Accumulation of acaricide resistance mechanisms in *Rhipicephalus (Boophilus) microplus* (Acari: Ixodidae) populations from New Caledonia Island

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**Abstract**

*Rhipicephalus (Boophilus) microplus* has been pesticide-controlled for several decades in the pacific island of New Caledonia. Since 1996, pesticide-control has been based on either deltamethrin (Butox\(^b\)) or amitraz (Taktic\(^b\)) in herds harbouring deltamethrin-resistant ticks. In this island, the first *R. microplus* deltamethrin- and amitraz-resistances were detected in 1992 and 2003, respectively. Using LPT bioassays, we have undertaken to update data regarding the geographical distribution and the physiological diversity likely to be involved in these resistances. We confirmed that after 17 years of intensive use of deltamethrin, several resistances of moderate levels (\(<30\)-fold) have evolved and/or diffused in any part of the island. We also evidenced that amitraz-resistant phenotypes have recently evolved in diverse western tick populations, although none has reached fixation in any tick population yet. According to synergists bioassays, the physiological changes involved in amitraz-resistance in New Caledonia would involve target modification and detoxifying P450 cytochrome oxidase(s). It may also involve detoxifying esterase(s) although this later point will need confirmation on samples bearing higher frequency of resistant phenotypes. Results are discussed with regard to the local evolutionary dynamics of resistance.

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**Keywords:** *Rhipicephalus (Boophilus) microplus*; Resistance; Deltamethrin; Amitraz; Synergists; Bioassays

*Rhipicephalus (Boophilus) microplus* has become a major pest in tropical and sub-tropical agrosystems (Frisch, 1999). This is partly due to the recurrent introductions of *Bos taurus* European breeds in the tropical belt (Frisch, 1999). Indeed, contrary to the tropical host of this tick *B. indicus*, such European hosts are unable to mount efficient immune responses to *R. microplus* infestation (Frisch, 1999). Tick-burdens on *B. taurus* have thus been controlled for decades through intensive pesticide programs. The situation is worsened by the capacity of *R. microplus* to quickly cope with new environments and new environmental heterogeneity. Invasive in many areas (McCoster, 1979), *R. microplus* has recurrently succeeded to develop resistances to any pesticide used in tick-control.
programs indeed (e.g. Aguirre et al., 2000; Bianchi et al., 2003; Ducornez et al., 2005; Foil et al., 2004; He et al., 1999; Li et al., 2004; Miller et al., 1999 and references therein).

The history of *R. microplus* control has been particularly complex in New Caledonia Island (164–168° East; 20–22° South). *R. microplus* was introduced in this Pacific Island in 1942 via the importation of Australian animals (Rageau and Vergent, 1959). In this island of about 18,575 km², there are about 150,000 cattle formed of three *B. taurus* breeds (Limousin, Charolais and Hereford) and their inter-crosses. The high susceptibility of such *B. taurus* breeds to *R. microplus*’s infestations (Barré, 2003) coupled with the climatic conditions allowing a year-around activity of the tick (Bianchi et al., 2003) had jointly favoured a rapid demographic increase of that parasite. Fortunately, the microbial diseases transmitted by *R. microplus* were not imported in New Caledonia along with the tick. However, blood loss and reduction in weight resulting from blood feeding represent one of the most important pathological constraints to the island livestock production (Daynes et al., 1984). As a result, the territorial authorities decided to take the responsibility of cattle tick control by freely delivering acaricides to cattle producers. The first chemicals used were arsenic (1943–1950) and DDT (1947–1973). Then, distributions of organophosphate (OP) and synthetic pyrethroid (SP) chemicals were generalized over the entire island according to the following sequence: diethylethion (OP, 1973–1980s), ethion (OP, 1986–1994), chlorpyrifos (OP, 1994–1996), and deltamethrin (SP, 1986–present). The apparition of OP-resistance in the 1980s (Brun et al., 1983) and its generalization over New Caledonian tick populations drove the replacement of OPs by deltamethrin (SP) in 1986. In turn, the detection of diverse SP-resistances in 1992 (Beugnet and Chardonnet, 1995) forced the Veterinary Services to redefine tick-control programs. Since 1996, either deltamethrin (Pyr, Butox®) or amitraz (formamidine, Taktic®) have been distributed to farmers, with farmers receiving Taktic® if only their herd was convinced to harbour deltamethrin-resistant ticks by the Parasitology Unit of the Agronomic Institute of New Caledonia (IAC). In 1998, IAC performed an extensive survey in order to characterize the agronomic risk factors of deltamethrin-resistance (Bianchi et al., 2003). Geography emerged as a significant factor: a primary decreasing gradient in deltamethrin-resistance frequency was evidenced from South to North, and a secondary decreasing gradient from West to East. This geographical heterogeneity was mostly explained by the technical practices of the farmers resulting in differences in the intensity of acaricide selection (Bianchi et al., 2003). In one extreme, the use of bush fires, mostly performed in the Melanesian farms from the Eastern coast, was associated to a lower cattle density, lower acaricide selection and lower resistance frequency. In the other extreme, the belonging to cattle farmers’ organization, a common trait among the farmers from the south-west of the island, appeared as a resistance risk factor of resistance probably because of an unreasonable promotion of pesticide use within these organizations. Meanwhile, such a geographical heterogeneity in deltamethrin-resistance had mechanically induced a geographical heterogeneity in the intensity of amitraz-selection. Unsurprisingly thus, amitraz-resistance was firstly detected in the south-western end of cattle distribution (Ducornez et al., 2005). This amitraz-resistance was detected after 86 amitraz treatments performed in five and a half years (i.e. with ca. 1.3 treatments per month), and apparently occurred within only two neighbour farms in 2003 (Ducornez et al., 2005).

