International Journal for Parasitology 41 (2011) 895–903

Contents lists available at ScienceDirect

International Journal for Parasitology

journal homepage: www.elsevier.com/locate/ijpara

Evolution of acaricide resistance: Phenotypic and genotypic changes in field populations of *Rhipicephalus* (*Boophilus*) *microplus* in response to pyrethroid selection pressure

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ARTICLE INFO

Article history:
Received 21 January 2011
Received in revised form 12 March 2011
Accepted 14 March 2011
Available online 10 May 2011

Keywords:
Rhipicephalus (*Boophilus*) *microplus*
Cypermethrin
Resistance
Phenotypic
Genotypic

ABSTRACT

There have been few, if any, studies of arthropod field populations quantifying the kinetics of evolution of phenotypic and genotypic resistance to chemicals in response to the presence or absence of selection pressure. A prospective intervention study was undertaken over 2 years in Mexico to measure changes in resistance phenotype and genotype in the presence or absence of pyrethroid selection pressure on field populations of *Rhipicephalus* (*Boophilus*) *microplus* ticks on 11 farms with varying degrees of pyrethroid resistance. The resistance phenotype was evaluated by bioassay in a larval packet test expressed as the resistance factor (RF) derived from probit analysis of dose mortality regressions, and resistance genotype by an allele-specific PCR (AS-PCR) to determine the frequency of a sodium channel mutation (F1550I) associated with pyrethroid resistance. To validate the AS-PCR, a Pyrosequencing™ method was developed to detect the F1550I mutation. There was good concordance with the genotypes identified by both Pyrosequencing™ and AS-PCR (*Kappa*: 0.85). On five farms cypermethrin (CY) was exclusively used at intervals and on six farms amitraz was used. On two of the five CY-treated farms, the experiment was prematurely terminated due to unacceptably high levels of tick resistance. For all five farms, after 8–24 months of continued selection pressure with CY, the RF had increased 2–125-fold. The frequency of the resistance allele increased on all five farms from a starting range of 5–46% to a range of 66–95% after 8–24 months. On six farms treated with amitraz neither the RF nor the frequency of the resistance allele changed. A clear correlation between the phenotype and genotype was found in three of four treated farms confirming that the F1550I mutation is a major cause of synthetic pyrethroid resistance in Mexico. These results show that the pyrethroid resistance trait is stable (> 2 years) and that resistance is acquired much faster than it is lost. Hence, alternation of pyrethroid acaricide with other chemicals is likely to lead to the stepwise acquisition of synthetic pyrethroid resistance but not additional prolongation of its efficacious lifespan.

1. Introduction

Resistance to synthetic pyrethroids (SP) is one of the most serious problems in ticks worldwide, particularly in countries where the species *Rhipicephalus* (*Boophilus*) *microplus* is endemic such as Australia, Africa, North America and South America (Kunz and Kemp, 1994; Jonsson et al., 2000; Rodriguez-Vivas et al., 2006a,b, 2007). This has led to extensive studies of resistance mechanisms in ticks. To date, 10 mutations resulting in single amino acid substitutions in the voltage-gated sodium channel gene have been confirmed to be responsible for pyrethroid resistance in many arthropods (Dong, 2007; Soderlund, 2008) and in parallel with this, SP resistance studies in *R. microplus* populations have primarily focused on identifying single nucleotide polymorphisms (SNP) in this gene.

American strains of *R. microplus* were found to possess one such SNP in the S6 transmembrane segment of the domain III of the sodium channel gene (F1550I, He et al., 1999). Guerrero et al. (2001) developed an allele-specific PCR assay (AS-PCR) to genotype pyrethroid resistant isolates of *R. microplus* which was subsequently used to demonstrate the same mutation in six pyrethroid-resistant populations of *R. microplus* from Mexico (Rosario-Cruz et al., 2005) and is implicated as the major mechanism of resistance in Mexican isolates of this tick species (Rosario-Cruz et al., 2009). Recently, other mutations corresponding to the domain II S4–5 linker region (L64I and G72V) have been identified and associated with SP resistance in populations of *R. microplus* in Australia (Morgan et al., 2009; Jonsson et al., 2010), raising the possibility that different

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0020-7519/$36.00 © 2011 Published by Elsevier Ltd. on behalf of Australian Society for Parasitology Inc.
doi:10.1016/j.ijpara.2011.03.012

resistance mechanisms have developed independently between Australian and Mexican isolates.

Investigations into the relationship between resistance genotype or alleles and phenotype, as defined by response to lethal concentrations (LC) of pyrethroids, have been found in many insects: *Plutella xylostella* (Kwon et al., 2004), *Culex pipiens pallens* (Song et al., 2007) and *Anopheles gambiae* (Martinez-Torres et al., 1998; Ranson et al., 2000). 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 *Rhipicephalus decoloratus* occurred during an 18-month period (in five to six tick generations). Furthermore, Davey and George (1999) were able to select an *R. microplus* strain for resistance to permethrin by treating larvae with increasing doses through successive generations (generations F_2–F_7). Each generation was defined phenotypically by comparison with a susceptible reference isolate, recorded as a resistance factor (RF), which increased with each successive generation from 5.4 in the F_2 to 20.9 in the F_7. These results demonstrate that under laboratory conditions, continuous selection pressure results in an increase in resistance phenotype in a relatively short space of time. Similarly, a correlation between resistance phenotype (LC_{50}, % survival at discriminating doses) and genotype (resistant allele/genotype frequency) has been reported for a variety of laboratory and field isolates (Morgan et al., 2009; Jonsson et al., 2010). However, to our knowledge the evolution of a resistance phenotype and simultaneous monitoring of genotype in the presence or absence of SP selection pressure on field populations of *R. microplus* has not been reported. Indeed we believe there are few, if any, intervention studies that correlate phenotypic and genotypic changes in parasite populations under field conditions.

