Mutation in the *RmβAOR* gene is associated with amitraz resistance in the cattle tick *Rhipicephalus microplus*

Sean W. Corley[a], Nicholas N. Jonsson[b,1], Emily K. Piper[a], Christian Cutullé[a,c], Michael J. Stear[b], and Jennifer M. Seddon[a]

[a]School of Veterinary Science, University of Queensland, Gatton, QLD 4343, Australia; [b]University of Glasgow College of Medical, Veterinary and Life Sciences, Bearsden G61 1QH, United Kingdom; and [c]Instituto de Patobiología, Centro de Investigación en Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuaria, B1712WAA Buenos Aires, Argentina

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We aimed to describe the evolution of resistance to amitraz in *Rhipicephalus microplus* in the field and to test the association between amitraz resistance and the frequency of a mutation in the β-adrenergic octopamine receptor gene (*RmβAOR*). We established six populations of *Rhipicephalus microplus* ticks in similar paddocks by the admixture of ticks from strains known to be susceptible and resistant to amitraz and synthetic pyrethroids. Each population was managed using one of three acaricide treatment regimes: always amitraz, always spinosad, or rotation between amitraz and spinosad. We used microsatellites to elucidate population structure over time, an SNP in the *para*-sodium channel gene previously demonstrated to confer resistance to synthetic pyrethroids to quantify changes in resistance to synthetic pyrethroids over time, and a nonsynonymous SNP in the *RmβAOR*, a gene that we proposed to confer resistance to amitraz, to determine whether selection with amitraz increased the frequency of this mutation. The study showed panmixia of the two strains and that selection of ticks with amitraz increased the frequency of the *RmβAOR* mutation while increasing the prevalence of amitraz-resistance. We conclude that polymorphisms in the *RmβAOR* gene are likely to confer resistance to amitraz.

Amitraz has been one of the most important acaricides for the control of tick (*Rhipicephalus microplus*) infestation of cattle in Australia and many other countries since its introduction in the early 1970s (1). In Australia, resistance to amitraz in *R. microplus* was first detected in 1980 among ticks on cattle in central Queensland (1). In 1992 a field isolate that was resistant to amitraz and to all available synthetic pyrethroids (SPs) was identified in Central Queensland (2) and subsequently maintained in culture by the Queensland Government as the Ultimo laboratory strain. Since first detection in Australia, the number of cattle properties with ticks resistant to amitraz increased slowly and fewer than 150 cases had been diagnosed up to 2006 (3). This result contrasts with the rapid spread of resistance to amitraz among ticks on Mexican cattle (4) and has been suggested to be because of a lack of fitness and a recessive mode of inheritance in resistant Australian isolates (3).

The target of amitraz has been proposed to be one of the biogenic amine receptors, most likely the adrenergic or octopaminergic receptors (5, 6). Previous investigations of the role of the octopamine receptor in amitraz resistance in *R. microplus* (7, 8) were not fruitful, but it seems likely that the studies investigated a tyramine receptor, rather than a true octopamine receptor (9). We have recently identified several G protein-coupled receptors in *R. microplus* (9) and identified polymorphisms in resistant populations in the β-adrenergic octopamine receptor (*RmβAOR*, SNP designated *I61F*), which we propose might be associated with resistance to amitraz in *R. microplus*.

The introgression of alleles conferring resistance to antiparasitic and antibiotic chemicals is an important mechanism for the spread of resistance into susceptible populations. Subsequent rapid increase in the frequency of resistance alleles is largely driven by the selection pressure conferred by the use of the chemical, balanced by the fitness cost associated with the resistance-conferring mutation through direct or pleiotropic effects (10). Compensatory mutations at second mutational sites may modify the fitness cost and the epistatic effect modulates the overall fitness of the individual (11, 12). Nonetheless, fitness costs may be sufficiently high to erode the frequency of the resistance allele in the absence of chemical control (13).

The aim of the field study was to describe the evolution of acaricide resistance in populations of *R. microplus* ticks that were created by the admixture and hybridization of amitraz and SP-resistant and susceptible ticks, and subsequently placed under three divergent acaricide treatment regimes using amitraz and spinosad. Initially, in the absence of any molecular tools for population analysis, we intended to determine whether there was a loss of amitraz resistance when amitraz was withdrawn from use on a population of ticks, and whether a rotation strategy using amitraz and spinosad could be effective when resistance-conferring genes were already present in a population. These findings have been reported elsewhere (14). Subsequently, as we have developed molecular tools for analyzing the *R. microplus* populations (9, 15–17), we examined population dynamics on a molecular genetic basis. The primary objectives of this work were to: (*i*) determine whether intensive selection with amitraz resulted in an increased frequency of the *I61F* SNP in the β-adrenergic octopamine receptor (*RmβAOR*); (*ii*) undertake a detailed analysis of the *R. microplus* population dynamics in the field in the first generations after admixture of two distinct strains of cattle tick (susceptible and multiple resistant); and (*iii*) characterize the relationship between resistance to amitraz and resistance to SPs derived from a single multiresistant strain of ticks.

