Veterinary Parasitology 214 (2015) 233–241

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Ten years later: Evaluation of the effectiveness of 12.5% amitraz against a field population of *Rhipicephalus (Boophilus) microplus* using field studies, artificial infestation (Stall tests) and adult immersion tests

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ARTICLE INFO

Article history:
Received 17 June 2015
Received in revised form 22 October 2015
Accepted 23 October 2015

Keywords:
Artificial infestations
Natural infestation
Resistance
Spray formulations
Stall test

ABSTRACT

Using field trials, artificial infestations (Stall tests) and *in vitro* adult immersion tests, the present study evaluated the acaricidal efficacy of 12.5% amitraz administered *via* whole body spraying against a *Rhipicephalus (Boophilus) microplus* population that did not have any contact with chemical products belonging to this acaricide family for 10 years (approximately 40 generations). Two natural infestation trials, two artificial infestation trials (Stall tests) and two adult immersion tests were performed in two different stages in 2005 and 2015. Between 2002 and 2015, the bovine herd of this property was formed by approximately 450 animals from the Simmental breed that were divided into nine paddocks formed by *Cynodon dactylon* (L.) Pers. For the natural infestation experiments in 2005 and 2015, we selected nearly 70 animals naturally infested with ticks from the same herd that belonged to the "São Paulo" farm located in São José do Rio Pardo, São Paulo, Brazil. Field studies were performed in the same paddock (9). To evaluate anti-*R. (B.) microplus* activity in the artificially infested cattle (Stall tests) and adult immersion tests, two experiments of each methodology were performed at CPPAR (the Center of Research in Animal Health located on the FCAV/UNESP campus in Jaboticabal, São Paulo, Brazil) in 2005 and 2015. *R. (B.) microplus* used in the artificial infestation, and adult immersion test experiments were obtained from paddocks 1–9 in 2005 and 2015 from the commercial farm where the field studies were performed. Based on the obtained results, it was possible to conclude that amitraz use in rotation with pyrethroids every 28 days for three consecutive years (2002–2004) previous to the beginning of the first trial (2005) was sufficient to generate a *R. (B.) microplus* strain resistant to amitraz. Moreover, using field trials, artificial infestations (Stall tests) and adult immersion tests, we verified that 40 generations of the tick species with no contact to the aforementioned compound (amitraz) were not sufficient to revert or modify the efficacy/resistance of amitraz for this analyzed *R. (B.) microplus* strain. The reversion of amitraz efficacy values in *R. (B.) microplus* may only occur when resistance of the field strain is incipient. Alternatively, the differences in the results may be due to differences in the *Rhipicephalus* spp. species between current study locations. Therefore, future studies must be performed to prove this hypothesis.

1. Introduction

Rhipicephalus (Boophilus) microplus is a transformer agent of blood in eggs and represents a major problem for the cattle industry (Maza et al., 2013). As a consequence of its feeding habits (hematophagy), this ectoparasite can lead to severe reductions in

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http://dx.doi.org/10.1016/j.vetpar.2015.10.024
0304-4017/

livestock productivity and cattle breeding, mainly due to anemia, disease transmission, reduced weight gain, excessive cost to the producers and even death of highly infested animals (Lopes et al., 2013).

The main tool available to control this ectoparasite is the use of synthetic chemical compounds (acaricides) (Pereira et al., 2008). In accordance with Cruz et al. (2015) and Gomes et al. (2015), the control of this ectoparasite using these chemical products is a key factor in improving production, but efforts to combat ticks on most cattle farms are performed incorrectly and are characterized by the excessive and disordered use of therapeutic approaches. In turn, this excessive usage increases production costs and accelerates the selection of parasite resistance, which represents a very significant (if not the primary) economic issue in animal production (Corrêa et al., 2015).

Amongst the available chemical groups, organophosphates, pyrethroids, amidines, macrocyclic lactones, phenylpyrazole and benzoylphenyl urea derivates stand out (Cruz et al., 2014a,b). Amitraz is a formamidine acaricide that is applied to cattle in plunge dips and spray races to control the cattle tick *R. (B.) microplus*. This compound is the most commonly used acaricide in some regions of Brazil (Vargas et al., 2003; Farias et al., 2008; Santos et al., 2009). It has some advantages over other acaricides, such as low toxicity to cattle and short withdrawal periods for milk (one day) and meat (14 days).

Several researchers (Foil et al., 2004; Furlong et al., 2007; Jonsson et al., 2010; Spagnol et al., 2010) demonstrated that the only acaricide group in which a resistance reversion was possible was the amidine class. According to these authors, there is a possibility for the reutilization of this formamidine after a few tick generations (15–20 generations) without contact with products of this family or following the use of amitraz in a rotation system with other acaricide classes. For this reason, the present study used field trials, artificial infestations (Stall tests) and adult immersion tests to evaluate the acaricidal efficacy of 12.5% amitraz administered through whole body spraying against a *R. (B.) microplus* population that lacked any contact with active principles belonging to this ectoparasiticide class for 10 years (approximately 40 generations).

