finney, 1971). A $\log_{10}$ probit slope is the number of geometric standard deviation of the log-normal distribution of susceptibilities per 1 $\log_{10}$ change in dosage. The confidence limits indicate a range within which the probit line of the true response percentage is almost certain to lie; the most common used for statistical purpose is the CL 95 %. The usual way to compare lethal doses or other point estimates is to examine their CL 95 % confidence limits. If the limits overlap, then the lethal doses do not differ significantly except under unusual circumstances. However, the error rate for this procedure is only approximately 5 % (Finney, 1971; LeOra Software. 2004).

The analysis used in this study included probit transformation of percentage mortality and natural logarithm transformation of dose to establish the lethal concentration at 50 % and 99 % value (LC_{50}, LC_{99}) and the respective 95 % confidence limits (95 % CL). Resistance factors (RF) were calculated by taking into account the LC_{50} of the tested populations divided by the LC_{50} of a susceptible reference strain (Media Joya-CENAPA strain; data provided by the National Centre of Parasitology-SENASICA-SAGARPA in Mexico). This procedure provided an estimate of the relative level of phenotypic resistance in each tick population. Although the use of LC_{50} has provided estimates of relative levels of resistance in insect populations (Kwon et al., 2004; Song et al., 2007), Cameron et al. (1995) and Jonsson et al. (2007) recommended the use of other LCs (LC_{90}, LC_{95} or LC_{99}) to determine a global comparative picture of the level of resistance. In this study additional RF was judged by LC_{99}, dividing the LC_{99} of the tested population by the LC_{99} of the reference strain. In the present study, we added a new alternative to analyze RFs by using RFs judged by both LCs (LC_{50} + LC_{99}). Populations were considered susceptible when both RF values (judged by LC_{50} and LC_{99}) were < 3.0 and resistant populations when RF values were ≥ 5.0. Tolerant populations were considered when one or both RF values were 3-5.

CHAPTER 3

Modification and validation of the Allele-Specific Polymerase Chain Reaction to detect the presence a target SNP associated with pyrethroid resistance in *Rhipicephalus* (*Boophilus*) *microplus* using Pyrosequencing™.

3.1. Introduction

The voltage-gated sodium channel is the primary target of pyrethroid insecticides (Soderlund and Knipple, 2003) and its insensitivity is known to be associated with pyrethroid resistance in several *kdr* insect species, including ticks (Dong, 2007). He et al. (1999a) investigated the molecular mechanism of resistance to pyrethroids in *R. (B.) microplus* and obtained and sequenced a partial *para*-homologous sodium channel gene from susceptible and pyrethroid-resistant tick strains. A point mutation that results in an amino acid change Phe → Ile was identified in a highly conserved domain III segment 6 (IIIS6) of the homologous sodium channel gene from ticks that were highly resistant to pyrethroids. Based on this finding, Guerrero et al. (2001) developed an allele specific polymerase chain reaction (AS-PCR-68 bp) to detect a mutation in the sodium channel gene of *R. (B.) microplus*. However, limitations of this assay are the poor visualization of the small size 68 bp PCR product, and the lack of genotyped controls. Thus, the development of a new AS-PCR with longer PCR product or a high throughput quantitative method of detecting pyrethroid resistance *R. (B.) microplus* is needed.

Pyrosequencing™ is a non-electrophoretic realtime DNA sequencing technology. It is a novel method of nucleic acid sequencing by synthesis that is based on the detection of released pyrophosphate (PPi) during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides (Ronaghi et al., 1998). The method is optimal for sequencing of short sequences (up to 100 bases of a DNA) rapidly and in a semiautomated format (Ronaghi, 2001; Gharizadeh et al., 2002). Several reports have been published addressing different applications of Pyrosequencing™ technique for mutation analyses. These include SNP discovery, genotyping, allele frequency in pooled samples (Gruber et al., 2002), methylation analyses (Uhlmann et al., 2002), molecular haplotyping (Ahmadian et al., 2000), and identification of short DNA sequences in bacterias, virus and parasites (Gharizadeh et al., 2003; Hodgkinson et al., 2008).

3.2. Experimental Aims and Methods

3.2.1. Aim of the Study

The specific aims of the work presented in this section were: a) to modify the AS-PCR and develop a novel Pyrosequencing™ method to detect the presence of a target SNP associated with pyrethroid resistance in *R.* (*B.*) *microplus*, and b) to validate the ability of the AS-PCR to genotype individual larvae using Pyrosequencing™ method as a gold standard.

3.2.2. Tick Populations

Nineteen different field populations of *R.* (*B.*) *microplus* were collected in Yucatan, México (**Figure 3.1., see section 2.3.**). Different acaricide treatments (OPs, amitraz and SPs) had been regularly used on farms where ticks were collected. Tick collection on each farm and production of *R.* (*B.*) *microplus* larvae were carried out as described in **section 2.4.**

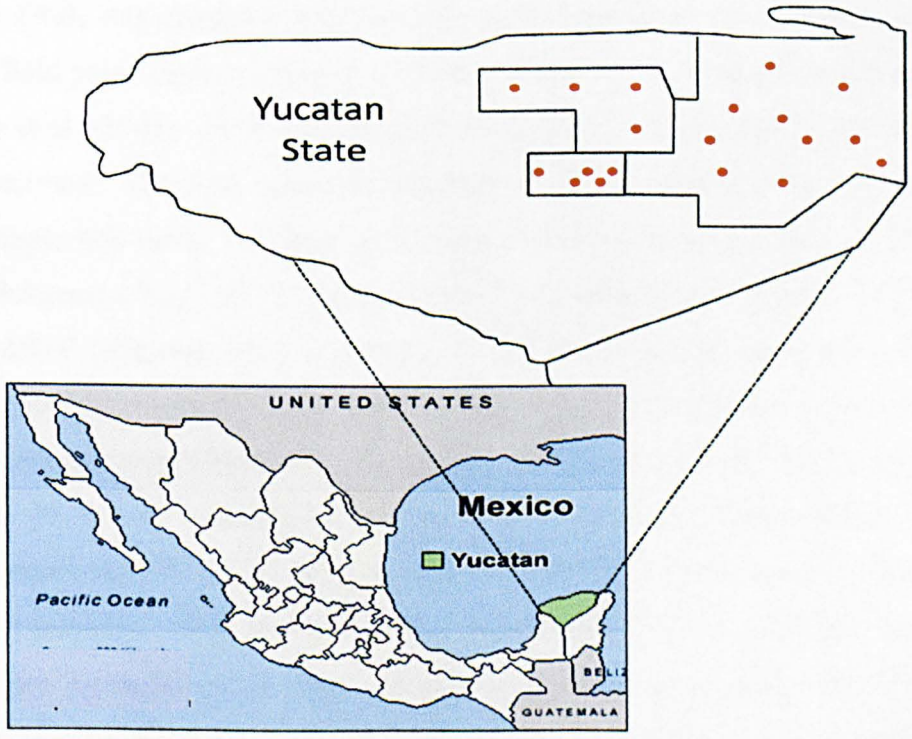

Figure 3.1. Map showing the locality of 19 farms where tick populations of *Rhipicephalus* (*Boophilus*) *microplus* were obtained for this study.

3.2.3. Amplification of Genomic DNA by Polymerase Chain Reaction, Cloning and Sequencing of a Partial Sodium Channel Gene of Rhipicephalus (Boophilus) microplus

a). Amplification of Genomic DNA by Polymerase Chain Reaction

Following a search of the NCBI database (http://www.ncbi.nlm.nih.gov/) no genomic DNA (gDNA) sequence information for the sodium channel of *R.* (*B.*) *microplus* was found, only complementary DNA (cDNA) (GenBank accession #: AF134216). In order to develop a new AS-PCR and a Pyrosequencing method it was first necessary to amplify the target gene sequence from a gDNA template. To facilitate efficient design of primers for AS-PCR and Pyrosequencing™, cloning of this DNA was performed. We used two primers, forward 5'-GCAACATTCAAAGGCTGGA-3' (NB-F) and reverse 5'-TTGTTCATTGAAATTGTCGA-3' (FG-R227, Guerrero et al., 2001) to amplify a region around the SNP associated with pyrethroid resistance, of the sodium channel gene of *R.* (*B.*) *microplus* (**Figure 3.2., see Appendix IV**).

Genomic DNA was extracted from six individual larvae of *R.* (*B.*) *microplus* belonging to three field populations in Yucatan, Mexico (**Table 3.1.**), as previously described by Guerrero et al. (2001). PCR was performed in thin-walled microcentrifuge tubes using 50 µl reactions. The final optimised reaction conditions used 2 µl of gDNA solution from a single tick larva, 1 X high performance buffer (ABgene, Surrey, UK), 1.5 mM $MgCl_2$ (ABgene, UK), 1.25 U Thermo-Start DNA polymerase (ABgene, UK), 200 µM of each dNTP (ABgene, UK), 2 µM NB-F (SIGMA, Genosys, Haverhill, UK), 2 µM FG-227 (SIGMA Genosys, UK) and distilled water. Amplification was carried out using a thermocycler (Biometric, Göttingen, Germany) and the following protocol: Initiation: 95 °C, 15 minutes, 40 cycles each consisting of denaturation: 95 °C, 15 seconds, annealing: 57 °C, 30 seconds, and extension: 72 °C, 1 minute. Final extension: 72 °C, 10 minutes. PCR products were separated on 2.0 % Eurogentec agarose with Hyperladder II (Bioline, UK). DNA visualization was achieved using SYBR®safe DNA gel stain (Invitrogen, UK), Syngene Safe Imager™ (Invitrogen, UK) with the Gene Snap Syngene Program (Cambridge, UK). The expected size of the PCR product based

on the published cDNA (He et al., 1999a) sequence was 186 bp; however, two sizes of products around 1000 pb were produced from gDNA.

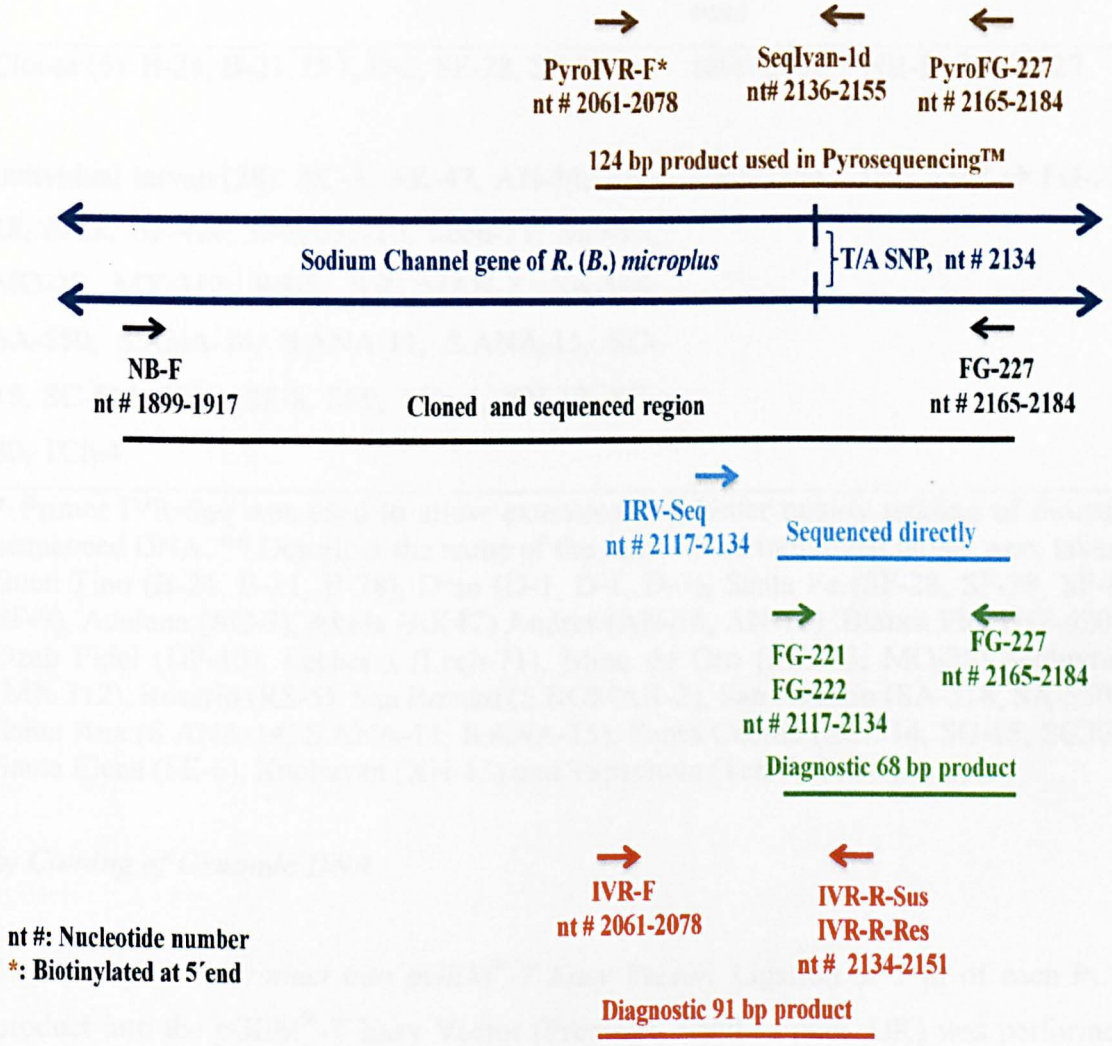

Figure 3.2. Diagrammatic representation of the *Rhipicephalus* (*Boophilus*) *microplus* partial sodium channel gene and locations of oligonucleotide primers used to amplify, clone and sequence a conserved region around the SNP target (T → A) detected by He et al. (1999a). Primers depicted in brown (PyroIRV-F, PyroFG-227) were used to amplify a region for Pyrosequencing™ (primer SeqIvan-1d was used as sequencing primer in pyrosequencing reaction), primers in black (NB-F, FG-227) were used to amplify, clone and sequence a conserved region of gDNA, primer in light-blue (IVR-Seq) was used as sequencing primer to allow extension of directly sequenced gDNA, primers (FG-221, FG-222, FG-227) in green were used for AS-PCR-68 bp developed by Guerrero et al. (2001), and primers in red (IRV-F, IRV-R-Sus, IRV-R-Res) were used to develop an AS-PCR-91 bp.

Table 3.1. Tick populations selected to sequence a region of the sodium channel gene of 34 *Rhipicephalus* (*Boophilus*) *microplus* larvae using gene specific primers NB-F, IVR-Seq and FG-227.

Tick larvae selected for sequencing**	Nucleotide read	PCR primer used
Clones (6): B-24, B-21, D-1, D-2, SF-28, SF-29	1899-2184	NB-F → FG-227
Individual larvae (28): AC-3, AK-47, AN-14, AN-18, B-28, BF-420, D-3, DF-10, Lech-71, MO-13, MO-25, MX-312, RS-5, S.ROMAN-2, SA-518, SA-550, S.ANA-14, S.ANA-11, S.ANA-15, SC-15, SC-514, SE-6, SF-8, SF9, TCh-5, XN-13, SC-30, TCh-4	2066-2184	IRV-Seq* → FG-227

* Primer IVR-Seq was used to allow extension and better quality reading of directly sequenced DNA. ** Describes the name of the farm where individual larvae were taken: Buen Tino (B-24, B-21, B-28), Dino (D-1, D-1, D-3), Santa Fe (SF-28, SF-29, SF-8, SF-9), Aculena (AC-3), Akula (AK47) Andres (AN-14, AN-18), Blanca Flor (BF-420), Dzab Fidel (DF-10), Lecheria (Lech-71), Mina de Oro (MO-13, MO-25) Moluxtun (MX-312), Rosario (RS-5), San Roman (S.ROMAN-2), San Agustin (SA-518, SA-550), Santa Ana (S.ANA-14, S.ANA-11, S.ANA-15), Santa Cecilia (SC-514, SC-15, SC30), Santa Elena (SE-6), Xnohayan (XN-13) and Tapachula (Tch-5, Tch-4).

b) Cloning of Genomic DNA

Ligation of PCR Product into pGEM®-T Easy Vector. Ligation of 3 µl of each PCR product into the pGEM®-T Easy Vector (Promega, Southampton, UK) was performed as per the manufacturer's protocol. Briefly, reactions were prepared with 2 X rapid ligation buffer, 50 ng of pGEM®-T Easy Vector and 3 U T4 DNA ligase (Promega, UK) and incubated overnight at 4 °C.

Transformation of Plasmids into JM109 Competent Escherichia coli cells. Ligation reactions were transformed into JM109 competent *E. coli* cells with 10^8 colony forming units/µg (Promega, UK). Briefly, cells were thawed on ice and mixed. Reactions were made up in 1.7 ml eppendorf tube. Two µl of each ligation reaction was added to 50 µl of cells, mixed gently and incubated on ice for 20 minutes. The cells were heat shocked

at 42 °C for 45 seconds and immediately returned to ice for two minutes. 950 µl of LB medium was added to the reaction, which was then incubated for one hour at 37 °C. To allow the detection of colonies containing the recombinant plasmid, plates were prepared with LB agar, 100 µg/ml ampicillin (Sigma-Aldrich, UK), 500 µM isopropyl β-D-1-thiogalactopyranoside, (IPTG, Invitrogen, Paisley, UK) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, Bioline, London UK), dissolved in dimethylformamide (DMF, Fisher Scientific, Loughborough UK) to a final concentration of 80 µg/ml. These plates allowed colorimetric detection of recombinant colonies (white) from non-recombinant colonies (blue). 100 µl of the transformation reaction was spread aseptically on a plate and left to dry for 10 minutes at room temperature before being incubated overnight at 37 °C. Plates were stored at 4 °C.

Small Scale Culture and Purification of Plasmid DNA. For each PCR product, four recombinant colonies were removed from the plate and inoculated into 2 ml of LB medium containing 100 µg/ml ampicillin. Cultures were incubated at 37 °C overnight in a shaking incubator (Townson and Mercer, Manchester, UK) at 210 rpm. One ml of each culture was placed in a 1.7 ml eppendorf tube and centrifuged at 13,000 rpm for 2 minutes followed by removal of the supernatant and resuspension of the pellet. 300 µl of TENS (1 x TE, 0.5 % SDS, 0.1 M NaOH) was added to the reaction followed by vortexing to lyse the cells. DNA was then isolated by: addition of 150 µl of 3 M sodium acetate pH 5.4, vortexing briefly and then adding 200 µl of phenol:chlorofom:isoamyl alcohol mixture (25:24:1, Sigma-Aldrich, UK), with vigorous shaking and centrifugation at 10,000 rpm for 3 minutes. The upper aqueous layer was transferred to a fresh eppendorf and DNA was precipitated by adding 900 µl of 100 % molecular grade ethanol and storing the reaction at -80 °C for a minimum of 10 minutes followed by centrifugation at 4 °C, 13,000 rpm for 15 min. The pellet was washed by adding 200 µl of 70 % ethanol and centrifugation at 10,000 rpm for 10 min. The supernatant was removed and the pellet was left to air dry for approximately 10 minutes. Samples were resuspended in 30 µl of TE/RNase (100 µg/ml, Qiagen, West Sussex, UK).

Digestion of Recombinant Plasmids with EcoR1 to Confirm the Presence of the Insert. Three µl of each plasmid sample was mixed with 1 X Buffer H (Promega, UK) and 12

U of EcoR1 (Promega, UK) made up to a 10 µl volume using dd H_2O in a 0.6 ml eppendorf. After incubation at 37 °C for 1 hour to allow digestion, the reaction was stopped by adding 2 µl of 10 X orange loading dye and fractionating on 1.0 % Eurogentec agarose with Hyperladder II (Bioline, UK). DNA visualization was achieved using SYBR®safe DNA gel stain (Invitrogen, UK) and Syngene Safe Imager™ (Invitrogen, UK) with the Gene Snap Syngene Program (Cambridge, UK).

Large Scale Culture and Purification of DNA. Large scale culture and purification were carried out using 500 µl of the remaining small scale DNA culture to inoculate 25 ml of LB medium with 100 µg/ml ampicillin. Cultures were incubated at 37 °C overnight in a shaking incubator at 210 rpm. Plasmid purifications were carried out using HiSpeed® Plasmid Midi Kit (Qiagen, UK) according to the manufacturer's protocols.

c) Sequencing of Genomic DNA

Two clones (B-24 and B-21) with different size products were sent to GATC-Biotech (Konstanz, Germany) to be sequenced in the forward and reverse directions. Sequences were edited and a consensus sequence for each clone was created using BioEdit (v7.0.8) Create Consensus Sequence (Hall, 2007). To verify gDNA sequence results manual inspection of the chromatogram was carried out. The sequence of the two clones and the cDNA reported in GenBank were aligned and a primer (IRV-Seq) was selected to use in a further sequencing reaction (**see Figure 3.2.**).

To allow amplification of the target region from multiple different isolates and facilitate the design of conserved primers for Pyrosequencing™, an additional 28 individual larvae from 19 field populations of ticks from Yucatan, Mexico were used for gDNA isolation/extraction **(Table 3.1.)**. DNAs were extracted, PCR amplified (using gene specific primers: NB-F and FG-R227, **see Figure 3.2.**) and purified (QIA-quick PCR Purification Kit, Quiagen, UK). The 28 individual larvae and four remaining clones (D-1, D-2, SF-28 and SF-29, **see Table 3.1.**) were sequenced directly only in the forward direction by GATC-Biotech. On this occasion the IRV-Seq reading primer was used for sequencing to allow extension and better quality reading. In each PCR product a

sequence of 76 nucleotides was obtained and aligned together with the sodium channel cDNA and the two gDNA clones (B-24, B-21) in BioEdit software.

3.2.4. Design of a New Allele-Specific Polymerase Chain Reaction to Detect the Presence of a Target SNP in the Sodium Channel Gene of Rhipicephalus (Boophilus) microplus

Based on the gDNA sequences of the two (B-24, B-21) clones and comparing with published cDNA sequence, the point mutation at nucleotide # 2,134 of the tick sodium channel sequence reported by He et al. (1999a) is situated close to an intron/exon boundary. To develop a new AS-PCR method to genotype pyrethroid resistant populations of *R. (B.) microplus* with a longer PCR product, only 91 bp could be amplified hence this AS-PCR was designated as AS-PCR-91 bp (**see Appendix V**). Reaction products were separated on 3.0 % Eurogentec agarose with Hyperladder II (Bioline, UK). DNA visualization was achieved using SYBR®safe DNA gel stain (Invitrogen, UK), Syngene Safe Imager™ (Invitrogen, UK) with the Gene Snap Syngene Program (Cambridge, UK). The gDNA from the 34 individual larvae were tested by both AS-PCR-68 bp (Guerrero et al., 2001) and AS-PCR-91 bp (**See Apendix III**).

3.2.5. Design of a novel Pyrosequencing™ Technique to Detect the Presence of a Target SNP in the sodium channel gene of Rhipicephalus (Boophilus) microplus

a) PCR Conditions for Pyrosequencing

A PCR assay was designed using PSQ™ 96MA assay design software (Biotage AB, Sweden). The assay was designed based on cDNA (He et al., 1999a) and 34 gDNA sequences isolated in this study. The assay used two PCR primers to amplify 124 bp including the SNP of interest (**Figure 3.2.**). PCR was performed in thin-walled microcentrifuge tubes using 50 µl reactions. The final optimised reaction condition used 1 µl of gDNA solution from a single tick larva, 1 X high performance buffer (ABgene, Surrey, UK), 1.5 mM $MgCl_2$ (ABgene, UK), 1.25 U Thermo-Start DNA polymerase (ABgene, UK), 200 µM of each dNTP (ABgene, UK), 2 µM of biotinylated forward primer: 5′-BioGGACCAACCGGAATACGA-3′ (PyroIRV-F, SIGMA,

Genosys, Haverhill, UK), 2 μM of non-biotinylated reverse primer: 5′-TTGTTCATTGAAATTGTCGA-3′ (PyroFG-227, SIGMA Genosys, UK) and milliQ water (**see Appendix VI**). Amplification was carried out using a thermocycler (Biometric, Göttingen, Germany) and the following protocol: Initiation: 95°C, 15 minutes, 40 cycles each consisting of denaturation: 95°C, 15 seconds, annealing: 58°C, 30 seconds, and extension: 72°C, 1 minute; final extension: 72°C, 7 minutes. Reaction products were separated on 2.0 % Eurogentec agarose with Hyperladder II (Bioline, UK). DNA visualization was achieved as described in **section 3.2.4**.

b) Pyrosequencing™ Method

Pyrosequencing™ was conducted according to the manufacturer's protocols (Biotage, AB, Sweden) using a PSQ™ HS-96A instrument (**see Appendix VII**). Briefly, for each reaction, 30 μl of biotinylated PCR product was prepared for sequencing by immobilizing with 3 μl of Streptavidin Sepharose™ high performance beads (Amersham Biosciences, Sweden) in an 80 μl reaction volume including 1 X binding buffer for 5 min at room temperature. Denaturation and washing were performed using the Vacuum Prep Tool and solutions according to the manufacturer's protocol (Biotage AB, Sweden). Pyrosequencing™ was carried out on the PSQ 96MA automated 96-well Pyrosequencer (according to the manufacturer's protocol) using the genotyping mode and standard factory parameters, the PSQ SNP 96 Reagents Kit (Biotage AB, Sweden). We used a degenerate sequencing primer (SeqIvan-1d) derived from the nucleotide sequence results of the 34 sequenced individual larvae (5′-CGATGAA/TTAGATTCAAGGTG-3′) (**see Appendix VIII**). This is a reverse assay (**Figure 3.3.**). This primer is used as a starting point for the pyrosequencing reaction. The nucleotide dispensation order was carried out manually (CAGCTAGAGAGTCGAGA), adding three nucleotide dispensations that should not generate specific signal, in order to detect possible background noise and to provide an internal quality-control measure (control nucleotides are depicted in red). Initially, a series of reactions using template, sequencing primer and biotinylated primer were used to confirm that each had a negligible effect on baseline signal. Negative controls (without DNA template) were run in all PCRs and were then subjected to pyrosequencing. Genotypes were accepted when the negative control failed and the

individual larva passed the PSQ quality control criteria. Pyrosequencing™ was used to genotype the 34 individual larvae for the T → A polymorphism at the sodium channel gene of *R.* (*B.*) *microplus*.

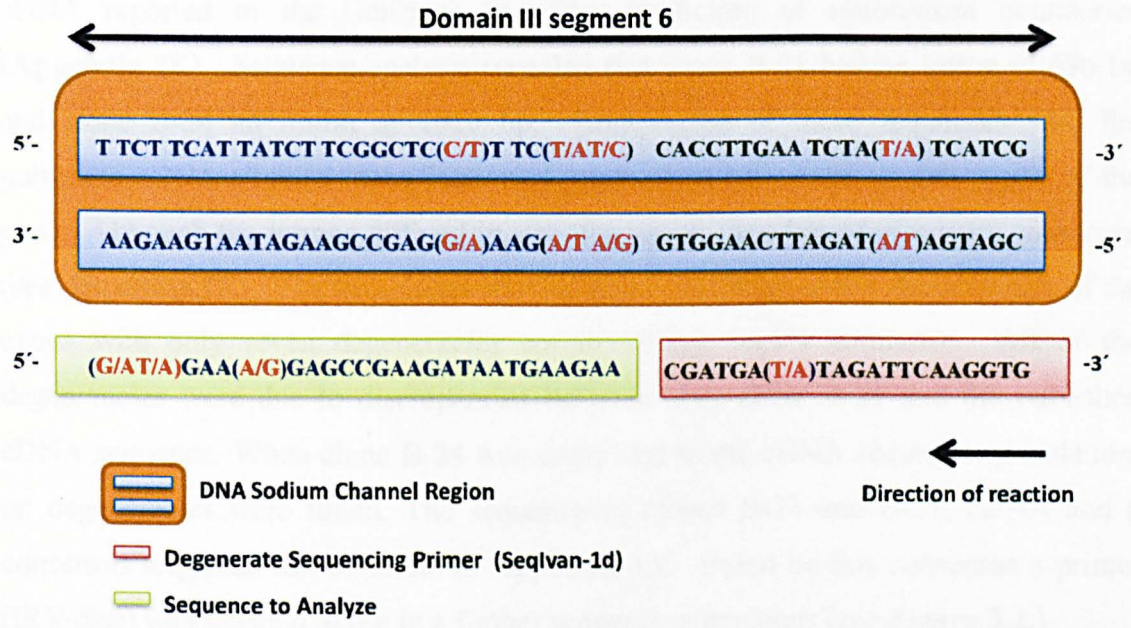

Figure 3.3. Nucleotide sequence for a region of the sodium channel gene of *Rhipicephalus* (*Boophilus*) *microplus*, sequencing primer (SeqIvan-1d) and sequence to analyze in Pyrosequencing™ methodology to detect a SNP associated with pyrethroid resistance are shown.

3.2.6. Statistical Analysis

Comparisons of the frequency of the R allele between Pyrosequencing™, AS-PCR-68 bp and AS-PCR-91 bp were made as a contingency table using chi-square tests.

3.3. Results

3.3.1. Amplification of gDNA by Polymerase Chain Reaction, Cloning and Sequencing of a Partial Sodium Channel Gene of Rhipicephalus (Boophilus) microplus

To amplify the region of the sodium channel gene of *R.* (*B.*) *microplus* containing the SNP thought bto be associated with pyrethroid resistance, primers NB-F and FG-227 were used (**see Figure 3.2.**). The expected size of this product based on the published

cDNA (He et al., 1999a) sequence was 186 bp; however, two different sized (~700-1000 bp) were produced from gDNA (**Figure 3.4.**). This was confirmed when clones B-24 and B-21 were sequenced in both directions and aligned (BioEdit, v7.0.8) with cDNA reported in the GenBank to allow prediction of intron/exon boundaries (**Appendix IX**). Sequence analysis revealed that clone B-21 had an intron of 696 bp and clone B-24 an intron of 1020 bp. Comparison of these sequences with the published cDNA allowed identification of intron-exon boundaries in this region of the gene. Although the introns differed in size, the intron-exon boundaries were conserved (**see Appendix IX**). The two clones showed good homology within the sequence of the exons with only seven degeneracies out of 180bp: 96.1% similarity. All of the degeneracies were due to discrepancies between only clone B-21 and the published cDNA sequence. When clone B-24 was compared to the cDNA sequence in isolation, no degeneracies were found. The sequence of clones B-24 and B-21, cDNA and a consensus sequence can be found in **Appendix IX**. Based on this consensus a primer (IRV-Seq) was selected to use in a further sequencing reactions (**see Figure 3.2.**).

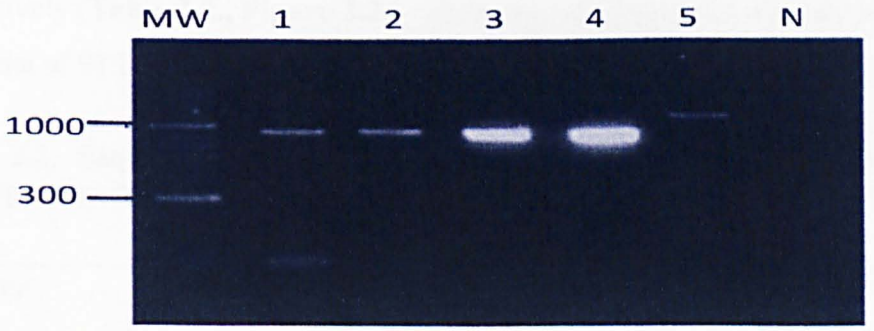

Figure 3.4. Picture of agarose gel electrophoresis showing five PCR products using NB-F and FG-227 primers (see Figure 3.2.). Lane MW is a molecular weight hyperladder, lanes 1-5 are individual larval samples and N is a negative control.

A further 28 PCR products were amplified from individual larvae and purified. The remaining four clones and the 28 purified PCR products were sequenced directly in the

forward direction using the IRV-Seq primer (**see Figure 3.2.**). From each PCR product a sequence of 76 bp was obtained. Sequences of all individual larvae (34) in the coding region surrounding the SNP of interest were aligned together with the cDNA sodium channel (**Figures 3.5., 3.6.**) using BioEdit (v7.0.8). Based on these sequences, six SNPs were identified, two at DIIIS6 codon # 1550 (Phe → Ile previously reported by He et al., 1999a, and Phe→Ser), one at DIIIS6 codon # 1555 (Phe → Ile), one at DIVS1 codon 1566 (Gln→Ser) and two silent SNPs (**Figure 3.6.**).

3.3.2. Allele-Specific Polymerase Chain Reaction (AS-PCR- 68 bp and ASPCR-91 bp) to Detect a Target SNP in the sodium channel gene of Rhipicephalus (Boophilus) microplus

Once the location of the intron sequence was clear it was possible to identify the nucleotide identity on the sense strand, the AS-PCR-91 bp assay was developed. Primer IRV-F was designed to anneal to both the wild type (susceptible) and the resistant-.,specific alleles upstream of the nucleotide substitution site, and primers IRV-R-Sus and IRV-R-Res were designed as diagnostic primers to detect the identity of variant nucleotide by hybridizing to the antisense strand of the wild type and resistant alleles, respectively (**Table 3.2., Figure 3.2.**). With this set of primers we were able to amplify a product of 91 bp.

