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A study of horn fly, Haematobia irritans (L.) (Diptera: Muscidae), target-site sensitivity, susceptibility, and resistance management at selected sites in Louisiana

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A STUDY OF HORN FLY, *HAEMATOBIA IRRITANS* (L.) (DIPTERA: MUSCIDAE), TARGET- SITE SENSITIVITY, SUSCEPTIBILITY, AND RESISTANCE MANAGEMENT AT SELECTED SITES IN LOUISIANA

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DEDICATION

This work is dedicated to my wife, Laura Younger, and to my children Stephanie Kelly and Colten Younger.

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ABSTRACT

The purpose of the first study was to evaluate the effectiveness of the use of a mid-season avermectin pour-on to cattle for managing OP resistance in horn fly populations at the Rosepine Louisiana Agricultural Research Station. During an eight year study, ivermectin treatments were made three times (when the treatment threshold of less than five weeks of control was reached), and in the following years, the number of weeks of control increased to 9 to 10 weeks each time. The purpose of the second study was to test a two-year OP/one-year pyrethroid rotation strategy as a strategy for maintaining susceptibility in horn fly populations to both pyrethroids and organophosphates. A seven year study was conducted at the Agricultural Research Stations at Iberia and Hill Farm, Louisiana. At Iberia, 6, 10 and 7 weeks of control were recorded when pyrethroid ear tags were used every third year. When OP tags were used, horn fly control was maintained at 9 to 7 weeks throughout the 4 years of use. At Hill Farm, when pyrethroid ear tags were used, weeks of control reduced from 11 to 6 to 2 weeks of control. When OP ear tags were used, weeks of control reduced from 12 to 3 in the 4 years of use. The objective of the third study was to determine the rate of change in *kdr* and *skdr* allele ratios and genotype proportions in horn fly populations in the absence of pyrethroid pressure at the Louisiana Agricultural Research Stations in Winnsboro and St. Joseph. Allele ratios decreased about every 45 days from July to September by an average 20% for *skdr* and 10% for *kdr*. The number of *skdr* homozygous susceptible horn flies increased significantly and the number homozygous resistant horn flies increased significantly. We showed for the first time that the allele ratio changes were related primarily to the RR *kdr* and *skdr* genotypes and that the heterozygote likely had no or reduced biotic fitness costs.

CHAPTER 1

LITERATURE REVIEW

The horn fly, *Haematobia irritans irritans* (L.), belongs to the family Muscidae, of which there are more than 4000 species worldwide and more than 700 species in North America. The genus *Haematobia* is a genus of haematophagous flies that includes the species *H. irritan*s that has two closely related subspecies that were once considered two separate species: *H. irritans irritans*, the horn fly, and *H. irritans exigua*, the buffalo fly. The horn fly is widely distributed in Europe, North America, and parts of South America. The buffalo fly's distribution includes Asia and Australia. The horn fly is an Old World species native to Europe that arrived in North America at Philadelphia from southern Europe in 1885-1886 (Riley 1889, Marlatt 1910). The horn fly quickly spread to other parts of the country with the transportation of cattle, and by the 1900's the horn fly was reported in most of the United States, Canada and Puerto Rico (Hargett and Goulding 1962). The horn fly entered South America via Venezuela in 1977 (Graham and Hourrigan 1977) and spread to Brazil, Argentina, and other South American countries (Anziani et al. 1993).

The horn fly is considered a major economic pest of cattle throughout Europe, North Africa, Asia Minor, and the Americas. In the United States, annual economic losses due to horn fly damage and control efforts have been estimated at $876 million (Kunz et al. 1991). In Brazil, economic losses have been estimated at $150 million annually (Grisi et al. 2002). Damage to cattle from horn fly feeding is associated with irritation and blood loss which results in economic losses due to decreased weight gains in beef cattle, decreased weaning weights of calves, and decreased milk production of dairy cows (Palmer et al. 1981).

The horn fly has been called, “the easiest fly to kill, but the hardest to control” by J.G. Matthysse (Butler and Okine 1999). Several factors that contribute to the difficulty of controlling the horn fly include its reproductive potential, its behavior, and its dispersal tendencies (Wilkerson 1974). Both male and female horn flies are obligate haematophagous ectoparasites of cattle that can take as many as 20-30 blood meals per day (Foil and Hogsette 1994). In the United States, horn fly populations peak during the summer months, and populations can average from a few hundred to twenty thousand per animal (Bruce 1964). Horn flies normally feed on the backs and sides of cattle, although they have been observed around the base of the horns of cattle, hence the name horn fly. Adult horn flies have been reported to live from 4-8 weeks (Bruce 1964). Adult horn flies spend most of their life on their host; the female only leaves the host briefly to lay eggs in or under fresh manure pats. A female horn fly can lay multiple egg batches of 14-24 eggs per batch and lay up to 200-360 eggs during her lifetime (Palmer and Bay 1983). The eggs are small (1-2 mm), elliptical in shape, white when first laid, and then turn brown upon tanning. The eggs hatch in 11 to 20 hours (Melvin and Beck 1931), and larvae feed in the manure and go through three instars in about three days (Bruce, 1964). Pupation occurs in four to five days in or near the aged manure patty (Butler et al. 1981) and eclosion occurs in five to seven days when conditions are favorable. In unfavorable conditions, the horn fly can enter into diapause, which is induced, by a combination of reduced photoperiod and temperature (Wright 1970). During the winter, horn flies maintain low population levels on cattle in the southern regions (Wilkerson 1974). In the temperate regions, horn flies readily go into diapause and emerge again the following spring. The horn fly survives winter by diapausing as a pharate adult in its puparium. Diapause induction varies among latitudes. In colder climates, diapause begins in late July and early August in Alberta, Canada (Depner 1961), and in

more temperate climates such as Texas, Louisiana, and Mississippi diapause occurs around October (Thomas and Kunz 1986, Hoelscher and Combs 1971). Depner (1961) compared the effects of temperature changes on horn fly development in the field and laboratory in Canada. He reported that photoperiod created a predisposition to begin diapause and that as temperature increased, time until diapause increased. He also reported that diapause did not occur at 24° and 30°C in pupae from eggs laid in early to mid-September. Lysyk and Moon (1994) reported that the length of the photoperiod or day-night cycles was unrelated to the incidence of diapausing horn flies, but that diapause induction was related to the temperature that the immatures were exposed to. March and Bay (1983) studied the thermotactic responses of 3rd instar larvae for survival of temperature extremes, and reported that larvae exhibited a positive thermotaxis toward warmer, but nonlethal temperatures, in the upper strata of the manure pat, and they exhibited a negative thermotaxis to lethal temperatures.

Factors that affect horn fly dispersal include age of fly, fly density, sex, and weather conditions. A four-year study to evaluate the effects of environmental factors on dispersal of horn flies was done (Chamberlain 1984). Chamberlain (1981) reported that laboratory-reared horn flies moved downwind more in relatively slow wind velocities, horn flies moved toward the sun in the morning, and recapture rates were higher in wet conditions than in dry conditions. Guillot et al. (1988) reported that newly emerged unfed adult horn flies disperse more readily than older horn flies. Adult horn flies are capable of dispersing 8-16 kilometers in search of a host. Once a fly has found a host, it is highly mobile within the host's herd, although the horn fly typically does not disperse again unless fly density within the herd becomes too high. Marley et al. (1991) evaluated the dynamics of natural dispersal of horn flies by comparing fly counts of insecticide treated and untreated herds; they reported that the rate of dispersal was

either independent or inversely related to horn fly population increases, that newly emerged flies were more likely to disperse, and that horn flies were more attracted to cattle that had fewer flies.

There have been several studies that report the economic benefits of applying insecticides to cattle to control horn flies on yearling cattle, calves, and stockers. Calves from beef cows treated with pyrethroid ear tags for horn flies gained 6.7 kg more than calves from untreated cows in a growing season (Quisenberry and Strohbehn 1984). Haufe (1982) reported that yearling cattle treated with ear tags containing fenvalerate gained 18.5% more weight compared to untreated cattle. Derouen et al. (1995) reported that yearling cattle in Louisiana treated for horn flies gained 12 kg or 17% more weight than untreated yearlings. A three-year study at multiple locations reported that total weight gain was 14% higher for replacement heifers with horn fly control than untreated, and the authors also reported that nonpregnant treated heifers gained 33% more weight than nonpregnant untreated heifers (Derouen et al. 2003).

The economic threshold is the population density at which control action should be initiated to prevent an increasing pest population from reaching the economic injury level. The economic threshold for horn flies can vary based on cattle prices, age of animal, and cost of treating animals (Gordon et al. 1984). Schreiber et al. (1987) monitored cattle aggregation behavior which has been reported to cause weight loss in response to horn fly numbers. They reported that horn fly levels <200 per animal did not elicit aggregation behavior; and thus, they suggested that the economic injury level is >200 horn flies per cow. Hogsette et al. (1991) reported that cattle in Florida could tolerate >200 flies for 70 days with no detrimental effects occurring. They hypothesized that cattle in geographic areas with long fly seasons would develop higher tolerance thresholds and that maintaining populations below a level of 50 flies per animal was not feasible.

Attempts at biological control of horn flies have focused on the immature stages of the horn fly and have included the use of natural predators, parasites, parasitoids, pathogens, and competitors. Hu (1995) identified 226 species of arthropods associated with the cow-dung community. Many species have been tested for their biotic potential of controlling horn flies. Dung beetles were introduced successfully into Hawaii from Mexico in 1923 to aid in the control of the horn fly (Waterhouse 1974). Dung beetles were imported from Africa to the United States in the 1970s in an attempt to control horn flies (Butler and Okine 1999), but no evidence was produced that dung beetles were effective at reducing horn fly populations. The biotic potential of dung beetles as dung competitors of horn flies was evaluated and found to be not only ineffective at reducing horn fly numbers but to cause an increase in the number of horn flies due to the beetles disturbance of the manure, which interfered with the native predators of the horn fly (Macqueen 1975, Legner 1978, Roth et al. 1983). A study to investigate the causes of horn fly natural mortality found that predatory insects, especially staphalinid beetles, were the largest contributing factor of mortality to the immature stages of horn flies (Harris 1981). In Louisiana, the red imported fire ant, *Solenopsis invicta*, was recognized as a major predator of horn fly immature stages in manure pats (Marley et al. 1991). Harris (1981) also found hymenopterous pupal parasitoids to be important in horn fly control, although their role was less significant than predators. Gingrich (1984) demonstrated the bacteria pathogen *Bacillus thuringiensis* was effective against horn fly larvae.

Mechanical control efforts for the horn fly have been directed at both the larval and adult stages. Garzo (1985) demonstrated disturbing manure pats by dragging pastures was effective at reducing horn fly larval survival and adult emergence. A walk-through trap described by Bruce (1940) brushed off adult horn flies as cattle walked through. Hall and Doisy (1989)

improved the trap design and reported 73% control in pastured cattle. Watson et al. (2002) added electrocution grids and black lights to the Bruce walk-through type trap and conducted a two-year evaluation using dairy cattle that were forced to walk through the trap twice a day. They reported that horn fly numbers decreased from greater than 1400 to less than 200 per animal.

Control strategies related to genetics include using the sterile male technique and selecting cattle breeds less susceptible or less attractive to horn flies. Kunz et al. (1974) used the sterile male technique in Texas, and they reported that control could not be achieved because the target area could not be isolated. Eschele et al. (1977) used the sterile male technique in Hawaii along with methoprene administered orally to cattle, and they reported horn fly populations were eliminated for two weeks.

Tugwell et al. (1969) conducted tests using two-year-old heifers to determine the attractiveness or repellency of Brahman cattle for horn flies. The authors eliminated differences in color by dyeing Brahman cattle black and compared percent Brahman versus horn fly burden as percent Brahman increased fly numbers decreased. The fewest flies were counted on the white cattle with 0% Brahman and the most flies were counted on the black with the lowest percent Brahman. Steelman et al. (1991) compared horn fly attractiveness to different beef cattle breeds and they reported that Charolais and Chianina had significantly fewer horn flies than Angus, Hereford, Polled Hereford, and Red Poll breeds. Stellman et al. (1994) documented that using Brahman breeds that they could manage insecticide-resistant populations of horn flies as effectively as insecticides.

Primarily, insecticides are used to control horn flies, and they have been applied using several different application methods. Common application methods that have been used to

apply insecticides included back rubbers, dust bags, pour-ons, dips, sprays, and ear tags (Foil and Hogsette 1994). In the 1970's, ear tags made of poly-vinyl impregnated with an insecticide were introduced. The physical properties of the ear tags allowed insecticides to be released at low levels for extended periods of time, which provided horn fly control for up to 20 weeks (Harvey and Brethour 1970, Ahrens 1977, Ahrens and Cocke 1979, Sparks et. al 1985, Kunz et al. 1991). A topical application pour-on method where an insecticide is poured on the back of cattle developed by Rogoff and Kohler (1961) to apply systemic insecticides to control bot fly cattle grubs is commonly used today to apply avermectins, spinosad, and pyrethroids for horn fly control.

Many different insecticides from different classes have been used over the decades for controlling horn flies and the use of insecticides has led to resistance to chlorinated hydrocarbons, organophosphates (OPs), pyrethroids, and carbamates (Harris 1964, Sheppard 1983, Quisenberry et al. 1984). In the 1940's, the organochlorine DDT was commonly used for horn fly control (Laake 1946). Resistance to DDT was first recorded in 1959 (Burns et al. 1959). Organophosphates were used for horn fly control in the 1960's and provided effective economic season-long horn fly control in back rubbers. Soon after that, the first report of organophosphate (OP) resistance in horn flies in the early 1960's was associated with the use of back rubbers that contained fenchlorphos (Burns and Wilson 1963). With the use of ear tags, the first reports of horn fly OP resistance were for stirofos and tetrachlorvinphos in the 1970's (Sheppard 1983, Harvey et al, 1984).

Pyrethroids were discovered in the 1970's and were more photolabile and less toxic to mammals than organophosphates. Pyrethroids have been formulated for use as sprays, pour-ons and ear tags for fly control. Ear tags containing pyrethroids initially provided 17-20 weeks of

horn fly control, but resistant horn flies were selected for in only 2-4 years in the southeastern United States (Miller et al. 1983, Quisenberry et al. 1984, Schmidt et al. 1985). A study in Louisiana tested horn flies collected from three locations suspected of having pyrethroid resistant populations and compared them to flies from an untreated location (Quisenberry et al. 1984). They reported the horn flies exposed to pyrethroid ear tags were 10 to 30 times more resistant to fenvalerate and permethrin than flies from the control herd. They attributed the resistance to extensive DDT use and cross-resistance among pyrethroids.

Avermectins were discovered in 1976 and developed as an antihelmenthic. Ivermectin was the first avermectin marketed as an antiparasitic agent in 1981, and by the mid-1980's ivermectin had been used to treat hundreds of millions of livestock in 77 countries (Campbell 1988). Avermectins have had a significant impact on the animal health industry. Avermectins have been useful for controlling horn flies because avermectin acts both as an adulticide and a larvicide. Avermectin readily passes through a bovine's gastrointestinal system where it accumulates in the manure and prohibits the development of horn fly larvae for up to four weeks (Floate et al. 2001). Furthermore, reduced fecundity has been observed for horn flies that have fed on ivermectin-treated animals (Miller et al. 1986). Lancaster et al. (1991) applied ivermectin to cattle as a pour-on for adult horn flies, and they reported four weeks of control. They also reported horn fly control was better maintained when ivermectin applications followed a mid-season application of diazinon ear tags. Foil et al. (1998) reported an avermectin pour-on of all cattle at a location was effective at providing eight weeks of horn fly control when used in conjunction with permethrin ear tags. To date, no substantial resistance to avermectins has been recorded. One objective of this study was to further evaluate the effectiveness of the use of a mid-season avermectin pour-on to cattle for managing OP resistance in horn fly populations.

The avermectins are macrocyclic lactones that were isolated from natural products from the soil microorganism *Streptomyces avermitilis*. Avermectins have a broad-spectrum of activity at extremely low dosages. Avermectins act in the central nervous system of insects by binding and activating glutamate-gated chloride channels (GluCls). Avermectins are highly toxic to the invertebrate nervous system, where GluCls are readily accessible, and avermectins are relatively non-toxic to vertebrates, where GluCls are found only in the central nervous system, which is protected by a blood-brain barrier that avermectins cannot readily penetrate (Wright 1985). Avermectins used for horn fly control include ivermectin, moxidectin, and eprinomectin.

The mode of action of organochlorines and pyrethroids are similar in acting upon axonal sodium channels by delaying their inactivation after an action potential, which causes a continuous influx of sodium ions causing nerves to fire repeatedly, leading to paralysis. Pyrethroids are synthetic derivatives of pyrethrins present in pyrethrum extract of *Chrysanthemum* flower species (Elliott 1977). Pyrethroids are commonly grouped into two categories (I and II) based on their distinct poisoning symptoms in mammals, effects on nerve preparations, and their chemical structures. Type I pyrethroids structurally lack a cyano group at the phenylbenzyl alcohol moiety which is present in Type II pyrethroids. Type I pyrethroids produce repetitive discharge in response to a single stimulus, and Type II pyrethroids cause a membrane depolarization accompanied by action potential suppression (Clements et al. 1977, Lund and Narahashi 1983). Both types of pyrethroids cause prolonged opening of sodium channels by inhibiting channel deactivation and stabilizing the open sodium channel, although experiments have shown that type II pyrethroids inhibit the deactivation of sodium channels to a greater extent than Type I pyrethroids (Narahashi 1986). Permethrin is a Type I pyrethroid most

commonly used for horn fly control, and Type II pyrethroids commonly used include cypermethrin, fenvalerate, zeta-cypermethrin, beta-cyfluthrin, and lambda-cyhalothrin.