To achieve the objectives, we established six populations of *R. microplus* ticks in similar paddocks by the artificial infestation of cattle with equal numbers of tick larvae derived from a fully susceptible strain (nonresistant field strain, NRFS) and a multiresistant strain (Ultimo: resistant to amitraz and SP). Each

Significance

Amitraz is a widely used acaricide for the control of the cattle tick *Rhipicephalus microplus*, an important parasite of cattle in the tropics and subtropics. Here we describe in detail the evolution of amitraz resistance in replicated populations of ticks in the field, using divergent selection pressures with amitraz. We also demonstrate a close association between resistance to amitraz and a specific allele of the β-adrenergic octopamine receptor gene, which we propose confers resistance to amitraz.

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[1]To whom correspondence should be addressed. E-mail: nicholas.jonsson@glasgow.ac.uk.

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population was managed independently using one of three acaricide treatment regimes: always amitraz, always spinosad, rotation between amitraz and spinosad, according to consistent treatment criteria based on a threshold number of ticks per animal. We measured the level of resistance to amitraz and SP at least once per generation of ticks using the larval packet test (LPT). We extracted DNA from tick larvae that were surplus to requirements for the LPT on at least three occasions: the first or hybrid generation; the second generation in the winter of the first year; a collection in the winter of the second year of the study, estimated to be the seventh generation of ticks generated by the admixture and hybridization. The larvae used for DNA extraction and molecular genetic analysis had not been subjected to the LPT. We used a nonsynonymous SNP in the β-adrenergic octopamine receptor gene (*RmβAOR*), which we proposed to confer resistance to amitraz, to determine whether selection with amitraz increased the frequency of this mutation. In addition, we used microsatellites to elucidate population structure over time and an SNP (*BmNa L64I*) in the *para*-sodium channel gene *(BmNa)* previously demonstrated to confer resistance to SP to describe changes in resistance to SP over time.

Results

Initial Differentiation of the Susceptible and Resistant Strains Used for Infestations. Cattle were initially infested with equal proportions of two laboratory strains, the susceptible NRFS strain and the amitraz- and SP-resistant Ultimo strain. Using the panel of seven microsatellite markers, these strains showed a high level of diversity (expected heterozygosity 0.69 and 0.72 and allelic richness 5.1 and 6.7, respectively) (Table S1) and were significantly differentiated from each other (Table S2) (F_{ST} 0.102, $P < 0.001$). The Ultimo strain, which is subjected to occasional selection with acaricides during strain maintenance, showed a significant departure from Hardy–Weinberg equilibrium (HWE) (Table S1).

Changes in Acaricide Resistance Phenotypes and Genotypes Over Time. The changes in the SP and amitraz-resistance phenotype measured using the LPT and the proportion of resistant homozygotes for the *BmNa L64I* and *RmβAOR I61F* SNP during the 3-y field study under the three acaricide treatment groups are shown in Fig. 1. At generation 1, there were relatively low levels of resistance (21–58%) in all groups. By the final time point, there was a reduction in mortality on exposure to amitraz in the two amitraz treatment groups, consistent with increased resistance to amitraz in this treatment. There was a reduction in resistance to amitraz in both of the spinosad-treated groups, which had not received amitraz treatment, and in the rotation group, which had received several amitraz treatments. There were, however, fluctuations in mortality across time points and treatment groups. Resistance to SP did not follow a consistent path, increasing in some populations while decreasing in others.

RmβAOR* Sequence and Genotyping.** ***Sequencing the coding region. Sequencing the entire coding region from the resistant (Ultimo) and susceptible (NRFS) strains identified a single nonsynonymous SNP (A181T), which resulted in the substitution of an isoleucine with a phenylalanine (*I61F*) in the first transmembrane domain. A comparison of 16 β-adrenergic-like octopamine receptors from nine invertebrate species in three phyla showed that isoleucine at this site was highly conserved in a diverse range of organisms (Fig. S1).

