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ARTHROPODS AND MEDICAL ENTOMOLOGY - ORIGINAL PAPER

Impacts of long-term insecticide treatment regimes on *skdr* and *kdr* pyrethroid resistance alleles in horn fly field populations

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Abstract

We evaluated the effects of four different 6-year duration control strategies on the resistance levels and frequency of the pyrethroid target site resistance alleles, *superkdr* (*skdr*) and *kdr*, at four field populations of *Haematobia irritans irritans* (Linnaeus, 1758) (Diptera: Muscidae) in Louisiana, USA. Consecutive use of pyrethroid ear tags for 6 years caused a significant increase in the resistance ratio to pyrethroids as well as the frequencies of both *skdr* and *kdr* resistance alleles. After 3 years of consecutive use of pyrethroid ear tags, followed by 1 year with no treatment, and followed by 2 years with organophosphate ear tags, the resistance ratio for pyrethroid was not significantly affected, the %R-*skdr* significantly dropped while the %R-*kdr* allele remained relatively high and stable. Similar results were observed when pyrethroid ear tags were used for three consecutive years, followed by 1 year with no treatment, and followed by 2 years with endosulfan ear tags; however, this treatment resulted in a slight increase in the resistance ratio for pyrethroids. In a mosaic, the resistance ratio for pyrethroids showed a 2.5-fold increase but the *skdr-kdr* genetic profiles did not change, as the %R alleles (*skdr* and *kdr*) remained low and stable through the 6 years. Lack of exposure to pyrethroid insecticides for 3 years significantly affected the *skdr* mutation but not the *kdr* mutation, preventing re-establishment of susceptibility to pyrethroids. SS-SR (*skdr-kdr*) individuals were responsible for the maintenance of the *kdr* mutation in two of the populations studied, and fitness cost seems to strongly affect the SR-RR genotype. None of the four treatment regimens evaluated in the study had satisfactory results for the management of *kdr* resistance alleles.

Keywords *Haematobia irritans* · Control · Management · Fitness cost · Target site resistance

Introduction

The horn fly, *Haematobia irritans irritans* (Linnaeus, 1758) (Diptera: Muscidae), is an important economic pest of cattle worldwide. Due to its hematophagous behavior, the horn fly causes considerable irritation to animals, leading to decreased meat and milk production, which in turn leads to increased production costs as producers attempt to control horn fly populations (Kunz et al. 1991; Oyarzún et al. 2008). Horn fly control is mainly based upon application of insecticides; however, after many years of exposure, data from several countries show that horn fly populations have developed resistance to many of the commercially available products, including organochlorines (DDT), organophosphates, pyrethroids, and cyclodienes (endosulfan) (Guglielmone et al. 2002; Oyarzún et al. 2008; Oyarzún et al. 2011; Domingues et al. 2013).

The target site of pyrethroid insecticides is the sodium channel, which is a transmembrane voltage-gated protein responsible for conduction of electrical signals in neurons. Pyrethroids bind to these proteins, prolonging the opening of channels and causing repetitive nerve firing leading to paralysis and insect death (Soderlund and Kniple 2003; Dong 2007). Pyrethroid target site insensitivity, also called knockdown resistance, is caused by nucleotide substitutions leading

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to amino acid changes in the sodium channel protein. The resulting structural changes to the sodium channel prevent pyrethroid binding, which reduces the sensitivity of arthropods to these insecticides (Soderlund and Kniple 2003; Dong 2007). Guerrero et al. (1997) identified two mutations in the horn fly sodium channel gene, *superkdr* (*skdr*) and *kdr*, which were associated with pyrethroid target site resistance. The *skdr* mutation results from a nucleotide substitution that leads to a methionine to threonine change (M918T) in the S4-S5 transmembrane segment of domain II (Guerrero et al. 1997). The *kdr* mutation is a nucleotide substitution leading to a leucine to phenylalanine amino acid substitution (L1014F) in the transmembrane segment S6 of domain II. Jamroz et al. (1998) and Foil et al. (2005) confirmed that *skdr* and *kdr* mutations confer significant levels of pyrethroid resistance in horn flies. Target site insensitivity, mainly through *kdr*, also has been associated with resistance of horn fly to pyrethroids in other countries including Argentina (Guglielmone et al. 2002), Mexico (Li et al. 2003), Brazil (Guerrero and Barros 2006; Barros et al. 2013), and Chile (Oyarzún et al. 2011).

It is important to determine the influence that various insecticide treatment regimes and rotations have on resistance development. Byford et al. (1999) evaluated different control strategies including consecutive use of the same insecticide for several generations or years, mixture, rotation, and mosaic regimes under both laboratory and field conditions. The authors showed that mixtures and mosaic had the best results regarding delay of resistance development under laboratory and field conditions, respectively (Byford et al. 1999). Barros et al. (1999) evaluated yearly alternate use of pyrethroid and organophosphate ear tags and showed that 7 years of rotation did not improve pyrethroid efficacy or prevent the development of resistance to either pyrethroids or organophosphates. Oremus et al. (2006) evaluated the effect of mid-season doramectin treatments on the level of resistance to pyrethroids and the frequency of *skdr* and *kdr* in three populations of horn fly that were subjected to pyrethroid ear tag treatment during the fly season. The mid-season doramectin treatment increased the weeks of control after the treatment in one of the horn fly populations, but the increase in weeks of fly control did not persist for the remaining years of the study when only pyrethroids were used. Li et al. (2009) evaluated the use of pyrethroid pour-ons for 1 year followed by organophosphate ear tags for 4 years and reported that the use of organophosphate in a pyrethroid-resistant population provided sustainable control of the horn fly population; however, the *skdr* and *kdr* mutations persisted in the population even after the use of pyrethroids was halted and organophosphate tags were used.

Knowledge of the effect of selection pressure on the phenotypes and genotypes of resistant horn fly populations should help in the development of rational insecticide treatment regimens to control external parasites and in designing novel strategies to prevent or minimize the development of resistance in livestock pests. The aim of the present study was to evaluate the effects of different 6-year duration treatment regimes on the resistance levels and frequency of *skdr* and *kdr* mutations in four horn fly populations from the state of Louisiana, USA.

