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Diagnosis of amitraz resistance in *Boophilus microplus* in New Caledonia with the modified Larval Packet Test

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Abstract

The tick *Boophilus microplus* represents a serious pathological constraint to livestock production in New Caledonia. Cattle ticks are controlled by chemical application of two acaricides that are currently used in New Caledonia; deltamethrin is used at 46% of the cattle production facilities and amitraz at the remaining 54% premises where resistance to deltamethrin has been identified. In 2003, a modified Larval Packet Test (LPT) was used to conduct a survey for amitraz resistance. Ticks were collected from 29 farms, including farms using deltamethrin ($n = 8$) or amitraz ($n = 21$). Of eighteen different tick populations, sixteen populations were defined susceptible to amitraz and two populations were considered amitraz-resistant. This is the first report of populations of *B. microplus* being resistant to amitraz, using the modified LPT in New Caledonia. A thorough survey of tick susceptibility to amitraz in cattle farms of the country should be conducted to assess the presence of amitraz-resistant populations. The emergence of amitraz resistance so soon after its introduction has some important implications for the strategy and organisation of tick control in New Caledonia, and this paper discusses some of the urgent actions that should be undertaken.

Keywords: *Boophilus microplus*; Modified Larval Packet Test; Amitraz; Resistance; New Caledonia

1. Introduction

The tick *Boophilus microplus* (Canestrini) was introduced into New Caledonia (between 20° and 22° South) in 1942 via importation of animals from Australia (Rageau and Vervent, 1959). This one-host-tick is the principal ectoparasite of Caledonian cattle. Climatic conditions favour *B. microplus* activity all year, and the tick can complete at least four generations per year (Bianchi et al., 2003). Fortunately, the microbial diseases transmitted by *B. microplus* were not imported into New Caledonia along with the tick. However, blood loss and reduction in weight gain resulting from tick feeding represents one of the most important pathological constraints to livestock production in the country (Daynes et al., 1984).

There are about 150,000 cattle belonging to 1200 breeders in New Caledonia (Barré, 2003). The main

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cattle breeds include Limousin, Charolais and Hereford. These breeds, being *Bos Taurus*, are highly susceptible to ticks (Wharton et al., 1973; Pegram et al., 1993; de Castro and Newson, 1993). Tick control in New Caledonia is the responsibility of territorial authorities, and acaricide is freely delivered to cattle producers. The tick control program is based exclusively on the use of chemicals applied on a regular basis every 4–5 weeks (Bianchi et al., 2003). In the past, arsenic (1943–1950), DDT (1947–1973) and diethylethion (1973–1980s) were used for tick control until resistance to these compounds made further use impractical (Brun et al., 1983). Diethylethion was replaced by the synthetic pyrethrinoid (SP) deltamethrin (Butox ND). Six years after introduction, the first cases of deltamethrin resistance were reported by Beugnet and Chardonnet (1995). The organophosphate (OP) chlorpyriphos-ethyl (Dursbel ND) was introduced into New Caledonia in 1994 but replaced in 1996 by amitraz (Taktic ND) for use on deltamethrin-resistant farms. Since that time, only amitraz and deltamethrin are provided by Veterinary Services to producers for the control of *B. microplus* throughout the country. The emergence of tick populations resistant to amitraz was predictable after several years of use.

Currently, suspicion of resistance to deltamethrin in a cattle production facility is confirmed by a bioassay conducted at the Laboratory of Parasitology of IAC at Port-Laguerre. The bioassay technique used in New Caledonia was developed by Stone and Haydock (1962), and was subsequently adopted by the FAO as a standard method to determine the susceptibility of tick populations to acaricides as the Food and Agriculture Organization Larval Packet Test (LPT). This bioassay is based on the observation of larval mortality after placement in a paper packet treated with a known concentration of acaricide. Unfortunately, the LPT technique does not measure the susceptibility of ticks to amitraz because no dose related response is produced (Kemp et al., 1998; Miller et al., 2002). Recently, a modification of the LPT for the determination of amitraz resistance in *B. microplus* was developed at the United States Department of Agriculture, Cattle Fever Tick Research Laboratory (USA) by Miller et al. (2002), and it was decided to conduct a survey for amitraz resistance in New Caledonia using this test.