Association of SNP frequency with amitraz resistance in the LPT. Fig. 1 shows genotype and acaricide-resistance prevalence (LPT-derived percentage resistant, using amitraz 0.1%). DNA was not available for *RmβAOR* genotyping from larvae in the rotation treatment groups. The frequency of the homozygous susceptible genotype decreased from 40% and 59% on the two amitraz-treated farmlets to 0% and 7%, respectively, by the end of the second year, whereas the homozygous *I61F* resistant genotype increased from 10% and 21% to 97% and 93% over the same period. In contrast, the frequency of the *I61F*-resistant genotype decreased from 77% and 50% to 41% and 40% on farmlets where spinosad was used for tick control and no amitraz was applied. Fig. S2 shows an increase over time in the frequency of the resistant homozygous *I61F* genotype in the farmlets on which amitraz was used regularly, contrasted with relatively static frequency of the *I61F* homozygous genotype in the farmlets on which amitraz was never used. The REML model showed highly significant effects of genotype ($F = 12.34$, $P < 0.0001$) and a genotype*generation interaction ($F = 254.81$, $P < 0.0001$) on the percentage of individuals resistant to amitraz in the LPT, consistent with increasing frequency of amitraz resistance with increasing generation number only in the *I61F*-resistant genotype. There was higher resistance in the *I61F* homozygote but the heterozygote and homozygote-susceptible genotypes did not differ from each other. The effect of the replicate (farmlet) was not significant in this model ($F = 0.84$, $P = 0.3599$). There was a close and positive correlation between the frequency of the *I61F*-resistant homozygous genotype and resistance to amitraz ($r = 0.90$) (Fig. S3), which contrasts with the strong negative association of resistance with the heterozygote and with the wild-type. Six of the samples obtained from the amitraz-treated farmlets showed significant heterozygote deficiency, whereas the remaining five were in HWE (Table S3). In contrast, among the samples from the spinosad-treated farmlets, eight were in HWE, two in heterozygote deficiency, and one in excess. The probability that the deviations from HWE were a chance effect because the large number of comparisons was 7.1×10^{-10} for the amitraz-treated populations and 1.3×10^{-5} for the spinosad-treated populations.

Changes in *BmNa L64I* Frequencies Over Time. The Ultimo strain that was used has phenotypic resistance to both cypermethrin and amitraz and showed a high percentage of *BmNa L64I* homozygous-resistance genotypes (87.5%, with 10% heterozygotes and 2.5% susceptible homozygotes), in contrast to the susceptible NRFS strain, which contained only homozygous susceptible individuals (Fig. 1). Using this marker to assess the relative contributions of the NRFS and Ultimo strains to the first generation showed that populations in the first generation had heterozygote proportions ranging from 0.40 to 0.77, where 0.50 would be expected. These proportions of *L64I* heterozygosity in the first or hybrid generation suggest hybridization of the strains and panmixia rather than assortative mating within the strains. In the absence of selection with SP, after the first generation there was no consistent trend over time, with frequency of *BmNa L64I* varying from 0.27 to 0.73 by the third time point (homozygote proportion ranging from 0.03 to 0.53). This observation is consistent with an absence of linkage between amitraz and SP resistance.

Genetic Diversity Over Time. Generation 1 individuals, the F1 progeny of the two laboratory strains, showed greater observed heterozygosity across the seven microsatellite loci than either laboratory strain in five of six populations (H_e generation 1: 0.71–0.82; H_e laboratory strains: 0.64–0.65) (Table S1). By Winter 2005, only one of the six populations showed significant departures from HWE. All populations had indications of a bottleneck, with six or seven loci showing expected heterozygosity significantly greater than expectations at mutation-drift equilibrium (bilateral Wilcoxon's signed rank test, $P < 0.05$) (Table S4). There was no obvious trend in the change of number of alleles per locus over time or treatment group for the microsatellites. Changes in allele frequency across the three time points are shown for the *BmM1* microsatellite locus for two treatment replicates in Fig. S4. Eighteen alleles were found in the latter two time points that were not detected in generation 1, occurring at a frequency of 1.67–17.50 (median 1.67 ± 3.21) (Table S5). This result might suggest either incomplete sampling at early time points or that there were ticks of different genotypes than those we used for infestations in the pastures before commencement of the study.