2. Material and methods

2.1. Location and history of chemical compound use against R. (B.) microplus in the farm where the field trials were performed

From 2002 to 2015 when the second field trial was conducted, the bovine herd of this particular farm was formed by approximately 450 animals from the Simmental breed. These animals were housed in nine paddocks formed by Coast cross grass, as seen in Fig. 1. This farm has not purchased animals from other properties since 1994, and each paddock is occupied and grazed continuously without any animal-free paddocks during the entire year.

According to the owner's records, *R. (B.) microplus* control with chemical products started in a significant manner in 2001. Any bovine that reached high infestation levels was treated with a cypermethrin-based pulverization (Barrage®, Zoetis Animal Health). In an attempt to initiate strategic control measures, all animals from the herd were treated against this tick species every 28 days between January 2002 and December 2004 with intercalated whole body spraying of amitraz (Triatox®, MSD Animal Health) and cypermethrin (Barrage®, Zoetis Animal Health) independent of each animal's infestation level. This strategic control scheme was finalized in December 2004. Between 2005 and 2015 (when the second stage of the present study was performed), the farm started to use different chemical products against the Southern Cattle Tick that were based on phenylpyrazole (fipronil), macrocyclic lactones (mainly ivermectin and doramectin) and benzoylphenyl ureas (fluazuron). These treatments were performed only when employees visually detected ticks present on the animals and lots. No amidines (amitraz) were administered in any treatments performed between 2005 and 2015.

2.2. Field trials with natural R. (B.) microplus infestations

Two different trials were conducted to evaluate the effect of 12.5% amitraz administered *via* whole body spraying against *R. (B.) microplus* in naturally infested cattle: the first in February 2005 and the second in January 2015. For each experiment, a single paddock (9) that belonged to the "São Paulo" farm (located in São José do Rio Pardo, São Paulo, Brazil) was utilized. This paddock contained approximately 50 animals, and all animals were naturally infested with ticks. We selected 20 animals in both experiments; all of the animals were 12- to 13-month-old Simmental males. Animal selection was based on an average of three consecutive counts of fully engorged *R. (B.) microplus* females between 4.5 and 8.0 mm in length located on the left side of each animal's body (Wharton and Utech, 1970). Selected bovines had no contact with any antiparasitic drugs for a minimum of 90 days prior to the beginning of the trials. Animals were randomly designated to treatment groups according to a randomized block design. The block formation was based on the arithmetic means of female ticks (4.5–8.0 mm long) counted on three consecutive days (-3, -2, and -1). Cattle were divided into 10 blocks with 2 animals each, and inside each block the animals were randomly placed into one of the treatment groups: 10 animals were selected to be untreated controls and 10 animals were treated *via* whole body spraying with 12.5% amitraz. Prior to the pre-treatment tick counts, the cattle were maintained in a common paddock. However, after treatment paddock 9 was subdivided into two equal parts so that none of the groups would have any form of contact with animals from the other group during the entire post-treatment period. Both experiments (2005 and 2015) were conducted in the same paddock (number 9, displayed in Fig. 1).

To determine the therapeutic and residual efficacies obtained by the tested formulation, tick counts were performed on days 3, 7, 14, 21 and 28 post-treatment (Wharton and Utech, 1970).

2.3. Stall tests with artificial R. (B.) microplus infestations

To evaluate the anti-*R. (B.) microplus* activity in artificially infested cattle, two experiments were performed at CPPAR (the Center of Research in Animal Health), which is located on the FCAV/UNESP campus in Jaboticabal, São Paulo, Brazil.

In each experiment (2005 and 2015), nearly 500 fully engorged female ticks were collected from the body surfaces of bovines from all paddocks of the property (1–9) during the tick counts performed prior to treatment (days -3, -2 and -1). These specimens were transported to CPPAR/FCAV/UNESP, where a laboratory colony of this particular *R. (B.) microplus* strain was initiated for posterior use in the artificial infestation trials. One colony was started in 2005 and used in the first study, while a second colony was started in 2015 and used for the second trial. During the experiments, the ticks were maintained in CPPAR using experimental bovines and a BOD incubator.

