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Evidence of Permethrin Resistance and Fipronil Tolerance in *Rhipicephalus sanguineus* s.l. (Acari: Ixodidae) Populations From Florida and California

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Abstract

Rhipicephalus sanguineus s.l. (Latreille), is a vector of multiple disease-causing pathogens to humans and dogs. Permethrin and fipronil are two acaricides used to manage *R. sanguineus* s.l. infestations. Homeowners have reported treatment failures in managing brown dog ticks using permethrin and fipronil based products. Previous studies demonstrated that high permethrin resistance in some *R. sanguineus* s.l. populations was due to metabolic detoxification and target site insensitivity. In this study, three *R. sanguineus* s.l. strains, one from a laboratory colony (NC) and two colonies originally collected from Florida (FL) and California (CA), were evaluated for resistance expression against permethrin and fipronil. Metabolic detoxification mechanisms were evaluated in the FL strain using three synergists, while a polymerase chain reaction assay was used to detect a resistance mutation in all strains. The NC strain was susceptible to both permethrin and fipronil, while both the FL and CA strains exhibited high resistance to permethrin and tolerance to fipronil. The synergist tests and PCR results indicated that the FL strain utilized both metabolic resistance and target site insensitivity against permethrin, while the CA strain was documented to have the target-site insensitivity resistant allele. This study confirmed permethrin resistance in both California and Florida populations and its persistence in Florida populations, although its susceptibility can potentially be increased by adding a synergist. Fipronil resistance was not detected suggesting this acaricide may provide suitable tick control.

Key words: brown dog tick, resistance, metabolic, sodium channel mutation, synergist

Rhipicephalus sanguineus s.l. (Latreille) is a complex of three-host tick species that primarily feeds on dogs (Dantas-Torres 2008) and has been documented as a vector of multiple disease-causing pathogens in both humans and other animals (Groves et al. 1975, Demma et al. 2005, Chao et al. 2017). Effective management of *R. sanguineus* s.l. involves multiple control methods, but primarily relies on the utilization of chemical acaricides as both on-host and off-host treatments (Dantas-Torres and Figueredo 2006). Permethrin and fipronil are two common active ingredients used in multiple commercial products that target ticks (Otranto et al. 2005, Roma et al. 2009).

Permethrin acts on the voltage-gated sodium channel in insects (Field et al. 2017) and its extensive use to manage multiple indoor pests may have aided in the development of high levels of resistance in some *R. sanguineus* s.l. populations. Permethrin resistance has been reported in many *R. sanguineus* s.l. populations from the U.S. (Eiden et al. 2015, Tucker et al. 2021), Panama (Miller et al. 2001), and the Caribbean (Tucker et al. 2021). Fipronil blocks both γ-aminobutyric acid (GABA)-gated chloride channels and glutamate-gated chloride channels, causing neuroexcitation (Zhao et al. 2004). The susceptibility to fipronil in some *R. sanguineus* s.l. populations has decreased (Eiden et al. 2015, Becker et al. 2019) and fipronil resistance has been reported in other tick species, such as *R. (Boophilus) microplus* Canestrini (Acari: Ixodidae) from Brazil (Castro-Janer et al. 2010) and Mexico (Miller et al. 2013).

Two common resistance mechanisms detected in ticks are target site insensitivity and metabolic detoxification (Guerrero et

al. 2014), both of which have been detected in *R. sanguineus* s.l. populations (Eiden et al. 2017, Tucker et al. 2017). In *R. sanguineus* s.l. populations, a single point mutation affecting the domain III, S6 region of the sodium channel (Klafke et al. 2017) was detected at a high rate in permethrin-resistant populations collected from Florida and Texas, but not in susceptible populations (Tucker et al. 2017). Amrutha et al. (2021) reported that a single point mutation in the domain II, S3-4 linker gene region as well as the aforementioned S6 mutation were associated with deltamethrin resistance. In a fipronil-resistant *R.* (*B.*) *microplus* population, a mutation affecting the GABA-gated chloride channel was detected (Janer et al. 2019). In addition to target site insensitivity, metabolic detoxification has contributed to acaricide resistance as well. Increased esterase activity has been documented as the major metabolic detoxification mechanism in some permethrin-resistant tick populations, but not in fipronil-tolerant tick populations (Miller et al. 2001, Eiden et al. 2017). However, it has been suggested as a contributing factor to fipronil resistance in other tick and insect species (Tang et al. 2010, Gondhalekar and Scharf 2012, Miller et al. 2013).

