THE ECOLOGY OF STABLE FLIES (DIPTERA: MUSCIDAE) ASSOCIATED WITH EQUINE FACILITIES IN FLORIDA

By

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To my family and friends for their overwhelming support

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THE ECOLOGY OF STABLE FLIES (DIPTERA: MUSCIDAE) ASSOCIATED WITH EQUINE FACILITIES IN FLORIDA

By

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Beginning in November 2007 and continuing until December 2009, a series of field- and laboratory-based studies were conducted to increase our understanding of stable fly ecology associated with Florida equine facilities. Weekly stable fly surveillance was conducted at four equine facilities near Ocala, Florida using alsynite sticky traps for adults and by searching larval developmental sites for pupae. Although stable flies were collected throughout the year, most were captured between January and April. Correlation analysis suggested that adult stable fly presence to predict future stable fly breeding cannot be used for parasitoid releases on equine farms.

In contrast to studies of other livestock installations, such as cattle and poultry, 99.9% of all parasitoids collected were *Spalangia* spp., consisting of *Spalangia cameroni* Perkins (56.5%), *Spalangia nigroaenea* Curtis (34.0%), *Spalangia endius* Walker (5.8%), and *Spalangia nigra* Latreille (3.7%). In addition, laboratory evaluations assessing the ability of *S. cameroni*, *S. endius*, and *Muscidifurax raptorellus* Kogan and Legner to locate and attack stable fly hosts in a field-collected fly-breeding substrate, suggest that *Spalangia* spp. are more suited to searching in habitats created by equine husbandry.

To assess potential movement between farms, a multiplex polymerase chain reaction (PCR) was developed to identify the blood meal host source of stable flies, specifically those taken from cattle, horse, human, and dog hosts. Over 60% of all blood meals identified were from stable flies that had previously fed on cattle, with less than 30% having fed on horses. In addition, pastures enclosing cattle were up to 1.5 km from stable fly collection areas within each equine facility.

Stable fly susceptibility to the commonly used pyrethroid, permethrin, was determined to assess possible resistance development in this pest. Stable fly survival after exposure to permethrin was up to 15-fold higher in field-collected flies than in those of a susceptible colony. Diagnostic concentration evaluations demonstrated 40 and 10% stable fly survival to permethrin residues of 3X and 10X the LC_{99} of a susceptible strain. In addition, a laboratory-generated, permethrin-resistant stable fly strain demonstrated 15-fold greater resistance expression over that observed for the parental field-collected stable flies, after only five permethrin selections.

CHAPTER 1
LITERATURE REVIEW OF THE STABLE FLY, *STOMOXYS CALCITRANS* (L.)

Life History

The stable fly, *Stomoxys calcitrans* (L.), is a haematophagous muscoid fly that feeds on humans and a wide variety of other animals. Although both sexes feed on blood, female stable flies require blood proteins for egg development. Anderson and Tempelis (1970) found that female stable flies required three to seven blood meals for oviposition to occur. Stable flies feed on an array of hosts and information regarding the reproductive potential of different host blood types has been investigated. DuToit (1975) found that egg production was highest in female stable flies fed cattle blood when compared to those that fed on blood of the pig. A study by Sutherland (1978a) confirmed the increased nutritional value of bovine blood to the stable fly, concluding that the blood of herbivores including donkeys, sheep, and horses resulted in higher egg production and survivability than that of omnivores such as pigs and dogs. Stable flies that fed on chicken blood were unable to produce eggs.

Anderson and Tempelis (1970) reported that, on average, the time required to fully digest a blood meal was 24-36 hours when stable flies were fed on citrated human blood and maintained at temperatures of 20-21 ºC. Digestion in stable flies fed cattle blood was longer, taking 46 and 70 hr when maintained at maximum and minimum temperatures of 21 and 15 ºC, respectively. Anderson and Tempelis (1970) also noted a decreased digestion time of 10 hr when cattle blood-fed flies were held at 25 ºC, as well as a 2 d decrease in pre-oviposition period in stable flies held at 30 ºC than those held at 22 ºC. These findings are consistent with a review by Lehane (2005), who proposed a rough average time of 48 hr for complete digestion, but noted that many

factors can alter this time frame, including temperature, blood meal size and host type, age, and mating and gonotrophic status.

Depending on the host from which a blood meal was taken, female stable flies will enter a 5- to 10-day pre-oviposition period (Sutherland 1978a). After this period, stable flies will seek an appropriate medium on which to oviposit. Females utilize a variety of organic larval media including spilled livestock feeds, manure, compost piles, and other mixtures of decaying organic matter (Meyer and Petersen 1983, Broce and Haas 1999). Lysyk (1998) reported that the number of eggs produced by a single female varied depending on the temperature at which females were maintained. The highest egg production averaged 26.22 at 30º C, and was lowest at temperatures of 15 and 35º C. Lifetime fecundity was estimated to be <30 eggs for females maintained at 15 or 35 ºC, with a potential of >700 eggs for females maintained at 25 ºC.

Eggs generally hatch 12 to 24 hours after they are deposited (Foil and Hogsette 1994). Survival of the resultant larvae is most dependent upon the breeding media selected by the female stable fly and the environmental conditions during development such as temperature, humidity, and pH. For example, Gilles et al. (2005) demonstrated that larval development varied between 13 and 71 days at 30 and 15 ºC, respectively. Sutherland (1978b) found the dung of various livestock, with the exception of the chicken, as well as several types of vegetable matter, to be suitable for larval development. A study by Boire et al. (1988) also demonstrated larval development in a variety of media including the dung of horses, cattle, swine, and chickens. They found that adding bermudagrass hay to manure increased the numbers and weight of surviving pupae for all manure types except that of cattle. The addition of hay to larval

substrates may play a role in increasing microbial fermentation and bacterial populations. Data collected by Romero et al. (2006) suggest that a symbiosis occurs whereby larval development is dependent upon bacterial populations. Furthermore, results indicated that female stable flies are capable of detecting bacterial cues that indicate the suitability of the larval medium. After the development period, larvae purge the gut and begin wandering throughout the media to locate an adequate pupariation site (McPheron and Broce 1996). The pupal stage generally lasts 5-7 days, with commencement of eclosion dependent on temperature.

Economic Importance

The stable fly is a cosmopolitan pest of humans and an assortment of other animals. The economic losses caused by this pest to livestock producers, especially those of cattle, are well documented. Bruce and Decker (1958) found that monthly reductions in milk and butterfat content averaged 0.7% per fly per cow in response to stable fly pressure. It was also shown that depressed rates of production persisted in the weeks and months after the fly season had ended. Miller et al. (1973) found that despite the annoyance behavior of cattle, tremendous fly loads of 650-1600 per animal significantly decreased milk production in only one of six trials. The behavioral responses of dairy cattle to stable flies were also monitored in a more recent study conducted by Mullens et al. (2006). Although much time was initially spent fighting stable flies with head throwing, stomping, skin twitching, and tail switching, this behavior decreased over time. The results of this study suggest that cattle acclimated to the pain caused by biting flies. It was also concluded that peak stable fly loads of 3.0-3.5 per leg were not adequate to elicit a loss in production. Although these results are conflicting,

they attest to the potential detriment to cattle production systems due to annoyance behaviors caused by stable fly feeding activity.

Stable flies have been shown to affect weight gain and feed efficiency of both confinement and pastured beef cattle. Campbell et al. (1977) observed a 13.2 and 20% decrease in weight gain when growing calves were exposed to 50 and 100 stable flies, respectively. A subsequent study conducted by Campbell et al. (1987) demonstrated the economic threshold of stable flies to be less than two per front leg when weight gain, feed efficiency, and costs of fly control were considered. Catangui et al. (1993) observed an average reduction of 10.6% in weight gains when Brahman-crossbred and English-cross exotic heifers were exposed to 32 flies per foreleg. Furthermore, Campbell et al. (2001) determined the costs incurred by producers of grazing cattle due to stable fly attacks. In this study, untreated control steers averaged 2.79 more stable flies per leg than those treated with permethrin. Flies on control animals resulted in a 19% weight gain reduction compared to those that were treated with insecticide. The overall cost to producers due to attacks by flies was $33.26 per animal, or 2.33 cents per fly.

Although stable flies readily attack horses, little information exists regarding the ecology of this pest associated with equine facilities. This may be due in part to the difficulty in ascertaining losses to horse producers. However, expenses to control stable fly populations on these farms are incurred through the purchase of mechanical and chemical control devices, as well as commercially available pteromalid pupal parasitoids. Stable flies have also been associated with disease transmission and other disorders in horses. Gortel (1998) states that stable flies are often implicated in

arthropod hypersensitivity reactions in horses. These reactions can cause pruritis, which worsens with age, resulting in selling or euthanizing the animal (Fadok and Greiner 1990). Gortel also reports that stable flies often transmit habronemiasis, a condition more commonly known as “summer sores.”

Control

Insecticides and Traps

Stable fly control, as is the case with other muscoid flies, relies heavily on sanitation and pesticide use. Unlike the horn fly, *Haematobia irritans* (L.), stable flies only approach hosts two or three times a day to feed. Furthermore, stable flies prefer to feed on the forelegs of cattle, a site not usually frequented by horn flies (Dougherty et al. 1995, Guglielmone et al. 2004). These behaviors increase the difficulty of controlling stable flies on livestock with insecticides. However, Hogsette et al. (1987) state that pesticides applied to cattle do provide some control of stable fly populations, although it is slower and less dramatic than that observed for horn flies.

Several chemicals with different application methods have been evaluated for controlling the stable fly. Mount et al. (1966) found Baygon (Bayer 39007, methyl carbamate), naled, and fenthion to be highly effective when used against stable flies as a thermal fog. They also noted that the concentrations needed for 80% and greater mortality were as little as 1, 2, and 4%, for Baygon, naled, and fenthion, respectively. Hogsette and Ruff (1986) observed that flucythrinate-impregnated ear tags and permethrin ear tapes were effective against stable flies for 10 weeks. A more recent study conducted by Guglielmone et al. (2004) involving the abundance of stable flies on cattle treated with oganophosphate-impregnated ear tags, found no significant decrease in stable fly numbers. They concluded that pyrethroids, such as permethrin, act as

repellents to stable flies, whereas organophosphates do not. This may account for the effectiveness of permethrin ear tapes observed by Hogsette and Ruff (1986).

Efforts to control stable flies have also been attempted with several traps. The evolution of the cross-configuration alsynite trap developed by Williams (1973), to the cylinder type trap developed by Broce (1988) as reported by Hogsette and Ruff (1990), is in itself evidence to the utility of this material to attract stable flies. Several variations of the alsynite trap have been used to try to enhance collection and control of stable flies. Hogsette and Ruff (1996) found that permethrin-impregnated yarn wrapped in a vertical fashion across the length of the trap did not alter trap attractiveness, and permethrin efficacy lasted for 6-8 weeks.

Other sticky trap configurations, such as the Bite Free™ and EZ Trap™ developed by Farnam (Phoenix, AZ), have proven effective in trapping stable flies (Taylor and Berkebile 2006). Adhesive-coated plasticized corrugated boards in a range of colors have also been shown to effectively collect stable flies (Cilek 2003). Beresford and Sutcliffe (2006) compared the efficiency of colored corrugated plastic coated with adhesive to that of alsynite. They found white and blue plastic boards captured more flies in general, but attributed the increase to numbers of males and nulliparous females collected.

Newer control devices, such as the treated targets developed by Foil and Younger (2006), may prove to be more efficient in controlling stable fly populations than traditional traps. While alsynite is effective in monitoring stable fly populations, the numbers they collect have not been shown to sufficiently control this pest. Treated targets collected 6-fold the number of stable flies caught with alsynite sticky traps. Foil

and Younger (2006) suggested that the attractiveness of blue and black contrasting panels treated with insecticide may be a viable addition to stable fly control programs.

Biological Control

Increases in filth fly insecticide resistance have only compounded the pressures put on confinement livestock producers by neighboring urban communities to decrease both chemical control and fly populations. This problem has led many producers to seek alternate control methods, such as the use of hymenopterous pupal parasitoids. Many studies have been conducted to determine the parasitoid species that prefer different filth fly species, as well as their ability to locate and control fly populations. However most of this work has been carried out within confinement beef and dairy cattle facilities.

A commonly used method to determine the parasitoid species composition in a given region has been to collect naturally-occurring pupae. In addition, sentinel pupae can be placed at these sites to concurrently determine parasitoid preference for a fly species, and parasitism rates after release. Greene et al. (1989) collected house fly, *Musca domestica* L., and stable fly pupae from northwest Florida dairies to determine parasitism rates during the winter months. They found *Spalangia cameroni* Perkins to be the dominant species, accounting for 76 and 58% of the total parasitized stable flies and house flies, respectively. To a lesser extent, *Muscidifurax* spp. accounted for 11 and 36% of parasitized stable fly and house fly pupae, respectively. Differences in rates of parasitism were observed depending on habitat and substrate, with greater numbers occurring in silage than in hay or manure. A study conducted by Jones and Weinzierl (1997) found that 93 and 86% of parasitized stable fly and house fly pupae collected, respectively, resulted in a *Spalangia* spp.

Field-collected stable fly and house fly pupae from dairies in California during the summer also yielded significantly greater numbers of *Spalangia* spp. (Meyer et al. 1990). It was noted that *Muscidifurax* spp. parasitized sentinel house fly pupae more than three times the rate of *Spalangia* spp. This may have occurred because *Muscidifurax* spp. have been shown to forage closer to the substrate surface than *Spalangia* spp. (Legner and Brydon 1966, Skovgård and Jespersen 1999). A subsequent study conducted by Meyer et al. (1991) again revealed a greater proportion of *Spalangia* spp., in particular *Spalangia endius* Walker, attacking filth fly pupae than any other pteromalid species collected. Results also indicated that *Spalangia nigroaenea* Curtis parasitized more house fly pupae than those of the stable fly. Furthermore, data suggested a preference for stable flies by *S. cameroni*, although the difference in parasitism rate was attributed to the decomposing straw or hay substrate in which stable fly pupae were most often found. The authors surmised that ovipositional experience might have driven this species to preferentially forage in these areas for hosts.

Many livestock producers have used mass releases of commercially available hymenopterous parasitoids in attempts to control filth fly populations. However, studies conducted to evaluate the efficacy of this method have provided conflicting results. Petersen et al. (1992) used freeze-killed sentinel house fly pupae to determine mortality caused by field-reared *Muscidifurax zaraptor* Kogan and Legner on cattle feedlots. Mean parasitism was 37.3 and 25.9% in sentinel pupae placed at two treatment feedlots, but only 3.9% was in the two untreated feedlots. Parasitism by this species was relatively high after biweekly releases of up to 29,000 wasps. Petersen et al.

(1992) also documented the capacity of *M. zaraptor* to locate hosts as far as 60-70 m from rearing sites. Andress and Campbell (1994) attained dissimilar results from mass releases of *Muscidifurax raptor* Girault and Saunders and *S. nigroaenea* purchased from a commercial insectary. Parasitoids were released at rates five-fold the insectary recommendation, with no significant effect on adult stable fly populations. Furthermore, it was determined that the cost of this treatment was greater than the losses sustained from stable fly feeding activity. Scientists evaluating the ability of *Muscidifurax raptorellus* Kogan and Legner to parasitize filth fly pupae found this species to utilize sentinel pupae at substantially higher rates than those pupae occurring naturally (Petersen and Cawthra 1995, Petersen and Currey 1996). However, the rates of parasitism in naturally occurring pupae were up to 15.5 and 37.2% for stable flies and house flies, respectively, were the highest observed in a field-based parasitoid study to date. In addition to the ease and cost-effective nature of rearing this species, Floate et al. (2000) concluded that *M. raptorellus* could be released at 200 m intervals for uniform coverage of a confinement facility. This is three-fold the distance observed for *M. zaraptor* by Petersen et al. (1992). Weinzierl and Jones (1998) found that weekly releases of *S. nigroaenea* and *M. zaraptor* nearly doubled parasitism rates, and increased total stable fly and house fly mortality by approximately 10%. However, total parasitism never exceeded 13% for either fly species in feedlots where wasps were released.

While data are conflicting in studies using mass parasitoid releases in open confinement animal operations, these practices have had little affect on fly populations in isolated environments as well. Kaufman et al. (2001c), using individual and paired

releases of *M. raptorellus* and *Nasonia vitripennis* Walker in poultry houses, found that parasitism of the house fly by the former did not significantly increase despite weekly releases. *Nasonia vitripennis* became established when released alone, but decreasing rates of parasitism were observed in the presence of other parasitoids. In a subsequent release study, Kaufman et al. (2001a) confirmed this behavior when *N. vitripennis* accounted for <1% of parasitized hosts in the presence of *M. raptorellus* and *M. raptor*.

Many factors can be attributed to the varied results obtained in studies using parasitoids to control filth fly populations. These can include season, temperature, type of substrate and moisture therein, host species composition, and the depth at which hosts are found. Geden (1999) found that differing moisture levels in poultry manure elicited strong responses in several parasitoid species. *Muscidifurax raptor*, for example, preferred manure with ≤ 75% moisture independent of host density, with the highest response to samples in substrates at 45% moisture. Three *Spalangia* spp. were shown to prefer manure with a moisture content of 45-65%, although they would attack pupae in wetter substrate when host numbers were low. These results suggest that releases of several parasitoid species with different moisture preferences may provide increased filth fly control over that of a single species in poultry facilities.

Meyer et al. (1991) found pure manure to be the most abundant stable fly breeding sites on dairies, although they observed no difference in abundance of seven parasitoid species at these locations. This suggests filth fly parasitoids may be more habitat-specific than host-specific. Because feed storage, waste removal, and unit design differ from one livestock facility to the next, different breeding habitats are created. Stable flies utilizing loose, wet alfalfa hay at one cattle feedlot, may be found readily in

compacted manure and grain at the next. Scenarios such as this may account in part for some of the varied success of parasitoid releases.

Insecticide Resistance

Muscoid flies, like many insect pests whose control relies heavily on the use of insecticides, have developed widespread resistance to a variety of chemicals. Insecticide resistance in the house fly and horn fly has been well documented. Both species have developed resistance to most insecticides used for their control, and the mechanisms behind their ability to do so are well established.

Over the past five decades, many insecticides, including chlorinated hydrocarbons such as DDT, organophosphates such as tetrachlorvinphos and naled, and more recently, pyrethroids such as permethrin and fenvalerate, have been used to control muscoid flies in Nebraska (Marçon et al. 2003). Shifting usage of pesticides such as these has occurred largely due to development of resistance in filth fly populations. However, the discontinued use of some insecticides, including organophosphates and carbamates, can be attributed to federal regulations, such as the Food Quality Protection Act of 1996 (Kaufman et al. 2001b).

Insecticide resistance, coupled with the difficulty and costs of developing new chemicals with unique modes of action that are safe, and effective, increases the need for information concerning the resistance status of these fly pests. Resistant house fly populations have been collected from cattle feedlot and dairy facilities, as well as from poultry units (Scott et al. 2000, Kaufman et al. 2001b, Marçon et al. 2003). Populations of insecticide resistant horn flies have also been collected from various confinement cattle facilities and pastures (Schmidt et al. 1985, Crosby et al. 1991, Cilek et al. 1991, Kaufman et al. 1999, Barros et al. 2001). In areas where the gap between residential

areas and confinement animal facilities continues to close, the mechanisms behind muscoid fly pesticide resistance must be determined as the potential for litigation looms.

Selection pressure, due to years of pesticide use, has driven the genetic forces of resistance. Because pyrethroids, such as permethrin, are used to control a wide variety of arthropod pests, much work has been conducted to understand the behavioral and physiological mechanisms behind resistance to this group of insecticides. Studies conducted by Lockwood et al. (1985) and Zyzak et al. (1996) documented that avoidance behaviors to pyrethroids displayed by horn flies, might be a cause of resistance to these compounds. Two additional resistance mechanisms are widely accepted: increased rates of metabolic detoxification, and mutations in pyrethroid target sites (Huang et al. 2004).

Distribution and Dispersal of Muscoid Fly Insecticide Resistance

Although resistant populations of the house fly are known to occur globally, their geographical distribution must also be determined, because resistant populations may be problematic far from areas that currently employ chemical pesticides. Learmount et al. (2002) stated that by 1978, populations of resistant house flies in the United Kingdom had been found for all major classes of pesticides used for their control. This included chlorinated hydrocarbons, organophosphates, organochlorines, pyrethrins and synthetic pyrethroids. Learmount et al. (2002) also found that resistant house fly populations were a widespread and increasing problem. A similar trend has been observed in the United States where permethrin has been the pesticide of choice to control muscoid flies for over 25 years.

Because information concerning the geographical limits of resistant house fly populations is important, studies have been conducted to obtain this information. One

way to obtain such data is to evaluate pesticide resistance in flies collected from various confinement animal facilities in different geographical locations. In a study conducted by Scott et al. (2000), house flies were collected from eight poultry facilities across New York state to evaluate their susceptibility to nine pesticides. Analysis revealed that resistant populations were highly correlated with those poultry units that used specific insecticides. It was also determined that dispersal of resistant house flies to other poultry facilities was limited. Scott et al. (2000) concluded that management strategies against resistant fly populations of poultry units were feasible due to the relative isolation of insects within such closed facilities.

In a similar study on New York state dairies, Kaufman et al. (2001b) evaluated resistant populations of house flies. House flies collected from several dairies were exposed to seven insecticides to evaluate resistance. In contrast to the study of poultry facilities in which units were relatively isolated, levels of resistance were similar for all dairies independent of pesticide use history. The results suggest that flies from these open-design facilities disperse, and thus, resistant population strains were found for all insecticides used collectively. It was also observed that resistance levels for permethrin had increased from those detected in a 1987 survey of house flies collected from New York dairies (Kaufman et al. 2001b).

Managers of cattle feedlot facilities also rely on the use of insecticides to control pest flies. Reduced weight gain and feed efficiency due to the landing or biting activity of muscoid flies, such as stable flies, can can result in severe economic impacts for producers (Campbell et al. 2001). Routine use of pesticides, such as permethrin, to control these pests has contributed to the resistant fly populations observed in

confinement animal operations. In a study conducted by Marçon et al. (2003), house flies collected from two cattle feedlots in Nebraska were evaluated for resistance to permethrin, stirofos, and methoxychlor. Although flies from both facilities demonstrated resistance to permethrin, resistance levels to stirofos and methoxychlor were greater still. Resistance to methoxychlor, an organochlorine, and stirofos, an organophosphate, may have established in these fly populations when they were exposed to these, or other compounds with similar modes of action.

It is surprising that stable flies collected from the same feedlots in Nebraska by Marçon et al. (2003) were highly susceptible to permethrin, methoxychlor, and stirofos (Marçon et al. 1997). Topical application of pesticides to cattle may limit chemical exposure to stable flies, which are normally found feeding on the forelegs. Data collected from cattle feedlots in Kansas by Cilek and Greene (1994), demonstrate that stable flies can become resistant to permethrin and stirofos, as well as dichlorvos. Resistant populations were found within facilities where pesticide use was infrequent or absent, suggesting dispersal of local populations from other installations. Cilek and Greene (1994) also found dichlorvos resistance at facilities where it had not been used. However, Naled, a related compound, had been applied, thus conferring resistance to dichlorvos, through a mechanism known as cross-resistance.

Mechanisms of Resistance

A number of resistance mechanisms have been identified in muscoid flies. Most of this work has been conducted on the genetic influences of pyrethroid resistance in house flies and horn flies. This is probably results from wide use of permethrin for controlling insect populations and the decreasing efficacy documented over the years. House flies have developed resistance to most insecticides used against them.

Therefore, determination of the mechanisms by which this occurs is important if future management systems are to be successful.

Knockdown pesticides, such as DDT and pyrethroids, result in rapid death of insects by acting on the nerve membrane voltage-sensitive sodium channels. However, long-term use of these classes of insecticides has led to "knockdown resistance" (*kdr*) within pest populations. A *kdr* mechanism was first demonstrated in horn flies using genetic crosses (Roush et al. 1986). Knockdown resistance target-site insensitivity was confirmed in horn flies when metabolic detoxification synergists failed to increase resistance in a laboratory colony (McDonald and Schmidt 1987). House flies expressing this trait were shown by Knipple et al. (1994) to harbor a *kdr* gene that is linked to the voltage-sensitive sodium channel gene. They concluded that a point mutation on the latter might be the basis of *kdr* in the house fly. Further study by Smith et al. (1997) confirmed that a substitution of phenylalanine for leucine at the 1014 position of the amino acid sequence, L1014F, resulted in increased decay rates of pyrethroid induced sodium currents. Smith et al. (1997) concluded that this mutation decreased the pyrethroid effects on sodium channels sufficiently to account for *kdr.* Lee et al. (1999) found that a second mutation of the *kdr* gene at the M918T location conferred even greater resistance to pyrethroids than that of the L1014F. Flies with this trait have been termed "*super-kdr*". The substitution of phenylalanine for leucine, as well as threonine for methionine, has also been shown in *kdr* and *super-kdr* horn flies, respectively (Guerrero et al. 1997). The frequencies of these point mutations vary with the level of resistance in house flies and horn flies (Jamroz et al. 1998, Huang et al. 2004).