Table 3.2. Sequences of PCR primers used to develop an AS-PCR-91 bp to genotype pyrethroid resistant populations of *Rhipicephalus* (*Boophilus*) *microplus*.

Primer ID	Sequence	Description	Annealing site
IRV-F	5'-GGACCAACCGGAATACGA-3'	Upstream non specific	nt # 2061-2078
IRV-R-Sus	5'-GAATAGATTCAAGGTGAA-3'	Wild type-specific antisense	nt # 2134-2151
IRV-R-Res	5'-GAATAGATTCAAGGTGAT-3'	Resistant-specific antisense	nt # 2134-2151

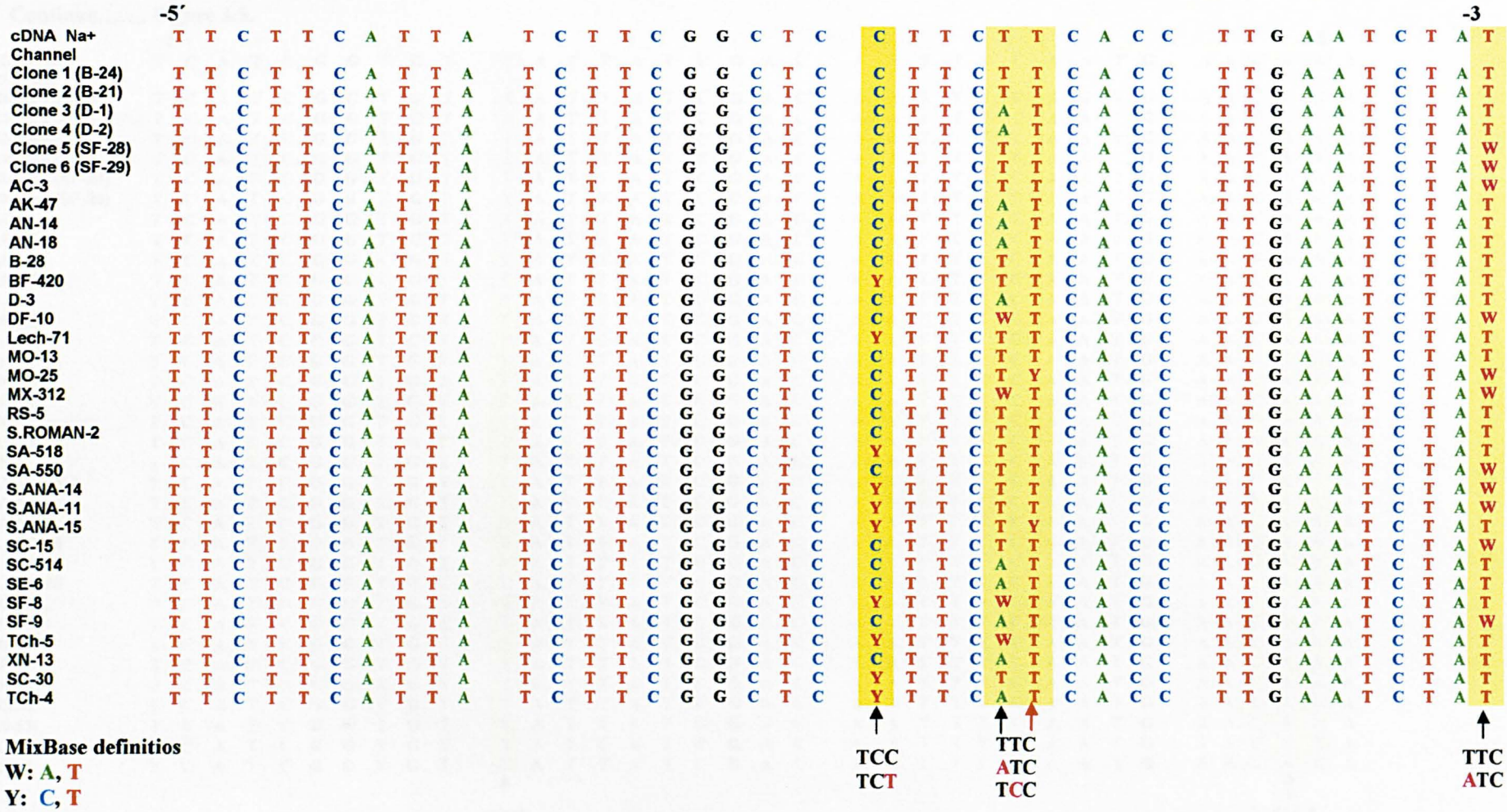

Figure 3.5. Partial DNA sequences of the sodium channel gene of 34 *Rhipicephalus* (*Boophilus*) *microplus* larvae to detect SNPs associated with pyrethroid resistance (see Table 3.1.). The first sequence is the partial sodium channel cDNA found in GenBank (accession # AF134216) (He et al., 1999a). Highlighted rows show the identified mutations, arrows show possible changes in nucleotide sequences (red arrow shows the known mutation).

Continue……. Figure 3.5.

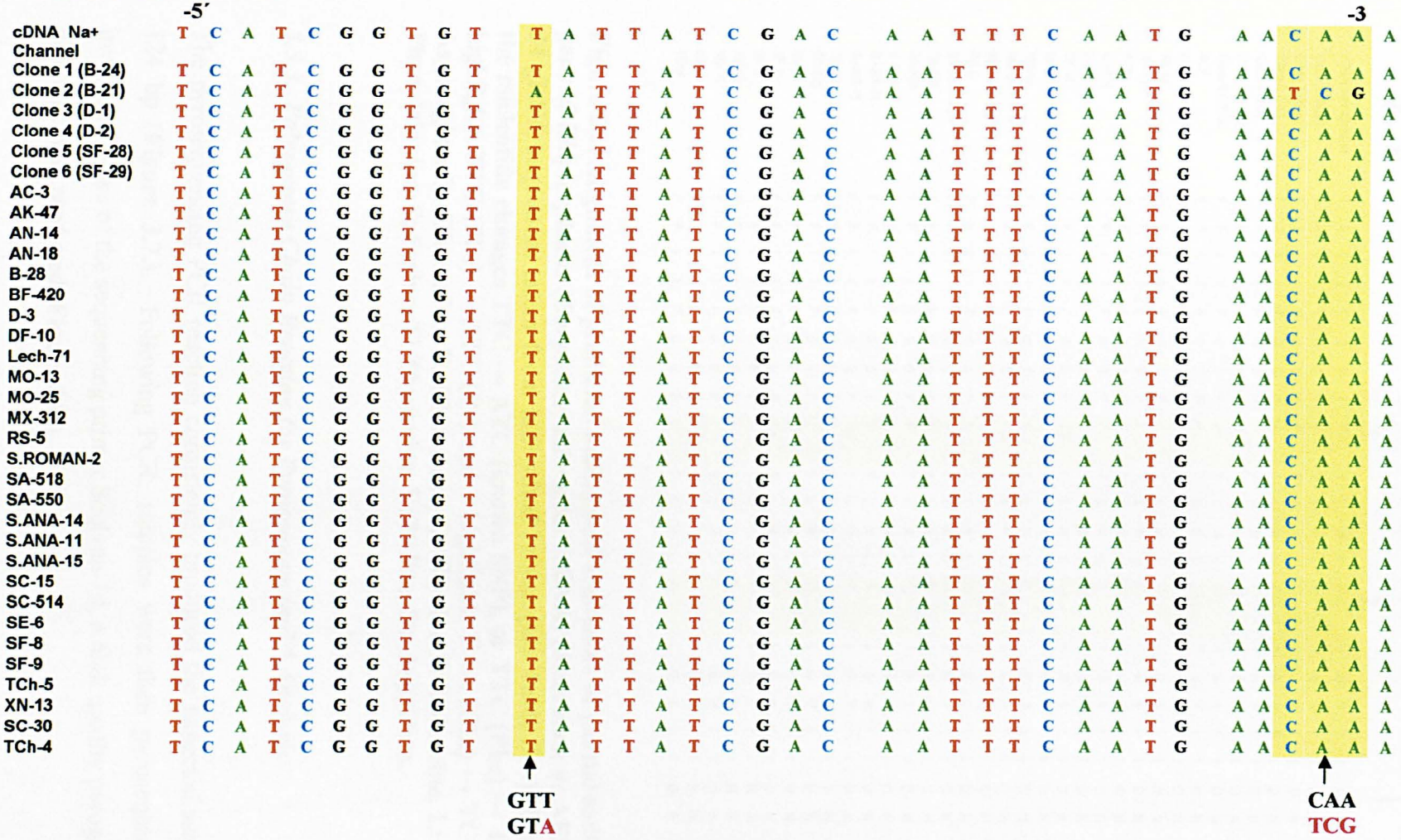

Figure 3.5. Partial DNA sequences of the sodium channel gene of 34 *Rhipicephalus* (*Boophilus*) *microplus* larvae to detect SNPs associated with pyrethroid resistance (see table 3.2.). The first sequence is the partial sodium channel cDNA found in GenBank (accession # AF134216) (He et al., 1999a). Highlighted rows show the identified mutations, arrows show possible changes in nucleotide sequences (red arrow shows the known mutation).

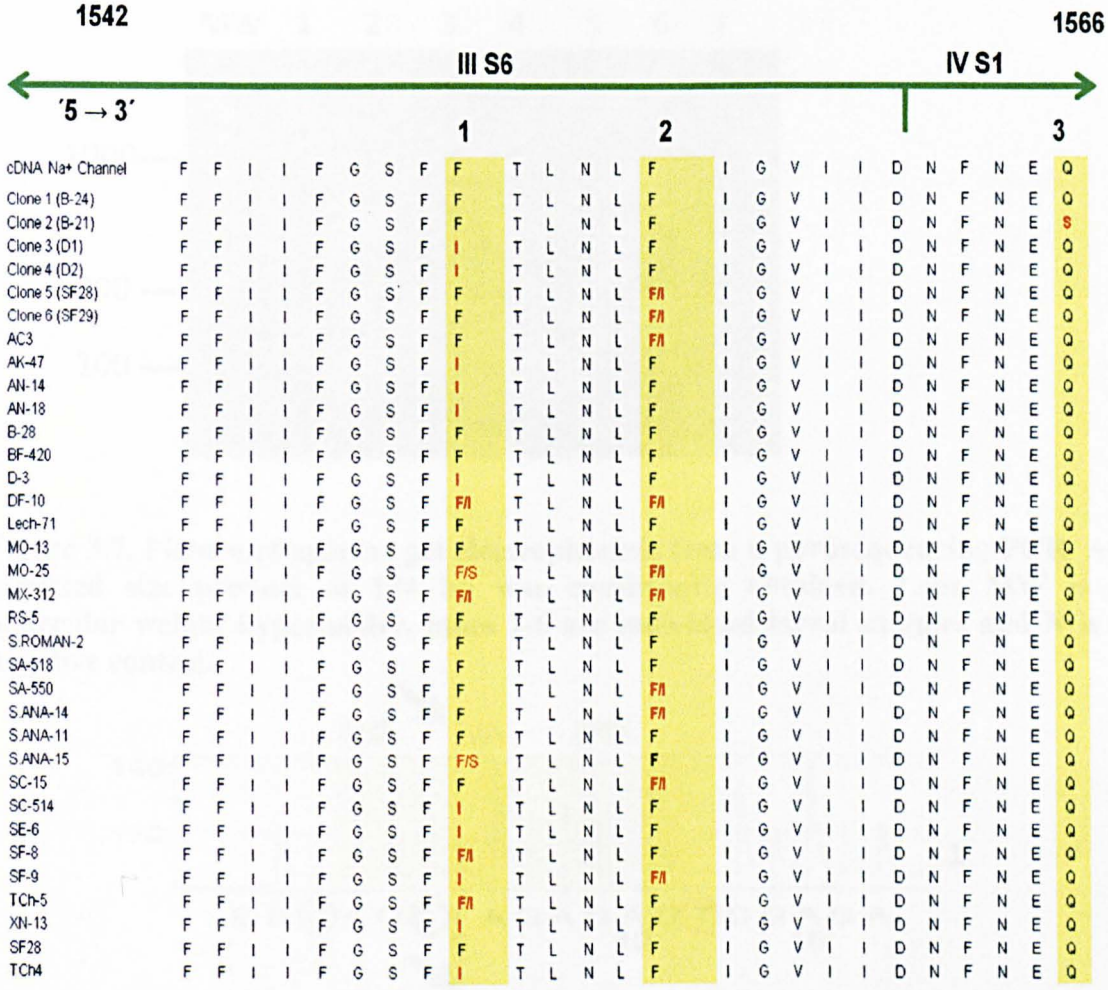

Figure 3.6. Alignment of predicted amino acid sequences of partial sodium channel gene of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA) (Accession #: AF134216) and 34 gDNA sequences taken from individual larvae (see Table 3.1.). One highlights the nucleotide changes TTC → ATC (known SNP), or TTC (Phe) → TCC (Ser), 2 highlights TTC (Phe) → ATC (Ile) and 3 highlights CAA (Gln) → TCG (Ser). N: Asparagine, Q: Glutamine, E: Glutamate, G: Glycine, I: Isoleucine, L: Leucine, F: Phenylalanine, S: Serine, T: Threonine, V: Valine, D: Aspartate.

3.3.3. Polymerase Chain Reaction for Pyrosequencing™ Analysis

The pyrosequencing PCR reaction consistently produced the expected size product of 124 bp (**Figure 3.7.**). Following PCR, samples were then pyrosequenced, which involved the use of the sequencing primer SeqIvan-1d, a high quality pyrogram for each genotype, was produced (**Figure 3.8.**).

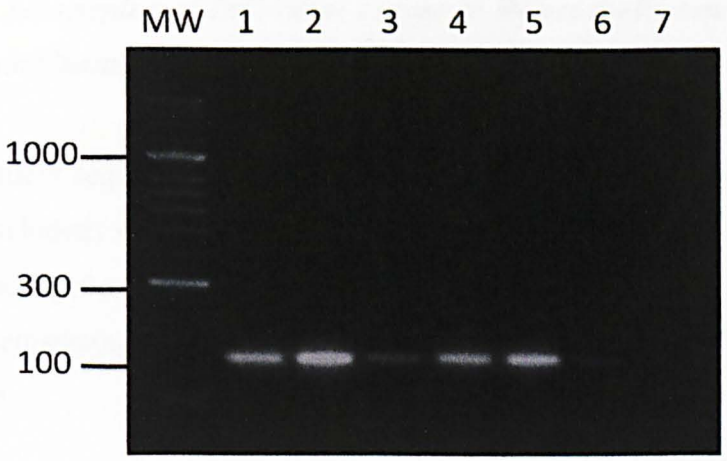

Figure 3.7. Picture of agarose gel electrophoresis from a pyrosequencing PCR. An expected size product of 124 bp was consistently obtained. Lane MW is a molecular weight hyperladder, lanes 1-6 are individual larval samples and N is a negative control.

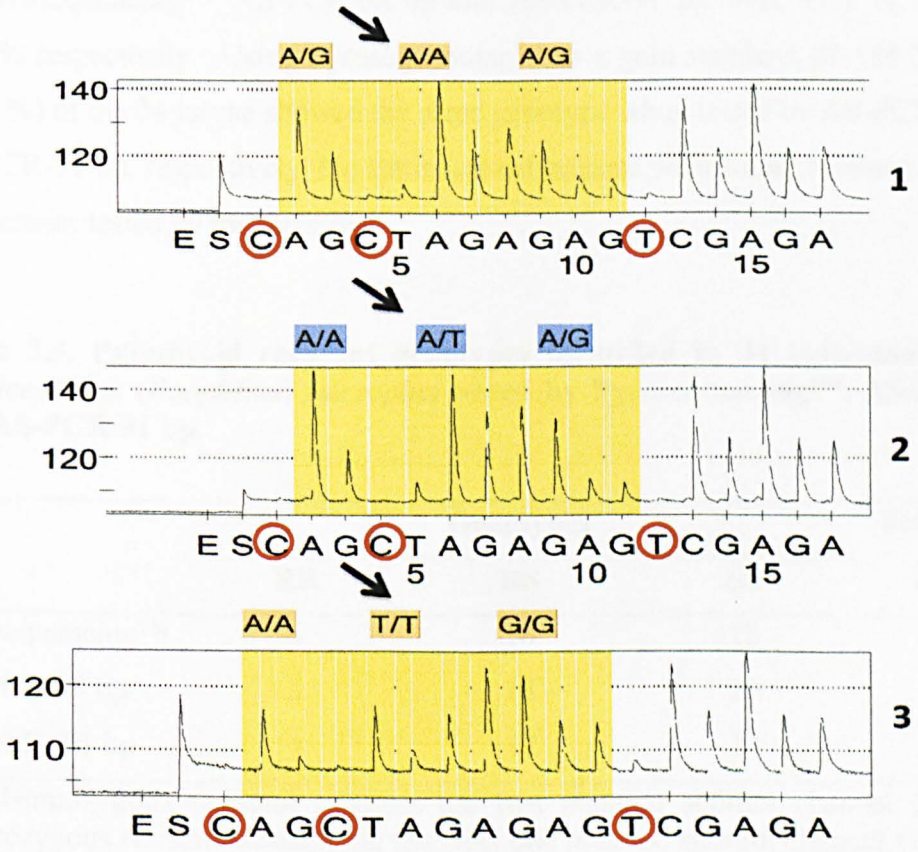

Figure 3.8. An example of a pyrogram for each of the genotypes: 1) arrow shows A/A = homozygous resistant, no T peak and double height A peak, 2) = arrow shows A/T = heterozygous, one A peak and one T peak, 3) arrow shows T/T = homozygous susceptible, no A peak and double height T peak. Red circles depict control nucleotides (no peaks were registered).

3.3.4. Genotyping of Individual Larvae to Detect thePresence of a Target SNP in the Sodium Channel Gene of Rhipicephalus (Boophilus) microplus

The direct sequence data generated from the 28 individual larvae (the six clones were not included) was used to infer genotype at the T → A polymorphism at the sodium channel gene of *R. (B.) microplus.* When the same ticks were genotyped by Pyrosequencing™, the results of the two methods concurred for 19 (67.8 %) of these larvae.

Genotypes of the 34 individual larvae (including the six larvae that were cloned) identified using Pyrosequencing™, AS-PCR-68 bp, and AS-PCR-91 bp are showed in **Table 3.3**. The frequency of the resistant allele of the 34 individual larvae determined by Pyrosequencing™, AS-PCR-68 bp and AS-PCR-91 bp were 41.1 %, 35.2 % and 36.7 % respectively. Using Pyrosequencing™ as a gold standard, 30 (88.2 %) and 31 (91.1 %) of the 34 larvae showed the same genotype when tested by AS-PCR-68 bp and AS-PCR-91 bp, respectively. No statistical differences were found between the R allele frequencies tested by the three tests.

Table 3.3. Pyrethroid resistant genotypes identified in 34 individual larvae of *Rhipicephalus* (*Boophilus*) *microplus* tested by Pyrosequencing™, AS-PCR-68 bp and AS-PCR-91 bp.

Test	Genotypes			Frequency of
	RR	RS	SS	R allele*
Pyrosequencing™	6	16	12	41.1 %a
AS-PCR-68 bp	3	18	13	35.2 %a
AS-PCR- 91 bp	4	17	13	36.7 %a

RR: Homozygous resistant (possess the two mutated sodium channel alleles), RS: Heterozygous resistant-susceptible (possess one mutated sodium channel allele and one wild-type allele). SS: Homozygous susceptible (possess the two wild-type alleles), R allele frequency: Percentage of mutated sodium channel allele (R) in the total number of alleles assayed (2 alleles per individual). *Figures with different letters are significantly different.

3.4. Discussion

In this study we sequenced for the first time gDNA around the target SNP (thought to be) associated with pyrethroid resistance in 34 individual larvae of *R.* (*B.*) *microplus* from 19 tick populations. To date only partial cDNA sequence of the sodium channel gene of *R.* (*B.*) *microplus* has been reported (He et al., 1999) using information generated from four *R.* (*B.*) *microplus* reference strains. The sequences of both cDNA and gDNA are essential to elucidate the molecular bases of pyrethroid resistance. Cecherine et al. (1996) mentioned that when a candidate gene approach is undertaken in order to elucidate the molecular bases of an inherited disorder, the determination of the intron-exon boundaries of a gene usually represents the first step toward the detection of point mutations. A number of different strategies can be used to determine the genomic structure of a gene, depending on several factors such as its genomic length, the availability of its cDNA sequence and the availability of information deriving from other species (Cecherine et al., 1996).

Comparison of the gDNA sequences of the two clones (B-24, B-21) with the published cDNA allowed us to identify the intron-exon boundaries in this region of the gene. Although the introns differed in size, the intron-exon boundaries were conserved. The two clones showed good homology within the sequence of the exons with 96.1 % similarity. However, it cannot be confirmed, based on the analysis carried out here, whether these two products represent the same gene. The question of how many copies of the sodium channel gene *R.* (*B.*) *microplus* has remains unanswered. Genetic and molecular studies have identified two different sodium channel genes in *Drosophila melanogaster*, *para* (paralytic) and DSC1 (*Drosophila* sodium channel). The first gene was identified on the basis of mutation causing a paralytic phenotype (Ramaswami and Tanouye, 1989; Linsay et al., 2008) and the importance of gene DSC1 to neuronal excitability is not known because mutations affecting its function have not been isolated (Hong and Ganetzky, 1994). The *An. gambiae* sodium channel was physically mapped to chromosome 2L, division 20C using *in situ* hybridization (Ranson et al., 2000). The authors concluded that this position corresponds to the location of a major quantitative trait locus determining resistance to permethrin in strains of *An. gambiae*. As the genome project for *R.* (*B.*) *microplus* is currently underway (Guerrero et al., 2005,

2006), it is hoped that gene discovery will determine the number of sodium channel genes that exist in *R. (B.) microplus.*

Standardized bioassays for determining the resistance level of a tick population, such as the larval packet test (Stone and Haydock, 1962), are valuable in that they offer a method for phenotyping a population in response to acaricides. However, bioassays often require large numbers of larvae and several weeks can elapse before results are available. The AS-PCR developed by Guerrero et al. (2001) can genotype pyrethroid resistant populations of *R. (B.) microplus* and provides information about the frequency of resistant or susceptible alleles in the tick population. However, until now this assay has not been applied to large numbers of tick populations. The AS-PCR developed by Guerrero et al. (2001) amplifies a product of only 68 bp. This size of product is difficult to resolve via agarose gel electrophoresis (Hawcroft, 1997). The assay can sometimes be subjective as to whether a band is actually there or not, and given that it was designed on cDNA sequence requires further validation for use with gDNA template. Based on the partial sequence of the sodium channel gene at the gDNA level of *R. (B.) microplus*, we showed that the SNP associated with pyrethroid resistance as identified by He et al. (1999a), is located close to an intron/exon boundary. With this information, we developed the AS-PCR-91 bp that amplifies a 91 bp product. This assay was able to genotype pyrethroid resistant larvae of *R. (B.) microplus*; however, the proximity of the intron did not allow the amplification of a larger product, hence it did not resolve the issue of visualizing the product by electrophoresis.

Though the majority of DNA sequencing techniques are gel-based and electrophoretic, there are high-throughput techniques that are more suitable for other applications than long sequence reads (Ronaghi, 2001; Gharizadeh et al., 2002). Pyrosequencing™ is a quantitative real-time sequencing reaction, well suited for de novo and short-readsequencing. Pyrosequencing™ has been demonstrated to allow sequencing of up to 100 bases (Gharizadeh et al., 2002). In this study, we investigated Pyrosequencing™ as a high throughput method for genotyping SNPs (T → A) at the sodium channel gene associated with pyrethroid resistant in *R. (B.) microplus*. Using Pyrosequencing™ as a gold standard, the results obtained by direct sequencing and Pyrosequencing™ agree in 67.8 % cases. Differences between the results of these methods were most likely to be

due to inaccurate determination of sequences derived from the direct sequencing of PCR products. This is because ambiguities arise during direct sequencing whenever the two alleles of each gene differ (Clark, 1990). Caution is needed when using direct sequences produced by automated sequencers. We noticed that many times the DNA sequencer would choose the stronger of two overlapping signals, rather than assign an undetermined "N" for any nucleotide. This process can result in an incorrect determination for that site and mis-determination of the genotype. A visual inspection of the interested site(s) on the chromatogram is necessary for proper assignment in such cases. This inspection is particularly important for the assignment of heterozygote individuals. Clark (2006) investigating the association of mutations at codon 167 and 200 of the beta-tubulin isotype 1 gene with benzimidazole resistance phenotype of mixed species populations of cyathostomins, found 74 % (at codon 167) and 95 % (at codon 200) concordance between direct sequencing and Pyrosequencing™. However, in the Clark study the beta-tubulin gene was sequenced in both directions, while in this study the partial sequence of the sodium channel gene was amplified in one direction only (forward strand). It is probable, that amplification of both strands will increase the concordance between both methods. Pyrosequencing™, however, was specially designed as a quantitative direct sequencing technology that produces a clear distinction between genotypes, to make genotyping accurate and straightforward and is therefore likely to produce more robust genotyping information (Lavebratt and Sengul, 2006).

The genotypes identified by Pyrosequencing™ showed good agreement with the AS-PCR-91 bp (91.1 %) and the AS-PCR-68 bp (88.2 %). Furthermore, no statistical differences were found in the ability of the three tests to determine the frequency of the R allele involved with pyrethroid resistance in *R.* (*B.*) *microplus* larvae. Pyrosequencing™ has a number of advantages over AS-PCR and other sequencing methods: it removes the need for labeled primers, labeled nucleotides and gel electrophoresis which were needed for previous DNA sequencing methods; due to the nature of addition of one nucleotide iteratively it is possible to insert control nucleotides which should not produce PPi and thus no peaks on the program. An automated microtiter-based pyrosequencer instrument has been produced by Biotage and this allows for high throughput of samples. It has been reported that Pyrosequencing™ has the ability to sequence up to 100 nucleotides (Ringquist et al., 2002). Furthermore,

Pyrosequencing™ is quantitative and can be used to estimate allele frequency in pooled samples; Gruber et al. (2002) and Lavebratt and Sengul (2006) found that the difference between true allele frequencies and those estimated from pools of DNA by Pyrosequencing™ has been shown to vary by 1.1–6.5 % and the correlation between true and estimated allele frequencies was good (r^2 = 0.92–0.99). Recently, real time PCR amplification of a specific allele has been developed to detect resistance in a pooled sample of 30 *An. sinensis* with good correlation between samples which were individually genotyped and then pooled and genotyped (Kim et al., 2007).

Comparison of gDNA sequences obtained from 34 individual larvae from 19 different field populations of *R.* (*B.*) *microplus* identified for the first time three new SNPs in the sodium channel gene coding region which results in amino acid changes from Phe to Ile or from Phe to Ser (both in the same codon), from Phe to Ile and from Gln to Ser. Additional mutations at other sites in the sodium channel gene (domain I, II and III) may also contribute to resistance since several point mutations have been indentified in other insects showing insensitivity to pyrethroids (Dong, 2007; Soderlund, 2008). Despite the proximity of the SNPs Pyrosequencing™ detected these nucleotide substitutions and was able to identify the target SNP. The evaluation of these three SNPs in association with pyrethroid resistance of *R.* (*B.*) *microplus* is underway.

The present study has shown that Pyrosequencing™ is a reliable and high-throughput method that could be used as an alternative method for genotyping pyrethroid resistant populations of *R.* (*B.*) *microplus*. In accordance with studies on other viruses, bacteria, fungi and parasites (Gharizadeh et al., 2005; Edvinsson et al., 2007; Hodgkinson et al., 2008), the data presented show that Pyrosequencing™ is an efficient method for fast genotyping. Further pyrosequencing studies including larger number of larvae and pooled samples are suggested. For the purposes of this project the collection of ticks and detection of genotypes was conducted in the Veterinary Parasitology Laboratory at the Campus de Ciencias Biológicas y Agropecuarias de la Universidad Autónoma de Yucatán (CCBA-UADY), in Mérida, Yucatán, Mexico. It was not possible to carry out Pyrosequencing™ due to the lack of equipment and high cost of reagents; hence all genotyping was performed by AS-PCR-68 bp. However, as an additional validation of the published method, the Pyrosequencing™ method developed at the University of

Liverpool was used to genotype a number of adult *R.* (*B.*) *microplus.* These were subsequently used as positive controls (known genotypes, SS, RS, RR) in a subset of AS-PCR-68 bp reactions (10 % of all reactions performed). In the future, the genotyping of *R.* (*B.*) *microplus* larvae will be carried out at both sites using the AS-PCR-68 bp and Pyrosequencing™ in Yucatan, Mexico and Liverpool, UK, respectively.

CHAPTER 4

Demonstration of association between survival of larvae and genotypes: Association between survival of larvae exposed to cypermethrin and pyrethroid resistance genotypes in *Rhipicephalus* (*Boophilus*) *microplus*

4.1. Introduction

In the previous study it was found that Pyrosequencing™ is a reliable and high-throughput method that could be used as an alternative for genotyping pyrethroid resistant populations of *R. (B.) microplus*. Furthermore, the ability of the AS-PCR for genotyping pyrethroid resistant populations was validated. Due to the lack of a Pyrosequencer in Mexico, the larvae genotyping in this study was carried out by the AS-PCR-68 using positive controls tested by Pyrosequencing™.

Both DDT and pyrethroids act on the same target site protein, the insect voltage-gated sodium channel (responsible for generating action potentials in insect nerve cells), modifying the gating kinetics and resulting in the prolonged opening of individual channels, leading to paralysis and death of the insect (Soderlund and Knipple, 2003). *Kdr* occurs as the result of a change in the affinity between the insecticides and their binding site(s) on the channel, caused by mutations in the sodium channel in several insect species (Dong, 2007). Lee et al. (2000) and Gao et al. (2003) demonstrated that presence of *Pediculus capitis* with homozygote resistant alleles (mutations T929I and L932F) correlated well with increased survival of lice in bioassays. Recently, Hoti et al. (2006) using bioassays exposed a field population of mosquitoes (*An. culicifacies*) to DDT (4 %) and found that survivors posses higher frequency of the R allele (*kdr*) (80 %) in comparison with mosquitoes that died (11 %) in the bioassay. This association between larvae survival exposed to cypermethrin in bioassay and the presence of the target SNP associated with pyrethroid resistance has not been investigated in field populations of *R. (B.) microplus.*

4.2. Experimental aims and methods

4.2.1. Aim of the study

The specific aim of the work presented in this section was to determine the association between survival of larvae exposed to cypermethrin and pyrethroid resistance genotypes in 10 populations of *R. (B.) microplus.*

4.2.2. Study Population

Ten farms were studied in three areas of Yucatan, Mexico (**Figure 4.1., see section 2.3.**) and a sample of 30-50 engorged adult female *R.* (*B.*) *microplus* ticks was collected from at least 10 animals on each farm (**see section 2.4.**). Tick collection on each farm and production of *R.* (*B.*) *microplus* larvae were carried out as was described in **section 2.1.**

4.2.3. Phenotypic Analysis by Dose-Response Bioassay and Genotypic Analysis by Allele-Specific Polymerase Chain Reaction

The phenotypic response of *R.* (*B.*) *microplus* to cypermethrin as determined by dose-response bioassays were carried out using the modified larval packet test (**see section 2.5.**). A discriminating dose of the technical grade acaricide was used and was calculated by doubling the mean lethal dose 99.9 % derived from the series of tests conducted with a susceptible strain (Kemp et al., 1998). The cypermethrin discriminating dose used was 0.05 % (Santamaria, 1992). Three replicates and a control (filter paper with trichloroethylene and olive oil) were used. The treated larvae were exposed for 24 h to the acaricide and the numbers of live and dead larvae were counted to calculate the percentage of larval mortality. If one or more larvae were found alive, the population was considered as resistant. When bioassays were read, "survivors" and "dead" larvae were separated with a vacuum and immediately frozen at -70 °C for PCR analysis.

To examine the genotypic response of *R.* (*B.*) *microplus* to cypermethrin the AS-PCR (Guerrero et al., 2001) method was carried out as described in **section 2.6.** Interpretation of the AS-PCR assay using gel analysis was based on whether or not the diagnostic product (68 bp) for mutation was detectably amplified to allow designation of a genotype to individual larvae (**see section 2.6.**). Where possible, on each farm 30 (15 "dead" and 15 "survivor") individual larnbvae were genotyped.

