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Research paper

Survey of cattle tick, *Riphicephalus (Boophilus) microplus*, resistance to amitraz and deltamethrin in New Caledonia

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ABSTRACT

The evolution of tick resistance to acaricides in New Caledonia was followed in two ways: through two large scale surveys in 1998 and 2014 and through the routine analysis of samples of ticks with suspected resistance. The results of the two approaches were qualitatively similar though analysis of ticks with suspected resistance gave higher frequencies of resistance, as expected of a biased sample.

Resistance tests using a larval packet test have been conducted since 1993 for deltamethrin and 2003 for amitraz. Deltamethrin was used in country-wide control from 1986 to 2003 and amitraz since 1996. This study analyzed the variation of resistance parameters such as lethal concentration 50 (LC50) and the resistance factor over 21 years for deltamethrin and LC99 over 11 years for amitraz. There was an increase in deltamethrin's annual mean LC50 from 1993 to 2004 when it reached 2.9 g/l and then a progressive decrease, reaching a level consistently below 1 g/l since 2007. Even though breeders stopped using deltamethrin in New Caledonia in 2003, the percentage of susceptible strains has remained below 30% since 1998. Amitraz's LC99 mean increased significantly from 0.31 g/l in 2003 to 2.96 g/l in 2014. Whereas all tested strains in 2003 were susceptible, only 40% of strains tested at the request of farmers were susceptible in 2014. The recent territory-wide survey showed that 76.7% of strains are still susceptible. This study established that resistance to amitraz has developed slowly in New Caledonia. A reversion phenomenon may have occurred concerning deltamethrin resistance, visible through the decrease of LC50 mean and the decreased proportion of very resistant strains; however the proportion of susceptible strains remains at a low level and there is anecdotal evidence that high resistance can re-emerge rapidly.

1. Introduction

Rhipicephalus (*Boophilus*) *microplus* is an important cattle tick widely distributed in most countries with a tropical or subtropical climate (Cutullé et al., 2009; Estrada-Peña et al., 2006). This tick was introduced into New Caledonia in 1942 via the import of animals from Australia (Rageau and Vervent, 1959) and is nowadays the main ectoparasite of Caledonian cattle (Bianchi et al., 2003). Blood loss and reduction in weight gain resulting from tick feeding are of the major factors that affect cattle production in the territory (Daynes and Gutierrez, 1980).

In New Caledonia, the extensive use of acaricides has led to resistance to the successive classes of acaricide and the regular introduction of another class of chemicals. Pyrethroid acaricides were introduced in late 1986 to control organophosphorus resistant ticks (Beugnet, 1995). In New Caledonia, the only pyrethroid used was deltamethrin as a dip. Farmers have never used either pour on formulations or other pyrethroids such as flumethrin. After only 5 years of use, the first case of pyrethroid resistance was detected in 1991 (Beugnet and Chardonnet, 1995; Brun, 1992). Amitraz (Taktic®) was used for the first time in 1996 to control pyrethroid-resistant ticks and the first case of amitraz resistance was reported in 2003 (Ducornez et al., 2005).

For a long time, the local strategy of chemical use was decided by the Caledonian government, which ordered, paid for and distributed acaricides to the cattle owners, in consultation with farmers' associations. Until now, the chemicals are free of charge, except for macrocyclic lactones. As farmers have to pay for macrocyclic lactones and not for other products, they used the former only once or twice a year. Macrocyclic lactones were used for the first time in 2003 in New Caledonia. Long lasting acaricides like long acting ivermectin and insect growth regulator (fluazuron) are now used in an integrated pest management program controlled by governmental structures. Fipronil has never been used in New Caledonia as the authorities consider it causes too much damage to the

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environment and its use was not strictly necessary at the moment in New Caledonia as other products remain efficient. Due to extensive nature of cattle production, acaricides have to be easy to use by dipping or spraying. A class of chemical is used until resistances appear and is then replaced by a new one throughout the territory when resistance becomes widespread.