A total of 12 crossbred animals that were approximately seven months old and had not received any type of antiparasitic medication for a minimum of 120 days before the experiment were selected for each trial; the animals were identified using individually numbered ear tags. Each animal was kept in an individual suspended box that was appropriate for the Stall tests starting 27 days before treatment to allow for acclimatization. After the acclimation period, all animals were infested with 5000 *R. (B.) microplus*

larvae (0.25 g of eggs) with ages ranging from 14 to 28 days on the following experimental dates: -24, -21, -19, -17, -14, -12, -10, -7, -5, -3 and -1 (considering day 0 as the treatment day) (Brazil, 1997). The infestation of cattle was performed using the following procedure. The stall door was opened and the animal was identified. Then, the animals were humanely restrained with a halter and infested with ticks in the stall. The syringes containing the larvae were applied over the dorsal line of the animal, and the larvae were allowed to move into the coat to settle. The total confinement time for each animal was approximately 60 min. On days -3, -2 and -1 (with day 0 defined as the treatment day), counts of fully engorged female ticks that naturally detached from each animal were performed.

The cattle were randomized and distributed to treatment groups according to the average number (arithmetic means) of fully engorged *R. (B.) microplus* females that detached from each animal. The animals were separated into 6 blocks with two animals each; within each block, individuals were randomly allocated to one of the following groups: 6 animals were used as untreated controls and 6 animals were treated *via* whole body spraying with 12.5% amitraz. The blocks were assigned to 2 sets of nearby pens, and animals within a block were allocated to pens within the set at random.

In both experiments with experimental infestations, daily counts of fully engorged female ticks that detached from each animal were performed from day 1 until day 28 post-treatment, always between 08:00 and 09:00 a.m. Additionally, all experimental animals were infested twice per week (Tuesdays and Thursdays) with approximately 5000 viable unfed larvae following the recommendations of Holdsworth et al. (2006).

2.4. Treatment

Treatment of the experimental animals in both trials (natural and artificial tick infestations) was performed using a hand pump with pressure in the "triangle format" so that each animal received treatment for 5 min with 5 L of solution containing 12.5% amitraz (Triatox®, MSD Animal Health). For product administration, the animals were individually restrained with the aid of a rope that was tied so that there was no risk of hanging.

Products were diluted at a ratio of 40 mL of amitraz per 20 gallons of water according to the manufacturer's recommendations. After preparation, the dilution was subdivided into equal parts containing 5 L, and the slurry was mixed thoroughly. The entire body of each animal was treated, including inside the ears, base of the horns, base of the tail, dewlap and inner face of the limbs and udder; all animals were dosed uniformly according to the product label specifications. Each animal received a mean dosage of 19 mL/kg of the chemical compound.

After treatment and throughout the experiment, all animals were evaluated regarding the presence of clinical signs of systemic poisoning, such as ataxia, sialorrhea, prostration, excitement, convulsions, dysphonia, diarrhea, dyspnea, jaundice, cough, head tremors, skin tremors, and hyperesthesia.

2.5. Efficacy

For field trials with naturally infested animals and Stall tests evaluating artificially infested cattle, arithmetic means were selected to calculate the acaricide efficacies rather than geometric means according to the recommendations of Dobson et al. (2009), Vercruysse et al. (2011) and Lopes et al. (2014) using the following formula:

$$\text{Efficacypercentage} = [1 - \frac{\text{Ta} \times \text{Cb}}{\text{Tb} \times \text{Ca}}] \times 100$$

In this equation, "Ta" is the number of fully or partially engorged female ticks counted on the treated animals after medication, "Tb" is the number of ticks counted on the treated animals during the 3 days prior to treatment, "Ca" is the number of fully or partially engorged female ticks counted on untreated control animals after

Fig. 1. Satellite photograph (Google Earth) of the trial site showing the paddocks numbered. Paddock 9 site where were performed the field studies, this paddock was subdivided in two for spearing the groups after the treatments; paddocks 1–9 were used for collecting the fully engorged females used in the artificial infestations (Stall tests) and in the adult immersion tests.

Table 1
Mean counts of female *R. (B.) microplus* (4.5–8.0-mm long) for control and treated cattle groups; efficacy percentages. Arithmetic means. Field study performed in 2005.

Study performed in 2005									
Day of the study	T01: control (water)			T02: amitraz 12.5%[b]			ANOVA		Efficacy(arithmetic mean)
	Mean count[a]/range			Mean count[a]/range			CV[c]	Pr < F[d]	
0	42.4	11.3–104.7	A	42.1	12–104.7	A	16.79	0.9956	–
3	37.5	16–93	A	12.4	0–40	B	29.09	0.0021	67
7	37.3	18–103	A	8.6	4–12	B	15.48	<0.0001	77
14	43.4	22–94	A	12.4	0–20	B	32.12	0.0025	71
21	45.0	26–100	A	27.4	8–42	B	12.76	0.0302	39
28	46.2	23–103	A	39.4	20–56	A	10.33	0.4682	14

[a] Means values followed by the same letter on the same line do not differ significantly at a 95% reliability level.
[b] Commercial formulation purchased in the local market.
[c] Coefficient of variation.
[d] Probablility of significance *F*.

the treatment date, and "Cb" is the number of ticks counted on untreated control animals on the three days preceding treatment.