In this study, three *R. sanguineus* s.l. strains were examined for their susceptibility to permethrin and fipronil, followed by synergist bioassays and polymerase chain reactions (PCR) to identify potential metabolic detoxification mechanisms and the domain III S6 target site mutations, respectively. The results from this study provide updated information about the development of acaricide resistance in populations of *R. sanguineus* s. l. from Florida and California.

Materials and Methods

Ticks

Three *R. sanguineus* s.l. strains (Tian et al. 2022) were evaluated for acaricide susceptibility in this study. A long-standing laboratory strain from North Carolina (NC) was obtained from Ecto Services, Inc. (Henderson, NC). It was used as an acaricide-susceptible strain in earlier studies (Eiden et al. 2015). Several hundred adult ticks collected from dogs are introduced to this colony annually. The CA strain was obtained from a colony reared at the Centers for Disease Control and Prevention (Atlanta, GA), which was originally collected in California in 2017. The FL strain was collected from a residence at Port St. Lucie, Florida in 2018 and colonized in the Veterinary Entomology Laboratory at the University of Florida. These three *R. sanguineus* strains are referred to as NC, CA, and FL in the following text, respectively. Ticks were held at standard conditions: 25 ± 1°C, 92% relative humidity, and 12:12 light cycle (Eiden et al. 2015).

Bioassay

The larval packet test was used to evaluate the susceptibility of each *R. sanguineus* strain against permethrin and fipronil, as described in Eiden et al. (2015). Technical grade permethrin (98.8%, cis: trans, 40.1:58.7; ChemService Inc., West Chester, PA) and fipronil (98.3%; ChemService Inc., West Chester, PA) were used to prepare the treatment solutions. The highest concentrations utilized ranged from 0.3 to 30.0% (W/V) for permethrin and 0.1 to 0.4% (W/V) for fipronil (Supp Table 1 [online only]). Tick mortality was examined after a 24 hr exposure. A tick was defined as dead when it no longer exhibited movement after being exposed to human exhaled breath twice and probed twice using a watercolor brush. Ticks were frozen at –80°C after counting.

Synergist Bioassay

Metabolic detoxification mechanisms were evaluated for the NC and FL strains using three synergists paired with permethrin: diethyl maleate (DEM) (ChemService, Inc., West Chester, PA); piperonyl butoxide (PBO) (Sigma-Aldrich Corp., St Louis, MO); and triphenyl phosphate (TPP) (ChemService, Inc., West Chester, PA), as described in Eiden et al. (2017). The CA strain was lost during the course of this research, so we were unable to include this population. The same larval packet test protocol was used in the synergist bioassays with the strain-specific permethrin concentrations (0.025–0.3% for NC strain, 1.875–30% for FL strain) and with the following modifications: 1) A synergist was added in both the highest concentration solution and the diluent solutions at a constant rate of 2% before serial dilution, thus maintaining a 2% synergist concentration in all dilutions; 2) Diluent with synergist was used as a control solution in addition to the diluent without synergist.

Polymerase Chain Reaction (PCR)

An allele-specific PCR assay was used to detect the domain III, S6 sodium channel mutation (Klafke et al. 2017) in all three *R. sanguineus* s.l. strains using the DNA extraction and PCR assay methods described by Tucker et al. (2017). One modification to the DNA extraction protocol was the addition of 25 mM NaOH or 100 mM tris-HCl to the DNA isolation buffer to adjust the pH to 8.3. For each tick, two PCRs were used to detect presence of the susceptible or resistant allele using the RSSC-SUS-F/BDT-227 or RSSC-RES-F/BDT-227 primer combinations, respectively (Tucker et al. 2017).

Statistics

Lethal concentrations at 50 and 90% for each strain were estimated using PoloPlus (LeOra Software Company, Petaluma, CA). The susceptibility of tick strains to an acaricide was considered significantly different if the 95% confidence intervals of the LC values did not overlap (Eiden et al. 2015). The resistance ratio (RR) was calculated by dividing the LC_x of each strain by the LC_x of the most susceptible strain to determine the RR_x for that strain. Tolerant and resistant classifications were defined with RR ≥ 2 and < 10, and RR ≥ 10, respectively (Gondhalekar et al. 2011). The effect of a synergist was examined by comparing the mean mortality between acaricide with and without synergist using a nonparametric Kruskal-Wallis test one-way analysis of variance, followed by a Dunn's test. For each strain, the proportion of resistant alleles was calculated by dividing the sum of the resistant alleles by the total number of alleles in the sample, which was the total number of ticks tested multiplied by two (Tucker et al. 2017).