Materials and methods

Animal treatments

The study was performed at four experimental research stations in Louisiana, USA. From 2006 to 2011, cattle herds at Red River Research Station (Bossier City, LA), Macon Ridge Research Station (Winnsboro, LA), Saint Joseph Research Station (St. Joseph, LA), and Saint Gabriel (St. Gabriel, LA) were tagged according to manufacturer's recommendations in the spring, and the tags were removed in the following fall (4 to 5 months of treatment). The cattle at each of these stations were crossbred reproductive herds maintained in groups of 20–25 during the horn fly season with at least 100 animals per station. The treatment scheme is shown in Tables 1 and 2. All the cattle at the Red River Research Station (6PYR) were tagged in the spring of all 6 years with two PYthon® ear tags (10% zetacypermethrin and 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY) (Table 1). At the St. Joseph Research Station (3PYR-1No-2OP) and the Macon Ridge Research Station (3PYR-1No-2CYC), the animals were tagged with PYthon® ear tags in 2006, 2007, and 2008. In 2009, these animals received no treatment to control horn fly, and in 2010 and 2011, the animals at the St. Joseph Research Station received one Warrior® ear tag (30% diazinon, 10% chlorpyrifos, Y-Tex Corporation, Cody, WY), while the animals at the Macon Ridge Research Station were treated with one Avenger® ear tag (30% endosulfan, KMG Chemicals, Houston, TX) (Table 1). From 2006 to 2011, a mosaic (different treatments for different groups of cattle) was used at Saint Gabriel Research Station (Mosaic) (Table 2). Each year, at least one group of 20–25 animals was maintained without any treatment, and at least 40 cows were treated with either Ivomec® (0.5% *w*/*v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w*/*v* eprinomectin, Merial, Duluth, GA) pour on as their fly control measure. In 2006, different groups were treated with either Warrior® ear tags, Vapona® spray (1% dichlorvos, PBU Gordon Corp, Kansas City, MO), or PYthon® ear tags. In 2007, treatments included Patriot® ear tags (40% diazinon, Bayer AG, Leverkusen, Germany) and Guard Star plus® ear tags (10% permethrin, Y-Tex Corporation, Cody, WY). In 2008, 2009, and 2010, treatments included Patriot®, Guard Star plus®, and XP 820® ear tags (8% Abamectin, 20%

Table 1 History of insecticide use for horn fly control at four experimental research stations in Louisiana, USA, from 2006 to 2011

Year	Treatments			
	Red River (6PYR)	Saint Joseph (3PYR-1No-2OP)	Macon Ridge (3PYR-1No-2CYC)	Saint Gabriel (Mosaic)
2006	Pyrethroid[a]	Pyrethroid	Pyrethroid	Mosaic[e]
2007	Pyrethroid	Pyrethroid	Pyrethroid	Mosaic
2008	Pyrethroid	Pyrethroid	Pyrethroid	Mosaic
2009	Pyrethroid	None[b]	None	Mosaic
2010	Pyrethroid	Organophosphate[c]	Cyclodiene[d]	Mosaic
2011	Pyrethroid	Organophosphate	Cyclodiene	Mosaic

[a] PYthon® ear tags (10% zetacypermethrin and 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)

[b] No insecticide was used

[c] Warrior® ear tags (30% diazinon, 10% chlorpyrifos, Y-Tex Corporation, Cody, WY)

[d] Avenger® ear tag (30% endosulfan, KMG Chemicals, Houston, TX)

[e] See Table 2 for full description of each treatment group for each year

piperonyl butoxide, Y-Tex Corporation, Cody, WY). In 2011, treatments included Warrior®, PYthon®, Guard Star plus®, and Avenger® ear tags (Table 2).

Bioassays

Flies were collected for bioassays and subsequent genomic analysis before treatment in the spring (PRE), and approximately 2 weeks after the tags were removed in the following fall (POST). At each research station, horn flies were collected from the dorsal region of the cattle using an aerial hand net and tested for susceptibility to permethrin using the impregnated filter paper method (Sheppard and Hinkle 1987). Technical grade permethrin (FMC, Philadelphia, PA) was used to make stock solutions for 2-fold serial dilutions in pesticide grade acetone of 10–11 concentrations ranging from 0.2 to 400 μg/cm^2. Three replicates were used for each insecticide treatment. Fly mortality was determined after a 4-h exposure period with flies unable to walk considered dead. A subsample of the collected flies was stored at − 80 °C for later genomic testing using PCR.

Detection of *superkdr* and *kdr*

To determine the frequency of the *skdr* and *kdr* mutant alleles in the different horn fly populations, genomic DNA was isolated from individual frozen adult flies (20 males and 20 females) from each of the 48 collections (pre- and post-treatment, 4 locations, 6 years) by an adaptation of a method used for DNA purification from *D. melanogaster* (Czank 1996). Briefly, individual flies were pulverized using microtube pestles in 1.5-ml centrifuge tubes prechilled on dry ice. Twenty-five microliters of PCR buffer II (Applied Biosystems Inc., Foster City, CA) were added to each tube and grinding continued for approximately 20 s. Tubes were briefly centrifuged and then placed in a boiling water bath for 3 min. After a 5-min centrifugation at 14,000 rpm, an aliquot was diluted 1:10 in PCR grade water and stored at −20 °C.

PCR was performed as described by Oremus et al. (2006), using 20-μl reactions optimized for primer annealing temperature and sequence and $MgCl_2$ concentration to allow for the consistent discrimination between the susceptible (S) and resistant (R) sodium channel *skdr* and *kdr* alleles. Two reactions were done per fly using the primers described in Table 3, with one reaction (S) used to detect the presence of the susceptible allele and the second reaction (R) to detect the resistant allele. Final optimized reaction conditions used 1 μl of diluted genomic DNA from a single fly, 2 μl of 10× PCR buffer II (Applied Biosystems Inc.), 2 mM $MgCl_2$, 0.05 mM of each dNTP, and 0.2 μl of a 1:1 vol:vol mix of Ampli*Taq* (Applied Biosystems) and TaqStart antibody (Clontech, Mountain View, CA). To assay for the presence of susceptible alleles (S reaction), 1 μM of each primer FG154, FG235, FG130, FG138, FG243, and FG234 were included in the mix (Table 3). To assay for resistant alleles (R reaction), 1 μM of each primer FG155, FG235, FG134, FG138, FG243, and FG234 were included in the mix (Table 3). Primers FG234 and FG243 serve as PCR positive controls and amplify a portion of the *GAPDH* gene-coding region. Amplification was carried out using a DNA Engine (MJ Research, Watertown, MA) programmed for 96 °C for 2 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. A final extension at 72 °C for 7 min was also included.