Evidence exists that cytochrome P450's, in particular CYP6D1, play a role in pesticide resistance of muscoid flies as well (Seifert and Scott 2002). This cytochrome acts to detoxify pyrethroids in the house fly through a monooxygenase-mediated response (Liu and Scott 1997, 1998). This metabolic resistance mechanism was also demonstrated in horn flies by Sheppard and Joyce (1992), when pyrethroids were synergized with piperonyl butoxide. In flies that express this mechanism of resistance, an upregulation in transcription of CYP6D1 is observed. Liu and Scott (1998) found that flies of the Learn Pyrethroid Resistant (LPR) strain had 10-fold greater transcription levels of cytochrome CYP6D1 than did susceptible strains.

The discovery of this cytochrome may also contribute to understanding the geographical extent of resistant house fly populations. In a study by Seifert and Scott (2002), *CYP6D1* was sequenced from two different house fly populations. Populations from Georgia and the original LPR strain discovered from a New York state dairy were found to have identical CYP6D1, suggesting the possibility of dispersal. However, a study by Rinkevich et al. (2007) found that resistance allele frequency varied greatly between house flies collected at New York state dairies and those from Florida. The authors surmised therefore, that local factors, such as selection pressure, are the driving forces behind resistance expression in house fly populations.

Resistance to other pesticides, such as thiodicarb and fipronil, has been observed in house fly populations. Karunaratne and Plapp (1993) found that resistance to thiodicarb pesticides might be due to alterations in the activity of cholinesterases. Although this may be the mechanism behind detoxification of the chemical, cross-resistance may be the cause. In a study by Wen and Scott (1999), it was determined

that the LPR strain of house fly was resistant to the relatively new chemical, fipronil. The cause of cross-resistance in this case was contrary to previous results where cyclodiene-resistant insects were shown to be susceptible to fipronil. The LPR strain used in these experiments had not before been associated with the cyclodiene chemical.

Scott (1998) evaluated the effect of a newer chemical, spinosad, on both susceptible and resistant house fly populations. The results of this study demonstrated that the insecticide was highly toxic to both populations, although the activity of the compound was relatively slow. It was also determined that the chemical could be synergized with piperonyl butoxide and S,S,S,-tributylphosphorotrithioate to increase its toxicity, while diethyl maleate synergism resulted in no effect. When synergized, it was concluded that house flies may have expressed a slight monooxygenase-mediated detoxification of this newly-developed chemical. More recently, Kristensen and Jespersen (2004) observed that field-collected house fly populations expressed small resistance factors to spinosad. However, they concluded that cross-resistance to other chemical classes should not initially play a major role. Therefore, spinosad may offer an alternative to other conventional insecticides such as permethrin.

Although spinosad could be used with permethrin in an alternating pesticide management program, Barros et al. (1999) found that such a regimen was ineffective in controlling pyrethroid-resistant horn flies. Organophosphate and pyrethroid ear tags were used in a rotational management strategy, but did not improve or delay the effects of pyrethroid resistance. This is contrary to the laboratory findings of Byford et al. (1999), where rotations of diazinon, permethrin, and ivermectin, as well as mixtures of

these chemicals delayed the onset of resistance for up to 12 generations. Furthermore, a mosaic strategy employing pyrethroid and organophosphate ear tags did not result in decreased efficacy during a three-year period.

Research Objectives

Research studies concerning various aspects of stable fly biology and ecology associated with cattle production are numerous. However, a large void exists in the literature exists for research concerning stable flies and other biting fly pests affecting equine production systems. Therefore, field and laboratory studies investigating several aspects of stable fly ecology associated with equine facilities in Florida were undertaken to bridge this gap, as well as to provide a basis for further research in this area. My specific research objectives include:

1) The concurrent study of on-site adult and pupal stable fly populations to predict the presence of pupal populations, and thus the appropriate timing of pteromalid parasitoid releases. Determine the species composition and seasonal distribution of parasitoids attacking filth flies at Florida equine facilities.

2) Evaluation of the efficiency of stable fly host location and attack by selected pteromalid pupal parasitoids to determine potential intra- and interspecies differences in attack rates of hosts within a substrate and hosts made freely accessible.

3) Develop a multiplex polymerase chain reaction blood meal analysis tool to detect the hosts of stable flies collected from Florida equine facilities and determine whether stable fly host blood meal identification can be used to indicate their short-term localized movement.

4) Conduct a critically needed update on the status of permethrin susceptibilities of field-collected stable flies using diagnostic concentrations, and determine the rate at which stable flies express resistance to this insecticide by selecting for this trait in a laboratory colony.

CHAPTER 2
SEASONAL ABUNDANCE OF STABLE FLIES AND THEIR PUPAL PARASITOIDS (HYMENOPTERA: PTEROMALIDAE) AT EQUINE FACILITIES

Introduction

Studies concerning the impact of stable flies, *Stomoxys calcitrans* (L.), on livestock are numerous (Bruce and Decker 1958, Miller et al. 1973, Campbell et al. 1977, Mullens et al. 2006). However, control of this pest has been difficult using traditional tactics such as traps and insecticides. Whether this is due to factors such as the repellency effects of some pesticides (Hogsette and Ruff 1986), the relatively short time period spent on the host compared to other biting flies such as the horn fly, *Haematobia irritans* (L.) (Crosby et al. 1991), or their ability to disperse to other locations (Bailey et al. 1973, Hogsette and Ruff 1985, Chapter 4) remains to be seen. In addition, the modest control achieved using insecticides may become increasingly limited due to their expression of insecticide resistance (Chapter 5), a problem most noted in the house fly, *Musca domestica* L.

Pteromalid pupal parasitoids are often utilized as an alternative filth fly control measure in an integrated pest management program. However, studies at cattle feedlot and dairy facilities report mixed success using parasitoids (Andress and Campbell 1994, Petersen and Cawthra 1995, Petersen and Currey 1996). Reasons for this may include differences in season, temperature, moisture, host density and depth, and manure-substrate types utilized by filth flies. Meyer et al. (1991) found no significant difference in the abundance of seven parasitoid species utilizing stable fly pupae in dairy cattle manure. They concluded that habitat, rather than host, played the primary role in rates of parasitism. In a study conducted by Skovgård and Jespersen (1999), more *Spalangia cameroni* Perkins had parasitized stable flies buried deep in feed or manure,

whereas *Muscidifurax raptor* Girault and Saunders parasitized the majority of house flies located closer to the substrate surface. It was surmised that this difference was due to accessibility of pupae and substrate rather than parasitoid preference for a particular fly species. Smith and Rutz (1991) also noted the habitat preferences of *M. raptor*, most often found in outdoor feed and straw, and *S. cameroni* most often found in loose indoor substrates. Preference for a particular habitat has been demonstrated in other *Muscidifurax* and *Spalangia* spp. using different moisture levels in poultry manure (Geden 1999).

Other factors, such as species competition or dispersal patterns may also affect the outcome of a parasitoid release program. Kaufman et al. (2001a, 2001c) found that *Nasonia vitripennis* Walker parasitized more house fly sentinel pupae when it was utilized alone. However, when released with *Muscidifurax raptorellus* Kogan and Legner, host utilization by *N. vitripennis* decreased. While Tobin and Pitts (1999) found that *M. raptorellus* dispersed distances of 2 m or less in poultry facilities, Floate et al. (2000) recorded dispersal distances of up to 200 m from release sites at cattle feedlots. The seeming disparity in dispersal between these studies however, may have been due to the facility types in which they were conducted. The open nature of cattle feedlots may increase movement of insects such as parasitoids, as they are more exposed to weather and environmental factors such as wind. The confined nature of poultry facilities would inhibit such assisted movement.

A more simple explanation for the disparities in success using parasitoids to control filth flies may be due to the difficulty in predicting the appropriate time for their release. A fly problem based on the presence of adult filth flies may not take into

consideration the presence of pupae. Often, assuming migration is not the primary cause, a sudden increase in adult flies indicates the eclosion of on-site pupae, thereby rendering immediate releases of parasitoids an ineffective control measure. Furthermore, depending on environmental conditions (Lysyk 1993), if a parasitoid release is conducted at that time, it may be weeks before suitable host pupae are present. By this time, natural mortality and dispersal of released parasitoids may result in insignificant control of any subsequent pupal populations (Skovgård 2002). This is especially true for the stable fly, as immature development can take as long as 71 d under cool temperatures (Gilles et al. 2005). In general, under optimum conditions, the stinging activity of some parasitoids ends when pupae reach 4 d in age (Kaufman et al. 2001a). This can further complicate control if parasitoids are purchased from commercial insectaries, as shipment time may delay their release to a point when on-site hosts are no longer suitable.

These factors necessitate the need for preliminary investigations of parasitoid activity within a particular facility prior to using pteromalid releases as a filth fly control measure (Greene et al. 1989). Differences in habitat type and suitable breeding substrate may exist between livestock installations, affecting the abundance, species composition, and parasitism rates of resident parasitoids. Although many equine producers utilize them for filth fly control, I was unable to find any study on the effectiveness or species composition of pupal parasitoids at equine facilities. Therefore a study was initiated in December 2007 near Ocala, Florida, to help fill this void. The primary objectives of this study were to 1) use concurrent study of adult and pupal stable fly populations to predict pupal presence, and thus appropriate timing of

pteromalid parasitoid release and 2) determine the species composition and seasonal distribution of parasitoids attacking filth flies at equine facilities.

Materials and Methods

Equine Facilities. In November 2007, a two-year study of stable fly and house fly pupal parasitoid abundance of Florida equine facilities was initiated. The four facilities used in the study were located approximately 12 km north and/or west of Ocala, Florida, and approximately 8 km from each other. Each farm varied in acreage and number of horses: Farm 1; 1,800 acres, 350 horses, Farm 2; 200 acres, 110 horses, Farm 3; 4,500 acres, 250 horses, Farm 4; 320 acres, 120 horses. Farms 1, 3, and 4 used large wood chips (2-3 cm) and straw as bedding in stalls, while Farm 2 used small particle (0.1-0.3 cm) wood shavings. All farms used alfalfa hay as feed for horses, but only Farm 4 utilized round hay bales in pastures during the winter. Though daily removal of waste and debris from stalls was practiced at each farm, the methods used for its disposal differed. Farms 1 and 3 disposed of accumulated horse wastes using large composting areas, with a central dumping location from which compost windrows were made. Farms 2 and 4 utilized manure spreaders to distribute horse waste products throughout pastures. All farms were chosen because they were large tracts of land with at least 100 horses, and were an appreciable distance (>0.5 km) from nearby cattle facilities, which facilitated the completion of my objectives in Chapter 4.

Adult Stable Fly Surveillance. Adult stable fly populations were monitored weekly at each farm using corrugated alsynite cylinder traps placed in different locations throughout each farm. Three traps were placed similarly on all farms, with one trap near pastured horses, a second near barns with stabled horses, and a third near composting or manure spreading areas. Each trap was set up similarly to those

described by Hogsette and Ruff (1990). Briefly, a corrugated alsynite cylinder (Olson Products Inc., Medina, OH) approximately 30 cm in height and 20 cm in diameter was mounted in a slit cut into a 5 x 5 x 122 cm wooden stake. Each wooden stake was driven 30 cm into the ground so that trap height was approximately 90 cm (Williams and Rogers 1976) (Fig 2-1). A translucent adhesive-coated propylene sleeve (Olson Products Inc., Medina, OH) was attached to the outer surface of each trap with metal clips and left in place for 7 d. Sleeves were replaced weekly and stable fly numbers were recorded for each facility by date. Stable fly counts did not include sex-determination as the characters used for this identification were unreliable due to damage caused by the adhesive glue and time that captured flies spent on the traps.

Pupal Collection. Weekly filth fly pupal collections began in December 2007 and continued until December 2009. Initially, an attempt was made to use the sentinel pupae technique of Rutz and Axtell (1979) to assess pteromalid pupal parasitoid activity. However, this effort was abandoned as local red imported fire ant, *Solenopsis invicta* Buren, populations destroyed most sentinels. Thereafter, each week an attempt was made to collect at least 50 pupae from expected breeding areas at 3-5 sites within each farm, collecting more pupae when possible. Regardless of availability, searching ceased after 30 minutes and the resultant pupae were removed from collected debris by floatation. All pupae were returned to the University of Florida Veterinary Entomology Laboratory and sorted to remove any pupae that were previously eclosed, had been damaged, or had parasitoid emergence holes using the intact pupae method of Petersen and Meyer (1985). This method was also used to calculate the percent parasitism. Immediately after their collection, any intact pupae that were dark in color

were placed individually into #0 gelatin capsules. At this time, all other intact pupae were placed into 120 ml plastic soufflé cups with covers and held for 3- to 5-d at 26 ºC, 12:12 LD, and 70% RH for adult fly eclosion. After the 3- to 5-day holding period, any remaining uneclosed pupae were placed individually into #0 gelatin capsules and held for 40 d at 26 ºC, 12:12 LD, and 70% RH for parasitoid emergence (Fig. 2-2). Pupae that did not produce a fly or a parasitoid were dissected to determine if adult fly mortality was due to an aborted parasitoid. The keys of Rueda and Axtell (1985a), and the pictorial guide by Gibson (2009) were used to identify all pteromalid pupal parasitoids.

Statistical Analysis. Stable fly trap collection data were subjected to analysis of variance (ANOVA) using the PROC GLM procedure of SAS® 9.2 (SAS Institute 2004) to determine differences in adult stable fly activity by month and by farm. Stable fly numbers by trap/month and by trap/farm were transformed using $\ln(n + 1)$, using month and farm as fixed effects in their respective analysis. These results are presented as untransformed data. Stable fly trap collections were also subjected to the PROC CORR procedure to determine the correlation in stable fly seasonal distribution between farms. The percent contribution of each trap collection to the total number of stable flies collected on each farm was subjected to an arcsine square root transformation prior to this analysis. Multiple mean comparisons were conducted with the Ryan-Einot-Gabriel-Welsh (REGW) multiple range test ($\alpha = 0.05$). To assist in interpretation of stable fly activity, weekly precipitation and temperature data was collected from the National Oceanic and Atmospheric Administration (NOAA, 2009) (Fig. 2-3, untransformed data) site, located in Ocala, Florida.

An attempt to correlate stable fly pupal collections with those of adult stable flies, as well as rainfall and temperature was made using the PROC REG procedure (stepwise multiple regression). Only weeks in which at least one stable fly pupa could be found were included in the analysis. Total adult and pupal stable fly collections from all four farms during a given week were transformed using $\ln(n + 1)$, and are presented using transformed data.

The percent parasitism was calculated as the number of emerged (identifiable) and aborted (unidentifiable) parasitoids divided by the number of intact pupae collected during a given week, at a given farm. These data were subjected to ANOVA to determine differences in parasitism rates between farms, as well as differences in parasitoid species composition during a given week, at a given farm. The data were analyzed separately for each fly species. There were only two weeks in which 50 house fly pupae were collected from Farm 4, resulting in the subsequent removal of this farm from the analysis for that fly species.

Two additional ANOVAs were conducted to determine if differences between inter- and intra-species parasitism occurred during a given month. Data from both stable fly and house fly pupal collections were pooled for this analysis. Only months in which 50 stable fly or house fly pupae had been collected on at least five occasions were included in the analysis, resulting in the subsequent removal of June, July, August, November, and December. All parasitoid data were transformed using an arcsine square root of the percent parasitism for a given week, at a given farm, and are presented in tables as back-transformed means. Both farm and month were included

as fixed effects in their respective analysis. Multiple mean comparisons were conducted with the Ryan-Einot-Gabriel-Welsh (REGW) multiple range test ($\alpha = 0.05$).

Results

Adult Stable Fly Surveillance. A total of 104,718 stable flies were collected from four equine facilities between November 2007 and December 2009. Although stable flies were captured in every month, significantly more ($F_{11, 1263} = 75.98$; $P = <0.0001$) were collected during March (74 flies/trap/week) and April (76 flies/trap/week) than any other month. With the exception of Farm 2 in 2008, stable fly collections gradually increased beginning in January, with peak collections occurring at the end of April in both years (Fig. 2-4a, b). In 2008, peak stable fly collections occurred in January at Farm 2, although trap captures from this farm had been increasing when the study began in November 2007. Results from the mean separation test indicated that significantly more stable flies were collected from Farm 2 than any other farm, followed by Farms 4, 3, and 1, respectively. During this study, stable fly population dynamics were highly correlated ($P <0.0001$) between farms, with r-values ranging from 0.66-0.95 between years, and from 0.82-0.92 for both years combined.

Stable Fly Parasitism. During the two-year study 12,675 stable fly pupae were collected from sites located within the four equine facilities. From these, 1,928 adult and aborted pteromalid pupal parasitoids were produced (Table 2-1). With the exception of one pupa producing a *M. raptor*, all Pteromalidae collected from stable flies were *Spalangia* spp. No significant differences were detected between farms in overall stable fly percent parasitism or in the percent composition of *Spalangia nigroaenea* Curtis or *Spalangia endius* Walker. Significant differences between farms in percent composition were detected for *S. cameroni* ($F_{3, 56} = 3.80$; $P = 0.0303$) and *Spalangia*

nigra Latreille ($F_{3, 56} = 6.18$; $P = 0.0011$). A significantly greater percentage of the parasitoids collected from Farm 4 were *S. cameroni* than at Farm 1. However, a significantly lower *S. nigra* parasitism rate was observed at Farm 4, as compared to Farms 1 and 3. Although no significant differences were detected between farms in the percent composition of *S. endius*, this species was recovered more often from stable fly pupae collected at Farm 1 than at Farms 2 and 3.

House Fly Parasitism. A total of 12,841 house fly pupae were collected from four equine facilities between December 2007 and December 2009, producing a total of 1,331 emerged and aborted pteromalid pupal parasitoids (Table 2-1). With the exception of one house fly pupa yielding a *M. raptor* and one pupa producing a *Phygadeuon* spp. (data not shown), all resultant parasitoids were *Spalangia* spp. No significant differences in percent house fly parasitism were detected in overall percent parasitism between farms or in the percent composition of *S. nigra* and *S. endius*. Significant differences between farms were detected for percent composition of *S. cameroni* ($F_{2, 25} = 3.80$; $P = 0.0362$) and *S. nigroaenea* ($F_{2, 25} = 3.98$; $P = 0.0315$). On Farms 1 and 2, significantly more *S. nigroaenea* were recovered from house fly pupae than on Farm 3. Conversely, significantly more *S. cameroni* were collected from Farm 3 than Farms 1 and 2 (Table 2-1).

Monthly Parasitoid Abundance. Pteromalid pupal parasitoids were recovered during every month of the study with the exception of November, when no stable fly or house fly pupae could be recovered from any farm (Table 2-2). Our intra-species analysis indicated that there were no significant differences between months in overall percent parasitism by all *Spalangia* spp. or in percent composition of *S. nigra*.

Significant differences between months were detected in percent species composition of *S. cameroni* ($F_{6, 79}$ = 6.21; *P* = 0.0001), *S. nigroaenea* ($F_{6, 79}$ = 6.94; *P* = 0.0001), and *S. endius* ($F_{6, 79}$ = 8.06; *P* = 0.0001). A significantly greater proportion of *S. cameroni* were collected between January and May than during September and October. Significantly more *S. nigroaenea* were collected during September and October than in February or March. Significantly more *S. endius* were collected in September than any other month during the study.

Significant differences were detected in our interspecies abundance analysis for all months in which sufficient pupal recovery was achieved: January ($F_{3, 32}$ = 69.40; *P* < 0.0001), February ($F_{3, 56}$ = 106.86; *P* < 0.0001), March ($F_{3, 80}$ = 69.05; *P* < 0.0001), April ($F_{3, 92}$ = 32.78; *P* < 0.0001), May ($F_{3, 20}$ = 5.89; *P* = 0.0047), September ($F_{3, 20}$ = 11.22; *P* = 0.0002), and October ($F_{3, 16}$ = 7.17; *P* = 0.0029). Between January and April, significantly more *S. cameroni* were recovered from filth fly pupae than any other species, followed by *S. nigroaenea*, *S. nigra*, and *S. endius*. During May, significantly more *S. cameroni* and *S. nigroaenea* were collected than *S. endius*. Significantly more *S. nigroaenea* were collected from filth fly pupae than any other species during the months of September and October.

Correlating Pupal and Adult Stable Flies. The results of the multiple regression analysis indicated that precipitation, temperature, and adult stable fly trap collections were not significant factors in determining our ability to locate pupae from filth fly breeding sites. However, a significant ($F_{1, 37}$ = 108.18; *P* = 0.0001) relationship was identified between adult stable fly trap collections and pupal collections from 2 weeks prior, r^2 = 0.74 (Fig. 2-5).

Discussion

Adult stable flies were collected from Florida equine facilities every month during the two-year study. Significant differences were detected in the number of stable flies collected from each farm throughout the study. On average, traps at Farm 2 yielded the most stable flies per week, followed by Farms 4, 3, and 1. Although Farm 2 used a manure spreading technique to dispose of stall waste each day, the small particle size of the wood shavings and the continuous reuse of the same waste disposal sites, created an optimum breeding substrate for stable flies. Farm 4 also used a manure spreader, but here debris was cast out over long distances and did not accumulate as it did at Farm 2. However, Farm 4 was the only site to use round hay bales to feed horses, a widely recognized developmental site for stable fly larvae on cattle farms (Broce et al. 2005, Talley et al. 2009). Not surprisingly, stable fly trap collections were greatest at Farms 2 and 4. Stable fly production at Farm 3 was apparent, but did not seem to justify the numbers of stable flies collected on traps. My results from Chapter 4 suggest that many of the flies collected at Farm 3 could be occurring as emigrants from nearby cattle farms. Whether off-site breeding is the cause of the stable fly population on this farm remains unclear. Historically, stable fly breeding and host-feeding have been most often associated with cattle (Hogsette et al. 1987). Stable fly activity at Farm 1 was significantly lower than any other farm. This is probably due to the Farm's intense practice of daily cultural controls and composting activity. It also may be noted that this is the only farm in which chemical insecticides were not used, adding to the utility of cultural techniques as a stable fly control method.

Stable fly population dynamics between farms was highly correlated suggesting that adult stable fly activity is driven by similar factors at each farm, such as weather

(Lysyk 1993). This is particularly evident in stable fly collections during 2009, when a non-typical peak in stable fly activity occurred in June at all farms (Fig 2-4b). Average temperatures between both years during the months preceding June were similar. However, in May 2009, accumulated precipitation was 31 cm, compared to 0.5 cm in May 2008 (Fig. 2-3). Therefore, it is likely that precipitation played a larger role in the increased stable fly activity later in 2009 (Hogsette et al. 1987). This is likely to have provided a late-season developmental opportunity for stable flies otherwise maintained at minimal levels under average May precipitation conditions (3.2 cm) (NOAA 2009).

Although several studies of stable fly activity in Florida have been conducted, most have been in an attempt to identify their breeding sites (Fye et al. 1980) and sudden appearance in areas such as the Gulf coast beaches (King and Lenert 1936, Hogsette and Ruff 1985). In Florida, seasonal stable fly activity is usually greatest between the months of January and April, although stable flies occur throughout the year (Gentry 2002). Multiple peaks in stable fly abundance during these months can occur, depending on precipitation and available breeding habitats. This is similar to results of the present study, where stable fly collections began to increase steadily in January, with peak collections occurring in April. By early May, stable fly collections declined dramatically and were minimal for the remainder of the year, except for a single late-season peak in June of 2009 (Fig 2-4a, b).

The seasonal stable fly distribution observed in our study does corroborate research conducted by others for the state, but differs dramatically from studies in other areas of the U.S. Mullens and Meyer (1987) reported that peak stable fly activity at California dairies occurred in May and June, whereas, Burg et al. (1990) found that

stable fly populations in Kentucky began to rise in May, with peak collections occurring during the summer months of June, July, and August. This is similar to results obtained by Broce et al. (2005) in Kansas, although bimodal peaks in stable fly activity were observed in June and again during the months of September and October. Increased stable fly activity noted by Lysyk (1993) working in Alberta, Canada, was later still, occurring during the months of August and September. The later dates for more northern areas are not surprising, given the seasonal temperature patterns of those areas.

To my knowledge, yearlong stable fly seasonal surveillance data have not been published for Florida, nor has it been used to indicate the presence of stable fly pupae. An attempt to correlate both stable fly life stages was made to give livestock producers a practical method to more closely predict appropriate times for release of pupal parasitoids. Regression analysis demonstrated that while the presence of adult and pupal stable flies were highly correlated (Fig. 2-5), the relationship was only valid when adult trap collections were associated with pupae from 2 weeks prior. Such knowledge does little to assist in the preemptive management of this pest.

There are several potential reasons for this occurrence. In the present study, stable fly populations gradually increased throughout the season; it is likely their resultant breeding also produced gradually increasing pupal numbers within breeding areas. The significant relationship between adult stable flies and pupae from two weeks prior does correspond with the average time period for stable fly development from egg to pupa (Gilles et al. 2005). Additionally, the average temperatures during the months when stable flies are most active in Florida are similar to those used for colony rearing

in our laboratory. Furthermore, this analysis was dependent on my ability to locate pupae scattered throughout a substrate, and their numbers likely varied due to the methods used for their collection. However, stable fly pupae were found in 39 of over 100 sampling attempts, which is consistent with the number of weeks (16-18/year) that stable flies are primarily active in Florida. Although house flies were not the target of this study, they were opportunistically collected as pupae. Further research using the concurrent trapping of both fly species and different pupal collection methods may provide a relationship that could be used to predict the presence of subsequent pupa.