Organophosphates and carbamates act by inhibiting acetylcholinesterase in the insect's central nervous system by binding to the enzyme at its hydroxyl moiety of the serine amino acid. The overstimulation of the post synapse with acetocholine leads to tremors and eventual death of an insect. Organophosphates are more toxic than carbamates because a phosphorylated cholinesterase is more tightly bound by a covalent bond than a carbamylated cholinesterase which is readily reversible (Eldefrawi 1985). Some OPs used in the past for horn fly control or presently used include stirofos, coumaphos, dichlorvos, fenthion, phosmet, tetrachlorvinphos, chlorpyrifos, diazinon, and malathion.

Resistance is a forced change in the genetic makeup of a population in response to selection of a resistant gene pool by pesticide exposure(s), and the elimination of those not having the selected genes (Ware 1993). Insecticide use can select for flies with rates of absorption, distribution, biotransformation, and excretion of insecticides that promote resistance. Two important biological attributes that contribute to horn flies becoming resistant to insecticides within a few years of use are that adult horn flies spend the majority of their lives on cattle, and horn flies have a relatively short life. Studies that investigate insecticide resistance in horn flies suggest there are three mechanisms; biochemical, physiological, and behavioral (Sparks et al. 1985); regardless of the mechanism leading to resistance, a genetic change in fly populations in response to selection by the toxicant occurs. To detect and monitor insecticide resistance in horn flies, a filter paper bioassay exposing horn flies in petri dishes to serial dilutions of insecticides was developed by Sheppard and Hinkle (1987). However, the filter paper bioassay provides no information about the genotypes of individual horn flies within a

population. Guerrero et al. (1997) developed a polymerase chain reaction (PCR)-based rapid screening procedure to test individual horn flies for the presence of specific nucleotide substitutions in the sodium channel gene sequence known as the "knock down resistance" *kdr* and *super-kdr* mutations to determine the genotype of horn flies with target-site pyrethroid resistance.

Increased metabolism can cause changes in distribution, biotransformation and excretion of insecticides in horn flies. Detoxification enzymes that can prevent an insecticide from reaching its target site include esterases, oxidases, or glutathione S-transferases (GST). Esterases detoxify insecticides by hydrolyzing ester linkages. Oxidases metabolize insecticides through O-, S-, and N-alkyl hydroxylation, aliphatic hydroxylation and epoxidation, aromatic hydroxylation, ester oxidation, and nitrogen and thioether oxidation (Wilkinson 1976). The GSTs detoxify insecticides by O-alkyl, O-aryl, and phosphonate conjugates (Motoyama 1980). Byford et al. (1985) used piperonyl butoxide (PBO) to block oxidative metabolism and reported that horn flies resistant to pyrethroids had an increased metabolism. Byford et al. (1985) reported pyrethroid cross-resistance in horn flies by testing pyrethroid resistant flies with a broad spectrum of pyrethroids. Guerrero et al. (1997) also used PBO in combination with pyrethroids and demonstrated that PBO increased susceptibility of pyrethroid resistant flies to pyrethroids. Guerrero et al. (1999) compared esterase levels of susceptible laboratory-reared horn flies to field-collected flies; they detected 28 different esterase bands and concluded that a number of specific esterases may contribute to OP resistance in horn flies. Guerrero et al. (1999) showed that horn flies from field populations resistant to OPs had higher esterase levels and organophosphate-insensitive acetylcholinesterase than susceptible laboratory colonies. Li et al. (2007) investigated diazinon resistance in horn flies and the effects of using PBO on diazinon

toxicity in horn flies. They reported that PBO had no effect on esterase activity in horn flies and it had a biphasic response: PBO inhibited diazinon toxicity at higher concentrations and enhanced toxicity at lower concentrations. The authors suggested that a low concentration of PBO facilitated diazinon cuticle penetration and that horn fly survival in OP resistant flies was associated with enhanced esterase activity.

Physiological resistance can contribute to the level of resistance of horn flies by increased excretion, reduced penetration of insecticide through its exocuticle, and target site insensitivity (Miller and Adams 1982). In pyrethroid resistant horn flies, target-site insensitivity is the most important contributing factor (Bull et al. 1988). Target-site insensitivity occurs due to a structural point-mutation in the voltage-gated sodium channel gene where the nucleotide phenylalanine is substituted with leucine (L1014F) in domain II of the transmembrane segment II-S6. This mutation is known as "knock down resistance" (kdr) (Miller & Adams 1982) and was described in horn flies by Jamroz et al. (1998) and Guerrero et al. (1998). This identical *kdr* mutation has been recorded in other insects that are resistant to pyrethroids, including *Musca domestica* (L.) (Knipple et al. 1994), *Blatella germanica* (L.) (Miyazaki et al. 1996) and *Drosophilia melanogaster* (Meigan) (Amichot et al. 1992). Another point-mutation that occurs in the sodium channel gene where the amino acid threonine is substituted for methionine (M918T) infers 3-20 fold higher pyrethroid resistance than the *kdr* level is known as *super-kdr* (Williamson et al. 1996a,b). In horn flies, the *super-kdr* mutation described by Guerrero et al. (1998) has never been found without the *kdr* mutation being present (Jamroz et al. 1998, Guerrero et al. 2002, and Foil et al. 2005). Foil et al. (2005) compared the allelic frequencies of *superkdr-kdr* genotypes in field populations of horn flies and found that pyrethroid susceptibility increased from RR-RR>SR-RR>SR-SR>SS-RR>SS-SR>SS-SS. The authors also reported that

a dose of 400 μg/cm^2 for permethrin and 160 μg/cm^2 of λ-cyhalothrin discriminated against SS-SS flies. In addition, the authors reported that the frequency of homozygous-resistant (RR) *kdr* genotypes was a relative predictor of potential fly control.

Temeyer et al. (2008) developed a PCR-based assay for detection of OP target-site resistance in horn flies, and they reported that horn flies had a point mutation in the structural gene encoding AChE from glycine to alanine that occurred at position 262 (G262A) in the HiAchE amino acid sequence. Foil et al. (2010) developed a multiplex PCR-based assay to detect both the G262A mutation and the *kdr* mutation simultaneously. The authors screened horn flies using discriminating concentrations of diazinon at four locations in Louisiana using the discriminating dose bioassay developed by Foil et al. (2005) and genotyped dead and living flies. They reported that horn flies that survived a discriminating concentration had a higher percent of resistant alleles for G262A than those that died.

One way behavioral resistance represents events when insects detect a harmful toxin and move to an area where the toxin is not as concentrated. Horn flies that normally congregated on the backs of cattle have been reported to redistribute on the ventral surfaces of cattle treated with pyrethroid ear tags (Quisenberry et al. 1984, Lockwood et al. 1985). Lockwood et al. (1985) attributed behavioral resistant flies to having a hypersensitivity to pyrethroids, a lowered sensitivity threshold, or both.

Biotic fitness is the result of one or more genetic changes conferring adaptation to a new environment that may induce a fitness cost in the previous environment. Many studies have shown that there are biotic fitness costs associated with insecticide resistance (Ferrari and Georghiou 1981, Argentine et al. 1989, Parello and Trumble 1989, White and Bell 1990). For example, resistant mosquitoes were found to have lower mating success (Berticat et al. 2002), be

more susceptible to natural enemies (Agnew et al. 2004, Foster et al. 1999), more prone to mortality during over-wintering (Foster et al. 1996).

Several studies have described the biotic fitness costs in horn flies resistant to pyrethroids. Scott et al. (1997) reported that susceptible horn flies emerged sooner than resistant flies, susceptible flies pupated two times faster than resistant flies, and resistant flies produced half as many eggs as susceptible flies. Guerrero et al. (2002) compared the effects of rotating pyrethroid and OP ear tags annually in a seven-year study on *kdr* allele frequencies. They reported that rotating ear tag chemistries annually was not effective at controlling development of resistant horn fly populations; weeks of control decreased from 7 to 2 using pyrethroids and 15 to 3 using OPs. The authors proposed that a fitness cost must be associated with the pyrethroid resistant allele because the percentages of resistant genotypes were reduced in the absence of pyrethroid ear tags. One objective of this study was to determine the rate of change in *kdr* and *skdr* allele ratios in horn fly populations in the absence of pyrethroid pressure.

Many different strategies have been proposed for managing horn flies to increase the duration of control and/or to help reduce insecticide resistance levels in horn fly populations. Some strategies that have met with varied levels of success include rotation of insecticide classes within years or between years, the use of synergists, and delivery system modifications. Byford et al. (1987) used pyrethroid/OP mixtures and pyrethroids synergized with piperonyl butoxide and reported poor efficacy, minimal control, and only a marginal decrease in resistant horn flies. Barros et al. (1999) compared different insecticide treatment strategies in the field and their effects on horn fly resistance development over a three-year period. The authors reported that when the same chemistry ear tags were used consecutively, the number of weeks of control dropped from 13 to 3 and from 16 to 6 for pyrethroids and OPs, respectively. Byford et al.

(1987) reported using two insecticides in a mosaic strategy was effective at maintaining efficacy over a 3-year period, but they indicated that the success of using mosaics was limited by horn fly dispersal and dependent upon area-wide cooperation of producers.

Pyrethroid resistant horn flies have been shown to have an enhanced susceptibility to OPs, and under field conditions OPs have been shown to provide adequate control of pyrethroid resistant populations (Byford et al. 1998). Barros et al. (1999) in a seven-year study tested the effects of rotating OP and synergized pyrethroid ear tags annually in two pyrethroid-resistant populations and used efficacy as a measure of resistance. The authors reported efficacy decreased using this strategy for both treatments; the number of weeks with fewer than 50 horn flies per side decreased from 7 to 2 and 4 to 0 using synergized pyrethroid ear tags and 15 to 3 and 10 to 7 using OP ear tags. Foil et al. (1998) evaluated the benefits of using a topical application of ivermectin in conjunction with permethrin ear tags for controlling horn flies. The authors reported that an ivermectin pour-on in conjunction with permethrin ear tags provided 11 weeks of control compared with eight weeks of control provided when pour-on or ear tags were used individually. In a ten-year study, Barros et al. (2001) monitored the development of OP resistance in a population of horn flies. Each year cattle were treated with OP ear tags and the number of weeks of control declined in four years from greater than 20 to 1 week. The cattle were treated with an avermectin the fourth year of the study, and the authors reported 12 weeks of control the next year. The authors also indicated that pre-season resistance ratios to diazinon were not reliable indicators of product efficacy. However, they reported that a discriminating concentration (DC) of 1.72 $\mu g/cm^2$ was a good indicator of product efficacy; when no flies survived the DC OP ear tags provided <10 weeks of control and when 5% of horn flies survived the DC greater than 8 weeks of control were recorded. Oremus et al. (2006) evaluated the effects

of a mid-season avermectin treatment for controlling pyrethroid resistant horn flies in a four-year study at three locations in Louisiana. The authors reported this strategy was effective at one location and provided 11 weeks of control in the year following a mid-season avermectin treatment, and they attributed the lack of efficacy at the other two locations to the high fixed frequency of the *kdr* mechanism in the horn fly populations. One objective of this study was to further test the two-year OP/one-year pyrethroid rotation strategy as a strategy for maintaining susceptibility in horn fly populations to both pyrethroids and organophosphates.

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CHAPTER 2

THE EFFECTS OF APPLYING MID-SEASON AVERMECTIN TREATMENTS TO CATTLE FOR MANAGEMENT OF ORGANOPHOSPHATE RESISTANT HORN FLY (DIPTERA: MUSCIDAE) POPULATIONS

2.1 Introduction

The horn fly, *Haematobia irritans* (L.), is considered to be a major economic pest of cattle throughout Europe, North Africa, Asia Minor, and the Americas. In the United States, annual economic losses due to horn fly damage and control efforts have been estimated at $876 million (Kunz et al. 1991). Damage to cattle from feeding horn flies is due primarily to irritation and blood loss which results in decreased weight gains in growing cattle, decreased weaning weights of calves, and decreased milk production of dairy cows (Campbell 1976, Kinzer et al. 1984).

Many different insecticides from different chemical classes have been used for controlling horn flies (Burns and Wilson 1963, Sheppard 1983, Quisenberry et al. 1984, Kunz and Schmidt 1985). The use of insecticides for horn fly control has led to resistance to insecticides in a number of classes including, organochlorines, organophosphates, pyrethroids, and carbamates (Harris 1964, Sheppard 1983, Quisenberry et al. 1984). Horn fly insecticide resistance remains a major challenge for the cattle industry today. Adult horn flies spend the majority of their lives on cattle, and horn flies from temperate regions have a relatively short life cycle of 10-14 days, which allows them to have 15 or more generations per year (Quisenberry et al. 1984). Therefore, selection for resistant populations of horn flies can occur rapidly.

Organophosphates (OPs) have been used for about 50 years for horn fly control in the United States. The OPs are known to inhibit acetylcholinesterase in insects by binding to the serine hydroxyl at the enzymes active site. In insects, this causes over stimulation of the post-

synaptic nerve with acetylcholine, which leads to tremors and death. The first record of OP resistance in horn flies was associated with the use of back rubbers that contained fenchlorphos (Burns and Wilson 1963). Subsequently, resistance of horn flies to the OP tetrachlorvinphos was shown to occur with the use of insecticide-impregnated ear tags (Sheppard 1983, Harvey et al. 1984).

Several resistance mechanisms to OPs have been reported for horn flies. Until recently, OP resistance in horn flies has been primarily attributed to having an increased metabolism. Guerrero et al. (1999) conducted esterase and acetylcholine esterase (AChE) sensitivity dot blot assays on individual horn flies from populations with varying degrees of OP resistance. They reported that horn flies resistant to diazinon had higher general esterase levels than OP susceptible horn flies. Guerrero et al. (1999) also reported that OP resistant horn flies had multiple specific esterases that were not found in OP susceptible horn flies. Li et al. (2007) reported that horn flies that were resistant to diazinon had higher esterase levels, but also showed evidence of enhanced cytochrome P450 levels. Recently, Temeyer et al. (2008) reported that a point mutation of glycine to alanine at position 262 in the structural gene encoding AChE in the HiAchE amino acid sequence was associated with OP target-site resistance in horn flies collected at Rosepine in 2003 during the time of this study at the same location. Guerrero et al. (1999) reported that OP resistant horn flies had higher AChE insensitivity than susceptible horn flies.

Different horn fly resistance management strategies have been tested including: the use of mixtures of insecticides, rotation of insecticide classes within years or between years, and the use of synergists in combination with insecticides. Little success has been reported using these strategies to prevent resistance development in horn fly populations (Byford et al. 1999, Barros et al. 1999). However, Foil et al. (1998) and Barros et al. (2001) demonstrated that an ivermectin

pour-on in conjunction with insecticidal ear tags increased weeks of control compared to ear tag and pour-on only groups. Foil et al (1998) reported that cattle that were treated with pyrethroid ear tags that had an ivermectin pour-on in mid-summer gained an additional four weeks of control compared to ear tag and pour-on only groups. Barros et al. (2001) demonstrated that using a mid-season avermectin pour-on of all cattle at a location with OP resistant horn flies was associated with increased OP product efficacy in the following year. The study was not repeated, and the authors indicated that the observed increase in control associated with the ivermectin pour-on could have been due to a shorter time of ear tag exposure in the previous year. The purpose of this study was to further evaluate the effectiveness of the use of a mid-season avermectin pour-on to cattle for managing OP resistance in horn fly populations.

2.2 Materials and Methods

Trials were conducted from 1998-2005 at the Louisiana State University Agricultural Center, Rosepine Research Station (30°55'23.4552", -093°16'58.3716"), Rosepine, LA. Approximately 300- 350 adult crossbred cattle and their progeny were maintained each year at this site, and all cattle were treated with OP ear tags, excluding a herd of 20-40 untreated cattle that were used for control counts. A group of 20-40 cattle was tagged with two 40% diazinon ear tags (Patriot®, Boehringer Ingelheim, Ingelheim or Cutter 1® Bayer Animal Health, Shawnee, KS). Certain groups of cattle were treated with ear tags containing diazinon provided by other manufacturers. Treatment groups and the control group were maintained in nonadjacent pastures. All treatment groups were tagged each year in mid-May when horn fly numbers exceeded 50 horn flies per side, and the ear tags were removed the first week of August each year. In years when the treatment threshold of <5 weeks of control (mean number horn flies <50 per side) was exceeded, a mid-season 0.5% ivermectin pour-on (Ivomec® Merial

limited Duluth, GA) was administered to all cattle at the research station at 1 ml/10kg body weight in 1998, 2000 and 2004..

Fly populations on treated and untreated groups were monitored weekly by counting the number of flies per side on ten randomly selected cows from each group with the aid of binoculars before 0830 h. The number of weeks of fly control provided by the diazinon ear tags was established in two ways: number of weeks of product efficacy (WPE) was determined by the number of weeks the mean number of horn flies from treated herds and control herds were significantly different using ANOVA ($p=0.05$). The number of weeks of control (WOC) was determined by the number of weeks the mean number of horn flies from treated herds averaged <50 per side when control numbers averaged >50 per side.

The impregnated filter paper method (Sheppard and Hinkle 1987) was used to determine relative insecticide susceptibility of horn fly populations. Pre-season (PRE) horn fly bioassays were conducted in May, prior to ear tag application, and post-season (POST) bioassays were done in September at least two weeks after ear tags were removed. Technical grade diazinon (Boehringer Ingelheim Animal Health; St. Joseph, MO), and permethrin (FMC; Philadelphia, PA) were used to make stock solutions for two-fold serial dilutions in pesticide grade acetone of 10-11 concentrations ranging from 0.2-400 $\mu g/cm^2$ for permethrin and 0.03-13.76 g/cm^2 for diazinon. One milliliter of each of the insecticide-acetone solutions was applied to filter papers (Whatman no. 1, Whatman, Maidstone, England) and allowed to dry for at least two hours prior to placement into petri dishes. Three sub-replicates were made for each concentration. Horn flies were collected for bioassays from the backs of untreated cattle using an aerial hand net, and approximately 25 flies were aspirated and blown into each dish. Fly mortality was determined after a four-hour exposure period; flies that were unable to walk were considered dead. A

reference susceptible strain obtained from Knipling-Bushland US Livestock Insects Research Laboratory, USDA-ARS (Kerrville, TX) was used each year for comparison. Estimates of 50% mortality (LC_{50}) were made using probit transformation using POLO-PC (LeOra Software 1987), and differences were considered significant when 95% fiducial limits did not overlap. Bioassay data for permethrin and diazinon of PRE, POST, and the reference susceptible population were converted to probits and plotted with concentration on a Log scale for visual comparisons of data. Permethrin and diazinon bioassay data were plotted using probit regression line plots for a year with and without an ivermectin treatment. Comparisons were made between PRE, POST, PRE the following spring and a combined susceptible composite plot for all years. The composite susceptible probit plot was made separately for permethrin and diazinon by combining data from 1998 to 2005. All probit data were corrected for control mortality using Abbot's formula.