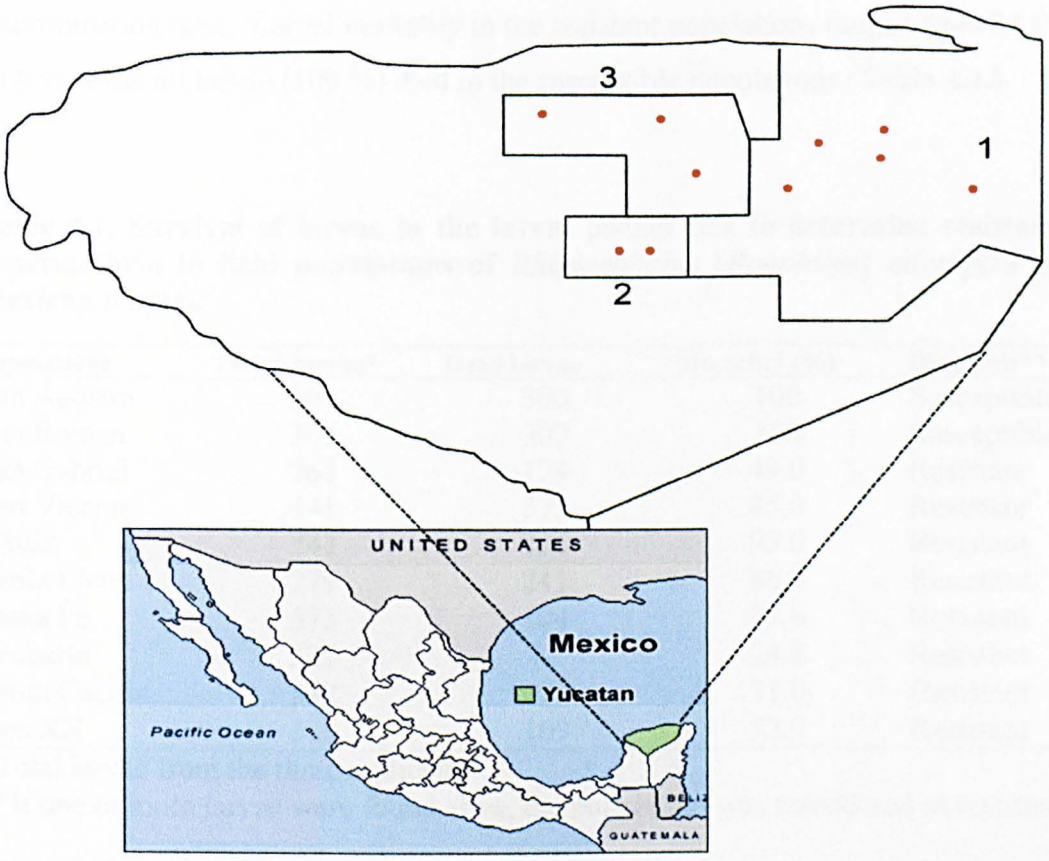

Figure 4.1. Map showing the locality of 10 farms (red dots) in three areas of Yucatan, Mexico (1. Tizimin, 2. Sucila, 3. Panaba), where tick populations were taken to determine the association between survival of larvae exposed to cypermethrin and a SNP associated with pyrethroid resistance in *Rhipicephalus* (*Boophilus*) *microplus*.

4.2.4. Data Analysis

Comparisons of the different genotypes between "dead" and "survivor" larvae were made as a contingency table using chi-square tests.

4.3. Results

From the 10 *R.* (*B.*) *microplus* populations studied, eight were resistant and two were susceptible to cypermethrin by the larval packet test using cypermethrin 0.05 % as a

discriminating dose. Larval mortality in the resistant populations ranged from 24.8 % to 93.0 % while all larvae (100 %) died in the susceptible populations (**Table 4.1.**).

Table 4.1. Survival of larvae in the larval packet test to determine resistance to cypermethrin in field populations of *Rhipicephalus* (*Boophilus*) *microplus* in the Mexican tropics.

Populations	Tested larvae*	Dead larvae	Mortality (%)	Diagnosis**
San Agustin	300	300	100	Susceptible
San Roman	302	302	100	Susceptible
San Gabriel	263	129	49.0	Resistant
San Vicente	441	375	85.0	Resistant
Akula	243	226	93.0	Resistant
Santa Elena	279	241	86.4	Resistant
Santa Fe	373	104	27.8	Resistant
Lechería	327	81	24.8	Resistant
Santa Cecilia	342	106	31.0	Resistant
Dos XX	319	169	53.0	Resistant

*Total larvae from the three replicates

**If one or more larvae were found alive, the population was considered as resistant

Table 4.2. shows the results of AS-PCR on field populations which survived or were killed by cypermethrin 0.05 % in the larval packed test. All larvae in the two susceptible populations that were killed by cypermethrin possessed the S allele (SS genotype). In both resistant and susceptible populations larvae that were killed possessed 96 % of at least one copy of the S allele (SS: 49.3 %, RS: 46.7 %). Six of the 150 larvae (4.0 %) that were killed by cypermethrin possessed the RR genotype (**Table 4.2.**). All surviving larvae had the R allele (RS: 41.7 % and RR: 58.3 %). The Lecheria population showed the lowest mortality when larvae were exposed to cypermethrin 0.05 % and 100 % of the surviving larvae possessed two copies of the R alleles (RR).

Chi-square tests of significance showed there was a statistical significant association ($p< 0.05$) of larval survival with the presence of the R allele (dead larvae 27.3 % vs. survivor larvae 78.3 %). There was a higher probability that larvae with one mutated allele (RS) survived exposure to cypermethrin when compared to homozygous susceptible (SS) larvae, and larvae with two R mutated alleles (RR) were more likely to survive than those with one R allele (RS) ($p< 0.05$).

Table 4.2. Genotypes of *Rhipicephalus* (*Boophilus*) *microplus* larvae surviving and killed by a discriminating dose of cypermethrin in the larval packet test. Genotypes were determined by the AS-PCR.

			Genotype			
	Larval condition	**No. larvae**	**SS +(%)**	**RS +(%)**	**RR +(%)**	**R Allele (%)**
Susceptible populations						
San Agustin*	Dead	15	15(100)	0(0.0)	0(0.0)	0.0
San Roman*	Dead	15	15 (100)	0 (0.0)	0(0.0)	0.0
Sub-total	Dead	30	30 (100)	0(0.0)	0(0.0)	0.0
Resistant populations						
San Gabriel	Dead	15	9 (60.0)	6 (40.0)	0 (0.0)	20.0
	Survivor	15	0 (0.0)	7 (46.7)	8 (53.3)	76.7
San Vicente	Dead	15	2 (13.3)	13 (88.7)	0 (0.0)	43.3
	Survivor	15	0 (0.0)	11 (73.3)	4 (26.7)	63.3
Akula	Dead	15	4 (26.7)	11 (73.3)	0 (0.0)	43.3
	Survivor	15	0 (0.0)	12 (80.0)	3 (20.0)	60.0
Santa Elena	Dead	15	8 (53.4)	7 (46.6)	0 (0.0)	23.3
	Survivor	15	0 (0.0)	7 (46.6)	8 (53.4)	76.7
Santa Fe	Dead	15	5 (33.3)	8 (53.3)	2 (13.3)	40.0
	Survivor	15	0 (0.0)	2 (13.3)	13 (88.7)	93.3
Lecheria	Dead	15	5 (33.3)	10 (66.7)	0 (0.0)	33.3
	Survivor	15	0 (0.0)	0 (0.0)	15 (100)	100
Santa Cecilia	Dead	15	3 (20.0)	10 (66.7)	2 (13.3)	46.7
	Survivor	15	0 (0.0)	6 (40.0)	9 (60.0)	80.0
Dos XX	Dead	15	8 (53.4)	5 (33.3)	2 (13.3)	30.0
	Survivor	15	0 (0.0)	5 (33.3)	10 (66.7)	83.3
Sub-total	Dead	120	44 (36.7)	70 (58.3)	6 (5.0)	34.2
	Survivor	120	0 (0.0)	50 (41.7)	70 (58.3)	78.3
Total	Dead**	150	74^{a} (49.3)	70^{a} (46.7)	6^{b} (4.0)	27.3
	Survivor **	120	0^{a} (0.0)	50^{b} (41.7)	70^{c} (58.3)	78.3

*No survivors

**Figures within row (total) with different letters are significantly different ($p < 0.05$).

SS denoted the homozygous susceptible (possess the two wild-type alleles)

RS denoted the heterozygous resistant-susceptible (possess one substituted sodium channel allele and one wild-type allele). RR denoted the homozygous resistant (possess the two substituted sodium channel alleles). R denoted the resistance allele in the total number of alleles assayed (assuming two alleles per individual).

4.4. Discussion

In this study, there was a clear relation between larval survival and the presence of the R allele (dead larvae 27.3 % vs. survivor larvae 78.3 %, $p< 0.05$) associated with

pyrethroid resistance in *R.* (*B.*) *microplus*. This association between the presence of a mutated allele (*kdr*) in insects that survived pyrethroid or DDT treatments has been detected in populations of *An. gambiae* (Fanello et al., 1999; Kolaczinski et al., 2000), *An. culicifacies* (Hoti et al., 2006), *Haematobia irritans* (Foil et al., 2005) and *Pediculus capitis* (Lee et al., 2000; Gao et al., 2003). Furthermore, Rosario-Cruz et al. (2005) working with *R.* (*B.*) *microplus* found a relation between the larval survival and the percentage of the R allele involved in pyrethroid resistance.

In the two susceptible tick populations (San Agustin and San Roman) all larvae were killed by 0.05 % of cypermethrin and all tested showed the SS genotype. However, this situation is unlikely to occur in field conditions, because in most cases, it is likely that genes that confer resistance are already present at very low levels in the tick population before the introduction of a new acaricide (ffrench-Constant, 2006). The rate at which a resistant allele becomes established in the population is dependent upon many factors (frequency of the mutation before treatment, mode of inheritance of the resistant allele, frequency of acaricide treatment, the concentration gradient of the acaricide and proportion of ticks in refugia (Nari et al., 2000). Conversely, in farms with high pyrethroid selection pressure the selection for resistant allele would be greater. This was conformed in the present study when 58.3 % of survivors had the RR allele (in Lecheria population all survivors showed the RR allele). In this tick population lack of pyrethroid efficacy was claimed by the farmer and another acaricide family is used to control ticks.

The AS-PCR data showed that all surviving larvae possessed at least one copy of the mutated allele. This finding shows that the mutated allele is present in those larvae and the SNP identified by He et al. (1999a) is associated with larval survival. However, in both resistant and susceptible populations larvae that were killed possessed 96 % of at least one copy of the S allele. This means that the presence of the mutated allele in one copy of the gene does not always confer resistance. Kunz and Kemp (1994) mentioned that pyrethroid resistance in *R.* (*B.*) *microplus* is inherited as recessive or semirecessive. Recently, Aguilar-Tipacamu et al. (2008) found that pyrethroid resistance (deltamethrin, flumethrin and cypermethrin) is inherited as a "partially dominant" when *R.* (*B.*) *microplus* female is resistant. However, when the male is resistant for deltamethrin and

flumethrin the resistance is inherited as complete recessive (partially dominance for cypermethrin). The same authors, using Mendelian predictions (effective dominance) found that one single mutation at the sodium channel can produce a multiple resistance to pyrethroids phenotype (deltamethrin, flumethrin and cypermethrin), since no mortality was produced in the resistant strain when increasing concentrations of the pyrethroids were used, probably due to the high frequency of the homozygous resistant genotype. Furthermore, based on phenotype results and Mendelian predictions (larval mortality), Tapia-Pérez et al. (2003) inferred that resistance to flumethrin in *R. (B.) microplus* might be controlled by more than one gene. To date, the mutation in the sodium channel is the only SNP associated with pyrethroid resistance in ticks; however, it might be possible that other mutations are present in the sodium channel (possibly at low level) as it was found in other insects (Souderlund and Knipple, 2003). Recently, Li et al. (2008) investigated the genetic basis of permethrin resistance by cross-mating experiments as well to determine the mechanism of permethrin resistance through synergist bioassays and biochemical analysis of esterase profile, and suggested that other mechanism, including a possible new sodium channel mutation that is different from the one currently known, may be responsible for permethrin resistance in the Santa Luzia strain of *R. (B.) microplus*. However, using molecular methods, only one sodium channel gene has been identified in *R. (B.) microplus* (He et al., 1999). The absence of dominant trait in pyrethroid resistance in *R. (B.) microplus* might explain why the presence of the mutated allele in one copy of the gene is not always expressed as survival.

The clear association between survival of larvae exposed to cypermethrin and pyrethroid resistance genotypes suggested that the target SNP is one of the most important mechanisms that confer pyrethroid resistance in the studied *R. (B.) microplus* populations. However, other mechanisms of resistance to pyrethroids have been reported in ticks. Enhanced carboxylesterase-mediated metabolic detoxification has been indicated in both OP and SP resistance in *R. (B.) microplus* ticks (Rosario-Cruz et al., 1997; Jamroz et al., 2000). Jamroz et al. (2000) identified a pyrethroid-resistant *R. (B.) microplus* strain (Cz) having high esterase-hydrolytic activity (CzEST9) compared to a susceptible strain of *R. (B.) microplus*. The same high hydrolytic activity was found following purification of CzEST9 and therefore it was hypothesized CzEST9 is

associated with permethrin resistance in the Cz strain (Pruett et al., 2002). Recently, Baffi et al. (2008) working with OP and SP resistant Brazilian strains of *R.* (*B.*) *microplus*, found that metabolic detoxification by two acetylcholinesterases contributed toward the development of resistance of these tick populations. This metabolic resistance mechanism was not evaluated in the present study; however, future studies are needed to evaluate the role of metabolic resistance mechanisms in these tick populations.

The discriminating dose is a single dose that will theoretically affect a high percentage of the susceptible genotypes in a population without affecting resistant genotypes in a resistant population (Roush and Miller, 1986). However, it is difficult to establish a single dose that discriminates all of the possible genotypes (Denholm, 1990). The use of a too high discriminating dose can kill the heterozygote resistant individuals and result in a missed diagnosis when the frequency of resistant genes in a population is low. The discriminating dose used in the larval packet test (cypermethrin 0.05 %) and AS-PCR allowed the separation of functionally resistant individual larvae from susceptible. Furthermore, the AS-PCR is efficient in picking up very low frequencies of resistance genes, which are mainly presented in heterozygous form. This means resistance management can be implemented at an early stage, if such genotypes are detected.

It conclusion, there was a high association between survival of larvae exposed to cypermethrin and the presence of the target SNP associated with pyrethroid resistance in *R.* (*B.*) *microplus*, suggesting that this target SNP is one of the most important mechanism that confer pyrethroid resistance in *R.* (*B.*) *microplus* populations from Yucatan, Mexico.

CHAPTER 5

Cross Sectional Study: Determination of prevalence of pyrethroid resistance phenotype and genotype in *Rhipicephalus* (*Boophilus*) *microplus* in Yucatan, Mexico

5.1. Introduction

In Mexico, resistance to pyrethroid was first detected in 1993 (Fragoso et al., 1995) by the larval packet test with discriminating doses (Santamaria, 1992). *R. (B.) microplus*-resistant strains were identified as co-resistant to pyrethroids and organophosphates, with a wide geographical distribution in the livestock regions of the Gulf of Mexico, northeastern and southern Tamaulipas, eastern San Luis Potosi, southeastern Tabasco, northeastern Chiapas, and all of the State of Veracruz (Ortiz et al., 1995). The prevalence of farms with pyrethroid resistant *R. (B.) microplus* in the Mexican states of Yucatan, Quintana Roo, Tabasco, and Chiapas is 66 %, 95 %, 94 %, and 90.8 %, respectively (Rodriguez et al., 2006a, 2007) as shown by the larval packet test using discriminating doses for the diagnosis of resistant or susceptible populations. These results provide a general idea of pyrethroid resistance problems in southern Mexico; however, they do not provide the level of resistance in those populations. In order to know about the level of phenotypic resistance of *R. (B.) microplus* populations to acaricides, the RF_{50} (Beugnet and Chardonnet, 1995; Bianchi et al., 2003) is typically determined and interpreted. The RF_{50} is an indicator of the phenotypic response (i.e. larval mortality) in half of the population exposed to an acaricide in comparison to reference strain values. Nevertheless, the RF_{50} does not explain population behavior. In order to more objectively evaluate the behavior of the majority of an insect population to an acaricide, the use of other LCs (i.e. LC_{90}, LC_{95}, or LC_{99}) (Cameron et al., 1995; Miller et al., 2007) as well as the slope on probit analysis (Robertson and Preisler, 1992) are recommended. In the present study, we added a new alternative to analyze RFs by using RFs judged by both LCs (LC_{50} + LC_{99}).

Little is known about the *R. (B.) microplus* resistance level and frequency of resistant alleles on field populations and how these populations change with local environmental and selection pressure. Given the association demonstrated in **chapter 4** it is now possible to detect the target SNP in field populations and make inference about resistant levels. Due to the increasing incidence of acaricide control failures in Mexican cattle farms and the ecological factors in the Yucatan state which are so favorable to successful tick infestation of cattle, we conducted a cross sectional study using the AS-

PCR validated in this thesis (**Chapter 3**) and the larval packet test using different dilutions of cypermethrin to obtain the RFs.

5.2. Experimental Aims and Methods

5.2.1. Aim of the Study

The specific aim of the work presented in this section was to determine the prevalence of pyrethroid resistance phenotype and genotype on field populations of *R.* (*B.*) *microplus* using a cross sectional study in Yucatan, Mexico.

5.2.2. Study Population, Tick Collection and Production of Larvae

A cross sectional study was carried out on farms in three areas of Yucatan, Mexico as was described in **section 2.3.** The sample size (49 farms) was calculated, considering (with a confidence level of 90 % and an error of 10 %) an expected prevalence of 77 % of *R.* (*B.*) *microplus* resistant to pyrethroids (Rodriguez-Vivas et al., 2005b) and a total number of cattle farms of 4,629. Farms were randomly selected from a list provided by the Cattlemen's Association of the state of Yucatan. Logistical difficulties to sample eight of those farms led to their replacement by eight other farms, also randomly selected. Each farm was visited once or twice to collect ticks. A sample of 30-50 engorged adult female *R.* (*B.*) *microplus* ticks was collected at each visit from at least 10 animals on each farm. Tick collection on each farm and production of *R.* (*B.*) *microplus* larvae were carried out as was described in **section 2.4.**

5.2.3. Phenotypic Analysis by Dose-Response Bioassay and Genotypic Analysis by Allele-Specific Polymerase Chain Reaction

The phenotypic response of *R.* (*B.*) *microplus* to cypermethrin bioassays was carried out by the modified larval packet test using different dilutions of technical grade cypermethrin to test the susceptibility (**see section 2.5.**). To genotype *R.* (*B.*) *microplus* to cypermethrin the AS-PCR to detect the target SNP in the sodium channel gene was carried out as described in **section 2.1.** Genotype interpretation of PCR assay using gel

analysis was based on whether or not the diagnostic product (68 bp) for the target SNP was detectably amplified (**see section 2.6.**). On each farm 27-34 individual larvae were randomly selected and genotyped.

5.2.4. Data Analysis

Probit analysis was performed on dose-response bioassay results using *Polo-Plus* (LeOra Software, 2003) and RF judged by LC_{50} and LC_{99} were calculated (**see section 2.7.**). Populations were considered susceptible when RF values (judged by LC_{50}) were < 3.0, tolerant 3-5 and resistant ≥ 5.0 (Beugnet and Chardonnet, 1995). Additional phenotype classification was considered as follows: susceptible populations when both RF values (judged by LC_{50} and LC_{99}) were < 3.0 and resistant populations when RF values were ≥ 5.0. Tolerant populations were considered when one or both RF values were 3-5. Spatial distributions of phenotypes were georeferenced using a map of Yucatan, Mexico. The proportion of phenotypes explained by LC_{50} and by both LCs (between LCs and across the three areas in Yucatan) was assessed by the chi-square.

To investigate the correlation between the level of cypermethrin resistance and the frequency of the mutated allele, the RFs judged by LC_{50} and LC_{99} of field populations were plotted against the frequency of the R allele, and linear regression lines were generated.

5.3. Results

Populations of *R.* (*B.*) *microplus* from 49 farms were examined from three areas of Yucatan, Mexico (20 in Tizimin, 16 in Sucila and 13 in Panaba). Judged by LC_{50}, the prevalence of *R.* (*B.*) *microplus* susceptible, tolerant and resistant populations to cypermethrin were 65.3 %, 2.0 % and 32.6 % respectively (**Table 5.1., 5.2.**). Judged by both LCs, the prevalence of susceptible, tolerant and resistant populations were 26.5 %, 40.8 %, 32.6 % respectively (**Tables 5.1., 5.3.**). No statistical differences were found in

the proportion of phenotypes between the three areas when the RFs were judged by LC_{50} (**Table 5.2.**) and both LCs (**Table 5.3.**).
There was a significant difference ($p < 0.001$) to phenotype susceptible populations using alone LC_{50} (65.3 %, 32/49) or using both LCs (26.6 %, 13/49).

A substantial inter-population variation in the level of pyrethroid resistance was evident (RFs judged by LC_{50} ranged from 0.31 to 2599.0 and by LC_{99} ranged from 0.7 to >5000) in the three studied areas. Among field populations, ten populations (20.40 %) showed a high level of resistance (LC_{50}: >11-fold, LC_{99}: >169-fold). Six of these populations were found to exhibit very high resistance (LC_{50}: >41-fold, LC_{99}: >542-fold) (**Table 5.1.**). The most cypermethrin resistant tick population (Africa population) was found in the area of Sucila (RF of 2599 judged by LC_{50} and > 5000 by LC_{99}). For all cypermethrin-susceptible populations (judged by both LCs) the dose-responses (probit line) were accurately fitted by slopes ≥2.42 (**Table 5.1.**). By contrast, resistant populations exhibited slopes <1.97 (**Table 5.1.**). The spatial distribution of *R. (B.) microplus* resistant, tolerant and susceptible populations to cypermethrin judged by LC_{50} and both LCs are presented in **Figures 5.1** and **5.2.**

Detection of the target SNP associated with pyrethroid resistance revealed that the mutation in *R. (B.) microplus* was present in all areas studied. AS-PCR assays revealed clear differences in overall allelic frequency between resistant and susceptible *R. (B.) microplus* populations (**Table 5.4.**). The frequency of the R allele in populations judged by LC_{50} ranged in the resistant, tolerant and susceptible populations 46.7-95.0 %, 21.7 % (only one value), and 1.7-51.7 % respectively. The frequency of the R allele in populations judged by both LCs ranged in the resistant, tolerant and susceptible populations was 46.7-95.0 %, 1.7-51.7 % and 1.7-16.7 % respectively.

To investigate the correlation between the level of cypermethrin resistance phenotype and the frequency of the mutated genotype, the RFs to cypermethrin judged by LC_{50} and LC_{99} of field populations were plotted against the frequency of the R allele (**Figures 5.3., 5.4.**), and linear regression lines were generated. The increasing presence of the R allele correlated well with increased levels of resistance at LC_{50} ($r^2 = 0.667$) and LC_{99} ($r^2 = 0.684$) to cypermethrin in the three areas.

Table 5.1. Levels of cypermethrin in field populations of *Rhipicephalus* (*Boophilus*) *microplus* in three areas of Yucatan, Mexico.

Populations	50 %			99 %			Slope	Phenotype	
	LC	CI95 %	RF	LC	CI95 %	RF		LC_{50}	LC_{50}+LC_{99}
Tizimin									
San Agustín	0.019	0.018-0.021	1.4	0.098	0.082-0.121	2.1	3.29	S	S
Palomar	0.019	0.016-0.022	1.4	0.166	0.127-0.236	3.6	2.42	S	S
San Roman	0.021	0.020-0.023	1.6	0.119	0.099-0.150	2.5	3.12	S	S
Blanca Flor	0.022	0.017-0.027	1.6	0.199	0.134-0.364	4.3	2.41	S	T
Kantok	0.022	0.018-0.026	1.6	0.147	0.105-0.243	3.1	2.80	S	T
San Isidro	0.016	0.011-0.021	1.2	2.502	1.256-6.866	54.3	1.05	S	T
San Diego	0.017	0.010-0.024	1.3	2.832	1.179-12.066	61.5	1.04	S	T
San Pedro Tigre	0.004	0.002-0.008	0.3	1.239	0.577-4.670	26.9	0.95	S	T
Santa Fe	0.026	0.014-0.039	2.0	8.866	2.505-104.17	192.7	0.92	S	T
Dino	0.033	0.022-0.046	2.5	1.593	0.688-7.525	34.6	1.38	S	T
Dos equis	0.013	0.008-0.018	1.0	3.556	1.776-9.914	77.3	0.95	S	T
San Vicente Tiz	0.018	0.011-0.024	1.3	0.601	0.329-1.675	13.0	1.51	S	T
Rosales	0.031	0.027-0.036	2.3	0.367	0.266-0.564	7.9	2.17	S	T
Alamo	0.185	0.150-0.234	13.2	10.08	4.484-35.953	219.1	1.33	R	R
Santa Cruz Cul	0.159	0.114-0.240	12.2	9.910	3.318-73.794	215.4	1.29	R	R
Xnohuayan	0.133	0.094-0.220	10.2	6.851	2.221-53.347	148.9	1.35	R	R
Poop	0.201	0.169-0.242	15.4	7.814	3.873-23.417	169.8	1.46	R	R
Moluxtun	0.591	0.422-1.074	45.4	24.95	7.50-257.452	542.4	1.43	R	R
Andres	7.171	4.901-12.91	551.6	688.49	202.43-4995.3	>5000	1.17	R	R
Santa Cecilia	8.92	4.31-33.265	686.1	4116.6	3.656-65.285	>5000	0.87	R	R
Sucila									
Actunchacmol	0.008	0.006-0.009	0.6	0.044	0.036-0.060	0.9	3.16	S	S
San Gregorio	0.011	0.009-0.012	0.8	0.088	0.071-0.117	1.9	2.54	S	S
Estrella de Ori	0.013	0.012-0.015	1.0	0.090	0.077-0.108	1.9	2.82	S	S
Chembech	0.016	0.013-0.019	1.2	0.125	0.092-0.198	2.7	2.63	S	S
Dzadz Fidel	0.017	0.009-0.024	1.3	0.108	0.058-0.721	2.3	2.86	S	S
Los Pirules	0.022	0.020-0.026	1.6	0.094	0.095-0.174	2.0	3.66	S	S
Roma	0.019	0.016-0.021	1.4	0.166	0.128-0.235	3.6	2.45	S	T
Santa Ana	0.021	0.008-0.035	1.6	1.988	0.744-16.155	43.2	1.17	S	T
Tapachula	0.006	0.003-0.010	0.4	0.449	0.256-1.139	9.7	1.25	S	T
Mina de Oro	0.036	0.032-0.040	2.7	0.458	0.369-0.595	9.9	2.10	S	T
Las Palmas	0.007	0.004-0.010	0.5	3.069	1.584-7.951	66.7	0.87	S	T
Santa María	0.015	0.008-0.022	1.1	0.812	0.397-2.929	17.6	1.33	S	T
El Platanal	0.013	0.010-0.019	1.1	0.409	0.254-0.840	8.8	1.60	S	T
San Vicente Suc	0.067	0.051-0.084	5.1	1.735	0.982-4.223	37.7	1.64	R	R
San Gabriel	0.155	0.107-0.253	11.9	124.83	20.4-4318.7	2713.7	0.80	R	R
África	33.787	4.35-182.82	2599.0	2599	ND	>5000	0.56	R	R
Panaba									
El Rosario	0.012	0.011-0.013	0.9	0.032	0.028-0.038	0.7	5.44	S	S
Concepcion	0.018	0.016-0.021	1.3	0.155	0.121-0.215	3.3	2.51	S	S
Akulá	0.027	0.018-0.035	2.0	0.230	0.160-0.537	5.0	2.45	S	S
Tino	0.022	0.020-0.026	1.6	0.094	0.093-0.172	2.0	3.64	S	S
Tabasco	0.035	0.032-0.039	2.6	0.408	0.326-0.534	8.8	2.19	S	T
Rancho nuevo	0.031	0.015-0.049	2.3	1.989	0.655-26.090	43.2	1.28	S	T
Santa Cruz	0.042	0.035-0.050	3.2	0.602	0.379-1.155	13.0	2.01	T	T
Lechería	0.087	0.066-0.110	6.6	1.316	0.779-3.045	26.6	1.97	R	R
Santa Isabel	0.155	0.106-0.254	11.9	120.01	20.7-4316.41	2608.9	0.92	R	R
Santa Elena 2	0.187	0.148-0.253	14.3	9.781	3.805-49.711	212.6	1.35	R	R
Aculena	0.305	0.187-0.435	23.4	123.26	32.66-1522.61	2679.6	0.89	R	R
Yaxcabá	0.542	0.443-0.704	41.6	9.28	5.220-20.731	201.7	1.88	R	R
Maravillas	0.590	0.419-0.988	45.3	22.87	8.13-214.31	497.1	1.57	R	R

LC: Lethal concentration. CI: Confidence Interval. RF: Resistance Factor (Cenapa strain was used as a reference: LC_{50}: 0.013 y LC_{99}: 0.046). ND: Not determined due to high resistance level. Susceptible: Both RF < 3.0 (judged by LC_{50} and LC_{99}), Tolerant: One or both RF 3-5, Resistant: Both RF ≥ 5.0. S: Susceptible, T: tolerant, R: resistant.

Table 5.2. Populations of *Rhipicephalus* (*Boophilus*) *microplus* susceptible, tolerant and resistant to cypermethrin (determined by the LC_{50}) on 49 farms in three areas of Yucatan, Mexico.

Areas	No.	Phenotype*		
	Populations	Susceptible No. (%)*	Tolerant No. (%)*	Resistant No. (%)*
Tizimin	20	13 (65.0)a	0 (0.0)**	7 (35.0)a
Sucila	16	13 (81.2)a	0 (0.0)**	3 (18.7)**
Panaba	13	6(46.1)a	1 (7.7)**	6 (46.1)a
Total	49	32(65.3)	1 (2.0)	16 (32.6)

Susceptible: RF < 3.0, Tolerant: RF 3-5, Resistant: ≥ 5.0. *Figures within column with different letters are significantly different ($p < 0.05$). **Insufficient data to conduct a test (one or more cells with less than five counts).

Table 5.3. Populations of *Rhipicephalus* (*Boophilus*) *microplus* susceptible, tolerant and resistant to cypermethrin (determined by both LC_{50} and LC_{99}) on 49 farms in three areas of Yucatan, Mexico.

Areas	No.	Phenotype*		
	Populations	Susceptible No. (%)*	Tolerant No. (%)*	Resistant No. (%)*
Tizimin	20	3 (15.0)**	10 (50.0)a	7 (35.0)a
Sucila	16	6 (37.5)**	7 (43.7)a	3 (18.7)**
Panaba	13	4 (30.7)**	3 (23.0)**	6 (46.1)a
Total	49	13 (26.5)	20 (40.8)	16 (32.6)

*Susceptible: Both RF < 3.0 (judged by LC_{50} and LC_{99}), Tolerant: One or both RF 3-5, Resistant: Both RF ≥ 5.0. *Figures within column with different letters are significantly different ($p < 0.05$). **Insufficient data to conduct a test (one or more cells with less than five counts).

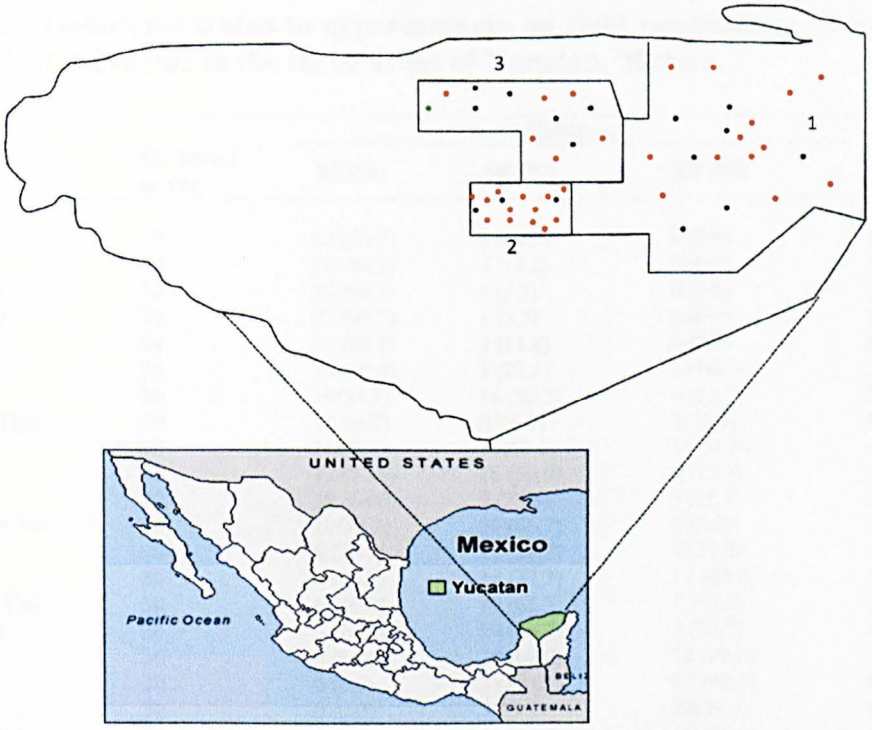

Figure 5.1. Spatial distribution of *Rhipicephalus* (*Boophilus*) *microplus* resistant (black dots), tolerant (green dots) and susceptible (red dots) populations to cypermethrin (judged by LC_{50}) in Tizimin (1), Sucila (2) and Panaba (3), Yucatan, Mexico.