Population Structure Over Time. Bayesian clustering analysis in STRUCTURE was used to determine the degree of admixture

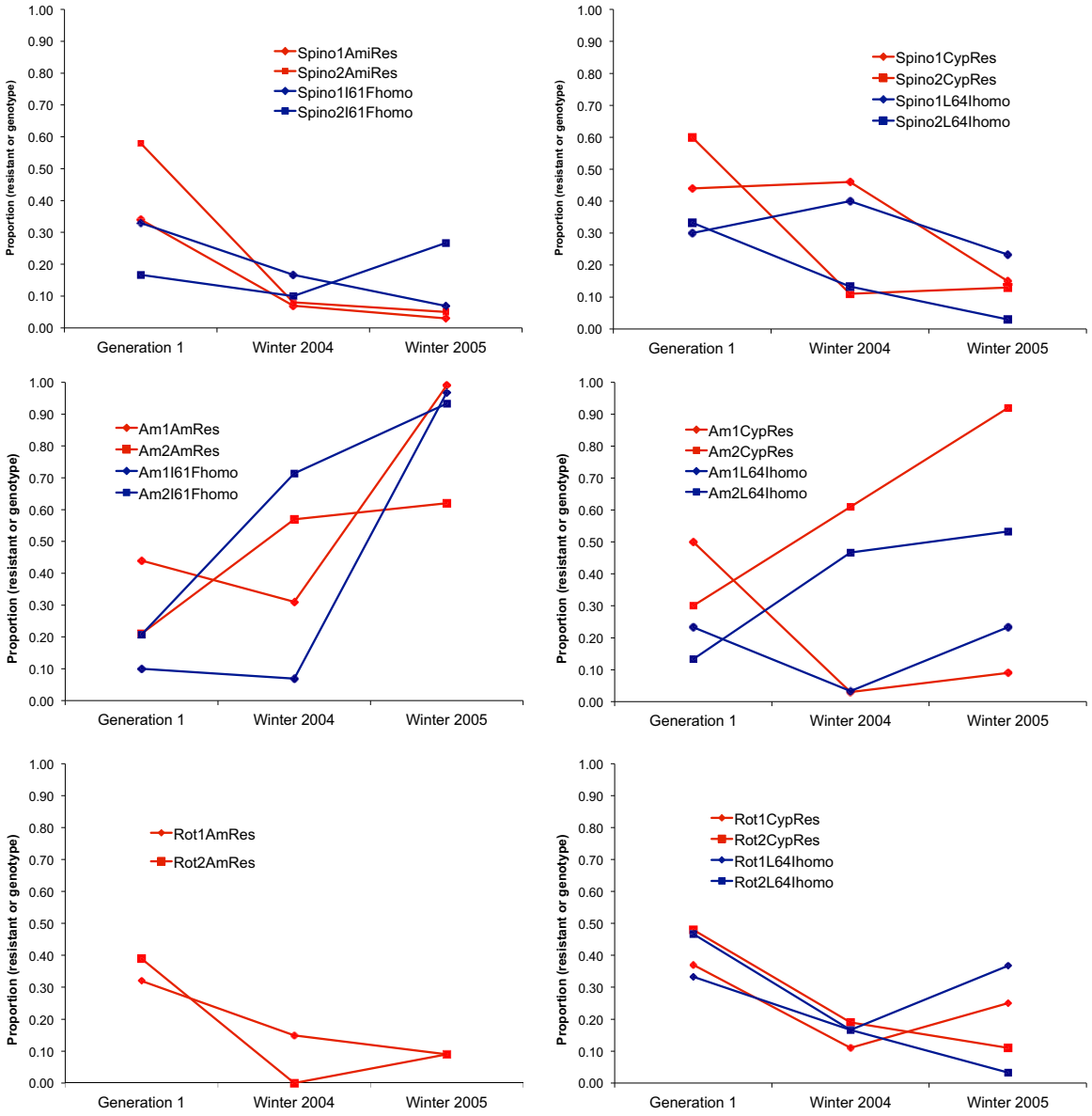

Fig. 1. (*Left*) Phenotypic proportion of resistance to amitraz (red lines) and the proportion of ticks homozygous for the resistant allele *RmβAOR I61F* SNP (blue lines). (*Right*) Phenotypic proportion of resistance to cypermethrin (red lines) and the proportion of ticks homozygous for the *BmNa L64I* SNP (blue lines). Rows show the different treatments from spinosad (*Top*) to amitraz (*Middle*), and rotation (*Bottom*) at each of three time points and for each of the farmlets. In all cases, LPT results are derived from duplicate observations of each acaricide concentration and the genotypic proportion is based on 30 larvae.