Resistance ratio (RR) values were calculated from LC_{50} (treated)/LC_{50} (susceptible). The discriminating concentration (DC) was designated as the lowest concentration of diazinon and permethrin for which 100% mortality was observed for the reference susceptible strain. The percentage of field-collected flies that survived the DC of 1.72 $\mu g/cm^2$ was designated as the resistance frequency (RF) of the population and resistance frequency calculated (RFC) was the percentage of field-collected flies that survived the DC for diazinon and permethrin from each year. Correlation analysis (GraphPad Software Inc. 2000) was done to determine if PRE LC_{50} values, PRE RR or RF for permethrin and diazinon from 1998-2005 were correlated to weeks of control.

2.3 Results

During this study there were no records of ear tag manufacturing problems, and the control achieved using other manufacturers' ear tags containing diazinon did not exceed the level of control for the 40% diazinon ear tags. Ivermectin was administered in 1998, 2000 and 2004. Throughout the study, WPE and WOC were similar, and in six out of eight years, there was not more than a three week difference between the two. In the years following ivermectin treatment, control increased each time from <5 weeks to 6-12 WPE and 9-10 WOC (Table 2.1). For years when ivermectin was not used, WPE and WOC decreased in sequential years of use with the exception of an increase that occurred from 2001 to 2002 for WPE.

The mean number of horn flies in control groups was higher than treated groups in the years before ivermectin treatments were made and counts averaged 77, 39, and 145 for treated groups and 186, 210, and 236 for control groups (Figure 2.1). Each year following an ivermectin treatment, fly counts from the cattle with diazinon ear tags remained low throughout the season; counts averaged 15, 23, and 33 in 1999, 2001, and 2005 while the fly counts from control herds averaged 220, 75, and 250, respectively.

For this study, LC_{50} values for diazinon ranged from 0.3 to 5.8 $\mu g/cm^2$ and goodness of fit χ^2 values were significant PRE four out of eight years and POST six out of eight years, which indicated the data did not fit the binomial model (Table 2.1). In the five years without ivermectin treatments, LC_{50} values for diazinon from PRE to POST increased numerically three years (two significantly) and decreased in two years. When ivermectin was used, LC_{50} values for diazinon increased significantly PRE to POST two years and decreased one year. Each year ivermectin was applied, LC_{50} values for diazinon increased two out of three years POST to PRE although there were no significant changes. In years without ivermectin treatments, LC_{50} values for

diazinon POST to PRE decreased numerically three times (two significant) and increased significantly one year.

From 1998 to 2004, RR for diazinon PRE ranged from 0.8 - 11.3 and for diazinon POST ranged from 1.1- 6.9 (Table 2.2). The DC for diazinon ranged from 0.43-1.72 µg/cm^2. Two RF values were used for comparison: RFC-

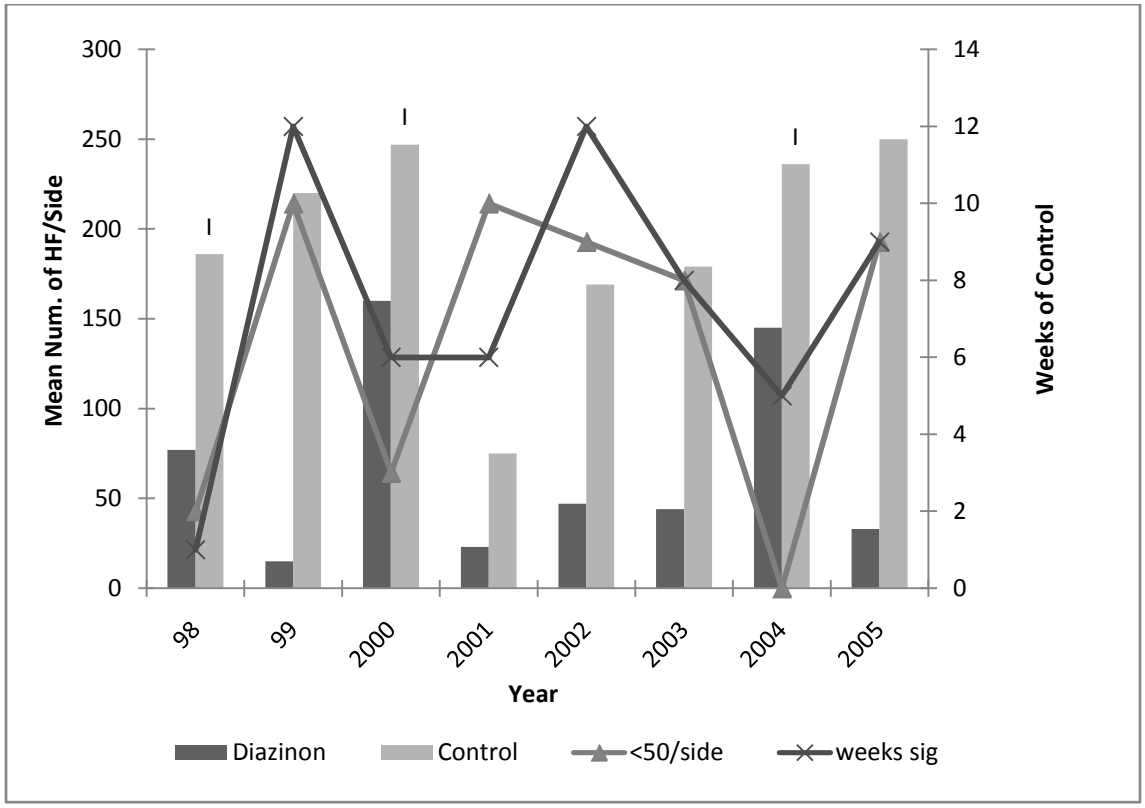

Figure 2.1. Mean horn fly counts from 1998-2005 with and without 40% diazinon ear tags, years ivermectin was applied (I=trt with ivermectin), number of weeks the mean number of horn flies were <50/side and number of weeks treatments were significantly different (p=0.05).

Table 2.1 Horn fly susceptibility and weeks of control at Rosepine using diazinon ear tags

Year	Time	Diazinon LC_{50} (95% FL) µg/cm^2	χ^2	DF	RR[a]		Perm LC_{50} (95% FL) µg/cm^2	χ^2	DF	RR[a]	WOC (WPE)[b]
1998[c]	PRE[d]	0.8 (0.6-1.1)	2.9	4	3.0		5.5 (4.3-6.8)	4.1	5	5.9	2(1)
	POST[e]	1.6 (1.4-1.9)	3.5	4	5.9		4.3 (3.6-5.1)	9.4	5	4.6	
1999	PRE	1.7 (1.6-1.8)	6.7	6	3.6		1.7 (1.5-2.0)	1.0	4	1.6	10(12)
	POST	1.8 (1.6-2.1)	19.6[g]	6	3.5		1.7 (1.4-1.9)	12.1[g]	5	1.6	
2000[c]	PRE	0.3 (0.2-0.4)	2.6	5	1.8		2.9 (2.0-3.9)	41.9[g]	8	2.0	3(6)
	POST	0.7 (0.5-0.8)	12.4[g]	5	1.1		2.5 (1.8-3.3)	15.7[g]	6	1.7	
2001	PRE	0.4 (0.3-0.5)	13.4[g]	5	1.8		1.4 (0.5-3.3)	165.8[g]	7	6.4	10(6)
	POST	2.6 (2.3-3.0)	10.8	7	6.9		4.5 (4.1-5.0)	5.1	5	20.5	
2002	PRE	1.2 (1.0-1.4)	14.9[g]	6	3.1		2.6 (2.2-2.9)	1.3	5	11.8	9(12)
	POST	2.5 (1.6-3.2)	27.4[g]	6	6.5		2.7 (1.2-5.3)	141.7[g]	7	12.2	
2003	PRE	1.7 (1.1-2.7)	223.3[g]	28	5.2		2.1 (1.6-2.7)	83.4[g]	34	7.1	6(8)
	POST	0.7 (0.4-1.1)[f]	178.4[g]	28	2.1		1.1 (0.9-1.4)	51.5[g]	34	3.7	
2004[c]	PRE	3.5 (3.1-3.9)	25.5	28	11.3		7.9 (3.1-18.9)	672.3[g]	34	13.0	0(5)
	POST	1.0 (0.4-3.2)	659.7[g]	28	3.2		5.6 (4.0-7.9)	144.8[g]	34	9.2	
2005	PRE	5.8 (2.1-20.0)	4622.2[g]	28	3.8		4.4 (2.4-7.7)	489.7[g]	34	3.3	9(9)
	POST	4.8 (3.9-5.6)	70.8[g]	28	3.1		3.9 (3.0-4.9)	43.1	34	3.0	

[a] Resistance Ratio (LC_{50} from field population/ LC_{50} from Kerrville reference susceptible colony strain)
[b] WOC= number of weeks horn fly means were less than 50 flies/side; WPE =number of weeks mean number of horn fly counts were significantly different p=0.05.

[c] Designates treatment of ivermectin pour-on
[d] Flies collected before treatment of the animals with ear tags (PRE)
[e] Flies collected at least two weeks after tags are removed (POST)
[f] Data are too heterogeneous to calculate 95% fiducial limits: 90% fiducial limits used instead (LeOra Software, 1987)
[g] χ^2 significant at p=0.05

Table 2.2. Resistance frequency, discriminating concentration, resistance ratio, and weeks of control of horn flies at Rosepine using diazinon ear tags

Year	DC[a] µg/cm^2	RFC[b] %	RF[b] %	Diazinon PRE RR[c]	Permethrin PRE RR[c]	WOC[f]
1998[d]	0.86	1.7	7.6	3.0	5.9	2
1999	1.72	42.9	42.9	3.6	1.6	10
2000[d]	1.72	0.8	0.8	0.8	2.0	3
2001	0.43	21.0	0	1.8	6.4	10
2002	0.86	84.1	11.5	3.1	11.8	9
2003	1.72	64.3	64.3	5.2	7.1	6
2004[d]	0.86	92.5	88.1	11.3	13.0	0
2005	1.72	—[e]	—[e]	—[e]	3.3	9

[a] Discriminating concentration (DC) lowest dose at which 100% mortality was observed from Kerrville reference susceptible strain

[b] Resistance frequency (RFC), percentage of flies surviving a DC for diazinon for each year from Kerrville reference susceptible strain, RF calculated using DC of 1.72 µg/cm^2

[c] PRE RR=(LC_{50} from field population/ LC_{50} from Kerrville reference colony strain)

[d] Ivermectin pour-on applied after ear tags were removed

[e] Not calculated

[f] the number of weeks the mean number of horn flies from treated herds averaged <50 per side when control numbers averaged >50 per side.

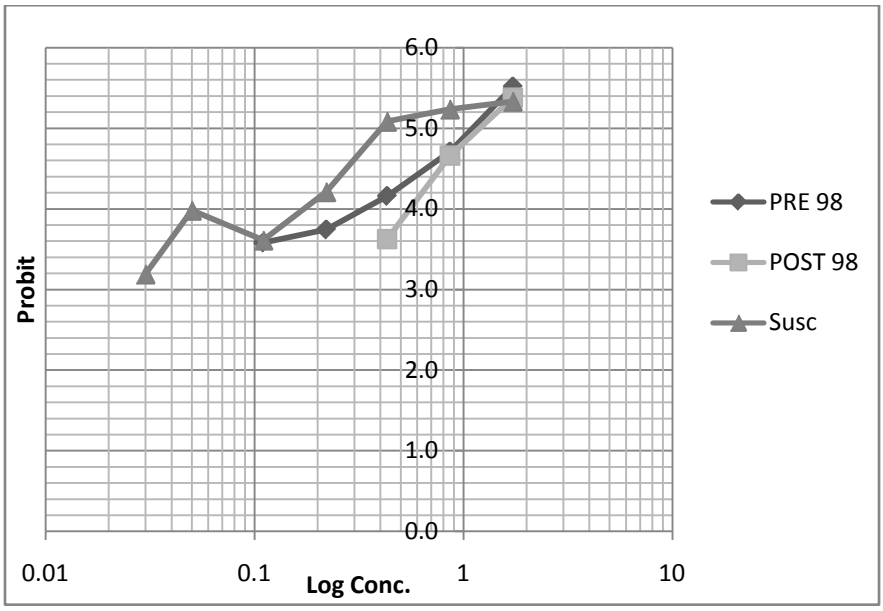

Figure 2.2. Probit plots of diazinon bioassay mortality data for horn flies collected in the field PRE and POST at Rosepine a year cattle were treated with a mid-season ivermectin treatment, PRE the following year and a reference susceptible strain

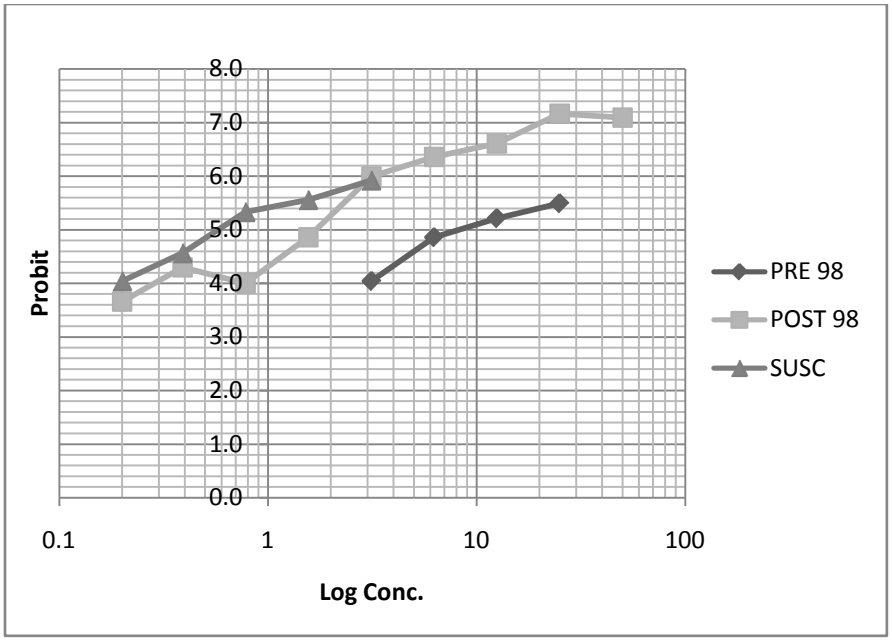

Figure 2.3. Probit plots of permethrin bioassay mortality data for horn flies collected in the field PRE and POST at Rosepine a year cattle were treated with a mid-season ivermectin treatment, PRE the following year and a reference susceptible strain

LC_{50} values for permethrin decreased numerically PRE to POST. In years without ivermectin treatments, two years LC_{50} values for permethrin increased POST to PRE significantly and decreased

for two years (one significantly). Values for LC_{50} for permethrin decreased from POST to PRE each year ivermectin was applied and the decrease was significant one year.

Resistance ratios for permethrin from 1998 to 2005 for PRE ranged from 1.6 - 13.0 and POST 1.6 - 20.5 (Table 2.1). Correlations for PRE RRs for permethrin and weeks of control were not significant (p=0.61). There was a significant negative correlation (p=0.0055) for PRE LC_{50} values for permethrin to weeks of control (r= -0.87) with 95% confidence intervals (-0.98- -0.42) r^2 = 0.75.

Correlations were not significant for PRE LC_{50} values between diazinon and permethrin (p=0.27), PRE LC_{50} values for diazinon and WOC (p=0.53), PRE RR values for diazinon and WOC (p=0.53), diazinon RF and WOC (p=0.45), or diazinon RFC and WOC (p=0.99).

The slopes from probit plots of mortality data for flies exposed to diazinon when ivermectin was applied increased each year from PRE to POST as in Figure 2.2. When no ivermectin treatments were made the slopes decreased each year PRE to POST except in 2001 slopes increased 37% from 1.21 to 1.66. The slopes from probit plots of mortality data for permethrin PRE to POST for years when ivermectin was applied as in Figure 2.3 increased two years and decreased in one year. For years when no ivermectin treatments were made slopes for probits from permethrin data numerically increased three and decreased two years PRE to POST.

2.4 Discussion

The strategy of applying a mid-season avermectin pour-on to cattle to manage OP resistance in horn flies was shown to be effective at Rosepine. The mean number of horn flies on cattle the

spring after ivermectin treatments was made decreased by an average 73% compared to the year before. Following an ivermectin treatment, the number of WPE and WOC increased an average 370% and 511%, respectively, when compared to the years before. When ivermectin treatment was not made, the number of WPE and the number of WOC decreased the following year in all instances except for one (Table 2.1). The increase in the number of WOC and decrease in number of horn flies suggests that mid-summer treatment of cattle with ivermectin is a viable way to manage OP resistant horn fly populations. Lysyk and Colwell (1996) reported that treatment of cattle with diazinon ear tags and an ivermectin pour-on did not increase the level of control of horn flies when compared to tags alone, although the duration of control increased for that year. Barros et al. (2001) reported (at Rosepine) a 50% increase in the number of weeks of control above the previous year to 14 weeks using 40% diazinon ear tags following an ivermectin treatment.