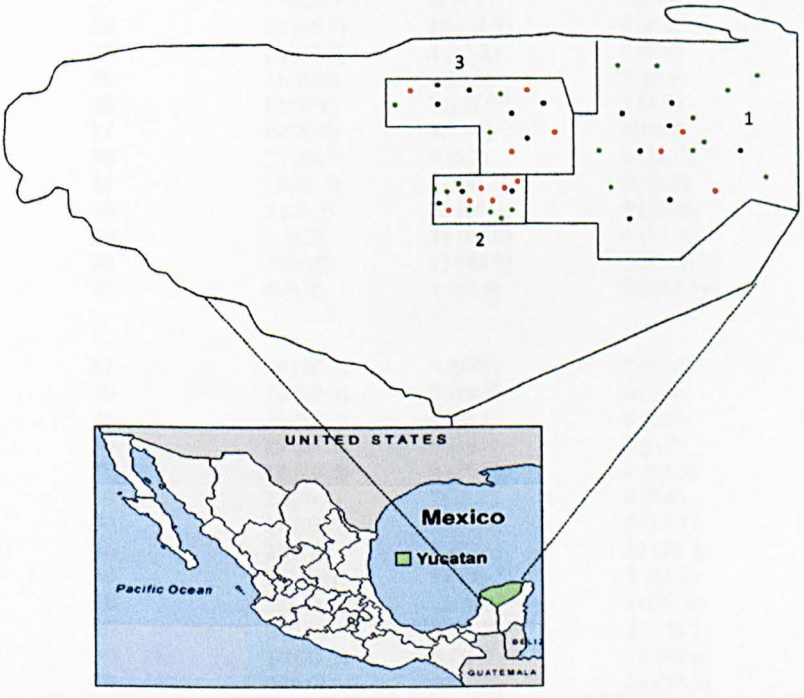

Figure 5.2. Spatial distribution of *Rhipicephalus* (*Boophilus*) *microplus* resistant (black dots), tolerant (green dots) and susceptible (red dots) populations to cypermethrin (judged by LC_{50}+LC_{99}) in Tizimin (1), Sucila (2) and Panaba (3), Yucatan, Mexico.

Table 5.4. Genotypic status to cypermethrin on field populations of *Rhipicephalus* (*Boophilus*) *microplus* in the three areas of Yucatan, Mexico.

Populations	No. tested larvae	Genotype			R allele (%)
		SS (%)	SR (%)	RR (%)	
Tizimin					
San Agustín	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0
Palomar	30	26 (86.6)	4 (13.3)	0 (0.0)	6.7
San Roman	30	29 (96.7)	1 (3.3)	0 (0.0)	1.7
Blanca Flor	30	29 (96.7)	1 (3.3)	0 (0.0)	1.7
Kantok	34	30 (88.2)	4 (11.8)	0 (0.0)	5.9
San Isidro	30	18 (60.0)	7 (23.3)	5 (16.7)	28.3
San Diego	30	10(33.3)	16 (53.3)	4 (13.3)	36.6
San Pedro Tigre	30	17(56.7)	8(26.7)	5(16.7)	30.0
Santa Fe	29	2(6.9)	17(58.6)	10(34.5)	63.8
Dino	32	12 (37.5)	16 (50.0)	4 (12.5)	37.5
Dos equis	30	18 (60.0)	7 (23.3)	5 (16.7)	28.3
San Vicente Tiz	30	10(33.3)	20 (66.7)	0 (0.0)	33.3
Rosales	30	6 (20.0)	17 (56.7)	7 (23.3)	51.7
Alamo	30	8 (26.7)	10 (33.3)	12 (40.0)	56.7
Santa Cruz Cul	30	6 (20.0)	19 (63.3)	5 (16.6)	48.3
Xnohuayan	30	6 (20.0)	20 (66.7)	4 (13.3)	46.7
Poop	30	0 (0.0)	18 (60.0)	12 (40.0)	50.0
Moluxtun	30	0 (0.0)	3 (10.0)	27 (90.0)	95.0
Andres	30	0 (0.0)	7(23.3)	23(76.7)	88.3
Santa Cecilia	30	0 (0.0)	11 (36.7)	19 (63.3)	81.7
Sucila					
Actunchacmol	30	27(90.06)	1(3.3)	2(6.7)	8.3
San Gregorio	30	20 (66.7)	10 (33.3)	0. (0.0)	16.7
Estrella de Oriente	30	25 (83.3)	4 (13.3)	1 (3.3)	8.3
Chembech	30	26 (86.7)	3 (10.0)	1 (3.3)	8.3
Dzadz Fidel	29	19(65.5)	7(24.1)	1(3.4)	15.5
Los Pirules	30	20 (66.7)	10 (33.3)	0 (0.0)	16.6
Roma	30	26 (86.6)	4 (13.3)	0 (0.0)	6.7
Santa Ana	30	21(70.0)	6(20.0)	3(10.0)	20.0
Tapachula	28	14(50.0)	12(42.9)	1 (3.5)	25.0
Mina de Oro	27	19(70.4)	4(14.8)	4(14.8)	22.2
Las Palmas	30	20 (66.7)	2 (6.7)	8 (26.7)	30.0
Santa María	27	11(40.7)	11(40.7)	5(18.5)	38.9
El Platanal	30	7 (23.3)	20 (66.6)	3 (10.0)	43.3
San Vicente Suc	30	2 (6.7)	24 (80.0)	4 (13.3)	53.3
San Gabriel	30	9 (30.0)	13 (43.3)	8 (26.7)	48.3
África	32	0 (0.0)	4 (12.5)	28 (87.5)	93.8
Panaba					
El Rosario	32	29 (90.6)	3 (9.40)	0 (0.0)	4.7
Concepcion	30	26 (90.0)	3 (10.0)	0 (0.0)	5.0
Akulá	34	30 (88.2)	4 (11.8)	0 (0.0)	5.8
Santa Cruz	30	19 (63.3)	9 (30.0)	2 (6.7)	21.7
Tabasco	30	18 (60.0)	8 (26.7)	4 (13.3)	26.7
Tino	30	23(76.7)	7(23.3)	0 (0.0)	11.6
Rancho nuevo	30	19 (63.3)	7 (23.3)	4 (13.3)	25.0
Lechería	30	2 (6.7)	7 (23.3)	21 (70.0)	81.7
Santa Isabel	30	9 (30.0)	14 (46.7)	7 (23.3)	46.7
Santa Elena 2	28	1(3.6)	13(46.4)	14(50.0)	73.2
Aculena	30	0 (0.0)	8 (26.7)	22 (73.3)	86.7
Yaxcabá	30	10 (33.3)	8 (26.7)	12 (40.0)	53.3
Maravillas	30	0 (0.0)	6 (20.0)	24 (80.0)	90.0

RR: Homozygous resistant (possess the two mutated sodium channel alleles), RS: Heterozygous resistant-susceptible (possess one mutated sodium channel allele and one wild-type allele). SS: Homozygous susceptible (possess the two wild-type alleles). R allele frequency: Percentage of mutated sodium channel allele (R) in the total number of alleles assayed (2 alleles per individual).

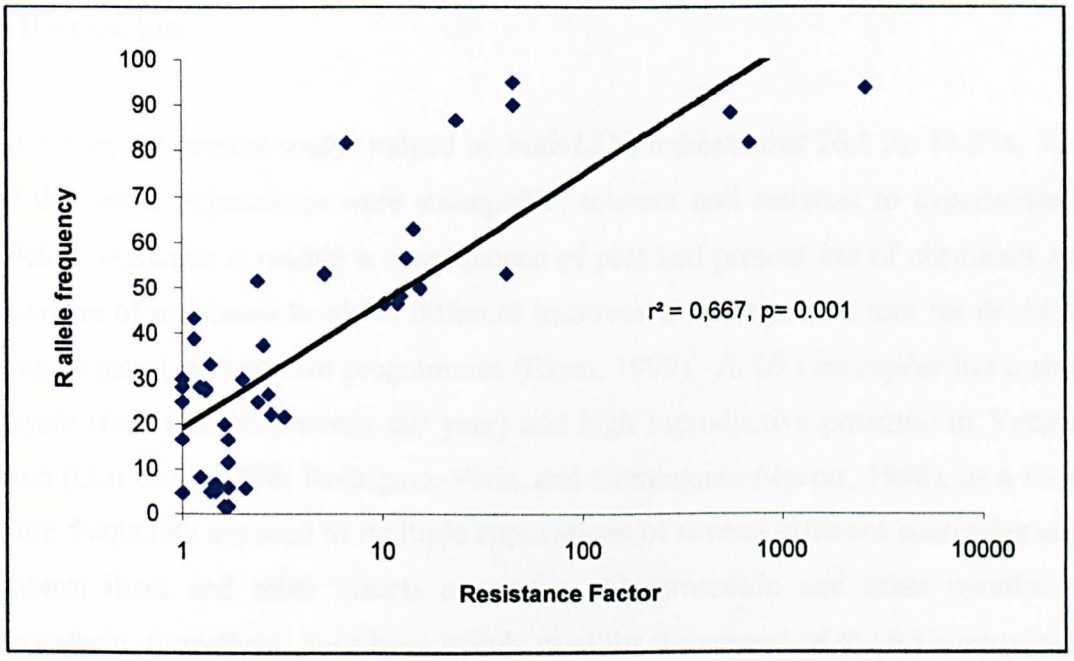

Figure 5.3. Correlation between the level of pyrethroid resistance (resistance factor) and the frequency of the R allele. RFs to cypermethrin judged by LC_{50} of field populations were plotted against the frequency of the R allele and linear regression line was generated.

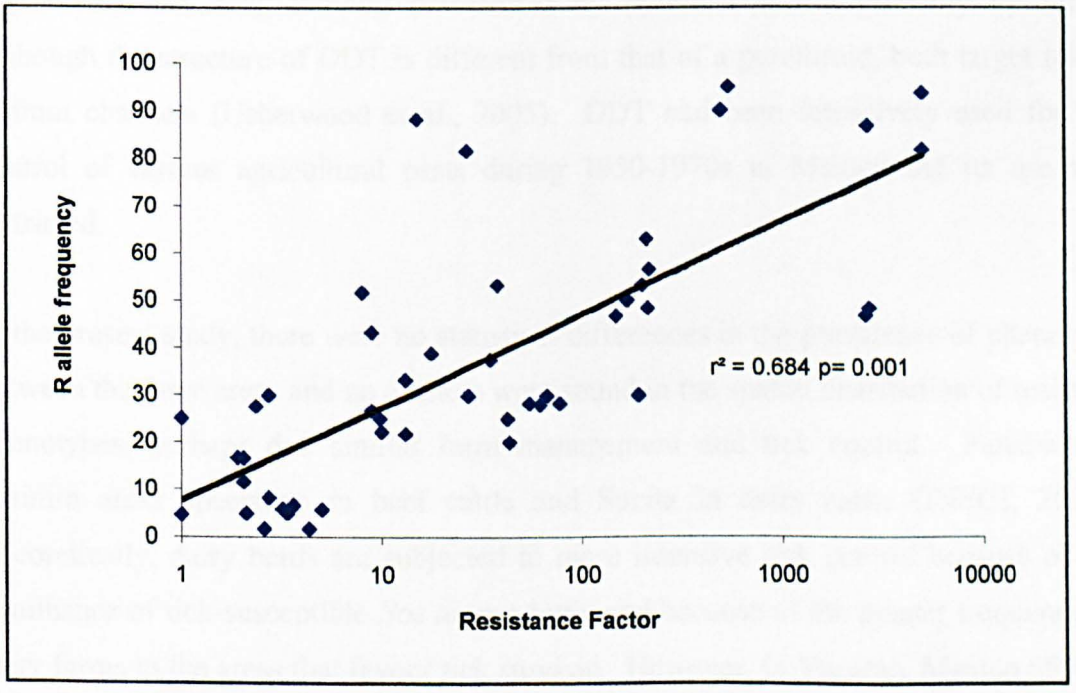

Figure 5.4. Correlation between the level of pyrethroid resistance (resistance factor) and the frequency of the R allele. RFs to cypermethrin judged by LC_{99} of field populations were plotted against the frequency of the R allele and linear regression line was generated.

5.4. Discussion

Results from the present study (judged by both LCs) indicate that 26.5 %, 40.8 %, 32.6 % of the tested populations were susceptible, tolerant and resistant to cypermethrin. Acaricide resistance is mainly a consequence of past and present use of chemicals and comparison of resistance levels in different locations is an important tool for decision-making in insect pest control programmes (Ekesi, 1999). *R. (B.) microplus* has a short life cycle (four tick generations per year) and high reproductive potential in Yucatan Mexico (Cen et al., 1998; Rodriguez-Vivas and Dominguez-Alpizar, 1998), as a result they are frequently exposed to multiple applications of several different acaricides used to control them and other insects on cattle. Cypermethrin and other pyrethroids (deltamethrin, flumethrin) have been widely used for the control of *R. (B.) microplus* in Yucatan, Mexico since 1982 (Rodriguez-Vivas et al., 2006b) and it is therefore not surprising that resistance to cypermethrin is developing in these three areas studied. Forty one percent of the populations studied (judged by both LCs) were classified as tolerant to cypermethrin. This finding suggests that a high percentage of the populations had been initially preselected by pyrethroids and probably by DDT. Although the structure of DDT is different from that of a pyrethroid, both target insect sodium channels (Usherwood et al., 2005). DDT had been intensively used for the control of various agricultural pests during 1950-1970s in Mexico but its use was restricted.

In the present study, there were no statistical differences in the prevalence of phenotype between the three areas and no clusters were found in the spatial distribution of resistant phenotypes, perhaps due similar farm management and tick control. Panaba and Tizimin areas specialize in beef cattle and Sucila in dairy cattle (INEGI, 2002). Theoretically, dairy herds are subjected to more intensive tick control because of the dominance of tick-susceptible *Bos taurus* cattle and because of the greater frequency of dairy farms in the areas that favour tick survival. However, in Yucatan, Mexico 65% of the farms uses acaricides >6 times/year and pyrethroids have been intensively used since 1986. Despite this difference in cattle production between areas the presence of *R. (B.) microplus* resistant to cypermethrin in the livestock zone of Yucatan, seems to be similar. Nevertheless, among field populations, ten populations showed a high level of

resistance and six of these populations were found to exhibit very high resistance. In 1995, Rodriguez-Vivas et al. (2005b) reported in Yucatan that 65 % of the cattle farms used pyrethroids (cypermethrin, deltamethrin and flumethrin) to control ticks; however, after 10 years (2005) only 21 % of the farms used pyrethroids (Rodriguez-Vivas et al., 2006a). This intensive use of pyrethroids in Yucatan in the last decade, may have contributed to the selection of resistance to cypermethrin in field individual populations of *R. (B.) microplus*. Other main risk factors associated with pyrethroid resistance in *R. (B.) microplus* are type of acaricides used, type of application, fly control and grazing management (Jonsson et al., 2000; Bianchi et al., 2003; Rodriguez-Vivas et al., 2006b). Furthermore, white-tailed deer (*Odocoileus virginianus*), elk (*Cervus canadensis*), and European red deer (*Cervus elaphus*) are all known carriers of *R. (B.) microplus* and *R. (B.) annulatus* ticks. In southern Texas, white-tailed deer are the most important alternative hosts for cattle ticks. In 2004, deer were associated with *R. (B.) annulatus* infestations in a cluster of ranches in the tick-free zone in Texas, USA. In the buffer zone (tick-free zone between Texas and Mexico) on a ranch in Starr County, 19 of 25 white-tailed deer captured were infested with *R. (B.) microplus*. Similar incidents occurred in 2005 in Maverick County and in 2006 in Webb County in which ticks were found on cattle (George, 2008). However, Cooksey et al. (1989) and Davey (1990) evaluated the role of the white-tailed deer (*Odocoileus virginianus*) as an alternate host for *R. (B.) annulatus* and they found that deer are biologically suitable hosts for *R. (B.) annulatus*, but significantly fewer ticks complete engorgement and those that complete engorgement have reduced fecundity. In a preliminary study carried out in Yucatan, Mexico, to evaluate the role of ungulates (*Odocoileus virginianus*, *Mazama americana*, *Pecari tajacu*) as carriers of *R. (B.) microplus*, Mukul et al. (2006) found that *Amblyomma* spp is the only tick species parasitizing those animals. The role of ungulates as carriers of *R. (B.) microplus* and *R. (B.) annulatus* ticks in Mexico need to be elucidated. Regional cooperation of tick control is essential, because resistance can be disseminated rapidly on transported cattle (Jonsson and Hope, 2007); however, stringent controls on the movement of cattle might alleviate this effect by using effective acaricides.

In the previous study (**Chapter 4**) we found a high association between survival of larvae exposed to cypermethrin and the presence of the target SNP associated with

pyrethroid resistance, suggesting that this target SNP is one of the most important mechanisms that confer pyrethroid resistance in *R.* (*B.*) *microplus* populations from Yucatan, Mexico. Based on this conclusion and the clear difference ($p > 0.001$) to classify tick susceptible populations between the LC_{50} and both LCs (65.3 % vs. 24.4 %), we compared the frequency of genotypes and the RFs judged by the LC_{50} and both LCs. The frequency of the R allele in susceptible populations judged by LC_{50} and by both LCs ranged from 1.7 % to 51.7 % and from 1.7 % to 16.7 % respectively. These results show that in bioassays analysis the best way to classify a susceptible population is when the RF value is <3 considering both LCs. This cut-off point to differentiate between susceptible and tolerant populations will allow identifying *R.* (*B.*) *microplus* populations with high susceptibility to pyrethroids and possessing a reduce proportion of the R allele.

Additional information obtained from this study is that all susceptible populations (judged by both LCs) in the three studied areas exhibited the highest slopes. These results are in agreement with the statement of Robertson and Preisler (1992) who argued that data homogeneity as well as high-slope linked with both low-LC_{50} and low-LC_{99} values suggested that most individuals have the wild- type allele. Theoretically a susceptible strain composed of totally susceptible individuals will produce the highest slope for a regression line of dose-response data. With selective pressure from exposure to insecticides, a population will become heterozygous for resistant genotypes and as the frequency of resistant genotypes increases, the slope of the regression line will drop off and the line will shift to the right (Robertson and Preisler, 1992). Chevillon et al. (2007) found in amitraz-susceptible *R.* (*B.*) *microplus* strains slopes ranging 3-6 and resistant strains with slopes < 2. However, the importance of the slope in individual bioassays has been questioned by Chilcutt and Tabashnik (1995), who suggested that the slope of the concentration-mortality line is an indicator of the phenotypic variation, which includes environmental as well as genetic variation.

The frequencies of the mutated allele (R) correlated well with the level of pyrethroid resistance (RF) in the three areas. This significant correlation between mutated alleles and LC to pyrethroids has been found in other insects. Kwon et al. (2004) found in *Plutella xylostella* that the increasing presence of the T929I mutation correlated well

with increased levels of resistance to both cypermethrin (r^2= 0.912) and fenvalerate (r^2: 0.810). Song et al. (2007) working with *C. pipiens pallens* found significant correlation between *kdr* allelic frequency and the LC_{50} estimates of Es-bioallethrin (r^2= 0.947), deltamethrin (r^2= 0.747) and betacypermethrin (r^2= 0.967).

While we have shown that the target SNP associated with pyrethroid resistance is very important in the evolution of pyrethroid resistance development in field populations of *R. (B.) microplus* in Yucatan, Mexico, it is unlikely to be the sole mechanism responsible for such a widespread phenomenon as knock down-mediated resistance. In particular, more than 10 sodium channel mutations have already been identified as being involved in reducing channel sensitivity to insecticides (Soderlund and Knipple, 2003). It has previously been reported that resistance to pyrethroids has been associated with enhanced carboxylesterase-mediated metabolic detoxification in several insect pests including *R. (B.) microplus* (Rosario-Cruz et al., 1997). However, we did not test this hypothesis in our population of *R. (B.) microplus* in this study. Further studies to verify the role of this metabolic mechanism in pyrethroid resistance are recommended.

The RFs as well as the frequencies of resistance vary greatly between populations. This situation makes resistance management much more difficult over large geographic areas in the Mexican tropics. Based on the high frequency of the R allele in the tolerant and resistant populations, continued intensive use of pyrethroids to control *R. (B.) microplus* will severely aggravate the resistance problem by further selecting against the remaining susceptible alleles. Since the SNP target is expected to confer cross-resistance to all other pyrethroids, switching to alternative pyrethroids may not be a useful option.

The AS-PCR assays can quickly yield information about the current status of genotypic resistance to cypermethrin in field populations of *R. (B.) microplus*. Furthermore, the AS-PCR was able to detect the presence of the R allele in susceptible populations, which are unlikely to be revealed by standard bioassays and which can lead to product failure in subsequent generations following selection pressure by pesticide applications. The AS-PCR may constitute a valuable molecular tool for the rapid monitoring of the frequency of the target SNP associated with pyrethroid resistance in field populations of *R. (B.) microplus*. This would allow an early detection of the target SNP to prevent the

spread of such a resistant phenomenon at the state level. It would also improve the control of the populations that have already developed this highly specific resistance to pyrethroid by recommending the use of other compounds or alternative control methods such as biological control (Alonso et al., 2007), anti-tick vaccine (de la Fuente et al., 2007a), resistant breeds (Bianchi et al., 2003) and farm management (Kunz and Kemp, 1994). Regular monitoring of phenotype and genotype would help the National Tick Campaign to identify and recommend the best available treatment of *R.* (*B.*) *microplus* in the Mexican tropics.

In conclusion, both bioassay and AS-PCR clearly show that the prevalence of *R.* (*B.*) *microplus* resistant-tolerant to cypermethrin (judged by LC_{50} and LC_{99}) is high in Yucatan. A clear correlation between the RF and the frequency of the R allele was found, confirming for the second time (**see chapter 4**) that the target SNP is one of the most important mechanism that confer pyrethroid resistance in the studied *R.* (*B.*) *microplus* populations.

CHAPTER 6

Prospective interventional study: Changes in pyrethroid resistance phenotype (resistance factor) and genotype (frequency of the resistance allele) in response to presence or absence of pyrethroid selection pressure in field populations of *Rhipicephalus* (*Boophilus*) *microplus*

6.1. Introduction

The development of acaricide resistance in a tick population is dependent on the frequency of occurrence of resistant individuals in the population and the intensity of chemical selection pressure (Kunz and Kemp, 1994). In epidemiological studies to determine the risk factors associated with *R. (B.) microplus* resistance to pyrethroids, Jonsson et al. (2000) and Rodriguez-Vivas et al. (2006a) found higher probability of resistance to deltamethrin, flumethrin and cypermethrin in tick populations when pyrethroids were used >5 times/year. Rapid onset and development of *R. (B.) microplus* resistant to pyrethroids have been observed in controlled laboratory trials (Coetzee et al., 1987; Davey and George, 1998). Harris et al. (1988) and Davey et al. (2003) conducted studies to generate resistance in *R. (B.) microplus* to OPs under laboratory conditions, they found during the selection process that the OP strains became 38 and 12-times more resistant than the control groups, respectively. Li et al. (2004) developed a selection pressure using amitraz on larvae of a *R. (B.) microplus* strain. The strain was challenged with various concentrations of amitraz after its establishment in the laboratory. The strain responded to selection quickly, and the RF was elevated from 13.3 in F_1 to 154 in F_6. Recently, in a field study conducted in the Mexican tropics, Rosado-Aguilar et al. (2008) applied selection pressure to three field populations of *R. (B.) microplus* with amitraz. After 15 months of amitraz selection pressure all populations increased their RF (from 1 to 13, from 1 to 22, and from 2 to 6).

Rapid onset and development of SP resistance in controlled field trials was observed by Coetzee et al. (1987) who reported that the development of resistance to fenvalerate in *R. (B.) decoloratus* occurred during an 18-month period (in 5-6 tick generations). Furthermore, in another controlled pen trial, Davey and George (1998) were able to select an *R. (B.) microplus* strain for resistance to permethrin by treating larvae with increasing doses through successive generations (generations F_2-F_7). At the beginning of the selection process (F_2), the SP resistant strain was 5.4 times more resistant to permethrin than the SP susceptible strain, and the level of resistance increased in each successive generation of the SP resistant strain, reaching a RF of 20.9 in the F_7 generation. The results demonstrated that under continuous selection pressure the tick population increased resistance in a relatively short time. However, the development of

SP resistance in populations under normal field conditions has not been reported to our knowledge.

Fitness costs associated with insecticide resistance have been documented in many pest species (Roush and McKenzie, 1987; Coustau et al., 2000; Oliveira et al., 2007). The reproductive fitness of *R.* (*B.*) *microplus* strains resistant to OPs, SPs or amitraz was compared to an acaricide-susceptible strain to determine whether the acquisition of resistance affected reproductive fitness in the resistant strains (Davey et al., 2006). The authors found that the OP-resistant strain produced 30% fewer eggs than the susceptible strain indicating the acquisition of resistance placed the OP-resistant at a selective disadvantage relative to the susceptible strain. The fitness cost of amitraz and SP-resistant strains was not found. However, in Mexico, the level of resistance of *R.* (*B.*) *microplus* to amitraz in the San Alfonso strain decreased from 42-fold to 10-fold after six generations on laboratory colonization without selection (Soberanes et al., 2002). Given the high prevalence of SP resistance in *R.* (*B.*) *microplus* reported in **chapter 5** it is important to appreciate the effect of continuous use of SP in these tick populations in Mexico. The present study evaluates the evolution of resistance phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *R.* (*B.*) *microplus.*

6.2. Experimental Aims and Methods

6.2.1. Aim of the Study

In this section we present a prospective controlled intervention study to measure the evolution of the resistant phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *R.* (*B.*) *microplus.*

6.2.2. Study Design

Based on the results of Rodriguez-Vivas et al. (2006a), 11 cattle farms (**Figure 6.1.**) from the state of Yucatan, Mexico (**see section 2.3.**) with different larval mortality in

the larval packet test exposed to a discriminating dose of cypermethrin were selected (**Figure 6.1.**). On each farm a sample of 30-50 engorged adult female *R.* (*B.*) *microplus* ticks was collected from at least 10 animals (**Section 2.4.**). The phenotypic response of each *R.* (*B.*) *microplus* population to cypermethrin was measured by dose-response bioassays by the modified larval packet test using different dilutions of technical grade cypermethrin (**see section 2.5.**). For genotypic response of *R.* (*B.*) *microplus* to cypermethrin the AS-PCR was carried out as described in **section 2.6** on 30-42 larvae from each farm. The RFs judged by LC_{50} and LC_{99} and the frequency of the R allele was calculated as described in **section 2.7.** The pyrethroid resistance phenotype and genotype of the 11 tick populations are present in **Table 6.1.**

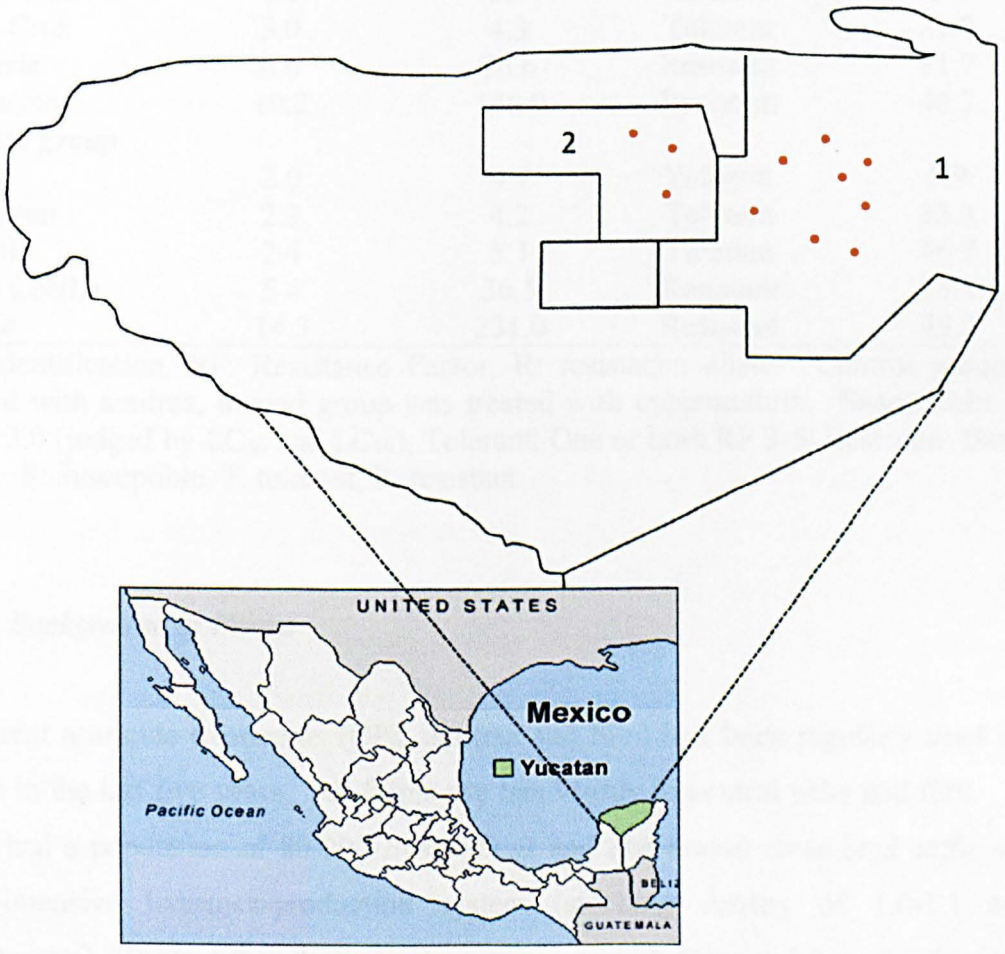

Figure 6.1. Map showing the locality of 11 farms (red dots) in Yucatan, Mexico (1. Tizimin, 2. Panaba), where tick populations were taken to evaluate phenotypic (resistance factor) and genotypic (frequency of the resistance allele) changes of resistance to cypermethrin in response to presence or absence of selection pressure on field populations of *Rhipicephalus* (*Boophilus*) *microplus*.

Based on the phenotype and genotype results, two groups of populations were formed. San Agustin, Blanca Flor, San Roman Santa Cruz, Lecheria and Xnohayan tick populations were selected as a control group, and Akula, Moluxtun, Kantok, Santa Cecilia and Alamo tick populations as a treated group.

Table 6.1. Phenotype and genotype resistance to cypermethrin in 11 populations of *Rhipicephalus* (*Boophilus*) *microplus* from Yucatan, Mexico, before the trial started.

ID of tick population	RF (LC_{50})	RF (LC_{99})	Phenotype	R allele Frequency
Control group				
San Agustin	1.0	1.1	Susceptible	0
Blanca Flor	1.1	2.9	Susceptible	0
San Roman	1.1	3.5	Tolerant	1.6
Santa Cruz	3.0	4.3	Tolerant	21.7
Lecheria	6.6	26.6	Resistant	81.7
Xnohayan	10.2	148.9	Resistant	46.7
Treated group				
Akula	2.0	4.7	Tolerant	5.9
Moluxtun	2.3	4.2	Tolerant	33.3
Kantok	2.4	5.1	Tolerant	46.7
Santa Cecilia	5.4	36.5	Resistant	35.5
Alamo	14.3	231.0	Resistant	45.2

ID: Identification, RF: Resistance Factor, R: resistance allele. Control group was treated with amitraz, treated group was treated with cypermethrin. Susceptible: Both RF < 3.0 (judged by LC_{50} and LC_{99}), Tolerant: One or both RF 3-5, Resistant: Both RF ≥ 5.0. S: Susceptible, T: tolerant, R: resistant.

6.2.3. Background of Farms

Different acaricide treatments (OPs, amitraz and SPs) had been regularly used on all farms in the last five years. All farms use hand-spray to control ticks and flies. Each farm had a population of 80-200 *Bos indicus* and *Bos taurus* cross-bred cattle with a semi-intensive livestock-production system (stocking density of 1.0-1.1 animal unit/hectare) (**Table 6.2.**). Individual pastures on each farm and between farms were divided by fences. During the study, movement of animals carrying ticks (>5 animals) between neighboring farms was not reported.

Table 6.2. Characteristics of the farms selected to study cypermethrin resistance on field populations of *Rhipicephalus* (*Boophilus*) *microplus* ticks in the state of Yucatan, Mexico.

Farms	Land Area	No. Cattle	Stocking Density	No. of paddocks	Acaricide used in the last five years
Control group					
San Agustin	440	300	1.46	10	OP, amitraz
Blanca Flor	450	320	1.40	12	OP, amitraz
San Roman	430	300	1.43	12	OP, amitraz
Santa Cruz	350	340	1.02	7	SP, OP, amitraz
Lecheria	120	120	1.00	7	SP, OP, amitraz
Xnohuayan	320	270	1.18	10	SP, OP, amitraz
Treated group					
Akula	300	280	1.07	12	OP, amitraz
Moluxton	170	160	1.06	8	OP, amitraz
Kantok	370	310	1.19	11	SP, OP, amitraz
Santa Cecilia	140	135	1.03	7	SP, OP, amitraz
Alamo	320	295	1.08	10	SP, OP, amitraz

OP: Organophosphate, SP: Synthetic pyrethroid. Control group was treated with amitraz, treated group was treated with cypermethrin.