of the populations over time. At both generation 1 and Winter 2004, there were only two clusters detected ($k = 2$) and these largely corresponded to the NRFS and Ultimo strains (Fig. S5). The treatment group populations showed admixture of these two clusters. However, by the Winter 2005 time point, three clusters were identified. The NRFS population had a high, inferred ancestry to one of the clusters (cluster 1, 72.2%) (Fig. S5*C*, black) and the Ultimo population had a mean 54.3% inferred ancestry to cluster 2 (Fig. S5*C*, dark gray). The treatment group populations did not show a fixed trend toward any of the clusters with, for example, spinosad replicate 1 having greatest inferred ancestry (60.7%) to cluster 3 (Fig. S5*C*, light gray) and amitraz replicate 1 greatest ancestry (50.8%) to cluster 2. The degree of population structure among treatment groups was noted to increase over time using F_{ST} analysis (Table S1). Among populations at generation 1, there were 6 of 15 (40%) among-population comparisons that were significantly differentiated, but this number rose to 12 (80%) by Winter 2005. The amitraz replicate 2 population at the Winter 2004 time point and the amitraz replicate 1 population at the Winter 2005 time point were significantly differentiated from all other populations, coincident with the reductions in mortality seen in the LPT in these populations (Fig. 1). An AMOVA showed that populations were not structured by treatment within time point or by time point within treatment group (Table S6). The majority of variation was found within individuals, and low but significant levels of structuring were identified among replicates within treatment group (2.14–3.49%).

Field Samples for *RmβAOR* SNP *I61F*. Thirty larvae from each of four regions were sequenced. The *I61F* SNP was found in samples submitted from central Queensland but was absent in tick samples from north Queensland, southeast Queensland, and northern New South Wales. The resistant laboratory strain Ultimo, which has the *I61F* SNP, originates from central Queensland. In a sample of 30 larvae from one susceptible central Queensland population, 10 heterozygote *I61F* ticks were found, with the

remainder being susceptible homozygotes, and in a sample of 30 ticks from a central Queensland population estimated to be 73% resistant from the bioassay, two homozygote *I61F* ticks were found, with 26 heterozygotes and only two homozygote wild types. A further nonsynonymous SNP (T185C) resulting in the substitution of a highly conserved isoleucine with a threonine (*I62T*) (Fig. S1) was identified in larvae from southeast Queensland.

Discussion

In this study we have shown a strong association between a polymorphism in a highly conserved region of the *RmβAOR* gene of *R. microplus* and resistance to amitraz in the LPT bioassay, and demonstrated that the mutation is selected for by treatment with amitraz over seven generations in the field. This mutation was identified in a small number of susceptible and resistant field populations, but not in all resistant populations, indicating that there must be other mechanisms capable of conferring resistance to amitraz. In the first generation (i.e., the first hybrid generation) of susceptible and resistant ticks, genotyping of the putative amitraz resistance-conferring SNP and the previously identified *para*-sodium channel gene SNP showed no evidence of different contributions from the susceptible and resistant strains. We take this result to suggest that there was no detectable difference in the ability of the two strains of ticks to produce progeny, and hence no evidence of a lack of fitness in the resistant genotypes. However, over the entire study period there was some evidence of loss of resistance to amitraz in populations of ticks on the farmlets where cattle were treated with spinosad. There was no evidence of assortative mating of ticks according to their acaricide-resistance genotype.

Three distinct classes of octopamine receptor have been identified in arthropods. The first of these, α-adrenergic-like octopamine receptors (αAORs), shows the greatest structural similarity to vertebrate α-adrenergic receptors. This similarity is reflected by their pharmacology in that they are likely to mediate their effects via elevations in intracellular calcium concentrations (18). A putative αAOR (*Rmα2AOR*) has been isolated in the cattle tick (9). The second group includes the octopamine/tyramine receptors, which are preferentially activated by tyramine over octopamine in most cases (19, 20). The first octopamine-like receptor isolated in the cattle tick (7) was recently shown to group with the octopamine/tyramine receptors on phylogenetic analysis of insect and arachnid octopamine receptors (9). The third group includes β-adrenergic-like octopamine receptors (βAOR), which show the greatest structural similarity to vertebrate β-adrenergic receptors. These receptors are preferentially activated by octopamine rather than tyramine, causing increases in intracellular cAMP concentration rather than calcium (21). Because amitraz is an octopaminergic agonist that interacts with octopamine receptors to cause increases in cAMP levels, it should be expected that the most likely target for amitraz would be a βAOR and it is therefore unsurprising that the octopamine/tyramine receptor isolated by Baxter and Barker (7) failed to show any sequence difference between susceptible and resistant tick strains. At the putative βAOR gene (*RmβAOR*) recently isolated in the cattle tick (9), a nonsynonymous SNP (A181T; *I61F*), situated in the first transmembrane domain (TM1), distinguished the amitraz-susceptible (NRFS) strain and an amitraz-resistant strain (Ultimo). Isoleucine at this codon is highly conserved in βAORs across a diverse range of species, suggesting that a mutation at this site might be functionally important, despite both amino acids being neutral and nonpolar. The functional role of this mutation in conferring resistance was supported by the strong positive correlation identified over five time points in a 2-y period between the frequency of the homozygous *I61F* genotype and amitraz resistance determined by LPT, with strong negative correlation identified between resistance and the heterozygous and homozygous susceptible genotypes. Furthermore, increasing frequency of the putative resistance-conferring mutation *I61F* over generations of selection with amitraz contrasted with decreasing frequency of the mutation on the farmlets in which amitraz was not used. Heterozygote deficiency in the majority of amitraz-treated populations provided further indications of selection acting on this gene. Taken together, these findings provide strong circumstantial support that the *I61F* SNP might contribute to resistance or is closely associated with a gene that confers the mechanism for amitraz resistance, and that resistance is recessively inherited. Functional studies and evaluation of a broader range of field populations of resistant and susceptible ticks are necessary to confirm this theory. The existing observations on field populations reported here are consistent with an association of the *I61F* SNP with resistance to amitraz but clearly show that there must also be other mechanisms of resistance, as the same SNP was not seen in resistant populations from other regions.