The present study evaluates the concurrent phenotypic and genotypic changes in *R. microplus* populations from 11 farms in Yucatan, Mexico. Throughout the 2 year field trial five farms were regularly treated with cypermethrin (CY) whilst six farms were exposed to amitraz (AM). Adult female ticks were recovered from animals at regular intervals and their progeny subjected to phenotypic analysis by larval packet test (LPT) and genotypic analysis by AS-PCR for the mutation previously associated with *R. microplus* populations in Mexico, F1550I. Furthermore, in the process of this study a novel Pyrosequencing approach to detect the F1550I mutation was developed and validated.

2. Materials and methods

2.1. Area of study

Yucatan State is located between 19°30′ and 21°35′ north latitude and 90°24′ west longitude of the Greenwich meridian. The climate is sub-humid tropical with a summer rainy season. The State has 4629 cattle farms with 624,488 head of cattle (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 nlemfuensis*), with supplementary feeding during the dry season. The use of acaricides is common (Solorio-Rivera et al., 1999). Twenty-one percent of the farms in Yucatan State use pyrethroids to control ticks and 42% of the farms applied acaricides >12 times/year (Rodriguez-Vivas et al., 2006a).

2.2. History of parasite populations, study design and acaricide treatments

Eleven cattle farms with different levels of pyrethroid resistance (Table 1) were selected for inclusion in the study based on the results of Rodriguez-Vivas et al. (2006a). All farms had between 120 and 340 *Bos indicus* and *Bos taurus* cross-bred cattle within a semi-intensive livestock production system, with stocking density between 1.0 and 1.4 animal unit/hectare and 7–12 paddocks to pasture animals. In both groups, individual pastures on each farm and between farms were divided by fences. During the study, in the 11 studied farms movement of cattle carrying ticks from neighbouring farms was not reported.

At the start of the experiment farms were allocated to CY ($n = 5$) or AM ($n = 6$) groups to give similar ranges and means with respect to RF, frequency of the resistance allele and a similar range of histories of acaricide usage (Table 1). All cattle on farms assigned to the CY treatment group received CY (Ticoff®, Lapisa, Mexico) at the recommended dose (0.2% active). Under Mexican field conditions it would be inappropriate to leave a group untreated due to the high level of tick infestation and risk of tick-borne disease transmission (Solorio-Rivera et al., 1999; Rodríguez-Vivas et al., 2004) thus the other group was treated with AM (Trak®, Lapisa, Mexico) at the recommended dose (12.5% active). Acaricides were administered topically by whole body spray using 4 L of the total finished spray volume per animal every 30–45 days. Observations were continued for up to 24 months but on two farms in the CY group the experiment was suspended early due to treatment failure, which was reasonably attributed to resistance.

2.3. Tick collection and production of R. microplus larvae

At 4–6 month intervals a sample of 30–50 engorged adult female *R. microplus* ticks was collected from at least 10 animals on each farm and transported to the parasitology laboratory at the Campus de Ciencias Biológicas y Agropecuarias of the Universidad Autónoma de Yucatán (CCBA-UADY), Mexico. Engorged adult females were placed individually in Petri dishes and incubated at 27 ± 1.5 °C and a relative humidity of 85–86% (Cen et al., 1998). After oviposition (14–18 days), eggs from all females were randomly divided into two 3 ml glass vials and plugged with a cotton cap. Larvae hatched approximately 30 days after collection of en-

Table 1
Resistance status and treatment history for parasite populations on 11 farms included in this study.

Amitraz group				Cypermethrin group			
	RF	R allele frequency	Treatment history		RF	R allele frequency	Treatment history
Farm 1	1	0	OP, AM	Farm 7	2	5	OP, AM
Farm 2	1	0	OP, AM	Farm 8	2	33	OP, AM
Farm 3	1	1	OP, AM	Farm 9	2	46	OP, AM, SP
Farm 4	3	21	OP, AM, SP	Farm 10[a]	5	35	OP, AM, SP
Farm 5[a]	6	81	OP, AM, SP	Farm 11[a]	14	45	OP, AM, SP
Farm 6[a]	10	46	OP, AM, SP				

RF, resistance factor; OP, organophosphate; AM, amitraz; SP, synthetic pyrethroid.
[a] Defined as tick populations resistant to cypermethrin based on criteria described in Beugnet and Chardonnet (1995).

gorged females; 7–14 days after larval eclosion, one vial of larvae was used for bioassays (Kemp et al., 1998) and the other was frozen at −70 °C for DNA isolation and PCR.

2.4. Determination of CY resistance phenotype by bioassay

Briefly, dose–response bioassays were carried out using the modified LPT. Technical grade CY was diluted in a mixture of trichloroethylene and olive oil (2:1 ratio) (CY dilutions from 2.0% to 0.007%). Treated filter papers were dried for 2 h in a fume hood before being folded into packets using bulldog clips. Approximately 100 *R. microplus* larvae (taken randomly from the vial) were added to the treated filter paper packets, which were then sealed with additional bulldog clips and placed in an incubator (27 °C and 85–86% relative humidity). Three replicates of each acaricide-treated dilution and a control (papers treated with dilutants only) were used. The treated larvae were exposed to CY for 24 h and the numbers of live (those that could walk) and dead larvae were counted to calculate the percentage of larval mortality. Live but inactive larvae were stimulated to move. A discriminating dose of 0.05% CY concentration (commonly used in Mexico in the LPT to detect CY resistance) was used to calculate the percentage of larval survival.