2.6. R. (B.) microplus *adult immersion tests*

To evaluate the sensitivity of the population of *R. (B.) microplus* from the farm where the field studies were performed, two *in vitro* tests (one in 2005 and the second in 2015) were conducted with the tested 12.5% amitraz formulation using fully engorged female ticks belonging to different paddocks (1–9) from the experimental farm.

Approximately 80 fully engorged females were separated during the collection of ticks to form the laboratory colonies. In each test (2005 and 2015), 40 ticks were used for each group: 20 (with two replications) were subjected to treatment with 12.5% amitraz and the remaining 20 (with two replications) were used as negative untreated controls. Subsequently, specimens were taken to the laboratory (CPPAR/FCAV/UNESP, Jaboticabal, São Paulo, Brazil), washed with running water and dried using paper towels. Then, the ticks were placed on a white tray for selection based on visual inspection of those that showed good mobility and repletion. Ticks that showed anomalies in size or shape were immediately discarded. To form groups for both tests, female ticks were ordered from the biggest to the smallest individuals, thereby allowing a similar final weight for each group to be determined.

Female ticks from each group for each test (2005 and 2015) were placed in 50 mL Falcon tubes and immersed in 20 mL solutions containing diluted 12.5% amitraz for 5 min.

Next, the ticks were recovered on plastic strainers, dried using paper towels at room temperature for approximately 5 min and then transferred to plastic Petri dishes and moved to a biochemical oxygen demand (BOD) incubator at a temperature of 27 °C and approximately 85% relative humidity to stimulate oviposition. Twenty days after oviposition in the BOD incubator, the egg masses of engorged female ticks were recorded for each group. Subsequently, the egg masses of each group were transferred to adapted 3 mL syringes and returned to the BOD incubator (at 27 °C and approximately 85% relative humidity) to stimulate larval hatching. After 20 days of larval hatching in the BOD incubator, the percentage of hatchability was calculated according to a methodology described by Gonzales et al. (1993). The percentage of hatched eggs from each sample group and each test (2005 and 2015) was visually estimated using a stereo-microscope with an ocular grid to compare the proportion of larvae in relation to the proportion of unhatched eggs for each group and post-treatment day (Drummond et al., 1973; Gonzales et al., 1993; George and Davey, 2004; Holdsworth et al., 2006). The error of this hatching percentage analysis was estimated to be 5%.

2.7. Data analysis

A "Split Plot in Time" design was used to analyze the tick counts obtained in the field trials and Stall tests. Treatments were considered the main parcels (with 10 or 6 replications each, depending on the trial), and the observational dates were considered the secondary parcels (Banzatto and Kronka, 1989). Data were examined using a methodology described by Little and Hills (1978), in which the data were log transformed ($x+1$). Analyses were conducted using the F Test, and means were compared using the Tukey Test (SAS Institute, 1996).

2.8. Criteria for the diagnosis of resistance or susceptibility of a tick population to amitraz

According to EMEA (2004) and Holdsworth et al. (2006) with some exceptions, the control of a tick population by a specific formulation is considered unsatisfactory when the efficacy values of the compound are inferior to 90%. Moreover, the Brazilian Ministry of Agriculture, Livestock and Food Supply (Ministério da Agricultura, Pecuária e Abastecimento; MAPA) through Ordinance N° 48 (Brazil, 1997) states the importance of considering the average efficacy of a formulation between days 7 and 14 post-treatment for field studies. In artificial infestation studies (Stall tests), the mean efficacy of a spray formulation should be calculated between days 1 and 23 post-treatment. A mean efficacy inferior to 90% between these dates was the criteria adopted to designate *R. (B.) microplus* as resistant to 12.5% amitraz (spray).

3. Results

No signs of abnormalities or systemic intoxication were observed in the experimental animals before or after the administration of 12.5% amitraz in all experiments. Moreover, no bovines died and no concomitant medications were administered during these experiments.

3.1. 2005 trials

The results of the 12.5% amitraz efficacy against *R. (B.) microplus* obtained from the 2005 field trial are described in Table 1. The analysis of these results demonstrated that amitraz reached efficacy indices of 67, 77 and 71% on the 3rd, 7th and 14th days post-treatment (DPT), respectively. The acaricidal efficacy of the chemical decreased to 39 and 14% on days 21 and 28 post-treatment, respectively, when the tick counts for this study were concluded (Table 1). Based on the results of the statistical analysis, animals from the untreated control group were verified to present an average number of ticks that was significantly superior ($P \leq 0.05$)

Table 2
Average number of engorged *Rhipicephalus (Boophilus) microplus* females naturally detached from control and treated cattle and percent efficacy (arithmetic means). Artificial infestation (Stall test) of study performed in 2005.