Over the course of the two-year study, nearly 100% of all pteromalid pupal parasitoids recovered from filth fly pupae were *Spalangia* spp., with more than 90% of the parasitoids being either *S. cameroni* or *S. nigroaenea*. This is similar to results from across the U.S., where these species made up a significantly larger proportion of recovered parasitoids than others (Greene et al. 1989, Meyer et al. 1990, Jones and Weinzierl 1997). Several studies also attest to the propensity of members of this genus to search deep within substrates for both stable fly and house fly hosts, compared to other parasitoids such as *Muscidifurax* spp. (Rueda and Axtell 1985b, Greene et al. 1989, Skovgård and Jespersen 1999). In addition, most studies have been conducted at cattle feedlots or dairies (Seymour and Campbell 1993, Meyer et al. 1991), and poultry facilities (Rutz and Axtell 1981, Kaufman et al. 2001c) where fly breeding habitats can differ greatly depending on the cultural management practices of each farm. These studies often report the common occurrence of *Muscidifurax* spp. recovered from pupae within different livestock facilities. In the present study, pupae were never located in substrates at depths less than 3 cm, and in most cases were

collected at greater depths. In addition, most of the breeding areas sampled in the current study contained porous, loose debris; sites favorably searched by *Spalangia* spp. (Smith and Rutz 1991). Stable fly pupae from all farms with the exception of Farm 4, were most often collected from within horse dung found in discarded horse bedding. Most pupae collected from Farm 4 were located deep within decomposing alfalfa hay, near round bale feeding sites. These pupae were located at distances from round bales similar to observations of Talley et al. (2009). House fly pupae were most often located deep within the sandy soil beneath discarded horse bedding. These factors may explain the overwhelming occurrence of *Spalangia* spp. and the absence of other genera, such as *Muscidifurax* spp.

Overall parasitism rates varied between farms from approximately 7-18% and 5-18% for stable flies and houses flies, respectively, but were not significantly different. Estimates of parasitism determined by published studies vary widely, but several report parasitism rates similar to those of the present study. Petersen and Cawthra (1995) and Petersen and Currey (1996) observed parasitism rates of *M. raptorellus* as high as 15.5 and 37.2% for stable flies and house flies, respectively. Weinzierl and Jones (1998) determined that weekly releases using *S. nigroaenea* and *Muscidifurax zaraptor* Kogan and Legner resulted in parasitism rates of 11.6 and 13% for stable flies and house flies, respectively. In Florida, Greene et al. (1989) found that parasitism was as high as 61 and 71% for stable flies and house flies, respectively, depending on the substrate from which pupae were collected. However, parasitism in the Greene et al. study was calculated using only pupae that resulted in a fly or a parasitoid, which may have inflated parasitism estimates. The use of the intact pupa method in the present

study may have underestimated parasitism rates, as some of the pupae held for parasitoid emergence may have been suitable for parasitoid attack when they were removed from the equine environment. However, my technique does provide a moderately accurate assessment, according to Petersen and Meyer (1985).

All four *Spalangia* spp. collected during this study were recovered from both stable fly and house fly pupae (Table 2-1). Significant differences in parasitoid species recovered from house fly pupae were detected only for Farm 3. Here, the percent species composition of *S. cameroni* was greater at Farm 3 than the other farms, while that for *S. nigroaenea* was lower at Farm 3 than the other farms, for the entire sampling period. Analysis of the seasonal distribution for these species makes it unlikely that this is due to species competition, and instead suggests the differences may be due to my inability to locate pupae at Farm 3 during times when *S. nigroaenea* were most abundant. The significant differences determined in percent composition of *Spalangia* spp. were similar for parasitoids emerging from both stable fly and house fly pupae.

In general, *S. cameroni* made up a larger proportion of the parasitoids recovered from stable flies and house flies from Farms 3 and 4, although there was no significant difference between these farms and Farm 2. *Spalangia nigroaenea* made up a larger proportion of the parasitoids recovered from Farms 1 and 2, although no significant differences were detected in percent composition. These differences may be explained through examination of the filth fly immature developmental habitat on the four farms. Although they differed in equine waste disposal methods, the developmental habitats created by the moistened alfalfa hay and straw at Farm 3, and round hay bale debris at Farm 4, were actually quite similar. However, in the absence of rain events, the lack of

straw and hay accumulation areas at Farms 1 and 2 left horse manure debris as the only suitable area available for larval development. It may be that when encountered at equine facilities, these species demonstrate a preference for a particular habitat, the presence of which is dependent on a combination of the cultural management practices of each farm and weather patterns of a given year. These results are consistent with many studies demonstrating that parasitoid occurrence is not dependent on the host species, but rather the habitat in which that host is located (Rueda and Axtell 1985b, Meyer et al. 1991, Smith and Rutz 1991, Geden 1999).

Analysis of the months in which *Spalangia* spp. are active at Florida equine facilities demonstrated that *S. cameroni* were most often recovered in pupae collected between January and May, while *S. nigroaenea* were most often recovered in September and October. Although collected less often, most *S. endius* were also recovered in September. Based on this information, one may speculate that the difference in seasonal distribution may be a method to avoid competition by utilizing a different temporal niche.

There were only two occurrences of *M. raptor* throughout the study. This is probably due to the nature of the habitats created by equine husbandry that are suitable for filth fly breeding, and the unwillingness of this genus to search within those habitats. One *Phygadeuon* spp. was recovered from a house fly pupae, and may be the first reported occurrence of this genus from a Florida livestock facility. Although not included in any statistical analysis, Staphylinidae (82%) were recovered from pupae collected during October, December, and January. No adult beetle was recovered from any

pupae making identification beyond family difficult. Because *Aleochara* spp. have been frequently identified from filth fly pupae, it is likely that our samples were of this genus.

Our results demonstrate that the composition of pteromalid pupal parasitoids occurring at Florida equine facilities is unique among similar studies. It is likely that horse producers utilizing commercially available parasitoids or parasitoid mixtures containing *Muscidifurax* spp. will attain little control, if any with those species. Therefore, further research utilizing releases of only *Spalangia* spp. are needed to determine if increased fly management is possible. Our data concerning the seasonal distribution of filth fly parasitoids should assist in temporal releases of particular *Spalangia* spp. as well.

Table 2-1. Total pupae collected from four Florida equine facilities between December 2007 and 2009, with their respective mean percent parasitism rates and mean percent *Spalangia* spp. composition[1].

Farm	No. Parasitoids (No. UID)[2]	%Parasitism (95% CI)[3]	% *Spalangia* spp. (95% CI)[4]			
			S. cameroni	*S. nigroaenea*	*S. nigra*	*S. endius*
Stable Fly Host[5]						
1	209(9)	12.0(8.8-15.7)a	55.7(42.4-68.6)b	17.7(10.6-26.2)a	6.3(2.3-12.0)a	1.7(0.2-4.5)a
2	480(41)	17.7(13.6-22.3)a	77.1(68.1-85.0)ab	15.8(9.1-23.9)a	2.2(0.9-4.1)ab	<0.1(0-0.2)a
3	705(17)	17.1(12.4-22.4)a	68.1(58.4-77.2)ab	13.9(7.9-21.3)a	8.7(5.5-12.6)a	<0.1(0-0.1)a
4	448(19)	7.3(5.7-9.0)a	89.4(85.6-92.5)a	8.4(5.7-11.6)a	<0.10 (0-0.2)b	0.2(0.1-0.4)a
Total	1,842(86)					
House Fly Host[6]						
1	473(23)	5.1(2.8-8.3)a	33.4(19.2-49.5)b	60.3(44.6-75.0)a	0.0 (0)a	0.4(0-1.7)a
2	599(34)	17.7(9.0-28.6)a	21.8(9.8-37.8)b	69.9(54.1-83.6)a	<0.1(0-.03)a	1.8(0.3-4.4)a
3	87(5)	5.6(3.6-8.0)a	92.9(82.4-98.9)a	4.4(0.6-11.3)b	0.9(0-3.4)a	0.3(0-1.0)a
4	107(3)	2.6 n/a	92.8 n/a	7.2 n/a	0.0 n/a	0.0 n/a
Total	1,266(65)					

[1] Means in each column within host-type followed by the same letter are not significantly different (Ryan-Einot-Gabriel-Welsh multiple range test ($\alpha = 0.05$). Insufficient house fly pupae were collected at Farm 4 to be included in the analysis (n/a).

[2] UID represents aborted parasitoids recovered during pupal dissections where identification was not possible.

[3] Percent parasitism was calculated as the number of emerged and aborted parasitoids recovered divided by the total intact pupae collected at any given farm, during any given week.

[4] Percent *Spalangia* spp. was calculated as the total number of a given species collected from a farm during a given week, divided by the total parasitoids recovered for that week.

[5] A total of 12,675 stable fly pupae collected: Farm 1 = 1,677, Farm 2 = 2,149, Farm 3 = 3,788, Farm 4 = 5,061.

[6] A total of 12,841 house fly pupae collected: Farm 1 = 7,164, Farm 2 = 3,646, Farm 3 = 1,226, Farm 4 = 805.

Table 2-2. Mean percent parasitism rates and mean percent *Spalangia* spp. composition recovered from stable fly and house fly pupae collected from four equine facilities near Ocala, Florida[1].

		% *Spalangia* spp. (95% CI)[4]			
Month[2]	% Parasitism (95% CI)[3]	*S. cameroni*	*S. nigroaenea*	*S. nigra*	*S. endius*
Jan	6.5 (3.3-10.6)A	60.1 (53.6-66.4) Aa	38.3 (34.4-45.5)BCb	<0.1 (0-0.1) Ac	0.5 (0.1-1.1)Bc
Feb	17.8 (12.8-23.5)A	84.4 (79.6-88.7) Aa	9.2 (6.1-12.8)Cb	3.2 (1.8-5.1) Ab	<0.1 (0-0.07)Bc
Mar	8.0 (6.2-10.1) A	85.6 (79.0-91.0) Aa	4.8 (2.3-8.0)Cb	2.7 (1.2-4.9) Ab	0.1 (0-0.5)Bb
Apr	13.7 (10.2-17.7) A	66.8 (57.4-75.6) Aa	25.6 (17.2-35.0)BCb	1.3 (0.4-2.5) Ac	<0.1 (0.02-0.2)Bc
May	8.9 (4.7-14.2) A	62.1 (39.5-82.2) Aa	31.7 (12.8-54.6)BCab	1.2 (0.04-3.9) Ab	0.2 (0-0.6)Bb
Sep	6.5 (3.3-10.6) A	8.7 (2.6-18.1)Bb	71.3 (56.0-84.4)ABa	<0.1 (0-0.06) Ab	14.3 (6.4-24.6)Ab
Oct	1.8 (1.2-2.6) A	9.6 (0-34.6)Bb	90.4 (65.5-100)Aa	0.0 (0) Ab	0.0 (0) Bb

[1] Means in each column followed by the same capital letter are not significantly different, while means in each row followed by the same lower-case letter are not significantly different (Ryan-Einot-Gabriel-Welsh multiple range test (α = 0.05)).

[2] Insufficient pupae were collected in June, July, and Aug, and December to be included in the statistical analysis. No pupae were recovered from any farm in November.

[3] Within farm and collection week, percent parasitism was calculated as the number of emerged and aborted parasitoids recovered, divided by the total intact pupae collected.

[4] Within farm and collection week, percent *Spalangia* spp. was calculated as the total number of a given species collected, divided by the total parasitoids recovered.

Figure 2-1. Corrugated alsynite sticky trap mounted at a height of 90 cm near horse pasture, Ocala, Florida.

Figure 2-2. Plastic soufflé cup containing stable fly or house fly pupae individually placed into #0 gelatin capsules and held at 26 ºC, 12:12 LD, and 70% RH for parasitoid emergence.

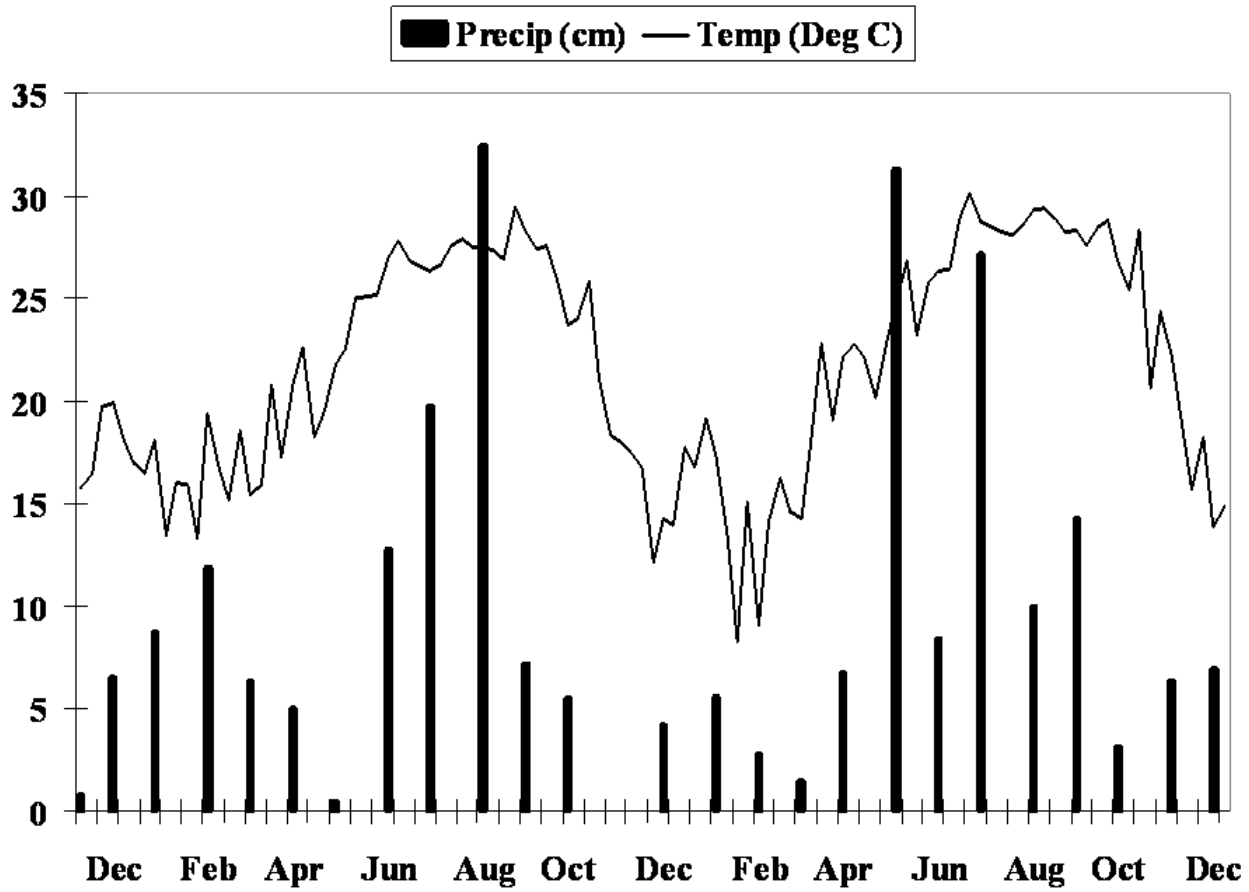


Figure 2-3. Mean weekly temperatures and accumulated monthly precipitation for Ocala, Florida, occurring between November 2007 and December 2009. Data obtained from the National Oceanic and Atmospheric Administration (NOAA, 2009) site in Ocala, Florida.

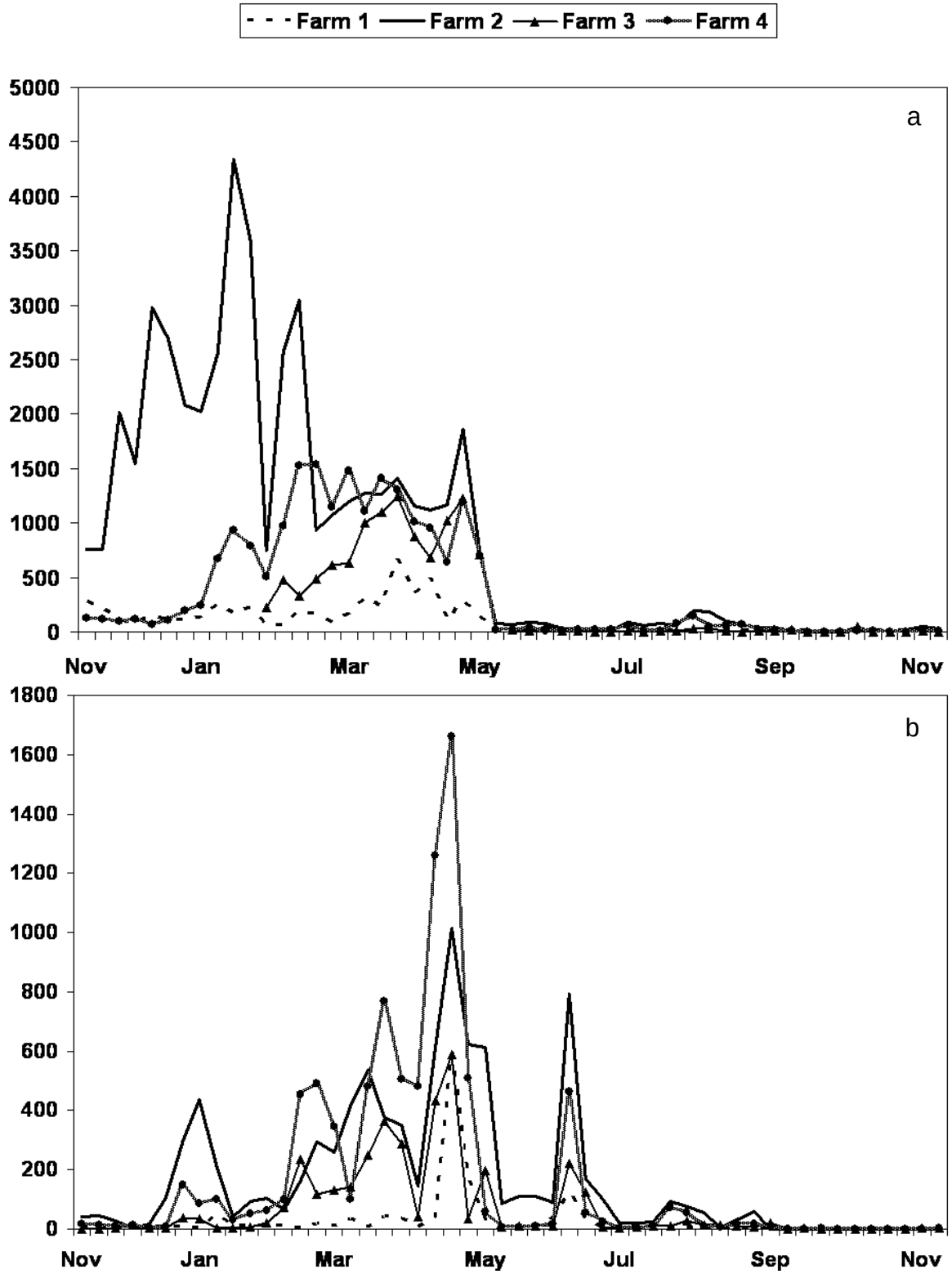


Figure 2-4. Weekly stable fly trap collections from four equine facilities during a.) November 2007-2008 and b.) November 2008-2009. The x-axis represents month of collection and the y-axis represents the total stable fly captures from three traps at each farm, each week.

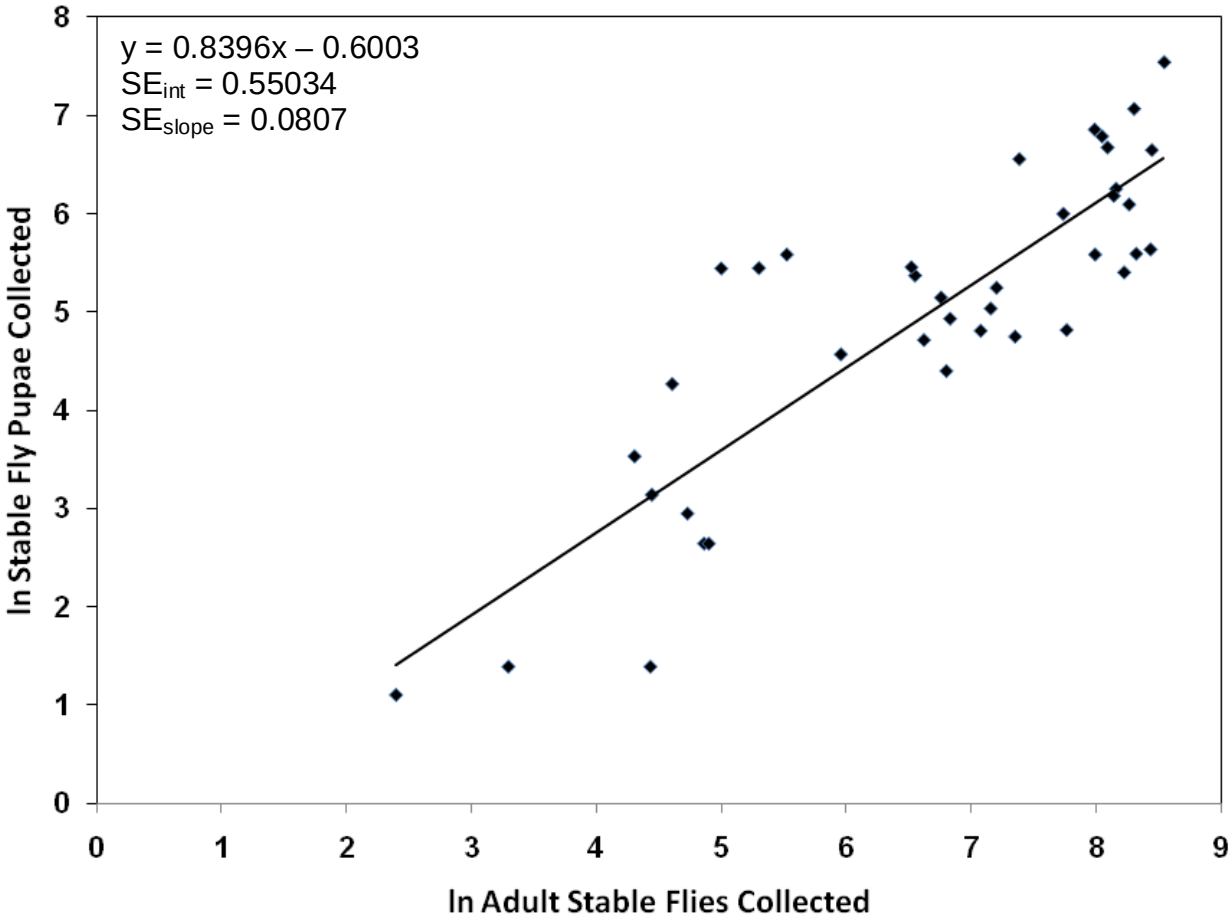


Figure 2-5. Relationship of adult stable fly trap captures for a given date, and stable fly pupae collected 2 wk prior. Stepwise multiple regression documented that adult stable fly trap captures were strongly correlated with stable fly pupal numbers 2 wk earlier (r^2 = 0.74, $F_{1, 37}$ = 108.18, P = <0.0001).

CHAPTER 3
THE ABILITY OF SELECTED PUPAL PARASITOIDS (HYMENOPTERA: PTEROMALIDAE) TO LOCATE STABLE FLY HOSTS IN EQUINE HUSBANDRY-GENERATED SUBSTRATES

Introduction

Filth flies, in particular the stable fly, *Stomoxys calcitrans* (L.), continue to be a significant pest of confined and pastured livestock. Furthermore, the insecticides available for control of pests such as the stable fly result in modest control, and are becoming increasingly limited due to federal regulation such as the Food Quality Protection Act of 1996 (Kaufman et al. 2001b) and resistance expression in some populations (Cilek and Greene 1994, Marçon et al. 1997, Chapter 5). In addition, pressure on livestock producers to control dispersing fly populations is mounting, as human population growth continues to decrease the gap between residential areas and nearby livestock operations. This can cause a greater quandary, as the flies' nuisance behavior and potential for disease transmission to urban areas may result in litigation (Tobin and Pitts 1999).

Many studies have been conducted to determine the effects of parasitoids, both released and naturally-occurring, as an alternative method for filth fly control. However, the results of such studies have provided conflicting data. Geden et al. (1992) observed sentinel pupae parasitism rates as high as 65% on dairies in New York state with a parasitoid release program, compared to 30% on control farms. Similarly, Petersen et al. (1992) noted up to 37% mortality of sentinel pupae on cattle feedlots where parasitoid releases occurred, while mortality at control feedlots was 4%. These results are dissimilar to results of a parasitoid study conducted on California dairies, where sentinel pupal parasitism increased from approximately 10% to only 20% when wasps

were released (Meyer et al. 1990). Concurrent evaluation of field-collected pupae indicated parasitism rates of only 4.4 and 12.5% for stable flies and house flies, respectively. In Nebraska, a study using parasitoid release-rates five-fold greater than the insectary-recommended amount were ineffective in reducing adult stable fly populations (Andress and Campbell 1994).