2.5. Determination of CY resistance genotype by AS-PCR

Larval genomic DNA isolation and an AS-PCR to detect the SNP (resistance allele R) in the sodium channel gene of *R. microplus* was carried out following a published method (Guerrero et al., 2001) with the following modifications: 2 μl homogenate from each single tick larva, 0.25 mM of each dNTP (ROCHE), 2.25 mM $MgCl_2$ (QIAGEN) and 0.25 μl of 1:1 vol:vol mix of hotStartTaq DNA polymerase (5 U/μl stock), and TaqStart antibody (1.1 μg/μl stock) (QIAGEN). The published primers FG221 (TTA-TCT-TCG-GCT-CCT-TCT), FG222 (TTA-TCT-TCG-GCT-CCT-TCA) and FG227 (TTG-TTC-ATT-GAA-ATT-GTC-GA) were used in the PCR, however the number of cycles was increased to 42 and the 68 bp PCR products were separated on 3.0% NuSieve agarose tris/borate/ethylene diamine tetra acetic acid (TBE) gels. Larvae were identified as one of three genotypes: homozygous susceptible (S allele only); homozygous resistant (R allele only) and heterozygous (one S and one R allele). Genotypes were identified on the presence or absence of a 68 bp diagnostic amplicon. Larvae of known genotype ((susceptible–susceptible (SS), resistant–susceptible (RS), resistant–resistant (RR)) generated from Pyrosequencing™ (one larva of each genotype) were used in the AS-PCR as positive controls in a proportion of AS-PCRs. To date only one sodium channel gene of *R. microplus* has been reported (He et al., 1999) hence the frequency of the R allele was determined as follows: percentage of the mutated sodium channel allele (R) in the total number of alleles assayed (assuming two alleles per individual).

2.6. Development of a pyrosequencing assay to determine the CY resistance genotype

During the course of this study the lack of validation of the published AS-PCR for use with a genomic DNA template and problems with visualisation of the 68 bp product were addressed by the development of a robust pyrosequencing assay. The assay was designed based on published cDNA sequence (He et al., 1999) and de novo genomic DNA sequence for 34 larvae from 19 field populations of *R. microplus* from Yucatan, Mexico, generated herein (data not shown). This included a subset of six larvae for which a 1000 bp genomic DNA fragment flanking the SNP of interest was amplified using a novel primer NBF, 5′-GCAACATTCAAAGGCTG-GA-3′ in combination with FG227. The pyrosequencing assay used two PCR primers to amplify 124 bp products. PCR was performed in thin-walled microcentrifuge tubes or 96-well PCR plates using 50 μl reactions, using 1 μl of homogenate from a single tick larva, 1X 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′ (PyroFG227, SIGMA Genosys, UK) and milliQ water. Amplification was carried out using a thermocycler (Biometric, Göttingen, Germany) with an intial 95 °C, 15 min, followed by 40 cycles of 95 °C for 15 s, 58 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 7 min. PCR products were separated on 2.0% Eurogentec agarose with Hyperladder II (Bioline, UK). Pyrosequencing™ was conducted according to the manufacturer's protocols (Biotage, AB, Sweden) using a PSQ™ HS-96A instrument. Briefly, for each reaction 30 μl of biotinylated PCR product were prepared for sequencing by immobilizing with 3 μl of Streptavidin Sepharose™ high performance beads (Amersham Biosciences, Sweden) in an 80 μl reaction volume including 1X binding buffer for 5 min at room temperature. 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 designed and used a degenerate sequencing primer (SeqIVR, 5′-CGATGAA/TTA-GATTCAAGGTG-3′) derived from the nucleotide sequence results of the 34 sequenced individual larvae. 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 Pyrosequencing™ quality control criteria. The assay was used to genotype the same 34 individual *R. microplus* larvae previously subjected to both AS-PCR and genomic DNA sequencing.

2.7. Data analyses

Probit analysis was performed on dose–response bioassay results using Polo-Plus (LeOra Software, 2004). The analysis involved probit transformation of percentage mortality and natural logarithm transformation of dose to establish the lethal concentration to kill 50% of the sample (LC_{50}) and the respective 95% confidence limits (95% CL). RF were calculated by dividing the LC_{50} of the tested populations 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). The RF estimates the magnitude of tolerance or resistance in relation to a susceptible reference strain (Robertson et al., 2007). The CY LC_{50} of the susceptible strain was 0.013 (95% CL: 0.011–0.014). This procedure provided an estimate of the relative level of phenotypic resistance in each tick population. Differences between LC_{50} estimates between times of sampling in each tick population were designated as statistically significant if their 95% CLs did not overlap.

To evaluate changes in allele frequency over time for each tick population a contingency table using chi-square tests was used. To investigate the correlation between the level of phenotypic pyrethroid resistance and the mutated allele frequency, the RF of each population were plotted against the frequency of the R allele and linear regression lines were generated. Concordance between the AS-PCR and Pyrosequencing to detect genotypes (RR, RS, SS) was calculated by using *kappa* analysis (no agreement beyond chance gives a *kappa* of 0, and a *kappa* of 1 indicates perfect agreement) (Thrusfield, 2007). Field populations from each farm and month of sampling were tested to determine whether allele frequencies conformed to Hardy–Weinberg equilibrium (HWE) using GENEPOP 4.0 (Raymond and Rousset, 1995). Those that did not conform were further tested to determine whether the deviation

from HWE was due to heterozygote deficiency or excess. Significance was defined at the $P \leqslant 0.05$ level.