The presence of a fitness cost associated with acaricide resistance will theoretically reduce the survival or reproduction of resistant ticks and limit the spread of resistance. In the absence of treatment with amitraz, a fitness cost would be expected to reduce the survival and reproduction of Ultimo genotype ticks. However, in this study, we noted that there was no difference between strains in reproductive performance in the field over a single generation and in the absence of selection with any acaricides. Observations on the frequency of the cypermethrin resistance-associated SNP (*BmNa L64I*) showed no loss of heterozygotes or *BmNa L64I* homozygotes among the surviving progeny of the admixed population in generation 1. Although this is not the SNP leading to amitraz resistance, it indicates the "amitraz resistance-type" (at least in the first generation, before substantial impact of recombination or chromosome sorting). A similar effect was seen in the Bayesian clustering of microsatellite markers, presumed to be spread across the genome. In later generations, particularly by Winter 2005, a fitness cost to amitraz resistance became evident, as documented in more detail elsewhere (14). This result was shown by a substantial loss in phenotypic amitraz resistance in the spinosad-treated groups and in the rotation groups. The rotation groups had received amitraz treatment but the loss of phenotypic resistance suggested there was insufficient selection pressure to overcome the fitness cost. The absence of a detectable fitness cost at generation 1—but its stronger presence in later generations, especially in Winter 2005—could be because of the interplay between fitness cost and environmental conditions. Such interaction has been found in mutant mosquitos, *Culex pipiens*, bearing the resistance gene Ace^R, in which survival costs in these insects during winter were estimated to be in the order of 70% (22). In *Drosophila melanogaster*, mutations in the *para* sodium channel gene have been shown to cause cold-induced paralysis (23). In our study cold weather conditions were encountered between the Winter 2004 and Winter 2005 collection time points. Alternative explanations for loss of fitness in amitraz-resistant ticks in the absence of amitraz treatment would be either multiple mutations, leading to amitraz resistance, or a combination of resistance and compensatory mutations. Compensatory mutations, if present, would ameliorate the fitness cost of resistance but the process of introgression following gene flow may, over time, uncouple a resistance-compensatory mutation set that is not in genetic linkage. In the absence of strong selection pressure, the maintenance of a set of mutations would be difficult because of recombination and chromosome sorting during meiosis. Mutants with compensatory mutations are unlikely to arise unless resistance is common (12), which would lead to the low fitness of ticks with the amitraz-resistance mutation but without the compensatory mutations, or to ticks with an incomplete set of mutations leading to resistance. However, there was no indication that "hybrid" ticks in generation 1 showed reduced fitness, which might be expected if there was a simple separation of resistance and compensatory mutations on different chromosomes. In neither the amitraz nor rotation replicates was there a consistent relative increase in the microsatellite cluster that predominates in the Ultimo strain, which may be indicative of the resistance genotype. Nor was there a preference for the NRFS type over the Ultimo type seen for spinosad (nonamitraz) -treated populations, which may suggest that the resistance mutation has been inherited independently to the Ultimo genomic background by Winter 2004 (7 mo later)

because of chromosomal mixing in meiosis or recombination. Furthermore, the overall mixing of genomes, coupled with genetic drift, is evident in the finding of a third Bayesian cluster in the analysis of the microsatellite genotypes.

It was surprising that the NRFS and Ultimo strains showed relatively high levels of diversity, despite being repeatedly inbred for several years under laboratory conditions. The heterozygosity levels of these strains were only slightly lower than those (H_e 0.75–0.78) found in cattle tick populations in field samples from Queensland in a previous study (15). Although maintaining tick strains in culture, with the expected founder effects, bottlenecks, and drift generally associated with small population size is expected to lead to a loss of diversity, high levels of genetic diversity have been maintained in these populations. NRFS is reared without acaricide selection, whereas Ultimo is occasionally selected with amitraz 0.05% whenever resistance wanes. Selection with a synthetic pyrethroid has not been considered necessary because the strain has shown consistently high levels of resistance to SP. This reduction of in vitro estimates of amitraz resistance in the Ultimo strain provides further empirical evidence of a fitness cost associated with resistance in the absence of selection.