Several factors may account for the contrasting results of parasitoid release studies conducted in different geographical areas or livestock facilities. Habitat has been shown to play a large role in the host-location success of many parasitoids. Filth fly breeding sites such as tightly packed feed and manure at one facility may cause wandering maggots to pupate closer to the surface or in easily accessible cracks, favoring attack by *Muscidifurax* spp., whereas conditions at another facility may require that parasitoids search at greater depths to locate pupae, thereby favoring attack by *Spalangia* spp. (Rueda and Axtell 1985b, Meyer et al. 1991, Smith and Rutz 1991). Furthermore, filth fly breeding substrates differing in abiotic factors such as moisture (Geden 1999) or light (Smith and Rutz 1990) may influence the success of a parasitoid release program.

Research studies concerning stable flies in Florida are numerous (King and Lenert 1936, Fye et al. 1980, Hogsette and Ruff 1985). However, most of this research has been directed towards non-animal breeding sites and dispersal patterns, with limited research on livestock facilities (Greene et al. 1989). A large gap in research concerning equine facilities and filth flies also exists, particularly in the area of resident pupal parasitoid populations. Additionally, in a previous study, I demonstrated that the unique

filth fly breeding habitats created by equine husbandry practices influence the pteromalid pupal parasitoid species composition on these farms (Chapter 2).

Herein, an experiment was conducted to determine the ability of *Spalangia cameroni* Perkins, *Spalangia endius* Walker, and *Muscidifurax raptorellus* Kogan and Legner to locate and attack hosts in a standard substrate collected from a Florida equine facility. The primary goals of this study were to: 1) determine if the lack of field-collected *Muscidifurax* spp. is linked to searching behavior and host-location within substrates found at Florida equine facilities; and 2) determine the intra- and interspecies differences in attack rates of hosts both within a substrate and those made freely accessible.

Materials and Methods

Stable Flies. A stable fly colony was established from wild individuals collected in February of 2007, at the University of Florida Dairy Research Unit, in Hague, FL (UFD strain). The UFD colony flies were maintained at 26 ± 2º C, 12:12 LD, and 70 ± 5% RH. Citrated bovine blood was provided daily via saturated cotton in a 120 ml plastic soufflé cup. Gatorade® was provided *ad-libitum* in a 500 ml cup fitted with dental wick as a sugar and electrolyte source. Eggs were collected one to two times weekly and added to a larval medium similar to that described by McPheron and Broce (1996). A modification of this diet was made using maple wood chips in place of vermiculite. Briefly, the diet was comprised of 2.8 L water, 4.0 L wheat bran, 1.2 L Teklad maple sani-chips (Harlan™ Laboratories, Inc., Tampa, FL) and 0.4 L fishmeal (Nelson and Sons Inc., Murray, UT). After a development period of approximately 14 d, pupae were extracted from the rearing medium by floatation, dried, and placed in a clean 45 x 45 x 45 cm aluminum screened cage.

Pupal Parasitoids and Test Substrate. The pteromalid pupal parasitoids chosen for this experiment were *S. cameroni*, *S. endius*, and *M. raptorellus*. *Both S. cameroni* and *S. endius* were obtained for this project on the day of their intended use in the experiment, from colonies maintained by Dr. Christopher Geden at the USDA-ARS Center for Medical Agriculture, and Veterinary Entomology in Gainesville, Florida. The gregarious strain of *M. raptorellus* used in this study was obtained from a colony maintained at the University of Florida Veterinary Entomology Laboratory in Gainesville, Florida.

In our field studies, more stable flies and parasitoids were recovered from traps and pupal collections, respectively, at Farm 2, an equine facility northwest of Ocala, Florida, than from the other equine farms evaluated (Chapter 2). Therefore, Farm 2 served as the source for the soiled horse bedding substrate chosen for our laboratory assays. Soiled horse bedding at Farm 2 was composed of small particle (0.1-0.3 cm) wood shavings, and always contained varying amounts of discarded alfalfa hay, horse manure, and horse urine. To obtain uniform samples for our assays, large, 60 L plastic bins of soiled horse bedding were thoroughly mixed by shovel and divided among 3.75 L plastic zipper bags. These bags were frozen for 1-week prior to use to kill any arthropods present at the time of collection.

Parasitoid Release Chambers. Cylindrical plastic bins (chambers) having a 26 cm diameter, a 9 cm height, and a total volume of 4.8 L, were used as arenas for the soiled horse bedding habitats for the experiment. The chambers had tight-fitting lids modified with an 80-mesh screened area. Fifty, post-feeding, UFD stable fly larvae were released on the center surface of 16 release chambers previously filled with

approximately 3.7 L (habitat depth, 7 cm) soiled horse bedding, (Fig 3-1) as well as on 16, 120-ml plastic soufflé cups (cups) containing dry maple wood chips (Fig 3-2). Each set of 16 chambers or cups was randomly assigned to one of four treatment groups which included: *S. cameroni*-release, *S. endius*-release, *M. raptorellus*-release, and a no-parasitoid control. This arrangement resulted in treatments containing both 4 chambers (with bedding) and 4 cups (no bedding) per experimental setup. The cups served three purposes: 1) determination of the time of pupariation, and thus the appropriate time for parasitoid release, 2) a way to compare parasitoid efficiency between searching for dispersed pupae in release chambers and freely accessible pupae in cups, and 3) a way to ensure that the pupae were suitable for parasitization.

When at least 90% of the larvae contained in plastic cups had pupated, five female parasitoids of the appropriate species were introduced into each chamber or cup of their respective treatment (Geden 2002). Parasitoids were allowed 72 hr to search for and attack hosts (Kaufman et al. 2001a). At this time, the pupae from each chamber were recovered from the soiled horse bedding using a #6 brass sieve, and placed into new 120-ml plastic cups. Pupal recovery rates were approximately 92-100% in the chambers and 100% in the cups. Parasitoids were removed from treatment cups and all cups were held at 26 ºC, 12:12 LD, and 70% RH for 3-5 days for adult fly eclosion. Following the 5 day holding period, remaining uneclosed pupae were placed individually into #0 gelatin capsules and held at the same conditions for 40 d to allow parasitoid emergence (Mann et al. 1990, Lysyk 2001). Any pupae not producing an adult stable fly or parasitoid were dissected to determine the presence or absence of partially-developed parasitoids. This experiment was replicated three times, for a total of 12

release chambers and 12 cups for each treatment group. For each replication, five samples of the soiled horse bedding were weighed and dried to assess the moisture content of the substrate.

Statistical Analysis. Three factors were subjected to statistical analysis to determine if differences in searching behavior and parasitism existed between parasitoid species. These factors included: 1) the percent whole pupae, or pupae that did not produce an adult stable fly divided by the total pupae recovered from a particular container, 2) the percent parasitism, or pupae that produced an adult or partially-developed parasitoid divided by the total pupae recovered from a particular container, and 3) the percent parasitoid-induced mortality (PIM) (Petersen et al. 1991), or difference between percent whole pupae and percent parasitism. Stable fly control mortality was assessed in release chambers and plastic cups designated as no-parasitoid treatments. Therefore, Abbott's correction (Abbott 1925) was applied to the percent whole pupae to adjust for natural mortality factors.

Each of the three response variables listed above were subjected to analysis of variance (ANOVA) using the PROC GLM procedure of SAS® 9.1 (SAS Institute 2004) to determine differences between species. For each ANOVA, species and replication were included as fixed effects in the model, with container type as a variable. An additional ANOVA was conducted for each parasitoid to assess any within species differences in the efficiency of the aforementioned factors due to container type. All data were transformed using an arcsine square root of the percent whole pupae, percent parasitism, and percent PIM. Multiple mean comparisons were conducted with

the Ryan-Einot-Gabriel-Welsh (REGW) multiple range test (α = 0.05). All data presented in figures are back-transformed means.

Results

Percent Whole Pupae. Significant differences in percent whole pupae recovered were detected between parasitoid species in both the chambers ($F_{4, 31}$ = 80.90; *P* <0.0001) and cups ($F_{4, 31}$ = 6.09; *P* <0.0059) (Table 3-1). There was a significant difference ($F_{2, 31}$ = 5.28; *P* = 0.0106) in percent whole pupae between replications in the cups, but not between replications in the chambers. In both the chambers and cups, significantly fewer whole pupae were recovered for *M. raptorellus* (3 and 76%, respectively) than either *Spalangia* spp., with no difference between *S. cameroni* (73 and 93%, respectively) and *S. endius* (69 and 96%, respectively) (Fig. 3-3a, b). The intraspecies analysis revealed that significantly fewer whole pupae were recovered from chambers than from cups for all species: *S. cameroni* (73 and 93%, respectively), $F_{1, 22}$ = 13.15; *P* = 0.0015, *S. endius* (69 and 96%, respectively), $F_{1, 22}$ = 19.80; *P* = 0.0002, and *M. raptorellus* (3 and 76%, respectively), $F_{1, 22}$ = 76.25; *P* <0.0001 (Table 3-2, Fig. 3-4).

Percent Parasitism. Significant differences in percent parasitism were detected only between parasitoid species in chambers ($F_{4, 31}$ = 57.16; *P* <0.0001), with significantly more pupae parasitized by *S. cameroni* (56%) and *S. endius* (54%), than by *M. raptorellus* (1%) (Table 3-1, Fig. 3-3a,b). No significant difference was detected in parasitism in the within species analysis of *S. cameroni* or *S. endius*. However, significantly more ($F_{1, 22}$ = 138.15; *P* < 0.0001) pupae were parasitized in cups (49%) than release chambers by *M. raptorellus* (Table 3-2, Fig. 3-4).

Percent PIM. Significant differences in percent PIM were detected between parasitoid species in both the chambers ($F_{4, 31} = 11.16$; $P = 0.0002$) and cups ($F_{4, 31} = 10.18$; $P = 0.0004$). There was also a significant difference ($F_{2, 31} = 5.35$; $P = 0.0101$) (Table 3-1) in percent PIM between replications for the cups, but not in the chambers. In both the release chambers and cups, percent PIM was significantly less in treatments containing *M. raptorellus* (2 and 20%, respectively) than either *S*. *cameroni* (14 and 39%, respectively) or *S. endius* (10 and 31%, respectively), with no significant difference between the two *Spalangia* spp. (Fig. 3-3a,b). The intraspecies analysis revealed that percent PIM was significantly less in chambers than in cups for all species: *S. cameroni* (14 and 39%, respectively), $F_{1, 22} = 33.81$; $P < 0.0001$, *S. endius* (10 and 31%, respectively), $F_{1, 22} = 25.00$; $P < 0.0001$, and *M. raptorellus* (2 and 20%, respectively), $F_{1, 22} = 29.36$; $P <0.0001$ (Table 3-2, Fig. 3-4).

Discussion

Our laboratory findings support those of our field studies (Chapter 2) conducted in Ocala, Florida, where nearly 100% of all pteromalids recovered were *Spalangia* spp. This is particularly evident in the results observed for percent parasitism between container types (Fig. 3-3a,b). When parasitoids were forced to search for hosts buried in soiled horse bedding, pupae in release chambers containing either *Spalangia* spp. reproduced at a significantly higher level than *M. raptorellus*. This was likely due to the depths at which most stable fly maggots ultimately pupated, and the ability of *Spalangia* spp. to search in the substrate, as parasitism rates between species were not significantly different in cups where pupae were freely accessible. The cylindrical release chamber was chosen to inhibit larval aggregation, as flies in our facility often pupate in the rearing container corners. This behavior would potentially alter the results

of the assay. Preliminary tests (data not shown) were conducted to ensure larvae distributed more or less evenly, and that aggregation was minimal. Although the depth at which every pupa recovered was not recorded from the chamber portion of the experiment, preliminary studies of stable fly maggot dispersal indicated that most pupation in these containers occurred at depths of 3-7 cm. Taken together, this further corroborates my findings in Chapter 2, where field-collected stable fly pupae were collected at depths of 3 cm or greater, and nearly 100% of all parasitoids recovered were *Spalangia* spp.

Several studies demonstrating the effects of host dispersal and abiotic factors on pupal parasitoid activity may in part explain the findings of our laboratory experiments. A field study conducted by Rueda and Axtell (1985b) demonstrated that most *M. raptor* were recovered from pupae collected at depths of 3 cm or less, whereas most *Spalangia* spp. were collected between depths of 5 and 10 cm. King (1997), conducting laboratory evaluations of both *M. raptor* and *S. cameroni*, determined that host burial greatly reduced parasitism by the former species, whereas that of the latter was relatively unaffected. This is similar to results obtained by Floate and Spooner (2002), when pupal parasitism by three *Muscidifurax* spp. greatly decreased if hosts were located at depths of 1 cm or greater. In a study conducted by Geden (2002), both *S. cameroni* and *S. endius* searched uniformly through a commonly used fly rearing medium, and regularly located hosts at 6 cm depths in this porous, relatively loose substrate. Pupae were also attacked by *M. raptor* at 6 cm depths, but only half as often as *Spalangia* spp. The substrate used in our assays more closely approximates the fly rearing medium than the dense sandy soil or manure also evaluated by Geden (2002).

In the present study, the soiled horse bedding used in release chambers also included horse manure, areas from which stable fly pupae were regularly recovered. This behavior was also noted in my field studies assessing pteromalid pupal parasitoids attacking naturally-occurring stable fly pupae. Although one or two stable fly pupae could be found in these areas, aggregation in horse manure was inhibited in release chambers due to its smaller particle size after mixing. The dense nature of the horse manure may have dissuaded attack by *M. raptorellus* at any depth in our study, while favoring attack by either *S. cameroni* or *S. endius*.

Smith and Rutz (1990) and Geden (1999) also have demonstrated the impact of both light and moisture on *Spalangia* spp. and *Muscidifurax* spp. In both studies, *Muscidifurax* spp. preferred drier substrates, whereas *Spalangia* spp. preferred those with higher moisture content. However, both species have been shown to prefer dimly lit conditions (Smith and Rutz 1990). Because light conditions in the present study were similar for all treatments, negating its effect, it is likely that the 60% average moisture content in the current study is another factor that resulted in the increased searching activity of *Spalangia* spp. over that of *M. raptorellus*.

In the present study, percent whole pupae and percent PIM were significantly greater for *S. cameroni* and *S. endius* than for *M. raptorellus* for both the chambers and the cups, although differences were greatest between species in the cups. The percent whole pupae were those pupae that did not initially produce an adult fly, or the overall killing effect of parasitoids. The percent PIM, or pupal mortality due to some factor other than the emergence of a parasitoid, such as host feeding, was significantly higher in release chambers containing either *Spalangia* spp. than those with *M. raptorellus*.

Causes for increased PIM other than host feeding may include differences in ovipositional restraint. Wylie (1971, 1972a) demonstrated that *Muscidifurax zaraptor* Kogan and Legner discriminated against previously stung hosts more often than *S. cameroni*. Petersen et al. (1991) also described this cause of PIM in detail with several *Muscidifurax* spp. and *Spalangia nigroaenea* Curtis. Under various conditions, Petersen et al. (1991) found that *Muscidifurax* spp. displayed great ovipositional restraint when encountering previously stung hosts. However, *S. nigroaenea* demonstrated little ovipositional restraint under similar conditions. Therefore, differences in ovipositional restraint may be the cause behind differences in the levels of PIM between the two genera used in the present study.

Differences in PIM between the two genera were also greater in cups, than in chambers. The difference in frequency of host encounters was greater in smaller cups where pupae were more freely accessible than in chambers, which required greater searching effort. This may have further accentuated differences in ovipositional restraint between the two genera. Furthermore, the gregarious nature of *M. raptorellus* may be similar to that shown by *Nasonia vitripennis* (Walker) (Wylie 1972b), where eggs of other species are not generally attacked. Instead, the speed and efficiency of *N. vitripennis* host utilization often results in starvation of competing species. Under field conditions, this may also be true for *M. raptorellus*. The stinging of pupae containing previously developing parasitoid larvae would not result in increased PIM, but fewer offspring, due to insufficient resources. This may be a potentially disadvantageous situation for livestock producers releasing *M. raptorellus* during times when parasitoid to host ratios are high. Under our conditions, if *M. raptorellus* did sting pupae with

conspecific eggs, it also would not have increased the percent PIM, but rather increased the numbers of subsequent adult parasitoids. It is possible that the temporal window in the present study (3 d) would not be as adequate for decreased adult production as under field conditions, where hosts and immature parasitoids of all ages are available.

Further evidence of increased host encounter frequency in cups compared to chambers is provided by our intra-species analysis of the aforementioned factors (Fig. 3-4). No significant differences in parasitism were detected between container types for either *S. cameroni* or *S. endius*, suggesting that these species located similar numbers of hosts regardless of the searching effort required. However, this analysis revealed that *M. raptorellus* was less efficient in locating hosts when required to search in release chambers. The percent whole pupae recovered and percent PIM was significantly less in chambers than in cups for all species. This further confirms the likelihood that host encounter frequency was greater in cups than in the chambers. This would accentuate differences in host-feeding and ovipositional restraint behavior between the two genera.

In a few cases, significant differences were detected between replications for percent whole pupae recovered and percent PIM. However, these differences only occurred between replications of cups. This discrepancy can be explained by our use of a newly established stable fly colony. Although it was our intent, it was rather difficult to collect only post-feeding third-instar stable flies from our rearing media. Pupariation in this colony is not completely synchronized and takes place continually between days 10 and 14 post-oviposition. In general the feeding status of larval stable flies was determined by their aggregation behavior. Because some larvae were still feeding, higher control mortality resulted from placing larvae in substrate free cups than in the

chambers. Those larvae still requiring nutrition to pupate were able to continue feeding in substrate-containing chambers, resulting in lesser and more uniform control mortality.

Several studies evaluating the behavior or ability of pteromalid parasitoids to locate hosts under laboratory conditions have been used as supporting evidence for the findings in the present study. However, most of these studies utilized small (approximately 100 ml) release chambers, or artificially-made substrate conditions to evaluate parasitoid activity. Furthermore, all of these studies utilize previously-pupariated pupae, placed in the release chamber at pre-determined locations. This study is, to my knowledge, the first evaluation of pteromalid parasitoid host location using sizeable chambers that more closely approximate natural field conditions. In addition, this is the first study allowing third-instar filth flies to wander and pupate as they would under field conditions using a substrate where known filth fly breeding occurred.

My studies clearly demonstrate the ability of *S. cameroni* and *S. endius* to search more efficiently for hosts than *M. raptorellus*, using a substrate where fly breeding occurred at a livestock farm. This experiment also corroborates our findings that host depth, due to the fly breeding substrates generated by equine husbandry practices (Chapter 2), is likely the cause for the high proportions of *Spalangia* spp. found in those field studies. Further experiments are needed to determine if releases of *Spalangia* spp. at equine facilities in Florida can increase filth fly control.

Table 3-1. Analysis of variance (ANOVA) F values for interspecies percent whole pupae, percent parasitism, and percent parasitoid-induced-mortality (PIM) of stable fly pupae in chambers containing soiled horse bedding, and cups with no bedding.

	Chambers		Cups	
	Species[1]	Replication	Species[1]	Replication
% Whole Pupae[2]	80.90**	0.30 NS	6.09**	5.28*
% Parasitism[3]	57.16**	0.88 NS	2.26 NS	2.06 NS
% PIM[4]	11.16**	0.28 NS	10.18**	5.35*

[1] Species evaluated included Muscidifurax raptorellus, Spalangia cameroni, and Spalangia endius.

[2] The percent whole pupae were those pupae from which an adult stable fly did not emerge.

[3] The percent parasitism were those pupae from which an adult or aborted parasitoid was recovered.

[4] The percent PIM was the difference between percent whole pupae and percent parasitism.

**, $P \leq 0.01$; *, $P \leq 0.05$; NS, $P > 0.05$.

Table 3-2. Analysis of variance (ANOVA) F values for and intraspecies percent whole pupae, percent parasitism, and percent parasitoid-induced-mortality (PIM) of stable fly pupae in chambers containing soiled horse bedding, and cups with no bedding.

	M. raptorellus	*S. cameroni*	*S. endius*
% Whole Pupae[1]	76.25**	13.15**	19.80**
% Parasitism[2]	138.15**	0.78 NS	0.62 NS
% PIM[3]	29.36**	33.81**	25.00**

[1] The percent whole pupae were those pupae from which an adult stable fly did not emerge.

[2] The percent parasitism were those pupae from which an adult or aborted parasitoid was recovered.

[3] The percent PIM was the difference between percent whole pupae and percent parasitism.

**, $P \leq 0.01$; NS, $P > 0.05$.

M. = *Muscidifurax*; *S*. = *Spalangia*.

Figure 3-1. Chambers (26 cm diameter, 9 cm height) filled with 3.7 L (7 cm) soiled horse bedding.

Figure 3-2. Soufflé cups (120 ml) containing 10 ml maple wood chips used to assess parasitism when stable fly pupae were freely accessible.

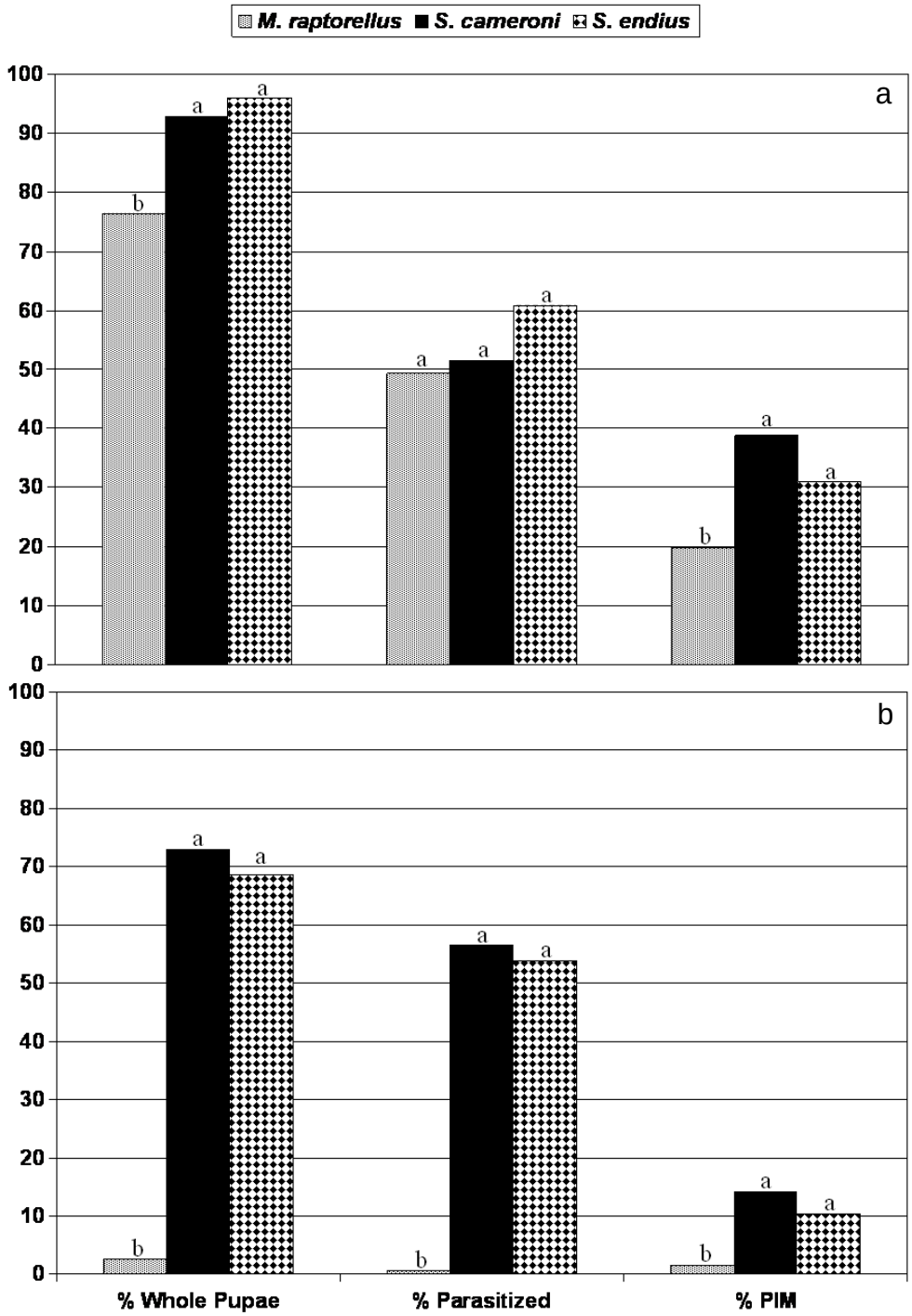


Figure 3-3. Comparison of three parasitoid species host attack parameters expressed as mean percent whole pupae, percent successful parasitism, and percent parasitoid-induced mortality (PIM) exposed to stable fly pupae a) freely accessible in cups and b) dispersed in chambers containing 3.7 L (7 cm) of soiled horse bedding substrate. Within evaluation parameter, columns with the same letter are not significantly different by the Ryan-Einot-Gabriel-Welsh multiple range test ($\alpha = 0.05$) following an analysis of variance. *M.* = *Mucidifurax*, *S.* = *Spalangia.*