The diagnostic PCR products were visualized by agarose gel electrophoresis using 4% NuSieve agarose TBE gels (Lonza Walkersville Inc., Rockland, ME) followed by staining with GelStar (Lonza Walkersville Inc.). Detectable amplification of a 72 bp *skdr* diagnostic product in only the S reaction indicated the individual was homozygous susceptible at

Table 2 Description of mosaic insecticide treatments for controlling horn flies at Saint Gabriel Research Station, Louisiana, USA, from 2006 to 2011

Year	Group	Insecticide commercial name (active ingredient and manufacturer)
2006	Untreated	None[a]
	OP	Warrior® (30% diazinon, 10% chlorpyrifos, Y-Tex Corporation, Cody, WY)
	OP	Vapona® (1% dichlorvos, PBU Gordon Corp, Kansas City, MO)
	PYR	PYthon® (10% zetacypermethrin and 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)
2007	Untreated	None
	OP	Patriot® (40% diazinon, Bayer AG, Leverkusen, Germany)
	PYR	Guard Star plus® (10% permethrin, Y-Tex Corporation, Cody, WY)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)
2008	Untreated	None
	OP	Patriot® (40% diazinon, Bayer AG, Leverkusen, Germany)
	PYR	Guard Star plus® (10% permethrin, Y-Tex Corporation, Cody, WY)
	ML	XP 820® (8% Abamectin, 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)
2009	Untreated	None
	OP	Patriot® (40% diazinon, Bayer AG, Leverkusen, Germany)
	PYR	Guard Star plus® (10% permethrin, Y-Tex Corporation, Cody, WY)
	ML	XP 820® (8% Abamectin, 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)
2010	Untreated	None
	OP	Patriot® (40% diazinon, Bayer AG, Leverkusen, Germany)
	PYR	Guard Star plus® (10% permethrin, Y-Tex Corporation, Cody, WY)
	ML	XP 820® (8% Abamectin, 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)
2011	Untreated	None
	OP	Warrior® ear tag (30% diazinon, 10% chlorpyrifos, Y-Tex Corporation, Cody, WY)
	PYR	PYthon® (10% zetacypermethrin and 20% piperonyl butoxide, Y-Tex Corporation, Cody, WY)
	PYR	Guard Star plus® (10% permethrin, Y-Tex Corporation, Cody, WY)
	CYC	Avenger® ear tag (30% endosulfan, KMG Chemicals, Houston, TX)
	ML	Ivomec® (0.5% *w/v* ivermectin, Merial, Duluth, GA) or Eprinex® (0.5% *w/v* eprinomectin, Merial, Duluth, GA)

[a] No insecticide was used

OP organophosphate, *PYR* pyrethroid, *ML* macrocyclic lactones, *CYC* cyclodiene

the *skdr* locus. A 72 bp product in only the R reaction indicated a *skdr* resistant homozygote and a 72 bp product in both the S and R reaction indicated a *skdr* heterozygote. Detection of *kdr* susceptible and resistant alleles was similar, with homozygous susceptible, homozygous resistant, and heterozygous individual diagnosed by detectable amplification of the 285 bp diagnostic product in only the S reaction, only the R reaction, or both the S and R reactions, respectively. The size of the *GAPDH* positive control PCR product is 154 bp.

Data analysis

The concentration required to kill 50% of the flies (LC_{50}) in the bioassays was calculated by Probit analysis (LeOra Software 1987), and differences between LC_{50} were considered significant when their 95% fiducial limits did not overlap. Resistance ratios (RF) were calculated by dividing the LC_{50} from field populations by the LC_{50} from the reference susceptible strain from the Knipling-Bushland US Livestock Insects Research Laboratory, USDA-ARS, Kerrville, TX, for the

Table 3 Primer sequences for detection of *kdr* and *superkdr* alleles

Primer	Sequence	Description
FG154	5′-ACCCATTGTCCGGCCCA-3′	Sus downstream *skdr* diagnostic primer
FG155	5′-ACCCATTGTCCGGCCCG-3′	Res downstream *skdr* diagnostic primer
FG235	5′-CTTCGTGTATTCAAATTGGC A-3′	Sus/Res upstream *skdr* primer
FG130	5′-TACTGTTGTCATCGGCAATC-3′	Sus upstream *kdr* diagnostic primer
FG134	5′-TACTGTTGTCATCGGCAATT-3′	Res upstream *kdr* diagnostic primer
FG138	5′-CAATATTACGTTTCACCCAG-3′	Sus/Res downstream *kdr* primer
FG243	5′-GGCATGGCTTTCCGTGTCC-3′	GAPDH PCR positive control primer
FG234	5′-CTTCTTCATCGGTGTAGC-3′	GAPDH PCR positive control primer

respective year, with the exception of 2010, when the Kerrville susceptible strain was not tested and the resistance ratio was calculated using the average of the LC_{50} of years 2006, 2007, 2008, 2009, and 2011.

The frequency of the R allele (%R allele) was calculated by dividing the number of R alleles by the total number of alleles (S and R) and multiplying the result by 100. Fisher's exact test was used to compare the %R allele in time for both the *skdr* and *kdr* mutations at each research station as well as genotypes (homozygous susceptible—SS, heterozygous—SR, homozygous resistant—RR) using the software GraphPad (GraphPad Prism 7.0 for Windows, GraphPad Software Inc., La Jolla, CA, USA). Differences were considered significant at $p \leq 0.05$.

Results

Bioassays

All bioassay results are summarized in Table 4. Comparing the pre-treatment to the post-treatment data, the permethrin resistance ratios (RF) of the horn fly population from the Red River Research Station (6PYR) increased in all years after treatment with pyrethroid ear tags, except in 2007. However, the decrease from PRE to POST in 2007 and the increase from PRE to POST in 2009 were not statistically significant. The permethrin resistance ratio decreased during the 8-month interval with no insecticide exposure (from the POST sampling point to the following year's PRE sampling), except from POST 2006 to PRE 2007 when the resistance ratio increased; however, this increase was not statistically significant. Over the entire study, after 6 years of exposure to only pyrethroid ear tags, the permethrin resistance ratio significantly increased 26-fold (2006 PRE vs. 2011 POST Table 4).

Comparing the pre-treatment to the post-treatment data at the St. Joseph Research Station (3PYR-1No-2OP), the resistance ratio to permethrin had a statistically significant increase of approximately 3-fold in 2006, 2007, and 2008 (see PRE vs POST in Table 4) after the animals were treated with pyrethroid ear tags. In 2009, when no insecticide was used for a whole year, the permethrin resistance ratio had a 1.3-fold decrease, which was not statistically significant (95% FL overlap). In both years when organophosphates were used, the permethrin resistance ratio increased but not significantly. For the POST to PRE comparisons at this location, the permethrin resistance ratios decreased 1.5- to 4-fold for all years, but the only statistically significant differences were observed following years when the animals were treated with pyrethroid ear tags (POST 2006 vs. PRE 2007, POST 2007 vs. PRE 2008, and POST 2008 vs. PRE 2009). Although there was a 2.5-fold decrease in the permethrin resistance ratio over the course of the study (PRE 2006 to POST 2011), this difference was not statistically significant (95%FL overlap, Table 4).

At the Macon Ridge Research Station (3PYR-1No-2CYC), the permethrin resistance ratio increased approximately 7-fold from PRE to POST 2006, but significantly decreased in 2007 and 2008, although pyrethroid ear tags were used in all three years. In 2009, when no insecticides were used, the resistance ratio remained stable. In 2010 and 2011, when the animals were treated with endosulfan ear tags, the permethrin resistance ratio significantly increased and remained stable, respectively. No significant difference was observed for any of the POST to PRE comparisons. Over the entire study (PRE 2006 to POST 2011), the permethrin resistance ratio had a significant increase of approximately 2-fold (Table 4).