Historically herds were dipped every 3 to 4 weeks to prevent tick infestations but since the beginning of the 2000s, recommendations changed and farmers were asked to treat animals only when ticks were observed.

As New Caledonia has a tropical climate, ticks are present all year around and depending on weather conditions and herd management, breeders can dip animals up to 10 times a year.

The objective of the present study was to analyze the evolution of tick resistance over 21 years for deltamethrin including the 10 years following the discontinuation of its use. The evolution of amitraz use was followed for 11 years. These results are used to define the local strategy of tick control by governmental officers and associations of farmers.

2. Materials and methods

2.1. Area description

New Caledonia is a French island in the South Pacific Ocean with a dry tropical climate, 1200 km to the west of Australia and 2000 km to the north of New Zealand, approximately 400 km long and 50 km wide, with about 600 farms running about 80,000 cattle under extensive conditions. The main breed, Limousin cattle, was introduced in 1905 but its breeding and performance are compromised by its high susceptibility to tick infestation and the development of acaricide resistance.

2.2. Tick collection

Ticks that were to undergo resistance tests were collected in two ways. Firstly, two large surveys were conducted, one in 1998 to assess the resistance level to deltamethrin after it had been used for 10 years and one in 2014 to assess the resistance level to amitraz after 11 years of exclusive use. For these surveys, ticks were collected directly on farms or at the slaughterhouse. About 20% of Caledonian farms were sampled during each survey. Secondly, breeders may ask for a resistance test if they observe a decrease in the efficiency of a treatment. In this case, they send the ticks directly to the laboratory. These resistance tests are referred to as diagnostic tests. The number of tests recorded each year depended on requests from breeders. Interpretation of the results depended on the origin of the tick samples. During surveys, the farms were chosen using random selection, whereas when tests were conducted at the request of farmers, there was already a suspicion of resistance and the prevalence of resistant strains was expected to be higher.

2.3. Bioassays

2.3.1. Deltamethrin test

Routine tests to determine resistance to deltamethrin were established in New Caledonia in 1993 (Beugnet and Chardonnet, 1995). The modified larval packet test (LPT) (Stone and Haydock, Stone and Haydock, 1962) was used to test the *in vitro* efficiency of this acaricide on *R. microplus* populations. Briefly, a commercial deltamethrin solution (50 g/l) (Butox 50%0®, Intervet) was diluted in olive oil to generate a solution of 12.8 g/l deltamethrin. Serial dilutions in two parts acetone and one part olive oil generated the testing doses. Eleven doses, including the control (diluents only) were prepared for each bioassay and each dose had two replicates (Mendes et al., 2011; Stone and Haydock, 1962). Packets were prepared by placing 670 μl of testing dose on a 7.5 cm x 8.5 cm piece of filter paper (Whatman #541, Techmed) before allowing acetone evaporation for one hour in an extractor hood. Treated papers were folded in half and sealed on the sides with clips to form packets. Approximately 100 *R. microplus* larvae were placed into each treated filter paper packet, which was then sealed with additional clips and placed in an incubator (27 °C and 85% of relative humidity (RH)) for 24 h. After that time, mortality was determined.

For each test the concentrations (LC) causing 50, 90 and 99% mortality were calculated. The LC50 of each sample was compared to the LC50 of a reference susceptible strain to determine its level of resistance. The reference strain used was Yeerongpilly, with an LC50 of 0.044 g/l. A resistance factor (RF), which is the ratio between the LC50 of each tested strain and the reference strain, was calculated. If RF was below 3, the strain was classified as susceptible, between 3 and 5, the strain was intermediate, between 5 and 50, the strain was resistant and when RF was above 50, very resistant (Bianchi et al., 2003). The LC50 concentrations corresponding to each RF threshold were 0.132 g/l, 0.22 g/l, 2.2 g/l respectively.