The present survey was undergone in 2005 with the main objectives to (i) test the potential diffusion of the first detected amitraz-resistance, (ii) to determine the physiological diversity involved in amitraz-resistance, and (iii) to update data regarding deltamethrin-resistance distribution. Accordingly, we designed tick-sampling in order to cover the various geographical parts of the island, and increased sampling efforts in the south-western end of cattle distribution when looking at amitraz-resistance cases. We based our survey on acaricide-bioassays so that the mortality-responses of field-collected ticks were compared to that of strains selected for acaricide-resistance or -susceptibility. We completed our investigation by analysing how three synergists affected acaricide-mortality response to evaluate the possible implication of esterases, glutathione-transferases (GSTs) and/or P450 oxidases in amitraz- and/or deltamethrin-resistances. Results are discussed with regard to the long-term evolution of pesticide resistance and the possible means to manage such resistance diversity.

1. **Material and methods**

1.1. **Tick strains**

Two strains are currently selected in IAC onto distinct and isolated steers: Tiquotine for deltamethrin and amitraz-susceptibility, and Vache3 for
amitraz-resistance. At each tick generation, fully engorged females from each strain have been collected when falling onto the soil and allowed to lay eggs in an environmental chamber (temperature $T = 26 \pm 1^\circ C$; room humidity RH: 80–92%) where offspring hatched. The larvae selected to generate the next tick-generation were placed back on steer when they were from 14 to 20 days old.

In 2003, the field ancestors of Tiquotine were collected as fully-engorged females within the northeastern F1 herd (Fig. 1). At collection time, this tick population remained successfully controlled by deltamethrin and had never faced amitraz-based control yet. Fully-engorged females were brought to IAC to lay eggs. After two generations of mass-rearing, 93 mature females were individually dispatched in glass-tubes to lay eggs. Two sub-groups of 50 larvae each were collected per brotherhood to be exposed to either 0 or 0.2 g/l deltamethrin in a standard deltamethrin-bioassay (see below). Unexposed larvae from the 23 most susceptible brotherhoods were selected to create Tiquotine. Their descent had experienced two additional generations when the study began.

The Vache3 strain was created from the descent of Gadjii collected in A1 herd (Fig. 1), with Gadjii being the first amitraz-resistance sample detected in the island (Ducornez et al., 2005). When the study began, this strain had experienced five subsequent generations of selection designed as follows. At each generation, a subset ($600 < N < 2000$) of 14 days old larvae were engaged in a standard amitraz-bioassay (see description below) aimed at evaluating the amitraz dose inducing 50% mortality (LC$_{50}$). The remaining larvae were then challenged for 24 h at LC$_{50}$ before completing their development on bovine host.

1.2. Tick sampling

Thirty-five tick samples were performed in New Caledonian cattle farms in April–May 2005 according to the sampling geography described in Fig. 1. Samples A5, A6, B11, C4 and E2 were sent to IAC by farmers for resistance diagnosis. Other samples were collected during visits to farms and/or slaughter houses. In any case, from 30 to 80 fully engorged females were collected in early morning on a minimum of 5–10 cows per herd, and brought to IAC to lay eggs in an acaricide-free environmental chamber ($T = 26 \pm 1^\circ C$; RH: 80–92%). The hatching process was surveyed every 2 days to reduce as much as possible the error on larval age estimates. In a few cases, a sub-sample of mature females had spent from 5 to 10 days at 10°C before reaching the development chamber. This allowed stopping egg development without hurting females,
and hence post-poning the dates of bioassay without affecting results (N.B. unpublished results).