There are at least two reasons why ivermectin treatments could result in increased horn fly control in the following season: 1) ivermectin acts both as an adulticide and larvicide against the horn fly (Lysyk and Colwell 1996) and 2) ivermectin has a different mode of action than diazinon. Miller et al. (1981) reported seven weeks of larval control when cattle were treated with an ivermectin pour-on, and Meyer et al. (1980) reported adult horn fly control for up to eight weeks using ivermectin. Target-site cross-resistance between OPs and avermectins is not likely since OPs act by inhibiting the action of acetylcholinesterase in nerve cells, and avermectins act as an antagonist for the neurotransmitter glutamate. Therefore, a population of adult OP resistant horn flies and their progeny could theoretically be eliminated by an ivermectin treatment and any population resurgence that occurred would be from susceptible horn flies. Two possible sources for susceptible horn flies in the years following an ivermectin treatment would be from immigration of susceptible horn flies from surrounding farms or susceptible horn flies from the local population.

Since all cattle were treated with ivermectin pour-on at Rosepine, the former was the more probable source.

Similar to this study, Oremus et al. (2006) tested the effectiveness of using a mid-season avermectin (doramectin) pour-on of all cattle to manage pyrethroid resistant horn flies at three locations. Oremus et al. (2006) reported significant gains were not detected in the number of WOC at two out of three locations, but at one location a significant increase in the number of WOC from 2 to 13 was reported following a doramectin treatment. The lack of control at the other two locations was attributed to the absence of a susceptible refugia of horn flies at the farm or from nearby farms.

There were no trends in diazinon changes in bioassay results that matched the changes in the levels of control of flies using diazinon ear tags or the expected changes when the OP tags and ivermectin were used. In the five years that ivermectin was not used, LC_{50} values for diazinon spring to fall numerically increased three years and decreased two years. Other studies also have shown that during diazinon ear tag exposure periods, diazinon susceptibility levels measured by bioassays do not change significantly. Barros et al. (2001) conducted a ten year study at Rosepine with cattle treated yearly with diazinon ear tags and reported that LC_{50} values for diazinon increased from spring to fall in six out of ten years; the increases were significant in only two years. During this study, when ivermectin treatments were made, LC_{50} for diazinon values increased significantly in two out of three years. Therefore, in none of these studies did the differences in diazinon bioassay LC_{50} values from spring to fall correspond with expected changes associated with insecticide use.

From spring to fall, the bioassay LC_{50} values for permethrin did not have any noticeable patterns in relation to cattle treated annually with diazinon ear tags or ivermectin treatments.

Susceptibility to permethrin increased each year ivermectin treatments were made, although the decreases in LC_{50} values were not significant. In the five years that ivermectin was not used, LC_{50} values for permethrin from spring to fall numerically increased three years and decreased two years. When horn flies are exposed to cattle with pyrethroid ear tags, pyrethroid susceptibility generally decreases. For example, Guerrero et al. (2002) conducted an ear tag rotation study using cattle tagged in alternating years with OP and pyrethroid ear tags; the authors reported that during each pyrethroid exposure period, pyrethroid susceptibility decreased from spring to fall.

From fall to spring, susceptibility to permethrin increased four out of seven years and diazinon susceptibility increased three out of seven years. Barros et al. (2001) reported LC_{50} values for diazinon decreased POST to PRE each year and three years the decreases were significant. The authors attributed the observed decreases to a biotic fitness costs associated with horn flies resistant to diazinon or to an increase in the number of immigrating susceptible horn flies. Similarly it is probable that during this study, the observed increased susceptibility from fall to spring could be due to biotic fitness costs associated with resistant horn flies and/or an influx of susceptible horn flies.

Bioassay data (PRE LC_{50}s, PRE RRs, RFCs and RF values) for diazinon and permethrin were not useful as a predictor of diazinon ear tag efficacy for number of WOC or WPE with the exception of PRE LC_{50} values for permethrin. There was a significant negative correlation between PRE LC_{50} values for permethrin and number of WOC. A possible reason that PRE LC_{50} values for permethrin were negatively correlated to number of WOC could be due to positive cross-resistance for OPs and pyrethroids, which would indicate permethrin susceptibility was equal to diazinon susceptibility, possibly due to levels of cytochrome P450s or esterases. Byford et al. (1987)

reported that horn flies resistant to pyrethroids were cross-resistant to stirofos and the authors attributed the cross-resistance to increased levels of detoxifying enzymes.

There are several possible reasons that the diazinon bioassay data was not correlated to the number of weeks of control for cattle treatment data. First, horn fly exposure to diazinon on the cattle is very different than that in a petri dish, and for different substrates like hair and filter paper compounds may release at different rates. Second, due to insecticide concentration gradients that exist on animals treated with ear tags (Miller et al. 1983), horn flies on treated cattle are not confined to a single concentration. Horn flies exposed to concentration gradients of diazinon or other xenobiotics over an extended period of time could be protected by induced metabolic activity. Exposure of horn flies to a high fixed concentration of diazinon for a short period potentially may not allow time for induction of metabolic enzyme production. Also, quantitative age-related differences may exist for resistant horn flies for responding to xenobiotics. If inducible metabolic enzymes allow resistant flies to live on treated cattle, then newly emerged resistant horn flies would likely have higher metabolic enzyme levels than would unchallenged older horn flies. Additionally, behavioral resistance of horn flies in response to concentration gradients of insecticides that exist on the cow also could contribute to a lack of correlation between bioassay and control data. In the petri dish, the horn flies are exposed to high concentrations of diazinon with no option to move away from it. On the animal, resistant horn flies may be able to move away from toxic concentrations of insecticides (Sparks et al. 1985). Barros et al. (2001) reported that the diazinon bioassay was not a reliable indicator of product efficacy in the field. Barros et al. (2001) evaluated other OPs in addition to diazinon bioassays of horn flies and they reported that the bioassay data for all OPs tested were similar. Therefore, the choice of diazinon other than another OP for the bioassays was not a factor in the low utility of the assays.

The χ^2 goodness-of-fit test demonstrated for most years in the spring and fall that the populations were genetically heterogeneous. Therefore, bioassay data were plotted using probit regression lines, which could reveal additional information about the horn fly population's response to treatments. For years that ivermectin treatments were made, slopes from the probit plots increased for both diazinon and permethrin from spring to fall with the exception of one year for permethrin. The increase in slope indicated that the population had become more homogenous, probably due to the reduction in resistant horn flies due to the ivermectin treatments. Robertson (2007) stated, "a probit regression line with a plateau is characteristic of genetically heterogeneous populations and is often observed in groups that contain resistant and susceptible individuals". In years when no ivermectin treatments were made, results were variable and there were no repeatable patterns of interest for either diazinon or permethrin.

We found that mid-season ivermectin treatments of cattle were useful in managing OP resistant horn fly populations. Since both larval and adult horn flies are controlled when all cattle are treated with ivermectin in mid-season, resistant horn fly populations can be suppressed for up to 10 weeks. If susceptible flies from nearby farms subsequently repopulate the cattle, then control can be restored. Since this study and Barros et al (2001) were both conducted at Rosepine, future studies at other locations on the effects of mid season treatment of cattle with avermectins to manage OP resistant horn fly populations should be conducted. Studies to determine the biotic fitness costs associated with OP target-site resistance also would be useful for developing effective control strategies.

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CHAPTER 3

EVALUATION OF A TWO-YEAR ORGANOPHOSPHATE/ONE-YEAR PYRETHROID EAR TAG ROTATION STRATEGY FOR RESISTANCE MANAGEMENT OF HORN FLIES

3.1 Introduction

The horn fly, *Haematobia irritans* (L.), is an important insect pest of cattle, which costs producers annually an estimated $876 million due to costs of control and economic loss (Kuntz et al. 1991). Damage to cattle from feeding horn flies is due primarily to irritation and blood loss which results in decreased weight gains in growing cattle, decreased weaning weights of calves, and decreased milk production of dairy cows (Campbell, 1976, Kinzer et al., 1984).

Horn flies spend the majority of their adult lives on cattle, and historically efforts to control the horn fly have been primarily from insecticide use. Insecticides are applied to cattle primarily by direct application or use of insecticidal ear tags. Insecticide-impregnated ear tags were first used for horn fly control in the 1970's (Harvey and Brethour 1970). The use of ear tags was proposed to be a more environmentally friendly method of treating cattle for ectoparasites than the use of sprays or whole-body dips because of reduced environmental contamination. Further, the sustained insecticide release rate from ear tags also provided longer periods of control (Kunz and Schmidt 1985). The first ear tags used for horn fly control contained stirofos, which provided up to 20 weeks of control (Ahrens and Cocke 1979). However, resistance to stirofos was reported for horn fly populations within a few years after the first use of the ear tags (Shepard 1983). Subsequently, pyrethroid ear tags were developed and provided up to 16 weeks of horn fly control, but pyrethroid resistance in horn fly populations was reported within five years of tag use (Schmidt et al. 1985).

Factors that have contributed to the selection of resistant horn fly populations to multiple chemistries include using insecticides of the same chemical class each year, failure to use the

recommended number of ear tags per animal, and failure to remove ear tags when they were no longer effective (Burns and Wilson 1963, Shepard 1983, Quisenberry et al. 1984, Kunz and Schmidt 1985). The biological characteristics of horn flies that contribute to the selection of resistant horn fly populations with insecticide use include: 1) adult horn flies spend the majority of their lives on cattle and 2) horn flies can have as many as 15 generations per year (Quisenberry et al. 1984).

Different insecticide management strategies have been proposed to manage resistance in horn flies including: using mixtures of insecticides, synergists in combination with insecticides, mosaics of insecticides (different chemistries for different herds within a production area), and rotation of insecticide classes within years or between years. Byford et al. (1987) reported poor efficacy, minimal control, and only a marginal decrease in resistant horn flies using pyrethroid/organophosphate mixtures and pyrethroids synergized with piperonyl butoxide. Byford et al. (1987) reported that using mosaics of pyrethroids and organophosphates (OPs) was preferred to sequential use of only one chemistry or mixtures of chemistries for controlling horn flies, but they indicated that the success of using mosaics was limited by horn fly dispersal and dependent upon area-wide cooperation of producers. Pyrethroid resistant horn flies have been shown to have enhanced susceptibility to OPs; and under field conditions, OPs have been shown to provide partial reversion of pyrethroid resistant populations (Byford et al. 1999).

The management strategy of rotating between pyrethroids and OPs each year for controlling horn flies was evaluated in a seven-year study (Barros et al. 1999), and the authors reported that the number of weeks of control steadily declined from 5 to 3 to 1 and 7 to 2 to 0 when λ-cyhalothrin ear tags were used at two locations in spite of OP ear tag use in alternate years. In the alternate years when OP ear tags were used, the number of weeks of control decreased from 15 to 4 to 3 at one

location, while the number of weeks of control decreased from 10 to 5 and then increased to 7 at the other location. Since annual rotations were found not to be effective at preventing the development of permethrin or OP resistance in horn flies, Byford et al. (1998) recommended a rotation strategy of using OP ear tags in two consecutive years followed by pyrethroid ear tags in one year. Byford et al. (1998) reported that a partial reversion of susceptibility to permethrin occurred and the number of weeks of control with permethrin ear tags increased from zero to four after using OP ear tags for the two years between. The purpose of this study was to test the two-year OP/one-year pyrethroid rotation strategy as a strategy for maintaining susceptibility in horn fly populations to both pyrethroids and organophosphates.

3.2 Materials and Methods

Trials were conducted from 1999-2006 at two Louisiana State University Agricultural Center Stations; Hill Farm Research Station (33°01'37.5168", -093°03'15.1164"), Homer, La , and New Iberia Research Station (29°54'53.8668", -091°39'48.3372"), Jeanerette, La. Adult crossbred cattle and their progeny (approximately 300-350) were maintained each year at each site. All cattle at each location were treated with either OP or pyrethroid ear tags each year excluding a herd of 20-40 untreated cattle, which were used for control counts. Control cattle were kept in pastures that were not adjacent to the treated cattle. At Hill Farm, pyrethroid ear tags were used in 2000, 2003, and 2006 and OP ear tags were used in 2001, 2002, 2004, and 2005. Additional treatments were made at Hill Farm in 2005 and 2006 in July, August, and September; in 2005 cattle were also treated with a 1% dichlorvos spray (Vapona® Insecticide Dairy Cattle Spray, PBI/ Gordon Corp, Kansas City, MO) and in 2006 cattle were treated with a pyrethroid pour-on containing 1% permethrin and 1.0% piperonyl butoxide (Control Solutions, Inc., Pasadena, TX). At Iberia, permethrin ear tags were used in 1999, 2002, and 2005 and OP ear tags were used in 2000, 2001,

2003, and 2004. Depending upon the chemical rotation, a treatment group of 20-40 cattle was tagged with either one 40% diazinon (Patriot®, Boehringer Ingelheim, Ingelheim) ear tag or two 10% permethrin (Gardstar Plus®, Y-Tex Corp, Cody, WO) ear tags. In 2000 at Hill Farm, different 10% permethrin (Atroban®, Schering-Plough, Kenilworth, NJ) ear tags were used. All treatment groups were tagged each year in mid-May when horn fly numbers exceeded 50 horn flies per side (except in 2006 at Hill Farm when cattle were tagged the 15th of June), and ear tags were removed in September of each year. The rotation schedule at both locations began with permethrin ear tags followed by two years of OP ear tags; permethrin ear tags were used every third year until the end of the study. The trials ran from 1999 to 2005 at Iberia and 2000 to 2006 at Hill Farm.

Weekly observations of fly populations on treated and untreated groups were made by counting the number of flies per side on ten randomly selected cows from each group with the aid of binoculars before 0830 h. The number of weeks of fly control provided by the ear tags was established two ways: number of weeks of product efficacy (WPE) was determined by the number of weeks the mean number of horn flies/side from treated and control cattle were significantly different using ANOVA ($p<0.05$), and the number of weeks of control (WOC) was determined by the number of weeks the mean number of horn flies/side were <50 per side in treated herds and >50 per side in the control herds.

The impregnated filter paper method (Sheppard and Hinkle1987) was used to determine relative insecticide susceptibility of horn fly populations. Pre-season (PRE) horn fly bioassays were conducted in May, prior to insecticide exposure, and post-season (POST) bioassays were done at least two weeks after ear tags were removed in September. Stock solutions were made from the technical grade insecticides diazinon (Boehringer Ingelheim Animal Health, St. Joseph, MO), permethrin (FMC, Philadelphia, PA) and in 2006 *lambda*-cyhalothrin (Schering-Plough) in

pesticide-grade acetone. Two-fold serial dilutions of 10-11 concentrations for each insecticide were made ranging from 0.2-400 µg/cm^2 for permethrin and 0.03-13.76 µg/cm^2 for diazinon. One milliliter of the insecticide-acetone solutions was applied to filter papers (Whatman no. 1, Whatman, Maidstone, England), which were allowed to dry for at least two hours prior to placement into petri dishes. Three sub-replicates were made for each concentration. Horn flies were collected for bioassays from the backs of untreated cattle using an aerial hand net and approximately 25 flies were aspirated and blown into each dish. Fly mortality was determined after a four-hour exposure period; flies unable to walk were considered dead.

Data were analyzed by probit analysis using POLO-PC (LeOra Software 1987). The lethal concentration that would kill 50% of the population (LC_{50}) was calculated and differences were considered significant when 95% fiducial limits did not overlap. Resistance ratios were calculated by LC_{50} (treatment)/LC_{50} (susceptible) using a reference susceptible strain obtained from Knipling-Bushland US Livestock Insects Research Laboratory, USDA-ARS (Kerrville, TX). Linear regression and correlations (InStat®, 2009) were made between pre-season RR's, RF's and LC_{50}s for permethrin and diazinon and number of weeks of control. Bioassay data were plotted using probit regression line plots. All susceptible bioassay data during this study were combined to make a composite probit plot for comparison.

3.3 Results

During this study, the mean number of horn flies on the untreated control herds was significantly higher at Hill Farm ($p<0.0001$) and Iberia ($p=0.0041$) when compared to the mean number of horn flies on cattle treated with diazinon or permethrin ear tags. The mean number of horn flies per side for each group from greatest to least at both locations were untreated control

herds > diazinon > permethrin; at Hill Farm, the means were 109 > 41 > 33 (Figure 3.1) and at Iberia the means were 86 > 30 > 16 (Figure 3.2).

At Hill Farm when permethrin ear tags were used, WPE reduced from 11 to 6 to 2 and WOC reduced from 11 to 5 to 3 in spite of two years of OP ear tag use in between the second and third use of permethrin ear tags (Figure 3.1). When OP ear tags were used at Hill Farm in 2 consecutive years, WPE decreased from 12 to 8 and then from 8 to 3 weeks and WOC reduced from 9 to 6 and then increased 10 to 5 weeks (Figure 3.1). At Iberia, when permethrin ear tags were used, WPE increased each year permethrin ear tags were used from 5 to 7 to 10, and WOC increased from 6 to 10 weeks and then decreased to 7 (Figure 3.2). When OP ear tags were used at Iberia in 2 consecutive years, WPE increased from 6 to 9 and then decreased from 8 to 3, and WOC decreased from 9 to 7 and then remained unchanged at 7 weeks in 2003 and 2004.