3. Results

3.1. CY resistance (phenotype and genotype)

In the populations of *R. microplus* not exposed to CY there was no significant difference in either the RF or frequency of the R allele between the start and the end of the 24 month study period on any farm, irrespective of the RF and R allele frequency at the beginning of the study (Fig. 1A). In populations of *R. microplus* treated with CY at regular intervals both the RF and frequency of the R allele changed significantly on each farm during the study period (Fig. 1B). The time points at which the starting values were significantly exceeded varied from 14 to 24 months for RF and 8–19 months for frequency of the R allele (indicated by asterisks in Fig. 1B) and this equated to 14–19 and 8–19 treatments, respectively. The RF for the five populations increased 2–125-fold and the frequency of the R allele increased from a range of 5–46% at the start to 66–95% at the end. Two populations which were already resistant to CY at the beginning of the study showed rapid increases in the RF (Farm 10: 125-fold and Farm 11: twofold) and CY treatments were prematurely suspended (after 8 and 19 months for Farms 11 and 10, respectively) when *R. microplus* engorged females were observed after treatments and a lack of efficacy was claimed by owners.

Fig. 2 shows the sequential determinations of RR genotype to CY in *R. microplus* on farms where AM and CY were used for tick control. Only farms where CY (Farms 7–11, Fig. 2B) was used for tick control increased the frequency of RR genotype ($P < 0.05$).

On three farms where CY was used the increasing presence of the R allele correlated well with an increased level of resistance to CY (Farm 7: $r^2 = 0.875$, $P = 0.006$; Farm 8: $r^2 = 0.841$, $P = 0.006$; and Farm 10: $r^2 = 0.720$, $P = 0.042$). On Farm 9 ($r^2 = 0.051$, $P = 0.668$) the correlations were not significant. Correlation for