Study performed in 2005									
Day of the study	T01: control (water)			T02: amitraz 12.5%[b]			ANOVA		Efficacy (arithmetic mean)
	Mean count[a]/range			Mean count[a]/range			CV[c]	Pr < F[d]	
0	81.67	31.67–119.0	A	80.11	30.67–154.0	A	13.30	0.9752	–
1	28.67	12–50	A	17.33	2–23	A	23.69	0.2320	38
2	40.17	21–67	A	20.50	14–32	A	11.28	0.0601	48
3	91.33	30–197	A	43.83	11–123	A	21.13	0.0860	51
4	332.33	42–867	A	110.83	11–358	B	24.20	0.0070	66
5	580.00	162–1175	A	164.00	45–423	B	16.09	0.0181	71
6	711.33	208–1549	A	183.50	68–452	B	14.03	0.0172	74
7	748.33	204–1518	A	199.83	93–380	B	13.24	0.0216	73
8	647.50	181–1386	A	140.17	76–225	B	12.74	0.0103	78
9	606.17	177–1179	A	121.50	20–234	B	15.67	0.0042	79
10	159.00	32–198	A	58.50	17–89	B	17.54	0.0268	62
11	114.17	36–252	A	43.17	9–87	B	21.13	0.0435	61
12	99.83	34–178	A	38.00	10–61	B	17.92	0.0433	61
13	168.83	58–296	A	60.50	22–96	B	13.00	0.0171	63
14	161.83	61–350	A	90.83	24–161	B	14.53	0.0472	43
15	138.50	56–235	A	84.17	23–177	B	16.11	0.0349	38
16	140.67	41–295	A	72.83	12–141	B	20.25	0.0045	47
17	170.33	44–316	A	77.33	21–181	B	17.17	0.0290	54
18	151.83	45–292	A	68.83	5–179	B	24.33	0.0312	54
19	240.67	125–454	A	109.00	5–236	B	21.78	0.0326	54
20	226.83	90–435	A	121.50	45–232	B	12.68	0.0217	45
21	203.00	78–431	A	104.83	32–145	B	13.38	0.0189	47
22	201.50	79–421	A	108.17	39–158	B	17.88	0.0321	45
23	198.50	81–401	A	106.00	41–143	B	13.47	0.0489	45
24	200.83	84–412	A	110.17	44–165	B	21.35	0.0239	44
25	203.83	89–425	A	113.00	48–178	B	18.21	0.0431	43
26	200.33	75–418	A	112.67	45–179	B	14.59	0.0318	43
27	197.33	71–404	A	113.67	48–182	B	12.39	0.0194	41
28	197.17	65–409	A	114.00	46–186	B	11.32	0.0324	41
1–23	255.74	12–1549	A	96.74	2–452	B	12.36	0.0021	61

[a] Means values followed by the same letter on the same line do not differ significantly at a 95% reliability level.
[b] Commercial formulation purchased in the local market.
[c] Coefficient of variation.
[d] Probablility of significance *F*.

Table 3
Results of the reproductive parameters of engorged *Rhipicephalus (Boophilus) microplus* females from groups of control and treated with 12.5% amitraz in the study of adult immersion tests. Reduction of oviposition, hatchability, reproductive efficiency and reproductive capacity.

Study performed in 2005										
Test	Group	Repetition	Number of engorged female pre-selected	Engorged female weigth (g)	Egg mass weight (g)	Hatchability (%)	%Reduction		Reproductive efficiency	Reproductive capacity (%)
							Oviposition (%)	Hatchability (%)		
A	Control	1	10	2.021	0.521	99.6	–	–	513524	–
		2	10	2.045	0.512	99.4	–	–	497729	–
	Mean		10	2.033	0.517	99.5	–	–	505576	–
	12.5% amitraz	1	10	2.029	0.334	34.0	36.0	66.0	111937	–
		2	10	2.115	0.435	46.0	17.0	54.0	189220	–
	Mean		10	2.072	0.385	40.0	26.0	60.0	150578	70

to the average amount of *R. (B.) microplus* quantified in bovines treated with amitraz between the 3rd and 21st DPT (Table 1).

The results obtained from the artificial infestation trial conducted in 2005 with this same tick strain reiterated the efficacy values against *R. (B.) microplus* obtained from the field trial. In the Stall test, amitraz reached a maximum efficacy index of 79% on the 9th DPT. Subsequent evaluation dates saw a constant decrease in efficacy, which reached 41% on the 28th DPT, leading to the end of this experiment (Table 2). Mean tick counts from the control group were significantly superior ($P \leq 0.05$) to the mean *R. (B.) microplus* counts from the animals treated with 12.5% amitraz between days 4 and 28 post-treatment (Table 2).