6.2.4. Acaricide Management and Sampling

All animals (cows, heifers, steers, and bulls) from the control group were treated with amitraz over 24 months (Trak®, Lapisa, Mexico) at the recommended dose (12.5 % active ingredient). This group of farms was used as control, because in Mexican field conditions it would be inappropriate to leave an untreated group due to the high tick infestation level and risk of tick-borne disease transmission (Solorio-Rivera et al., 1999; Rodriguez-Vivas et al., 2004). Amitraz is the main member of the formamidines acaricides and causes neural hyperexitability and death in ticks (George et al., 2004). There is strong evidence that the octopamine receptor is the target site of amitraz (Baxter and Barker, 1999) and no cross-mechanisms of resistance in *R.* (*B.*) *microplus* between amitraz and pyrethroids has been reported, for that reason, amitraz treatment was used as control in this study. Animals from the treated group were treated with cypermethrin over 23 months (Ticoff®, Lapisa, Mexico) at the recommended dose (0.2 % active ingredient). Both groups were treated as a whole body spray using at least 4 l of total finished spray volume per animal, every 30-45 days. During the entire duration of the study (24 months) eight *R.* (*B.*) *microplus* generations will have occurred (Rodriguez-Vivas and Dominguez-Alpizar, 1998). A sample of 30-50 engorged adult

female *R.* (*B.*) *microplus* ticks was collected every 6-4 months from at least 10 animals on each farm (**Section 2.4.**). Tick collection on each farm and production of *R.* (*B.*) *microplus* larvae were carried out as was described in **section 2.4.**

6.2.5. Phenotypic Analysis by Dose-Response Bioassay and Genotypic Analysis by Allele-Specific Polymerase Chain Reaction

The phenotypic response of each *R.* (*B.*) *microplus* population to cypermethrin was measured by dose-response bioassays by the modified larval packet test using different dilutions of technical grade cypermethrin (**see section 2.5.**). For genotypic response of *R.* (*B.*) *microplus* to cypermethrin AS-PCR was carried out as described in **section 2.6.** The RFs judged by LC_{50} and LC_{99} and the frequency of the R allele were calculated as described in **section 2.7.** On each farm, at each time of sampling 30-37 individual larvae were genotyped.

6.2.6. Data Analysis

Probit analysis was performed on dose-response bioassay results using *Polo-Plus* (LeOra Software, 2003) and RF judged by LC_{50} and LC_{99} were calculated (**see section 2.7.**). Susceptible populations were considered when both RF values (judged by LC_{50} and LC_{99}) were < 3.0 and resistant populations when RF values were ≥ 5.0 (Beugnet and Chardonnet, 1995). Tolerant populations were considered when one or both RF values were 3-5. At the beginning of the study, in the control tick populations (receiving amitraz treatment), four populations were classified as susceptible or tolerant (San Agustin, Blanca Flor, San Roman and Santa Cruz) to cypermethrin and two populations as resistant (Lecheria and Xnohuayan); in the treated populations (receiving cypermethrin treatment), three populations were tolerant (Akula, Moluxtun and Kantok) and two resistant (Santa Celilia and Alamo).

To evaluate changes in allele frequency over time on each tick population a contingency table using chi-square test was used. To investigate the correlation between the level of phenotypic pyrethroid resistance and the mutated allele frequency, the RFs of each

population were plotted against the frequency of R allele, and linear regression lines were generated.

6.3. Results

The probit analysis results to determine susceptibility to cypermethrin in farms with *R. (B.) microplus* populations receiving amitraz treatment are shown in **Table 6.3.** At the beginning of the study, the tick populations of *R. (B.) microplus* classified as susceptible or tolerant to cypermethrin show the slope of the probit line >2. After 24 months without cypermethrin treatment, those populations remained with the same status with few changes to their RFs to cypermethrin (range from 0.5 to 3.0 judged by LC_{50}, and from 0.3 to 4.3 judged by LC_{99}) and slopes > 2 (from 2.0 to 6.5) (**Figures 6.2.** and **6.3.**). In the two resistant populations at the beginning of the study, after 24 months without cypermethrin treatment, their RF values to cypermethrin ranged from 3.3 to 13.6 judged by LC_{50} and from 21.8 to 285.2 judged by LC_{99}, with slopes of the probit line <2.

The probit analysis results to determine susceptibility to cypermethrin in farms with *R. (B.) microplus* populations receiving cypermethrin are shown in **Table 6.4.** After 8-23 months of cypermethrin treatments, in all populations (Akula, Alamo, Moluxtun, Kantok and Santa Cecilia) the changes in the RF values judged by LC_{50} and both LCs, although not always uniform, were steadily upward (**Figures 6.4** and **6.5.**). The slopes of the probit line decreased, indicating that a higher concentration of cypermethrin is required to kill 99 % of the tick population in those populations after selection pressure. Populations at the beginning of the study that were susceptible or tolerant (Akula, Moluxtun and Kantok), after 23 months of cypermethrin treatment, increased their RFs (Akula: 6.4-fold, Moluxtun: 19.7-fold and Kantok: 2.1-fold) and became resistant. Two populations at beginning of the study that were resistant (Santa Cecilia and Alamo) developed high resistance rapidly and cypermethrin treatments were suspended after a short period of time when *R. (B.) microplus* engorged females were observed after treatments and a lack of efficacy was claimed by owners. Santa Cecilia population reached a very high cypermethrin resistance level. After continued selection pressure with cypermethrin the RFs in the five populations had increased 2-125 folds judged by LC_{50}.

Table 6.3. Sequential determinations of resistance phenotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where amitraz was exclusively used for tick control for 24 months.

Populations	Bioassay						
	50 %			99 %			
Month of Sampling	LC	CL95%	RF**	LC	CL95%	RF**	Slope
San Agustin							
0*	0.014	0.007-0.022	1.0a	0.054	0.044-0.075	1.1a	4.04
4	0.009	0.008-0.010	0.6ab	0.027	0.023-0.044	0.5a	5.17
8	0.019	0.018-0.021	1.4ac	0.098	0.082-0.121	2.1b	3.29
14	0.008	0.007-0.009	0.6ab	0.018	0.016-0.022	0.3c	6.51
19	0.008	0.007-0.009	0.6ab	0.055	0.045-0.075	1.1a	2.75
24	0.014	0.013-0.016	1.0ac	0.059	0.046-0.086	1.2a	3.79
Blanca Flor							
0*	0.015	0.007-0.022	1.1a	0.133	0.115-0.325	2.9a	2.21
4	0.022	0.017-0.027	1.6ab	0.199	0.134-0.364	4.3a	2.41
8	0.020	0.018-0.023	1.5ab	0.096	0.073-0.146	2.0a	3.43
14	0.007	0.005-0.008	0.5ac	0.031	0.026-0.041	0.6b	3.45
19	0.008	0.007-0.009	0.6ac	0.030	0.026-0.037	0.6b	4.23
24	0.021	0.018-0.024	1.6ab	0.115	0.760-0.173	2.5a	3.30
San Roman							
0*	0.015	0.007-0.022	1.1a	0.163	0.115-0.325	3.5a	2.21
4	0.021	0.020-0.023	1.6ab	0.119	0.099-0.150	2.5a	3.12
8	0.019	0.016-0.021	1.4ab	0.166	0.128-0.235	3.6a	2.45
14	0.008	0.007-0.009	0.6ac	0.055	0.045-0.071	1.1b	2.81
19	0.008	0.007-0.009	0.6ac	0.055	0.045-0.075	1.1b	2.75
24	0.019	0.014-0.023	1.4ab	0.189	0.125-0.367	4.1a	2.31
Santa Cruz							
0*	0.040	0.035-0.050	3.0a	0.202	0.179-1.155	4.3a	2.01
4	0.024	0.022-0.026	1.8bc	0.131	0.105-0.179	2.8a	3.16
8	0.019	0.015-0.022	1.4bc	0.164	0.078-0.267	3.5a	2.02
14	0.028	0.025-0.031	2.1bd	0.183	0.145-0.246	3.9a	2.83
19	0.012	0.009-0.014	0.9be	0.168	0.124-0.255	3.6a	2.02
24	0.028	0.025-0.031	2.1bd	0.160	0.127-0.217	3.4a	3.04
Lecheria							
0*	0.087	0.066-0.110	6.6a	1.316	0.779-3.045	26.6a	1.97
4	0.051	0.035-0.068	3.9a	1.007	0.608-2.244	21.8a	1.79
8	0.043	0.033-0.055	3.3b	1.344	0.765-3.132	29.2a	1.55
14	0.046	0.031-0.063	3.5b	3.120	1.324-13.86	67.8a	1.26
19	0.046	0.032-0.063	3.5b	3.397	1.451-14.43	73.8a	1.24
24	0.048	0.034-0.066	3.6a	3.051	1.430-10.24	66.3a	1.29
0	0.052	0.041-0.067	4.0a	2.731	1.468-6.770	59.3a	1.35
Xnohuayan							
0*	0.133	0.094-0.220	10.2a	6.851	2.221-53.34	148.9a	1.35
4	0.178	0.149-0.213	13.6a	7.259	4.261-35.00	157.8a	1.44
8	0.107	0.076-0.142	8.2a	6.47	29.00-650.7	140.6a	0.78
14	0.102	0.053-0.158	7.8a	10.35	4.496-44.69	225.0a	1.16
19	0.096	0.050-0.149	7.3a	13.12	5.516-58.66	285.2a	1.09
24	0.115	0.067-0.168	8.8a	10.86	5.016-38.95	236.7a	1.17

*Bioassay obtained immediately before amitraz introduced. **Figures within column with different letters are significantly different based on the failure of 95% confidence limit to overlap. LC: Lethal concentration, CL95%: 95% confidence limit. RF: Resistance factor (evaluated population LC_{50}/ Media Joya-CENAPA susceptible reference strain LC_{50}). In the bioassay the Media Joya-CENAPA strain had the following values: LC_{50}: 0.013 (CL95%: 0.011-0.014) and LC_{99}: 0.046 (CL95%: 0.011-0.014).

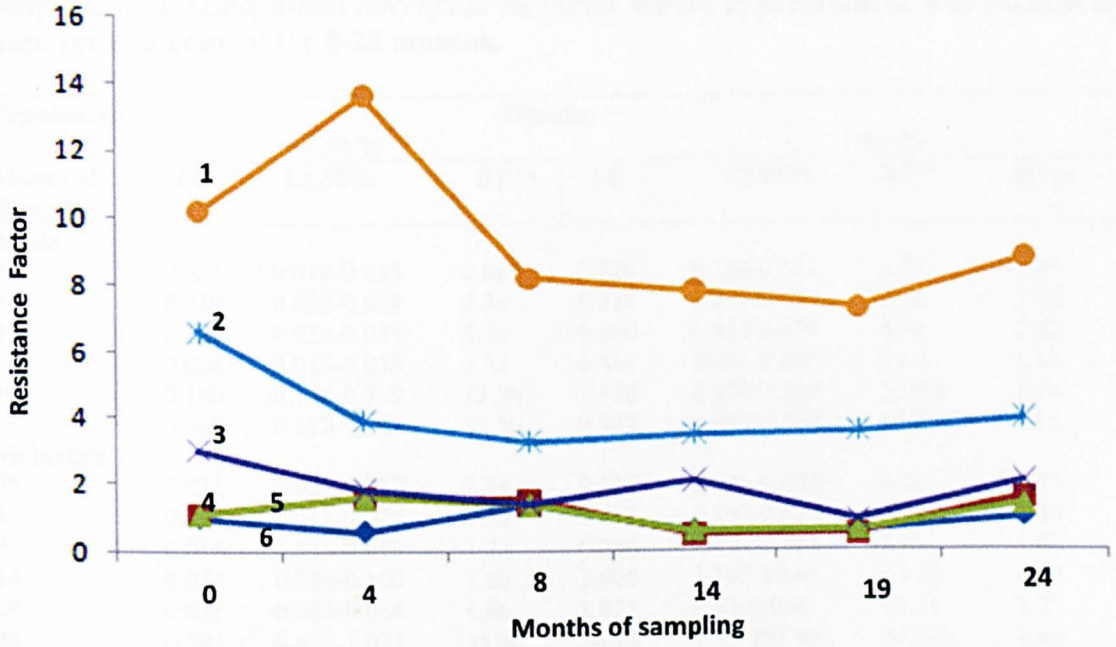

Figure 6.2. Sequential determination of resistance factors (Determined by the LC_{50}) of *Rhipicephalus* (*Boophilus*) *microplus* on farms where amitraz was exclusively used for tick control for 24 months. 1) Xnohuayan, 2) Lecheria, 3) Santa Cruz, 4) Blanca Flor, 5) San Roman and 6) San Agustin.

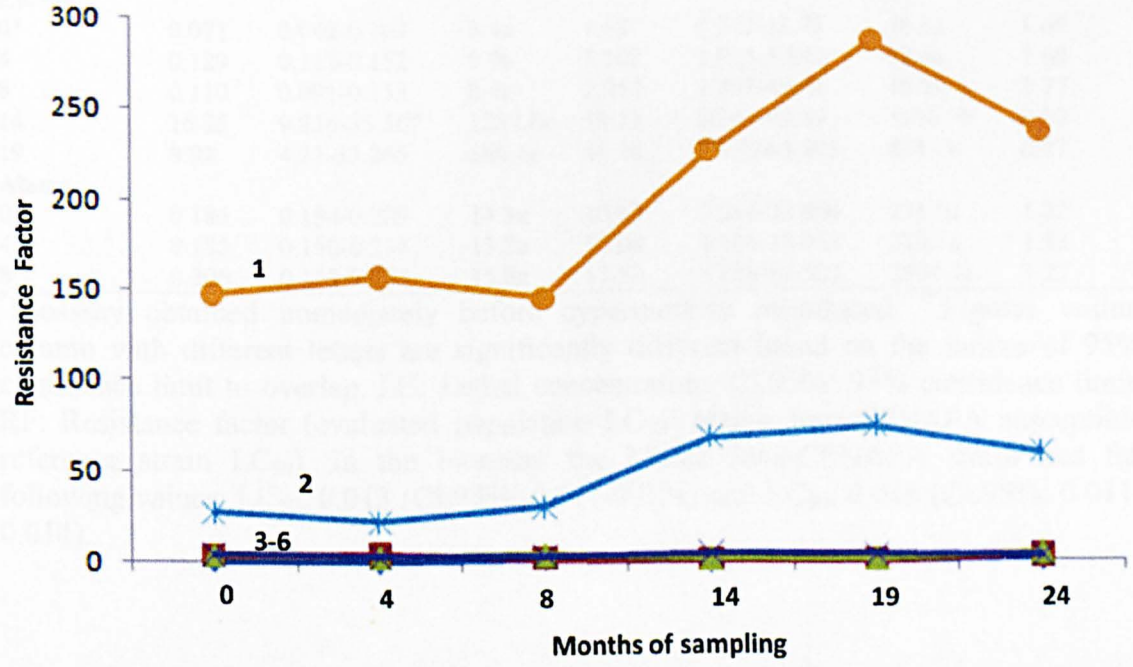

Figure 6.3. Sequential determination of resistance factors (Determined by the LC_{99}) of *Rhipicephalus* (*Boophilus*) *microplus* on farms where amitraz was exclusively used for tick control for 24 months. 1) Xnohuayan, 2) Lecheria, 3-6) San Agustin, Blanca Flor, San Roman and Santa Cruz.

Table 6.4. Sequential determinations of resistance phenotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where cypermethrin was exclusively used for tick control for 8-23 months.

Populations	Bioassay						
	50 %			99 %			
Month of Sampling	LC	CL95%	RF**	LC	CL95%	RF**	Slope
Akula							
0*	0.027	0.018-0.035	2.0a	0.220	0.160-0.537	4.7a	2.45
4	0.030	0.026-0.034	2.3a	0.274	0.211-0.384	5.9a	2.42
8	0.029	0.024-0.033	2.2a	0.300	0.215-0.470	5.5a	2.28
14	0.024	0.016-0.035	1.8a	0.661	0.305-2.886	14.3a	1.62
19	0.180	0.156-0.209	13.8b	1.150	0.890-3.220	25.0.b	1.14
23	0.167	0.112-0.203	12.8b	2.982	1.880-6.207	64.8b	1.85
Moluxtun							
0*	0.031	0.026-0.037	2.3a	0.197	0.196-0.539	4.2a	2.37
4	0.018	0.013-0.022	1.3a	0.312	0.197-0.630	6.7a	1.86
8	0.016	0.013-0.019	1.2a	0.296	0.198-0.518	6.4a	1.81
14	0.073	0.056-0.100	5.6b	2.906	1.297-10.45	64.3b	1.45
19	0.053	0.044-0.064	4.0b	3.821	0.41-6.068	83.0b	1.25
23	0.591	0.422-1.074	45.4c	24.95	7.50-257.45	542.3c	1.43
Kantok							
0*	0.032	0.023-0.039	2.4a	0.235	0.162-0.460	5.1a	2.67
4	0.022	0.018-0.026	1.6a	0.147	0.105-0.243	3.1a	2.80
8	0.005	0.002-0.007	0.3b	0.050	0.040-0.074	1.0b	2.27
14	0.005	0.001-0.010	0.3b	0.207	0.118-0.662	4.5a	1.45
19	0.021	0.018-0.025	1.6a	0.145	0.104-0.238	3.1a	2.79
23	0.066	0.050-0.090	5.0c	3.212	1.369-12.663	69.8c	1.37
Santa Cecilia							
0*	0.071	0.042-0.103	5.4a	1.68	0.713-11.73	36.5a	1.69
4	0.129	0.112-0.152	9.9b	3.102	1.925-5.840	67.4a	1.68
8	0.110	0.091-0.133	8.4a	2.253	1.357-4.641	48.9a	1.77
14	16.35	9.816-35.567	1257.8c	53.22	36.43-99.39	1156.9b	0.92
19	8.92	4.31-33.265	686.1c	41.16	3.656-65.285	894.7b	0.87
Alamo							
0*	0.186	0.154-0.229	14.3a	10.63	5.014-32.864	231.0a	1.32
4	0.185	0.150-0.234	13.2a	10.08	4.484-35.953	219.1a	1.33
8	0.206	0.162-0.275	15.8a	13.52	5.188-67.503	2939.1a	1.27

*Bioassay obtained immediately before cypermethrin introduced. **Figures within column with different letters are significantly different based on the failure of 95% confidence limit to overlap. LC: Lethal concentration, CL95%: 95% confidence limit. RF: Resistance factor (evaluated population LC_{50}/ Media Joya-CENAPA susceptible reference strain LC_{50}). In the bioassay the Media Joya-CENAPA strain had the following values: LC_{50}: 0.013 (CL95%: 0.011-0.014) and LC_{99}: 0.046 (CL95%: 0.011-0.014).

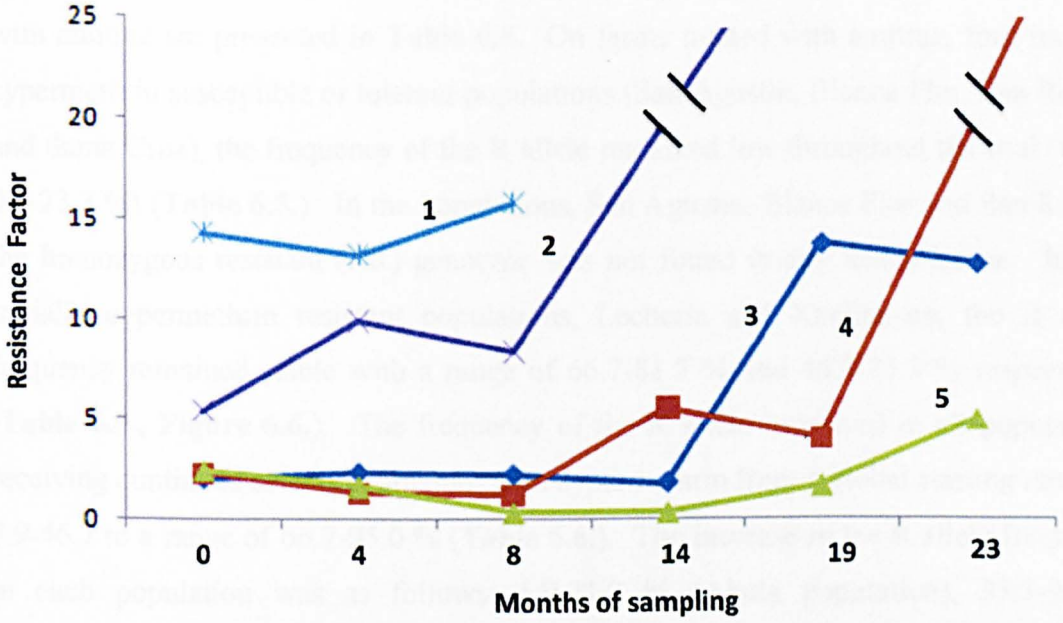

Figure 6.4. Sequential determination of resistance factors (Determined by the LC_{50}) of *Rhipicephalus* (*Boophilus*) *microplus* on farms where cypermethrin was exclusively used for tick control up to 23 months. 1) Alamo, 2) Santa Cecilia, 3) Akula, Moluxtun, 5) Kantok.

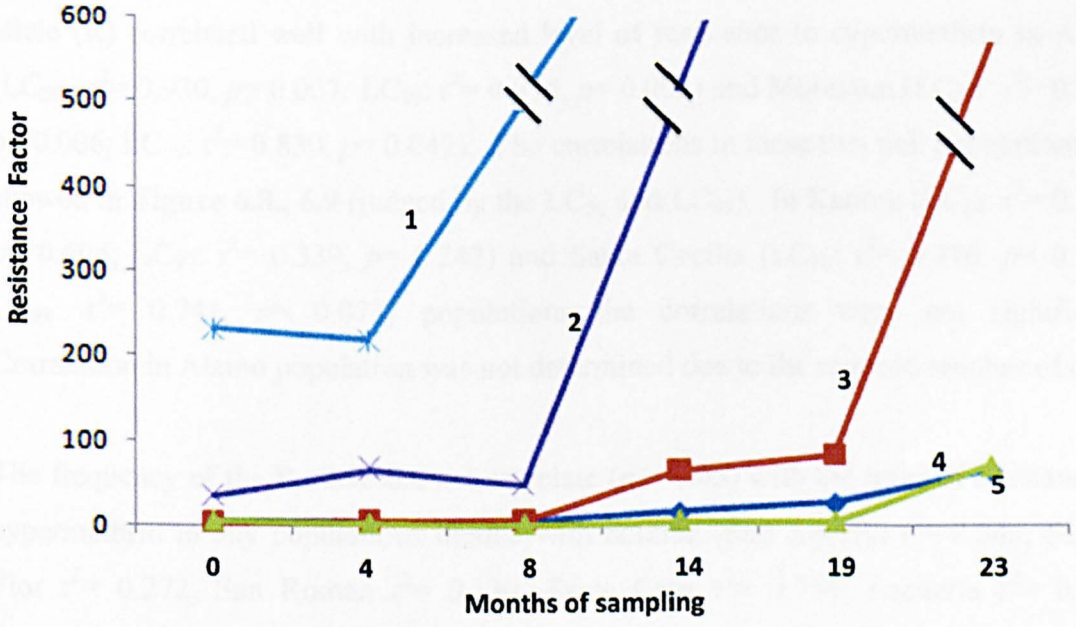

Figure 6.5. Sequential determination of resistance factors (Determined by the LC_{99}) of *Rhipicephalus* (*Boophilus*) *microplus* on farms where cypermethrin was exclusively used for tick control up to 23 months. 1) Alamo, 2) Santa Cecilia, 3) Akula, 4) Moluxtun, 5) Kantok.

The *R. (B.) microplus* genotype associated with pyrethroid resistance, expressed as the presence of substituted sodium channel or wild-type alleles recorded in farms treated with amitraz are presented in **Table 6.5.** On farms treated with amitraz, four initially cypermethrin susceptible or tolerant populations (San Agustin, Blanca Flor, San Roman and Santa Cruz), the frequency of the R allele remained low throughout the trial (range 0.0-23.3 %) (**Table 6.5.**). In the populations, San Agustin, Blanca Flor and San Roman the homozygous resistant (RR) genotype was not found in any tested larvae. In two initially cypermethrin resistant populations, Lecheria and Xnohuayan, the R allele frequency remained stable with a range of 66.7-81.7 % and 46.7-73.3 % respectively (**Table 6.5., Figure 6.6.**). The frequency of the R allele increased in all populations receiving continued selection pressure with cypermethrin from a global starting range of 5.9-46.7 to a range of 66.7-95.0 % (**Table 6.6.**). The increase in the R allele frequency in each population was as follows: 5.9-71.9 % (Akula population), 33.3-95.0% (Moluxtun population), 46.7-71.7% (Kantok population), 35.5-81.7 (Santa Cecilia population) and 45.2-66.7 % (Alamo population) (**Table 6.6.** and **Figure 6.7.**).

On two farms where cypermethrin was used the increasing presence of the mutated allele (R) correlated well with increased level of resistance to cypermethrin in Akula (LC_{50}: $r^2= 0.930$, $p= 0.007$; LC_{99}: $r^2= 0.918$, $p= 0.002$) and Moluxtun (LC_{50}: $r^2= 0.993$, $p= 0.006$; LC_{99}: $r^2= 0.830$, $p= 0.042$). The correlations in these two tick populations are showed in **Figure 6.8., 6.9** (judged by the LC_{50} and LC_{99}). In Kantok (LC_{50}: $r^2= 0.270$, $p= 0.604$; LC_{99}: $r^2= 0.339$, $p= 0.242$) and Santa Cecilia (LC_{50}: $r^2= 0.776$, $p= 0.127$; LC_{99}: $r^2= 0.741$, $p= 0.073$) populations the correlations were not significant. Correlation in Alamo population was not determined due to the reduced number of data.

The frequency of the R allele did not correlate ($p > 0.05$) with the level of resistance to cypermethrin in any populations treated with amitraz (San Agustin $r^2= 0.343$, Blanca Flor $r^2= 0.272$, San Roman $r^2= 0.130$, Santa Cruz $r^2= 0.731$, Lecheria $r^2= 0.671$, Xnohuayan $r^2= 0.063$).

Table 6.5. Sequential determinations of resistance genotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where amitraz was exclusively used for tick control for 24 months.

Months of sampling	PCR genotype assay				
	No. Tested Larvae	SS Total (%)	RS Total (%)	RR Total (%)	Frequency R allele**
San Agustín					
0*	30	30 (100)	0 (0.0)	0 (0.0)	0.0a
4	31	31 (100)	0 (0.0)	0 (0.0)	0.0a
8	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0a
14	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0a
19	30	29 (96.7)	1 (3.3)	0 (0.0)	1.6a
24	30	26 (86.7)	4 (13.3)	0 (0.0)	6.7a
Blanca Flor					
0*	30	30 (100)	0 (0.0)	0 (0.0)	0.0a
4	32	31 (96.9)	1 (3.1)	0 (0.0)	0.0a
8	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0a
14	30	28 (93.3)	2 (6.6)	0 (0.0)	3.3a
19	30	25 (83.3)	5 (16.7)	0 (0.0)	8.3a
24	30	26 (86.7)	4 (13.3)	0 (0.0)	6.7a
San Roman					
0*	30	29 (96.7)	0 (0.0)	0 (0.0)	1.6a
4	30	29 (96.7)	1 (3.3)	0 (0.0)	1.6a
8	30	26 (86.7)	4 (13.3)	0 (0.0)	6.7a
14	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0a
19	30	28 (93.3)	2 (6.7)	0 (0.0)	3.3a
24	30	25 (83.3)	5 (16.7)	0 (0.0)	8.3a
Santa Cruz					
0*	30	19 (63.3)	9 (30.0)	2 (6.7)	21.7a
4	30	23 (76.7)	7 (23.3)	2 (6.7)	18.3a
8	30	22 (73.3)	7 (23.3)	1 (3.3)	15.0a
14	30	18 (60.0)	10(33.3)	2 (6.7)	23.3a
19	30	22 (73.3)	6 (20.0)	2 (6.7)	16.6a
24	30	19 (63.3)	9 (30.0)	2 (6.7)	21.7a
Lecheria					
0*	30	2 (6.7)	7 (23.3)	21 (70.0)	81.7a
4	30	2 (6.7)	11(36.7)	17 (56.7)	75.0a
8	32	3 (9.4)	13(40.6)	16 (50.0)	70.3a
14	30	2 (6.7)	13(43.3)	15 (50.0)	71.7a
19	30	3 (9.4)	14(46.7)	13 (43.3)	66.7a
24	30	1 (3.3)	17(53.1)	12 (40.0)	68.3a
	30	3 (9.4)	14(46.7)	13 (43.3)	66.7a
Xnohuayan					
0*	30	6 (20.0)	20(66.7)	4 (13.3)	46.7a
4	30	0 (0.0)	10(33.3)	16 (53.3)	70.0b
8	30	1 (3.3)	20(66.7)	9 (30.0)	63.3a
14	30	1 (3.3)	14(46.7)	15 (50.0)	73.3b
19	31	5 (16.1)	16(53.3)	10 (32.3)	60.0a
24	30	1 (3.3)	21(70.0)	8 (26.6)	61.6a

RR: resistance-resistance genotype, RS: resistance-susceptible genotype, SS: susceptible-susceptible genotype. *Genotypes obtained immediately before amitraz introduced. **Figures within column with different letters are significantly different based on the failure of 95% confidence limit to overlap.

Table 6.6. Sequential determinations of resistance genotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where cypermethrin was exclusively used for tick control for 8-24 months.

Months of sampling	PCR genotype assay				
	No. Tested larvae	SS Total (%)	RS Total (%)	RR Total (%)	Frequency R allele**
Akula					
0*	34	30 (88.2)	4 (11.8)	0 (0.0)	5.9a
4	30	22 (73.3)	8 (26.7)	0 (0.0)	13.3a
8	30	19 (63.3)	10 33.3)	1 (3.3)	20.0b
14	30	20 (66.7)	10 33.3)	2 (6.7)	21.9b
19	30	10 (31.2)	8 (26.7)	12 40.0)	53.3c
23	32	10 (31.2)	2 (6.3)	22 68.8)	71.9d
Moluxtun					
0*	30	10 (33.3)	20(66.7)	0 (0.0)	33.3a
4	30	12 (40.0)	18(60.0)	0 (0.0)	30.0a
8	30	11 (36.7)	19(63.3)	0 (0.0)	31.7a
14	30	14 (46.7)	9 (30.0)	7 (23.3)	38.3a
19	30	0 (0.0)	25(83.3)	5 (16.7)	58.3b
23	30	0 (0.0)	3 (10.0)	27(90.0)	95.0c
Kantok					
0*	30	7(23.3)	18(60.0)	5 (16.7)	46.7a
4	30	4(13.3)	21(70.0)	5 (16.7)	51.7a
8	30	2(6.7)	19(63.3)	9 (30.0)	61.7ab
14	30	2(6.7)	18(60.0)	10(33.3)	63.3ab
19	30	1(3.3)	17(56.7)	12(40.0)	66.3ab
23	30	1(3.3)	15(50.0)	14(46.7)	71.7b
Santa Cecilia					
0*	31	10 (32.3)	20(64.5)	1 (3.2)	35.5a
4	37	18 (48.7)	14(37.8)	5 (13.5)	32.4a
8	30	6 (20.0)	17(56.7)	7 (23.3)	51.7a
14	32	2 (6.3)	16(50.0)	14(43.8)	68.8b
19	30	0 (0.0)	11(36.7)	19(63.3)	81.7c
Alamo					
0*	42	16 (38.1)	14(33.3)	12(28.6)	45.2a
4	30	8 (26.7)	10(33.3)	12(40.0)	56.7a
8	30	6 (20.0)	8 (26.7)	16(53.3)	66.7b

RR: resistance-resistance genotype, RS: resistance-susceptible genotype, SS: susceptible-susceptible genotype. *Genotypes obtained immediately before cypermethrin introduced. **Figures within column with different letters are significantly different based on the failure of 95% confidence limit to overlap.