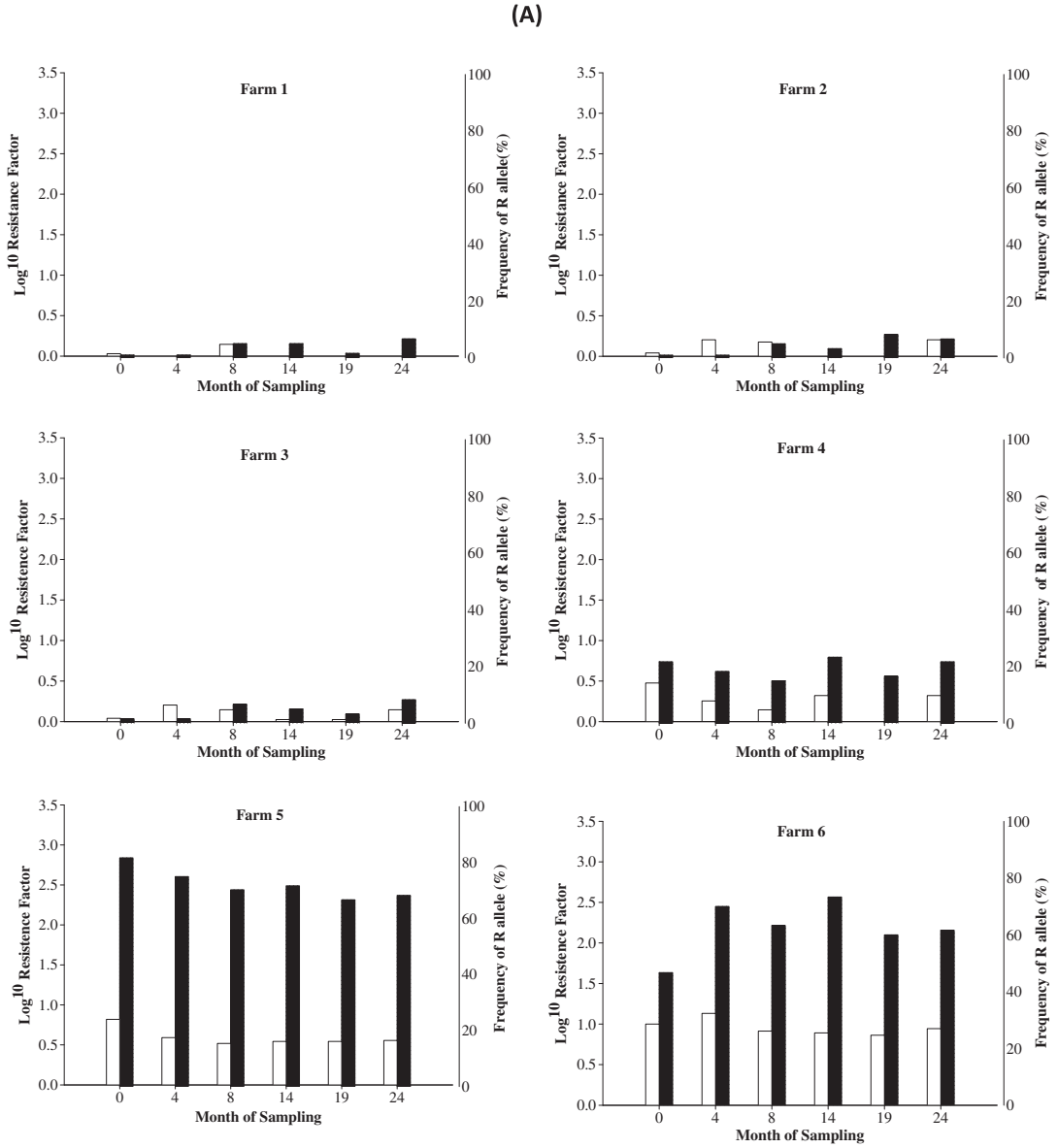

Fig. 1. Sequential determinations of resistance phenotype (resistance factor) and resistance genotype (frequency of R allele) to cypermethrin (CY) in *Rhipicephalus* (*Boophilus*) *microplus* on Farms 1–6 (A) and 7–11 (B) where amitraz (AM) and CY were used for tick control, respectively. Bold bars are the frequency of the R allele (%) and white bars are the $\log^{10}$ resistance factor (RF). No statistical changes in RF (failure of 95% confidence limit to overlap) and frequency of the R allele (chi-square test) between the beginning and end of the experiment were found in Farms 1–6. *Month of sampling where the first statistical difference was found on Farms 7–11.

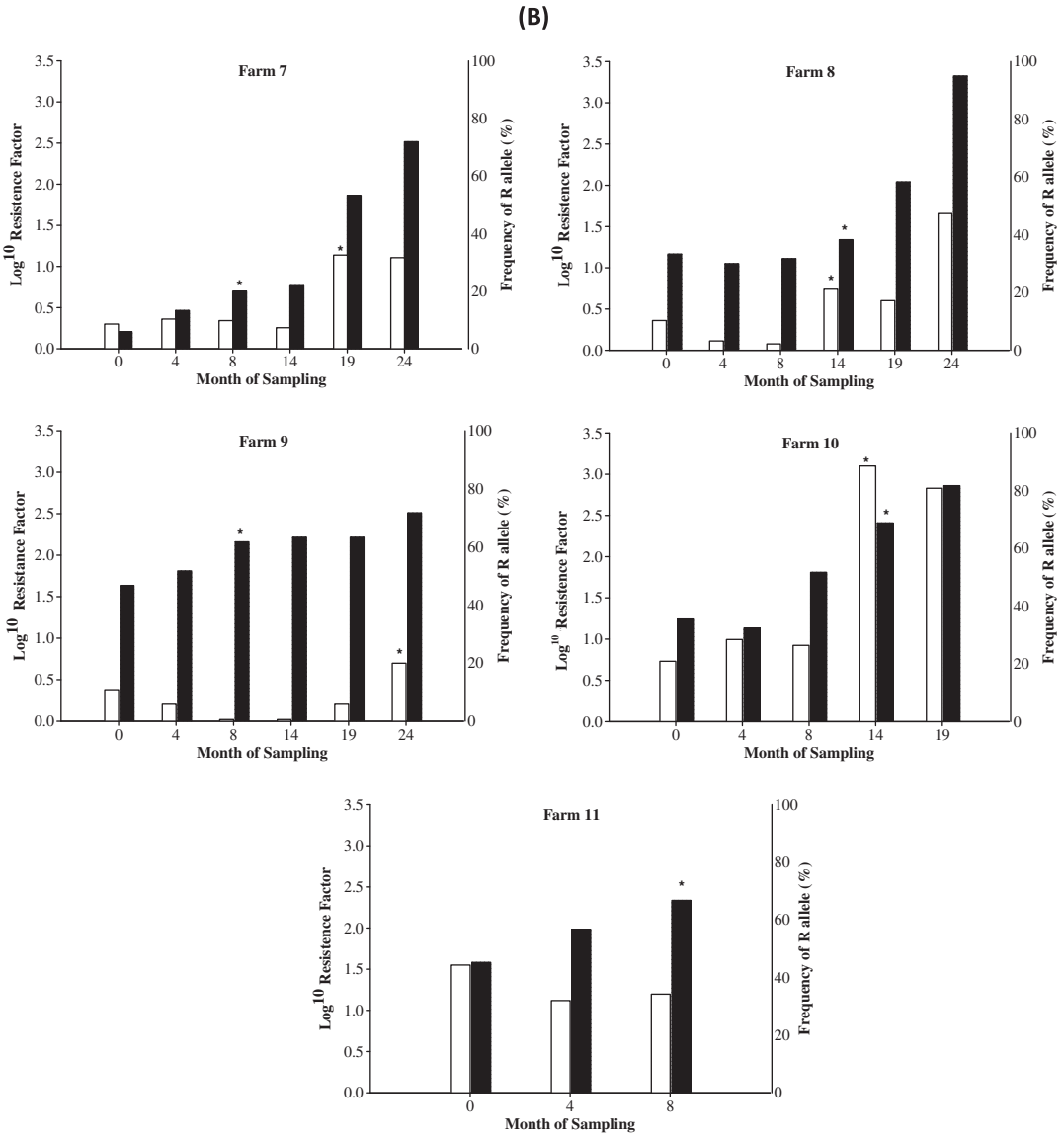

Fig. 1 (*continued*)

the Farm 11 population was not determined due to the reduced number of time points. The frequency of the R allele did not correlate ($P > 0.05$) with the level of resistance to CY in any populations treated with AM (Farm 1: $r^2 = 0.169$, $P = 0.419$; Farm 2: $r^2 = 0.520$, $P = 0.664$; Farm 3: $r^2 = 0.015$, $P = 0.813$; Farm 4: $r^2 = 0.584$, $P = 0.077$; Farm 5: $r^2 = 0.625$, $P = 0.061$; and Farm 6: $r^2 = 0.001$, $P = 0.950$). There was a strong correlation between the percentage of homozygous resistant ticks and the proportion of larval survival for three farms; Farm 7: $r^2 = 0.932$, $P = 0.002$; Farm 8: $r^2 = 0.851$, $P = 0.009$ and Farm 10: $r^2 = 0.9135$, $P = 0.011$; however, either no or a very weak relationship between the proportion of heterozygotes and percentage survival was observed for all farms.