2.3.2. Amitraz test

Tests to determine resistance to amitraz were set up in New Caledonia in 2003 (Ducornez et al., 2005). The modified LPT was conducted following the procedures described in Miller et al. (2002). In this test, nylon fabric (Type 2320, Cerex Advanced Fabrics, Pensacola, FL) was used as a substrate. Serial dilutions from a maximum dose of amitraz (10 g/l) were made using a 2:1 acetone and olive oil diluent. Formulated amitraz (Taktic ND 12.5% EC, Intervet) was used. Twelve doses, including the control (diluents only) were prepared for each bioassay and each dose had three replicates (Miller et al., 2002). A volume of 670 μl of each dilution was applied to a piece of nylon fabric (7.5 cm × 8.5 cm). After 2 h in an extractor hood, the acetone evaporated and pockets were made with the treated fabrics. Approximately 100 *R. microplus* larvae were placed into each treated filter paper packet, which was then sealed with additional clips and placed in an incubator (27 °C and 85% RH) for 24 h. After incubation, the live and dead larvae were counted in each packet to determine mortality.

As there are no official criteria to define resistance, LC99 is used to define the level of resistance to amitraz in this study (Miller et al., 2002). Limits of LC99 used in this study where those proposed by Barré (2006). When LC99 is below 1.0 g/l, the strain was classified as susceptible. If LC99 was between 1.0 and 2.0 g/l, the strain was intermediate and when LC99 was over 2.0 g/l, the strain was considered to be resistant to amitraz.

2.3.3. Statistical analysis

Probit analysis was run on the bioassay results using Polo-Plus (LeOra Software, 1987). The log-probit model estimated by Polo-Plus gives regression line representing the relationship between the percentage of larval mortality and the acaricide dose. Deviation from linearity of log-probit transformed data was assessed through a chi-square test (Robertson and Preisler, 1992). For each test the following parameters were estimated: LC50, 90, 99 and the slope of the regression line.

Differences between years concerning the number of resistant, intermediate or susceptible strains were tested with a chi-square test. The difference in mean of LC50 or LC99 between years was tested with a non-parametric Wilcoxon test. Statistical analyzes were done using R® software.

3. Results

Since the beginning of resistance tests to deltamethrin, 963 tests have been conducted: 114 tests during the survey of 1998, 104 dur-

Table 1
Number of resistance tests registered each year for deltamethrin and amitraz at the laboratory of parasitology of New Caledonia (for 1998 and 2014, the number of tests includes tests done for the survey).

Number of test for deltamethrin	12	2	7	10	51	206	72	96	58	30
Year	1993	1994	1995	1996	1997	1998	1999	2000	2001	2002

Year	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014
Number of test for deltamethrin	26	4	24	25	28	36	27	45	43	25	32	104
Number of test for amitraz	53	13	51	60	71	47	30	46	56	31	33	123

ing the last study of 2014 and 772 tests at the request of breeders. From 1993 to the end of its extensive use in 2003, routine tests only targeted this acaricide as more and more resistance was observed in the field. Even after the use of deltamethrin was discontinued in Caledonian farms in 2003, resistant tests to deltamethrin were maintained routinely to follow a potential reversion phenomenon (Table 1).

In 2004, when deltamethrin was no longer used, the first cases of resistance to amitraz were recorded. Since then, requests from breeders were more targeted to putative resistance to amitraz. Since 2003, 454 tests for amitraz resistance have been conducted: 364 were done at the request of farmers and 90 were done during the 2014 survey (Table 1).

3.1. Resistance to deltamethrin

As the resistance factor is based on LC50 for deltamethrin, we choose to follow this parameter to study the evolution of deltamethrin resistance. The evolution of the LC50 mean between 1993 and 2014 is presented in Fig. 1. The LC50 mean reached its highest level in 2004 with a value of 2.98 g/l. It rapidly decreased over the following years to reach a low point of 0.33 g/l in 2008. The difference between 2006 (2.36 g/l) and 2007 (0.81 g/l) is significant ($p < 0.001$). Then it remained between 0.34 and 0.91 g/l from 2008 to 2014. The distribution of strains per class and per year is presented in Fig. 2.