2. Materials and methods

2.1. Choice of farms

A total of 29 farms were visited including (1) farms where deltamethrin was still used and where animals have never been treated with amitraz ($n = 8$) and (2) farms where amitraz was used regularly (10–12 times a year) for 3–10 years ($n = 21$). Among these farms, one breeder had observed a lack of efficacy of the treatment.

2.2. Collecting of ticks

At each farm, ≈30 engorged females of *B. microplus* were collected with a maximum of five ticks from any one animal. Ticks were transported to the Laboratory of Parasitology of Port-Laguerre and held in a rearing room at 26–27 °C and 80–92% RH for 2 weeks. Twelve to 16-day-old larvae were used for testing.

2.3. Bioassays

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 top dose of amitraz (1%) were made using a 2:1 trichloroethylene and oil diluent. Formulated amitraz (Taktic ND 12.5% EC, product of Intervet) was used. Twelve doses, including the control (diluent only) were prepared for each bioassay and each dose had three replicates. A volume of 0.67 ml of each dilution was applied to a piece (7.5 cm × 8.5 cm) of nylon fabric. After 2 h in a fume hood, to allow for the trichloroethylene to evaporate, pockets were made with the treated fabrics. Approximately 100 larvae were placed into each pocket before they were placed in an incubator at 27 °C and 85–92% RH for 24 h. After incubation the live and dead larvae were counted in each packet to determine mortality.

2.4. Statistical analysis

Probit analysis was run on the bioassay results using Polo-PC (Le Ora Software, 1987). The log-probit model estimated by Polo-PC is illustrated by a

regression line representing the relationship between the percentage of larval mortality and the acaricide dose. For each test, the following parameters were estimated: $LC_{50,90}$ (Lethal Concentration is the concentration for which 50 or 90% mortality of larvae is expected) and the slope of the regression line.

The resistance ratio (RR) is a measure of the comparative susceptibility of an unknown population to a susceptible reference population. We used the ratio between the LC_{50} (or LC_{90}) for the population under examination and the LC_{50} (or LC_{90}) for the reference population. Resistance ratios for comparison at LC_{50} (or LC_{90}) and their confidence intervals were generated using the formula described by Robertson and Preisler (1992, pp. 42–44). Significance of each comparison was determined when the number 1 was not included in the confidence interval.

3. Results

3.1. Interpretable tests

The tests could not be conducted due to insufficient numbers of larvae or not validated due to high control mortality or no dose–mortality relationship observed, for 11 populations. Forty-five tests concerning 18 different populations produced results suitable for statistical analysis. However, in some of these tests, there was a patterned divergence from the probit model at the extremely low concentrations, but the estimated line fitted the observed data through the important and interesting part of the dose–response (from 20 to 95% mortality in the *Nassandou* population, for example, Fig. 1).

3.2. Susceptible populations and resistant populations

The following parameters were estimated for 18 populations (Table 1), LC_{50}, LC_{90} and slope of the regression line. Sixteen populations were defined susceptible and two populations were considered amitraz-resistant (*Gadji* and *Néty*). The slopes generated from the susceptible populations were relatively high (Fig. 1; *Nassandou* and *Foué* populations). The slopes (S.E.) ranged from 2.0 ± 0.09 (*Boghen* population) to 7.1 ± 0.6 (*La Pouéo 1* population) and the mean value (S.E.) was 3.8 (± 0.4). The LC_{90} estimates ranged from 0.0054 to 0.024% with a mean value (95% CL) of 0.013% (0.0098–0.016). Mortality of 100% was observed in the three replicates at 0.03125% amitraz