1.3. Bioassays

1.3.1. Deltamethrin bioassay

Deltamethrin-mortality responses were characterized by modifying the larval packet test (LPT, FAO, 1984) so that the insecticide source was Butox® (5% EC, Intervet) rather than technical grade deltamethrin. Butox® (5% EC, Intervet) was diluted in olive oil to generate a stock solution at 12.8 g/l deltamethrin. Serial dilutions in two parts trichloroethylene and one part olive oil generated the testing doses. Packets were prepared by depositing 0.67 ml of a testing dose on a 7.5 cm × 8.5 cm piece of filter paper (Whatman #541, Techmed) before allowing trichloroethylene evaporation for 1h in fume hood. Treated papers were folded on half and sealed on the sides with bulldog clips to form packets. About 100 larvae were placed within packets half and sealed on the sides with bulldog clips to form packets. About 100 larvae were placed within packets secondarily sealed with a bulldog clip. Larvae were generally 21 days old when engaged into deltamethrin-bioassays, with minimal and maximal age of 19 and 30 days, respectively. Filled packets were held for 24 h at bioassays, with minimal and maximal age of 19 and 30 generally 21 days old when engaged into deltamethrin-secondarily sealed with a bulldog clip. Larvae were placed within packets. About 100 larvae were placed within packets half and sealed on the sides with bulldog clips to form packets. About 100 larvae were placed within packets secondarily sealed with a bulldog clip. Larvae were generally 21 days old when engaged into deltamethrin-bioassays, with minimal and maximal age of 19 and 30 days, respectively. Filled packets were held for 24 h in an environmental chamber reserved to deltamethrin bioassays before counting dead and alive larvae. In those conditions, deltamethrin-susceptible ticks are expected to die at any dose equal or higher than 0.8 g/l deltamethrin. A standard bioassay involved a control duplicate – where papers had received dilutants only – and duplicates of nine treating doses (0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8 g/l active ingredient, AI).

1.3.2. Amitraz bioassay

Amitraz-mortality responses were characterized as in Miller et al. (2002). Testing doses were prepared as serial dilutions of a commercial formulation (Taktic®, ND 12.5% EC, Intervet) in two parts trichloroethylene and one part olive oil. Amitraz bioassay was usually performed within the 24 h following testing doses preparation, and never with solutions older than 36 h. Packets were prepared by depositing 0.67 ml of amitraz dose onto a 7.5 cm × 8.5 cm piece of filter paper (Type 2320, Cerex Advanced Fabrics, Pensacola, FL), and allowing trichloroethylene evaporation for 2 h in fume hood. The age of the larvae engaged in amitraz bioassay was strictly controlled as ranging between 14 and 15 days. Filled packets were held for 24 h in an environmental chamber (T = 27 ± 1 °C, RH: 85–92%) reserved to amitraz bioassays before counting dead and alive larvae. A standard bioassay involved a control duplicate and triplicates of eight treating doses distributed in a 2-fold scale between 0.001953% (w/v) and 0.25% AI.

1.3.3. Synergist studies

The role of metabolic enzyme in acaricide resistance was assessed by comparing the mortality responses obtained either in presence or in absence of a synergist compound. Technical grade of three synergists were used. Triphenylphosphate (TPP, 97% pure, Sigma) inhibits esterases activity. Pyperonyl butoxide (PBO, 98% pure, Sigma) inhibits the activity of cytochrom P 450 oxydases. Diethyl maleate (DME, 97% pure, Aldrich) inhibits the activity of glutathione-S-trans- feres (GST). In either case, the synergist was added in the olive oil used to prepare acaricide serial dilutions at 2% (w/v) relatively to olive oil volume, i.e. a concentration that was not expected to induce high mortality (Miller et al., 1999; Crampton et al., 1999). This hypothesis was a posteriori confirmed by looking at the mortality observed in ‘synergist-controls’ where larvae were only exposed to solvents and synergists. Aside a duplication of ‘synergist-controls’, the syner-gists’ bioassays included either duplicates of nine deltamethrine concentrations or triplicates of eight amitraz concentrations.

1.3.4. Statistical analysis

Mortality responses were analyzed using Polo-PC (LeOra Software, 1987) accordingly to the stepwise procedure described by Robertson and Preisler (1992). Abbott correction was applied to take into account the acaricide-independent mortality detected in controls. Corrected mortalities were probit-transformed, and acaricide doses were transformed into their natural logarithm. Transformed data were adjusted to a linear model in order to estimate LC50. Deviation from linearity of log-probit transformed data was assessed through a chi-square test (Robertson and Preisler, 1992). As deviation from linearity occurs whenever the individuals exposed are heterogeneous for their response to acaricide, it is likely to indicate population admixture of resistant and susceptible genotypes. Accordingly, we chose acaricide-susceptible references as exhibiting the weakest possible (if any) deviation from log-probit linear fits.