In the population of ticks collected by breeders, the proportion of strains resistant or very resistant to deltamethrin increased from 1994 to a prevalence of over 96.5% in 2001 and 2002. This population is biased as these ticks were sampled on farms having problems with acaricide, so this proportion may be higher than that of the overall population of ticks on the territory. Indeed, in 1998, the proportion of resistant or very resistant strains in diagnosis tests was 87.0% whereas it was 45.6% in the territorial survey and the distribution of tick strains between each class was significantly different ($p < 0.001$) between the diagnosis tests and the survey. The proportion of very resistant strains reached a peak at 75.0% in 2004, but these results should be interpreted with caution as there were only 4 tests conducted during that year. However, this proportion was 63.8% in 2001, but quickly decreased after 2004 under 10% in 2009 where it remains. The proportion of susceptible or intermediate strains regularly decreased in diagnostic tests from 1993 to 2001 and was 54.4% in the survey of 1998. This proportion varies after deltamethrin use was discontinued but never reached the level of 1998 during the last survey of 2014.

Although the difference of LC50 is not significant between the surveys of 1998 and 2014, the distribution of strains between the classes is significantly different for the 1998 survey and the one of 2014 which shows more intermediate and resistant strains but only 0.03% very resistant strains.

3.2. Resistance to amitraz

Since initiating resistance tests to amitraz in 2003, the mean of LC99 increased steadily and reached 2.9 g/l in 2014; with a significant increase ($p < 0.001$) of the LC99 mean between 2003 (0.32 g/l) and 2014 (2.96 g/l). The evolution of this mean is presented in Fig. 3.

Considering the parameters of LC99 used in this study, the tested strains are distributed in three classes and the results over 11 years are presented in Fig. 4.

The proportion of tick strains resistant to amitraz in diagnosis tests began to increase in 2005 and varied from year to year to a maximum of 53.3% in 2014. However, a large difference ($p < 0.001$) is observed between results obtained through diagnostic tests and the survey. While only 40.0% of strains tested at the request of farmers were considered susceptible, the territorial survey indicated that 76.7% of tested strains were still susceptible to amitraz.

4. Discussion

Deltamethrin was first introduced in 1986 in New Caledonia when resistance to organophosphorus was widespread. The first resistance was observed in New Caledonia in 1991 (Brun, 1992), after 5 years of use and resistance to deltamethrin rapidly spread over the whole territory. In a widespread survey in 1998, Bianchi et al. (2003) found that 57% of tested strains were no longer susceptible to this acaricide. The proportion of susceptible strains fell under 5% in diagnosis tests between 2001 and 2003. The low prevalence during these 3 years is probably an overestimate as only tick strains coming from farms with suspicion of acaricide resistance were tested. This is confirmed by the difference between proportion of resistant and very resistant strains in 1998 during the survey and routine diagnostic tests, which were respectively 45.6% and 87.0%. But resistance to deltamethrin became more and more problematic in farms. In 2003 it was decided to cease use of this acaricide, 17 years after its introduction, and the replace it with amitraz and macrocyclic lactones. These results are consistent with other studies that highlighted the quick spread of synthetic pyrethrinoid (SP) resistant strains 10 to 15 years after its first use in a country. Fernández-Salas et al. (2012) observed that 90.6% of tested strains in the state of Veracruz (Mexico) were resistant to cypermethrin 18 years after the introduction of SP in this country. Mendes et al. (2011) found that resistance to deltamethrin in the state of Sao Paulo (Brazil) reached 86.4% after more than 20 years of use.

As deltamethrin has not been used since 2003, tick strains tested for diagnosis after 2003 did not come specifically from farms facing trouble with this acaricide. However, the LC50 mean stayed constant for two years and then began to decrease in 2006 to quickly drop in 2007. Since then, the proportion of resistant strains stayed at a relatively high level: between 47.2% to 81.4% depending on the year, without any clear trend. In contrast, the proportion of very resistant strains decreased significantly in 2007 to below 10% in 2008, where it has stayed. As deltamethrin was removed from farms between 1996 and 2003 when resistance phenomena were diagnosed, the decrease of resistance is, in this study, observed 4 to 11 years later, equivalent to 16 to 44 generations of ticks in New Caledonia (De MeeÛs et al., 2010).