The LC_{50} values for permethrin at Hill Farm during this study ranged from 1.8 to >400 µg/cm^2 and RRs ranged for permethrin from 1.8 to >3333.0 (Table 3.1). Values for χ^2 from bioassay data for permethrin at Hill Farm were significant (p=0.05) PRE and POST each year with the exception of POST 2006 (Table 3.1). When permethrin ear tags were used at Hill Farm, LC_{50} values for permethrin increased (significantly 2 out of 3 years) from PRE to POST from 3- to 50- to >222-fold. When OP ear tags were first used at Hill Farm following permethrin ear tags in 2001 and 2004, LC_{50}s for permethrin decreased significantly PRE to POST 6- and 3-fold and RRs decreased 71- and 3-fold. The second year OP ear tags were used at Hill Farm following permethrin ear tags, LC_{50} values and RRs for permethrin increased PRE to POST. At Hill Farm for years diazinon ear tags were used, LC_{50} values for permethrin decreased POST to PRE 3 out of 4 years, and the decreases were significant in 2002 and 2005 each time OP

Table 3.1. Summary of bioassay data and weeks of control at Hill Farm

Year	Ear Tag	Bioassay Time[a]	Permethrin LC_{50} µg/cm^2 (95% FL)	χ^2	DF	RR[a]	Diazinon LC_{50} µg/cm^2 (95% FL)	χ^2	DF	RR	Weeks Control[b]
2000	PYR	Pre[c]	3.5 (1.9-5.8)	88.5 [f]	8	-	0.9 (0.8-1.0)	9.7	6	1.5	11 (11)
		Post[d]	10.4 (4.6-19.3)	59.1 [f]	0	-	1.0 (0.7-1.3)	15.1 [f]	6	1.6	
2001	OP	Pre	44.8 (31.4-64.5)	24.0 [f]	0	128	0.8 (0.4-2.0)[e]	196.6 [f]	6	4.0	12 (9)
		Post	8.1 (3.6-16.0)	47.7 [f]	0	1.8	2.1 (1.5-3.0)	17.8 [f]	7	10.5	
2002	OP	Pre	9.7 (5.2-16.2)	63.0 [f]	7	54.5	2.3 (1.8-3.0)	4.5	3	5.8	8 (6)
		Post	12.1 (7.6-17.6)	2.3 [f]	5	59.0	4.0 (2.2-6.3)[f]	9.8 [f]	2	10.0	
2003	PYR	Pre	3.6 (1.6-6.4)	216.4 [f]	4	12.0	1.4 (1.0-1.8)	91.4 [f]	8	4.7	6 (5)
		Post	178.2 (133.1-255.8)	52.4 [f]	4	594.0	1.2 (1.0-1.5)	86.1 [f]	8	4.0	
2004	OP	Pre	141.2 (90.9-247.7)	54.5 [f]	1	235.3	6.7 (4.0-11.8)	90.2 [f]	8	-	8 (11)
		Post	52.0 (33.9-76.7)	65.9 [f]	4	86.7	3.1 (2.3-3.8)	43.4 [f]	3	10.3	
2005	OP	Pre	19.7 (5.2-57.2)	160.5 [f]	4	15.2	7.9 (3.5-70.7)	237.4 [f]	8	5.3	3 (5)
		Post	29.8 (23.1-38.2)	69.3 [f]	4	22.9	6.3 (NC)	1074.1 [f]	8	4.2	
2006	PYR	Pre	1.8 (0.9-3.1)	112.6 [f]	4	14.7	0.7 (0.6-0.8)	105.2 [f]	8	4.7	2 (3)
		Post	>400	49.0 [f]	4	>3,333.0	5.7 (4.8-6.5)	33.1	8	38.2	

[a] Resistance ratio (LC_{50} from field population/ LC_{50} from Kerrville reference colony strain)
[b] Number of weeks mean horn fly counts were significant from controls (p=0.05), ( ) = less than 50 flies/side.
[c] Flies collected before treatment of the animals with ear tags (PRE)
[d] Flies collected at least two weeks after tags are removed (POST)
[e] Data are too heterogeneous to calculate 95% fiducial limits 90% fiducial limits used instead (LeOra Software, 1987)
[f] χ^2 significant at p=0.05

Table 3.2. Summary of bioassay data and weeks of control at Iberia

Year	Ear Tag	Bioassay Time[a]	Permethrin LC_{50} (95% FL) µg/cm^2	χ^2	DF	RR[a]	Diazinon LC_{50} (95% FL) µg/cm^2	χ^2	DF	RR	Weeks Control[b]
1999	PYR	Pre[c]	6.3 (3.8-9.7)	44.8[f]	9	6.1	0.8 (0.7-0.9)	6.9	6	5.4	6 (5)
		Post[d]	7.8 (5.2-11.7)	39.3[f]	9	7.5	2.3 (1.9-2.8)	6.5	6	4.2	
2000	OP	Pre	15.8 (8.4-23.5)	25.7[f]	9	10.7	0.3 (0.2-0.5)	18.2	5	0.3	9 (6)
		Post	2.4 (1.6-3.4)	8.0	9	1.6	0.6 (0.4-0.7)	19.6	6	0.9	
2001	OP	Pre	17.3 (14.4-20.5)	16.6	0	-	0.5 (0.4-0.6)	9.1	4	3.0	7 (9)
		Post	10.0 (6.3-15.0)	40.0 f	10	-	1.7 (1.6-1.9)	3.6	6	10.6	
2002	PYR	Pre	20.7 (12.0-33.2)	91.9[f]	0	93.7	2.5 (1.9-3.5)	10.3	6	6.5	10 (7)
		Post	55.6 (28.3-80.2)[e]	65.1[f]	9	252.0	2.7 (2.0-4.0)	33.3[f]	7	7.0	
2003	OP	Pre	52.2 (35.0-83.0)	238.6[f]	34	175.3	3.1 (2.7-3.5)	62.7[f]	28	9.4	7 (8)
		Post	50.7 (12.1-134.7)	166.3[f]	4	170.3	0.6 (0.5-0.7)	23.9	28	1.8	
2004	OP	Pre	54.9 (40.0-75.3)	57.9[f]	34	90.4	2.7 (1.7-4.7)	177.7[f]	28	8.8	7 (3)
		Post	30.4 (22.8-39.5)	32.9	30	50.0	1.9 (1.7-2.2)	32.6	28	6.3	
2005	PYR	Pre	9.5 (5.7-13.9)	81.2[f]	4	7.2	4.8 (3.1-5.7)	88.1[f]	28	53.0	7(10)
		Post	83.3 (61.8-107.7)	43.7	4	63.1	4.0 (3.2-4.7)	49.3[f]	28	2.6	

[a] Resistance ratio (LC50 from field population/ LC_{50} from Kerrville reference colony strain)
[b] Number of weeks average horn fly counts were less than 50 flies/side, ( ) weeks of control significant p=0.05.
[c] Flies collected before treatment of the animals with ear tags (PRE)
[d] Flies collected at least two weeks after tags are removed (POST)
[e] Data are too heterogeneous to calculate 95% fiducial limits 90% fiducial limits used instead (LeOra Software, 1987)
[f] χ^2 significant at p=0.05

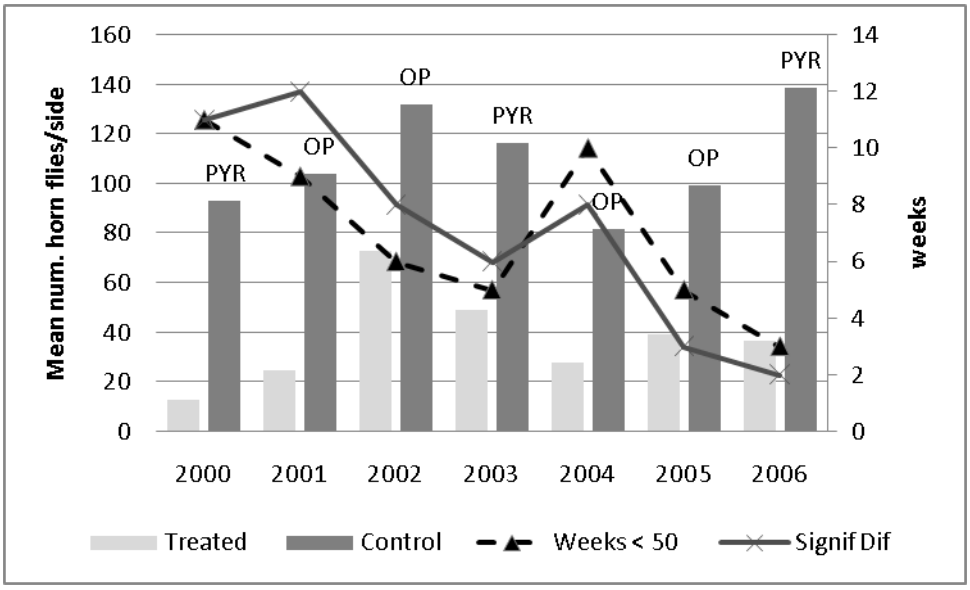

Figure 3.1. Hill Farm horn fly treatments, mean number of horn flies per side, number of weeks mean number of horn flies < 50 per side and number of weeks mean number of horn flies were significantly different among treatments

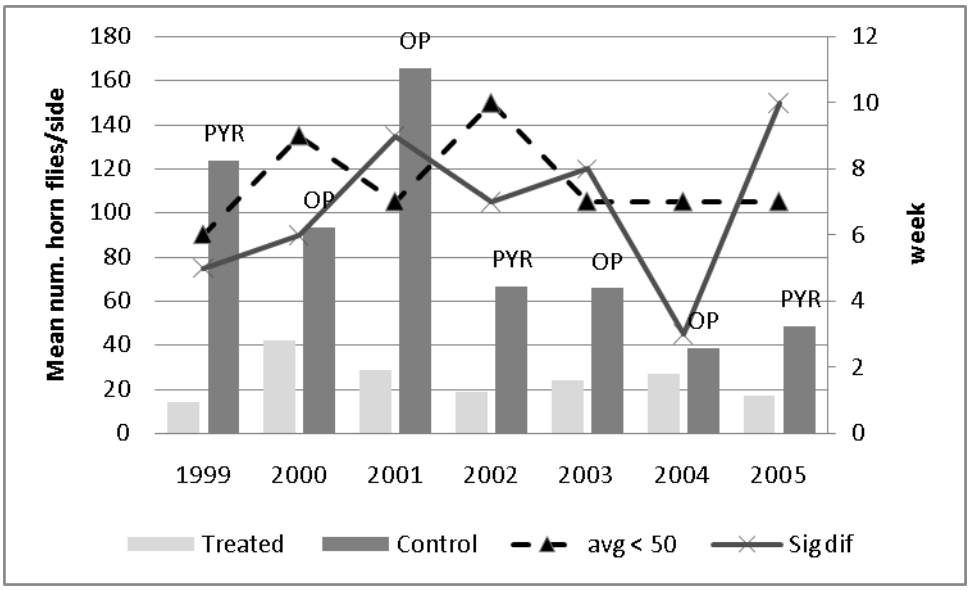

Figure 3.2. Iberia horn fly treatments, mean number of horn flies per side, number of weeks mean number of horn flies < 50 per side, and number of weeks mean number of horn flies were significantly different among treatments

ear tags were used two years consecutively. When pyrethroid ear tags were used, the POST to PRE LC_{50} values for permethrin increased significantly the first year permethrin ear tags were used and decreased the second year. increased PRE to POST. At Hill Farm for years diazinon ear tags were used, LC_{50} values for permethrin decreased POST to PRE 3 out of 4 years, and the decreases were significant in 2002 and 2005 each time OP ear tags were used two years consecutively. When pyrethroid ear tags were used, the POST to PRE LC_{50} values for permethrin increased significantly the first year permethrin ear tags were used and decreased the second year.

During this study at Hill Farm, LC_{50} values for diazinon ranged from 1.5 to 7.9 $\mu g/cm^2$, and RRs ranged from 0.7 to 38.2 (Table 3.1). Values for χ^2 from bioassay data for diazinon at Hill Farm were significant ($p=0.05$) five out of seven years PRE and six out of seven years POST. When permethrin ear tags were used, no significant changes occurred PRE to POST for LC_{50} values for diazinon with the exception of 2006 when an eight-fold increase occurred. When permethrin ear tags were used, POST to PRE LC_{50} values for diazinon decreased the first year used and increased significantly the second. When OP ear tags were first used following permethrin ear tags in 2001 and 2005, POST to PRE LC_{50} values for diazinon increased three-fold and decreased two-fold. The second years OP ear tags were used following permethrin ear tags, changes in LC_{50} values POST to PRE were not significant.

At Hill Farm, there was no significant correlation between diazinon and permethrin for PRE LC_{50} data ($p=0.20$). For years that permethrin ear tags were used, there were no significant correlations between WOC and diazinon for PRE LC_{50} data ($p=0.99$) nor WOC and permethrin for PRE LC_{50} data ($p=0.54$). For years when OP ear tags were used, there were no significant

correlations between WOC and PRE LC_{50} data for permethrin (p=0.11) or WOC and PRE LC_{50} data for diazinon (0.89).

The LC_{50} values for permethrin at Iberia during this study ranged from 2.4-83.3 µg/cm^2, and RRs ranged from to 1.6-252.0 (Table 3.2). Values for χ^2 from the probit analysis for permethrin at Iberia were significant (p=0.05) for PRE six out of seven years and POST four out of seven years (Table 3.2). Each time permethrin ear tags were used at Iberia, LC_{50} values for permethrin increased PRE to POST. The largest increase was a nine-fold significant increase that occurred in 2005. Each year when OP ear tags were first used at Iberia following permethrin ear tags in 2001 and 2004, LC_{50}s and RRs for permethrin decreased PRE to POST with a seven-fold significant decrease in 2000. The second years OP ear tags were used at Iberia in 2001 and 2004 following permethrin ear tags, LC_{50} values and RRs for permethrin decreased PRE to POST each time and the decrease was significant in 2004. When pyrethroid ear tags were used in 1999 and 2002, LC_{50} values POST to PRE for permethrin increased and decreased. For years when diazinon ear tags were used, LC_{50} values POST to PRE for permethrin increased numerically three years one year significantly and decreased significantly one year.

The LC_{50} values from diazinon bioassay data at Iberia during this study ranged 0.3-8.9 µg/cm^2, and RRs ranged 0.3-53.0 (Table 3.2). Values for χ^2 from probit analysis for diazinon were significant (p=0.05) for PRE the last three years and POST only two out of seven years. When permethrin ear tags were used, PRE to POST LC_{50} values for diazinon increased two out of three years and the increase was significant in 1999. When OP ear tags were first used following permethrin ear tags in 2000 and 2003, PRE to POST LC_{50} values for diazinon increased two fold and significantly decreased five- fold. The second years OP ear tags were used following permethrin ear tags, PRE to POST LC_{50} values for diazinon increased

significantly three-fold and decreased one-fold. When pyrethroid ear tags were used, LC_{50} values POST to PRE for diazinon decreased significantly in 1999 and increased in 2002. For years when diazinon ear tags were used, LC_{50} values for diazinon POST to PRE increased three out of four years with increases significant in 2004 and 2005.

At Iberia, correlations between diazinon and permethrin for PRE LC_{50} data (p=0.95) were not significant. When permethrin ear tags were used, there were no significant correlations between WOC and diazinon for PRE LC_{50} data (p=0.95). When OP ear tags were used, there were no significant correlations for WOC and permethrin for PRE LC_{50} data (p=0.40) or WOC and diazinon for PRE LC_{50} data (p=0.38). However, there was a significant positive correlation between WOC and PRE LC_{50} for permethrin data when permethrin ear tags were used (p=0.02)(R^2 =0.99).

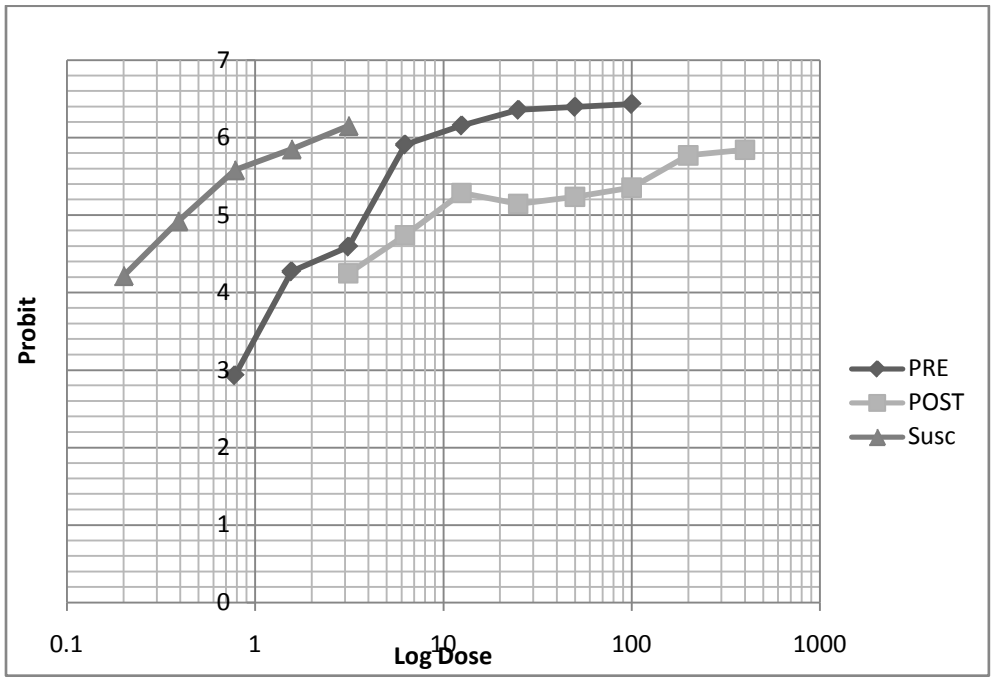

Figure 3.3 Probit plots of permethrin bioassay mortality data for horn flies at Hill Farm PRE and POST 2000 when pyrethroid ear tags were used compared to susceptible horn fly probit plots combined for all years.

Slopes from plotted probit lines for permethrin bioassay data for Iberia increased from PRE to POST each year pyrethroid ear tags were used, and for years when OP ear tags were used, slopes decreased PRE to POST the first year OP ear tags were used and increased PRE to POST each time OP ear tags were used consecutively. At Hill Farm, each year pyrethroid ear tags were used the slopes from probit lines plotted from permethrin bioassay data decreased PRE to POST each time, and when OP ear tags were used the slopes for permethrin increased for all years except in 2001 a decrease occurred.

The slope of the probit line plotted from diazinon bioassay data for Iberia increased PRE to POST two out of three years pyrethroid ear tags were used, and decreased each year PRE to POST when OP ear tags were used, except in 2000 when it increased. At Hill Farm, each year pyrethroid ear tags were used, the slope of the probit line for diazinon decreased PRE to POST each time, and when OP ear tags were used the slope for diazinon decreased for all years except in 2005 when an increase occurred.