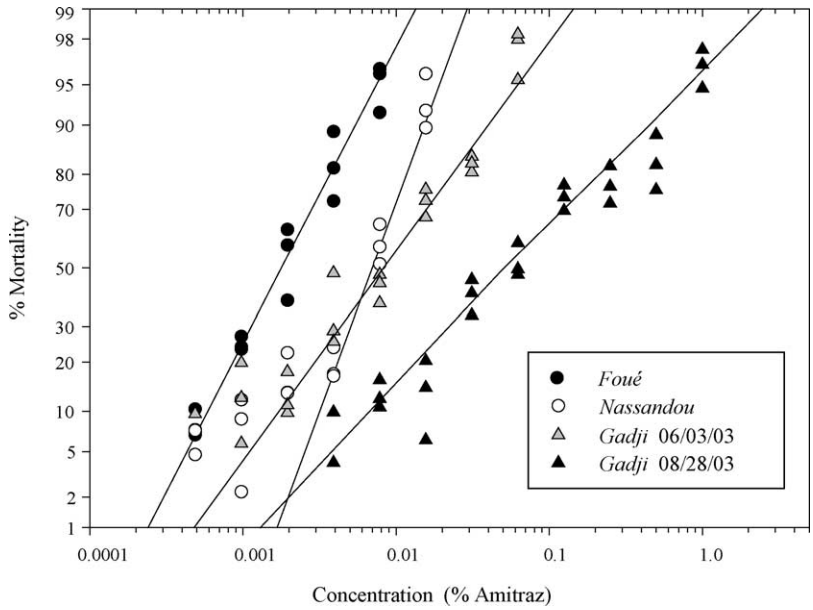

Fig. 1. Bioassays of amitraz susceptible populations (*Nassandou* and *Foué*) and resistant population (*Gadji*), 2 tests done at 3 months interval.

Table 1
Bioassays results for *Boophilus microplus* populations collected in New Caledonia