Nevertheless, this reversion is limited as the proportion of resistant strains remains at a constant high level 11 years after

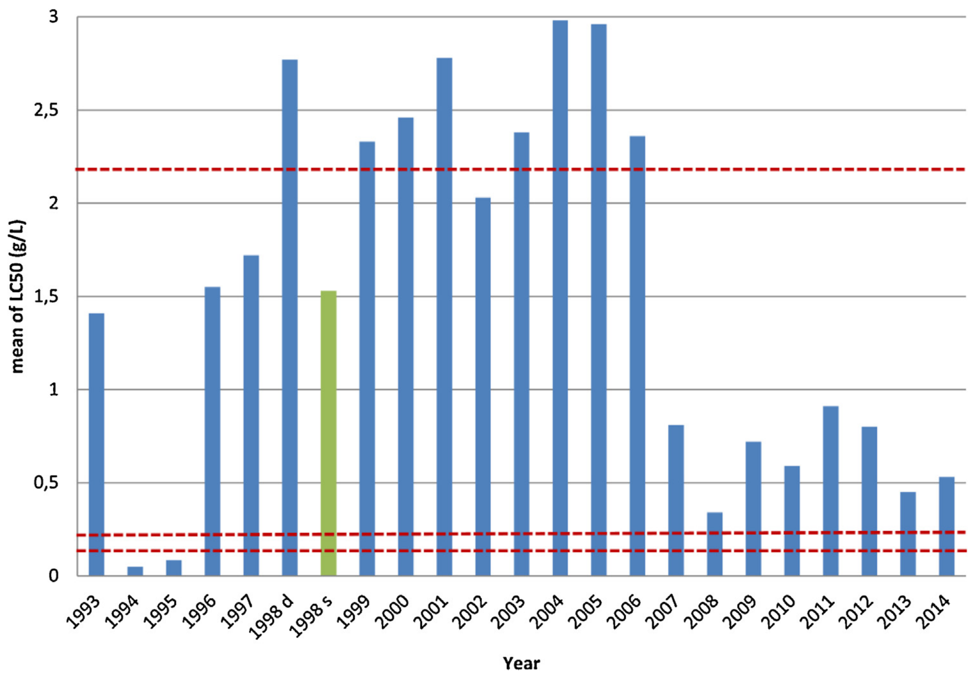

Fig. 1. Evolution of mean LC50 (g/L) between 1993 and 2014 for deltamethrin ("1998d": bioassays done at the request of farmers, "1998s": bioassays done for the survey). Dotted lines indicate the values of resistance thresholds (susceptible/intermediate/resistant/very resistant).