At the St. Gabriel Research Station (Mosaic), where a mosaic of treatments was used, the permethrin resistance ratio significantly increased in 2006 (4-fold), 2010 (4-fold), and 2011 (3-fold), while decreasing in 2008 (1.75-fold). Unfortunately, the Probit analysis did not generate a LC_{50} for the PRE 2007 and POST 2009 data, so permethrin resistance ratio comparisons could not be performed for those years. In the POST × PRE comparisons, the only significant difference observed was a 3-fold increase from POST 2008 to PRE 2009, despite the absence of insecticidal pressure for 6 months. Over the course of the study (PRE 2006 to POST 2011) at this location, the permethrin resistance ratio significantly increased by 2.5-fold (Table 4).

Table 4 Permethrin bioassay data and frequency of *superkdr* and *kdr* resistance alleles of horn fly populations under four long-term insecticide treatment regimens in Louisiana, USA, from 2006 to 2011

Research station	Year	Treatment[a]	Season	Slope ± SE	LC_{50}[b] (95% FL)	RF[c]	Significance PRE × POST[d]	Significance POST × PRE[e]	*superkdr*			*kdr*		
									%R allele[f]	PRE vs POST *p* value[g]	POST vs PRE *p* value[h]	%R allele	PRE vs POST *p* value	POST vs PRE *p* value
Red River (6PYR)	2006	PYR	PRE[i]	2.07 ± 0.16	0.48 (0.32–0.67)	4.00	Yes		0	ns		7.5	< 0.05	
			POST[j]	1.52 ± 0.08	5.42 (1.91–12.75)	45.17		No	2.5		ns	23.8		ns
	2007	PYR	PRE	2.46 ± 0.18	6.11 (4.92–7.51)	55.55	No		3.8	ns		11.3	< 0.001	
			POST	1.82 ± 0.12	5.92 (4.07–8.28)	53.82		Yes	8.8		ns	35		< 0.0001
	2008	PYR	PRE	2.13 ± 0.22	2.04 (1.34–2.77)	9.27	Yes		1.3	< 0.0001		7.5	< 0.0001	
			POST	0.79 ± 0.06	53.28 (36.06–84.47)	242.18		Yes	21.3		< 0.0001	72.5		< 0.0001
	2009	PYR	PRE	2.20 ± 0.15	3.23 (1.89–4.89)	17.94	No		0	< 0.0001		11.3	< 0.0001	
			POST	0.67 ± 0.07	5.51 (0.86–18.74)	30.61		No	26.3		< 0.0001	83.8		< 0.0001
	2010	PYR	PRE	1.68 ± 0.13	1.18 (0.44–2.51)	4.92	Yes		1.3	< 0.05		8.8	< 0.0001	
			POST	0.95 ± 0.07	76.82 (55.07–113.66)	320.08		Yes	13.8		< 0.05	48.8		< 0.001
	2011	PYR	PRE	1.47 ± 0.15	6.35 (3.12–10.31)	19.24	Yes		2.5	< 0.05		22.5	< 0.0001	
			POST	0.91 ± 0.07	34.59 (24.01–49.86)	104.82			12.5			63.8		
	PRE 2006	×	POST 2011						0.0014			0.00		
Saint Joseph (3PYR-1No-2-OP)	2006	PYR	PRE	0.84 ± 0.05	12.86 (8.20–20.11)	107.17	Yes		38.8	ns		75	ns	
			POST	1.15 ± 0.07	51.23 (38.06–69.85)	426.92		Yes	45		< 0.05	83.8		ns
	2007	PYR	PRE	1.32 ± 0.11	12.58 (8.79–16.96)	114.36	Yes		28.8	ns		72.5	< 0.05	
			POST	1.02 ± 0.06	38.29 (25.84–56.72)	348.09		Yes	38.8		< 0.05	88.8		< 0.05
	2008	PYR	PRE	1.37 ± 0.09	17.84 (13.88–22.35)	81.09	Yes		18.8	ns		71.3	< 0.05	
			POST	0.76 ± 0.05	57.60 (27.36–158.04)	261.82		Yes	27.5		ns	90		< 0.05
	2009	None	PRE	1.24 ± 0.08	14.33 (10.33–20.21)	79.61	No		15	ns		71.3	ns	
			POST	1.26 ± 0.09	10.68 (7.25–15.06)	59.33		No	10		ns	56.3		ns
	2010	OP	PRE	1.48 ± 0.10	9.53 (6.30–13.99)	39.71	No		7.5	ns		51.3	ns	
			POST	1.19 ± 0.07	20.07 (12.84–30.56)	83.63		No	15		< 0.05	65		< 0.05
	2011	OP	PRE	1.38 ± 0.14	10.04 (4.75–16.70)	30.42	No		1.3	ns		41.3	< 0.05	
			POST	1.11 ± 0.08	14.16 (9.18–20.45)	42.91			6.3			63.8		
	PRE 2006	×	POST 2011						0.00			ns		
Macon Ridge (3PYR-1No-2-CYC)	2006	PYR	PRE	0.88 ± 0.06	0.94 (0.52–1.51)	7.83	Yes		18.8	< 0.05		45	ns	
			POST	1.78 ± 0.10	6.24 (4.62–8.18)	52.00		No	6.3		ns	40		ns
	2007	PYR	PRE	1.84 ± 0.11	5.97 (4.08–8.29)	54.27	Yes		5	ns		40	ns	
			POST	1.51 ± 0.08	2.47 (1.78–3.35)	22.45		No	6.3		ns	43.8		< 0.05
	2008	PYR	PRE	1.54 ± 0.09	3.52 (2.58–4.64)	16.00	Yes		5	ns		25	< 0.05	
			POST	1.07 ± 0.07	1.54 (0.90–2.46)	7.00		No	7.5		ns	46.3		ns
	2009	None	PRE	1.50 ± 0.11	2.96 (1.92–4.27)	16.44	No		3.8	ns		55	ns	
			POST	1.30 ± 0.10	4.20 (2.34–6.64)	23.33		No	3.8		ns	45		ns
	2010	CYC	PRE	1.58 ± 0.10	1.84 (1.40–2.38)	7.67	Yes		3.8	ns		32.5	< 0.0001	
			POST	1.31 ± 0.07	14.43 (10.21–20.48)	60.13		No	6.3		ns	65		< 0.05
	2011	CYC	PRE	2.29 ± 0.35	8.07 (5.91–10.18)	24.45	No		1.3	ns		47.5	ns	
			POST	1.89 ± 0.12	5.58 (4.18–7.25)	16.91			1.3			40		
	PRE 2006	×	POST 2011						0.00			ns		
Saint Gabriel (Mosaic)	2006	Mosaic	PRE	1.84 ± 0.15	1.62 (0.45–3.33)	13.5	Yes		0	ns		21.3	ns	
			POST	1.99 ± 0.13	6.61 (3.46–10.52)	55.08		–	1.3		ns	22.5		ns

Table 4 (continued)