Populations	*n*	Slope (S.E.)	LC_{50} (95% CL)	RR_{50} (95% CI)	LC_{90} (95% CL)	RR_{90} (95% CI)	χ^2 (d.f.)
Susceptible							
Nassandou (*reference*)	1647	3.7 (±0.4)	0.0070 (0.0050–0.0083)	1	0.015 (0.012–0.025)	1	64 (13)
Foué	3589	2.7 (±0.1)	0.0018 (0.0016–0.0019)	0.25 (0.22–0.29)	0.0054 (0.0049–0.0061)	0.35 (0.30–0.41)	27* (34)
Daoui	3682	4.5 (±0.4)	0.0097 (0.0071–0.011)	1.38 (1.23–1.54)	0.019 (0.015–0.032)	1.21 (1.03–1.43)	226 (34)
Poya	1940	3.8 (±0.3)	0.0036 (0.0028–0.0043)	0.45 (0.38–0.52)	0.0079 (0.0064–0.011)	0.44 (0.39–0.50)	106 (16)
La Pouéo 1	2858	7.1 (±0.6)	0.0043 (0.0034–0.0050)	0.61 (0.54–0.67)	0.0065 (0.0054–0.012)	0.42 (0.36–0.48)	218 (16)
Port-Laguerre 1	1980	3.6 (±0.2)	0.0093 (0.0080–0.011)	1.33 (1.17–1.51)	0.021 (0.018–0.026)	1.37 (1.16–1.61)	51 (19)
La Pouéo 2	2479	6.0 (±0.5)	0.0097 (0.0082–0.011)	1.38 (1.23–1.56)	0.016 (0.013–0.022)	1.03 (0.88–1.20)	117 (19)
Ouégoa	1906	2.2 (±0.1)	0.0018 (0.0014–0.0022)	0.26 (0.23–0.29)	0.0070 (0.0052–0.011)	0.46 (0.38–0.55)	75 (13)
Fonwary	3373	4.9 (±0.4)	0.0061 (0.0056–0.0065)	0.87 (0.79–1.03)	0.011 (0.010–0.013)	0.73 (0.63–0.85)	42* (34)
Ouenghi	1664	3.0 (±0.3)	0.0044 (0.0032–0.0055)	0.63 (0.54–0.74)	0.012 (0.0096–0.017)	0.78 (0.65–0.94)	42 (14)
Boghen	2490	2.0 (±0.09)	0.0018 (0.0015–0.0020)	0.25 (0.22–0.29)	0.0077 (0.0067–0.0091)	0.51 (0.42–0.60)	27* (22)
Moindam	965	2.5 (±0.2)	0.0022 (0.0016–0.0028)	0.32 (0.26–0.38)	0.0073 (0.0057–0.010)	0.47 (0.38–0.59)	30 (13)
Boulouparis	1403	2.6 (±0.2)	0.0047 (0.0037–0.0056)	0.66 (0.57–0.77)	0.014 (0.012–0.019)	0.93 (0.78–1.12)	46 (16)
La Taraudière	3241	3.7 (±0.6)	0.011 (0.0048–0.014)	1.56 (1.33–1.83)	0.024 (0.020–0.046)	1.57 (1.34–1.83)	139 (34)
Néhoué	1714	4.6 (±0.4)	0.0097 (0.0089–0.011)	1.39 (1.22–1.57)	0.018 (0.017–0.021)	1.20 (1.02–1.41)	18* (19)
Port-Laguerre 2	3332	4.6 (±0.4)	0.0067 (0.0061–0.0073)	0.98 (0.88–1.09)	0.013 (0.012–0.014)	0.84 (0.72–0.98)	37* (34)
Resistant							
Gadji, 3 June 2003	2621	1.9 (±0.1)	0.0083 (0.0065–0.010)	1.18 (0.99–1.42)	0.040 (0.033–0.050)	2.60 (2.16–3.20)	49 (25)
Gadji, 28 August 2003	2336	1.4 (±0.07)	0.057 (0.045–0.069)	8.09 (6.58–9.95)	0.45 (0.36–0.58)	29.4 (23.1–37.3)	42* (34)
Néty	1658	3.4 (±0.2)	0.017 (0.014–0.019)	2.36 (2.07–2.69)	0.040 (0.034–0.049)	2.57 (2.20–3.00)	47 (16)

n: number of larvae; S.E.: standard error; 95% CL: 95% confidence limits; RR_{50}: resistance ratio at the LC_{50} estimate; 95% CI: 95% confidence interval; RR_{90}: resistance ratio at the LC_{90} estimate.

* The data followed the probit model ($p < 0.05$).

for 13 populations. At the same concentration, for the three other populations, 98–100% of larvae were killed and all the larvae were dead in the three replicates in the next higher concentration. A first test performed in June 2003 on the *Gadji* population produced LC_{50} and LC_{90} estimates (95% CL) of 0.0083% (0.0065–0.010) and 0.040% (0.033–0.050), respectively. The LC_{50} estimate was lower than the LC_{50} estimates of five susceptible populations (*Daoui*, *Port-Laguerre 1*, *La Pouéo 2*, *La Taraudière* and *Port-Laguerre 2* populations), indead the LC_{90} estimate was higher than the highest estimate of 0.024% (0.020–0.046) obtained from *La Taraudière*. At 0.03125% amitraz, the mortality observed in the three replicates ranged from 82.6 to 85.9% and at 0.0625% a few larvae were live. Furthermore, a decrease in the slope (S.E.) was observed 1.9 ± 0.1 (Fig. 1). These results indicate that this population contained individuals resistant to amitraz.

A test conducted 3 months later on larvae obtained from ticks collected from the same farm confirmed this suspicion of amitraz resistance. The range of amitraz dilutions used was increased to 4%. At 0.03125% amitraz, the percentage mortality observed in the three replicates was under 50% and it was only at 2% amitraz that 100% mortality was observed in the three replicates. The slope (S.E.) produced was low 1.4 ± 0.07. The LC_{50} and LC_{90} estimates (95% CL) were 0.057% (0.045–0.069) and 0.45% (0.36–0.58), respectively, and were much higher than those produced by the different susceptible populations tested (Table 1). The regression line produced was representative of a heterozygous resistant population.