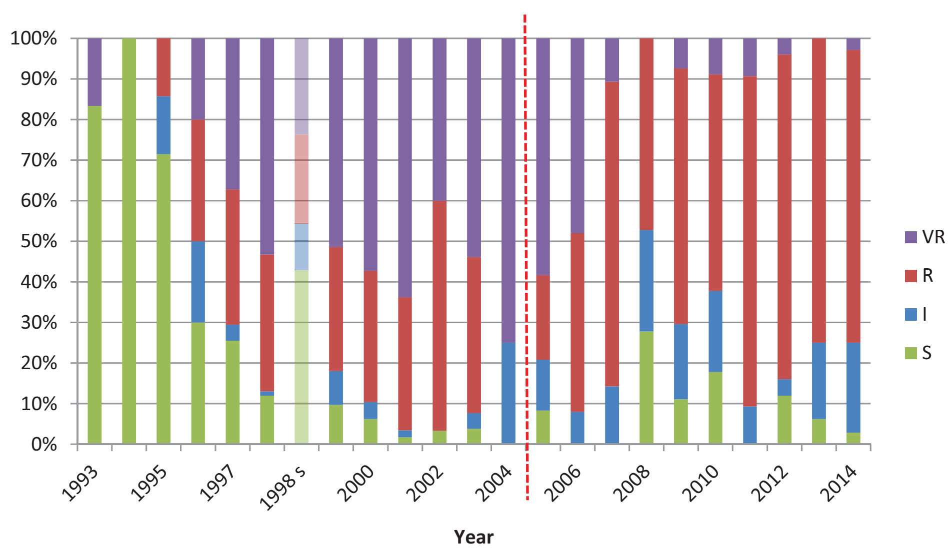

Fig. 2. Evolution of deltamethrin resistant classes (S:susceptible, I:intermediate, R:resistant, VR:very resistant) between 1993 and 2014 in New Caledonia ("1998d":bioassays done on the request of farmer, "1998s":bioassays done for the survey). The dotted line indicates the end of the use of deltamethrin in New Caledonia.

the cessation of use of this acaricide. During the survey of 2014, more than three quarters of tested strains were still resistant to deltamethrin. When compared to the survey of 1998, recent results indicate that the level of resistance is higher 11 years after the removal of this acaricide than 12 years after its first use on the territory and is now at a stable level.

The phenomenon of reversion has been mentioned in several publications but rarely described. Stone (1972) reported that reversion of resistance to DDT appeared in 13 generations of ticks to fall from 100% to 55% resistance but there was no further decline for at least another 18 generations. This reversion was inconsistent with organophosphates as, depending on pesticide, important reversion was recorded for a BHC-dieldrin resistant strain after 24 generations but no changes were observed after 5 generations for one organophosphosphate-resistant strain.

Rodriguez-Vivas et al. (2011) observed that, over a period of 2 years, the SP resistant traits remain fixed and concluded that acquisition of resistance is faster than any loss in resistance.

An important practical question is whether deltamethrin could be used at all without rapidly increasing the prevalence of very resistant populations. A potential strategy might be to use deltamethrin once or twice a year, alternating with amitraz and long lasting acaricides in an integrated tick management program, given that it would not be possible to use this acaricide as sole means of chemical control. Resistance gene frequency is known to increase rapidly when selection pressure is reinstated (Stone, 1972).

In New Caledonia, in some farm experiencing amitraz resistance and with a resistance factor to deltamethrin lower than 5, breeders started to re-use deltamethrin. The product seemed quite effective