Using the efficacy indices of the 12.5% amitraz formulation obtained from the adult immersion tests, we verified that this compound showed 70% efficacy towards the reproductive parameters of fully engorged *R. (B.) microplus* females (Table 3).

Analysis of these results based on the resistance diagnosis criteria pre-established for this experiment demonstrated that the tested *R. (B.) microplus* population was resistant to 12.5% amitraz administered through whole body spraying in all three trials conducted (field study, Stall test and adult immersion test).

Table 4
Mean counts of female *R. (B.) microplus* (4.5–8.0-mm long) for control and treated cattle groups; efficacy percentages. Arithmetic means. Field study performed in 2015.

Study performed in 2015									
Day of the study	T01: control (water)			T02: amitraz 12.5%[b]			ANOVA		Efficacy(arithmetic mean)
	Mean count[a]/range			Mean count[a]/range			CV[c]	Pr < F[d]	
0	36.9	22.1–77.0	A	37.3	22.4–77.7	A	12.89	0.9550	–
3	38.6	18–98	A	18.5	10–57	B	16.06	0.0084	52
7	40.4	21–103	A	17.9	10–58	B	16.89	0.0060	56
14	43.6	18–90	A	20.2	7–51	B	15.26	0.0051	53
21	46.3	21–101	A	30.3	17–54	B	11.38	0.0387	34
28	47.5	19–98	A	40.5	25–75	A	11.00	0.4143	14

[a] Means values followed by the same letter on the same line do not differ significantly at a 95% reliability level.
[b] Commercial formulation purchased in the local market.
[c] Coefficient of variation.
[d] Probablility of significance *F*.

Table 5
Average number of engorged *Rhipicephalus (Boophilus) microplus* females naturally detached from control and treated cattle and percent efficacy (arithmetic means). Artificial infestation (Stall test) of study performed in 2015.

Study performed in 2015									
Day of the study	T01: control (water)			T02: amitraz 12.5%[b]			ANOVA		Efficacy(arithmetic mean)
	Mean count[a]/range			Mean count[a]/range			CV[c]	Pr < F[d]	
0	49.23	34.70–74.70	A	49.78	29.70–74.30	A	8.56	0.9908	–
1	50.50	21–83	A	49.33	27–103	A	15.35	0.9164	3
2	51.17	29–76	A	43.83	22–98	A	12.58	0.4761	15
3	52.33	32–82	A	39.00	17–84	A	12.79	0.2530	26
4	53.17	27–91	A	31.00	13–69	A	15.75	0.1328	42
5	53.33	19–96	A	24.50	11–63	A	19.37	0.0790	55
6	54.50	23–102	A	20.83	8–58	B	21.62	0.0400	62
7	55.17	23–99	A	18.83	5–54	B	24.13	0.0285	66
8	55.00	26–82	A	27.50	8–61	B	19.43	0.0444	51
9	55.67	28–88	A	33.50	11–73	A	17.20	0.1370	40
10	55.33	29–81	A	33.50	13–74	A	15.40	0.1182	40
11	55.83	26–87	A	35.83	13–76	A	14.61	0.1682	37
12	55.67	28–118	A	30.67	10–72	A	20.42	0.2696	46
13	55.00	25–105	A	29.17	9–75	A	21.73	0.2248	48
14	55.83	16–123	A	24.00	6–67	A	25.06	0.1356	57
15	55.33	21–112	A	33.00	8–58	A	18.82	0.4411	57
16	53.50	26–103	A	37.67	6–98	A	23.34	0.4034	49
17	53.33	28–102	A	39.17	4–98	A	29.85	0.3144	50
18	52.17	28–95	A	35.33	11–99	A	18.91	0.2212	45
19	52.83	26–98	A	38.00	8–88	A	19.25	0.3049	40
20	53.50	31–93	A	29.17	6–87	A	21.08	0.0805	46
21	52.83	27–88	A	32.50	12–56	A	14.25	0.1407	37
22	57.67	21–98	A	34.17	13–65	A	21.03	0.3021	38
23	56.50	23–93	A	32.67	15–54	A	17.04	0.1234	35
24	59.00	20–90	A	34.67	16–65	A	32.45	0.3256	34
25	54.00	24–98	A	36.17	14–76	A	18.56	0.4532	34
26	55.00	27–94	A	36.00	13–76	A	24.56	0.2876	32
27	58.00	23–97	A	36.67	16–77	A	23.32	0.2945	32
28	56.67	26–90	A	36.33	18–73	A	25.31	0.4567	37
1–23	53.75	16–123	A	31.74	4–103	A	23.43	0.4921	42

[a] Means values followed by the same letter on the same line do not differ significantly at a 95% reliability level.
[b] Commercial formulation purchased in the local market.
[c] Coefficient of variation.
[d] Probablility of significance *F*.