Research station	Year	Treatment[a]	Season	Slope ± SE	LC_{50}[b] (95% FL)	RF[c]	Significance PRE × POST[d]	Significance POST × PRE[e]	*superkdr*			*kdr*		
									%R allele[f]	PRE vs POST *p* value[g]	POST vs PRE *p* value[h]	%R allele	PRE vs POST *p* value	POST vs PRE *p* value
	2007	Mosaic	PRE	2.57 ± 0.17	–	–	–		1.3	ns		11.3	ns	
			POST	2.31 ± 0.15	5.27 (2.70–8.93)	47.91		No	1.3		ns	12.5		ns
	2008	Mosaic	PRE	3.02 ± 0.27	4.18 (3.40–5.07)	19.00	Yes		1.3	ns		17.5	ns	
			POST	1.41 ± 0.11	2.38 (1.66–3.31)	10.82		Yes	0		ns	21.3		ns
	2009	Mosaic	PRE	1.39 ± 0.06	7.40 (5.65–9.64)	41.11	–		0	ns		23.8	< 0.05	
			POST	3.39 ± 0.29	–	–	–		0		ns	8.8		ns
	2010	Mosaic	PRE	1.49 ± 0.15	1.08 (0.70–1.55)	4.50	Yes		0	ns		6.3	ns	
			POST	1.86 ± 0.21	4.33 (2.26–7.14)	18.04		No	0		ns	11.3		ns
	2011	Mosaic	PRE	1.93 ± 0.15	3.89 (3.07–4.80)	11.79	Yes		0	ns		16.3	ns	
			POST	1.64 ± 0.10	11.27 (8.10–15.04)	34.15			0			30		
	PRE 2006	×	POST 2011						ns			ns		
Reference strain	2006	–	–	1.96 ± 0.30	0.12 (0.02–0.22)	–	–	–	–	–	–	–	–	–
	2007	–	–	1.63 ± 0.24	0.11 (0.04–0.17)	–	–	–	–	–	–	–	–	–
	2008	–	–	4.99 ± 0.77	0.22 (0.19–0.25)	–	–	–	–	–	–	–	–	–
	2009	–	–	4.53 ± 0.82	0.18 (0.15–0.21)	–	–	–	–	–	–	–	–	–
	2010	–	–	–	–	–	–	–	–	–	–	–	–	–
	2011	–	–	2.94 ± 0.26	0.33 (0.29–0.37)	–	–	–	–	–	–	–	–	–
	Average	–	–	–	0.24 (0.18–0.31)	–	–	–	–	–	–	–	–	–

[a] See Tables 1 and 2 for detailed information on yearly insecticide use at each location

[b] Permethrin concentration required to kill 50% of the flies calculated by Probit analysis (LeOra Software 1987), expressed in microgram per square centimeter

[c] Resistance ratio to permethrin was calculated by dividing the permethrin LC_{50} from field populations by the permethrin LC_{50} from the reference susceptible strain from the Knipling-Bushland US Livestock Insects Research Laboratory, Kerrville, TX for each year, except in 2010 when the average LC_{50} of the reference strain from the other 5 years was used

[d] Comparison of permethrin LC_{50} PRE and POST-treatment in the same year. Differences between LC_{50} were considered significant when their 95% fiducial limits did not overlap

[e] Comparison of permethrin LC_{50} POST and PRE in the following year. Differences between LC_{50} were considered significant when their 95% fiducial limits did not overlap

[f] Calculated by dividing the number of R alleles by the total number of alleles (S + R) and multiplying by 100

[g] Comparison of %R allele of PRE to POST data in the same year by Fisher's exact test

[h] Comparison of %R allele of POST treatment data to PRE data from the following year by Fisher's exact test

[i] Permethrin bioassay performed before spring ear tag treatment

[j] Permethrin bioassay performed approximately two weeks after year tags were removed following the end of the fly season

Frequency of *kdr* and *skdr* genotypes

The frequencies of the *skdr* and *kdr* mutations at each research station throughout the 6 years of the study are shown in Fig. 1 and Online Resource 1. The *skdr-kdr* genotypes SR-SS, RR-SS, and RR-SR were not found in any of the flies and, therefore, are not listed in neither the figure nor the table.

At the Red River Research Station (6PYR), the frequency of the homozygous susceptible genotype (SS-SS) ranged from 85% (PRE 2006) to 5% (POST 2009), always decreasing from spring to fall each year coinciding with the use of pyrethroid ear tags and increasing from fall to the next spring after 6 months without insecticide pressure. Comparing the pre-treatment to the post-treatment data, statistically significant differences were observed in all years, except 2006 (which only showed a small decrease with $p > 0.05$). During the interval without insecticide treatment (from the POST sampling point to the following year's PRE sampling), statistically significant differences were observed in the POST 2007 vs. PRE 2008, POST 2008 vs. PRE 2009, and POST 2009 vs. PRE 2010 comparisons. The other two POST vs. PRE comparisons showed small increases ($p > 0.05$). The frequency of the SS-SR genotype (*skdr* homozygous susceptible-*kdr* heterozygous) showed small fluctuations that were only statistically significant during the 2010 PRE vs. POST treatment period comparison (a decrease) and in the POST 2010 vs. PRE 2011 interval without insecticide pressure (an increase). The SS-RR genotype (*skdr* homozygous susceptible and *kdr* homozygous resistant) showed a frequency pattern nearly opposite to the SS-SS genotype. The use of pyrethroid ear tags resulted in a significant increase of this genotype (PRE vs. POST) in all years except 2007 (which also increased but not statistically significant). During the intervals without insecticide pressure (POST vs. PRE comparisons), a significant decrease in this genotype was found in all comparisons except for the POST 2006 vs. PRE 2007 and POST 2007 vs. PRE 2008 (these also showed decreased frequencies but without statistical significance). The frequency of the SR-SR genotype (heterozygous for both *skdr* and *kdr*) ranged from 2.5 to 10% throughout the study, but no statistical differences were detected for any of the comparisons in any year. For the SR-RR genotype (*skdr* heterozygous and *kdr* homozygous resistant), statistically significant increases in the PRE vs POST comparisons were observed in 2008, 2009, and 2010. The PRE vs. POST comparisons for 2006 and 2007 also showed increases but were not statistically significant. During the intervals without insecticide pressure, a significant decrease in this genotype was found in the POST 2008 vs. PRE 2009 and POST 2009 vs. PRE 2010 comparisons. The RR-RR genotype, which corresponds to homozygous resistant for both *skdr* and *kdr* alleles, was only observed in the POST 2008, POST 2009, and POST 2010 samples. However, statistically significant differences were not observed in any of the data comparisons at this location. Over the 6-year course of this study at Red River, we observed a statistically significant decrease in SS-SS genotype and an increase in the SS-RR and SR-RR genotypes (Fig. 1, Online Resource 1).