Results

Toxicity of permethrin to the NC, FL, and CA strains is summarized in Table 1. The FL strain LC_{50} and LC_{90} values could not be calculated due to low mortality (10.64%) at the highest concentration (30%) tested. To estimate a lower limit, we used 30% as the LC_{50} to calculate the RR_{50} of the FL strain, resulting in an RR_{50} >379. The CA strain had a permethrin LC_{50} value of 1.795 and an LC_{90} value of 4.425, which generated an RR_{50} of 22.72 and an RR_{90} of 23.04. There were significant differences between these strains for both lethal concentration levels indicated by nonoverlapping 95% CIs. Toxicity of fipronil to the NC, FL, and CA strains is summarized in Table 2. The 95% CI of FL and CA strains overlapped for both LC_{50} and LC_{90}. Confidence intervals of both strains did not overlap with the NC strain.

In the NC and FL strains (Fig. 1a and b), the addition of DEM did not significantly increase larval mortality at any concentration. The addition of PBO significantly increased permethrin toxicity to *R.*

Table 1. Lethal concentration (%) and resistance ratio of permethrin for three *Rhipicephalus sanguineus* s.l. strains

Strain	*n*	Slope (SE)	LC_{50} (95% CI)	LC_{90} (95% CI)	RR_{50}	RR_{90}
NC	10,106	3.325 (0.061)	0.079 (0.073–0.085)	0.192 (0.170–0.221)	1.0	1.0
FL	5,837	ND[a]	>30[a]	ND[b]	>379	–
CA	6,458	3.270 (0.082)	1.795 (1.504–2.106)	4.425 (3.596–5.960)	22.72	23.04

n is individual ticks tested in all bioassays: 4 bioassays for NC, 2 for FL, and 3 for CA.

Lethal concentration (LC) values represent the percentage of active ingredient.

RR_x: resistance ratio = LC_x of strain X/ LC_x of NC strain.

NC: tick strain obtained from Ecto Services Inc. and was used as susceptible strain; FL: tick strain collected from Port St Lucie, FL; CA: tick strain collected from Imperial Co., California.

[a]Low mortality (<50%) at 30% active ingredient.

[b]ND = Not determined due to the low mortality at the highest permethrin concentration.

Table 2. Lethal concentration (%) and resistance ratio of fipronil for three *Rhipicephalus sanguineus* s.l. strains

Strain	*n*	Slope (SE)	LC_{50} (95% CI)	LC_{90} (95% CI)	RR_{50}	RR_{90}
NC	11,264	4.277 (0.086)	0.021 (0.019–0.024)	0.043 (0.037–0.051)	1.0	1.0
FL	14,981	5.162 (0.086)	0.042 (0.037–0.047)	0.075 (0.066–0.082)	2.00	1.74
CA	3,260	8.181 (0.347)	0.046 (0.043–0.049)	0.066 (0.061–0.072)	2.19	1.53

n is individual ticks tested in all bioassays: 4 bioassays for NC, 6 for FL, and 1 for CA.

Lethal concentration (LC) values represent the percentage of active ingredient.

RR_x: resistance ratio = LC_x of strain X/ LC_x of NC strain.

NC: tick strain obtained from Ecto Services Inc. and was used as susceptible strain; FL: tick strain collected from Port St Lucie, FL; CA: tick strain collected from Imperial Co., California.

sanguineus s.l. larvae, however, 100% mortality was only achieved in the NC strain at the highest concentration (0.3%). For both NC and FL strains, larval mortality was significantly increased by adding TPP to permethrin and reached 100% at nearly all concentrations.

Genotype and allele frequencies of the sodium channel domain III, S6 mutation for the NC, FL, and CA strains are summarized in Table 3. Although highly resistant, the CA strain had only 5% Res/Res and 25% Sus/Res. The resistant allele was detected in the susceptible NC strain as 35% Sus/Res, while the FL strain showed 100% Res/Res.