At Hill Farm, to further analyze the large increases that occurred in permethrin resistance PRE and POST probit lines were plotted from bioassay data (Fig 3.3-3.5). In 2000, the PRE LC_{50} for permethrin from the probit figure was about 3.8 $\mu g/cm^2$ and the slope of the probit line was 0.69 and R^2=0.83. The 2000 POST LC_{50} value increased by about 123% to 8.5 $\mu g/cm^2$. In 2003, the PRE LC_{50} for permethrin from the probit figure was about 6.0 $\mu g/cm^2$ and R^2=0.72. The 2003 POST LC_{50} value increased by >4000% to about 250 $\mu g/cm^2$ and R^2=0.85. In 2006, the PRE LC_{50} for permethrin from the probit figure was about 2.0 $\mu g/cm^2$ and the slope of the probit line was 0.40 and R^2=0.88. The 2006 POST LC_{50} value could not be calculated (probit line never intercepted the probit value of five) and the slope was 0.17 with R^2=0.67.

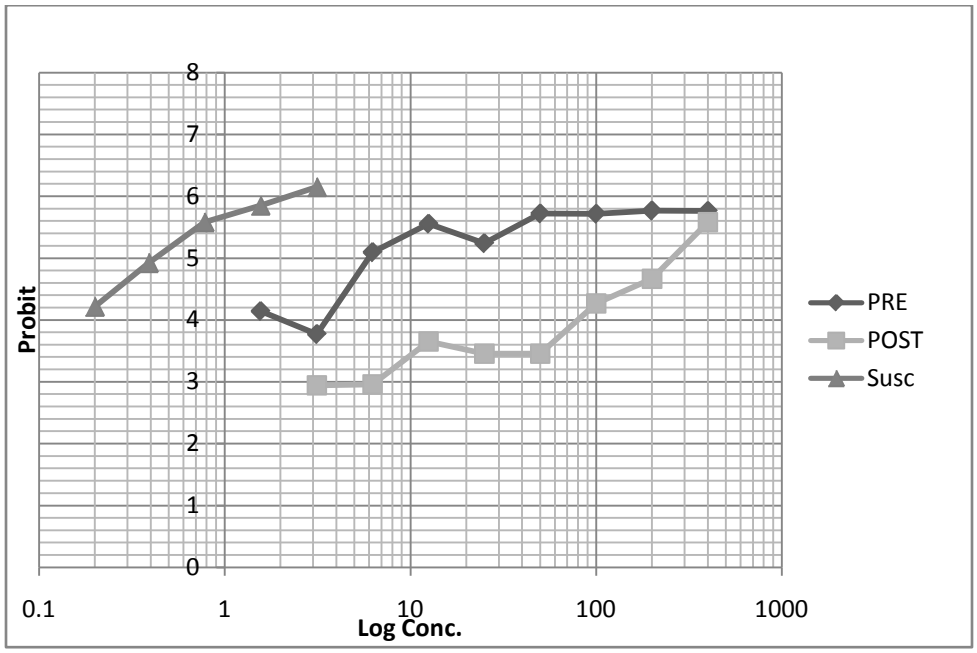

Figure 3.4. Probit plots of permethrin bioassay mortality data for horn flies at Hill Farm PRE and POST 2003 when pyrethroid ear tags were used compared to susceptible horn fly probit plots combined for all years.

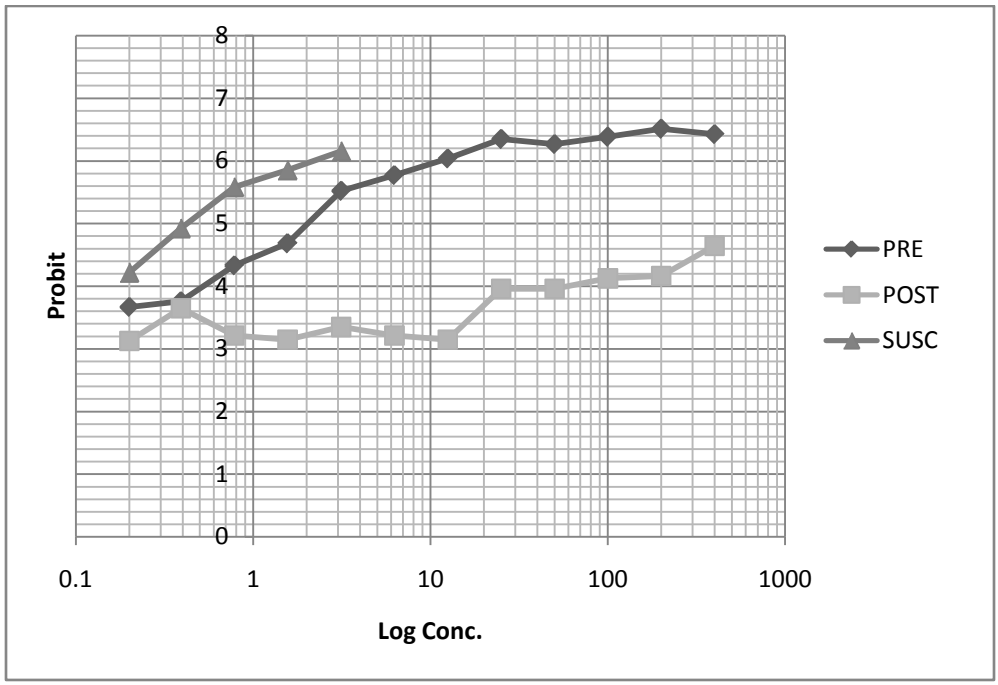

Figure 3.5. Permethrin probit plots at Hill Farm PRE and POST 2006 when pyrethroid ear tags were used compared to susceptible horn fly probit plots combined for all years

3.4 Discussion

The horn fly management strategy of using pyrethroid ear tags followed by two years of OP ear tags maintained horn fly control better at Iberia than at Hill Farm. At both locations, the first year OP ear tags were used following a year of pyrethroid ear tags, length of control increased each time by one to two weeks compared to the year in which OP ear tags were used before, with the exception of a seven week increase that occurred at Iberia in 2003. The second year OP ear tags were used consecutively, weeks of control at both locations decreased or remained the same. When pyrethroid ear tags were used following two years of OP ear tags, no increase in weeks of control was observed at either location. Overall, this strategy maintained acceptable levels of control using pyrethroid ear tags at Iberia of 6 to 10 weeks and weeks of control increased by 17% using pyrethroid ear tags. At Hill Farm, control was not maintained using this strategy and the length of control decreased by 82%. Preliminary data from Byford et al. (1999), demonstrated that this strategy had potential for maintaining control in pyrethroid resistant horn fly populations. Byford et al. (1999) reported that after zero weeks of control occurred using pyrethroid ear tags, an increase in control of four weeks occurred when pyrethroid ear tags were used again following two years of OP ear tags. Guerrero et al. (2002) reported the strategy of alternating between pyrethroid and OP ear tags each year was ineffective at slowing or preventing pyrethroid resistance and they reported overall weeks of control for pyrethroid ear tags decreased by 71% in the seven year study.

Several factors that could have contributed to the observed differences between the farms for maintenance of control include differences in climates, farm management practices, the number of immigrating horn flies, and resistance mechanisms present in the fly populations. Differences in climates could affect the number of generations that occur each year; the farm

located further south, Iberia, would probably have more generations per year compared to Hill Farm located further north. More generations in the absence of insecticide pressure would allow more time for a resistant horn fly population to be diluted naturally by genetics from susceptible horn flies from the local population, from immigrating susceptible horn flies, and/or by a biotic fitness cost that may be associated with resistant horn flies. Different climates would also affect the number of horn flies that enter diapause, how soon they enter diapause and how soon they would emerge from diapause which would also affect the number of generations per year. Klein and Lancaster (1992) reported that horn flies entered diapause in north Arkansas in mid-September and in late September in south Arkansas.

During this study, differences for the amount of rainfall received at each location, pasture topography and drainage could have had an effect on horn fly population size and genetic composition. Foil et al. (1998) reported that the pastures where the cattle were held had a tendency to flood at Iberia. Flooding of the pastures could drown the local resistant horn fly population, which could significantly reduce the size of the local horn fly population and decrease the number of resistant horn flies. Also, differences in the amount of immigrating horn flies and the percentage of horn flies immigrating that were susceptible could affect differences observed between the two farms. Oremus et al. (2006) tested the effectiveness of using a mid-season avermectin (doramectin) pour-on of all cattle to manage pyrethroid resistant horn flies at three locations. Oremus et al. (2006) reported significant differences in weeks of control at two locations, which they attributed to the absence of a susceptible refugia of horn flies at the farms or from nearby farms.

Different cattle management practices at each farm could also have contributed to the differences observed for weeks of control and susceptibility levels. The number of generations

that a horn fly population is exposed and not exposed to insecticide treatments could account for observed differences. Differences in herd composition, types of breeds, number of bulls and ages of cattle could all have significant impact on weeks of control at each farm. Some breeds of cattle have higher numbers of horn flies than other breeds, which could have an effect on the observed mean number of horn flies. This phenomenon would affect the number of weeks the mean number of horn flies was less than 50 per side, and different from controls, but statistical difference between control and treated would not be affected (Schreibera and Campbell 1986, Steelman et al. 1993).

Different resistance mechanisms present at each farm and the percentage or horn flies that had one or more resistance mechanism could also have contributed to observed differences in weeks of control between farms. Oremus et al. (2006) reported that different levels of pyrethroid resistance at three sites was due to different suites of resistance mechanisms. Target-site resistance has been reported in horn flies for both pyrethroids and OPs (Bull et al 1988, Byford et al. 1985, Guerrero et al. 1999, Li et al. 2007, Temeyer et al. 2008). Therefore, it is possible that the loss of horn fly control at Hill Farm during this study reflected horn fly population having both the pyrethroid and OP target-site mutations, while the population at Iberia did not.

The permethrin bioassay used during this study was useful for monitoring changes in permethrin resistance levels using this rotation strategy. When pyrethroid ear tags were used, permethrin resistance levels varied greatly overall between locations and also from spring to fall. Overall at Iberia, spring and fall permethrin resistance levels increased by 51% and 968%, respectively, during the seven year study. Overall at Hill Farm, spring permethrin resistance levels decreased by 49% and fall permethrin resistance levels increased by >3,700%. Using this

rotation strategy the spring following two years of OP ear tags, susceptibility to permethrin was the highest. Barros et al. (1999) reported similar results in a seven year study where pyrethroid and OP ear tags were rotated annually; they reported λ-cyhalothrin overall resistance levels increased in the spring by 159% and in the fall by 1600%.

When OP ear tags were used at Iberia, resistance to permethrin decreased from spring to fall each time, and at Hill Farm resistance decreased the first year following pyrethroid ear tags and increased the second year OP ear tags were used consecutively. At both locations, the first year OP ear tags were used following a year of pyrethroid ear tags the largest decrease from spring to fall in permethrin resistance occurred. The first year OP ear tags were used, permethrin resistance decreased by 82-85% at both sites. Similarly, Barros et al. (1999) reported that at two sites permethrin resistance levels dropped substantially the first year OP ear tags were used following pyrethroid ear tags, but the pattern was not repeated for subsequent years. The results of our assays suggest diazinon-negative cross resistance for pyrethroid resistant flies was likely involved at both locations the first year. Cilek et al. (1995) reported that diazinon negative-cross resistance in horn flies was likely due to the mixed function oxidase system. The second year OP ear tags were used consecutively, resistance to permethrin decreased at Iberia but increased at Hill Farm. Differences observed at Hill Farm for resistance to permethrin the second year OP ear tags were used could have been due to selection for horn flies with target-site resistance at Hill Farm. Foil et al. (2005) reported that horn lies that had the *kdr* and *skdr* mutation were not cross-resistant to diazinon, which would explain the observed exponential increase in resistance to permethrin during this study at Hill Farm. Therefore, it was possible at Hill Farm that horn flies that were target-site resistant may have been selected for because exposure to the OP ear tags killed more metabolic resistant horn flies but not the target-site resistant horn flies, which

then rapidly selected for horn flies with the *kdr* and *skdr* mutation. Oremus et al. (2006) reported at one location using pyrethroid ear tags >95% of horn flies had the *skdr* mutation when LC_{50} values for permethrin were >200 μg/cm^2. Therefore, it is possible that using this rotation strategy at Hill Farm where an LC_{50} of >400 was recorded in 2006, a horn fly population was selected for that was >95% *skdr*.

The diazinon bioassay used during this study was not very useful for detecting any noticeable patterns in response to pyrethroid or OP ear tags used. Similar results were found in Chapter 2. From fall to spring permethrin susceptibility significantly decreased at Iberia each time OP ear tags were used following a year of pyrethroid ear tags and at Hill Farm each time OP ear tags were used consecutively. Using OPs, Barros et al. (2001) reported LC_{50} values for diazinon decreased from fall to spring each year and three years the decreases were significant. The authors attributed the observed decreases to a biotic fitness costs associated with horn flies resistant to diazinon or there was an increase in the number of immigrating susceptible horn flies. Similarly it is probable that during this study, the observed increases from fall to spring could be due to biotic fitness costs associated with resistant horn flies and/or an influx of susceptible horn flies.

When pyrethroid ear tags were first used at Hill Farm, slopes were equal and probit lines were parallel for permethrin and the susceptible plots (Figure 3.3). From spring to fall each year pyrethroid ear tags were used, the slopes of probit lines for permethrin decreased by 60%, increased by 49% and decreased by 58%, respectively, which indicated changes in heterogeneity that could not be ascertained from bioassay data alone. Interestingly, the increase in slope and thus increased population heterogeneity that occurred in 2003 from spring to fall occurred following a significant decrease in permethrin resistance from fall to spring the preceding year.

Three possible reasons for the significant decrease in permethrin susceptibility and decreased slope at Hill Farm could be: 1) using this rotation strategy it took two years of consecutive OP ear tag exposure to have a significant impact on permethrin resistance levels 2) a surge of immigrating susceptible horn flies occurred in the spring of 2003, or 3) biotic fitness costs associated with harsh conditions. The first scenario is most likely because there were no significant decreases for permethrin resistance from fall to spring again until preceded by two years of OP ear tags, and population heterogeneity increased from fall 2000 to spring 2003 the next time pyrethroid ear tags were used by 17% and from fall 2003 to spring 2006 by 18%.

The strategy of using OP ear tags for two years followed by permethrin ear tags to maintain horn fly control was somewhat effective at Iberia and ineffective at Hill Farm. The observed differences for insecticide susceptibility at each farm were probably due to the type of resistance mechanisms and percentage of horn flies that had each type of resistance mechanism. Future rotation studies using chemistries with different modes of action other than OPs and pyrethroids should be conducted to examine usefulness in reducing or maintaining OP and/or permethrin susceptibility. Assays that could detect OP and pyrethroid target-site resistance may provide a better explanation for apparent treatment effects and could be used to predict product efficacy better than the filter paper bioassay.

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CHAPTER 4

BIOTIC FITNESS COSTS OF THE *SKDR-KDR* ALLELES IN HORN FLIES IN THE ABSENCE OF PYRETHROID PRESSURE

4.1 Introduction

The horn fly, *Haematobia irritans*, is a major pest of cattle in the United States and other countries (Hargett and Goulding, 1962, Graham and Hourrigan 1977, Anziani et al. 1993). The horn fly is considered to be the most economically important pest of cattle in the United States, and annual losses associated with decreased weight gains and milk production of cattle caused by the constant biting and irritation of horn flies are estimated to be $876 million (Kuntz et al. 1991).

Efforts to control horn flies have been primarily based on the use of insecticides. Many different insecticides from different classes have been used over the decades for horn fly control, and the use of insecticides has led to resistance in horn fly populations to insecticides in a number of classes including chlorinated hydrocarbons, organophosphates, pyrethroids, and carbamates (Harris 1964, Sheppard 1983, Quisenberry et al. 1984). When horn fly populations become resistant to an insecticide, control can be minimized or lost, which results in economic losses.

Since insecticide resistance is widespread in horn fly populations, studies have been conducted to test different resistance management strategies. Knowledge of the resistance mechanisms of horn flies and ways to counter those mechanisms is important for selecting appropriate management techniques. Knowing whether horn flies are positively or negatively cross resistant to insecticides and conditions that exploit the reversal of resistance is important. If there is a biotic fitness cost to resistance mechanisms, the termination of insecticide treatments

could result in reversal of fly populations to susceptible levels. For example, Emeka-Ejiofor et al. (1983) reported that adult *Anophles gambiae* mosquitoes that possessed the semi-dominant dieldrin resistance gene emerged more slowly than susceptible mosquitoes. Ferrari and Georghiou (1981) reported that *Culex quinquefaciatus* mosquitoes that were homozygous OP resistant had lowered fecundity, reduced viability and longer developmental times compared to susceptible mosquitoes. Halliday and Geoghiou (1984) reported that *C. quinquefaciatus* that were *kdr* homozygous resistant or susceptible had increased developmental periods compared to the heterozygotes. Sayyed et al. (2008) reported that *Heliothis virescens* that had the pyrethroid *kdr* mutation laid fewer eggs, had decreased egg hatch, decreased growth rate, and decreased body weight compared to susceptible moths

Biotic fitness has been described in horn flies resistant to pyrethroids in both laboratory and field colonies. In a laboratory, pyrethroid susceptible horn flies emerged sooner than pyrethroid resistant flies, pupated two times faster than resistant flies, and resistant flies produced half as many eggs as susceptible flies (Scott et al. 1997). In a seven year field study, Guerrero et al. (2002) reported that the frequencies of *super kdr-kdr* resistant genotypes increased when pyrethroid ear tags were used and decreased in the absence of pyrethroid ear tags, potentially due to biotic fitness costs.