At the St. Joseph Research Station (3PYR-1No-2OP), the frequency of the SS-SS genotype decreased with the use of pyrethroid tags for the PRE vs. POST comparisons in 2006, 2007, and 2008, although these changes were not statistically significant. During the year without insecticide use (2009), there was a small, though not statistically significant, increase in the SS-SS genotype frequency. The frequency remained steady during the first year of organophosphate usage (PRE 2010 vs. POST 2010) and then dropped significantly in 2011. During the intervals without treatment (POST vs. PRE comparisons), there was a statistically significant difference only in the first year (POST 2006 vs. PRE 2007). Over the course of the study, the SS-SS genotype frequency did not change significantly. With the SS-SR genotype, the frequency varied somewhat, but the only significant frequency difference was an increase in the comparison of POST 2007 vs. PRE 2008. The frequencies of the SS-RR and SR-SR genotypes did not show significant differences at this study location. The SR-RR genotype showed frequency increases in the second and third year of pyrethroid use and the first year of organophosphate use, though these differences were not statistically significant. In the comparisons between POST and PRE, a significant decrease was observed in POST 2007 vs. PRE 2008 and the POST 2010 vs. PRE 2011. The RR-RR genotype was only seen during the first 4 years of the study at this location, the 3 years of pyrethroid use (2006–2008) and the single year of no treatment (2009). This genotype was not found at the POST 2009 sampling point or at any sampling point afterwards. Over the course of this study at Saint Joseph, there was a significant increase in the frequency of SS-SR and decreased the frequency of the SR-RR and RR-RR genotypes (*skdr-kdr*) (Fig. 1, Online Resource 1).

At the Macon Ridge Research Station (3PYR-1No-2CYC), the frequency of the SS-SS genotype in the PRE vs. POST comparisons only showed statistical differences in the third year of pyrethroid ear tag usage (2008) and the first year of the endosulfan usage (2010); both of these were decreases in SS-SS frequency. In the POST vs. PRE comparisons, there was no consistent pattern or significant differences. For the SS-SR genotype, there were slight increases in frequency during the 3 years of pyrethroid ear tag usage, but these were not statistically significant. None of the other comparisons (PRE vs. POST or POST vs. PRE) or frequencies showed a pattern, and the resultant data comparisons were not statistically significant. For the SS-RR genotype, the only significant difference was an increase in 2010 (PRE vs. POST) after the first season of endosulfan. The SR-SR genotype did not show any statistically significant differences in any comparisons during the entire study. For the SR-RR genotype, there was a

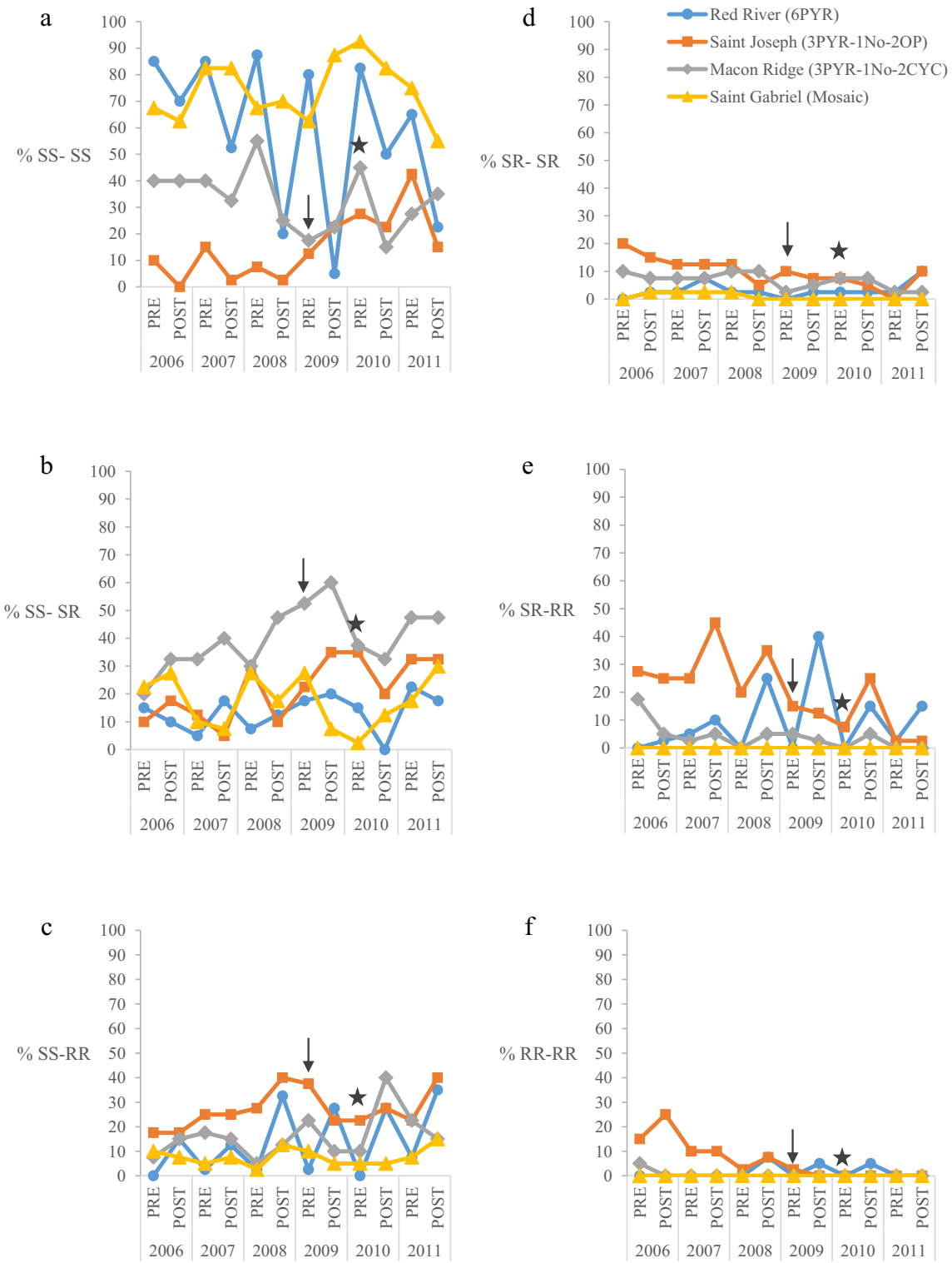

Fig. 1 Horn fly *superkdr-kdr* genotype frequencies from long-term insecticide treatment regimens in four study locations in Louisiana, USA, from 2006 to 2011. **a** Frequencies of SS-SS (*skdr-kdr*) genotype, **b** Frequencies of SS-SR (*skdr-kdr*) genotype. **c** Frequencies of SS-RR (*skdr-kdr*) genotype. **d** Frequencies of SR-SR (*skdr-kdr*) genotype. **e** Frequencies of SR-RR (*skdr-kdr*) genotype. **f** Frequencies of RR-RR (*skdr-kdr*) genotype. Arrow indicates when no insecticide was used at Saint Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC) research stations. Star indicates the first year when either organophosphate or cyclodienes ear tags were used at Saint Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC) research stations, respectively

decrease in frequency, though not statistically significant, during the first year of the study. Following the POST 2006 sampling, this genotype was infrequent or absent and no significant differences were seen in any comparisons. The RR-RR allele was only found in two (5%) individuals in the PRE sample taken in the first year of the study, and this allele was absent after this point. Over the course of the study, the SS-SR genotype increased significantly and the SR-RR genotype decreased significantly (Fig. 1, Online Resource 1).