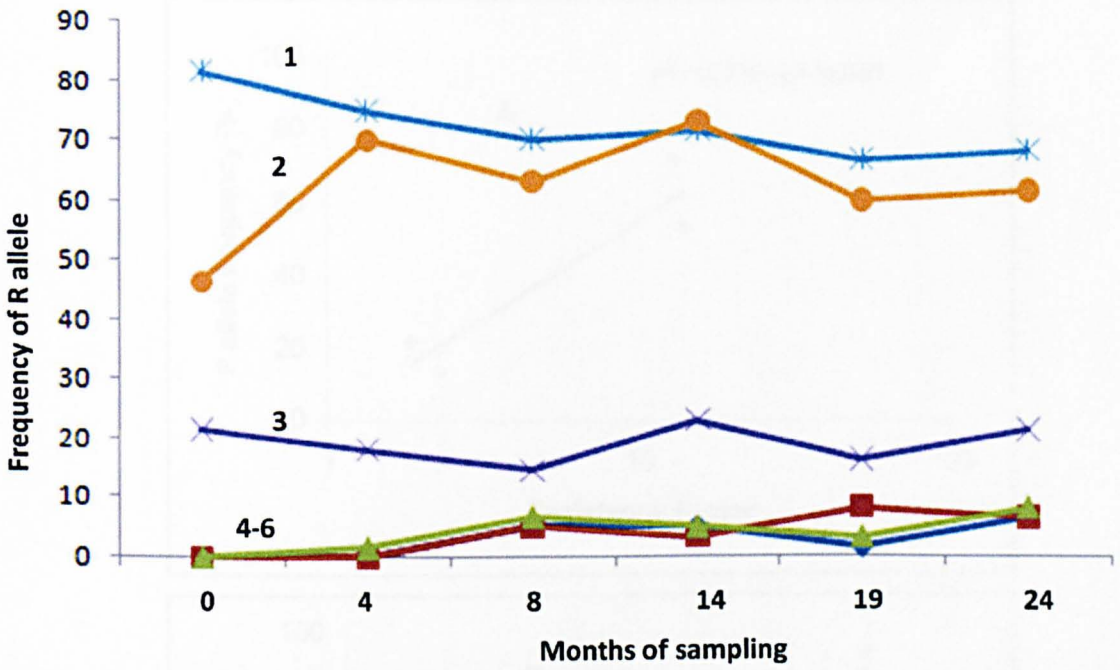

Figure 6.6. Sequential determination of resistance genotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where amitraz was exclusively used for tick control for 24 months. 1) Lecheria, 2) Xnohuayan, 3) Santa Cruz, 4) Blanca Flor, 5) San Roman and 6) San Agustin.

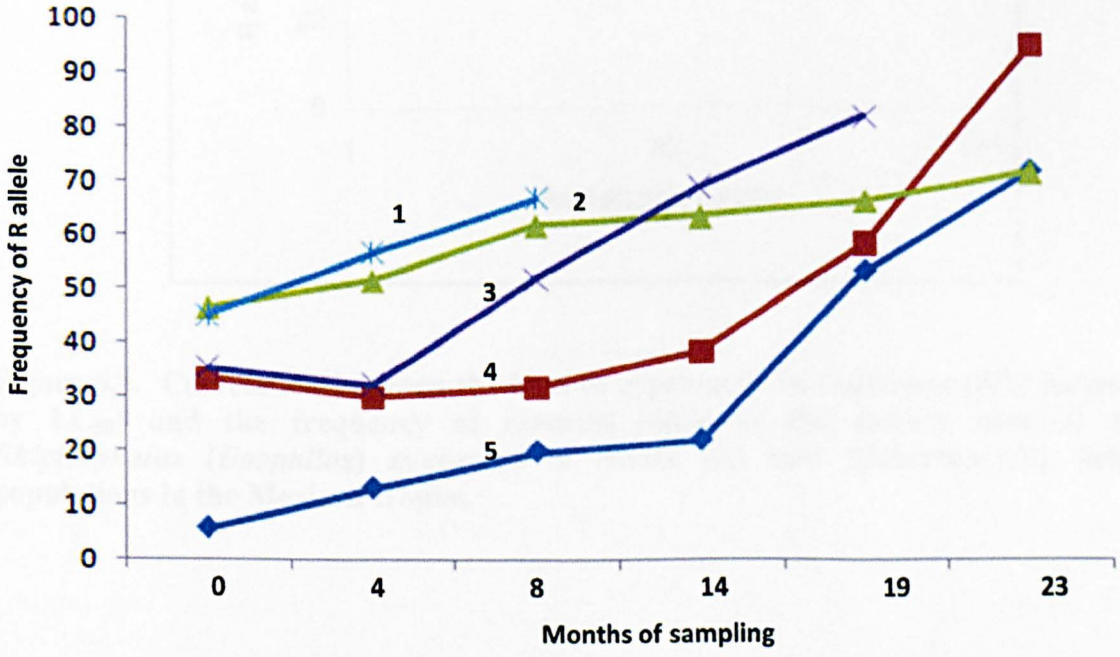

Figure 6.7. Sequential determination of resistance genotype to cypermethrin in *Rhipicephalus* (*Boophilus*) *microplus* on farms where cypermethrin was exclusively used for tick control for 8-24 months. 1) Alamo, 2) Kantok, 3) Santa Cecilia, 4) Moluxtun, 5) Akula.

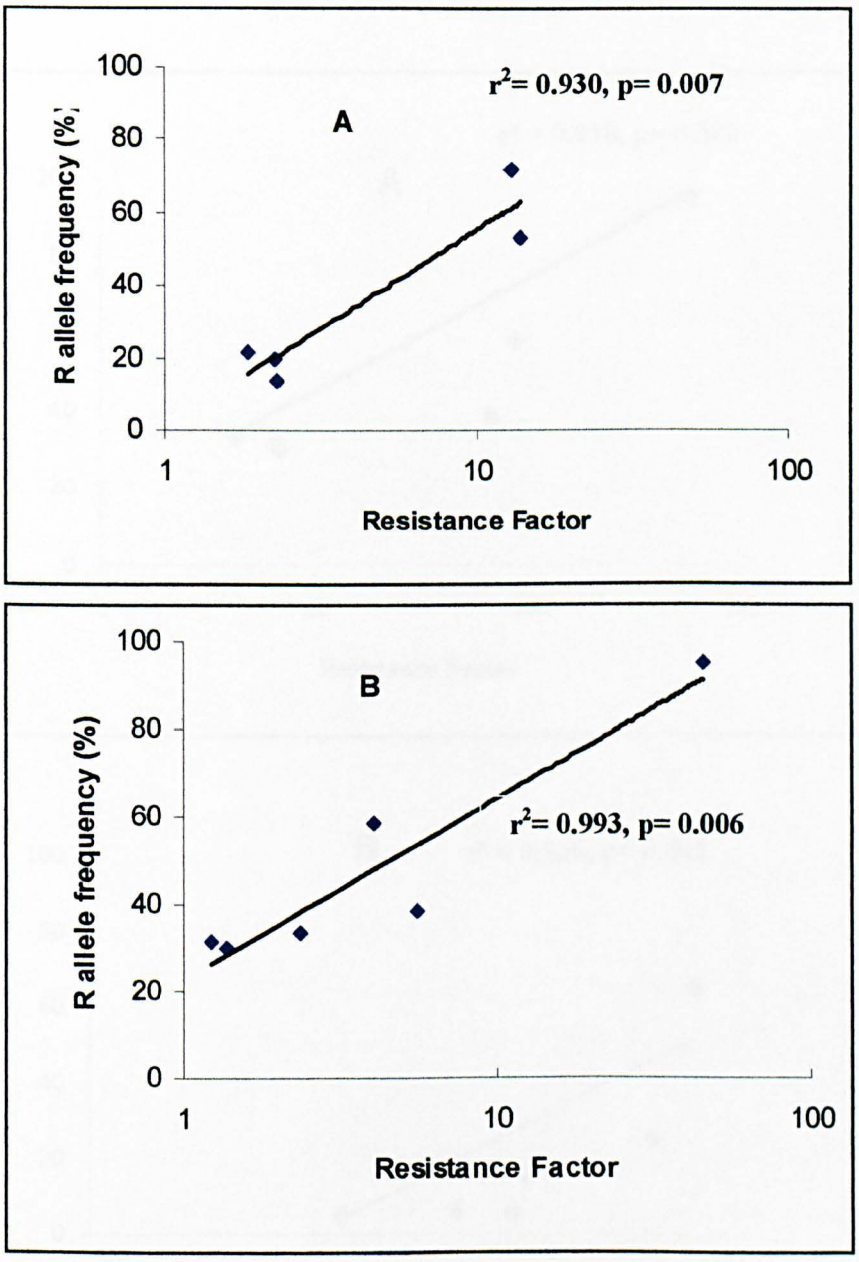

Figure 6.8. Correlation between the level of cypermethrin resistance (RFs judged by LC_{50}) and the frequency of mutated allele in the sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* in Akula (A) and Moluxtun (B) field populations in the Mexican tropics.

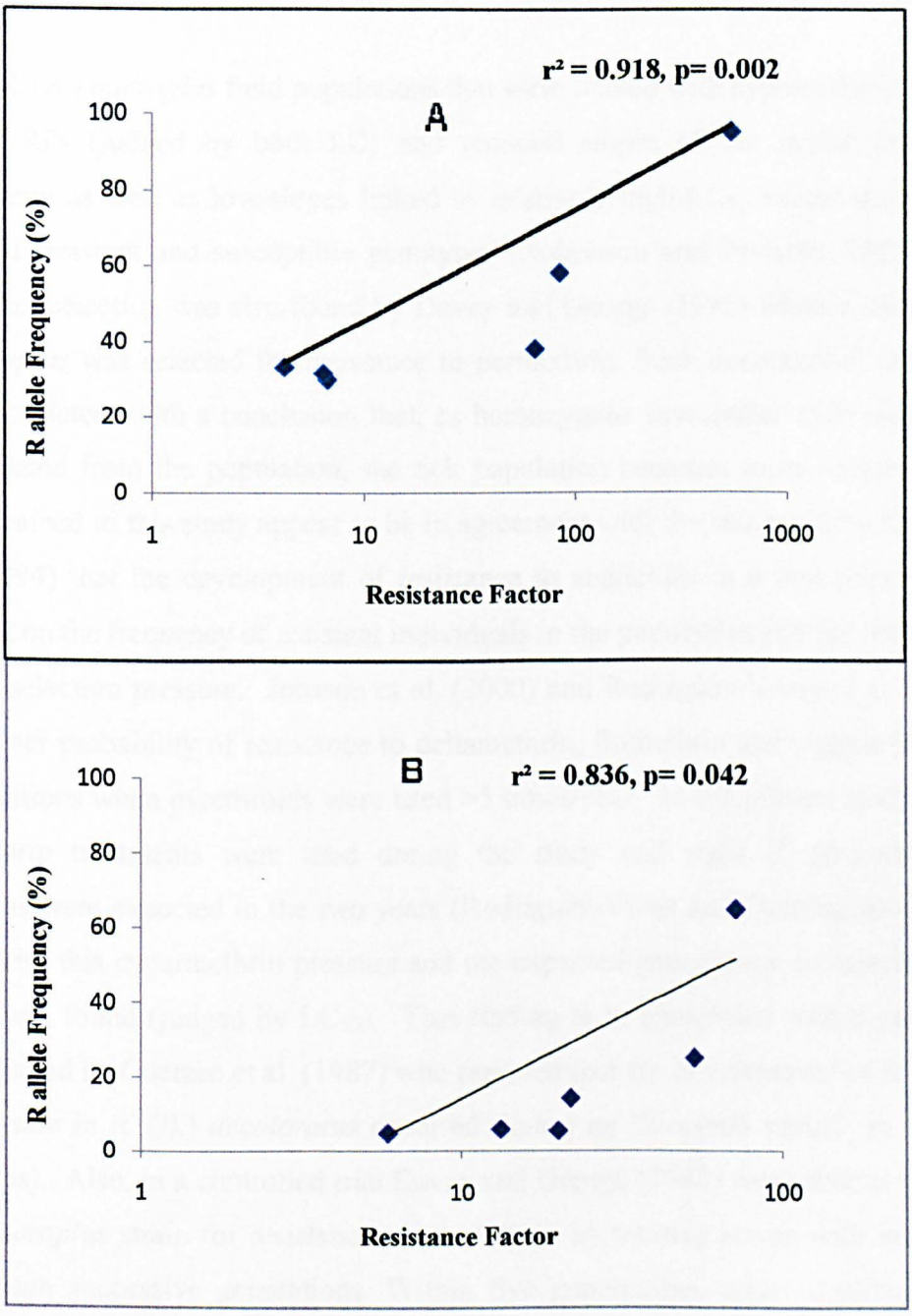

Figure 6.9. Correlation between the level of cypermethrin resistance (RFs judged by LC_{99}) and the frequency of R allele in the sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* in Akula (A) and Moluxtun (B) field populations in the Mexican tropics.

6.4. Discussion

The five *R.* (*B.*) *microplus* field populations that were treated with cypermethrin showed increased RFs (judged by both LC) and reduced slopes of the probit line. Data heterogeneity as well as low-slopes linked to relatively high-LC_{50} values suggested a mixture of resistant and susceptible genotypes (Robertson and Preisler, 1992). This pattern after selection was also found by Davey and George (1998) when a strain of *R.* (*B.*) *microplus* was selected for resistance to permethrin. Such decreases of the probit line are consistent with a conclusion that, as homozygous susceptible (SS) individuals are eliminated from the population, the tick population becomes more variable. The results obtained in this study appear to be in agreement with the statement by Kunz and Kemp (1994) that the development of resistance to acaricides in a tick population is dependent on the frequency of resistant individuals in the population and the intensity of chemical selection pressure. Jonsson et al. (2000) and Rodriguez-Vivas et al. (2006a) found higher probability of resistance to deltamethrin, flumethrin and cypermethrin on field conditions when pyrethroids were used >5 times/year. In the present study, 16-24 cypermethrin treatments were used during the study and eight *R.* (*B.*) *microplus* generations were expected in the two years (Rodriguez-Vivas and Dominguez-Alpizar, 1998). With this cypermethrin pressure and the expected generations an increase in 2-125-fold was found (judged by LC_{50}). This finding is in agreement with a controlled trial conducted by Coetzee et al. (1987) who reported that the development of resistance to fenvalerate in *R.* (*B.*) *decoloratus* occurred during an 18-month period (in 5-6 tick generations). Also, in a controlled trial Davey and George (1998) were able to select an *R.* (*B.*) *microplus* strain for resistance to permethrin by treating larvae with increasing dose through successive generations. Within five generations, under continuous and increasing selection pressure, the RF was increased 4-fold. The high RF reached in the Santa Cecilia population was of the magnitude of >100 reported for some *R.* (*B.*) *microplus* pyrethroid resistance strains (Nolan et al., 1989; Miller et al., 1999

The increases in RF after cypermethrin treatment, although not always uniform, were steadily upward; this pattern might be due to the environmental and biological factors on field interventional studies. These factors include generation time, offspring per generation, mobility, migration, host range, fortuitous survival and refugia which

regulate the proportion of total population selected with insecticides and the selection pressure exerted on treated populations (Kunz and Kemp, 1994).

In laboratory studies untreated control groups are required to compare RF values with treated groups; however, on field conditions of the Mexican tropics it would be inappropriate to leave an untreated group due to the high tick infestation level and risk of tick-borne disease transmissions (Solorio-Rivera et al., 1999; Rodriguez-Vivas et al., 2004, 2005a). In *R. (B.) microplus* there is no cross-resistance between pyrethroids and amitraz (Kunz and Kemp, 1994). For that reason, in the present study, animals in six farms with different pattern of susceptibility to cypermethrin were treated with amitraz and used as a control group. After 24 months, the six populations did not change their RFs (judged by both LCs) and slopes or LC values were of minimal resistance significance. It was clear that tick populations with no history of pyrethroid use in the last five years have low levels of resistance (RF) and low frequency of the R allele (San Agustin, Blanca Flor, San Roman and Moluxton populations). However, resistance to some acaricides persist in the field for many years (Willadsen, 2006), especially pyrethroids (Kunz and Kemp, 1994). Low fitness cost of *R. (B.) microplus* resistant to OPs and amitraz has been reported (Davey et al., 2006; Soberanes et al., 2002). In this study, relaxing of selection pressure with cypermethrin for 24 months did not lead to reversion to susceptibility and the R allele frequency remained remarkably stable. For this reason, strategies to manage resistance need to be aimed at reducing the selection pressure to a minimum while still achieving control.

The frequency of the resistance allele increased on all five farms treated with cypermethrin from a starting range of 5.9-46.7 % to a range of 66.7-95.0 %, whereas on six farms treated with amitraz the frequency of the resistance allele did not change during the present field study. Thus the increase in frequency of the R allele was attributed to use of cypermethrin. The natural population of ticks might maintain several alleles at the sodium channel locus, with alleles providing the best selective advantage under natural conditions being dominant. The pressure from acaricides could act as selection for an allele that might not be optimal in the absence of acaricide, but which provides the capacity to survive in the presence of acaricide (Kunz and Kemp, 1994).

The clear correlation between the phenotype and genotype found in Akula and Moluxtun populations indicates that the sodium channel SNP is one of the most important mechanisms that confer pyrethroid resistance in the studied *R. (B.) microplus* populations, as it was found and discussed in **chapter 4**. The increased RFs in Kantok and Santa Cecilia populations due to de intensive cypermethrin treatment were evident. However, this phenotype expression did not correlate with the frequency of the R allele, because in Kantok population the RF increased only 2-fold (LC_{50}) and in Santa Cecilia a very high fold increase was found in the two last measures. Despite this, in Santa Cecilia a positive correlation was found judged by the LC_{99} ($r^2 = 0.741$); however, this correlation was close to be significant ($p = 0.073$). In both Santa Cecilia and Alamo populations the lack of cypermethrin efficacy was evident in the field and treatments with this acaricide were replaced by another chemical before the end of this prospective interventional study.

The mode of inheritance of a resistant allele has significant implications for resistance management strategies, the most important of which would be on the rate of resistance development (Kranthi et al., 2006). The rate of change in the resistance allele frequency in field populations over time depends largely on the extent of selection pressure on *R. (B.) microplus*, and is significantly influenced by the mode of inheritance at it was discussed in **chapter 4**. Furthermore, in the first study (**Chapter 3**) we found in the sodium channel of *R. (B.) microplus* three new mutations that might be involved in pyrethroid resistance. It is interesting to note that there is evidence for carboxylesterase-mediated mechanisms of pyrethroid resistance in Mexican ticks (Jamroz et al., 2000), but this mediated mechanism was not evaluated in populations from the present study. Although the structure of DDT is different from that of a pyrethroid, both target insect sodium channels (Usherwood et al., 2005). It was shown some time ago that the L1014F mutation reduces sensitivity of houseflies (*Musca domestica*) to DDT by 10-fold (Farnham, 1977) and more recent discoveries of *kdr* mutations (including L1014F) in mosquitoes (*An. gambiae*) are not surprising (Martinez-Torres et al., 1998; Ranson et al., 2000). However, because DDT was employed years ago in the Mexican tropics to control some pests, it would be important

to know if this insecticide is related with the sodium channel mutations identified in tick resistant to pyrethroids.

In this prospective interventional study, it is concluded that cypermethrin selection pressure on field populations of *R.* (*B.*) *microplus* produced a rapid development of resistance with increases of RF which correlate with increased frequencies of the resistance allele. In populations in which cypermethrin was substituted with amitraz RFs and frequencies of the resistance allele remained stable over 24 months.

CHAPTER 7

Tactical Management: Introduction of pyrethroid-susceptible *Rhipicephalus* (*Boophilus*) *microplus* population into pyrethroid-resistant *Rhipicephalus* (*Boophilus*) *microplus* resistance population

7.1. Introduction

Resistance to acaricides has been a major incentive to the development of alternative tick control measures. Overwintering and migration influence the dynamics of resistance in the cotton bollworm (*H. armigera*). In eastern Australia, high densities of pyrethroid-resistant pupae diapausing beneath cotton stubble during winter ensure effective carryover of resistance between seasons. This effect is countered to some extent by spring immigration of susceptible moths from alternative hosts, causing a temporary reversion in resistance level (Daly and Fitt, 1990). Conversely, the wet weather during spring, which promotes emigration of resistant moths from cotton, has led to a gradual increase in resistance level on unsprayed hosts (Gunning and Easton, 1989). Tactics that promote the survival of susceptible homozygote in resistance pest populations can contribute to reversion of resistance (Kunz and Kemp, 1994). This tactic management was inspired by the theory of the "refuge" in helminthes and agricultural pest control (Nari et al., 2000).

Resistance to some acaricides persists in the field for many years, especially pyrethroid resistance (Kunz and Kemp, 1994). On field populations of *R.* (*B.*) *microplus*, Rodriguez-Vivas et al. (2005b) found persistent resistance to OP for more than four years. This type of resistance has been a major incentive to the development of alternative tick control measures. In the prospective intervention study (**Chapter 6**) we found that relaxing of selection pressure with cypermethrin (using amitraz) for 24 months did not lead to reversion to susceptibility and the R allele frequency remained remarkably stable in the six populations studied. Attempts to regenerate susceptibility by management of field populations of *R.* (*B.*) *microplus* resistant to acaricides have not been reported. For that reason, this study looks at a tactical management strategy to reduce the cypermethrin resistance on field populations of *R.* (*B.*) *microplus* in the Mexican tropics.

7.2. Experimental Aims and Methods

7.2.1. Aim of the Study

The specific aim of the work presented in this section was to evaluate a tactical management strategy to introduce a pyrethroid-susceptible *R. (B.) microplus* population into a pyrethroid-resistant *R. (B.) microplus* population.

7.2.2. Study Design

Based on the results of Rodriguez-Vivas et al. (2006a, 2007), four cattle farms (**Figure 7.1.**) from the state of Yucatan, Mexico (**see section 2.3.**), with different larval mortality in the larval packet test exposed to a discriminating dose of cypermethrin, were selected (**Figure 7.1.**). On each farm a sample of 30-50 engorged adult female *R. (B.) microplus* ticks was collected from at least 10 animals (**Section 2.4.**). The phenotypic response of each *R. (B.) microplus* population to cypermethrin was measured by dose-response bioassays by the modified larval packet test using different dilutions of technical grade cypermethrin (**see section 2.5.**). For genotypic response of *R. (B.) microplus* to cypermethrin the AS-PCR was carried out as described in **section 2.6** on 30-32 larvae from each farm. The RFs judged by LC_{50} and LC_{99} and the frequency of the R allele was calculated as described in **section 2.7.** The pyrethroid resistance phenotype and genotype of the four tick populations are presented in **Table 7.1.**

Based on the phenotype and genotype results, $Farm_1$ and $Farm_2$ were classified as resistant (RFs > 5.0 judged by both LCs) and $Farm_3$ and $Farm_4$ as susceptible (RFs < 3.0 judged by both LCs) (**see section 2.7.**) Additional information about the larval mortality obtained by the larval packet test using pyrethroids discriminating doses (flumethrin: 0.01 %, deltamethrin: 0.009 % and cypermethrin: 0.05 %) in the four farms was obtained from previous studies (Rodriguez-Vivas et al., 2006a, 2007) and is presented in **Table 7.2.**

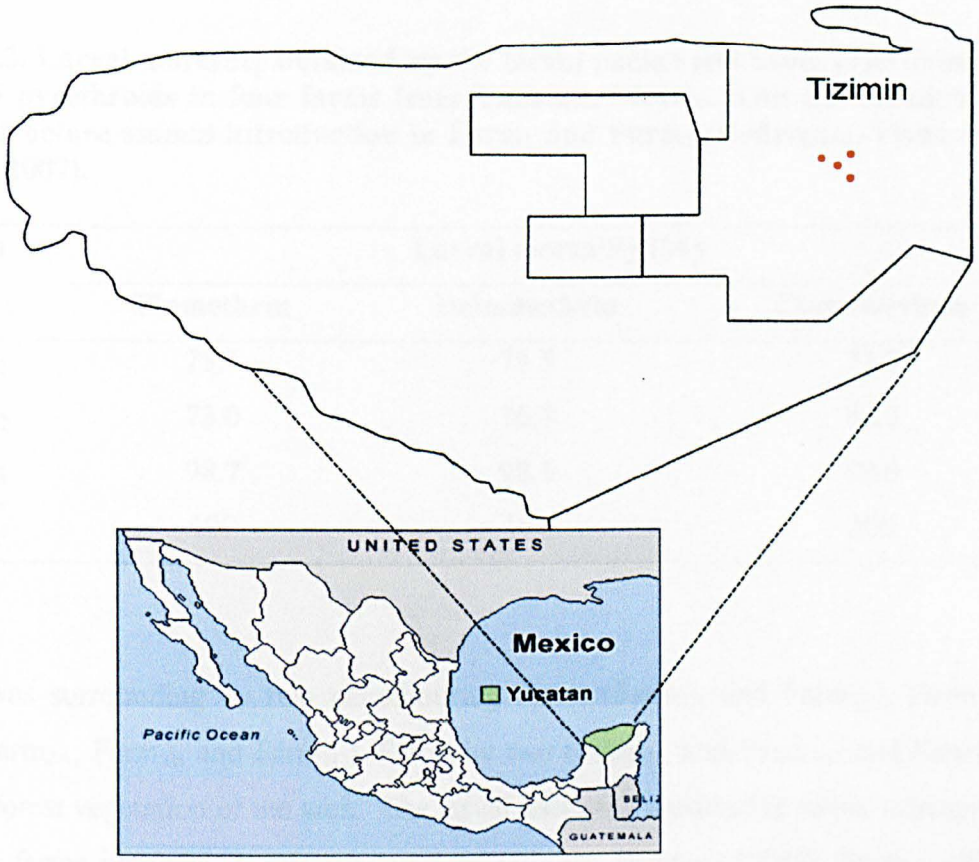

Figure 7.1. Map showing the locality of four farms (red dots) with known pyrethroid-resistant tick populations in the zone of Tizimin, Yucatan, Mexico, selected for the introduction of pyrethroid-susceptible tick populations.

Table 7.1. Phenotype and genotype resistance to cypermethrin in four populations of *Rhipicephalus* (*Boophilus*) *microplus* from Yucatan, Mexico, before the trial started.

ID of tick population	RF (LC_{50})	RF (LC_{99})	R allele Frequency
$Farm_1$	14.2	217.4	56.7
$Farm_2$	12.3	122.2	57.8
$Farm_3$	1.6	2.5	1.6
$Farm_4$	1.0	1.9	8.3

ID: Identification, RF: Resistance Factor, R: Resistance allele.

Table 7.2. Larval mortality obtained by the larval packet test using discriminating doses of pyrethroids in four farms from Yucatan, Mexico. This information was obtained before animal introduction in $Farm_1$ and $Farm_2$ (Rodriguez-Vivas et al., 20006b, 2007).

Farm	Larval mortality (%)		
	Flumethrin	Deltamethrin	Cypermethrin
$Farm_1$	75.4	71.5	71.7
$Farm_2$	73.0	76.3	80.0
$Farm_3$	98.7	98.1	98.6
$Farm_4$	100	100	100

$Farm_1$ was surrounding by two neighbouring farms ($Farm_{1A}$ and $Farm_{1B}$), $Farm_2$ by three ($Farm_{2A}$, $Farm_{2B}$ and $Farm_{2C}$), $Farm_3$ by two ($Farm_{3A}$ and $Farm_{3B}$) and $Farm_4$ by natural forest vegetation of the area. The larval mortality reported in farms surrounding the four farms involved in this trial was as follows: $Farm_{1A}$ (100%), $Farm_{1B}$ (82%), $Farm_{2A}$ 100%, $Farm_{2B}$ 100%, $Farm_{2C}$ 73%, $Farm_{3A}$ 100% and $Farm_{3B}$ 100%.

7.2.3. Background of Farms

Different acaricide treatments (OPs, amitraz and SPs) had been regularly used in the four farms in the last five years. All farms use hand-spray to control ticks and flies. Undefined cross bred cattle (*Bos indicus* x *Bos taurus*) were used in all farms. The land area, number of cattle, stocking density and number of paddocks in the four farms studied are presented in **Table 7.3.** Maximum distances between the four farms were approximately 40 km. Individual paddocks on each farm and between farms were divided by fences. During the study, movement of animals (>5 animals) carrying ticks between neighbouring farms was not reported.

Table 7.3. Characteristics of the farms selected to study the effect of the introduction of cypermethrin susceptible *Rhipicephalus* (*Boophilus*) *microplus* populations on field populations of *Rhipicephalus* (*Boophilus*) *microplus* known to be resistant to cypermethrin in the Mexican tropics.

Farm	Land area	No. Cattle	Stocking density	No. of paddocks	Acaricide used in the last five years
$Farm_1$	440	300	1.46	10	SP, OP, amitraz
$Farm_2$	430	300	1.43	12	SP, OP, amitraz
$Farm_3$	450	320	1.40	12	OP, amitraz
$Farm_4$	350	340	1.02	7	OP, amitraz

SP: Synthetic pyrethroids, OP: Organophosphates

7.2.4. Farm Management

During the study, cattle introduction was performed at different times in $Farm_1$ and $Farm_2$. Every 4-6 months, cattle from $Farm_4$ naturally infested with *R.* (*B.*) *microplus* pyrethroid susceptible ticks were transported to $Farm_1$ and $Farm_2$. On the basis of "standard" counts of 4.5-8.0 mm engorging female *R.* (*B.*) *microplus* (Wharton and Utech, 1970), the tick burden was calculated on all animals entering the $Farm_1$ and $Farm_2$.

Numbers of "standard" engorged ticks were determined on one side of each animal and multiplied by two. Such "standard" counts provide an accurate estimate of the number of ticks that should engorge within 24 h (Wharton and Utech, 1970). $Farm_3$ was used as control and together with the $Farm_4$ no cattle introduction was carried out during the study. For tick control all animals (cows, heifers, steers, and bulls) on the four premises were treated by spray with amitraz (Trak® Lapisa, Laboratory, Mexico, 12.5% active ingredient) at the recommended dose using at least four liters per animal. During the study, treatments at the four farms were carried out every two months. Animals that were introduced in $Farm_1$ and $Farm_2$ were treated with amitraz after 1-2 months of arrival.

7.2.5. Sampling

To evaluate susceptibility of *R.* (*B.*) *microplus* to cypermethrin in $Farm_1$, $Farm_2$ and $Farm_3$, every 8-9 months a sample of 20-30 engorged females was collected from at least 10 cattle from each farm (**Section 2.4.**). In farm $Farm_4$, engorged females were collected at the beginning and middle of the study. Tick collection on each farm and production of *R.* (*B.*) *microplus* larvae were carried out as was described in **section 2.4.**

7.2.6. Phenotypic Analysis by Dose-Response Bioassay and Genotypic Analysis by Allele-Specific Polymerase Chain Reaction

For resistance dynamics in all farms ($Farm_1$-$Farm_4$), the phenotypic response of *R.* (*B.*) *microplus* to cypermethrin were carried out as described in **section 2.5.** For genotypic response of *R.* (*B.*) *microplus* to cypermethrin the AS-PCR (Guerrero et al., 2001) was carried out as described in **section 2.6.** On each farm and time of sampling 27-32 individual larvae were genotyped.

7.2.7. Statistical Analysis

For all farms probit analysis at each time of sampling was performed on dose-response bioassay results using *Polo-Plus* as described in **section 2.7.** Differences between LC_{50} and LC_{99} estimates of studied populations at different times of sampling were designated as significant if their 95% confidence limits did not overlap. Susceptible populations were considered when both RF values (judged by LC_{50} and LC_{99}) were < 3.0 and resistant populations when RF values were ≥ 5.0 (Beugnet and Chardonnet, 1995). Tolerant populations were considered when one or both RF values were 3-5. To evaluate changes in allele frequency over time in tick populations of each farm a contingency table using chi-square test was used.

7.3. Results

Table 7.4. shows the number of cattle and the average of "standard" *R. (B.) microplus* engorged females per animal introduced in $Farm_1$ and $Farm_2$ during cattle introduction. A total of 208 and 174 cattle were introduced to $Farm_1$ and $Farm_2$ respectively during the time of the study. When cattle were introduced in $Farm_1$ and $Farm_2$ they had an average tick burden of 130.2 and 122.7 respectively.

Table 7.4. Number of cattle and "standard" engorged female *Rhipicephalus (Boophilus) microplus* per animal introduced in $Farm_1$ and $Farm_2$.

Times of animal introduction (Months)	No. of animals introduced	Mean engorged females/animal ± SD*
$Farm_1$		
0**	32	152.3 ± 34.2
4	17	104.4 ± 25.9
8	35	178.6 ±36.3
11	29	94.2 ± 17.5
16	22	142.1 ± 42.8
21	26	153.2±27.3
24	18	109.3± 32.3
28	29	107.6±22.7
$Farm_2$		
0**	16	147.4 ± 28.2
4	22	121.8 ±23.7
8	35	98.9 ±19.3
11	29	115.1 ±29.4
16	22	148.3 ±47.1
20	20	137.2±25.6
24	21	99.8± 30.3
28	9	113.4±20.7

SD: Standard deviation of the mean. * The "standard" engorged female was calculated using the methodology described by Wharton and Utech (1970). ** First introduction of animal infested with ticks.

The probit analysis results to determine susceptibility of *R. (B.) microplus* to cypermethrin in the four farms are shown in **Table 7.5.** In $Farm_1$ the RFs decreased significantly (LC_{50}= 14.2-1.3 and LC_{99}= 217.4-9.9) after 33 months of introducing cattle

with ticks susceptible to cypermethrin. The same behavior was shown in $Farm_2$ (LC_{50}= 12.3-1.6 and LC_{99}= 122.2-16.2). In $Farm_1$ the slope of the probit line increased from 1.33 to 2.10; however, in $Farm_2$ the slope remained stable.

Table 7.5. Levels of cypermethrin resistance in *Rhipicephalus* (*Boophilus*) *microplus* on four farms over a period of 33 months. The resistance factor was determined by the larval packet test taking into account the LC_{50} and LC_{99} of the tested population divided by the LC_{50} and LC_{99} of a susceptible reference strain.