Resistance ratios (RRs) and their confidence intervals (95% CI) were computed for each sample relatively to the susceptible reference by taking into account the variance and covariance of the slopes and intercepts of both regressions (Robertson and Preisler, 1992). The synergist effect on the mortality response of a particular
sample was investigated by computing means and 95% CI of synergism ratios (SRs) between the results acquired in presence and in absence of synergists. As for RRs, computations took into account the variance and covariance of the slopes and intercepts of both regressions (Robertson and Preisler, 1992). The comparison in SRs between resistant and susceptible samples are rather indirect comparison whenever the mortality responses do not correspond to parallel linear regressions, and testing field-samples we expected recurrent rejection of the hypothesis of parallel linear regressions. This is the reason why we also computed additional RRs between resistant and susceptible samples exposed to the same synergist to investigate how the synergist presence modified these RRs relatively to those computed in regular bioassays.

Given the absence of parallelism between the compared mortality responses, RRs and SRs were computed at the lethal concentrations inducing either 0.50 mortality (LC50) or 0.90 mortality (LC90) to give a more global comparative picture. For amitraz-bioassays a focus on subsets of data around either LC50 and LC90 can provide better estimates (Miller et al., 2007), we also investigated submitting data subsets or the entire datasets modified the results.

2. Results

2.1. Deltamethrin mortality curves

The panel of concentrations surveyed resulted in 3 to 6 concentrations leading to a mortality ranging between 0.1 and 0.9, and at least one dose leading to a mortality of 1. The log-probit linear model did not fit well the deltamethrin-mortality-response in 15 cases out of the 19 bioassays performed, including those of VACHE3 and Tiquotine strains (χ² test, P < 0.05, see Table 1). A focus on either the lowest or the highest deltamethrin doses did never succeed to eliminate the heterogeneity observed on deltamethrin-mortality responses (details unshown).

For B3, B13, C2, E1 and F2 samples, log-probit linear model fitted well the deltamethrin-mortality-response. F2 displayed the highest susceptibility to deltamethrin, characterized by LC50 = 0.22 g/l AI. Relatively to the F2-reference, B3 was the only

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bioassay characteristics</th>
<th>LC50</th>
<th>95% CI</th>
<th>RR50</th>
<th>95% CI</th>
<th>RR90</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1-T (VACHE3)</td>
<td>1437</td>
<td>2.4 ± 0.2</td>
<td>34 (14)**</td>
<td>0.56</td>
<td>0.42–0.68</td>
<td>2.5</td>
<td>2.1–3.0</td>
</tr>
<tr>
<td>A2-T</td>
<td>1077</td>
<td>1.5 ± 0.1</td>
<td>48 (8)**</td>
<td>0.54</td>
<td>0.20–0.90</td>
<td>2.7</td>
<td>2.1–3.0</td>
</tr>
<tr>
<td>B1-T</td>
<td>1633</td>
<td>1.6 ± 0.1</td>
<td>31 (8)**</td>
<td>0.64</td>
<td>0.42–0.85</td>
<td>3.1</td>
<td>2.6–3.7</td>
</tr>
<tr>
<td>B2-T</td>
<td>1149</td>
<td>1.3 ± 0.1</td>
<td>4 (4)**</td>
<td>0.43</td>
<td>0.13–0.78</td>
<td>2.5</td>
<td>2.1–2.9</td>
</tr>
<tr>
<td>B3-T</td>
<td>1892</td>
<td>1.4 ± 0.1</td>
<td>14 (6)**</td>
<td>0.38</td>
<td>0.25–0.50</td>
<td>1.8</td>
<td>1.4–2.4</td>
</tr>
<tr>
<td>B4-B</td>
<td>1462</td>
<td>2.4 ± 0.1</td>
<td>18 (5)**</td>
<td>2.46</td>
<td>2.00–3.05</td>
<td>8.5</td>
<td>7.6–9.4</td>
</tr>
<tr>
<td>B5-T</td>
<td>1588</td>
<td>2.2 ± 0.2</td>
<td>16 (10)**</td>
<td>0.36</td>
<td>0.22–0.49</td>
<td>1.7</td>
<td>1.4–2.0</td>
</tr>
<tr>
<td>B6-T</td>
<td>1597</td>
<td>2.2 ± 0.1</td>
<td>49 (8)**</td>
<td>1.27</td>
<td>0.98–1.67</td>
<td>5.7</td>
<td>5.1–6.5</td>
</tr>
<tr>
<td>B13-B</td>
<td>1707</td>
<td>5.4 ± 0.4</td>
<td>14 (14)</td>
<td>3.45</td>
<td>3.22–3.68</td>
<td>16</td>
<td>14–17</td>
</tr>
<tr>
<td>C1-T</td>
<td>1198</td>
<td>1.6 ± 0.1</td>
<td>33 (8)**</td>
<td>0.60</td>
<td>0.29–0.93</td>
<td>2.7</td>
<td>2.1–3.4</td>
</tr>
<tr>
<td>C2-T</td>
<td>1721</td>
<td>4.3 ± 0.2</td>
<td>4 (14)</td>
<td>3.85</td>
<td>3.63–4.35</td>
<td>12</td>
<td>11–14</td>
</tr>
<tr>
<td>C3-T</td>
<td>1324</td>
<td>1.2 ± 0.1</td>
<td>25 (7)**</td>
<td>0.20</td>
<td>0.01–0.50</td>
<td>2.2</td>
<td>1.4–3.4</td>
</tr>
<tr>
<td>C8-B</td>
<td>1590</td>
<td>2.3 ± 0.1</td>
<td>51 (8)**</td>
<td>0.35</td>
<td>0.27–0.45</td>
<td>1.6</td>
<td>1.4–1.8</td>
</tr>
<tr>
<td>C9-B</td>
<td>1505</td>
<td>3.6 ± 0.2</td>
<td>39 (14)**</td>
<td>3.76</td>
<td>3.24–4.37</td>
<td>18</td>
<td>16–21</td>
</tr>
<tr>
<td>D1-T</td>
<td>1172</td>
<td>2.4 ± 0.2</td>
<td>84 (8)**</td>
<td>2.22</td>
<td>1.41–3.18</td>
<td>10</td>
<td>8.8–11</td>
</tr>
<tr>
<td>E1-T</td>
<td>1380</td>
<td>5.3 ± 0.3</td>
<td>25 (8)</td>
<td>1.50</td>
<td>1.14–2.00</td>
<td>13</td>
<td>12–14</td>
</tr>
<tr>
<td>E7-B</td>
<td>1427</td>
<td>2.2 ± 0.1</td>
<td>35 (14)**</td>
<td>0.69</td>
<td>0.67–0.83</td>
<td>3.1</td>
<td>2.7–3.6</td>
</tr>
<tr>
<td>F1-B (Tiquotine)</td>
<td>1338</td>
<td>2.9 ± 0.2</td>
<td>28 (13)**</td>
<td>0.17</td>
<td>0.14–0.19</td>
<td>0.74</td>
<td>0.66–0.84</td>
</tr>
<tr>
<td>F2-B</td>
<td>1449</td>
<td>4.2 ± 0.2</td>
<td>15 (14)</td>
<td>0.22</td>
<td>0.20–0.24</td>
<td>0.42</td>
<td>0.35–0.50</td>
</tr>
</tbody>
</table>