A second population (*Néty*) was also considered amitraz-resistant. For this population, the LC_{50} estimate (95% CL) was high 0.017% (0.014–0.019) and LC_{90} estimate (95% CL) was similar to LC_{90} estimate produced by *Gadji* population in 3 June 2003 test, but the slope (S.E.) produced was high 3.4 ± 0.2 as well. At 0.03125% amitraz the percentage of mortality observed ranged from 76.6 to 89.6% and it was only at 0.25% amitraz that 100% mortality was observed in all three replicates.

3.3. Resistance ratio

The *Nassandou* population had never been exposed to amitraz and was used as a susceptible reference population. A comparison between the *Nassandou* and the other susceptible populations showed that for ten populations the RR produced (at the LC_{50} or LC_{90} estimates) was less than 1 and for five populations it ranged from 1.03 to 1.57 (Table 1).

Concerning the population *Gadji*, for the first test (conducted on 3 June 2003) the RR at the LC_{50} estimate showed that there was no significant difference between the two populations (one was included in the confidence interval of the RR). A RR (95% CI) of 2.60 (2.16–3.20) was measured at the LC_{90} estimate. This value was higher than those produced with the susceptible population. For the test performed on 28 August 2003 concerning the same population, the RR produced (95% CI) at the LC_{50} and LC_{90} estimates were 8.09 (6.58–9.95) and 29.4 (23.1–37.3), respectively. For the *Néty* population at the LC_{50} and LC_{90} estimates, the RR produced (95% CI) were 2.36 (2.07–2.69) and 2.57 (2.20–3.00), respectively.

4. Discussion

Several types of bioassays have been developed to measure amitraz susceptibility in *B. microplus* (Kemp et al., 1998; Drummond et al., 1973). The modified-LPT was chosen for this study on amitraz resistance because it is based on the same technique and expertise as the standard LPT, which was used at this laboratory for many years. In this study, the laboratory performed multiple bioassays on a single population and produced repeatable results (data not shown). However, it is necessary to strictly standardize: (1) the scoring of dead larvae, (2) age of larvae and (3) conditions of the incubator room (temperature and relative humidity).

The RR at the $LC_{50,90,99 \text{ or } 99.9}$ estimates are usually used for the interpretation of in vitro tests. Some reference values are available for each acaricide and each type of test, to determine the susceptibility or resistance of the population tested (Kemp et al., 1998). The modified-LPT is a recent test for which there is not a great deal of data available to provide a reference value for the LC estimates. In different studies LC_{50} estimates (Miller et al., 2002; Li et al., 2004) or LC_{90} estimates (Miller et al., 2003) were used to measure resistance to amitraz in laboratory or field collected populations of *B. microplus*. In this study a resistant