3.2. 2015 trials

After 10 years, the results from the second field trial conducted showed that 12.5% amitraz obtained efficacy indices of 52, 56, 53, 34 and 14% on days 3, 7, 14, 21 and 28 post-treatment, respectively. The statistical analysis verified that the average tick counts from the control group animals were significantly superior ($P \leq 0.05$) to those obtained from bovines that belonged to the group treated with amitraz between the 3rd and 21st DPT (Table 4).

In the Stall tests conducted with artificially infested bovines, amitraz reached maximum efficacy indices of 66 and 66% on the 6th and 7th DPT, respectively. Between the 9th and 20th DPT, the chemical compound presented efficacy values between 36 and 57% (Table 5). Between days 21 and 28 post-treatment, the amitraz efficacy remained approximately 30%, with 37% obtained on the 28th DPT when the trial was concluded. Statistical analysis of the data verified that the untreated animals presented *R. (B.) microplus* averages that were significantly superior ($P \leq 0.05$) to those obtained

Table 6
Results of the reproductive parameters of engorged *Rhipicephalus (Boophilus) microplus* females from groups of control and treated with 12.5% amitraz in the study of adult immersion tests. Reduction of oviposition, hatchability, reproductive efficiency and reproductive capacity.

Study performed in 2015										
Test	Group	Repetition	Number of engorged female pre-selected	Engorged female weigth (g)	Egg mass weight (g)	Hatchability (%)	%Reduction		Reproductive efficiency	Reproductive capacity (%)
							Oviposition (%)	Hatchability (%)		
A	Control	1	10	1.984	0.492	99.8	–	–	494976	–
		2	10	1.903	0.491	99.7	–	–	514425	–
	Mean		10	1.944	0.492	99.8	–	–	504498	–
	12.5% amitraz	1	10	1.932	0.321	47.0	35.0	53.0	156180	–
		2	10	1.980	0.378	58.0	23.0	42.0	221660	–
	Mean		10	1.956	0.350	52.2	29.0	47.0	188920	62

from bovines treated with amitraz between post-treatment days 6 and 8 (Table 5).

In the *in vitro* adult immersion tests, the formulation containing 12.5% showed 62% efficiency over the reproductive parameters of *R. (B.) microplus* females (Table 6).

Using the resistance diagnosis criteria adopted for the present study, we verified through trials conducted after a 10-year gap (2005–2015, comprising approximately 40 tick generations with no contact to amidines) that this *R. (B.) microplus* population remained resistant to 12.5% amitraz administered *via* whole body spraying in all of the trials conducted (field study, Stall test and adult immersion test).

4. Discussion

Brazil is a huge country with very different climatic regions (South, Southeast, Center–West, North and Northeast) where the temperature, relative humidity and pluviometric precipitation indices are different. These conditions directly alter the number of *R. (B.) microplus* generations in each of these regions. The location in which the present study was conducted (Southeast region of Brazil) was characterized by two distinct climate phases: one rainy season with high temperatures and humidity, which occurs during the spring and summer (October and March), and one season with inferior temperatures with low rain and relative humidity, which occurs during autumn and winter (April and September). *R. (B.) microplus* population dynamic studies in this same region of Brazil were conducted by Kasai et al. (2000) and Pereira et al. (2008). Both studies demonstrated that this tick species presented an average of 4 generations per year. Gomes (2014) evaluated the population dynamic of this same tick species in Minas Gerais, a state also located on the Southeast region of Brazil, and reported 5 generations per year. This result demonstrated that nearly 40 generations of this tick species remained free from any contact with amitraz and/or any other formamidine derivates between 2005 and 2015, which was period between both parts of this study.

Amitraz has been in use since 1975 in Australia, and the first case of resistance to this chemical in this country was reported in 1981 (Nolan, 1981). In Mexico, amitraz was introduced in 1986 and the first case of resistance was diagnosed in 2001 (Soberanes Cespedes et al., 2002). In Brazil, this compound was introduced in 1987 and the first case of resistance was diagnosed in 1994 by Francisco Branco (reported by Martins et al., 2003). Resistance to amitraz is now a problem for cattle producers around the world and has been reported in various locations including Australia (Jonsson and Hope, 2007), Mexico (Rodriguez-Vivas et al., 2006), Brazil (Li et al., 2005; Martins et al., 2003; Santos et al., 2013) and New Caledonia (Chevillon et al., 2007). The prevalence of resistance to amitraz was estimated to be 11% in Australia (Jonsson and Hope, 2007) and 19.4% in Mexico (Rodriguez-Vivas et al., 2006). Although resistance to the product was not widespread, it was the mainstay of tick control in many countries due to its high efficacy and relatively low cost (Jonsson and Matschoss, 1998).