Samples are labeled according to the area where their field-ancestors were collected (Fig. 1) and the acaricide control experienced by these ancestors (B or T for Butox® and Taktic®, respectively). Bioassays are characterized by the number of larvae involved (N), slope estimate, the chi-square value evaluating the goodness of fit of the linear regression to log-probit transformed data, and the concentration inducing 50% mortality (LC50). The among-sample differences in deltamethrine-induced mortality are summarized by resistance ratios computed relatively to F2-B larvae for 0.50 and 0.95 mortality rates.

*a in AI g/l.

*P < 0.05.

**P < 0.01.
susceptible sample (Table 1). The level of deltamethrin-resistance was about 12-fold in C2 and E1 at LC50 and LC90, and reached 16-fold in B7 at LC50 (Table 1). Among the mortality-responses that were not well fitted by linear models, the highest susceptibility was observed in TIQUOTINE strain (0.21 \,<\, RR90 \,<\, 0.87), the highest resistance in the C9 Butox-controlled population (17 \,\leq\, RR90 \,\leq\, 25), and an intermediate answer was observed in VACHE3 strain (3.6 \,\leq\, RR90 \,\leq\, 5.3, Table 1).

The action of synergists could be investigated for TIQUOTINE and VACHE3 strains as well as for the B13, C2, C9 and E1 field-samples (Table 2 and Fig. 2). The deltamethrin-resistance observed in C9 was synergized neither by DME nor by PBO (SR \,\approx\, 1), and was much less synergized by TPP (SR \,\leq\, 2) than the mortality response of the TIQUOTINE susceptible strain (SR \,\leq\, 10). This indicates that, in C9 population, deltamethrin-resistance is unlikely to involve detoxification mechanisms. In other samples, the effect of PBO and TPP was significant (SR \,\leq\, 1) but lower than in TIQUOTINE susceptible strain indicating that neither esterases nor P450 oxydases were involved in the resistant phenotypes present. Finally, DME synergized the deltamethrin-mortality of B13 and VACHE3 larvae (SR \,\leq\, 1) but not that of any other sample including TIQUOTINE susceptible strain. We completed the investigation by re-computing the resistance ratios for B13 and VACHE3 larvae relatively to the TIQUOTINE susceptible ones exposed to the same environment. A slight drop in the resistance level of both B13 and VACHE3 larvae relatively to TIQUOTINE was observed when all samples were exposed to DME (9.0 \,\leq\, RR50 \,\leq\, 10.5 and 7.1 \,\leq\, RR90 \,\leq\, 0.7 for B13; 0.7 \,\leq\, RR50 \,\leq\, 1.0 and 1.3 \,\leq\, RR90 \,\leq\, 2.5 for VACHE3) relatively to synergist-free bioassays (18.8 \,\leq\, RR50 \,\leq\, 23.3 and 11.2 \,\leq\, RR90 \,\leq\, 14.9 for B13; 2.8 \,\leq\, RR50 \,\leq\, 4.1 and 3.5 \,\leq\, RR90 \,\leq\, 5.1). Overall, these comparisons suggest that some of the resistant individuals present within B13 and VACHE3 bear particular GST phenotypes whereas target modification is involved in C9-resistance.