Discussion

Permethrin resistance in *R. sanguineus* s.l. from North America and the Caribbean has been variable over populations (Miller et al. 2001, Eiden et al. 2015, Tucker et al. 2021). Permethrin resistance in the FL strain reported in this study is consistent with Eiden et al. (2015) using the same susceptible NC strain for RR calculation. To our knowledge, this is the first report of permethrin resistance in a California-origin population. The FL strain RR_{50} was at least 17 times higher than the RR_{50} of the CA strain, which is likely a result of acaricide exposure history. The recent heavy use of acaricides before our collection of the FL strain likely contributed to the selection pressure on this strain. The CA strain had not been exposed to any acaricide during the two-year laboratory rearing between the original collection and the acquisition of ticks for this study. Acaricide exposure history of the CA strain prior to collection is unknown, but given the comparably lower resistance of this strain it is likely that selection pressure was lower. The NC strain has been maintained in the laboratory without exposure to acaricides, which has facilitated its high susceptibility to permethrin; however, the consistent addition of field-collected ticks to this strain after initial use in 2012 by Eiden et al. (2015) may have inadvertently shifted the genetic make-up of this tick strain. In the current study, the resistant allele was detected at a 0.175 frequency in the NC population, but was not detected in Tucker et al. (2017) where all 20 sampled larvae were homozygous susceptible, using tick samples preserved from Eiden et al. (2015). The three-fold LC_{50} increase of the NC strain (LC_{50} = 0.027 in Eiden et al. (2015) to the current LC_{50}=0.079) would shift the RRs lower for the FL and CA strain in the current study. However, the RR values of the FL and CA strain were much higher than 10, indicating high resistance in both FL and CA strains. For comparison, should the Eiden et al. (2015) susceptible LC_{50} value be applied to the FL and CA strains, these RR's would be over 1,000 and 66, respectively. The LC_{50} concentration estimate for the NC strain in Eiden et al. (2015) was done in 2012, and preserved tick samples from that time were used in Tucker et al. (2017), who reported 100% Sus/Sus genotypes. The work reported here was conducted in 2018, thus the introduction of resistance alleles likely occurred in one or more of the five colony addition events between 2012 and 2018, as reported by Ecto Services.

The FL and CA strains were categorized as tolerant to fipronil, which is consistent with other *R. sanguineus* s.l. strains collected from Florida (Eiden et al. 2015). However, an *R. sanguineus* s.l. strain from Brazil was reported with RR_{50} >10 (Becker et al. 2019), suggesting resistance to fipronil is possible in this tick. In this study, both the FL and CA strains were more susceptible to fipronil than permethrin, which could be due to the different label uses of permethrin (on- and off-host, indoor, outdoor) and fipronil (on-host, outdoor) as well as the dual mode of action of fipronil (Zhao et al. 2004).

The synergist results suggested that increased general esterase and to a lesser extent, cytochrome P450 activities were the major metabolic resistance mechanisms used by the FL strain, while GST had little involvement in permethrin resistance expression. These results support the findings in Eiden et al. (2015). However, cytochrome P450 activity may not be a major mechanism in every *R.*