population could not be consistently discriminated from a susceptible one using the LC_{50} estimate. The *Néty* population, showing a low resistance to amitraz, produced a RR (95% CI) at the LC_{50} estimate of 2.57 (2.20–3.00). The test performed on 3 June 2003 on the *Gadji* population, also amitraz-resistant, produced a RR (95% CI) at the LC_{50} estimate of 1.18 (0.99–1.42) that meant that there was no difference between the LC_{50} estimates of the two populations, even though a RR (95% CI) at the LC_{90} estimate of 2.60 (2.16–3.20) was obtained. Miller et al. (2003) discussed the results of the modified-LPT conducted in different laboratories. In Brazil, a susceptible reference population produced a LC_{90} estimate (95% CL) of 0.015% (0.012–0.023), and the comparison of a resistant population to this reference population at LC_{90} estimate produced a RR (95% CI) of 24.2 (19.9–29.5). These values were similar to those produced at the Laboratory of Port-Laguerre with the *Nassandou* and *Gadji* populations (test conducted on 28 August 2003). But unlike *Gadji* and *Nassandou* populations, in Brazil the slope of the regression lines obtained for the two populations named before were similar (2.2 ($\pm$0.08) and 1.9 ($\pm$0.13)). The steeper slopes measured in the Brazilian populations may indicate that the populations tested were more homogenous for the trait(s) that lead to amitraz resistance. For the modified-LPT bioassays performed at the Laboratory in New Caledonia, the following parameters have been retained to evaluate the resistance of an unknown population: (1) LC_{90} estimate $\geq$0.040% of amitraz, (2) a slope of the regression line $<$2 and (3) at 0.03% of amitraz, a percentage of mortality $<$95%. These criteria should not be applied strictly since a few susceptible populations may meet some of the above criteria (for example, population *La Taraudière*). However, the presence of the resistance in every suspected population should be carefully investigated. Finally, due to the limited knowledge of amitraz resistance, it is necessary to conduct a full bioassay (13 doses, 3 replicates) in order to determine the susceptibility of a population under examination.

In recent years, resistance to amitraz was found in *B. microplus* populations from South Africa (Taylor and Oberem, 1995), Brazil (Martins et al., 1995; Furlong, 1999) and Colombia (Benavides et al., 2000). The first cases of resistance to amitraz were reported after 10 years of use in Australia (Nolan, 1981) and 7 years of use in Mexico (Soberanes et al., 2002). The *Gadji* population was collected from a ranch on the west coast in the town of Païta. In this semi-intensive farm of 600 Charolais and Hereford cattle, the tick infestation was controlled by acaricide treatments applied on a regular basis using a spray-race unit. The control of tick infestation by acaricides began in the 1970s with the OP diethylethion (Rhodiacide ND). From 1992 to the end of 1997 deltamethrin (Butox ND) was used, but replaced by amitraz (Taktic ND) when the tick population was declared resistant to deltamethrin in October 1997. Resistance to amitraz appeared after a relatively short time on this ranch (5 years). This could be explained by the intensive use of amitraz. The number of amitraz treatments per year from 1998 to 2003 was 12, 11, 14, 15, 15 and 19, respectively. Therefore, resistance to amitraz was reported after 86 treatments with this acaricide. In 2003, the number of acaricide applications increased, because of a lack of efficacy of the acaricide. The second amitraz resistant population (*Néty*), was collected at a farm adjacent to the *Gadji* ranch. Ivermectin was recommended for control of amitraz resistant ticks on these two premises. Although untreated wild deer may be a secondary hosts for the ticks (Barré et al., 2001) compromising this strategy.

A thorough epidemiological survey of tick susceptibility to amitraz in cattle farms of New Caledonia should be conducted as soon as possible to determine if the two cases of amitraz resistance reported in this study are an indication of a larger problem that has not yet been discovered. The presence of amitraz-resistant populations in other areas is conceivable, and in order to prolong the use of amitraz in New Caledonia, it is important to detect and eradicate amitraz resistant populations as quickly as possible.

Cattle producers raise tick susceptible cattle, *B. taurus*, in an environment that sustains tick development all year. Therefore, producers are forced to apply acaricides often in order to maintain tick numbers at acceptable levels. This tick control practices in New Caledonia strongly favour the development of resistance (Beugnet et al., 1994). In view of the scarcity of acaricides available, at a reasonable price, and with low residues, tick control should not be based solely on acaricide use. Other tick control strategies can be combined with acaricide applications to reduce

tick populations and reduce the number of acaricide applications per year. Agronomic measures such as rotation are not well adapted to Caledonian farming system and the use of tick vaccination (TickGard ND) in New Caledonia produced very disappointing results. Finally, a very successful strategy used in many tropical areas of the world is the introduction of resistant cattle breeds or the selection of pure taurin cattle more tolerant to tick infestation.

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