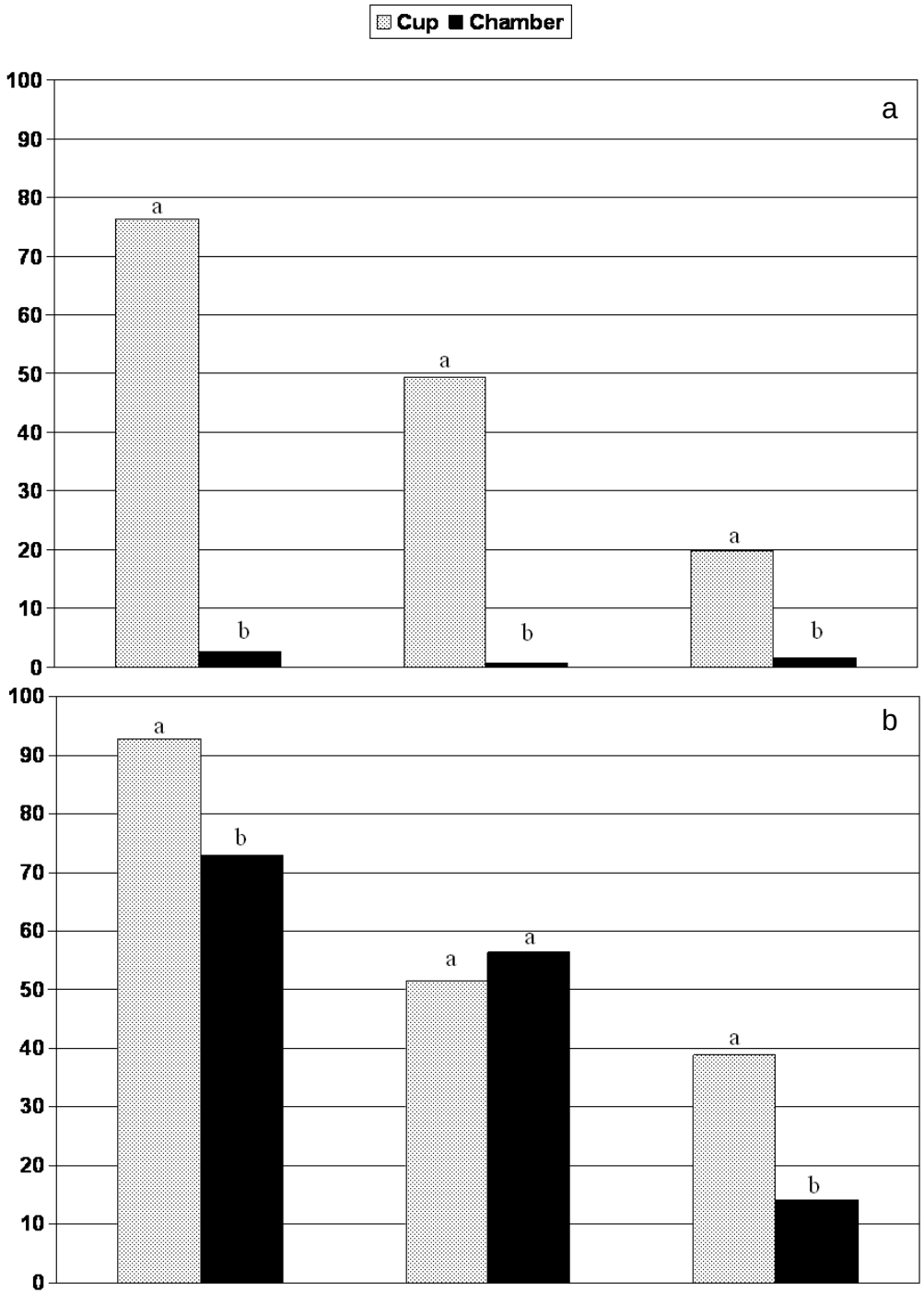


Figure 3-4. Evaluation of a) *Muscidifurax raptorellus* b) *Spalangia cameroni* and c) *Spalangia endius* searching ability on host attack rates expressed as percent whole pupae, percent successful parasitism, and percent parasitoid-induced mortality (PIM). Stable fly host pupae were either freely accessible in cups or required searching in chambers containing 3.7 L (7 cm) of soiled horse bedding substrate. Within evaluation criteria, columns with the same letter are not significantly different by the Ryan-Einot-Gabriel-Welsh multiple range test ($\alpha = 0.05$) following analysis of variance.

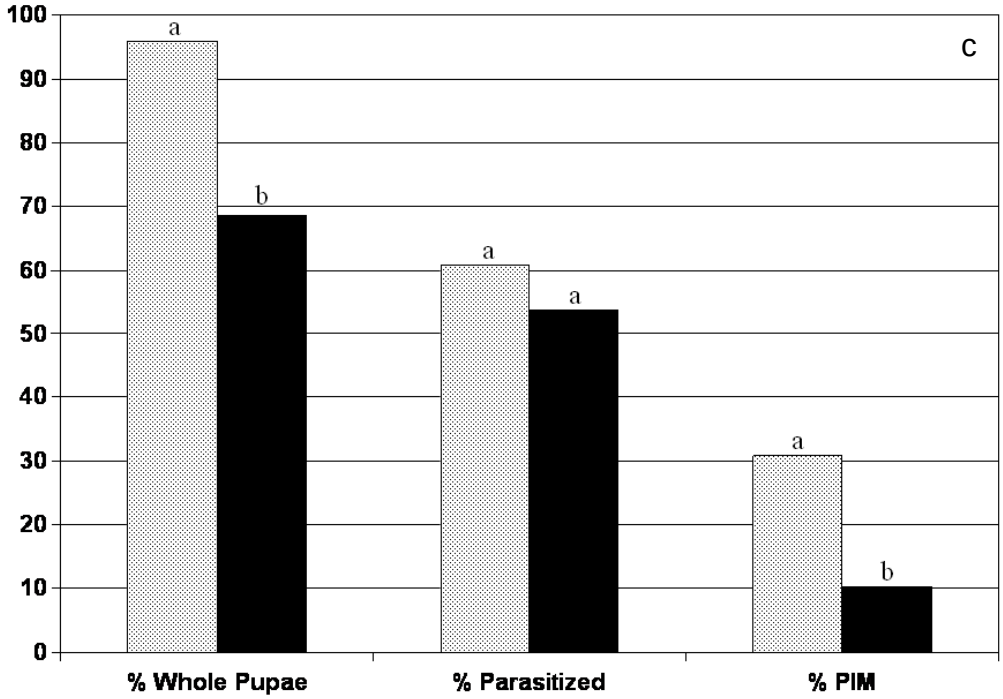


Figure 3-4. Continued.

CHAPTER 4
HOST BLOOD MEAL IDENTIFICATION OF STABLE FLIES COLLECTED FROM FLORIDA EQUINE FACILITIES USING A MULTIPLEX POLYMERASE CHAIN REACTION

Introduction

The detrimental effects of stable flies, *Stomoxys calcitrans* (L.), to confinement animal producers are well documented. Many studies have been conducted to ascertain the costs of stable fly attacks on dairy and beef cattle (Bruce and Decker 1958, Miller et al. 1973, Campbell et al. 1977, Mullens et al. 2006). The effect of stable flies on other animals, such as pigs (Moon et al. 1987) and chickens (Anderson and Tempelis 1970), has also been determined. However, data concerning the ecology of this pest associated with equine facilities is limited. Sutherland (1978a) found that survival and fecundity of the stable fly was increased when fed bovine blood, although it was determined that they could survive and oviposit when fed equine blood. Furthermore, Sutherland (1978b) found that stable fly immature mortality was less in horse manure than that of cattle manure.

Although stable flies can survive under conditions provided by equine facilities, their presence at these facilities may be at least partially due to dispersal from other animal installations. Bailey et al. (1973) found that stable flies traveled up to 3.2 km in search of a blood meal. In a study conducted by Gersabeck and Merritt (1985), stable flies readily traveled upwind 0.8 km to confined horses from four release sites. Similarly to observations of Bailey et al. (1973), these flies remained in the general area because potential hosts and suitable larval substrates were available. Flies released from sites where hosts could not be easily found were collected at distances up to 3.2 km. Additionally, Hogsette and Ruff (1985) documented that beach populations of stable

flies in Florida migrated as far as 225 km, possibly due to weather fronts. However, the understanding of stable fly ecology and their seasonal distribution continues to be the subject of considerable debate.

According to the American Horse Council (AHC 2009), horses are found in every state of the U.S., with 45 states having more than 20,000 horses. Florida ranks as having the third largest horse industry in the U.S., with 500,000 horses, contributing over $3.0 billion in goods and services, and maintaining over 38,000 full-time employees. In addition, approximately 60% of these animals are used in showing and recreational events. Stable flies are a common pest to horses and vector various pathogens of veterinary importance, including those that cause pruritis and habronemiasis, the latter of which can lead to summer sores and secondary infections (Gortel 1998). Both of these conditions result in decreased overall animal aesthetics, and thus, their show value. The tremendous effort to control fly pests of horses is apparent in the plethora of chemical and mechanical products available to horse owners. Therefore, it is surprising that research concerning stable flies and horse production is sparse, at best.

Florida's rich horse industry provides a particularly suitable situation in which to monitor the localized movement of stable flies through host blood meal identification. Analysis of blood meals taken by haematophagous arthropods has played an important role in ascertaining host preferences and capacity to vector blood-borne pathogens to both animals and humans (Abbasi et al. 2009, Kent 2009). The precipitin method has been used to successfully identify hosts of mosquitoes (Bertsch and Norment 1983) as well as stable flies (Anderson and Tempelis 1970). Enzyme-linked immunosorbent

assays also have been used with success in mosquito blood meal identification (Zinser et al. 2004). However, problems including the need for high quality antisera for the selected species and the need to refine antibodies to prevent cross-reactivity, as well as loss of blood quality over time, compound the technical difficulties of these assays themselves (Ngo and Kramer 2003).

More recently, the overwhelming increase in available DNA sequence data of various vertebrates has opened the door for newer molecular-based blood meal analysis approaches, such as polymerase chain reaction (PCR) (Kent 2009). Although the use of PCR for host blood meal identification in stable flies has not been reported, the blood meals of other haematophagous arthropods have been successfully identified using a variety of laboratory techniques. Polymerase chain reaction utilizing mitochondrial DNA, where many copies of the genome are present, can alleviate the problems of other blood-based techniques that rely on more rarely encountered nuclear DNA of mammalian white blood cells, providing a more direct approach to host blood meal identification. Furthermore, primers targeting cytochrome *b* can be species specific, and have been used in the successful host blood meal identification of mosquitoes (Boakye et al. 1999, Kent and Norris 2005).

Although stable flies are known pests of horses, the reasons for their occurrence in these areas, either due to available breeding areas or dispersal from neighboring livestock facilities, are largely unknown. In Ocala, Florida, the large number of horse producing units offers the potential to demonstrate stable fly movement between cattle and horse installations using PCR blood meal identification. Beginning in November 2007, a study was undertaken to 1) determine the hosts of stable flies collected from

Florida equine facilities and 2) determine whether stable fly host blood meal identification can be used to describe their short-term localized movement from off-farm sites.

Materials and Methods

Stable Fly Collection. Between November 2007 and December 2009, weekly attempts were made to collect 10 adult blood-fed stable flies from each of four equine facilities located near Ocala, Florida as described in Chapter 2. Live adult flies were collected from fence lines and barn walls of horse enclosures using a sweep net. Blood-fed individuals were identified by applying light pressure to the sides of each fly, inducing production of a fecal droplet (Fig. 4-1). Flies were considered suitable for analysis if they produced fecal droplets that were dark in color, suggesting digestion of a recently acquired blood meal. These flies were placed individually into clean, labeled 1.5-ml microcentrifuge tubes and held on ice to slow further digestion prior to processing. Retained stable flies were returned to the University of Florida (UF) Veterinary Entomology Laboratory and stored at -80 ºC until blood meal analysis could be performed.

Time Course Blood Meal Analysis. Approval was granted from the appropriate UF committee for the collection of blood from each mammalian species used in stable fly feeding assays prior to beginning this study. Approval was granted from the UF Institutional Review Board (#342-2008) for collection and use of human blood in feeding assays (Appendix A). Cattle blood used in this project was collected with the approval of the UF Animal Research Committee (#018-ANS08). Horse blood used in this project was obtained from the UF Horse Teaching Unit as part of a routine Coggin's test

performed at the facility. The UF Institutional Animal Care and Use Committee approval was necessary for collection of dog blood (#200801760) (Appendix B).

Previously non-blood-fed female stable flies (3-5 day-old) were engorged on blood of individual selected hosts, which included cattle, horse, dog, and human, as well as a blood mixture of all four hosts, to assess the time-dependent detection limits of the developed multiplex PCR. Stable flies from a colony established in February 2007 from individuals collected at the UF Dairy Research Unit in Hague, Florida, were reared according to the methods described in Chapter 3. Adult stable flies were mechanically aspirated from colony cages and placed into 120-ml plastic feeding chambers in groups of 10 (Fig. 4-2). A 200-µl sample of blood from each host was added individually by micropipette to the cap of a 1.5-ml microcentrifuge tube and attached to a screened area of each feeding chamber with a rubber band (Fig. 4-3). For the mixed-host feeding chambers, 50 µl of blood from each host was mixed and added to a microcentrifuge cap and attached to its respective feeding chamber. Stable flies were allowed to feed for 20 min, and held for 0, 8, 16, 24, and 48 hr post-blood-feeding at 26 ºC, 12:12 LD, and 70% RH. At the appropriate time, flies from each designated feeding chamber were individually placed into 1.5-ml microcentrifuge tubes and held at -80 ºC until blood meal analysis could be performed. Only stable flies that had fully engorged during the initial 20-min feeding period were analyzed. Experiments were conducted three times to ensure the accuracy of blood meal determinations.

DNA Extraction. Stable fly host DNA extractions were performed with the QIAGEN® DNeasy® Blood and Tissue Kit (QIAGEN, Valencia, CA). The abdomen of a previously frozen blood-fed stable fly was removed and placed in a 1.5-ml

microcentrifuge tube with 180 µl of phosphate-buffered saline, pH 7.4, and homogenized with a micro-tube pestle. At this point, DNA extractions were carried out by following the insect DNA extraction protocol provided with the kit, with the only modification being the use of 150 µl of elution buffer, rather than the prescribed 200 µl.

Primer Design and PCR. Primers (Sigma Genosys, St. Louis, MO) targeting the cytochrome *b* region of the mitochondrial genome of selected hosts were designed manually using a multiple alignment of sequences (CLUSTALW 2009) selected from GenBank®. These included: cattle (*Bos taurus*; Accession #DQ186290), horse (*Equus caballus*; Accession #NC001640), dog (*Canis lupus familiaris*; Accession #NC002008), human (*Homo sapiens*; Accession #NC012920), and stable fly (*Stomoxys calcitrans*; Accession #DQ533708). The horse, dog, and human hosts were selected as potential targets for PCR as they are commonly associated with equine facilities in Florida. The equine facilities in this study were chosen because they were large tracts of land (Chapter 2), known to be in the vicinity of cattle facilities. Therefore, cattle were selected as a fourth host due to their occurrence around, but not on the selected equine facilities. Blood-feeding on this host was used as an indicator of potential stable fly movement between farms. Four host-specific forward primers were designed to have at least three nucleotide differences in the 3' region in the cytochrome *b* gene between each other, as well as the stable fly sequence. A universal reverse primer was designed in a consensus region to all four hosts, and all primers were checked for melting temperature compatibility. Additionally, these primers were selected to have expected product sizes that differed by approximately 100 bases (Table 4-1), making the source identification more certain when visualized on an agarose gel.

Each multiplex PCR contained final concentrations of: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.05 U/µl Taq polymerase (Invitrogen, Carlsbad, CA), 0.2 pM HorseF and CattleF, 0.1 pM DogF and HumanF, 0.6 pM UnivRev, 1 µl eluted DNA, and brought to a final volume of 10 µl with deionized, autoclaved water. Conditions were optimized for target DNA amplification using a touchdown PCR procedure with the following conditions: initial denaturation for 5 minutes at 95 ºC, followed by 12 cycles of 94 ºC for 30 seconds, 57 ºC for 30 seconds, and 72 ºC for 50 seconds. Two additional sets of 12 cycles each followed, using decreasing temperatures of 56 and 55 ºC as annealing temperatures, respectively. A final elongation was performed at the end of the 36-cycle program for 5 minutes. Amplification of stable fly host DNA by PCR was performed in a Bio-Rad® DNA Engine Peltier thermal cycler (Bio-Rad, Hercules, CA), followed by gel electrophoresis using an ethidium bromide stained agarose gel (1.5%) and a 100-base pair molecular weight standard (Invitrogen, Carlsbad, CA) to confirm amplification product size.

To confirm that amplification products were from the selected hosts, four target bands comprising each host type from both control experiments and field-collected stable flies, were removed from agarose gels and prepared for sequencing. DNA was extracted from agarose bands with the QIAGEN® QIAquick® Gel Extraction Kit following the instructions included with the kit. Gel extractions were carried out on samples from both the time course control experiments, as well as samples from each host collected during the field portion of the project. The DNA obtained from gel extractions was sequenced using the Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) using the same primer sets as in the initial multiplex PCR.

The amplification products obtained through this procedure were sent to the UF Interdisciplinary Center for Biotechnology Research (ICBR) to be analyzed using the 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The sequences determined by the ICBR through this analysis were returned as electropherograms and edited using Sequencher 4.8 software (Gene Codes Corp., Ann Arbor, Michigan). The edited sequences of stable fly hosts from both lab and field specimens were confirmed using the Basic Local Alignment Search Tool (BLAST) available on the National Center for Biotechnology Information (NCBI 2009) web site.

Statistical Analysis. Data from blood meal identification of field-collected specimens were subjected to analysis of variance (ANOVA) using the PROC GLM procedure of SAS® 9.2 (SAS Institute 2004) to determine differences in host-feeding frequencies by stable flies at Florida equine facilities, as well as differences in host-feeding frequencies between farms. Any farm in which at least five blood-fed stable flies had been collected during a weekly attempt was included in the analysis. A two-sample t-test was conducted to determine if differences between host detections existed between sample collections of four or fewer flies and five or more flies. Due to the difficulty in collecting blood-fed stable flies from the facility, there was only one week in which Farm 1 met the five-fly criterion. Therefore, Farm 1 was removed from the between-farm host-feeding frequency analysis. Data were transformed using an arcsine square root of the percent host type composition. All data are presented in figures as back-transformed means. Both host and farm were included as fixed effects in their respective analysis. Multiple mean comparisons were conducted with the Ryan-Einot-Gabriel-Welsh (REGW) multiple range test ($\alpha = 0.05$).

To assist in interpretation of any differences in stable fly host-feeding frequency between farms, an effort was made to identify areas within 3 km of each farm in which horses and/or cattle could be found. Any pasture or fenced enclosure holding either of these animal types was noted on a map created using Google™ Earth v. 5.0 (Fig. 4-4a, b, c, d).

Results

Time Course Blood Meal Analysis. The multiplex PCR designed for stable fly blood meal analysis was used successfully in identification of selected hosts in the laboratory time course evaluation. Animal specific amplification products were detected with 100% efficiency up to 16 hr post-blood-feeding, with no cross amplification of non-target hosts, or amplification of any host in non-blood-fed stable flies (Fig. 4-5, 4-6). Host identification was also possible at 24 hr, but in only approximately 50% of the flies assayed (Fig. 4-7). Furthermore, these amplification products were difficult to visualize. No amplification products resulted from stable flies assayed at 48 hr post-blood-feeding (data not shown). Using the described methods, the time course experiment demonstrates that between 24 and 48 hr post-feeding, insufficient host DNA for successful amplification remains in the stable fly gut. However, this technique is 100% reliable if a stable fly is tested within 16 hr post-feeding.

Host DNA sequences obtained through extraction and sequencing of bands from agarose gels were entered into the BLAST program found on the NCBI website to confirm host specificity of the selected primers. All hosts were confirmed using this method, sharing 98-99% identities with the intended targets in both laboratory and field-tested stable flies.

Field-Collected Stable Fly Hosts. A total of 595 field-collected and, presumptive by visual inspection, blood-fed stable flies were subjected to blood meal analysis during this study. Of those, host amplification products were successfully detected in 350 flies, representing a detection efficiency of 58.8%. However, only 291 of the 350 positive flies collected during the collection period were used in the analysis due to our statistical requirements. A two-sample t-test between collections of four or less flies and five or more flies showed no significant differences in cattle (65.8 and 85.8%, respectively) or horse (25.8 and 12.7%, respectively) host utilization. Multiple-host-feeding was detected in 26 of the 350 stable flies analyzed (7.4%), with three of those flies having sufficient DNA from three host types. When overall host-feeding frequency of stable flies collected from equine facilities was examined, significantly more had fed previously on cattle (65%) than any other host ($F_{3,68} = 43.90$; $P = <0.0001$) (Fig. 4-8). Significantly more stable flies had fed previously on horses (28.8%) than dogs (0.5%) or humans (5.3%), and no significant difference was detected between dog and human feedings.

Significant differences in host-feeding frequency between farms were also detected for cattle ($F_{2,32} = 18.18$; $P = <0.0001$) and horses ($F_{2,32} = 19.27$; $P = <0.0001$) (Fig. 4-9). Significantly more stable flies that had previously fed on horses were collected from Farm 4 (46.3%) than from any other farm. Significantly more stable flies collected from Farm 3 (98.0%) than flies collected at any other farm had previously fed on cattle. No significant differences were detected in dog or human host feeding by stable flies between farms, and these detections were infrequent. Although data from Farm 1 were not included in the analysis, there were 13 cattle and three human blood-

fed stable flies collected from this facility, with no host-identifications from the horse or dog.

The results of the effort to identify cattle- and horse-populated pastures encircling the four horse farms where stable flies were collected are shown in Fig. 4-4a, b, c, d. There were at least two pastures holding cattle within 2 km of all farms. In general, Farm 3, which had 98% cattle blood meals, was associated with the most cattle pastures, having a total of 20-cattle populated pastures within 3 km of fly collection sites. Farm 4, with the highest horse blood meals (46.3%) was associated with the fewest nearby cattle-populated pastures, having a total of five within 3 km. Horse-populated pastures were abundant around all four farms where stable flies were collected.

Discussion

To my knowledge, stable fly host blood meal identification using multiplex PCR has not been documented. My post-feeding, time-series assays demonstrate that successful amplification using host-specific primers targeting cytochrome *b* is 100% reliable up to 16 hr post-feeding under laboratory conditions, and suggests this efficiency continues to some point before 24 hr. Similarly, between 24 and 48 hr post-feeding, DNA degradation in the stable fly gut decreases the detection efficiency from approximately 50% to 0. This is similar to mosquito host blood meal detection as performed by Kent and Norris (2005). The authors surmised that differences in detection limits between their assay and others could be due to differences in extraction protocol, arthropod digestive systems, or the hosts upon which the insect had fed. Ngo and Kramer (2003) were able to detect mosquito blood meals taken from quail for up to 3 d post-blood-feeding. However, their assay utilized both primers specific to

cytochrome *b*, and a restriction enzyme digestion to distinguish different avian hosts. Although not used in the study by Ngo and Kramer (2003), the efficiency of primers targeting nuclear DNA in birds might be greater because avian blood is nucleated, and thus provides more DNA amplification than similar primers for mammalian blood.

The results reported here for host blood meal identification are consistent with those of other studies regarding the fate of a blood meal in the stable fly gut. Anderson and Tempelis (1970) reported that on average, the time required to fully digest a blood meal was 24-36 hours when fed on citrated human blood. However, stable flies used in that study were maintained at temperatures of 20-21 ºC. Digestion of cattle blood was longer still, taking 46-70 hr when held at cycling temperatures between 21 and 15 ºC (max/min). Hafez and Gamal-Eddin (1959), as reported by Anderson and Tempelis (1970), demonstrated similar results for human blood at 21 ºC, but noted the decreased digestion time of 10 hr when cattle blood-fed flies were held at 25 ºC. Hafez and Gamal-Eddin (1959) also demonstrated a 2 d decrease in pre-oviposition period in stable flies held at 30 ºC than those held at 22 ºC. These findings are consistent with a review by Lehane (2005), who proposed a rough average time of 48 hr for complete digestion, but noted that many factors can alter this time frame, including temperature, blood meal size and host type, age, and mating and gonotrophic status. The holding conditions in my time-series analysis may explain the reason the assay became relatively unreliable at 24 hr. However, the holding conditions in the current study more closely represent those present when stable flies are active in Florida, making any conclusions drawn from these field-collected specimens appropriate.