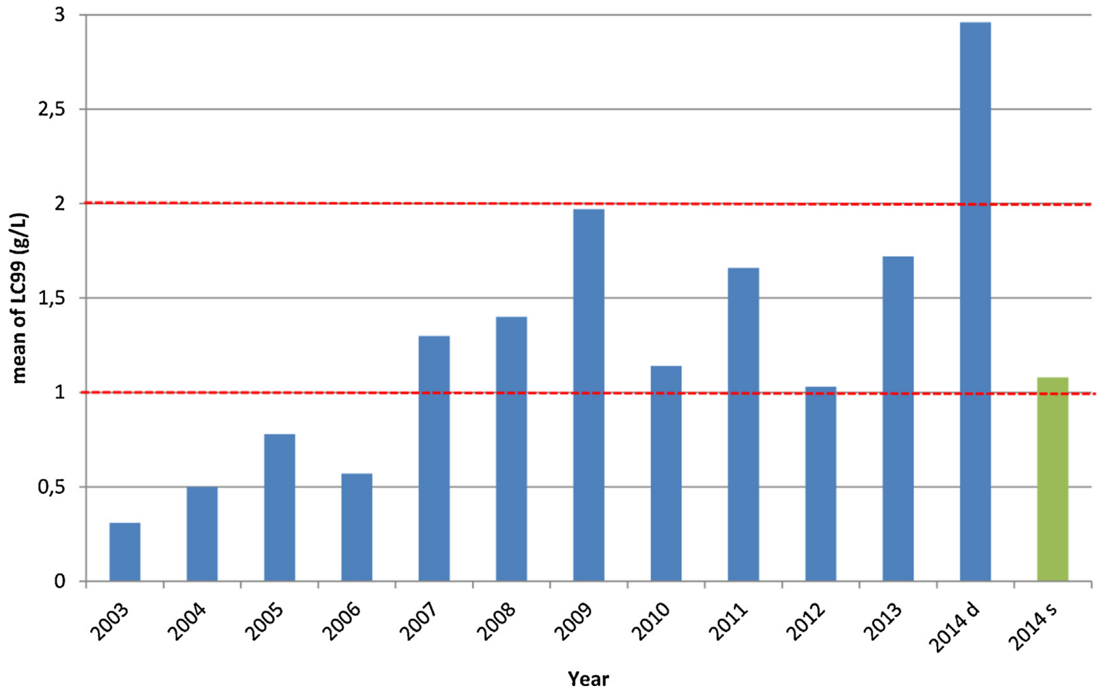

Fig. 3. Evolution of LC99 means (g/L) from 2003 to 2014 for amitraz ("2014d":bioassays done at the request of farmers, "2014s":bioassays done for the survey). Dotted lines indicate the values of resistance thresholds.

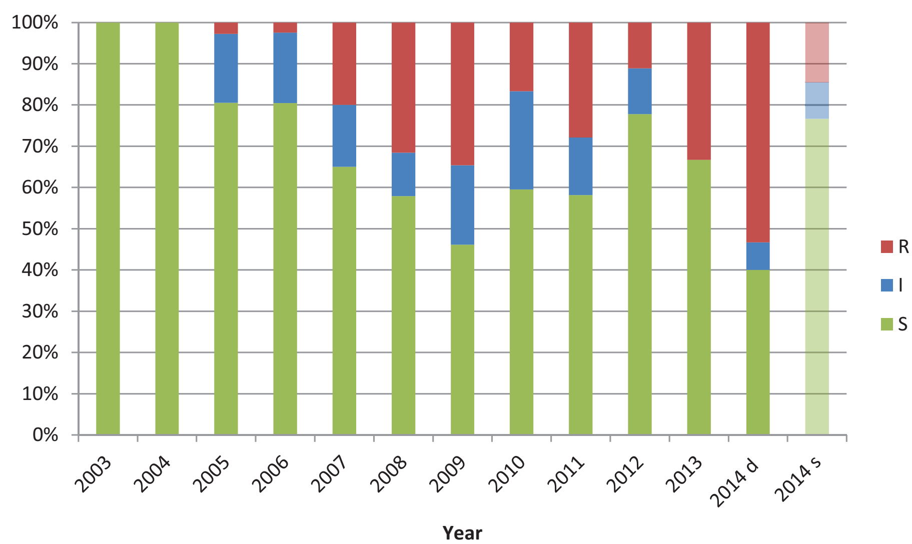

Fig. 4. Evolution of amitraz resistant classes (S:susceptible, I:intermediate, R:resistant) between 2003 and 2014 in New Caledonia ("2014d":bioassays done at the request of farmers, "2014s":bioassays done for the survey).

initially then lost efficacy again after only three treatments with this acaricide (unpublished data). While this has not been assessed on a large scale, the anecdotal results are not encouraging.

Amitraz was used for the first time in New Caledonia in 1996 as a reaction to deltamethrin resistance. As the use of deltamethrin was discontinued in 2003, amitraz has since been the main acaricide used by breeders. This is the first large survey on amitraz resistance on the island, and 18 years after its introduction and 11 years after its exclusive use prevalence of resistant strains remains low at 14.4%. Tick resistance to amitraz is documented in Australia and in Central and South America. Several studies assess the onset of resistance development with diverging results between countries. In Mexico, Rosado-Aguilar et al. (2008) found that resistance to this acaricide developed after only 15 treatments and 5 generations of ticks. In the same country, Rodriguez-Vivas et al. (2006) observed a prevalence of 19.4% of resistant strains after about 10 years of use alternating with other acaricides and, Fernández-Salas et al. (2012) reported that 54.7% of tested strains in the same country were resistant to amitraz eighteen years after its first use. In Brazil, amitraz has been available since 1977 (Martins et al., 2008). Mendes et al. (2001) studied the efficacy of amitraz in the Sao Paulo state in Brazil. Resistance varied from 74.4% to 95.8%, whereas Machado et al. (2014) recorded that amitraz efficacy was 54.1% in the state of Rio Grande do Sul in South of Brazil.