3.2. Hardy–Weinberg equilibrium

For the AM-treated populations, the majority of time points sampled on Farms 1, 2, 3 and 4 were found to be in HWE. Tick populations on farms 5 and 6 showed a distortion from HWE but only two were statistically significant (Farm 6: 8th, 24th month of sampling) and those both displayed heterozygote excess. For the CY-treated population the majority of time points sampled on all five farms showed deviation from HWE, although this was statistically significant on only nine sampling occasions (Table 3).

3.3. Development of a pyrosequencing assay

A well validated AS-PCR was used to determine the frequency of the R allele in this study, however pyrosequencing provides significant advantages in that it sequences the target SNP, hence we developed a pyrosequencing assay for the F1550I mutation. Amplification of the genomic region flanking the SNP of interest was performed on six larvae and in all except one case a 186 bp product was produced. Following cloning and sequencing of these PCR products they were aligned with the published cDNA sequence to allow prediction of intron/exon boundaries in this region of the gene which revealed an exon/intron boundary 78 bases upstream of the target SNP. The sequence of a 76 bp coding region surrounding the SNP of interest generated from these six larvae and a further 28 individuals from multiple field locations was compared with the cDNA sequence of the sodium channel to facilitate the design of a conserved sequencing primer for pyrosequencing. The pyrosequencing PCR consistently produced the expected size product of 124 bp. There was good concordance (*Kappa* value of 0.85)

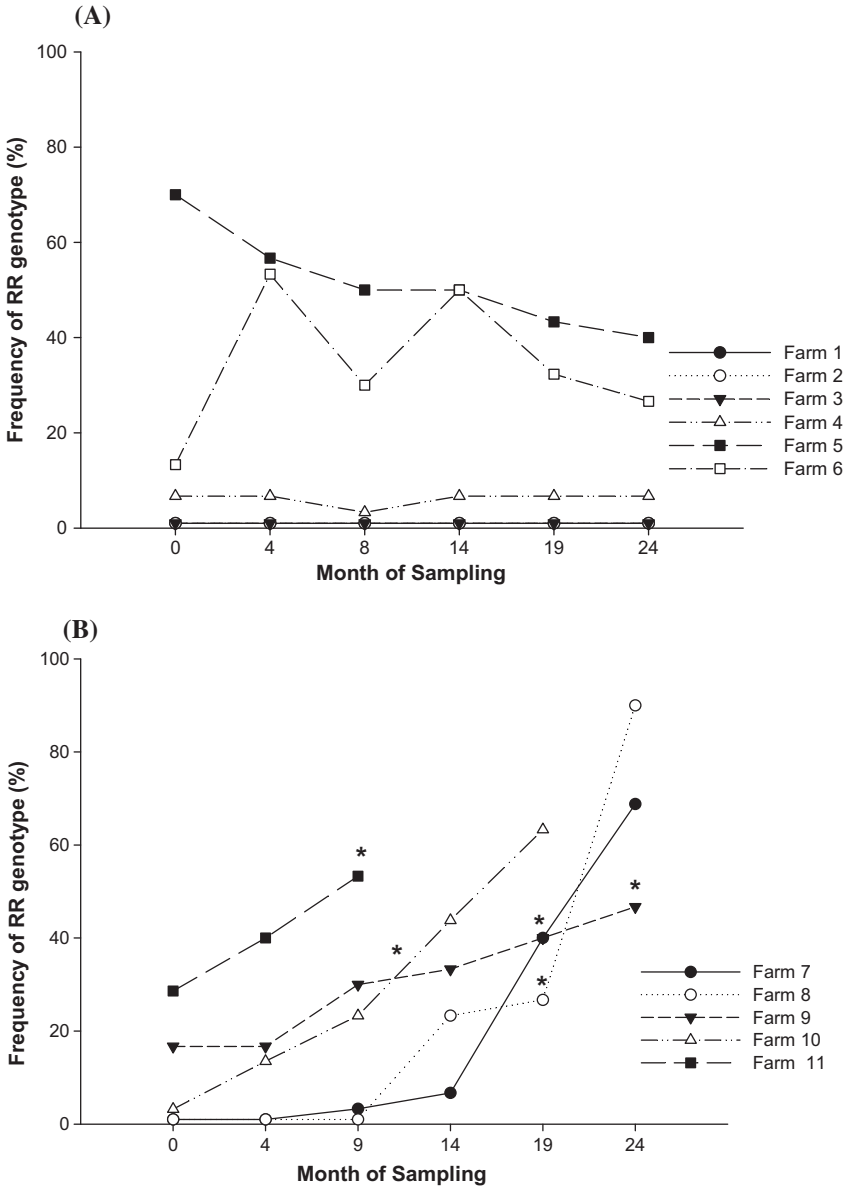

Fig. 2. Sequential determinations of resistance-resistance genotype to cypermethrin (CY) in *Rhipicephalus* (*Boophilus*) *microplus* on Farms 1–6 (A) and 7–11 (B) where amitraz (AM) and CY were used for tick control respectively. *Month of sampling where the first statistical difference was found.

with the genotypes of the 34 individual larvae identified by both pyrosequencing and AS-PCR (28/34 larvae (82.3%) showed the same polymorphism by both methods) and no statistical differences were found between the R allele frequencies tested by the two tests (Table 2).