In the present study, analysis of field-collected stable flies resulted in approximately 59% detection efficiency. Many studies have documented the response of cattle to the painful bites of stable flies. Dairy cattle exhibit various defensive behaviors including stomping, head throwing, skin twitching, and tail switching (Miller et al. 1973, Mullens et al. 2006). These behaviors, including bunching, are not unique to cattle and are often observed by horse producers. Defense behaviors may, in part, explain the disparity between the laboratory and field efficiency of our assay. In the laboratory assays, stable flies were allowed to feed to repletion, and in theory, imbibed the maximum amount of DNA possible in a single feeding event. Defensive behaviors by livestock in response to painful bites often dislodge stable flies and end a feeding event abruptly, causing them to move to another host or a nearby resting site. This activity is expressed in my data by the numerous multiple host detections among the field-collected stable flies. However, interrupted feeding may also result in lower volume blood meals, and therefore, decrease the time taken for digestion and DNA degradation. In addition, pressure induction of a fecal droplet to assess blood-feeding status did not guarantee the fly was actively digesting a recent blood meal, and instead, may have been within a few hours of complete digestion. The fecal droplet method was developed to thwart the problem of actively selecting flies having no visible signs of a blood meal, as well as a low labor alternative to dissections. Although flies having distended, red abdomens were rarely encountered, they were also avoided, as they were likely to have fed on nearby horses. These stable flies would not provide information regarding short-term localized movement between equine and nearby cattle farms.

Among the primary goals of this study was an attempt to determine the hosts utilized by stable flies collected from Florida equine facilities. Significantly more stable flies were collected that had previously fed on cattle than any other host, and on average made up 65% of the detections for any given week, at any given farm. This is surprising, as all of the stable flies analyzed were collected in the direct vicinity of and at a central location within each horse facility. These findings do not necessarily suggest that cattle are the preferred host of stable flies. Rather, the large proportion of cattle feedings may be due to their tolerance of stable fly biting activity over time.

Most studies involving stable flies and livestock have been conducted in an attempt to quantify losses in cattle production due to the relentless biting activity of pests. While studies of growing beef cattle have demonstrated economic losses due to 10-20% in weight-gain reductions (Campbell et al. 1977, 2001, Catangui et al. 1993), results of studies concerning dairy cattle have been conflicting. Bruce and Decker (1958) reported monthly reductions in milk and butterfat content of 0.7% per stable fly, per cow. Alternatively, Miller et al. (1973) and Mullens et al. (2006) demonstrated that defensive behaviors by dairy cattle did not affect production and furthermore, that these behaviors decreased over time.

A search of the literature provided little in the area of biting fly pests and horse behavior. However, Keiper and Berger (1992) documented defensive behavior including tail switching and alternative refuge seeking by feral horses in response to horse flies and other biting fly pests. They also noted that this behavior continued whenever biting activity occurred. Although speculation, this would suggest that horses

do not acclimate to stable fly biting activity as cattle do, and may have resulted in fewer collections of flies that had previously fed on horses.

A study of muscoid flies found at equine facilities by Burg et al. (1990), indicated that stable flies were regularly captured using CO_2-baited traps, but were not observed on pastured horses. DuToit (1975) and Sutherland (1978a) determined that while stable fly longevity and reproduction was maximized when reared on cattle blood, only moderate performance was achieved when reared on horse blood. In Chapter 2 of this dissertation, we document the availability and use of husbandry generated equine waste products as breeding sites for stable flies. Additionally, a study by Sutherland (1978b) demonstrated that immature stable fly survival was greatest using horse manure substrates over other types, including cattle. This was confirmed in tests conducted by Boire et al. (1988), demonstrating equal or better immature stable fly performance on horse manure when compared to that of cattle. Further study by Jeanbourquin and Guerin (2007) found that in all tests, stable flies chose horse dung when given a choice between cattle or horse dung types. These results, as well as those of the present study, suggest that horse feeding by stable flies is infrequent. Whether this phenomenon is due to defensive behavior or host preference remains to be determined and requires further study.

These data also suggest that stable flies are emigrating from nearby cattle installations. Although they may be breeding on off-farm sites as well, stable fly production did occur on each farm. It may be that stable flies are highly attracted to on-site breeding areas due to their preference for horse manure substrates, occurring as intermittent biting pests that emigrate back to cattle farms for their primary blood source.

Because stable fly activity was not monitored at nearby cattle farms, this theory remains to be proven. However, the results of this study do prove stable fly movement in some capacity, between farm types.

The second goal of this study was to determine whether differences in host feeding existed between farms. Although differences in cattle-feeding frequency between farms were detected, collections of stable flies previously fed on cattle blood predominated other blood types on all farms, with the exception of Farm 4. These results are similar to findings by Anderson and Tempelis (1970), documenting 98% of the stable flies captured on poultry farms to have previously fed on cattle. The other 2% were blood-fed on two horses and a dog, with no blood meals taken from chickens. The authors documented the nearest cattle to any poultry ranch in the investigation varied in distance from 0.4-0.8 km.

Maps were created noting cattle and horse pastures to a distance of 3 km to assist in interpretation of findings in the present study (Fig. 4-4a, b, c, d). The nearest cattle pastures were located approximately 1.5, 0.8, 0.8, and 1.5 km from Farms 1, 2, 3, and 4, respectively. However, Farm 3 was associated with the most cattle pastures, followed by Farms 2, 1, and 4, respectively. That Farm 4 had the fewest cattle and the highest horse-feeding results suggests that cattle distance and abundance plays a significant role in fly movement from cattle to horse farms. Although stable fly breeding sites were located on each farm in the study, Farm 4 was also the only facility that maintained horses on round hay bales, a preferred breeding substrate for stable flies (Broce et al. 2005, Talley et al. 2009). Furthermore, stable flies from Farm 4 were most often collected during times when on-site fly production occurred. Stable fly breeding

was encountered at other farms, but less often. However, this does not mean that breeding attempts by flies were not as numerous. Flies were abundant and present in numbers not generally justified by the breeding activity observed, suggesting that those farms may not be producing all resident stable flies. The observation of regularly practiced cultural controls at other farms may have also limited or destroyed stable fly development areas.

Stable fly dispersal has been documented in several studies (Bailey et al. 1973, Gersabeck and Merrit 1985, Hogsette and Ruff 1985), and the distances potentially traveled in the present study are not out of line with these reports. However, this is the first documentation of a method to monitor stable fly dispersal within a 24 hr time frame. The multiplex PCR developed in this study was 100% reliable when stable flies were tested within 16 hr post-blood-feeding. Therefore, we propose a relationship between the reliability of our PCR method and the distance traveled by stable flies from nearby cattle pastures. The results from Farm 4 and Farm 1 would suggest that within 16 hr, stable flies traveled at least 1.5 km in search of hosts or breeding sites.

The results of this study have far-reaching implications. One potentially important impact of this project will be in the area of integrated pest management. It is apparent that stable flies readily travel between horse and cattle farms and are capable of such dispersal within a 24 hr period. Therefore, it is likely that equine facilities are not solely responsible for the production of all on-site stable flies. In addition, other filth flies, such as the house fly, *Musca domestica* (L.), have demonstrated their ability to become resistant to many of the chemical controls used against them (Scott et al. 2000, Marçon et al. 2003). Kaufman et al. (2001b) determined that house fly insecticide resistance

was widespread among dairies in New York state, and that resistance levels were similar, indicating between-farm dispersal. House fly dispersal between livestock units in Florida may also occur, compounding fly control efforts among livestock producers. Some livestock producers utilize commercially available pteromalid pupal parasitoids to manage fly populations, with many using chemical control measures. These management practices on equine facilities may be ineffective if on-site fly breeding is limited and between-farm dispersal is occurring.

This study provides a foundation for further research. Blood meal analysis of stable flies collected from cattle farms may provide additional evidence of dispersal between livestock facilities, and may direct research to areas where substantial fly breeding occurs. Modification of our procedure to include the use of microsatellites may pinpoint origins of different stable flies due to off-site developmental areas or dispersal, or identify farms and pastures having particularly attractive hosts.

Table 4-1. Primer sequences targeting the cytochrome *b* region of the mitochondrial genome of mammals used to identify stable fly hosts with a multiplex polymerase chain reaction.

Primer	5'-3' Sequence	Melting Temp. ºC	Product Size
Cattle	ttatcatcatagcaattgcc	57.6	400
Horse	ccctacatcggtactaccc	58.3	499
Dog	agcctatattacggatcctatg	57.7	658
Human	ctcggcttacttctcttcc	58.2	273
UnivRev	agtgggygraatattatgc	58.9	-

Figure 4-1. Pressure induction of a fecal droplet from a blood-fed stable fly.

Figure 4-2. Feeding chambers used for stable fly host blood meal identification time course analysis. Each chamber is 120 ml and contains 10 female stable flies (3-5 day-old). Chambers were inverted during blood-feeding.

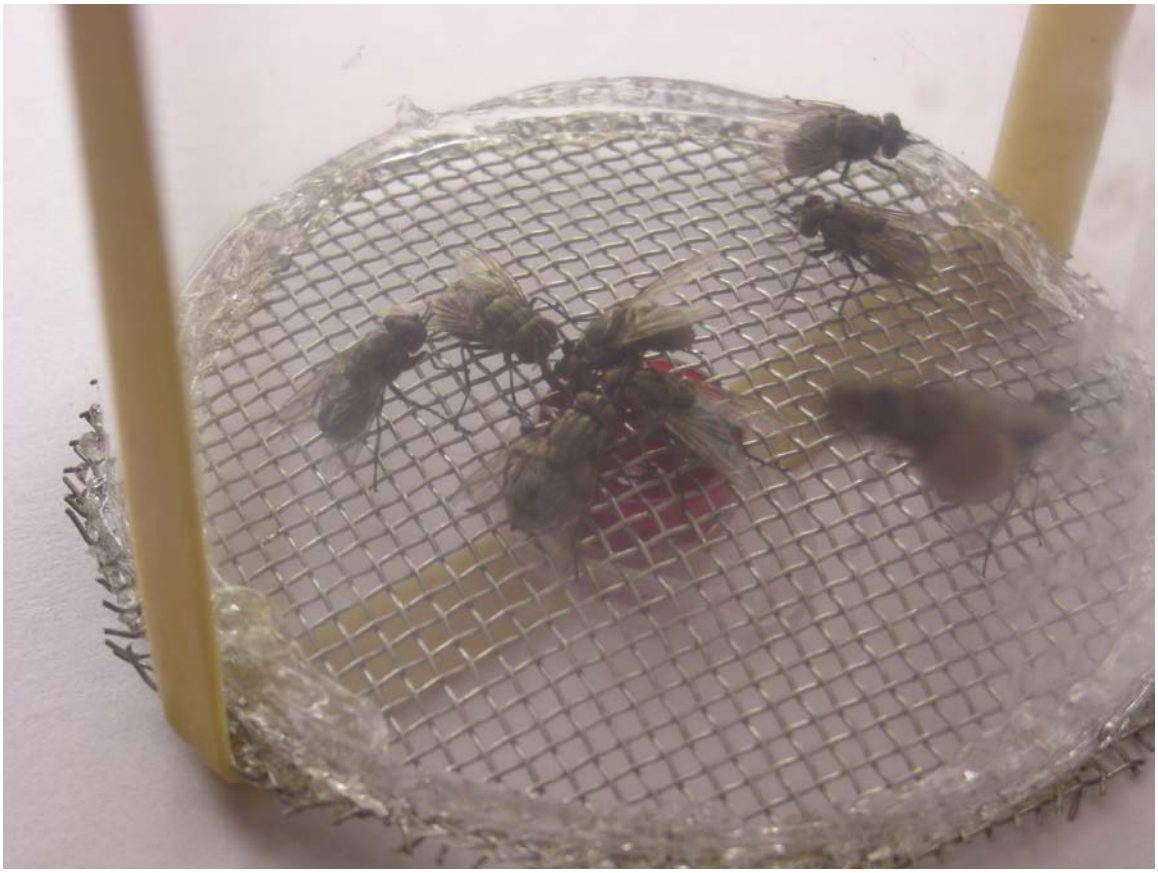
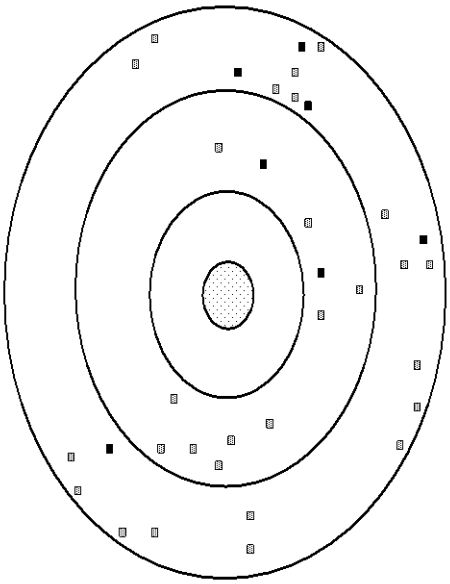


Figure 4-3. Stable fly feeding chambers with attached cap from a 1.5 ml microcentrifuge tube filled with 200-µl host blood.

a



b

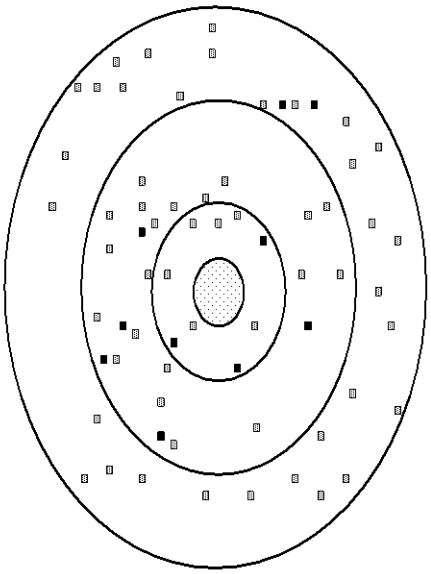
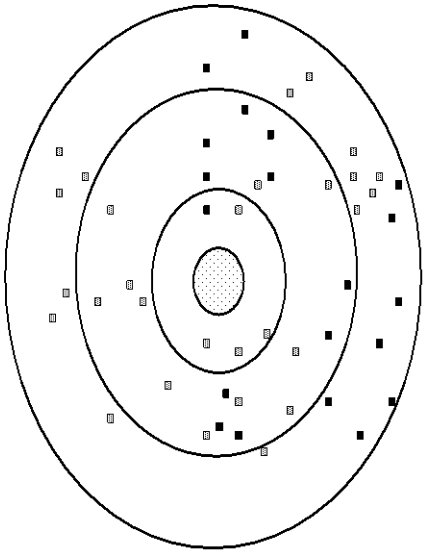


Figure 4-4. Pastures enclosing horses (dotted) and cattle (solid) within 3 km of each equine facility. Rings following the "bull's eye" represent 1-km distances from each farm: a) Farm 1, b) Farm 2, c) Farm 3, and d) Farm 4.

c



d

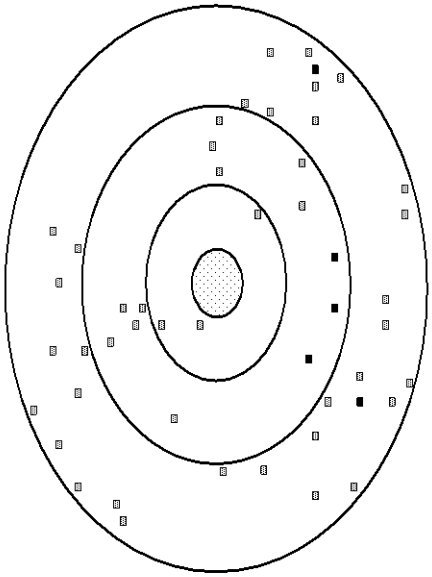


Figure 4-4. Continued.

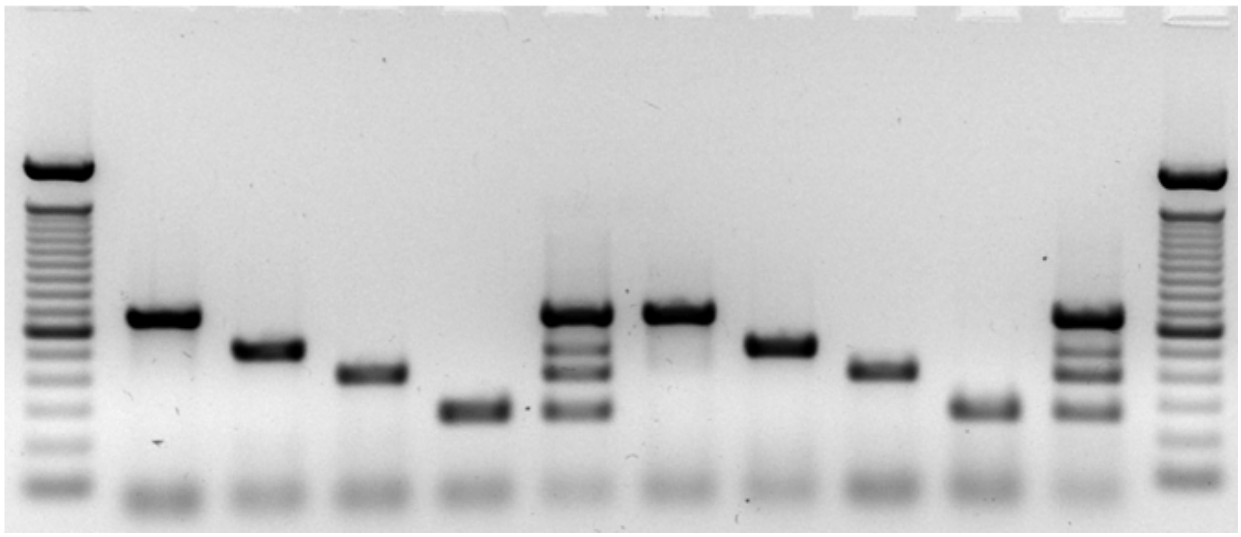


Figure 4-5. Agarose gel results of time course blood meal analysis. Outer lanes (left and rightmost) are 100-base pair molecular weight standards. Lanes 2-6 represent stable fly host blood meal identification at 0 hr post-blood-feeding on dog, horse, cattle, human, and mixed-blood types, respectively. Lanes 7-11 represent blood meal identification at 8 hr post-blood-feeding on the same host blood types.

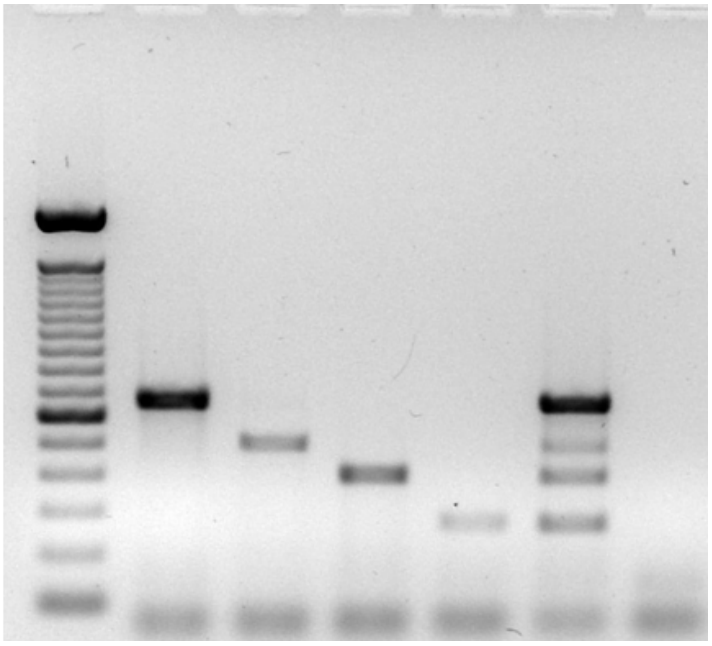


Figure 4-6. Agarose gel results of time course blood meal analysis performed at 16 hr post-blood-feeding. Lane 1 (leftmost) is a 100-base pair molecular weight standard. Lanes 2-6 represent blood meal identification of stable flies fed on dog, horse, cattle, human, and mixed blood types, respectively. Lane 7 demonstrates the absence of non-target amplification from a non-blood-fed stable fly.

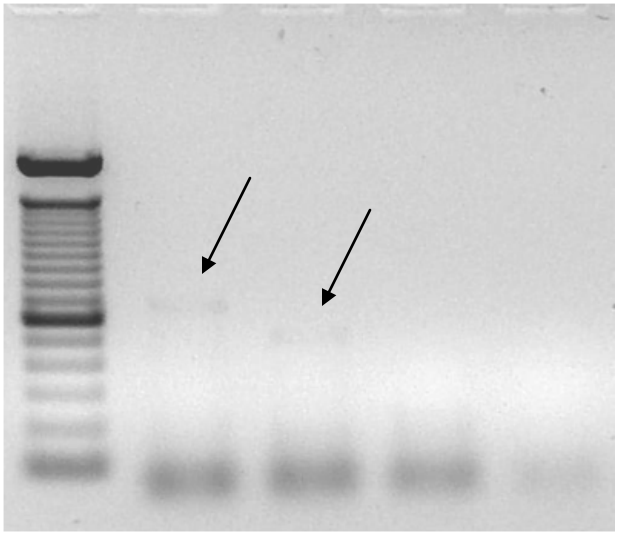


Figure 4-7. Agarose gel results of time course blood meal analysis performed at 24 hr post-blood-feeding. Lane 1 (leftmost) is a 100-base pair molecular weight standard. Lanes 2-5 represent blood meal identification of stable flies fed on dog, horse, cattle, human, and mixed blood types, respectively. Black arrows indicate faint bands present in lanes 2 and 3.

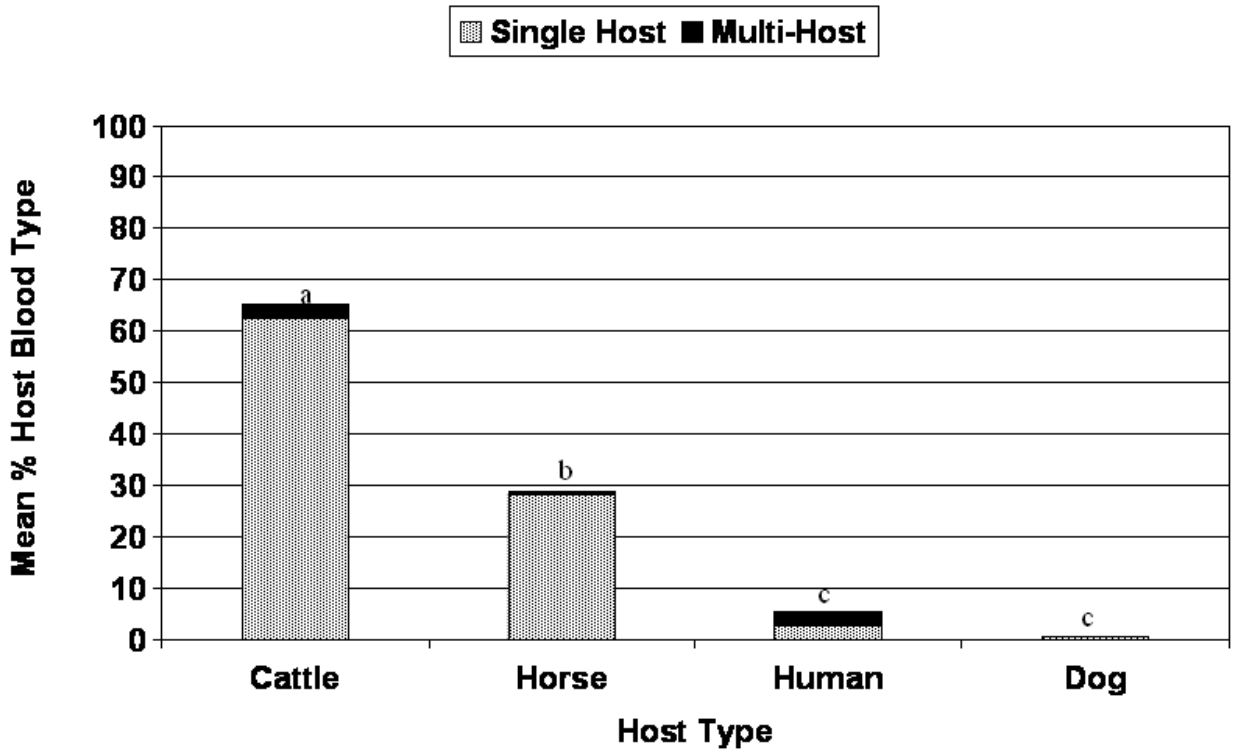


Figure 4-8. Mean percent blood type composition determined by stable fly blood meal identification using a multiplex polymerase chain reaction. Means represent percent host blood meal composition of stable flies collected across farms and dates. Means with the same letter are not significantly different (Ryan-Einot-Gabriel-Welsh multiple range test, $\alpha = 0.05$). $F_{3,68} = 43.90$; $P = <0.0001$, $n = 291$.