At the St. Gabriel Research Station (Mosaic), the only genotypes present in the horn fly population were SS-SS, SS-SR, SS-RR, and SR-SR. The only significant differences observed in the genotype frequency comparisons were during the fourth year of the mosaic (2009). In this year, the PRE vs. POST comparison showed a statistically significant increase in SS-SS following the treatment season and an accompanying significant decrease in SS-SR genotype. After 6 years of mosaic use, the frequency of the genotypes at the St. Gabriel Research Station did not change significantly (Fig. 1, Online Resource 1).

Frequency of the R allele (%R allele)

The frequency of the R allele (%R allele) for each mutation is shown in Table 4.

At the Red River Research Station (6PYR), the %R-*skdr* allele significantly increased PRE to POST treatments, except in the initial 2 years (2006 and 2007) and significantly decreased in the POST vs. PRE comparisons except for the first two comparisons (POST 2006 vs. PRE 2007 and POST 2007 vs. PRE 2008). There was a significant increase from PRE 2006 (0%) to POST 2011 (12.5%) ($p = 0.0014$). The %R-*kdr* allele ranged from 7.5 to 83.8%. The %R-*kdr* allele significantly increased after treatment in every year. Comparing the POST vs. PRE data, the %R-*kdr* allele significantly decreased in each comparison, except POST 2006 ×PRE 2007 (which also showed a decrease but $p > 0.05$). From the initial PRE 2006 sampling to the final POST 2011 sampling, the %R-*kdr* allele significantly increased from 7.5% to 63.8% ($p = 0.00$) (Table 4).

At the St. Joseph Research Station (3PYR-1No-2OP), the %R-*skdr* allele was not significantly affected by the use of either pyrethroid or organophosphate in any of the PRE vs. POST comparisons. However, %R-*skdr* allele significantly decreased in the POST 2006 vs. PRE 2007, POST 2007 vs. PRE 2008, and POST 2010 vs. PRE 2011 comparisons. There was also a decrease in 2008 POST vs. 2009 PRE though not statistically significant. Comparing the initial PRE 2006 data to the POST 2011 data, the %R-*skdr* significantly decreased ($p = 0.00$). The %R-*kdr* allele increased in the 3 years of treatment with pyrethroid ear tags, although the increase in 2006 was not statistically significant ($p > 0.05$). The %R-*kdr* allele then decreased during the year without insecticide treatment (2009), although not statistically significant. Interestingly, during the following 2 years of organophosphate use, the %R-*kdr* allele significantly increased in 2011. In the POST vs. PRE comparisons (the period where insecticide pressure was absent), the %R-*kdr* allele decreased in every comparison, although the POST 2006 vs. PRE 2007 and the POST 2009 vs. PRE 2010 decreases were not significant. The %R-*kdr* allele did not change significantly comparing the 2006 PRE beginning sample to the 2011 POST final sample (Table 4).

At the Macon Ridge Research Station (3PYR-1No-2CYC), the %R-*skdr* allele significantly decreased in the PRE 2006 vs. POST 2006 comparison only and remained stable in all the other years. The POST vs. PRE comparisons did not change significantly at any point. Comparing the initial PRE 2006 sample to the final POST 2011 sample, the %R-*skdr* allele showed a significant decrease ($p = 0.00$). The %R-*kdr* allele significantly increased only after the third year of pyrethroid use (2008) as well as following the first year of endosulfan use (2010). For the POST to PRE comparisons, the only significant differences were the decreases observed in POST 2007 vs. PRE 2008 and POST 2010 vs. PRE 2011 data. Following the 6 years of the study, the %R-*kdr* allele did not change significantly (Table 4).

At the St. Gabriel Research Station, the %R-*skdr* allele was rare and was only present in the first 3 years. The %R-*kdr* allele remained very stable for the first 3 years in both the PRE to POST and the POST to PRE comparisons. Then, in the fourth year (2009), the %R-*kdr* allele decreased significantly in the PRE 2009 vs. POST 2009 comparison while the POST 2009 vs. PRE 2010 comparison remained stable. This was followed by 2 years of slight increases in the %R-*kdr* allele for the PRE vs. POST comparisons, though with no statistical significance. Neither the %R-*skdr* nor the %R-*kdr* showed statistical differences when the first (PRE 2006) and final (POST 2011) sample data were compared (Table 4).

Discussion

During the 6-year study, the resistance ratios of the four horn fly populations either significantly increased (Red River Research Station—6PYR, Macon Ridge Research Station—3PYR-1No-2OP, St. Gabriel Research Station—Mosaic) or remained stable (St. Joseph Research Station—3PYR-1No-2OP) (Table 4). At the Red River Research Station (6PYR), the %R alleles of both mutations significantly increased, while at St. Joseph (3PYR-1No-2OP), Macon Ridge (3PYR-1No-2CYC), and St. Gabriel (Mosaic), the %R-*skdr* allele either dramatically dropped or remained stable, and the %R-*kdr* allele remained stable and above 30% (Table 4). Byford et al. (1999) showed that the consecutive use of a single insecticide greatly contributes to the development and persistence of resistance and an eventual control failure, which also was shown

in our study for the Red River (6PYR) population. Under field conditions, Byford et al. (1999) also found that the efficacy of pyrethroid and organophosphate insecticides did not change after 3 years of use in a mosaic. Our results showed that at St. Gabriel (Mosaic), the resistance ratios increased after 6 years of mosaic as well as the %R-*kdr* allele, although not significantly, indicating that this strategy did not outperform those used at St. Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC). Barros et al. (1999) reported that the yearly rotation of pyrethroid and organophosphate for 7 years did not improve pyrethroid efficacy or prevent further development of resistance to either class of insecticide. Oremus et al. (2006) reported that a mid-season treatment with doramectin improved horn fly control, but did not improve pyrethroid efficacy overall. The results reported by these authors and our findings suggest that once the target site pyrethroid resistance has established in a horn fly population, switching to an insecticide with a different mode of action will not eliminate the *skdr* and *kdr* pyrethroid resistance genes and pyrethroids will not provide satisfactory control. Therefore, insecticides from other classes must be used always with caution and avoiding the consecutive use of the same class to delay the development of resistance, especially target site resistance.