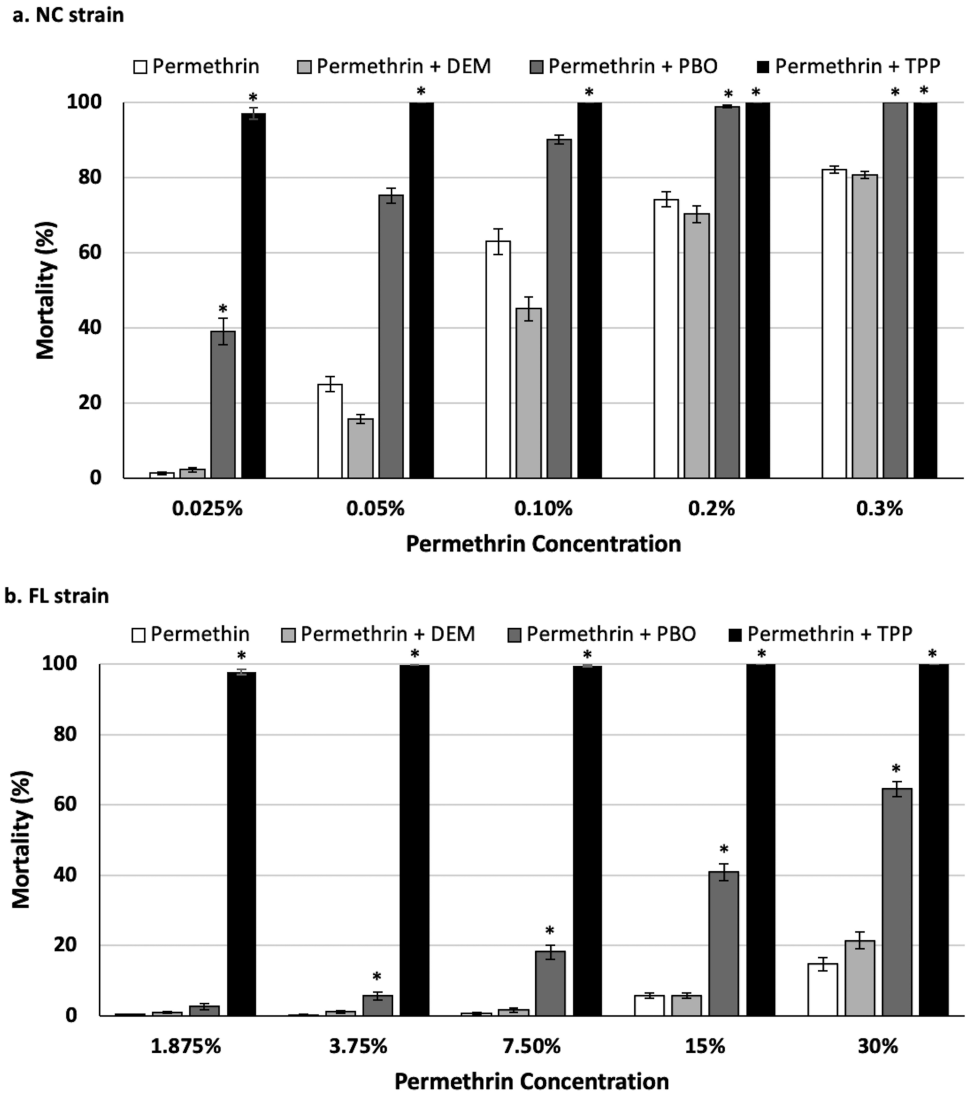

Fig. 1. The mean percent (±SE) mortality in *Rhipicephalus sanguineus* s.l. strains with and without the inclusion of one of three synergists added at a constant 2% synergist across five permethrin concentrations (Supp Table 2 [online only]). a. susceptible NC strain; b. permethrin-resistant FL strain. Note different concentrations used for the two strains. * Indicates significant difference ($\alpha = 0.05$) in tick mortality between permethrin alone and permethrin with synergist within a specific concentration.

Table 3. The genotype and resistant allele proportion of three *Rhipicephalus sanguineus* s.l. strains to permethrin

Strain	Sample size	Genotype			Resistant allele proportion[a]
		Sus/Sus	Sus/Res	Res/Res	
CA	20	14	5	1	0.175
NC	20	13	7	0	0.175
FL	20	0	0	20	1.00

[a]Resistant allele proportion = number of allele Res/ total number of alleles.

sanguineus s.l. population, as documented by Miller et al. (2001). Besides metabolic detoxification, the reported domain III, S6 sodium channel mutation was detected in all three strains, albeit at very different frequencies (Table 3). This indicates that both metabolic detoxification and target site insensitivity are used by the FL strain to enhance permethrin resistance. The CA strain harbors the domain III, S6 resistant allele, but because the strain was lost it is unclear what contribution metabolic resistance may have made to the observed phenotypic resistance.

This study identified the presence and levels of permethrin resistance and fipronil tolerance in two *R. sanguineus* s.l. field-collected populations, which support the results from Eiden et al. (2015). However, the susceptible strain used in this study had a slightly increased LC_{50} for permethrin and an increased ratio of Sus/Res genotypes, which was likely due to the introduction of field-collected ticks to this colony and should serve as a warning in longitudinal studies. There is strong evidence for metabolic detoxification as well as target site insensitivity in the permethrin-resistant

FL population and this result is consistent with Eiden et al. (2017) and Tucker et al. (2017). Although esterases and cytochrome P450s play a major role in permethrin resistance, only PBO has been commercialized to use with pyrethroids (Guerrero et al. 2014) due to the inherent challenges in mammals if general esterases were blocked. Studies examining the contribution of each resistance mechanism can further our understanding of tick resistance evolution and provide insight to novel control strategies targeting these mechanisms. The monitoring and prevention of fipronil resistance are critical as previous studies indicated that it is possible for *R. sanguineus* s.l. populations to develop resistance, although the results from this study do not indicate increases in resistance in Florida populations.

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Author Contributions

YT was the lead author and primarily designed and performed the research as well as data analysis. CET performed the polymerase chain reaction for the evaluation of sodium channel mutation and assisted on larval packet tests. PEK and CCL assisted in the experimental design, analysis, and manuscript writing. All authors have read and approved this manuscript and the authors report no conflict of interest.

Supplementary Data

Supplementary data are available at *Journal of Medical Entomology* online.

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