On the other hand, different Australian surveys indicated that resistance prevalence stayed relatively low. Jonsson et al. (2000) pointed out a prevalence of farms with tick resistant strains of 10% in 1997 after more than 20 years of use of this molecule, and Jonsson and Hope (2007) related that this prevalence was still at 10.8% 10 years later, in the same country.

These differences may be partly due to the conditions of the surveys and the parameters selected to estimate resistance level. There are no officially defined parameters to classify tick strains as susceptible or resistant. Rodriguez-Vivas et al. (2006) and Fernández-Salas et al. (2012) used the larval immersion test and mortality at a discriminating dose whereas Rosado-Aguilar et al. (2008) used the LC50 with the same test and Ducornez et al. (2005) performed a larval packet test and selected LC50, the slope and mortality at a discriminating dose to define a resistant strain.

Our results are closer to the Australian ones and our study confirms that the development of resistance to amitraz in *R. microplus* populations has been slower than that to previous pesticides used as control measures (Li et al., 2005).

All these investigations are important in order to define the future strategy of tick control. Development of amitraz resistance increases slowly but is becoming more and more problematic for Caledonian farmers.

Nowadays, there are 10% of farms with ticks resistant to both deltamethrin and amitraz. The use of amitraz began when a large majority of farms were resistant to deltamethrin. So, the development of amitraz resistance happened in farms with resistance to deltamethrin. It is very likely that this situation was not due to the development of cross resistance between deltamethrin and amitraz which has not been described yet. But, for breeders with multiresistant tick strains, treatment has become a challenge. These farms have still some tick control possibilities with long lasting acaricides and with genetics and agronomic options.

Long lasting acaricides, such as long lasting ivermectin or an insect growth regulator like fluazuron are now used in amitraz resistant farms under the control of government agencies. However long withholding periods complicate herd management and resistance to these products is already known in other countries and will inevitably develop within few years in the territory. Resistance to ivermectin was already reported in Brazil in 2001 (Martins and Furlong, 2001) and more recently in Mexico (Perez-Cogollo et al., 2010). A first case of resistance to fluazuron was also recorded in Brazil in 2014 (Reck et al., 2014).

Apart from farmers who have enough technical and financial support to maintain susceptible cattle breeds, such as Limousine or Charolais cattle, in these tick infested areas, local breeders are increasingly encouraged toward genetic transition, using tick resistant breeds and hence reducing the impact of ticks on their animals. Over the past decades the control of tick infestation in order to maintain populations of susceptible breeds of cattle was based on acaricide use alone; however in the future for those wishing to maintain susceptible breeds, tick control will need to combine different approaches in an integrated pest management strategy. There are some other ways to manage tick control like the elimination of more sensible animals in susceptible cattle breeds (Frisch et al., 2000), the use of vaccination, natural plant extracts or entomopathogenic species (Borges et al., 2011; De la Fuente et al., 2007; Samish et al., 2004). Vaccination has been used in Australia and is still used in South America with a BM86 based vaccine and there are some research projects in order to find other tick protein suitable for vaccination (Jonsson, 2004). Some molecules extracted from plants have showed efficacy in lab test (Jonsson, 2004). Some treatments with these molecules may now be developed. It is a new challenge for breeders to combine this possibility in order to control cattle ticks infestation.

This study demonstrates the slow but patent development of amitraz resistance and the possibility of reversion for deltamethrin resistance. These results are important to the design of strategies for tick management on a territorial scale and points to the need for regular surveys of acaricide resistance.

Tick resistance to acaricides is based on multiple mechanisms and molecular tools to examine these mechanisms are not yet routinely available (Rosario-Cruz et al., 2009). Once they are, it will be interesting to study the development of resistance and the reversion phenomena and their association with the evolution of resistant gene frequency in tick populations.

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