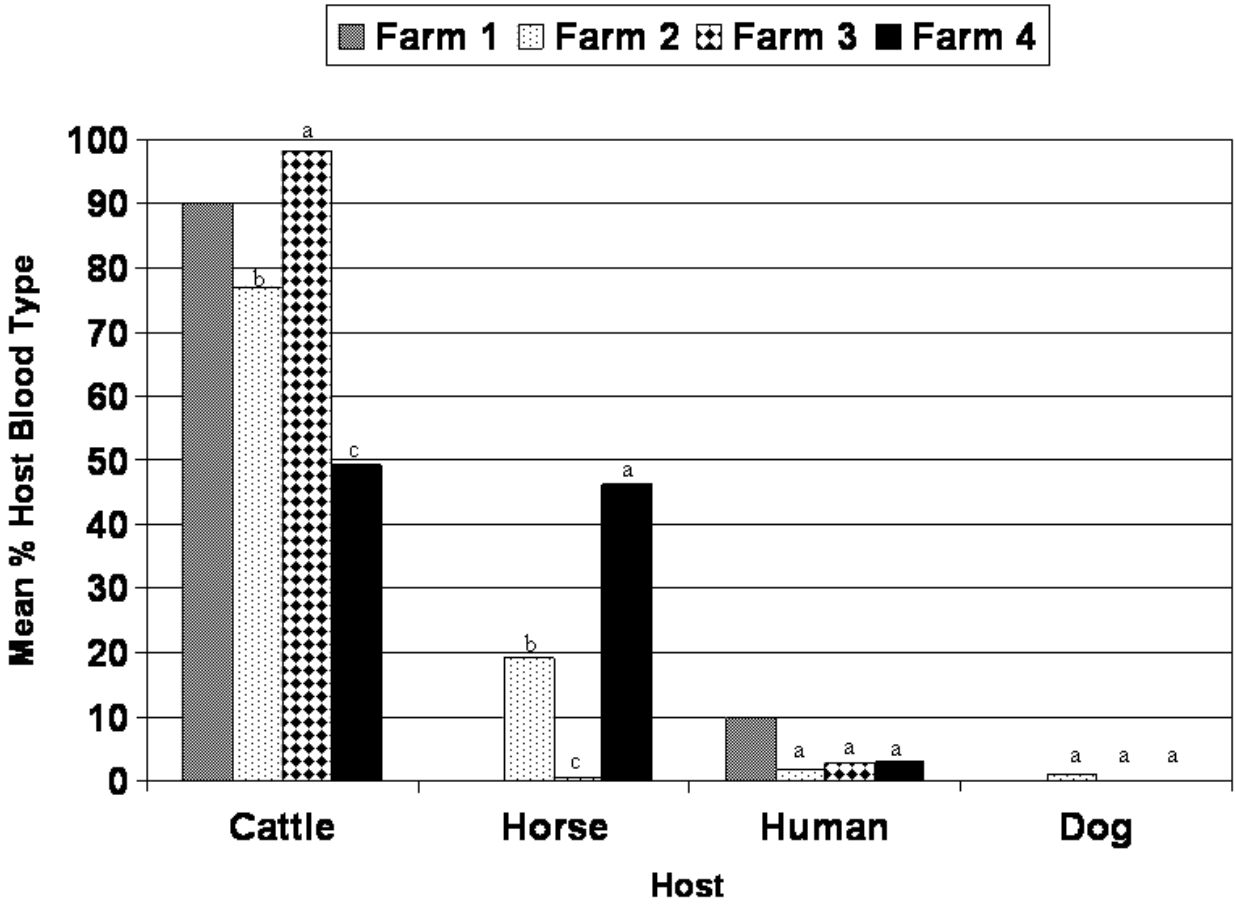


Figure 4-9. Mean percent blood type composition determined by stable fly blood meal identification using a multiplex polymerase chain reaction. Means represent percent host blood meal composition of stable flies collected from all collection events. Due to the statistical constraints, Farm 1 contained insufficient data and was removed from the analysis. Within host means with the same letter are not significantly different between farms (Ryan-Einot-Gabriel-Welsh multiple range test, $\alpha = 0.05$). Cattle; $F_{2,32} = 18.18$; $P = <0.0001$, Horse; $F_{2,32} = 19.27$; $P = <0.0001$.

CHAPTER 5
PERMETHRIN RESISTANCE STATUS OF THE STABLE FLY IN FLORIDA: A CRITICAL UPDATE USING LABORATORY SELECTIONS AND FIELD EVALUATIONS

Introduction

Populations of insecticide resistant muscoid flies, especially house flies, *Musca domestica* L., and horn flies, *Haematobia irritans* (L.), are known to occur throughout the world. Scott el al. (2000) found resistant populations of house flies in poultry units to be highly correlated with insecticide use history. While the isolated nature of poultry houses may inhibit dispersal of resistant flies to other facilities, Kaufman et al. (2001b) surmised that open cattle dairies may readily promote dispersal of potentially resistant flies. Although resistant populations of horn flies are found across the United States, dispersal of this pest may be limited due to its ectoparasitic feeding behavior (Schmidt et al. 1985, Crosby et al. 1991, Cilek et al. 1991, Kaufman et al. 1999, Barros et al. 2001). Reports of horn fly dispersal and resultant spread of resistance genes are sparse (Sheppard and Joyce 1992).

Of the predominant muscoid livestock pests including the horn fly, house fly, stable fly, *Stomoxys calcitrans* (L.), and face fly, *Musca autumnalis* De Geer, only the horn fly and house fly have been pressured enough to demonstrate their ability to become resistant to a wide array of insecticides. Furthermore, the mechanisms behind insecticide resistance in both species appear to be similar and include expression of knockdown resistance (*kdr*) and metabolic detoxification. Knockdown resistance is conferred through point-source mutations on the sodium channel gene resulting in target-site insensitivity. This mechanism has been demonstrated in house flies and horn flies using metabolic detoxification synergists such as piperonyl butoxide and diethyl maleate (McDonald and Schmidt 1987, Scott 1998). These point mutations have

also been shown to increase resistance expression relative to their increasing frequency (Guerrero et al. 1997, Smith et al. 1997, Jamroz et al. 1998, Lee et al. 1999).

If the mechanisms behind insecticide resistance are similar in house flies and horn flies, the closely related stable fly may also possess such abilities against insecticidal control. However, only one incidence of insecticide resistant, field-collected stable flies has been reported (Cilek and Greene 1994). Marçon et al. (1997) found small resistance factors in field populations of stable flies, but noted they were only as large as those observed between laboratory colony generations. The small amount of time spent on the host, coupled with a preference for the forelegs of animals may considerably limit stable fly exposure to applied insecticides.

Insecticide resistance in the stable fly has not been detected to a large degree in the U.S. However, a large void in the literature exists for pesticide evaluations of field-collected stable flies. Therefore, it is possible that insecticide resistance in this pest is occurring, and has merely been overlooked due to the general difficulty in controlling this pest with currently used techniques. Furthermore, it has been over a decade since an attempt has been made to evaluate the susceptibility of stable flies to insecticides, in particular permethrin. A study was initiated to address the need for information concerning the permethrin susceptibility of stable flies. The primary objectives for the study were to 1) determine the ability of the stable fly to become resistant to the commonly used pyrethroid, permethrin, using laboratory selections, and 2) evaluate field-collected stable fly susceptibility to permethrin susceptibilities using diagnostic concentrations.

Materials and Methods

Stable Flies. Two stable fly strains were used to generate preliminary dose response values used for these experiments. A stable fly colony maintained at the USDA-ARS-CMAVE in Gainesville, FL, served as the baseline susceptible strain (USDA-S) from which all resistance ratios were calculated. This strain has not been exposed to pesticides for approximately 30 years. A second stable fly colony, maintained at the University of Florida (UF) Veterinary Entomology Laboratory, was established from wild flies collected from the UF Dairy Research Unit in Hague, FL (UFD-07) in February of 2007. This colony, maintained under conditions described in Chapter 3, served as the parental strain from which permethrin selections were conducted. Wild stable flies, collected as pupae and evaluated using diagnostic permethrin concentrations, provided flies at a similar life stage to prevent discrepancies in survival due to age. These sites included equine Farms 1 and 4 located near Ocala, Florida (Chapter 2), and the UF Horse Teaching Unit (HTU) in Gainesville, Florida.

Insecticide-Coated Glass Jars. Glass jars (60 ml), having a 4-cm diameter, 4.4-cm height, and 67.86-cm^2 inside surface area, were treated with 1 ml of serially diluted technical grade permethrin (98%, *cis:trans* 47.6:50.4, Chem Service, West Chester, PA) in acetone approximately 1 hour prior to fly exposure. All dilutions were prepared from a stock solution the day prior to testing. The jars were rolled on an unheated electric hot dog cooker for 30 min to allow for evaporation of acetone and uniform pesticide coverage. After the allotted drying time, 20, 3- to 5-day-old female stable flies were anesthetized with CO_2 and transferred to each treated jar for a 4 hr exposure period. Following exposure, flies in each treated jar were again anesthetized, transferred to a clean 60 ml glass jar, and fed through a screened lid with a 2 cm length of dental wick

soaked with Gatorade®. Mortality was assessed at 4, 24, and 48 hr after the initial exposure, scoring ataxic flies as dead. Coated glass jar assays contained 6-8 concentrations, each with 4 jars. Jars coated with acetone-only served as the untreated control. The entire experiment was replicated three times for a total of 240 female flies tested per dilution.

Topically-Applied Insecticide. A set of 1:1 serial dilutions of 6-8 concentrations was prepared from a stock solution the day prior to testing. For each experiment, five samples of 15 female stable flies were weighed to obtain an average fly weight for the assay. Female stable flies were aspirated from colony cages, anesthetized with CO_2, and sorted into groups of 15. All 15 flies in each group were transferred to a Petri dish placed on ice and administered 1 µl of their respective permethrin concentration (98%, *cis:trans* 47.6:50.4, Chem Service, West Chester, PA) to the thorax using a Hamilton PB-600 repeating dispenser (micro-applicator) (Hamilton, Reno, NV). These flies were transferred to clean 60 ml glass jars and assessed for handling mortality, scoring ataxic flies as dead. There were four groups for each dilution contained four groups, and each experiment was replicated three times for a total of 180 female flies per dilution. Because the same micro-applicator syringe was used for the entire assay, topical applications began with the acetone-only control groups and proceeded from low to high concentrations. In addition, the micro-applicator was cleaned in triplicate with acetone between each 15-fly group. Mortality was assessed at 4 and 24 hr, scoring ataxic flies as dead.

Diagnostic Insecticide Exposure. In addition to assays used to generate standard dose-mortality data, field-collected and laboratory stable fly strains were

evaluated with diagnostic permethrin concentrations applied to coated glass jars using the technique of Scott et al. (2000). These assays were performed similarly to those described for the insecticide-coated glass jar technique. However, this assay contained only four diagnostic dilutions (98%, *cis:trans* 47.6:50.4, Chem Service, West Chester, PA) derived from the USDA-S stable fly colony. These dilutions included four jars each of 1-, 3-, 10-, and 30X concentrations, where X was the previously established LC_{99} determined from the USDA-S colony. This assay was replicated three times when possible, for a total of 240 female stable flies tested for each dilution. Because of the difficulty in collecting enough pupae to evaluate field strains, we required that each replication have three jars per dilution, for a total of 120 stable flies of either sex, using more when possible. When field fly populations were evaluated for permethrin susceptibility, concurrent evaluation of the USDA-S stable fly strain was conducted to ensure dilution accuracy. Abbott's correction (Abbott 1925) was applied to all data from this assay to adjust for control mortality.

Selection for Permethrin Resistance. The UFD-07 stable fly strain previously colonized in February 2007 was used in an attempt to generate a permethrin-resistant stable fly strain (UFD-PR). This colony had completed 30 generations between the time it was collected and when permethrin selections began. Stable fly selections were conducted using the LC_{70} value previously determined for the UFD-07 strain from dose-mortality data generated with treated glass jars.

Generations produced from surviving selected individuals were inadequate to complete both subsequent selections and three insecticide-treated glass jar replications to re-evaluate permethrin susceptibility with the F_{x+1} generation. Therefore, only every

other stable fly generation was subjected to permethrin selections. Additionally, due to low adult fly numbers resulting from the high selection pressure, it was often not possible to conduct three experimental replications to precisely determine permethrin susceptibility prior to the next selection. This problem was alleviated using a standard set of six dilutions derived during preliminary topically-applied insecticide assays. This allowed for the testing of fewer individuals to generate complete dose-response data. However, only two replications using this method were performed prior to the next selection (120 female flies per dilution tested).

Before each selection, a new LC_{70} value was estimated through conversion of the values determined using the topical-applicator results, to those estimated for insecticide-treated glass jars. Using the relationship determined between the LD and LC values of previous evaluations with the USDA-S and UFD-07 colonies, topical-applicator LD values (µg permethrin/mg insect) were multiplied by a mean factor of 33 to obtain equivalent values for treated glass (µg permethrin/cm^2).

Stable fly selections were carried out using permethrin (98%, *cis:trans* 47.6:50.4, Chem Service, West Chester, PA) applied to the internal surface of 1.06 L glass canning jars. Jar dimensions were 7.8 cm diameter, 12.5 cm height, and 339 cm^2 inside surface area. Ten jars served as permethrin-treated controls to evaluate gender selection efficiency, with five each designated to assess male and female mortality separately. Each of these jars contained 250 individuals and was monitored for mortality separately from all other selection jars. In all cases, stable flies were exposed to permethrin-treated jars for a 4 hr period, at which time they were anesthetized and transferred to a rearing cage. Flies in the gender-designated jars were transferred to a

30 x 30 x 30 cm rearing cage and held for 48 hr after the initial exposure prior to mortality assessment. After the 48 hr mortality assessment, surviving flies were transferred to the primary rearing cage with those from the remaining jars. The majority of the selected flies were held in jars containing 250-300 mixed-sex stable flies aspirated from rearing cages with a graduated vacuum tube. Mortality of stable flies in these jars was not assessed. Approximately 10,000 individuals were exposed during each selection assay. All surviving flies were held together following mortality assessment of gender-sorted treatment jars.

Statistical Analysis. All dose-response data was subjected to standard probit analysis using the PROC PROBIT procedure of SAS® 9.2 (SAS Institute 2004) to generate LC/LD values used for resistance ratio calculations, permethrin selections, and diagnostic concentrations. Values for LC/LD determined by this analysis were considered significantly different if no overlap occurred between the 95% confidence intervals (CI). Comparisons were made at both the LC/LD_{50} and LC/LD_{90} values. Resistance ratios were calculated as the LC/LD value of a particular strain, divided by that of the susceptible USDA strain.

Results

Colonized Stable Fly Strains. The LC/LD_{50} and LC/LD_{90} values determined for all fly strains evaluated is presented in Table 5-1 and Table 5-2. The LC_{70} concentrations used for each permethrin selection and the subsequent respective percent mortality are shown in Table 5-3.

Using treated-glass jars, the LC_{50} and LC_{90} values for the USDA-S colony were 0.0013 and 0.0022 µg/cm^2, respectively. Due to the non-overlap of the 95% CI, significant differences were identified between both LC values for the UFD-07 parental

colony, and the resultant UFD-PR following the fifth selection. The resistance ratios for the UFD-07 colony following the fifth permethrin selection had increased by 7- and 12-fold at LC_{50} and LC_{90}, respectively, over that of the USDA-S colony. However, because low-level resistance previously existed in the parental UFD-07 colony, resistance levels in the UFD-PR had only increased by approximately 3-fold over that of the parental strain.

Similar to experiments using insecticide-coated glass, resistance ratios for the field strains as well as those of the UFD-PR generations evaluated prior to permethrin selection, demonstrated significantly higher resistance levels than the USDA-S strain. Prior to selecting for permethrin resistance, experiments using topically-applied insecticide demonstrated that the UFD-07 strain possessed approximately 4- and 6-fold resistance levels over the USDA-S strain at the LD_{50} and LD_{90}, respectively (Table 5-2). As expected, the five selections conducted at increasing concentrations (Table 5-3), resulted in fairly consistent increases in the resistance ratio as compared to the USDA-S strain.

With the exception of the final selection, our methods to convert micro-applied insecticide LD values to those used for coated glass were accurate. Preliminary tests of the expected values for the fifth selection resulted in 100% fly survival at 4 hr, prompting an increase in the concentration from the determined 0.016 µg/cm^2, to 0.044 µg/cm^2. Overall, five laboratory selections resulted in a 15-fold increase in resistance levels, as compared to the USDA-S strain at both LD_{50} and LD_{90}, respectively, were measured using the topical applicator. The UFD-PR strain had also increased resistance levels to 5- and 3-fold that of the parental UFD-07 strain at LD_{50} and LD_{90}, respectively.

Field-Collected Stable Flies. Initially, an attempt was made to colonize four stable fly strains collected from each farm used in our field studies (Chapter 2). However, rearing success was achieved only in those flies collected from Farm 1 (Chapter 2) where sufficient stable fly numbers were obtained to conduct three replications using the topically-applied insecticide technique. To obtain the needed numbers of stable flies for assays, flies were reared in the laboratory using the procedure described in Chapter 3. Resistance ratios for flies from Farm 1 after five generations were approximately 9- and 14-fold that of the USDA-S strain at LD_{50} and LD_{90}, respectively.

Stable flies from the UFD-07, UFD-PR, Farms 1 and 4, and the HTU strains, were also evaluated using diagnostic concentrations (Fig. 5-1). Survival at 1X LC_{99} USDA-S ranged between 59 and 93%. Although decreased at 3X LC_{99} USDA-S, survival ranged from 10% for the UFD-07 colony to 57% in stable flies collected from Farm 4. Survival at 10X LC_{99} USDA-S, was greatest for flies collected at Farm 4 and the HTU. In all evaluations only one fly, collected from the HTU, survived at 30X LC_{99} USDA-S.

Discussion

My laboratory and field evaluations provided a critically needed update on the status of permethrin susceptibility in stable flies. To my knowledge, only two studies, conducted more than a decade ago by Cilek and Greene (1994) and Marçon et al. (1997) document the possibility that stable flies may express insecticide resistance as do their muscoid counterparts, the house fly and horn fly (Scott et al. 2000, Kaufman et al. 2001b, Marçon et al. 2003). The LC_{50} values determined in the present study were 3.5 and 13-fold greater than those used by Cilek and Greene (1994), for our UFD-07 and UFD-PR colonies, respectively. Our results also differ from those of Marçon et al.

(1997). Furthermore, evidence from our selection experiments demonstrate that under heavy selection pressure, as few as five permethrin selections can increase resistance levels up to 5-fold that of the parental strain, with a comparative total increase of 15-fold that of a susceptible strain.

Several known mechanisms, such as detoxification by P450 monooxygenases or hydrolases (Liu and Yue 2000), and target site mutations, such as those observed in the voltage-sensitive sodium channel gene (Smith et al. 1997, Lee et al. 1999) are possibly the cause of stable fly pyrethroid insecticide resistance. Unfortunately, most research in this area has been conducted on the house fly and horn fly. Further study is needed to determine which, if any resistance mechanisms occur in the stable fly. However, during our assays using insecticide-coated glass jars, it was often difficult to achieve results that fit the model under the assumptions of probit analysis. I believe this is largely due to the behavioral avoidance displayed by stable flies, particularly in the post-selection evaluation of the UFD-PR strain, where stable flies were observed on the untreated lids of the glass jars. This behavior is not uncommon and has been reported as a possible explanation for resistance observed in the horn fly (Lockwood et al. 1985, Zyzak et al. 1996). This cannot completely account for the cause of resistance in the stable fly determined in the present study, as even greater resistance ratios were determined using topically-applied insecticides, a technique that mitigates bias introduced by fly behavior.

The differences in resistance ratios between resistant and susceptible house fly strains of other studies (Scott and Georghiou 1985, Liu and Yue 2000) have been greater and more dramatic than those demonstrated for the stable fly in the present

study. However, resistance ratio increases over the parental house fly strains used in those selection studies are similar. One reason for this occurrence may be due to differences in the level of exposure between the two pests. Stable fly control has been historically difficult, with causes stemming from the repellent effects of pesticides (Hogsette and Ruff 1986), to their potential dispersal habits (Hogsette and Ruff 1985, Chapter 4). Undoubtedly, selection pressure on house flies, and particularly horn flies, has been greater than that of stable flies. Besides the small amount of time stable flies spend in commonly pesticide-treated areas such as building resting sites and on animals, few pesticide applications are made to animals specifically for stable fly control. Resistance development in the horn fly has been particularly problematic (Cilek et al. 1991, Byford et al. 1999, Foil et al. 2005), but is due essentially to the constant selection pressure that horn flies receive as a result of their continuous on-animal behavior. My studies of dispersal based on blood meal analysis of stable flies (Chapter 4) also suggest that stable flies may attack hosts and breed in different locations, increasing the difficulty of their control, and provide a means for dispersal of resistance genes in the population.

The potential control problems attributed to stable fly dispersal have been observed in populations of the house fly. Studies of insecticide resistance in house flies of poultry facilities in New York state, demonstrated that resistance was highly correlated with the pesticide use history of each facility, possibly due to limited house fly dispersal from the relatively isolated environment of poultry houses (Scott et al. 2000). However, study of more easily accessible dairy facilities may readily permit dispersal of resistant house flies, impacting the effectiveness of their chemical control at nearby

dairies (Kaufman et al. 2001b). Evidence for this phenomenon has also been observed in the present study. Stable flies were collected from Farm 1 and evaluated both topically and with diagnostic surface concentrations. In topical assays, this colony demonstrated 9- and 14-fold resistance ratios over the USDA-S strain at LD_{50} and LD_{90}, respectively. In addition, this colony demonstrated 20% survival at the 3X LC_{99} USDA-S. This is surprising, as this farm is the only one of those used in our field studies where facility managers reported that insecticides were not used. This suggests that stable flies arriving from other areas are likely the cause for the resistance observed in flies collected at Farm 1.

Additional data corroborating this hypothesis were the results of our host blood meal analysis of stable flies (Chapter 4), which revealed that all flies collected from Farm 1 that had not fed on humans had fed on cattle, an animal not present within 1.5 km of this facility. Although likely, this does not necessarily mean that stable flies on Farm 1 were predominantly from other sources within a few kilometers. Stable fly dispersal and movement continues to perplex veterinary entomologists, as data from various studies are conflicting. Stable fly dispersal of up to 225 km in Florida has been demonstrated by Hogsette and Ruff (1985), possibly due to incoming weather fronts. Because occurrence and dispersal are not static factors in stable fly biology, interbreeding populations of this pest may be more widespread than previously acknowledged. This is supported by evidence in the present study, where the UFD-07, Farm 1, and HTU stable fly strains all shared similar diagnostic dose susceptibilities across a region spanning 91 km. Further research investigating widespread stable fly susceptibilities, is needed to support these findings.

A study by Kunz (1991) demonstrated that permethrin resistance in colonized horn flies decreased 7-fold in as few as four generations when selection pressure ended. This may also account in part for the disparity between the increase in resistance of stable flies in the present study, and that observed by others in the house fly. In the current study, the number of offspring produced from the surviving adults of a previous selection was insufficient to re-evaluate permethrin susceptibility levels and perform a selection on their progeny. Therefore, an intermediate generation was used to generate numbers adequate to perform the entire procedure. My high-pressure selection approach inadvertently may have lessened the overall selection pressure, slowing the rate of increase in resistance expression. Although further study is required to determine if this effect occurs in the stable fly, the resistance ratios for Farm 1 may have been greater had the strain been evaluated sooner. Stable flies of the HTU strain were evaluated at the time of their capture in the present study, and demonstrated greater survivability than those colonized from Farm 1 when used in diagnostic dose experiments (Fig 5-1). Like the horn fly, resistance in the stable fly may decrease in the absence of selection pressure.

The results of this study demonstrate that insecticide resistance occurs in field populations of the stable fly. Although the resistance ratios determined in our selection and field evaluations are relatively low, the rate at which stable flies attained resistance was similar to that reported for house flies. Unlike the horn fly, it is possible that the dispersal habits of the stable fly have permitted the immigration of not only resistant, but also susceptible individuals within the population, decreasing the progression of resistance in this pest. Although speculation, the results of our topically-applied

insecticide assays suggest that in the period between colonization in 2007, a more than 2-fold increase in resistance had occurred between the UFD-07 stable flies, and those collected from Farm 1 in 2009. Because these stable flies were collected from two different geographical regions, further research will be required to fully support this hypothesis.

The permethrin resistance levels determined in the present study are certainly lower than those reported for house fly populations collected from other parts of the U.S. Therefore, timely research of insecticide resistance in stable flies is imperative, as this problem is still at a relatively manageable level. Further research, including continued re-evaluations of permethrin susceptibilities of Florida stable flies, are needed to fully understand the present rates of increase in resistance expression of this pest. Furthermore, investigations of the mechanisms involved in stable fly resistance may elucidate information that can be used to develop newer management strategies, including the development of insecticides with alternate modes of action that will act to slow or inhibit already developing resistance expression.

Table 5-1. Permethrin susceptibility for several stable fly strains evaluated using insecticide-treated glass jars.