### 2.2. Amitraz mortality curves

The panel of concentrations surveyed usually resulted in 4 to 9 doses where samples experienced a mortality ranging from 0.1 to 0.9, except for B9 larvaee and of 16 days old B8 larvae where only three doses

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**Table 2**

<table>
<thead>
<tr>
<th>Synergist</th>
<th>N</th>
<th>Slope</th>
<th>(\chi^2) (d.f.)</th>
<th>LC50</th>
<th>95% CI</th>
<th>SR50</th>
<th>95% CI</th>
<th>SR90</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>DME</td>
<td>1721</td>
<td>6.6 ± 0.6</td>
<td>1.9 (14)</td>
<td>2.09</td>
<td>1.98–2.22</td>
<td>1.6</td>
<td>1.5–1.8</td>
<td>1.8</td>
<td>1.6–2.1</td>
</tr>
<tr>
<td>C2-T</td>
<td>1389</td>
<td>4.3 ± 0.3</td>
<td>56 (13)**</td>
<td>1.96</td>
<td>1.59–2.40</td>
<td>1.2</td>
<td>0.9–2.0</td>
<td>1.0</td>
<td>0.9–1.2</td>
</tr>
<tr>
<td>C9-B</td>
<td>1567</td>
<td>3.4 ± 0.2</td>
<td>17 (16)</td>
<td>3.60</td>
<td>3.26–3.97</td>
<td>1.1</td>
<td>0.9–1.2</td>
<td>1.0</td>
<td>0.8–1.3</td>
</tr>
<tr>
<td>E1-T</td>
<td>1266</td>
<td>2.8 ± 0.2</td>
<td>153 (14)**</td>
<td>1.88</td>
<td>1.28–2.75</td>
<td>0.8</td>
<td>0.7–0.9</td>
<td>0.7</td>
<td>0.6–0.9</td>
</tr>
<tr>
<td>TIQUOTINE</td>
<td>1318</td>
<td>4.7 ± 0.3</td>
<td>10 (13)</td>
<td>0.22</td>
<td>0.20–0.23</td>
<td>0.8</td>
<td>0.7–0.9</td>
<td>1.1</td>
<td>0.9–1.4</td>
</tr>
<tr>
<td>VACHE3</td>
<td>1210</td>
<td>2.1 ± 0.2</td>
<td>18 (10)</td>
<td>0.18</td>
<td>0.15–0.23</td>
<td>3.1</td>
<td>2.5–3.8</td>
<td>2.6</td>
<td>1.9–3.8</td>
</tr>
</tbody>
</table>

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* in Al g/l.
** P < 0.05.
*** P < 0.01.

The number of larvae (N), the slope and the goodness of fit of log-probit regressions as well as the new LC50 and the SRs computed at LC50 and LC90 (SR50 and SR90) are detailed per synergist-bioassay. Bold characters indicate significant synergism (synergist ratio > 1).
described the 0.1–0.9 mortality interval. The log-probit linear model does not fit the amitraz-mortality-response obtained for Tiquotine strain ($P = 0.004$). Similar bad adjustmements were observed for 16 of the 36 bioassays performed on field samples (Table 3). Among the mortality responses well-adjusted by linear models, the highest susceptibility to amitraz was observed in E3; with enhanced susceptibility and homogeneity noticed on 16 days old relatively to 12 days old larvae (Table 3). Therefore, we chose the mortality-response observed...
among 16 days old E3 ticks as the amitraz-susceptible reference. Relatively to that reference, the resistance ratios obtained for Tiquotine larvae remained lower than 2.5 all over the surveyed mortality range (1.1 ≤ RR$_{50}$ ≤ 1.5; 1.3 ≤ RR$_{90}$ ≤ 1.8 and 1.3 ≤ RR$_{95}$ ≤ 2.4). Accordingly, we arbitrarily considered amitraz-resistance to be defined by resistance ratios higher than the 2.5 threshold. With that criterion, apparent resistance cases were geographically aggregated along the western coast. Amitraz-resistance was detected in 3 out of the 5 samples from area A, in 3 out of the 11 samples from area B, in 2 out of the 7 samples from area C, and in none of the 9 samples from the more northern and/or eastern areas (see bold characters in Table 3).