To date, loss of resistance to amitraz has not been documented after removal of selection pressure. The only two studies that examined the effect of selection with amitraz in the field were published by Rosado-Aguilar et al. (2008) and Jonsson et al. (2010). In the first study, a monthly application of amitraz was shown to increase resistance ratios from one- or two-fold to over 10-fold within 15 months. The effect after this time period was not reported. The second trial was performed by Jonsson et al. (2010) and evaluated the rotation of treatments between spinosad and amitraz to control *R. (B.) microplus* populations with amitraz resistance.

In this case, the study was conducted over 4 years in which three treatments were applied to 6 groups of Holstein dairy calves. Standard counts of all ticks between 4.5 and 8.0 mm in diameter on one side of each animal were made each week, and treatment was applied when the tick numbers exceeded a threshold of 25 engorged adults per side. The three treatments were: 1, spinosad spray whenever tick numbers exceeded the threshold; 2, amitraz spray whenever tick numbers exceeded the threshold; and 3, spinosad whenever tick numbers exceeded the threshold. These treatments were performed for the first 2 months, followed by amitraz for 2 months, with alternations every subsequent 2 months. The larval packet test bioassay was used to diagnose acaricide resistance to amitraz. These authors concluded that treatment with spinosad or with a rotation between spinosad and amitraz every 2 months resulted in the loss of evidence of amitraz resistance on the LPT and a return to full or almost full susceptibility to amitraz. In accordance with these results, rotation strategies using amitraz should be developed with caution because secondary mutations could negate the measured loss in amitraz resistance shown in the study. It should also be noted that a similar loss of resistance is measurable in the geographical location where the rotation program will be implemented. Areas with little seasonal climatic variation might not drive similar selection pressures against amitraz-resistant ticks (Jonsson et al., 2010).

In addition to the aforementioned field trials, the possibility of reutilizing amitraz in a field strain has been proposed. Furlong et al. (2007) stated that the amidine group was the only acaricide group that allowed for a regression of resistance. The authors claimed that nearly 15–20 generations of ticks not exposed to selection pressure by the use of products of this family would allow for a reutilization of these chemicals. Spagnol et al. (2010) correlated the difference in efficacy values obtained by amitraz use in Brazil (49–99%) with a possible amidine resistance reversion after a period without the administration of this product against a field strain.

The results described in the present study regarding the reutilization of amitraz against *R. (B.) microplus* differed from those obtained by Jonsson et al. (2010) in Australia. One possibility that

might justify this discrepancy was provided by Estrada-Peña et al. (2012). These authors reported that the Australian cattle tick *Rhipicephalus australis* Fuller was reinstated based on adults and larvae redescribed from material collected in Australia.

This long ignored boophilid was previously known as *Rhipicephalus microplus* Canestrini for specimens reported in Australia and New Caledonia. The use of principal components analysis on body measurements led to a clear separation of larvae of both taxa. A phylogenetic analysis based on 12S- and 16S-rDNA gene sequences supports the conspecific nature of the neotype material on which the reinstatement of the species is proposed and of the specimens used for previous interspecific crosses. *R. australis* is now known to be present in Australia, New Caledonia, the island of Borneo, Philippines, Sumatra, Java, New Guinea, Cambodia, and Tahiti. Finally, the authors conclude that despite these consequences, the lack of reliable data on Madagascan specimens and the limited number of strains used for the molecular and crossbreeding work by Labruna et al. (2009), there is now more than sufficient supporting evidence for the taxonomic separation of *R. australis* and *R. microplus* (Estrada-Peña et al., 2012). However, future studies are needed to confirm this hypothesis.

Based on the obtained results, it is possible to conclude that a period of three consecutive years (2002–2004) of rotation of amitraz and pyrethroids every 28 days before the initiation of the present study was sufficient to create an amitraz resistance situation for this particular *R. (B.) microplus* strain. Moreover, with the aid of field trials, artificial infestation Stall tests and adult immersion tests, we verified that nearly 40 tick generations without any contact to the tested compound (amitraz) were not sufficient to revert or modify the resistance/efficacy situation of this *R. (B.) microplus* strain against amitraz. It is possible that reversion in amitraz efficacy values in a *R. (B.) microplus* population occurs when resistance of this field strain is incipient in a tick population. An alternative explanation is that perhaps the tested *Rhipicephalus* spp. species are different in the trials conducted to date. Therefore, future studies should be performed to confirm these hypotheses.

Conflicts of interest

There were no conflicts of interest that may have biased the work reported in this paper.

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