Materials and Methods

Field Study. Ticks were sourced from a 3-y field study investigating the management strategies for controlling amitraz resistance using amitraz and spinosad, commencing in 2003 at the University of Queensland Pinjarra Hills Campus (14). Detailed information on field study and the use of acaricides is provided in the *Supporting Information*. The study was conducted with the approval of the University of Queensland Production and Companion Animals Animal Ethics Committee.

Tick Sampling. DNA was extracted from ticks collected for genetic analyses at three time points from spring–summer 2003 through to winter 2005. Additional samples were used to examine the time course of changes in the frequency of the *RmβAOR I61F* SNP. Engorged females were collected and as many larvae as required for the bioassay were assayed using the LPT for acaricide resistance (24). The LPT was applied using discriminating concentrations of analytical grade cypermethrin (0.3%, 24 h incubation) and amitraz (0.5%, 48 h incubation) (14). Larvae that were not required for the bioassay were frozen at −20 °C and DNA extracted. Hence, DNA was extracted from tick larval progeny from the same sample of females as the larvae submitted to the LPT bioassay. The larvae used for DNA extraction were not treated with acaricide. On several occasions there were no surplus tick larvae and it was not possible to genotype larvae from all collection times for all farmlets. This was a more common occurrence in the last year and a half of the study (2005 and 2006) when the farm was affected by drought conditions, resulting in a reduction of the number of animals in each paddock to two or three, with consequent lower levels of infestation and fewer ticks for the bioassay. Genomic DNA was extracted from 30 larvae from all treatment groups and time points, using the procedure described by Cutullé et al. (15).

β-Adrenergic octopamine receptor (*RmβAOR*) SNP *I61F*. ***Identification of amitraz-resistance mutation.*** The βAOR was sequenced using gDNA prepared from a pool of 30 larvae for each of the NRFS and the Ultimo strains using the method described by Corley et al. (9). PCR primers (F1 5′-AGTGCCCTC-TAGGCGATACA-3′ and R1 5′- TGTTGCTACGGTTGGCTATG-3′), designed using Primer3 software (24), were used to amplify a 1,644-bp fragment containing the entire coding sequence, which is a single exon. PCR amplification was performed in a 25-μL reaction volume containing; 0.2 μM each primer, 2 μL genomic DNA, 1× KAPA2G Buffer B, 1× KAPA Enhancer 1, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.05 U μL KAPA2G Robust DNA Polymerase. PCR conditions were: initial denaturation 95 °C for 2 min, followed by 30 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 2 min, followed by a final extension of 72 °C for 7 min. Unused dNTPs and primers were removed from the PCR product using Exosap-it (USB). Sequencing was performed using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit Version 3.1 (PE Applied Biosystems) and separation was performed on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems). Forward and reverse sequences were aligned and edited using ChromasPro (Technelysium).

***Genotyping of identified SNP* I61F.** Following identification of the SNP *I61F*, a 183-bp fragment containing the SNP was amplified using primers F2 FAM-5′-GAAATCTGACGGACGAGGAA-3′ and R2 5′-GCGACACGATGAAGTAGTTG-3′. Genomic DNA was amplified in a 10-μL reaction volume using conditions described above. Thirty individual larvae were genotyped at each of five time points for which sufficient larvae were available. Genotyping was carried out by digesting the fluorescently labeled PCR product with the restriction endonuclease *BCCI* (New England Biolabs) using 2.5 U of enzyme, 1× NE Buffer 1 supplemented with 100 μg/mL BSA at 37 °C, overnight. The digested product was separated on an ABI 3130xl Genetic Analyzer. The *I61F* (R allele) remained undigested (183-bp product), whereas the S allele was digested into two products (93 bp and 90 bp). Because only the forward primer was fluorescently labeled, only the 183-bp and 93-bp products were fluorescently labeled and could be visualized.