Genetically homogeneous strains generally, although not obligatorily, provide parallel mortality-responses in arthropods (Robertson and Preisler, 1992). In the present study, amitraz-susceptible mortality-responses were more or less accurately fitted by slopes ranging between 3 and 6. By contrast, resistance detection was associated with slopes lower than 2, with
a general tendency to reject linear adjustments. Such a contrast persisted when focusing on data subsets. It suggests that none of the resistant-classified populations had fixed resistance phenotypes yet. However, C4 sample merits attention as the estimated slope is high enough to rise the lethal amitraz doses LC_{50} 10 to 30-fold above the E3-reference. This suggests that C4 involved a significant percentage of amitraz-resistant ticks.

2.3. Synergists effects on amitraz-induced mortality

Table 4 and Fig. 3 summarized the effects of synergists on the susceptible reference E3, on the A2 and B11 field samples, and on the Tiquotine and Vache3 strains. DME had a similar effect on the samples including resistant phenotypes than on E3 and Tiquotine susceptible samples; indicating an absence of GST-based resistance. Indeed, the same RRs were computed from DME-bioassays (i.e. relatively to the mortality responses of E3 DME-exposed larvae) than from regular bioassays. PBO has got an enhanced effect on the mortality responses of A2 and Vache3 than on all other ones (3 ≤ SR_{90} ≤ 5 versus SR_{90} ≤ 2.2); suggesting the presence of P450 oxydases resistant phenotypes in these samples. The inhibition of P450 oxydases in all larval samples suppressed the resistance previously detected in A2 (1.1 ≤ RR_{90} ≤ 1.7 between A2 and E3 PBO-exposed larvae versus 2.9 ≤ RR_{90} ≤ 4.4 in regular bioassays), drastically reduced the resistance detected in Vache3 (RR_{90} ≤ 21 in PBO-bioassays versus 57 ≤ RR_{90} in regular bioassays) but did not affect B11 resistance (7.3 ≤ RR_{90} ≤ 12 in PBO-bioassays versus 5.9 ≤ RR_{90} ≤ 11 in regular ones). TPP has got a slightly enhanced effect on Tiquotine and A2 larvae (2 ≤ SR_{90} ≤ 4) than on Vache3 larvae (1.4 ≤ SR_{90} ≤ 2.2) and on E3 and B11 larvae (SR_{90} ≈ 1). Relatively to E3 larvae exposed to the same bioassay conditions, the inhibition of esterases suppressed resistance in A2 (0.9 ≤ RR_{90} ≤ 1.2) and decreased it in B11 and Vache3 (3.3 ≤ RR_{90} ≤ 5.0 and 30 ≤ RR_{90} ≤ 44, respectively). Taking Tiquotine as a new susceptible reference, the inhibition of esterases decreased resistance in A2 (1.4 ≤ RR_{90} ≤ 1.8 between A2 and Tiquotine TPP-exposed larvae versus 2.0 ≤ RR_{90} ≤ 2.8 in regular bioassays) but affected resistance neither in Vache3 (46 ≤ RR_{90} ≤ 64 in
TPP-bioassays versus 34 ≤ RR₉₀ ≤ 51 in regular ones) nor in B11 (5.0 ≤ RR₉₀ ≤ 7.4 in TPP-bioassays versus 4.0 ≤ RR₉₀ ≤ 6.9 in regular ones). Altogether, these results indicate a variability in amitraz-resistant phenotypes. First of all, resistant phenotypes that are insensitive to the three synergists were present in B11 and VACHE3 samples. In addition, some of VACHE3 and A2 larvae resisted to amitraz because of modifications in the activity of P450 oxydase(s). Finally, it is also possible that modifications in the activity of esterase(s) defined some of the amitraz-resistant phenotypes present in the A2 population.

3. Discussion

Our survey confirmed that deltamethrin-resistance phenotypes remain widely distributed among the New Caledonian tick populations. Synergist bioassays indicated that two physiological mechanisms are likely to contribute to deltamethrin-resistance there. Indeed, changes in the molecular target of deltamethrin is the most likely genetic basis of resistance in C9. Meanwhile, GSTs can partially explained the deltamethrin-resistance observed in B13 and VACHE3 samples. Observing different resistance physiological bases in New Caledonia after 17 years of intensive deltamethrin-selection is not surprising, and recalls the situation observed among Mexican tick populations after a long and intensive use of pyrethrinoids and/or OPs (Miller et al., 1999). Interestingly though, the levels of deltamethrin-resistance remained moderate in New Caledonia (RR < 30). For the sake of comparison, mutations in the pyrethrinoids target, the voltage-dependent sodium channel, was evidenced to confer resistance above 1000-fold in Mexican ticks (He et al., 1999; Miller et al., 1999). Surveying the polymorphism of that gene using Guerrero et al. (2001)’s test could help settling whether such mutations concurring high deltamethrin-resistance level are absent from New Caledonia or if they remain at low frequency there.