Fly strain	*n*	LC_{50} (µg/cm^2)[a] (95% CI)	LC_{90} (µg/cm^2)[a] (95% CI)	RR_{50}[b,d]	RR_{90}[c,d]	Slope (SEM)
USDA-S	1,380	0.0013 (0.0012-0.0014)	0.0022 (0.0021-0.0024)	1.00	1.00	5.43 (0.32)
UFD-07[e]	1,920	0.0024 (0.0022-0.0027)	0.0101 (0.0090-0.0116)	1.85*	4.59*	2.08 (0.09)
UFD-PR[f]	1,440	0.0092 (0.0077-0.0108)	0.0264 (0.0216-0.0345)	7.08*	12.00*	2.81 (0.28)

[a] Values represent micrograms permethrin per cm^2 applied to the inside surface area of glass jars.
[b] Resistance ratios at LC_{50} were calculated as the LC_{50} of any fly strain divided by that of the USDA-Susceptible strain.
[c] Resistance ratios at LC_{90} were calculated as the LC_{90} of any fly strain divided by that of the USDA-Susceptible strain.
[d] Resistance ratios were considered significantly different if the 95% confidence interval (CI) was non-overlapping.
[e] Parental stable fly strain colonized from individuals collected at the University of Florida Dairy Research Unit in Hague, Florida.
[f] Resultant permethrin resistant offspring after five permethrin selections targeting each generation's estimated LC_{70} value.
n = total number of female stable flies evaluated for permethrin susceptibility, * = significant difference between resistance of a stable fly strain compared to the USDA-S strain

Table 5-2. Permethrin susceptibility for several stable fly strains evaluated using topically-applied insecticide.

Fly strain	*n*	LD_{50} (µg/g)[a] (95% CI)	LD_{90} (µg/g)[a] (95% CI)	RR_{50}[b,d]	RR_{90}[c,d]	Slope (SEM)
USDA-S	1,200	0.0311 (0.0292-0.0333)	0.0603 (0.0548-0.0674)	1.00	1.00	4.47 (0.27)
UFD-07[e]	1,080	0.1178 (0.1066-0.1296)	0.3884 (0.3394-0.4555)	3.79*	6.44*	2.47 (0.14)
UFD-PR_1[f]	720	0.0714 (0.0618-0.0812)	0.2457 (0.2063-0.3066)	2.30*	4.07*	2.39 (0.19)
UFD-PR_2	839	0.1596 (0.1398-0.1807)	0.5487 (0.4638-0.6749)	5.13*	9.10*	2.39 (0.17)
UFD-PR_3	840	0.2839 (0.2295-0.3458)	1.1037 (0.8256-1.6896)	9.13*	18.30*	2.17 (0.25)
UFD-PR_4	720	0.3130 (0.2813-0.3451)	0.6827 (0.6009-0.8037)	10.06*	11.32*	3.78 (0.32)
UFD-PR[g]	1,080	0.5844 (0.5433-0.6285)	1.2788 (1.1510-1.4503)	18.79*	21.21*	3.77 (0.22)
Farm 1	1,080	0.2767 (0.2523-0.3029)	0.8492 (0.7453-0.9904)	8.90*	14.08*	2.63 (0.14)

[a] Values represent micrograms permethrin per gram insect applied by topical-applicator

[b] Resistance ratios at LC/LD_{50} were calculated as the LD_{50} of any fly strain divided by that of the USDA-Susceptible strain.

[c] Resistance ratios at LC/LD_{90} were calculated as the LD_{90} of any fly strain divided by that of the USDA-Susceptible strain.

[d] Resistance ratios were considered significantly different if the 95% confidence interval (CI) was non-overlapping.

[e] Parental stable fly strain colonized from individuals collected at the University of Florida Dairy Research Unit in Hague, Florida.

[f] Subsequent generations from surviving stable fly adults of the selected parental UFD-07 strain.

[g] Resultant permethrin resistant offspring after five permethrin selections targeting each generation's LC_{70} value.

n = total number of female stable flies evaluated for permethrin susceptibility, * = significant difference between resistance of a stable fly strain compared to the USDA-S strain

Table 5-3. Concentrations used and stable fly mortality results at each permethrin selection.

Selected Generation[a]	Selecting Conc. ($\mu g/cm^2$)[b]	% Male Mortality[c]	% Female Mortality[c]	% Total Mortality[d]
UFD-07	0.0044	76.3	76.7	76.5
UFD-PR_1	0.0080	80.7	78.6	79.7
UFD-PR_2	0.0088	87.8	86.3	87.0
UFD-PR_3	0.0160	92.0	90.5	91.2
UFD-PR_4	0.0442	94.7	88.2	91.4

[a] Stable fly colony obtained from adult flies collected at the University of Florida Dairy Research Unit in Hague, FL. Selections were performed on the parental strain, and every other generation thereafter using the estimated LC_{70} value of each generation. Therefore, the selection denoting F_1 was actually the F_2 according to a rearing schedule.

[b] Selection concentrations were applied to 1.06-L glass canning jars, in which 250-300 mixed-sex adult stable flies were exposed for a 4 hr period.

[c] Percent male and female mortality was determined at 48 hr, from five jars of each sex containing 250 stable flies.

[d] Percent total mortality was the total number of dead stable flies at 48 hr divided by a total of 2,500 separately monitored individuals used to determine sex-dependent mortality.

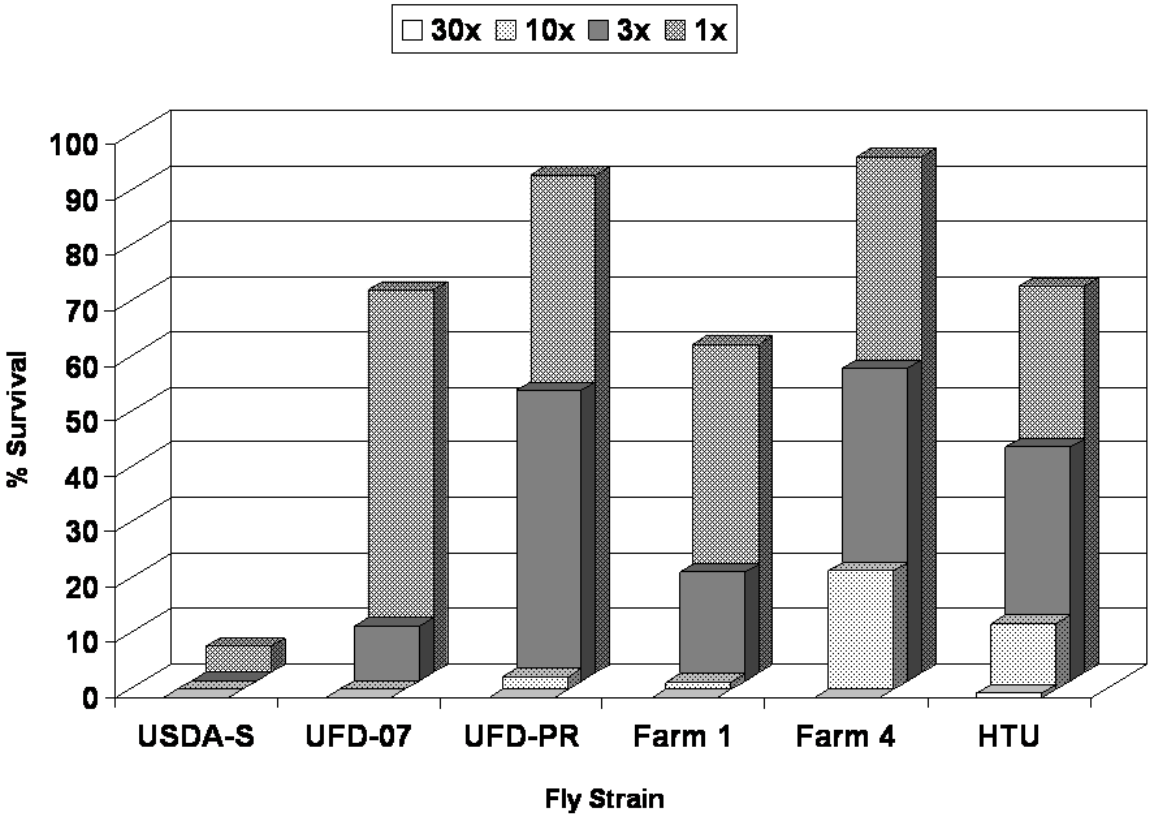


Figure 5-1. Mean percent survival of several stable fly strains exposed to diagnostic permethrin concentrations applied to 60 ml glass jars at 1-, 3-, 10-, and 30x the LC_{99} of a susceptible stable fly strain (USDA-S).

CHAPTER 6
IMPLICATIONS AND FUTURE DIRECTIONS FOR RESEARCH REGARDING STABLE FLIES AND EQUINE FACILITIES

The future success of stable fly, *Stomoxys calcitrans* (L.), management within livestock facilities likely will depend on our ability to develop and use integrated pest management (IPM) programs. The problems associated with house fly control due to expression of insecticide resistance (Scott et al. 2000, Kaufman et al. 2001b) and potential dispersal of resistance genetics (Learmount et al. 2002, Marçon et al. 2003) are particularly troubling when stable fly biology is considered. Stable flies have been shown to travel up to 3.2 km in search of a blood meal (Bailey et al. 1973, Gersabeck and Merritt 1985) and as far as 225 km depending on weather conditions (Hogsette and Ruff 1985). My study of stable fly host blood meal analysis clearly demonstrates dispersal as far as 1.5 km in as little as 16 hr, as well as the magnitude of the movement with 60% of the flies having come from cattle farms. Therefore, stable fly dispersal may greatly affect the success of current control methodologies such as the release of pteromalid pupal parasitoids or adult-directed insecticide use if these pests are breeding or immigrating from off-farm sites. Further research using polymerase chain reaction with microsatellite technology may elucidate the genetic relationship of stable fly populations in different geographic locations, thereby accounting for dispersal and potential similarities in insecticide resistance expression. Another avenue of research may involve using microsatellites of potential hosts at one farm, to directly pinpoint the origins of stable flies collected at another.

Stable fly insecticide resistance has been demonstrated twice, and only in the Midwestern U.S. (Cilek and Greene 1994, Marçon et al. 1997). However, those studies, conducted over a decade ago, document only moderate resistance ratios to permethrin

when compared to laboratory strains. Furthermore, Marçon et al. (1997) demonstrated it was likely the observed resistance levels in field-collected stable flies were no different than the varying levels detected between laboratory colony generations. My study on the permethrin susceptibility status of Florida stable flies provides a critically needed update in this area, and demonstrated up to 7- and 9-fold greater LC/LD values than those of Cilek and Greene (1994) and Marçon et al. (1997), respectively.

Stable flies from an equine facility with no reported insecticide use demonstrated approximately 20% survival with diagnostic concentrations at 3X LC_{99} that of a susceptible strain. Additionally, survival profiles of three field-collected stable fly strains using diagnostic concentrations were similar, despite a 91-km range between their collection sites. This further supports the hypothesis of long range stable fly dispersal, and supports the possibility of interbreeding populations that are separated geographically. Therefore, further research to determine the widespread insecticide susceptibility status of stable flies is needed, if future efforts utilizing chemical control methods are to succeed.

My studies also have shown that stable flies can readily develop resistance to a widely used pyrethroid insecticide, permethrin, in as few as five laboratory selections. Furthermore, a 15-fold increase in resistance expression resulted even when selecting every other stable fly generation. This likely decreased the potential gains in resistance had selections been performed on successive generations. Although it is likely that stable fly resistance is due to mechanisms previously demonstrated in the house fly, such as target site insensitivity (*kdr*) (Smith et al. 1997, Lee et al. 1999) and metabolic

detoxification (Liu and Yue 2000), identification of these mechanisms is imperative to the effectiveness of chemical control development and use for this pest.

My studies concerning the ability of stable flies to develop resistance to permethrin are similar to those observed in the house fly and horn fly, and provide a grim reminder of the importance in developing effective IPM programs. However, research involving the use of pteromalid pupal parasitoids as an alternative filth fly control measure provides conflicting data (Meyer et al. 1990, Geden et al. 1992, Petersen et al. 1992). Many factors can affect the success of parasitoid releases including immature filth fly breeding substrate light and moisture conditions (Smith and Rutz 1990, Geden 1999), host depth and density (Rueda and Axtell 1985b, King 1997), and substrate type (Geden 2002). My study concerning pupal parasitoids under both field and laboratory conditions provide supporting evidence to the importance of assessing conditions of potential fly breeding areas prior to selecting a parasitoid species for release. In addition, some parasitoids may occupy different temporal niches, whereby species specific releases may be dependent on season as well. During my field study, nearly all parasitoids collected were *Spalangia* spp., and consisted of *Spalangia cameroni* Perkins, *Spalangia nigroaenea* Curtis, *Spalangia nigra* Latreille, and *Spalangia endius* Walker. The hypothesis that this occurrence was due to the unique filth fly larval breeding habitats created by equine husbandry practices was corroborated under laboratory conditions. In all laboratory assays, *Spalangia* spp. located and attacked naturally-pupating stable fly hosts up to 50-fold more often than *Muscidifurax raptorellus* Kogan and Legner. Therefore, under the conditions provided by equine facilities in Florida, it is likely that *Muscidifurax* spp. will be ineffective in increasing filth-fly control.

The areas of research detailed in this dissertation are inter-related by the underlying hypothesis that stable fly dispersal and interbreeding population dynamics are more widespread than previously thought. Therefore, insecticidal, cultural, and biological control tactics may be rendered ineffective if off-site breeding is the source of on-site stable fly activity. Instead, other elements of an IPM program such as educating nearby managers of cattle producing facilities in the areas of stable fly larval biology and proper livestock husbandry-generated waste management, should become the forefront for control of this pest. Because control of this pest may also be limited to adult stable flies due to their dispersal behavior, an increase in research concerning the development of newer and more effective trapping devices such as the treated and electrified targets designed by Foil and Younger (2006) is warranted.

Most stable fly activity in Florida occurs in the early spring months between January and April. Therefore, Florida equine facilities with known stable fly breeding activity may benefit from pteromalid pupal parasitoid releases during these months, in addition to strong cultural management of livestock-generated waste. My field and laboratory studies of resident parasitoids suggest that *Spalangia* spp.-only releases may be more effective than those of other genera, such as *Muscidifurax* spp. Furthermore, these releases should be conducted with *S. cameroni*, as this was the most abundant species during the early spring months at Florida equine facilities. The utility of continued education to livestock producers concerning the cultural control of husbandry-generated wastes was demonstrated in my field studies. In those studies, the only farm with no reported insecticide use also accounted for the lowest adult stable fly trap

captures. This was probably due to the intense daily sanitation and composting activities practiced by the farm.

My studies of the ecology of stable flies associated with equine facilities in Florida should provide a basis for further research in an area relatively uninvestigated by the entomology field. Additionally, evidence provided by this dissertation suggests that the problems most often associated with horn flies and house flies, such as insecticide resistance, are increasing in the stable fly. Similar study of other livestock facilities, such as dairy and feedlot cattle systems may assist in increasing our understanding, and therefore, our chances of effectively controlling this economically important pest.

APPENDIX A
UNIVERSITY OF FLORIDA HEALTH CENTER INSTITUTIONAL REVIEW BOARD
#342-2008

UF | Institutional Review Board
UNIVERSITY of FLORIDA

INFORMED CONSENT FORM
to Participate in Research

University of Florida
Health Center
Institutional Review Board
APPROVED FOR USE
From 7/7/08 Through 7/6/09
cd

INTRODUCTION

Name of person seeking your consent: Phillip Kaufman

Place of employment & position: Graduate Research Assistant Entomology & Nematology Dept. UF.

This is a research study of stable fly host blood meal analysis.

Could participating in this study offer any direct benefits to you? Yes, as described on page 3.

Could participating cause you any discomforts or are there any risks to you? Yes, as described on page 3.

Please read this form which describes the study in some detail. I or one of my co-workers will also describe this study to you and answer all of your questions. Your participation is entirely voluntary. If you choose to participate you can change your mind at any time and withdraw from the study. You will not be penalized in any way or lose any benefits to which you would otherwise be entitled if you choose not to participate in this study or to withdraw. If you have questions about your rights as a research subject, please call the University of Florida Institutional Review Board (IRB) office at (352) 846-1494. If you decide to take part in this study, please sign this form on page 6.

GENERAL INFORMATION ABOUT THIS STUDY

1. Name of Participant ("Study Subject")

Jimmy Pitts

2. **What is the Title of this research study?**

   STABLE FLY HOST BLOOD MEAL ANALYSIS USING A MULTIPLEX POLYMERASE CHAIN REACTION

3. **Who do you call if you have questions about this research study?**

   Jimmy Pitzer (575)-571-6980

4. **Who is paying for this research study?**

   The sponsor of this study is University of Florida

5. **Why is this research study being done?**

   The purpose of this research study is to determine hosts of stable flies using a polymerase chain reaction-based blood meal analysis.

   You are being asked to be in this research study because humans are a potential host of stable flies. To ensure the proposed molecular technique is satisfactory, control experiments using blood-fed flies are needed. Therefore, blood collected by a medical professional can be used rather than allowing flies to feed directly on the subject. This alleviates any danger posed by contacting the fly directly. These initial control experiments will be used to validate the process for use in field-collected stable flies.

WHAT CAN YOU EXPECT IF YOU PARTICIPATE IN THIS STUDY?

6. **What will be done as part of your normal clinical care (even if you did not participate in this research study)?**

   No normal clinical care will be conducted.

7. **What will be done only because you are in this research study?**
   This study requires the periodic drawing of blood from the study subject by a medical professional for the purpose of conducting control experiments. The volume of blood needed is no greater than 80 ml during a 4 week period.

   If you have any questions now or at any time during the study, please contact Jimmy Pitzer in question 3 of this form.

8. **How long will you be in this research study?**

   This study will require subject participation for up to a year. During this time, blood will be drawn at no more than 20 ml per week during any 4 week period. The blood

drawing process should take no longer than is usually customary for this common procedure.

9. How many people are expected to take part in this research study?

Only one person, the principal investigator, will be required to participate in this study.

WHAT ARE THE RISKS AND BENEFITS OF THIS STUDY AND WHAT ARE YOUR OPTIONS?

10. What are the possible discomforts and risks from taking part in this research study?

The risks of venipuncture for the purpose of drawing blood include discomfort at the site of puncture; possible bruising and swelling around the puncture site; rarely an infection; and, uncommonly, faintness from the procedure.

This study may include risks that are unknown at this time.

Participation in more than one research study or project may further increase the risks to you. If you are already enrolled in another research study, please inform Jimmy Pitzer (listed in question 3 of this consent form) or the person reviewing this consent with you before enrolling in this or any other research study or project.

Throughout the study, the researchers will notify you of new information that may become available and might affect your decision to remain in the study.

If you wish to discuss the information above or any discomforts you may experience, please ask questions now or call the PI or contact person listed on the front page of this form.

11a. What are the potential benefits to you for taking part in this research study ?

Because the principal investigator is the only study participant, the primary benefit will be due to continuation of the project. This will allow me to determine the possible hosts, including humans, that stable flies feed on.

11b. How could others possibly benefit from this study?

This research may provide a way to indirectly determine the source of stable flies occurring on a particular livestock facility using blood meal analysis. For example, if flies captured on a horse farm are found to primarily feed on cattle, it may be that they

are immigrating from a nearby dairy. This information is a critical part of pest management, as pesticide failures at the horse farm may be due to chemical usage at the dairy. Therefore, costs may be reduced if the horse farm were to invest in cultural and biological control methods, rather than ineffective chemical use.

11c. How could the researchers benefit from this study?

In general, presenting research results helps the career of a scientist. Therefore, Jimmy Pitzer may benefit if the results of this study are presented at scientific meetings or in scientific journals.

12. What other choices do you have if you do not want to be in this study?

The option to taking part in this study is doing nothing. If you do not want to take part in this study, tell the principal investigator and do not sign this Informed Consent Form.

13a. Can you withdraw from this study?

You are free to withdraw your consent and to stop participating in this study at any time. If you do withdraw your consent, you will not be penalized in any way and you will not lose any benefits to which you are entitled.

If you decide to withdraw your consent to participate in this study for any reason, please contact Jimmy Pitzer at (575)-571-6980. They will tell you how to stop your participation safely.

If you have any questions regarding your rights as a research subject, please call the Institutional Review Board (IRB) office at (352) 846-1494.

13b. If you withdraw, can information about you still be used and/or collected?

Yes

13c. Can the Principal Investigator withdraw you from this study?

You may be withdrawn from the study without your consent for the following reasons:

If you do not follow the instructions given to you by the investigator.

WHAT ARE THE FINANCIAL ISSUES IF YOU PARTICIPATE?

14. If you choose to take part in this research study, will it cost you anything?

The only costs incurred for participation involve those for drawing blood. These costs will be paid by the supervisor, Phillip Kaufman.

15. Will you be paid for taking part in this study?

No.

16. What if you are injured because of the study?

Please contact the Principal Investigator listed in question 3 of this form if you experience an injury or have questions about any discomforts that you experience while participating in this study.

17. How will your privacy and the confidentiality of your research records be protected?

Information collected about you will be stored in locked filing cabinets or in computers with security passwords. Only certain people have the legal right to review these research records, and they will protect the secrecy (confidentiality) of these records as much as the law allows. These people include the researchers for this study, certain University of Florida officials, the hospital or clinic (if any) involved in this research, and the Institutional Review Board (IRB; an IRB is a group of people who are responsible for looking after the rights and welfare of people taking part in research). Otherwise your research records will not be released without your permission unless required by law or a court order.

Researchers will take appropriate steps to protect any information they collect about you. However there is a slight risk that information about you could be revealed inappropriately or accidentally. Depending on the nature of the information such a release could upset or embarrass you, or possibly even affect your insurability or employability.

If the results of this research are published or presented at scientific meetings, your identity will not be disclosed.

SIGNATURES

As an investigator or the investigator's representative, I have explained to the participant the purpose, the procedures, the possible benefits, and the risks of this research study; the alternatives to being in the study; and how privacy will be protected:

Signature of Person Obtaining Consent _____ Date _____

You have been informed about this study's purpose, procedures, possible benefits, and risks; the alternatives to being in the study; and how your privacy will be protected. You have received a copy of this Form. You have been given the opportunity to ask questions before you sign, and you have been told that you can ask other questions at any time.

You voluntarily agree to participate in this study. By signing this form, you are not waiving any of your legal rights.

Signature of Person Consenting _____ Date 7-14-08

APPENDIX B
UNIVERSITY OF FLORIDA INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
#200801760

UF | UNIVERSITY of FLORIDA

The Foundation for The Gator Nation

Notification of Initial Approval

From: William Buhi

To: Phillip Kaufman

CC:

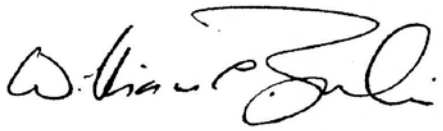
Re: IACUC Protocol #: 200801760

Title: Stable Fly Host Blood Meal Analysis Using a Multiplex Polymerase Chain Reaction

The above referenced study was APPROVED on 9/19/2008 . This approval has been granted by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida. Animals may be purchased and used for the above project and housed in any IACUC approved facility. Any significant change in this approved animal project must occur through a modification procedure and be approved by the IACUC before instituting the change. You are required to return to this site 60 days prior to 9/18/2009 and file a Continuation. If you fail to submit your Continuation and have it approved before this date, your study will automatically expire, and you will no longer be able to work with these animals. The study can only be reactivated with submission and approval of a Continuation.

If this IACUC protocol pertains to a sponsored research project it is the responsibility of the PI to forward a copy of IACUC approval and associated PeopleSoft Project number to the Office of Award Administration via Fax at (352)392-4522 or email at ufawards@rgp.ufl.edu

Sincerely,



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BIOGRAPHICAL SKETCH

Jimmy Pitzer was born in Tracy, California, where he resided for 22 years. After graduating high school in 1997, Jimmy began his undergraduate education at San Joaquin Delta College in Stockton, California. Although he was accepted for admission to the Animal Biology program at the University of California at Davis, Jimmy took the opportunity to travel to New Mexico with his parents in September of 2000. He decided to enroll at New Mexico State University in Las Cruces, New Mexico in July of 2001.

During his undergraduate study at NMSU, Jimmy enrolled in Parasitology, where he met Dr. Ronnie Byford. His overwhelming interest and enthusiasm for the field of veterinary entomology, prompted Dr. Byford to hire Jimmy as a laboratory aide at the NMSU Veterinary Entomology Laboratory. Jimmy graduated with a B.S. in Animal Science in December of 2003, and continued study as a master's student under the instruction of Dr. Byford. Jimmy's master's research focused on determining the potential vectors of West Nile virus in Doña Ana County New Mexico. His work in this area was completed in December of 2006, for which he received an M.S. degree in animal science.

Jimmy directed his focus on the field of veterinary entomology and pursuit of further education in this area of research. In January of 2007, Jimmy was accepted as a Ph.D. student at the University of Florida's Entomology and Nematology Department, under the instruction of Dr. Phillip Kaufman. Jimmy's research involves the ecology of stable flies associated with Florida equine facilities, including the study of their preferred hosts, insecticide susceptibility, and pupal parasitoids. He expects to graduate in May of 2010.

Jimmy has presented the findings of his research at various scientific conferences including the annual meetings of the Entomological Society of America, the Southeastern Branch of the Entomological Society of America, and the Livestock Insect Worker's Conference. He has also participated in the Student Science Training Program offered by the University of Florida for two years as mentor of high school students wishing to gain experience in scientific research at the collegiate level. His ultimate career goal is to earn a position as a research scientist and professor at a major university.