At the Macon Ridge (3PYR-1No-2OP) and St. Gabriel (Mosaic) research stations, the permethrin resistance ratio increased, despite the fact that the %R-*kdr* and %R-*skdr* either remained stable or dropped significantly from 2006 to 2011 (Table 4). This increase was likely caused by a resistance mechanism other than the target site. Metabolic resistance has been previously described in horn fly populations (Sheppard 1995; Guerrero and Barros 2006; Li et al. 2009). The PYthon® ear tags contain a component (piperonyl butoxide) that acts to depress cytochrome P450-based metabolic resistance. However, the prolonged insecticide pressure could have selected for metabolic esterases or other metabolic enzymes or transporters. The resistance ratio is a value that includes all mechanisms of resistance present in a population whether target site insensitivity, metabolic-based resistance, or other.

Guerrero et al. (2002) used PCR to screen the flies collected by Barros et al. (1999) and showed an increase in the percentage of susceptible alleles at the *kdr* locus during the interval with no pyrethroid use, concluding that susceptible alleles are selected for in the absence of PYR pressure. Li et al. (2009) showed that *skdr* and *kdr* alleles were maintained mostly in the heterozygous form when no pyrethroid was used for 1 year. The same trend for the SS-SS genotype was observed in our study, particularly for the Red River (6PYR) horn fly population exposed to pyrethroid for six consecutive years (Fig. 1, Online Resource 1). At the other three research stations, no significant differences were observed for POST × PRE comparisons for the SS-SS genotype, except POST2006 vs PRE 2007 at St. Joseph (3PYR-1No-2OP), which significantly increased (Fig. 1, Online Resource 1). On the other hand, the %SS-SR genotype significantly increased at St. Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC) from 2006 to 2011 (Fig. 1, Online Resource 1), showing that it was not negatively affected by the lack of exposure to PYR and was responsible for the maintenance of the *kdr* mutation at those two research stations.

After 6 years of consecutive use of pyrethroid ear tags, the %SR-RR genotype significantly increased at the Red River research station (6PYR), while at St. Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC), the %SR-RR genotype significantly dropped (Fig. 1, Online Resource 1), indicating that there are fitness costs for the SR-RR genotype. Biotic fitness deficits have been described in pyrethroid resistant horn flies in both laboratory and field studies. In the laboratory, pyrethroid susceptible horn flies pupated nearly twice as successful as resistant flies and produced more than twice as many progeny (Scott et al. 1997). In field trials, Guerrero et al. (2002), Oremus et al. (2006), and Li et al. (2009) reported that the frequencies of *kdr* and *skdr* resistant alleles increased when pyrethroid ear tags were used and decreased in the absence of pyrethroid ear tags. Kliot and Ghanim (2012) suggested that using isogenic laboratory strains differing only in the resistance trait would be the most accurate way to characterize resistance trait fitness cost. The populations evaluated in our study did not have the same genetic profile at the beginning of the study (Table 4); yet, we believe that the significant decrease of the %SR-RR genotype in the two populations with no pyrethroid use for 3 years and the increase at Red River (6PYR) is a robust indication of a strong fitness cost associated with the SR-RR genotype, corroborating with Scott et al. (1997), Guerrero et al. (2002), Oremus et al. (2006), and Li et al. (2009).

Guerrero et al. (2002), Oremus et al. (2006), and Li et al. (2009) suggested that although fitness cost appears to be associated with the *kdr* and *skdr* resistant alleles; once these alleles are established in a population, the use of insecticides from different classes (with different mode of action) do not eliminate the resistant alleles. According to Curtis et al. (1978), in the case of *Anopheles culicifacies* and DDT, once the frequency of the resistant gene reaches 20% or more, treatments influencing for further loss of resistance are weak or even non-existent. Our results corroborate their findings, especially for the *kdr* mutation, which remained stable at the St. Joseph (3PYR-1No-2OP) and Macon Ridge (3PYR-1No-2CYC) stations even after 3 years of no pyrethroid use (Table 4). On the other hand, for the *skdr* allele, the %R allele that allows a reverse of resistance may be higher than the 20% proposed by Curtis et al. (1978). At the St. Joseph Research Station, the %R-*skdr* allele significantly decreased from 38.8% (PRE 2006) to 6.3% (PRE 2011) (Table 4). Thus, this mutation seems to be more sensitive to the lack of insecticide

exposure than the *kdr* mutation. However, since the *skdr* mutation does not occur in horn flies that do not possess the *kdr* mutation (Online Resource 1; Jamroz et al. 1998; Guerrero et al. 2002; Foil et al. 2005), the fact that *skdr* may disappear after withdrawal of pyrethroid insecticides for several years will only contribute to a partial reversion of resistance because the *kdr* mutation will still be present.

Interestingly, in house flies (*Musca domestica*), two *kdr* haplotypes had different responses to the absence of insecticide pressure under laboratory conditions, showing that factors other than the L1014F mutation may affect the fitness of a mutation and the *skdr* mutation had a strong fitness disadvantage in the absence of insecticide pressure (Rinkevich et al. 2013). The same was reported in a field population of the state of New York where the frequency of *skdr* has remained constant since its first detection in mid-2003, despite a strong selection with pyrethroids and the high levels of resistance conferred by *skdr* (Rinkevich et al. 2007; Scott et al. 2013). On the other hand, Scott et al. (2013) showed a significant frequency of *skdr* in field populations of the states of Nebraska and Kansas and suggested that environmental conditions also are important to determine the fitness of the *skdr* mutation. An important aspect that must be considered in field studies is the immigration of flies from surrounding herds, which may affect the genetic makeup of a population. Oyarzún et al. (2011) reported the presence of the *kdr* mutation in horn flies from farms where insecticides had not been used for 5 years, which was attributed to the migration of resistant flies from surrounding herds. Horn flies can migrate up to 1.7 km within just 6 days (Byford et al. 1987).

We did not record the number of weeks of control achieved with each treatment regimen, which would better demonstrate the control efficacy of the different strategies within the years (Guerrero et al. 2002, Oremus et al. 2006), because that was not the point of the study. Thus, our results do not provide a final response on which treatment is more efficient in reducing the number of flies and controlling pyrethroid-resistant horn fly populations in the field. However, the results of the study do demonstrate that the regimens evaluated did not reduce the *kdr* mutation to a level that would allow the successful use of pyrethroids for horn fly control, which is consistent with other studies.

Conclusions

We believe our study brings important findings on how different control strategies affect the *skdr* and *kdr* mutations. Corroborating other studies, we showed that once the *kdr* mutation has established in a population, changing to a different class of insecticide or using a mosaic strategy will not eliminate this mutation, not even after 3 years without pyrethroid use. On the other hand, lack of exposure to pyrethroid insecticides significantly affects the *skdr* mutation. The RR-RR genotype was rare in all four populations evaluated and the *skdr* mutation was never found in the absence of the *kdr* mutation. Individuals that are homozygous susceptible for *skdr* and heterozygous for *kdr* (SS-SR) were responsible for the maintenance of the *kdr* mutation in two of the populations studied, and the SR-RR (*skdr-kdr*) genotype seems to be strongly affected by fitness cost.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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