Table 2
Pyrethroid-resistant genotypes identified in 34 individual larvae of *Rhipicephalus* (*Boophilus*) *microplus* tested by Pyrosequencing and allele specific (AS)-PCR (concordance between both tests: *kappa* value of 0.85).

Method	Genotypes			Frequency of R allele[a] (%)
	RR	RS	SS	
Pyrosequencing™	6	16	12	41.1
AS-PCR	3	18	13	35.2

RR, homozygous resistant; RS, heterozygous; SS, homozygous susceptible.
[a] R allele frequency is the percentage of mutated sodium channel allele (R) in the total number of alleles assayed (two alleles per individual).

4. Discussion

Due to the importance of chemicals in the control of parasites and the serious problems of acquired resistance compromising that control, there have been numerous studies documenting the prevalence and distribution of phenotypic resistance to insecticides, acaricides, anthelmintics and antiprotozoal agents. There have also been a number of studies in recent years investigating the molecular basis for resistance in, for example, insects (e.g. Dong, 2007), nematodes of veterinary importance (von Samson-Himmelstjerna, 2006; Hodgkinson et al., 2008; Leignel et al., 2010) and ticks (He et al., 1999; Morgan et al., 2009). However, to our knowledge there are few, if any, studies of the evolution of resistance phenotype and underlying genotype in wild parasite populations be those insects, helminths, protozoa or acarines of veterinary or medical significance. In this prospective intervention study we investigated the response of a phenotypic measure of resistance (RF determined by bioassay) and a relevant genotypic measure (allele frequency

Table 3
Sequential cypermethrin (CY) resistance phenotype defined by larval packet test (LPT, % survival), genotypes and Hardy–Weinberg equilibrium (HWE) for *Rhipicephalus* (*Boophilus*) *microplus* populations on five farms included in this study, following CY exposure.

Farm and month of sampling	CY-resistance (% survival)	Genotype			HWE	Heterozygote status
		SS	RS	RR		
Farm 7						
0[a]	15.5	30(88)	4(12)	0(0)	1	–
4	29.3	22(73)	8(27)	0(0)	1	–
8	22.7	19(63.3)	10(33.3)	1(3.3)	1	–
14	19.0	20(63)	10(31)	2(6)	0.6102	–
19	66.0	10(33)	8(27)	12(40)	0.0115[a]	Deficit
24	80.0	10(29)	2(6)	22(65)	0.0000[a]	Deficit
Farm 8						
0[a]	18.7	10(33)	20(67)	0(0)	0.0104[a]	Excess
4	8.7	12(40)	18(60)	0(0)	0.0320[a]	Excess
8	9.1	11(37)	19(63)	0(0)	0.0283[a]	Excess
14	36.2	14(47)	9(30)	7(23)	0.0528	–
19	53.5	0(0)	25(83)	5(17)	0.0001[a]	Excess
24	85.7	0(0)	3(10)	27(90)	0.0139[a]	Deficit
Farm 9						
0[a]	18.2	7(23)	18(60)	5(17)	0.4604	–
4	5.5	4(13)	21(70)	5(17)	0.0657	–
8	5.9	2(7)	19(63)	9(30)	0.1223	–
14	5.9	2(7)	18(60)	10(33)	0.2317	–
19	5.2	1(3)	17(57)	12(40)	0.2017	–
24	33.1	1(3)	15(50)	14(47)	0.3741	–
Farm 10						
0[a]	30.0	10(32)	20(65)	1(3)	0.0468[a]	Excess
4	51.4	18(49)	14(38)	5(13)	1	–
8	68.8	6(20)	17(57)	7(23)	0.7150	–
14	92.7	2(6)	16(50)	14(44)	0.6772	–
19	97.9	0(0)	11(37)	19(63)	0.5499	–
Farm 11						
0[a]	69.4	16(38)	14(33)	12(29)	0.7040	–
4	67.1	8(27)	10(33)	12(40)	0.1318	–
8	68.3	6(20)	8(27)	16(53)	0.0344[a]	Deficit

RR, resistant-resistant genotype; RS, resistant-susceptible genotype; SS, susceptible-susceptible genotype. Genotypes are shown as the number of individuals (%).
[a] Significant at $P \leqslant 0.05$.

determined by an allele-specific PCR) in farm populations of *R. microplus* under SP selection pressure over a 2 year period. *Rhipicephalus microplus* is not only a major ectoparasite of cattle in warm climates worldwide but acaricide resistance, and particularly SP resistance, is common in this species. In Australia, SP resistance was estimated to exceed 50% (Jonsson et al., 2000) and in Mexico, 59% of 98 field populations studied in Yucatan were resistant to CY (Rodriguez-Vivas et al., 2006a). Under these circumstances knowledge of the effect of selection pressure on the kinetics of the evolution of resistance and the kinetics of reversion, if any, to susceptibility in the absence of selection pressure, is germane to the development of rational acaricide usage. In particular, recommendations on the rotation of acaricides are currently made without such knowledge.

Whilst the involvement of esterase or other mechanisms may contribute to SP resistance, in Mexico target site insensitivity appears to be the major mechanism involved in conferring resistance to SPs (Rosario-Cruz et al., 2009) and, in particular, a SNP in the domain III S6 segment of the sodium channel gene (F1550I, He et al., 1999). Accordingly, we used the AS-PCR developed by Guerrero et al. (2001) based on this polymorphism to assess genotype.