Association of *RmβAOR* SNP *I61F* with Resistance. Based on observed fluctuations in the numbers of ticks counted each week on experimental animals (14), each collection of ticks for LPT was assigned to a putative generation from 0 (ticks applied in the artificial infestation) to 7 (collection made in Winter 2005). The effects of treatment*generation (0–7), replicate (1, 2) and genotype (*RmβAOR* SNP *I61F*) on the percentage resistance calculated by LPT were tested using the mixed procedure of SAS (v9.2, SAS Institute), using restricted maximum-likelihood as the parameter estimation method. Hardy–Weinberg exact tests for heterozygote deficiency and excess were conducted using Genepop 4.0.1 (25, 26). A metaanalysis of the Hardy–Weinberg exact tests was undertaken. When a large number of comparisons are made, low probabilities may arise by chance alone. We carried out a metaanalysis of probabilities (27) to determine whether the low probabilities were meaningful. The test statistic $-2 \Sigma \ln(p)$ is distributed as χ^2 with twice as many degrees-of-freedom as probabilities tested.

Field Samples for *RmβAOR* SNP *I61F*. To confirm that the SNP identified in this study exists in populations more broadly, field samples of tick larvae submitted to the Queensland government for acaricide-resistance testing by the LPT were screened using the sequencing and genotyping protocols described above. Samples obtained from four regions were surveyed: north Queensland, central Queensland, southeast Queensland, and northern New South Wales.

Genotyping of *para*-Sodium Channel Gene SNP *BmNa L64I*. Thirty larvae from each replicate farmlet were genotyped for *BmNa L64I* on each of the three time points: generation 1, Winter 2004, and Winter 2005. The genotype assay was a dual-probe quantitative PCR diagnostic assay described in Morgan et al. (16). Excepting the addition of 4% (vol/vol) DMSO to the PCR, concentrations of primers and Taqman MGB probes, and amplification conditions were the same as described previously. Note that the North American mutation in the *para*-sodium channel gene (28) has never been identified in Australia (3).

Microsatellites. Thirty larvae from each replicate farmlet were genotyped for the microsatellite markers on each of the three time points: generation 1, Winter 2004, and Winter 2005. Seven microsatellites (BmCO7, BmD10, BmM1, BmCO3, BmA12, BmCC1, and RJM451) (15) were genotyped in all samples. Except for BmM1, which was directly labeled, one primer of each pair was labeled with fluorescent dyes using an M13-tailing process (29). PCR reactions used the Qiagen Multiplex PCR kit in a 10-μL final volume with 0.03–0.2 μM of each M13-tailed forward primer and fluorescently labeled M13 primer and 0.08–0.4 μM of the nontailed reverse primer. PCR conditions contained an initial denaturation step of 95 °C for 15 min, 40 cycles of 94 °C for 30 s, 60 °C for 30 s (or a 58 °C to 48 °C touchdown for BmCO3, BmA12, and RJM451) and 72 °C for 1 min 30 s, followed by final extension of 72 °C. Microsatellite alleles were sized by capillary electrophoresis (ABI 3130xl Genetic Analyzer, Applied Biosystems).

Analysis of Microsatellite Data. Each treatment group, replicate, and time point was analyzed independently as a "population." Following procedures adopted in a previous analysis using tick larvae (15), we removed clusters of highly related individuals within populations, which might represent siblings or half-siblings from single engorged female ticks. To do this, we performed a Bayesian clustering analysis using the program STRUCTURE (30), as described below, on each population and calculated pairwise relatedness values using the Queller and Goodnight (31) estimator in GENALEX 6 software (32). Clusters with average relatedness greater than 0.25 were removed from the analysis. Final sample numbers for each population ranged from 20 to 30 and are shown in Table S1. Population genetic parameters including allele frequencies for each population by locus, observed and unbiased expected heterozygosity (H_o and H_e) were calculated using the Excel Microsatellite Toolkit v3.1 (33). The number of alleles per population corrected for sample

size (allelic richness) was estimated using FSTAT v2.9.3.2 (34). HWE was tested using Genepop [http://genepop.curtin.edu.au/genepop_op1.html (35)]. Recent reductions in effective population size were detected by departures of heterozygosity from the mutation-drift equilibrium using BOTTLENECK v1.2.02 (36) and an infinite allele model, with significance tested by a Wilcoxon's signed rank test. Population structure was determined by both traditional *F* statistics and Bayesian clustering techniques. Wright's *F* statistics (F_{ST}) using the method of Weir and Cockerham (37) and F_{IS} values were computed in GENETIX v4.05.2 (38). Without ascribing populations a priori, population structure was detected using the Bayesian clustering method implemented in STRUCTURE 2.01 (30) with a discarded burn-in and Markov Chain Monte Carlo chain of 120,000 permutations each. The number of clusters (*k*) was determined from 20 iterations and ΔK method of Evanno et al. (39). A hierarchical analysis of molecular variance (AMOVA) was performed using ARLEQUIN v3.11 (40) to examine the partitioning of genetic variation within and among time points and treatments.

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