It is important to know if a resistance mechanism has an associated biotic fitness cost, what effect the biotic fitness cost has, and what period of time it takes the mechanism to revert back to susceptible once the insecticide pressure is removed. Understanding biotic fitness costs in horn flies would be important to improve management and control of resistant horn fly populations. The objective of this study was to determine the rate of change in *kdr* and *skdr* allele ratios in horn fly populations in the absence of pyrethroid pressure. This information could

indicate whether the reduction in R alleles in a population is due to immigration of susceptible flies or reduced biotic fitness of resistant flies due to reduced fecundity, adult survival, or reduced ability of resistant flies to enter or survive diapause.

4.2 Materials and Methods

Trials were conducted in Louisiana from 2004-2005 at two Louisiana State University, Agricultural Research Stations; Macon Ridge Research Station (32°06'34.2252", -091°42'25.6140") at Winnsboro and Northeast Research Station (31°58'45.5088", -091°13'50.5128") at St. Joseph. The number of cattle maintained annually at these sites ranged 60-200. All cattle at each station were tagged in May with one ear tag per head containing 10% zeta-cypermethrin + 20% piperonyl butoxide, Python®, Y-Tex (Cody, WY), and ear tags were removed about the first week of August.

Horn flies were collected approximately every 45 days from herds at Winnsboro and St. Joseph on 29Jul04, 27Sep04, 11Nov04, and 05Apr05. A sample of horn flies was collected from the backs of the cattle using an aerial hand net and stored in 95% ethanol or at -80°C for later genomic testing using PCR. The polymerase chain reaction method (PCR) was done at Louisiana State University and the USDA-ARS Livestock Insect laboratory in Kerrville, TX to determine the frequency of *kdr* and *superkdr* mutant allele frequencies in different horn fly populations. Horn fly samples that were collected were stored at -80°C or in 95% ethanol for later genomic analysis. Individual flies were homogenized in prechilled 1.5ml centrifuge tubes on dry ice. PCR buffer (Perkin Elmer PCR buffer II) was added to each tube (25µl) and homogenized for at least 15 seconds per tube. Tubes were then heated for six minutes on a dry block heater at 110ºC to denature and separate the DNA strands. Samples were centrifuged for 8

minutes at 10,000g and then diluted 1:10 in PCR grade water. Two reactions were done per fly to detect both resistant and susceptible alleles. Reaction mixtures to detect susceptible and resistant alleles were prepared using the following measurements: 13.2µl of PCR grade water, 1.6µl of $MgCl_2$, 2.0µl of PCR buffer II and 0.4µl of dNTP mix. The primers (Table 4.1) described by Guerrero (1997) were used in both reactions at the following concentrations: FG (138, 234, 235, 243=0.2µl) FG 236=0.1µl.

Table 4.1. Primer sequences used for resistant and susceptible alleles

Allele	Primer	Sequence
Susceptible	FG130	5'-TACTGTTGTCATCGGCAATC
	FG154	5'-ACCCATTGTCCGGCCCA
	FG238	5'-CGCCACAAATGAAACC
Resistant	FG134	5'-TACTGTTGTCATCGGCAATT
	FG155	5'-ACCCATTGTCCGGCCCG
	FG239	5'-GCCACAAATGAAACG
	FG240	5'-GCCACAAATGAAACA
	FG241	5'-GCCACAAATGAAACT
Both	FG138	5'-CAATATTACGTTTCACCCAG
	FG234	5'-CTTCTTCATCGGTGTAGC
	FG235	5'-CTTCGTGTATTCAAATTGGCA
	FG236	5'-TTGTTGTCATGCTGCCTCC
	FG243	5'-GGCATGGCTTTCCGTGTCC

To assay for the presence of susceptible alleles, only primers FG (130, 154=0.2µl, and 238=0.1µl) were included in the primer mix. To assay for resistant alleles, only primers FG (134, 155=0.2µl, and 239, 240, 241=0.1µl) were used for the primer mix. A 0.3µl 1:1 vol:vol mix of AmpliTaq DNA Polymerase (5 units/µl stock; Perkin-Elmer) and TaqStart Antibody (1.1µg/µl stock; Clontech) was mixed and stored at room temperature for 5 minutes before

adding it to the mixtures. For each reaction, 20.0µl of the reaction mixture and 2.0µl of the 1:10 genomic DNA solution was mixed gently by thumping and then set in thermocycler. Amplification was done using a DNA Engine (MJ Research, Watertown, MA) programmed to run 96ºC for 2 minutes followed by 35 cycles, each consisting of denaturation at 94ºC for 1 min, annealing at 60ºC for 1 min, and an extension at 72ºC for 1 min followed by a final extension step at 72ºC for 7 min to cool down. The reaction products were then fractionated by electrophoresis through a 3.5% agarose TBE gel for 45 min at 165 volts and then stained by soaking for 30 minutes in a solution of GelStar DNA Staining Dye (FMC Bioproducts, Rockland, ME) prepared by adding 25.0µl gel stain to 250ml TBE. Fractionated DNA was then UV illuminated and photographed.

To determine if there was a difference between sexes in the rate that %R decreased in the absence of insecticide pressure, linear regression and ANOVA were used to detect statistical differences and means were separated using Tukeys ($p=0.005$). Correlation analysis was done to look for relationships between %R in time for both the s*kdr* and *kdr* mutation. An arc-sin transformation was done on percentage data prior to analysis. Fisher's exact test was used to look for significant relationships between genotypes. A chi square test for independence analysis was made using observed *skdr* genotypes for each month at both locations to compare if expected Hardy-Weinberg expected proportions were significantly different from observed. Proportions were significant if $p<0.05$. A comparison of the number of horn flies for each *skdr* genotype in July was compared to the number observed in May using step-wise binary logistic regression. Temperature data for Winnsboro and St. Joseph came from the nearest Louisiana Agricultural Center weather station.

4.3 Results

After the pyrethroid ear tags were removed from the cattle, the percentage of horn flies with the *kdr* and *skdr* mutation decreased from July to May at both locations. The larger decrease occurred at Winnsboro for *skdr* and *kdr* %R. About every 45 days at Winnsboro, %R allele ratios *skdr* and *kdr* decreased by 21% and 9% and at St Joseph by 18% and 10% (Table 4.2). At Winnsboro from July to May, %R decreased by 62% for *skdr* from 85% to 32% and 28% for *kdr* from 100% to 72%. In July, 100% of horn flies had the *skdr* mutation and 72% were RR-RR. The largest decreases occurred from July to September for both *skdr* and *kdr* of 33% and 15%, respectively. In September, only 6% of horn flies did not have the *skdr* mutation. From September to November, the number of horn flies that did not have the *skdr* mutation increased by 328% from 6% to 30%.

From July to May, %R for *skdr* and *kdr* decreased by 18% and 10%, respectively at St. Joseph. The largest decrease in %R for *skdr* was 15% that occurred from July to September. At St. Joseph, %R for *skdr* and *kdr* decreased from July to May by 50% for *skdr* from 86% to 37% and 37% for *kdr* from 100% to 64%, respectively (Table 4.2). In July, only 4% of horn flies did not have the *skdr* mutation and 73% were RR-RR. The largest decrease for *skdr* that occurred was 28% (from 86% to 62%) that occurred from July to September, and the largest decrease for *kdr* was 24% from April to May (from 84% to 64%). From July to May, the number of horn flies that did not have the *skdr* mutation increased 875% from 4% to 39% and increased an average 77% every 45 days; the 175% increase from July to September and was the highest.

Data from July to May, were combined for Winnsboro and St. Joseph because using step-wise binary logistic regression no significant differences in genotypes ($p=0.98$) due to location were found (Table 4.3). The number of horn flies for *skdr* that were RR decreased significantly

($p<0.0001$), and the number of horn flies that were SS increased significantly ($p=0.0008$) (Table 4.3). There was no significant change for the number of horn flies that were SR from July to May. For all collections, the percentage of *skdr* genotypes present did not significantly differ from expected Hardy-Weinberg proportions ($p=0.05$), but Hardy-Weinberg expected proportions were significantly different than observed genotypes at both locations in September.

There was no difference in the percentage of male and female horn flies for the different genotypes at each farm (Table 4.4) with the exception that St Joseph had significantly fewer RR male horn flies compared to female horn flies ($p=0.00042$). When data were combined for both farms there were no significant differences due to sex for each genotype (Table 4.4).

A comparison of male and female horn fly *skdr-kdr* genotypes for each location was made (Table 4.5). At Winnsboro after the ear tags were removed, 100% of horn flies had the *skdr* mutation, and of horn flies sampled, 65% of the males and 64% of the females were RR-RR. In September at Winnsboro, only 4% of the male horn flies and 6% of female horn flies did not have the *skdr* mutation, and the percentage of male and female horn flies that were RR-RR decreased by 68% for males and 67% for females from July to May. From July to May, the number of male horn flies that were RR-RR decreased by 86%; and the number of male horn flies that were SR-SR increased from 0 to 32%, and SR-RR decreased by 49%. At St. Joseph after the ear tags were removed, only 4% of horn flies did not have the *skdr* mutation, and of the 26 females sampled 73% were RR-RR. In July at St. Joseph, no data were collected for male horn flies. In September at St. Joseph, only 4% of the female horn flies did not have the *skdr* mutation, and the percentage of female horn flies that were RR-RR decreased by 40% from July

Table 4.2. The change in *skdr* allele frequencies and proportions in time at St. Joseph and Winnsboro

Location	Month	Num.	Genotypes of sampled flies % (Num. Observed)(Hardy-Weinberg expected)			p value[a]	*skdr* %R[b]	*kdr* %R
			SS	SR	RR			
St. Joseph								
	Jul	26	4% (1)	23% (6)	73% (19)	*	86	100
	Sep	66	11%c (7)(0.6)	55% c (36)(6.8)	35% c (23)(18.6)	0.196	62	90
	Nov	59	10% (6)(9.5)	58% (34)(31.1)	32% (19)(25.5)	0.104	60	92
	Apr	63	27% (17)(9.0)	57% (36)(28.1)	16% (10)(22.0)	0.212	45	84
	May	57	39% (22)(19.4)	51% (29)(31.1)	11% (6)(12.4)	0.430	37	64
Winnsboro								
	Jul	61	0% (0)	28% (17)	72% (44)	*	85	100
	Sep	63	6%c (4)(1.2)	73% c (46)(14.6)	21% c (13)(45.2)	*	57	85
	Nov	76	30% (23)(11.6)	46% (35)(30.9)	24% (18)(20.6)	0.514	47	75
	Apr	49	35% (17)(21.6)	49% (24)(37.8)	16% (8)(16.6)	0.923	42	73
	May	77	43% (33)(17.2)	49% (38)(23.7)	8% (6)(8.2)	0.271	32	72

[a] Hardy-Weinberg proportions were significantly different from HW expected if $p<0.05$.

[b] R allele frequency (%) = [No. of R alleles/(No. of S alleles + No. of R alleles)]*100

* Insufficient numbers for comparison if number of horn flies were <5 for one or more genotypes

[c] Observed values were significantly different from Hardy-Weinberg expected proportions using Chi Square test for independence $p<0.05$.

Table 4.3. Step-wise logistic regression summary from July to May for *skdr* genotypes combined for St. Joseph and Winnsboro

	Genotypes for *skdr* allele		
	SS	**SR**	**RR**
Mean Num Observed July to May	1-28	12-34	32-6
p value[a]	0.0008	0.58	<0.0001
Slope	0.30	0.94	-2.91
Slope C.I.	(0.15-0.60)	(0.76-1.17)	(-2.11- -4.01)

[a] binary step-wise logistic regression was done for number observed for each genotype from July to May. Data was combined for both locations due to no significant difference between locations (p= 0.9801).

Table 4.4. Genotypes for *skdr* of male and female horn flies at Winnsboro and St. Joseph.

			Genotype *skdr* (num observed)(%)			P value[a]	
Location	**Sex**	**N**	**SS**	**SR**	**RR**	**SS vs. SR**	**SR vs. RR**
Winnsboro	M	135	35(25)	70(51)	31(23)	0.47	0.54
	F	142	34(24)	84(59)	30(21)		
St. Joseph	M	119	36(30)	84(70)	25(21)	0.17	0.00042
	F	160	23(14)	83(52)	56(35)		
Combined	M	254	71(28)	154(61)	56(22)	0.49	0.40
	F	302	57(19)	167(55)	86(28)		

[a] Fisher's exact test two-sided

Table 4.5. Genotypes (*skdr-kdr*) of horn flies collected at Winnsboro and St. Joseph.

Station	Month	Sex	Num	Genotype *skdr-kdr* Num observed (%)						*skdr* %R[a]	*kdr* %R
				SS-SS	SS-SR	SS-RR	SR-SR	SR-RR	RR-RR		
Wins	July 29	M	17	0	0	0	0	6(35)	11(65)	82	100
		F	14	0	0	0	0	5(36)	9(64)	82	100
	Sep 24	M	29	0	2(7)	0	8(28)	13(45)	6(21)	34	83
		F	34	0	1(3)	1(3)	8(24)	17(50)	7(21)	32	87
	Nov 18	M	36	3(8)	4(11)	3(8)	12(33)	5(14)	9(25)	49	69
		F	40	2(5)	6(15)	5(13)	6(15)	12(30)	9(23)	45	80
	April 5	M	17	3(18)	1(6)	3(18)	5(28)	0	5(29)b	44	65
		F	32	1(3)	4(13)	5(16)	6(19)	13(41)	3(9)	39	81
	May 26	M	37	4(11)	10(27)	2(5)	12(32)	9(24)	0b	28	59
		F	22	0	0	9(41)	7(32)	4(18)	2(9)	34	84
St. Joseph	July 29	M	0	-	-	-	-	-	-	-	-
		F	26	0	0	1(4)	0	6(23)	19(73)	85	100
	Sep 24	M	32	0	1(3)	2(6)	6(19)	15(47)	8(25)b	58	89
		F	34	0	0	4(12)	6(18)	9(26)	15(44)	66	91
	Nov 18	M	25	0	0	4(16)	5(20)	13(52)	3(12)b	48	90
		F	34	0	0	2(6)	5(15)	11(32)	16(47)	71	93
	April 6	M	32	2(6)	3(9)	7(22)	2(6)	14(44)	4(13)	38	86
		F	31	1(3)	3(10)	1(3)	7(23)	13(42)	6(19)	52	81
	May 26	M	30	9(30)	3(10)	2(7)	7(23)	7(23)	2(7)	30	53
		F	27	1(4)	3(11)	4(15)	9(33)	6(22)	4(15)	43	74

[a] R allele frequency (%) = [No. of R alleles/(No. of S alleles + No. of R alleles)]*100

[b] male and female horn flies from same collection were significantly different for SR vs.RR, Fishers exact test (p=0.05)

to May. The number of horn flies that were RR-RR decreased by 80% for females from July to May and 72% for males from September to May. The percentage of male horn flies that were SR-SR decreased from September to May by 51% and then percentage of females decreased by 4% percent from July to May.

From July to May, there were significant differences between farms in the rate of change for the *skdr* SR and RR genotypes due to sex. At Winnsboro, the percentage of male and female horn flies that were RR decreased from July to May from 65% to 0% and 64% to 9%, respectively (Figure 4.5). At Winnsboro from July to May of the 119 males and 128 females sampled 26% and 23% were SR, respectively. At Winnsboro from July to May of the 119 males and 128 females sampled 58% and 60% were RR, respectively. At St. Joseph, there were fewer males than females with the RR genotype and the percentage of males decreased from September to May from 25% to 7% and females decreased (Figure 4.5) from 73% to 15%. At St. Joseph from July to May of the 119 males and 152 females sampled, 58% and 47% were SR, respectively. At St. Joseph from July to May of the 119 males and 128 females sampled 14% and 40% were RR, respectively. At Winnsboro, there were significant differences for the percentage of male and female horn flies that were SR or RR in April and May. At St. Joseph, the percentage of male and female horn flies that were SR or RR were significantly different in September and November.

During this study, average monthly temperatures were in the low 30's C° for the first few months, decreased to the lowest recorded in November at 24 C°, and then began increasing again in the spring months to 29 C°. There was no correlation between monthly temperature averages and changes in genotypes at either location (Figure 4.1 and 4.2).

At both locations for both males and females, as RR decreased SS increased (Figures 4.3 and 4.4). At Winnsboro, there were no SS male or female horn flies in July and the largest decrease in the percentage of RR flies for both male and females occurred from July to September. At St. Joseph, there were fewer RR males than females and there were fewer SS females than males.

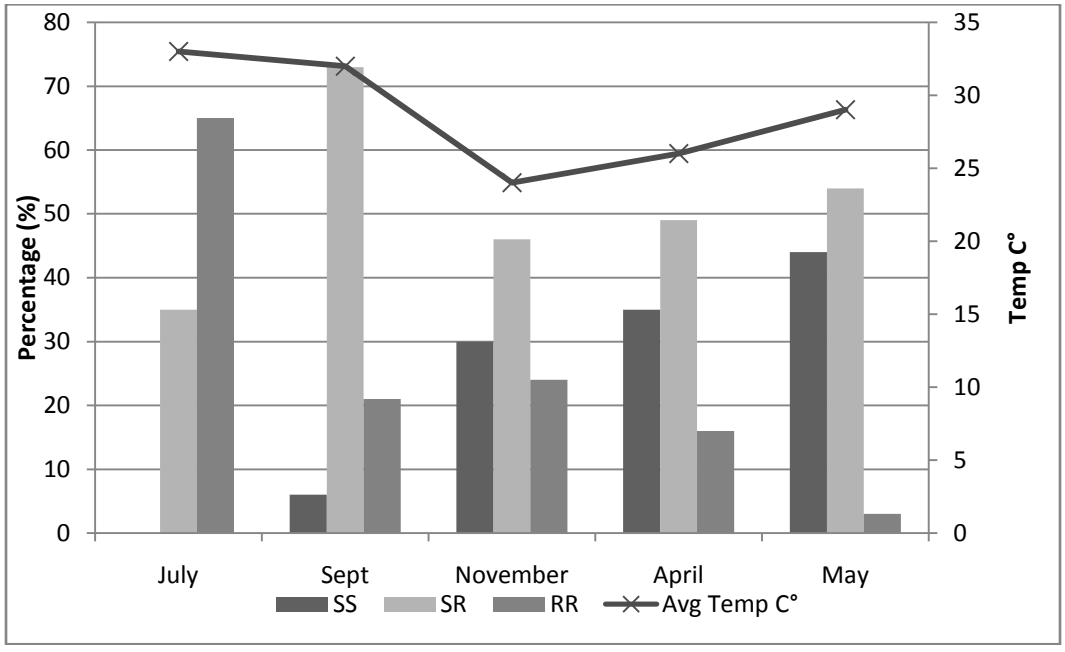

Figure 4.1. The change in horn fly genotypes for *skdr* at Winnsboro from July to May with monthly temperature averages.