Month of	50 %			99 %			
Sampling	LC	CL95%	RF*	LC	CL95%	RF*	Slope
$Farm_1$							
0**	0.185	0.150-0.243	14.2a	10.083	4.484-35.953	217.4[a]	1.33
8	0.187	0.148-0.247	14.8a	10.652	4.229-49.969	231.5[a]	1.32
16	0.035	0.023-0.039	2.6b	1.697	0.943-4.022	36.8b	1.34
24	0.018	0.007-0.029	1.3b	0.466	0.218-2.691	10.1b	1.64
33	0.017	0.005-0.021	1.3b	0.458	0.369-0.595	9.9c	2.10
$Farm_2$							
0**	0.161	1.131-1.196	12.3a	5.622	0.196-3.215	122.2a	1.50
8	0.131	0.105-0.162	10.0a	3.692	2.135-8.186	80.2a	1.60
16	0.116	0.097-0.137	8.9a	2.372	1.557-4.208	51.5b	1.77
24	0.060	0.40-0.080	4.6b	1.223	0.660-3.659	26.5c	1.77
33	0.021	0.010-0.033	1.6b	0.748	0.418-2.205	16.2c	1.50
$Farm_3$							
0**	0.021	0.020-0.023	1.6a	0.119	0.099-0.150	2.5a	3.12
8	0.019	0.016-0.021	1.4a	0.126	0.118-0.235	2.7a	2.45
16	0.008	0.007-0.009	0.6b	0.055	0.045-0.071	1.1b	2.81
24	0.008	0.007-0.009	0.6b	0.055	0.045-0.075	1.5b	2.75
33	0.019	0.014-0.023	1.4a	0.129	0.115-0.367	2.8a	2.31
$Farm_4$							
0**	0.013	0.012-0.015	1.0a	0.090	0.077-0.108	1.9a	2.82
16	0.014	0.011-0.016	1.0a	0.092	0.076-0.109	2.0a	2.84

LC: Lethal concentration. CI: Confidence Interval. RF: Resistance Factor (Media Joya-CENAPA strain was used as a reference: LC_{50}: 0.013, LC_{99}: 0.046). *Figures within column with different letters in the same farm are significantly different based on the failure of 95% confidence interval to overlap. **Bioassay obtained before animal introduction in $Farm_2$ and $Farm_3$.

$Farm_3$ remained susceptible with few changes on their RF to cypermethrin (range: LC_{50}= 0.61-1.61, LC_{99}= 1.1-2.8) and slopes from 2.31 to 3.12. In $Farm_4$ tick populations remained susceptible with RFs of 1.0 for both LCs and slope ranged from

2.82 to 2.84. In $Farm_1$ and $Farm_2$, the frequency of the R allele decreased from 56.7 % to 15.5 % and from 57.8 % to 18.3 % respectively (**Table 7.6.**) after introducing cattle with susceptible ticks to cypermethrin over a period of 33 months. In $Farm_3$ and $Farm_4$ the frequency of the R allele remained stable (≤8.3 %).

Table 7.6. Genotypic status to cypermethrin of four population of *Rhipicephalus* (*Boophilus*) *microplus* in the Yucatan, Mexico, over a period of 33 months.

Month of Sampling	No.	S-S	S-R	R-R	Frequency of R allele*
$Farm_1$					
0**	30	8 (26.7)	10 (33.3)	12 (40.0)	56.7a
8	30	6 (20.0)	17 (56.7)	7 (23.3)	51.7a
16	30	10(33.3)	20 (66.7)	0 (0.0)	33.3b
24	27	19(70.4)	4(14.8)	4(14.8)	22.2b
33	28	23 (82.1)	3(10.7)	2(7.1)	15.5bc
$Farm_2$					
0**	32	7 (21.8)	12 (37.5)	13 (40.6)	57.8a
8	31	5 (16.1)	18 (58.0)	8 (25.8)	54.8a
16	30	10(33.3)	18 (60.0)	2 (6.6)	36.6b
24	31	17(54.8)	12(38.7)	2(6.4)	25.8b
33	30	19 (63.3)	10(33.3)	1(3.3)	18.3b
$Farm_3$					
0**	30	29 (96.7)	1 (3.3)	0 (0.0)	1.6a
8	30	26 (86.7)	4 (13.3)	0 (0.0)	6.7a
16	30	27 (90.0)	3 (10.0)	0 (0.0)	5.0a
24	30	28 (93.3)	2 (6.7)	0 (0.0)	3.3a
33	30	25 (83.3)	5 (16.7)	0 (0.0)	8.3a
$Farm_4$					
0**	30	26 (86.7)	3 (10.0)	1 (3.3)	8.3a
16	31	29(93.5)	2 (6.45)	0(0.0)	3.2a

R-R: Homozygous resistant (possess the two mutated sodium channel alleles), R-S: Heterozygous resistant-susceptible (possess one mutated sodium channel allele and one wild-type allele). S-S: Homozygous susceptible (possess the two wild-type alleles), R allele frequency: Percentage of mutated sodium channel allele (R) in the total number of alleles assayed (assuming two alleles per individual). *Figures within column of the same farm with different letters are significantly different. **Bioassay obtained before animal introduction in $Farm_2$ and $Farm_3$.

7.4. Discussion

Resistance develops more rapidly with dominance, higher resistant allele frequencies and higher fitness advantage to resistant genotype (Georghiou and Taylor, 1977). One objective of resistance management is to maintain resistance alleles at very low frequencies. Thus, resistance management tactics should be aimed at reducing allele frequencies and minimizing the fitness of resistant genotypes. Fitness costs associated with pesticide resistance have been documented in many pest species (Roush and McKenzie, 1987; Coustau et al., 2000; Oliveira et al., 2007); however, fitness cost in *R. (B.) microplus*-pyrethroid resistance has not been reported (Davey et al., 2006). Resistance to pyrethroid in *R. (B.) microplus* persists in the field for many years and this type of resistance has been a major incentive to the development of alternative control measures (Kunz and Kemp, 1994). Tactics that promote introduction and survival of susceptible homozygotes include: a) leaving areas unsprayed thus providing untreated refugia, b) using higher action thresholds that tend to reduce the number of insecticide applications, c) applying short residual compounds that reduce the effective exposure time, d) using selective acaricides that do not exert pressure on other species, e) relying on non-chemical control, and f) promoting the introduction of susceptible individuals (Kunz and Kemp, 1994).

Regeneration of susceptibility to acaricides is difficult; however, in some insects, such as mosquitoes, mass release of susceptible males could dilute the resistance (May and Dobson, 1986). Migration can also greatly influence the reversion in pyrethroid-resistance in the cotton bollworm, *H. armigera* (Daly and Fitt, 1990) and OP-resistant in the spider mite *Tetranychus urticae* (Sites and Cone, 1985; Flexner at al., 1991). The present study is the first attempt to use a tactical management strategy to reduce pyrethroid resistance on field populations of *R. (B.) microplus*.

The present results show that tactical management to introduce cypermethrin-susceptible *R. (B.) microplus* into cypermethrin-resistant tick populations after 31 months caused significant reduction in RFs in $Farm_1$ (LC_{50}= from 14.2 to 1.3 and LC_{99}= from 217.4 to 9.9) and $Farm_2$ (LC_{50}= from 12.3 to 1.6 and LC_{99}= from 122.2 to 16.2). According to Beugnet and Chardonnet (1995) criteria the revertant *R. (B.) microplus*

populations in $Farm_1$ and $Farm_2$ changed their status from resistant to tolerant populations. However, several factors may limit the effectiveness of this strategy. Firstly, the decline in resistance (RFs) judged by both LCs was evident after approximately ten tick generations produced during the entire study period (Rodriguez-Vivas and Dominguez-Alpizar, 1998); however, in both farms high concentration of cypermethrin (≥0.458 %) were needed to kill the 99% of revertant populations; secondly, the slope of the probit line in both populations did not reach the value of > 2.4 found in several susceptible field populations of *R.* (*B.*) *microplus* (see cross sectional study in **chapter 5**); thirdly, the frequency of the R allele in $Farm_1$ and $Farm_2$ decreased from 56.7 % to 15.5 % and from 57.8 % to 18.3 % respectively; however the presence of the R allele in both tick populations is still high. Hoy (1995) mentioned that in insects it is difficult to manage pesticide resistance once resistant individuals make up more than 5 to 10 % of the population.

Tabashnik et al. (1994) mentioned that revertant strains responded rapidly to reselection if susceptibility was not fully restored. If account is taken of previous findings implying that resistance to pyrethroids is inherited as partially dominant when *R.* (*B.*) *microplus* females are resistant (Aguilar-Tipacamu et al., 2008) and resistance is controlled by more than one gene (Tapia-Pérez et al., 2003), it is highly probable that the continued application of pyrethroids against these tick populations would lead to a rapid resurgence of pyrethroid resistance because frequently genes that confer resistance become fixed in the population and are rapidly expressed under repeated selection pressure.

The regeneration of susceptibility is variable and somewhat difficult to quantify in field conditions, particularly since one needs to know not only whether individuals disperse but also what genetic impact they have when they join a population (Roush and McKenzie, 1987). In field studies, environmental and biological factors play an important role in acaricide resistance. These factors include generation time, offspring per generation, mobility, migration, host range, fortuitous survival, refugia and cost of fitness associated with resistance to insecticides (Georghiou and Taylor 1977). In the present study *R.* (*B.*) *microplus* populations of $Farm_3$ and $Farm_4$ did not change their RFs and the frequency of R allele over 33 months despite being surrounding by some

farms with pyrethroid-resistant tick populations. In the prospective intervention study (**Chapter 6**) we found that relaxing of selection pressure with cypermethrin (using amitraz) for 24 months did not lead to reversion to susceptibility and the R allele frequency remained remarkably stable in the six populations studied, for this reason in this study we used a susceptible farm to see possible changes in phenotype and genotype. Sutherst and Comins (1979) mentioned that it is difficult to conserve susceptible tick populations for long if neighbours do not cooperate using effective acaricides. Regional cooperation is essential, because resistant ticks can be disseminated rapidly on transported cattle (Jonsson and Hope, 2007). Stringent controls on the movement of cattle might alleviate this effect, but the cattle would have to be cleaned of ticks with an effective acaricide.

To decrease the multi-resistant tick population in a farm in New Caledonia it was suggested to replace this population by an introduced susceptible one collected in a susceptible farm (laboratory test confirmed), in order to dilute the remaining resistant gene of the original population. This strategy was inspired by the theory of the "refuge" in helminths and agricultural pest control (Flexner at al., 1991; Nari et al. 2000), but was not accepted by producers to which it was proposed (personal communication, Barré, N. Institut de Recherche Agronomique néo-Calédonien, New Caledonia). In Yucatan, Mexico, the strategy to introduce ticks from one farm to another was accepted, possibly be due to the short distance of farms and because $Farm_1$, $Farm_2$ and $Farm_4$ belonged to the same producer. Introducing ticks from one farm to another in the Mexican tropics might have some risks to change the endemic stability (a condition that implies a high incidence of organism in cattle, but rarely the presence of clinical disease, Mahoney and Ross, 1972) of *B. bovis*, *B. bigemina* and *A. marginale* in cattle populations and lead to fatal cases of babesiosis and anaplasmosis. This risk might be possibly due the introduction of more pathogenic isolates of *B. bovis*, *B. bigemina* and *A. marginale*, and the introduction of *R.* (*B.*) *microplus* with different inoculation rates of these haemoparasites. However, in Yucatan, Mexico the risk to change the endemic stability might be difficult because on most of the farms > 88 % of calves show protective antibodies to *B. bovis*, *B. bigemina* and *A. marginale* (Solorio-Rivera et al., 1998; Rodriguez-Vivas et al., 2004). This statement is supported by Cen et al. (1998)

who found that 20 % of *R.* (*B.*) *microplus* engorged females in farms from Yucatan, Mexico, are infected with *Babesia* spp.

At the end of the trial in $Farm_1$ and $Farm_2$ we observed an increase in tick burden on animals; however, this was not measured. In $Farm_1$ and $Farm_2$ approximately 22,000 and 27,000 "standard" engorged females were introduced respectively over a period of 31 months. These tick burdens of *R.* (*B.*) *microplus* and the immature stages of ticks that reached mature stage certainly contributed to increase the amount of ticks in both farms and the impact of this was not evaluated in this study. In future studies the impact of introducing cattle with ticks in farms must be determined (tick burden and tick borne-diseases). Incorporating susceptible ticks into integrated resistance management programs that utilize a combination of non-chemical resistance management tactics such as biological control (Alonso et al., 2007), anti-tick vaccine (de la Fuente et al., 2007a), resistant breeds (Bianchi et al., 2003) and farm management (Kunz and Kemp, 1994) in order to reduce acaricide pressure and to prolong as long as possible SPs life in most of the farms are needed. It is absolutely essential to quickly detect emerging resistance and to avoid the spread of ticks from SP resistant to SP susceptible farms.

In this study we can conclude that tactical management to introduce cypermethrin-susceptible *R.* (*B.*) *microplus* into cypermethrin-resistance tick populations promoted reversal of resistance.

CHAPTER 8

General Discussion

Rhipicephalus (*Boophilus*) *microplus* (Canestrini) is a haematophagous arachnid (Acari: Ixodidae) and has become a major cattle pest in tropical and subtropical agrosystems of the world, because of the direct effects of infestation and the diseases these ticks transmit (i.e. *B. bovis*, *B. bigemina* and *A. marginale*) (Solorio-Rivera et al., 1999; Rodriguez-Vivas et al., 2004).

Acaricides (OPs, SPs and amitraz) have played a pivotal role in the control of *R.* (*B.*) *microplus* in Mexico. As a consequence of extensive use *R.* (*B.*) *microplus* has developed resistance to all major classes of acaricides (Rodriguez-Vivas et al., 2007). Recently, Rodriguez-Vivas et al. (2006a, b) studied field populations of *R.* (*B.*) *microplus* and found that SP resistance was the most serious problem (66 % of prevalence) in southern Mexico. However, SPs have shown excellent efficacy (> 98%) to control ticks and flies in 34 % of the farms. A major imperative in tick control in Mexico is the need to conserve and use SPs in such a way that they are retained for effective use when necessary. For that reason, the present work was focused on SPs to control ticks.

The rate at which a resistant allele becomes established in the population and the time it takes for the control of ticks to break down is dependent upon many factors. These include the frequency of the original mutation in the population before treatment, the mode of inheritance of the resistant allele, the intensity of chemical selection pressure, and the proportion of the total tick population in refugia (Kunz and Kemp, 1994; Nari et al., 2000). Pyrethroids act on the insect voltage-gated sodium channel (generating action potentials in insect nerve cells), modifying the gating kinetics and resulting in the prolonged opening of individual channels, leading to paralysis and death of the insect (Soderlund and Knipple, 2003). *Kdr* occurs as the result of a change in the affinity between the insecticides and their binding site(s) on the channel, caused by mutations in the sodium channel in several insect species, including ticks (Dong, 2007).

Standardized bioassays for determining the resistance level of a tick population, such as the larval packet test (Stone and Haydock, 1962), are valuable in that they offer a method for phenotyping a population in response to acaricides. Guerrero et al. (2001)

developed an AS-PCR-68 bp to genotype pyrethroid resistant strains of *R.* (*B.*) *microplus* (amino acid substitution Phe→Ile in the sodium channel).

In order to delay the development of resistance and establish a reliable resistance monitoring system, it is important to understand how *R.* (*B.*) *microplus* become resistance and the impact of this process in the cattle industry. In this thesis we addressed the following research problems related with *R.* (*B.*) *microplus* resistance to pyrethroids.

Modification and validation of AS-PCR using Pyrosequencing™. In this study we sequenced for the first time gDNA around the target SNP associated with pyrethroid resistance in 34 individual larvae of *R.* (*B.*) *microplus* from 19 tick populations. To date only cDNA of partial sequence of the sodium channel gene of *R.* (*B.*) *microplus* has been reported (He et al., 1999). Comparison of gDNA sequences obtained from 34 individual larvae identified for the first time three new SNPs in the sodium channel gene coding region which result in amino acid changes. The possible associations of these new mutations in the sodium channel gene of *R.* (*B.*) *microplus* and pyrethroid resistance need to be studied by using a new set of primers to design a new AS-PCR or modifying the Pyrosequencing methodology (i.e. sequencing primer). In other insect such as *Drosophila melanogaster* (Meigen) (Amichot et al., 1992), and *Haematobia irritans irritans* (L.) (Guerrero et al., 1997) two mutations in the sodium channel gene (designated *kdr* and *superkdr*) have been identified and associated with pyrethroid target site resistance. Furthermore, Foil *et al.* (2005) found that when *Haematobia irritans irritans* possessed the SS–SS alleles (*kdr* and *superkdr*) it was more susceptible to pyrethroids than the other genotype combinations.

Limitations of the AS-PCR-68 bp are poor visualization of the small size 68 bp PCR product, and the lack of genotyped controls. Based on the partial sequence of the sodium channel gene at the gDNA level of *R.* (*B.*) *microplus*, we showed that the SNP associated with pyrethroid resistance as identified by He et al. (1999a), is located close to an intron/exon boundary. With this information, we developed the AS-PCR-91 bp that amplifies a 91 bp product. This assay was able to genotype pyrethroid resistant larvae of *R.* (*B.*) *microplus*; however, the proximity of the intron did not allow the

amplification of a larger product, hence it did not resolve the issue of visualizing the product by electrophoresis. In this study, we developed Pyrosequencing™ as a high throughput method for genotyping SNPs (T → A) at the sodium channel gene associated with pyrethroid resistant in *R. (B.) microplus*. Using Pyrosequencing™ as a gold standard, the results obtained by direct sequencing and Pyrosequencing™ correlated in 67.8 %. The genotypes identified by Pyrosequencing™, AS-PCR-68 bp and AS-PCR-91 bp showed good agreement and no differences were found in the ability to determine the frequency of the R allele. Despite these results, Pyrosequencing™ has a number of advantages over AS-PCR (i.e. it removes the need for labeled primers, labeled nucleotides and gel electrophoresis) (Ronaghi, 2001; Gharizadeh et al., 2002). The present study has shown that Pyrosequencing™ is a reliable and high-throughput method that could be used as an alternative method for genotyping pyrethroid resistant populations of *R. (B.) microplus*. Due to technical reasons in field studies in Mexico the validated AS-PCR-68 bp was used to genotype ticks; however, positive controls tested by Pyrosequencing™ were used.

In future studies the Pyrosequencing® technique will be evaluated using pooled samples. DNA from two individual larvae, one carrying TT alleles (two wild-type alleles) and one the AA alleles (mutated alleles) will be cloned as described in **chapter 3**. The DNA concentration of both clones will be normalized to 10 ng/µl by diluting with buffer. Pools will be created by titrating DNA samples in various proportions and subjected them to pyrosequencing. The true allele frequencies and those estimated from pools of DNA by Pyrosequencing™ will be estimated. Gruber et al. (2002) and Lavebratt and Sengul (2006) found that the difference between true allele frequencies and those estimated from pools of DNA by Pyrosequencing™ has been shown to vary by 1.1–6.5 % and the correlation between true and estimated allele frequencies was good ($r^2 = 0.92–0.99$). The pool estimates will be validated using pooled samples from the field. With this novel technique we will be able to genotype in one run a large number of samples (over 150). In the five studies carried out in this thesis ~9,000 PCR reactions (two reactions for each diagnosis) were run using ~375 agarose gels. With the use of pooled samples in Pyrosequencing the number of larvae that can be tested is far greater, for example for each farm 100 larvae will be tested (100 will be ideal). In further studies other states from the southern of Mexico need to be studied and

Pyrosequencing will be an important tool to genotype ticks and determine the frequency of the R allele associated with pyrethroid resistance in *R. (B.) microplus.*

Demonstration of association between survival of larvae and genotypes. In this work we investigated the association between larvae survival exposed to cypermethrin and the target SNP associated with pyrethroid resistance in *R. (B.) microplus.* All surviving larvae from the cypermethrin resistant populations possessed at least one copy of the mutated allele. However, in both resistant and susceptible populations larvae that were killed possessed 96 % of at least one copy of the S allele. This means that the presence of the mutated allele in one copy of the gene does not always provide a mechanism for surviving. The clear relation between larval survival and the presence of the R allele (dead larvae 27.3 % vs. survivor larvae 78.3 %) shows that the target SNP has a clear association with pyrethroid resistance; however, other mutations and metabolic mechanisms might be involved. In future studies linkage between both metabolic detoxification of carboxylesterase (Rosario-Cruz et al., 1997; Jamroz et al., 2000; Pruett et al., 2002) and the increase of monooxygenase (Cossio-Bayugar et al., 2008) activities, and pyrethroid acaricide resistance need to be evaluated in these tick populations. In conclusion, there was a high association between larvae survival exposed to cypermethrin and the presence of the target SNP associated with pyrethroid resistance in *R. (B.) microplus,* suggesting that this target SNP is one of the most important mechanism that confer pyrethroid resistance in *R. (B.) microplus* populations from Yucatan, Mexico. Recently a new molecular technology called RNA interference (RNAi) has been applied in tick research (de la Fuente et al., 2007b). RNAi is a nucleic acid-based reverse genetic approach that involves disruption of gene expression to determine gene function or its effect on a metabolic pathway. This technology has provided valuable information about the function of genes involved in the regulation of tick feeding, reproduction, the characterization of the tick-pathogens interface, and the screening and characterization of tick protective antigens (de la Fuente et al., 2007b; Sparagamo and De Luna, 2007). RNAi will probably provide a comprehensive contribution to study the resistance in ticks to acaricides and might have an impact on the identification of resistance mechanisms.

Cross sectional study. Little is known about the *R. (B.) microplus* resistance levels and the frequency of resistant alleles in field populations, or how these variables change with local environmental and selection pressures. We conducted a survey on 49 cattle farms to determine the prevalence of pyrethroid resistance phenotype and genotype on field populations of *R. (B.) microplus* using a cross sectional study in Yucatan, Mexico. Results from the present study (judged by both LCs) indicate that 26.5 %, 40.8 % and 32.6 % of the tested populations were susceptible, tolerant and resistant to cypermethrin. *R. (B.) microplus* has been exposed to multiple applications of pyrethroids in the last decade (Rodriguez-Vivas et al., 2006a, b) and this intensive use of pyrethroids in Yucatan, has contributed to select individual resistance to cypermethrin on field conditions. The presence of resistant and tolerant populations varied markedly over relatively short distance in Yucatan, Mexico (no statistical differences in the tick population phenotype between the three areas), perhaps due similar farm management and tick control. However, in this study we did not evaluate factors related with pyrethroid resistance. In future studies will be necessary evaluate risk factor related with farms, animals and management (stocking density, number of cattle on farm, farm size, treatment interval, number of acaricide treatments in one year, type of treatment, family of acaricide, non-chemical control, farm management, use of acaricide to control fly, proximity to another farm, alternative hosts for cattle ticks, cattle transportation, etc.).

All susceptible populations (judged by LC_{50} and LC_{99}) exhibited the highest slopes (≥2.41) and the lowest frequency of the R allele (1.7 % to 16.7 %). These findings are in agreement with the statement of Robertson and Preisler (1992) who argued that data homogeneity as well as high-slope linked with both low-LC_{50} and low-LC_{99} values suggested that most individuals have the wild- type allele. Furthermore, the RF determined by both LC_{50} and LC_{99} was able to discriminate phenotypic and genotypic cypermethrin-susceptible *R. (B.) microplus* populations from those resistance populations. The frequencies of the R allele correlated well with the level of pyrethroid resistance (RF), as it was found and discussed in **chapter 4**.

The AS-PCR may constitute a valuable molecular tool for rapid monitoring of the frequency of the target SNP associated with pyrethroid resistance on field populations of *R. (B.) microplus*. This would allow an early detection of the target SNP to prevent the spread of such a resistant phenomenon at the state level. It would also improve the control of the populations that have already developed this high specific resistance to pyrethroid by recommending the use of other compound or alternative control method such as biological control (Alonso et al., 2007), anti-tick vaccine (de la Fuente et al., 2007a), resistant breeds (Bianchi et al., 2003) and farm management (Kunz and Kemp, 1994). In conclusion, by both bioassay and AS-PCR clearly show that the prevalence of *R. (B.) microplus* resistant-tolerant to cypermethrin (judged by LC_{50} and LC_{99}) is high in Yucatan, Mexico and possess high frequencies of R allele.

Prospective interventional study. In this study we presented a prospective controlled intervention study to measure the evolution of resistance phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *R. (B.) microplus*. The populations that were treated with cypermethrin increased their RFs (judged by both LC) and the frequency of the resistance allele (from 5.9-46.7 % to 66-95 %), and their slopes of the probit line decreased. These results are in agreement with the statement by Kunz and Kemp (1994) that the development of resistance to acaricides in a tick population is dependent on the frequency of resistant individuals in the population and the intensity of chemical selection pressure. The increases in RF under cypermethrin pressure, although not always uniform, were steadily upward; this non-uniform pattern might be due to the environmental and biological factors affecting field interventional studies (tick generation time, offspring per generation, mobility, migration, host range, fortuitous survival and refugia). Similar non-uniform pattern in RFs was found in prospective interventional study conducted in the Mexican tropics (Rosado-Aguilar et al., 2008). The authors treated three field populations of *R. (B.) microplus* with amitraz and after 15 months of amitraz selection pressure all populations increased their RFs (from 1 to 13, from 1 to 22 and from 2 to 6). In laboratory and controlled trials Coetzee et al. (1987) and Davey and George (1998) who reported that the development of resistance to fenvalerate and permethrin in *R. (B.) decoloratus* and *R. (B.) microplus* respectively. The present study is the first to report the generation of resistance in *R. (B.) microplus* under field conditions.

It is interesting to note that the Kantok population at the beginning of the study did not show the expected phenotype and genotype, it was considered as tolerant (RFs of 2.4 and 5.1 judged by the LC_{50} and LC_{99} respectively) with the highest frequency of the R allele (46.7 %). After selection pressure with cypermethrin the Kantok population increased the RFs and the frequency of the R allele; however, other resistance mechanisms may be involved in this population. In further studies the role of other mutations and metabolic detoxification (Jamroz et al., 2000; Pruett et al., 2002) in pyrethroid resistance in this population need to be confirmed.

The tick populations used as control (treated with amitraz) in this study, after 24 months, did not change their RFs (judged by both LC) and slopes or LC values were of minimal resistance significance. These results showed that relaxing of selection pressure with cypermethrin for 24 months did not lead to reversion to susceptibility and the R allele frequency remained remarkably stable. The lack of fitness cost of *R. (B.) microplus* resistant to pyrethroids (Davey et al., 2006), make pyrethroid resistance difficult to manage under field condition. For this reason, strategies to manage resistance need to be aimed at reducing the selection pressure to a minimum while still achieving control.

There are several strategies to manage resistance and the general consensus is to reduce frequency of acaricide use, the strategies include: a) leaving areas unsprayed thus providing untreated refugia, b) avoid partial treatment systems (i.e. acaricide impregnated ear tag), c) using higher action thresholds that tend to reduce the number of insecticide applications, by using strategic or seasonal application acaricides, d) applying short residual compounds that reduce the effective exposure time (long acting products most be used in special cases), e) using selective acaricides that do not exert pressure on other species, f) use non-chemical control, g) promote the introduction of susceptible individuals, h) avoid the use of combination of acaricide, specially those in different chemical groups because this can induce resistance developing (mixture of acaricide families most be used in special cases), i) acaricide rotation, j) control of tick before cattle transportation, k) use of computer simulation to predict high tick infestation and use strategic treatment, and l) national and mandatory system of

registration of acaricides (George, 1990; Kunz and Kemp, 1994; Estrada-Peña, et al., 2006; Willadsen, 2006).

The frequencies of the R allele correlated well with the level of pyrethroid resistance (RF) in two tick populations treated with cypermethrin. Also, in the remaining tick populations lack of cypermethrin efficacy was evident and treatments with this acaricide were replaced by another chemical before the end of this study. The correlation between the R allele and the level of resistance have been identified in laboratory strain of *R.* (*B.*) *microplus* other insects. Kwon et al. (2004) found in *Plutella xylostella* that the increasing presence of the T929I mutation correlated well with increased levels of resistance to both cypermethrin and fenvalerate. Song et al. (2007) working with *C. pipiens pallens* found significant correlation between *kdr* allelic frequency and the LC_{50} estimates of Es-bioallethrin, deltamethrin and betacypermethrin. Also, evidence of a strong correlation between the expression of the resistance phenotype (as measured by insecticide exposure assay) and genotype at the *kdr* locus was presented in West African (Martinez-Torres et al., 1998) and East African (Ranson et al., 2000) *Anopheles gambiae* mutations. N'Guessan et al. (2007) found that a high frequency of *kdr* correlates to reduced efficacy of pyrethroid-based vector control efforts (*An. gambiae*) using insecticide-treated bed nets and indoor residual spraying. However, there may be other mechanisms to confer resistances to pyrethroids (Jamroz et al., 2000). It is concluded that cypermethrin selection pressure on field population of *R.* (*B.*) *microplus* produces a rapid development of resistance with increases of RF which correlate with increased frequencies of the resistance allele. In populations in which cypermethrin was substituted with amitraz RFs and frequencies of the resistance allele remained stable over 24 months.

Tactical management. We looked at a tactical management strategy to evaluate a tactical management strategy to introduce a pyrethroid susceptible *R.* (*B.*) *microplus* population into a pyrethroid-resistant *R.* (*B.*) *microplus* population. The introduction of susceptible ticks into resistance tick populations after 33 months caused significant reduction in RFs (changed their cypermethrin susceptibility status from resistant to tolerant). However, several factors may limit the effectiveness of this strategy. Firstly, the decline in resistance (RFs) judged by both LCs was evident after approximately ten

tick generations produced during the entire study in the Mexican tropics (Rodriguez-Vivas and Dominguez-Alpizar, 1998); however, high concentration of cypermethrin (≥0.458 %) were needed to kill the 99% of the population; secondly, the slope of the probit line in both populations did not reach the value of > 2.4 found in several susceptible field populations of *R. (B.) microplus* found in the cross sectional study; and thirdly, the frequency of the R allele decreased from 57.8 % to 15.5 %; however the presence of the R allele in the tick populations studied is still high. It is highly probable that the continued application of pyrethroids against these tick populations would lead to a rapid resurgence of pyrethroid resistance because frequently genes that confer resistance become fixed in the population and are rapidly expressed under repeated selection pressure (Tabashnik et al., 1994) and environmental and biological factors play an important role in acaricide resistance (Georghiou and Taylor 1977). In the present study, the pyrethroid-susceptible tick population that was introduced to a pyrethroid-resistant population was also susceptible to amitraz and OP; however, we did not evaluate the effect on reducing the resistance levels of *R. (B.) microplus* to those acaricides. The lower reproductive potential of amitraz resistant ticks compared with amitraz susceptible ticks (Soberanes et al., 2002) will promote faster revision of resistance. In future studies, the integral evaluation of *R. (B.) microplus* resistant to OPs, SPs and amitraz is recommended.

Introducing ticks from one farm to another might have some risks to change the endemic stability of haemoparasites in cattle populations and lead to fatal cases of tick-borne diseases. However, in Yucatan, Mexico the risk of affecting endemic stability might be low because in most farms > 88 % of calves show antibodies to *B. bovis, B. bigemina* and *A. marginale* (Solorio-Rivera et al., 1998; Rodriguez-Vivas et al., 2004).

Incorporation susceptible ticks into integrated resistance management program that utilize a combination of acaricides (amitraz, OP), MLs (ivermectin, moxidectin, doramectin), and non-chemical management such as anti-tick vaccine and biological control (*Metarhizium anisopliae*) (Alonso et al., 2007, de la Fuente et al., 2007a; Bianchi et al., 2003; Kunz and Kemp, 1994) is needed. Furthermore, it is essential to quickly detect emerging resistance and to avoid the spread of ticks from SP resistant to SP susceptible farms. In this study we conclude that tactical management to introduce

cypermethrin-susceptible *R.* (*B.*) *microplus* into cypermethrin-resistance tick populations promoted reversal of resistance.

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10. APPENDICES

APPENDIX I. Nucleotides of the partial wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GenBank accession number AF134216. The domain III segment 6 is depicted in red. The blue codon "ttc" is presents en susceptible ticks to pyrethroids. In resistant ticks this codon changes "atc" (He et al., 1999a).