Farms were selected for the study which had different levels of SP resistance at the start of the experiment including some phenotypically classified as having resistant populations. However, starting R allele frequency was ⩽81% and in most cases ⩽46%. Irrespective of the starting levels of phenotypic and genotypic resistance, on treated farms measures of each rose significantly within 8–19 months equivalent to some 8–19 treatments, demonstrating the very rapid change in response to selection pressure. Even on farms with resistance phenotypes at the beginning there was potential for the RF to increase substantially and significantly, reflecting the <100% R allele-frequency in these populations. The data from the farms treated with AM showed that both phenotypic and genotypic measures of resistance remained remarkably stable over 2 years and there was no significant change in either key variable. Notably on the two farms with starting resistant populations there was no evidence of any reduction in RF or R allele frequency in the absence of specific selection pressure. This finding suggests that the SP resistance trait remains fixed in wild populations for some time and that rotation of acaricides is unlikely to allow reversion to susceptibility within realistic time scales. Specifically, the acquisition of resistance (in <2 years) is faster than any loss in resistance (here >2 years). This suggests that rotation of acaricide type between SP and alternatives would lead to a stepwise acquisition of SP resistance. Whilst this might cause a delay in critical levels of SP resistance being reached it would not increase the net efficacious life of the SP. Contrary to our study, in a laboratory trial Thullner et al. (2007) kept a *R. microplus* strain from Costa Rica (resistant to deltamethrin but only a very low resistance to organophosphates) under selection pressure for 9–11 generations by using deltamethrin or coumaphos, either exclusively or in rotation. In the substrain selected with deltamethin, RF to deltamethrin increased from 2.1 to 756.0; however, in the substrain selected with deltamethin and then coumaphos in rotations, RF to deltamethrin was stable (1.0–4.2). These laboratory results suggested that rotation of pyrethroid with coumaphos could delay the development of pyrethroid resistance. The difference between these two sets of data is difficult to explain but may relate to the fact that one was

a field study and the other a laboratory study. The results obtained in our study were derived in a situation of minimal cattle – and hence tick – movement, in the absence of significant alternative hosts and where all animals on each farm were treated. Thus there were minimal refugia populations, but there would still be more than in a laboratory situation.

There was a close correlation between homozygote resistance frequency and larval survival in the bioassay on three farms exposed to CY ($r^2 = 0.932$, $r^2 = 0.851$, $r^2 = 0.9135$, for Farms 7, 8 and 10, respectively) and for two of these farms there was an observed shift from HWE or heterozygote excess to heterozygote deficiency (Farms 7 and 8, respectively, Table 3). These observations support the hypothesis that the F1550I allele is associated with CY resistance and that it may be recessively inherited. Similar to studies at other loci involved in SP resistance (Morgan et al., 2009), it also suggests that on exposure to CY this locus is under selection. The clear correlation between the phenotype and genotype found in three of four treated farms where there were a meaningful number of observations confirms that the sodium channel SNP is a major cause of SP resistance in Mexico, as shown by Rosario-Cruz et al. (2009), and supports the decision to use this particular polymorphism in this study. Subsequent to the execution of this project other mutations have been identified and associated with SP resistance (Morgan et al., 2009; Jonsson et al., 2010).

Comparison of the genomic DNA sequences with 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 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 products represent the same gene. The question of how many copies of the sodium channel gene exist in *R. microplus* remains unanswered. Genetic and molecular studies have identified two different sodium channel genes in *Drosophila melanogaster*, *para* (paralytic) and DSC1 (*Drosophila* sodium channel) (Ramaswami and Tanouye, 1989; Lindsay et al., 2008). As the genome project for *R. 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. microplus*. In the present study the AS-PCR was validated using pyrosequencing. Positive controls (known genotypes, SS, RS, RR) generated from pyrosequencing reactions were used in the AS-PCR. Pyrosequencing is an accurate and efficient method offering distinct advantages over AS-PCR, in particular detection of a genotype in a real time sequencing reaction rather than based on the presence and/or absence of a PCR amplicon. As such pyrosequencing can be considered a 'gold standard' giving confidence in the AS-PCR used here, as good agreement (*Kappa* value of 0.85) was seen between the two methods and no statistical differences were found between the R allele frequencies. However, pyrosequencing appeared more reliable in the detection of homozygous resistant genotypes (Table 2). In accordance with studies on other parasites (Hodgkinson et al., 2008; von Samson-Himmelstjerna et al., 2009), the data presented shows that pyrosequencing is an efficient, accurate genotyping method favoured for the analysis of large numbers of larvae. The assay we have developed provides the potential for high-throughput detection of the F1550I mutation and supports this technique for the detection of other SNP involved in pyrethroid resistance in the future.

In conclusion, this study has demonstrated the rapidity with which resistance to SP can develop in *R. microplus*, and that both phenotype and genotype remain stable for at least 2 years in the absence of selection pressure. These data suggest that rotation of SP with alternative chemicals would lead to a stepwise acquisition of SP resistance and no net increase in duration of useful life, so the preservation of SP acaricide efficacy should concentrate on other strategies.

Acknowledgments

We are very grateful to PROMEP-SEP-Mexico for supporting R.I. Rodriguez-Vivas in his PhD program at Liverpool University, UK. This project was funded by CONACYT-Complementario (No. 52212), Bayer HealthCare, Animal Heath, U.K. and Laboratorio Lapisa, S.A. of Mexico. Thanks are also due to Ford-Dodge laboratory in Mexico who kindly provided the technical grade of cypermethin. We thank Sarah Lake, Catherine Guy and Nicola Beesley for technical support.

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