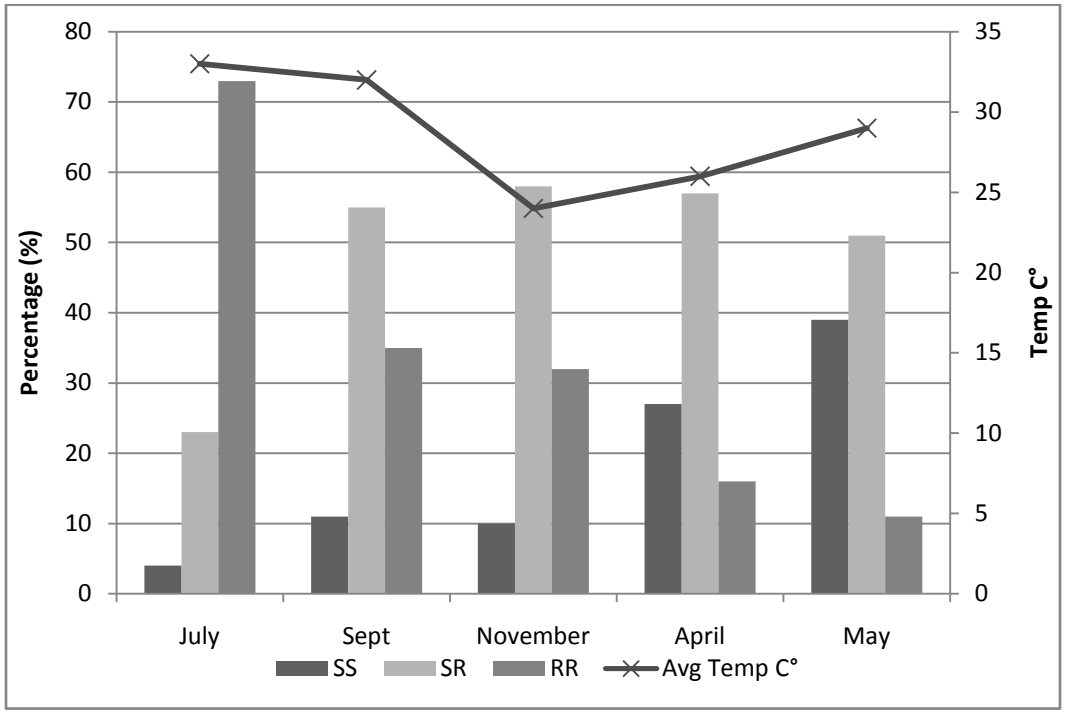

Figure 4.2. The change in horn fly genotypes for *skdr* at St. Joseph from July to May with monthly temperature averages.

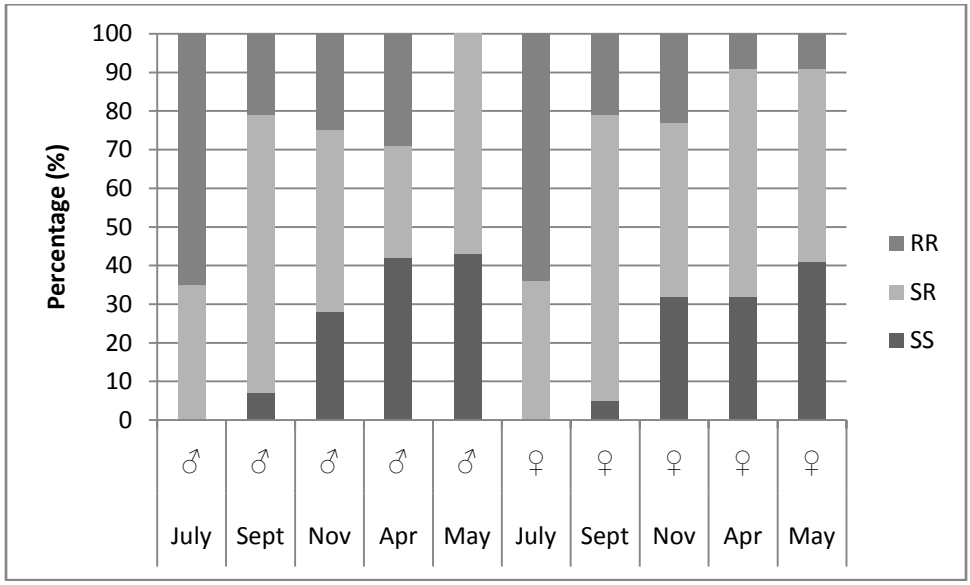

Figure 4.3. The change in time of the percentage of male and female horn flies for *skdr* genotypes at Winnsboro.

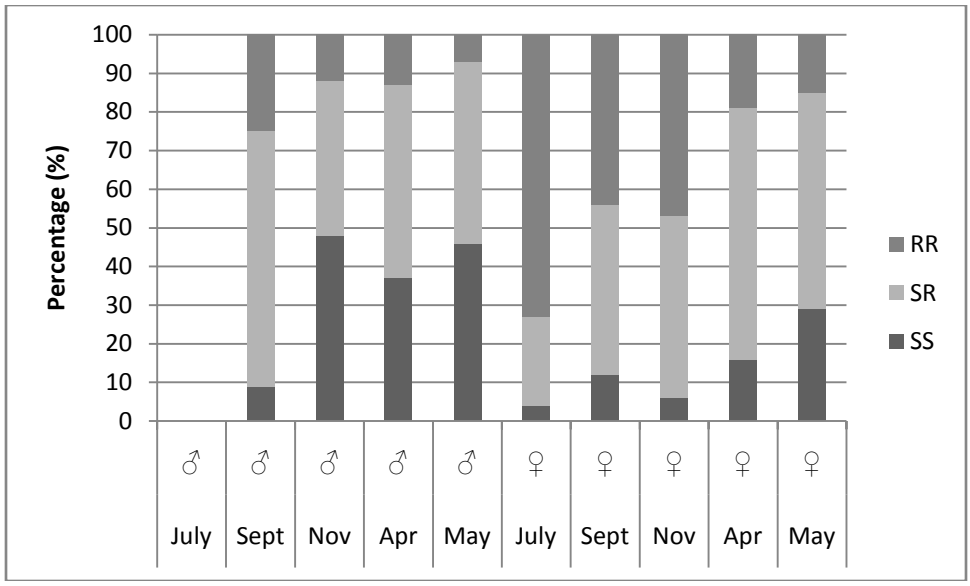

Figure 4.4. The change in time of the percentage of male and female horn flies for *skdr* genotypes at St. Joseph.

4.4 Discussion

When pyrethroid ear tags were removed at both locations, the percentage of horn flies that had the *skdr* or *kdr* mutation decreased every 45 days from July to May. Guerrero et al. (2002) and Oremus et al. (2006) reported that the percentage of horn flies with the *skdr* and *kdr* mutation decreased from fall to spring each year, which they attributed to biotic fitness costs. Oremus et al. (2006) was the first to report the change in allele ratios from fall to spring for both *skdr* and *kdr* in horn flies. Oremus et al. (2006) reported *kdr* allele ratios decreased by an average of 46% at Red River and 14% at St. Joseph, and for *skdr* allele ratios decreased by an average of 85% at Rev River and 24% at St. Joseph. Similarly, during this study from July to May, the percent *skdr d*ecreased by an average of 60% and *kdr* decreased an average 32% at St. Joseph. The results of this study also show that about every 45 days allele ratios decreased by an average of 20% for *skdr* and 10% for *kdr*. The larger allele ratio decreases for *skdr* observed during this study agree with those reported by Guerrero et al. (2002) and Oremus et al. (2006).

Therefore, the observed gradual decrease that occurs for *skdr* and *kdr* allele ratios when insecticide pressure is removed is likely not from biotic fitness costs associated with climate or diapause, which would be apparent by changes in allele ratios in response to climate changes that were not gradual from fall to spring. The observed biotic fitness cost during this study could be related to reduced fecundity of flies with resistant alleles. Scott et al. (1997) reported that pyrethroid resistant horn flies produced half as many eggs as susceptible horn flies and resistant horn flies developed slower. Similarly, Sayyed et al. (2008) reported that *H. virescens* that had the pyrethroid *kdr* mutation laid fewer eggs, had decreased egg hatch, decreased growth rate, and decreased body weight compared to susceptible moths. If fecundity differences do account for the biotic fitness costs, then potential differences in bloodmeal size between susceptible and resistant flies might be a potential cause. Homozygous resistant females laying fewer eggs or having reduced fertility could be due to resistant females having a smaller bloodmeal size than susceptible or heterozygous females. The correlation of bloodmeal size to increased egg production has been described for the mosquito *Aedes aegypti* and the horse fly *Tabanus fusciostatus* (Colless and Chellapah, 1960 and Leprince and Foil, 1993).

During this study, at both locations after pyrethroid ear tags were removed, the percentage of horn flies that were homozygous resistant *skdr* decreased significantly, the percentage of heterozygous horn flies did not change, and the percentage of homozygous susceptible horn flies increased significantly (Table 4.3). From July to May, the percentage of homozygous resistant *skdr* horn flies decreased by 85% at St. Joseph and by 89% at Winnsboro, and the percentage of horn flies that were homozygous susceptible increased from 0% to 43% at Winnsboro and increased from 4% to 39% at St. Joseph (Table 4.2). Oremus et al. (2006) reported similar changes from fall to spring for all *skdr* genotypes. They reported at one location

the percentage of homozygous resistant horn flies decreased by an average of 96% and at St. Joseph an average of 81% and the percentage of homozygous susceptible horn flies increased at one location from 4% to 84% and at St. Joseph from 14.9% to 51%. During this study and for Oremus et al. (2006), the percentage of heterozygotes were equal to normal expected genetic ratios. Guerrero et al. (2002) reported that when the pyrethroid target-site resistance becomes fixed in a population that only marginal control can be achieved. The constant presence of the heterozygous genotypes observed during this study would explain this phenomenon, and potentially indicate that there are no biotic fitness costs for the SR genotype. Ferrai and Georghiou (1981) reported that OP resistant heterozygous *C. quinquefasciatus* did not have any biotic fitness costs. The increase in susceptible horn flies beyond normal expected genetic ratios could be from immigrating susceptible horn flies from untreated farms. However, this observation could indicate that there are fitness costs for the SR genotype that are not expressed as rapidly as for the RR genotype. If this is a factor, then these traits would be detected during longer duration studies. Since the percentage of homozygous resistant horn flies decreased more than was expected in normal genetics, it is obvious that the homozygous resistant genotype has a significant biotic fitness cost. Such a biotic fitness cost could be related to reduced fecundity of homozygous resistant females.

There were significant differences between farms for the percentage of homozygous resistant *skdr* males compared to females. At Winnsboro, the percentage of male and female horn flies that were *skdr* homozygous resistant was equal and at St. Joseph there were significantly less male homozygous resistant horn flies compared to females. Guerrero et al. (2002) reported that in all years of a seven year study at St. Joseph there were fewer *kdr* homozygous resistant male horn flies compared to females and more heterozygous males than

females. The sex differences for *skdr* described in this study are the first reported for that genotype.

We showed that %R for *skdr* and *kdr* declined at a predictable rate through time indicating that the changes are not associated with climatic changes or diapause. Therefore, the reduced biotic fitness of the RR genotype is likely related to reduced fecundity which is recognized as a major element of relative fitness in genetics of populations (Hedrick 2000). We showed for the first time that the changes in percent of resistant alleles actually are related primarily to the RR *kdr* and *skdr* genotypes and that the heterozygote likely has no or reduced relationship to the observed decrease.

The under representation of homozygous resistant male horn flies for both *kdr* and *skdr* at different locations is not just due to random effects. If there was a lethal effect of the RR genotype for males expressed as embryonic death, then the number of eggs laid by resistant females would be less than the number laid by susceptible females and would be grossly recognized as a fecundity fitness deficit. If this were the case, then the rate of decline of the male RR genotype would be faster than the RR female. Since the rates of change were similar for RR males and females in this study, the RR genotype likely is not differentially expressed between sexes.

Other observed differences in the percentage of male and female *skdr* horn flies between farms could be related to collection methods or time of collection. At Winnsboro in some years when the bulls had heavy fly loads, horn flies were collected primarily from a single bull, and at St. Joseph horn fly samples were always collected from multiple cows. The difference in the disturbance of the flies on the cattle during collection could change the distribution of the flies. Male horn flies have been reported to inhabit the lower extremities and legs of cows more than

females (Witherspoon, 1968), which could affect collected male to female ratios. Differences between farms for time of collection also could possibly explain observed differences. Horn flies were always collected at Winnsboro first, usually early in the morning, and due to driving time between locations, horn flies were sampled at St. Joseph about one to two hours later. Therefore, the temperature difference could have affected the distribution of the flies on the cattle. Schrieiber and Campbell (1986) reported that horn fly distribution on cattle changes during the day from the morning distribution. Foster et al. (2003) reported that house flies that had the *kdr* mutation showed no positional preference along a temperature gradient and susceptible house flies exhibited a strong preference for warmer temperatures.

At both locations, we found that the percentage of horn flies with the *skdr* and *kdr* mutations decreased every 45 days by about 20% for *skdr* and 10% for *kdr* when pyrethroid selection pressure was removed, which is likely due to biotic fitness costs in male or female horn flies. We did find differences for the percentage of male and female with RR genotype of skdr and this is consistent with the findings of Guerrero et al. (2002) and Oremus et al. (2006). A potential factor for underrepresentation of the RR genotype for males could be from a sex-linked lethal effect of the RR genotype. However, behavioral differences of the RR males and females also could contribute to the under representation due to sampling procedures. Future studies on the effects of bloodmeal size and its effects on fecundity would be useful in determining the cause of the decrease in *kdr* and *skdr* homozygous resistant horn flies in the absence of insecticidal pressure.

When the flies were sampled from the treated cattle in July, the only represented genotypes were SR-RR and RR-RR (except for 1 out of 57 flies that were genotyped) which have been shown to be the least susceptible of the six possible genotypes (Foil et al. 2005).

Therefore, the horn fly populations were under an extreme selective pressure from pyrethroid exposure. After the tags were removed, the horn fly populations returned to Hardy-Weinburg equilibrium. The changes in the genotype proportions of the fly populations that followed indicated that there was a biotic fitness cost associated with the RR genotypes of both *skdr* and *kdr*. Future studies on the mechanisms of this biotic fitness cost are warranted. Areas of particular interest would be comparison of behavior and fecundity of the different genotypes.

4.5 References

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CHAPTER 5

CONCLUSIONS

We found that a mid-season ivermectin treatment of all cattle was useful in managing OP resistant horn fly populations. The mean number of horn flies on the cattle the spring after ivermectin treatments were made decreased by an average 73% compared to the year before, and up to 10 weeks of horn fly control was provided. We propose that control was regained because ivermectin acts both as an adulticide and a larvicide against the horn fly and ivermectin has a different mode of action than diazinon, which allows for control of the resistant population and repopulation by susceptible flies.

The horn fly management strategy of using pyrethroid ear tags followed by two years of OP ear tags was evaluated for maintaining susceptibility in horn fly populations to both pyrethroids and organophosphates. At Iberia, 6, 10 and 7 weeks of control were recorded when pyrethroid ear tags were used every third year. When OP tags were used at Iberia, horn fly control was maintained at 9 to 7 weeks throughout the 4 years of use. At Hill Farm, when pyrethroid ear tags were used, weeks of control reduced from 11 to 6 to 2 weeks of control. When OP ear tags were used at Hill Farm, weeks of control reduced from 12 to 3 in the 4 years of use. Several factors that could have contributed to the observed differences between the farms for weeks of control include differences in; climates, farm management practices, the amount of immigrating horn flies, and resistance mechanisms present. At both locations, the largest decrease from spring to fall in permethrin bioassay data occurred in the first year OP ear tags were used following a year of pyrethroid ear tags. The results of the bioassays suggest diazinon-negative cross resistance of pyrethroid resistant horn flies was likely involved at both

locations the first year OP ear tags were used. The second year OP ear tags were used, resistance to permethrin decreased at Iberia but increased at Hill Farm. The differences observed at Hill Farm for resistance to permethrin in the second year OP ear tags were used could have been due to selection for horn flies with *kdr* target-site resistance at Hill Farm.

After pyrethroid ear tags were removed from cattle at Winn and St Joseph, the rate of change of alleles for the *skdr* or *kdr* mutation decreased every 45 days by an average 20% for *skdr* and 10% for *kdr* over a period of 10 months. At the beginning of the study, nearly 100% of horn flies had the *skdr* mutation and 72% were homozygous resistant. The largest decrease for *skdr* occurred from July to September likely due to biotic fitness costs. From July to May, the number of homozygous susceptible horn flies increased significantly, and the number of homozygous *skdr* resistant horn flies increased significantly. There were significant differences between farms for the percentage of homozygous resistant *skdr* males compared to females. At Winnsboro, the percentage of male and female horn flies that were *skdr* homozygous resistant was not different but at St. Joseph, there were significantly fewer male homozygous resistant horn flies compared to females. We showed that %R for *skdr* and *kdr* declined at a predictable rate through time, indicating that the changes are not associated with climatic changes or diapause and is likely related to reduced fecundity or due to behavioral differences of the resistant horn flies. We showed for the first time that the changes in percent of resistant alleles actually are related primarily to the RR *kdr* and *skdr* genotypes and that the heterozygote likely has no biotic fitness costs.

VITA

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