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   1 tacttccgcg aaggctggaa tatcttcgat ttcctcatcg tcgcgctctc cttaatcgaa
  61 ctaagtttgg aaaacgtcca aggattgtct gtgctacgtt cgtttcgtct gctacgtgtg
 121 ttcaagctag ccaaatcgtg gcctaccctt aacctgctca tctctatcat ggggaaaacc
 181 atcggtgccc tcgggaactt gacctttgtc ctgggaatca tcatcttcat cttcgccgtg
 241 atgggaatgc aactctttgg caagaactac gaagaaagta aacacaagtt caaagataac
 301 atggttcctc ggtggaactt tgttgacttc atgcattcgt tcatgattgt gtttcgagtg
 361 ttgtgcggcg agtggatcca gtccatgtgg gactgcatgt gggtctcagg ctggccctgc
 421 atccccttct ttctcgctac tgtagtcatc gggaaccttg tggtgctcaa ccttttcctc
 481 gccttgctgc tgtcctcgtt cggggcgtcc aatctgtccc aagcgaatcc cgacagcggc
 541 gacacaaaga aactacaaga agccatcgac cggtttcacc gggccagtcg gtggatcaag
 601 tccaactcta tgaaactttt caagagcttc cgtcggaaac cacgcaacca gatcggggac
 661 cagacaacag acattcgtgg tggcggggca ggcgaagagt tggaggctga cccgggcgtc
 721 gcaggggaag tggttctcct cgacggtcgg gtgccaatgc gagacagaaa gccccaacac
 781 aacaacgacc ttgaggttgt cgttggggac ggcctcgata tcgccattca gggtgatggc
 841 aaggccgtta aaatgaagtt gaaaaacaac tcaaagcctg tgatgaattc tgtttgggtg
 901 ggacctatga tcgagcctaa gaacaagcag ctagaaaaag acaacaagga aaaggagaaa
 961 gaagcgcagg gcaataaggt gtacccgcaa aaggacgagg ataccctcag cgaaaagtca
1021 gcgtccagcc ccaaggagaa ggtgctcctt gggaacaaac cgtccaaaga tcttagcaac
1081 agttccctgt acctggggaa caaccttgag gaggagaaga aggacgccag caaagaggac
1141 ctcggtacta aagaaggaga ggaggccccc accgaagagc ccatcaaccc ggacacggaa
1201 gatgtggaca cagacaagct ggaaacggcc acctcggaca ttatcatccc cgagatgccg
1261 gccgactgct gccccgactg gtgttacacg cgattcgcgt tcgcctgctt ttttgatgag
1321 aacaagattt tttggcagcg ctacaagatt gtgcgcacca aggcgtacgc ccttgtagag
1381 cacaagtact tcgaaaccat tgtcgtcgtt ctcatcctca ccagcagctt ggcgctggcg
1441 cttgaagacg ttaacctgaa agaccggccg acgctcaagg cagtgctcac atatatggac
1501 aagaccttca cagtgatctt tttctttgaa atgatgctca agtggcttgc ctttggattc
1561 aagaaatatt tcacaaatgc ctggtgctgg ctcgactttg tcatcgtact cgtgtccttc
1621 tttaacatgg ccgtagccat gatgggctac ggacgaatcc ccgcctttaa aaccatgcga
1681 accctccgag cactcagacc tttgagggcg atgtcccgcc tggagggaat gcgcgttgtt
1741 gtcaacgccc tggtgcaagc catcccagcc atcttcaacg tgctgctggt gtgtctcatc
1801 ttttggctca tcttctccat catgggcgtc cagatgcttg cgggaaagtt ctaccgctgc
1861 gtcgatggaa acggcacgcg cttgaacagc acacacgtcc caaacagaaa ggcgtgtgaa
1921 gccaacaact tcacttggga caaccccatg atcaacttcg acaacgtcct caacgcatat
1981 ttggcccttt tccaagtggc aacattcaaa ggctggacgg acattatgga caatgcgatc
2041 gactccaggg gcggaaaaga ggaccaaccg gaatacgagg ctaacatcta catgtaccta
2101 tacttcgtgt tcttcattat cttcggctcc ttcttcacct tgaatctatt catcggtgtt
2161 attatcgaca atttcaatga acaaaagaag aaggctggag gatcattaga aatgttcatg
2221 acagaagacc aaaagaaata ctataacgcc atgaagaaaa tgggatccaa aaagccagcc
2281 aaggcaattc caagaccccg gttcaaactt caagcaatgg tcttcgacct gactacaaac
2341 aaaatgtttg acatggcgat catgatattt attgtcctca acatgacagt catggcactc
2401 gaccactata agcagtccag gctgttcgag tccatcctag aacggctcaa catcttcttc
2461 atcgctgtct tcacagccga atgcctgctc aaaatattcg ccctgcgctg gcactacttt
2521 cgagagccat ggaacatgtt cgacttcgta gttgtcatat tatctattct aggtacggtg
2581 ctaaaggacc tgatcgcggc ctacttcgtg tcgcccacgc ttctccgtgt ggtgcgtgtc
2641 gtgaaagtgg gccgcgtgct tcggctggtg aagggcgcgc ggggcatccg gaccctgctg
2701 ttcgccctgg ccatgtcatt gccggcgctg ttcaacatct gcctgctcct gttccttgtg
2761 atgttcatct acgcaatctt cggcatgtct ttcttcatgc acgtcaagca ccgctacggc
2821 gtcgacgaga acttcaactt cgagacgttc ggccagtcga tgatcctgct atttcaaatg
2881 tgcacgtccg ccggctggga cggtgtgttg gccgctatca tggacgagca cgactgcaac
2941 cggcccaccg acgaatccga gggcaactgc ggcaagcggg gcatcgcggt cgcctacctc
3001 gtctcgtacc tcatcatcag cttcctcgtc atcataaaca tgtatatcgc cgtcatcctc
3061 gagaactaca gccaggccac cgaggacgtg caggagggcc tgaccgacga cgactacgac
3121 atgtactacg agatctggca gcagttcgac ccgaagggca cccagtacgt ggcctactcc
3181 aacctgacca acttcgtgaa cgcgctggag gagcctctgc agatcccaaa gcccaacaag
3241 tacaagctga tcgccctgga catacccatc tgcaaggacg acatggtcta ctgcgtcgac
3301 atcttggacg ccctgacgcg ggacttcttc gcccgcaagg ggcacgccat cgaggaaccg
3361 ccgcgatttt tctctcttcc aagacaaaaa tgaaagattg aaacgccgcc gcacgacgtt
3421 gcacacctga tgatgatgcg gcctccttac ctccgccgtc accaacccct tcgggactgc
3481 ctaccagtgc gcgagtgtgc agcgtgcaac tactcaccgc cagatggggc cgcctgatgg
3541 ggacccggta ccgtcgccgg ctgcggcaaa gcctgcttgg accttacgcg cacgctccg
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APPENDIX II. Animo acids of the partial wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GeneBank accession number AF134216. The domain III segment 6 is depicted in red. The blue F is the Phenilalanine amino acid presents en susceptible ticks to pyrethroids. In resistant ticks this amino acid changes to Isoleucine (He et al., 1999a).

YFREGWNIFDFLIVALSLIELSLENVQGLSVLRSFRLLRVFKLAKSWPTLNLLISIMGKTIGAL
GNLTFVLGIIIFIFAVMGMQLFGKNYEESKHKFKDNMVPRWNFVDFMHSFMIVFRVLCGEWIQS
MWDCMWVSGWPCIPFFLATVVIGNLVVLNLFLALLLSSFGASNLSQANPDSGDTKKLQEAIDRF
HRASRWIKSNSMKLFKSFRRKPRNQIGDQTTDIRGGGAGEELEADPGVAGEVVLLDGRVPMRDR
KPQHNNDLEVVVGDGLDIAIQGDGKAVKMKLKNNSKPVMNSVWVGPMIEPKNKQLEKDNKEKEK
EAQGNKVYPQKDEDTLSEKSASSPKEKVLLGNKPSKDLSNSSLYLGNNLEEEKKDASKEDLGTK
EGEEAPTEEPINPDTEDVDTDKLETATSDIIIPEMPADCCPDWCYTRFAFACFFDENKIFWQRY
KIVRTKAYALVEHKYFETIVVVLILTSSLALALEDVNLKDRPTLKAVLTYMDKTFTVIFFFEMM
LKWLAFGFKKYFTNAWCWLDFVIVLVSFFNMAVAMMGYGRIPAFKTMRTLRALRPLRAMSRLEG
MRVVVNALVQAIPAIFNVLLVCLIFWLIFSIMGVQMLAGKFYRCVDGNGTRLNSTHVPNRKACE
ANNFTWDNPMINFDNVLNAYLALFQVATFKGWTDIMDNAIDS

RGGKEDQPEYEANIYMYLYFVFFIIFGSFFTLNLFIGVIIDNFNEQKKKAGGSLEMFMTEDQKK
YYNAMKKMGSKKPAKAIPRPRFKLQAMVFDLTTNKMFDMAIMIFIVLNMTVMALDHYKQSRLFE
SILERLNIFFIAVFTAECLLKIFALRWHYFREPWNMFDFVVVILSILGTVLKDLIAAYFVSPTL
LRVVRVVKVGRVLRLVKGARGIRTLLFALAMSLPALFNICLLLFLVMFIYAIFGMSFFMHVKHR
YGVDENFNFETFGQSMILLFQMCTSAGWDGVLAAIMDEHDCNRPTDESEGNCGKRGIAVAYLVS
YLIISFLVIINMYIAVILENYSQATEDVQEGLTDDDYDMYYEIWQQFDPKGTQYVAYSNLTNFV
NALEEPLQIPKPNKYKLIALDIPICKDDMVYCVDILDALTRDFFARKGHAIEEPPRFFSLPRQK

APPENDIX III. *Rhipicephalus* (*Boophilus*) *microplus* sodium channel mutation Allele Specific Polimerase Chain Reaction.

DNA extraction

1. For preparing larvae, label 1.5 ml microcentrifuge tubes. Set on dry ice prechill a few minutes.
2. Prechill a Petri plate for a minute on dry ice.
3. Pour ticks onto plate and quick transfer single larvae to the labeled prechilled tubes and turn to -70 °C.
4. Prechill disposable pestle tips on dry ice.
5. Set tubes containing larvae on dry ice. Pulverize larvae on dry ice by grinding around 15 sec.
6. Remove tube with pestle inside from dry ice and add 25 µl of DNA extraction buffer to sample and grind about 15 more minutes. Remove and discard pestle (make sure droplet of liquid are not adhering to tip when it is remove; if so, swirl the pestle against the inside of the tube to remove as much as possible.
7. Return tube to ice dry.
8. Once several tubes have been prepared, quickly bump liquid down for 2-5 sec in a microcentrifuge and place all tubes on a float and set in a boiling water bath for 3-5 min. Place on ice after boiling.
9. Centrifuge at 14,000 rpm at 4 °C for 5 minutes.
10. Set tubes at -20 in freezer boxes. All samples may be stored at this stage for an extended period at -70° C or -20 °C.

Extraction buffer can be purchased from Perkin-Elmer (PCR buffer II) of prepare as follows:

DNA extraction buffer (10 ml)

1667 µl of 3 M KCl

600 µl of 1 M Tris-Cl, pH 8.5

400 µl of 1 M Tris-Cl, pH 8.0

7333µl water

Vortex

Reagents

Stock reagents

PCR Buffer II (without $MgCl_2$)	10X
dNTPs	2.5 mM each dNTPs
$MgCl_2$	25 mM
Primer 221 (5'-TTA-TCT-TCG-GCT-CCT-TCT-3')	100 µM
Primer 222 (5'-TTA-TCT-TCG-GCT-CCT-TCA-3')	100 µM
Primer 227(5'-TTG-TTC-ATT-GAA-ATT-GTC-GA-3')	100 µM
HotStarTaq DNA Polymerase	5U/ µl

Reaction concentrations (20 µl per reaction)

Water (RNAs free)	14.55µl
$MgCl_2$	1.4 µl
PCR Buffer II	1.0 µl
dNTPs	0.4µl
Primer 221 (Forward Susceptible Allele)	0.2µl
Primer 222 (Forward Resistant Allele)	0.2µl
Primer 227 (reverse)	0.2µl
HotStarTaq DNA polymerase	0.25µl
DNA Extraction	2.0µl

NOTE: For AS-PCR-91 bp the following primers must be used (**see section 3.3.2.**):

Primer ID	Sequence	Description
IRV-F	5′-GGACCAACCGGAATACGA-3′	Upstream non specific
IRV-R-Sus	5′-GAATAGATTCAAGGTGAA-3′	Wild type-specific antisense
IRV-R-Res	5′-GAATAGATTCAAGGTGA′T-3′	Resistant-specific antisense

PCR Amplification

1- For each DNA sample, two reactions are required. Determine the number of reactions to work (in this examples, prepare 50 reactions plus an extra reaction).
2- Into a PCR hood, identify two sets of 200 µl PCR-vial (one set with "R" (1-50), and the other with "S" (1-50).
3- One MasterMix is required to identify the susceptible allele and other for the resistant. Label two microcentrifuge tubes (1.5 ml), one with "R" (resistant) and the other with "S" (susceptible)
4- With the help of the following table, add reagents to each microcentrifuge tubes.

		MasterMix	**MasterMix**
		Susceptible(S)	**Resistant (R)**
	µl/1 reaction	* µl/Rx	* µl/Rx
No. Reactions		**51**	**51**
Reagents			
RNAs	14.55	742.05	742.05
$MgCl_2$ (1.75mM)	1.4	71.40	71.40
Buffer PCR 10X (0.7 X)	1.0	51.00	51.00
dNTPs (0.20 mM)	0.4	20.40	20.40
Primer 221-S (1µM)	0.2	**10.20**	
Primer 222-R (1µM)	0.2		**10.20**
Primer 227 (1µM)	0.2	10.20	10.20
DNA Polymerase (5 U/µl)	0.25	12.75	12.75
Total volumen		18.00	18.00

5- Mix microcentrifuge tubes slowly (MasterMix).
6- Add 18 µl of MasterMix S (susceptible) to each 200 µl PCR-vial (50 in this examples).
7- Add 18 µl of MasterMix R (resistant) to each 200 µl PCR-vial (50 in this example).

8- Add 2 µl of DNA larvae sample (DNA extraction) to both 200 µl PCR-vial (R and S). This step must be done out of the PCR hood.

9- Place each 200 µl PCR-vial in the Thermocycler.

10- Turn on Thermocycler and program the following steps:

Thermocycler Program

Block

Step 1: 96°C 2 min

Step 2: 94°C 1 min

Step 3: 60°C 1 min

Step 4: 72°C 1 min

Step 5: Go to step 2, 42 times

Step 6: 72 °C 7 min

Step 7: 10 °C forever

Step 8: End

11- Start program.

APPENDIX IV. Wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GenBank accession number AF134216. Primers NB-F in red (data not published) and FG-227 in blue (Guerrero et al., 2001) were used to amplified an expected region of 186 bp. After amplification amplicons of ~ 1000 bp were found, cloned and sequenced.

```
   1 tacttccgcg aaggctggaa tatcttcgat ttcctcatcg tcgcgctctc cttaatcgaa
  61 ctaagtttgg aaaacgtcca aggattgtct gtgctacgtt cgtttcgtct gctacgtgtg
 121 ttcaagctag ccaaatcgtg gcctaccctt aacctgctca tctctatcat ggggaaaacc
 181 atcggtgccc tcgggaactt gacctttgtc ctgggaatca tcatcttcat cttcgccgtg
 241 atgggaatgc aactctttgg caagaactac gaagaaagta aacacaagtt caaagataac
 301 atggttcctc ggtggaactt tgttgacttc atgcattcgt tcatgattgt gtttcgagtg
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 601 tccaactcta tgaaactttt caagagcttc cgtcggaaac cacgcaacca gatcggggac
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 841 aaggccgtta aaatgaagtt gaaaaacaac tcaaagcctg tgatgaattc tgtttgggtg
 901 ggacctatga tcgagcctaa gaacaagcag ctagaaaaag acaacaagga aaaggagaaa
 961 gaagcgcagg gcaataaggt gtacccgcaa aaggacgagg ataccctcag cgaaaagtca
1021 gcgtccagcc ccaaggagaa ggtgctcctt gggaacaaac cgtccaaaga tcttagcaac
1081 agttccctgt acctggggaa caaccttgag gaggagaaga aggacgccag caaagaggac
1141 ctcggtacta aagaaggaga ggaggccccc accgaagagc ccatcaaccc ggacacggaa
1201 gatgtggaca cagacaagct ggaaacggcc acctcggaca ttatcatccc cgagatgccg
1261 gccgactgct gccccgactg gtgttacacg cgattcgcgt tcgcctgctt ttttgatgag
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1381 cacaagtact tcgaaaccat tgtcgtcgtt ctcatcctca ccagcagctt ggcgctggcg
1441 cttgaagacg ttaacctgaa agaccggccg acgctcaagg cagtgctcac atatatggac
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3481 ctaccagtgc gcgagtgtgc agcgtgcaac tactcaccgc cagatggggc cgcctgatgg
3541 ggacccggta ccgtcgccgg ctgcggcaaa gcctgcttgg accttacgcg cacgctccg
```

APPENDIX V. Wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GenBank accession number AF134216. Primers IVR-F in red, and IVR-R-Sus ("t" end) and IVR-R-Res (end "a") in blue were used in PCR to amplified 91 bp (AS-PCR-91 bp).

```
   1 tacttccgcg aaggctggaa tatcttcgat ttcctcatcg tcgcgctctc cttaatcgaa
  61 ctaagtttgg aaaacgtcca aggattgtct gtgctacgtt cgtttcgtct gctacgtgtg
 121 ttcaagctag ccaaatcgtg gcctaccctt aacctgctca tctctatcat ggggaaaacc
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 241 atgggaatgc aactctttgg caagaactac gaagaaagta aacacaagtt caaagataac
 301 atggttcctc ggtggaactt tgttgacttc atgcattcgt tcatgattgt gtttcgagtg
 361 ttgtgcggcg agtggatcca gtccatgtgg gactgcatgt gggtctcagg ctggccctgc
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```

APPENDIX VI. Wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GenBank accession number AF134216. Primers PyroIRV-F (biotinylated) in red and FG-227 in blue (Guerrero et al., 2001) were used to amplified an expected region of 124 bp. Amplicons were used for Pyrosequencing™.

```
   1 tacttccgcg aaggctggaa tatcttcgat ttcctcatcg tcgcgctctc cttaatcgaa
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3241 tacaagctga tcgccctgga catacccatc tgcaaggacg acatggtcta ctgcgtcgac
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3361 ccgcgatttt tctctcttcc aagacaaaaa tgaaagattg aaacgccgcc gcacgacgtt
3421 gcacacctga tgatgatgcg gcctccttac ctccgccgtc accaacccct tcgggactgc
3481 ctaccagtgc gcgagtgtgc agcgtgcaac tactcaccgc cagatggggc cgcctgatgg
3541 ggacccggta ccgtcgccgg ctgcggcaaa gcctgcttgg accttacgcg cacgctccg
```

APPENDIX VII. Steps in Pyrosequencing™ assay to detect a sodium channel single nucleotide polymorphism (Phe → Ile) associated to pyrethroid resistance in *Rhipicephalus (Boophilus) microplus.*

NOTE: General procedures of Pyrosequencing™ were prepared by Dr. Sarah Lake

Immobilization of PCR product to beads

a. Flick mix streptavidin beads until homogenous solution obtained
b. Prepare a master mix of 3µl of bead and 37µl of binding buffer per PCR product/control.
c. Mix well and aliquot 40 µl per well to a PCR plate
d. Add 30 µl of each PCR reaction (**NOTE:** when we add 30 µl we saw clear peaks at the Pyroprogram), add milliq water to make total volume 80µl per well. (40ul of beads+binding buffer plus 30 µl of PCR product/control mix+ 10 µ milliq water)
e. Seal the plate.
f. Using a shaker, incubate at room temperature for 1hr (can increase this time if necessary) ensuring continuous agitation of beads. Secure the plate with the elastic bands provided.

Summary:

3µl of bead

37µl of binding buffer

30 µl of each PCR reaction

10 µ milliq water

Preparing the PSQ Plate

PSQ plates are the flat bottomed 96 well plates.

- For each well prepare a master mix containing 0.4 µM sequencing primer, in a total volume of 40 µl per well with annealing buffer (1.6µl of **10µM** (Dilute 10 times) sequencing primer and 38.4µl of annealing buffer.
- Move plate to vacuum prep work station, as shown in figure 10.1.

Summary:

1.6µl of **10µM** (Dilute 10 times) sequencing primer

38.4µl of annealing bfr

Strand Separation

1. Turn on the grey thermocycler heating to 80°C (User: Sarah program :80°C)
2. Place five troughs on the Vacuum Prep Worktable as suggested below in Fig 1.
3. Prepare approx 200ml 70% ethanol and 1x washing buffer using milliq water and molecular grade ethanol.
4. Fill the troughs with approximately 180 ml of the respective solution (which is almost to the top of the trough); expect the trough for the denaturation solution, which should be filled with approximately 120 ml (up to first indent). Refill the troughs with the appropriate solution whenever needed. Troughs can be washed out with milliq and reused.

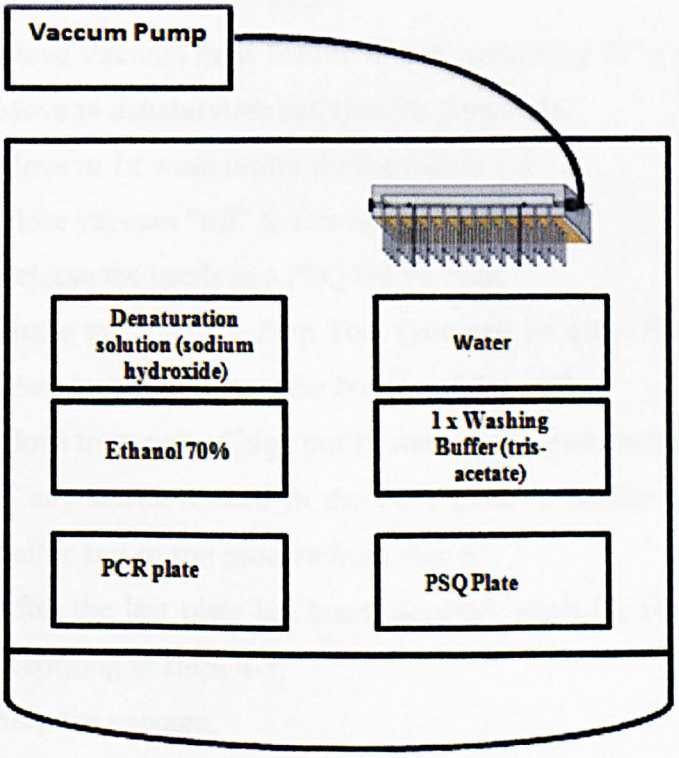

Figure 10.1. Vacuum Prep Worktable Layout for Pyrosequencing™ method.

5. Close the vacuum switch "off" and start the vacuum pump.
6. Apply the vacuum to the Vacuum Prep Tool by opening the vacuum switch "on".
7. Wash the probes of the Vacuum Prep Tool by lowering tool into milliq water (Parking position) for 20 seconds. Ensure vacuum working properly. When using the Vacuum Prep Worktable, check that the arm of the vacuum gauge has moved beyond the red zone.
8. Move 96 well PCR plate which contains beads + PCR reaction from the shaker onto PCR Plate position on Vacuum Prep Worktable.
9. Capture the beads containing immobilized templates on the filter probes by lowering Prep Tool into PCR plate. (Beads sediment quickly so this step must be done as soon as PCR plate removed from shaker).
10. Ensure liquid aspirated from all wells and that all beads have adhered to probe
    a. can see tiny granulations on probe when beads have adhered
    b. Any beads left in the plate can be re-suspended in 40µl of binding buffer and captured after dispensing the beads already on the probe. Repeat process from step 9.
11. Move Vacuum Prep Tool to trough containing 70 % ethanol for 5 seconds.
12. Move to denaturation solution for 5 seconds.
13. Move to 1x wash buffer for 5 seconds.
14. Close vacuum "off" to release the vacuum.
15. Release the beads in a PSQ HS 96 Plate
    Shake the Vacuum Prep Tool (you can be quite firm with this) while allowing filter probes to rest on the bottom of the wells.
16. Move to trough of high purity water and agitate tool for 10 seconds.
17. If any beads remain in the PCR plate re-suspend then with 40µl of binding buffer and re-run process from step 6.
18. After the last plate has been prepared, wash the filter probes with milliq water according to steps 4-5.
19. Stop the vacuum.

Primer Annealing

- Heat the PSQ plate without sealing at 80°C for two-three minutes using the flat bottomed adapter.
- Remove the plate from the heating block and let the samples cool to room temperature for 10 mins and then continue with the sequencing reaction. This can be done by putting the plate into the pyrosequencer.

Reagents

- Reconstitute the enzyme and substrate mixtures by adding milliq water (18.2 MΩ) at room temperature to the vials, as per instructions on side of vial.
- Carefully resuspend the mixture by gentle stirring.
- Remove the plastic needle cover from the underside of the reagents cartridge.
- Place reagents cartridge with label facing you. Pipette recommended volume of solutions into the cartridge into the following positions:

	G	
C		T
S	A	E

Before Starting the Run

1. *Loading the PSQ 96 Plate.*
    - Open the instrument lid.
    - Open the process chamber lid.
    - Open the plate-holding frame.
    - Place the PSQ 96 well plate on the heating block.
    - Close the plate-holding frame and the process chamber lid.

2. *Load the reagent cartridge.*
    - Open the dispensing unit cover; release the latch, then open the cover.
    - Insert the filled reagent cartridge into position. It will only fit if the label and slot face the user.
    - Close the dispending unit cover. Ensure that the latch snaps into its locked position.
    - Close the instrument lid.

Starting the Run

- You can start the run from the Run Setup window once you have entered all the required information (To open a previously entered Run-Setup, double-click the Run name in the appropriate tree-view area).
- Verify that the instrument name at the bottom of the window is correct. If necessary, choose the corresponding instrument from the drop down list.
    - Currently there is only 1 option.
- Click Run at the bottom of the Run Setup Window. The Instrument Status window is shown on top of the Run Overview window and the machine noise starts.

Monitoring the Run

- When the run is underway, the Run Overview window is displayed on the screen.
- The Instrument Status window is displayed on the top of the Run Overview during the run. A progress bar indicates the time elapsed and remaining.
- The following texts describing the instrument status are shown in the following order during the run:
    - *Well Overview:* A graphical representation of the wells indicates the current dispensation in each well. Click on a well to display the pyrogram in real time in the graph area.

- *Graph Area:* The pyrogram, or a selected process parameter, is shown in the graphical display. Dispensed nucleotides are indicated below the graph. Right click in the graph view to display the pop up menu. To view graphs for the block temperature, environmental temperature, pressure or mixer speed, select Environment and choose the desired option. To return to the pyrogram display click well again. The current values for the parameters (except for environment temp) are displayed on the Instrument Status tab.
- *Web browser area:* Click in the Web browser area to display information about the run.

Post Run Procedures

Analysis of the Results

- When a run is finished, a Close button appears on the instrument status dialog. Click close.
- Double click the Run name in the tree view. The SNP Analysis window opens.
- Click Analyse: All or mark desired wells and click Analyse Selected. The program will analyse the samples with default analysis criteria.

APPENDIX VIII. Wild type sodium channel of *Rhipicephalus* (*Boophilus*) *microplus* (cDNA fragment) reported by He et al. (1999a) with GenBank accession number AF134216. Region depict in red was amplified and sequenced to see SNPs in the gDNA of 34 individual larvae. The information generated in this region was used to design the Pyrosequencing™ assay.

```
   1 tacttccgcg aaggctggaa tatcttcgat ttcctcatcg tcgcgctctc cttaatcgaa
  61 ctaagtttgg aaaacgtcca aggattgtct gtgctacgtt cgtttcgtct gctacgtgtg
 121 ttcaagctag ccaaatcgtg gcctaccctt aacctgctca tctctatcat ggggaaaacc
 181 atcggtgccc tcgggaactt gacctttgtc ctgggaatca tcatcttcat cttcgccgtg
 241 atgggaatgc aactctttgg caagaactac gaagaaagta aacacaagtt caaagataac
 301 atggttcctc ggtggaactt tgttgacttc atgcattcgt tcatgattgt gtttcgagtg
 361 ttgtgcggcg agtggatcca gtccatgtgg gactgcatgt gggtctcagg ctggccctgc
 421 atccccttct ttctcgctac tgtagtcatc gggaaccttg tggtgctcaa ccttttcctc
 481 gccttgctgc tgtcctcgtt cggggcgtcc aatctgtccc aagcgaatcc cgacagcggc
 541 gacacaaaga aactacaaga agccatcgac cggtttcacc gggccagtcg gtggatcaag
 601 tccaactcta tgaaactttt caagagcttc cgtcggaaac cacgcaacca gatcggggac
 661 cagacaacag acattcgtgg tggcgggca ggcgaagagt tggaggctga cccgggcgtc
 721 gcaggggaag tggttctcct cgacggtcgg gtgccaatgc gagacagaaa gccccaacac
 781 aacaacgacc ttgaggttgt cgttggggac ggcctcgata tcgccattca gggtgatggc
 841 aaggccgtta aaatgaagtt gaaaaacaac tcaaagcctg tgatgaattc tgtttgggtg
 901 ggacctatga tcgagcctaa gaacaagcag ctagaaaaag acaacaagga aaaggagaaa
 961 gaagcgcagg gcaataaggt gtacccgcaa aaggacgagg ataccctcag cgaaaagtca
1021 gcgtccagcc ccaaggagaa ggtgctcctt gggaacaaac cgtccaaaga tcttagcaac
1081 agttccctgt acctggggaa caaccttgag gaggagaaga aggacgccag caaagaggac
1141 ctcggtacta aagaaggaga ggaggccccc accgaagagc ccatcaaccc ggacacggaa
1201 gatgtggaca cagacaagct ggaaacggcc acctcggaca ttatcatccc cgagatgccg
1261 gccgactgct gccccgactg gtgttacacg cgattcgcgt tcgcctgctt ttttgatgag
1321 aacaagattt tttggcagcg ctacaagatt gtgcgcacca aggcgtacgc ccttgtagag
1381 cacaagtact tcgaaaccat tgtcgtcgtt ctcatcctca ccagcagctt ggcgctggcg
1441 cttgaagacg ttaacctgaa agaccggccg acgctcaagg cagtgctcac atatatggac
1501 aagaccttca cagtgatctt tttctttgaa atgatgctca agtggcttgc ctttggattc
1561 aagaaatatt tcacaaatgc ctggtgctgg ctcgactttg tcatcgtact cgtgtccttc
1621 tttaacatgg ccgtagccat gatgggctac ggacgaatcc ccgcctttaa aaccatgcga
1681 accctccgag cactcagacc tttgagggcg atgtcccgcc tggagggaat gcgcgttgtt
1741 gtcaacgccc tggtgcaagc catcccagcc atcttcaacg tgctgctggt gtgtctcatc
1801 ttttggctca tcttctccat catgggcgtc cagatgcttg cgggaaagtt ctaccgctgc
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1981 ttggcccttt tccaagtggc aacattcaaa ggctggacgg acattatgga caatgcgatc
2041 gactccaggg gcggaaaaga ggaccaaccg gaatacgagg ctaacatcta catgtaccta
2101 tacttcgtgt tcttcattat cttcggctcc ttcttcacct tgaatctatt catcggtgtt
2161 attatcgaca atttcaatga acaaaagaag aaggctggag gatcattaga aatgttcatg
2221 acagaagacc aaaagaaata ctataacgcc atgaagaaaa tgggatccaa aaagccagcc
2281 aaggcaattc caagaccccg gttcaaactt caagcaatgg tcttcgacct gactacaaac
2341 aaaatgtttg acatggcgat catgatattt attgtcctca acatgacagt catggcactc
2401 gaccactata agcagtccag gctgttcgag tccatcctag aacggctcaa catcttcttc
2461 atcgctgtct tcacagccga atgcctgctc aaaatattcg ccctgcgctg gcactacttt
2521 cgagagccat ggaacatgtt cgacttcgta gttgtcatat tatctattct aggtacggtg
2581 ctaaaggacc tgatcgcggc ctacttcgtg tcgcccacgc ttctccgtgt ggtgcgtgtc
2641 gtgaaagtgg gccgcgtgct tcggctggtg aagggcgcgc ggggcatccg gaccctgctg
2701 ttcgccctgg ccatgtcatt gccggcgctg ttcaacatct gcctgctcct gttccttgtg
2761 atgttcatct acgcaatctt cggcatgtct ttcttcatgc acgtcaagca ccgctacggc
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2881 tgcacgtccg ccggctggga cggtgtgttg gccgctatca tggacgagca cgactgcaac
2941 cggcccaccg acgaatccga gggcaactgc ggcaagcggg gcatcgcggt cgcctacctc
3001 gtctcgtacc tcatcatcag cttcctcgtc atcataaaca tgtatatcgc cgtcatcctc
3061 gagaactaca gccaggccac cgaggacgtg caggagggcc tgaccgacga cgactacgac
3121 atgtactacg agatctggca gcagttcgac ccgaagggca cccagtacgt ggcctactcc
3181 aacctgacca acttcgtgaa cgcgctggag gagcctctgc agatcccaaa gcccaacaag
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3361 ccgcgatttt tctctcttcc aagacaaaaa tgaaagattg aaacgccgcc gcacgacgtt
3421 gcacacctga tgatgatgcg gcctccttac ctccgccgtc accaacccct tcgggactgc
3481 ctaccagtgc gcgagtgtgc agcgtgcaac tactcaccgc cagatggggc cgcctgatgg
3541 ggacccggta ccgtcgccgg ctgcggcaaa gcctgcttgg accttacgcg cacgctccg
```

APPENDIX IX. Sequences obtained from cloning aligned with the *Rhipicephalus* (*Boophilus*) *microplus* cDNA sequence (He et al., 1999a) to produce a consensus sequence and determine intron size.