Amitraz usage in New Caledonian tick controls is far more recent than other chemical usages, as it only started in 1996. Amitraz toxicity in arthropods is believed to be due to its interaction with the octopamine receptor in the central nervous system (Evans and Gee, 1980), and possibly also to its inhibition of monoamine oxydases (Atkinson et al., 1974). It has been claimed that the development resistance to amitraz in R. microplus has been slower than those of previous pesticide used in control (Li et al., 2005). New Caledonia Island looks as the exception to this rule since 6 years of amitraz-use was enough to detect the first failure of amitraz-control in the field-ancestors of the VACHE3 strain (Ducornez et al., 2005). Nonetheless, five additional generations of amitraz-selection at LC₅₀ were not enough to render the VACHE3 strain homogenous regarding amitraz-mortality response. This may suggest that this amitraz-resistance is too moderate to properly discriminate resistant genotypes from susceptible ones, that different genes are involved in that trait and/or that this resistance is a recessive trait. Synergists bioassays suggest the contribution of two physiological mechanisms to amitraz-resistance in VACHE3 strain. Indeed, the effect of PBO indicated the likely involvement of P450 cytochrom oxydase(s). Complementarily, the fact that no synergist succeeded to suppress amitraz-resistance suggests the contribution of a modification in the amitraz-target to that resistance. Interestingly, the same characteristic of a not entirely synergized amitraz-resistance was evidenced in the Brazilian strain Santa Luisa (Li et al., 2004) with, in that case, a clear evidence that such a resistance is inherited as a recessive trait with marked maternal effects (Li et al., 2005). Further amitraz-selection of the VACHE3 strain is required to fix the mutations involved and hence to identify their mode of inheritance.

The ancestral population of the VACHE3 strain, A1, was not the only place where failures of amitraz-based control of tick demography has been encountered in New Caledonia Island. The present survey confirmed the presence of amitraz-resistant phenotypes in western tick populations, with resistance level ranging from 4 to 7-fold (in B11, A2, A5 and A6) to more than 15-fold (in A5, B12 and C4). Such a geographical concentration of amitraz-resistances recalls the situation previously observed for deltamethrin-resistances and is likely to result from the same causes, i.e. an overall increase in acaricide-selection in the south-western end of the cattle distribution (Bianchi et al., 2003). Resistant phenotypes had not reached fixation in any of these tick populations yet making thus a correct characterization an even more complex task than in amitraz-selected strains (see Miller et al., 2007 for a review of the difficulties encountered in strains). We tempted nonetheless to characterize the physiological changes involved in amitraz-resistance in two of these field-samples. In A2, there were some PBO-synergized resistant phenotypes, i.e. probably resulting from modification in the activity of P450 oxydases. The enhanced TPP-synergist effect on A2 mortality response relatively to those of E3 and TIQUOTINE susceptible larvae also suggests the possible presence of resistant phenotypes with modified esterase activity in A2. This later point will require confirmation by re-performing the analysis on
A2-descent after a few generations of amitraz-selection aimed at increasing resistance frequency among bioassayed larvae. In B11 by contrast, amitraz-resistance seems resulting from target modification since it was not synergized by either PBO, TPP or DME. Regrouping the results acquired on A2, B11 and A1-descent (i.e. VACHE3) makes thus emerging a mosaic pattern with different physiological bases of resistance having been selected for within and among nearby populations within a few tick generations.

The fact that diverse phenotypes have quickly answered to acaricide-selection is compelling. This suggests a lower probability for resistance genes to invade the island than to recurrently appear by mutation.
A population genetics study based on non-selected polymorphism brought an independent support to that hypothesis. Indeed, Koffi et al. (2006) evidenced that, in New Caledonia, R. microplus gene dispersal was not greater than a few hundreds meters per tick generation and that the density in reproducing adults remains as large as a few hundreds per square kilometer there. In turn, the rapid accumulation of resistant phenotypes is not without consequence on the long-term evolution of resistance. First, such an accumulation is likely to slow down the fixation of any resistant phenotype within tick populations. Second, as observed in the mosquito Culex pipiens (Chevillon et al., 1999; Raymond et al., 2001), this opens the opportunity to ultimately select for the phenotypes associated to the weakest fitness cost. As no-costly resistant phenotypes are unlikely to disappear from large tick populations, this would thus strongly argue for the urgent need to introduce alternative means of tick control in New Caledonia, such as a better rotation of cattle among pastures and/or the introduction of B. indicus × B. taurus hybrid cattle (Frisch, 1999).

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